Blinking and the Brain

Pathways and Pathology

Albertine Ellen Smit

Blinking and the Brain ▪ Pathways and Pathology
Dissertation; Erasmus MC – Erasmus University Rotterdam, The Netherlands

Cover art work: A.E. Smit
Design and Layout: A.E. Smit
Printed by: Haveka BV

Financial support for printing of this thesis was kindly provided by Remmert Adriaan Laan Fonds and Erasmus University Rotterdam

© A.E. Smit, 2008
All rights reserved. No part of this thesis may be reproduced, distributed, stored in a retrieval system or transmitted in any form or by any means, without permission of the author, or, when appropriate, of the publishers of the publications.

Blinking and the Brain
Pathways and Pathology

Knipperen en het Brein
Projecties en Aandoeningen

Proefschrift
ter verkrijgen van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. S.W.J Lamberts

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

woensdag 21 januari 2009 om 13.45 uur
door

Albertine Ellen Smit

geboren te Hilversum

PROMOTIECOMMISSIE

Promotor:	**Prof.dr. C.I. De Zeeuw**
Overige leden:	**Prof.dr. G.P. Krestin**
	Dr. T.J. Ruigrok
	Dr. R. Willemsen
Copromotoren:	**Dr. F. van der Werf**
	Dr. J.N. van der Geest

CONTENTS

PART I

PREFACE

A blink is a rapid bilateral eyelid closure and co-occurring eye movement. The eyes rotate down towards the tip of the nose and back up again. Seemingly, this generally unnoticed often repeated action is not very spectacular. However, if not for the occasional blink we would all be blind. A blink is an interesting phenomenon worth investigating.

Through its role as a protective barrier for the eye and as a distributor of the eye's tearfilm, blinking is a necessity for our well-being but is also an important tool for neuroscience research and physicians. It is an extremely useful model to study motor performance, motor control, synaptic plasticity and is an excellent physiological instrument for the assessment of internal networks and nuclei [Nishimura, T. and Mori, K, 1996]. The blink rate, reflex blink characteristics and learned blinks are the three main parameters that can be studied to this end. The blink rate is the frequency with which spontaneous blinks occur and can give information about the dopaminergic system [Karson 1988] and might even be usable as a measure of fatigue [Stern et al. 1994]. The reflex blink is a rapid involuntary response evoked by external stimulation of the eye or eyelid. It has a protective function and is for instance used in research and physiological tests that use the blink to provide important information on the integrity of afferent and efferent pathways. However, important gaps remain in the knowledge of pathways underlying blinking, and aberrations in pathways or compensatory mechanisms are not fully understood. The learned blink is acquired during eyeblink or eyelid conditioning in which an involuntary blink-evoking stimulus is repeatedly combined with a neutral stimulus. After training the neutral stimulus can evoke the learned blink.

In the general introduction of this thesis an overview is presented on today's knowledge on various blink-related subjects. The chapter ends with a description of the techniques that are used in the experiments described in this thesis. In the research chapters several questions will be addressed which focus on blink pathways and cerebellar and cerebral pathologies.

CHAPTER 1

General Introduction

1.1 BLINK BEHAVIOR

The blink is part of every minute of everybody's everyday life. The eyelids constitute a protective barrier between the cornea and anything that might inflict damage from the outside world. If the cornea looses its transparency, eyesight will be compromised or altogether destroyed. In order to prevent such a thing from happening we are equipped with several types of blinks. Blinks can occur either voluntarily, spontaneously or as a reflex in response to external stimulation. In addition, blinks can also be a learned response, like in eyeblink conditioning. Blinking is then associated with a preceding tone. After repeated exposure to a paired tone and blink-eliciting stimulus subjects are conditioned to close the eyes at the time the puff or unconditioned stimulus is presented. Different pathways control the different types of blinks although some overlaps occur (see also chapter 1.3). The kinematic properties of voluntary, spontaneous and reflex blinks are very similar (see chapter 1.4).

Table 1. Various types of blinks and their initiators.

			Elicited through
Reflex	Trigeminal	Corneal reflex	Air puff, tactile / electrical stimulation
		Supra-orbital reflex	Glabellar tapping, electrical stimulation
		Ciliary reflex	Air puff, tactile / electrical stimulation
	Non-trigeminal	Acoustic (startle) reflex	Loud sounds
		Somatosensory reflex	Touch
		Photic reflex	Light flash
		Optic reflex	Menacing visual input
Spontaneous			Generator
Voluntary			Conscious thought

Reflex blinks are the fastest types of blinks and can be evoked in numerous ways. All reflex blinks are elicited by external stimuli; the three primary sensory modalities activated in reflex blinking include tactile, optic and acoustic sensations. Several reflex blinks can be distinguished (table 1). Reflex blinks can be divided into two categories, the trigeminal and non-trigeminal blinks. Tactile threats to the eye are likely to be perceived in the facial area which is innervated by the trigeminal nerve. The corneal reflex, ciliary (eyelash) reflex and supra-orbital reflex, evoked by electrical stimulation or glabellar tapping, are trigeminal reflexes. Corneal and ciliary reflexes can also be induced by tactile stimulation or air puffs. Tactile stimuli outside the face and non-tactile stimuli can also threaten the eye, which is then protected by non-trigeminal reflex blinks. Blink reflexes evoked by non-trigeminal inputs are the somatosensory, acoustic, photic and optic blink reflexes. A somatosensory reflex can be induced by an electrical stimulus to peripheral nerves, for example the median nerve at the wrist [Valls-Solé et al. 1994, Miwa et al. 1995]. Sudden loud sounds can evoke an acoustic blink reflex. When additional muscle contractions occur in the rest of the body the reaction is a startle reflex [Yeomans and Frankland 1995]. The earliest part of the orbicularis oculi contraction in such a startle response is argued to be an acoustic blink reflex [Brown et al. 1996, Aramideh et al. 2002]. The latency of the acoustic blink reflex and the orbicularis oculi component of the acoustic startle reflex are similar and two different orbicularis oculi startle components can sometimes be distinguished with electromyographic recordings. The true auditory startle response seems to begin where the auditory blink reflex ends, however after several stimuli the startle habituates but the acoustic blink reflex remains [Brown et al. 1991]. Photic blinks are

elicited after exposure to light stimuli. An optically evoked blink reflex is the menace reflex (perception of a sudden motion in depth towards the eye). In clinical practice blink reflexes are usually evoked by mechanical stimulation of the cornea or eye lashes or with glabellar tapping or electrical stimulation of the supra-orbital branch of the trigeminal nerve.

Spontaneous blinks spread a protective tear film over the cornea in order to prevent the ocular surface from drying. This pre-ocular tear film contains a lipid outer layer which prevents evaporation of the underlying aqueous layer and innermost mucous layer [Lemp and Wolfley 1992]. The mucous layer provides a physical and chemical barrier that protects the ocular surface from viral and bacterial pathogens, desiccation, chemical accidents, mechanical trauma, and thermal burns [Dartt 2004]. The rate with which spontaneous blinks occur is thought to be determined by a generator. FMRI studies show activation in the right medial frontal gyrus possibly corresponding to the supplementary motor area when compared to blink inhibition activation [Yoon et al. 2005] and in Brodmann areas 7, 17 and 19 compared to keeping the eyes closed during spontaneous blinking [Baker et al. 2003]. This implicates a role for these areas in the generation of spontaneous blinks. Independent of the exact location, the generator controlling the spontaneous blink rate appears to be influenced by several internal and external factors.

A first factor is age. Remarkably, the average blink rate is constant for a given individual [Ponder and Kennedy 1928]. However, if you study the blink rate over shorter periods, fluctuations in the blink frequency are observed. Four blinks per minute would suffice for pre-ocular tear film maintenance, which is about the frequency with which neonates blink [Lawrenson et al. 2005]. That means adults blink several redundant blinks per minute as an average resting person blinks about 10 to 20 times per minute [Delgado-Garcia et al. 2003, Esteban et al. 2004, Karson et al. 1981]. Karson (1988) considers this a protective redundancy of function and is therefore an example of natures tendency to better be safe than sorry. However, nature does apparently not like to overexaggerate either as this is compensated by a steady decrease in the reflex blinking frequency during childhood [Zametkin et al. 1979]. The blink rate and blink properties can be influenced by various other factors like the time of day, environment, emotional state, mental load or activity. The spontaneous blink rate increases in the evening. Presumably this change is correlated with central dopamine activity [Barbato et al. 2000, Taylor et al. 1999]. Thermal factors, like for instance low relative humidity and high room temperature can cause the pre-ocular tear film to dry fast, inducing an increase in the blink frequency [Wolkoff et al. 2006]. Emotional state has been found to influence the amplitude of acoustic blink reflexes of women when tested with pleasant, neutral and unpleasant visual input [Ruiz-Padial et al. 2003]. Intense visual input [Veltman and Gaillard 1998] as well as auditory memory tasks [Karson et al. 1981] and speech [Hall 1945, Karson et al. 1981] increase the blink rate. During speaking, articulatory difficulty does seem to influence the blink rate as Von Cramon and Schuri (1980) discovered that counting from 1 to 100 significantly increases the blink frequency, whereas recital of the alphabet did not. The eyeblink frequency decreases when there is a great demand on visual attention, e.g. during a complex air traffic control simulation [Karson et al. 1981, Brookings et al. 1996] or when children are playing video games [Yamada 1998]. Iwanaga et al. (2000) confirmed this when they found a decreased blink rate when male undergraduates performed a visual search and color-color word distinction task. The blink rate can also be changed by numerous neuropathological conditions, which is discussed in chapter 1.5. Additionally, gender also influences blinking. Karson et al. (1981) found that men can blink faster

Box 1. Blinking throughout the animal kingdom

Blinking is not an exclusively mammalian phenomenon. Birds also blink, however, contrary to mammals, they blink with one eye at a time. This way they will not loose a moment's visual input. Mammals generally blink virtually simultaneous with both eyes, possibly to minimize the downtime of binocular vision [Burr 2005].
Rodents, cats and dogs have low blink rates as they blink less than 3 times per minute whereas primates blink much more frequently (about 10 times per minute). Blink rates of other animals are given in table 2 [Blount 1928]. Interestingly, hunting animals have lower blink rates, which might be explained by the demand on visual attention when looking for a prey. On average a blink of nocturnal animals takes longer than a blink of diurnal animals, which makes sense as nocturnal animals often rely on other senses for their safety whereas diurnal animal are very dependent on their visual input. Diurnal animals are also likely to use blinking to shift attention from one object to another. In another study not the blink frequencies but blink oscillation frequencies were compared between species [Gruart et al. 2000]. An oscillator was suggested to define this species' specific frequency underlying eyelid movements [Domingo et al. 1997]. A relationship was found between a species' mean body weight and the mean oscillation frequency [Gruart et al. 2000, Koekkoek et al. 2002].

Table 2. Duration and average number of blinks per minute (Avg. b/m) of several noctural and diurnal animals [adapted from Blount 1928].

Nocturnal animals	duration	Avg. b/m	Diurnal animals	duration	Avg. b/m
Water possum	6.6		Enclosed pig	1.9	34
Giant armadillo	6.1		Horse	0.8	28
Opossum	5.6		Cow	0.7	22
European hedgehog	4.1		Vervet	0.6	20
Big brown bat	3.9		Gibbon		20
Cat	3.6	2	Open-field pig		20
Hamster	3.4	2	Baboon	0.7	18
Nine-banded armadillo	3.1		Patas monkey	0.9	17
Desert hedgehog	2.7		Man	1.9	14
Rat	2.6	0	Chimpanzee	1.4	14
Dog	2.6	2	Camel		12
Tree shrew	2.6	0	Gorilla		12
Red fox	2.4	4	Asian elephant	1.8	10
Tenrec	2.3		Gray squirrel		8
Little brown bat	2.0		Yak		7
Musk shrew	2.0		Bison		6
Phalanger	1.8	0	Antelope		4
Chinchilla	1.5	0	Goat	0.5	1
Short-tailed shrew	1.4		Sheep	0.6	0.5
Mouse	1.3	0	Giraffe	0.3	0
Galago	1.2		genet	1.3	
Tapir	1.0	6			
Rabbit	0.9	0			
Guinea pig	0.8	0			
Lion		0			
Zebra		2			
Mean	2.73	1.13		1.03	13.38
SD	1.57	1.73		0.54	8.96

and better suppress blinks than women. Humans blink with an average frequency compared to other diurnal animals (Box 1). Thus we can conclude that the blink rate is determined by many different internal and external factors.

Voluntary blinks are blinks initiated by conscious thought. Blinks are used to communicate, for instance to emphasize innocence or indicate that what you said is not very serious. Voluntary blinks are even used to replace speech by patients with severe motor paralysis [Hori et al. 2004]. Several MRI studies have been conducted investigating blink-related neuronal activation. Subjects showed bilaterally increased activation in the primary visual cortex, central thalamus, posterior putamen, and supplementary and primary motor cortex and cerebellum during voluntary blinking [Dimitrova et al. 2002].

1.2 BLINK ANATOMY

A blink consists of simultaneous eyelid and eye movement. Two antagonistic muscles are involved in the performance of the eyelid component of a blink; the orbicularis oculi muscle (OO muscle) and levator palpebrae superiorus muscle (LPS muscle) (fig. 1). The OO muscle, a flat broad elliptical sphincter-like muscle, induces eyelid closure. The tonically active LPS muscle serves to elevate the upper eyelid. A third muscle is the sympathetically innervated tarsal or Müller's Muscle which can adjust the width of the palpebral fissure and aids the LPS muscle in keeping the eyelid open. The eye component of a blink is performed with all 6 extraocular muscles. Lower mammals such as rabbits and cats have an additional muscle which retracts the eye into the orbit during blinking, the retractor bulbi muscle. Reflex blinks have short circuits in the brainstem; other blinks are evoked through projections linked to these circuits. The current knowledge on the pathways underlying different blinks is described in chapter 1.3.

For all muscle contractions proper conduction of signals through a nerve and muscle is imperative. Essential for efficient transfer of a signal from nerve to muscle are well-functioning motor units. A motoneuron and the muscle fibers it innervates constitute a motor unit. When a motoneuron enters a muscle it splits into numerous unmyelinated branches. One neuron can therefore innervate many muscle fibers. The synaptic contacts formed between the branches and muscle fibers are called neuromuscular junctions. For proper innervation only one such contact is made per muscle fiber [Schwartz and Westbrook 2000]. Each region of the OO muscle contains motor units of different sizes. The size of the motor unit determines the precision with which muscle force can be increased or decreased during different types of blinks.

Orbicularis oculi muscle

In the OO muscle three separate portions have been distinguished based on anatomical as well as functional characteristics. The pretarsal and preseptal portion, together known as the palpebral portion, and the orbital portion are depicted in figure 1. The pretarsal portion of the OO muscle contains relatively short muscle fibers and motor units [Lander et al. 1996] and consists for 90% of fibers that are capable of making fast contractions (fast twitch fibers). These fibers have a relatively low threshold and can therefore induce fast precise eyelid closure needed for spontaneous and reflex blinks. The orbital portion is involved in blinking, winking and more forceful eyelid closure [Aramideh et al. 1995] and contains relatively large muscle fibers and motor units which allows for more forceful, yet slower contractions [Gordon 1951, Lander et al. 1996]. The preseptal portion is active during both blinking and sustained activity. During a blink the OO motor units, predominantly in the palpebral portion, discharge brief high frequency bursts.

Levator palpebrae superioris muscle

The LPS muscle is ontogenetically an extraocular muscle that is closely related to the superior rectus muscle. The tonically active LPS muscle has motoneurons with a regular discharge pattern during steady eyelid position which changes linearly related to eye and eyelid position [Fuchs 1992]. Contrary to the other extraocular muscles the LPS muscle is not divided into an orbital and global layer. The muscle contains three types of singly innervated fibers also found in other extraocular muscles and the unique levator slow-twitch fiber type [Porter et al. 1989]. The LPS muscle is connected to the eyelid by

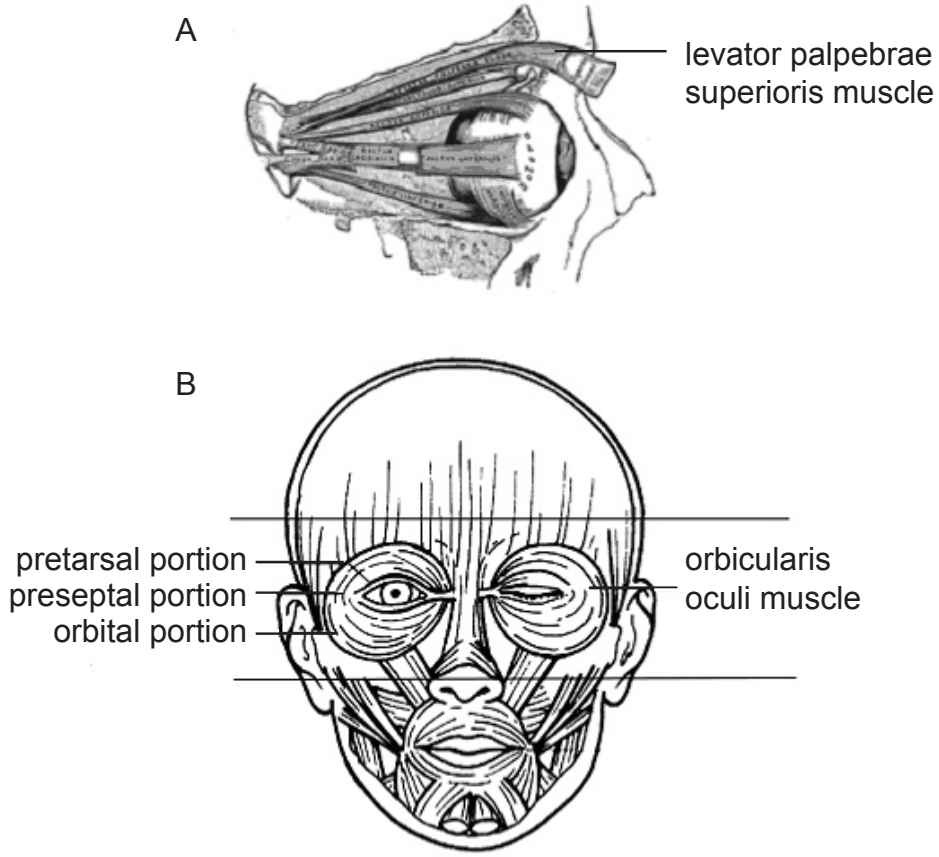


Figure 1. The eye and eyelid musculature. A: the 6 muscles that facilitate eye movements as well as the levator palpebrae superioris muscle. B: the orbicularis oculi muscle and its three subdivisions: the pretarsal, preseptal and orbital portions.

the levator aponeurosis which consists of two layers that contain smooth muscle components in their proximal portions [VanderWerf et al. 1993]. During blinking LPS muscle activity is inhibited. The LPS muscle acts in synergy with the superior rectus muscle. This means when the eye rotates upward, the eyelid is raised so vision is not blocked. Likewise, on downward gaze, the lid comes down to protect the superior part of the eyeball. Rotations of the eye are mediated by the extraocular muscles [Spencer and Porter 2006].

Extraocular muscles

The extraocular muscles are four recti and two oblique muscles that serve to move the eyeball in different directions. The primary movement of the medial, lateral, inferior, superior rectus and inferior and superior oblique muscles is adduction, abduction, depression, elevation, excycloduction and incycloduction of the eyeball, respectively [Burde and Feldon 1992]. Furthermore, extraocular muscles hold the eye steady during fixation. Extraocular muscles have a complicated make-up, in accordance with the range of movements they have to make, varying from high velocity saccades or blinks to

slow smooth pursuit movements. In order to provide the necessary contraction speeds and fatigue resistances the eye muscles contain several fiber types. All extraocular muscles consist of a orbital (facing the orbital wall) and global (near the eye) layer. The orbital layer contains a highly fatigue-resistant singly innervated fiber type and multiple innervated type, possibly with twitch characteristics in the middle of the muscle belly but with tonic characteristics at the proximal and distal ends of individual fibers. The global layer contains three singly innervated, fast-twitch fiber types and one multiply innervated fiber type [Porter and Hauser 1993, Spencer and Porter 2006]. The extraocular muscles have small motor units (approximately 10 muscle fibers per motoneuron), which corresponds perfectly with the small increments in muscle tension necessary for accurate eye movements [Porter et al. 1995, Spencer and Porter 2006].

1.3 BLINK PATHWAYS

Neuronal structures in the brainstem, cerebrum and cerebellum regulate and coordinate the blink. The precise projections and locations of areas involved in blinking within these neuronal structures have only been established to a certain extent. Seven of the cranial nerves are involved in blinking; the optic, oculomotor, trochlear, trigeminal, abducens, facial and vestibulocochlear nerves. In table 3 a short description is given of the 12 cranial nerves and their functions.

Reflex blinks are basal and fast reactions and are regulated in the brainstem. In the cerebrum voluntary blinks are initiated. The cerebellum is involved in the precise timing and coordination of blinks and plays an eminent role in associative learning of a conditioned response. Although a conditioned blink is not the same as a reflex blink there are arguments that the cerebellum might also be involved in reflex blinking [Evinger personal communication]. Freed and co-workers (1981) found that cerebellectomized rats displayed increased blink rates. Imaging studies even showed cerebellar activation during spontaneous blinking [Evinger and Perlmutter 2003].

Most of the influence exerted by the higher order or supranuclear areas modulates the excitability of the blink, through for instance, descending cortical projections via the thalamus and superior colliculus (SC) via tecto-reticular projections [Basso et al. 1996]. Supranuclear projections involved in blinking are distributed over several regions. Imaging studies revealed that the primary motor cortex, supplementary motor cortex, cingulated motor cortex and the central thalamus are active during spontaneous and voluntary blinking [Evinger and Perlmutter 2003]. Using rabies virus as a transneuronal tracer, the hypothalamus and especially the parietal areas of the cerebral cortex were labeled as part of the eyelid premotor circuit [Morcuende et al. 2002]. Direct projections have been described from the motor cortex to the facial nucleus and lateral medullary reticular formation [Jenny and Saper 1987, Kuypers 1958]. Grinevich et al. (2005) also showed monosynaptic projections from the rat vibrissae motor cortex to facial motoneurons using lentivirus-based axonal tracing methods. This monosynaptic connection was, however, expected as a result of ongoing development of specialized movements of the whiskers. Blinks do not require as much skill, though it is possible that monosynaptic projections from the motor cortex to the facial motoneurons involved in blinking exist. Tracing studies in the monkey showed projections from the motor cortex to the intermediate facial motor nucleus [Morecraft et al. 2001]. The basal ganglia can also influence reflex blinking through different pathways [Esteban 1999].

Table 3. Cranial nerves and their functions (adapted from Kandell 2000), blink-related functions are bold.

Nerve		Function
I	Olfactory	Sensory: Smell
II	**Optic**	**Sensory: Vision**
III	**Oculomotor**	**Motor: Innervation of the medial, inferior and superior recti and inferior oblique extraocular muscles, striated muscle of the eyelid.** Autonomic innervation of the pupil and lens.
IV	**Trochlear**	**Motor: Innervation of the superior oblique muscle.**
V	**Trigeminal**	**Sensory: Cutaneous and proprioceptive sensation from skin, muscles** and joints **of the face** and mouth and sensory innervation of teeth and jaws. Motor: Innervation of the mastication, tensor tympani, tensor veli palatine, mylohyoid and the anterior belly of the digastric muscles.
VI	**Abducens**	**Motor: Innervation of the lateral rectus muscle.**
VII	**Facial**	Sensory: Sensation from the skin of the external ear and taste from the anterior two thirds of the tongue. **Motor: Innervation of the muscles of facial expression** and stylohyoid, stapedius and posterior belly of the digastric muscles. Autonomic innervation of the salivary glands (except the parotid and lacrimal glands and cerebral vasculature).
VIII	**Vestibulocochlear**	**Sensory: Hearing and sense of motion** (angular and linear acceleration).
IX	Glossopharyngeal	Sensory: Taste from the posterior third of the tongue and sensation from the posterior palate and tonsillar fossae and carotid sinus. Motor: Innervation of the stylopharyngeus muscle. Anatomic innervation of the parotid gland.
X	Vagus	Sensory: Sensation from the posterior pharynx, visceral sensation from the pharynx, larynx, thoracic and abdominal organs, taste from the posterior tongue and oral cavity. Motor: Innervation of the striated muscles of the larynx and pharynx. Autonomic innervation of smooth muscle and glands of the gastrointestinal, pulmonary and cardiovascular systems in the neck, thorax and abdomen.
XII	Spinal Accessory	Motor: Innervation of the trapezius and sternocleidomastoid muscles.
XII	Hypoglossal	Motor: Innervation of the intrinsic muscles of the tongue.

Most experimental studies described in this thesis focus on reflex and conditioned blinks, the pathways of these two types of blinks are discussed in more detail. During reflex blinking the eyes and eyelids are innervated by their individual circuits which are discussed separately. For conditioned blinks the eyes and eyelids are partially innervated by the same pathways. The exact projections that enable this form of associative learning are still extensively investigated although some consensus has been reached, which is discussed in the last part of this chapter. Box 2 provides additional information on the main neuronal structures involved in the blink.

Pathways for eyelid movement

The eyelids receive motor, sensory, sympathetic and parasympathetic innervation [Holstege 1986, Hart 1992, Pellegrini et al. 1995, Van Ham and Yeo 1996, VanderWerf et al. 1998, Gong et al. 2003, Zerari-Mailly et al. 2003]. During a blink the tonically active eye-opening LPS muscle is temporarily inhibited to allow the eye-closing OO muscle to contract. The OO muscle is innervated by the zygomatic branch of the facial nerve (fig. 2). The cell bodies of the facial nerve fibers that innervate the OO muscle lie within the intermediate subnucleus of the facial motor nucleus [Holstege et al. 1986, VanderWerf et al. 1998]. The LPS muscle is innervated by the oculomotor nerve [Büttner-Ennever 2006]. LPS muscle motoneurons can be found in the central caudal nucleus of the oculomotor complex [Fuchs et al. 1992, Porter et al. 1989, VanderWerf et al. 1997]. Most blink studies focus on innervation of the OO muscle as this is the active component of the blink. Obviously this active component needs to be coordinated with

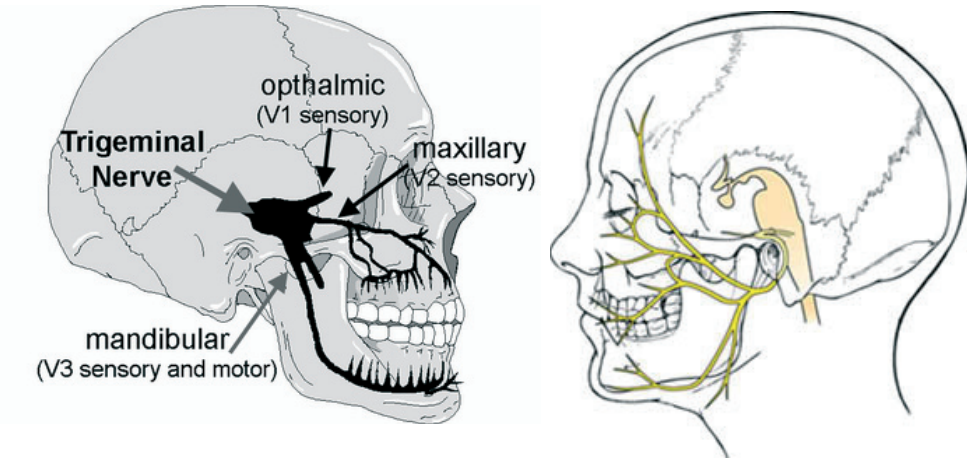


Figure 2. Schematic representation of the localization of the different facial (right) and trigeminal (left) nerve branches.

the inhibition and resumption of LPS muscle activity. During blinking LPS muscle activity is inhibited by activation of local inhibitory premotor neurons or inhibitory premotor neurons in the sensory trigeminal complex (STC) [Van Ham and Yeo 1996]. Sensory innervation of the skin occurs through the trigeminal nerve (fig. 2). A spider web of projections has been revealed for all the different blink reflexes. The reflex blink can be divided in trigeminal and non-trigeminal reflex blinks.

Trigeminal reflex blinks

For trigeminal reflex blinks there is a simple direct circuit from the trigeminal nerve through the Gasserian ganglion, which contains the cell bodies of trigeminal primary afferent neurons, to the secondary sensory neurons in the STC which directly projects to the facial motor nucleus [Jacquin et al. 1993; Van Ham and Yeo 1996b] (fig. 3 pathway 1). From the STC two indirect pathways arise. The first by way of the reticular formation, the second via the rostral cervical spinal cord (C1) [Zerari-Mailly et al. 2003] (fig. 3 pathways 2 & 3). The indirect pathways were studied in the cat [Holstege et al. 1986b], guinea pig [Pellegrini et al. 1995], rabbit [Van Ham and Yeo 1996a,b] and rat [Zerari-Mailly et al. 2003]. For all other trigeminal blink reflexes this circuit is the same though the input into the Gasserian ganglion differs. The ganglion is organized somatotopically in three main divisions. Cell bodies from the ophthalmic division are located anteromedially, cell bodies from the mandibular division posterolaterally and cell bodies forming the maxillary division predominantly in between (fig. 4).

In his studies on the somatotopy of afferent cell bodies of trigeminal subdivisions in the Gasserian ganglion of the cat, Marfurt (1981) found cell bodies from the supra-orbital nerve and the lateral branch of the infra-orbital nerve concentrated in the dorsal part of the trigeminal ganglion. Cell bodies from the medial branch of the infra-orbital nerve, the inferior alveolar nerve and mental nerve were mostly located in the ventral part of the ganglion. Hereby he demonstrated that the Gasserian ganglion somatotopy of the cat is not only from medial to lateral for the main divisions of the trigeminal nerve but also from dorsal to ventral. The ophthalmic division of the trigeminal nerve comprises the afferent pathway of the corneal, supra-orbital and ciliary reflexes.

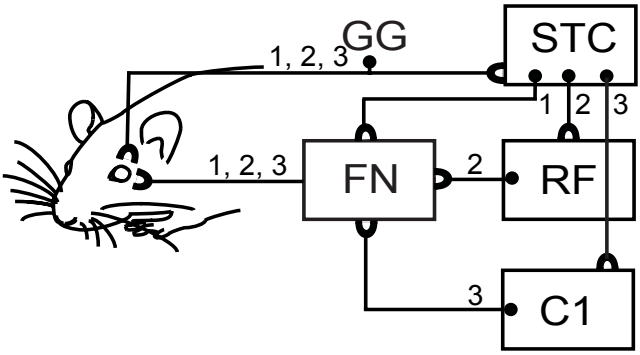


Figure 3.
The trigeminal blink reflex circuitry.
Abbreviations: Gasserian ganglion (GG), sensory trigeminal complex (STC), facial motor nucleus (FN) reticular formation (RF) and upper part of the cervical spinal cord (C1).

1. Short direct pathway via the STC and FN
2. Indirect pathway via the STC, RF and FN
3. Indirect pathway via the STC, C1 and FN

Non-trigeminal reflex blinks

Visual, acoustic, mechanical and electrical stimuli can induce blink reflexes that are mediated by non-trigeminal circuits. The optic nerve is the afferent pathway for blink reflexes evoked by visual stimuli (menace and dazzle reflexes). There is a direct visual subcortical input to the supraoptic nuclei and superior colliculus. The efferent pathway is mediated by association fibers of the facial nuclei. The menace reflex appears to be more cortically dependent as it requires stimulus processing in the visual cortex and its associative connection to the rolandic area [Hart 1992]. The acoustic blink reflex, or startle reflex, has a very short latency (5-10 ms depending on the muscle recorded from) and therefore has a short circuit. The reflex is mediated by the cochlea and vestibulocochlear nerve, pontine reticular formation, facial nucleus and facial nerve [Koch and Schnitzler 1997]. Further projections have been found from the cochlear nuclei to the ventrolateral tegmental nuclei. The somatosensory reflex can be

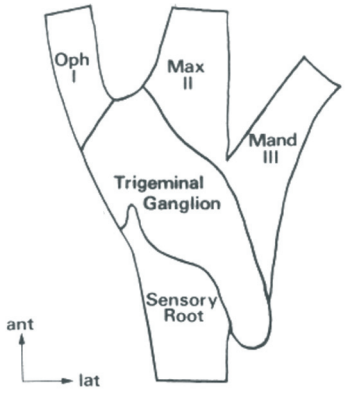


Figure 4. Schematic diagram of the cat Gasserian ganglion in the horizontal plane. Oph = Ophthalmic, Max = Maxillary, Mand = Mandibular, Ant = Anterior, Lat = Lateral [adapted from Marfurt 1981].

evoked by brief mechanical or electrical stimulation of peripheral nerve afferents from the upper and lower limbs. This stimulation facilitates the early orbicularis oculi response R1 and inhibits the second orbicularis oculi R2 (orbicularis oculi responses are further explained on page 26). This suggests that processing of sensory inputs from the face and the limbs occurs at the brainstem. Here they are probably integrated in a network of interneurons which influences the excitability of facial motoneurons [Valls-Solé et al. 1994].

Pathways for eye movement

Eye movement is mediated by the extraocular muscles which are innervated by three different cranial nerves. Five different types of eye movement can be distinguished: Saccades, smooth pursuit eye movements, vestibulo-ocular reflex, optokinetic response and convergence. Eye muscles are also required to keep the eye still for gaze holding or fixation [Büttner and Büttner-Ennever 2006]. Each eye movement has its own circuit but they all converge at the level of the motoneuron. The lateral rectus is innervated by the abducens nerve, the superior oblique is innervated by the trochlear nerve and the medial, inferior and superior recti and inferior oblique muscles are all innervated by the oculomotor nerve [Burde and Feldon 1992]. Neither the circuitry for voluntary eye movement nor the circuitry for eye movement during blinking is exactly known. Several models have been developed throughout the years to describe this complicated combined effort of the eye muscles to move the eyeball [Miller and Robinson 1984, Leigh and Zee 1999, Munoz and Fecteau 2002, Scudder et al. 2002, Haslwanter et al. 2005].

The major areas that are involved in visual fixation and saccadic initiation are illustrated in figure 5. Saccades are accompanied by eyelid movement and, likewise, a blink is accompanied by a saccade-like eye movement. The pathway facilitating the typical eye movement of both eyes during blinking is unknown, though a neuronal structure should link the well-coordinated eye and eyelid movement. Eye and eyelid movement might be connected through the oculomotor system, although projections have not yet fully been established. According to Goossens et al. (2000a,b) a direct relation between the superior colliculus (SC) and reflex blinking exists, and therefore the SC could be a generator of eye- and eyelid coordination during the blink reflex. The SC has been considered part of the visual pathway concerned with the analysis of visual pace and directing of orienting movements. The presence of a somatosensory map in register with the visual map indicates that the SC might also be involved in the control of tactually elicited orienting movements [Finlay et al. 1978]. The SC appears to be a structure in which several aspects of vision and eye movement converge. A possible role for the SC in blinking was also considered by Evinger and co-workers (1994) and Basso and co-workers (1996) in humans and in monkeys [Goossens and van Opstal, 2000a,b]. Physiological recordings of awake trained monkeys showed suppression of neuron firing of the intermediate and deeper SC layers during blink-interrupted saccadic movement [Goossens and Van Opstal, 2000b]. The latency of this suppression was relatively short (10-30 ms) after an initiated reflex blink, therefore a direct connection between the sensory trigeminal complex and SC was suggested [Ndiaye et al. 2002].

Pathways for conditioned responses

Conditioned blink responses are generated through a different circuit, depending on the type of conditioning stimuli used. Normal reflex blinks or unconditioned responses (UR) with trigeminal blinks are mediated by the trigeminal circuit as previously described. However, by combining this blink

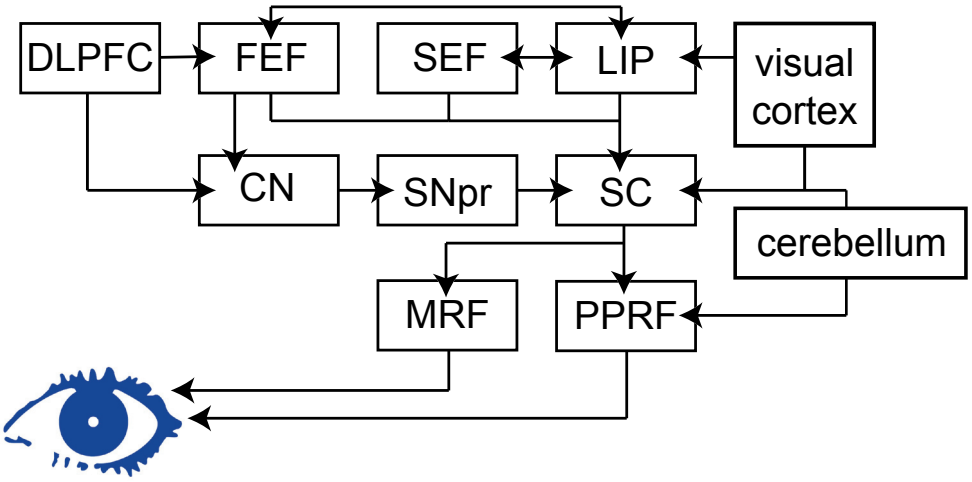


Figure 5. Brain areas and projections involved in visual fixation and saccadic initiation in monkeys [adapted from Munoz and Fecteau 2002]. CN = caudate nucleus, DLPFC = dorsolateral prefrontal cortex, FEF = frontal eye fields, LIP = lateral intraparietal area, MRF = mesencephalic reticular formation, PPRF = paramedian pontine reticular formation, SC = superior colliculus, SEF = supplementary eye fields, SNpr = substantia nigra pars reticulata.

eliciting unconditioned stimulus (US) with a biologically insignificant conditioned stimulus (CS) another circuit is activated simultaneously. Initially the result will be as if only the trigeminal circuit was active but gradually the timing and execution of the response will change due to the additional circuit.

Two frequently used types of eyeblink conditioning are delay and trace conditioning. During delay conditioning the US is presented at the end of the CS, whereas during trace conditioning there is a gap between the end of the CS and the US. The principle of conditioning is further explained in chapter 1.7.

There is general consensus that the cerebellum is essential for learning of the delay conditioned response [Raymond et al. 1996, Van Ham and Yeo 1996, Ito 2002, Lavond 2002, DeZeeuw and Yeo 2005, Christian and Thompson 2005]. However, the big question is: "where in the cerebellum does learning occur?" Acquisition of the conditioned response can be prevented by inactivation of the inferior olive, cerebellar cortex and cerebellar nuclei [De Zeeuw and Yeo 2005]. These findings confirm the need for an intact olivo-cortico-nuclear loop for conditioned response (CR) acquisition, though do not reveal what the exact learning site is. Some authors state that cerebellar Purkinje cell parallel fiber synapses in the cerebellar cortex are sites where changes occur due to conditioning [Attwell et al. 2002]. These changes could be reinforced by climbing fiber olivary input. Other authors suggest a strengthening of input from precerebellar nuclei to the cerebellar interpositus nucleus is what enables us to make conditioned responses [Kleim et al. 2002]. Lavond (2002) proposes the interpositus nucleus as the primary learning site of the association between the conditioned and unconditioned stimulus.
Short-term (for acquisition) and long-term (for saving) motor memory exist, shown by retention of a

performance over several trials. Where the long-term memory is retained is yet another issue of debate [Krakauer and Shadmehr 2006]. A second learning site might exist in the cerebellar nuclei [Attwell et al. 2002] like the interpositus nucleus [Christian and Thompson 2005]. This location outside the cerebellar cortex might contribute to relearning, or saving, of the conditioned response [Medina et al. 2001]. This hypothesis is supported by experiments with animals without cerebellar cortex in which a previously acquired conditioned response was not completely abolished [Ohyama et al. 2003]. Unlearning or extinction of the CR is a third conditioning parameter. Inhibition of climbing fiber inferior olive input is thought to be the mechanism through which extinction is achieved [Medina et al. 2002]. Further research will have to reveal whether the cerebellar cortex, nuclei or even the hippocampus are also essential for extinction of the conditioned response [Robleto et al. 2004].

The pathways hypothesized to underlie eyeblink conditioning are summarized in figure 6 [Van Ham and Yeo 1996, Attwell et al. 2002, De Zeeuw and Yeo 2005]. The auditory conditioning stimulus enters the cerebellum through the cochlear nucleus and pontine nuclei. The unconditioned stimulus reaches the cerebellum through the cornea, Gasserian ganglion, STC and the inferior olive. The unconditioned response is mediated disynaptically through the Gasserian ganglion (fig. 3 and 6.) and polysynaptically through the reticular formation and rostral C1 (fig. 3). Eyelid closure is enabled by OO motoneurons in the facial motor nucleus via projections from the STC or red nucleus.

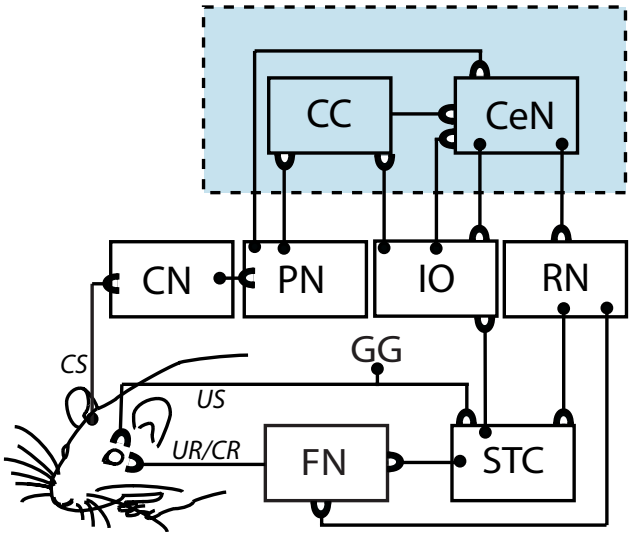
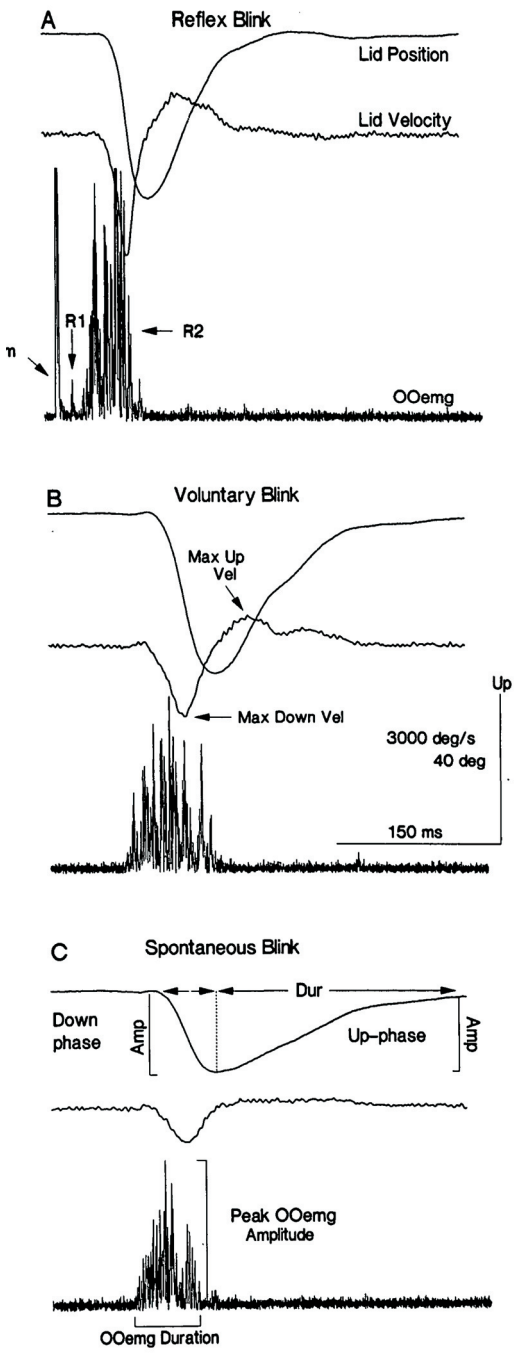


Figure 6. Diagram of the structures and projections thought to be involved in conditioning. The conditioned stimulus (CS), unconditioned stimulus (US), unconditioned response (UR) and conditioned response (CR) pathways can be distinguished. Abbreviations: CC = cerebellar cortex, CeN = cerebellar nuclei, CN = cochlear nucleus, FN = facial motor nucleus, GG = Gasserian ganglion, IO = inferior olive, PN = pontine nuclei, RN = red nucleus, STC = sensory trigeminal complex.

Box 2. Background information on the main structures involved in blinking.

Supra-orbital nerve
The supra-orbital nerve of the 5th cranial nerve contains sensible fibers innervating the skin and oral and nasal mucosa. The trigeminal nerve has its origin in the trigeminal complex and gives off three main branches after the Gasserian ganglion: the ophthalmic, maxillary and mandibulary. The supra-orbital nerve originates from the ramus tentorii, a split-off of the ophthalmic branch, which gives off the frontal nerve, which splits into the cochlear and supra-orbital nerve. It serves predominantly as sensory nerve for the upper eyelid and forehead skin [Kahle W, 2001] (fig.2).
Gasserian ganglion
The Gasserian ganglion, otherwise known as trigeminal ganglion or semilunar ganglion, is located at the end of the sensory radix of the trigeminal complex in the trigeminal cave. This sensory ganglion of the trigeminal nerve was described in 1765 by Antonius Hirsh in his dissertation for the medical graduation. Hirsch named the ganglion in honor of his professor, Johann Lorenz Gasser [Hirsch ABR, 1765].
Sensory trigeminal complex
The sensory trigeminal complex extends from the midbrain to the upper cervical spinal cord and is divided into three main subnuclei: the mesencephalic nucleus (Me5), the main or principal nucleus (Pr5), and the spinal trigeminal nucleus (Sp5). Sp5 is subdivided into oral (Sp5O), interpolar (Sp5I) and caudal (Sp5C) parts. Anatomical studies, using transganglionic tracing methods, have shown central projections of afferent fibers from the SO-nerve within the sensory trigeminal complex. In cats [Marfurt 1981, Panneton 1981], rabbits [Van Ham and Yeo 1996b] and guinea pigs [Pellegrini et al. 1995] primary afferent projections from the eyelids terminate predominantly in the Sp5C and Sp5I whereas only few projections could be found in the Pr5 and Sp5O regions. Furthermore in monkeys [May, 1998] and rats [Ndiaye et al. 2002] projections from the eyelids were observed in the ventrolateral part of the Sp5C and ventrally in the caudal Pr5 and some additional projections in the Sp5O and Sp5I.
Facial motor nucleus
The facial motor nucleus consists of 5 subnuclei, the lateral, ventrolateral, ventromedial, dorsomedial and intermediate. Motoneurons innervating the orbicularis oculi can be found in the dorsal part or intermediate subnucleus [Watson et al. 1982, Martin and Lodge 1977, Holstege et al. 1986].
Facial nerve
The seventh cranial nerve or facial nerve contains nerve fibers of the facial motor nucleus and innervates facial muscles, among which the orbicularis oculi muscle. The facial nerve has three large collaterals with motor fibers, the ophthalmic, maxillary and mandibulary branches. The facial nerve also contains two sensory branches, the intermediate nerve-innervating taste buds, and a small parasympathic nerve [Frotscher 2001] (fig. 4).
Orbicularis oculi muscle (OO muscle)
The OO muscle is a sphincter-like muscle, its main function is to close the eyelid (thereby protecting the eye). In addition, this muscle, together with other facial muscles, plays a role in expression of emotions [Holstege et al. 1986].
Reticular Formation
The reticular formation is a netlike structure of cell bodies and nerve fibers and is located medially in the brain stem and is divided into 3 main fields. From caudal to rostral they are the medullar, pontine and mesencephalic reticular formation, which consist of their own divisions based on cell density, size and function. The lateral reticular nucleus, nucleus reticularis tegmenti pontis of Bechterew and paramedian nucleus are sometimes not included as they project to the cerebellum in contrast to the rest of the reticular formation [Brodal 1981].
C1
The C1 or rostral part of the cervical spinal cord is part of several pathways among which the blink reflex. Premotor afferent neurons to the facial motor nucleus were identified in the C1 (Tanaka et al., 1978; Pellegrini et al., 1995). Pellegrini (1995) suggested that the R2 component is conveyed through the C1. Furthermore C1 can modulate cornea evoked blink reflexes [Henriquez 2005].
Superior colliculus (SC)
The SC is a layered structure which is located dorsally in the rostral pontine reticular formation. Each layer can be distinguished on the basis of its cyto- and myelo-architectonical characteristics. In rats and cats SC has been found to consist of seven laminae (Kanaseki 1974, Killackey 1981). From dorsal to ventral, in the rat superior colliculus the first layer is the stratum zonale, a thin layer which contains small neurons; the second layer or stratum griseum superficiale is a relatively thick layer; layer three or stratum opticum is composed of fibrous elements with a few scattered neurons; layer four, otherwise known as stratum griseum intermediale is a densely packed layer with a dorsal band of large neurons; layer five or stratum album intermediale is a layer with fiber bundle patches mixed with cellular elements; layer six or stratum griseum profundum consists of a layer of large neurons at its dorsal border. Finally, layer seven or stratum album profundum, is a fibrous layer that separates the SC from the periaquaductal gray [Ndiaye 2002, Sparks and Hartwhich-Young 1989].
Oculomotor muscles
Humans have 6 muscles that enable eye movement. The oculomotor nuclei in the brainstem innervate these muscles. The purpose of the oculomotor system is to bring the eyes into a chosen position in an efficient and coordinated manner [Büttner and Büttner-Ennever 2006].



1.4 BLINK PHYSIOLOGY

Blink kinematics

Eyelid kinematics

For all types of blinks, muscle activity onset precedes eyelid movement onset with about 12 ms and the maximum velocity of the closing eyelid (down phase) is generally twice as high as the maximum velocity during eyelid opening (up phase) [Evinger et al. 1991]. Reflex, voluntary and spontaneous blinks also have their own characteristics. Figure 7 shows eyelid kinematics and orbicularis oculi muscle electromyogram (OO-EMG) for a typical reflex blink, voluntary blink and spontaneous blink. For the reflex blink the largest burst of muscle activity is generated by very efficient simultaneous motorunit activation resulting in fast powerful eyelid closure and somewhat slower eyelid opening. The voluntary blink has a less powerful but longer contraction of the OO muscle which creates a slower eyelid movement with an amplitude similar to a reflex blink. The spontaneous blink has the smallest muscle activity and therefore the slowest and smallest eyelid movement
[Evinger et al. 1991, VanderWerf et al. 2003].

Eye kinematics

During blinking the eye rotates down towards the nose and back up again (see fig. 8) [Collewijn 1985, Evinger 1984]. The blink appears to be a symmetric movement of both eyes and eyelids. Although when measured electrophysiologically, eye movement during voluntary blinks as well as reflex blinks is slightly asymmetric [Kassem and Evinger

Figure 7. Eyelid kinematics and OO muscle electromyogram (OO-EMG) measured in humans for a A. reflex blink, B. voluntary blink and C. spontaneous blink. n: stimulus artifact from supra-orbital nerve stimulation, Dur = duration, Amp = amplitude, Vel = velocity [Evinger et al. 1991].

2006]. Besides the rotation the eye is also displaced 1-2 mm back into the orbit during blinking [Evinger 1984]. Eye movement during blinking is achieved through co-contraction of all extraocular muscles except the superior oblique. The exact shape of the rotation is dependent on the start position of the eye [Bour et al. 2000, Evinger et al. 1993]. The eye always returns to the initial gaze position after a blink. Bour and co-workers (2000) found that for reflex blinks this was independent of the intensity of the stimulus that evoked a blink. In rabbits the latency for extraocular muscle activation is longer than for OO muscle activation and varies depending on the muscle and blink-evoking stimulus [Evinger and Manning 1993].

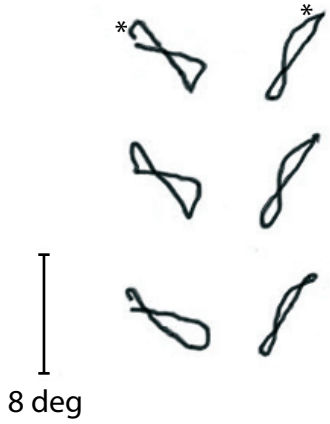


Figure 8. Rotation of the left and right eye during three consecutive blinks in a healthy subject. (*) indicates the startpoint of the eye movement

Blink electrophysiology

In most neurophysiologic studies of blinking, blink reflexes are evoked by electrical sensory stimulation. Electromyogram (EMG) responses are measured on the orbicularis oculi muscle (OO muscle) and/or LPS muscle, with either small surface electrodes or needle electrodes. Trigeminal nerve stimulation is most frequently used. The supra-orbital (SO) branch of the ophthalmic division of the trigeminal nerve is stimulated at the supra-orbital foramen, and by way of the short loop (fig. 3) a blink reflex is elicited. The stimulation intensity is generally three times the threshold which evokes a blink also called supramaximal. During a blink the OO and LPS muscles have to act antagonistically: the LPS muscle relaxes, followed by OO muscle contraction. As the OO muscle ceases to contract the LPS muscle resumes its tonic activity. LPS muscle inhibition precedes and outlasts OO muscle activity by about 10 ms [Schmidtke and Büttner-Ennever 1992, Aramideh et al. 1994].

The orbicularis oculi response

Blink reflexes evoked by electrical stimulation of the SO nerve can yield three different responses of the OO muscle known as the R1, R2 and R3 (fig. 9). In humans the R1 is an ipsilateral fast response whereas the R2 is a bilateral slow response that is responsible for eyelid closure. The R1 has a stable onset latency of about 10 ms whereas the R2 typically has more variable latencies. R2 is a larger response than R1 and has a lower threshold [Esteban 1999]. In contrast to human subjects, the R1 is

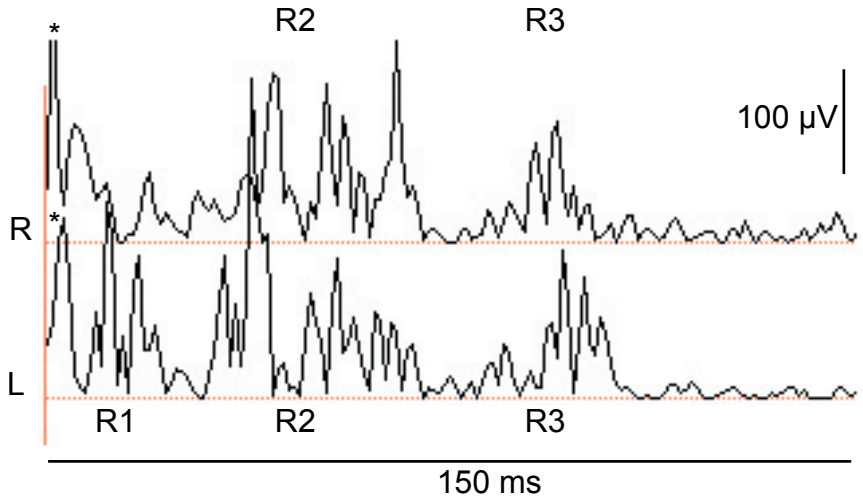


Figure 9. Electromyographic recording of blink responses (rectified, integrated) of the right (R) and left (L) orbicularis oculi muscle after electrical stimulation of the left supra-orbital nerve. The responses consist of an early R1 response ipsilateral to the stimulated side followed by bilateral R2 and R3 responses. The traces start with a stimulus artefact (*).

the major and R2 the small response in rats. The R1 is thought to be mediated through the short circuit (no. 1) described in figure 3 [Pellegrini et al. 1995, Holstege et al. 1986b, Van Ham and Yeo 1996a,b, Cruccu et al. 2005]. Modulation and regulation of the R2 is thought to involve the indirect pathways via the reticular formation [Kimura and Lyon 1972, Hiraoka and Shimamura 1977, Holstege et al. 1986, Tamai et al 1986, Cruccu 2005] and C1 [Pellegrini 1995] (indirect pathways no. 2&3, fig. 3). Pellegrini et al. (1995) showed that in guinea pigs the spinal trigeminal nucleus produces the R1, whereas the R2 originates from the C1 region of the spinal cord.

The R3 was not noticed until 20 years after the first electrical stimulation study. The R3 response is obtainable from supra-orbital, infra-orbital and mental nerve stimulation. The R3 can clearly be distinguished after low intensity stimulation and increases parallel to R1 and R2 after higher intensity stimulation at which levels it becomes more difficult to separate R2 from R3 [Esteban 1999]. It were Penders and Delwaide (1972) that first described this bilateral response. Further research from Rossi and co-workers (1989) suggested that the R3 is mainly triggered by nociceptive input and has a mean onset latency of around 80 ms. Ellrich and co-workers (1996, 1997) found a similar R3 latency (84 ms) but stated that the origin of the R3 is still largely unclear. The long latency suggests that the R3 is mediated by a long reflex arc possibly via ophthalmic nerve nociceptive fibers to the spinal trigeminal subnucleus and the high reflex threshold argue in favour of a nociceptive origin.

Other methods used in electrophysiological studies of OO muscle responses are facial, infra-orbital and mental nerve stimulation. After a percutaneous electrical stimulation of the facial nerve at the pretragal area of the face two OO muscle responses can be distinguished: a direct ipsilateral motor response and a second bilateral response. This second response is considered a facio-facial reflex [Willer and Lamour 1977]. After electrical stimulation of the infra-orbital nerve an R1 component is sometimes observed in the OO muscle response. After electrical stimulation of the mental nerve the

R1 component is rare or even absent [Kimura et al. 1969, Esteban 1999]. An R2 component is found both after infra-orbital and mental nerve stimulation. Required stimulus intensity is usually higher than for SO nerve stimulation and onset latency for the R2 is often longer.

The levator palpebrae superioris response
Stimulation of the SO-nerve evokes two silent periods in the LPS muscle, the first or early silent period, SP1 and the second or late period SP2. In contrast with the R1 response of the OO muscle, SP1 occurs bilaterally regardless of the stimulated site. The latency of SP1 varies between 9-13 ms and is thus slightly shorter than the latency for the R1 of the OO muscle. The duration of SP1 is 12 to 15 ms. The latency of SP2 varies from 27 to 35 ms and has a total duration of 32 to 50 ms. There is however some variability in the LPS muscle response which depends on the prestimulus contraction of the LPS muscle and the stimulus intensity. Weak contraction of the LPS muscle before the stimulus can induce one large inhibitory period. After a low intensity stimulus the SP2 occurs and after supramaximal stimulation of the SO-nerve SP1 becomes visible and latency shortens and the duration lengthens of both responses [Aramideh et al. 2002, Esteban 1999].

The eye response
Unilateral low current SO nerve stimulation elicits bilateral eye movements. At stimulus intensities over 2 times the threshold value for eyelid movement an additional ipsilateral early eye movement can be observed which corresponds to the eyelids R1. The latency of the early eye movement component is about 15 ms. With an increasing stimulus intensity the latency of the late bilateral eye movement decreases from approximately 85 ms to 35 ms [Bour et al. 2000].

1.5 BLINK PATHOLOGY

The brain and especially nerve branches are exposed to many different threats like numerous types of neural damage, caused by trauma, surgery, tumours, toxins, compression or inflammation. Disorders of ocular and/or eyelid motility are therefore rather common. They include a wide variety of conditions from cranial nerve neuropathies to transmission problems, lesions and movement disorders. Eye and eyelid movements are mediated by the oculomotor and facial nerves and their associated nuclei which are influenced by many different structures in the brain. Dysfunction of any of these nerves / neuronal structures will affect blinking. Some of these pathologies will specifically affect one or several of the blink nerves, others will affect transmission and yet other blink abnormalities are caused by lesions or movement disorders. In the following paragraphs an impression will be given of various blink-related pathologies (see also Aramideh et al. 2002).

Neuropathies

Trigeminal neuropathy
Trigeminal sensory nuclei and reflex circuits are present throughout the brainstem and even extend into the rostral spinal cord. Therefore the blink reflex is often affected by brainstem disorders. Moreover the Gasserian ganglion, where the first order neurons for the trigeminal blink reflexes lie, is not fully

protected by the blood-brain-barrier, making it more vulnerable to circulating antibodies or pathogens [Aramideh et al. 2002]. Conditions include trigeminal neuralgia [Bhatti and Patel 2005, Manzoni and Torelli 2005], focal lesions of peripheral branches or ganglia [Valls-Solé 2005] and migraine [Buzzi and Moskowitz 2005].

Facial neuropathy

Like trigeminal neuropathies facial neuropathy can originate from many sources. The most common agents of facial paralysis are herpes simplex and varicella-zoster. Illnesses like Guillain-Barré, Lyme disease (caused by Borrelia burgdorferi) and sarcoidosis can also cause bilateral facial palsies. Peripheral nerve palsy accompanied by an erythematous vesicular rash on the ear or in the mouth is called Ramsay Hunt syndrome [Sweeney and Gilden 2001].

For about half of the patients with facial paralysis the cause is unknown. This idiopathic facial paralysis is also named Bell's palsy and primarily occurs unilaterally. Facial nerve paralysis often results in synkinesis of facial muscles and facial weakness [Aramideh et al. 2002]. These sequelae may be caused by aberrant regeneration of the facial nerve or an alteration in the facial nucleus. The sequelae of Bell's palsy could also be caused by plasticity of higher order structures. A prolongued lack of feedback to the motor cortex in the most severe cases of this paralysis, who cannot move their facial muscles for about 4 months, might lead to changes in the motor cortex.

The most severe complication of facial paralysis is the postparalytic facial syndrome, characterized by muscular pain, tension and spasms induced by common activities and has been found up to 16 years after onset of the palsy. Hemifacial spasms can occur during the recovery process of facial paralysis but is also a facial neuropathy in its own right. The spasms are characterized by involuntary muscle twitches on one side of the face, involving synchronous contraction of upper and lower facial muscles. This mostly idiopathic condition can be induced by hyperventilation and compression syndromes at the facial root entry zone [Valls-Solé 2002].

Oculomotor neuropathy

Pathologies can occur in any of the oculomotor nerves and affect the eye movement component of the blink. This could lead to a less effective spread of the preocular tear film over the cornea. Unilateral paresis of the oculomotor nerve can be caused by schwannoma's in patients with neurofribromatosis type 2. Acute abducens nerve palsy can occur due to vitamin A treatment of xerophthalmia multiple sclerosis and intracranial plasmocytoma [Szatmary and Leigh 2002].

Polyneuropathy

Oculomotor, trigeminal or facial nerve abnormalities may be an indicator of a systemic disorder. In Bell's palsy, earlier described as a facial neuropathy, other nerves can also be involved [Adour et al. 1975, Benatar 2004]. Peripheral neuropathy is one of the late complications of diabetes mellitus [Kazem and Behzad 2006]. Cranial nerves III, V, and VII are most frequently affected in diabetic patients [Cruccu et al. 1998, Kazem and Behzad 2006]. Disorders in which demyelination occurs often result in polyneuropathies also affecting the blink reflex [Cruccu et al. 1998, Yamamoto et al. 2004, Vucic et al. 2004]. One of the subtypes of Guillain-Barré (acute peripheral neuropathy) is also characterized by severe demyelination. This leads to slower conduction and therefore a significant delay of all blink reflexes [Aramideh et al. 2002]. In all subtypes of Guillain-Barré, including Miller

Fisher syndrome (acute ophthalmoplegia, ataxia and areflexia), reflex blinking is disturbed [Jamal and Ballantyne 1988, Urushitani et al. 1995, Calleja et al. 1998, Hughes and Cornblath 2005]. Furthermore several hereditary conditions affect multiple neurons and reflex blinking like the hereditary sensory and autonomic neuropathies [Esteban-Garcia et al. 2004, Ishpekova et al. 2005]. Various infectious agents can also cause polyneuropathy that severely affect blinking, as is for instance the case in herpes zoster infection of the head and limbs [Mondelli et al. 2002] and meningitis. Other pathologies that are known to induce polyneuropathy include AIDS, Burkin's Lymphoma, sickle cell disease and rheumatic arthritis [Aramideh et al. 2002].

Transmission

Problems with neuronal transmission can be caused by motoneuron diseases and neuromuscular transmission defects. Motoneuron diseases are often very severe as, due to progressive failure of muscle functioning, they will inevitably lead to death. Amyotrophic lateral sclerosis (ALS) and the juvenile and adult types of progressive bulbar palsy are examples of such progressive motoneuron diseases. The vagal, glossopharyngeal and hypoglossal brainstem nuclei are most frequently affected. Myastenia gravis, of which also an ocular form exists, is characterized by muscle weakness due to abnormal neuromuscular transmission. In general, neck and proximal limb muscles are most severely affected [Aramideh et al. 2002]. In several forms of mental retardation transmission is also affected. Neuron-neuron transmission can be altered due to a genetic defect. People with the fragile X syndrome have altered spine formation and as a result synaptic transmission is affected. Whether these people have aberrant blink behavior is not known.

Lesions

Brainstem lesions

Depending on the location of the lesion abnormalities in reflex blinking might occur (fig. 10). Lesions can be caused by a traumatic event but also by pathological conditions like ischemic infarctions, hemorrhages, tumors or multiple sclerosis (MS). In MS the electrically evoked, photic and corneal blink reflexes can be affected. Kimura (1975) found that the R1 is abolished in 40% of the patients tested that did not display clinical signs of brainstem damage. The corneal reflex can also be affected by a tumor in the vicinity of the cerebellopontine angle. Loss of this sensitive reflex often precedes further manifestation of trigeminal nerve damage [Hart 1992]. Most people with Wallenberg's syndrome, which affects the lateral medulla, have an abnormal R2 and normal R1. Abnormalities of blink responses can however vary according to how rostrally or caudally the lesion is located in the lateral medulla [Aramideh et al. 2002].

Lesions in higher order brain areas

Hemispheric lesions have been found to alter some components of the corneal reflex blink. After a stroke a transient delay in the R1 can occur. During chronic states changes in R2 may remain longer. In hemispheric disorders, corneal and late blink reflex responses may be absent or diminished bilaterally when the face is stimulated.

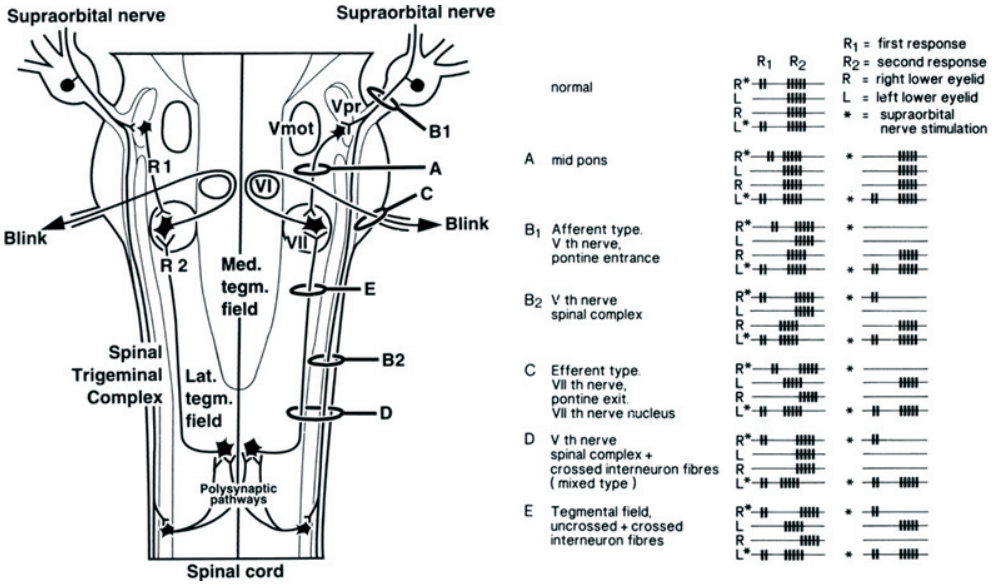


Figure 10. Various possible brainstem lesions in humans and their consequences on the R1 and R2 of the orbicularis oculi muscle. On the left: Schematic representation of the location of the lesions. On the right: abnormalities in blink reflex responses after lesions A-E in the right (R) and left (L) OO muscles after stimulation (*) of the supra-orbital nerves. Blink reflexes are either delayed (left column) or absent (right column). Abbreviations: abducens nucleus (VI), facial nucleus (VII), principal trigeminal nucleus (Vpr), trigeminal motor nucleus (Vmot), lateral tegmental field (Lat. Tegm. Field), medial tegmental field (Med.Tegm. field) [Aramideh et al. 1997].

Large cortical areas influence the blink reflex but the biggest influence is likely exerted from the lower postcentral area corresponding to the sensory area of the face. A hemispheric infarction can cause hemiplegia. If the representative area of the face in the motor cortex of hemiplegia patients is activated with transcranial magnetic stimulation, delayed or no motor potentials are measured. Contrary to these inhibitions of reflex blinking, lesions in the pyramidal tract may facilitate the R1 on the paretic side [Aramideh et al. 2002]. Lesions of the putamen cause ipsilateral deviation of the eyes because descending projections from the frontal eyefield are disrupted [Burst 2000].

Movement disorders

Eyelid movement disorders can affect the blink rate, eyelid opening or eyelid closure. The blink rate can for example be reduced to 0 - 3 blinks per minute in patients with Steele-Richardson-Olszewski syndrome or increase to over 50 blinks per minute in cranial dystonia patients. Patients with apraxia of eyelid opening have difficulty opening their eyes on command and can often not keep their eyes open for prolonged periods of time, which is caused by involuntary inhibition of the LPS muscle. Apraxia of eyelid opening can occur simultaneously with blepharospasm. Blepharospasm is a focal dystonia of the eyelids. Symptoms include tremulous, phasic and/or clonic discharges in the OO muscle. Blepharospasm may occur singularly or as part of a spectrum of dystonic movements. When

for instance lower facial or neck muscles are involved, the disorder is referred to as Meige's syndrome [Jordan et al. 1989]. Eekhof et al. (2001) showed that, at least during spasm-free periods, the afferent and efferent pathways of the reflex arc as well as suprasegmental control of the reflex are working properly. Quarterone and co-workers (2006) recently suggested that blepharospasm is caused by abnormal plasticity in the neuronal circuit mediating the trigeminal blink reflex.

Difficulty with eyelid opening is also induced by orbicularis oculi motor persistence. This abnormality is linked to an inability to suppress the activity of the orbicularis oculi muscle. Patients with apraxia of eyelid closure experience problems with voluntary eyelid closure. This condition is often associated with parietal lobe lesions [Aramideh et al. 2002].

Blink responses with short latencies, like the R1, are relatively mildly affected by extrapyramidal disorders. Responses with longer latencies involve more synapses and are under stronger suprasegmental influence and more likely to be altered by changed excitability of the interneurons. Parkinson's disease, probably the most famous example of an extrapyramidal disorder, is characterized by enhanced excitability of brainstem interneurons. As a consequence the recovery time after a blink is much shorter for these patients and the blink rate is increased [Korosec et al. 2006]. This phenomenon is also seen in several parkinsonian disorders, such as progressive supranuclear palsy, striatonigral degeneration and multiple system atrophy. Progressive supranuclear palsy is somewhat different as the somatosensory reflex is also abolished in these patients. Patients have a limited range of voluntary downward gaze, but have no problems with their reflexes which rely on lower eye movement areas. However, the acoustic reflex is abnormally reduced in progressive nuclear palsy patients and is delayed in patients with Parkinson's disease, but is normal in patients with multiple systems atrophy and striatonigral degeneration. In Parkinson's disease patient's prepulse inhibition with auditory stimuli is less effective whereas for somatosensory prepulses no abnormalities are observed [Aramideh et al. 2002].

Gilles de la Tourette syndrome also affects eyelid movement. This stress-sensitive neurologic disorder can induce a range of vocal and motor tics (involuntary movements). Tics that affect the eyelids include excessive blinking, squinting and exaggerated eyelid opening and closing [Berardelli et al. 2003, Kovacich 2008].

Another well-known movement disorder is Huntington's disease. As a consequence of degeneration of the striatal cells, that have inhibitory projections into the globus pallidus, blinking is affected. For instance the R2 latency is increased [Aramideh et al. 2002].

Finally, the spinocerebellar ataxias are a group of inheritable autosomal dominant neurodegenerative diseases, which are characterized by dysfunction and atrophy in the cerebellum and often brainstem along with additional pathological foci throughout the brain [Manto 2005]. In this patient group we recently found large delays and high variability in the onset of reflex blinks.

1.6 SCOPE OF THE THESIS

This thesis revolves around the neuronal circuitry of blinking and several of the pathologies that affect it. Despite the seemingly extensive knowledge on blink behavior, anatomy, pathways, physiology and pathology, as described in the previous sections, many questions remain.

Numerous projections have been revealed in the blink circuits, nonetheless the overview of the circuits is far from complete. For instance, although physiological evidence indicates the presence of a blink generator which should initiate the eye and eyelid movement during blinking in the reticular formation, the exact location of the generator is still not known. The reticular formation is an interesting structure as projections from many brain areas cross, converge and integrate in this area. Areas in the lateral reticular formation project to the eyelid movement-facilitating facial motor nucleus, but not to any structures involved in eye movement. On the other hand, specific areas in the mesencephalic reticular formation and paramedian pontine reticular formation project only to the eye movement-facilitating oculomotor nuclei. Within the midbrain, the superior colliculus is involved in different types of eye movements. Physiological experiments have also shown superior colliculus activity during blinking and indicate that a pathway through the superior colliculus might exist. Thus the question is whether blink-related projections from the reticular formation to the superior colliculus exist and whether these projections might be part of a possible blink generator. In Part II anatomical circuitries underlying blinking are described and extended with new findings on the location of common neuronal structures generating and coordinating eye and eyelid movement during blinking.

Mental retardation principally acts on the whole central nervous system, as is demonstrated in people with fragile X syndrome who lack the expression of the fragile X mental retardation protein. The consequential effect on cerebral learning and memory is clear; It is unclear to what extent cerebellar functioning is disturbed in the fragile X syndrome. Would cerebellar learning be aberrant in these people or are there compensatory mechanisms through which this learning process can still occur as in unaffected subjects? Part III discusses cerebellar dysfunction and blinking for which the fragile X syndrome is used as a model. Their ability for cerebellar-dependent associative learning and unlearning is described and compared to control subjects.

A disease which often affects the peripheral nervous system directly and the central nervous system indirectly is hemi-facial paralysis. Over 50% of the hemi-facial paralysis cases are idiopathic, this disorder is also named Bell's palsy. Bell's palsy patients suffer from a sudden loss of facial nerve function possibly combined with additional alteration in other cranial nerves. Most patients and especially the severely affected patients never completely recover. The cause of the disease and the incomplete recovery has not yet been elucidated. Whether the peripheral or central nervous system is the mayor player during the recovery of Bell's palsy also remains a question. Possible changes during recovery in cortical areas involved in blinking might provide answers. Part IV focuses on the slow and incomplete recovery of eye and eyelid movement of Bell's palsy patients. Changes in the motor cortex, sensory cortex and cerebellum in the neuronal activation induced by lip pursing and blinking as well as eye and eyelid kinematics are monitored in a longitudinal study of this patient group.

In Part V the main findings of the previous chapters are reviewed and discussed, and some recommendations for future research are given.

1.7 METHODOLOGY

Multiple neuroanatomical, neurophysiological and neurobehavioral approaches were used to address the questions raised in this thesis. Neuroanatomy is the study of neuronal tissues and structures and the relations between these entities. Neurophysiology studies the functions of living organisms or any of its parts at the system level. Behavioral studies are employed to deduct functional properties in in vivo systems from their behavior.

In order to investigate the topics that are relevant for this thesis we employed several techniques; With neuronal tracing we studied the neuroanatomy underlying the blink reflex. We performed classical eyeblink conditioning experiments to study associative motor learning in people with the fragile X syndrome. Using the magnetic distance measurement technique we were able to monitor learning abilities and motor control by registering the eyelid responses. In order to follow the re-innervation of facial muscles in Bell's palsy patients, electromyographic (EMG) measurements and a Remmel setup were employed to reveal the precise muscle activity and eyelid movements of Bell's palsy patients during blinks in a longitudinal study. Functional magnetic resonance imaging (fMRI) was used to record influences of facial recovery on cortical areas.

Neuroanatomy

The lines of communication between subnuclei within the brain have been studied at length. The first neuro-anatomical studies were purely descriptive. Later, lesion studies were introduced by the English neurophysiologist Augustus Volney Waller. Wallerian degeneration, named after him, is degeneration of the distal segment of a peripheral axon that has been lesioned, without local inflammation [Waller 1850]. In 1892 retrograde nerve degeneration was demonstrated in the facial nucleus after transection of the nerve by Nissl. Methylene blue was introduced in the same paper as a stain for chromatin material. Thereafter Wallerian degeneration became a popular technique applied to study oculomotor projections by looking at degenerated nerve endings after a precise lesion using light and later electron microscopes [Warwick 1950, Ruskell 1970, 1974, 1976]. Adamük (1870), and Henderson and Völckers (1878) were the first to use intracranial electrical stimulation of the hindbrain of dogs to study the functional organization of the oculomotor complex. The most famous example is probably the electrophysiological experiments of Penfield and Rasmussen (1950) in which they studied the motor and sensory cortex somatotopy creating the homunculi. More recently patch clamping and imaging techniques like CT, PET, EEG, MEG and MRI have been added to the list of possible neuro-anatomical techniques as well. However, for the past 25 years anatomical studies on neuronal projections and circuits were predominantly tracing studies [Vercelli et al. 2000]. Classical neuronal tracing techniques allowed researchers to inject into a site and then follow the projections. Depending on their characteristics, tracer substances can be transported over the axons either anterogradely (toward the axon terminal) or retrogradely (toward the cell body) or both. Several examples are listed in table 4. Horseradish peroxidase is the most widely used retrograde tracer. After uptake these tracers remain within the neuron. Transneuronal tracers, often viruses like rabies or herpes, are capable of crossing synapses thereby identifying neuronal networks. The main disadvantage of retrograde viral tracers is that transsynaptic transport occurs in all directions. As a consequence the investigated neuronal

circuitry is masked by the large amount of aspecifically labeled branches. Tracer is visualized with immunohistochemical procedures, immunolabeling or autoradiography [Oztas 2003]. Often tracer injections are combined with other techniques. For instance with expression of the immediate early gene c-Fos, and its protein Fos, which were introduced in 1988 as tools for determining activity changes within neurons [Sagar et al. 1988, Hoffman et al. 2002].

Table 4. Examples of methods and materials used for neuronal tracing [Oztas 2003].

Method	Material
Anterograde tracing	
Machi method	OsO4 after tract of nerve lesion
Nauta Method - Fink Heimer Method	Reduced Silver Method after tract lesion
Autoradiography with Radiolabelled Amino Acid	Tritiated Glycine / Leucine
Anterograde Tracer	Phageolus Vulgaris Leucoagglutinin (PHA-L), BDA
Retrograde tracing	
Nissl Reaction	Methylene blue, Toluidine blue, Thionin, Cresyl violet
Retrograde Tracer	Horseradish Peroxidase (HRP), Wheat Germ Agglutinin (WGA)
Fluorescence Tracer	Lucifer Yellow, Fast Blue, Nuclear Yellow
Viruses and Toxoids	Herpes Simplex Virus, Adeno Virus, Pseudo-rabies, Cholera toxin-B chain (CTB), Tetanus, Lentivirus

Neurophysiology

Physiology, including studies on eyelid behavior, employs a broad range of techniques which have changed throughout the years. The British physician Overend (1896) was the first to study a blink reflex by tapping on one side of the forehead. It was Kugelberg (1952) that performed the first electromyographic study of the blink induced by glabellar tapping. He recognized an early ipsilateral response (R1) and late bilateral response (R2). Ten years later Rushworth (1962) started to employ supra-orbital nerve stimulation to evoke reflex blinks. In 1969, Kimura published an article on the orbicularis oculi response to supra-orbital nerve stimulation which description is still today's standard for supra-orbital nerve stimulation studies [Kimura 1969].

Electromyography

Ocular electromyography (EMG) was introduced by Björk and Kugelberg (1953) as a technique with clinical applications in man. EMG is an electrical registration of muscle activity. Recordings are made with small surface electrodes or needle electrodes. EMG recordings of the orbicularis oculi (OO-EMG) and levator superior (LPS-EMG) muscles give information about timing and intensity of muscle activity and indirectly on eyelid movement. Onset latencies can give information on conductance in the circuitries and the number of neurons involved. Aramideh and co-workers (1994) showed that the orbicularis oculi reflex can be examined together with inhibitory reflex of the LPS muscle. The

combination of supra-orbital stimulation and OO-EMG gives information on the workings of the entire facial nerve, whereas other methods like determining nerve excitability or terminal latency only evaluate the status of the distal end (segment distal to stylomastoid foramen) of the nerve. This is a disadvantage when trying to diagnose facial palsy patients [Kimura 1969].

Behavioral studies

Search coils in a double magnetic field

This equipment is used for monitoring eye movements. Ronald S. Remmel (1984) adapted the setup from the design of Robinson (1963). It consists of two Helmholtz field coils, creating a double magnetic field, developed by Collewijn and co-workers (1975), which is used to detect scleral search coil motion. A scleral search coil is a coiled wire that is either incorporated in a contact lens, as is used in studies with humans, or implanted surgically around the eye. This coil provides high resolution measurements, linearity, low signal drift and has low noise compared to techniques using electro-oculogram recordings or light reflecting from different parts of the eye which it replaced. During measurements subjects are placed in a horizontal and vertical magnetic field oscillating at two different frequencies. The two frequencies allow separation of vertical and horizontal coil motion. The scleral coil acts as a transformer of magnetic to electrical energy. A voltage is induced in the scleral coil by the magnetic flux that traverses the coil. This voltage is proportional to the sine of the angle between the axis of the scleral coil and the axis of the fields. For angles up to 30° this relationship is linear. Eyelid movement can be calculated by combining the horizontal and vertical displacement data. This accurate and inexpensive technique contributed greatly to eye movement research.

Magnetic Distance Measurement Technique

OO-EMG is the standard technique for measuring eyelid movement. Recently a new blink registration technique has been developed. The magnetic distance measurement technique (MDMT) was created to enable eyelid measurements in mice and was later adapted for use in humans [Koekkoek et al. 2002]. The MDMT directly measures the actual eyelid displacement using a field-sensitive chip or receiver coil (sensor) to detect motion of a local magnetic field generated by a magnet or second transmitter coil. In mice the sensor is attached to a pedestal mounted on the head, placing the sensor adjacent to the upper eyelid and a magnet is placed on the lower eyelid or a receiver coil is implanted in the upper eyelid. For studies with human subjects the sensor is attached to the lower orbit of the eye and a NIB (neodymium, iron, boron) magnet is fixed close to the lashes of the upper eyelid. With this technique it is possible to record eyelid movement with a high spatiotemporal resolution in freely moving subjects without artefacts like aspecific muscle activation (which can be a problem during EMG measurements in mice) or restraint-induced experimental stress. The MDMT technique is very suitable for eyelid movement registrations during eyelid conditioning.

Conditioning

Ever since Pavlov noticed his drooling dogs, behavioral conditioning has been used to experimentally study associative or adaptive learning. It was little over a hundred years ago that Pavlov and also Thorndike performed the early studies on learning. In a typical classical eyeblink conditioning paradigm, a subject is trained with an innocuous stimulus, such as a tone and a biologically significant stimulus

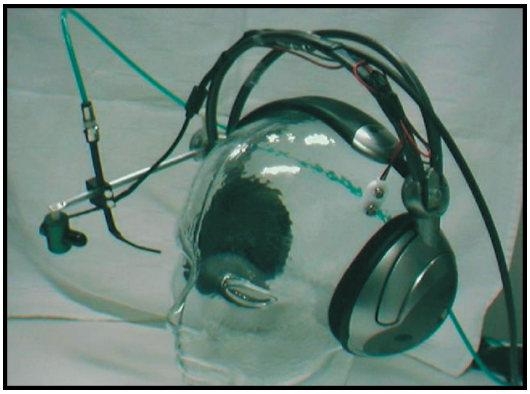


Figure 11. Headphone with a camera and air-puff-tube used for eyeblink conditioning.

such as a weak electric shock to the supra-orbital nerve or air puff on the cornea. Such a significant stimulus, usually referred to as the unconditioned stimulus (US), elicits a reflex blink or unconditioned response (UR). During conditioning subjects are exposed to a US presented together with the tone or conditioned stimulus (CS). As US-CS pairings continue, the CS becomes capable of eliciting a blink in the absence of a US. This response is a conditioned response (CR). A second type of conditioning is operant or instrumental conditioning. Like classical conditioning a US is paired with a CS, however contrary to the classical conditioning UR, an operant conditioning UR is not automatically elicited but reinforced by positive or negative consequences to voluntary behavior. A subject learns to expect a consequence to a certain behavior. For instance, a rat can learn to turn left in a T-maze (voluntary behavior) for a food reward (consequence) [Mowrer and Klein 2001].

In conditioning two more important phenomena exist; saving and extinction. Saving is retrieval of a previously acquired conditioned response, displayed in relearning faster than in the first training session [Medina et al. 2001]. Extinction is the result of continued exposure to the CS until the CR ceases to occur. There is an ongoing debate whether this is mediated through new learning or unlearning [Mauk and Ohyama 2004]. The conditioning experiments were performed with the headphone in figure 11. An air-puff-tube and camera are attached to the headphone for air-puff stimuli and eyelid recordings, respectively.

Functional Magnetic Resonance Imaging

Nowadays neuroimaging is indispensable in both diagnostic and research studies of the brain. Until the early seventies only conventional x-ray techniques, with rather low soft tissue resolution and harmful properties, were available. New techniques made their entrance among which nuclear magnetic resonance (NMR). The first successful NMR experiments were made in 1946 by Felix Bloch and Edward Purcell. In 1984 the name NMR was changed into magnetic resonance imaging (MRI) [no authors listed 1984] in order to improve marketing after which the technique became widely available for diagnostic purposes (see box 3 for a short explanation of the MRI technique). In the Science Museum in London an early example of a MRI scanner with examples of the first head coils are exhibited (fig. 12). The (functional) MRI scanner used for the studies described in this thesis is shown next to the old scanner.

Figure 12. An old MRI-scanner is part of the collection of the science museum in London (left). In the background primitive coils can be seen used for brain MRI scans. These coils made at London's Hammersmith hospital were mounted on bicycle helmets and got the nick-name 'jedi' helmets. On the right the (functional) MRI scanner used to gather data for this thesis is shown.

Box 3

Magnetic resonance imaging

MRI images are constructed with a static magnetic field around the subject to which nuclei of hydrogen atoms within a subject will align. A radio frequency pulse is used to excite the protons, i.e. tipping them out the alignment to the main magnetic field. The weak second gradient magnetic field in the length of the scanner (z axis) will only allow a specific slice of protons to fall back according to their place within the gradient after removal of the radio frequency. During the return to the alignment to the main magnetic field, or relaxation, the protons emit energy which is captured by coils placed close to the subject. The location of protons within the 2D slice can be determined with the use of two more gradients along the x and y axis affecting the spin frequency and phase of the spinning respectively. A series of these 2D slices can be combined into a 3D volume.

Functional magnetic resonance imaging (fMRI) is a relatively new development in the still young magnetic resonance imaging field. The first fMRI scans were performed in 1990 [Ogawa et al. 1990a,b, Turner et al. 1991]. Exactly 100 years earlier Charles Sherrington demonstrated that stimulation of the brain caused local increase in blood flow and that the relative oxygen uptake from this blood was reduced. FMRI makes use of the local change in the ratio between paramagnetic deoxyhemoglobin and diamagnetic oxyhemoglobin also called blood oxygenation level. Activation of the brain as a result of specific tasks performed while subjects are scanned can be measured in this fashion. The measured response is the blood oxygenation level-dependent (BOLD) contrast. This is an indirect measure to estimate neuronal activation. The activity measured during a rest condition is subtracted from the activity measured during performance of tasks which leaves task-specific local increases in the BOLD response indicating increased activity. Instead of this indirect measurement it is also possible to use direct imaging of the blood flow using perfusion fMRI, but this is not widely used due to the low sensitivity of this technique [Jezzard 1998].

The future prospects of this fast-developing non–invasive imaging technique are very promising as it provides a view on the in vivo workings of the brain previously impossible, especially in combination with techniques with a high temporal resolution like MEG and EEG to complement the high spatial resolution of the fMRI technique.

REFERENCES

Adour, K. K., D. N. Bell, et al. (1975). Herpes simplex virus in idiopathic facial paralysis (Bell palsy). JAMA 233(6): 527-30.

Aramideh, M., B. W. Ongerboer de Visser, et al. (1994). Electromyographic features of levator palpebrae superioris and orbicularis oculi muscles in blepharospasm. Brain 117 (Pt 1): 27-38.

Aramideh, M., J. L. Eekhof, et al. (1995). Electromyography and recovery of the blink reflex in involuntary eyelid closure: a comparative study. J Neurol Neurosurg Psychiatry 58(6): 692-8.

Aramideh, M., B. W. Ongerboer de Visser , et al. (1997). The late blink reflex response abnormality due to lesion of the lateral tegmental field. Brain 120 (Pt 9): 1685-92.

Aramideh M., V.-S. J., Cruccu G, Ongerboer de Visser BW (2002). Disorders of the cranial nerves. Neuromuscular function and disease. B. C. Brown WF, Aminoff MJ. Philadelphia, WB Saunders: 433-453, 757-780.

Attwell, P.J., M. Ivarsson, et al. (2002). Cerebellar mechanisms in eyeblink conditioning. Ann N Y Acad Sci 978:79-92.

Baker, R.S., A.H. Andersen, et al. (2003). A functional magnetic resonance imaging study in patients with benign essential blepharospasm. J Neuroophthalmol 23(1):11-5.

Barbato, G., G. Ficca, et al. (2000). Diurnal variation in spontaneous eye-blink rate. Psychiatry Res 93(2): 145-51.

Basso, M. A., A. S. Powers, et al. (1996). An explanation for reflex blink hyperexcitability in Parkinson's disease. I. Superior colliculus. J Neurosci 16(22): 7308-17.

Benatar, M. and J. Edlow (2004). The spectrum of cranial neuropathy in patients with Bell's palsy. Arch Intern Med 164(21): 2383-5.

Berardelli A., A. Currà, et al. (2003). Pathophysiology of tics and Tourette syndrome. J Neurol. 250(7): 781-7. Review.

Bhatti, M. T. and R. Patel (2005). Neuro-ophthalmic considerations in trigeminal neuralgia and its surgical treatment. Curr Opin Ophthalmol 16(6): 334-40.

Bour, L. J., M. Aramideh, et al. (2000). Neurophysiological aspects of eye and eyelid movements during blinking in humans. J Neurophysiol 83(1): 166-76.

Brodal, A. (1981). Neurological Anatomy. New York, Oxford University Press.

Brookings, J. B., G. F. Wilson, et al. (1996). Psychophysiological responses to changes in workload during simulated air traffic control. Biol Psychol 42(3): 361-77.

Brown P., J. C. Rothwell, et al. (1991) New observations on the normal auditory startle reflex in man. Brain 114 (Pt 4): 1891-902.

Burde R. M., Feldon. S. (1992). The extraocular muscles. Adler's physiology of the eye. J. W. M. Hart: 101-197.

Burr, D. (2005). Vision: in the blink of an eye. Curr Biol 15(14): R554-6.

Burst, J. (2000). Circulation of the brain. Principles of neural science. E. Kandel, Schwartz, JH., Jessel, TM., McGraw-Hill: 1302-1316.

Büttner, U., J. Büttner-Ennever (2006). Present concepts of oculomotor organization. Progress in brain research: Neuroanatomy of the oculomotor system. B.-E. JA. Amsterdam, Elsevier. 151: 1-42.

Büttner-Ennever, J. (2006). The extraocular motor nuclei: organization and functional neuroanatomy. Neuroanatomy of the oculomotor system. B.-E. JA. amsterdam. 151: 95-126.

Buzzi, M. G. and M. A. Moskowitz (2005). The pathophysiology of migraine: year 2005. J Headache Pain 6(3): 105-11.

Calleja, J., A. Garcia, et al. (1998). [Miller-Fisher syndrome: electrophysiological serial study of five patients]. Rev Neurol 27(155): 60-4.

Christian, K.M. and R.F. Thompson (2005). Long-term storage of an associative memory trace in the cerebellum. Behav Neurosci. 119(2):526-37.

Collewijn, H., F. van der Mark, et al. (1975). Precise recording of human eye movements. Vision Res 15(3): 447-50.

Collewijn, H., J. van der Steen, et al. (1985). Human eye movements associated with blinks and prolonged eyelid closure. J Neurophysiol 54(1): 11-27.

Cruccu, G., R. Agostino, et al. (1998). Mandibular nerve involvement in diabetic polyneuropathy and chronic inflammatory demyelinating polyneuropathy. Muscle Nerve 21(12): 1673-9.

Cruccu, G., G.D. Iannetti (2005). Brainstem reflex circuits revisited. Brain 2005 Feb;128(Pt 2):386-94.

Dartt, D. A. (2004). Control of mucin production by ocular surface epithelial cells. Exp Eye Res 78(2): 173-85.

Dimitrova, A., Weber, J., et al. (2002). Eyeblink-related areas in human cerebellum as shown by fMRI. Human brain mapping 17, 100-115.

Domingo, J. A., Gruart A., et al. (1997). Quantal organization of reflex and conditioned eyelid responses. J Neurophysiol 78(5): 2518-30.

Eekhof, J. L., M. Aramideh, et al. (2001). Orbicularis oculi and orbicularis oris reflexes in blepharospasm and torticollis spasmodica during spasm-free intervals. Eur Neurol 46(2): 75-8.

Ellrich, J., B. Bromm, et al. (1997). Pain-evoked blink reflex. Muscle Nerve 20(3): 265-70.

Ellrich, J. and H. C. Hopf (1996). The R3 component of the blink reflex: normative data and application in spinal lesions. Electroencephalogr Clin Neurophysiol 101(4): 349-54.

Esteban, A. (1999). A neurophysiological approach to brainstem reflexes. Blink reflex. Neurophysiol Clin 29(1): 7-38.

Esteban, A., A. Traba, et al. (2004). Eyelid movements in health and disease. The supranuclear impairment of the palpebral motility. Neurophysiol Clin 34(1): 3-15.

Esteban-Garcia, A., E. Salinero-Paniagua, et al. (2004). [Hereditary sensory and autonomic neuropathies. The neurophysiological and pathological aspects of two cases with congenital insensitivity to pain]. Rev Neurol 39(6): 525-9.

Evinger, C. and K. A. Manning (1993). Pattern of extraocular muscle activation during reflex blinking. Exp Brain Res 92(3): 502-6.

Evinger, C., K. A. Manning, et al. (1994). Not looking while leaping: the linkage of blinking and saccadic gaze shifts. Exp Brain Res 100(2): 337-44.

Evinger, C., K. A. Manning, et al. (1991). Eyelid movements. Mechanisms and normal data. Invest Ophthalmol Vis Sci 32(2): 387-400.

Evinger, C. and J. S. Perlmutter (2003). Blind men and blinking elephants. Neurology 60(11): 1732-3.

Evinger, C., M. D. Shaw, et al. (1984). Blinking and associated eye movements in humans, guinea pigs, and rabbits. J Neurophysiol 52(2): 323-39.

Finlay, B. L., S. E. Schneps, et al. (1978). Topography of visual and somatosensory projections to the superior colliculus of the golden hamster. Brain Res 142(2): 223-35.

Freed, W. J., C. N. Karson, et al. (1981). Increased spontaneous eye-blinks cerebellectomized rats. Biol Psychiatry 16(8): 789-92.

Fuchs, A. F., W. Becker, et al. (1992). Discharge patterns of levator palpebrae superioris motoneurons during vertical lid and eye movements in the monkey. J Neurophysiol 68(1): 233-43.

Gong, S., Q. Zhou, et al. (2003). Blink-related sensorimotor anatomy in the rat. Anat Embryol (Berl) 207(3): 193-208.

Goossens, H. H. and A. J. Van Opstal (2000). Blink-perturbed saccades in monkey. II. Superior colliculus activity. J Neurophysiol 83(6): 3430-52.

Goossens, H. H. and A. J. Van Opstal (2000). Blink-perturbed saccades in monkey. I. Behavioral analysis. J Neurophysiol 83(6): 3411-29.

Gordon, G. (1951). Observations upon the movements of the eyelids. Br J Ophthalmol 35(6):339-51.

Grinevich, V., M. Brecht, et al. (2005). Monosynaptic pathway from rat vibrissa motor cortex to facial motor neurons revealed by lentivirus-based axonal tracing. J Neurosci 25(36): 8250-8.

Gruart, A., B. G. Schreurs, et al. (2000). Kinetic and frequency-domain properties of reflex and conditioned eyelid responses in the rabbit. J Neurophysiol 83(2): 836-52.

Hall, A., (1945). The origin and purposes of blinking. Br J Ophtalmol 29: 445.

Hart, W. (1992). The eyelids. Adler's physiology of the eye. W. Hart: 1-17.

Haslwanter, T., M. Buchberger, et al. (2005). SEE++: a biomechanical model of the oculomotor plant. Ann N Y Acad Sci 1039: 9-14.

Henriquez, V. M. and C. Evinger (2005). Modification of cornea-evoked reflex blinks in rats. Exp Brain Res 163(4): 445-56.

Hiraoka, M. and M. Shimamura (1977). Neural mechanisms of the corneal blinking reflex in cats. Brain Res 125(2): 265-75.

Hoffman, G. E. and D. Lyo (2002). Anatomical markers of activity in neuroendocrine systems: are we all 'fos-ed out'? J Neuroendocrinol 14(4): 259-68.

Holstege, G., J. Tan, et al. (1986). Anatomical observations on the afferent projections to the retractor bulbi motoneuronal cell group and other pathways possibly related to the blink reflex in the cat. Brain Res 374(2): 321-34.

Holstege, G., J. J. van Ham, et al. (1986). Afferent projections to the orbicularis oculi motoneuronal cell group. An autoradiographical tracing study in the cat. Brain Res 374(2): 306-20.

Hori J., Sakano K., et al. Development of communication supporting device controlled by eye movements and voluntary eye blink. Conf Proc IEEE Eng Med Biol Soc. 2004;6:4302-5.

Hughes, R. A. and D. R. Cornblath (2005). Guillain-Barre syndrome. Lancet 366(9497): 1653-66.

Ishpekova, B. A., L. G. Christova, et al. (2005). The electrophysiological profile of hereditary motor and sensory neuropathy-Lom. J Neurol Neurosurg Psychiatry 76(6): 875-8.

Ito, M. (2002). Historical review of the significance of the cerebellum and the role of Purkinje cells in motor learning. Ann N Y Acad Sci 978:273-88.

Iwanaga, K., S. Saito, et al. (2000). The effect of mental loads on muscle tension, blood pressure and blink rate. J Physiol Anthropol Appl Human Sci 19(3): 135-41.

Jacquin, M. F., W. E. Renehan, et al. (1993). Morphology and topography of identified primary afferents in trigeminal subnuclei principalis and oralis. J Neurophysiol 70(5): 1911-36.

Jamal, G. A. and J. P. Ballantyne (1988). The localization of the lesion in patients with acute ophthalmoplegia, ataxia and areflexia (Miller Fisher syndrome). A serial multimodal neurophysiological study. Brain 111 (Pt 1): 95-114.

Jenny, A. B. and C. B. Saper (1987). Organization of the facial nucleus and corticofacial projection in the monkey: a reconsideration of the upper motor neuron facial palsy. Neurology 37(6): 930-9.

Jezzard, P. (1998). Advances in perfusion MR imaging. Radiology 208(2): 296-9.

Jordan, D.R., J.R. Patrinely, et al. (1989). Essential blepharospasm and related dystonias. Surv Ophthalmol 34(2):123-32.

Kanaseki, T. and J. M. Sprague (1974). Anatomical organization of pretectal nuclei and tectal laminae in the cat. J Comp Neurol 158(3): 319-37.

Karson, C. N. (1988). Physiology of normal and abnormal blinking. Adv Neurol 49: 25-37.

Karson, C. N., K. F. Berman, et al. (1981). Speaking, thinking, and blinking. Psychiatry Res 5(3): 243-6.

Karson, C. N., R. A. Staub, et al. (1981). Drug effect on blink rates in rhesus monkeys: preliminary studies. Biol Psychiatry 16(3): 249-54.

Kassem, I. S. and Evinger, C. (2006). Asymmetry of blinking. Invest Ophthalmol Vis Sci 47(1): 195-201.

Kazem, S. S. and D. Behzad (2006). Role of Blink Reflex in Diagnosis of Subclinical Cranial Neuropathy in Diabetic Mellitus Type II. Am J Phys Med Rehabil 85(5): 449-52.

Killackey, H. P. and R. S. Erzurumlu (1981). Trigeminal projections to the superior colliculus of the rat. J Comp Neurol 201(2): 221-42.

Kimaid, P. A., L. A. Resende, et al. (2002). Blink reflex: comparison of latency measurements in different human races. Arq Neuropsiquiatr 60(3-A): 563-5.

Kimura, J., J. M. Powers, et al. (1969). Reflex response of orbicularis oculi muscle to supraorbital nerve stimulation. Study in normal subjects and in peripheral facial paresis. Arch Neurol 21(2): 193-9.

Kimura, J. (1975). Electrically elicited blink reflex in diagnosis of multiple sclerosis. Review of 260 patients over a seven-year period. Brain 98(3): 413-26.

Kitazawa, S. (2002). Neurobiology: ready to unlearn. Nature 416(6878): 270-3.

Kleim, J.A., J.H. Freeman, et al. (2002). Synapse formation is associated with memory storage in the cerebellum. Proc Natl Acad Sci U S A 99(20):13228-31.

Koch, M. and H. U. Schnitzler (1997). The acoustic startle response in rats--circuits mediating evocation, inhibition and potentiation. Behav Brain Res 89(1-2): 35-49.

Koekkoek, S. K., W. L. Den Ouden, et al. (2002). Monitoring kinetic and frequency-domain properties of eyelid responses in mice with magnetic distance measurement technique. J Neurophysiol 88(4): 2124-33.

Korosec, M., I. Zidar, et al. (2006). Eyelid movements during blinking in patients with Parkinson's disease. Mov Disord 21(8): 1248-51.

Kugelberg, E. (1952). [Facial reflexes.]. Brain 75(3): 385-96.

Kovacich, S. (2008). Tourette syndrome and the eye. Optometry 79(8): 432-5.

Krakauer, J.W. and R. Shadmehr (2006). Consolidation of motor memory. Trends Neurosci 29(1):58-64.

Kugelberg, E. (1953). Clinical electromyography. Prog Neurol Psychiatry 8: 264-82.

Lander, T., J. D. Wirtschafter, et al. (1996). Orbicularis oculi muscle fibers are relatively short and heterogeneous in length. Invest Ophthalmol Vis Sci 37(9): 1732-9.

Lavond, D.G. (2002). Role of the nuclei in eyeblink conditioning. Ann N Y Acad Sci 978:93-105.

Lawrenson, J. G., R. Birhah, et al. (2005). Tear-film lipid layer morphology and corneal sensation in the development of blinking in neonates and infants. J Anat 206(3): 265-70.

Leigh, R.J. and Zee D.S. (1999). The Neurology of eye movements. 3rd ED, New York, Oxford University Press.

Lemp MA, Wolfley. D. (1992). The lacrimal apparatus. Adler's physiology of the eye. St. Louis, Mosby Year Book.

Manto MU (2005). The wide spectrum of spinocerebellar ataxias (SCAs). Cerebellum 4(1): 2-6.

Manzoni, G. C. and P. Torelli (2005). Epidemiology of typical and atypical craniofacial neuralgias. Neurol Sci 26 Suppl 2: s65-7.

Marfurt, C. F. (1981). The somatotopic organization of the cat trigeminal ganglion as determined by the horseradish peroxidase technique. Anat Rec 201(1): 105-18.

Martin, M. R. and D. Lodge (1977). Morphology of the facial nucleus of the rat. Brain Res 123(1): 1-12.

Mauk, M. D. and T. Ohyama (2004). Extinction as new learning versus unlearning: considerations from a computer simulation of the cerebellum. Learn Mem 11(5): 566-71.

May, P. J. and J. D. Porter (1998). The distribution of primary afferent terminals from the eyelids of macaque monkeys. Exp Brain Res 123(4): 368-81.

Medina, J. F., K. S. Garcia, et al. (2001). A mechanism for savings in the cerebellum. J Neurosci 21(11): 4081-9.

Medina, J. F., W. L. Nores, et al. (2002). Inhibition of climbing fibres is a signal for the extinction of conditioned eyelid responses. Nature 416(6878): 330-3.

Miller, J. M. and D. A. Robinson (1984). A model of the mechanics of binocular alignment. Comput Biomed Res 17(5): 436-70.

Miwa, H., N. Imamura, et al. (1995). Somatosensory evoked blink response: findings in patients with Miller Fisher syndrome and in normal subjects. J Neurol Neurosurg Psychiatry 58(1): 95-9.

Mondelli, M., C. Romano, et al. (2002). Herpes zoster of the head and limbs: electroneuromyographic and clinical findings in 158 consecutive cases. Arch Phys Med Rehabil 83(9): 1215-21.

Morcuende, S., J. M. Delgado-Garcia, et al. (2002). Neuronal premotor networks involved in eyelid responses: retrograde transneuronal tracing with rabies virus from the orbicularis oculi muscle in the rat. J Neurosci 22(20): 8808-18.

Morecraft, R. J., J. L. Louie, et al. (2001). Cortical innervation of the facial nucleus in the non-human primate: a new interpretation of the effects of stroke and related subtotal brain trauma on the muscles of facial expression. Brain 124(Pt 1): 176-208.

Mowrer, R. R. and S. B. Klein (2001). Handbook of contemporary learning theories. Mahwah, New Jersey, Lawrence Erlbaum Associates, Inc.

Munoz, D. P. and J. H. Fecteau (2002). Vying for dominance: dynamic interactions control visual fixation and saccadic initiation in the superior colliculus. Prog Brain Res 140: 3-19.

Ndiaye, A., G. Pinganaud, et al. (2002). Organization of trigeminocollicular connections and their relations to the sensory innervation of the eyelids in the rat. J Comp Neurol 448(4): 373-87.

Nishimura, T. and K. Mori (1996). Blink reflex in meningomyelocele, with special reference to its usefulness in the evaluation of brainstem dysfunction. Childs Nerv Syst 12(1): 2-12.

Ogawa, S., T. M. Lee, et al. (1990). Brain magnetic resonance imaging with contrast dependent on blood oxygenation. Proc Natl Acad Sci U S A 87(24): 9868-72.

Ogawa, S., T. M. Lee, et al. (1990). Oxygenation-sensitive contrast in magnetic resonance image of rodent brain at high magnetic fields. Magn Reson Med 14(1): 68-78.

Overend, W. (1896). Preliminary note on a new cranial reflex. Lancet 1: 619.

Oztas, E. (2003). Neuronal tracing. Neuroanatomy 2: 2-5.

Panneton, W. M. and H. Burton (1981). Corneal and periocular representation within the trigeminal sensory complex in the cat studied with transganglionic transport of horseradish peroxidase. J Comp Neurol 199(3): 327-44.

Pellegrini, J. J., A. K. Horn, et al. (1995). The trigeminally evoked blink reflex. I. Neuronal circuits. Exp Brain Res 107(2): 166-80.

Penders, C. A. and P. J. Delwaide (1972). [Electrophysiological analysis of the blinking reflex in the normal human being and in clinical neurology]. Rev Electroencephalogr Neurophysiol Clin 2(3): 354-61.

Porter, J. D., R. S. Baker, et al. (1995). Extraocular muscles: basic and clinical aspects of structure and function. Surv Ophthalmol 39(6): 451-84.

Porter, J. D., L. A. Burns, et al. (1989). Morphological substrate for eyelid movements: innervation and structure of primate levator palpebrae superioris and orbicularis oculi muscles. J Comp Neurol 287(1): 64-81.

Ponder, E. and W.P. Kennedy (1928). On the act of blinking. Q J Exp Physiol 18: 89.

Porter, J. D. and K. F. Hauser (1993). Diversity and developmental regulation of extraocular muscle: progress and prospects. Acta Anat (Basel) 147(4): 197-206.

Quartarone, A., A. Sant'Angelo, et al. (2006). Enhanced long-term potentiation-like plasticity of the trigeminal blink reflex circuit in blepharospasm. J Neurosci 26(2): 716-21.

Raymond, J.L., S.G. Lisberger, et al. (1996). The cerebellum: a neuronal learning machine? Science 272(5265):1126-31.

Remmel, R. S. (1984). An inexpensive eye movement monitor using the scleral search coil technique. IEEE Trans Biomed Eng 31(4): 388-90.

Robinson, D. A. (1963). A Method of Measuring Eye Movement Using a Scleral Search Coil in a Magnetic Field. IEEE Trans Biomed Eng 10: 137-45.

Robleto, K., A. M. Poulos, et al. (2004). Brain mechanisms of extinction of the classically conditioned eyeblink response. Learn Mem 11(5): 517-24.

Rossi, B., R. Risaliti, et al. (1989). The R3 component of the blink reflex in man: a reflex response induced by activation of high threshold cutaneous afferents. Electroencephalogr Clin Neurophysiol 73(4): 334-40.

Ruiz-Padial, E., J. J. Sollers, 3rd, et al. (2003). The rhythm of the heart in the blink of an eye: emotion-modulated startle magnitude covaries with heart rate variability. Psychophysiology 40(2): 306-13.

Rushworth, G. (1962). Observations on blink reflexes. J Neurol Neurosurg Psychiatry 25: 93-108.

Ruskell, G. L. (1970). An ocular parasympathetic nerve pathway of facial nerve origin and its influence on intraocular pressure. Exp Eye Res 10(2): 319-30.

Ruskell, G. L. (1974). Ocular fibres of the maxillary nerve in monkeys. J Anat 118(Pt 2): 195-203.

Ruskell, G. L. (1976). The source of nerve fibres of the trabeculae and adjacent structures in monkey eyes. Exp Eye Res 23(4): 449-59.

Sagar, S. M., F. R. Sharp, et al. (1988). Expression of c-fos protein in brain: metabolic mapping at the cellular level. Science 240(4857): 1328-31.

Schmidtke, K. and J. A. Buttner-Ennever (1992). Nervous control of eyelid function. A review of clinical, experimental and pathological data. Brain 115 Pt 1: 227-47.

Schwartz, J.H. and Westbrook G.L. (2000). The cytology of neurons. In: Principles of neural science, 4/e Ch4 p 67-87 eds Kandel ER, Schwartz JH, Jessel TM printed at: McGraw-Hill.

Scudder, C. A., C. S. Kaneko, et al. (2002). The brainstem burst generator for saccadic eye movements: a modern synthesis. Exp Brain Res 142(4): 439-62.

Sparks, D. L. and R. Hartwich-Young (1989). The deep layers of the superior colliculus. Rev Oculomot Res 3: 213-55.

Spencer, R. Porter., JD. (2006). Biological organization of the extraocular muscles. Neuroanatomy of the oculomotor system. J. A. Buttner-Ennever. Amsterdam, Elsevier. 151: 43-80.

Stern, J. A., D. Boyer, et al. (1994). Blink rate: a possible measure of fatigue. Hum Factors 36(2): 285-97.

Sweeney, C. J. and D. H. Gilden (2001). Ramsay Hunt syndrome. J Neurol Neurosurg Psychiatry 71(2): 149-54.

Szatmáry, G. and R.J. Leigh (2002). Peripheral and central eye movement disorders. Curr Opin Neurol 15(1):45-50.

Tamai, Y., M. Iwamoto, et al. (1986). Pathway of the blink reflex in the brainstem of the cat: interneurons between the trigeminal nuclei and the facial nucleus. Brain Res 380(1): 19-25.

Tanaka, T., Y. Takeuchi, et al. (1978). Cells of origin of the spino-facial pathway in the cat: a horseradish peroxidase study. Brain Res 142(3): 580-5.

Taylor, J. R., J. D. Elsworth, et al. (1999). Spontaneous blink rates correlate with dopamine levels in the caudate nucleus of MPTP-treated monkeys. Exp Neurol 158(1): 214-20.

Turner, R., D. Le Bihan, et al. (1991). Echo-planar time course MRI of cat brain oxygenation changes. Magn Reson Med 22(1): 159-66.

Urushitani, M., F. Udaka, et al. (1995). Miller Fisher-Guillain-Barre overlap syndrome with enhancing lesions in the spinocerebellar tracts. J Neurol Neurosurg Psychiatry 58(2): 241-3.

Valls-Sole, J. (2002). Facial palsy, postparalytic facial syndrome, and hemifacial spasm. Mov Disord 17 Suppl 2: S49-52.

Valls-Sole, J. (2005). Neurophysiological assessment of trigeminal nerve reflexes in disorders of central and peripheral nervous system. Clin Neurophysiol 116(10): 2255-65.

Valls-Sole, J., A. Cammarota, et al. (1994). Orbicularis oculi responses to stimulation of nerve afferents from upper and lower limbs in normal humans. Brain Res 650(2): 313-6.

van Ham, J. J. and C. H. Yeo (1996a). Trigeminal inputs to eyeblink motoneurons in the rabbit. Exp Neurol 142(2): 244-57.

van Ham, J. J. and C. H. Yeo (1996b). The central distribution of primary afferents from the external eyelids, conjunctiva, and cornea in the rabbit, studied using WGA-HRP and B-HRP as transganglionic tracers. Exp Neurol 142(2): 217-25.

VanderWerf, F., B. Baljet, et al. (1993). Innervation of the superior tarsal (Muller's) muscle in the cynomolgus monkey: a retrograde tracing study. Invest Ophthalmol Vis Sci 34(7): 2333-40.

VanderWerf, F., M. Aramideh, et al. (1997). A retrograde double fluorescent tracing study of the levator palpebrae superioris muscle in the cynomolgus monkey. Exp Brain Res 113(1): 174-9.

VanderWerf, F., M. Aramideh, et al. (1998). Retrograde tracing studies of subdivisions of the orbicularis oculi muscle in the rhesus monkey. Exp Brain Res 121(4): 433-41.

VanderWerf, F., P. Brassinga, et al. (2003). Eyelid movements: behavioral studies of blinking in humans under different stimulus conditions. J Neurophysiol 89(5): 2784-96.

Veltman, J. A. and A. W. Gaillard (1998). Physiological workload reactions to increasing levels of task difficulty. Ergonomics 41(5): 656-69.

Vercelli, A., M. Repici, et al. (2000). Recent techniques for tracing pathways in the central nervous system of developing and adult mammals. Brain Res Bull 51(1): 11-28.

Von Cramon, D. and U. Schuri (1980). Blink frequency and speed motor activity. Neuropsychologia 18(4-5): 603-6.

Vucic, S., K. D. Cairns, et al. (2004). Neurophysiologic findings in early acute inflammatory demyelinating polyradiculoneuropathy. Clin Neurophysiol 115(10): 2329-35.

Warwick, R. (1950). A study of retrograde degeneration in the oculomotor nucleus of the rhesus monkey, with a note on a method of recording its distribution. Brain 73(4): 532-43.

Watson, C. R., S. Sakai, et al. (1982). Organization of the facial nucleus in the rat. Brain Behav Evol 20(1-2): 19-28.

Willer, J. C. and Y. Lamour (1977). Electrophysiological evidence for a facio-facial reflex in the facial muscles in man. Brain Res 119(2): 459-64.

Wolkoff, P., J. K. Nojgaard, et al. (2006). The modern office environment desiccates the eyes? Indoor Air 16(4): 258-65.

Yamada, F. (1998). Frontal midline theta rhythm and eyeblinking activity during a VDT task and a video game: useful tools for psychophysiology in ergonomics. Ergonomics 41(5): 678-88.

Yamamoto, T., Y. Oya, et al. (2004). [Palpable orbital subcutaneous masses in chronic inflammatory demyelinating polyneuropathy. MRI and neurophysiological study of multiple peripheral nerve swelling]. Rinsho Shinkeigaku 44(4-5): 286-90.

Yoon, H.W., J.Y. Chung, et al. (2005). Neural correlates of eye blinking; improved by simultaneous fMRI and EOG measurement. Neurosci Lett 381(1-2):26-30.

Zametkin, A. J., J. R. Stevens, et al. (1979). Ontogeny of spontaneous blinking and of habituation of the blink reflex. Ann Neurol 5(5): 453-7.

De Zeeuw, C.I., C.H. Yeo (2005). Time and tide in cerebellar memory formation. Curr Opin Neurobiol 15(6):667-74.

Zerari-Mailly, F., C. Dauvergne, et al. (2003). Localization of trigeminal, spinal, and reticular neurons involved in the rat blink reflex. J Comp Neurol 467(2): 173-84.

PART II

Blinking
and
Brainstem pathways

Chapter 2

Reticulo-collicular projections: a neuronal tracing study in the rat

ABSTRACT

Neuroanatomical tract-tracing methods were used to study the topography of the reticulocollicular projections. Injections of gold-HRP or BDA tracers into the medial and/or central portions of the superior colliculus resulted in labelled neurones mainly in the medial reticular formation, whereas injections into the lateral portion of the superior colliculus showed labelling in the medial and lateral reticular formation. When tracer was injected into the lateral portion of the caudal superior colliculus, extensive lateral labelling was observed in the contralateral parvocellular reticular nucleus and the contralateral dorsal medullary reticular nucleus, two areas involved in reflex blinking. The present study shows that these reticular areas project to the lateral superior colliculus, which is known to be involved in the coordination of eye and eyelid movements.

INTRODUCTION

The superior colliculus (SC), known as a key structure of sensory-motor integration, receives visual and somatosensory afferent inputs and provides premotor messages for gaze movements. Recently, evidence was obtained indicating that the lateral portion of the SC participates in the regulation of reflex blinking as well [1,5,6,13]. Numerous studies have been conducted in order to gain more information about the premotor neuronal circuits of the blink reflex. Neuronal tracing studies in rats suggest a premotor role for the reticular formation (RF) in the regulation of the blink reflex [10,15,16]. Two “RF blinking reflex areas” have been determined in the lateral RF, the pontine parvocellular field (PCRt) and the dorsal medullary reticular nucleus (MdD). The pontine PCRt is located around the fibres of the facial nerve between the superior olivary nucleus and the principal nucleus of the sensory trigeminal complex. The MdD area lies adjacent to the caudal subnucleus of the sensory trigeminal complex. Up to now, few studies examined ascending pathways from the RF to the SC. In rats, projections from the pontine and mesencephalic RF have been observed mainly in the intermediate layers of the ipsilateral SC [14]. In the monkey, RF projects bilaterally towards SC [7] and nerve endings originating from the medial RF were observed mainly in the medial and central SC portions [3]. In contrast, connections between lateral parts of RF, the PCRt and the MdD, and the lateral SC portion are not yet fully understood. In the current study, we determined the pontomedullary ascending RF projections towards the SC by means of retrograde tracing experiments in the rat.

MATERIALS AND METHODS

Biotylinated dextran amine (BDA) or gold apohorseradish peroxidase complex (gold-HRP) injections were performed in the right SC of 14 Sprague–Dawley rats. Gold-HRP [12] was pressure injected in 12 rats. After a survival time of 4–7 days, the animals were sacrificed and perfused with 500 ml of phosphate buffer saline (PBS, 0.1 M, pH 7.4) followed by 500 ml 4% paraformaldehyde fixative soluted in PBS. Serial 40 μm thick sections were processed with a silver intensification method in a dark room to reveal the protein gold complex. After a brief rinse in sodium citrate buffer (1 M, pH 3.8), the sections were dipped in a developer solution during 60 min. This solution (100 ml) consisted of: (i) 60 ml 50%

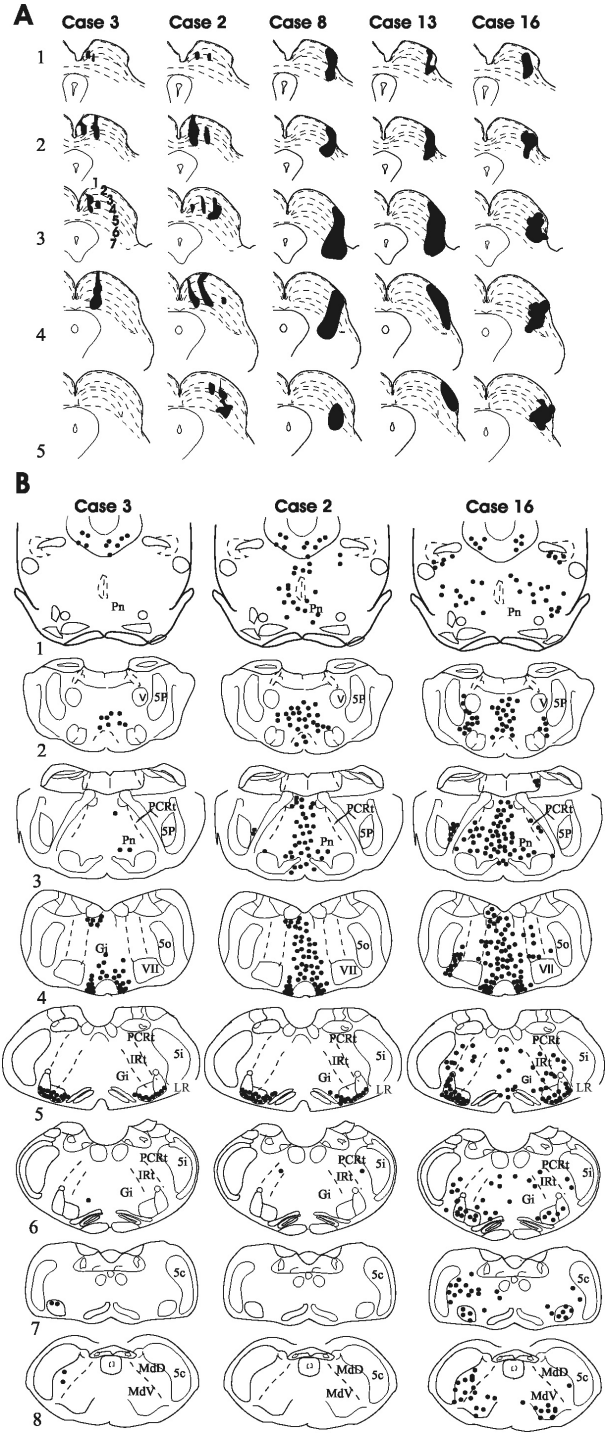


Figure 1.

Distribution of labelled neurones in the reticular formation (RF) following injections in the medial, central or lateral superior colliculus (SC) portions. (A) Diagrammatic representation of transverse sections through the SC (from rostral 1 to caudal 5) showing injection sites in cases 3, 2, 16 (Gold HRP) and in cases 8, 13 (BDA). (B) Camera lucida drawings of transverse sections through the pontomedullary RF (from rostral 1 to caudal 8) showing the distribution of labelled neurones in cases 3, 2 and 16. Gi, gigantocellular reticular nucleus; IRt, intermediate reticular nucleus; LR, lateral reticular nucleus; MdD and MdV, dorsal and ventral medullary reticular nucleus; PCRt, parvocellular reticular nucleus; Pn, pontine reticular nucleus; V, trigeminal motor nucleus; VII, facial motor nucleus. The principal nucleus (5P), the pars oralis (5o), the pars interpolaris (5i) and the pars caudalis (5c) of the sensory trigeminal complex are indicated. Pn; pontine reticular nucleus.

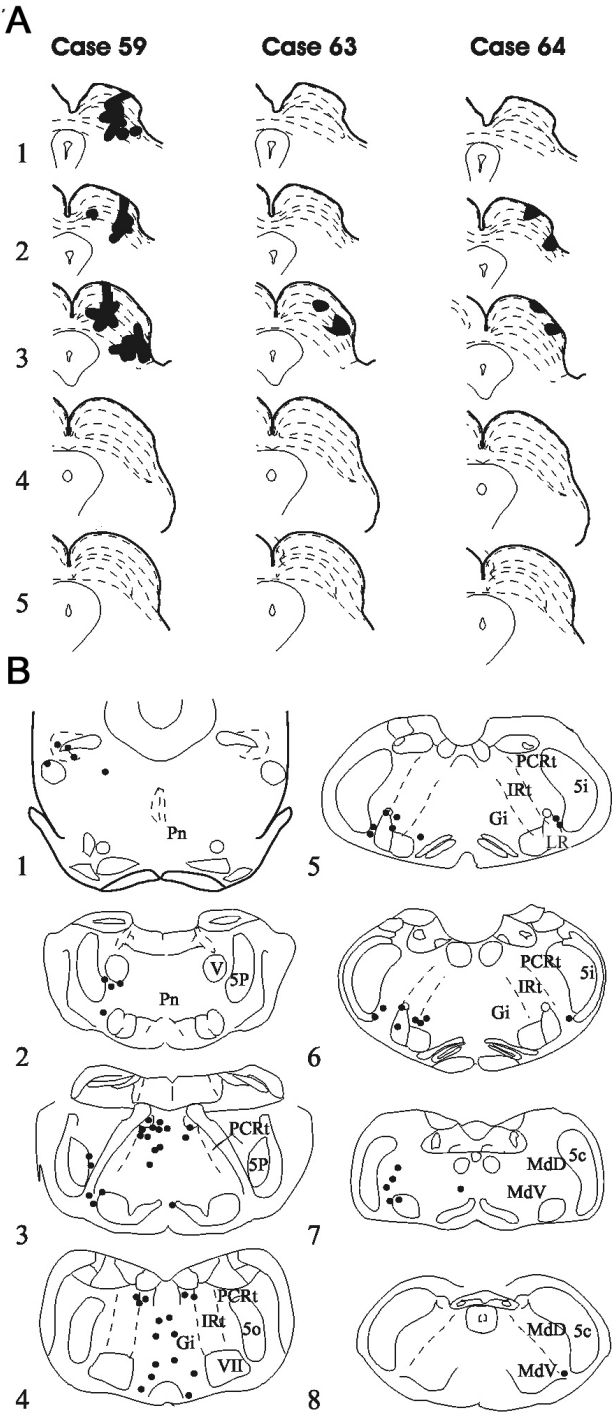


Figure 2.
Distribution of retrogradely labelled neurones in the reticular formation (RF) following injections in the anterior part of the superior colliculus (SC). (A) Diagrammatic representation of transverse sections through the SC (from rostral 1 to caudal 5) showing the gold-HRP injections sites in cases 59, 63, 64. (B) Labeled neurons (cumulative results of cases 59, 63, 64) are plotted on camera lucida drawings of transverse sections through the pontomedullary RF (from rostral 1 to caudal 8). Abbreviations as in Fig. 1.

Arabic Gum distilled water solution, (ii) 10 ml citrate buffer solution (1 M, pH 3.5), (iii) 15 ml 5.5% hydroquinone in distilled water solution and (iv) 15 ml 0.7% silver lactate in distilled water solution. After development, the sections were rinsed in PBS, put into a 2.5% sodium thiosulphate PBS solution for 5 min, and rinsed in PBS.

A 10% solution of BDA (3000 MW) in phosphate buffer (PB, pH 7.4) was iontophoretically injected (7 A, 7s on—7 s off) in two rats. Following 6–7 survival days, the animals were perfused with 500 ml of PBS followed by 500 ml of cold 4% paraformaldehyde in PBS. Transverse sections (40 μm thick) were incubated in an avidin–biotin complex (ABC, Vector Laboratories, Burlingame, CA) in PBS overnight at room temperature. The peroxidase component of the ABC was visualized using 0.05% diaminobenzidin (DAB) and 0.03% H2O2.

All sections were mounted and counterstained with neutral red, cover slipped and examined under a light microscope.

RESULTS

In a first series of six gold-HRP and two BDA experiments, large injections were made impregnating SC layers 1–7 within its rostrocaudal extent. In all experiments, retrograde labelled neurons were found in the RF, with preponderance at the contralateral side. Amongst them, five experiments presented selective tracer injections either into the medial SC (case 3), or into the medial/central SC (case 2) or into the lateral SC (cases 8, 13, 16) (Fig. 1A).

a) In experimental case 3, limited to the medial SC (Fig. 1A), relatively few labelled neurones were observed medially in the rostral gigantocellular field (Gi), in the ventral part of the lateral reticular nucleus (LR) and in the pontine RF (Fig. 1B, levels 3–5). Following an injection limited to the medial/central SC (Fig. 1A, case 2) an increased number of labelled neurones was found in the rostral Gi, the LR and the pontine RF (Fig. 1B, levels 1–5). No or few labelled neurones were detected in the MdD.
b) In one gold-HRP experiment (Fig. 1A, case 16) and in two BDA experiments (Fig. 1A, cases 8, 13) the injection sites were limited to the lateral SC. These experiments exhibited similar results as illustrated in Fig. 1B by the representative case 16. Many labelled neurones appeared in the Gi and in the ventral part of the LR. In addition, labelled neurones were found in the lateral pontine RF, some wedged between the principal and the motor trigeminal nuclei in what is known as the intertrigeminal region (Fig. 1B, case 16, levels 1–3). Interestingly, these experiments also revealed many labelled neurones in the contralateral PCRt around the fibres of the facial nerve, as well as in the caudal MdD (levels 3 and 7–8). These latter results demonstrated that neurones in the "RF blinking reflex areas" reached the lateral SC portion.

In a second series of six gold-HRP experiments, injections were made predominantly in the rostral or caudal SC. The injections performed in the rostral SC, regardless their medio-lateral localisation, showed only few labelled neurones distributed throughout the RF. In cases 59, 63 and 64 (Fig. 2A) the number of labelled RF neurones was 25, 6 and 5, respectively. In order to provide a more accurate localisation of the RF neurones projecting to the rostral SC, the cumulative results of these three experiments are illustrated in Fig. 2B.

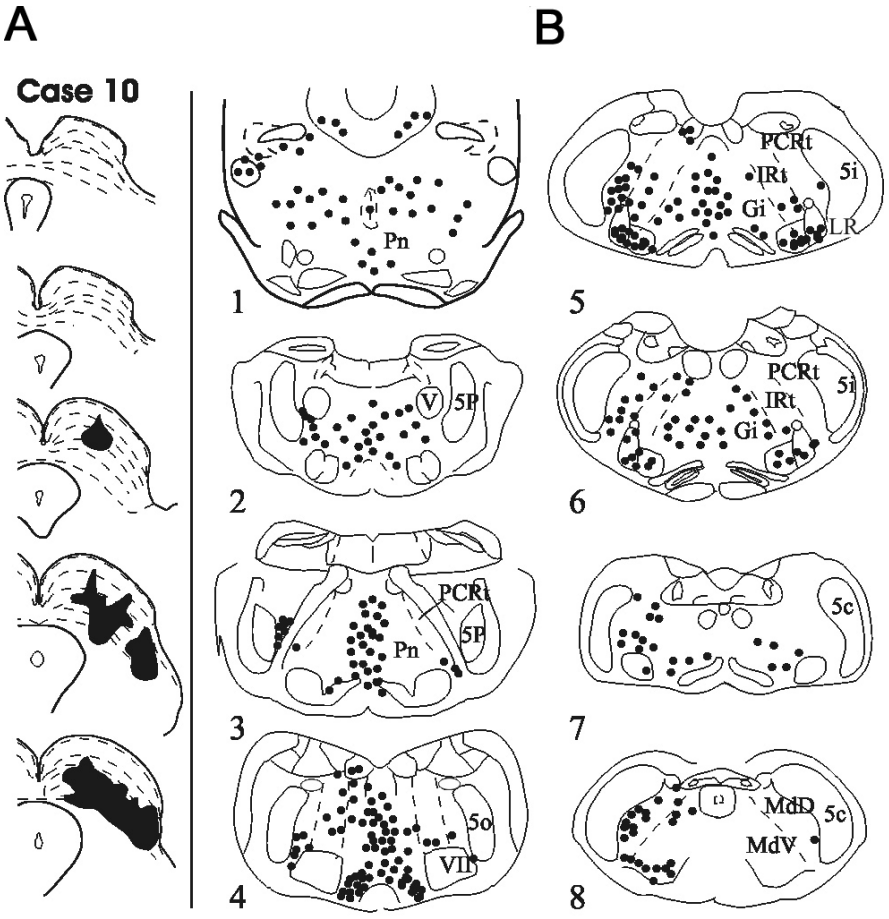


Figure 3. Distribution of retrogradely labelled neurones in the reticular formation (RF) following injections in the posterior part of the superior colliculus (SC). (A) Diagrammatic representation of transverse sections through the SC (from rostral 1 to caudal 5) showing gold-HRP injection site in case 10. (B) Camera lucida drawings of transverse sections through the pontomedullary RF (from rostral 1 to caudal 8) showing the distribution of labelled neurones in case 10. Abbreviations as in Fig. 1.

Most of the labelled neurones were observed bilaterally throughout the Gi (Fig. 2B, level 4) and in the dorsal part of the contralateral pontine RF (Fig. 2B, level 3). Only few labelled neurones appeared in the PCRt wedged between the trigeminal principal nucleus and the facial nerve (Fig. 2B, level 3) or in the MdD (Fig. 2B, levels 7–8).

Striking extensive labelling of RF neurones was seen in case 10 where injections comprised the central and lateral portions of the caudal SC (Fig. 3A). Numerous labelled neurones appeared in the PCRt and in the MdD (Fig. 3B). The distribution pattern of labelled neurones in the pontomedullary RF is very similar to that observed following injections restricted to the lateral SC portion (Fig. 1C, case 16). It appears that the RF neurones project mainly to the caudal part of the SC. The current study also shows

that only few RF neurones project to the medial and intermediate SC (Fig. 1A, cases 3 and 2).

In conclusion, it can be assumed that neurones in the “RF blinking reflex areas” mainly project to the lateral portion of the caudal SC.

DISCUSSION

The present data show the detailed relationships between the diverse RF fields and the SC, demonstrating that most of the reticulocollicular neurons are located throughout the Gi of the pontomedullary RF and within the ventral part of the LR. These neurones reach the entire SC, projecting moderately to the medial/central SC and strongly to the lateral SC portions. The Gi mainly receives afferent projections from intermediate and deep SC layers, a major head-movement area [4]. The same study also showed efferent projections from Gi towards the ipsilateral facial motor nucleus and the ipsilateral trigeminal motor nucleus, indicating Gi is also involved in whisker movements [8]. Moreover, it is known that neurones in the medial Gi, by their projections to the spinal cord [9] and to the oculomotor nuclei, are involved in the motor command of coordinated movements of the eye, neck and trunk [11]. In addition, the LR, by its strong projection to the cerebellar cortex participates in the control of the vestibular system [2], motor activity and coordination. Accordingly, the present study demonstrates a new pathway through which Gi and LR may act upon orienting behaviours, such as coordination of gaze, eye and head, movements. A clear projection from neurones located within the lateral RF fields was also observed. A new interesting finding is that neurones in the PCRt and the MdD, i.e. in the “RF blinking reflex areas”, project to the SC and essentially, if not exclusively, to the lateral SC portion. Interestingly, this SC region was shown to receive sensory information from the eyelids [13] and to contain neurons projecting to the facial motor nucleus [5]. In the same paper a new somatosensory motor function of SC has been proposed, in which SC modulates eye movement during reflex blinking.

Acknowledgement
This work was supported by NWO grant number VGP 95-398.

REFERENCES

[1] M.A. Basso, A.S. Powers, C. Evinger, An explanation for reflex hyperexcitability in Parkinson's disease. I. Superior colliculus, J. Neurosci. 16 (1996) 7308–7317.

[2] J.A. Büttner-Ennerver, A review of otolith pathways to brainstem and cerebellum, Ann. Acad. Sci. 871 (1999) 51–64.

[3] B. Chen, P.J. May, The feedback circuit connecting the superior colliculus and central mesencephalic reticular formation: a direct morphological demonstration, Exp. Brain Res. 131 (2000) 10–21.

[4] R.J. Cowie, M.K. Smith, D.L. Robinson, Subcortical contributions to head movements in Macaques. II. Connections of a medial pontomedullary head-movement region, Neurophysiolgy 72 (1994) 2665–2683.

[5] C. Dauvergne, A. Ndiaye, C. Buisseret-Delmas, P. Buisseret, F. Vanderwerf, G. Pinganaud, Projections from the superior colliculus to the trigeminal system and facial nucleus in the rat, J. Comp. Neurol. 478 (2004) 233–247.

[6] J.W. Gnadt, S.M. Lu, B. Breznen, A. Basso, M. Henriquez, C. Evinger, Influence of the superior colliculus on the primate blink reflex, Exp. Brain Res. 116 (1997) 389–398.

[7] R. Hartwich-Young, J.S. Nelson, D.L. Sparks, The perihypoglossal projection to the superior colliculus in the rhesus monkey, Vis. Neurosci. 4 (1990) 29–42.

[8] A.M. Hattox, C.A. Priest, A. Keller, Functional circuitry involved in the regulation of whisker movements, J. Comp. Neurol. 442 (2002) 266–276.

[9] G.E. Hermann, G.M. Holmes, R.C. Rogers, M.S. Beattie, J.C. Bresnahan, Descending spinal projections from the rostral gigantocellular reticular nuclei complex, J. Comp. Neurol. 455 (2003) 210–221.

[10] G. Holstege, J.J. Van Ham, J. Tan, Afferent projections to the orbicularis oculi motoneuronal cell group: an autoradiographical tracing study in the cat, Brain Res. 374 (1986) 320–360.

[11] B.E. Jones, Reticular formation: cytoarchitecture, transmitters, and projections, in: G. Paxinos (Ed.), The Rat Nervous System, vol. III, Academic Press, London, 1995, pp. 155–171.

[12] D. Ménétrey, A.I. Basbaum, Spinal and trigeminal projections to the nucleus of the solitary tract: a possible substrate for somatovisceral and viscerovisceral reflex activation, J. Comp. Neurol. 255 (1987) 439–450.

[13] A. Ndiaye, G. Pinganaud, C. Buisseret-Delmas, P. Buisseret, F. VanderWerf, Organization of trigeminocollicular connections and their relations to the sensory innervation of the eyelids in the rat, J. Comp. Neurol. 448 (2002) 373–387.

[14] R.P. Vertes, G.F. Martin, Autoradiographic analysis of ascending projections from the pontine and mesencephalic reticular formation and the median raphe nucleus in the rat, J. Comp. Neurol. 275 (1988) 511–541.

[15] F.P. Zemlan, M.M. Behbehaniand, R.M. Beckstead, Ascending and descending projections from nucleus reticularis magnocellularis and nucleus reticularis gigantocellularis: an autoradiographic and horseradish peroxidase study in the rat, Brain Res. 292 (1984) 207–220.

[16] F. Zerari-Mailly, C. Dauvergne, P. Buisseret, C. Buisseret-Delmas, Localization of trigeminal, spinal and reticular neurons involved in the rat blink reflex, J. Comp. Neurol. 467 (2003) 173–184.

CHAPTER 3

Reticulo-collicular and spino-collicular projections involved in eye and eyelid movements during the blink reflex

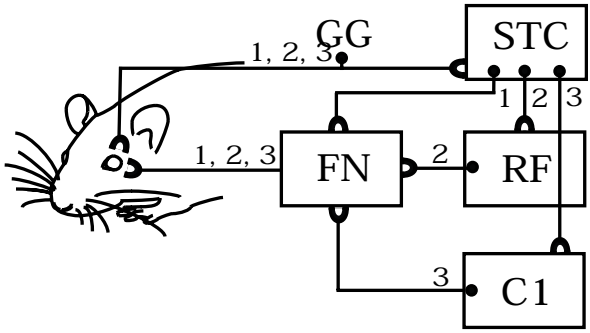
ABSTRACT

Reflex blinking provides a useful experimental tool for various functional studies on the peripheral and central nervous system, yet the neuronal circuitry underlying this reflex is not precisely known. In the present study we investigated as to whether neurons in the reticular formation and rostral cervical spinal cord (C1) may be involved in the blink reflex in rats. To this end we investigated c-Fos expression in these areas following supraorbital nerve stimulation combined with retrograde tracing of gold conjugated horse radish peroxidase (Gold-HRP) from the superior colliculus. We observed many double labeled neurons in the parvocellular reticular nucleus, medullary reticular formation, and laminae IV and V of C1. Thus, these brain regions contain neurons that may be involved in blink reflexes as well as eye movements, because they both can be activated following peri-orbital stimulation and project to the superior colliculus. Consequently, we suggest that the medullary reticular formation and C1 region play a central role in the coordination of eye and eyelid movements during reflex blinking.

INTRODUCTION

Blinking is a process in which eyes and eyelids act in concert. After onset of eyelid closure the eyes move characteristically nasal downward and then lateral upward (Evinger et al. 1984; Collewijn et al. 1985). The pre-motor neuronal circuits controlling the simultaneous eye and eyelid movements during the blink reflex have been investigated intensively (Holstege et al. 1986[a]; Van Ham and Yeo 1996[a,b]; Morcuende et al. 2002; Zerari-Mailly et al. 2003; Cruccu et al. 2005). Trigeminal blinks can be elicited by supraorbital (SO) nerve or corneal stimulation (Evinger et al. 1984; Gruart et al. 1995; VanderWerf et al. 2003). In humans, EMG recordings of the eyelid-closing orbicularis oculi muscle shows two responses after electrical stimulation of the SO nerve (Kugelberg 1952; Aramideh et al. 2002). The brief early response R1 is unilateral and the large late response R2 bilateral and corresponds with the actual eyelid movement in humans. Guinea pigs, cats and rats however show bilateral R1 and R2 responses upon moderate-intensity electrical stimulation. In guinea pigs both the R1 and R2 contribute significantly to eyelid movement during the blink (Pellegrini et al. 1995). The circuitry regulating trigeminal blinks is short, nonetheless not uncomplicated. The orbicularis oculi motoneurons in the facial motor nucleus are innervated through three different pathways. The shortest (Fig.1, pathway 1), direct circuit involves the sensory trigeminal complex (STC) which directly projects to the facial motor nucleus (Jacquin et al. 1993; Van Ham and Yeo 1996[b]). From the STC the other two indirect pathways arise. The first by way of the reticular formation (Fig.1, pathway 2), the second (Fig.1, pathway 3) via the rostral cervical spinal cord (C1) (Zerari-Mailly et al. 2003). The indirect pathways were studied in the cat (Holstege et al. 1986[b]), guinea pig (Pellegrini et al. 1995), rabbit (Van Ham and Yeo 1996[a,b]) and rat (Zerari-Mailly et al. 2003). Higher brain regions, like the basal ganglia, can also influence reflex blinking through different brainstem structures (Basso et al. 1996).

Excitability of the blink can be modulated by descending cortical projections via the thalamus and superior colliculus (SC) via tecto-reticular projections (Basso et al. 1996). The SC is important for eye and head movements as well as for many other sensory motor functions including the blink reflex (Goosens and Van Opstal 2000; Ndiaye et al. 2002; King 2004). SC involvement in the blink reflex was also shown with experiments in the monkey (Gnadt et al. 1997) and rat (Basso et al. 1996) where



1. Short direct pathway via the STC and FN.
2. Indirect pathway via the STC, RF and FN.
3. Indirect pathway via the STC, C1 and FN.

Figure 1
The blink reflex circuit. Three pathways can be distinguished (1, 2, 3). Abbreviations: gasserian ganglion (GG), sensory trigeminal complex (STC), facial motor nucleus (FN) reticular formation (RF) and rostral part of the cervical spinal cord (C1).

electrical micro-stimulation of the SC suppressed the trigeminal blink reflex. Hemi-facial paralysis patients have impaired eye and eyelid movements during blinking, while the eye moves normal during saccades and smooth pursuit (unpublished results VanderWerf et al.). This implies that the SC as eye movement generator is not affected; therefore a separate structure projecting to the SC must initiate eye movement during the blink. Recently, the latero-caudal SC was shown to receive input from two reticular areas; the ventral part of the parvocellular reticular nucleus (PCRt) and the dorsal part of the medullary reticular nucleus (MdD) (Smit et al. 2005). Subsequently, the question arose whether the coordination of eye movements during blinking is regulated by the reticular formation and/or C1 and thus how the projections towards the SC are arranged.

Since timing of the stereotypical eye and eyelid movement during the blink is known to be very precise (Bour et al. 2000) a common neuronal structure must initiate the coordination of both movements. We hypothesize that different areas in the RF can generate eye and eyelid movement associated with the blink reflex. A reticular area that receives SO nerve input and contains neurons that project to the facial motor nucleus as well as neurons that project to the SC could be the common neuronal structure regulating this phenomenon.

The aim of the present study was to clarify the role of the reticular formation, spinal cord and SC in eye movement during reflex blinks and define the location of a central neural origin of the eye and eyelid components of the blink reflex. To this end we examined the functional and topographical relationships between the spino- and or reticulo-collicular connections and the primary afferent inputs from the eyelids (i.e., the SO nerve). Localization of cells in functional pathways in the nervous system was achieved through immunohistochemical detection of cellular counterpart of the immediate early gene c-Fos. The c-Fos gene encodes the nuclear protein c-Fos that is rapidly and transiently expressed in neurons in response to various peripheral stimuli (Hunt et al. 1987; Sheng and Greenberg 1990). The development and use of this method has been reviewed extensively (Armstrong and Montminy

1993; Hoffman and Lyo 2002; Munglani and Hunt 1995; Sheng et al. 1990). Since c-Fos activation is a well established high resolution metabolic marker for polysynaptic pathway tracing in the brain (Dragunow and Faull 1989), in the current study expression of the c-Fos protein was evaluated after electrical SO nerve stimulation normally eliciting trigeminal blinks. In addition to this experimental series, a retrograde tracer, the colloidal gold apo-horseradish peroxidase complex (Gold-HRP), was also employed (Ménétrey 1985). The combination of c-Fos expression following electrical SO nerve stimulation and Gold-HRP micro-injections in the SC were used to identify reticular and spinal neurons involved in the trigeminal blink reflex and neurons that project to the SC, respectively. These experiments may identify neurons in the pontine and medullary reticular formation involved in the organisation of the blink reflex.

MATERIALS AND METHODS

Animals

For the experiments 9 adult Sprague-Dawley rats were used. Animals were anesthetized with an intraperitoneal sodium pentobarbital injection (50mg/kg), a very suitable anesthetics for c-Fos expression studies in rats (Takayama et al. 1994). During surgery animals were placed in a stereotaxic apparatus. Gold-HRP injections were made in the right side of the brain, electrical SO nerve stimulation on the left. The microinjection were aimed at the latero-caudal SC, since it has been shown that this portion of the SC has reciprocal connections with the STC and is involved in the blink reflex (Ndiaye et al. 2002; Dauvergne et al. 2004). To determine the effects of anaesthetics and surgical procedures on c-Fos expression, three control animals were anesthetized and sham operated similar to the study of Zerari-Mailly and co workers (2003). Six animals received both a Gold-HRP injection and electrical stimulation. All experiments were conducted following the “principles of laboratory animal care” (NIH publication No. 86-23, revised 1985) and French law on the protection of animals.

Experimental procedures

Colloidal gold apo-horseradishperoxidase complex (Gold-HRP) injection. Gold-HRP solution (Ménétrey 1985) was pressure injected into the SC according to stereotaxic coordinates defined by Paxinos and Watson (1986). Six microinjections (0.1–0.15 µl) were made into the right SC three days prior to electrical stimulation of the SO nerve. The exact locations of the injections are given in figure 2.

Electrical stimulation. In 6 rats, the main branches of the left SO nerve were dissected, cut distally, placed over a pair of silver hook electrodes and covered with mineral oil. The nerve was stimulated with 3 pulse bursts (300Hz, 0.1ms duration; repeated every 900ms for 1 hour). The intensity of the stimulation was 1.5 times the blink reflex threshold value (0.1-1.0 mA; up to 1mA in case 18). Presumably nociceptive A and C fibers were not stimulated at this intensity as Ellrich and co workers (2001) showed that the nociceptive R3 component of the blink was not elicited after stimulation under 2.9 times the detection threshold of a blink. Furthermore no neck movement was observed at the chosen stimulus. One hour after stimulation the animals were perfused with 500 ml phosphate-buffered saline (PBS, pH 7.4) and 500 ml cooled fixative (4% paraformaldehyde in PBS). The brains were stored in a 30% sucrose PBS solution at 4°C for 48 hours. 40µm thick slices were cut with a freezing microtome.

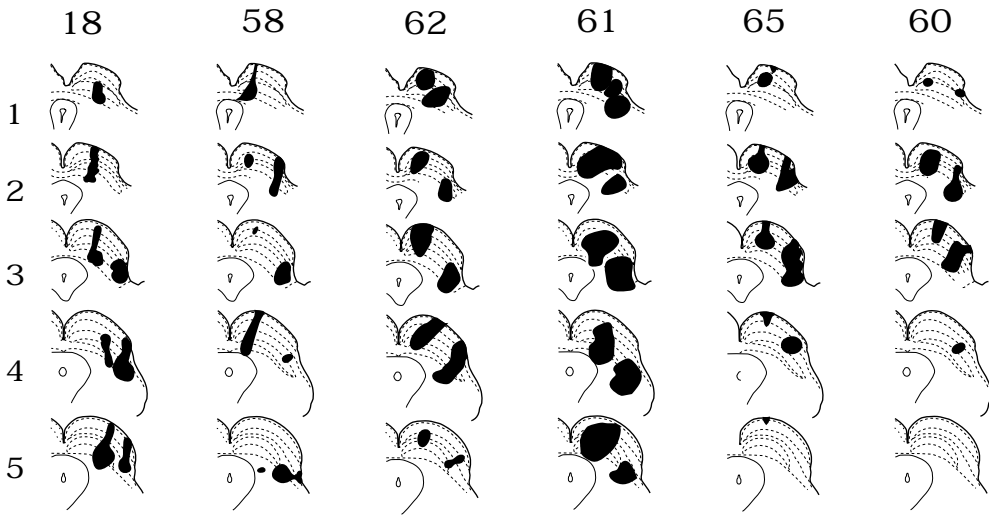


Figure 2 Schematic drawing of transverse sections of the superior colliculus (from rostral 1 to caudal 5) depicting the Gold-HRP injection sites in the six rats.

Histochemical procedures

Gold-HRP histochemistry. Sections were processed with a silver intensification method to reveal the protein gold complex. The procedures have been described by Zerari-Mailly and co workers (2003).

C-Fos immunohistochemistry. Detailed procedures for visualization of c-Fos expression were previously described (Zerari-Mailly et al. 2003).

Data analysis

Illustrations. Drawings were made with a camera lucida and imported into Adobe Illustrator 10.0. Photomicrographs were taken with a Leitz Diaplan photomicroscope and processed with Adobe Photoshop 6.0 (Adobe systems Inc., San Jose, CA).

Cell counting. The number of single labeled c-Fos and Gold-HRP labeled neurons as well as double labeled neurons was counted in three control and six stimulated rats. Labeled neurons were counted in the ponto-medullary reticular formation. Slices were included from the rostral facial motor nucleus until just above the spino-medullary junction. Seven sections were selected just below the spino-medullary junction, for counting c-Fos neurons in the C1.

RESULTS

C-Fos labeling

SO nerve stimulation induced a large increase in the number of c-Fos positive neurons compared to control animals and numerous c-Fos positive neurons were observed throughout the pontomedullary reticular formation (table 1; Fig.3) and C1 segment of the spinal cord (table 1; Fig. 4). The locations of labeled areas were similar on the ipsi-and contralateral sides, however the number of c-Fos positive neurons was consistently higher at the stimulated side. Large numbers of c-Fos positive neurons were

found in three reticular areas: the PCRt, the MdD, and the C1; 700, 475 and 934, respectively (table 1). Whereas, in control animals these numbers were 64, 185 and 676, respectively (table 1). The highest increase of c-Fos positive neurons was found in the PCRt (1000%, table 1).

Table 1. Number of c-Fos positive neurons found in the parvocellular reticular nucleus (PCRt), dorsal medullary reticular formation (MdD) and rostral part of the cervical spinal cord (C1) in control and experimental animals. Counting's were made ipsilateral to the stimulated side.

	PCRt	MdD	C1
Control			
22	34	197	659
24	43	162	644
36	114	195	725
Average	**64**	**185**	**676**
Experiment			
18	850	418	1200
58	450	302	739
60	457	363	879
61	819	633	918
62	913	632	-
65	713	502	-
Average	**700**	**475**	**934**
% increase vs. control	**1000**	**157**	**38**

In the pontine reticular formation (Fig. 3, cases 18A, 62A, 65A, level 1-3) c-Fos positive neurons were concentrated ventral and medial to the STC. Smaller groups of c-Fos positive neurons were located around the trigeminal motor nucleus and medial to the VIIth cranial nerve. In addition, c-Fos labeled neurons were observed near the oral subnucleus of the STC. In the medullary reticular formation (Fig. 3, cases 18A, 62A, 65A, level 4-6), most c-Fos positive neurons were found ventral in the caudal PCRt, between the interpolaris subnucleus of the STC and lateral reticular nucleus, and in the lateral portion of the MdD. C-Fos positive neurons were also seen laterally within the lateral reticular nucleus. In addition, some c-Fos positive neurons were observed in the intermediate reticular field just above the facial motor nucleus and/or the lateral reticular nucleus in the gigantocellular reticular nucleus, adjacent to the inferior olive, and in the ventral medullary reticular nucleus (Fig. 3, cases 18A, 62A, 65A).

In the C1 (Fig. 4, cases 18A, 58A, 61A) c-Fos positive neurons were found in lamina I to VI with a concentration of labeled neurons ventro-lateral in the superficial laminae. A comparison with control animals revealed that most c-Fos positive neurons were located within laminae III, IV and V.

Gold-HRP labeling

In six experiments, Gold-HRP injections were made in the SC contralateral to the side of SO nerve stimulation (Fig. 2). In four injections the rostrocaudal extent of the SC was impregnated by the tracer: the injection sites comprised the central and caudo-lateral (case 18), medial and caudo-lateral (case 58) and entire SC (cases 62 and 61). In two experiments, the injection sites were restricted to the rostral SC (cases 65 and 60). When the injection site encompassed the caudo-lateral portion of the SC numerous Gold-HRP labeled neurons were located throughout the pontomedullary reticular formation

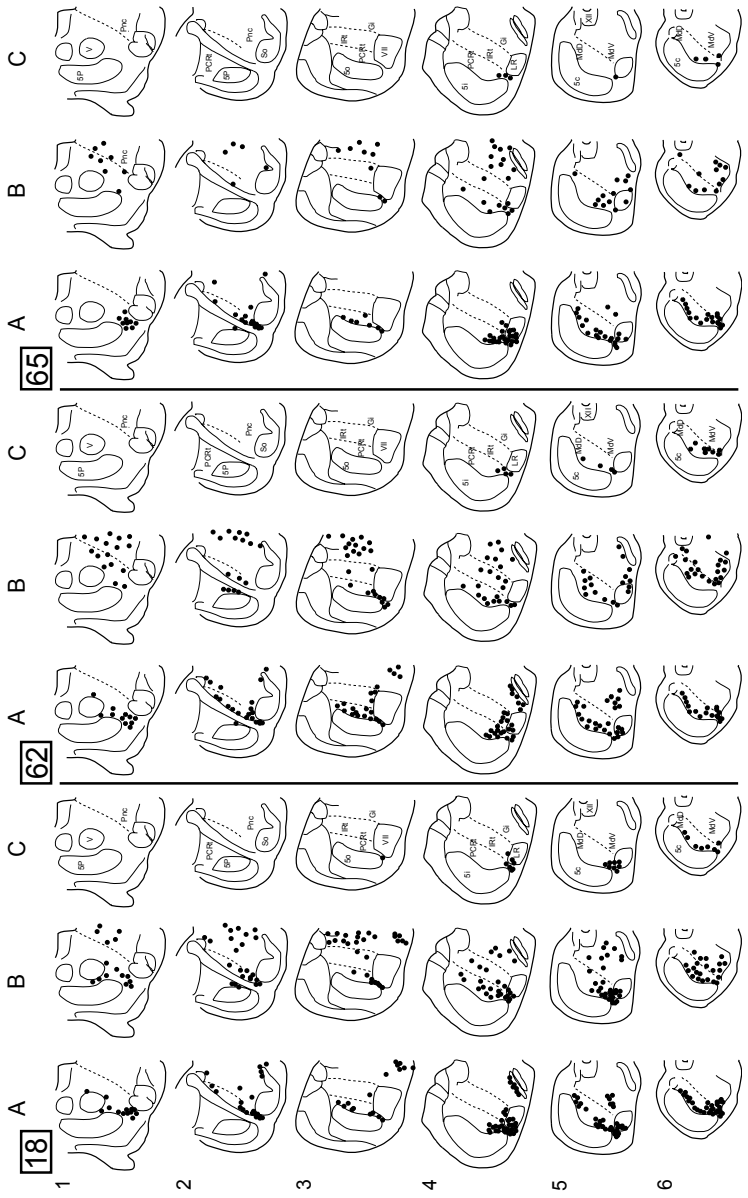


Figure 3
Schematic drawing of the pontomedullary reticular formation from rostral level 1 to caudal level 6. Black dots indicate the location in which labeled neurons were found in case 18, 62 and 65; total numbers of labeled neurons are shown in table 1.

A: The distribution of c-Fos positive neurons after supraorbital (SO) nerve stimulation.

B: The distribution of Gold-HRP labeled neurons following micro-injections in the caudo-lateral portion of the superior colliculus (SC).

C: The distribution of c-Fos positive/Gold-HRP double labeled neurons following SO nerve stimulation and micro-injections in the caudo-lateral SC portion.

Abbreviations: principal nucleus of the sensory trigeminal complex (5P), oral subnucleus of the sensory trigeminal complex (5o), interpolar subnucleus of the sensory trigeminal complex (5i), caudal subnucleus of the sensory trigeminal complex (5c), gigantocellular reticular nucleus (Gi), intermediate reticular nucleus (IRt), lateral reticular nucleus (LR), dorsal medullary reticular nucleus (MdD), ventral medullary reticular formation (MdV), parvocellular reticular nucleus (PCRt), caudal pontine reticular nucleus (Pnc), superior olivary nucleus (So), trigeminal motor nucleus (V), facial motor nucleus (VII), hypoglossal motor nucleus (XII).

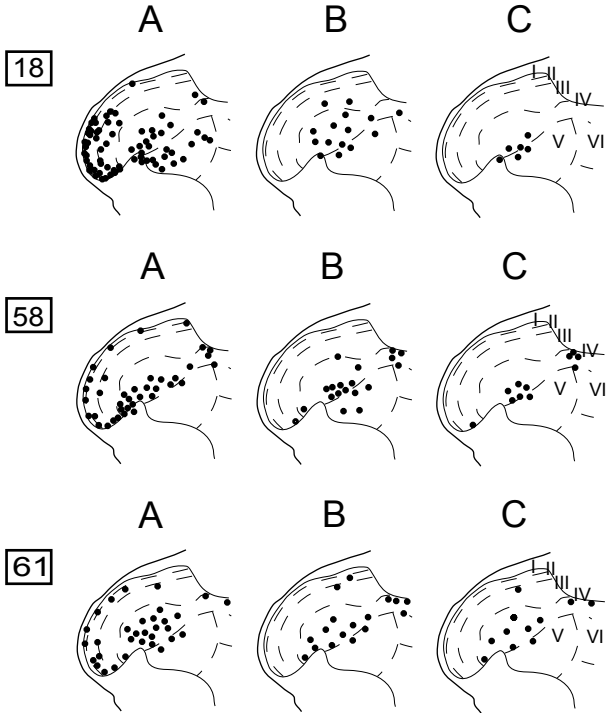


Figure 4. Schematic drawing of the rostral part of the cervical spinal cord showing the distribution of labeled neurons in case 18, 58 and 61. Black dots represent the location of labeled c-Fos positive or Gold-HRP neurons from the dorsal layer I to the ventral layer VI; total numbers of neurons are shown in table 1.
A: The distribution of c-Fos positive neurons after supraorbital (SO) nerve stimulation.
B: The distribution of Gold-HRP labeled neurons following micro-injections in the caudo-lateral portion of the superior colliculus (SC).
C: The distribution of c-Fos positive/Gold-HRP double labeled neurons following SO nerve stimulation and micro-injections in the caudo-lateral SC portion.

(table 2; Fig. 3, cases 18B, 62B, 65B) and C1 (table 2; Fig. 4, cases 18B, 58B, 61B). Labeled neurons were predominantly found contralateral to the side of Gold-HRP injection. The number of labeled neurons increased with a larger injection site. The PCRt as well as the MdD contained approximately three times more labeled neurons than C1 (table 2).

In the pontine reticular formation (Fig. 3, cases 18B, 62B, 65B, level 1-3) numerous labeled neurons were observed around the trigeminal motor nucleus, around the VIIth nerve, and wedged between the oral subnucleus of the STC and the facial motor nucleus. Cases 60 and 65 demonstrate that if the injection site does not comprise the most caudal lateral SC portion less neurons are labeled, which is most obvious in the small amount of labeled neurons in the PCRt (Fig 3, case 65B, level 1-4).

In the medullar reticular formation (Fig. 3, cases 18B, 62B, 65B, level 4-6), labeled neurons were predominantly found in the PCRt and the MdD. Neurons were also labeled in the medial and intermediate reticular formation. In case 60, which was injected in the lateral portion of the rostral SC, numerous labeled neurons occupied the medial reticular formation. Few labeled neurons were found in

the lateral reticular formation, i.e. in areas involved in the blink reflex.

In the C1 (Fig. 4, cases 18B, 58B, 61B) Gold-HRP labeled neurons were observed in laminae III to VI, with predominance in laminae III and IV. Gold-HRP labeled neurons were virtually absent in laminae I and II.

Double labeling

C-Fos/Gold-HRP double labeled neurons were observed primarily in the ventral PCRt, MdD (Fig.3, cases 18C, 62C, 65C) and C1 (Fig. 4, cases 18C, 58C, 61C). In case 60, with a rostral injection (Fig. 2), no double labeled neurons were present. The distribution of double labeled neurons in the reticular formation was bilateral. The largest number of double labeled neurons was found contralateral to the side of injection and ipsilateral to the side of SO nerve stimulation. The MdD not only contained the highest number of double labeled neurons but the relative proportion of the two neuron populations that are either activated by SO nerve stimulation or project to the SC was also the highest (i.e. twice the amount of C1 and 1.5 that of PCRt; table 2).

In the pontine reticular formation (Fig. 3, cases 18C, 62C, 65C, level 1-3) practically no double labeled neurons were observed. Some double labeled neurons were observed between the oral subnucleus of STC and facial motor nucleus (Fig. 3, case 18C, level 3).

In the medullary reticular formation (Fig. 3, cases 18C, 62C, 65C, level 4-6) double labeled neurons are observed in the PCRt (Fig. 5a) and MdD (Fig. 5b). In the PCRt, double labeled neurons are found in the ventral part, mostly between the interpolar subnucleus of the STC and the lateral reticular nucleus. In the MdD, double labeled neurons were found adjacent to the lateral reticular nucleus and medial to the caudal subnucleus of the STC. In case 65, relatively few double labeled neurons were found in the MdD.

In the C1, double labeled neurons were mainly found in laminae IV and V (Fig. 5c), additionally a few double labeled neurons were found in lamina III (Fig. 4, cases 18C, 58C, 61C).

Table 2. Number of Gold-HRP and double labeled neurons in the parvocellular reticular nucleus (PCRt), dorsal medullary reticular nucleus (MdD) and rostral part of the cervical spinal cord (C1) counted ipsilateral to the stimulated side. % of Gold-HRP = percentage of Gold-HRP labeled neurons that was double labeled, % of c–Fos = percentage of c-Fos positive neurons that was double labeled.

	Gold - HRP			Double labeled		
Experiment	PCRt	MdD	C1	PCRt	MdD	C1
18	215	195	51	76	85	7
58	160	99	46	35	55	11
60	6	0	1	0	0	0
61	177	134	44	62	84	10
62	135	168	-	28	62	-
65	105	150	-	25	15	-
Average	**133**	**124**	**36**	**38**	**50**	**7**
% of Gold-HRP				**28**	**40**	**20**
% of c-Fos				**5**	**11**	**0.7**

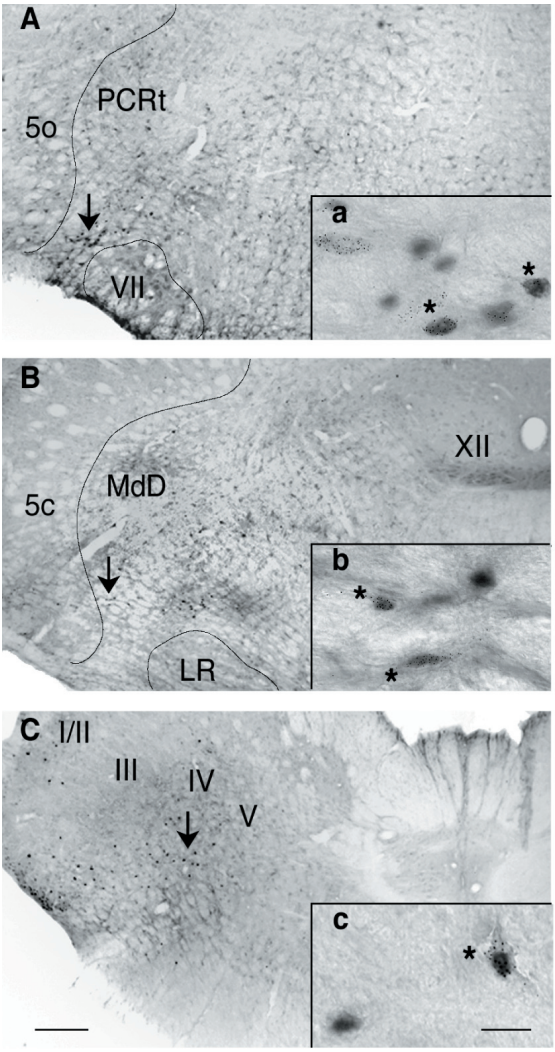


Figure 5
Photomicrographs of transverse slices from case 18 containing double labeled neurons in (A) the parvocellular reticular nucleus, (B) dorsal medullary reticular nucleus and (C) rostral part of the cervical spinal cord. a, b and c are magnifications of areas in A, B and C indicated by arrows. Asterisks are placed near double labeled neurons in the magnifications. Abbreviations: oral subnucleus of the sensory trigeminal complex (5o), caudal subnucleus of the sensory trigeminal complex (5c), lateral reticular nucleus (LR), parvocellular reticular nucleus (PCRt), dorsal medullary reticular nucleus (MdD). facial motor nucleus (VII), hypoglossal motor nucleus (XII), laminae of the C1 cervical spinal cord (I-V). Scale bar = 200 µm for A, B and C / 10 µm for a, b, and c.

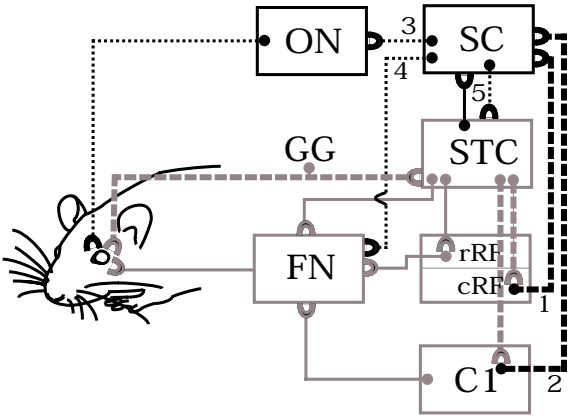
DISCUSSION

In the current study we aimed to reveal reticulo-collicular (Fig. 6 pathway 1) and spino-collicular (Fig. 6 pathway 2) projections involved in reflex blinking. In these experiments, c-Fos immunohistochemistry following SO nerve stimulation (Fig. 6 thick grey dashed lines) and Gold-HRP injections into the SC (Fig. 6 thick black dashed lines) were combined. We determined specific areas in the reticular formation and C1.

The distribution patterns of c-Fos neurons following SO nerve stimulation were determined in the pontomedullary reticular formation and the C1. C-Fos neurons were predominantly observed in the lateral reticular formation and laminae IV and V of C1. Possible c-Fos inducers other than electrical stimulation of SO nerve were not found: anaesthesia, surgical manipulation and placement of electrodes

upon the SO nerve or tracer injections into the SC did not induce c-Fos expression in the examined reticular formation areas. In addition, c-Fos expression after SO nerve stimulation was not induced by a non-specific effect like nociceptive stimulation of C fibers. If unmyelinated C fibers were stimulated most c-Fos would have been expressed in lamina II of the C1, where very little c-Fos expression was found in this study (Pellegrini et al. 1995).

In the present experiments the SO nerve stimulation induced c-Fos expression in trigeminal neurons, confirming labeled areas described by Ndiaye et al. (2002) and Zerari-Mailly et al. (2003). However, despite known disynaptical facial motoneuron activation during experimental conditions, no c-Fos labeling was observed in the facial motor nucleus. Absence of labeling in regions that are activated has been shown in several studies such as motoneurons or other neurons in the dorsal root ganglia or the substantia nigra (Hunt et al. 1987; Dragunow and Faull 1989; Carr et al. 1995; Dai et al.



1. Reticulo-collicular projection.
2. Spino-collicular projection.
3. Colliculo-occulomotor projection.
4. Colliculo-facial projection.
5. Colliculo-trigeminal projection.
   Trigemino-collicular projection.

Figure 6. The blink reflex circuit (grey) expanded with structures/projections involved in eye movement during the blink reflex (black). The projections examined in the current study are indicated by thick grey (supraorbital nerve stimulation) and black (Gold-HRP injection) dashed lines. Double labeled neurons are located in caudal reticular formation (cRF) and cervical spinal cord (C1). The projections in the scheme are based on findings of the current and other studies of Smit et al 2005, the reticulo-collicular and spino-collicular projections (1,2), of Goossens and van Opstal 2000[b], the colliculo-oculomotor projections (3), of Dauvergne et al , the colliculo-trigeminal and colliculo-facial projections (4, 5), of Ndiaye et al 2002, the colliculo-trigeminal and trigemino-collicular projections (5), and of Zerrari et al 2003, the trigemino-facial, trigemino-reticular and reticulo-facial projections (grey lines).
Abbreviations: gasserian ganglion (GG), sensory trigeminal complex (STC), facial motor nucleus (FN), rostral reticular formation (rRF), caudal reticular formation (cRF), rostral part of the cervical spinal cord (C1), superior colliculus (SC), oculomotor nuclei (ON).

2005). Moreover, Dragunow and Faull (1989) suggested that (moto)neurons might lack the required biochemical messengers regulating c-Fos activation. Subsequently, facial motoneurons that are active under specific conditions can fail to express c-Fos.

The present data demonstrated that most of the labeled c-Fos neurons appeared rostrally around the motor trigeminal nucleus, wedged between the facial nerve and the superior olivary nucleus, and in the PCRt. Caudally, labeled c-Fos neurons were observed in the ventral PCRt and scattered within the MdD. In the C1, c-Fos neurons were found in laminae I to VI concentrated in lamina IV and V. In rats, some of these structures are pre-motor areas for reflex blinking.

In a previous neuroanatomical study on the eyelid movement during reflex blinking the SO nerve was stimulated and the facial motor nucleus injected with Gold-HRP (Zerari-Mailly et al. 2003). Double labeled neurons, which receive SO nerve input and both project to the facial motor nucleus, were found in the ponto-medullary reticular formation and dorsal horn of the C1. A summary diagram of the distribution pattern of these double labeled neurons is given in figure 7 (grey areas). When the injection sites comprised the ipsilateral dorsal facial motor nucleus, containing orbicularis oculi motoneurons, the double labeled neurons were found in three different reticular areas which might hence be part of the indirect pathway of the trigeminal blink reflex (Fig.1, pathways 2 and 3, Fig. 6 thick grey dashed lines). The first area was in the pontine reticular formation, rostral around fiber bundles of the VIIth cranial nerve and the trigeminal motor nucleus (Fig. 7, grey area, level 1-2). This pontine area near the trigeminal motor nucleus is similar to the pre-motor area found by Holstege et al. (1986[b]). In agreement with a study by Mogoseanu et al. (1994) monosynaptic projections were shown from the PCRt to facial motoneurons, confirming the idea of a specific eyelid control area in the pontine reticular formation of rats. The second reticular area was the caudal MdD in the medullary reticular formation. (Fig. 7, grey area, level 5 and 6). The third area was in C1 spinal cord, comprising the inner lamina IV and outer lamina V (Fig. 7, grey area, level 7).

In the present study, c-Fos immunohistochemistry following SO nerve stimulation and Gold-HRP injections in the SC were combined. The distribution pattern of c-Fos neurons which receive SO nerve input and project to the SC is given in figure 7 (hatched areas). Double labeled neurons were found in the medullary reticular formation, but rarely in the pontine reticular formation. In the medullary reticular formation two areas contained a population of double labeled neurons, the ventral PCRt (Fig.7, hatched area, level 3 and 4), the ventral MdD (Fig.7, hatched area, level 5), and more caudal the ventral as well as the dorso-lateral MdD (Fig.7, hatched area, level 6). Double labeled neurons were also found in laminae III-V of the C1 (Fig.7, hatched area, level 7).

Comparison of the distribution of c-Fos reticulo-facial and reticulo-collicular neurons shows that two neuronal populations can be distinguished within the PCRt. A rostral area, which projects to the facial motor nucleus (Fig. 6, level 1-2), confirms that the PCRt is involved in eyelid movements. An area in the ventrocaudal PCRt projects to the SC (Fig. 7, level 3-4), indicating that the PCRt is also involved in eye movements during blinking. Thus pre-motor areas of the eye and eyelid movement during reflex blinking are separated in the PCRt. The lack of a substantial number of neurons in the PCRt that both project to the SC and the facial motor nucleus and are innervated by the SO-nerve in the current study makes it unlikely that this area is a candidate for a common eye and eyelid movement generator.

Most c-Fos labeled neurons that project to the SC were found in the MdD, implying an important role for this area in the eye movement during the blink. In the rostral MdD, neurons projecting to the SC are

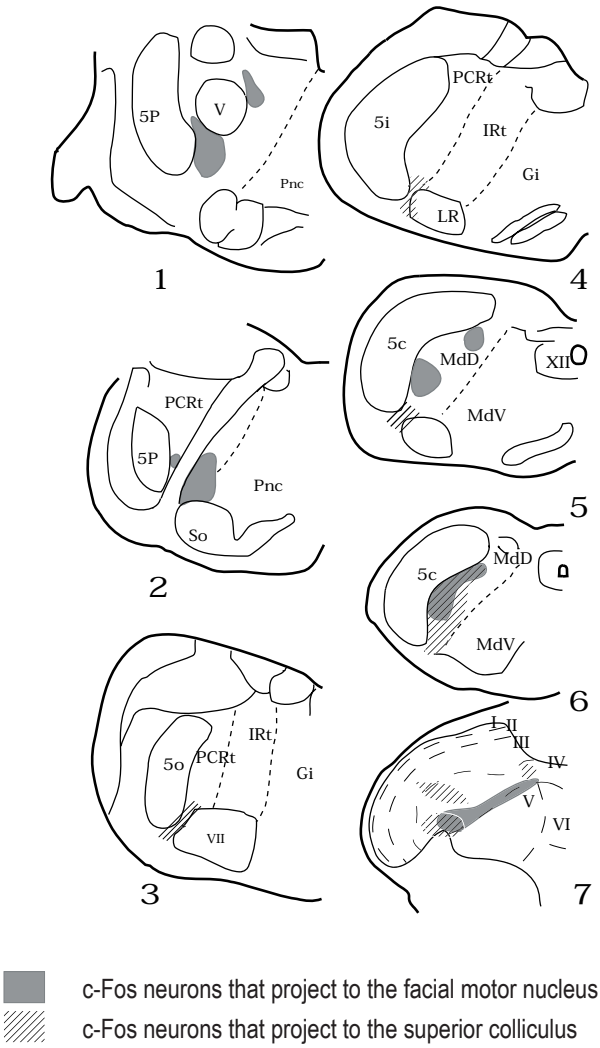


Figure 7. Summary diagram showing the pontomedullary reticular formation and rostral part of the cervical spinal cord (C1) from rostral level 1 to caudal level 7. The location of neurons that receive supraorbital nerve input and project to the facial motor nucleus are indicated by grey areas, the location of neurons projecting to the superior colliculus are indicated by hatched areas. Partial overlap of two areas is indicated; one in the dorsal medullary reticular nucleus and the other in laminae IV and V of the C1. Abbreviations: principal nucleus of the sensory trigeminal complex (5P), oral subnucleus of the sensory trigeminal complex (5o), interpolar subnucleus of the sensory trigeminal complex (5i), caudal subnucleus of the sensory trigeminal complex (5c), gigantocellular reticular nucleus (Gi), intermediate reticular nucleus (IRt), lateral reticular nucleus (LR), dorsal medullary reticular nucleus (MdD), ventral medullary reticular formation (MdV), parvocellular reticular nucleus (PCRt), caudal pontine reticular nucleus (Pnc), superior olivary nucleus (So), trigeminal motor nucleus (V), facial motor nucleus (VII), hypoglossal motor nucleus (XII).

located ventrally and those projecting to the facial motor nucleus are located dorsally (Fig.7, level 5). In the caudal MdD the neuron populations of the two studies overlap (Fig. 7, level 6). This overlapping area might be involved in motor control of the orbicularis oculi muscle as well as in eye movement control through the SC projection. Another possibility is that this area might be involved in the control system during saccadic movement. It has been suggested that blinking is linked to saccadic gaze shifts by a common premotor drive (Evinger et al. 1994) which might be comprised by this area.

The ventral MdD is a functionally complex and heterogeneous brain area which is involved in autonomic functions, motor reactions and pain responses (Cobos et al. 2003). This area encompasses main autonomic functions like regulation of cardio-vascular and respiratory functions. The caudal ventro-lateral medulla also projects to orofacial motor nuclei and participates in motor control through cerebellar and rubral connections (Jones 1995). A recent MRI study by Cruccu et al. (2005) on patients with brainstem lesions gave new insights into the roles of different structures during the early (R1) and late (R2) components of blinking. Most lesions affecting the R2 response were found in the dorso-lateral medulla at the level of the inferior olive. This area corresponds precisely with the area containing most double labeled neurons in the MdD in the current study.

A second overlapping area which contains neurons that are innervated by the SO nerve and project to the SC or facial motor nucleus is located in laminae IV and V of the C1 (Fig. 7, level 7). Like the MdD, this area might be involved in the coordination of eye and eyelid movement during blinking or even saccadic gaze shifts. The trigeminal system was proposed by Goossens and Van Opstal (2000[a,b]) as a candidate structure for saccade inhibition during reflex blinking, because the short latency with which a saccadic perturbation occurs indicates a short circuit. This pleads for a role of the non-overlapping hatched area in the C1 (Fig. 7, level 7) in saccadic control, as C1 is the caudal prolongation of the STC.

The C1 receives direct trigeminal eye blink input (Van Ham and Yeo 1996[b]) and is an important area for blink initiation and modulation, which can be demonstrated by suppression of corneal-evoked blinks through micro stimulation of the C1 area. (Cruccu et al. 2005; Henriquez and Evinger 2005). The C1, like the MdD, is also a relay centre in the R2 response; Pellegrini and co workers (1995) observed in guinea pigs that the R2 blink response, but not the R1 response, was eliminated after hemi-section at the level of the C1. As blink related C1 projections towards the SC were found in the current study, the C1 might, serve as an initiator of the eye movement accompanying the eyelid movement of the R2 component.

The distribution of reticulo-collicular and reticulo-facial neurons receiving input from SO nerve overlap in the MdD and the C1. This mixed group of reticulo-collicular and reticulo-facial neurons is a good candidate to constitute a “blink generator” regulating the precise timing of eye and eyelid movement. An anterograde tracing study in the rat which revealed projections from the red nucleus, *the* output nucleus of the cerebellum, towards the MdD, supports this hypothesis (Cobos et al., 2003). The generator might be composed of different premotor structures located in the brainstem; another possibility is one area stretching from the MdD to the C1. This area would correspond with the area described by Kimura and Lyon (1972) responsible for the R2 response, conducted through the descending spinal tract, in the lower part of the medulla oblongata. Future studies will have to reveal whether the overlapping areas described here in the caudal MdD and C1 are indeed common areas from where projections originate to facilitate both the eye and eyelid component of the blink reflex.

REFERENCES

Aramideh, M., Cruccu, G., Valls-Solé, J., Ongerboer de Visser, B.W., 2002. Cranial nerves and brainstem reflexes: electro-diagnostic techniques, physiology, and normative data. In: Brown, W.F., Bolton, C.F., Aminoff. M.J. (Eds.), Neuromuscular Function and Disease, vol. 1. Saunders, Philadelphia, pp 433-453.

Armstrong, R.C., Montminy, M.R., 1993. Transsynaptic control of gene expression. Annu. Rev. Neurosci. 16, 17-29.

Basso, M.A., Powers, A.S., Evinger, C., 1996. An explanation for reflex blink hyperexcitability in Parkinson's disease. I. Superior colliculus. J. Neurosci. 16, 7308-7317.

Bour, L.J., Aramideh, M., Ongeboer de Visser, B.W., 2000. Neurophysiological aspects of eye and eyelid movements during blinking in humans. J. Neurophysiol. 83, 166-176.

Cobos, A., Lima, D., Almeida, A., Tavares, I., 2003. Brain afferents to the lateral caudal ventro-lateral medulla: a retrograde and anterograde tracing study in the rat. Neuroscience 120, 485-498.

Collewijn, H., Van der Steen, J., Steinman, R.M., 1985. Human eye movements associated with blinks and prolonged eyelid closure. J. Neurophysiol. 54, 11-27.

Cruccu, G., Ferracuti, S., Leardi, M.G., Fabbri, A., Manfredi, M., 1991. Nociceptive quality of the orbicularis oculi reflexes as evaluated by distinct opiate- and benzodiazepine-induced changes in man. Brain Res. 556, 209-217.

Cruccu, G., Iannetti, G.D., Marx, J.J., Thoemke, F., Truini, A., Fitzek, S., Galeotti, F., Urban, P.P., Romaniello, A., Stoeter, P., Manfredi, M., Hopf, H.C., 2005. Brainstem reflexes revisited. Brain 128, 386-394.

Dauvergne, C., Ndiaye, A., Buisseret-Delmas, C., Buisseret, P., VanderWerf, F., Pinganaud, G., 2004. Projections from the superior colliculus to the trigeminal system and facial nucleus in the rat. J. Comp. Neurol. 478, 233-247.

Dragunow, M., Faull, R., 1989. The use of c-Fos as a metabolic marker in neuronal pathway tracing. J. Neurosci. Methods 29, 261-265.

Ellrich, J., Katsarava, Z., Przywara, S., Kaube, H., 2001. Is the R3 component of the human blink reflex nociceptive in origin? Pain 91, 389-395.

Evinger, C., Manning, K.A., Pellegrini, J.J., Basso, M.A., Powers, A.S., Sibony, P.A., 1994. Not looking while leaping: the linkage of blinking and saccadic gaze shifts. Exp. Brain Res. 100, 337-344.

Evinger, C., Shaw, M.D., Peck, C.K., Manning, K.A., Baker, K., 1984. Blinking and associated eye movements in human, guinea pigs and rabbits. J. Neurophysiol. 52, 323-339.

Gnadt, J.W., Lu, S.M., Breznen, B., Basso, M.A., Henriquez, V.M., Evinger, C., 1997. Influence of the superior colliculus on the primate blink reflex. Exp. Brain Res. 116, 389-398.

Goossens, H.H.M.L., Van Opstal, A.J., 2000[a]. Blink-perturbed saccades in monkey. I. Behavioral analysis. J. Neurophysiol. 83, 3411-3429.

Goossens, H.H.M.L., Van Opstal, A.J., 2000[b]. Blink-perturbed saccades in monkey. II. Superior colliculus activity. J. Neurophysiol. 83, 3440-3452.

Gruart, A., Blázquez, P., Delgado-Garcia, J.M., 1995. Kinematics of spontaneous, reflex, and conditioned eyelid movements in the alert cat. J. Neurophysiol. 74, 226-248.

Henriquez, M., Evinger, C., 2005. Modification of cornea-evoked reflex blinks in rats. Exp. Brain Res. 163, 445-456.

Hoffman, G.E., Lyo, D., 2002. Anatomical markers of activity in neuroendocrine systems: are we all fos-ed out? J. Neuroendocrin. 14, 259-268.

Holstege, G., Tan, J., Van Ham, J., 1986[a]. Anatomic observations on afferent projections orbicularis oculi and retractor bulbi motoneural cell groups and other pathways possibly related to the blink reflex in the cat. Brain Res. 374, 306-320.

Holstege, G., Van Ham, J.J., Tan, J., 1986[b]. Afferent projections to the orbicularis oculi motoneuronal cell group: an autoradiographical tracing study in the cat. Exp. Brain Res. 374, 321-334.

Hunt, S.P., Pini, A., Evan, G., 1987. Induction of c-Fos-like protein in spinal cord neurons following sensory stimulation. Nature 328, 632-634.

Jacquin, M.F., Renehan, W.E., Rhoades, R.W., Panneton, W.M., 1993. Morphology and topography of identified primary afferents in trigeminal subnuclei principalis and oralis. J. Neurophysiol. 70, 1911-36. Jones, B.E., 1995. Reticular formation: cytoarchitecture, transmitters, and projections. In: Paxinos. G. (Ed), The Rat Nervous System, 2nd ed. Academic Press, San Diego, pp. 155-171.

Kimura, J., Lyon, L.W., 1972. Orbicularis oculi reflex in the Wallenberg syndrome: alteration of the late reflex by lesions of the spinal tract and nucleus of the trigeminal nerve. J. Neurol. Neurosurg. Psychiatry 35, 228-233.

King, A.J. 2004. The superior colliculus. Curr. Biol. 14, R335-R338.

Kugelberg, E., 1952. Facial reflexes. Brain 75, 385-396.

Marfurt, C.F., Rajchert, D.M., 1991. Trigeminal primary afferent projections to "non-trigeminal" areas of the rat central nervous system. J. Comp. Neurol. 303, 489-511.

Ménétrey, D., 1985. Retrograde tracing of neural pathways with a protein-gold complex. I. Light microscopic detection after silver intensification. Histochemistry 83, 391-395.

Mogoseanu, D., Smith, A.D., Bolam, J.P., 1994. Monosynaptic innervation of facial motoneurons by neurons of the parvocellular reticular formation. Exp. Brain Res. 101, 427-438.

Morcuende, S., Delgado-Garcia, J.M., Ugolini, G., 2002. Neuronal premotor networks involved in eyelid responses: retrograde transneuronal tracing with rabies virus from the orbicularis oculi muscle in the rat. J. Neurosci. 22, 8808-8818.

Munglani, R., Hunt, S.P., 1995. Proto-oncogenes: basic concepts and stimulation induced changes in the spinal cord. Prog. Brain Res. 104, 283-298.

Ndiaye, A., Pinganaud, G., Buisseret-Delmas, C., Buisseret, P., VanderWerf, F., 2002. Organisation of trigeminocollicular connections and their relations to the sensory innervation of the eyelids in the rat. J. Comp. Neurol. 448, 373-387.

Paxinos, G., Watson, C., 1986. The Rat Brain in Stereotaxic Coordinates. 2nd ed. Academic Press, Sydney.

Pellegrini, J.J., Horn, A.K.E., Evinger, C., 1995. The trigeminally evoked blink reflex. I. neuronal circuits. Exp. Brain Res. 107, 166-180.

Sheng, M., Greenberg, M.E., 1990. The regulation and function of c-Fos and other immediate early genes in the nervous system. Neuron 4, 477-485.

Smit, A.E., Zerari-Mailly, F., Buisseret, P., Buisseret-Delmas, C., VanderWerf, F., 2005. Reticulo-collicular projections: a neuronal tracing study in the rat. Neurosc. Lett. 380, 276-279.

Sugimoto, T., 1998. c-Fos induction in the subnucleus oralis following trigeminal nerve stimulation. Brain Res. 183, 158-162.

Takayama, K., Suzuki, T., Miura, M., 1994. The comparison of effects of various anesthetics on expression of Fos protein in the rat brain. Neurosc. Lett. 176, 59-62.

VanderWerf, F., Brassinga, P., Reits, D., Aramideh, M., Ongerboer de Visser, B., 2003. Eyelid movements: Behavioural studies of blinking in humans under different stimulus conditions. J. Neurophysiol. 89, 2784-2796.

Van Ham, J.J., Yeo, C.H., 1996[a]. The central distribution of primary afferents from the external eyelids, conjunctiva, and cornea in the rabbit, studied using WGA-HRP and B-HRP as transganglionic tracers. Exp. Neurol. 142, 217-225.

Van Ham, J.J., Yeo, C.H., 1996[b]. Trigeminal inputs to eye blink motoneurons in the rabbit. Exp. Neurol. 142, 244-257.

Zerari-Mailly, F., Dauvergne, C., Buisseret, P., Buisseret-Delmas, C., 2003. Localization of trigeminal, spinal, and reticular neurons involved in the rat blink reflex. J. Comp. Neurol. 467, 173-184.

PART III

Blinking
and
Cerebellar pathology

CHAPTER 4

Deletion of FMR1 in Purkinje Cells Enhances Parallel Fiber LTD, Enlarges Spines, and Attenuates Cerebellar Eyelid Conditioning in Fragile X Syndrome

SUMMARY

Absence of functional FMRP causes Fragile X syndrome. Abnormalities in synaptic processes in the cerebral cortex and hippocampus contribute to cognitive deficits in Fragile X patients. So far, the potential roles of cerebellar deficits have not been investigated. Here, we demonstrate that both global and Purkinje cell-specific knockouts of Fmr1 show deficits in classical delay eyeblink conditioning in that the percentage of conditioned responses as well as their peak amplitude and peak velocity are reduced. Purkinje cells of these mice show elongated spines and enhanced LTD induction at the parallel fiber synapses that innervate these spines. Moreover, Fragile X patients display the same cerebellar deficits in eyeblink conditioning as the mutant mice. These data indicate that a lack of FMRP leads to cerebellar deficits at both the cellular and behavioral levels and raise the possibility that cerebellar dysfunctions can contribute to motor learning deficits in Fragile X patients.

INTRODUCTION

Fragile X syndrome is the most common, known monogenic cause of mental retardation (De Vries et al., 1997; Turner et al., 1996). Clinically the syndrome is characterized by mental retardation, hyperactive behavior, attention deficits, facial abnormalities, and macroorchidism (Hagerman and Hagerman, 2002). The gene involved is the Fragile X mental retardation 1 gene, FMR1, which contains in the 5# UTR region a polymorphic CGG repeat (Fu et al., 1991; Verkerk et al., 1991). In Fragile X patients, this repeat spans more than 200 CGG units, which, in turn, causes methylation of the promoter region of FMR1 and thereby functionally inactivates the gene. Due to inactivation of FMR1, its protein, FMRP, is absent in patients, while it is normally expressed in a panneuronal fashion (Verheij et al., 1993; Bakker et al., 2000).

A mouse model for Fragile X syndrome has been created by interruption of the mouse Fmr1 gene (The Dutch-Belgian Fragile X Consortium, 1994). This knockout mouse shows behavioral and cognitive abnormalities comparable to the symptoms found in Fragile X patients, and several of these symptoms can be linked to a dysfunction of a particular brain region. For example, their enhanced startle responses to auditory stimuli and their reduced freezing behavior in response to both contextual and conditional fear stimuli indicate a malfunction of the amygdala (Chen and Toth, 2001; Nielsen et al., 2002; Paradee et al., 1999). Similarly, the tendency of the knockout mice to show a deficiency in their ability to learn the position of a hidden escape platform in a water maze task suggests hippocampal dysfunction (D'Hooge et al., 1997; Dobkin et al., 2000). To date, a potential contribution of cerebellar dysfunctions to the deficits in Fragile X patients has not been elucidated.

The pathological cellular mechanisms that may underlie the cortical behavioral and cognitive deficits described above are probably related to dysfunctions at the level of dendritic spines and their input. The dendritic spines of pyramidal cells of both Fragile X patients and Fmr1 knockout mice are unusually long and irregular (Comery et al., 1997; Irwin et al., 2001; Rudelli et al., 1985). Since these spines appear morphologically immature, FMRP has been suggested to be involved in spine maturation and pruning, as well as in synaptogenesis (Comery et al., 1997). Indeed, FMRP and Fmr1 mRNA are present in spines and/or dendrites, and FMRP is translated in response to activation of the type 1 metabotropic

glutamate receptors (mGluR-1) in synaptoneurosomes (Weiler et al., 1997). The function of FMRP as an inhibitor of translation of bound mRNAs in vitro, including its own mRNA and that of proteins involved in microtubule-dependent synapse growth and function, indicates that FMRP may act as a regulator of activity-dependent translation in synapses (Brown et al., 2001; Li et al., 2001; Miyashiro et al., 2003). This possibility is supported by the finding that the induction of mGluR1-dependent long-term depression (LTD) is enhanced in pyramidal cells of the hippocampus in Fmr1 knockout mice (Huber et al., 2002). Thus, altered hippocampal LTD in Fragile X patients may interfere with normal formation and maintenance of synapses required for particular cognitive functions.

Metabotropic GluR1-dependent LTD, which appears to require rapid translation of mRNA, can also be induced at the parallel fibers (PF) to Purkinje cell (P cell) synapses in the cerebellum (Coesmans et al., 2003; Karachot et al., 2001). Absence of FMRP in cerebellar P cells could, therefore, similarly to the consequences of its absence in the pyramidal cells in the hippocampus, cause spine abnormalities in its dendrites, alter LTD induction at its PF inputs, and elicit abnormalities in motor learning behavior that specifically depends on intact cerebellar P cells, such as associative eyeblink conditioning, (Mauk and Donegan, 1997; Kim and Thompson, 1997; Yeo and Hesslow, 1998). Moreover, because the PF inputs to P cell spines compete to some extent with the climbing fiber (CF) input to their dendrites (Ichikawa et al., 2002), absence of FMRP may also affect the normal mono CF innervation of adult P cells. Thus, to investigate the possibility that cerebellar deficits contribute to Fragile X syndrome, we tested the cerebellar learning capabilities of global and P cell-specific Fmr1 knockout mice as well as those of Fragile X patients using classical eyeblink conditioning procedures. In addition, we investigated whether such behavioral deficits might be correlated to morphological and/or cell physiological abnormalities of the PF and CF input to P cells.

RESULTS

Eyeblink Conditioning Is Affected in Both Global *Fmr1* Null Mutants and Purkinje Cell-Specific *L7-Fmr1* Knockouts

To find out whether a lack of FMRP can cause deficits in cerebellar eyeblink conditioning, we first subjected global Fmr1 null mutant mice (n = 10) and wild-type littermates (n = 9), during four paired training sessions, to a classical eyeblink conditioning task (Koekkoek et al., 2002). The percentage of conditioned responses (CRs) in the global mutants was significantly reduced at sessions T2, T3, and T4 ($p < 0.05$, Student's t tests; $p < 0.005$, MANOVA) (Figure 1A). In addition, the global mutants showed significant deficits in both the peak amplitude and peak velocity of their CRs during training sessions T3 and T4, but not during sessions T1 and T2 (for both parameters at both T3 and T4, $p < 0.05$, Student's t tests) (Figures 1B and 1C). In contrast, the latencies to the onset and peak amplitude of the CRs were not significantly affected in the global null mutant (latency to onset: 109 ± 8 ms in mutants versus 102 ± 5 ms in wild-types; $p > 0.4$, Student's t test; latency to peak amplitude: 295 ± 14 ms in mutants versus 315 ± 15 ms in wild-types; $p > 0.3$, Student's t test). After two sessions of extinction, the percentages of CRs in both wild-types and global Fmr1 null mutants were significantly reduced (in both cases, $p < 0.01$, Student's t tests). Moreover, when the conditioned stimulus (CS) and unconditioned stimulus (US) were randomly paired, virtually no CRs were observed. Finally, to determine possible effects of a lack of FMRP on the eyeblink reflex itself, we further analyzed the kinetics of the unconditioned responses

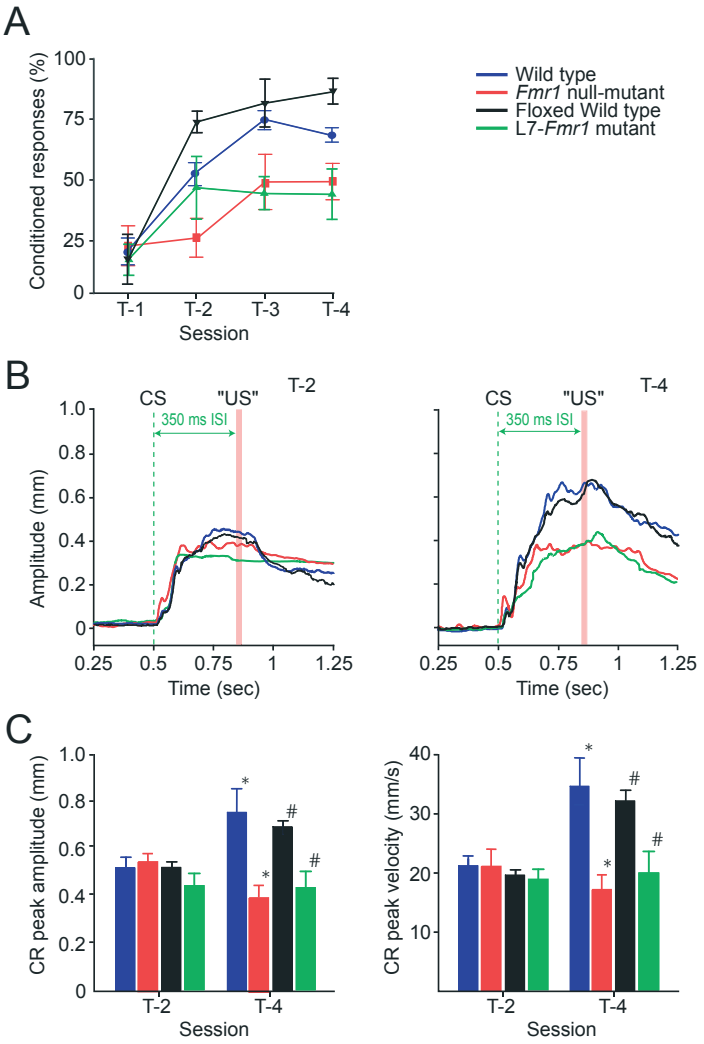


Figure 1. Eyeblink Conditioning Is Impaired in Both Global Fmr1 Null Mutants and P Cell-Specific L7-Fmr1 Mutants.
(A) Mean percentages (± SEM) of significant CRs over 4 days of paired training for global Fmr1 null mutants (red; n = 10) and wild-type littermates (blue; n = 9) as well as for L7-Fmr1 mutants (green; n = 7) and floxed controls (black; n = 8). These data show that Fmr1 mutants cannot improve the percentage of their CRs during the training as well as wild-types can. (B) Examples of data sets for training sessions T-2 (left) and T-4 (right), showing the average amplitude of CS-only responses of a representative global Fmr1 null mutant (red), wild-type littermate (blue), L7-Fmr1 mutant (green), and floxed control (black). Note that at T-4, both the wild-type animals and Fmr1 mutants show reasonably well-timed responses around the moment when the US is supposed to take place ("US"), while the sizes of the responses of the Fmr1 mutants remain fixed in amplitude over the training sessions. (C) Histograms showing average peak amplitudes and peak velocities of global Fmr1 null mutants (red), wild-type littermates (blue), L7-Fmr1 mutants (green), and floxed controls (black) at T-2 and T-4. In contrast to the Fmr1 mutants, wild-type animals show a significantly increased peak amplitude and peak velocity at T-4 (for all comparisons,*$p < 0.05$ and # $p < 0.05$), but not at T-2. Error bars indicate SEM.

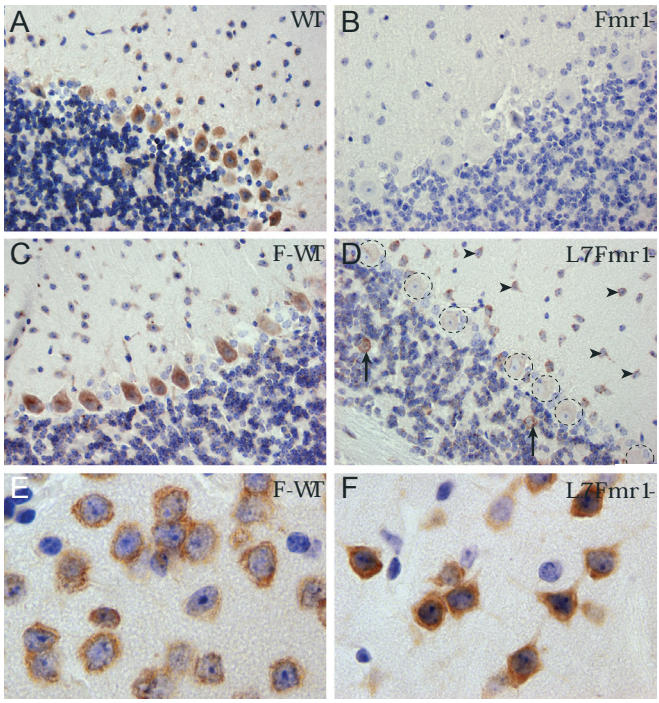


Figure 2. FMRP Expression in Wild-Type, Global Fmr1 Null Mutant, Floxed Control, and L7-Fmr1 Mutant Mice.
FMRP expression in wild-type (A), global Fmr1 null mutant (B), floxed control (C and E), and L7-Fmr1 mutant (D and F) mice, using immunoperoxidase staining on paraffin sections. Note in (D) that the P cells of the L7-Fmr1 mutant are not labeled (dashed circles), while Golgi cells (arrows) and stellate cells (arrowheads) are positively stained for FMRP in the same section. In contrast, in cerebellar sections of the global Fmr1 null mutant (B) or the wild-types (A and C), none or all of these types of neurons are labeled, respectively. In the cerebral cortex virtually all neurons were positively labeled both in the floxed wild-type (E) and the L7-Fmr1 mutant (F).

(URs). The amplitudes (0.68 ± 0.03 mm) and peak velocities (44.9 ± 2.07 mm/s) of the URs in the mutants were not significantly different from those (0.73 ± 0.03 mm and 47.3 ± 2.04, respectively) in wild-types ($p > 0.25$ for both comparisons; Student's t tests). Thus, it appears unlikely that differences in sensitivity to the US among Fmr1 null mutants and wild-types contribute to the differences in CRs. Because the eyeblink paradigm in mice is largely controlled by the cerebellum (Koekkoek et al., 2003), the data described above suggest that a lack of FMRP in cerebellar neurons is at least partly responsible for the behavioral deficits. We therefore investigated to what extent the abnormal conditioning behavior can be explained by a lack of FMRP specifically in P cells, which form the main site of integration for the PF and CF inputs and are the sole output of the cerebellar cortex.

P cell-specific Fmr1 knockout mice were created using crossbreedings of L7-cre mice and floxed Fmr1 mutants. Immunohistochemical analysis demonstrated that the L7-Fmr1 knockout mice did not express FMRP, while their surrounding neurons in the cerebellum, as well as neurons outside the cerebellum, did (Figure 2). Like the global Fmr1 null mutants the P cell-specific L7-Fmr1 mutants (n = 7) showed

a significantly reduced percentage of CRs on days T-3 and T-4 in comparison with both the wild-type littermates of the global Fmr1 mutants and their floxed controls (n = 8 for both sessions and both control groups; $p < 0.05$, Student's t tests) (Figure 1A). Moreover, the positive CRs showed significantly lower amplitudes and velocities on days T-3 and T-4 (for both parameters, $p < 0.05$, Student's t tests; Figures 1B and 1C), while the timing properties were unaffected (latency to onset: 115 ± 18 ms in mutants versus 111±8 ms in wild-types; $p > 0.7$, Student's t test; latency to peak amplitude: 296 ± 17 ms in mutants versus 302 ± 17 ms in wild-types; $p > 0.6$, Student's t test). In addition, the amplitudes and velocities of the URs did not differ from those in their controls ($p > 0.1$ for all comparisons; Student's t test). These data demonstrate that a lack of FMRP in P cells alone is sufficient to replicate the deficits in eyeblink conditioning described above for the global knockout.

Startle Responses Are Enhanced in Global Fmr1 Null Mutants but Not in Purkinje Cell-Specific L7-Fmr1 Knockouts

Since enhanced startle responses to auditory stimuli have also been associated with Fragile X syndrome (Chen and Toth, 2001; Nielsen et al., 2002), we also analyzed the initial 60 ms periods, following the onset of the tone, of the eyeblink responses in both the global Fmr1 mutants and P cell-specific L7-Fmr1 mutants, as well as in their controls. This analysis showed that during all training sessions the peak amplitudes of the startle responses in the global Fmr1 mutants, but not those in the P cell-specific L7-Fmr1 mutants, were significantly higher than those of wild-types (for all sessions T1–T4, $p < 0.001$, Student's t tests) (Figure 3). Moreover, the percentage of startle responses was significantly increased during all sessions in the global Fmr1 mutants, but not in the P cell-specific L7-Fmr1 mutants

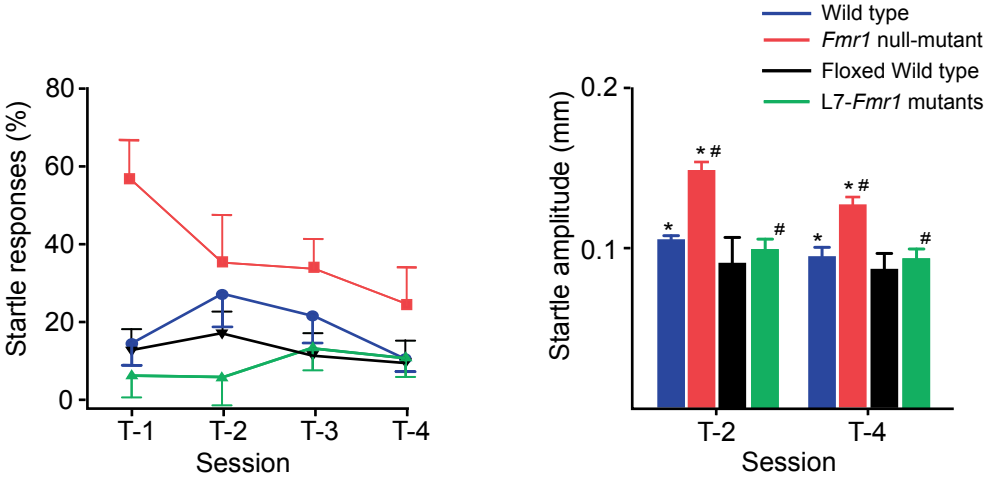


Figure 3. Startle Responses Are Enhanced in Global Fmr1 Null Mutants, but Not in P Cell-Specific L7-Fmr1 Mutants.
(Left panel) The percentage of startle responses in wild-types and mutants during the initial 60 ms period of the eyeblink responses. Note that this percentage was significantly increased during all sessions in global Fmr1 null mutants (red) as compared to wild-type littermates (blue), L7-Fmr1 mutants (green), and floxed wild-type controls (black). (Right panel) Peak amplitudes of the startle responses of Fmr1 null mutants were significantly higher than those of wild-type littermates, L7-Fmr1 mutants, and floxed controls ($p < 0.001$ [* and #]; Student's t tests). Error bars indicate SEM.

($p < 0.05$, MANOVA). These differences between the global and P cell-specific mutants suggest that a lack of FMRP in regions outside of the cerebellum can enhance the startle response, and therefore follows the notion that startle responses are controlled primarily by higher brain structures such as the amygdala (Paradee et al., 1999). In addition, these differences once again illustrate the sensitivity and specificity of the magnetic distance measurement technique (MDMT) recording method that we employ for eyeblink conditioning (Koekkoek et al., 2002; De Zeeuw et al., 2004).

LTD Is Enhanced in Purkinje Cells of both Global Fmr1 Mutants and L7-Fmr1 Mutants

Because deficits in eyeblink conditioning have been associated with cell physiological deficits of the PF-P cell synapse (Shibuki et al., 1996; Koekkoek et al., 2003), we investigated the induction of LTD and the efficacy of this synapse in slices of Fmr1 mutants. LTD was induced by conjunctively applying PF stimuli and depolarizing pulses (single pulses, 140 ms duration; from -70 to +10 mV) at 1 Hz for 5 min after reaching stable recordings of EPSCs during PF stimulation at 0.2 Hz for 10 min (Miyata et

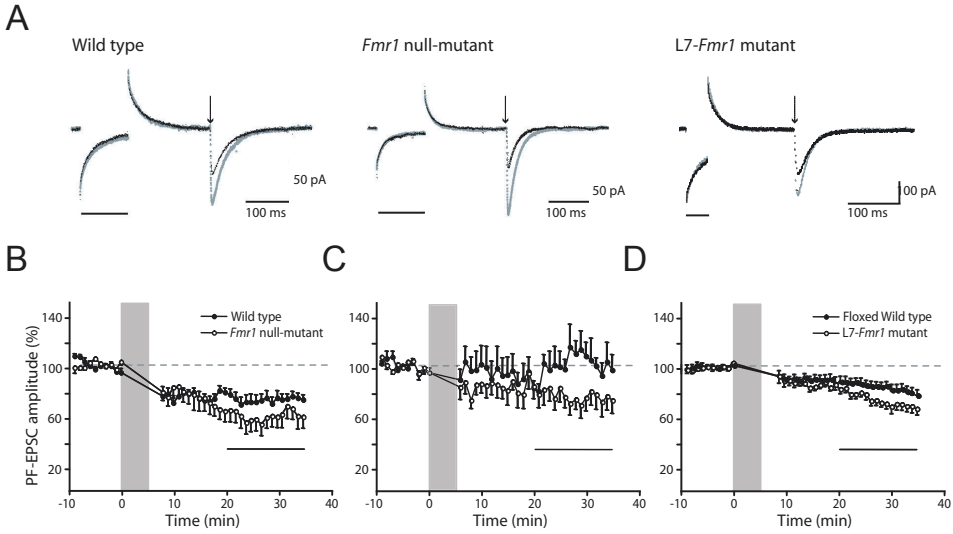


Figure 4. LTD Induction Is Enhanced in Purkinje Cells of Global Fmr1 Null Mutants and L7-Fmr1 Mutants.

(A) Superimposed PF-EPSCs in three of the P cells recorded before conjunctive stimulation and 33 min (wild-type), 31 min (global Fmr1 null mutant), or 32 min (L7-Fmr1 mutants) after conjunctive stimulation, each trace representing an average of 12 traces. Note the stronger reduction of the PF-EPSCs in the mutants. Horizontal bars indicate hyperpolarizing pulses (2 mV, 100 ms) used for monitoring access resistance and input resistance, while downward arrows indicate moments of PF stimulation. (B) PF-EPSC amplitude is plotted against time before and after conjunctive stimulation averaged for 10 cells from seven wild-type mice and 10 cells from six global Fmr1 null mutant mice. In each of these cells, 12 records successively acquired at 0.2 Hz were averaged to obtain PF-EPSC values for every minute. The shaded column indicates the period of conjunctive stimulation. (C) PF-EPSC amplitude is plotted against time for repetitive stimulation of PFs only in global Fmr1 null mutants. (D) PF-EPSC amplitude is plotted against time before and after conjunctive stimulation averaged for seven cells from seven floxed wild-type mice and six cells from six L7-Fmr1 mutant mice. In (B)–(D), vertical bars extending either upward or downward from the plotted points indicate SEM.

al., 1999). Figure 4A shows that this conjunctive stimulation induced LTD in both wild-type (n = 7) and global Fmr1 mutant mice (n = 6), as represented by a significant reduction in PF-EPSC (in both cases $p < 0.01$, Student's t tests). Hyperpolarizing pulse-evoked currents hardly changed in both wild types and global Fmr1 mutants ($p > 0.5$ in both cases, Student's t tests), implying that conjunctive stimulation does not affect access resistance, input resistance, or membrane capacitance. The change in access resistance after conjunctive stimulation was no more than 2% on average in the cells used for analyses (10 wild-type and 10 mutant cells). During the 15 min period from 21 to 35 min after the onset of conjunctive stimulation, the mean amplitude of the PF-EPSCs was reduced to 70.4% ± 1.3% in wild-types and to 60.7% ± 2.3% in global Fmr1 mutant mice. Thus, the induction of LTD in P cells of global Fmr1 null mutants was significantly enhanced compared with that in wild-types ($p < 0.01$; -Duncan's New Multiple Range Test) (Figure 4B). LTD was also induced using double shock stimulation of PFs at a 50 ms interval combined with the depolarizing pulse. The depression of PF-EPSC obtained with this double shock protocol in the global mutant mice was 72.5% ± 1.1% (n = 5), which was relatively modest. Nevertheless, it was still enhanced compared to the depression of 81.8% ± 0.9% (n = 4) ($p < 0.01$, ANOVA) obtained in wild-type mice with the same double shock protocol.

To find out whether the difference in LTD induction between wild-types and global mutants is specific to conjunctive stimulation, we also tested the effect of repetitive stimulation of PFs only at 1 Hz for 5 min. Indeed, this stimulus paradigm induced depression to 75.6% ± 2.5% of the baseline values in global Fmr1 mutants, while it did not cause a significant reduction in PF-EPSCs of P cells in wild-types. In these experiments too, the difference between wild-types and mutants was greatest 20 min after onset of the tetanus protocol. Thus, LTD induction following repetitive stimulation of PFs alone in global mutant mice is comparable to that following conjunctive stimulation in wild-type mice ($p > 0.05$, ANOVA), but smaller than that following conjunctive stimulation in global Fmr1 mutants ($p < 0.01$, ANOVA) (Figures 4B and 4C).

The differences in LTD induction between global mutants and wild-types raise the question of whether the general synaptic efficacy of the PF-P cell synapse is also affected in Fmr1 mutants. We therefore investigated the relationships between stimulation strength and PF-EPSC amplitude; paired-pulse facilitation of PF-EPSCs, facilitation, and fatigue of PF-EPSCs; maximum amplitude of metabotropic glutamate receptor type 1 (mGluR1)-dependent slow PF-EPSCs, thresholds, and maximum firing rate of Na+-spikes; and maximum Ca2+ current and density of voltage-dependent Ca2+ channels in P cells. None of these parameters differed between global Fmr1 mutants and wild-type littermates (see Table 1 in the Supplementary Data available with this article online). Thus, these control data suggest that the basic synaptic efficacy of the PF-P cell synapse is not affected in Fmr1 mutants.

Since the PF input to the dendrites of a P cell competes with its CF input (Cesa et al., 2003; Kakizawa et al., 2000), the affected level of PF-LTD in Fmr1 mutants may possibly be related to an abnormal CF input. We therefore investigated the strength and depression of CF-EPSCs, and we examined whether the global Fmr1 mutants suffer from a persistent multiple CF input (Kano et al., 1998). The CF-EPSCs in mutant P cells did not show any significant anomaly in that both the absolute strength and paired-pulse depression in null mutants (n = 7) were indistinguishable from those in wild-types (n = 7) ($p > 0.4$ and $p > 0.16$, respectively, Student's t tests). The paired-pulse depression of the CF-EPSCs was 81.7% ± 1.8% in P cells of the mutant mice compared with 77.5% ± 2.3% in the wild-type mice. In regard to the number of CF inputs per P cell, we found that 60.4% of the P cells (n = 48) in wild-types tested at

P21–48 showed a single-CF innervation, while double- and triple-CF innervations were observed in 35.4% and 4.2% of the cases, respectively. In mutant mice, single-, double-, and triple-CF innervations were observed in 75.6%, 22.2%, and 2.2% of the P cells (n = 45) tested, respectively. The percentage of single-CF innervation was in fact higher in the mutants than in the wild-types ($p < 0.01$, .2 test). Thus, the CF input to P cells in Fmr1 mutants does not show any sign of a pre- or postsynaptic deficit, and their development does not show any sign of a delay; in contrast, the normal development from multiple to mono-CF innervation is accelerated.

Finally, to investigate whether the enhancement in LTD induction is due solely to an intrinsic abnormality of P cells or whether it results from an interactive process between P cells and their surrounding neurons, we also investigated LTD of the PF-P cell synapse in cerebellar slices of the P cell-specific L7-Fmr1 mutants. Here too, conjunctive stimulation induced LTD in both the P cell-specific L7-Fmr1 mutant mice (n = 6) and the floxed controls (n = 7), as represented by a significant reduction in PF-EPSC (in both cases, $p < 0.01$, Student's t tests) (Figure 4D). Hyperpolarizing pulse-evoked currents changed minimally in both controls and mutants ($p > 0.35$ in both cases, Student's t tests), implying that conjunctive stimulation does not affect access resistance, input resistance, or membrane capacitance. The change in access resistance after conjunctive stimulation was no more than 3% on average in the cells used for analyses (seven control and six mutant cells). During the period from 21 to 35 min after the onset of conjunctive stimulation, the mean amplitude of the PF-EPSCs was significantly more reduced in L7-Fmr1 mutant mice (72.9% ± 1.3%) than in controls (83.2% ± 0.8%) ($p < 0.01$; Duncan New Multiple Range Test). Thus, the enhancement in LTD induction found in global Fmr1 mutants was also found in P cell-specific L7-Fmr1 mutants, indicating that the difference with the wild-types can indeed be attributed to an intrinsic effect of the P cells themselves.

Morphology of Purkinje Cells

The finding that P cells in the cerebellum of Fmr1 mutants show an enhanced level of LTD induction at the PF input to their dendritic spines raises the question of whether the dendritic tree of P cells in Fmr1 mutants shows morphological abnormalities. As revealed by both calbindin immunocytochemistry and intracellular labeling with biotinylated dextran amine (BDA), the dendrites and axons of P cells of Fmr1 null mutants appeared normal at the light microscopic level (Figure 5). The ramifications of the dendrites were not significantly different ($p > 0.6$, Student's t test) when analyzed with topological analyses for symmetry of arborizations (Van Pelt et al., 1992). The spine densities of distal dendrites with an average diameter smaller than 1.5 μm were 1.22 ± 0.30 spines/μm (mean ± SD) and 1.18 ± 0.27 spines/μm (mean ± SD) in Fmr1 null mutants (n = 7) and wild-types (n = 7), respectively. Likewise, the spine density in dendritic fragments with an average diameter bigger than 1.5 μm (proximal category) was 1.26 ± 0.27 spines/μm (mean ± SD) in Fmr1 null mutants and 1.22 ± 0.20 spines/μm (mean ± SD) in wild-types. Thus, unlike the pyramidal cells in cerebral cortical areas, the spine density of cerebellar P cells in Fmr1 null mutants did not differ significantly from that in their wild-type littermates (distal versus distal, $p > 0.5$; proximal versus proximal, $p > 0.5$; total versus total, $p > 0.5$; Student's t tests).

In contrast, the shape of the spines of P cells in Fmr1 null mutants differed from that in wild-types. Electron microscopic analysis of calbindin-stained P cells showed that their spines were more irregular and longer (Figure 6A). The average lengths of the spine head and spine neck in Fmr1 null mutants (0.56 ± 0.05 μm and 0.63 ± 0.18 μm, respectively; n = 4) were significantly greater than those in wild-types (0.50 ± 0.04 μm and 0.46 ± 0.15 μm, respectively; n = 4) ($p < 0.05$ and $p < 0.001$ for heads

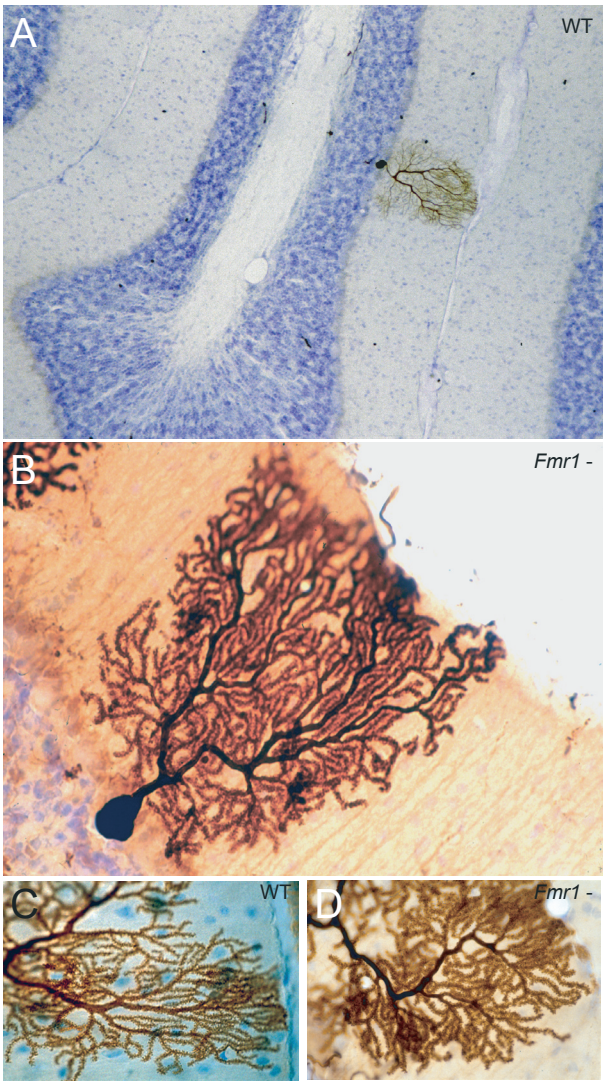


Figure 5.
Dendritic Arborization of the Purkinje Cells in Global Fmr1 Null Mutants Is Normal.
Light microscopic images of the dendritic trees of P cells in wild-types (A and C) and Fmr1 null mutants (B and D) that are retrogradely labeled with BDA. Both the topology of the P cell dendrites and the density of their spines appear normal.

and necks, respectively; Student's t tests). The spine head diameter, spine head length/ spine head diameter ratio, average spine neck diameter, and minimal spine neck diameter of Fmr1 null mutants were not significantly different from those of wild-types (for all parameters, $p > 0.2$, Student's t tests). Finally, electron microscopic analyses of the spine densities did not reveal any difference, either, between mutants and wild-types ($p > 0.6$; Student's t test).

To find out whether the differences in lengths of spine heads and necks were due to intrinsic changes of the P cells rather than an interaction with the environment, we also investigated the P cell spines in the L7-Fmr1 mutant mice at the ultrastructural level (Figure 6B). The average lengths of the spine head and spine neck in L7-Fmr1 mutants (0.57 ± 0.07 µm and 0.70 ± 0.1 µm, respectively;n=4) were significantly greater than those in the floxed controls (0.45 ± 0.05 µm and 0.42 ± 0.08 µm, respectively; n=4) ($p< 0.05$ and $p < 0.02$ for heads and necks, respectively; Student's t tests) (Figure 6C). No

differences were observed in the densities of the spines ($p > 0.4$; Student's t test). Thus, similar to the global mutants, the P cell-specific L7-Fmr1 mutants showed longer spines in which the necks were particularly elongated, while the number of spines appeared normal.

Eyeblink Conditioning Following Lesions of Cerebellar Nuclei in Trained Animals

The eyeblink data of the Fmr1 mutants indicate that the output of the cerebellum to a large extent controls the conditioning process. However, since the mutants do not express FMRP in their P cells in early development or thereafter, we cannot exclude the possibility that secondary developmental

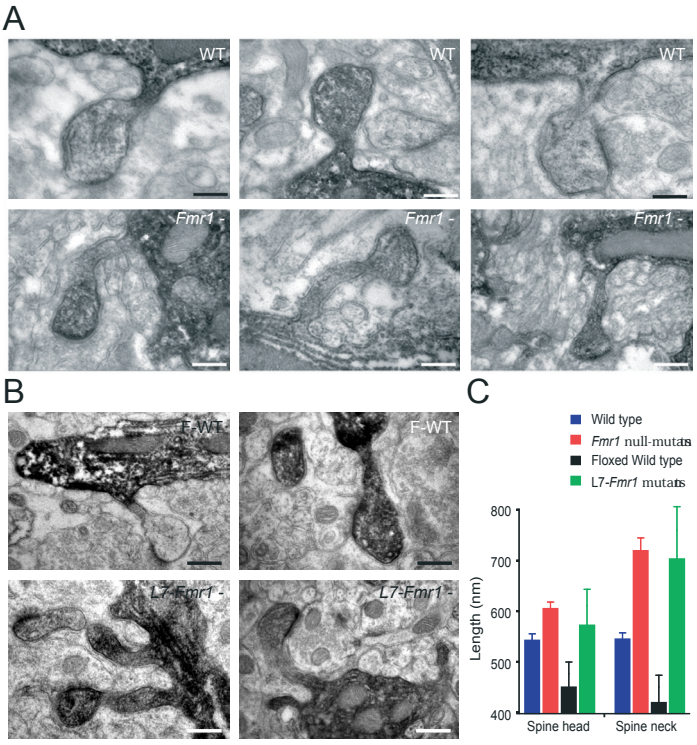


Figure 6. Ultrastructural Characteristics of Purkinje Cell Spines in Fmr1 Null Mutants and L7-Fmr1 Mutants Are Abnormal.

(A) Electron microscopic images of the morphology of individual P cell spines in wild-types (WT; top panels) and global Fmr1 null mutants (Fmr1-; bottom panels) that are labeled following immunocytochemistry with an antibody against calbindin. Note the longer and more irregularly shaped spines in Fmr1 null mutants. Scale bars in micrographs of the wild-types represent 271 nm, 283 nm, and 260 nm, respectively (left to right). Scale bars in micrographs of the Fmr1 mutants represent 297 nm, 309 nm, and 321 nm, respectively (left to right).

(B) Electron micrographs of individual P cell spines in floxed wild-types (F-WT; upper panel) and P cell-specific L7-Fmr1 mutants (L7-Fmr1-; bottom panel) that are labeled following calbindin immunocytochemistry. Scale bars in micrographs of the floxed wild-types represent 279 nm and 257 nm, respectively (left to right). Scale bars in micrographs of the L7-Fmr1 mutants represent 307 nm and 342 nm, respectively (left to right).

(C) Histograms of average lengths (+SD) of spine heads and spine necks in Fmr1 mutants (n = 194), wild-type littermates (n = 204), L7-Fmr1 mutants (n = 124), and floxed wild-type controls (n = 113).

aberrations downstream of the P cells do occur. This possibility may be especially valid for the global mutants, because the neurons involved in the eyeblink pathway downstream of the P cells also lack FMRP. We therefore investigated the change in CRs in the global Fmr1 mutants (n = 4) and in their wild-type littermates (n = 4) after bilateral lesions of the anterior interposed nuclei, which form the ultimate cerebellar output mediating control signals for eyeblink responses (Yeo and Hesslow, 1998;

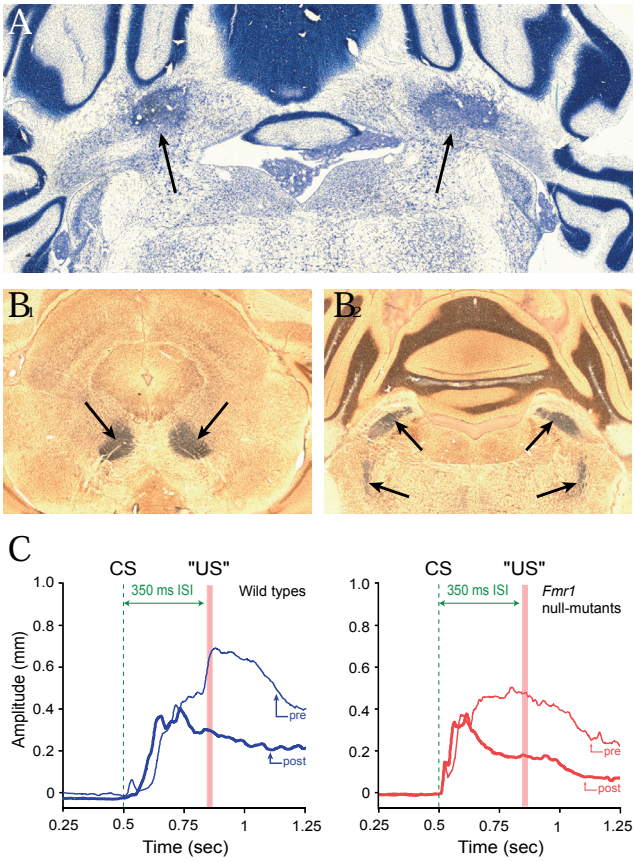


Figure 7.
Bilateral Lesions of Anterior Interposed Nuclei Lead to Relatively Comparable Eyeblink Traces in Trained Wild-Types and Fmr1 Null Mutants.
(A) Example of bilateral lesion (arrows) of anterior interposed nuclei in Nissl-stained section of Fmr1 null mutant.
(B) Example of degenerated axonal fibers (silver staining is indicated by arrows) in the superior cerebellar peduncles (left) and ipsilateral descending tracts (right); the latter are indicative for lateral damage to the anterior interposed nucleus (Teune et al., 2000).
(C) Traces showing the average amplitudes of the CRs in wild-types (left, blue) and Fmr1 null mutants (right, red) before (thin line) and after (thick line) the lesions.

Koekkoek et al., 2003). Figures 7A and 7B show an example of such lesions in an Fmr1 mutant and their impact on the number of degenerating fibers in the superior cerebellar peduncles and the ipsilateral descending tracts, which are indicative for abundant damage in the anterior interposed nuclei (Teune et al., 2000). Following such lesions in trained wild-types and trained global Fmr1 mutants, the percentages of CRs were reduced by 36% ± 11% and 21% ± 6%, respectively. Both changes were significant ($p < 0.01$ and $p < 0.05$, respectively; Wilcoxon rank-sum test). In addition, the remnant responses showed a reduction in amplitude (for wild-types and global Fmr1 mutants: 42% ± 9% and 25% ± 8%, respectively) and latency to peak amplitude (for wild-types and global Fmr1 mutants: 35% ± 1% and 30% ± 8%, respectively) (Figure 7C). Both changes were significant for both wild-types and Fmr1 mutants (for all comparisons, $p < 0.05$, Wilcoxon rank-sum tests). While percentages of CRs and CR amplitude values were significantly different between wild-types and null mutants after training session T-4 before the lesion ($p < 0.05$ and $p < 0.05$, respectively; Student's t tests), these differences

disappeared after the lesions (p = 0.25 and p = 0.3, respectively; Student's t tests). The results of these experiments confirm that the main differences in eyeblink conditioning parameters, such as the changes in peak amplitude and peak velocity that we observed between unlesioned Fmr1 mutants and

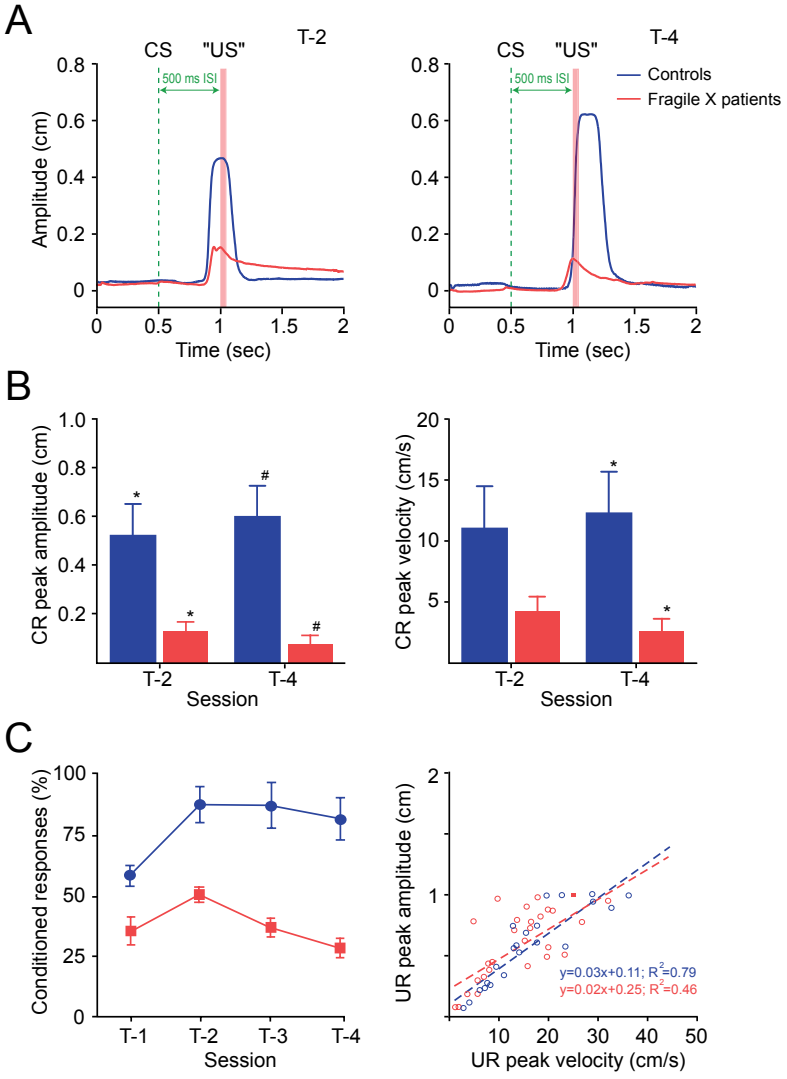


Figure 8. Eyeblink Conditioning Is Impaired in Fragile X Patients.
(A) Examples of data sets for training sessions T-2 (left) and T-4 (right), showing the average amplitude of CS-only responses of a control subject (blue) and a Fragile X patient (red).
(B) Histograms showing average peak amplitudes (left) and peak velocities (right) for all tested controls (n = 6) and Fragile X patients (n = 6) at T-2 and T-4. * indicates p < 0.05 and # indicates p < 0.005. (C) While the mean percentages (± SEM) of CRs in control subjects are significantly higher than those in Fragile X patients after each of the four training sessions (T1–T4; left), the kinetics of their unconditioned responses are indistinguishable from each other (right). Error bars indicate SEM.

their wild-type littermates, are largely due to a direct difference in cerebellar control; in addition, they suggest that these differences are not due to secondary developmental aberrations downstream of their P cells.

Eyeblink Conditioning in Fragile X Patients

The data described above indicate that an animal model of Fragile X syndrome shows deficits in eyeblink conditioning and that this deficit is probably due largely to a lack of FMRP in cerebellar P cells. To find out whether a lack of functional FMRP in humans leads to the same deficits in cerebellar motor learning, we tested affected males (n = 6) and controls (n = 6), using an eyeblink conditioning task in which the eyelids are conditioned to a tone. The patients showed severe deficits (Figure 8). The peak amplitude (0.14 ± 0.03 cm) and peak velocity (3.3 ± 0.7 cm/s) in affected Fragile X males were, on average, significantly lower ($p < 0.001$ and $p < 0.001$, respectively; Student's t test) than those in normal subjects (0.54 ± 0.06 cm and 10.0 ± 1.5 cm/s, respectively). When separated according to training session (T1, T2, T3, and T4), peak amplitudes in Fragile X patients were significantly smaller than those in controls after T2, T3, and T4 (for T2 and T3, $p < 0.05$; for T4, $p < 0.005$; Student's t tests), while peak velocities in Fragile X patients were significantly smaller after T3 and T4 (in both cases, $p < 0.05$, Student's t tests).

In addition, Fragile X patients showed a robust decrease in the number of CRs that were acquired during and after training (Figure 8C). The percentage of CRs was on average 80.3% ± 4.0% in control subjects and 37.6% ± 2.7% in affected males. For all sessions, the differences in the percentage of CRs were significant (T1, $p < 0.005$; T2, T3, and T4, $p < 0.001$; Student's t tests). In contrast, neither the average onset latency (0.87 ± 0.05 s) nor the average latency to peak amplitude of the CRs (0.95 ± 0.06 s) in affected males ($p = 0.29$ and $p = 0.30$, respectively; Student's t tests) differed from those in controls (0.92 ± 0.02 s and 1.02 ± 0.03 s, respectively). UR kinetics were analyzed to check for possible deficits in reflex pathways that may contribute to reduced motor learning (Figure 8C). Neither the mean amplitude (0.59 ± 0.05 cm) nor the mean velocity (13.4 ± 1.5 cm/s) of the responses in Fragile X patients differed from those in controls (0.57 ± 0.06 cm and 15.9 ± 2.0 cm/s, respectively) ($p = 0.88$ and $p = 0.31$, respectively; Student's t tests). Finally, when we subjected Fragile X patients and controls to randomly paired training paradigms, no CRs were observed. From these data, we conclude that Fragile X patients show the same deficits in eyeblink conditioning as those that we observed in the animal models of Fragile X.

Modeling Deficits in Eyeblink Conditioning Associated with Enhanced LTD

The observation that Fmr1 mutants show both deficits in eyeblink conditioning and enhanced PF LTD raises the question of whether these two factors are causally related. Such a relationship seems counterintuitive because PF LTD is assumed to form an important memory trace for cerebellar conditioning (Koekkoek et al., 2003; Mauk and Donegan, 1997; Yeo and Hesslow, 1998). We therefore investigated whether enhanced LTD can lead to a diminished eyeblink response in a model (Figure 9). The model that we created is focused on the impact of P cells on cerebellar nuclei neurons and is based on the following assumptions: (1) during its time course, the CS activates consecutively different sets of granule cells (Medina and Mauk, 2000); (2) for each activated granule cell, the strength of LTD depends on the length of the time interval to the CF stimulus (Wang et al., 2000); (3) LTD at a PF synapse will decrease the response of a P cell to that PF input; (4) fast release from inhibition

drives cerebellar nuclei neurons effectively through postinhibitory rebound (Aizenman and Linden, 1999, 2000); (5) the strength of the response of a cere bellar nucleus neuron will be determined not only by instantaneous changes in firing rate of the afferent P cells but also by the steady-state level of the activity of cerebellar nuclei neurons (a depolarization of the average resting membrane potential of cerebellar nuclei neurons can, by diminishing deinactivation of T-type Ca2+ channels, reduce the number of neurons that are available for postinhibitory rebound); (6) increased LTD in Fragile X results in reduced P cell activity and decreased inhibition of cerebellar nuclei neurons at the resting level; and (7) the motor response during a CR is determined by the instantaneous firing rate of a subpopulation of neurons in the cerebellar nuclei (Gruart et al., 1997). The details and formulas of the model are presented in the Supplemental Data (see the Supplementary Data available with this article online) and outlined in Figure 9. In short, the model demonstrates that a change in balance between excitation and inhibition in the cerebellar nuclei neurons, resulting from enhanced LTD at the PF-P cell synapse, may cause a paradoxical impairment of the CR. The primary mechanism is exhaustion of the pool of cerebellar nuclei neurons capable of producing postinhibitory rebound when the CS relieves the cerebellar nuclei neurons of P cell inhibition.

DISCUSSION

The present study shows that cerebellar abnormalities in Fragile X syndrome can occur at the morphological level, cell physiological level, and behavioral level. We found that a lack of FMRP results in a unique phenotypical combination of elongated P cell spines, enhanced LTD at the PFs that innervate these spines, and an impaired motor learning capability that is controlled by P cells. This unique combination reveals not only the extent to which cerebellar deficits may contribute to abnormalities in Fragile X syndrome but also possible clues about cerebellar function in general. The abnormalities of dendritic spines that we observed in cerebellar P cells of both the global and cell-specific Fmr1 null mutants mimic only partially those that have been described for pyramidal cells in the cerebral cortex (Comery et al., 1997; Hinton et al., 1991; Irwin et al., 2002).

They follow the same pattern in the morphology of individual spines appearing as immaturely shaped processes with elongated necks and heads, but they differ in that their spine density is normal. Apparently, the density of spines in P cells is more tightly regulated by compensatory mechanisms than that in pyramidal cells is. The spine density in P cells is largely subject to a well-regulated process in which the CFs and PFs compete with each other for specific sites at the dendritic tree (Cesa et al., 2003; Kakizawa et al., 2000). It is therefore attractive to hypothesize that the accelerated elimination of multiple CF inputs that we found in our electrophysiological recordings of Fmr1 null mutants reflects a mechanism that compensates for a slowdown in spine maturation. Such a view is supported by recent data obtained by Strata and colleagues, who showed that at least two different mechanisms are responsible for spine density and spine pruning in P cells, i.e., one dependent on activity in the CFs and another one that is activity-independent (Bravin et al., 1999; Strata et al., 2000).

One of our major findings is that a lack of FMRP leads to enhanced PF LTD without affecting the basic electrical properties of P cells. Interestingly, this difference between Fmr1 mutants and their wild-type controls, which has not been described for any other cerebellar mutant before, occurs about 15 min after the offset of conjunctive stimulation of the PFs and CFs or about 15 min after repetitive

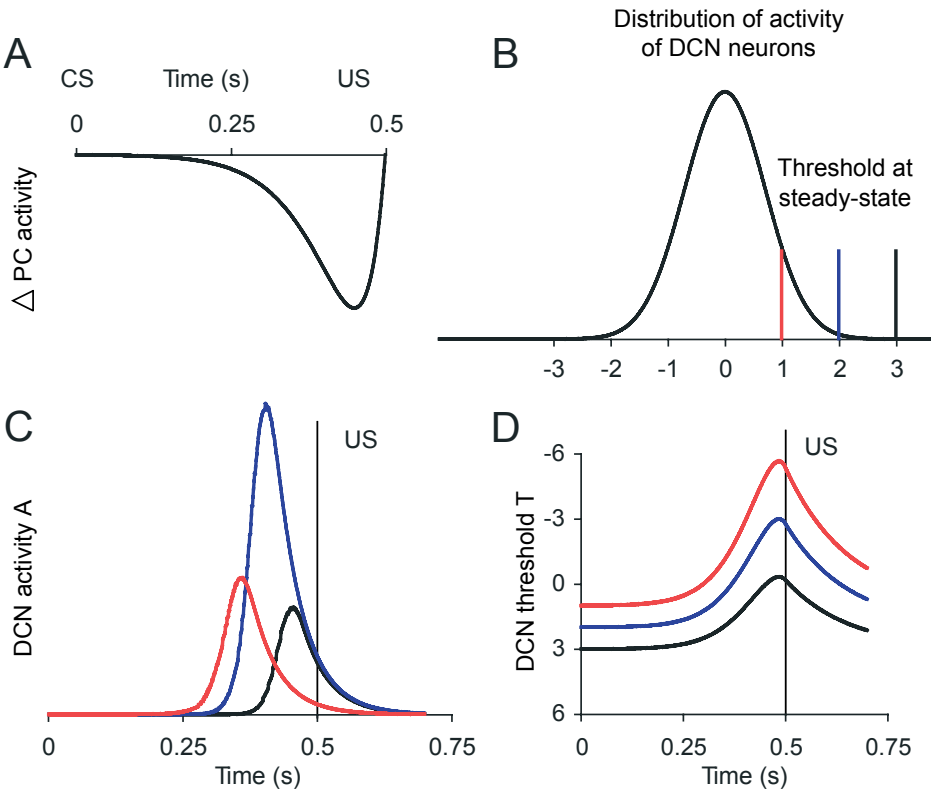


Figure 9. Mathematical Model Illustrating a Critical Sensitivity of the CR on the Steady-State Activity of P Cells (PCs) and Deep Cerebellar Nuclei (DCN) Neurons.

(A) Time course of the presumed decrease in PC activity during CS presentation. This curve reflects the dependency of LTD on the relative timing of spikes in parallel and CFs during training (Wang et al., 2000).

(B) Representation of the level of excitation in the population of DCN neurons. Excitation is distributed as depicted by the standard normal curve. The blue vertical line indicates the steady-state position of the firing threshold before CS presentation; changes in PC activity cause the threshold to move leftward (disinhibition) or rightward (inhibition). Subthreshold neurons are able to produce a rebound burst when disinhibited. The leftward-shifted steady-state threshold (red line, Fragile X) indicates the increased level of excitation that enhanced LTD is predicted to produce in the cerebellar nuclei (see Kenyon et al., 1998); the black-line threshold represents the case of a decreased steady-state excitation, due to decreased LTD or intrinsic excitability.

(C) Simulated rebound DCN activity during presentation of the CS. The decrease in PC activity (depicted in [A]) drives the threshold leftward, causing disinhibited neurons to produce a rebound response. The response is clearly strongest when starting from an optimal steady state (blue curve, wild-type). Both enhanced (red curve, Fragile X) and reduced (black curve) levels of steady-state DCN excitation reduce the response amplitude.

(D) Time evolution of the threshold (upward corresponding to disinhibition) during the responses computed in (C).

stimulation of PFs alone. This period directly follows the critical time period during which the presence and expression of one or more rapidly turned over protein(s) is/are required to induce LTD (Karachot et al., 2001). Thus, since FMRP can operate as a negative regulator of mRNA translation (Laggerbauer et al., 2001), one may assume that FMRP probably normally inhibits the translation of at least one of the proteins that is required for the expression of PF LTD 15 min after its induction. Similar time frames have been found for the impact of a lack of FMRP on the induction of LTD at the CA3-CA1 synapse in the hippocampus (Huber et al., 2002).

Based on their recordings in hippocampal slices, Bear and colleagues proposed a model in which they suggest that FMRP serves to limit expression of homosynaptic LTD by inhibiting mGluR-dependent translation of local synaptic mRNAs that are involved in the stabilization of endocytosed AMPA receptors. Because PF LTD is also driven by activation of metabotropic glutamate receptors (Coesmans et al., 2003) and because PF LTD is ultimately also expressed as an endocytosis of AMPA receptors (Xia et al., 2000; Linden, 2001), their hippocampal model may also be applicable to cerebellar P cells. Considering the common specificity of the electrophysiological effects in both the hippocampus and cerebellum in that a lack of FMRP causes enhanced homosynaptic LTD without affecting basic electrophysiological properties, one would expect that the specific behavioral consequence of such a unique defect is prominently present. Unfortunately, the hippocampal deficits that can be observed in Fmr1 null mutants subjected to spatial learning tests are partially controversial (see e.g., D'Hooge et al., 1997; Dobkin et al., 2000; Van Dam et al., 2000).

Here, we show that when subjected to an associative eyeblink test, which allows us to detect deficits specific for cerebellar motor learning, these global Fmr1 null mutants do have a robust phenotype and that the same behavioral phenotype can be observed in P cell-specific L7-Fmr1 mutants as well as in Fragile X patients themselves. All of them showed significantly less CRs, and they were all unable to increase the peak amplitude and peak velocity of their CRs during the training. In contrast, the latency to peak amplitude of the CRs was not significantly affected, indicating that learning-dependent timing is not severely impaired by a lack of FMRP. In this respect, the phenotype of LTD-enhanced Fmr1 mutants diverges from that of LTD-deficient mutants. Transgenic mice in which PF LTD is selectively blocked by P cell-specific expression of an inhibitory peptide against multiple isoforms of protein kinase C (De Zeeuw et al., 1998) cannot adjust the timing of their CRs to the moment of onset of the US (Koekkoek et al., 2003). On the other hand, these LTD-deficient mice show, like the Fmr1 mutants, a reduced percentage of CRs, and they are also unable to increase the peak amplitude and peak velocity of their CRs during the training. Thus, while the existence of PF LTD may be qualitatively necessary for the occurrence of learning-dependent timing of CRs, the exact level of PF LTD may be quantitatively responsible for the amount of CRs. Perhaps there is a level of expression of PF LTD that is optimal for attaining a maximum level of learned responses. Our model suggests that the CR may be impaired if the average level of the activity of cerebellar nuclei neurons is at a nonoptimal steady-state due to decreased inhibition by P cells. A mathematical model of cerebellar learning (Kenyon et al., 1998) predicted that enhanced LTD at the PF-P cell synapse would cause compensatory changes in the entire cerebelloolivary feedback loop. More particularly, an increased steady-state activity of cerebellar nuclei neurons would be needed to restore the balance between LTD and long-term potentiation (LTP) and to stabilize the weights of the PF-P cell synapses at nonsaturating values (see also Coesmans et al., 2004). The present model suggests that an altered steady-state level of the activity of cerebellar nuclei neurons may, in addition, impair the expression of the CR. Interestingly, the cerebellum may

not be the only brain region in which an optimal rather than a maximum level of cellular plasticity is necessary for effective learning behavior. Several studies have demonstrated that a relatively mild enhancement of LTP induction in the hippocampus can be associated with impaired fear conditioning or spatial learning (Gu et al., 2002; Migaud et al., 1998).

Due to the unique aberration of enhanced LTD in Fmr1 mutants and due to the unique combination of their deficits in classical conditioning, we have not only provided suggestive evidence for the potential importance of an optimal, instead of a maximum, level of PF LTD for cerebellar motor learning, but we have also shown that cerebellar deficits may be associated with learning deficiencies in Fragile X syndrome. Over the past decade, research on the potential roles of the cerebellum in cognitive processes has shown a remarkable advent. Investigations vary from transneuronal tracing studies, showing robust reciprocal and topographic connections between the cerebral and cerebellar cortex via the pons and thalamus (Kelly and Strick, 2003; Middleton and Strick, 1994), to clinical and neuropsychological studies, showing cognitive dysfunctions following cerebellar lesions (Leiner et al., 1993), and imaging studies, showing cerebellar activities correlated with cognitive activities (Kim et al., 1994; Vokaer et al., 2002). Thus, while a lack of FMRP in areas such as the cerebral cortex, amygdale, and hippocampus may induce cognitive symptoms in Fragile X syndrome, the current data allow us to conclude that a lack of functional FMRP in cerebellar P cells may equally well lead to deficits in motor learning in Fragile X patients.

EXPERIMENTAL PROCEDURES

Eyeblink Conditioning in Mice

Wild-type and Fmr1 mutant mice were prepared for eyeblink conditioning according to the MDMT procedure as described by Koekkoek et al. (2002). In short, mice were anesthetized, using a mixture of nitrous oxide and halothane, and a premade connector was placed on the skull. A sensor chip linked to the connector was placed over the upper eyelid, while a magnet was attached to the lower eyelid. Mice were subjected to either a paired or a randomly paired procedure in four sessions. During one session, the subject received 64 trials grouped in 8 blocks. The trials were separated by a random intertrial interval (ITI) ranging from 20 s to 40 s. In the procedure of paired training, each block consisted of one US-only trial, six paired trials, and one CS-only trial. After four sessions of paired trainings the subject was allowed to rest for 1 day, followed by two sessions of extinction. In the extinction procedure, each block consisted of one US-only trial and seven CS-only trials. In the randomly paired procedure, the US occurred randomly in the ITI, while the CS was given as described in the paired trials. In the analyses of the eyelid movements, we considered a movement as a significant eyelid response when its amplitude was greater than the mean + 3 SDs of the amplitude of the movements that occurred in the 500 ms period before the onset of the CS. Such a response was considered to contain a startle response when movement occurred within the 60 ms period directly after the onset of the CS; when significant movement occurred after this period, it was considered a CR.

Cell Physiology

Mice were anesthetized with ether and decapitated (for details, see Llano et al., 1991). The cerebellum was excised, and slices were prepared from the vermis. The recording chamber was perfused with

oxygenized saline containing 100 μM picrotoxin. Recordings of P cells were obtained at 31.0°C, using an upright Nikon or Zeiss microscope, and whole-cell patchclamp recordings were obtained with the use of borosilicate pipettes (resistance, 3–5 MΩ). Membrane current was recorded with a Multiclamp700A amplifier (Axon), while stimulation and online data acquisition were performed using pClamp 9 software (Axon). PFs and CFs were focally stimulated by applying pulses through glass pipettes positioned on the surface of the slice. Properties of voltage-gated Ca2+ channels in P cells were measured under voltage-clamp conditions. Slow EPSC caused by repetitive stimulation (8 pulses, 50 Hz) via type 1 metabotropic glutamate receptors (mGluR1) was measured in the presence of NBQX (10 μM) (Batchelor and Garthwaite, 1997).

Generation of Purkinje Cell-Specific Fmr1 Knockout Mice, L7-Fmr1 Mutant Mice

We generated conditional knockout mice in which the first coding exon of Fmr1 can be deleted through Cre-mediated recombination. In brief, the floxed Fmr1 allele contains a lox site 2800 bp in front of exon 1 of the Fmr1 gene and a second lox site 260 bp after exon 1 in intron 1 of the Fmr1 gene. Mice expressing a L7/PCP2-cre transgene (Barski et al. 2000) were subsequently crossed with the floxed Fmr1 mice to generate a P cell-specific Fmr1 knockout mouse. To confirm that FMRP was selectively not expressed in P cells, adult mice were sacrificed and processed for immunohistochemical analysis of FMRP expression (for details, see Bakker et al., 2000).

Cytology of Purkinje Cells

The morphology of P cells was investigated, using BDA injections or immunocytochemistry against calbindin. BDA injections (10% in 0.1 M phosphate buffer) were made following electrophysiological identification of the cerebellar nuclei. After the iontophoretic injections, the animals were allowed to recover for 5 days and then were subsequently anesthetized (Nembutal; 50 mg/kg) and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were removed and cut in sagittal sections, which were reacted with ABC complex and diaminobenzidine to visualize the BDA. Calbindin immunocytochemistry was performed by incubating the sections with rabbit anti-calbindin antibody, ABC, and diaminobenzidine. Some of the sections were investigated under the light microscope, while others were osmicated, embedded in Durcupan, and processed for electron microscopy (De Zeeuw et al., 1989). For analysis, Purkinje cell dendrites were divided into a proximal category of dendrites (with a diameter R 1.5 μm) and a distal category (with a diameter < 1.5 μm). Spine density was calculated by dividing the total number of spines per dendrite by the length of the dendrite. Total spine length was calculated by measuring the distance between the tip of the spine head and the base of the spine neck; spine head length was measured by multiplying the distance from the tip of the spine to the head diameter-intersection line by a factor of two; and spine neck length was calculated by subtracting the spine head length from the total spine length.

Cerebellar Lesions

The anterior interposed cerebellar nuclei were identified with the use of electrophysiological recordings in trained animals, and the lesions were subsequently made with the use of pressure injections of 200 nmol of N-methyl-D-aspartate. After recovery and new eyeblink recordings, the animals were anesthetized (Nembutal; 50 mg/kg) and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were removed and cut into sections, which were stained with silver reagents as described by Haasdijk et al. (2002).

Eyeblink Conditioning in Humans

Normal males and males with Fragile X syndrome were subjected to eyeblink conditioning procedures with the use of MDMT and video technology. MDMT as described by Koekkoek et al. (2002) was modified so it could be applied to human subjects. During MDMT recording, we simultaneously captured video frames for calibration purposes. The MDMT sensor was attached on the edge of the orbit below the right lower eyelid, while a NIB magnet was attached to the edge of the right upper eyelid. A headset containing the MDMT amplifier, MDMT power supply, puff nozzle, miniature camera, and headphones was mounted on the head of the subject. Subjects were seated before a monitor and allowed to watch a movie. The headset provided a head-free recording situation, which is necessary when dealing with mentally compromised patients. The puff nozzle was set to direct the air puff to the cornea close to the outer canthus of the eye at a distance of 15 mm. The puff had an intensity of 20 PSI at the source, while stimulus duration was set at 20 ms. The sound of the movie acted as background noise, and volume was adjusted to an average of 75 dB. The CS was a 650 Hz tone at 75 dB with a duration of 520 ms starting 500 ms prior to US delivery (interstimulus interval, 500 ms). The headset provided complete sound isolation from the environment. The training was divided into four training sessions with two blocks of eight trials each. Each block contained one CS-only and one US-only trial, which were randomly distributed. The ITI was randomly determined but always ranged from 10 s to 30 s. All data values were obtained from CS-only trials, with the exception of UR data values. In the randomly paired procedure, the US occurred randomly in the ITI, while the CS was given as in the paired trials. For analysis criteria, see Koekkoek et al. (2002).

Model

Simulations were performed with custom-written C code. Differential equations (see Supplemental Data) were integrated with the forward Euler method. In population models like the present one, there is an unavoidable lack of data to constrain all parameters. We therefore confirmed that the main finding, i.e., the critical dependence of the CR amplitude on the steady-state level of activity in P cells and neurons of the cerebellar nuclei, can be reproduced in models sharing these features: the instantaneous response of cerebellar nuclear neurons is dominated by rebound discharges, the rebound discharge depends on the level of inhibition, and the pool of neurons available for disinhibition, or the overall rebound response, can be exhausted.

Supplemental Data

Supplemental Data include a table and sections of text pertaining to cell physiology, the mathematical model used, and the results and can be found with this article online at http://www.neuron.org/ cgi/ content/full/47/3/339/DC1/.

Acknowledgments

We thank E. Dalm, Ir. J. v.d. Burg, I. Nieuwenhuizen, L. Severijnen, Dr. A.E. Smits, and Dr. L. Govaerts for their excellent assistance. The work was supported by the Dutch Organization for Medical Sciences (NWO-PIONIER and ZON-MW), Life Sciences (NWO-ALW), Neuro-Bsik consortium (Senter), the European Community (EC), NIH (NIH 5 RO1 HD38038), FRAXA Research Foundation, IUAP and FWO (Belgium).

REFERENCES

Aizenman, C.D., and Linden, D.J. (1999). Regulation of the rebound depolarization and spontaneous firing patterns of deep nuclear neurons in slices of rat cerebellum. J. Neurophysiol. 82, 1697–1709.

Aizenman, C.D., and Linden, D.J. (2000). Rapid, synaptically driven increases in the intrinsic excitability of cerebellar deep nuclear neurons. Nat. Neurosci. 3, 109–111.

Bakker, C.E., de Diego Otero, Y., Bontekoe, C., Raghoe, P., Luteijn, T., Hoogeveen, A.T., Oostra, B.A., and Willemsen, R. (2000). Immunocytochemical and biochemical characterization of FMRP, FXR1P, and FXR2P in the mouse. Exp. Cell Res. 258, 162–170.

Barski, J.J., Dethleffsen, K., and Meyer, M. (2000). Cre recombinase

expression in cerebellar Purkinje cells. Genesis 28, 93–98.

Batchelor, A.M., and Garthwaite, J. (1997). Frequency detection and temporally dispersed synaptic signal association through a metabotropic glutamate receptor pathway. Nature 385, 74–77.

Bravin, M., Morando, L., Vercelli, A., Rossi, F., and Strata, P. (1999). Control of spine formation by electrical activity in the adult rat cerebellum. Proc. Natl. Acad. Sci. USA 96, 1704–1709.

Brown, V., Jin, P., Ceman, S., Darnell, J.C., O'Donnell, W.T., Tenenbaum, S.A., Jin, X., Feng, Y., Wilkinson, K.D., Keene, J.D., et al. (2001). Microarray identification of FMRP-associated brain mRNAs and altered mRNA translational profiles in fragile X syndrome. Cell 107, 477–487.

Cesa, R., Morando, L., and Strata, P. (2003). Glutamate receptor delta2 subunit in activity-dependent heterologous synaptic competition. J. Neurosci. 23, 2363–2370.

Chen, L., and Toth, M. (2001). Fragile X mice develop sensory hyperreactivity to auditory stimuli. Neuroscience 103, 1043–1050.

Coesmans, M., Smitt, P.A., Linden, D.J., Shigemoto, R., Hirano, T., Yamakawa, Y., van Alphen, A.M., Luo, C., van der Geest, J.N., Kros, J.M., et al. (2003). Mechanisms underlying cerebellar motor deficits due to mGluR1-autoantibodies. Ann. Neurol. 53, 325–336.

Coesmans, M., Weber, J.T., De Zeeuw, C.I., and Hansel, C. (2004). Bidirectional parallel fiber plasticity in the cerebellum under climbing fiber control. Neuron 44, 691–700.

Comery, T.A., Harris, J.B., Willems, P.J., Oostra, B.A., Irwin, S.A., Weiler, I.J., and Greenough, W.T. (1997). Abnormal dendritic spines in fragile X knockout mice: maturation and pruning deficits. Proc. Natl. Acad. Sci. USA 94, 5401–5404.

De Vries, B.B., van den Ouweland, A.M., Mohkamsing, S., Duivenvoorden, H.J., Mol, E., Gelsema, K., van Rijn, M., Halley, D.J., Sandkuijl, L.A., Oostra, B.A., et al. (1997). Screening and diagnosis for the fragile X syndrome among the mentally retarded: an epidemiological and psychological survey. Collaborative Fragile X Study Group. Am. J. Hum. Genet. 61, 660–667.

De Zeeuw, C.I., Holstege, J.C., Ruigrok, T.J., and Voogd, J. (1989). Ultrastructural study of the GABAergic, cerebellar, and mesodiencephalic innervation of the cat medial accessory olive: anterograde tracing combined with immunocytochemistry. J. Comp. Neurol. 284, 12–35.

De Zeeuw, C.I., Hansel, C., Bian, F., Koekkoek, S.K., van Alphen, A.M., Linden, D.J., and Oberdick, J. (1998). Expression of a protein kinase C inhibitor in Purkinje cells blocks cerebellar LTD and adaptation of the vestibulo-ocular reflex. Neuron 20, 495–508.

De Zeeuw, C.I., Elgersma, Y., Hulscher, H.C., Dortland, B.R., Hensbroek, R.A., Ruigrok, T.J., and Koekkoek, S.K.E. (2004). Response to comment on "Cerebellar LTD and Learning-Dependent Timing of Conditioned Eyelid Responses". Science 304, 211C.

D'Hooge, R., Nagels, G., Franck, F., Bakker, C.E., Reyniers, E., Storm, K., Kooy, R.F., Oostra, B.A., Willems, P.J., and De Deyn, P.P. (1997). Mildly impaired water maze performance in male Fmr1 knockout mice. Neuroscience 76, 367–376.

Dobkin, C., Rabe, A., Dumas, R., El Idrissi, A., Haubenstock, H., and Brown, W.T. (2000). Fmr1 knockout mouse has a distinctive strain-specific learning impairment. Neuroscience 100, 423–429.

Fu, Y.H., Kuhl, D.P., Pizzuti, A., Pieretti, M., Sutcliffe, J.S., Richards, S., Verkerk, A.J., Holden, J.J., Fenwick, R.G., Jr., Warren, S.T., et al. (1991). Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox. Cell 67, 1047–1058.

Gruart, A., Pastor, A.M., Armengol, J.A., and Delgado-Garcia, J.M. (1997). Involvement of cerebellar cortex and nuclei in the genesis and control of unconditioned and conditioned eyelid motor responses. Prog. Brain Res. 114, 511–528.

Gu, Y., McIlwain, K.L., Weeber, E.J., Yamagata, T., Xu, B., Antalffy, B.A., Reyes, C., Yuva-Paylor, L., Armstrong, D., Zoghbi, H., et al. (2002). Impaired conditioned fear and enhanced long-term potentiation in Fmr2 knock-out mice. J. Neurosci. 22, 2753–2763.

Haasdijk, E.D., Vlug, A., Mulder, M.T., and Jaarsma, D. (2002). Increased apolipoprotein E expression correlates with the onset of neuronal degeneration in the spinal cord of G93A–SOD1 mice. Neurosci. Lett. 335, 29–33.

Hagerman, R.J., and Hagerman, P.J. (2002). The fragile X premutation: into the phenotypic fold. Curr. Opin. Genet. Dev. 12, 278–283.

Hinton, V.J., Brown, W.T., Wisniewski, K., and Rudelli, R.D. (1991). Analysis of neocortex in three males with the fragile X syndrome. Am. J. Med. Genet. 41, 289–294.

Huber, K.M., Gallagher, S.M., Warren, S.T., and Bear, M.F. (2002). Altered synaptic plasticity in a mouse model of fragile X mental retardation. Proc. Natl. Acad. Sci. USA 99, 7746–7750.

Ichikawa, R., Miyazaki, T., Kano, M., Hashikawa, T., Tatsumi, H., Sakimura, K., Mishina, M., Inoue, Y., and Watanabe, M. (2002). Distal extension of climbing fiber territory and multiple innervation caused by aberrant wiring to adjacent spiny branchlets in cerebellar Purkinje cells lacking glutamate receptor delta 2. J. Neurosci. 22, 8487–8503.

Irwin, S.A., Patel, B., Idupulapati, M., Harris, J.B., Crisostomo, R.A., Larsen, B.P., Kooy, F., Willems, P.J., Cras, P., Kozlowski, P.B., et al. (2001). Abnormal dendritic spine characteristics in the temporal and visual cortices of patients with fragile-X syndrome: a quantitative examination. Am. J. Med. Genet. 98, 161–167.

Irwin, S.A., Idupulapati, M., Gilbert, M.E., Harris, J.B., Chakravarti, A.B., Rogers, E.J., Crisostomo, R.A., Larsen, B.P., Mehta, A., Alcantara, C.J., et al. (2002). Dendritic spine and dendritic field characteristics of layer V pyramidal neurons in the visual cortex of fragile-X knockout mice. Am. J. Med. Genet. 111, 140–146.

Kakizawa, S., Yamasaki, M., Watanabe, M., and Kano, M. (2000). Critical period for activity-dependent synapse elimination in developing cerebellum. J. Neurosci. 20, 4954–4961.

Kano, M., Hashimoto, K., Watanabe, M., Kurihara, H., Offermanns, S., Jiang, H., Wu, Y., Jun, K., Shin, H.S., Inoue, Y., et al. (1998). Phospholipase cbeta4 is specifically involved in climbing fiber synapse elimination in the developing cerebellum. Proc. Natl. Acad. Sci. USA 95, 15724–15729.

Karachot, L., Shirai, Y., Vigot, R., Yamamori, T., and Ito, M. (2001). Induction of long-term depression in cerebellar Purkinje cells requires a rapidly turned over protein. J. Neurophysiol. 86, 280–289.

Kelly, R.M., and Strick, P.L. (2003). Cerebellar loops with motor cortex and prefrontal cortex of a nonhuman primate. J. Neurosci. 23, 8432–8444.

Kenyon, G.T., Medina, J.F., and Mauk, M.D. (1998). A mathematical model of the cerebellar-olivary system I: self-regulating equilibrium of climbing fiber activity. J. Comput. Neurosci. 5, 17–33.

Kim, J.J., and Thompson, R.F. (1997). Cerebellar circuits and synaptic mechanisms involved in classical eyeblink conditioning. Trends Neurosci. 20, 177–181.

Kim, S.G., Ugurbil, K., and Strick, P.L. (1994). Activation of a cerebellar output nucleus during cognitive processing. Science 265, 949–951.

Koekkoek, S.K.E., Den Ouden, W.L., Perry, G., Highstein, S.M., and De Zeeuw, C.I. (2002). Monitoring kinetic and frequency-domain properties of eyelid responses in mice with magnetic distance measurement technique. J. Neurophysiol. 88, 2124–2133.

Koekkoek, S.K., Hulscher, H.C., Dortland, B.R., Hensbroek, R.A., Elgersma, Y., Ruigrok, T.J., and De Zeeuw, C.I. (2003). Cerebellar LTD and learning-dependent timing of conditioned eyelid responses. Science 301, 1736–1739.

Laggerbauer, B., Ostareck, D., Keidel, E.M., Ostareck-Lederer, A., and Fischer, U. (2001). Evidence that fragile X mental retardation protein is a negative regulator of translation. Hum. Mol. Genet. 10, 329–338.

Leiner, H.C., Leiner, A.L., and Dow, R.S. (1993). Cognitive and language functions of the human cerebellum. Trends Neurosci. 16, 444–447.

Li, Z., Zhang, Y., Ku, L., Wilkinson, K.D., Warren, S.T., and Feng, Y. (2001). The fragile X mental retardation protein inhibits translation via interacting with mRNA. Nucleic Acids Res. 29, 2276–2283.

Linden, D.J. (2001). The expression of cerebellar LTD in culture is not associated with changes in AMPA-receptor kinetics, agonist affinity, or unitary conductance. Proc. Natl. Acad. Sci. USA 98, 14066–14071.

Llano, I., Marty, A., Armstrong, C.M., and Konnerth, A. (1991). Synaptic-and agonist-induced excitatory currents of Purkinje cells in rat cerebellar slices. J. Physiol. 434, 183–213.

Mauk, M.D., and Donegan, N.H. (1997). A model of Pavlovian eyelid conditioning based on the synaptic organization of the cerebellum. Learn. Mem. 4, 130–158.

Medina, J.F., and Mauk, M.D. (2000). Computer simulation of cerebellar information processing. Nat. Neurosci. 3 (Suppl.), 1205–1211.

Middleton, F.A., and Strick, P.L. (1994). Anatomical evidence for cerebellar and basal ganglia involvement in higher cognitive function. Science 266, 458–461.

Migaud, M., Charlesworth, P., Dempster, M., Webster, L.C., Watabe, A.M., Makhinson, M., He, Y., Ramsay, M.F., Morris, R.G., Morrison, J.H., et al. (1998). Enhanced long-term potentiation and impaired learning in mice with mutant postsynaptic density-95 protein. Nature 396, 433–439.

Miyashiro, K.Y., Beckel-Mitchener, A., Purk, T.P., Becker, K.G., Barret, T., Liu, L., Carbonetto, S., Weiler, I.J., Greenough, W.T., and Eberwine, J. (2003). RNA cargoes associating with FMRP reveal deficits in cellular functioning in Fmr1 null mice. Neuron 37, 417– 431.

Miyata, M., Okada, D., Hashimoto, K., Kano, M., and Ito, M. (1999). Corticotropin-releasing factor plays a permissive role in cerebellar long-term depression. Neuron 22, 763–775.

Nielsen, D.M., Derber, W.J., McClellan, D.A., and Crnic, L.S. (2002). Alterations in the auditory startle response in Fmr1 targeted mutant mouse models of fragile X syndrome. Brain Res. 927, 8–17.

Paradee, W., Melikian, H.E., Rasmussen, D.L., Kenneson, A., Conn, P.J., and Warren, S.T. (1999). Fragile X mouse: strain effects of knockout phenotype and evidence suggesting deficient amygdala function. Neuroscience 94, 185–192.

Rudelli, R.D., Brown, W.T., Wisniewski, K., Jenkins, E.C., Laure-Kamionowska, M., Connell, F., and Wisniewski, H.M. (1985). Adult fragile X syndrome. Clinico-neuropathologic findings. Acta Neuropathol. (Berl.) 67, 289–295.

Shibuki, K., Gomi, H., Chen, L., Bao, S., Kim, J.J., Wakatsuki, H., Fujisaki, T., Fujimoto, K., Katoh, A., Ikeda, T., et al. (1996). Deficient cerebellar long-term depression, impaired eyeblink conditioning, and normal motor coordination in GFAP mutant mice. Neuron 16, 587–599.

Strata, P., Morando, L., Bravin, M., and Rossi, F. (2000). Dendritic spine density in Purkinje cells. Trends Neurosci. 23, 198–198.

Teune, T.M., van der Burg, J., van der Moer, J., Voogd, J., and Ruigrok, T.J. (2000). Topography of cerebellar nuclear projections to the brain stem in the rat. Prog. Brain Res. 124, 141–172.

The Dutch-Belgian Fragile X Consortium. (1994). Fmr1 knockout mice: A model to study fragile X mental retardation. Cell 78, 23–33.

Turner, G., Webb, T., Wake, S., and Robinson, H. (1996). Prevalence of fragile X syndrome. Am. J. Med. Genet. 64, 196–197.

Van Dam, D., D'Hooge, R., Hauben, E., Reyniers, E., Gantois, I., Bakker, C.E., Oostra, B.A., Kooy, R.F., and De Deyn, P.P. (2000). Spatial learning, contextual fear conditioning and conditioned emotional response in Fmr1 knockout mice. Behav. Brain Res. 117, 127–136.

Van Pelt, J., Uylings, H.B., Verwer, R.W., Pentney, R.J., and Woldenberg, M.J. (1992). Tree asymmetry–a sensitive and practical measure for binary topological trees. Bull. Math. Biol. 54, 759–784.

Verheij, C., Bakker, C.E., de Graaff, E., Keulemans, J., Willemsen, R., Verkerk, A.J., Galjaard, H., Reuser, A.J., Hoogeveen, A.T., and Oostra, B.A. (1993). Characterization and localization of the FMR-1 gene product associated with fragile X syndrome. Nature 363,
722–724.

Verkerk, A.J., Pieretti, M., Sutcliffe, J.S., Fu, Y.H., Kuhl, D.P., Pizzuti, A., Reiner, O., Richards, S., Victoria, M.F., Zhang, F.P., et al. (1991). Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell 65, 905–914.

Vokaer, M., Bier, J.C., Elincx, S., Claes, T., Paquier, P., Goldman, S., Bartholome, E.J., and Pandolfo, M. (2002). The cerebellum may be directly involved in cognitive functions. Neurology 58, 967–970.

Wang, S.S., Denk, W., and Hausser, M. (2000). Coincidence detection in single dendritic spines mediated by calcium release. Nat. Neurosci. 2, 1266–1273.

Weiler, I.J., Irwin, S.A., Klintsova, A.Y., Spencer, C.M., Brazelton, A.D., Miyashiro, K., Comery, T.A., Patel, B., Eberwine, J., and Greenough, W.T. (1997). Fragile X mental retardation protein is translated near synapses in response to neurotransmitter activation. Proc. Natl. Acad. Sci. USA 94, 5395–5400.

Xia, J., Chung, H.J., Wihler, C., Huganir, R.L., and Linden, D.J. (2000). Cerebellar long-term depression requires PKC-regulated interactions between GluR2/3 and PDZ domain-containing proteins. Neuron 28, 499–510.

Yeo, C.H., and Hesslow, G. (1998). Cerebellum and conditioned reflexes. Trends Cogn. Sci. 2, 322–331.

CHAPTER 5

Savings and extinction of conditioned eyeblink responses in fragile X syndrome

ABSTRACT

The fragile X syndrome is the most widespread heritable form of mental retardation caused by the lack of expression of the fragile X mental retardation protein. This lack has been related to deficits in cerebellum-mediated acquisition of conditioned eyelid responses in individuals with fragile X syndrome. In the current behavioral study long term effects of deficiency of fragile X mental retardation protein were investigated, by examining the acquisition, savings and extinction of delay eyeblink conditioning in male individuals with fragile X syndrome.

In the acquisition experiment fragile X syndrome subjects displayed a significantly poor performance compared to control subjects. In the savings experiment performed at least 6 months later, fragile X syndrome and control subjects showed similar levels of savings of conditioned responses. Subsequently, extinction was faster in fragile X syndrome than in control subjects.

These findings confirm that absence of the fragile X mental retardation protein affects cerebellar motor learning. The normal performance in the savings experiment and aberrant performance in the acquisition and extinction experiments of individuals with fragile X syndrome suggests that different mechanisms underlie acquisition, savings and extinction of cerebellar motor learning.

INTRODUCTION

With an estimated prevalence of 1 in 4000 men and 1 in about 6000 women, the fragile X syndrome (FRAXA) is the most widespread heritable form of mental retardation. An elongated facial structure, large protruding ears, hyper extensible joints and macro-orchidism characterize individuals with FRAXA. The mental retardation can be accompanied by various degrees of hyper arousal, attention deficits, anxiety, social withdrawal, autistic behavior and seizure susceptibility (Beckel-Mitchener, 2004; Crawford, 1999; Jin et al., 2004; Turner, 1996; De Vries, 1997).

Several studies suggest that the cerebellum is involved in FRAXA (Gothelf et al., 2007; Hessl et al., 2004; Huber et al., 2006; Koekkoek et al., 2005). On a molecular level, the fragile X mental retardation protein (FMRP) is strongly expressed in the cerebellum of healthy individuals, especially in Purkinje cells which comprise the sole output of the cerebellar cortex (Tamanini et al., 1997). In FRAXA, hyper expansion of a trinucleotide CGG repeat in the fragile X mental retardation gene, FMR1, results in transcriptional silencing of FMRP (Verkerk, 1991). On a morphological level, FMRP is a translational suppressor at synaptic sites in dendrites and important for normal spine maturation and pruning (Bagni and Greenough, 2005; Comery, 1997; Galvez and Greenough, 2005). FMR1-null mouse mutants, in which the expression of FMRP is absent, have abnormally shaped spines on dendrites of Purkinje cells. However the spine density and shape of the dendritic tree appear to be normal (Koekkoek et al., 2005). In FRAXA patients, MRI revealed hypoplasia of the cerebellar vermis, in particular of lobules VI and VII (Gothelf et al., 2007; Hessl et al., 2004).

On a behavioral level, FRAXA patients show aberrant delay eyeblink conditioning (Koekkoek et al., 2005). In an eye blink conditioning test, repeated paired exposure to an air-puff on the cornea (US: unconditioned stimulus), which evokes a reflex blink, and a tone (CS: conditioned stimulus), which by itself does not evoke a blink, results in a blink not only in response to the air-puff but also to the

tone alone (CR: conditioned response) (Domjan, 2005). Delay eyeblink conditioning, in which the termination of the CS and US coincide, is largely dependant on cerebellar functioning (Bracha, 2004; Christian and Thompson, 2003, 2005; Woodruff-Pak and Papka, 1996), which is thought to control the precise timing of the CR (Gerwig et al., 2005; Koekkoek et al., 2003).

Acquisition of the CR is thought to be mediated by long-term depression of the parallel fiber-Purkinje cell synapses induced by concurrent stimulation of the parallel fiber system by the CS (tone) and the climbing fiber system by the US (air-puff) (Kitazawa, 2002). In a previous study we observed that in global and Purkinje cell specific knockout mice absence of FMRP leads to enhanced parallel fiber long-term depression (Koekkoek et al., 2005). A lack of FMRP suppression might lead to an exaggeration of translation responses linked to group I metabotropic glutamate receptors (mGluRs 1 and 5), which are involved in the consolidation of long-term depression (Bear et al., 2004; Vanderklish and Edelman, 2005).

Deviant cerebellar functioning could explain the lower performance of FMRP mouse mutants and FRAXA patients in the short-term acquisition of the delay conditioned eyeblink response (Koekkoek et al., 2005). In the present paper we investigated the long-term mechanisms of delay eye blink conditioning in individuals with FRAXA. Presence of an increased percentage of CRs a long time after CR acquisition (savings) would indicate that normal expression of FMRP in cerebellar Purkinje cells is necessary for learning, but not for remembering a conditioned eyeblink response. On the other hand, deviant savings behavior in individuals with FRAXA suggests that both learning and storage of the memory traces in delay eye blink conditioning are dependent on similar mechanisms. Likewise, FMRP may also be involved in the ability to actively forget the CR (extinction).

Thus in the present study we investigated savings and extinction behavior of the delay conditioned eyeblink response in individuals with FRAXA to study the long-term effects of disturbed FMRP expression.

MATERIALS & METHODS

Experimental subjects

Written informed consent was obtained from (the parents of) 11 male subjects with FRAXA (21-39 years of age: average 31 ± 6.5) and 14 healthy male control subjects (22-45 years of age: average 30 ± 6.0). The EramusMC Medical Ethics Committee (MEC-2004-205) approved the protocol. The FRAXA participants had full mutations as they had over 200 CGG repeats in their FMR1 gene (data not shown).

All subjects could distinguish at least six out of ten 650 Hz, 500 ms tones presented at different volumes from a continuous 60 dB white noise background, so their hearing was sufficient to participate. During the experiments, all participants displayed reflex blinks in response to an air-puff with an onset latency of less than 100 ms, which indicated that they all had normal eyelid motor function. Spontaneous blink rates did not differ between the FRAXA and the control group (6.0 ± 3.3 and 7.6 ± 5.0 blinks per minute, respectively, p=0.501).

Apparatus

All subjects were seated in front of a monitor and watched a movie of their own choice during the experiments. The eyeblink conditioning setup consisted of a headset with an air puff nozzle attached to the right side of the headphone and magnetic distance measurement technique (MDMT) equipment (Koekkoek et al., 2002). The headset allowed free head movement, and provided sound isolation from the environment. The setup was especially designed to be able to perform experiments with individuals with FRAXA. Free head movement and entertainment with the movie were essential to ensure that attention deficits and anxiety would not interfere with the eyeblink conditioning sessions. The air puff nozzle was directed at the cornea close to the outer canthus of the right eye at a distance of about 15 mm. It served to deliver a 20 ms air-puff with an intensity of 10-50 PSI at the source (the US: unconditioned stimulus). The intensity could be adjusted to elicit a single blink reflex. The sound of the movie (background noise) and the 650 Hz, 75 dB, 520 ms tones (the CS: conditioned stimulus) were presented bilaterally through the headphones. The MDMT equipment was originally designed for mice and is described in detail elsewhere (Koekkoek et al., 2002), and was modified for the present study in human subjects. In short, the MDMT consists of a giant magnetoresistive sensor attached to the edge of the lower orbit of the right eyelid. It measures the distance to two small gold plated neodymium magnets (N43 1.6 x 0.7 x 0.8cm, with the long side positioned parallel to the eyelid), attached to the edge of the right upper eyelid. The signal was amplified by a pre-amplifier close to the sensor (10x) and further amplification (between 10-50 times) could be adjusted per subject. The MDMT signal was digitized (1000 Hz) using National Instruments hardware. A custom made LabView (National Instruments Austin, Texas, USA) script controlled the monitoring of eyelid movement, presentation of the stimuli, and capturing of data on disk.

Procedure

Subjects participated in three experiments in which acquisition, savings and extinction of delay eyeblink conditioning was assessed. Eleven FRAXA subjects and 14 control subjects participated in the study. The protocol for the acquisition experiment was similar to our previous study (Koekkoek et al., 2005). In this experiment subjects received 10 blocks of eight 2-second trials. Each block consisted of one trial in which the air-puff was presented alone (US only), one trial in which the tone was presented alone (CS only) and six trials in which tone and air-puff were paired. In these paired trials the 20 ms air-puff was presented with a 500 ms delay after onset of the 520 ms tone, so the two stimuli ended simultaneously (delay eyeblink conditioning, see figure 1). The three types of trials (US only, CS only and paired) were presented in random order within each of the ten blocks. The trials were separated by a random interval of 20-30 seconds and blocks were separated by one minute.

The savings experiment was performed at least six months after the acquisition experiment (average 9.2 ± 3.4 months), using the same protocol. At the end of the savings experiment the extinction experiment was performed. In the extinction experiment each block consisted of seven CS only trials, yielding 70 trials in total, again randomly separated by 20-30 seconds.

Eyelid position was constantly monitored with the MDMT signal. Trials were started only when the eyelid was fully open, which was determined automatically using a threshold. This threshold was manually adjusted to above the position that was measured when the eye was open.

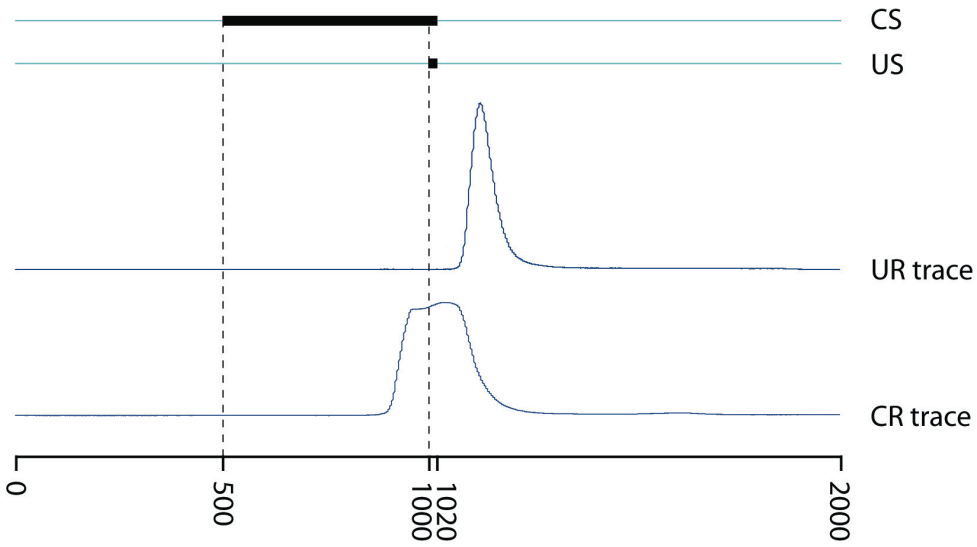


Figure 1.
Description of a trial. Each trial lasted 2000 ms, during which either an air puff alone (US only), a tone alone (CS only) or a paired CS and US stimulus could be presented. The 520 ms tone (CS) would start at 500 ms after trial onset, the 20 ms air-puff (US) would start at 1000 ms after trial onset, and both stimuli would end at 1020 ms in the trial. During each trial the eyelid movement (trace) is recorded and blinks are shown as peaks in the recording. Traces are shown of an unconditioned response (UR trace) and a conditioned response (CR trace). An eyelid response with an onset latency between 650 and 1025 ms with respect to the start of a CS-only or paired trial was considered a conditioned response.

Raven's tests

A rough estimate of non-verbal cognitive performance of the FRAXA subjects was obtained using Raven's Colored Progressive Matrices in 10 subjects and Raven's Standard Progressive Matrices in one subject. Raw scores were transformed into IQ scores (Raven, 2000).

Data analysis

For each CS only and paired trial the presence or absence of a conditioned response (CR) was determined semi-automatically using custom written scripts in Matlab (version 6.5: The Mathworks, Inc. Natick, Ma, USA) and LabView (version 7.1 and 8.2: National Instruments Austin, Texas, USA). First the MDMT data was filtered with a 7th order Butterworth 100 Hz low pass filter. The program looked for a maximum eyelid closure between 600 and 1300 ms and calculated the range between this maximum and the absolute minimum between 600 ms and the time of the maximum. Subsequently, the first local minimum before the maximum eyelid closure under 5% of this range was determined. The time point of this local minimum could be manually adjusted to ensure proper detection, and was taken as the onset of the eye blink.

A trial was considered invalid when the 2-second recording contained more than 3 peaks, or when the maximum eyelid closure during a blink occurred between 0 and 100 ms after CS onset. The latter was

considered a startle response to the tone. These trials were discarded from the analysis. A CR was defined as a blink with an onset latency between 150 ms (650 ms in the trial) and 525 ms (1025 ms in the trial) after the start of the CS, to exclude eyelid reflexes in response to the air-puff, which was presented 500 ms after the tone.

For each subject and experiment we calculated the percentage of CRs within the blocks, taking only valid trials into account. We compared the percentage of CRs between FRAXA and control subjects for each of the three experiments as a whole and for each block within an experiment. To assess overall learning we compared the percentages of CRs in the beginning of the acquisition experiment to the end of the savings experiment. To study savings in particular, we compared the percentage of CRs between the first block of the acquisition experiment to the first block of the savings experiment. To evaluate extinction we compared the percentage of CRs between the last block of the savings experiment and the last block of the extinction experiment for both the FRAXA and control group and looked at the differences between the groups. In the latter analysis only participants with scores over 50 percent CRs in the last two blocks of the savings experiment were included to ensure that we studied individuals who did acquire a delay conditioned eyeblink response.

All comparisons were done with the non-parametric Mann-Whitney test using SPSS (version 11.0: SPSS Inc. Chicago, Illinois, USA) and the level of significance in all analyses was set at $p<0.05$. In the results section, averages and standard deviations are presented.

RESULTS

Three experiments of delay eyeblink conditioning were performed to assess 1) acquisition, 2) saving and 3) extinction of a conditioned response (CR) in both male FRAXA subjects and male controls. Individual traces of eyelid movements of a FRAXA subject and control subject for each of the three experiments are shown in figure 2.

Acquisition of the CR

Both the FRAXA subject (figure 2a) and the control subject (figure 2d) did acquire the CR. However, the forward shift in the onset latency of this response does not start until the fourth block in the FRAXA subject.

The learning curves of the FRAXA group and the control group in the first delay eyeblink conditioning experiment are plotted in the first part of figure 3. Over the whole experiment, the FRAXA group exhibited less CRs than the control group (38.4 ± 8.2 % vs. 56.6 ± 12.6 % of the trials, respectively, $p = 0.106$). In the first two blocks the number of CRs was not different between the two groups, but the number of CRs in the FRAXA group was lower in block 3 ($p = 0.009$) but not significantly different in blocks 4 to 10. The increase in the percentage of CRs between the first and last block of the acquisition experiment was significant in controls (25.0 vs. 67.2, $p = 0.003$), but not in FRAXA subjects (23.9 vs. 45.5, $p = 0.501$). The average onset latency of the CRs was similar in FRAXA subjects (850 ± 45 ms) and controls (856 ± 37 ms, $p = 0.640$).

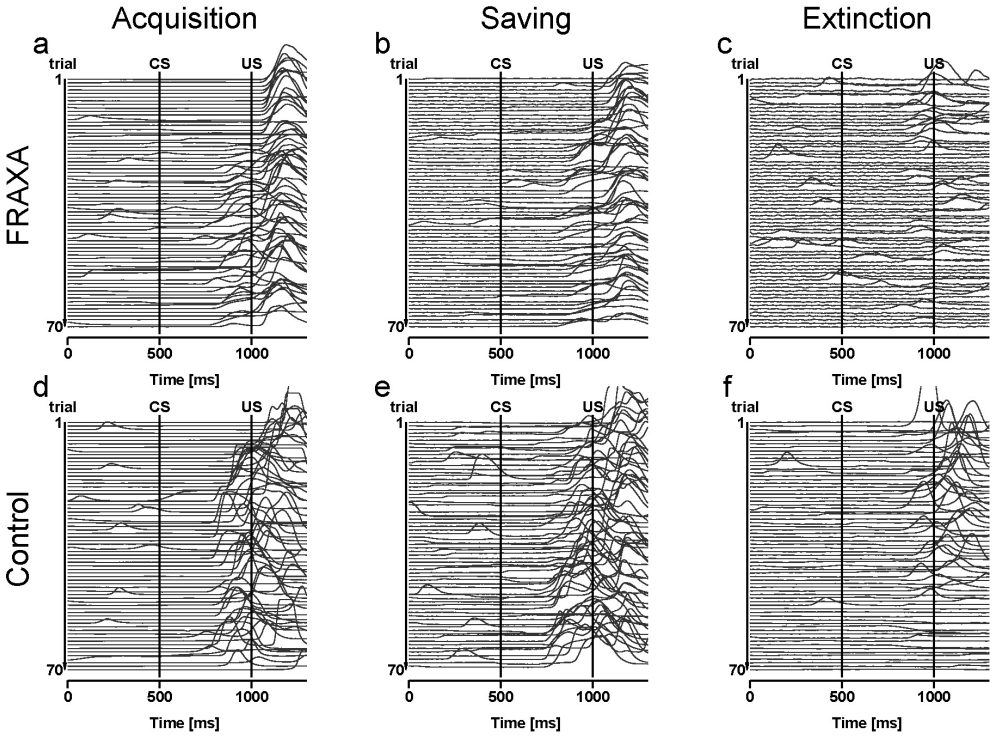


Figure 2. Exemplary raw eyelid movement data of a FRAXA subject (upper row) and a control subject (lower row) during the acquisition (a,d), savings (b,e) and extinction (c,f) experiments. Recordings of the 70 CS only and paired trials are shown in order, starting with the first trial at the top. The two vertical lines indicate CS onset (at 500 ms) and US onset (at 1000 ms).

Savings of the CR

Eyelid movements measured during the savings experiment of the two exemplary subjects are shown in figure 2b (FRAXA) and 2e (control). Both subjects clearly start displaying CRs earlier than they did in the acquisition experiment, but the FRAXA subject is slower. He starts showing CRs at the end of the second block and the control already starts in the first.

The percentages of CRs during the savings experiment after approximately six months are plotted in the second part of figure 3. In general, the FRAXA group exhibited slightly less CRs than the control group (53.6 ± 9.3 % vs. 69.4 ± 8.4 % of the trials, respectively, $p = 0.261$), and no significant differences between the two groups were observed for individual blocks as well. The differences in the percentage of CRs between the first block of the savings experiment and the first block of the acquisition experiment were not significant between FRAXA (33.9 ± 43.5 %) and controls (51.5 ± 38.6 %, $p = 0.143$). The average onset latency of CRs in the savings experiment was similar in FRAXA subjects (860 ± 51 ms) and controls (855 ± 46 ms, $p = 0.891$).

Repetition of the acquisition protocol induces a significant increase in the percentage of CRs, when we compared the end of the savings experiment with the beginning of the acquisition experiment in both the FRAXA group (24.6 ± 14.8 % vs. 58.4 ± 35.8 %, $p = 0.004$) and the control group (25.0 ± 29.5 %

vs. 76.5 ± 26.6 %, p < 0.001). However, the overall learning of CRs was not different between the two groups (p = 0.827, figure 4a).

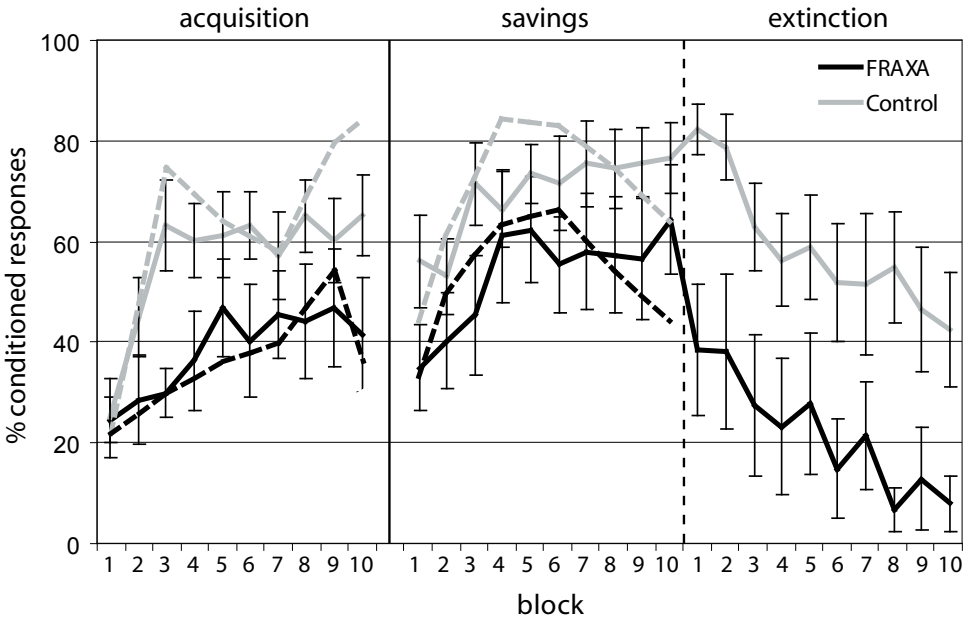


Figure 3. The average percentages of conditioned responses in CS only and paired trials per block for the FRAXA group (black line) and control group (gray line) group, for each of the three experiments (acquisition, savings and extinction) are displayed. Error bars represent standard error of the mean. For clarity of presentation conditioned responses in CS only trials are displayed as dashed lines without error bars. Both groups showed an increase in conditioned response incidence across the acquisition and savings experiment and a decrease in the extinction experiment.

Extinction of the CR

Eyelid movement traces of the extinction experiment of a FRAXA and control subject are shown in figure 2. The FRAXA subject (figure 2c) unlearns the CR within three blocks whereas the control subject (figure 2f) exhibits CRs until block seven. Five of the 11 FRAXA subjects and four of the 14 control subjects did not reach 50 percent CRs in the last two blocks of the savings experiment. These subjects were discarded from further analysis. The average learning curve of the remaining 6 FRAXA subjects and 10 control subjects is plotted in the third part of figure 3.

The average percentage of CRs in the whole extinction experiment was lower in the FRAXA group (21.8 ± 11.4 %) than in the control group (58.6 ± 13 %, p = 0.005). The difference between the groups was significant for the first two blocks (p = 0.0135, p = 0.030) and for block 4 and 8 (p = 0.024, p = 0.005). The difference in percentage of CRs between the last savings block and the last extinction block was not significantly different between the FRAXA group (75.4 ± 29.3 %) and the control group (46.2 ± 29.7 %, p = 0.095, figure 4b). The average onset latency of CRs in the extinction experiment was similar in FRAXA subjects (830 ± 38 ms) and controls (842 ± 38 ms, p = 0.594).

IQ of the FRAXA subjects

The IQ-scores of the FRAXA subjects were on average of 74 (± 6). We observed no significant correlation between IQ and the percentages of CRs in either the acquisition experiment ($r = 0.035$, $p = 0.460$) or the savings experiment ($r = -0.198$, $p = 0.279$).

DISCUSSION

Three sessions of delay eyeblink conditioning were performed to assess acquisition, savings and extinction of a conditioned response (CR) of FRAXA subjects and controls. Overall, FRAXA subjects displayed fewer CRs than controls. In the acquisition experiment the FRAXA group showed a slower increase in the percentage of CRs. In the savings experiment both FRAXA and control subjects showed saving of the CR. Especially the FRAXA group improved as the percentages of CRs no longer significantly differ from controls. Extinction of the learned behaviour in FRAXA subjects was characterized by a swift decline in the percentage of CRs in the first trials. After that, the shape of the extinction curve was similar for FRAXA and control subjects. We observed no correlation between IQ and performance during delay eyeblink conditioning. These new findings imply a long-term influence of the lack of FMRP on extinction but not on savings of the delay conditioned eyeblink response.

Acquisition of the CR

We confirmed the previously observed difference in delay eyeblink conditioning performance in the acquisition experiment (Koekkoek et al., 2005). This affirms the notion that individuals with FRAXA have impaired cerebellar motor learning. Compared to patients with hereditary ataxia and in patients with lesions restricted to the cerebellum the acquisition rate is higher in FRAXA subjects (Bracha et al., 2000, Timmann et al., 2005). The reason is that the cerebellar circuit in FRAXA subjects is intact in contrast to cerebellar ataxia patients.

Savings of the CR

In the savings experiment the difference in motor learning behavior between the FRAXA and control subjects was not significant. Moreover, both the FRAXA group and the control group had retained their increased percentage of CRs at the beginning of the savings experiment. This suggests that a long-term memory effect occurred in the FRAXA group, despite their impairments in CR acquisition. This new finding in humans seems to be at odds with repeated delay eyeblink conditioning experiments global FMR1 knockout mice. These mice did show improvement during repeated delay eyeblink experiments, although their percentage of CRs was always significantly lower than wild-type littermates (Koekkoek et al., 2005). Arguably no long-term savings-effect was noticed in this study as the experiments were performed in one day whereas in the present study the two experiments were separated by at least 6 months.

Extinction of the CR

The rapid decrease in the number of CRs of individuals with FRAXA during the extinction experiment is remarkable. The fast extinction of the FRAXA subjects in contrast to their slower acquisition implies that learning and unlearning of the CR are not direct opposites of the same mechanism. However, our

results do not reveal whether extinction requires formation of a separate memory trace or extinction is a mask of the CR. Regardless of the exact mechanism for this type of motor learning, our results suggest that extinction is accelerated by absence of FMRP.

IQ of the FRAXA subjects

The IQ-scores, estimated by the Raven test, are high compared to other studies (Eliez et al., 2001; Fisch et al., 2007). However, we suspect that our FRAXA subjects represent the high end of the spectrum of the FRAXA population, because parents are more likely to enroll their child in a study when they deem them able to participate successfully, thereby probably inducing a bias for individuals with higher IQs. The IQ-scores did not correlate with the delay eyeblink conditioning results, which is in line with other studies in retarded individuals (Ohlrich and Ross, 1968). More, elaborate IQ tests or larger group sizes could possibly change these outcomes.

Anatomical implications

Our findings that the percentage of CRs was impaired during the acquisition but normal during savings suggest that there are at least two different sites at which learning occur. One affected by absence of FMRP and another less affected by absence of FMRP. In the cerebellum FMRP is expressed in the cytoplasm of all neurons and strongest in Purkinje cells in which FMRP is also localized in the nucleolus (Bakker et al., 2000; Tamanini et al., 1997). Purkinje cells can only be found in the cerebellar cortex. Many authors suggest that during delay eyeblink conditioning plasticity occurs in both the cerebellar cortex and deep cerebellar nuclei (for reviews see: Attwell et al., 2002; Christian and Thompson, 2003; DeZeeuw and Yeo, 2005; Ito, 2002). Both parallel fiber-Purkinje cell synapses in the cerebellar cortex and cerebellar nuclei are vital for acquisition of the CR (Chen et al., 1996; Koekkoek et al., 2003), whereas only the interpositus nucleus (a deep cerebellar nucleus) is thought to be essential for long-term savings of the CR (Christian and Thompson, 2005). The results of the current study are in agreement with these ideas. Acquisition, dependant on FMRP deprived Purkinje cells, appears impaired in FRAXA subjects. During savings FRAXA subjects delay eyeblink conditioning resembles control subject performance. Savings could be mediated by the interpositus nucleus, which functioning is possibly less dependent on FMRP expression.

Contrary to normal performance in the savings experiment FRAXA subjects display abnormal extinction behavior. The anterior lobe of the cerebellum is necessary for extinction (Perrett and Mauk, 1995). No anatomical abnormalities have been reported about this region in individuals with FRAXA.

Behavioral implications

In the current study we show differences as well as similarities between FRAXA and control subjects. We state that these differences are due to a learning defect and are not a consequence of an inability to perform the task correctly. The FRAXA phenotype is characterized by attention deficits, which make many tasks difficult to perform. However, eyeblink conditioning is independent of awareness (Clark and Squire, 1998; Smith et al., 2005), so low attention cannot explain the differences we observe. In addition, FRAXA subjects have no difficulty hearing the tone (as tested before the experiments) or responding to the air-puff. Koekkoek and coworkers (2005) showed that though conditioned response amplitudes were affected in FRAXA subjects, unconditioned response kinematics were normal. On the other hand, acute stress has been shown to impair learning in female rats but not in male rats (Bangasser and Shors, 2004; Duncko et al., 2007; Shors, 2001). Individuals with FRAXA are often

hyper excitable and their mental state, induced by a new environment, new people and performing new tasks, could influence their learning abilities. This might explain the large increase in the percentage CRs during the savings experiment as they already know what is going to happen and are therefore likely to be more relaxed. Besides, in general, subjects appeared relaxed and involved in watching their favorite movie.

FRAXA is often associated with autism (Rogers et al. 2001). Several behavioral symptoms and cerebellar neuro-anatomical abnormalities are found in individuals in both disorders (Belmonte and Carper 1998, Kaufmann et al. 2003, Rogers et al. 2001). During eyeblink conditioning both subjects with autism (Sears et al. 1994) and FRAXA show faster extinction of the CR. However, subjects with autism also show faster acquisition of the CR contrary to the slower acquisition observed in the FRAXA subjects. Thus, although both disorders appear to have a cerebellar component, at present one cannot pinpoint a common pathological mechanism underlying the symptoms.

Clinical implications

To date, there is no drug available addressing or preventing the behavioral features of individuals with FRAXA. In a previous study we described an individual with FRAXA who presented with exceptionally good cognitive, motor and behavioral capacities and had reached a high level of self-sufficiency (Govaerts et al., 2007). His performance might be the result of intensive mental and physical training he received throughout childhood. The acquisition experiment suggested that cerebellar plasticity is affected in FRAXA patients, but our results also showed that a significant improvement in motor learning is achieved by repeating the same experiment. Therefore including more physical exercise in training programs could be beneficial to compensate for the reduced cerebellar plasticity. An important challenge remains to further understand this disorder to be able to improve guidance and treatment of individuals with FRAXA.

Conclusion

Different performance during acquisition and savings shows that the well-defined genetic mutation of FRAXA provides an interesting model to study location of plasticity involved in delay eyeblink conditioning. We can conclude that absence of FMRP significantly influences cerebellar motor learning in individuals with FRAXA. On the long term, FRAXA subjects can save and thereafter rapidly extinct the delay CR. Presence of savings and extinction behavior in FRAXA suggests that different sites or mechanisms of learning and storage of the memory traces are involved in these types of associative learning than in acquisition of the CR.

Acknowledgements

This study was supported by NWO: 91656179, ZonMw: 912-07-022, NIH: 5 RO1 HD38038, RdJ/OP05/16/100506.

We thank the participants and their families for their contribution.

REFERENCES

Attwell, P.J., Ivarsson, M., Millar, L. & Yeo, C.H. (2002) Cerebellar mechanisms in eyeblink conditioning. *Ann N Y Acad Sci* 978, 79-92.

Attwell, P.J.E., Rahman, S. & Yeo, C.H. (2001) Acquisition of eyeblink conditioning is critically dependent on normal function in cerebellar cortical lobule HVI. *Journal of Neuroscience* 21, 5715-5722.

Bagni, C. & Greenough, W.T. (2005) From mRNP trafficking to spine dysmorphogenesis: the roots of fragile X syndrome. *Nat Rev Neurosci* 6, 376-387.

Bakker, C.E., de Diego Otero, Y., Bontekoe, C., Raghoe, P., Luteijn, T., Hoogeveen, A.T. Oostra, B.A. & Willemsen, R. (2000) Immunocytochemical and biochemical characterization of FMRP, FXR1P, and FXR2P in the mouse. *Exp Cell Res* 258, 162-170.

Bangasser, D.A. & Shors, T.J. (2004) Acute stress impairs trace eye blink conditioning in females without altering the unconditioned response. *Neurobiol Learn Mem* 82, 57-60.

Bear, M.F., Huber, K.M. & Warren, S.T. (2004) The mGluR theory of fragile X mental retardation. *Trends in Neurosciences* 27, 370-377.

Beckel-Mitchener, A. & Greenough, W.T. (2004) Correlates across the structural, functional, and molecular phenotypes of fragile X syndrome. *Ment Retard Dev Disabil Res Rev* 10, 53-59.

Belmonte, M. & Carper, R. (1998) Neuroanatomical and neurophysiological clues to the nature of autism. In: Garreau, B., (ed) , *Neuroimaging in Child Neuropsychiatric Disorders.* Springer-Verlag, Berlin pp. 157-191.

Bracha, V. (2004) Role of the cerebellum in eyeblink conditioning. *Prog Brain Res* 143, 331-339.

Bracha, V., Zhao, L., Irwin, K.B., Bloedel, J.R. (2000) The human cerebellum and associative learning: dissociation between the acquisition, retention and extinction of conditioned eyeblinks. *Brain Res* 860: 87-94.

Chen, L., Bao, S., Lockard, J.M., Kim, J.K. & Thompson, R.F. (1996) Impaired classical eyeblink conditioning in cerebellar-lesioned and Purkinje cell degeneration (pcd) mutant mice. *J Neurosci* 16, 2829-2838.

Christian, K.M. & Thompson, R.F. (2003) Neural substrates of eyeblink conditioning: acquisition and retention. *Learn Mem* 10, 427-455.

Christian, K.M. & Thompson, R.F. (2005) Long-term storage of an associative memory trace in the cerebellum. *Behavioral Neuroscience* 119, 526-537.

Clark, R.E. & Squire, L.R. (1998) Classical conditioning and brain systems: the role of awareness. *Science* 280, 77-81.

Comery, T.A., Harris, J.B., Willems, P.J., Oostra, B.A., Irwin, S.A., Weiler, I.J. & Greenough, W.T. (1997) Abnormal dendritic spines in fragile X knockout mice: maturation and pruning deficits. *Proc Natl Acad Sci* U S A 94, 5401-5404.

Crawford, D.C., Meadows, K.L., Newman, J.L., Taft, L.F., Pettay, D.L., Gold, L.B., Hersey, S.J., Hinkle, E.F., Stanfield, M.L., Holmgreen, P., Yeargin-Allsopp, M., Boyle, C. & Sherman, S.L. (1999) Prevalence and phenotype consequence of FRAXA and FRAXE alleles in a large, ethnically diverse, special education-needs population. *Am J Hum Genet* 64, 495-507.

de Vries, B.B., van den Ouweland, A.M., Mohkamsing, S., Duivenvoorden, H.J., Mol, E., Gelsema, K., van Rijn, M., Halley, D.J., Sandkuijl, L.A., Oostra, B.A., Tibben, A. & Niermeijer, M.F. (1997) Screening and diagnosis for the fragile X syndrome among the mentally retarded: an epidemiological and psychological survey. Collaborative Fragile X Study Group. *Am J Hum Genet* 61, 660-667.

De Zeeuw, C.I . & Yeo, C.H. (2005) Time and tide in cerebellar memory formation. *Curr Opin Neurobiol* 15, 667-674.

Domjan, M. (2005) Pavlovian conditioning: A functional perspective. *Annual Review of Psychology* 56, 179-206.

Duncko, R., Cornwell, B., Cui, L., Merikangas, K.R. & Grillon, C. (2007) Acute exposure to stress improves performance in trace eyeblink conditioning and spatial learning tasks in healthy men. *Learn Mem* 14, 329-335.

Eliez, S., Blasey, C.M., Freund, L.S., Hastie, T. & Reiss, A.L. (2001) Brain anatomy, gender and IQ in children and adolescents with fragile X syndrome. *Brain* 124, 1610-1618.

Fisch, G.S., Carpenter, N., Howard-Peebles, P.N., Holden, J.J., Tarleton, J., Simensen, R. & Nance, W. (2007) Studies of age-correlated features of cognitive-behavioral development in children and adolescents with genetic disorders. *Am J Med Genet* A 143, 2478-2489.

Gerwig, M., Hajjar, K., Dimitrova, A., Maschke, M., Kolb, F.P., Frings, M., Thilmann, A.F., Forsting, M., Diener, H.C. & Timmann, D. (2005) Timing of conditioned eyeblink responses is impaired in cerebellar patients. *J Neurosci* 25, 3919-3931.

Gothelf, D., Furfaro, J.A., Hoeft, F., Eckert, M.A., Hall, S.S.,

O'Hara, R., Erba, H.W., Ringel, J., Hayashi, K.M., Patnaik, S., Golianu, B., Kraemer, H.C., Thompson, P.M., Piven, J .& Reiss, A.L. (2007) Neuroanatomy of fragile X syndrome is associated with aberrant behavior and the fragile X mental retardation protein (FMRP). *Ann Neurol* [Epub ahead of print].

Govaerts, L.C., Smit, A.E., Saris, J.J., VanderWerf, F., Willemsen, R., Bakker, C.E., De Zeeuw, C.I. & Oostra, B.A. (2007) Exceptional good cognitive and phenotypic profile in a male carrying a mosaic mutation in the FMR1 gene. *Clin Genet* 72, 138-144.

Hessl, D., Rivera, S.M. & Reiss, A.L. (2004) The neuroanatomy and neuroendocrinology of fragile X syndrome. *Ment Retard Dev Disabil Res Rev* 10, 17-24.

Huber, K.M. (2006) The fragile X-cerebellum connection. *Trends Neurosci* 29, 183-185.

Ito, M. (2002) Historical review of the significance of the cerebellum and the role of Purkinje cells in motor learning. *Ann N Y Acad Sci* 978, 273-288.

Jin, P., Alisch, R.S. & Warren, S.T. (2004) RNA and microRNAs in fragile X mental retardation. *Nat Cell Biol* 6, 1048-1053.

Kaufmann, W.E., Cooper, K.L., Mostofsky, S.H., Capone, G.T., Kates, W.R., Newschaffer, C.J., Bukelis, I., Stump, M.H., Jann, A.E., Lanham, D.L. (2003) Specificity of cerebellar vermian abnormalities in autism: A quantitative magnetic resonance imaging study. *J Child Neurol* 18, 463-470.

Kitazawa, S. (2002) Neurobiology: Ready to unlearn. *Nature* 416, 270-273.

Koekkoek, S.K., Den Ouden, W.L., Perry, G., Highstein, S.M. & De Zeeuw, C.I. (2002) Monitoring kinetic and frequency-domain properties of eyelid responses in mice with magnetic distance measurement technique. *J Neurophysiol* 88, 2124-2133.

Koekkoek, S.K., Hulscher, H.C., Dortland, B.R., Hensbroek, R.A., Elgersma, Y., Ruigrok, T.J. & De Zeeuw, C.I. (2003) Cerebellar LTD and learning-dependent timing of conditioned eyelid responses. *Science* 301, 1736-1739.

Koekkoek, S.K., Yamaguchi, K., Milojkovic, B.A., Dortland, B.R., Ruigrok, T.J., Maex, R., De Graaf, W., Smit, A.E., VanderWerf, F., Bakker, C.E., Willemsen, R., Ikeda, T., Kakizawa, S., Onodera, K., Nelson, D.L., Mientjes, E., Joosten, M., De Schutter, E. Oostra, B.A. & et al. (2005) Deletion of FMR1 in Purkinje cells enhances parallel fiber LTD, enlarges spines, and attenuates cerebellar eyelid conditioning in Fragile X syndrome. *Neuron* 47, 339-352.

Medina, J.F., Garcia, K.S. & Mauk, M.D. (2001) A mechanism for savings in the cerebellum. *J Neurosci* 21, 4081-4089.

Ohlrich, E.S. & Ross, L.E. (1968) Acquisition and differential conditioning of the eyelid response in normal and retarded children. *J Exp Child Psychol* 6, 181-193.

Perrett, S.P. & Mauk, M.D. (1995) Extinction of Conditioned Eyelid Responses Requires the Anterior Lobe of Cerebellar Cortex. *Journal of Neuroscience* 15, 2074-2080.

Raven, J. (2000) The Raven's progressive matrices: change and stability over culture and time. *Cognit Psychol* 41, 1-48.

Rogers, S.J., Wehner, E.A., Hagerman, R. (2001) The behavioural Phenotype in Fragile X: Symptoms of autism in very toung children with fragile X syndrome, idiopathic autism, and other developmental disorders. *J Dev Behav Pediatr* 22, 409-417.

Sears, L.L., Finn, P.R., Steinmetz, J.E. (1994) Abnormal classical eye-blink conditioning in autism. *J Autism Dev Disord* 24, 737- 751.

Shors, T.J. (2001) Acute stress rapidly and persistently enhances memory formation in the male rat. *Neurobiol Learn Mem* 75, 10-29.

Smith, C.N., Clark, R.E., Manns, J.R. & Squire, L.R. (2005) Acquisition of differential delay eyeblink classical conditioning is independent of awareness. *Behav Neurosci* 119, 78-86.

Tamanini, F., Willemsen, R., van Unen, L., Bontekoe, C., Galjaard, H., Oostra, B.A. & Hoogeveen, A.T. (1997) Differential expression of FMR1, FXR1 and FXR2 proteins in human brain and testis. *Hum Mol Genet* 6, 1315-1322.

Timmann, D., Gerwig, M., Frings, M., Maschke, M. (2005) Eyeblink conditioning in patients with hereditary ataxia: a one-year follow-up study. *Exp Brain Res* 162: 332-345.

Turner, G., Webb, T., Wake, S. & Robinson, H. (1996) Prevalence of fragile X syndrome. *Am J Med Genet* 64, 196-197.

Vanderklish, P.W. & Edelman, G.M. (2005) Differential translation and fragile X syndrome. *Genes Brain Behav* 4, 360-384.

Verkerk, A.J.M.H., Pieretti, M., Sutcliffe, J.S., Fu, Y.H., Kuhl, D.P., Pizzuti, A., Reiner, O., Richards, S., Victoria, M.F., Zhang, F.P., Eussen, B.E., van Ommen, G.B., Blonden, L.A.J., Riggins, G.J., Chastain J.L., Kunst, C.B., Galjaard, H., Caskey, C.T. & et al. (1991) Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster

region exhibiting length variation in fragile X syndrome. *Cell* 65, 905-914.

Villarreal, R.P. & Steinmetz, J.E. (2005) Neuroscience and learning: lessons from studying the involvement of a region of cerebellar cortex in eyeblink classical conditioning. *J Exp Anal Behav 84*, 631-652.

WoodruffPak, D.S., Papka, M. & Ivry, R.B. (1996) Cerebellar involvement in eyeblink classical conditioning in humans. *Neuropsychology* 10, 443-458. Attwell, P. J., M. Ivarsson, et al. (2002). «Cerebellar mechanisms in eyeblink conditioning.» Ann N Y Acad Sci 978: 79-92.

CHAPTER 6

Exceptional good cognitive and phenotypic profile in a male carrying a mosaic mutation in the *FMR1* gene

ABSTRACT

Fragile X (FRAX) syndrome is a common inherited form of mental retardation resulting from the lack of expression of the fragile X mental retardation protein (FMRP). It is caused by a stretch of CGG repeats within the fragile X gene, which can be unstable in length as it is transmitted from generation to generation. Once the repeat exceeds a threshold length, the *FMR1* gene is methylated and no protein is produced resulting in the fragile X phenotype. The consequences of FMRP absence in the mechanisms underlying mental retardation are unknown.

We have identified a male patient in a classical FRAX family without the characteristic FRAX phenotype. His IQ is borderline normal despite the presence of a mosaic pattern of a premutation (25%), full mutation (60%) and a deletion (15%) in the *FMR1* gene. The cognitive performance was determined at the age of 28 by the Raven test and his IQ was 81. However, FMRP expression studies in both hair roots and lymphocytes, determined at the same time as the IQ test, were within the affected male range. The percentage of conditioned responses after delay eyeblink conditioning was much higher than the average percentage measured in FRAX studies. Moreover, this patient showed no correlation between FMRP expression and phenotype and no correlation between DNA diagnostics and phenotype.

INTRODUCTION

The fragile X syndrome is the most common form of hereditary cognitive impairment, with a frequency of 1:4000 males and 1:6000 females [1], and is caused by expansion of a trinucleotide repeat (CGG) located upstream of the coding region in the fragile X mental retardation gene (*FMR1)* [2].

In the normal population, the repeat length varies from 5 to 50 CGG repeats [3]. Subjects with the fragile X syndrome have more than 200 CGG repeats (full mutation; FM) and, as a consequence, the *FMR1* promoter region, including the CGG repeat, is hypermethylated [4, 5]. As hypermethylation results in a lack of *FMR1* gene transcription, no *FMR1* protein (FMRP) is produced. The absence of FMRP in the brain (neurons) is responsible for the cognitive impairment in patients with the fragile X syndrome [6, 7].

In addition, some individuals have alleles between 50 and 200 CGG repeats (premutations; PM). PM alleles are unmethylated with FMRP biosynthesis, but are unstable during transmission to the next generation. Individuals with a premutation do not show the classic phenotype of the fragile X syndrome and were initially thought to be asymptomatic, although a number of studies have reported mild learning disabilities and social phobias or anxiety disorders in a small subgroup of premutation carriers [8]. Evidence gathered over the past 5 years shows that premutation carriers (especially males) are at risk of a neurodegenerative disorder, FXTAS [9, 10]. This disorder is completely distinct (from both clinical and molecular pathogenic perspectives) from the neurodevelopmental disorder, fragile-X syndrome. Up to 40% of affected males show a variable combination of PM and FM alleles, but with the usual predominance of FM [11]. In this mosaic genotype up to 30% of the cells can contain a PM allele. The main characteristic of male fragile X patients is mild to severe learning difficulties. The disorder is further characterized by some physical anomalies, including elongated facial structure, large protruding

ears, hyperextensible joints, and, in males specifically, macroorchidism [8]. In addition, the phenotype is characterized by other variable neurobehavioral indications, including hyperarousal, attention deficit, anxiety, social withdrawal, enhanced blink rate ([12], and seizure susceptibility.

Tassone et al [13] reported 3 non-retarded males with expression of FMRP in >50% of lymphocytes. One mosaic proband had 83.6 % premutation repeat length in lymphocytes, the other two had partially methylated full mutations. All three showed some manifestations of fragile X syndrome, both physical and behavioral characteristics.

Recently, we have reported that FRAX patients show cerebellar deficits in eyeblink conditioning [14]. The study showed aberrant delay eyeblink conditioning implicating cerebellar deficits in FRAX patients. This test has been shown clearly to discriminate between FRAX patients and control. Delay eyeblink conditioning is a useful tool to assess cerebellar functioning during an associative learning process [14]. During conditioning an individual learns to associate a conditioning stimulus (CS), like a tone, with an unconditioning stimulus (US), in this case a corneal air puff. Delay eyeblink conditioning is characterized by a delay between the onset of the CS and the US, but simultaneous end of both stimuli. First subjects will show reflex blinks in the paired CS/US trials. After repeated exposure to paired stimuli the onset of eyelid movement occurs earlier than a reflex, and eventually the subject should learn to precisely time closure of the eyelids in time to prevent the air puff from reaching the cornea. This eyelid closure is called a conditioned response (CR). Repetition of the same paradigm half a year after the initial training session enabled us to monitor saving of the previously learned behavior. Extinction or unlearning of the conditioned response was the third parameter.

Here we report about a male patient in a classical FRAX family without the characteristic FRAX phenotype. His IQ is in the borderline normal range despite the presence of a mosaic premutation and full mutation. The cognitive performance was determined by the Raven test and his IQ was 81. However, FMRP expression studies in both hair roots and lymphocytes were within the affected male range. The percentage of conditioned responses after the delay eyeblink conditioning showed no difference from that of healthy subjects.

MATERIALS AND METHODS

Family data

Individual E.C is a member of a family in which several relatives have the classical FRAX phenotype. His father is a flight controller at the national airport, giving training courses all over the world. His mother is a teacher by training. The mother dedicated several years of her live to an intensive training program for her son: 8 years of logopedic training, 8 years of physiotherapy, 6 years of private swimming lessons. The current age of E.C is 30 years.

Raven test

Individual E.C. completed the Standard Raven's Progressive Matrices (RPM) to measure the basic cognitive functioning.The RPM was constructed by Raven in 1954 and ever since has been widely applied in both clinical practice and research. The RPM measures the educative ability component of general intelligence (g) as defined in Spearman's theory of cognitive ability. Raw scores were transferred into IQ scores. [15].

FMRP expression in hair roots and lymphocytes

Determination of FMRP expression in lymphocytes and hair roots was carried out as described earlier [16, 17]. These diagnostic tests can identify fragile X patients based on the lack of FMRP in their lymphocytes and hair roots.

Delay eyeblink conditioning

Eyeblink conditioning is a useful tool to assess cerebellar function during an associative learning process [14]. The conditioning sessions consisted of 80 trials divided in 60 paired trials, 10 CS only trials and 10 US only trials. The stimuli were randomized over 10 blocks. The extinction session consisted of 75 CS-only trials. In the 2 second trials the tone CS (500 ms, 650 Hz, 80 dB) started after 500 ms and the air puff US (20 ms, 2 Bar) was presented at the end of the CS at 1000 ms. An eyelid response was considered a conditioned response (CR) if the onset of the eyelid movement occurred between 700 and 1050 ms and the amplitude exceeded the SD of the baseline at least twice. The SD of the baseline was determined with the first 500 ms of each recording. Trials were excluded when eyelid movement occurred within this period. The amplitude is expressed in percentage of eyelid closure to allow more accurate comparison between subjects.

DNA and Genotyping

DNA isolated from peripheral blood cells, from patient and his mother was analyzed by Southern blotting using probe pP2 [18] after double-digestion with *Hind*III plus the methylation sensitive enzymes *Eag*I or *BssH*II. The size of the CGG repeat was determined using the probe pP2 as described before [17]. Fragments expected in normal males and females are 5.2 kb after *Hind*III digest only, which will be digested to 2.8 kb by either *Eag*I or *BssH*II, depending on methylation status, i.e. due to CGG repeat expansion and/or (female) X-inactivation. Repeat length was also determined by PCR [3]. Deletion breakpoints were defined by PCR amplification of genomic DNA of patient and mother using primers #013 (Forward 5'CGC GTC TGT CTTTCG ACCCG) and #014 (Reverse 5' TAG GCG CTA GGG CCT CTC GG) [19] and direct sequencing.

RESULTS

Phenotypic and genotypic features

E.C. had his IQ tested at the beginning of primary school, as there was a family history of FRAX and the parents wanted to have the best education. The result, reported by the mother, was 90, with some problems with mathematics. No written report of the WAIS test was available. He needed remedial teaching at school. He has finished the secondary school, MAVO, which is the equivalent of second grade school. Now he has a regular job and is living on his own without assistance. E.C. makes good eye contact, he is appropriate in his social interactions, and he has no facial dysmorphisms, normal ears and no macroorchidism. There is no echolalia. According to the fragile X checklist of Hagerman E.C. scores only positive on family history of mental retardation [8]. Unfortunately, permission to publish his picture was denied.

DNA analysis was carried out in 1994 in the period after the identification of the CGG repeat in the *FMR1* gene. The mother of the proband has a premutation and a normal allele. A mosaic pattern

of a premutation (70 CGGs) and a full mutation (300 CGGs) was detected in the proband (data not shown). At a later stage when the other tests were performed a new blood was taken and on the newly isolated DNA the mutation in the *FMR1* gene was determined. In the proband a mosaic pattern of four discrete bands was detected (Fig 1). Two methylated bands with a full mutation size were detected that comprised more than 60 % of the *FMR1* alleles of 5.9 kb (230 repeats) and 7.3 kb (450 repeats) as determined by densitometry. Next a premutation size band (170 repeats; 25 %) as well as a band with a deletion of 175 bp (15%) was seen. This deletion removes the CGG repeat together with 61 bp in front of the CGG repeat and 27 bp after the repeat. Mosaic deletions have been described before in the CGG region [19].

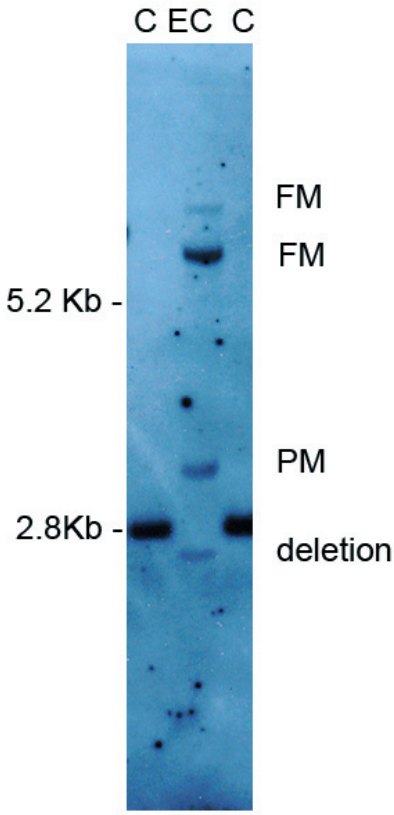


Figure 1. Southern blot of control DNA (C) and of E.C. digested with *Eco*RI and *Eag*I, and hybridized with probe pP2.

Raven test, FMRP expression in hair roots and lymphocytes of the proband

We subjected E.C. to the Raven test and found a raw score of 24, reflecting an IQ of 81. In order to try to search for an explanation for the discrepancy between the DNA genotype and the phenotype we decided to determine the FMRP expression both in blood and in hair roots. The FMRP expression in lymphocytes of E.C. was 18%, which is clearly in the range below 20% as seen in FRAX patients [16].

Control individuals show clear expression of FMRP in nearly every hair root, whereas male fragile X patients lack expression of FMRP in almost all their hair roots [17]. The FMRP expression in hair roots of E.C. was 6% and thus in the affected range.

Delay eyeblink conditioning

This test has been shown clearly to discriminate between FRAX patients and control. The profiles of the FRAX patient show fast learning of the CR during the first conditioning session. Six months later, the CR occurs earlier during the (saving) eyelid conditioning session and further improvement of the amplitude of lid movement was seen. During the third extinction session rapid unlearning of the CR was observed (Fig 2 and Table). The percentage of eyelid closure during CRs measured in the first session was 36%. During the second conditioning session the percentage of eyelid closure was 48% and during extinction 55%. The peak velocities were 6.29, 8.79 and 10.23 cm/s, respectively. In the first conditioning session 59% of the stimuli evoked a CR in the second conditioning session this percentage increased to 93%. During the extinction the patient showed CRs in 17% of the trials. Figure 3 shows these percentages in addition to the percentages previously found in FRAX patients and control subjects during conditioning. The average onset latencies of the CR of the subject were 0.88, 0.83, 0.87 seconds during the first, second and extinction session, respectively.

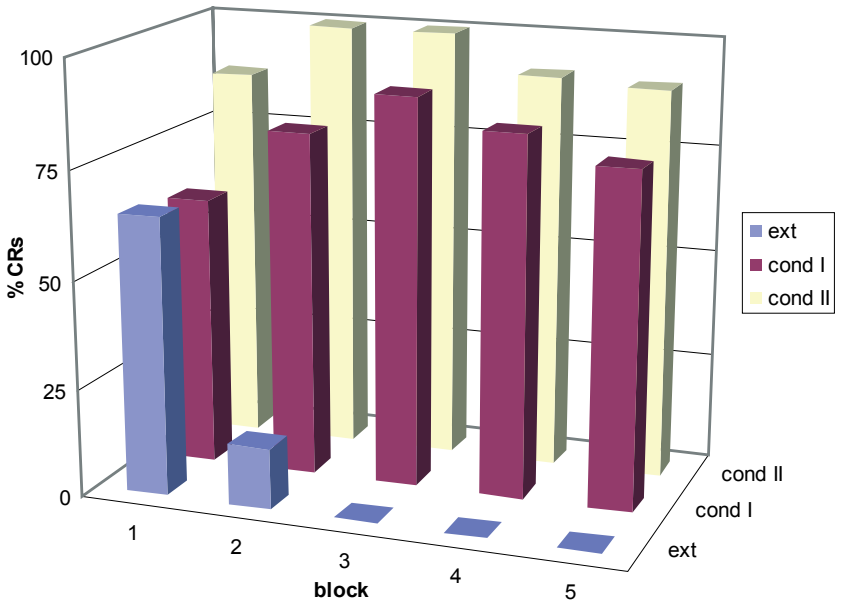


Figure 2. Percentage of conditioned responses during conditioning session I (cond I), conditioning session II (cond II) and extinction (ext). Each session has been divided into 5 blocks.

Table 1. Average values of the amplitude, velocity and onset of conditioned responses of E.C. during conditioning session I (cond I), conditioning session II (cond II) and extinction (ext). Standard deviations are given in parenthesis.

	cond I		cond II		ext	
Average Amplitude (%)	36	(16)	48	(11)	55	(7)
Average Velocity (cm/s)	6.29	(3.20)	8.79	(1.84)	10.23	(1.66)
Average Onset	0.88	(0.095)	0.83	(0.075)	0.87	(0.078)

DISCUSSION

Phenotype and genotype

The spectrum of the phenotype of males with a full mutation varies from mildly to severely retarded, and predictive protein testing is in most cases not informative for degree of retardation within the FRAX patients [20]. Protein testing shows a clear difference between controls and FRAX patients. However, E.C. showed no correlation between FMRP expression both on blood and in hair roots (both below 20%) and his phenotype; FMRP expression is clearly in the range found in affected males, while his IQ is in the borderline normal range [20]. There is a small difference between FMRP expression in blood and hair roots, but they are both in the very low range. Lymphocytes and hair roots cells have different origins. Lymphocytes originate from connective tissue, which arises from mesoderm. In contrast, both neurons and skin develop from the ectoderm. Therefore it was suggested that there might be a better correlation between (the lack of) FMRP expression in hair roots and mental retardation [17].

We also did not find a correlation between DNA genotype (60% methylated and 15% a deletion) and phenotype. The combination of a mosaic full mutation repeat length, FMRP expression in both hair roots and lymphocytes in the affected range and normal intelligence is very rare. Eyeblink conditioning results are very similar to controls. This procedure is a more direct way to interrogate neuronal function and offers additional information. Hagerman [8] reported a boy with 18% FMRP expression, and normal intellectual abilities, but with learning problems in the area of attention and concentration and visual motor coordination, in addition to academic problems in math.

The contribution of genetic background and environmental factors to cognitive functioning in female carriers of the FM was investigated by studying FMRP expression in hair roots and cognitive functioning of female FM carriers and a group female control relatives. No important role of genetic background and environmental factors could be determined [20]. A systematic analysis in affected males is not available in the literature. In females it is important which proportion of the normal X chromosome is inactivated in females with a full mutation.

E.C. is clearly a DNA mosaic, but the percentage of methylated alleles is more than 50% which has been found in FRAX patients to a mental retarded phenotype. The percentage of methylated (60%) and deleted (15%) alleles is in line with the FMRP expression in blood and hair roots (below 20%). Recently an individual with a relative high IQ (64-72) and a full mutation was described [21]. When a discrepancy between the IQ and the DNA test is identified Willemsen [17] recommended to carry out a hairroot test. In this way a number of cases were identified in which the FMRP test was in line with the IQ. E.C. is an exceptional finding.

Delay eyeblink conditioning

It was previously shown that people with FRAX display aberrant delay eyeblink conditioning profiles and that these deficits are probably largely due to a lack of FMRP in cerebellar Purkinje cells [14]. In that study peak amplitude (0.14 cm) and a peak velocity (3.3 cm/s) was found in FRAX males and that is on average significantly lower than those in normal subjects (0.54 and 10.0 cm/s, respectively). The peak amplitude and velocity values of the CR in this subject are comparable with values of normal subjects as found by Koekkoek *et al*. [14].

Moreover, 79% CRs is higher than the 37.6% CRs of FRAX males, found in that study and equal to the 80.3% CRs of normal subjects (Fig 3). The onset latency values are comparable to the previously found onset latencies of both affected males (0.87) and healthy males (0.92).

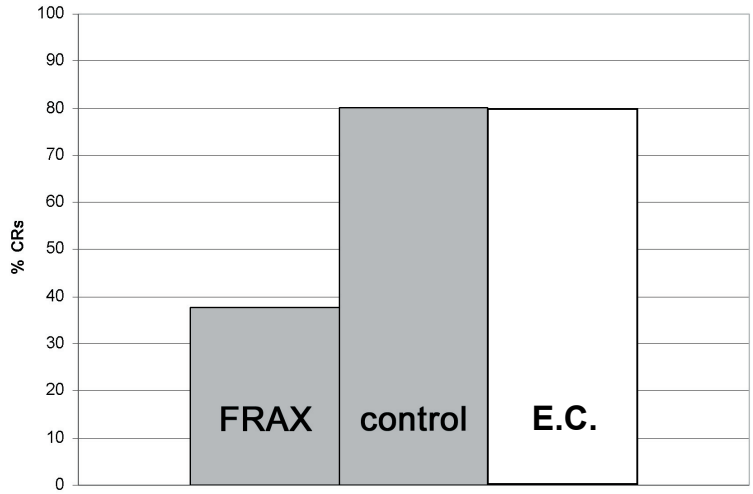


Figure 3. Average percentage of conditioned responses during conditioning session I of FRAX patients, control subjects and E.C.

Nature versus nurture

How can we explain the exceptionally mild phenotype of E.C. The relatively high IQ might be explained by the genetic background of E.C. He is born in a well educated family and a drop of IQ can lead to a still relatively high IQ. On the other hand there might be a relative increase in IQ points due to intense stimulation.

The parents were strongly engaged in educational intervention at an early stage, including adequate support and child adapted educational programs: he received years of training : 6 years of swimming lessons, it took years before he managed to ride a bicycle, 8 years of logopedy, 8 years of physiotherapy, especially for fine motor skills. They were convinced of the beneficial result of their efforts. Rat experiments show promising results of stimulation. Also in the mouse enrichment can reverse several structural and behavioral abnormalities resulting from the silencing of the *FMR1* gene [22]. The behavioral data are consistent with the observations by Reiss and coworkers [23, 24],

indicating that environmental factors positively influence the behavioral outcome in children with FRAX syndrome. Some mechanisms of neuronal plasticity are preserved in *FMR1*-KO mice and can be triggered by environmental stimulation [22]. However, intellectual training can not account for absence of phenotypic characteristics of the proband and a molecular explanation has to be taken into account too.

In conclusion, we have identified an individual with a FRAX genotype with an unexpected IQ in the normal range.

Acknowledgements

We are thankful for technical assistance by Liesanne Severijnen. This research was financially supported FRAXA, by Zon-MW (C.I de Z.), EU (SENSOPAC) (C.I de Z.), Prinses Beatrix Fonds (C.I de Z. and R.W.), NFXF (R.W.), NIH RO1 HD38038 (B.A.O). We like to thank Dr. F. Fanserine of the AMC for providing previous data about E.C.

REFERENCES

1. De Vries, B.A., A.M.W. van den Ouweland, S. Mohkamsing, et al. Screening and diagnosis for the fragile X syndrome among the mentally retarded: an epidemiological and psychological survey. Am J Hum Genet 1997: 61: 660-667.

2. Kooy, R.F., R. Willemsen, and B.A. Oostra Fragile X syndrome at the turn of the century. Mol Med Today 2000: 6: 193-198.

3. Fu, Y.H., D.P. Kuhl, A. Pizzuti, et al. Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox. Cell 1991: 67: 1047-1058.

4. Verkerk, A.J., M. Pieretti, J.S. Sutcliffe, et al. Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell 1991: 65: 905-914.

5. Oberlé, I., F. Rousseau, D. Heitz, et al. Instability of a 550-base pair DNA segment and abnormal methylation in fragile X syndrome. Science 1991: 252: 1097-1102.

6. Verheij, C., C.E. Bakker, E. de Graaff, et al. Characterization and localization of the FMR-1 gene product associated with fragile X syndrome. Nature 1993: 363: 722-724.

7. Devys, D., Y. Lutz, N. Rouyer, J.P. Bellocq, and J.L. Mandel The FMR-1 protein is cytoplasmic, most abundant in neurons and appears normal in carriers of a fragile X premutation. Nat Genet 1993: 4: 335-340.

8. Hagerman, R.J. The physical and behavioural phenotype. in Fragile-X syndrome: diagnosis, treatment and research. R.J. Hagerman and P. Hagerman. Eds: The Johns Hopkins University Press, Baltimore.2002: 3-109.

9. Hagerman, R.J., M. Leehey, W. Heinrichs, et al. Intention tremor, parkinsonism, and generalized brain atrophy in male carriers of fragile X. Neurology 2001: 57: 127-30.

10. Jacquemont, S., R.J. Hagerman, M. Leehey, et al. Fragile X Premutation Tremor/Ataxia Syndrome: Molecular, Clinical, and Neuroimaging Correlates. Am J Hum Genet 2003: 72: 869-78.

11. Nolin, S.L., A. Glicksman, G.E. Houck, W.T. Brown, and C.S. Dobkin Mosaicism in fragile X affected males. Am J Med Genet 1994: 51: 509-512.

12. Roberts, J.E., F.J. Symons, A.M. Johnson, D.D. Hatton, and M.L. Boccia Blink rate in boys with fragile X syndrome: preliminary evidence for altered dopamine function. J Intellect Disabil Res 2005: 49: 647-56.

13. Tassone, F., R.J. Hagerman, D.N. Ikle, et al. FMRP expression as a potential prognostic indicator in fragile X syndrome. Am J Med Genet 1999: 84: 250-61.

14. Koekkoek, S.K., K. Yamaguchi, B.A. Milojkovic, et al. Deletion of FMR1 in Purkinje Cells Enhances Parallel Fiber LTD, Enlarges Spines, and Attenuates Cerebellar Eyelid Conditioning in Fragile X Syndrome. Neuron 2005: 47: 339-52.

15. Raven, J. The Raven's progressive matrices: change and stability over culture and time. Cognit Psychol 2000: 41: 1-48.

16. Willemsen, R., S. Mohkamsing, B. De Vries, et al. Rapid antibody test for fragile X syndrome. The Lancet 1995: 345: 1147-1148.

17. Willemsen, R., B. Anar, Y. de Diego Otero, et al. Noninvasive test for Fragile X syndrome, using hair root analysis. Am J Hum Genet 1999: 65: 98-103.

18. Verkerk, A.J., B.B. de Vries, M.F. Niermeijer, et al. Intragenic probe used for diagnostics in fragile X families. Am J Med Genet 1992: 43: 192-196.

19. De Graaff, E., P. Rouillard, P.J. Willems, et al. Hotspot for deletions in the CGG repeat region of FMR1 in fragile X patients. Hum Mol Genet 1995: 4: 45-49.

20. Willemsen, R., A. Smits, L.A. Severijnen, et al. Predictive testing for cognitive functioning in female carriers of the fragile X syndrome using hair root analysis. J Med Genet 2003: 40: 377-9.

21. Han, X.D., B.R. Powell, J.L. Phalin, and F.F. Chehab Mosaicism for a full mutation, premutation, and deletion of the CGG repeats results in 22% FMRP and elevated FMR1 mRNA levels in a high-functioning fragile X male. Am J Med Genet A 2006: 140A: 1463-1471.

22. Restivo, L., F. Ferrari, E. Passino, et al. Enriched environment promotes behavioral and morphological recovery in a mouse model for the fragile X syndrome. Proc Natl Acad Sci U S A 2005: 102: 11557-11562.

23. Hessl, D., J. Dyer-Friedman, B. Glaser, et al. The influence of environmental and genetic factors on behavior problems and autistic symptoms in boys and girls with fragile x syndrome. Pediatrics 2001: 108: E88.

24. Dyer-Friedman, J., B. Glaser, D. Hessl, et al. Genetic and environmental influences on the cognitive outcomes of children with fragile X syndrome. J Am Acad Child Adolesc Psychiatry 2002: 41: 237-44.

PART IV

Blinking,
Lip pursing and
Central compensations

CHAPTER 7

Blink recovery in patients with Bell's palsy: a neurophysiological and behavioural longitudinal study.

ABSTRACT

Purpose: To examine the recovery process of blinking in a longitudinal study of 9 severely affected Bell's palsy patients.

Methods: Kinematics of bilateral eyelid and eye movements and concomitant orbicularis oculi activity during voluntary blinking and air puff and acoustic click induced reflex blinking were determined using the magnetic search coil technique and electromyographic recording of the orbicularis oculi muscle (OO-EMG).

Results: In the first three months absence of OO-EMG activity, reduced eyelid and eye movement of the palsied eyelid were observed during all types of blinking. First OO-EMG activity was determined three months after onset of the affliction. After one year OO-EMG activity was normalized and showed similar values to the non-palsied side. Clinically, eyelid movements were normal after one year, although corresponding maximal amplitudes and corresponding velocities were two times smaller, expressed in reduced eyelid motility. Directions of eye movement during reflex blinking were normal after one year, although maximal amplitudes were smaller at the palsied side. Eye movements during voluntary blinking remained impaired. A simultaneous horizontal upward shift of both eyes in the same direction was recorded throughout the study.

Conclusions: Although OO-EMG activity on the palsied side was normalized one year after onset of the affliction, the accompanying eyelid movements, their maximal amplitudes and velocities remained smaller throughout the study. The consistent impairment of eye movements in voluntary blinking during the study and reduced motility of eyelid movements indicates that higher brain structures, modifying eyelid and eye movement control during blinking, might be altered by the affliction.

INTRODUCTION

Blinking is imperative to protect the eye against corneal drying and damage[1]. The characteristic eye and eyelid movements during a blink obtain an optimal tear film distribution over the cornea. Disturbances in this blinking pattern can lead to inflammation of the eye and if not treated to functional blindness.
Eyelid movement during blinking is mainly mediated by the levator palpebrae superioris and orbicularis oculi (OO) muscles[2 - 4]. Humans exhibit three different types of blinks: spontaneous, voluntary and reflex blinks[3, 5]. During blinking stereotypical eye movements occur[6, 7]. In healthy subjects, the eyeball rotates from a straight-ahead position nasally downward, directly followed by laterally upward movement during blinking. A slight displacement of the eyeball occurs into the orbit[6] by performing a horizontal, vertical rotation[8, 9] and torsion[10, 11]. The underlying neuronal structure initiating eye movement during blinking is not known, but recently evidence was obtained which showed that specific areas in the lateral medullar reticular formation are involved in eyelid and eye movements during blinking[12, 13].

Patients with Bell's palsy, a form of unilateral facial nerve palsy, characteristically have unilateral peripheral facial weakness and are unable to blink at the palsied side[14]. Those with complete clinical facial paralysis also have decreased tearing at the palsied side[15, 16] and eye irritation. The worldwide incidence of this affliction is approximately 30 cases per 100,000 people per year[16]. Clinically, facial functions appear to recover completely in these patients, although a significant number have residual

facial weakness and synkinesia. Bell's palsy patients, classified as House-Brackmann grade VI[17], show complete absence of excitability of the facial nerve within one week. A main feature of this grade of affliction is that blink restoration will not occur sufficiently. Blink restoration remains completely absent in some patients and surgical interference is their only possibility to regain eyelid motility.

Research on blinking of Bell's patients has mainly concentrated on reflex blinks elicited by electrical stimulation of the supraorbital (SO) nerve[18 - 23]. Analysis of eyelid kinematics during spontaneous and voluntary blinks showed varying degrees of recovery in patients with unilateral facial nerve palsy[20]. In other studies the OO-EMG recording technique was used to investigate the recovery of OO-muscle activity at different stages of facial nerve palsy[18, 21 - 23]. Adaptive changes of eyelid movements at the non-palsied side were observed[24, 25]. Whether adaptive changes and normal kinematical values of eyelid movement during blinking will be reached after recovery is not known. In order to examine these phenomena during blink recovery, eye and eyelid kinematics and OO-EMG features were monitored in a longitudinal study.

MATERIALS AND METHODS

Subjects

Nine patients (6 men and 3 women) with Bell's palsy, House-Brackmann grade VI were included in the study (Table 1). Inclusion criteria were: acute unilateral facial paralysis, onset and deterioration within 48 hours, impossible to excite the facial nerve within 10 days after onset of the affliction. All patients were given prednisolone, 60 mg orally for 5 days, decreasing to 10 mg at day 10 after which medication was stopped. Exclusion criteria were: viral Herpes simplex I, Varicella zoster, and diabetic cranial neuropathy (all established with laboratory tests) otological disorders, history of cerebral vascular accident, psychiatric history and pregnancy. The mean age of the patients at the onset of palsy was 46 $\pm$ 12 (mean $\pm$ SD; range 29 - 67) years. The mean duration of disease was 16 $\pm$ 3.7 (mean $\pm$ SD) months. All subjects gave an informed consent according to the Declaration of Helsinki and the Medical Ethics Committee of Erasmus MC University approved the study.

Eyelid movements, eye movements and OO-EMG recordings

Movements of both eyelids were measured with the magnetic search coil technique[26, 27]. Detailed procedures have been described previously[5]. While the subject looked straight ahead, a search coil (2.2 mm diameter, 20 turns, 20 mg) of copper wire (0.1 mm diameter) was placed 1 mm above the rim of the upper eyelid above the centre of the pupil. Movements of both eyes were also measured with the magnetic search coil technique using standard scleral search coils (Skalar Medical BV, Delft, The Netherlands). Prior to insertion of the search coil, the eye was anaesthetised with 2 drops of the topical ophthalmic anaesthetic oxybuprocaine 0.4% (Novesin®). A previously described device was used to calibrate eye and eyelid rotations[5].

For OO-EMG recordings, two 6-mm-diam Ag/AgCl surface electrodes were used. The active recording electrode was placed about 10 mm below the margin of the lower eyelid and the reference-recording electrode was placed 10 mm lateral to the temporal margin of the eyelids. A self-attached circle electrode (10 x 10 mm) was positioned on the forehead and served as ground electrode.

Table 1 represents OO-EMG and eyelid kinematics at four moments during recovery of voluntary and reflex blinking. The differences between both eyelids and their significances are presented in Table 2.

Type of Blinking	OO-EMG and Eyelid Kinematics	Onset		18 Weeks		36 Weeks		84 Weeks	
		Non-palsied	Palsied	Non-palsied	Palsied	Non-palsied	Palsied	Non-palsied	Palsied
Voluntary									
	Integrated OO-EMG (μVs)	4.8 ± 0.7	0.7 ± 0.2	4.1 ± 0.9	2.1 ± 0.6	3.6 ± 0.5	3.1 ± 0.6	2.8 ± 0.3	3.5 ± 0.5
	Start Lid – Start OO-EMG (ms)	19 ± 5	indefinite	9 ± 6	15 ± 2	12 ± 3	16 ± 2	29 ± 5	30 ± 3
	Down Phase (ms)	77 ± 8	128 ± 6	69 ± 6	135 ± 12	84 ± 9	121 ± 8	65 ± 6	93 ± 4
	Up Phase (ms)	265 ± 40	171 ± 40	212 ± 19	173 ± 22	194 ± 21	175 ± 22	220 ± 13	160 ± 23
	Maximal Amplitude (°)	44.0 ± 6.7	4.8 ± 0.8	48.7 ± 3.7	19.7 ± 4.0	46.3 ± 2.9	31.2 ± 4.4	36.9 ± 2.0	27.1 ± 4.3
	Maximal Velocity (°/s)	1304 ± 145	65 ± 7	1359 ± 135	331 ± 87	1379 ± 78	650 ± 117	1204 ± 74	676 ± 120
Air Puff-induced at Non-palsied Eyelid									
	Start OO-EMG (ms)	27 ± 2	indefinite	32 ± 1	43 ± 5	31 ± 2	34 ± 2	27 ± 2	28 ± 2
	Integrated OO-EMG (μVs)	5.8 ± 1.2	0.7 ± 0.2	3.9 ± 0.7	2.4 ± 0.7	3.6 ± 0.6	2.9 ± 0.5	3.2 ± 0.4	3.0 ± 0.8
	Start Time (ms)	56 ± 4	75 ± 6	49 ± 3	60 ± 5	40 ± 1	44 ± 1	41 ± 4	40 ± 2
	Start Lid – Start OO-EMG (ms)	29 ± 7	indefinite	17 ± 2	17 ± 7	9 ± 1	10 ± 2	14 ± 2	11 ± 1
	Down Phase (ms)	90 ± 23	97 ± 21	65 ± 6	93 ± 16	78 ± 8	93 ± 12	63 ± 5	81 ± 8
	Up Phase (ms)	294 ± 20	213 ± 13	237 ± 17	184 ± 11	199 ± 18	176 ± 19	207 ± 28	171 ± 26
	Maximal Amplitude (°)	47.9 ± 5.0	5.1 ± 1.0	46.0 ± 2.6	18.4 ± 4.8	44.7 ± 3.1	27.1 ± 4.9	39.8 ± 4.1	24.4 ± 5.3
	Maximal Velocity (°/s)	1675 ± 109	109 ± 9	1638 ± 92	460 ± 150	1566 ± 123	715 ± 133	1569 ± 136	748 ± 201
	Time Maximal Amplitude (ms)	146 ± 27	172 ± 21	114 ± 3	153 ± 18	118 ± 8	137 ± 12	104 ± 3	121 ± 9
	Time Maximal Velocity (ms)	70 ± 1	98 ± 9	72 ± 2	88 ± 4	69 ± 3	90 ± 4	71 ± 3	82 ± 3
Air Puff-induced at Palsied Eyelid									
	Start OO-EMG (ms)	31 ± 2	indefinite	34 ± 2	41 ± 4	34 ± 2	31 ± 2	30 ± 3	26 ± 2
	Integrated OO-EMG (μVs)	7.7 ± 2.8	1.1 ± 0.2	4.5 ± 0.6	2.6 ± 0.6	3.0 ± 0.4	3.5 ± 0.9	3.7 ± 0.9	3.9 ± 1.3
	Start Time (ms)	57 ± 3	82 ± 6	46 ± 2	54 ± 4	41 ± 2	42 ± 2	40 ± 5	38 ± 4
	Start Lid – Start OO-EMG (ms)	26 ± 3	indefinite	12 ± 4	12 ± 7	7 ± 1	11 ± 2	10 ± 3	12 ± 4
	Down Phase (ms)	101 ± 25	124 ± 28	80 ± 11	115 ± 18	73 ± 6	85 ± 7	74 ± 6	99 ± 18
	Up Phase (ms)	285 ± 36	205 ± 30	273 ± 34	213 ± 30	224 ± 35	211 ± 36	205 ± 22	163 ± 13
	Maximal Amplitude (°)	45.3 ± 4.6	7.3 ± 2.5	44.3 ± 3.6	19.5 ± 3.9	40.7 ± 3.3	28.7 ± 4.8	38.7 ± 3.1	32.1 ± 4.6
	Maximal Velocity (°/s)	1596 ± 125	123 ± 37	1569 ± 126	395 ± 100	1532 ± 144	782 ± 138	1561 ± 120	923 ± 144
	Time Maximal Amplitude (ms)	152 ± 17	206 ± 33	126 ± 9	169 ± 17	114 ± 6	127 ± 7	114 ± 7	136 ± 20
	Time Maximal Velocity (ms)	72 ± 2	102 ± 12	73 ± 3	98 ± 7	72 ± 2	83 ± 4	72 ± 5	82 ± 5
Acoustic Click-induced									
	Start OO-EMG (ms)	39 ± 3	indefinite	39 ± 1	45 ± 1	41 ± 1	40 ± 2	37 ± 2	37 ± 1
	Integrated OO-EMG (μVs)	1.3 ± 0.1	0.2 ± 0.2	1.2 ± 0.2	1.0 ± 0.2	1.2 ± 0.2	1.2 ± 0.2	1.4 ± 0.1	1.3 ± 0.1
	Start Time (ms)	56 ± 4	62 ± 4	49 ± 2	54 ± 3	48 ± 2	50 ± 2	49 ± 4	51 ± 3
	Start Lid – Start OO-EMG (ms)	16 ± 8	indefinite	10 ± 2	11 ± 3	7 ± 2	10 ± 3	11 ± 6	14 ± 4
	Down Phase (ms)	52 ± 8	54 ± 13	53 ± 2	57 ± 5	53 ± 3	57 ± 6	54 ± 4	54 ± 6
	Up Phase (ms)	155 ± 28	116 ± 20	150 ± 17	140 ± 17	149 ± 15	151 ± 14	202 ± 28	179 ± 29
	Maximal Amplitude (°)	22.1 ± 5.3	3.0 ± 0.5	22.0 ± 4.3	5.6 ± 1.1	21.5 ± 4.8	12.2 ± 2.5	17.5 ± 5.4	12.5 ± 3.1

Experimental procedures

Patients were recorded every six weeks. Three types of blinks were recorded: 1) voluntary; 2) acoustic click induced and 3) corneal trigeminal reflex (air puff induced, ipsi- and contralateral stimulated) blinks. The mean number of recordings per subject was 13. Out of the 52 recordings, 16 were simultaneous eye and eyelid recordings. The subject was seated comfortably in a chair and the subject's head was stabilised with a head holder by fixing his/her chin at a standard position. The measured eyelid/eye was placed near the centre of the magnetic field.

Voluntary blinks

Subjects were asked to focus on a fixation point in the middle of a 2 x 2 m flat transparent screen at a distance of 82 cm and to respond with a gentle blink as short as possible every time the fixation point disappeared for 50 ms. We recorded at least 15 blinks with a 6-second interval between successive blinks.

Trigeminal reflex blinks

Trigeminal blink reflexes were elicited by an air puff stimulus (2 bar, 10 ms). For this purpose, the end of a silicon rubber tube (diameter 1 mm) was directed towards the lateral rim of the iris, 2-3 mm from the eyeball. Air puff induced blinks were recorded with random stimulus intervals between 25 and 30 seconds. At least 15 registrations were made both after stimulation of the non-palsied and the palsied side.

Acoustic click induced reflex blinks

Blinks were elicited by an acoustic click of 90 dB SPL; duration 10 ms. The source box was placed 60 cm from subject's head, at the level of the external ear at the non-palsied side. Ten registrations were made with random stimulus intervals between 30 and 40 seconds.

Data acquisition and statistical analysis

Data acquisition was done as described previously[5]. For each trial, the computer displayed bilateral OO-EMG and eyelid/eye movements in vertical and horizontal directions. The raw data were stored on disks for off-line analysis.

For eyelid movements, we analysed the start time, the down phase duration, the up phase duration, the maximal down phase amplitude, the maximal down velocity, the time maximal amplitude and the time maximal velocity. For OO-EMG onset latency and integrated OO-EMG until the time of maximal amplitude of the eyelid movement were analysed for voluntary, air puff and acoustic click induced blinks. We also determined the delay between the onset of OO-EMG and the onset of eyelid movement for all types of blinks. Eye movements during blinking were depicted by their angle and maximal amplitude according to a polar coordinate system.

In the text and tables standard errors of the mean (SEM) are indicated unless otherwise mentioned. If the data of chosen parameters were not normally distributed, they were logarithmically transformed after which statistical analysis was performed.

Ratios of the integrated OO-EMG of the palsied and non-palsied eyelid and kinematics were determined for the up phase, the maximal amplitude and velocity. Ratios were logarithmically averaged and values of $p< 0.05$ were considered significant.

RESULTS

There was a significant difference in eyelid kinematics between all types of blinks (Table 1). At onset of the affliction the OO-EMG activity was absent in the palsied eyelid, both eye and eyelid movements of both eyes were disturbed. Almost no recovery of eyelid movement at the palsied side was recorded within the first 18 weeks. At the end of the study the blink seems clinically recovered, though the maximal amplitude and maximal velocity remained different between both eyelids during voluntary and reflex blinking, this resulted in reduced eyelid motility (Figs. 1A, B, 2C and 2F). Eye movements remained impaired during voluntary blinking (Fig. 2C).

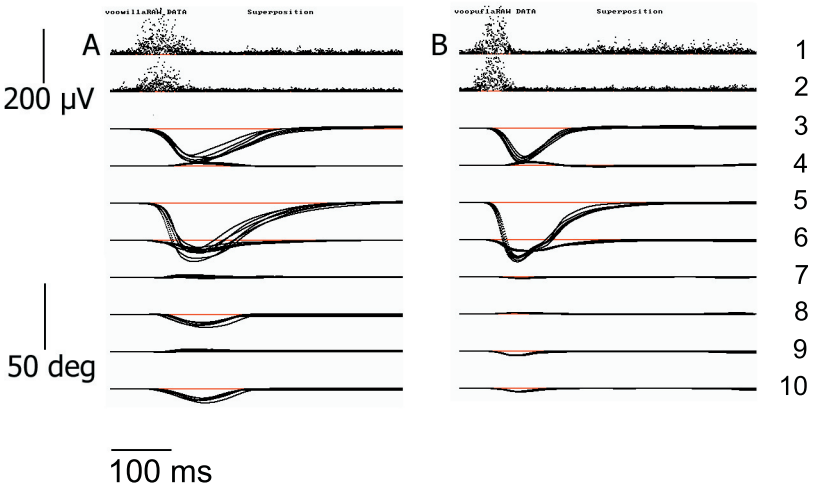


Figure 1. Simultaneously recorded OO-EMG activities, upper eyelid and eye movements during blinking in one subject. *A*: Superposition of six successive traces of voluntary blinks and their OO-EMG activities. *B*: Superposition of six successive traces of palsied corneal air puff-induced blinks and their OO-EMG activities. Lines 1 and 2 represent OO-EMG activities of the palsied and non-palsied eyelid, respectively; lines 3 and 5 represent the vertical displacement, lines 4 and 6 represent the horizontal eyelid displacement at palsied and non-palsied sides. Lines 7 and 9 represent the vertical eye displacement at palsied and non-palsied sides. Lines 8 and 10 represent the horizontal eye displacement at palsied and non-palsied sides. Note the abnormal vertical displacement (lines 7 and 9) and the large horizontal displacement (lines 8 and 10) of the eyes during voluntary blinking. The bar in front of lines 1 and 2 corresponds with a 200 µV OO-EMG signal. The bar in front of lines 3-10 corresponds with a 50 deg. rotation. The duration bar represents 100 ms.

Eyelid kinematics

The start times of the movement of both palsied and non-palsied eyelids showed a clear time delay in the first 18 weeks (Fig 3A, Table 1). The non-palsied eyelid exhibited a slight shortening of the start time until one year. The difference of start times of OO-EMG and eyelid movement shortened directly after onset of the affliction and reached normal values at 18 weeks. The mean start time difference for voluntary blinking, air puff (non-palsied/palsied side induced) and acoustic click induced of the non-palsied eyelid was 15.8 ± 2.8, 12.5 ± 1.4, 11.1 ± 1.3, 10.6 ± 1.1 ms, respectively. For the palsied eyelid

the mean values were: 13.8 ± 4.5, 13.7 ± 1.3, 12.5 ± 1.3 and 11.4 ± 0.9 ms, respectively. The values of both eyes are not significantly different.

In voluntary and air-puff induced reflex blinks, the down phase duration of the palsied side was prolonged during the first 18 weeks. After 18 weeks active eyelid movements reverted and the down phase duration became shorter, although measured values never reached values at the non-palsied side (Table 1). At the non-palsied side, the down phase duration slightly shortened from onset until one year. The up phase duration of the palsied eyelid was always shorter than that of the non-palsied eyelid except for acoustic click-induced blinking (Fig. 3B, Table 1). The maximal amplitude and velocity increased significantly at the palsied side at least until 84 weeks (Fig. 3C, D, Table 1). The largest velocity increase was measured within 36 weeks, followed by a small continuous increase from 782 to 923 degrees/s for the air puff induced blink stimulated at the palsied side at 84 weeks. However, these values never reached values measured at the non-palsied side. At the non-palsied side no significant changes in the maximal amplitude and velocity were measured during the study. At the palsied side the time of maximal amplitude and velocity shortened after 18 weeks until the end of the study, but they never reached the values measured at the non-palsied side except for acoustic click induced blinks (Fig. 3E, Table 1).

Voluntary blinks

The difference between the down phase duration of palsied and non-palsied eyelids, measured at the end of the study, was the largest of all types of blinks, e.g. 28 ms (Table 2). The down and up phase duration of the non-palsied eyelid ranged during the study between 65 and 84 ms and between 194 and 265 ms, respectively. The maximal amplitude and velocity of the palsied eyelid was increased at 18 weeks from 4.8 at onset to 19.7 degrees and from 65 to 331 degrees/s, respectively (Table 1).

Reflex blinks

Independent of the stimulation side, air puff induced blinks showed shortening of the start time of eyelid movement at 18 weeks. When the eye was stimulated at the non-palsied side, shortening of start time of the palsied eyelid continued until the end of the study from 49 ms at 18 weeks to 41 ms. At the non-palsied side the shortening was from 60 ms at 18 weeks to 40 ms at 84 weeks. Down phase and up phase duration of both eyelids slowly shortened during the study.

When the palsied eye was stimulated, the maximal amplitude and velocity of the palsied eyelid was increased at 18 weeks from 7.3 degrees at onset to 19.5 degrees and from 123 to 395 degrees/s, respectively (Table 1). After stimulation of the non-palsied side the maximal amplitude was 1.28 ± 0.04 ($p < 0.00002$) times larger than. The maximal velocity was 1.24 ± 0.05 ($p < 0.0002$) times larger.

When the non-palsied eye was stimulated, the maximal amplitude and velocity of the palsied eyelid was increased at 18 weeks from 5.1 degrees at onset to 18.4 degrees and from 109 to 450 degrees/s, respectively (Table 1). The maximal amplitude of the non-palsied eyelid was 1.10 ± 0.02 ($p < 0.0005$) times larger than the amplitude measured after stimulation at the palsied side; the maximal velocity was 1.11 ± 0.03 ($p < 0.005$) times larger.

In acoustic click-induced reflex blinks, the start times of both eyelids were comparable after 18 weeks (Table 1). Down phase durations of non-palsied and palsied eyelids ranged between 49 and 59 ms and between 53 and 64 ms, respectively, during the study. The up phase duration of the palsied eyelid

increased from 116 at onset to 179 ms until the end of the study. The maximal amplitude and velocity of the palsied eyelid was increased at 18 weeks from 3.0 to 5.6 degrees and from 107 to 146 degrees/s, respectively (Table 1).

Electromyography of the OO muscle

At onset of the affliction, start times of OO-EMG were not measurable at the palsied side, because integrated OO-EMG measured at the palsied side was at noise level in all types of blinking examined. Six weeks later, the start time of OO-EMG of the palsied eyelid was measurable in reflex blinks and shortened until the end of the study (Fig. 3H, Table 1). At the non-palsied side the start time of OO-EMG remained constant. At 36 weeks, start times of eyelid movement and OO-EMG and integrated OO-EMG's of both eyes were no longer significantly different (Table 2).

The integrated OO-EMG of the palsied eyelid increased in the first 18 weeks, followed by a continuous small increase until 84 weeks (Table 1). In reflex blinking, OO-EMG of the palsied eyelid was reset after one year and showed similar values to OO-EMG of the non-palsied eyelid (Fig. 3F). The integrated OO-EMG of the non-palsied eyelid slightly decreased during the study. In individual cases an overshoot of the integrated OO-EMG at the palsied side was recorded between 36 weeks and one year. After one year values were comparable with those measured at the non-palsied side. The sum of integrated OO-EMG of palsied and non-palsied eyelids in all measurements was almost constant throughout the study (Fig. 3G).

Voluntary blinks

At the non-palsied side the integrated OO-EMG only decreased from 4.8 to 2.8 μVs during the study. At the palsied side the integrated OO-EMG increased from 0.7 at onset to 3.5 μVs at 84 weeks (Table 1).

Reflex blinks

Although in all reflex blinks start times of OO-EMG of the palsied eyelid were not measurable at onset, OO-EMG continuously decreased from around 45 at 18 weeks to 28 ms at the end of the study. Start time of the non-palsied eyelid was measurable at onset and values were stable values after 18 weeks. Mean values measured from 18 weeks until the end of the study are: 28.9 ± 0.6, 33.1 ± 0.6, 38.5 ± 0.8 ms for air puff at non-palsied side, air puff at palsied side and acoustic click of the non-palsied eyelid, respectively. If the eye at the non-palsied side was stimulated with an air puff the start time of OO-EMG at the non-palsied side was 4.2 ± 0.7 ms ($p < 0.0001$) shorter then if the eye at the palsied side was stimulated.

The integrated OO-EMG of the non-palsied eyelid decreased independent of the stimulation side until 18 weeks and then ranged between 3.2 and 4.5 μVs. The integrated OO-EMG of the palsied eyelid, measured after air-puff stimulation at the non-palsied side, increased from 0.7 at onset to 3.0 μVs at 84 weeks. If the eye was stimulated with an air puff at the palsied side, integrated OO-EMG of the palsied eyelid was 1.19 ± 0.05 times ($p < 0.001$) larger than if the eye at the non-palsied side was stimulated.

In acoustic click induced blinks, the integrated OO-EMG of the palsied eyelid, increased until 36 weeks and then remained constant. The integrated OO-EMG of the non-palsied eyelid was at onset 1.3 μVs and was almost stable until the end of the study, 1.4 μVs.

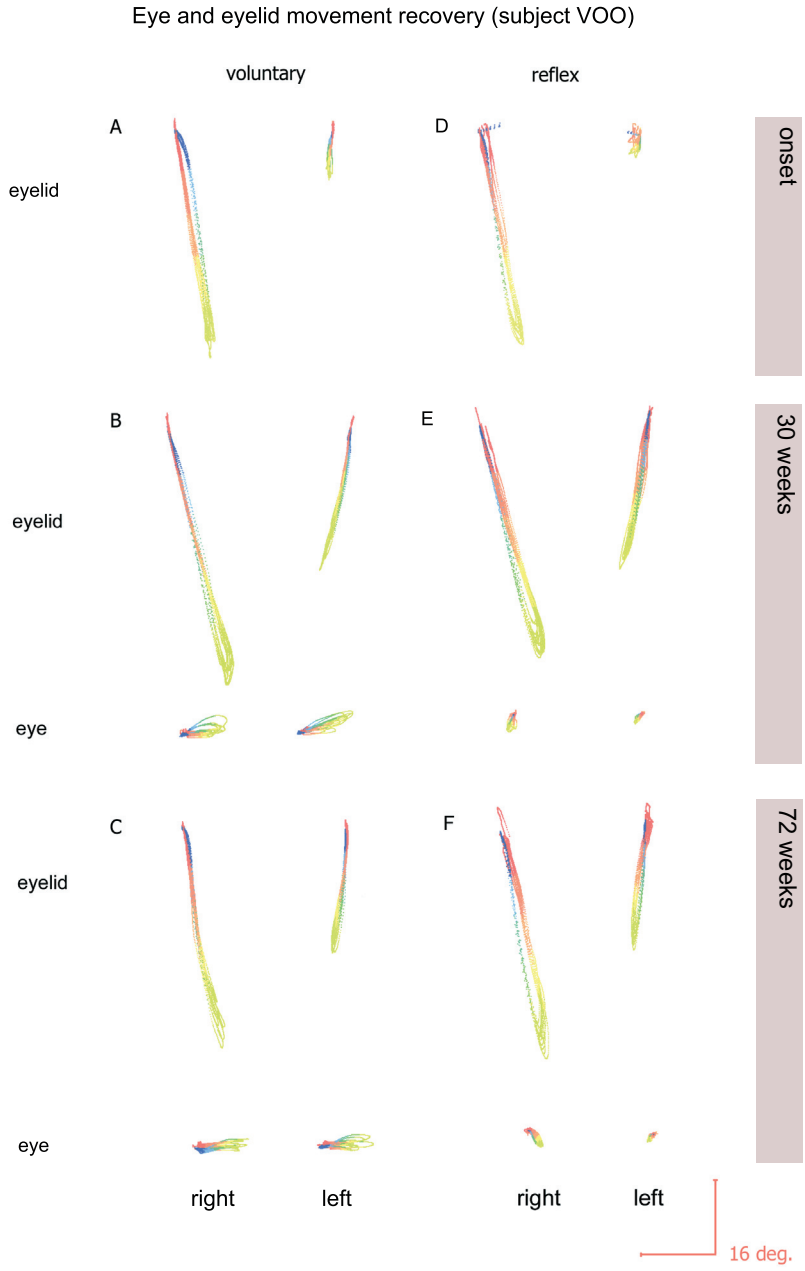


Figure. 2. Profiles of six superimposed successive traces of simultaneous recorded eyelid and eye movement during voluntary and reflex blinking measured from the same patient as in figure 1. *A*: Eyelid movement during voluntary blinking recorded at onset of the affliction. *B and* C: eyelid and eye movement during voluntary blinking recorded at 30 and 72 weeks, respectively. *D*: Eyelid movement after a corneal air puff at the palsied side recorded at onset of the affliction. *E and F*: Eyelid and eye movement after a corneal air puff at the palsied side recorded at 30 at 72 weeks, respectively. Note: Eyelid movement motility remained impaired in both types of blinking throughout the study. Both eyes move in the same abnormal direction during voluntary blinking, whereas in reflex blinking the direction of eye movement is normal after 72 weeks, however, the amplitude at the palsied side remains smaller.

Eye movements

The maximal amplitude and time of maximal amplitude of eye movement were recorded during voluntary blinking, air puff induced and acoustic click induced reflex blinking 30 and 72 weeks after onset of the affliction. In general, directly after onset impaired eye movements were observed during blinking.

In all types of blinking the maximal amplitude of eye movements measured at the palsied side always remained twofold smaller compared to the non-palsied side (Table 3). Maximal amplitudes did not change significantly between 30 and 72 weeks for both the palsied and non-palsied sides during reflex blinking.

After 72 weeks the direction of eye movement remained impaired in voluntary, but was normal in reflex blinking. Interestingly, at 30 weeks a shift in direction was observed in the rotation of both eyes during voluntary and reflex blinking (Figs. 2B and E). The vertical component was never more than 2 degrees; the horizontal component ranged between 5 and 10 degrees. The sweep of both eyes during voluntary blinking was much more pronounced than during reflex blinking (Figs. 2B, C, E and F, Table 3). No relation was found between the direction of the shift and the side of affliction. In 5 subjects eye movements during blinking were directed towards the palsied side, in the remaining 4 towards the non-palsied side.

Voluntary blinks

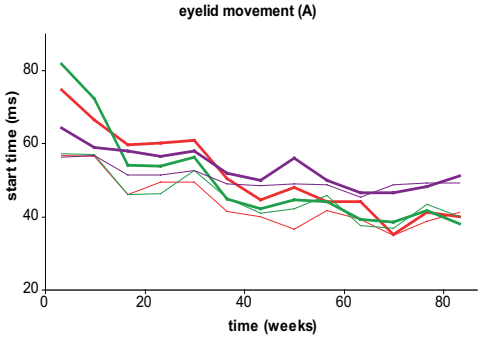
Time differences between the maximal amplitude of both eyes did not change significantly. The amplitude ratio slightly decreased from 30 until 72 weeks (Table 3). Aberrant eye movements of both eyes remained until the end of the study.

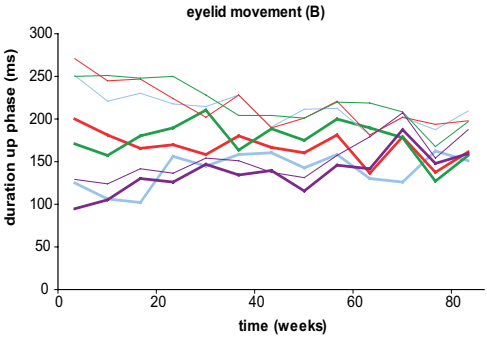
Reflex Blinks

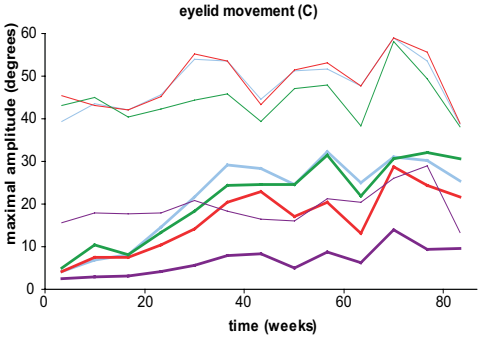
For air puff stimulation, maximal amplitudes of eye movements at the palsied side remained between 2.3 and 2.9 degrees and at the non-palsied side between 5.5 and 8.7 degrees during the study (Table 3). The time of maximal amplitude was the same for both eyes at 72 weeks. At the non-palsied side values measured at 30 and 72 weeks did not differ independent of the side of stimulation.

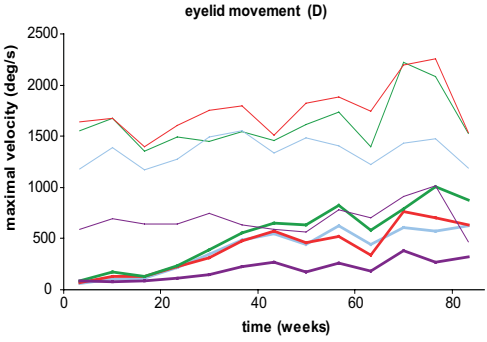
In acoustic click induced blinks the maximal amplitude increased slightly during the study. The time of maximal amplitude and time difference between maximal amplitudes remained constant.

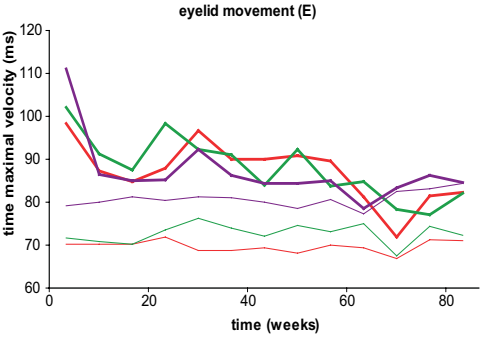
Figure 3.
Schematic representations of OO-EMG and eyelid kinematics during recovery. Light blue represents voluntary blinking, red corneal air-puff-induced blinking at the non-palsied side, and green corneal air puff-induced blinking at the palsied side, purple acoustic click-induced blinking. Thick lines represent values at the palsied side; thin lines represent values at the non-palsied side. Different parameters are plotted against the recovery time. In figures A, E and H values from voluntary blinking (light blue) are absent, as an exact start time of a “trigger” can not be determined for this type of blinking.

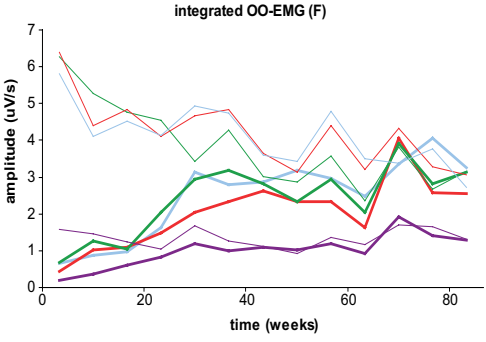

eyelid movement (A)
start time (ms)
time (weeks)

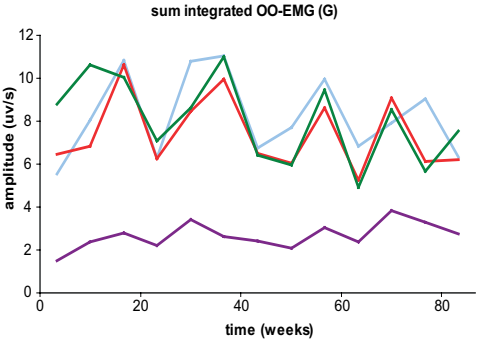

eyelid movement (B)
duration up phase (ms)
time (weeks)

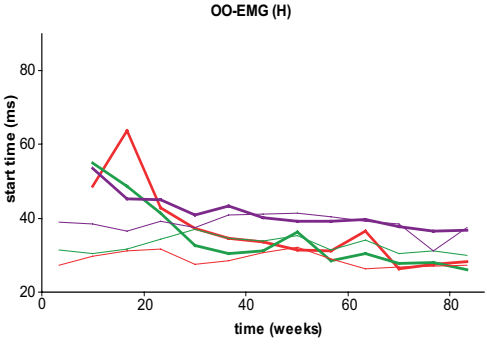

eyelid movement (C)
maximal amplitude (degrees)
time (weeks)


eyelid movement (D)
maximal velocity (deg/s)
time (weeks)


eyelid movement (E)
time maximal velocity (ms)
time (weeks)


integrated OO-EMG (F)
amplitude (uV/s)
time (weeks)


sum integrated OO-EMG (G)
amplitude (uv/s)
time (weeks)


OO-EMG (H)
start time (ms)
time (weeks)

DISCUSSION

The present study describes the blink recovery of 9 patients with a complete (House-Brackmann grade VI) Bell's palsy, who were given prednisolone. The recovery of OO-EMG and eyelid kinematics occurs in phases.

In the first phase start times of OO-EMG and lid movement synchronized between onset of the affliction and 18 weeks. During the second phase, which lasted from 18 weeks until 36 weeks after onset of the affliction, the palsied eyelid of these patients showed the first signs of OO-EMG activity and active eyelid movement at the same time. At the end of the second phase OO-EMG values of both eyes were no longer significantly different (Table 2). The third phase is characterized by overshoot of OO-EMG activity of the palsied eyelid. The overshoot was no longer measurable approximately one year after onset of the affliction. Together with the increase of OO-EMG activity on the palsied side, a decrease in OO-EMG activity was observed at the non-palsied side. The sum of OO-EMG of both eyelids remained almost constant throughout the study. In the fourth phase a subtle increase of maximal amplitude and velocity was found. However, these increases were not significant. Except for the start times of the eyelid movements, we found that recovery of eyelid movements at the palsied side during reflex blinking is incomplete at 84 weeks. Interestingly, the direction of the eye movements during voluntary blinking remained impaired, but the direction was normal in reflex blinking one year after onset of the affliction. The maximal amplitudes of eye movements remained aberrant throughout the study.

Eyelid kinematics

This longitudinal study suggests that the recovery of Bell's palsy induced adaptations in eyelid kinematics. Probably, motoneuron excitability was changed, which resulted in eyelid asymmetry during blinking[28]. At onset of the paralysis, the palsied eyelid only generated a passive down phase of eyelid movement during all types of blinking. The down phase of the palsied eyelid was not in concert with the down phase of the non-palsied eyelid. After 18 weeks signs of active down phase movement at the palsied side appeared for the first time, which is about one to two months later than is described in the literature[21, 22, 24].

The maximal amplitudes and velocities measured at the palsied side, never reached values of healthy volunteers[5]. This is another example of reorganisation of motor units in the OO muscle. At the palsied side this ongoing reorganization modifies motoneuron excitability[28]. Maximal amplitude and velocity values at the non-palsied side did not differ significantly from our previous study[5]. Huffman and co-workers[20] had similar observations in a group of un-recovered patients during spontaneous and voluntary blinking. On the other hand, they found normal amplitude and peak velocity values in a group of patients clinically recovered from Bell's palsy. The grade of affliction of those patients was not indicated and appearance of synkinesia was not mentioned, whereas we already observed synkinesia in all our patients after six months.

Table 2

The differences of the integrated OO-EMG, the up phase, the maximal amplitude and velocity are expressed in logarithmic ratios to normalize their distributions. The remaining parameters are expressed as time differences (ms). Apart from the Means and the standard errors of the means (SEM's) p-values are indicated to determine the significance of each parameter. P values above 0.05 were considered as non significant (n.s.).

Table 2 represents differences in OO-EMG and eyelid kinematics between non-palsied and palsied sides.

Type of Blinking	OO-EMG and Eyelid Kinematics	Onset			18 weeks			36 Weeks			84 Weeks		
		Mean	SEM	p <	Mean	SEM	p <	Mean	SEM	p <	Mean	SEM	p <
Voluntary	Start OO-EMG (ms)	indefinite			11	5	0.03	-1	4	n.s.	2	7	n.s
	Log (Integrated OO-EMG Ratio)	0.897	0.130	0.0005	1.053	0.162	0.001	0.101	0.071	n.s.	-0.080	0.061	n.s.
	Start Time (ms)	11	9	n.s.	6	3	0.04	3	2	n.s.	3	5	n.s
	Start Lid – Start OO-EMG (ms)	indefinite			-5	6	n.s.	4	5	n.s.	1	5	n.s.
	Down Phase (ms)	51	9	0.002	66	13	0.005	37	10	0.005	28	7	0.02
	Log (Up Phase Ratio)	0.228	0.090	0.03	0.137	0.014	0.00001	0.053	0.028	0.05	0.155	0.042	0.01
	Log (Maximal Amplitude Ratio)	0.950	0.118	0.00005	1.021	0.112	0.00002	0.202	0.044	0.001	0.153	0.089	n.s.
	Log (Maximal Velocity Ratio)	1.288	0.095	0.000002	1.378	0.070	0.0000002	0.381	0.059	0.0001	0.277	0.085	0.01
	Time Maximal Amplitude (ms)	58	23	0.02	27	5	0.0005	17	4	0.002	11	2	0.01
	Time Maximal Velocity (ms)	46	21	0.04	27	9	0.01	25	4	0.0002	14	7	n.s.
Air Puff-induced at Non-palsied Eyelid	Start OO-EMG (ms)	indefinite			11	5	0.03	3	2	n.s.	1	3	n.s
	Log (Integrated OO-EMG Ratio)	0.397	0.075	0.0005	0.436	0.112	0.005	0.145	0.052	0.02	0.082	0.081	n.s.
	Start Time (ms)	19	5	0.005	11	3	0.01	4	1	0.005	-1	5	n.s.
	Start Lid – Start OO-EMG (ms)	indefinite			0	6	n.s.	1	2	n.s.	-2	3	n.s.
	Down Phase (ms)	7	6	n.s	28	9	0.03	15	5	0.02	18	5	0.02
	Log (Up Phase Ratio)	0.104	0.041	0.02	0.110	0.035	0.01	0.057	0.017	0.005	0.086	0.039	0.04
	Log (Maximal Amplitude Ratio)	0.502	0.102	0.001	0.621	0.159	0.005	0.270	0.073	0.005	0.247	0.116	0.04
	Log (Maximal Velocity Ratio)	0.769	0.123	0.0002	0.846	0.188	0.001	0.412	0.103	0.002	0.379	0.157	0.03
	Time Maximal Amplitude (ms)	26	9	0.02	39	17	0.02	19	6	0.01	17	6	0.04
	Time Maximal Velocity (ms)	28	9	0.01	16	5	0.01	21	5	0.001	11	5	n.s.
Air Puff-induced at Palsied Eyelid	Start OO-EMG (ms)	indefinite			7	4	n.s.	-3	3	n.s.	-4	2	n.s.
	Log (Integrated OO-EMG Ratio)	0.101	0.071	n.s.	0.145	0.052	0.02	0.031	0.103	n.s.	0.007	0.089	n.s.
	Start Time (ms)	25	5	0.005	8	2	0.005	1	3	n.s.	-2	3	n.s
	Start Lid – Start OO-EMG (ms)	indefinite			0	5	n.s.	4	1	0.005	2	3	n.s.
	Down Phase (ms)	23	16	n.s.	35	11	0.01	12	4	0.01	25	13	n.s.
	Log (Up Phase Ratio)	0.053	0.028	0.05	0.057	0.017	0.005	0.036	0.017	0.04	0.094	0.016	0.001
	Log (Maximal Amplitude Ratio)	0.202	0.044	0.001	0.270	0.073	0.005	0.201	0.063	0.01	0.091	0.070	n.s.
	Log (Maximal Velocity Ratio)	0.381	0.059	0.0001	0.412	0.103	0.002	0.350	0.085	0.002	0.243	0.096	0.03
	Time Maximal Amplitude (ms)	54	15	0.02	43	12	0.005	13	2	0.0002	22	14	n.s.
	Time Maximal Velocity (ms)	30	13	0.03	25	9	0.02	11	3	0.002	10	5	n.s.
Acoustic Click-induced	Start OO-EMG (ms)	indefinite			6	2	0.005	-1	2	n.s.	0	1	n.s
	Log (Integrated OO-EMG Ratio)	-0.081	0.061	n.s.	0.082	0.081	n.s.	0.021	0.031	n.s	0.020	0.015	n.s.
	Start Time (ms)	6	6	n.s.	5	2	0.04	2	2	n.s.	2	2	n.s.
	Start Lid – Start OO-EMG (ms)	indefinite			-1	3	n.s.	3	3	n.s.	2	3	n.s.
	Down Phase (ms)	2	7	n.s	4	3	n.s.	4	3	n.s.	0	3	n.s.
	Log (Up Phase Ratio)	0.155	0.042	0.02	0.086	0.039	n.s	-0.007	0.021	n.s.	0.064	0.022	0.02
	Log (Maximal Amplitude Ratio)	0.153	0.089	n.s.	0.247	0.116	n.s.	0.291	0.077	0.005	0.135	0.165	n.s.
	Log (Maximal Velocity Ratio)	0.277	0.085	0.03	0.379	0.157	0.05	0.351	0.077	0.001	0.160	0.196	n.s.
	Time Maximal Amplitude (ms)	8	7	n.s.	9	6	n.s.	6	2	0.03	2	4	n.s.
	Time Maximal Velocity (ms)	32	26	n.s.	5	5	n.s.	4	2	0.04	1	1	n.s.

Aberrant regeneration of peripheral facial nerve palsy was earlier observed in a primate study[29] and in a study of 29 patients[21]. In the latter study of the OO-muscle activity was found at least four months after facial nerve degeneration. These authors also observed involuntary facial movement disorders, which were recently confirmed in a Bell's palsy patient who had signs of blepharospasm at the non-palsied side[30]. We did not find any sign of blepharospasm in our patients.

Voluntary blinks

From onset of the paralysis, the down and up phase duration and maximal amplitude at the non-palsied side showed clear fluctuations following the recovery. This means that the neural blinking circuits are activated at both the palsied and non-palsied side[31]. We found that these duration adaptations are transient and independent of incomplete recovery of kinematics of the palsied eyelid, although a reduced maximal amplitude and velocity was still present at the end of the study.

Several brain regions are involved in voluntary and spontaneous blinking[32]. Specific regions in the motor cortex, including regions in control of eyelid and mouth performance project directly or indirectly towards the facial motor nucleus[33-35]. Kaneko and co-workers[36] showed neural activity of the supplementary motor area during voluntary blinks. Transcranial magnetic stimulation showed that the cortical centre, initiating upper facial movements, including blinking, is located in the meso-frontal region rather than in the facial region of the primary motor cortex[37]. Sub-cortical dopaminergic pathways appear to play a role in the inhibition of the levator palpebrae superioris and/or inactivation of the OO muscle during voluntary blinks[2]. These pathways may change during the paralysis, because the representation fields of facial muscles like the OO and orbicularis oris muscles in the primary motor cortex probably alter in composition and size during recovery of this affliction.

Table 3 represents several eye movement parameters after 30 and 72 weeks of recovery. Time differences of voluntary blinking at 30 and 72 weeks and of air puff induced blinking at 72 weeks are not significant. Neither is the time of maximal amplitudes of air puff-induced blinking after 72 weeks.

Type of Blinking	Eye Movement Kinematics	30 Weeks		72 Weeks	
		Non-palsied	Palsied	Non-palsied	Palsied
Voluntary					
	Maximal Amplitude (°)	9.1 ± 1.7	3.4 ± 3.4	10.9 ± 3.1	5.6 ± 3.1
	Time Difference Maximal Amplitudes (ms)	10 ± 19		1 ± 10	
	Amplitude Ratio	2.68 ± 0.18		1.97 ± 0.09	
Air Puff-induced at Non-palsied Eyelid					
	Maximal Amplitude (°)	7.5 ± 1.2	2.3 ± 0.3	8.7 ± 1.3	2.5 ± 0.4
	Time Maximal Amplitude (ms)	99 ± 6	134 ± 6	100 ± 2	103 ± 6
	Time Difference Maximal Amplitudes (ms)	35 ± 7		3 ± 4	
	Amplitude Ratio	3.26 ± 0.10		3.52 ± 0.07	
Air Puff-induced at Palsied Eyelid					
	Maximal Amplitude (°)	5.5 ± 0.6	2.6 ± 0.2	6.9 ± 1.6	2.9 ± 0.6
	Time Maximal Amplitude (ms)	98 ± 4	137 ± 7	97 ± 2	98 ± 2
	Time Difference Maximal Amplitudes (ms)	39 ± 5		1 ± 3	
	Amplitude Ratio	2.13 ± 0.02		2.23 ± 0.12	
Acoustic Click-induced					
	Maximal Amplitude (°)	2.4 ± 0.2	1.1 ± 0.2	3.0 ± 1.2	1.5 ± 0.2
	Time Maximal Amplitude (ms)	96 ± 2	111 ± 5	96 ± 1	110 ± 4

Reflex blinks

A number of studies show that movements of palsied eyelids during reflex blinking are reduced during and after recovery[25, 29, 38, 39]. The characteristics of air puff and acoustic click induced reflex blinks found in the current study were consistent with each other and confirmed that reflex blinks have the shortest down and up phases of all types of blinks [5, 6, 28]. Interestingly, the current study showed that acoustic click-induced reflex blinks recovered fastest.

Schicatano and co-workers[25] observed a difference in synchronism of eyelid closure during contralateral SO stimulation at onset of unilateral facial nerve palsy. This a-synchronism was accompanied by blink oscillations that decreased during recovery. In that study, a reduced amplitude and a non-significant increased down phase duration of the eyelid movement was observed too. Stimulation at the palsied side prolonged the blink duration and often "blink oscillations" were seen. These oscillations may arise from oscillatory processes within trigeminal reflex blink circuits developed as a consequence of aging of the sensory trigeminal complex neurons or decreased lacrimation associated with aging[39]. We had similar observations after air puff stimulation at the palsied side, e.g. reduced motility and blink oscillations. However, when the non-palsied side was stimulated hardly any blink oscillations were found.

Electromyography of the OO muscle

Testing the blink reflex using OO-EMG may predict the recovery trajectory of Bell's palsy patients[22]. At onset of the affliction, OO-EMG activity during voluntary blinks and click and air puff induced blinks was at noise level. For all types of blinks we registered both integrated OO-EMG and OO-EMG start time at the palsied side did not show significant differences with the non-palsied side after 36 weeks. Interestingly, the sum of integrated OO-EMG of palsied and non-palsied eyelids was almost constant throughout the study indicating that both facial motoneuron pools possibly receive a common cortical input from representation fields M2 and M3 in the motorcortex[34].

Another negative effect, which was present in all patients, was the development of facial weakness during the disease. In individual patients, an overshoot of OO-EMG at the palsied side was found between 36 weeks and one year. Despite improvement in maximal amplitude and maximal velocity of the eyelid facial weakness remained. This together with synkinesia creates less effective eyelid movement during blinking[19]. Our finding is not in agreement with observations in a human Bell's palsy study where larger OO-EMG responses at the palsied side after recovery of the affliction were found even after 18 months[40].

In order to improve facial symmetry of late or partially recovered Bell's palsy patients non-surgical, with facial physiotherapy or botulinum toxin injections, or surgical treatment can be performed[41]. However, the result is often unsatisfactorily and alternatives are hardly available. Electrical stimulation of facial muscles during recovery is one of these alternatives to decrease facial weakness. Although a three months therapeutic electrical stimulation improved the eyelid movement, no significant improvement was recorded in spontaneous maximal amplitudes and maximal velocities[42]. An electrophysiological study in Bell's palsy patients showed that patients with residual facial weakness showed enhancement in their reflex blink recovery after electrical SO nerve stimulation at the palsied side[19]. Cossu and co-workers[23] suggested starting "treatment" about 3 months after onset of complete facial nerve palsy, the period of the very first connections between growing axons and denervated muscles. At this stage

very little muscle activity was detected in our patients. Starting therapy at that stage risks an over stimulation (activation) of facial muscles at the non-palsied side, which could result in pronounced facial asymmetry. In order to prevent the over stimulation, prudence should be taken with the start of the training. We recommend starting directly after the first signs of innervation of both the OO and orbicularis oris muscles.

Eye movements during blinking

It was sir Bell who first noticed that hemi-facial paralysis patients moved their eye upward during blinking at the palsied side[43]. Using standard scleral search coils, we recorded eye movements during voluntary, acoustic click and air puff induced blinks. Until 30 weeks eye movements during all types of blinking were disturbed, their direction was oblique upward parallel to each other, but not straight up as described previously[44]. After 72 weeks, eye movements during reflex blinking were normal in direction, but had smaller amplitudes at the palsied side. At the non-palsied side amplitudes were normal[6].

During voluntary blinking eye movements of both eyes were enlarged and the direction remained disturbed. Specifically the vertical eye component remained smaller compared to values measured in healthy subjects. The exceptional increase in amplitude of eye movements during voluntary blinking suggests that besides the facial nerve other cranial nerves are involved in the aetiology of Bell's palsy. This implies that Bell's palsy is a cranial neuropathy as was suggested by Adour and co-workers[15].
Contrary to our results a restraint eyelid study of Collewijn and co-workers[8] showed no alteration in eye movements during blinking. Voluntary blinking was recorded with one eyelid kept open by adhesive tape. Relatively large blink-related eye movements were observed. The relatively large eye movement during voluntary blinking in the lid restraint experiment may not be caused by a mechanical factor, as Bour and co-workers showed that the extent of eye rotation depends on the initial eye position[6]. In the current study large eye movements during blinking were seen as well, although the direction of eye movements was disturbed.

Adaptive changes

Bell's palsy can lead to changes in the blinks of the non-palsied eyelids[30, 45]. This may result in a Bell's palsy-induced blepharospasm, which was observed in the non-palsied eyelid. Fortunately, treatment with apomorphine or other dopamine receptor agonists' can reduce blepharospasm and blink excitability and possibly change motoneuron recruitment[30]. We observed blink oscillations at the palsied side, however none of the nine Bell's palsy patients' revealed signs of involuntary eyelid movement disorders at the non-palsied side.

It is stated in a lid restraint study that unilateral facial nerve palsy is a long-term version of the adaptive process of eyelid movement after lid restraint[25] and that hyper excitability of the neural circuit of the blink reflex during an acute period of facial palsy is an adaptive response to compensate impaired facial motor function[28]. We think that these functional changes are more complex and are also determined centrally, as voluntary and to a lesser extent reflex blinking remained disturbed in our group of Bell's palsy patients. This might be the result of changes in representative cortical areas of eye and eyelid movements developed during recovery[46]. Therefore, a proper facial function needs peripheral and central adaptations in facial motor control. Further studies are needed to elucidate the role of the sensory and motor cortex during the recovery process.

REFERENCES

1. Evinger C, Bao JB, Powers AS, Kassem IS, Schicatano EJ, Henriquez VM, Peshori KR. Dry eye, blinking, and Blepharospasm. *Mov Disord.* 2002; 17 (suppl 2): S75-S78.

2. Schmidtke K, Buttner-Ennever JA. Nervous control of eyelid function: a review of clinical, experimental and pathological data. *Brain* 1992; 115: 227-47.

3. Esteban A, Traba A, Prieto J. Eyelid movements in health and disease. The supranuclear impairment of the palpebral motility. *Neurophysiol Clin*. 2004; 34: 3-15.

4. Evinger C, Manning KA, Sibony PA. Eyelid movements. Mechanisms and normal data. *Invest Ophthalmol Vis Sci.* 1991; 32: 387-400.

5. VanderWerf F, Brassinga P, Reits D, Aramideh M, Ongerboer de Visser B. Eyelid movements: behavioral studies of blinking in humans under different stimulus conditions. *J Neurophysiol.* 2003; 89: 2784-96.

6. Bour LJ, Aramideh M, Ongerboer de Visser BW. Neurophysiological aspects of eye and eyelid movements during blinking in humans. *J Neurophysiol.* 2000; 83: 166-76.

7. Evinger C, Shaw MD, Peck CK, Manning KA, Baker K. Blinking and associated eye movements in human, guinea pigs and rabbits. *J Neurophysiol.* 1984; 52: 323-39.

8. Collewijn H, Van der Steen J, Steinman RM. Human eye movements associated with blinks and prolonged eyelid closure. *J Neurophysiol.* 1985; 54: 11-27.

9. Riggs LA, Kelly JP, Manning KA, Moore RK. Blink-related eye movements. *Invest Ophthalmol Vis Sci.* 1987; 28: 334-42.

10. Bergamin O, Bizzarri S, Straumann D. Ocular torsion during voluntary blinks in humans. *Invest Ophthalmol Vis Sci*. 2002; 43: 3438-43.

11. Straumann D, Zee DS, Solomon D, Kramer PD Validity of Listing's law during fixations, saccades, smooth pursuit eye movements, and blinks. *Exp Brain Res.* 1996; 112: 135-146.

12. Smit AE, Zerari-Mailly F, Buisseret P, Buisseret-Delmas C, VanderWerf F. Reticulo-collicular projections: a neuronal tracing study in the rat. *Neuroscience Lett*. 2005; 380:276-279.

13. Smit AE, Buisseret P, Buisseret-Delmas C, CI de Zeeuw, VanderWerf F, Zerari-Mailly F. Reticulo-collicular and spino-collicular projections involved in eye and eyelid movements during the blink reflex. *Neuroscience Res.* 2006 (in press).

14. Gilden DH. Clinical practice. Bell's Palsy. *N Engl J Med*. 2004; 351(13):1323-1331.

15. Adour KK, Byl FM, Hilsinger RL, Kahn ZM, Sheldon MI. The true nature of Bell's palsy: Analysis of 1000 consecutive patients. *Laryngoscope* 1978; 88: 787-801.

16. Adour KK, Wingerd J. Idiopathic facial paralysis (Bell's palsy): Factors affecting severuty and outcome in 446 patients. *Neurology* 24: 1112-1116.

17. House JW, Brackmann DE. Facail nerve grading system. *Otolaryngol Head Neck Surg.* 1985; 93: 146-147.

18. Manca D, Munoz E, Pastor P, Valldeoriola F, Valls-Sole J. Enhanced gain of blink reflex responses to ipsilateral supraorbital nerve afferent inputs in patients with facial nerve palsy. *Clin Neurophysiol*. 2001; 112: 153-156.

19. Syed NA, Delgado M, Sandbrink F, Schulman AE, Hallett M, Floeter MK. Blink recovery in facial weakness. *Neurology* 1999; 52: 834-838.

20. Huffman MD, Baker RS, Stava MW, Chuke JC, Rouholiman BR, Porter JD. Kinematic analysis of eyelid movements in patients recovering from unilateral facial nerve palsy. *Neurology* 1996; 46: 1079-1085.

21. Kimura J, Rodnitzky RL, Okawara SH. Electrophysiologic analysis of aberrant regeneration after facial nerve paralysis. *Neurology* 1975; 25: 989-993.

22. Kimura J, Giron LT, Young SM. Electrophysiological study of Bell's palsy. Electrically elicited blink reflex in assessment of prognosis. *Arch Otolaryngol.* 1975; 102: 140-143.

23. Cossu G, Valls-Sole J, Valldeoriola F, Munoz E, Benitez P, Aquilar F. Reflex excitability of facial motoneurones at onset of muscle reinnervation after facial nerve palsy. *Muscle & Nerve* 1999; 22: 614-620.

24. Valls-Sole J, Montero J. Movement disorders in patients with peripheral facial palsy. *Mov Disord.* 2003; 18: 1424-1435.

25. Schicatano EJ, Mantzouranis J, Peshori KR, Partin J, Evinger C. Lid restraint evokes two types of motor adaptation. *J Neuroscience* 2002; 22:569-576.

26. Robinson DA. A method of measuring eye movement using a scleral search coil in a magnetic field. *IEEE Trans Biomed Eng.* 1963; 10: 137-45.

27. Becker W, Fuchs AF. Lid-eye co-ordination during vertical gaze changes in man and monkey. *J Neurophysiol.* 1988; 60: 1227-52.

28. Kassem IS, Evinger C. Asymmetry of blinking. *Invest Ophthalmol Vis Sci.* 2006; 47: 195-201.

29. Baker RS, Stava MW, Nelson KR, May PJ, Huffman MD, Porter JD. Aberrant reinnervation of facial musculature in a subhuman primate: a correlative analysis of eyelid kinematics, muscle synkinesia, and motoneuron localization. *Neurology* 1994; 44: 2165-2173.

30. Cattaneo L, Chierici E, Pavesi G. Bell's palsy-induced blepharospasm relieved by passive eyelid closure and responsive to apomorphine. *Clin Neurophysiol.* 2005; 116: 2348-2353.

31. Toda N, Nakamura K, Takeda N. Blink reflex R2 recovery curves in patients with facial palsy within ten days after onset. *ORL.* 2005; 67: 16-22.

32. Evinger E, Perlmutter JS. Blind men and blinking elephants. *Neurology* 2003; 60: 1732-1733.

33. Grinevich V, Brecht M, Osten P. Monosynaptic pathway from rat vibrissa motor cortex to facial motor neurons revealed by lentivirus-based axonal tracing. *J Neuroscience* 2005; 25: 8250-8258.

34. Morecraft JR, Louie JL, Herrick JL, Stillwell-Morecraft KS. Cortical innervation of the facial nucleus in the non-human primate. A new interpretation of the effects of stroke ansd related subtotal brain trauma on the muscles of facial expression. *Brain* 2001; 124:176-208.

35. Morecraft JR, Stilwell-Morecraft KS, Rossing WR. The motor cortex and facial expression: new insights from neuroscience. *Neurologist* 2004 Sep;10(5):235-49.

36. Kaneko K, Mito K, Makabe H, Takanokura M, Sakamoto K. Cortical potentials associated with voluntary, reflex and spontaneous blinks as bilateral simultaneous eyelid movement. *Electromyogr Clin Neurophysiol.* 2004; 44: 455-462.

37. Sohn YH, Voller B, Dimyan M, St Clair Gibson A, Hanakawa T, Leon-Sarmiento FE, et al. Cortical control of voluntary blinking: a transcranial magnetic stimulation study. *Clin Neurophysiol.* 2004; 115: 341-347.

38. Somia NN, Rash GS, Epstein EE, Wachowiak M, Sundine MJ, Stremel RW, Barker JH, Gossman D. A computer analysis of reflex eyelid motion in normal subjects and in facial neuropathy. *Clin Biomechanics* 2000; 15: 766-771.

39. Peshori KR, Schicatano EJ, Gopalaswamy R, Sahay E, Evinger C. Aging of the trigeminal blink system. *Exp Brain Res.* 2001; 136: 351-63.

40. Valls-Sole J, Tolosa ES, Pujol M. Myokymic discharges and enhanced facial nerve reflex responses after recovery from idiopathic facial palsy. *Muscle & Nerve* 1992; 15:37-42.

41. Bulstrode NW, Harrison DH. The phenomenon of the late recovered Bell's palsy: Treatment options to improve facial symmetry. *Plast Reconstr Surg.* 2005; 115: 1466-1471.

42. Gittins J, Martin K, Sheldrick J, Reddy A, Thean L. Electrical stimulation as a therapeutic option to improve eyelid function in chronic facial nerve disorders. *Invest Ophthalmol Vis Sci.* 1999; 40: 547-554.

43. Bell C. *The nervous system of the human body [appendix; case 49].* London, Longman, Rees, Orme, Brown and Green, 1830: 85-87.

44. Miles WR. Elevation of the eyeballs on winking. *J Exp Psychol.* 1931; 14: 311-332.

45. Nacimiento W, Podoll K, Graeber MB, Töpper R, Möbius E, Ostermann H, Noth J, Kreutzberg GW. Contralateral early blink reflex in patients with facial nerve palsy: indication for synaptic reorganization in the facial nucleus during regeneration. *J Neurol Sci.* 1992; 109:148-155.

46. Sanes JN, Suner S, Donoghue JP. Dynamic organization of primary motor cortex input to target muscles in adult rats. I. Long-term patterns of reorganization following motor or mixed peripheral lesions. *Exp Brain Res.* 1990; 79: 479-491.

CHAPTER 8

Cortical plasticity after peripheral facial nerve injury; a longitudinal fMRI study I: lip pursing

ABSTRACT

Several studies revealed plasticity in the primary motor cortex following permanent damage due to chronic pathologies, such as stroke or lesions of the central nervous system or amputation of a limb. Here we investigated plasticity in the primary motor cortex, but also in the sensory cortex and the cerebellum induced by a transient peripheral nerve pathology. In nine patients with idiopathic unilateral facial nerve paralysis, also known as Bell's palsy, the brain activation related to lip movements was monitored across measurements at four different stages of their recovery process using functional magnetic resonance imaging. Despite good recovery of lip motor function, we found changes in the hemispherical lateralization of the activation in both primary motor cortex and cerebellum. Activation in the motor cortex shifted away from the side of the lesion, whereas activation in the cerebellum shifted towards the side of the lesion. In the primary sensory cortex no large changes in lateralization of activation appear to occur. The opposite shift in the primary motor cortex and cerebellum fit with the neuro-anatomical connections between the motor cortex and the cerebellum. The motor cortex in one hemisphere predominantly innervates the contralateral and a cerebellar hemisphere the ipsilateral facial musculature.

INTRODUCTION

It is well established that changes in activation patterns in the motor cortex of the human brain can occur as a result of chronic central pathology. Several studies in patients with lesions, as for instance caused by a stroke, of the central nervous system reported plasticity in the motor cortex (for reviews see: Dancause 2006, Rossini et al. 2007, Ward 2004). Changes in activation have also been reported after peripheral chronic pathologies, such as limb amputation (for review see: Reilly and Sirigu 2008). However, little is known about brain plasticity following transient peripheral pathologies. The current study aims to map cortical plasticity in the primary motor cortex, sensory motor cortex and cerebellum of patients with a transient peripheral paralysis. To this end we designed a longitudinal study to monitor changes in the cortical representation of lip movement of Bell's palsy patients using functional magnetic resonance imaging (fMRI).

Bell's palsy is an idiopathic unilateral facial nerve paralysis with a sudden onset (Gilden 2004). fMRI recordings were made of these patients in each of the four phases of recovery as described in the study of VanderWerf and co-workers (2007). In short; phase one is the period of deterioration of the facial nerve, which starts abrubtly but can continue for about 10 days. The second phase is the period of nerve outgrowth towards the target structures in the face and lasts approximately four months. In the third phase, the period between four months and a year, re-establishment of nerve/muscle contacts and motor unit formation occur. In phase four, which we monitored up to one and a half year, the newly-formed motor unit is being tuned, and, for instance, motor behavior of the eyelid, is characterized by subtle changes in amplitude and velocity of the blinks.

In healthy subjects, each half of the lip is innervated by motoneurones in the ipsilateral facial motor nucleus. Each nucleus receives contralateral projections from the primary motor cortex (Morecraft et al. 2001, 2004). The cerebellum and primary sensory cortex are activated during lip movements as

well (Dresel et al. 2005, Tamura et al. 2008). Since the paralysis affects only one side of the facial musculature, we hypothesized that changes in cortical and cerebellar activation are reflected by changes in lateralization between the two hemipsheres. More specifically, since the motor cortex on one-side is primarily innervating the contralateral facial musculature, we expected to see a shift in activation to the motor cortex ipsilateral to the paralysis, which innervates the unaffected side of the face (figure 1). Sensory input from the face is still intact, though lack of movement and therefore a decreased amount of input could induce changes within the primary sensory cortex.

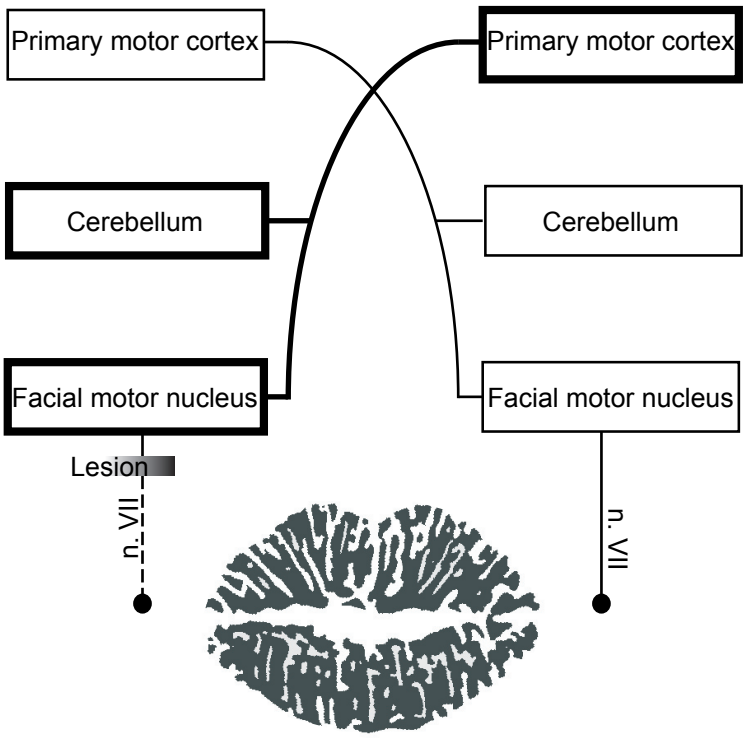


Figure 1. Schematic representation of the connections between the primary motor cortex, cerebellum and facial nucleus. The bold squares indicate that according to our hypothesis the paralysis of the facial nerve (indicated by the grey/black rectangle in the left n. VII) primarily affects the ipsilateral facial nucleus and cerebellum and the contralateral primary motor cortex.

Finally, given the crossed anatomical connections between the cerebellum and the motor cortex, as has been shown to control speaking and singing (Riecker et al. 2000), we expected to see the opposite in the lateralization within the cerebellum.

MATERIALS AND METHODS

Subjects

Nine Bell's palsy patients (four men and five women, average age at onset of the paralysis; 41.7 ± 12.3 years, range; 20 - 59 years) participated in this study. Bell's palsy patients were recruited through the Department of Otolaryngology of the Erasmus MC, University of Rotterdam, The Netherlands. Inclusion criteria were: acute idiopathic unilateral facial nerve paralysis diagnosed by an otolargyngologist, onset and deterioration within 48 hours, no medical treatment, complete facial nerve denervation as defined by House Brackmann grade V-VI (Berg et al. 2004). Patients who presented with viral Herpes simplex I, Varicella zoster or a history of neurological, psychiatric, otological disorders or cerebral vascular accident were excluded.

The patients participated in four fMRI scans throughout their recovery to monitor plasticity in motor areas. Functional recovery was assessed with the Sunnybrook grading system (Ross et al. 1996, Kanerva et al. 2006).

Fourteen healthy subjects (nine men and five women, average age; 39.6 ± 15.0 years, range; 25 - 64 years) were scanned once. They performed the same task as the patients. Subjects with MR contra-indications, such as pregnancy, claustrophobia, or permament (medical) implants were not included in this study. Before entering the study, all subjects gave informed consent to participate in this study that was approved by the local ethical board.

fMRI Data Acquisition

The experiments were performed on a 1.5 Tesla clinical MRI scanner (Sigma CV/I General Electric Milwaukee, USA) with a standard head coil. First, a T1 weighted SE (TR/TE/TI 9.8/1.9/400 ms, 224x320 matrix size, field of view 24 cm, 1.6 mm thick slices, no gap) anatomical image was acquired in the tansversal plane and covered the whole brain. Secondly, the functional scans were made. These T2* weighted single shot gradient echo EPI functional scans (TR/TE 3000/40 ms, field of view 26 cm, 96x96 matrix size, 5mm thick slices, 1 mm interslice gap) were scanned in the same plane as the anatomical images and covered the whole brain.

Experimental Design

The four fMRI scan sessions in patients were performed as soon as possible after onset (15 ± 12 days), after four months, after one year, and after one and a half year after onset of the paralysis. Prior to each session subjects received exact instructions and practiced the task to ensure correct task execution. The lip pursing task was a self paced repetitive kiss like movement with the lips. Subjects were instructed not to use other facial muscles.

During a scan session an anatomical scan and two functional scans were made. The lip pursing task was performed in a block paradigm and repeated twice. The block paradigm in a scan consisted of ten 30 second on and off periods in which the task was continuously performed during the on period and the subject was told to relax during the off period. During the experiment the subjects were asked to keep their eyes closed and lie as still as possible. Control subjects received only one scan session in which they performed the same experiment.

Sunnybrook scale

The Sunnybrook facial grading system was used to document clinical recovery of facial function in the Bell's palsy patients (Ross et al. 1996). The facial grading system is based on the evaluation of resting symmetry, degree of voluntary excursion of facial muscles, and degree of synkinesis associated with specific voluntary movements. Different regions of the face are examined separately with the use of five standard expressions. They include eyebrow lifting, gentle and forceful closing of the eyelids, wrinkling the nose, smiling with an open mouth and lip pursing. All items were evaluated and this resulted in a score between 0 (no facial muscle function) and 100 (perfect facial muscle function). For each patient a score was obtained parallel to each scan session.

Data Analysis

fMRI data was preprocessed and statistically analyzed with Matlab version 6.5 (Math Works Inc., Natick, MA) and the Statistical Parametric Mapping toolbox (SPM5, Welcome department of Cognitive Neurology, London, UK). Functional images were realigned and co-registered to the anatomical images. Anatomical and functional images were then normalized to a standard T1 image template based on the Montreal Neurological Institute (MNI) reference brain. Anatomical images were resampled to 1 x 1 x 1 mm^3 voxels. Functional images were resampled to 3 x 3 x 3 mm^3 voxels and smoothed with a gaussian kernel (full width half maximum (FWHM) 8 mm^3).

To reduce movement artifacts, the movement parameters, as determined during realignment, were included as regressors of no interest when the statistical parametric contrast maps were created. The model was estimated with a high-pass filter with a 128 second cut-off period.

The two separate fMRI scans of the lip-pursing task were pooled and analyzed resulting in a t-contrast map per subject and scan session. For proper interpretation of the data the contrast maps of the three patients presenting with a paralysis on the right side were flipped horizontally. As a consequence all patients are considered to be paralyzed on the "left" side. The contrast maps were used for comparisons per group for each task and scan session in second level analysis (one-sample t-tests, p=0.001 uncorrected for multiple comparisons).

To study changes in lateralization of the activation, we calculated a laterality index for each of the four measurements using the results of the group analyses. The laterality index (LI) was calculated as the contrast between the total number of significant voxels in each hemisphere for a particular region of interest using the formula $(N_{left} - N_{right}) / (N_{left} + N_{right})$. Therefore, a positive index will indicate that more voxels were significantly activated on the paralyzed left side than on the opposite site.

Since the current study aimed to understand the central motor recovery after a temporary peripheral nerve injury, we focused on three regions of interest (ROIs); the primary motor area, the sensory motor area and the cerebellum as defined by the automated anatomical labeling (AAL) program (Tzurio-Mazoyer et al., 2002).

RESULTS

Controls

Brain activation related to lip pursing in the fourteen control subjects was mostly found in the primary motor cortex (391 voxels p=0.001) (fig. 3 first row indicates distribution of activation in controls), cerebellum (457 voxels p=0.001) (fig. 4 first row indicates distribution of activation in controls) and sensory cortex (432 voxels p=0.001) in the whole brain group analysis. Most cerebellar activation was found in lobule VI and crus I.

Patient group comparisons

In general, the number of activated voxels in each of the three regions of interest was consistently lower in patients compared to controls in all four measurements. Changes in lateralization of activation across the four measurements are shown in figure 2, 3 and 4. The first measurement yielded more activation in the primary motor cortex ipsilateral to the paralysis. The lateralization within the primary motor cortex shifted in the second and third measurement towards the contralateral side. In the fourth measurement, 1.5 years after onset of the paralysis the lateralization was almost symmetrical with slightly more activation on the contralateral side (fig. 2, 3).

In the sensory cortex no large shifts in the lateralization of activation were observed. Across the four measurements the laterality index gradually changed from slightly ipsilateral to slightly contralateral (fig. 2).

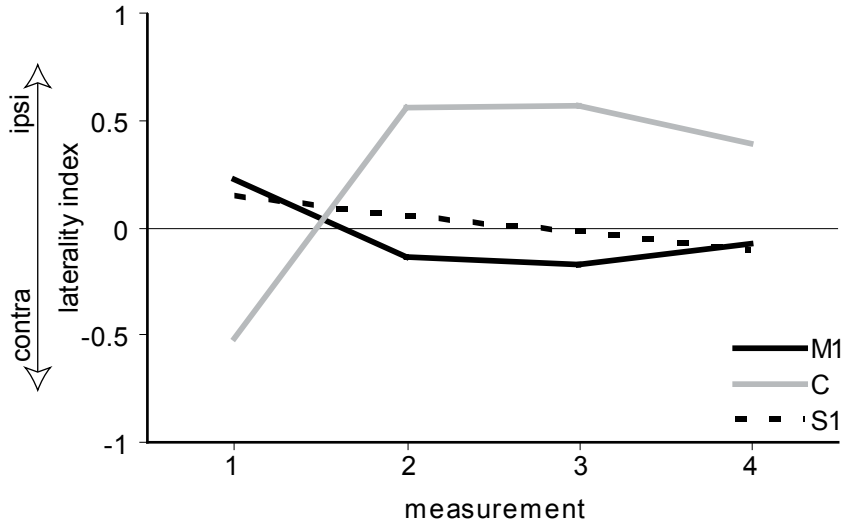


Figure 2. Changes in the laterality index (LI) in the primary motor cortex (M1), primary sensory cortex (S1) and cerebellum (C) measured at 4 time points throughout recovery of Bell's palsy patients. The laterality index shows the lateralization of the number of active voxels per ROI. Positive values indicate more active voxels ipsilateral (ipsi) to the paralyzed side and negative values indicate more active voxels contralateral (contra) to the paralyzed side.

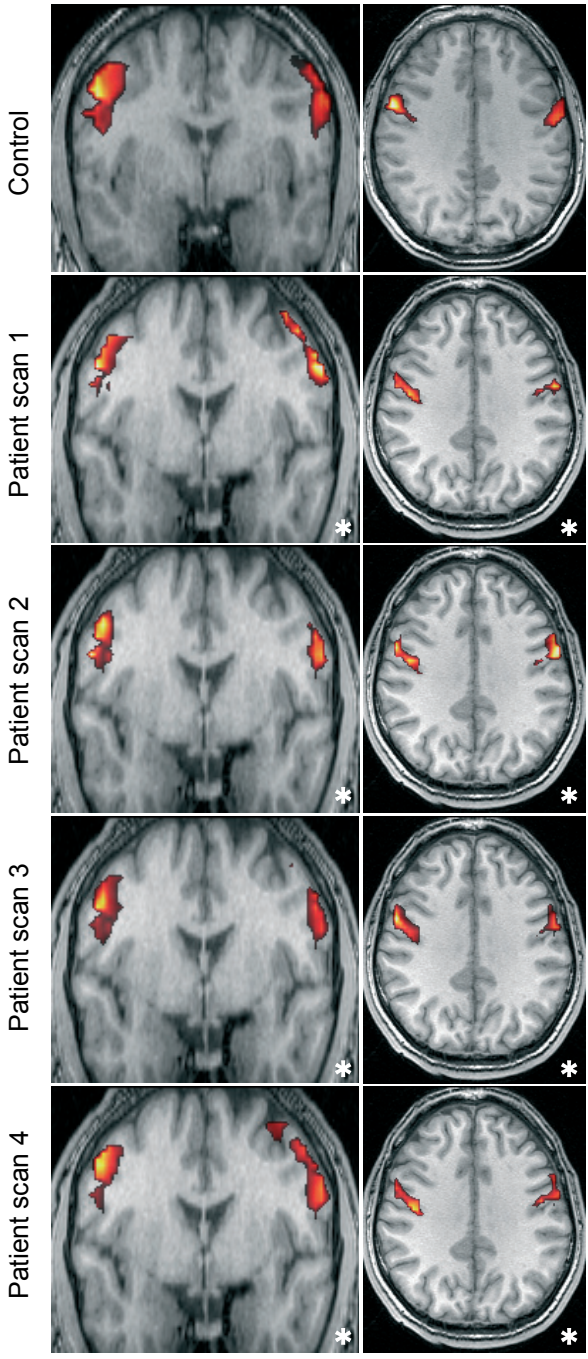


Figure 3. Coronal (left column) and transversal (right column) slices showing distribution of activation in the primary motor cortex during lip pursing in the control group (top row) and the four measurements in Bell's palsy patients (patient scan 1-4). Note the slightly larger activated areas in the contralateral motor cortex in scans 2 and 3. The (*) indicates the paralyzed side.

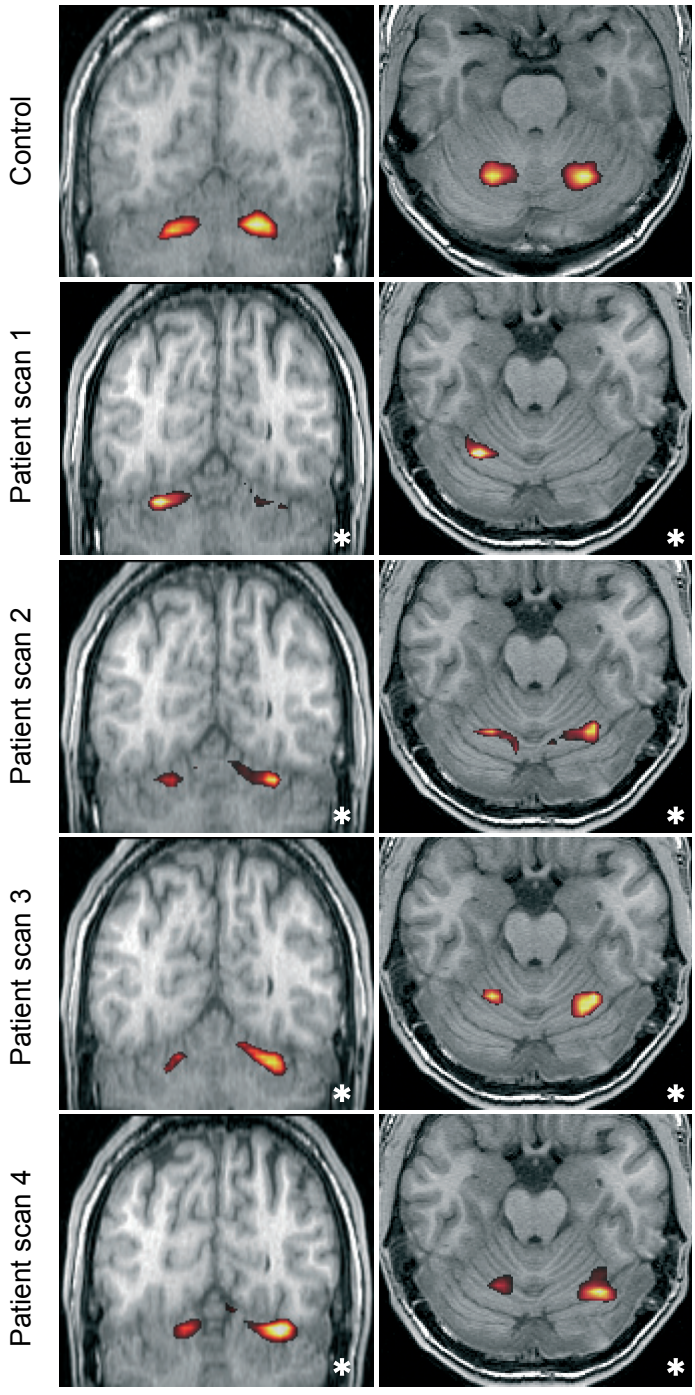


Figure 4. Coronal (left column) and transversal (right column) slices showing distribution of activation in the cerebellar hemispheres during lip pursing in the control group (top row) and in the four measurements in Bell's palsy patients (patient scan 1-4). The (*) indicates the paralyzed side.

In the cerebellum most activation was seen bilaterally in cortical lobules 6. The changes of the lateralization within the cerebellum were about twice as large and in the opposite direction compared to primary motor cortex. This translates in clear changes in lateralization in figures 2 and 4. Most activation was observed contralateral to the paralyzed side in the first measurement. Activation shifted ipsilateral to the paralyzed side in the second and third measurement. In the fourth measurement most activation was still found ipsilateral to the paralyzed side, although the difference in activation was less pronounced than in the third measurement (fig. 2, 4).

Peripheral Recovery

The Sunnybrook facial grading system revealed that facial muscle function was indeed severely affected in all patients directly after onset of the paralysis (figure 5). After 4 months, the time of the second measurement, there was large variability between subjects in the recovery process. Compared to the second measurement all patients showed improvement of facial muscle function. At the time of the fourth measurement, it was clear that none of the patients fully recover. Three patients even had lower scores after 1.5 year compared to 1 year after onset of the paralysis.

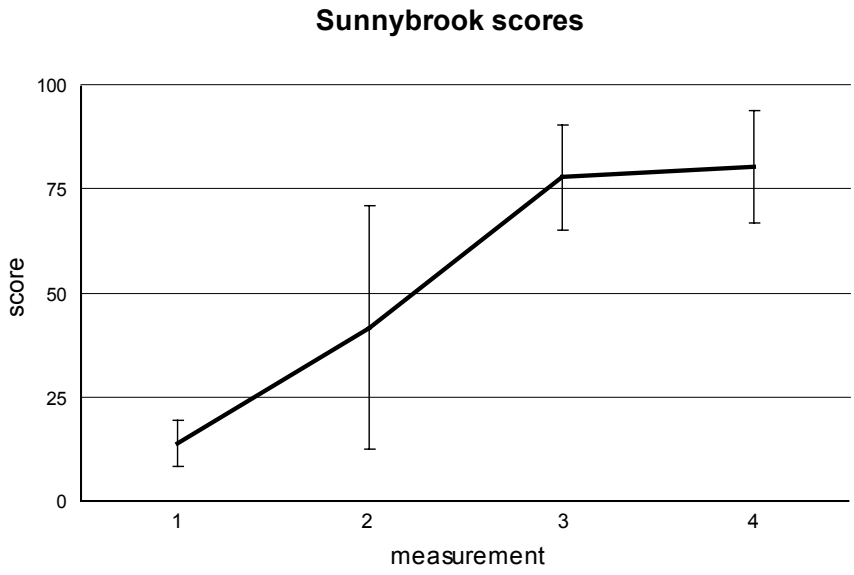


Figure 5. Functional recovery of the Bell's palsy patients. The average Sunnybrook score of 9 Bell's palsy patients scored parallel to the four fMRI measurements is indicated by the black line (error bars show the standard deviation).

DISCUSSION

As expected, group analysis of the control subject data revealed task related activation in the regions of interest in both the left and right hemisphere. The motor activation we found in the primary motor area and cerebellum are in good agreement with previous fMRI studies of lip movement (Dresel et al. 2005, Hesselmann et al. 2004).

The number of significantly activated voxels in the primary motor cortex, primary sensory cortex and cerebellum was consistently lower in patients. A preliminary analysis of data of a larger group of Bell's palsy patients, including additional data of 4 subjects who did not received all four measurements (yet) showed that the number of significantly activated voxels approached control subject level (data not shown). This suggests that the lesser activation in the 9 patients is likely to be caused by the smaller group size.

In the first measurement there was more primary motor cortex activation on the side of the paralysis during lip pursing. Morecraft and coworkers (2001, 2004) showed that the lower half of the face is almost exclusively controlled by the contralateral motor cortex. The lack of facial muscle activity on one side could well be related to the reduced activation within the contralateral motor cortex in patients. Whether this change is caused by lack of motor activity or lack of proprioceptive feedback that corresponds with the intended movement or both can not be distinguished.

No major changes were observed in distribution of significantly active voxels in the primary sensory cortex. The lack of proprioceptive input that confirms correct execution of a movement from the trigeminal nerve apparently does not influence the activation pattern in this area. This suggests that comparison of proprioceptive feedback with the original motor command occurs elsewhere. The 'error signal' of the discrepancy between intended movement and executed movement in Bell's palsy patients could be generated in the cerebellum. Shadmehr and Krakauer (2008) suggest that the cerebellum is able to correct motor commands using a prediction of the expected sensory feedback based on a copy of the motor command and the actual sensory feedback. Possibly the 'error signal' feedback from the cerebellum to higher structures is not sent to the sensory cortex but only to the primary motor cortex, in which changes are observed in the current study.

The observed changes in lateralization of brain activation during lip pursing in the cerebellum were in the opposite direction compared to the primary motor cortex. Whereas during the four measurements, activation in the motor cortex shifted to the contralateral side, activation in the cerebellum shifted towards the ipsilateral side. This observation corresponds well to the crossing projections between the primary motor cortex and the contralateral cerebellum (figure 1). If lateralization shifts to one side of the motor cortex, an opposite shift in the cerebellum is not unlikely.

The lateralization in the cerebellum is more pronounced than the lateralization in the primary motor cortex. Possibly the cerebellar hemisphere that projects to the paralyzed side plays an important role in the fine tuning of execution of lip movements in the whole recovery process, first when lip movement starts to occur again around measurement 2 and later around measurements 3 and 4, during synkinesis (co-contraction of for instance lip muscles during eyelid movement caused by misrouting of facial nerve fibers (Beurskens and Heymans 2004)). Indeed, several subjects showed a significant synkinesis, as reflected by the sub-optimal Sunnybrook scores for functional recovery at measurement

3 and 4. The plastic changes in the primary motor cortex could be more straightforward and therefore result in smaller changes in the lateralization of neuronal activation.

Just after onset of the paralysis, in the primary motor cortex and cerebellar cortex asymmetry was observed in the representation of lip pursing. The amount of activation in the regions in control of the paralyzed side was smaller than the amount of activation in the regions in control of the healthy side. Possibly, the reduced amount of activation was caused by the inability of the patients to perform the task correctly during the first measurement. However, studies on mental imagery have shown that activation in the motor cortex and cerebellum can already be induced by simply thinking about a movement (Stippich et al. 2002). In this case it is not just imagination. The patients are actually performing the motor task within their motor system, but lack proprioceptive feedback from the paralyzed side that corresponds with the given motor command. As a consequence plasticity in the sensory cortex seems likely, though, as mentioned above, we did not see changes in this region.

An explanation for the persistent asymmetry in activation between 4 months and 1.5 years in the cerebellum and to a lesser extent in the primary motor cortex, might be that the previously strictly contralateral projection is now more bilateral for the paralyzed half of the face. The additional ipsilateral input could arise from nerve fibers that originally projected to the eyelids. The eyelids receive bilateral input from the motor cortex (Morecraft et al. 2001, 2004).

It is known that the ipsilateral cerebellum is more involved during complex hand movements than in simple movements like unimanual tapping (Park et al. 2008). Possibly lip pursing becomes a rather complex task after hemifacial paralysis, which could explain the larger changes within the cerebellum.

Peripheral recovery

The functional recovery of the nine patients is in accordance with previous studies of Bell's palsy patients (VanderWerf et al. 2007). In both the primary motor cortex and the cerebellum the largest shift in lateralization of brain activation is observed between the first and second measurement. This period coincides with restoration of the facial nerve muscle contacts and lip movement in most of the patients. It could be that the paralyzed side requires more innervation than the other intact side of the face. However, few changes in the lateralization of activation are seen between the second and third measurements, when all patients are able to move their lips again. It appears that most activation is needed / generated when the muscle first becomes active rather than later during the recovery process.

REFERENCES

Berg, T., Jonsson, L., and Engstrom, M. (2004). Agreement between the Sunnybrook, House-Brackmann, and Yanagihara facial nerve grading systems in Bell's palsy. Otol Neurotol 25, 1020-1026.

Beurskens, C.H., Heymans, P.G. (2004). Physiotherapy in patients with facial nerve paresis: description of outcomes. Am J Otolaryngol. 25(6):394-400.

Dancause, N. (2006). Vicarious function of remote cortex following stroke: recent evidence from human and animal studies. Neuroscientist 12, 489-499.

Dresel, C., Castrop, F., Haslinger, B., Wohlschlaeger, A.M., Hennenlotter, A., and Ceballos-Baumann, A.O. (2005). The functional neuroanatomy of coordinated orofacial movements: sparse sampling fMRI of whistling. NeuroImage 28, 588-597.

Gilden, D.H. (2004). Clinical practice. Bell's Palsy. The New England journal of medicine 351, 1323-1331.

Hesselmann, V., Sorger, B., Lasek, K., Guntinas-Lichius, O., Krug, B., Sturm, V., Goebel, R., and Lackner, K. (2004). Discriminating the cortical representation sites of tongue and up movement by functional MRI. Brain topography 16, 159-167.

Kanerva, M., Poussa, T., and Pitkaranta, A. (2006). Sunnybrook and House-Brackmann Facial Grading Systems: intrarater repeatability and interrater agreement. Otolaryngol Head Neck Surg 135, 865-871.

Morecraft, R.J., Louie, J.L., Herrick, J.L., and Stilwell-Morecraft, K.S. (2001). Cortical innervation of the facial nucleus in the non-human primate: a new interpretation of the effects of stroke and related subtotal brain trauma on the muscles of facial expression. Brain 124, 176-208.

Morecraft, R.J., Stilwell-Morecraft, K.S., and Rossing, W.R. (2004). The motor cortex and facial expression: new insights from neuroscience. The neurologist 10, 235-249.

Park, J.W., Kwon, Y.H., Lee, M.Y., Bai, D., Nam, K.S., Cho, Y.W., Lee, C.H., and Jang, S.H. (2008). Brain activation pattern according to exercise complexity: a functional MRI study. NeuroRehabilitation 23, 283-288.

Reilly, K.T., and Sirigu, A. (2008). The motor cortex and its role in phantom limb phenomena. Neuroscientist 14, 195-202.

Riecker, A., Ackermann, H., Wildgruber, D., Dogil, G., and Grodd, W. (2000). Opposite hemispheric lateralization effects during speaking and singing at motor cortex, insula and cerebellum. Neuroreport 11, 1997-2000.

Ross, B.G., Fradet, G., and Nedzelski, J.M. (1996). Development of a sensitive clinical facial grading system. Otolaryngol Head Neck Surg 114, 380-386.

Rossini, P.M., Altamura, C., Ferreri, F., Melgari, J.M., Tecchio, F., Tombini, M., Pasqualetti, P., and Vernieri, F. (2007). Neuroimaging experimental studies on brain plasticity in recovery from stroke. Europa medicophysica 43, 241-254.

Shadmehr, R., Krakauer, J.W. (2008). A computational neuroanatomy for motor control. Exp Brain Res. 185(3): 359-81. Review.

Stippich, C., Ochmann, H., and Sartor, K. (2002). Somatotopic mapping of the human primary sensorimotor cortex during motor imagery and motor execution by functional magnetic resonance imaging. Neuroscience letters 331, 50-54.

Tamura, Y., Shibukawa, Y., Shintani, M., Kaneko, Y., and Ichinohe, T. (2008). Oral structure representation in human somatosensory cortex. NeuroImage.

Tzourio-Mazoyer, N., Landeau, B., Papathanassiou, D., Crivello, F., Etard, O., Delcroix, N., Mazoyer, B., and Joliot, M. (2002). Automated anatomical labeling of activations in SPM using a macroscopic anatomical parcellation of the MNI MRI single-subject brain. NeuroImage 15, 273-289.

VanderWerf, F., Reits, D., Smit, A.E., and Metselaar, M. (2007). Blink recovery in patients with Bell's palsy: a neurophysiological and behavioral longitudinal study. Investigative ophthalmology & visual science 48, 203-213.

Ward, N.S. (2004). Functional reorganization of the cerebral motor system after stroke. Current opinion in neurology 17, 725-730.

ADDENDUM CHAPTER 8

Cortical plasticity after peripheral facial nerve injury; a longitudinal fMRI study II: blinking

INTRODUCTION

In addition to lip pursing we also studied plasticity in the cortical representation of voluntary blinking throughout the recovery process of severely affected Bell's palsy patients. The methods we used for the four fMRI measurements were highly similar to the methods described in chapter 8. In this experiment, the same nine subjects participated, but during the active blocks they did not pout their lips but blinked. The subjects were instructed to perform fast but light blinks and not to squeeze the eyelids or include other facial muscles in the movement. When not performing the task subjects were asked to keep their eyes closed. The imaging data of the three patients with a unilateral facial paralysis on the right side were flipped so the paralysis was on the same side in all patients.

RESULTS

Group analysis of 14 control subjects revealed blink-related activation bilateral in the primary motor cortex (413 voxels) and in the cerebellum (1058 voxels in total, mostly in lobule 4, 5 and 6, vermis layers 4 to 6 and Crus 1). Little activation was observed in the sensory cortex (47 voxels).

The results of the group analyses for the 9 patients and the changes in lateralization in the primary motor cortex and cerebellum over the four measurements are presented in figure 1. In the first measurement bilateral activation was found in the primary motor cortex. In the cerebellum activity was mainly observed bilaterally in lobe 6 but also in vermis layer 6 to 9 and Crus 1. In both the primary motor

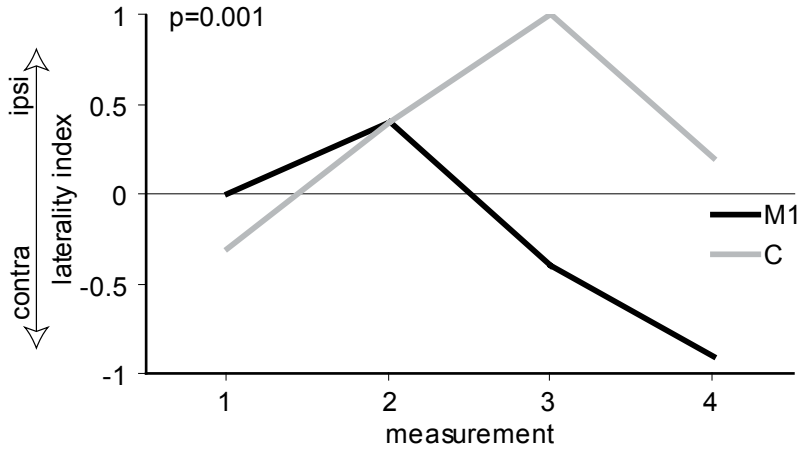


Figure 1. Changes in the laterality index for the activation in the primary motor cortex (M1) and cerebellum (C) across the four measurements made during the recovery process of nine Bell's palsy patients. The laterality index shows the lateralization of the number of active voxels per ROI: 1 = all activation ipsilateral, -1 is all activation contralateral to the paralyzed side. The statistical threshold was set at p=0.001.

cortex and the cerebellum the lateralization changed towards the paralyzed side during the recovery process. There appears to be a shift in the laterality of activation in the primary motor cortex between the second and fourth measurement towards the contralateral side. In the second, third and fourth measurement most activated voxels were found ipsilateral to the paralyzed side in the cerebellum. In these three measurements the total amount of significant voxels greatly decreased and remained low with an average number of significantly active voxels for both hemispheres of 45 in the primary motor cortex and 23 in the cerebellum. In patients, like in controls, hardly any blink-related activation was observed in the sensory cortex.

Compared to the lip pursing experiment the total amount of activation was low during blinking. When we reduced the threshold to p=0.01 the total amount of activation increased, which influenced the laterality index as well (figure 2).

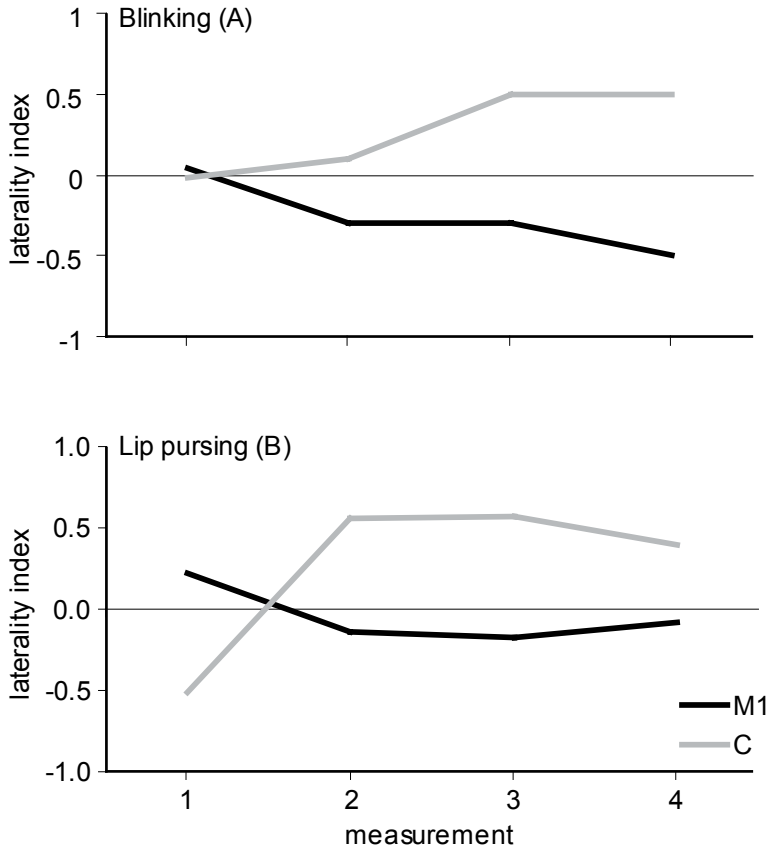


Figure 2. Changes in the laterality index (LI) in the primary motor cortex (M1) and cerebellum (C) throughout recovery of the Bell's palsy patients. Panel A shows of the blinking task using a threshold of p=0.01 in the second to fourth scans. In panel B the results of the lip pursing task are plotted (p=0.001). The laterality index represents the lateralization of the number of active voxels per ROI; 1 = all activation ipsilateral, -1 is all activation contralateral to the paralyzed side.

DISCUSSION

We found neuronal activation in the primary motor cortex and the cerebellum during blinking. This is in agreement with previous fMRI studies on blinking (Baker et al. 2003, Cheng et al. 2008, Chung et al. 2006, Dimitrova et al. 2002 and Kato and Miyauchi 2003). Compared to lip pursing, blinking does not induce a lot of neuronal activation in the sensory cortex. The lack of activation in the sensory cortex during blinking in all four patient measurements and very little activation in control subjects suggests that this task simply does not induce a lot of sensory feedback.

Similar to the lip pursing experiment, cortical changes are observed in the representation of blinking in the primary motor cortex and cerebellum. In the lip pursing task the changes in lateralization in the primary motor cortex and cerebellum were found to be in the opposite direction. This implies that these two structures innervate the mouth from opposite sides of the brain. However in the blinking task the lateralization within the primary motor cortex and the cerebellum are not consistently correlated at a statistical threshold of p=0.001.

An explanation for this inconsistency could be that the laterality index was calculated on the basis of a relatively low number of voxels. If the threshold is lowered to p=0.01 the results begin to resemble those in the lip pursing task (fig. 2). Now the average number of activated voxels determining the LI is 220 in the primary motor cortex and 335 in the cerebellum. Measurements in additional subjects are needed to reveal whether this trend can be confirmed with a stricter threshold.

On the other hand, differences in anatomical projections innervating the mouth and the eyelid may explain the difference in results between the two tasks. Contrary to the strictly contralateral innervation of the lower half of the face, innervation of one side of the upper half of the face is controlled by both hemispheres (Morecraft et al. 2001, 2004). Therefore paralysis of the orbicularis oculi muscle on one side is likely to induce changes in both hemispheres. This could lead to higher variability between patients in the changes in lateralization, making detection of common activations in the whole group statistically difficult. Furthermore the part of the facial motor nucleus that innervates the lower half of the face receives a strong projection from the primary motor cortex whereas the upper half of the faces receives most direct projections from the supplementary motor area en rostral cingulate cortex and only marginally from the primary motor cortex (Morecraft et al. 2001, 2004). On the other hand with transcranial magnetic stimulation of the primary motor cortex it is possible to evoke a response in the ipsilateral and contralateral oribicularis oculi muscle (Paradiso et al. 2005).

REFERENCES

Baker, R.S., Andersen, A.H., Morecraft, R.J., and Smith, C.D. (2003). A functional magnetic resonance imaging study in patients with benign essential blepharospasm. J Neuroophthalmol 23, 11-15.

Cheng, D.T., Disterhoft, J.F., Power, J.M., Ellis, D.A., and Desmond, J.E. (2008). Neural substrates underlying human delay and trace eyeblink conditioning. Proceedings of the National Academy of Sciences of the United States of America 105, 8108-8113.

Chung, J.Y., Yoon, H.W., Song, M.S., and Park, H. (2006). Event related fMRI studies of voluntary and inhibited eye blinking using a time marker of EOG. Neuroscience letters 395, 196-200.

Dimitrova, A., Weber, J., Maschke, M., Elles, H.G., Kolb, F.P., Forsting, M., Diener, H.C., and Timmann, D. (2002). Eyeblink-related areas in human cerebellum as shown by fMRI. Human brain mapping 17, 100-115.

Kato, M., and Miyauchi, S. (2003). Functional MRI of brain activation evoked by intentional eye blinking. NeuroImage 18, 749-759.

Morecraft, R.J., Louie, J.L., Herrick, J.L., and Stilwell-Morecraft, K.S. (2001). Cortical innervation of the facial nucleus in the non-human primate: a new interpretation of the effects of stroke and related subtotal brain trauma on the muscles of facial expression. Brain 124, 176-208.

Paradiso, G.O., Cunic, D.I., Gunraj, C.A., and Chen, R. (2005). Representation of facial muscles in human motor cortex. The Journal of physiology 567, 323-336.

PART V

Summary & Discussion

Lay summary

Lekensamenvatting

Chapter 9

Summary & Discussion

SUMMARY AND DISCUSSION

The goal of this thesis was to gain insight into the neuronal pathways and pathologies of the blink. The studies presented in this thesis focus on control of blinking in the brainstem, cerebellum and cerebrum. In the introduction of this thesis (**part I**), an overview is given of today's knowledge on the blink and blink-related pathologies. In the present thesis we have focussed on blinking and brainstem pathways (part II), blinking and cerebellar pathology (part III) and blinking and lip pursing and central compensations (part IV). In the remainder of this chapter reflex blinks and conditioned responses are considered and ideas for future blink studies are discussed.

BLINKING AND BRAINSTEM PATHWAYS

The brainstem is the part of the brain essential for execution of most cranial reflexes. Blink reflexes also depend on the brainstem. During a blink reflex the eye and eyelid movement have to be coordinated, which is the topic of **part II**. The short delay between reflex-evoking stimulus and the reflex itself indicates that few synapses are involved, thereby pointing towards the brainstem for a location where this coordination occurs. The reticular formation (RF) was the candidate we chose to study, as this area is known as an integrator that contains many ascending and descending tracts (Brodal 1981) and has been linked to eyelid movement (Holstege et al. 1986, Zemlan et al. 1984, Zerari-Mailly et al. 2003). To reveal whether projections to eye movement-controlling areas exist from the RF, neuro-anatomical retrograde tracing experiments were performed from the eye movement-coordinating part of the superior colliculus of rats.

Results of the study presented in chapter 2 reveal that cells were labelled in several areas of the RF. Injections of gold conjugated horseradish peroxidase (gold-HRP) or biotinylated dextran-amine (BDA) tracers into the medial and/or central portions of the superior colliculus resulted in labelled neurons predominantly in the medial RF, whereas after injections into the lateral portion of the superior colliculus labelling is observed in the medial as well as lateral RF. Most labelled neurons and thus reticulo-collicular projections were found when the injection was made in the lateral or caudal superior colliculus. Injections in this area revealed neurons that project to the superior colliculus in two areas previously shown as important for blinking, the dorsal medullary reticular nucleus and parvocellular reticular nucleus. Both areas were labelled predominantly contralateral to the side of the superior colliculus which was injected.

To confirm that the reticular neurons that project to the superior colliculus are indeed involved in eyelid movement, double labelling experiments were conducted. The results of this study are presented in chapter 3. In addition to the RF the rostral cervical spinal cord (C1) was studied, as the short circuit implied by the timing of reflex blinks could also include this area. In the RF and C1 labelling of c-Fos expression after supra-orbital nerve stimulation and Gold–HRP labelling after injection in the superior colliculus were studied. We observed many double labelled neurons in the parvocellular reticular nucleus, medullary RF, and laminae IV and V of C1. Thus, these brain regions contain neurons involved in blink reflexes as well as eye movements, as they are both activated following supra-orbital nerve stimulation and projecting to the superior colliculus. These data suggest that the parvocellular

reticular nucleus, medullary RF and C1 region play a central role in the coordination of eye and eyelid movements during reflex blinking.

However, how exactly RF and other brainstem structures organize the coordination between eye and eyelid movement is yet to be unravelled.

BLINKING AND CEREBELLAR PATHOLOGY

In **part III** we investigated the possible presence and effects of cerebellar abnormalities in people with fragile X syndrome (FRAXA). The blink was used to study cerebellar motor learning with classical delay eyeblink conditioning. The genetic cause of this disorder is well defined (Garber et al. 2008). Neuro-anatomical and functional consequences are still under investigation. Cerebellar neuro-anatomical abnormalities include irregular, elongated spines of Purkinje cells. Delay eyeblink conditioning revealed learning deficits in both mice with a global knockout and Purkinje cell-specific knockout for the fragile X mental retardation protein (FMRP).

In humans with FRAXA abnormal cerebellar learning was also found in the first conditioning session (chapters 4, 5). Subsequent testing of saving abilities revealed normal performance, implying that though short-term learning during the first conditioning session is disturbed, long-term memory processes seem to be intact (chapter 5). Unlearning of the conditioned response (CR) during extinction trials is significantly faster in individuals with FRAXA (chapter 5). These findings confirm that absence of FMRP affects cerebellar motor learning. The normal performance in the savings experiment and aberrant performance in the acquisition and extinction experiments of individuals with FRAXA suggests that different mechanisms or sites are likely to be involved in learning, remembering and forgetting CRs in delay eyeblink conditioning.

This study is not only interesting as a means to gain knowledge on the disease mechanism to help design a better treatment strategy for individuals with FRAXA, but also a tool to learn more about the exact role of the cerebellum during associative motor learning and study learning in general.

With the results of the current study we can for instance make suggestions about the site(s) at which learning occurs during eyeblink conditioning. As previously stated, the data imply that at least two different sites and/or mechanisms are involved in the different types of eyeblink conditioning: one affected by absence of FMRP and another less affected by absence of FMRP. In the cerebellum FMRP is expressed in the cytoplasm of all neurons and strongest in Purkinje cells, the only output cells of the cerebellar cortex (Bakker et al., 2000; Tamanini et al., 1997). During delay eyeblink conditioning plasticity is thought to occur in both in the cerebellar cortex (at the parallel fiber-Purkinje cell synapses) and deep cerebellar nuclei (for reviews see: Attwell et al., 2002; Christian and Thompson, 2003; De Zeeuw and Yeo, 2005). Both the cerebellar cortex and cerebellar nuclei are necessary for acquisition of the CR (Chen et al., 1996; Koekkoek et al., 2003). The interpositus nucleus (a deep cerebellar nucleus) is thought to be essential for long-term savings of the CR (Christian and Thompson, 2005). The results of the current study are in agreement with these ideas. Acquisition, dependent on FMRP-deprived Purkinje cells, appears impaired in FRAXA subjects. During savings FRAXA subjects delay eyeblink conditioning resembles control subject performance. Savings could be mediated by the interpositus nucleus, which functioning is possibly less dependent on FMRP expression.

It can be hypothesized that extinction, a short-term memory process, could again be dependent on the parallel fibre Purkinje cell synapses. If so, that would imply that extinction and acquisition occur in the same location. Whether extinction is formation of a new memory trace or a mask of an old memory trace is a much debated subject. If acquisition and extinction do occur in the same synapse our results would imply that after slow learning the poorly established memory trace is easily erased.

The exception

Learning is disturbed in most individuals with FRAXA but not all. In chapter 6 we describe a male patient in a classical FRAXA family without the characteristic FRAXA phenotype. His intelligence is borderline normal despite the presence of a mosaic pattern of a pre-mutation (25%), full mutation (60%) and a deletion (15%) in the FMR1 gene. However, FMRP expression in both hair roots and lymphocytes is very low. The percentage of CRs after delay eyeblink conditioning was much higher than the percentage found in the average individual with FRAXA. Also, savings were better and extinction was slower, compared to other individuals with FRAXA, and more like control subjects.

This combination of normal intelligence and normal cerebellar learning in an individual with subnormal threshold FMRP expression is unique. Does this mean that not only FMRP expression is disturbed in other individuals with FRAXA? That seems highly unlikely as previous studies have shown a tight correlation between levels of protein expression and severity of mental retardation (Tassone et al. 1999). One thing which distinguishes this individual from others is the intensive guidance / training he received throughout childhood. Also, in chapter 5 we showed that repetition of the same paradigm led to normal behavior. This could of course be true for many tasks and other types of learning.

BLINKING, LIP PURSING AND CENTRAL COMPENSATIONS

To better understand recovery of people with sudden onset unilateral facial nerve paralysis, Bell's palsy, we used blinking as a model in **part IV**. In chapter 7 we described peripheral recovery and in chapter 8 we studied central recovery of these patients using functional magnetic resonance imaging (fMRI).

Peripheral recovery

First, peripheral recovery was studied in nine severely affected Bell's palsy patients. The recovery process was examined by looking at eye and eyelid kinematics during voluntary blinks and air-puff and acoustic-click induced reflex blinks and associated orbicularis oculi activity. In the recovery process four phases could be recognized.

In the first phase, which comprised 3-4 months, the facial nerve grew towards the facial muscles and made its first contacts with the facial musculature. Very little orbicularis oculi activity was measured in this phase. During the second phase, which lasted until 9 months after onset of the paralysis, the orbicularis oculi activity of the affected eyelid increased. The onset of eyelid movement on the paralyzed and non-paralyzed side synchronized in this period. The third phase was characterized by overshoot of orbicularis oculi activity in the paralyzed eyelid. Together with the increase of orbicularis oculi activity on the paralyzed side, a decrease in orbicularis oculi activity was observed on the non-paralyzed side. Clinically, eyelid movement was normal after 1 year. In the fourth phase, which started 1 year after onset of the paralysis, subtle increases in maximum amplitude and velocity were found. Other than the

onset latency of eyelid movements, we found that recovery of eyelid movements during reflex blinking was incomplete on the 'paralyzed' side when we stopped measuring after over 1.5 years.

The direction of the eye movements during voluntary blinking remained impaired throughout recovery, but the direction was normal in reflex blinking 1 year after onset of the affliction. The maximum amplitudes of eye movements remained aberrant as well. The consistent impairment of eye movements in voluntary blinking during the study and reduced motility of eyelid movements indicates that higher brain structures, which modify eyelid and eye movement control during blinking, may be altered by the affliction.

Central recovery

In order to study whether central changes are induced by a peripheral paralysis like Bell's palsy we used fMRI in chapter 8. We monitored the cortical representation of lip and eyelid movement. Four measurements were performed at the beginning of the four phases we recognized in the recovery of patients with (almost) complete facial muscle paralysis.

In normal controls we observed that the left and right motor and sensory cortex and the left and right cerebellum are most prominently involved in regulating lip movements. In patients, we observed that the laterality of the activations in the primary motor cortex and cerebellum changed during their recovery period. Activation in the motor cortex shifted away from the side of the lesion, whereas activation in the cerebellum shifted towards the side of the lesion. Contrary to the motor cortex and cerebellum, no large shifts in the lateralization of activation induced by the lip pursing task were observed in the sensory cortex.

The adverse shift in the primary motor cortex and cerebellum is congruent with the neuro-anatomical connections between the motor cortex and the cerebellum. The motor cortex in one hemisphere predominantly innervates the contralateral side and a cerebellar hemisphere innervates the ipsilateral side of the facial musculature. This was for instance shown during speaking and singing (Riecker et al. 2000).

In the regulation of the blink the primary motor cortex and cerebellum are active and in the sensory cortex very little activation was observed in both patients and controls. In the motor cortex and cerebellum of patients scans changes were found in the lateralization of activation. To increase the number of voxels we lowered the threshold to derive a more reliable estimate of lateralization. Now the blink results resembled lip pursing results. Future measurements will have to disclose whether this trend can be confirmed. On the other hand similar changes in the representation of lip movement and eyelid movement would be surprising since Morecraft and co-workers (2001) showed that the lower half of the face is innervated by the contralateral motor cortex, whereas the upper half of the face is bilaterally innervated.

FUTURE STUDIES

Blink reflexes and conditioned responses

Technical considerations about the blink (reflex) for future studies

The blink is used for many purposes but there is not much consensus on its definition. In the preface of this thesis I described the blink as: 'a rapid bilateral eyelid closure and co-occurring eye movement. The eyes rotate down towards the tip of the nose and back up again.' Everybody agrees that a blink constitutes of closing and opening of the eyelids, though how far does the eye need to close in order for a blink to be complete? Likely all the way, though how do you know exactly, when only electromyographic recordings of the orbicularis oculi muscle are used? Also how long can a blink last until it should be called eyelid closure?

During a blink reflex the eyelids close and open after an external stimulus. The reflex should occur following a stimulus, after a short latency (depending on which stimulus is used). However, when is the latency too long? It can be argued that it should be below about 100 to 200 ms, the time after which we can make a voluntary eyelid movement? In this thesis, we defined a reflex blink as eyelid closure that started between 25 to 100 ms after the stimulus with a minimum amplitude of the average baseline position plus three times the baseline standard deviation.

Technical considerations about a conditioned response for future studies

For the conditioned blink or rather conditioned response (CR), as both the kinematic profiles and neuronal circuits are different for blinks and conditioned responses, there is not much consensus on the exact definition either. In practically all eyeblink conditioning studies, CRs are defined using the onset of the eyelid movement. The definition of the time window in which a CR is allowed to occur with respect to the CS is very important as it can have substantial influence on the outcome of the study. The onset of a CR should occur after conditioning stimulus (CS) onset. Most researchers choose not to include the first 50 to 150 ms as responses in this period could be acoustic startles (which have an onset latency of about 30-50 ms (Tackmann et al. 1982)). In delay eyeblink conditioning the CS usually lasts about 500 ms (though can vary from 350 ms (Christian and Thompson 2005) to over 1000 ms (Smith et al. 2005)) and the end of the CS coincides with the unconditioned stimulus (US) (for examples see: Kronenbuerger et al. 2008 (humans), Medina et al. 2002 (rabbits)). Many researchers use the end of the CS as the end of the window in which a CR is allowed to have its onset. This is a bit strict as it would at least take a subject 30 ms to close the eyes if the response were a reflex blink. Therefore we chose to allow CRs to have an onset between 150 ms after CS onset and 25 ms after the CS ended and the UR was given.

However, there is a problem when defining the CR using the onset of the eyelid movement. The behaviour that a subject should learn during conditioning is well-timed association between the CS and the US, in this case eyelid closure at the time the air puff reaches the eye. With a time window in which the onset of a CR is allowed to occur, a CR could already be finished when the UR occurs. According to the definition based on the onset latency that is as good a CR as when a subject closes its eyes exactly in time to prevent the air-puff from reaching the cornea. Alternatively, one could study whether or not the eye is closed when the US is given. Or one could use the percentage of eyelid closure at that time

point. That would solve the onset window problem and you would be looking at the actual behaviour you are studying.

Ideas for future blink studies

Brainstem: It would be useful to perform additional (transneuronal) tracing experiments to check if 'blink neurons' of the superior colliculus indeed project to the oculomotor nucleus. In addition it would be interesting to conduct physiological experiments to unravel how reticular formation (RF) and other brainstem structures organize the coordination between eye and eyelid movement. This way blinking can be used to study the integrative function of the RF. Possibly rats or mice can be used to make electrophysiological recordings in the structures we found during evoked blink reflexes. Timing of activity and fire patterns should reveal how coordination occurs between eye and eyelid movement and also give information about integration of signals in general.

In addition, it would be interesting to study brainstem structures known to be involved in the execution of saccades. During saccades eye and eyelid movement are not the same as during a blink. For instance, during a blink eyelid movement precedes eye movement whereas during a saccade it is the other way around. Nevertheless coordination between eye and eyelid movement still needs to occur. When the eye looks upward the eyelid also raises, otherwise the pupil would disappear behind the upper eyelid. Different brainstem structures are in control of eye and eyelid movement coordination during saccades compared to blinks (Büttner-Ennever 2008, Horn and Büttner-Ennever 2008) yet comparing the mechanisms controlling coordination of movement of the same two structures could be interesting.

Cerebellum: To learn more about mechanisms underlying delay eyeblink conditioning and normal learning, delay eyeblink conditioning experiments could be conducted with subjects with other types of X-linked mental retardation with specific genetic alterations. We know by studying FRAXA that FMRP is important for regulation of synaptic maturation, synaptic plasticity, axonal guidance and synaptic pruning (Willemsen et al. 2004, Hagerman et al. 2005). Other types of mental retardation affect other molecular processes and might provide us with new insights. For example mutation in the GDI1 gene also causes mental retardation (D'Adamo et al. 1998). Already it was shown that GDI1 knockout mice display deficient trace conditioning, but normal fear conditioning (D'Adamo et al. 2002).

Also remarkable is the relation between FRAXA and autism. Autistic features are found in 30 percent of individuals with FRAXA (Kaufmann et al. 2004, Hagerman et al. 2005). Clarification of the link between these two disorders could reveal more about the disease mechanisms of both disorders. In a previous study with delay eyeblink conditioning autistics learned faster than controls (Sears et al. 1994). Delay eyeblink conditioning with autistic Fragile-X subjects could clarify more about cerebellar functioning in this group.

Cerebrum: In the fMRI study of Bell's palsy patients we studied motor recovery and restricted the analysis to comparison of the number of activated voxels in the hemispheres. Of course lateralization is not the only interesting parameter to study. For future analysis the location and changes of location should be studied as well as the strength of the activation. Lack of motor innervation and movement of muscles also means proprioceptive feedback of this lack of movement. Cortical plasticity is therefore also likely to occur in premotor areas.

In this thesis a thorough description of the recovery of Bell's palsy patients is given. Depending on the progress of their recovery patients are referred to a mime-therapist. Here they are given exercises to improve facial motor skills and reduce possible synkinesis. Peripherally these exercises work. It is unknown what central changes are induced by these exercises. With fMRI it would be possible to monitor these changes and correlate them with the amount of synkinesis reduction and therapy participation of participants. It is important to take therapy participation into account, for although patients are referred and all given the same exercises, therapy loyalty differs greatly between patients.

Final Remarks

This thesis aimed to elucidate several important questions regarding blinking and the brain. We showed that the RF contains structures that could coordinate eye and eyelid movement during blinking. The data on individuals with FRAXA indicate that lack of FMRP leads to cerebellar deficits affecting eyeblink conditioning. We found that peripheral paralysis of mouth and eyelid muscles induces changes in activation in central motor areas at various stages in the recovery process. Finally, everybody agrees that we need blinks to function in our everyday life and I hope this thesis has also convinced you that 'A blink is an interesting phenomenon worth investigating'.

REFERENCES

Attwell, P.J., Ivarsson, M., Millar, L., and Yeo, C.H. (2002). Cerebellar mechanisms in eyeblink conditioning. Annals of the New York Academy of Sciences 978, 79-92.

Bakker, C.E., de Diego Otero, Y., Bontekoe, C., Raghoe, P., Luteijn, T., Hoogeveen, A.T., Oostra, B.A., and Willemsen, R. (2000). Immunocytochemical and biochemical characterization of FMRP, FXR1P, and FXR2P in the mouse. Experimental cell research 258, 162-170.

Brodal, A. (1981). Neurological Anatomy (New York: Oxford University Press).

Buttner-Ennever, J.A. (2008). Mapping the oculomotor system. Progress in brain research 171, 3-11.

Chen, L., Bao, S., Lockard, J.M., Kim, J.K., and Thompson, R.F. (1996). Impaired classical eyeblink conditioning in cerebellar-lesioned and Purkinje cell degeneration (pcd) mutant mice. Journal of Neuroscience 16, 2829-2838.

Christian, K.M., and Thompson, R.F. (2003). Neural substrates of eyeblink conditioning: acquisition and retention. Learning & memory (Cold Spring Harbor, N.Y 10, 427-455.

Christian, K.M., and Thompson, R.F. (2005). Long-term storage of an associative memory trace in the cerebellum. Behavioral neuroscience 119, 526-537.

D'Adamo, P., Menegon, A., Lo Nigro, C., Grasso, M., Gulisano, M., Tamanini, F., Bienvenu, T., Gedeon, A.K., Oostra, B., Wu, S.K., et al. (1998). Mutations in GDI1 are responsible for X-linked non-specific mental retardation. Nature genetics 19, 134-139.

D'Adamo, P., Welzl, H., Papadimitriou, S., Raffaele di Barletta, M., Tiveron, C., Tatangelo, L., Pozzi, L., Chapman, P.F., Knevett, S.G., Ramsay, M.F., et al. (2002). Deletion of the mental retardation gene Gdi1 impairs associative memory and alters social behavior in mice. Human molecular genetics 11, 2567-2580.

De Zeeuw, C.I., and Yeo, C.H. (2005). Time and tide in cerebellar memory formation. Current opinion in neurobiology 15, 667-674.

Garber, K.B., Visootsak, J., Warren, S.T., (2008). Fragile X syndrome. Eur J Hum Genet. 2008 Jun;16(6):666-72. Review

Hagerman, R.J., Ono, M.Y., and Hagerman, P.J. (2005). Recent advances in fragile X: a model for autism and neurodegeneration. Current opinion in psychiatry 18, 490-496.

Holstege, G., van Ham, J.J., and Tan, J. (1986). Afferent projections to the orbicularis oculi motoneuronal cell group. An autoradiographical tracing study in the cat. Brain research 374, 306-320.

Horn, A.K., and Buttner-Ennever, J.A. (2008). Brainstem circuits controlling lid-eye coordination in monkey. Progress in brain research 171, 87-95.

Kaufmann, W.E., Cortell, R., Kau, A.S., Bukelis, I., Tierney, E., Gray, R.M., Cox, C., Capone, G.T., and Stanard, P. (2004). Autism spectrum disorder in fragile X syndrome: communication, social interaction, and specific behaviors. American Journal of Medical Genetics. Part A 129A, 225-234.

Koekkoek, S.K., Hulscher, H.C., Dortland, B.R., Hensbroek, R.A., Elgersma, Y., Ruigrok, T.J., and De Zeeuw, C.I. (2003). Cerebellar LTD and learning-dependent timing of conditioned eyelid responses. Science (New York, N.Y 301, 1736-1739.

Kronenbuerger, M., Tronnier, V.M., Gerwig, M., Fromm, C., Coenen, V.A., Reinacher, P., Kiening, K.L., Noth, J., and Timmann, D. (2008). Thalamic deep brain stimulation improves eyeblink conditioning deficits in essential tremor. Experimental neurology 211, 387-396.

Medina, J.F., Nores, W.L., and Mauk, M.D. (2002). Inhibition of climbing fibres is a signal for the extinction of conditioned eyelid responses. Nature 416, 330-333.

Morecraft, R.J., Louie, J.L., Herrick, J.L., and Stilwell-Morecraft, K.S. (2001). Cortical innervation of the facial nucleus in the non-human primate: a new interpretation of the effects of stroke and related subtotal brain trauma on the muscles of facial expression. Brain 124, 176-208.

Riecker, A., Ackermann, H., Wildgruber, D., Dogil, G., and Grodd, W. (2000). Opposite hemispheric lateralization effects during speaking and singing at motor cortex, insula and cerebellum. Neuroreport 11, 1997-2000.

Sears, L.L., Finn, P.R., and Steinmetz, J.E. (1994). Abnormal classical eye-blink conditioning in autism. Journal of autism and developmental disorders 24, 737-751.

Smith, C.N., Clark, R.E., Manns, J.R., and Squire, L.R. (2005). Acquisition of differential delay eyeblink classical conditioning is independent of awareness. Behavioral neuroscience 119, 78-86.

Tackmann, W., Ettlin, T., and Barth, R. (1982). Blink reflexes elicited by electrical, acoustic and visual stimuli. I. Normal values and possible anatomical pathways. European neurology 21, 210-216.

Tamanini, F., Willemsen, R., van Unen, L., Bontekoe, C., Galjaard, H., Oostra, B.A., and Hoogeveen, A.T. (1997). Differential expression of FMR1, FXR1 and FXR2 proteins in human brain and testis. Human molecular genetics 6, 1315-1322.

Tassone, F., Hagerman, R.J., Ikle, D.N., Dyer, P.N., Lampe, M., Willemsen, R., Oostra, B.A., and Taylor, A.K. (1999). FMRP expression as a potential prognostic indicator in fragile X syndrome. American journal of medical genetics 84, 250-261.

Willemsen, R., Oostra, B.A., Bassell, G.J., and Dictenberg, J. (2004). The fragile X syndrome: from molecular genetics to neurobiology. Mental retardation and developmental disabilities research reviews 10, 60-67.

Zemlan, F.P., Behbehani, M.M., and Beckstead, R.M. (1984). Ascending and descending projections from nucleus reticularis magnocellularis and nucleus reticularis gigantocellularis: an autoradiographic and horseradish peroxidase study in the rat. Brain research 292, 207-220.

Zerari-Mailly, F., Dauvergne, C., Buisseret, P., and Buisseret-Delmas, C. (2003). Localization of trigeminal, spinal, and reticular neurons involved in the rat blink reflex. The Journal of comparative neurology 467, 173-18

LAY SUMMARY

LEKENSAMENVATTING

LAY SUMMARY

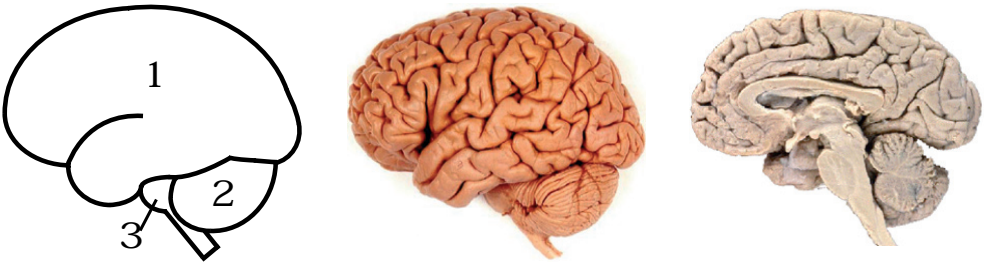


Figure. Three views of the brain. On the left the outlines of the brain with numbers indicating the cerebrum (1), cerebellum (2) and brainstem (3). In the middle the outside of the brain and on the right a sagital view through the middle of the brain.

The three main divisions of the brain are the large brain (cerebrum), little brain (cerebellum) and brainstem (see figure above). In the cerebrum processing and integration of complicated functions takes place. This can for instance be sensory (touch), motor (movement) or cognitive functions. The cerebellum is important for fine regulation of motor behavior. In the brainstem, the structure buried deep within the brain, vital functions and reflexes are controlled. Information of brain cells (neurons) from these areas for the head, neck, chest and most of the abdominal organs is transferred through the twelve cranial nerves (table 3, chapter 1), which originate from the brainstem.

This thesis focuses on blinking and the brain. Three different types of eyeblinks can be distinguished; reflex blinks, spontaneous blinks and voluntary blinks. These blinks serve to protect the eye and play a role in non-verbal communication. The seventh cranial nerve, or facial nerve, is the motor nerve that enables this quick eyelid movement. Confirmation of correct performance of the intended movement is relayed via the fifth cranial nerve or trigeminal nerve. Parts of the brainstem together with these nerves are necessary for the reflex blink. During spontaneous and voluntary blinks, other than the areas needed for reflex blinking, the large brain is involved as well.

The introduction **(Part I)** gives an overview of the current knowledge on the anatomy and characteristics of blinking with the eyes. Also, eyeblink-related diseases are described. In addition, the techniques used for the studies in this thesis are explained.

In the first studies **(Part II)**, neuroanatomical tracer techniques are used to find out in which area of the brainstem eye and eyelid movement are coordinated during blinking. With tracer injections, it is possible to study connections between brain structures. For this study we studied the connection between two brain areas; the superior colliculi (involved in eye movement) and parts of the reticular formation (involved in eyelid movement). Colliculus is latin for hill, the superior colliculi are the two upper of four small hills on top of the brainstem. Reticular formation means ‘area with network-like structure’, which is a complex neural network in the central core of the brainstem. We found two subnuclei within this structure that could play a role in the coordination of eye and eyelid movement during reflex blinking.

Blinks can also be used to study learning abilities. During a so-called conditioning experiment a small puff of air will be blown into the eye and a tone will be played. The natural response to the air-puff stimulus is a blink. The tone does not evoke any response. After repeated paired exposure to the

air-puff and tone normal subjects learn to associate these two stimuli. As a consequence, subjects no longer only blink when the air-puff is blown into the eye, but also when they hear just the tone. The tone is called a conditioning stimulus. Blinks evoked by this tone are called conditioned responses.

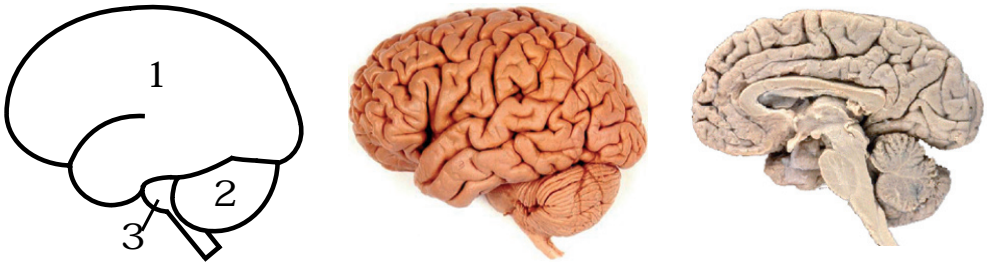
The little brain is involved in delay eyeblink conditioning, a specific type of eyeblink conditioning. During delay eyeblink conditioning the air-puff reaches the eye at the end of the tone. Damage to the little brain leads to diminution or even absence of the ability to associate the tone to the air-puff. This results in less or no conditioned responses during a delay eyeblink conditioning experiment.

We used this type of eyeblink conditioning to investigate how well the little brain of people with fragile X syndrome functions **(Part III)**. People with this hereditary syndrome lack a protein (FMRP), the absence of which leads to mental retardation. We studied learning, remembering and forgetting of conditioned responses. We found that people with fragile X syndrome learn slower, remember just as well but unlearn faster than normal subjects. This indicates that other than cognitive problems people with fragile X syndrome also have problems with cerebellar learning, but that processes important for long term memory are unaffected. We also describe an exceptional person with fragile X syndrome. He lacks the protein, though functions very well, which is confirmed by normal performance in the delay eyeblink conditioning experiment. There is no clear explanation for this. Possibly he has another genetic abnormality which compensates for the lacking protein. Other than that, the intensive training he received throughout childhood might play a role. This would confirm the importance of good guidance and stimulation of people with fragile X syndrome.

In further studies we investigated the recovery of people with sudden paralysis of facial muscles on one side of the face **(Part IV)**. These people are for instance no longer able to move the muscles around the eyes and mouth. When there is no explanation for this paralysis of the facial nerve it is called Bell's palsy or idiopathic unilateral facial nerve paralysis. We meticulously studied recovery of eye and eyelid movement in people with a severe grade of this affection. Four different phases could be distinguished in the recovery of this group of patients. The first phase is the (near) total paralysis of the nerve. The paralysation can occur within a couple of hours and can continue up to ten days after onset of the complaints. This is followed by outgrowth of the new nerve and establishment of new contacts with the facial muscles after about 3-4 months in the second phase. The third phase, which lasts till about a year after onset of the paralysis, is characterized by motor recovery. In the last phase, which we followed up to a year and a half, fine-tuning of facial muscle movement occurs. When people are in this phase it is generally no longer obvious that they have had Bell's palsy. Though with our measurements we could still observe abnormalities in eye and eyelid movements. An interesting finding is that other than the eyelid movement the eye movement is affected also. This implies that multiple cranial nerves are involved in this paralysis, since the eye muscles are not controlled by the recovering facial nerve.

In addition to studying functional recovery we also looked at changes in the brain of people with Bell's palsy. Changes in the brain could play a role during recovery of these patients. With functional MRI scans during each of the phases of the recovery process changes in brain activation during facial movements were mapped. Brain activation induced during blinking and lip pursing was studied. In the year and a half period we followed these patients, we found a shift in the proportion of brain activation in motor areas between the left and right side of the brain. This shift occured in opposite directions in the little brain and the large brain. These changes in brain activation might play a role in the recovery of Bell's palsy patients.

LEKENSAMENVATTING



Figuur. Drie keer het brein. Links de omtrek van het brein met daarin aangegeven het cerebrum (1), cerebellum (2) en de hersenstam (3). In het midden zie je de buitenkant van het brein en rechts een sagitale doorsnede door het midden van het brein.

Het brein is grofweg te verdelen in de grote hersenen (cerebrum), kleine hersenen (cerebellum) en hersenstam (zie bovenstaand figuur). In het cerebrum vindt verwerking en intergratie plaats van ingewikkelde processen en functies. Hierbij kan het bijvoorbeeld gaan om sensorische (gevoel), motorische (beweging) en cognitieve functies. Het cerebellum is belangrijk voor precieze motorische controle. In de hersenstam worden vitale functies en reflexen gereguleerd. Informatie van hersencellen (neuronen) uit deze gebieden voor het hoofd, de nek, borstkas en de meeste organen in de buik wordt doorgegeven via de twaalf hersenzenuwen (tabel 3, hoofdstuk 1), welke vanuit de hersenstam ontspringen.

Dit proefschrift gaat over knipperen en het brein. Er zijn 3 soorten knipperbewegingen van het oog te onderscheiden; reflex-knippers, spontane knippers en willekeurige knippers. Deze knippers dienen bijvoorbeeld om het oog te beschermen en kunnen een rol spelen bij non-verbale communicatie. De zevende hersenzenuw, de nervus facialis, is de motorzenuw die deze kortdurende ooglidbeweging mogelijk maakt. Via de sensorische vijfde hersenzenuw, de nervus trigeminus, wordt teruggekoppeld of de beweging daadwerkelijk uitgevoerd wordt. Delen van de hersenstam tezamen met deze zenuwen zijn nodig voor de reflex-knipper. Bij spontane en willekeurige knippers zijn ook gebieden in de grote hersenen betrokken.

In de introductie (**Part I**) wordt een uitgebreide beschrijving gegeven van de huidige kennis over anatomie en eigenschappen van het knipperen met de ogen. Daarnaast wordt een beschrijving gegeven van ziektebeelden die betrekking hebben op het knipperen met de ogen. Verder worden de technieken beschreven die voor de studies in dit proefschrift gebruikt zijn.

In de eerste onderzoeken (**Part II**) wordt met neuro-anatomische tracer-studies uitgezocht in welk gebied in de hersenstam de beweging van het oog en ooglid tijdens een knipper wordt gecoördineerd. Door middel van tracer-injecties is het mogelijk om verbindingen tussen verschillende hersenstructuren te bestuderen. Voor deze studie hebben we verbindingen tussen twee hersengebieden onderzocht: de hogere colliculi (belangrijk voor oogbeweging) en delen van de reticulaire formatie (betrokken bij ooglidbewegingen). Colliculus is latijn voor heuvel, de hogere colliculi zijn de twee bovenste van vier heuveltjes die op de hersenstam liggen. De reticulaire formatie betekent “gebied met de gedaante van

een netwerk", en is een langgerekte structuur in het midden van de hersenstam waar vele zenuwbanen kruisen. Wij vonden twee subkernen van deze structuur die een rol zouden kunnen spelen bij de coördinatie van oog- en ooglidbewegingen tijdens reflex-knippers.

Knippers kunnen ook gebruikt worden om het leervermogen mee te bestuderen. Tijdens een zogenaamde conditioneringsproef worden een luchtstroompje op de oogbol, welke van nature een knipperbeweging uitlokt, en een toon herhaald samen aan geboden. Indien het brein in staat is te leren worden deze stimuli met elkaar geassocieerd. Dit heeft als gevolg dat je na verloop van tijd niet alleen met je ogen knippert als er een luchtstroompje in je oog wordt geblazen, maar ook als je alleen de toon hoort. Deze toon noemen we de conditionerende stimulus, en de daardoor uitgelokte oogknippers worden dan ook geconditioneerde responsen genoemd.

De kleine hersenen zijn betrokken bij een speciale vorm van het conditioneren van de ooglid beweging, het zogenaamde 'delay eyeblink conditioning'. Hierbij valt het luchtstroompje op de oogbol samen met het einde van de toon. Schade aan de kleine hersenen leidt tot een verminderde of zelfs afwezige associatie tussen toon en luchtstroom en er treden dan ook minder of geen geconditioneerde responsen op.

Het op deze wijze conditioneren van de knipper hebben wij gebruikt om te weten te komen hoe goed de kleine hersenen van mensen met het fragiele X syndroom functioneren (**Part III**). Mensen met deze erfelijke aandoening missen een eiwit wat leidt tot verstandelijke beperkingen. We hebben gekeken naar het aanleren, onthouden en vergeten van de geconditioneerde responsen. Hier bleek dat ze langzamer aanleren, maar evengoed onthouden en zelfs sneller afleren dan proefpersonen zonder het fragiele X syndroom. Dit wijst er op dat er naast cognitieve leerproblemen ook cerebellaire leerproblemen zijn bij mensen met het fragiele X syndroom, maar dat processen die belangrijk zijn voor het onthouden op de lange termijn wel goed functioneren. Ook wordt een uitzonderlijk persoon met het fragiele X syndroom beschreven. Hij mist wel het eiwit, maar functioneert heel goed, wat bevestigd werd door zijn normale gedrag in de conditioneringsproef. De reden hiervoor is niet duidelijk. Mogelijk heeft hij nog een andere genetische afwijking waardoor de problemen veroorzaakt door het afwezige eiwit worden opgevangen. Daarnaast zou ook de intensieve training die hij heeft gehad in zijn jeugd een rol kunnen spelen, wat het belang zou bevestigen van goede begeleiding en stimulatie van mensen met het fragiele X syndroom.

Ook is er onderzoek gedaan naar het herstel van mensen met een plotselinge eenzijdige verlamming van het aangezicht (**Part IV**). Deze mensen zijn niet meer in staat bewegingen te maken met de aangezichtsspieren aan één zijde van het gezicht, zoals die rondom het oog en de mond. Als deze verlamming van de nervus facialis niet nader te verklaren is wordt dit ook een verlamming van Bell of idiopatische hemifacialisparese genoemd. Bij mensen met een ernstige vorm van deze aandoening hebben wij het functionele herstel van de ooglid- en oogbeweging nauwkeurig gemeten. Hierbij werd duidelijk dat er in het herstel van deze groep patiënten vier fases te onderscheiden zijn. De eerste fase is het (bijna) volledig uitvallen van de zenuw. Dit uitvalsproces kan plaatsvinden binnen een paar uur en kan ongeveer 10 dagen doorgaan. Deze fase wordt gevolgd door het uitgroeien van de zenuw en het maken van nieuwe contacten met de aangezichtsspieren na ongeveer 3-4 maanden in de tweede fase. De derde fase, die duurt tot een jaar na het begin van de verlamming, staat in het teken van motorisch herstel en in de laatste fase die wij gevolgd hebben tot anderhalf jaar vindt restherstel plaats. Vaak is in deze fase niet meer te zien dat de mensen een verlamming van Bell hebben gehad. Wij

vonden dat er toch nog afwijkingen te zien zijn in onze metingen van de ooglidbewegingen. Opvallend is hierbij dat naast de ooglidbeweging ook de oogbeweging is aangedaan. Dit impliceert dat meerdere hersenzenuwen betrokken zijn bij deze aandoening, aangezien de oogspieren niet door de zich herstellende nervus facialis aangestuurd worden.

Naast bestudering van het functionele herstel is er ook gekeken naar veranderingen in de hersenen bij mensen met een verlamming van Bell. Mogelijk spelen veranderingen in de hersenen een rol bij het herstel van deze patiënten. Met functionele MRI-scans in elk van de vier fases in het herstelproces is de activiteit van de hersenen tijdens aangezichtsbewegingen in kaart gebracht. We lieten de patiënten met hun ogen knipperen en met de lippen tuiten. Over een periode van anderhalf jaar vonden we een verschuiving in de verhouding van hoeveelheid hersenactiviteit in de motorische gebieden in de linker en rechter hersenhelft. Deze verschuiving was in de kleine hersenen tegengesteld aan de verschuiving in de grote hersenen. Deze veranderingen in hersenactiviteit kunnen een rol spelen bij het herstel van mensen met de verlamming van Bell.

PERSONAL

Dankwoord
Curriculum Vitae
List of publications

DANKWOORD

In een heerlijk herfstzonnetje zit ik nu in de tuin. In mijn luie stoel overdenk ik mijn promotieperiode. Een ding is mij al snel duidelijk: er zijn veel mensen die ik dankbaar ben.

Allereerst mijn promotor Prof. dr. Chris de Zeeuw. Chris, aan het einde van mijn master vroeg je mij te blijven werken op de afdeling. Ik stemde hier uiteraard mee in, waarna enkele jaren vol uitdagingen bij allerlei projecten volgden. Steeds heb jij ook meegedacht met de projecten, waar ik veel van geleerd heb. Dank je voor de steun en motivatie tijdens mijn promotietraject.

Dan Dr. Frans van der Werf. Frans, met jou heb ik tijdens mijn mini-stage in het AMC mijn eerste stapjes gezet in het wetenschappelijk onderzoek. Via jou heb ik virologisch onderzoek gedaan en ben ik vervolgens naar Parijs gegaan om neurowetenschappelijk onderzoek te gaan doen. Je hebt me tijdens het hele promotie traject up-close-and-personal meegemaakt, want je was zowel mij copromotor als kamergenoot. Jij staat dan ook met stip bovenaan in de theeleut-tijdens-werk top 10. Jouw kennis van fysiologische experimenten en neuro-anatomie was onmisbaar voor de onderzoeken die we gedaan hebben. Dank je voor alle ervaringen en alvast veel geluk in jullie nieuwe huis en in Noorwegen.

Vervolgens uiteraard verder met Dr. Jos van der Geest, copromotor nummer 2. Jos, van jou leerde ik werken met de fMRI en met allerlei vragen over data-analyse kon (en kwam) ik je lastig vallen. Iets wat je over jezelf afroept, als je Matlab-scripts kan schrijven met het gemak waarmee ik een boodschappenlijstje samenstel. Ook zorgde je voor de nodige frisse lucht als ik jou vergezelde op jouw rookpauzes voor efficiënt overleg. Dank je voor alles.

Prof. dr. Pierre Buisseret et Prof. dr. Catherine Buisseret-Delmas, Chers Pierre et Catherine, je tiens à vous remercier de m'avoir accueilli dans votre laboratoire pendant mon séjour à Paris. J'y ai appris des nouveaux techniques, et de parler la langue français. Malheureusement, actuellement ce n'est plus aussi facile de parler français que quand j'habitais dans votre studio. Je suis très heureuse que vous venir aux Pays-Bas pour faire partie du jury de ma soutenance de thèse. Veuillez envoyer des gros bisous aux autres: Fawzia, Gaby, Joceline, Monique, Awa et bien sur Céline.

Dr. Tom Ruigrok, Prof. dr. Gabriël Krestin en Dr. Rob Willemsen wil ik danken voor hun leeswerk en commentaar op het manuscript. Ook dank aan de andere leden van de commissie: Prof. dr. Rob Baatenburg de Jong en Prof. dr. Guillain Fernandez voor hun bijdrage aan mijn promotie.

… Hmm een wolk, toch nog iets in dit dankwoord waar ik niet dankbaar voor ben….

Wel ben ik heel dankbaar voor mijn paranimfen Janneke en José.
Janneke, helemaal aan het begin van mijn promotieperiode zaten Frans en ik bij jou op de kamer. Dat duurde niet lang maar toch waren we nooit verder dan 2 deuren van elkaar verwijderd. Jij bent dan ook een goede tweede in de theeleut top 10. Dank je voor alle leuke dingen die we samen hebben gedaan, niet op z'n minst de wintersportvakantie… Ik hoop echt dat ik op tijd terug ben om paranimf te zijn bij jouw promotie.
José, we kennen elkaar al ons hele leven. We moeten altijd lachen als we bedenken dat we zelfs samen in de wieg hebben gelegen, en wat mij betreft zijn we er nog steeds niet uit wie er ouder is. We hebben samen veel gedeeld en ik vind het dan ook super dat je ook tijdens de promotieceremonie weer mijn steun en toeverlaat bent.

Dan wil ik ook graag alle deelnemers aan mijn studies en hun familieleden, die vaak ook meekwamen, bedanken. Jullie waren natuurlijk onmisbaar, maar daarnaast trof ik het echt met de leuke, enthousiaste mensen die ik heb leren kennen omdat zij bereid waren hun tijd in wetenschappelijk onderzoek te investeren.

Bas, met jouw shebots heb ik heel wat mensen aan het knipperen gekregen op 650 Hz tonen. Je bent ongelooflijk handig, veel succes met al je wetenschappelijke uitdagingen. Chiel, van jou heb ik het eyeblink-stokje overgenomen, maar in de loop der jaren hebben we gelukkig wel contact gehouden.
Ben, Rob en Lutgarde, met jullie heb ik met veel plezier aan het fragiele X project gewerkt.
Mick, je bent heel belangrijk voor de Bell's palsy studies, zonder jou alleen al geen mensen om te bestuderen. Je bezoekjes aan ons lab waren altijd leuk.
Dik, jou heb ik al ontmoet tijdens mijn mini-stage in Amsterdam. Nog steeds ondersteun je het blink-lab. Je hebt hele brede interesses, zonder jou had ik bijvoorbeeld nooit geweten waar Helgoland ligt.
Hans, je hebt menig technisch probleem verholpen en dankzij jou weet ik nu dat de Oral-B Pulsar niet werkt in de MRI.
Caroline, we both love working with fMRI and have spent quite a few hours scanning together. You are always willing to help. Thank you for all the fun we had together and good luck with your jobs. Bij het scannen was ook Mijke onmisbaar, dank je voor de vele vermakelijke scanuurtjes.

Heel wat collega's op de 14e hebben in de loop der jaren de dagelijkse werkzaamheden en lunches van gezelligheid voorzien. Harm, van buurman naar RSI-collega, ik vind het mooi om je passie te zien voor de dingen die je doet. Veel succes in de toekomst. Inger, ontzettende lieve collega, dank je voor alle goede gesprekken die we gehad hebben in de loop der tijd. Met of zonder thee, bij jou kon ik altijd even binnen lopen. Het laatste jaar was je er niet veel maar nu promoveren we toch vlak na elkaar. Heel veel liefs en succes met je opleiding. Ook bedankt: Dieke (kopjes thee drinken was ook met jou heel gezellig!), Maureen en Joyce (het zingen in het koor was erg ontspannend), Michiel, Eelco, Hans, Janine, Mark, Durk, Marieke, Ans en alle anderen.

Debbie, wij hebben samen de Neuroscience master gedaan en van onze etentjes heb ik altijd erg genoten.
Susan, dat we nu zo fanatiek aan het sporten zijn is echt heerlijk en onwijs gezellig. De eindfase van mijn promotie is er een stuk leuker door geworden. Heel veel succes met jouw promotie!
En natuurlijk ook bedankt Michiel (met het schizofrenie-project komt het helemaal goed), Flip (veel succes met jouw promotie en ik hoop je een keer op te zien treden.), Bart en Beerend (het tripje van Las Vegas naar San Diego was geweldig en ook als flatmates waren jullie niet verkeerd), Maarten (altijd paraat met goede raad en erg gezellig; vooral Wenen was top).

Naast collega's zou deze tijd heel vervelend zijn geweest zonder mijn vrienden en familie. Ook al heb ik jullie de laatste jaren minder vaak gezien, ik wil wel kwijt dat ik heel erg blij met jullie ben. Enkelen van jullie wil ik in het bijzonder noemen:
Kate, I met you in Paris. Immediately we hit it off and soon the rosé and pizza evenings were a tradition. Over the years we have repeatedly established that Paris is only a three hour train ride away, although this fact can, I think, never be examined thouroughly enough. Thank you for being such a wonderful friend and for providing me with a nice pied-a-terre in Paris. Also special greetings to Mats and Peter, you have now moved away from Paris but I hope to visit you sometime soon.
De Ines, wat zijn (medisch) biologen toch leuk. Onze dineetjes zijn altijd een feest/kippenhok en het is

fijn om met een paar woorden uit te kunnen leggen waar je mee bezig bent en nog begrepen worden ook. Ook onze weekendjes weg zijn legendarisch, ik ben klaar voor de volgende!
Sietze, jij hebt mijn studie en de eerste jaren van mijn promotie van dichtbij meegemaakt. Ik ben trots op waar je nu bent, en ik hoop dat er nog veel moois komen gaat.

Lieve familieleden. Ik kijk er altijd naar uit als we elkaar weer gaan zien. Dank je voor wie jullie zijn, Jelta, Theo, Kiki (met jou is het altijd lachen, gieren, brullen)(en Bob), Eelco (en Quirien), Henny & Dick (ik vond het erg leuk om wat te vertellen over mijn werk bij de seniorenvereniging).
Oma, lieve omie, ik zal nooit vergeten hoe jij mij zei dat onderzoek doen en promoveren wordt gedaan door hele intelligente mensen, dat zal ik dus maar niet tegenspreken. Met veel plezier bel ik regelmatig om je op de hoogte houden van al mijn belevenissen en te horen over die van jou en om natuurlijk de nodige tûûtsen uit te wisselen. Je bent een fantastisch lieve Oma.
Steven, mijn “kleine” broertje. Je bent een eigenzinnig mens met een hart van goud. Alle liefs en veel succes van je zus.
Lieve pap en mam, jullie hebben me altijd enorm gesteund. De meeste dank gaat dan ook uit naar jullie. Dank je voor alles wat jullie me gegeven hebben, ik kan me geen betere ouders wensen.

Als laatste René, ik ben heel blij dat jij mijn leven bent komen binnenskiën, want met ‘gij kunneh de lacheh’. Ik kan me niemand voorstellen met wie ik liever op wereldreis zou gaan.

Dus collega’s, vrienden en familie, genoemd en niet genoemd, het moge duidelijk zijn dat ik me bewust ben dat het me zonder jullie niet was gelukt dit boekje te voltooien. Bedankt!

CURRICULUM VITAE

Personal

Name:	Albertine Ellen Smit
Born:	08-02-1981 in Hilversum, The Netherlands
Address:	Hoogstraat 2a
	5261 TH Vught
E-mail address:	a.e.smit@erasmusmc.nl
Phone:	+31-644345396

Education/Appointments

09-1993 - 09-1999	A-levels at the Sint Vitus College in Bussum
09-1999	start studying Medical Biology
11-2002 - 04-2003	Doctoral internship at the Virology Department of the Amsterdam Medical Centre
11-2003 - 06-2004	Doctoral internship at the Department of Functional Neuroanatomy for Sensory Motor Systems of Université Jussieu in Paris.
03-2005	**Msc in General Medical Biology** (University of Amsterdam)
09-2005	**Master of Neuroscience** (Erasmus University Rotterdam)
05-2005 - 04-2008	PhD student at the Department of Neuroscience of ErasmusMC, Rotterdam
05-2008 - current	Post-doc at the Department of Neuroscience of ErasmusMC, Rotterdam

LIST OF PUBLICATIONS

Smit AE, van der Geest JN, Metselaar MR, van der Lugt A, VanderWerf F, De Zeeuw CI. Long-term changes in cerebellar activity during functional recovery from transient peripheral motor paralysis. *Submitted*

Smit AE, van der Geest JN, Vellema M, Koekkoek B, Willemsen R, Govaerts LC, Oostra BA, De Zeeuw CI, Vanderwerf F. Savings and Extinction of Conditioned Eyeblink Responses in Fragile X Syndrome. *Genes Brain Behav. 2008 7:770-777*

Dauvergne C, **Smit AE**, Vallac J, Diagnec M, Buisseret-Delmas C, Buisseret P, Pinganaud G, VanderWerf F. Are locus coeruleus neurons involved in blinking? *Neurosci Res. 2008 Jun;61(2):182-191.*

VanderWerf F, **Smit AE.** The world according to blink: blinking and aging CH 20. In: Age-related changes of the human eye. Eds. Cavallotti C, Cerulli L. *Humana press USA 2008 ISBN: 978-1-934115-55-8*

Govaerts L, **Smit AE**, Saris J, Vanderwerf F, Willemsen R, Bakker C, De Zeeuw C, Oostra B. Exceptional good cognitive and phenotypic profile in a male carrying a mosaic mutation in the FMR1 gene. *Clin Genet. 2007 Aug;72(2):138-144*

VanderWerf F, Reits D, **Smit AE**, Metselaar M. Blink recovery in patients with Bell's palsy: a neurophysiological and behavioral longitudinal study. *Invest Ophthalmol Vis Sci. 2007 Jan;48(1):203-213*

Smit AE, Buisseret P, Buisseret-Delmas C, VanderWerf F, Zerari-Mailly F. Reticulo-collicular and spino-collicular projections involved in eye and eyelid movements during the blink reflex. *Neurosci Res. 2006 Dec;56(4):363-371.*

Koekkoek SK, Yamaguchi K, Milojkovic BA, Dortland BR, Ruigrok TJ, Maex R, De Graaf W, **Smit AE**, Vanderwerf F, Bakker CE, Willemsen R, Ikeda T, Kakizawa S, Onodera K, Nelson DL, Mientjes E, Joosten M, De Schutter E, Oostra BA, Ito M, De Zeeuw CI. Deletion of FMR1 in Purkinje Cells Enhances Parallel Fiber LTD, Enlarges Spines, and Attenuates Cerebellar Eyelid Conditioning in Fragile X Syndrome. *Neuron. 2005 Aug 4;47(3):339-352.*

Smit AE, Zerari-Mailly F, Buisseret P, Buisseret-Delmas C, VanderWerf F. Reticulo-collicular projections: a neuronal tracing study in the rat. *Neurosci Lett. 2005 Jun 3;380(3):276-279.*