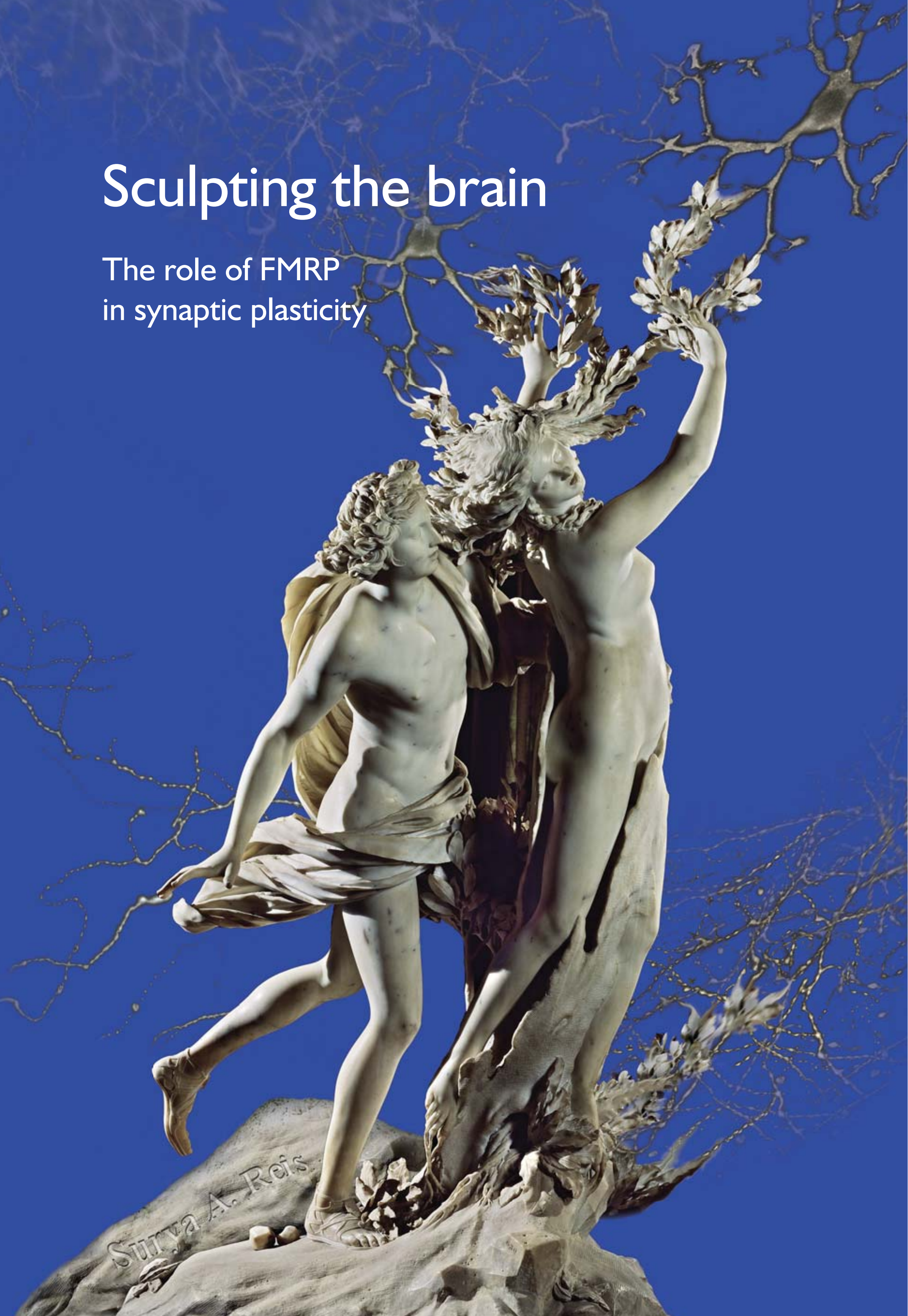
Sculpting the brain

The role of FMRP in synaptic plasticity



Sculpting the Brain:

the role of FMRP in synaptic plasticity

Structurering van de hersenen:

de rol van FMRP in synaptische plasticiteit

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Sculpting the Brain:

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Dare to be different

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General introduction

Für Mama

Chapter 1

The world of the brain

Chapter I

The world of the brain

Cogito ergo sum – I think, therefore I am.

If our human existence is philosophically reasoned since René Descartes' publication of *Meditationes* (1641) by our awareness of a thinking process, one of the most pressing questions is where and how this process takes place. The quest to identify the seat of our minds has intrigued philosophers and scientists for thousands of years. Hippocrates and Plato (387 B.C.) believed that the brain was responsible for intelligence, whilst Aristotle (335 B.C.) was convinced that the heart harboured the mental processes. However, it was Descartes who stirred up the fire and gave a significantly new direction in the ongoing debate about a philosophical distinction between mind and body. Seeing the rational soul as a separate entity from the body, he proposed a body/mind *interactionism* at the pineal gland: external events rearrange the peripheral end of the *nerve fibrils*, followed by a change of pattern of the *interfibrillar space*, finally leading to a specific flow of *animal spirits* into the apposite nerves (see figure 1; [1]).

Figure 1: proposed mechanism by Descartes for automatic reaction in response to external events; illustrated in *De homine*, 1662.

Descartes might not have been anatomically correct, but it is still our common understanding today that the brain and the nervous system as part of the physical world generate states of the mind, while thoughts, perceptions, and emotions have a direct affect on our bodies. The first endeavour to pinpoint specific mental functions to specific parts of the brain was undertaken by Franz Josef Gall, who correlated variations in character to variations in skull shape (see figure 2; [2].

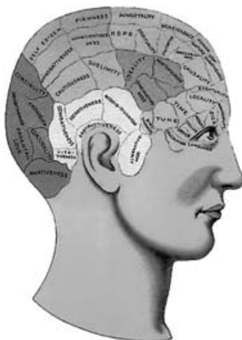


Figure 2: correlation of specific mental functions to specific areas in the brain according to Franz Gall; published in *Anatomie et physiologie du système nerveux en général*, 1810.

Though Gall's methods were almost immediately rejected as highly controversial, his work inspired Marie-Jean-Pierre Flourens to apply an experimentally based approach to demonstrate localization of function in the brain by removing portions of the brain until he identified the locus of a motor centre in the medulla oblongata [3]. The first link of a higher mental function to a particular brain region was achieved by Paul Broca. In 1861, one of his patients who was unable to articulate any kind of speech with the exception of a single syllable – tan – died. Performing a post-mortem examination, Broca found a superficial lesion in the left frontal lobe [4], a finding that was confirmed shortly afterwards by another case. Taking into account that language is solely a human skill and is therefore considered a higher mental function, the brain has finally been identified as the organ of mind.

The organ of mind – magnum opus of millions of years of vertebrate brain evolution

Life in the primordial sea some 500 million years ago was relatively simple so that the number of nerve cells of the earliest brains didn't exceed a few hundred. But with increasing diversity of the archaic sea fauna, the architecture of the brain became more elaborate. The most primitive brain within the subphylum *vertebrata,* developed in the class *Agnatha*. These were prehistoric marine animals lacking both jaws and paired fins. Their nerve cord had closed, thus forming a neural tube, the precursor to the nervous system. Around the neurons, insulating myelin sheaths had formed, so that the speed of signal transport increased to 120m/s. Grey and white areas existed, but were still indistinct. The only living descendants of the agnathans are the hagfishes and lampreys. A key event in 'pursuit of brain sophistication' coincides with the conquering of landmasses by amphibians. In comparison to the *Agnatha*, the *Amphibia* had an enlarged and complexified brain with a similar basic plan. Their need to assess the new environment, the requirement to adjust to new and varied climatic and geological surroundings, the increasing stimuli and sensory inputs, and the pressure of natural selection culminated in the creation of the reptilian brain, which has a design that is still present in modern reptiles and mammals. This development took place in form of size expansion and elaboration, as well as the addition of a new region, the cerebellum, which controlled movement and balance (see figure 3 and 4). The next big leap as a result of increased activity and flexibility, transpired with the growth of the cerebrum and the cortex, which signified the emerging of the early mammalian brain. The number of neurons had increased to hundreds of millions. In concordance with exponential growth and intricacy of the cerebrum, a new species was born about 5 million years ago, mankind.
Further complexity and augmentation of the cerebrum and especially the cerebral cortex led finally to the advent of the formation of consciousness and intelligence, two unique properties of *Homo sapiens* (http://www.duke.edu/~dbc4/resonance/literature/essays/sci/evolverb.htm). In the last 100000 years, the human brain almost tripled its weight (now roughly 1400 gram), the number of neurons has increased to 100 billion, whereas the number of synapses in the cerebral cortex climbed to 60 trillion, and the total surface area of the cortex has reached

$25m^2$ – accentuating the sophisticated architecture of the modern human brain [5].

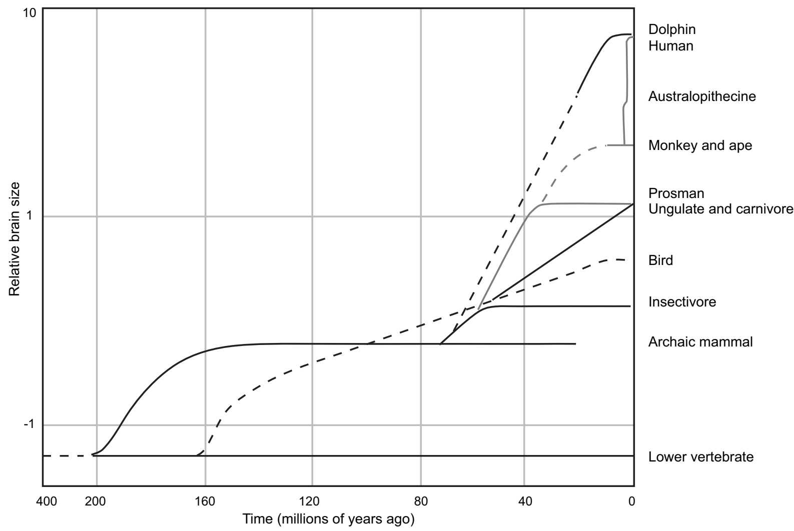


Figure 3: increase of brain size in different vertebrate. Adapted from [6].

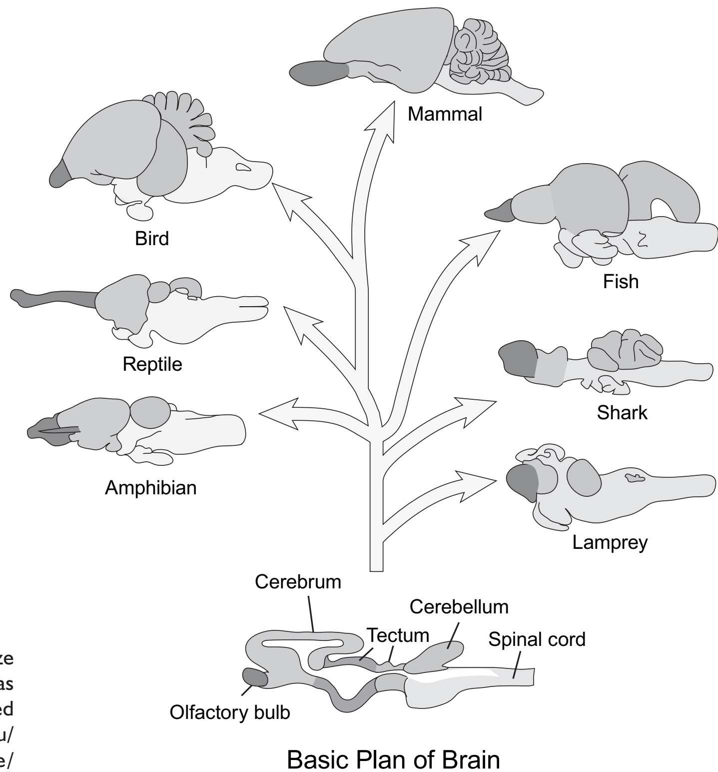


Figure 4: comparison of size relations of different brain areas in different vertebrates. Adapted from http://www.colorado.edu/epob/epob3730rlynch/image/figure5-4.jpg.

Functional architecture of the human brain

Based on phylogenetic considerations, Paul MacLean suggested that the human brain is not one, but actually consists of 3 different brains, where each brain has developed in response to evolutionary needs, with each brain being responsible for separate functions, but with all of them interacting considerably [7].

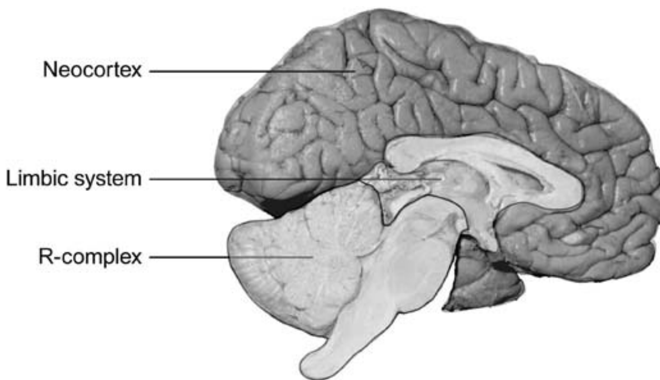


Figure 5: The Triune Model.
Innermost layer: R-complex - responsible for physical survival
Middle layer: Limbic system – responsible for emotions, memories
Outermost layer: Neocortex – responsible for higher mental functions

In his triune model (see figure 5) the first brain or first layer, the innermost and evolutionary oldest part of the brain, has developed in order to accommodate functions concerned with physical survival (such as breathing, generation of biorhythms, regulation of food and temperature, and digestion), defence mechanisms, threat display, and mating. This part of the brain entails the brainstem and the cerebellum. It is the most dominant brain part in reptiles, therefore giving rise to the name of 'the reptile brain' or the R-complex. R-complex behaviours are rigid, obsessive, compulsive, ritualistic, paranoid and highly resistant to change. Behaviours are repeated again and again, without learning from past mistakes. This layer is active even in deepest sleep. The second layer to evolve in MacLean's theory is the limbic system, which encloses the amygdala, the hippocampus, the hypothalamus and the thalamus. The limbic system is similar in all mammals. It's wrapped around the R-complex and is mainly concerned with emotions, often in form of suppression and control of the atavistic emotional responses of the R-complex. This hypothesis is supported by patients suffering from Klüver-Bucy syndrome who exhibit a high non-directed sexual drive in response to an impairment of the amygdala [8]. The limbic system is able to learn from experiences and to create recallable long term memories. It enables the mammals to socially bond, it is responsible for attention and sleep, it aids to develop concepts of value and truth, and it validates knowledge and understanding. The outermost layer, the 3rd brain of the triune model, is the neocortex. In proportion to body size and brain size, the surface of the

human cortex is double in comparison to other primates. Damage of the cortex often causes relatively sophisticated impairments of thought. Therefore the cortex is indeed involved with rationalization and logic, as MacLean suggested. As the limbic system controls the R-complex, the neocortex in turn controls the limbic system. It is in charge of higher mental processes such as language, voluntary movement, processing of sensory information, long range planning, deciphering relationships and recognition of patterns of meaning, creation of models for understanding, and some processing of emotions. The three brains of the triune model interact via an intricate network of nerves. Bi-directional communication between the neocortex and the limbic system influences thinking and emotions. The interplay between emotion, thought, memory and action is at the basis of our individual personality. While all three brain layers are probably active at all times, one or the other will dominate on occasion. However, the inherent plasticity of the brain throughout life empowers us to influence our behavioural responses through repeated training and education in its broadest sense. As Daniel Goleman puts it, instead of being a ‘passion’s slave’ who succumbs to ‘emotional hijacking’ by the amygdala (the emotional centre that triggers the most primitive survival response – fight or flight) into rage, paralysing fear, or high anxiety in response to an often insignificant trigger, the neocortex, when educated accordingly, enables us to suppress the first impulse and to rationalize over alternative modes of response [9]. Response to brain activity is communicated to the rest of the body via the spinal cord, which completes the constituents of the central nervous system (CNS). The spinal cord extends to the hip area and is protected by vertebrae. All the nerves connecting the CNS to the rest of the body form the peripheral nervous system (PNS), which is divided into the somatic and the autonomic nervous system (ANS). The ANS is responsible for involuntary mechanisms such as breathing, blood circulation and digestion, while the somatic nervous system is responsible for voluntary mechanisms. The brain is covered by 3 protective layers of membranes called meninges. Other means of protection for the brain and the spinal cord are provided by the cerebrospinal fluid (CSF), which buffers the CNS against jolts, and the blood-brain barrier, which hinders entrance of many harmful agents into the brain [10].

Research since the 19th century has impressively paid tribute to F.J. Gall’s idea of localization of function in the brain (see table 1 and figure 6 for main functional domains). However, already Karl Wernicke (1908) formulated the hypothesis that complex cognitive functions result from cooperation of different areas, where different components of a single activity are processed in different brain regions [11]. Despite this early notion of *distributed processing*, science has predominantly focused on functional segregation by means of looking at patients with specific cognitive impairments in order to identify the responsible brain areas. Yet traditional neuroanatomy and neurophysiology reveal that functional segregation by itself will not be sufficient to explain brain function in its whole.

Brain area	Function
Medulla oblongata	Responsible for vital functions: breathing, digestion, control of heart rate
Pons	Transfers information regarding movement between cerebellum and cerebral hemisphere
Cerebellum	Involved in movement control and learning of motor skills
Midbrain	Controls sensory and motor functions (eye movement, visual/auditory reflexes)
Diencephalon	Is divided into thalamus (information processing between cerebral cortex and rest of the CNS) and hypothalamus (regulates autonomic, visceral, and endocrine function).
Cerebral hemisphere	Consists of cerebral cortex (divided into 4 lobes – see below), basal ganglia (regulation of motor performance), hippocampus (learning and memory), and amygdaloid nuclei (emotional responses).
Temporal lobe	Hearing
Frontal lobe	Planning of future actions, control of movement
Parietal lobe	Somatic sensation, forming of body image
Occipital lobe	Vision

Table 1: Main functional domains of the brain

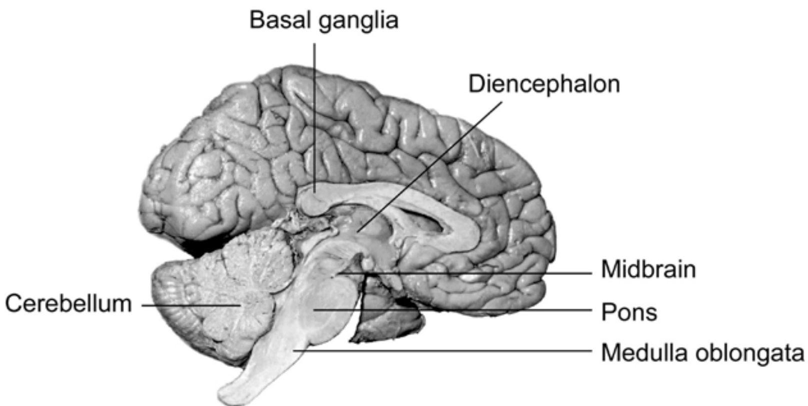


Figure 6: position of the main functional domains within the brain.

Therefore a complementary perspective has come into focus to shed light onto brain function: connectivity. Brain function is explained by bi-directional dynamic flow of information between numerous networks of interconnected brain areas. This approach to neuroscience was rendered possible with the advent of new imaging techniques such as functional magnetic resonance imaging (fMRI), diffusion tensor imaging (DTI), and positron emission tomography (PET) [12]. As suitable and necessary as these techniques are in deepening our understanding

of brain function, there is one drawback that should be taken into account when interpreting experimental data: neuroimaging techniques are applied in order to map localization of brain function by statistical analysis of groups of subjects. Sites of activation are 'identified' by comparison of patterns of brain activity between different individuals and identification of the common patterns within the group. This methodology to pinpoint functional domains or even functional networks within the brain doesn't consider the possibility that individual subjects may apply significantly different cognitive strategies to produce the same cognitive response [13]. Though nobody will dispute that there are domains, which are primarily contributing to a specific cognitive function, scientists should stay open minded about the possibility that every individual may have a very individual pattern of brain activity to perform a cognitive function with all its intricacies.

The principal players and processes for cognitive function

Conveyor of brain activity: the neuron

In the late 1800s, Camillo Golgi developed a method to stain cells with silver salts, a technique that was applied by Santiago Ramón y Cajal to show that the brain consists of networks of discrete cells, rather than being a continuous web [14]. Cajal was the first to provide evidence for Golgi's concept now known as the *neuron doctrine*: individual cells – the neurons – are the elementary signalling units of the brain [15]. Considering the vast array and immense diversity of sophisticated functions that the human brain orchestrates, it is quite amazing that this single cell type alone is sufficient to act as the basic unit of the brain. There are more than 1000 subtypes of neurons (see table 2 for examples), based on architectural considerations (number of processes) or their function (sensory neurons, interneurons, motor neurons), but they all share the same 4 morphological and functional regions: the cell body, dendrites, the axon, and presynaptic terminals (see figure 7).

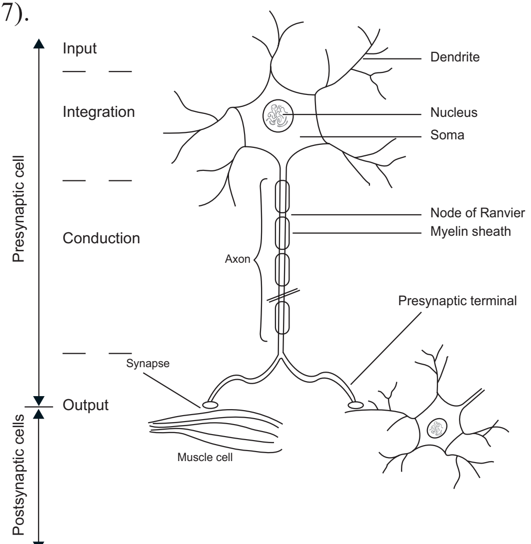


Figure 7: Scheme of a neuron, which forms synapses with a muscle cell and a second neural cell.

The cell body (*soma*) comprises the metabolic factory of the cell like in any other cell type. The other three features are unique in neurons. The axon is a single protrusion from the cell body, which is the key attribute responsible for signal conduction. In order to increase the speed of the signal, the axon is wrapped by insulating myelin sheaths, which in return are interrupted by the nodes of Ranvier. Axons may extend up to 3m, but eventually branch into the presynaptic terminals and form 'contact sites' with other nerve cells or muscles. However, the invention of the electron microscope made it possible to show that there is no physical contact between two communicating cells, but that both cells are separated by the *synaptic cleft*. This finding delivered the final proof for the first law of the neuron doctrine, stating that the neuron is an anatomical segregated unit without any cytoplasmic continuity among cells. The second law, the law of dynamic polarization, states that the flow of an electrical signal occurs in one direction from the receptive surface through the soma and axon to the terminal branches. Responsible for receiving signals are mainly the dendrites, which also extend from the soma. Dendrites branch significantly in tree-like fashion. The transmitting cell is called the *presynaptic cell,* while the receiving one is the *postsynaptic cell*. A further principle of the neuron doctrine is the principle of connectional specificity: nerve cells do not form random networks, but they form specific connections with certain target cells. The intricacy of human behaviour is not encoded by the complexity of the individual neurons, but rather by the pathway that the signal travels within the brain. Considering that there are 3.6 million different ways to create a network of 10 different cells connected by a simple path, the number of possibilities for the brain to encode information is so big, that it nearly becomes meaningless.

Brain region	Neuron type	Description/function
Cerebellum	Purkinje cells	Purkinje cell axons are the sole output of the cerebellar cortex. There are roughly $15x10^6$ in a human. The soma and the dendrites of these cells form distinct layers.
	Granule cells	Smallest and most numerous cells in the cerebellum; cells have 4/5 short dendrites.
	Golgi cells	These have extensive radial dendritic trees and they provide feedforward and feedback inhibition to granule cells.
Hippocampus	Pyramidal cells	The layer of pyramidal cells are divided into 3 regions: CA1, CA2, CA3 based on size and appearance of the neurons. These neurons have elaborate dendritic trees extending perpendicularly
Neocortex	Spiny neurons	Their dendrites have small protrusions called spines, which provide an increase of space for synapse formation

Table 2: Examples of neuron subtypes. Data based on [5].

Although the neuron is the principal functional unit, it is not the only cell type in the brain. In fact, the second class of cells, the glia, numerically exceed the neurons substantially. The

CNS houses three types of glia: (1) oligodendrocytes, which (like Schwann cells) provide the myelin sheath that insulates an axon; (2) astrocytes, which play a key role in the formation of the blood-brain barrier, which are involved in the glutamate response and the activation of inter neuronal communication, and which maintain the appropriate levels of potassium ions in the extracellular space between neurons; (3) microglia, these are macrophages which are involved in response to injury, infection, and disease. While the glial cells provide quite a number of different support functions for the neurons, they are somewhat the 'stepchildren' within the field of neuroscience, since they have not been directly implied in signal transduction [16].

The alphabet of brain-language: the action potential

In the 1780s, while conducting experiments with dissected frog legs, Luigi Galvani discovered that a frog's muscle would twitch in response to an electrical stimulus. Galvani concluded that the brain would secrete 'animal electricity' in form of a fluid, and that flow thereof through the nerves would activate the muscles [17]. While Galvani didn't possess sufficiently sensitive technical means to measure the small currents within the body, Emil Heinrich Du Bois-Reymond succeeded six decades later to provide experimental proof for Galvani's hypothesis. Du Bois-Reymond invented a sensitive nerve galvanometer and an induction coil to produce an electrical stimulus. Thus he was able to demonstrate that a nerve impulse is associated with an electrical discharge, the action potential, and that the electrical pulse travels with a speed of 120m/sec through a neuron [18]. Du Bois-Reymond had hence identified the alphabet of the brain's language. In recognition for his inventions of numerous electrical instruments and his contribution to our understanding of the electrical nature of muscles, neurons, and the brain, Du Bois-Reymond, is recognised today as the founder of electrophysiology.

The electrical properties of the neuron are based on three principles:

1. existence of electrochemical energy in form of ion gradients over the cell membrane
2. release of part of this energy in form of ion flow through selectively permeable membrane channels
3. passive electrical properties of the cell membrane (electrical conductivity and capacity)

An unstimulated cell maintains a so-called *resting membrane potential*. It is defined as the voltage difference between the inner and outer side of the plasma membrane. This difference is due to the unequal distribution of electrically charged ions such as Na^+, K^+, Ca^{2+}, Cl^-, and amino acids and proteins on both sides of the membrane. At rest, there is an excess of positively charged ions on the outer side of the membrane. This disequilibrium is maintained by ionic pumps and ionic channels, which are dispersed along the cell. The resting membrane potential in a typical neuron is -65mV, since the outside voltage has been defined as zero. An incoming signal changes the permeability of the voltage gated ion channels, thus increasing the flow of positively charged ions into the cells, which in return leads to a reduction of the membrane potential, a phenomenon called *depolarization*. If this depolarization reaches a

specific threshold, influx of the positive ions increases rapidly, so that the negative charge inside the cell membrane is neutralized and even becomes positive – the action potential (see figure 8).

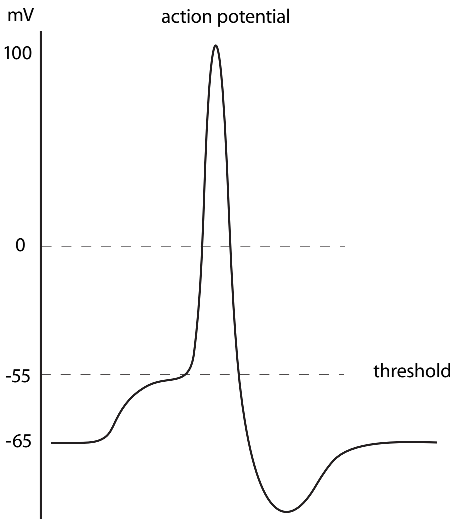


Figure 8: action potential
An incoming signal causes depolarization. If this depolarization reaches a threshold, membrane potential dramatically changes and becomes positive. In suite the potential drops under the resting potential., which will be reestablished.

The dominant ion in the generation of action potentials in axons and cell bodies is Na^+, while in somatic and dendritic regions voltage-gated Ca^{2+} currents play an important role. The amplitude of the action potential is 100mV, and it lasts for 1ms. This action potential is conducted along the axon towards the synaptic terminals, without change in the amplitude, since it is initiated in an all-or-nothing fashion. Conduction along the axon takes place at the nodes of Ranvier since only there ions can pass between the inside and outside of the membrane, so that the electrical signal jumps from node to node, which is known as *saltatory conduction*. Depolarized cells are called *exhibitory*, since the ability of the cell to create an action potential is enhanced. Cells where the membrane potential is increased, *hyperpolarized* cells, are *inhibitory*, since generation of an action potential is less likely [5, 19]. As astounding it is that the neuron suffices as the signalling unit for the brain, as remarkable it is that the signal itself, the action potential, hardly varies between neurons. The nerve cell employs a very simple, yet clever code to create different 'letters' of meaning: the number of action potentials and the time intervals between them.

From 'letters to meaning'

As mentioned earlier, the ingenuity of the brain to govern over the multifarious human behaviours roots in the formation of specific networks of neurons. A fundamental principle in biology for any given behaviour is to make use of a hierarchy of levels of organizations. That

this idea also holds true for the brain, was already proposed by John Hughlings Jackson in the 1870s. He suggested that there are specific cortical areas, which are exclusively involved in higher-order integrative functions. These *association areas* are in charge of interpreting sensory input, to compare it with previous experience, to devise a plan of action, and finally to coordinate the motor response [20]. However, imperative to the function and formation of any network or any level of organization are the 'intersection' points of two communicating cells – the synapses. An ordinary neuron forms approximately 1000 synaptic connections, while receiving even many more (in the cerebellum, Purkinje cells may receive up to 200,000 connections). There are three types of neuronal communication: (1) electrical via gap junctions (2) ephatical interactions due to physical proximity of two neurons, and (3) chemical. At electrical synapses, the two communicating cells are not physically separated, but they are connected via specialized channels called gap junctions. Ionic currents and small molecules can flow bi-directionally through these channels and cause a positive charge on the postsynaptic membrane, which in turn can generate an action potential. Electrical synapses are usually engaged in simple, yet rapid signalling, often used to connect large groups of neurons. In mammals, electrical synapses predominate in olfactory granule cells, the retina, and some nuclei of the brainstem. In brain regions where neurons are closely spaced, such as the cell bodies in the hippocampus and the cerebellum, or the dendritic bundles of the cerebral cortex, ephatic interaction takes place to synchronize activity between functionally related neurons. The physical proximity allows direct exchange of ionic currents. However, the most common neuronal communication takes place by chemical means between two cells, which are separated by a synaptic cleft. Chemical synapses can produce complex behaviours. Actions can be excitatory as well as inhibitory, and the signal is often amplified. Transmission at chemical synapses is unidirectional and is delayed in comparison to electric transmission (0.5-20ms). During this time a whole series of events takes place [5].

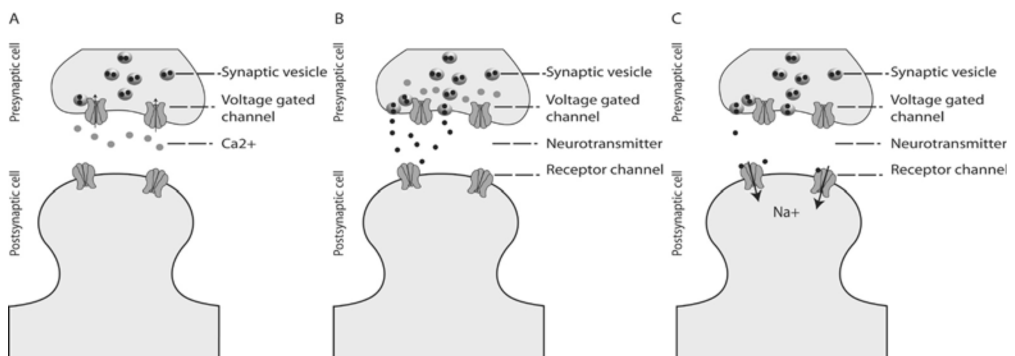
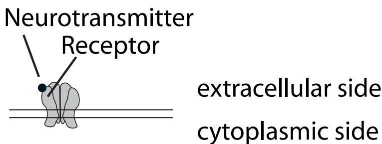


Figure 9: Events at chemical synapse.
A: Action potential at presynaptic terminal increases influx of Ca^{2+} into the cell.
B: Synaptic vesicle fuses with the membrane and releases neurotransmitter into synaptic cleft
C: Neurotransmitter binds to postsynaptic receptor, thereby changing membrane potential.

When an action potential reaches the presynaptic membrane, the depolarization thereof leads to an increase of Ca^{2+} ions in the presynaptic terminal, which in turn finally causes the fusion of a synaptic vesicle with the plasma membrane (figure 9A). Synaptic vesicles are discrete units filled with a chemical substance – a neurotransmitter. Upon fusion of the vesicle with the plasma membrane, the neurotransmitter is released into the synaptic cleft, where it diffuses towards specific receptors on the postsynaptic membrane (figure 9B). There are two groups of receptors for neurotransmitters. Firstly, there is the ionotropic receptor (figure 10A). Binding of the neurotransmitter to these ionotropic channels has an effect on the conductance, so that the potential of the postsynaptic membrane changes (figure 9C). In case the excitability of the postsynaptic cell is increased by depolarization, one speaks of an excitatory postsynaptic potential or EPSP. An IPSP or inhibitory postsynaptic potential is the definition of the opposite effect: a hyperpolarizing change decreases the excitability of the receiving cell. Ionotropically mediated chemical communication lies at the base of sensory perception, reflexes, and voluntary movements.

A ionotropic receptor



B metabotropic receptor

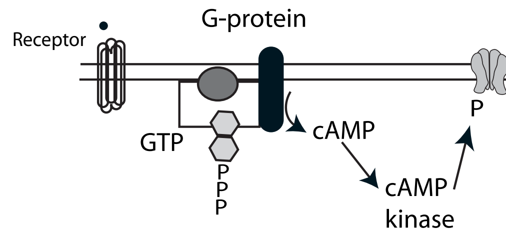


Figure 10: Types of postsynaptic receptors.
A - Neurotransmitter binds directly to the voltage gated ion channel
B - Binding of neurotransmitter to its receptor activates a second-messenger cascade. In this case: neurotransmitter binding activates a GTP-binding protein (G-protein), which activates adenylyl cyclase to convert ATP to cAMP. cAMP in turn activates cAMP-kinase, which leads to phosphorylation of the ion channel. Phosphorylation of the ion channel induces changes in the membrane potential.

The second type of receptors, the metabotropic receptor, can be subdivided into two groups: the G protein-coupled receptors and the receptor tyrosine kinases. The former initiates downstream effects by activating a GTP-binding protein (figure 10B), while the latter activates ion channels through a cascade of phosphorylation events. Both families of metabotropic receptors initiate a second-messenger pathway that consequently influences the ionotropic channels on the postsynaptic membrane. The metabolic effect of second-messengers on synaptic efficacy can be short- as well as long lasting. This seems to play an important role for learning and memory. Neurotransmitters can interact with both types of receptors. The variety of receptors allows a wide range of synaptic actions to be mediated by a small number of transmitters. Thus the combination of the numerical and temporal range of presynaptic action potentials, the number and specific architecture of axonal and dendritic branches, the

type of neuronal communication between cells, and the variety of the neurotransmitter and the postsynaptic receptor affords the brain to encode incoming and outgoing signals [5, 21].

Ex ovo omnia

As complex as the architecture of the brain is, it all starts from a single fertilized egg. Until there is a brain with all its association areas, functional regions, electrical circuits, different types of neurons, synapses, transmitters, receptors, axons, spines etc, this single cell has to undergo a rather unequalled metamorphosis in form of a series of proliferation, migration and specialization procedures. One of the first major steps during early embryogenesis is the formation of the three main cell layers, a period named *gastrulation*. During this phase, cells are assigned to a specific fate based on their location. The *endoderm*, the innermost layer, will develop into the gut, lungs, and liver. The *mesoderm*, the middle layer, provides the cells for the connective tissue, muscles, and the vascular system. In the outermost layer, the *ectoderm*, the nervous system is born as well as the skin. The first structure, which is designated to a CNS fate, is the *neural plate*, an area where ectodermal cells have been transformed to thickened neuroepithelial cells. At this stage, all cells lose there totipotent character. With further progress of development, cells become exceedingly restricted and specialized in their functional potential. Neural plate formation is the first major phase in the process of *neurulation*. Subsequently, the neural plate will lengthen and narrow as a result of size increase and reshuffling of the neuroepithelial cells until the lateral folding culminates in fusion of the most apical cells. The thus arising *neural tube* will separate from the overlying ectodermal sheet. At this point cells have been committed to contribute either to the spinal cord or the brain. The neural tube is now subjected to a progression of subdivisions, which bring about the foremost functional organization of the mature brain [22].

The gross developmental stages that a nerve cell has to master can be summed up as: (1) birth of the neuron (2) extension of axon and dendrites, (3) fine-tuning of synaptic connections. As described above, the first cells designated to establish the CNS, constitute the neuroepithelium. These CNS precursor cells experience a set of proliferation and migration steps, until they reach their final destination in respect to location and function. The migration pathway of neurons and their precursors is often guided by radial glia. Once the soma of a precursor cell arrives at its allocated region, the cell undergoes its final mitotic division and the newly born neuron begins to protrude an axon and dendrites, and the *neurite* (= axon or dendrite) prepares to find its proper target cell. Its 'pathfinder' is a *growth cone*: a flat hand-like enlargement at the tip of the developing neurite, which initially seems to crawl confused around its environment before it shifts into a higher gear and speeds off towards its target. On its scouting mission, the growth cone picks up a range of road signs prior to reaching the sought-for synaptic partner [23]. The mechanisms that make an axon chose one potential target cell over another cell, are still a relatively unexplored field. However, a number of models have been proposed, such as the time and site of the birth of the neuron, the local environment at the target area in form of chemoattraction, neural activity within the target area, formation of random connections

which will be fine-tuned in response to neural activity, or competition between innervating axons for limited space or limited so-called neurotrophic factor of the postsynaptic target cell [24, 25].
In order to assemble a synapse, three strategic actions have to be taken. Firstly, a selective connection between the future synaptic co-workers has to be established. Secondly, the growth cone has to mature into a presynaptic terminal. Thirdly, the target cell has to advance the postsynaptic machinery. With accomplishment of these developmental phases, the nervous system has reached a first milestone: institution of the first electrical circuits and commence of the neuronal communication [25, 26]. However, there are still some more steps to climb on the way to achieving the earlier described sophistication. Surprisingly, the expanding nervous system produces many more cells than needed for an efficient performance. Nearly 50% of the early embryonic neuron population will be eliminated by an active process, *apoptosis*. Reason for the overproduction is probably to ensure that there are sufficient cell numbers in order to create all the required networks, whereas the programmed cell death might serve to guarantee that information-processing will occur in the most economical fashion. A similar argumentation could be valid for the observation that the numbers of neuromuscular junctions (NMJ) present at birth are tremendously lessened postnatal, so that ultimately only one synapse persists per muscle fibre [27-29]. Synapse elimination during early postnatal life, which is not a result from neuronal apoptosis, but an independent mechanism, also takes place in the developing CNS [30, 31]. It is a dynamic, rapid, and competitive process involving the presynaptic adversaries and the prospective postsynaptic partner. Axons retract their branches asynchronously without a spatial bias, suggesting that competition takes place at every single synaptic site. Retraction of one presynaptic terminal leads to fortification of another terminal. Time-lapse imaging experiments with neo-natal mice expressing different spectral variants of fluorescent proteins in motor neurons have provided a unique opportunity to witness this process *in vivo* [32-34]. This remodelling is an activity-dependent process [35-38]. As axons branch and remodel, as synapses form and disassemble, the brain acquires the anatomical refinement of its hierarchical synaptic circuits necessary to perform all its complex tasks.

Mechanisms of learning and remembering

Cogito ergo (ego) sum. The definition of this *ego*, our individuality, and our perception of the world very much depend on three cognitive functions of the brain: its ability to learn, to store information, and to retrieve it. Most of our skills are acquired by learning: reading, the name of our friends, breathing, the periodic table of elements etc. It is therefore reasonable to perceive learning and remembering as the most important cognitive functions. As becomes apparent from this short list of skills, there are very distinctly different kinds of memory. Indeed, memory is not restricted to a single brain area, but to different regions, which are responsible for specific types of memories (see figure 11). The first category, *implicit memory*, concerns information that is unconsciously retrieved, such as reflexive motor and perceptual

skills. Information that is recalled consciously, such as the birthday of family, your first kiss, your favourite book, is defined as *explicit memory*.

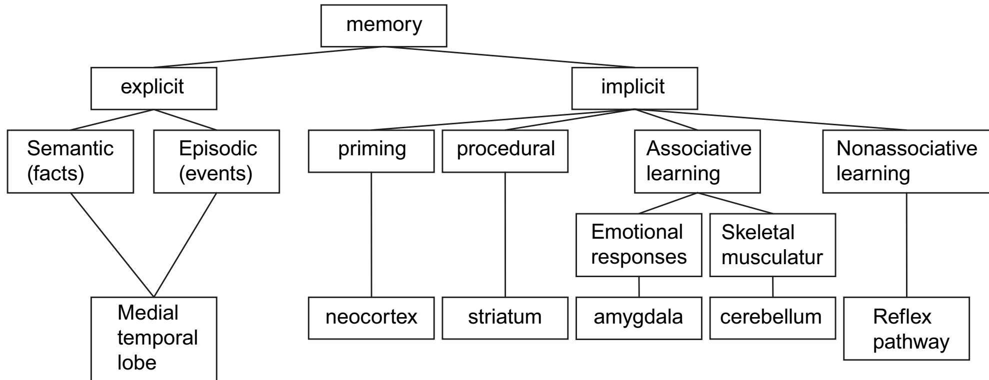


Figure 11: Different types of memory are processed in different parts of the brain. Based on. [39].

The molecular basis of learning and memory has been related to the brain's ability to change its anatomical shape, a process termed *plasticity*, based on the Greek word *plaistikos* – to form. Brain plasticity is the lifelong capacity of the brain to change its anatomical shape by reorganization of the neuronal pathways in response to environmental stimulation. Next to learning and memory it also has implications on recovery after injury. Reorganization of neuronal networks can occur in two ways: (a) the number of synapses and the number of neuritic branches is changed; (b) the strength of the synapse is shifted. Convincing support of the first mechanism has been provided by studies with children playing string instruments. The earlier the children started to play, the larger the cortical representation of the string playing hand was [40]. This indicates that continuous periods of activity can enhance functional ability by rearrangement of neuronal circuits. However, the primary mechanism of plasticity depends on the architecture of the synapse itself. Synaptic plasticity takes place through changes of the efficacy of synaptic transmission. A model for such an event was first postulated by Donald Hebb [41].

The synapses are weak during development. In order to cause a depolarization above the threshold level on the postsynaptic membrane, multiple firing inputs are necessary on the presynaptic site. The simultaneous firing of these cells will increase the strength of all the synapses. Future action potentials on the postsynaptic site will then be initiated by fewer simultaneous active inputs (see figure 12). Such a synaptic enhancement is known as *long-term potentiation* (LTP). Shift in the strength of a synapse can also take place in form of strength reduction, *i.e. long-term depression* (LTD).

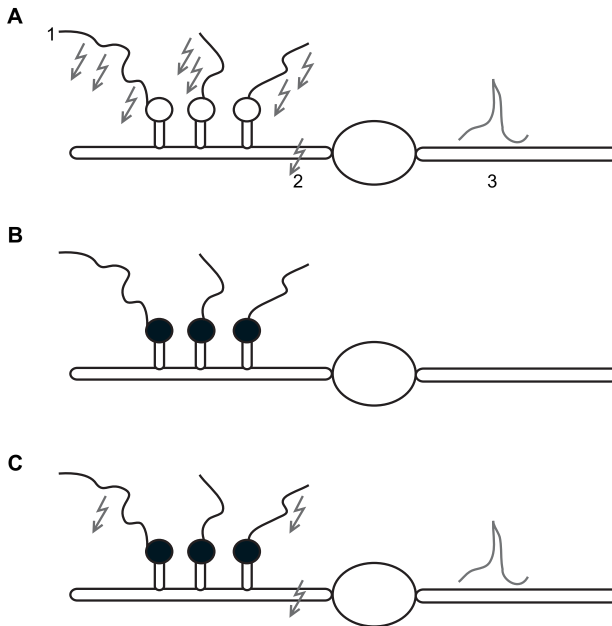


Figure 12: Hebb's model:
The strength of the connection between two synaptic partner cells increases in response to simultaneous firing of action potentials from both cells. A - Initially to cause a depolarization above the threshold level on the postsynaptic membrane of this weak synapse, multiple firing inputs are necessary on the presynaptic site. (1) Firing input cells. (2) Depolarization on postsynaptic membrane. (3) Action potential of postsynaptic cell. B - In response, the strength of all the synapses increases. C - Future action potentials on the postsynaptic site will then be initiated by fewer simultaneous active inputs. Figure adapted from Rosenzweig [42].

Both forms of synaptic plasticity are activity-dependent: neuronal activity strengthens immature synaptic connections, whereas inactive synapses weaken and eventually become eliminated ('use-it-or-lose-it'). LTD and LTP share a number of molecular features with opposite effects: both are induced by an influx of Ca2+ ions into the postsynaptic cell, NMDA receptors are involved, however the lasting effect is manifested by a change in the number of AMPA receptors on the postsynaptic membrane (in LTD number of AMPA receptors is reduced, while it is increase in LTP). LTP is not an increase in the strength of *every* synapse of a specific cell, but it causes selective synaptic weight changes. One branch of an axon may undergo LTP, while another one may undergo LTD. In this model, the process of changing the synaptic strength is considered learning, and the precise pattern of LTP and LTD distribution for all the synapses of a certain cell is considered the first step to memory encoding [43-47]. Today, the Hebbian theory is widely accepted as a model for associative learning through synaptic plasticity in form of LTP [48-50].

There are different mechanisms leading to LTP, which can change with age, such as increase of Ca2+ in pre-and postsynaptic compartments, pulse frequency, scaffolding proteins, phosphorylation of receptors, and neurogenesis [42, 51]. Not surprisingly, brain and synaptic plasticity is much more prone in the immature brain. As mentioned, the number of neurons and the number of synaptic connections are at a maximum in early postnatal life. There is more material for the creation/remodelling of networks as a response to environmental inputs, activity, and experience. With loss of neurons through apoptosis and with dendritic pruning (retraction of branches and spines in absence of neural activity), the ability decreases to reshape the anatomical architecture of the brain. However, recent research has shown that

even the adult and aging brain is plastic. There is evidence for the occurrence of neurogenesis. Proliferation has been noticed most markedly in the subventricular zone, from where newly born neurons migrate to the olfactory bulb and the subgranular zone of the hippocampus. Also survival of neurons born during adult life is activity-dependent. The proliferation and survival of these cells can be upregulated by a variety of environmental and behavioural factors, such as enriched environment, hippocampal-dependent learning, exercise, estrogens, and antidepressant treatment. Maturation of newly generated cells into functional and integrated neurons in the adult mammalian hippocampus circuitry has also been verified recently [52]. It follows that the persistence of neuronal stem cells into adult life increases the capacity of the mature brain for structural and functional plasticity to a greater degree than previously appreciated, which opens up a whole new avenue for functional improvement and repair of the adult brain.

As sketched in this chapter, the physical nature of the organ of mind is as manifold as the universe. However, if we ever want to grasp the concept of the mind, as well as the interaction between the two, we have to comprehend its physical nature. That an intimate relationship exists between these two entities, is plainly revealed in individuals with diminished mental capabilities caused by genetic mutations. The identification of these genetic aberrations will enable us to explore the physical features of human cognition.

Chapter II

A gene model to explore the brain: the fragile X syndrome

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Sum ergo cogito?

As described in the previous chapter, the brain is a highly complex organ. The genes provide the most basic level of organization within a cell. Although genetics surely will not be sufficient on its own accord to explain all the intricacies of the brain, it will provide insights into the most fundamental facets of cognitive functions by elucidating the molecular and pathophysiological processes. Currently, the most common approach to study a particular trait is to investigate a single gene. Of course no single gene product acts on its own, but rather in concert with other proteins. The deletion or the malfunction of a gene expressed in a neuron, if not lethal, will therefore result in an adjustment of the cell's faculties. The knowledge gained through deletion studies may consequently be more an indication of these adjustment capabilities, than an indication of the actual and precise function of the gene in question. Nevertheless, identification and understanding of disease genes will have profound consequences for a particular trait, such as potential therapeutic avenues, establishment of presymptomatic and prenatal diagnosis, provision of prognostic information, and it will advance our comprehension, conception, and perception of the brain, human behaviour, and our philosophical outlook on life.

Figure 13: Molecular pathways at pre- and postsynaptic sites relevant for neuronal processes. Disruption may lead to impaired cognition. Left side – pathways significant during development. Right side – pathways active throughout life. Adapted from Chechlacz and Gleeson, 2003 [53].

An impairment of cognitive functions, generally termed mental retardation (MR), is broadly defined as a "disability characterized by significant limitations both in intellectual functioning and in adaptive behaviour as expressed in conceptual, social, and practical adaptive skills" (American Association on Mental Retardation –
http://www.aamr.org/Policies/faq_mental_retardation.shtml, 2002). Causes of MR are both acquired (alcohol, infections, malnutrition, injury etc) and congenital. Since nearly 50% of the 25,000 human genes are expressed in the brain, MR is not a single disorder, but encompasses a wide variety of diverse phenotypes and severity. Impaired cognition is due to alterations at different sites within the molecular pathways of neuronal processing (see figure 13).

The most common inherited form of MR is the fragile X syndrome (FRAXA). It is a single gene disorder linked to a site at Xq27.3. Approximately 1 in 4000 men and 1 in 6000 women are affected [54]. The first description of a family with typical clinical features was given by Purdon Martin and Julia Bell in 1943 [55]. The affected site appears as a constriction or a gap – the fragile site - on the long arm of the X chromosome on chromosome spreads of patients' lymphoblasts or fibroblasts [56-58]. This gap gave rise to the current name - fragile X syndrome.

The fragile X syndrome

The phenotype

In the case of FRAXA, the MR can vary from mild to severe. In general, males are affected more severely than females, since females are heterozygous for the mutated gene (60% of carrier females develop MR). Depending on the ratio of mutated versus normal gene being active after random X-inactivation, cognitive impairment in females varies [59-62]. The cognitive disability in males seems to become more critical with age [63-65]. It is, however, not clear whether this regression is due to a progressive loss of mental function or whether the full extent of a delayed developmental process only becomes apparent during adulthood [66, 67]. Young boys with FRAXA often demonstrate speech and language delays, irritability, sensory regulation problems, spatial and mathematical deficits, and behavioural problems. The behavioural changes include hyperactivity, frequent tantrums, attention problems, aggression, self-injury, and mood swings [68-72]. FRAXA has often also been associated with autistiform behaviour, epilepsy, attention-deficit hyperactivity disorder, depression, anxiety, shyness, and social avoidance [73-76]. Recent advances in human brain imaging methods have significantly facilitated the unravelling of the relationship between the behaviours and the brain structure in fragile X patients (see table 3 - reviewed by Hessl *et al* [77]).

Brain structure	Size in FRAXA	Function related to the brain structure	Function affected in FRAXA
Cerebellar vermis	↓	Execution and regulation of motor functions, auditory processing, some aspects of language	Hyperactivity, repetitive movements, tactile defensiveness, attention deficits, language dysfunction
Fourth ventricle	↑		In patients, correlation between ventricle size and IQ decrease has been established
Lateral ventricle	↑		In patients, correlation between ventricle size and IQ decrease has been established
Superior temporal gyrus	With age ↓	Involved in auditory processing and speech	
Hippocampus	With age ↑	Learning, memory, processing visuospatial information	Decreased functioning in FRAXA patients
Amygdala	↑	Involved in emotional responses	Aggression, tantrums. Abnormal conditioned fear response in knock-out mice
Caudate nucleus	↑	Regulation, organization, filtering of information involved in shifting attention, motor planning, executive functions	Attention deficit
White matter tracts in frontostriatal pathways and parietal sensory-motor tracts	↓	Mediate sensory processing, executive function, regulation of affect, motor programming	

Table 3: Affected brain structures in FRAXA

Not only size differences of various brain regions were correlated to impaired functioning in fragile X patients, but functional Magnetic Resonance Imaging (fMRI) also revealed that female patients (studies not done on males yet) employ significantly different patterns of activation during different cognitive tasks [78]. These type of studies also demonstrated that the activation level in the frontal gyrus was significantly lower in female patients during a visuo-spatial working memory task [79], indicating that the phenotypes observed in this disorder result from disruption of different organisational levels in the brain.

Next to size and processing, the cellular anatomy is affected in FRAXA. Post-mortem microscopic examinations of patients have shown that the dendritic spines are abnormal

[80-82]. In addition, fragile X patients have a higher density of spines, and an increased number of long and thin immature spines, whereas the number of short, mature spines is decreased in the temporal and visual cortex. Spine abnormalities are a general phenomenon in disorders related to MR. Dendritic spines are the smallest functional and structural units within a neuron. The majority of postsynaptic sites have these small thorn-like protuberances. Especially the cerebellar cortex, basal ganglia, and cerebral cortex have neurons with spines. About 79% of all excitatory synapses within the cerebral cortex are made onto spines. A number of functions have been ascribed to the spines, such as site of synaptic connection, developmental synaptic target, unit for synaptic plasticity, information processing unit, and biochemical compartment (for an extensive reading see Shepherd 'The synaptic organization of the brain' [5]). Since no indication of cell migratory or neuronal proliferation failure has been reported for fragile X, it has been suggested that the root of the intellectual disability in this syndrome may result from an impairment of brain plasticity, in particular a flawed synaptic pruning and maturation process [82]. Since both synaptic pruning and maturation are events taking place pre- and perinatal, FRAXA is considered a neurodevelopmental and paediatric disorder. Non-neurological aspects of FRAXA include an elongated face, protruding ears, macro-orchidism in post-pubertal boys, and a decrease in body height and limb lengths [83, 84]. There seems to be a general impairment in the control of the expansion, maturation, and differentiation of neuronal and non-neuronal cell populations.

The gene

Nearly half a century has passed between the first description of the syndrome and the identification of the responsible gene: the Fragile X Mental Retardation gene 1 (*FMR1*) [85]. The 38 kb gene contains 17 exons [86]. Some exons are alternatively spliced, thus giving rise to different protein isoforms [87-89]. The 5' untranslated region of the gene contains a CGG repeat, which is polymorphic within the general population with a varying size between 5 and 50 repeat units [90]. Fragile X patients have in nearly all cases repeat sizes significantly exceeding 200 units. These so-called full mutation alleles can extent up to several thousand CGG repetitions. Expansion of the repeat over 200 units is usually correlated with methylation of the repeat and the upstream CpG islands, which suppresses transcription and translation of the gene [91-93]. The gene product of the *FMR1* gene is the fragile X mental retardation protein (FMRP). Its lack is hypothesised to instigate the phenotypic disposition involved in FRAXA [91, 94-96]. Full mutation alleles surface upon maternal transmission of CGG repeats within a range of 50 to 200 units, the so-called premutation range, since the repeat is instable within this range. Increasing size is concurrent with augmented risk of acquiring a full mutation and the resulting fragile X phenotype [97-100]. Premutation alleles are unmethylated, to the extent that carriers do not show any typical characteristics related to the fragile X phenotype [97, 98]. However, both female and male carriers of a premutation allele are at higher risk for a number of distinctly separate phenotypes (see below).
Instability of the repeat length is not restricted to germ cells. Indeed, 40 - 50% of male patients

have been shown to possess 'somatic length mosaicism': Southern blot and PCR analysis of leukocytes revealed the presence of different repeat lengths, both in the premutation and in the full mutation range [101-103]. In a minority of cases, mosaic expression pattern may based on the methylation status of the repeat: some males with a full mutation allele escape methylation in a significant number of their cells [104-111]. The term 'high-functioning-males' (HFM) originates in their ability to produce nearly normal levels of FMRP in concert with marginally impaired cognitive functioning.
Based on the research mentioned in table 3, it can be hypothesized that a correlation exists between the degree of change in brain architecture and the degree of cognitive function. However, a comparison of mosaic males with males, which have ubiquitously a full mutation, has indicated that there seems to be a threshold, under which there is no correlation between FMRP levels and cognitive function. Both groups performed similar in cognitive tasks, even though the mosaic males had 28% of FMRP-expressing blood cells [63, 94, 112-115]. As mentioned earlier, a mosaic expression pattern is also relevant in females who are heterozygous for the full mutation. Due to the random X-inactivation, on average, 50% of their cells are expected to express FMRP. Despite this relatively high expression level of the protein, 50-75% of these females show some degree of cognitive impairment [59, 112]. Both findings suggest that normal mental functioning requires a high number of cells expressing the FMRP protein. This notion is supported by findings that there is a direct correlation between the number of cells that are expressing FMRP and the degree of cognitive function of female full mutation carriers [116]. This finding may have imperative implications for developing a therapy.
DNA methylation is a common trigger of gene silencing [117]. In general, it coincides with a number of histone modifications [118, 119]. Usually, actively transcribing genes have unmethylated promoter sequences as well as acetylated lysine residues in the N-terminus of histones H3 and H4 [119, 120]. Epigenetic gene regulation requires a concoction of many interacting proteins. In the case of FRAXA, methylation of the promoter occurs, when the repeat number exceeds 200. How the expansion of the repeat takes place is still unknown. It has been hypothesised that the CGG repeat forms hairpins and that DNA polymerase slippage occurs during replication [121-126]. Repeat expansion *per se* is not sufficient to silence FMRP expression, HFM individuals are functioning normally, since they escape DNA methylation. As a rule, FRAXA is caused by the aforementioned hypermethylation of the promoter and repeat sequence. There are only a few case reports on patients, where lack of FMRP is owed to deletion of the promoter region, the flanking sequences, or the partial or complete *FMR1* gene [127-133]. Lack of FMRP has also been implied in two patients with mutations in the coding region of the gene [134]. To date only one missense mutation is known that leads to a modification in the protein product itself: De Boulle *et al* describe a severely affected male, who has a point mutation in one of the functional domains. This point mutation results in the substitution of an isoleucine with an asparagine amino acid at position 304 [135].

The structure and expression pattern of the protein

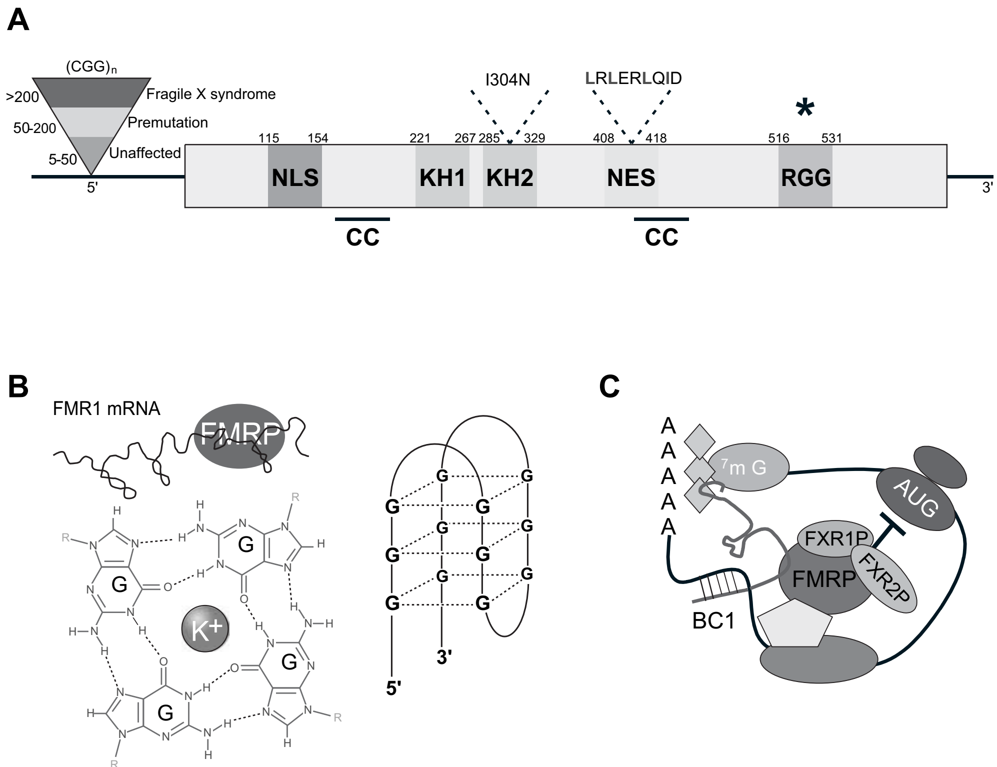

Figure 14: Schematic representation of the *FMR1* gene, FMRP, and two models of target recognition.
A – The *FMR1* gene and protein. The number of CGG repeat units in the 5' UTR is responsible for the different phenotypes: units between 5 and 50 are common in normal individuals. Premutation carriers have repeat units between 50 and 200. Repeat numbers exceeding 200 lead to hypermethylation and subsequent silencing of the gene, the primary cause for fragile X syndrome. Abbreviations: NLS – nuclear localisation signal; KH – K-protein homology domain; NES – nuclear export signal (the essential amino acids are indicated); RGG – Arg-Gly-Gly triplet; CC – coiled-coiled domain. The position of the I304N missense mutation found in a single, severely affected patient is within the second KH domain. * - G-quartet of target mRNAs binds to the RGG box.
B – The G-quartet structure responsible for mRNA recognition by the RGG box.
C – Model of interaction between FMRP and the neuronal BC1 RNA. The FMRP-containing RNP complex binds to BC1 RNA. BC1 RNA in return base-pairs with target mRNA and thereby initiates translation of the bound cargo.

The gene product of the fragile X gene, FMRP, has been classified as an RNA-binding protein based on the presence of two KH-domains and an RGG box. Both sequences have been shown to possess RNA-binding capacities (see figure 14) [136-138]. Furthermore, FMRP contains a Nuclear Localisation Signal (NLS) and a Nuclear Export Signal (NES), suggesting a shuttling function of FMRP between the nucleus and the cytoplasm. Four major isoforms have been detected, which have a molecular mass between 70 and 80kD [96]. However, no tissue-specific expression of these isoforms has been demonstrated [96]. The expression of FMRP is ubiquitous in the human organism, with high levels in the brain and testis. High

levels of *FMR1* mRNA have been found in the lung, kidney, and placenta, too, whereas there is little or no mRNA in the liver, pancreas, and skeletal muscle [139]. The cell-types bearing high levels of FMRP expression include CNS neurons, spermatogonia, and actively dividing cells in epidermal layers [97]. Subcellularly, FMRP mainly localises within the cytoplasm and is associated with (poly)ribosomes [96, 97]. However, also some nuclear localisation has been demonstrated [140-143]. Within the brain, most FMRP is found in the granular layers of the hippocampus, the cortex, motorneurons, and the Purkinje cells of the cerebellum [97, 139, 144]. The majority of neuronal FMRP is in the soma, along with dendritic, but no axonal expression [96, 97, 145, 146]. However, FMRP has been shown to be present also in oligodendrocytes [147].

The FXR-protein family

FMRP is a member of a small protein family, the fragile X related (FXR) family. The Fragile X Related Proteins 1 and 2 (FXR1P and FXR2P), interact with FMRP [148] in the brain, where the three proteins are co-expressed. Differential expression of the three proteins has been demonstrated in skeletal muscle tissue and testis [146]. The classification of these proteins is based on ontological considerations, since all three gene sequences are highly homologous (86% for FXR1 and 70% for FXR2) and share the same functional motifs. A common ancestor of the three genes seems likely, especially considering that in *drosophila*, there is only one gene – *dfxr* – with features of all three human genes [149]. Efforts to identify the similarities/dissimilarities in the function of the three proteins are on their way. One argument to envision specific rather than overlapping functions for all three proteins is, that some isoforms of FXR1P and FXR2P have an additional functional motif – a nucleolar targeting signal [150], suggesting that different physiological functions are divided among the FXR proteins [151]. Further support is derived from the identification of a novel RNA-binding nuclear protein [152] that interacts with FMRP, but not with the other two proteins. Knockout (ko) mouse models for *Fxr1* and *Fxr2* have been generated. While the *Fxr1* ko mouse shows a different phenotype (striated muscle), the *Fxr2* ko mouse displays also impaired learning capacities [153, 154]. Nevertheless partial functional compensation by FXR1/2P in absence of FMRP in the brain is imaginable. So far, no mutations are known for *FXR1/2* and no reports exist on patients with lack of FXR1/2P.

Involvement of the *FMR1* gene in phenotypes not related to fragile X syndrome

As explained earlier, premutation alleles of the *FMR1* gene are not subjected to methylation and can therefore produce FMRP. Thus premutation carriers were generally expected to function normally. However, nearly 20% of female premutation carriers enter menopause before the age of 40. This disorder, termed premature ovarian failure (POF), is caused

by loss of ovarian follicles [155]. A minority of female premutation carriers also exhibits mild learning difficulties and emotional problems such as anxiety and mood swings [156]. Currently, no models have been proposed on the involvement of the premutation allele in the molecular pathways leading to POF.
First indications of the effect of a premutation on the cellular biochemistry come from recent studies of *FMR1* mRNA expression levels in male premutation cells. Though the level of FMRP was close to normal, the mRNA levels were significantly elevated in the permutation cells. The amplitude of the increase was positively correlated with the size of the CGG repeat [110, 157, 158]. It has been postulated that conformational changes in the *FMR1* mRNA caused by the expansion of the CGG repeat would interfere with the translation machinery and would lead to a reduced FMRP production [110, 157, 158]. The elevated transcript levels could either be explained by a feedback mechanism to compensate for the translational inefficiency, or by up-regulation of the transcription due to a CGG repeat-induced enhancement of promoter accessibility. Hitherto no direct proof has been provided for either proposition.
Surprisingly, carriers of a premutation may develop a newly identified neurodegenerative disorder with advancing age: fragile X-associated tremor/ataxia syndrome (FXTAS). Main phenotypes in this disorder are progressive intention tremor and ataxia [159]. Other problems may be acquired with progression of FXTAS, such as memory and executive function impairment, essential tremor, autonomic dysfunction, parkinsonism, anxiety, and peripheral neuropathy [160-166]. Not only has volume loss in different brain regions been established [167], but also the presence of eosinophilic intranuclear inclusions in both neurons and astroglia throughout the brain has been demonstrated [168]. These inclusions have been shown to be positive for ubiquitin, proposing a link with the proteasome degredation pathway. Little is known on the molecular effects of raised mRNA levels in cells, but a direct link has been proposed between the mRNA levels and the occurrence of inclusion bodies. A mouse model with 98 units of a human CGG repeat, where an increase in the size and the number of ubiquitin-positive inclusions over time has been demonstrated [169], might provide an important tool to shed light on the molecular pathways involved in the pathogenesis of FXTAS.

A mouse model for fragile X syndrome

Various animal species (fly, rat, fish, frog *etc.*) have been employed in the quest to understand the normal function of FMRP and the fragile X phenotype. However, the most successful use of an animal model is the *Fmr1* knockout mouse, which was generated a decade ago [170]. Recollection of the FMRP-dependent physical, behavioural, and neuroanatomical features of the mouse is crucial to interpretation of experimental data and an extrapolation to the human situation. A discussion on the general suitability of the mouse as a model system for higher human functions can be found elsewhere [171].
The murine *Fmr1* gene shows 97% sequence identity with the human gene [88], which allows structural-related studies in the mouse. One of the most striking differences within the

sequence is the significantly lower number of CGG repeat units in the murine *Fmr1* gene. A naturally occurring model caused by the same mutation mechanism as in fragile X patients has not been noticed so far. Efforts to produce a CGG repeat length-induced shut down of murine FMRP are ongoing [169]. Disruption of the *Fmr1* gene led to the absence of Fmrp (see figure 15) [170].

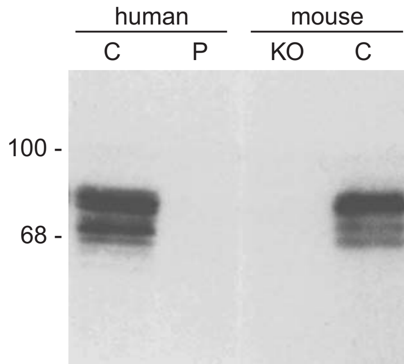

Figure 15: Western blot after immunoprecipitation (IP) of brain extracts from human individuals and mice with antibodies against FMRP. IP reveals presence of different isoforms of FMRP in normal individuals and wild-type mice (C), while no FMRP is present in homogenates from fragile X patients (P) and *Fmr1* knockout mice (KO).

In general, cognitive and behavioural characteristics are similar between mice and humans (see table 4) [170, 172-178].

Human fragile X patients	Fmr1 knockout mice
Mental retardation	Deficits in spatial learning
Aberrant spines	Aberrant spines
Aberrant behaviour: hyperactivity	Aberrant behaviour: hyperactivity?
Seizures	Audiogenic seizures
Macro-orchidism	Macro-orchidism
Overall decrease in functioning of neuronal network	Decreased reactivity to external stimuli
Abnormalities on MRI	Not observed
In early embryonic life, maybe FMRP expression, since methylation occurs at a later stage	No Fmrp from conception

Table 4: Comparison of symptoms between patients and knockout mice

However, the structural differences in the brain of fragile X patients, which are summarised in table 3, have not been confirmed in the mouse [179]. It is likely that the macroscopic anatomy differs between human and mouse, although similar microscopic neuropathology has been described, in particular the abnormal dendritic spine structure [81, 180, 181]

(Koekkoek pers. communication). In general, the lack of Fmrp seems to have a lesser impact on the mouse organism. This is supported by only slight increase in testes weight in knockout mice in comparison to male patients who may develop severe post-pubertal macro-orchidism [70, 182]. In some (if not most) aspects, such as immunocytochemistry and biochemistry, embryonic development, and experiments that require time curves, studies have to rely mainly/entirely on the use of model organisms. Thus most of the knowledge on temporal and spatial expression patterns of FMRP in different tissues was gained by examination of mice [97, 144, 183]: FMRP is ubiquitously expressed, with the highest levels in the central nervous system (CNS) and testes. Model organisms also supply sources of embryonic tissues for primary cell cultures. Since absence of FMRP in neurons is causative for the MR involved in the syndrome, primary neurons permit the investigation of neuronal differentiation, neuronal morphology, and many other cellular processes. Especially cultures of knockout cells can be transfected with green fluorescent protein (GFP)-fusion proteins to follow the cellular dynamics of a particular protein.
In summary, the *Fmr1* knockout mouse is a tremendously beneficial tool for unravelling FMRP function (see table 4 - for a review see [171]).

Based on the information given here (FRAXA is a single gene disorder, no neurodegeneration has been identified, different model organisms are available), the fragile X syndrome is a highly suitable model to study the physical anatomy of the brain (in particular at the synapses), and it will increase our knowledge of the mind by revealing mechanisms involved in learning and memory.

Chapter III

The role of FMRP in synaptic plasticity

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Neuronal mRNA targets of FMRP

The FMR1 protein is considered an RNA binding protein, based on its functional domains (two KH domains, one RGG box). Indeed, FMRP has been shown to bind to 4% of total human fetal brain mRNAs *in vitro* [136], suggesting a broad but selective RNA-binding role of FMRP. The presence of two further motifs, a nuclear localisation signal (NLS) and a nuclear export signal (NES), suggests that FMRP may has a shuttling function [150, 151]. FMRP is therefore deemed to enter the nucleus, to bind to a subset of mRNAs, to transport these into the cytosol and partially into the dendrites, and to act as an inhibitor on the translation of its mRNA cargo [184] (see figure 16).

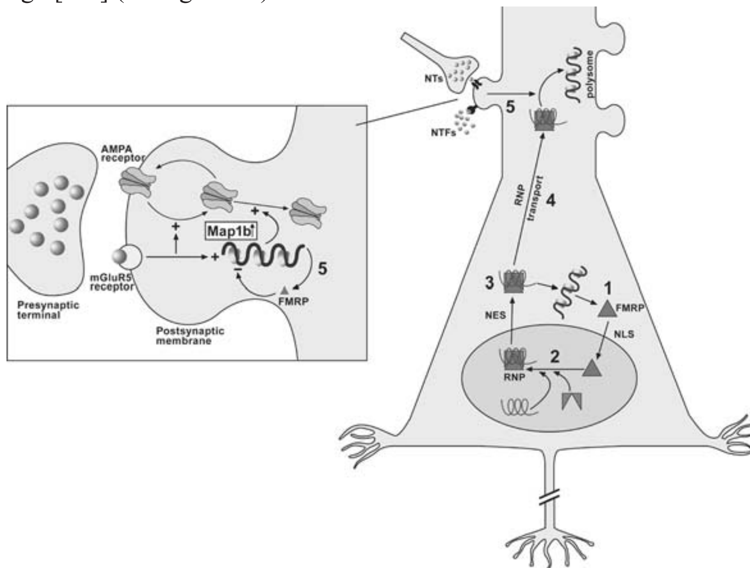

Figure 16: Schematic model of stepwise transport and translation of FMRP's mRNA cargos. (1) FMRP is synthesized in the cytoplasm and enters the nucleus *via* its NLS domain. (2) In the nucleus FMRP finds its target mRNAs and a number of proteins, thus forming an RNP particle. (3) The RNP particle is transported back into the cytosol by the exportin1 complex. The RNP complex suppresses translation of the bound mRNAs until an appropriate signal is given. Release of the mRNAs allows their translation and FMRP may re-enter the nucleus. (4) A small proportion of the RNP complexes associates with molecular motors such as kinesin and is transported to distal dendritic sites. (5) Upon synaptic activation FMRP localises at the post synaptic density of spines and releases its mRNAs, allowing rapid translation of proteins involved in the cyclic internalisation of AMPA receptors and other neuronal processes. One of the identified mRNAs involved, is MAP1B.

This model underscores the importance of discerning the mRNAs bound by FMRP to enable a meticulous insight into FMRP function and the downstream effects on the neuronal machinery. A number of single mRNAs bound to FMRP have been identified (see table 5).

mRNA bound by FMRP	Function of the mRNA/ corresponding protein	Possible involvement in fragile X related phenotype	references
Xenopus elongation factor 1 A, xEF-1A	In yeast, EF-1A overproduction is correlated with increased nonsense suppression and may be involved in cytoskeletal actin [185, 186]	Actin-dependent changes in dendritic spine morphology and stability [181, 187]	[188]
Drosophila futsch/ MAP1B	Futsch is a homologue of mammalian Map1b; MAP1B plays a role in the cytoskeleton and neurite outgrowth [189, 190]	The Drosophila model of fragile X, the dfxr null mutant, shows elevated Futsch levels and correlated structural and neurophysiological impairments [191]	[191, 192]
PSD95	A scaffolding protein involved in synaptic plasticity	Impaired synaptic architecture	[193]
Small GTPase Rac1	Promotes dendritic branching	Patients show increased denritic branching	[194]
Lgl	Cytoskeletal protein involved in cellular polarity	Impaired synaptic spines	[195]

Table 5:A selection of mRNAs bound by FMRP

Although identification of target mRNAs one-by-one will add valuable knowledge to our understanding of FMRP-related functions, it would be a very elaborate and time consuming attempt to comprehend the complete diversity of FMRP's roles in neuronal processes; especially taking into account that the numbers of mRNAs present in dendrites significantly exceeds previous expectations (for a review see [196]). Therefore, different methodologies have been developed and applied by various laboratories in order to identify 'global' FMRP-related mechanisms of mRNA-recognition. Sung *et al* used biotinylated-FMRP affinity resin to isolate RNAs from the parietal cortex of a normal female as well as the corresponding embryonic tissue in wild-type mice [197]. They identified 9 target mRNAs, including *FMR1*-mRNA, a neuronal NT2 EST, and Tip60a, which is a tat interactive protein. Microarray technology allowed Brown and colleagues the identification of 432 mouse mRNAs by co-immunoprecipitation with FMRP ribonucleoprotein complexes (RNP) and 251 human mRNAs that were differentially expressed in polysomal fractions of lymphoblasts [198]. Only 12 mRNAs were identified by both methods. The identified list of differentially expressed mRNAs can be subdivided into over- or underexpressed proteins in absence of FMRP. While identification of differentially expressed mRNAs by analysis of polysomes disregards if such

an mRNA is directly bound to FMRP-containing RNPs or if it is a downstream effect of lack of FMRP, co-immunoprecipitation should restrict identification of mRNAs to directly interacting targets of FMRP-containing RNP complexes. Bearing this assumption in mind, the discovery of underexpressed mRNAs in absence of FMRP by co-immunoprecipitation (immunoprecipitation with antibodies against one of the known components of FMRP-containing ribonucleoprotein complexes) disagrees with a pure suppressor function of FMRP on mRNA translation. Identified mRNAs with important roles in neuronal processes included semaphorin, the microtubule-associated protein MAP1B and NAP22, which is located in pre- and postsynaptic terminals. Eight of the 12 overlapping mRNAs identified in both approaches contained a G quartet structure (see figure 13B). G quartet-mediated binding of mRNAs to FMRP has been suggested as a mechanism for target identification by the RGG in the C-terminal end of FMRP. Supporting evidence for G quartet-induced target recognition was provided by Darnell *et al* [138] and Ramos *et al* [199], and it has been demonstrated particularly for binding of FMRP to its own messenger [200]. We suggest a second model for target recognition (see chapter IV). In our model, FMRP directly binds to a small non-translating RNA, *BC1* (see figure 13C). This small RNA has been implied in neuronal development as well as learning and memory functions (for review see [201]). *BC1* is able to associate with mRNAs via base-pairing, which constitutes the origin of our proposed targeting mechanism. We verified *BC1*-mediated targeting and translational control for the mRNAs encoding for Arc, MAP1B, and α-CaMKII. Although the precise mechanism of *BC1*-induced translational repression by FMRP is still unclear, additional steps within this process have been identified by Wang and colleagues, including translational repression by disruption of the 48S preinitiation complex and association of *BC1* with the poly(A)-binding protein and eIF4A [202, 203]. Support of a target recognition mechanism induced by *BC1* has been provided recently by Gabus *et al* [204], who indicate a novel (possibly BC1-mediated) function for FMRP as a nucleic acid chaperone. While no data have been published on the binding site for *BC1* to FMRP, a recent review provided by the Bagni lab hints at involvement of FMRP's N-terminus in specific binding to the 5'stem-loop of *BC1* [205].

If the identified target recognition mechanisms (G-quartet or BC1 mediated) work synergistically or mutually exclusive, remains unanswered so far. However, MAP1B has been implied both in *BC1*- and G quartet- mediated recognition. MAP1B's leading role in synapse formation and neurite development is well established. In the course of neuronal development, it controls neurite extension and growth cone motility (reviewed by [206]). However, MAP1B has been in the spotlight of fragile X-related research, since a tight developmental relationship between FMRP and MAB1 was discovered [207]. Concurringly with the peak of FMRP expression in the neonatal hippocampus and the cerebellum, Lu and colleagues demonstrate that FMRP-containing RNP complexes selectively bind MAP1B-mRNA. The level of MAP1B protein decreases gradually within the first two postnatal weeks. In the absence of FMRP, this decrease of the MAP1B protein is delayed, while the mRNA level remains comparable to the wild-type situation. These findings suggest that lack of FMRP causes elevated MAP1B expression in a crucial time frame for the dynamic organisation of the neuronal cytoskeleton.

Thus impaired MAP1B regulation may cause the abnormal spine morphologies observed in both fragile X patients and *Fmr1* knockout mice [207].
An *in situ* approach using antibody-positioned RNA amplification (APRA) has been developed by Miyashiro *et al* to pinpoint direct mRNA binding partners of FMRP-associated RNP particles [208]. Nearly 60% of the mRNAs pre-screened by APRA were shown to be directly associated with FMRP. A subset of these mRNAs displayed slight changes in expression levels and subcellular localisation in the brain of *Fmr1* knockout mice. An additional 81 mRNAs were identified, which encoded proteins involved in different neuronal mechanisms such as cytoskeleton structure and function, synaptic signalling events, and nuclear transport. One of the identified mRNAs encoded the glucocorticoid receptor α (GRα). This receptor had an altered dendritic expression pattern in the knockout hippocampus, which may explain the observed learning deficits in fragile X patients. The most recent effort of identifying neuronal mRNA targets of FMRP has been carried out by Sung *et al* [209]. The rationale behind use of synaptoneurosomes was to provide topologically restricted subsets of mRNAs in the rat. Fifteen mRNAs have been identified, which play roles in signal transduction, vesicle trafficking, lipid modification and cell shaping.
Rather than fishing for mRNA cargos of FMRP-containing RNP complexes, Zhang *et al* used two-dimensional difference gel electrophoresis to identify proteins with different expression profiles in *Drosophila* null mutants. Surprisingly, only a small number of proteins were identified with this approach. The 24 identified proteins could be classified into a number of functional groups. The biggest group contained proteins involved in energy metabolism, the second included enzymes involved in biogenic amine synthesis. The other groups included heat shock proteins, protein degradation proteins, and cytoskeletal proteins. Zhang and colleagues further showed a significant elevation of dopamine and serotonin in *dFxr* null mutants as well as an increase of the dense core vesicles that package these neurotransmitters. These findings suggest that altered levels of neurotransmitters play a role in the cognitive and behavioural aspects of fragile X patients [210]. Importantly, it should be noted that the fruitfly contains only a single *dFxr* gene, representing a homologue of the whole *FXR* gene family members.

Considering that a number of research groups have tackled the challenge of identifying the mRNA ligands of FMRP, it is surprising how little the data corroborate with each other. Possible arguments for the observed discrepancies may be the varying animal models, the different brain tissues, the diverse technical approaches, or simply a multitude of distinct and complex FMRP-induced neuronal processes beyond current expectations. Therefore, we developed a method, that will allow the identification of FMRP's target mRNA in a very constricted subcellular location, the neurites (see chapter VI). Confining experiments to a particular locus will enable the identification of distinctly local functions of FMRP.

FMRP's involvement in dendritic mRNA transport

Since the discovery of polyribosomes at the base of spines in hippocampal neurons [211], the idea of a cell-wide regulated protein expression gave way to the hypothesis of a locally regulated protein expression. While the asymmetric distribution of proteins may be explained by its fundamental role in the development and maintenance of cells and cellular processes, the necessity to distribute also mRNAs asymmetrically is less obvious. One of the most convincing arguments is that the transport of an mRNA and its spatially restricted translation reduces the chance of the corresponding protein to cause unwanted effects at inappropriate sites. In favour of this model is CNS myelin basic protein (MBP). This sticky protein is able to bind to many membranes and may possibly cause random aggregation. However, MBP mRNA is localized in the myelin of oligodendrocytes [212]. This subcellular specificity constrains MBP expression to myelin assembly sites, where the protein is inserted directly, thus limiting the possibilities for improper aggregation to other membranes. The theory of local protein synthesis became very attractive for the CNS, since it offered neurons a way of rapidly changing the protein pool in a spatially restricted fashion in response to a triggering signal. In the past years, site-specific regulation of protein expression has been shown to be important for a number of neuronal processes. For example, brain derived neurotrophic factor (BDNF)-induced potentiation of hippocampal Schaeffer collateral synapses depends on local protein synthesis. [213]. Other processes of synaptic plasticity have been identified to rely on local protein translation, such as serotonin-induced long-term facilitation of *Aplysia* sensory-motor synapses [214, 215]. A very sophisticated proof for local protein synthesis involvement in long term potentiation (LTP), long-term spatial memory, associative fear conditioning, and object recognition memory has been delivered by Miller and colleagues [216], who have generated a mouse model in which the CaMKIIα protein is fully functional. However, CaMKIIα mRNA is site-specifically suppressed in the dendrites. Removal of its dendritic mRNA caused a dramatic decrease of CaMKIIα in the post-synaptic density and resulted in malfunctioning of the listed processes.

Spatial distribution of specific mRNAs to distinct cellular compartments has been indicated in many cells. Dendrite specific mRNA expression has been demonstrated for *MAP2*, *Arc*, *CaMKII*, and glutamate receptors [217-220]. The dendrite specific localisation of these messengers has been suggested to be mediated by *cis*-acting signalling motifs in their 3'UTRs [221, 222]. Proteins such as MARTA1/2 and the zip-code-binding protein (ZBP1) have been demonstrated to be putative RNA binding proteins able to identify the aforementioned 3'UTR motifs [223, 224]. MARTA1 (rat) and ZBP2 (chicken) are both homologues of the human KH-type splicing regulatory protein (KSRP), a splicing factor involved in cytoplasmic localisation of β-actin [225]. β-actin mRNA is involved in cell motility [226]. In addition to the zip-code, a cytoplasmic polyadenylation element (CPE) has been identified as a *cis*-acting signalling motif [227] in mRNAs. (For a review on *cis* and *trans*-acting factors see [228]). The existence of local dendritic translation has been widely accepted. However, involvement of local protein synthesis in axons is still a much debated issue (for a review see [229]).

The presence of dendritic polyribosomes in close proximity to spines led to a hypothesized

role of local postsynaptic protein translation for correct synaptogenesis [230]. Provision of a local protein apparatus at synaptic sites enables neurons to quickly mediate mRNA-specific postsynaptic responses to signalling events. Therefore, efficient transport of particular messengers from the cytosol to the synaptic sites, storage of these inactive mRNAs at the synapses, and signal-related rapid initiation of translation is required for proper synaptic processes.

The vehicle of mRNA transport transpired to be a high molecular weight complex comprising mRNAs, RNA binding proteins, translational factors, and ribosomal subunits [231-233]. The trafficking of these RNPs over long distances depends on microtubules [231, 234, 235], while short distance movement is mediated by microfilaments and myosin [236, 237]. The previously discussed specificity requirement for dendritic mRNA translation is probably met by condition/function-dependent combination of diverse RNP-forming components with distinct specificity-promoting motifs. One striking feature of RNPs is their triple functional provision for synaptic processes: particular mRNAs are transported to appropriate sites, the protein synthesis machinery is offered, and translational control is conducted.

As might have been expected in view of the functional domains, the expression pattern, and its association to ribosomes, FMRP has been identified as an RNP component [140, 238-240] (chapter IV). The most recent evidence that Fmrp is associated with RNP particles in the brain was provided by Khandjian *et al* [241]. As a first attempt to shed light onto FMRP's part in dendritic mRNA transport *in vivo*, De Diego Otero and co-workers have generated a stably transfected PC12 cell line with an inducible expression system for regulated expression of an FMRP-enhanced green fluorescent protein (FMRP-GFP) fusion molecule [233]. Following induction, the fusion protein initially materialized in the cell soma, and thereafter FMRP-GFP granules could be observed in neurites. Time-lapse microscopy allowed visualisation of FMRP-GFP-positive granule movement through the neuron. This movement has been shown to be microtubule-dependent, and the average velocity (0.2 μm/s) was similar to that of other mRNA transport studies [231, 242-244]. It is noteworthy that although the net movement of the FMRP-GFP granules was towards the growth cones in neurites, bidirectional movement over small distances was also observed [233]. A rationale for this phenomenon remains elusive. However, trafficking between different individual spines may underly this movement.

De Diego Otero *et al* also identified a number of the RNP particle components by co-localisation experiments, including RNA, ribosomal subunits, FXR1P, and kinesin heavy chain. The list of FMRP-containing RNP particle components has recently been extended and corroborated by research groups which have chosen different experimental approaches; while Villace *et al* set out to identify partners of *Drosophila* Staufen involvement in RNA translation, Kanai and colleagues undertook an elaborate effort to reveal the role of kinesin in RNA transport [245, 246]. In total 42 proteins (among them proteins known to be involved in RNA transport - FMRP, FXR1/2P, Pur-α/β, Staufen, protein synthesis –EF-1 α, Hsp70, RNA helicases, and hnRNPs) and a number of mRNAs (including *CaMKIIα* and *Arc*) were identified by Kanai *et al* as elements of Pur α-containing granules. Immunoprecipitation experiments established that all tested proteins are within the same complex, since the identical pool of proteins was immunoprecipitated regardless of which antibody was used. Time-lapse studies find similar

RNA transport kinetics and bidirectional movement as reported by De Diego Otero *et al*. Furthermore, Pur α's presence in the granules seems to indicate that these particles are dendritic transport molecules. Further evidence for the contribution of kinesin to the transport of FMRP-containing RNP granules has been given by Ling *et al* [247], who concluded that *Drosophila dFxr*-containing RNP granules move *via* kinesin-1 and cytoplasmic dynein. Remarkably, all the groups conclude similar findings, regardless of experimental approach and model system used.

Interestingly, a mutated form of FMRP is unable to form granules. The most common mutation in the *FMR1* gene, an expansion of the CGG repeat, leads to absence of the FMR protein. However, one missense mutation has been described (I304N) in a patient with an extremely severe phenotype [135]. This mutation affects a highly conserved hydrophobic amino acid in the second KH domain. The RNA binding capacity and the folding stability of the mutated protein is compromised [199, 248-251]. Neither is the mutant protein able to homo-oligomerise, to function as a translational repressor, and to form normal RNP particles [184, 238, 251, 252]. We used an *FMR1* I304N-EGFP stably transfected PC12 cell line to study the transport kinetics of the mutated protein (see chapter V). We could verify the presence of the FMRP I304N-EGFP in neurites, which can either be explained by an active transport or by local *de novo* translation of *FMR1* mRNA. However, no FMRP I304N-EGFP-positive granules could be detected, while smaller complexes could still be identified by immunocytochemistry with antibodies against some of the known FMRP interactors (FXR1P, kinesin, P0). Photo bleaching experiments revealed an active microtubule-dependent transport of the mutant protein. Immunoprecipitation studies also revealed that FMRP I304N-EGFP co-precipitated with P0 and FXR1P. Taken together these findings suggest that mutant FMRP is still able to form small complexes and to locate in neurites, however, the formation of large complexes in the range of an RNP are compromised. We speculate that the severe phenotype caused by the I304N missense mutation may result from an impaired translation control of the abnormal FMRP-I304N-containing complexes.

FMRP as a translational regulator in lasting forms of synaptic plasticity

In addition to polyribosomes, many other components of the translational machinery are present in dendrites, such as initiation factors, elongation factors, poly(A) binding protein, transfer RNA (tRNA), aminoacyl-tRNA synthetase, microRNAs, and brain-specific small RNAs (for a review see [253]). In light of the localisation of a translational machinery and the active trafficking of specific mRNAs to the post synaptic density, assumption of local protein synthesis in dendrites is close at hand. Local translation has indeed been demonstrated by different groups in synaptosomal fractions [254, 255], while Torre and Steward [256] demonstrated *de novo* protein synthesis in dendrites physically separated from their cell bodies. The raison d'être of dendritic translation may be its prerequisite in long-lasting forms of synaptic plasticity.

Since synaptic plasticity and memory formation share a number of common cellular and

molecular features, a link between these two processes has long been hypothesized. Most strikingly, short-term forms of synaptic plasticity and memory do not require new synthesis of mRNAs and proteins, whereas long-lasting forms depend on newly synthesised macromolecules [257]. Considering that fragile X patients and *Fmr1* knockout mice display hippocampal-related memory deficits, the involvement of FMRP in transport of mRNAs, and differences in the dendritic mRNA pool in absence of FMRP, FMRP jumps out as a key candidate as a mediator of translation-dependent long-living forms of plasticity and memory. In fact, data has been gathered in recent years to strengthen a metabotropic glutamate receptor (mGluR)-dependent role of FMRP in synaptic plasticity. There are three families of mGluRs besides the ionotropic NMDA and AMPA receptors in the hippocampus. mGluRs have been indicated a role in both LTP and LTD (for reviews see [258, 259]. Weiler *et al* showed that increased *Fmr1*-mRNA associated with translational complexes in response to activation of mGluRs in synaptosomal fractions [260]. While no function of FMRP has been related to LTP in electrophysiological studies in hippocampal sections of the knockout mouse [261] and also late-phase hippocampal LTP could not be proven to be affected in *Fmr1* knockout mice [173], Li *et al* succeeded in demonstrating that LTP was reduced in the cortex of *Fmr1* knockout mice [262]. However, no studies have been performed to test for a possible LTD-related function of FMRP until recently. An LTD-related function of the mGluRs has been demonstrated in the cerebellum as well as in the hippocampus [263-265]. In the hippocampus, mGluR-dependent LTD requires rapid protein synthesis on postsynaptic sites [266], while the predominant NMDA-triggered LTD is initially unrelated to translational events [267, 268]. Huber and colleagues succeeded in demonstrating an enhancement of mGluR5-dependent LTD in absence of the FMRP protein [269]. As a result of activation of postsynaptic group 1 mGluRs (predominantly mGluR5), AMPA and NMDA receptors are internalised and FMRP is synthesised. The negative regulatory function of FMRP on mRNA translation ensures limited expression of the proteins required for permanent receptor endocytosis. Thus FMRP regulates the degree of LTD. In absence of FMRP, receptor intake is not negatively regulated, and consequently an exaggerated number of receptors are internalised. The decrease in receptor numbers on the postsynaptic membrane weakens the synapse and changes the morphology of the spines. The increase in the number of elongated and immature appearing spines that have been shown in fragile X patients is suggested to be a result of incomplete synapse elimination, thus the mental retardation is supposedly an effect of exaggerated LTD [269]. 'The mGluR theory of fragile X' has been reviewed by Bear [270].

In support of a role of mGluRs in FMRP-dependent synaptic plasticity, it has recently been shown that the activation of these receptors is crucial for localisation of FMRP into dendrites [192]. Neuronal activity mediated by mGluRs led to an overall significant rise of FMRP and *FMR1*-mRNA levels in the dendrites. The activity-dependent rise of FMRP was independent of protein synthesis, while an inrease in the granule trafficking could be observed. Surprisingly, there is a differential effect on FMRP in comparison to *FMR1*-mRNA site-specifically at the synapses in response to neuronal activity. While *FMR1*-mRNA levels remain high/increase, FMRP levels actually decrease at the synapse. The events at the synapse in response to neuronal activity might occur as follows: activity leads to either degradation or removal

of FMRP from the synapse (thus a temporarily site-specific decrease of FMRP levels); the mRNA cargos previously suppressed by FMRP are released and translated; remaining *FMR1*-mRNA at synaptic sites allows translation of FMRP and subsequent shut-down of the protein synthesis after appropriate postsynaptic response has been given. Weiler *et al* further delineated the role of FMRP in activity-dependent control of translation at synapses [271]. They reveal that in contrast to wild-type mice, in *Fmr1* knockout mice polyribosomes are not rapidly formed in response to neurotransmitter induction and protein synthesis is decreased, which suggests an impaired translational initiation in absence of FMRP.

The significance of FMRP-related LTD beyond the hippocampus has been emphasized in an elaborate effort by Koekoek and colleagues [272]. This group of investigators used classical delay eyeblink conditioning to test cerebellar dysfunction of fragile X patients and Fmrp-deficient mice. Patients as well as knockout mice showed reduced conditioned responses along with a reduction in peak amplitude and peak velocity, while unconditioned responses remained unaffected. Not only the well established 'global' *Fmr1* knockout mouse, but also a Purkinje cell specific knockout of *Fmr1* displayed the same cerebellar deficits in the kinetics of eyeblink conditioning as the patients. The Purkinje cells of the *Fmr1* deficient mice show elongated spines and enhanced LTD induction at the parallel fiber synapses that innervate these spines. The observed cerebellar deficits are probably independent of further developmental aberrations downstream, since bilateral lesions of the cerebellar nuclei affected wild-type and *Fmr1* knockout mice alike. These studies are the first to demonstrate a role for FMRP in cerebellar-related motor learning.

Both brain and synaptic plasticity are not restricted to the immature brain. Recent research has shown that even the adult and aging brain is plastic. There is evidence for neurogenesis. Proliferation has been noticed most markedly in the subventricular zone, from where newly born neurons migrate to the olfactory bulb and the subgranular zone of the hippocampus. As neurons during early development, neurons born during adult life undergo apoptosis. However, proliferation and survival of these cells can be upregulated by a variety of environmental and behavioural factors, such as enriched environment, hippocampal-dependent learning, exercise, estrogens, and antidepressant treatment. Maturation of newly generated cells into functional and integrated neurons in the adult mammalian hippocampus circuitry has also been verified recently (for review see [52]). It follows that the persistence of neuronal stem cells into adult life increases the capacity of the mature brain for structural and functional plasticity to a greater degree than previously appreciated. This may open up a whole new avenue for functional improvement and repair of the adult brain.

The ability to influence FMRP expression by experience has been demonstrated by Todd *et al*, who demonstrated that FMRP levels increase in the somatosensory cortex of the rat in response to unilateral whisker stimulation [273]. In an effort to further shed light onto the experience-dependent production of FMRP, the same group showed that inhibiting translation in barrel cortex synaptic fractions suppressed the whisker-induced production of FMRP. Also the levels of *Fmr1* mRNA remained unchanged in this scenario. Furthermore, FMRP production depended on the activation of both NMDA receptors and mGluR1s [274]. Additional evidence that experience regulates the expression levels of FMRP *in vivo* at the

level of translation was presented by Gabel *et al*: visual experience modulates the production of FMRP. Light exposure of dark reared rats rapidly (within 15 minutes after exposure) increases FMRP levels in the cell bodies and dendrites of the visual cortex. The upregulation occurs posttranscriptional and can be inhibited by NMDA receptor antagonists [275].

The involvement of FMRP in forms of LTD and LTP prompted us to develop a technique that would allow identification of site-specific mRNA partners of FMRP (see chapter VI). Knowing the mRNAs at the synapses will shed light onto the molecular pathways involved in synaptic plasticity.

Prospects for therapy

The ultimate goal of clinical genetic studies is to find cures for the human diseases. There are a number of aspects, which suggest that a remedy for fragile X may be possible: although FMRP is expressed embryonically, the highest levels have been observed neonatally. Therefore, postnatal treatment may be sufficient to treat most consequences of lack of FMRP. However, it cannot be excluded that spine abnormalities acquired during embryonic development may be irreparable after birth. The morphological modifications seem to be confined to volumetric changes of different brain regions, while no significant neurological degeneration has been observed. The foremost cause of FRAXA is the absence of the FMRP protein in the neurons of the CNS, suggesting that ‘simply’ replacing the protein may solve all the problems. However, there are a number of constraints imposed by the nature of the syndrome. FMRP is ubiquitously expressed throughout the brain, thus global delivery of the protein is necessary. Within the brain, mainly neurons depend on FMRP function, thus cell specific targeting is required. Within cells, FMRP has rather diverse functions. However, no functional assay exists currently. In an attempt to test the possibility of protein-based therapy for FRAXA, we fused FMRP to a protein transduction domain (TAT), which had previously been reported to deliver macromolecules into the brain. However, the biochemical properties of FMRP, namely propensity to aggregate and the difficulties in avoiding toxic effects due to overexpression, question the suitability of direct protein replacement (see chapter VII). Advances in molecular genetics initially led to the belief that gene therapy may be the answer for every disease. Yet the requirements for a suitable vector (transgene structure, expression pattern, packaging, target specificity *etc*) are far more complex than anticipated. The prospects for gene therapy in FRAXA have recently been reviewed [276]. Another avenue towards a ‘global’ therapy is still in its infancy: reactivation of the endogenous *FMR1* gene in patients. Expansion of the CGG repeat results in hypermethylation of the repeat and the promoter region of the gene, thus transcriptionally silencing the gene. Reversing the hypermethylation could be a possibility to reactivate FMRP expression. Advances in this respect were undertaken by Chiurazzi *et al*, who used 5-azadeoxycytidine (5-azadC) in lymphoblastoid cells of fragile X patients to overturn the hypermethylation and thus initiated mRNA and protein production of FMRP. These findings support the crucial role of hypermethylation in the shut-down of the *FMR1*

gene [277, 278]. In a first effort to identify the genetic factors involved in the methylation status of the *FMR1* gene, Stoyanova and co-workers fused cells of fragile X patients with cells of normal individuals or so-called high-functioning-males (individuals with unmethylated CGG repeat expansions above 200 units, who are expressing FMRP, thus functioning normally) [279]. Upon fusion, the hypermethylation of the *FMR1* promoter of the patients' cells was inverted in absence of DNA replication, thus suggesting that fragile X cells lack a factor(s), which is responsible for demethylation/suppression of methylation of the *FMR1* gene. These findings also suggest that the hitherto unidentified factor acts on the methylation status in a developmental time-independent manner, so that reactivation of the gene in adult life may be possible. As promising as these studies (5 azadC and identification of methylation factors) are for basic research, they require long and intense research before they will provide a therapy for patients. Currently, the only treatment available for fragile X patients is limited to symptom-based treatment of the behavioural problems by psychopharmaca (reviewed by [280]). The recent advances in understanding the mechanisms at the synapses, as discussed above, imply that glutamate receptors play a crucial role in transferring FMRP function to the neuronal machinery. Since exaggeration of mGluR activation may be linked to many phenotypes of the fragile X syndrome, antagonists of these receptors are prime candidates for compensating the loss of FMRP. The therapeutic implications of the mGluR theory of fragile X syndrome are reviewed by Bear [270]. Most promising in this respect has been the administration of MPEP to fragile X mice, which enabled the mice to escape from sound-induced seizures and subsequent death, while untreated *Fmr1* knockout mice died almost instantly upon signal induction (Bauchwitz, pers communication). An alternative to administration of mGluR antagonists would be to increase the sensitivity of the AMPA receptors to compensate for the diminished postsynaptic activity. Currently, clinical trials are taking place to test if ampakine indeed may increase the sensitivity of AMPA receptors [280]. Hence finding global mechanisms downstream of FMRP function within neuronal processes currently seems to be a more promising route to finding possible cures than replacing/reactivating the *FMR1* mutant gene. Notably, these therapeutic interventions will affect some of the behavioural problems in fragile X patients. For treatment of the MR in FRAXA other therapeutic approaches will be necessary. Non-medication-based hope is also provided by recent findings that enriched environment can improve the behavioural and morphological abnormalities in fragile X mice [281]. The authors show that an enriched environment rescues hyperactivity, altered open field exploration, and lack of habituation of *Fmr1* knockout mice. The enrichment also increased ionotropic GluR1 levels in the brain. These data clearly indicate that the brain is truly plastic even into adulthood and that cognitive functions rely on multiple independent pathways, thus allowing improvement of behaviour by alternative routes, in case the affected pathway cannot be repaired.

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Scope of the thesis

The present thesis thrives to further the knowledge of the mechanisms involved in cognitive functions, such as learning and memory, by studying the role of FMRP in synaptic plasticity. In **chapter I** a broad historical overview is given, how the brain was identified as the responsible organ for mental activity; the evolutionary events leading to the modern human brain are summarized, and the brain's principle physical and functional architecture is explained. Additionally, it is described how neurons, the basic units for electrical activity, communicate with each other. The term 'plasticity' is introduced: the lifelong ability of the brain to change its shape. Since plasticity plays predominantly a role in early life, the main developmental processes are recapitulated. Furthermore, the mechanisms involved in synaptic plasticity are portrayed, since they are significant for learning and memory processes. In **chapter II** an overview of fragile X syndrome, its symptoms, the gene defect, and the function of the involved protein is given. Also a mouse model for the syndrome is described. Taken together, these data exemplify that fragile X syndrome is a suitable model to study mental function. **Chapter III** focuses on the role of FMRP in neurons, in particular the neuronal mRNA targets of FMRP, how these mRNAs are transported to the dendrites, and the role of FMRP in the translational regulation of its cargo at synapses. The experimental work has been conducted in order to identify and understand the mechanisms involved in neuronal processes, especially mRNA trafficking to the dendrites and local translation. **Chapter IV** describes a model mechanism how FMRP finds its target mRNAs in the nucleus by using a non-coding RNA. The sequence of this RNA determines the specificity of the target RNAs. This non-coding RNA also plays a role in the translational control of the targets. In **chapter V** the dynamics of a mutated protein are examined in order to learn more about the (impaired) targeting and/or the translational efficiency of mRNAs by FMRP at specific sites in the dendrites. Although mutated FMRP is targeted to the dendrites, no formation of high molecular weight granules has been observed. With the aim to establish a link between the lack of FMRP and the observed phenotype (anatomically as well as behaviorally) in fragile X patients and *Fmr1* knockout mice, we tried to identify which mRNAs are bound by FMRP. For this purpose a cell culture system has been developed (**chapter VI**) to allow the isolation of mRNA in the dendrites. The described method enabled mRNA isolation from mouse neurites. This system provides the means to identify, which mRNAs are present in dendrites and if there are differences in the mRNA content between wild-type and disease-related mice. The preliminary results of testing an application for the developed system are depicted in the **appendix**. In addition to studying the neuronal function of FMRP, we looked into the possibilities to develop a therapy for fragile X syndrome based on protein replacement (**chapter VII**). FMRP demonstrated to be toxic when in excess. Since quantity regulation in protein replacement therapy is difficult to achieve, this may not be a suitable method to treat fragile X syndrome.

Experimental work

Für Papa

Chapter IV

The fragile X syndrome protein FMRP associates with BC1 RNA and regulates the translation of specific mRNAs at synapses

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The Fragile X Syndrome Protein FMRP Associates with *BC1* RNA and Regulates the Translation of Specific mRNAs at Synapses

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Summary

The Fragile X syndrome, which results from the absence of functional FMRP protein, is the most common heritable form of mental retardation. Here, we show that FMRP acts as a translational repressor of specific mRNAs at synapses. Interestingly, FMRP associates not only with these target mRNAs, but also with the dendritic, non-translatable RNA *BC1*. Blocking of *BC1* inhibits the interaction of FMRP with its target mRNAs. Furthermore, *BC1* binds directly to FMRP and can also associate, in the absence of any protein, with the mRNAs regulated by FMRP. This suggests a mechanism where *BC1* could determine the specificity of FMRP function by linking the regulated mRNAs and FMRP. Thus, when FMRP is not present, loss of translational repression of specific mRNAs at synapses could result in synaptic dysfunction phenotype of Fragile X patients.

Introduction

FMRP is an RNA binding protein highly expressed in the brain. Absence or mutation of FMRP leads to the Fragile X syndrome, an X-linked dominant disorder and the most frequent cause of inherited mental retardation (1 in 4000 males and 1 in 6000 females). The syndrome is characterized by mental retardation of variable severity, autistic behavior, macroorchidism in adult males, characteristic facial features, and hyperextensible joints (for recent reviews see Bardoni and Mandel, 2002; O'Donnell and Warren, 2002; Oostra, 2002). The syndrome is mostly associated with an unstable expansion of a CGG repeat located in the 5′ UTR of the fragile X mental retardation gene (*FMR1*), which leads to an abnormal methylation pattern that frequently causes transcriptional silencing of the gene (Verkerk et al., 1991; Oberlè et al., 1991; Sutcliffe et al., 1992). In addition, rare atypical cases of Fragile X syndrome have been reported that are not associated with an amplification of the trinucleotide repeat, but with deletions or single point mutations (Gedeon et al., 1992; Meijer et al., 1994; Hirst et al., 1995; De Boulle et al., 1993; Wohrle et al., 1992; Milà et al., 2000).

The FMRP protein contains several RNA binding domains including two KH motifs and one RGG box. As expected from this domain structure, FMRP binds RNA homopolymers and mRNAs in vitro (Ashley et al. 1993; Siomi et al., 1994; Brown et al., 1998, Adinolfi et al., 1999). In particular, FMRP associates with mRNAs and binds to 4% of human fetal brain mRNAs, including the myelin basic protein mRNA (Brown et al., 1998). Further mRNAs that may associate with FMRP and that compose a very heterogeneous family have been recently reported (Sung et al., 2000; Brown et al., 2001). Moreover, specific binding was demonstrated to its own mRNA in a region that may form a particular structure called the purine quartet (Ashley et al., 1993; Schaeffer et al., 2001; Darnell et al., 2001). In mammalian organisms, there are two FMRP homologs, FXR1P and FXR2P (Zhang et al., 1995), which are thought to have distinct but overlapping functions. For example, the pattern of expression in brain and testis as well as the subnuclear distribution mainly overlaps but shows some differences (Tamanini et al., 1997). Several proteins have been shown to interact with FMRP. In the cytoplasm, these include FXR1P and FXR2P and the two proteins CYFIP1 and CYFIP2 (Schenck et al., 2001), while different proteins interact with FMRP in the nucleus (Bardoni et al., 1999; Ceman et al., 1999, 2000). The presence of an NLS and an NES suggests that FMRP may function as a shuttle for mRNA export from nucleus to cytoplasm. Moreover, several lines of evidence suggest that FMRP could modulate stability and/or translation of its target mRNAs in the cell body and also at the synapses. First, FMRP was found to associate with ribosomes in the cell body and in dendrites (Eberhart et al., 1996; Khandjian et al., 1996; Tamanini et al., 1996; Corbin et al., 1997; Feng et al., 1997, Weiler et al., 1997; Greenough et al., 2001). Second, human mRNAs with altered polysomal profiles have been identified by probing microarrays using mRNA isolated from polyribosomes of a human fragile X lymphoblastoid cell line (Brown et al., 2001). Third, two different groups have shown that FMRP functions as a nonspecific repressor of translation in vitro (Li et al., 2001; Laggerbauer et al., 2001) and, more recently, in cotransfection experiments (Mazroui et al., 2002). Finally, it has been shown that the *Drosophila* FMRP regulates *futsch*, a homolog of the mammalian *MAP1B* mRNA, probably at the level of translation (Zhang et al., 2001). However, it is not understood how the translational regulation by FMRP works and whether it exhibits a selectivity for certain mRNAs in vivo.

Here, we show using the *FMR1* knockout (KO) mouse

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model that FMRP is a repressor of translation in vivo and regulates translation of specific dendritic mRNAs, including those encoding the cytoskeletal proteins Arc/Arg3.1 and MAP1B, and the kinase α-CaMKII. FMRP is found in a ribonucleoprotein complex that also contains the small dendritic non-translatable RNA *BC1*. *BC1* binds directly to FMRP and can also associate specifically with mRNAs regulated by FMRP. These results strongly suggest that *BC1* RNA recruites FMRP to the targeted mRNAs, thereby determining the specificity of FMRP action.

Results

FMRP Is a Repressor of Translation at Synapses

FMRP has recently been shown to be a translational repressor in reticulocyte extracts (Laggerbauer et al., 2001; Li et al., 2001). To address the function of FMRP in the neuronal cell, we analyzed the translational efficiency of specific neuronal mRNAs of wild-type as well as *FMR1* knockout mice (Bakker et al., 1994). We selected the mRNAs encoding the following proteins: MAP1B, the microtubule associated protein 1B, because its *Drosophila* homolog has recently been suggested to be translationally regulated by FMRP (Zhang et al., 2001); MAP2, the microtubule associated protein 2 (Garner et al., 1988); α-CaMKII, the α subunit of Ca^{2+}/calmodulin-dependent protein kinase II, whose function is associated to synaptic plasticity (Soderling, 2000) and which is highly expressed at the synapses where its mRNA is translationally regulated (Bagni et al., 2000); Arc, the activity-regulated cytoskeleton associated protein, also known as Arg3.1 (Lyford et al., 1995; Link et al., 1995); the two FMRP homologs, FXR1P and FXR2P; GlyRα1, the α 1 subunit of the glycine receptor, and GluR1, a subunit of the ionotropic glutamate receptor AMPA, because they are thought to play a role in synaptic plasticity (Muller et al., 2002); and, as a control, the small dendritic RNA *BC1* (Tiedge et al., 1991). Since *BC1* does not encode a protein, it should not associate with translating polyribosomes.

A reliable way to assess mRNA translational efficiency is to analyze its partitioning between actively translating polysomes and mRNPs that are not translated (Bagni et al., 2000). To gauge the translational efficiency, the percentage of a given mRNA associated with polysomes (PMP = Percent of Messenger on Polysomes) is quantified. Cytoplasmic extracts, prepared from a whole brain were fractionated through sucrose gradients (see Experimental Procedures). Ten fractions were collected from each gradient while recording the absorbance profile (Figure 1A). Total RNA was extracted from the gradient fractions and then analyzed by quantitative RT-PCR for specific mRNAs. To correct for variations in the efficiency of the RT-PCR reaction, the same amount of a synthetic RNA was added to each sample, amplified, and used for normalization. Exemplarily, the analysis of the α-*CaMKII* mRNA, *BC1*, and the synthetic control RNA is shown for the total brain extracts and the synaptoneurosomes preparation (see below). In each set of panels, the top row shows the distribution of the α-*CaMKII* mRNA, the middle row *BC1*, and the bottom the control RNA.

Quantification demonstrated that some of the dendritic messenger RNAs are translated more efficiently in the *FMR1* KO mice compared to the wild-type mice (Figure 1B). In particular, the PMP for *Arc* increases by 38% in KO mice while that for α-*CaMKII* and *MAP1B* increases by 13% and 17%, respectively. The mRNAs of the two *FMR1* homologs, *FXR1* and *FXR2*, are also translated more efficiently in *FMR1* KO mice compared to the wild-type, presenting a 22% increase in their PMP. Significantly, this is not a general phenomenon as the distribution of mRNAs encoding *GlyRα1*, *GluR1*, *MAP2*, β-*Actin*, and the non-translatable dendritic *BC1* RNA on the polysome gradient do not change significantly (Figure 1B). Moreover, *Ferritin* mRNA, which is involved in iron metabolism and translationally repressed (Preiss and Hentze, 1999), shows no difference in wild-type mice compared to *FMR1* KO mice.

The data are highly reproducible, and they strongly indicate that FMRP is a repressor of specific mRNAs in the brain. Since FMRP is present at synapses (Weiler et al., 1997), we wanted to verify if FMRP is also a translational repressor at synapses where protein synthesis is known to occur (for reviews see Martin et al., 2000; Steward and Schuman, 2001; Richter and Lorenz, 2002). Therefore, we performed polysome/mRNPs analysis of extracts from synaptoneurosomes as described above for the total brain. These preparations are highly enriched in synaptic termini and virtually devoid of cell body contamination (Bagni et al., 2000).

We found that the translation of some dendritically localized mRNAs is also increased in purified synaptoneurosomes and the increase is, in fact, even higher than in total brain. In particular, the PMPs of *Arc* and α-*CaMKII* mRNAs increase by 34% and 53%, respectively (Figure 1B). The *MAP1B*, β-*Actin*, and *Ferritin* mRNAs were not detectable in the synaptoneurosomes under the same PCR conditions used to amplify the dendritic mRNAs, while the analysis of *GlyRα1*, *GluR1*, and *MAP2* was not performed on the synaptoneurosomes because they did not show any significant difference in total brain extracts of wild-type and *FMR1* KO mice. In conclusion, several, but not all, mRNAs are translated more efficiently in *FMR1* KO mice, and this effect is significantly stronger in isolated synaptoneurosomes as compared to the total brain extracts.

To determine whether the translational upregulation also leads to higher levels of the proteins in question, we assayed equal amounts of the respective extracts by quantitative immunoblotting. β-Actin was used as a control; as expected, its abundance is not affected in the *FMR1* KO (Figure 1C). The level of the other assayed proteins reflects quite well the translational efficiency of their mRNAs. Thus, Arc, FXR2P, and MAP1B are more abundant in total brain extracts from *FMR1* KO versus wild-type mice, and the effect was much more pronounced in the synaptoneurosome preparation. For α-CaMKII, the increase was observed only in the synaptoneurosomes.

Surprisingly, a significant part of the synaptosomal *BC1* RNA cosediments with the polysomes of *FMR1* KO, but not of wild-type mice (Figures 1A and 1B). This shows a clear dysregulation of *BC1* RNA and demonstrates that *BC1* RNA has the ability to associate with large complexes, possibly the translating ribosomes,

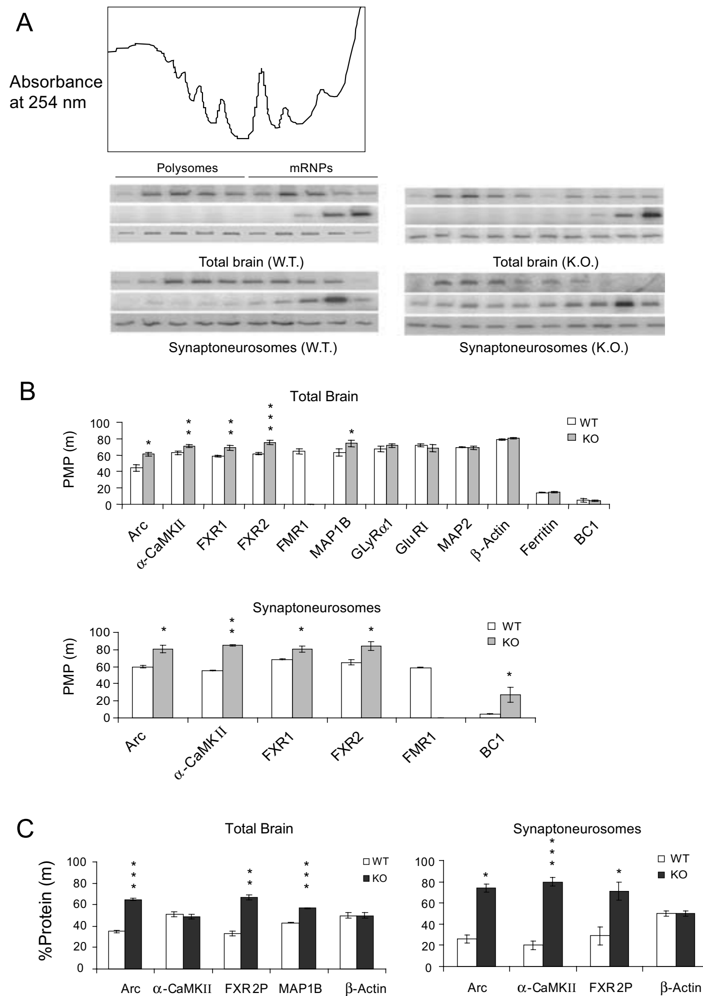

Figure 1. Polysome/mRNPs Distribution of Dendritic and Cell Body mRNAs

Mouse total brain and synaptoneurosomes extracts were fractionated by sucrose gradient centrifugation. Each gradient was collected in ten fractions, and an equal amount of synthetic RNA was added to each fraction and then amplified by radioactive RT-PCR. Radioactive signals in the polysomal and non-polysomal fractions were quantified, corrected versus control RNA, and expressed as PMP (Percentage Messenger on Polysome). (A) Typical polysomal profile from total brain cytoplasmic extracts and example of RT-PCR reaction performed to detect α-*CaMKII* mRNA and *BC1* RNA association with the polysomal gradient. (B) Polysome/mRNPs analysis in total brain extracts and in synaptoneurosomes. Values shown are the mean ± SEM ($n \geq 3$). (C) Protein level analysis in total brain extracts and synaptoneurosomes. Values shown are the mean ± SEM. The quantitation was repeated three times. *, $p < 0,05$; **, $p < 0,01$; ***, $p < 0,001$ for KO versus W.T. by Student's t test in all figures.

but this association is inhibited in wild-type mice by the FMRP protein.

FMRP Associates Mainly with Ribonucleoparticles

Previous studies on non-neuronal cell lines have shown FMRP to be present both in fractions containing the polyribosomes and in fractions containing the monomeric 80S ribosome (Eberhart et al., 1996; Feng et al., 1997; Khandjian et al., 1996; Tamanini et al., 1996; Siomi et al., 1996; Corbin et al., 1997; Brown et al., 2001; Mazroui et al., 2002). Considering the possibility of a brain-specific repressor complex, we investigated where FMRP sediments on sucrose gradients of brain extracts. We fractionated cytoplasmic extracts on a continuous sucrose gradient, as performed to study mRNA translation, and analyzed FMRP distribution by Western blot using polyclonal antibodies against the C terminus of the human FMRP. This region exhibits no homology to FXR1/2P, and the antibodies do not cross-react with the FXR proteins (see Experimental Procedures). Interestingly, FMRP mainly cosediments with the monomeric 80S ribosomes and with mRNPs in the upper fractions of the sucrose gradient (Figure 2). A very similar pattern of FMRP distribution was detected in a gradient derived from synaptoneurosomes (Figure 2). This distribution is

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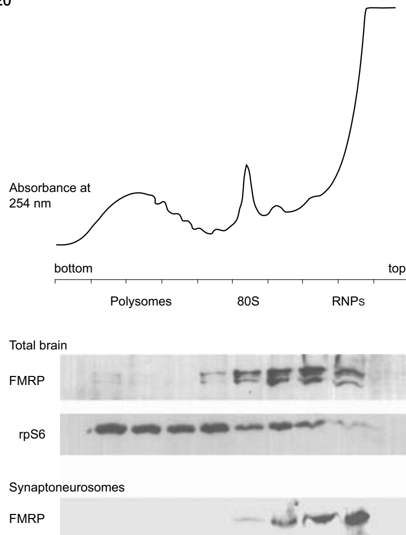

Figure 2. Polysome/mRNPs Distribution of FMRP Protein

Mouse total brain and synaptoneurosomes extracts were fractionated by sucrose gradient centrifugation. Each gradient was collected in ten fractions. Absorbance profile of the sucrose gradient from total brain showing the polysomal pattern is reported in the upper part of the figure. An aliquot (same volume) of each fraction was analyzed by Western blot using FMRP antibody (rAM1) and, as control, an antibody for the ribosomal protein S6.

consistent with a role of FMRP as repressor of translation. As control for our experiments, we used the ribosomal protein S6 that cosedimented perfectly with the polysomes and, as expected, with the 40S ribosomal subunit. This clearly shows that, in brain, FMRP forms complexes different from those previously reported for non-neuronal cell lines, leading to the initial hypothesis that in the latter cell lines, the repressor complex is absent or less stable.

The Small Dendritic RNA *BC1* Associates with FMRP

The finding that *BC1* significantly changes its partitioning on the polysome gradients in the absence of FMRP prompted us to investigate whether *BC1* is associated with FMRP. For this purpose, we specifically immunoprecipitated the FMRP-RNP particle. Antiserum directed against the FMRP C terminus, but not preimmune serum, efficiently immunoprecipitates FMRP from mouse brain extracts (Figure 3A). Efficient immunoprecipitation requires elevated salt concentrations possibly because the C terminus is masked at physiological salt concentrations; nevertheless, the antibodies precipitate a protein complex that also contains FXR1P, a known FMRP interactor (Figure 3A). To elucidate if the small dendritic RNA *BC1* is part of the FMRP complex, total brain extracts were used to immunoprecipitate FMRP. Analysis of coprecipitating RNA by RT-PCR showed that FMRP can specifically immunoprecipitate *BC1* RNA as well as *Arc* and *MAP1B* mRNAs, whereas the α-*Tubulin* mRNA is not immunoprecipitated (Figure 3B). The binding specificity was demonstrated by using extracts from the *FMR1* KO mice (lanes 6, 8, 10, 12) as well as from preimmune serum (data not shown), which did not immunoprecipitate any of the tested RNAs. For these experiments we used stringent salt conditions (250 and 350 mM NaCl). Thus, we conclude that *BC1* RNA, *Arc* and *MAP1B* mRNAs specifically associate with FMRP.

A potential *BC1* analog in primates is called *BC200* RNA (Martignetti and Brosius, 1993; Tiedge et al., 1993). *BC200* RNA, like rodent *BC1* RNA, associates with protein(s) to form an RNP complex (Cheng et al., 1997). Distribution of the human *BC200* reveals a neuron-specific expression and dendritic localization (Tiedge et al., 1993), suggesting a role in dendritic RNA transport and/or translation. This prompted us to investigate whether *BC200* is associated with human FMRP. We specifically immunoprecipitated the FMRP-RNP particle from human neuroblastoma, glioma, and lymphoblast cell line extracts (Figure 3C, left panel), and *BC200* was detected by RT-PCR in extracts from glioma and neuronal cell lines (Figure 3C, right panel, lanes 4 and 5), but not in extracts of lymphoblast cell lines (lane 6).

FMRP Binds Directly to *BC1*

To assess whether FMRP interacts directly with the *BC1* RNA, we performed electrophoretic mobility shift assays (EMSA) using in vitro transcribed, radiolabeled *BC1* RNA and purified, recombinant FMRP protein or brain extracts (Figure 4). Addition of FMRP protein to the RNA leads to formation of an RNP complex that migrates more slowly on the native polyacrylamide gel than the RNA alone (compare lanes 1 and 2). This complex indeed contains the FMRP protein since it is shifted by anti-FMRP antiserum (lane 3). The binding of FMRP to *BC1* RNA is specific and stoichiometric since the complex can be easily competed by a 100-fold excess of unlabeled *BC1* RNA, but not by nonspecific competitor tRNA (lanes 6 and 7). It should be noted that recombinant FMRP is stable only at 1 M salt concentrations. Since the protein preparation makes up 75% of the binding reactions, the binding occurs at 750 mM salt. This high salt stringency underlines the specificity of the interaction. At the same time, only a small part of the *BC1* RNA is bound (25%), despite the 2000-fold excess of the protein. While this may indicate either that the majority of the RNA is folded in an unfavorable structure or that the high concentration may be too far from physiological conditions, we believe that the binary complex has a rather high dissociation constant and needs to be stabilized by additional proteins (see Discussion). This notion is supported by our finding that *BC1* RNA predominantly forms a complex in brain extracts that is bigger than the binary complex (compare lanes 2 and 4). This complex contains FMRP protein, as judged by its shift upon addition of anti-FMRP antibodies.

***BC1* Mediates the Interaction between FMRP and the Regulated mRNAs**

Since *BC1* RNA associates with FMRP, it might well target, via base-pairing, FMRP to the mRNAs that are to be regulated. To support this hypothesis, we searched for regions of complementarity between *BC1* and the regulated mRNAs. We found that nt. 3-21 and nt. 47-70 of *BC1* RNA are predicted to basepair almost perfectly to *MAP1B* mRNA, while the overlapping region nt. 62-77 is predicted to basepair with α-*CaMKII* mRNA, and the region

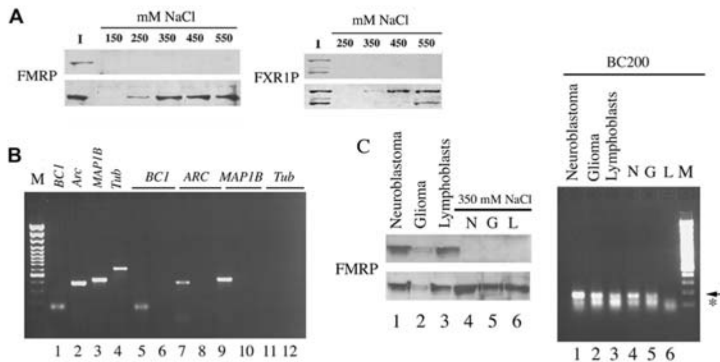

Figure 3. *BC1* RNA, *Arc*, and *MAP1B* mRNA Are Associated with FMRP Complex

(A) Brain lysates were prepared from wild-type mice and immunoprecipitated in presence of different salt conditions with rAM1 antibody and probed with 1C3 anti-FMRP. Preimmune serum was used for IP in the upper panel. Similar immunoprecipitates were probed with antibodies directed against the paralog FXR1P.

(B) Immunoprecipitations were performed from wild-type (lanes 5, 7, 9, 11) and *FMR1* KO mice extracts (lanes 6, 8, 10, 12). RNA was extracted, DNase-treated and RT-PCR was performed using specific oligos for *BC1* RNA, *Arc, MAP1B*, and α-*Tubulin* mRNAs. Shown is the product of the PCR reactions. Lanes 1, 2, 3, and 4 contain 1/3 of the input (the mRNAs are equally present in the W.T. as well as in the KO extracts).

(C) Immunoprecipitations were performed from human neuroblastoma, glioma, and lymphoblast cell line extracts with rAM1 antibody and probed with IC3 anti-FMRP (left, upper is preimmune, lanes 1, 2, and 3 contain 1/3 of the input). In a parallel experiment, RNA was extracted, Dnase treated, and RT-PCR was performed using specific oligos for *BC200* RNA. The arrow points to the specific *BC200* product while the asterisk points to a nonspecific product (oligo concatamers). M = 100 bp.

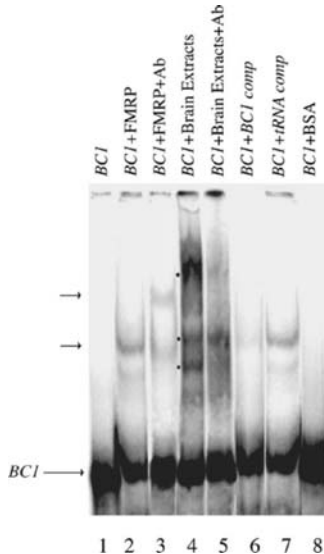

Figure 4. FMRP Binds Directly to *BC1* RNA

EMSA was performed with ^{32}P-labeled *BC1* RNA and purified FMRP or brain extracts. A retarded band is shown (lane 2) due to FMRP interaction with *BC1*. This band is supershifted after incubation with FMRP (rAM1) Ab (lane 3). Three major complexes (black dots) are formed incubating *BC1* with brain extracts (lane 4), and two of them are destroyed after incubation with rAM1 Ab (lane 5). Competition experiments are reported in lanes 6 and 7 with non-labeled *BC1* and *tRNA*, respectively. A reaction with BSA alone is shown in lane 8.

between nt. 2-20 of *BC1* RNA is predicted to basepair to *Arc* mRNA (Figure 5A). Interestingly, the sequence complementarity is found at the base of the longer stem loop, according to a stable predicted secondary structure of *BC1* RNA (Rozhdestvensky et al., 2001, Figure 5B). β-*Actin* mRNA, in contrast, is not regulated by FMRP and shows no significant complementary to *BC1* RNA.

For further support of a direct interaction between *BC1* RNA and the mRNA targets, we designed a 21mer DNA oligonucleotide that anneals to *BC1* RNA in the region of complementarity to *MAP1B* mRNA, which has the longest region of homology among the targeted mRNAs (the region covering 47-70, oligo *BC1*-sl-1). Total brain cytoplasmic extracts were incubated at 37°C with the *BC1* oligo. Then FMRP was precipitated, and the associated RNAs were analyzed by RT-PCR. Initially, two concentrations of oligos corresponding to an estimated 150 and 750 molar excess of *BC1* were used; since both gave the same result, we used the lower concentration in the subsequent experiments.

As shown in Figure 6A, annealing of the specific oligo, as opposed to a control oligo directed against β-*Actin* mRNA, reduced coprecipitation of *BC1* with FMRP. To verify whether this reduction was due to either a destruction of *BC1* by an RNaseH activity in the extract or to an interference of the oligo with FMRP binding to *BC1* RNA, we performed the same experiment using a 2′O-methylated RNA oligo (O-Me-*BC1* sl-1) that does not induce RNase H degradation of the target mRNA and observed the same reduction in FMRP-*BC1* complex (Figure 6A). Importantly, no *MAP1B* mRNA was detected under saturating PCR conditions when the extract was treated with the specific *BC1* DNA oligo sl-1, whereas treatment with the control oligo caused only a minor

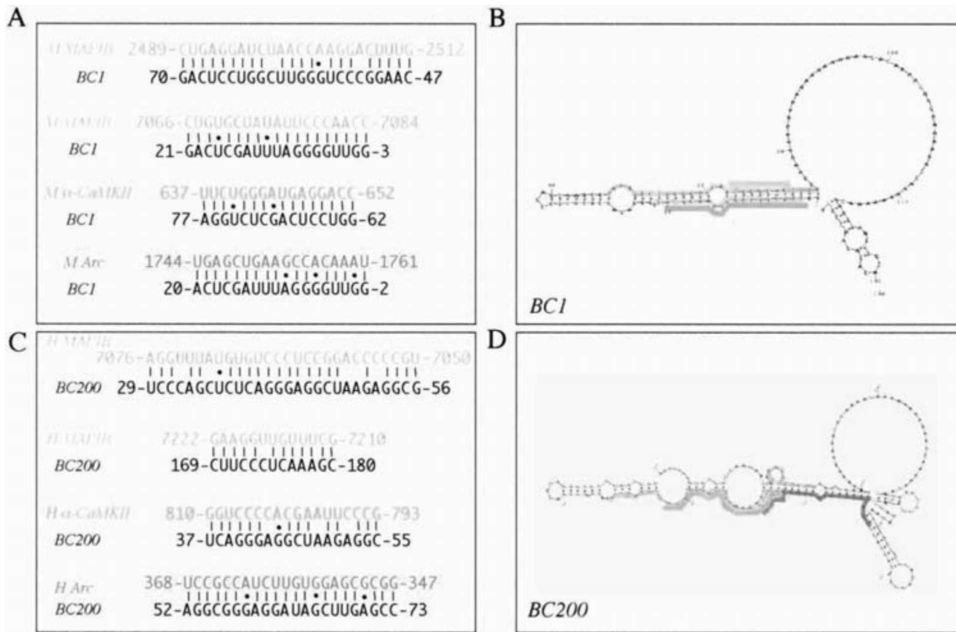

Figure 5. Homology between *BC1* RNA Sequence and the Regulated mRNAs

(A) Mouse *BC1* exhibits sequence complementarity to *Arc*, *α-CaMKII*, and *MAP1B* mRNAs.

(B) Secondary structure of *BC1* RNA has been performed using the program FoldRNA. *BC1* sequence is shown in black, while the complementarity of *Arc*, *α-CaMKII*, and *MAP1B* mRNAs with *BC1* RNA are reported in red, green, and blue, respectively.

(C) Sequence complementarity between human *BC200* and the human *Arc*, *α-CaMKII*, and *MAP1B* mRNAs.

(D) Secondary structure of *BC200* RNA has been performed using the program FoldRNA.

nonspecific reduction of *MAP1B* mRNA (Figure 6B). Therefore, we conclude that *BC1* is required for the association of *MAP1B* mRNA with FMRP. Moreover, the oligo sl-2 directed against the 3′ stem loop of *BC1* does not inhibit the FMRP/*MAP1B* association. These data demonstrate the importance of the *BC1* region that is complementary to the target mRNA.

To assay whether *BC1* RNA and FMRP-targeted mRNAs interact in the absence of proteins, an in vitro annealing reaction was performed with purified total brain RNAs and biotinylated *BC1* RNA. As shown in Figure 6C, *Arc*, *α-CaMKII*, and *MAP1B* mRNAs coprecipitated with biotinylated *BC1* RNA. The specificity of this experiment is shown by the absence of in vitro interaction between *BC1* and the neuronal mRNAs like *GluR1*, *GlyRα1*, and *MAP2* (neuronal mRNAs that are not regulated at the translational level by FMRP). This finding strongly suggests a base-pairing interaction between *BC1* and the mRNAs.

Discussion

FMRP Represses the Translation of Target mRNAs at Synapses

While it is clear that loss of FMRP protein is associated with the mental retardation of Fragile X (FRAXA) patients, it is still not understood how the lack of this protein causes such a severe clinical phenotype. The morphological anomaly in the brain of both FRAXA patients and *FMR1* knockout mice appears to be limited to the presence of abnormal dendritic spines, which is reminiscent of a delay in maturation (Hinton et al., 1991; Comery et al., 1997). In the *FMR1* KO mice, longer and denser dendritic spines are observed, consistent with the human phenotype (Nimchinsky et al., 2001). It has been shown that the level of FMRP increases near synapses in response to neurotransmitter activation (Weiler et al., 1997), thus FMRP is thought to have an effect on maturation and/or function of the synapses (Greenough et al., 2001). Since both mRNAs and the protein synthesis machinery are present in dendrites and near postsynaptic sites (Steward and Schuman, 2001), it is now clear that local and regulated synthesis of key synaptic proteins plays an important role in synapsis maturation and neuronal development.

Interestingly, we show that the mRNAs encoding FMRP and the two homologs FXR1P and FXR2P are translated at synapses (Figure 1B). The fact that these proteins are known to interact (Zhang et al., 1995; and Figure 3A) and the fact that all three proteins are synthesized locally at the synapses suggests that these complex(es) can also form in the distal region of the neuron.

FMRP Regulates mRNA Translation at the Synapses
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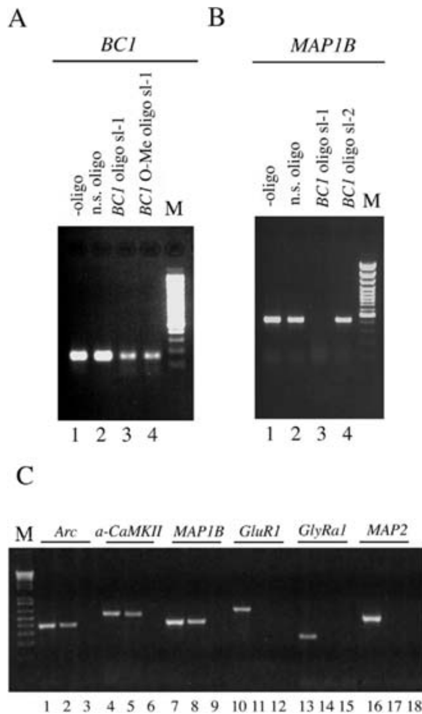

Figure 6. *BC1* RNA Interacts with FMRP-Targeted mRNAs

Total brain cytoplasmic extracts were incubated in the absence of oligonucleotides (lanes 1), in the presence of an oligo complementary to an unrelated RNA (lanes 2), of an oligo complementary to *BC1* RNA in the stem loop 1 (lanes 3), or of a modified RNA oligo O-Me-RNA (lane 4). Then, FMRP was immunoprecipitated and coprecipitating *BC1* RNA (A) or *MAP1B* mRNA (B) was amplified by RT-PCR. Shown is the product of the PCR reaction fractionated on an agarose gel and stained with ethidium bromide. Brain extracts were also incubated in presence of an oligo complementary to *BC1* RNA in the stem loop 2, FMRP was immunoprecipitated, and coprecipitating *MAP1B* mRNA amplified by RT-PCR (Figure 6B, lane 4). M = 100 bp. (C) Biotinylated *BC1* RNA was incubated with isolated total RNA from mouse brain and RNAs annealed to *BC1* RNA were selected on streptavidin beads. RNA was extracted and RT-PCR was performed using specific oligos for *Arc, α-CaMKII, MAP1B, GlyRα1, GluR1*, *and MAP2* mRNAs. Shown is the product of the PCR reactions (lanes 2, 5, 8, 11, 14, 17). Lanes 1, 4, 7, 10, 13, and 16 contain 1/10 of the input. Lanes 3, 6, 9, 12, 15, and 18 show the same experiment but performed in the absence of *BC1* RNA. M = 1 kb plus.

Recent evidence shows that FMRP is a dose-dependent inhibitor of mRNA translation in reticulocyte extracts and *Xenopus* oocytes (Laggerbauer et al., 2001; Li et al., 2001, Schaeffer et al., 2001). In vivo, FMRP could exert a more specific effect. In fact, elegant studies using *Drosophila* genetics showed that dFXR, the homolog of mammalian FMRP, negatively regulates the expression of Futsch, a homolog of the mammalian microtubule-associated protein MAP1B (Zhang et al., 2001). Here, we show that translation of specific mRNAs is up-regulated in *FMR1* knockout mice. Significantly, the translational repression by FMRP is much stronger in isolated synaptoneurosomes than in total brain extracts (Figure 1B). Interestingly, the translational repression acts specifically only on a subset of neuronal mRNAs and affects

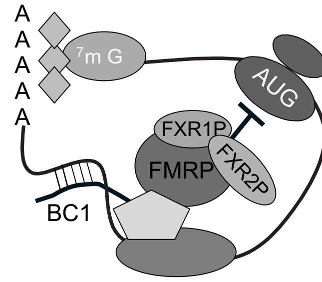

Figure 7. Model of FMRP Function

The model proposes a direct interaction between *BC1* RNA and the targeted regulated mRNAs. The FMRP RNP is thereby brought into vicinity of the initiation codon and blocks the translation. Green rhombi represent Poly(A) binding protein, while the orange and yellow figures represent unknown FMRP protein partners.

mRNAs encoding key regulatory proteins, which could explain how the lack of FMRP could impair the function of synapses.

BC1 RNA as a Guide to Target FMRP to the mRNAs that Are Regulated

Several studies have uncovered a variety of mechanisms through which mammalian gene expression can be regulated at the level of translation. In many cases, this happens at the initiation step, where factors can interfere, for example, by phosphorylation or proteolytic cleavage of initiation factors, or by binding to specific sequence elements in the mRNAs (Preiss and Hentze, 1999). Evidence has been reported that FMRP can prevent the formation of the initiation complexes (Laggerbauer et al., 2001).

Here, we present a model in which *BC1* RNA determines the specificity of FMRP repression (Figure 7). *BC1*, a non-translatable RNA, is part of an RNP in the brain (Cheng et al., 1996, 1997). The specific location of *BC1* RNA may indicate a functional role related to translational processes in the somatodendritic compartments of neurons (Tiedge et al., 1991). Part of *BC1* RNA, the ID region, is derived from $tRNA^{Ala}$, which has been recruited or adapted into a novel function (Brosius and Gould, 1992). Therefore, it is conceivable that *BC1* RNA may interact with ribosomes and may be involved in regulating protein synthesis. Moreover, tissue-specific expression of *BC1*-like RNA is conserved across mammalian species of considerable evolutionary distance, suggesting that the RNA products have an important cellular function.

BC1 RNA cofractionated to a significant degree with the polysome fraction in absence of the FMRP protein. This was observed only in the synaptosomal preparations (Figure 1B), thus linking *BC1* RNA to FMRP regulation at synapses. Further analysis demonstrated that *BC1* RNA coprecipitates with the FMRP protein (Figure 3B) and that it can bind directly to FMRP (Figure 4), establishing a physical link between the two molecules. This association appears to be rather tight, since it was observed at stringent salt concentrations in immunoprecipitation and band shift experiments. Thus, the interesting model arises in which *BC1* RNA binds to FMRP and

also associates with mRNAs, thus bridging FMRP and the mRNAs (Figure 7). In this way, *BC1* might well be responsible for targeting FMRP to the mRNAs that are to be regulated. Since FMRP is thought to inhibit translation at the initiation step, this model predicts that only a minor fraction of *BC1* coassociates with actively translating ribosomes and that this fraction should significantly increase when FMRP and, hence, the block of translation is removed. This is indeed the case. Second, the model predicts that *BC1* RNA would bind to those mRNAs regulated by FMRP. We found significant stretches of complementarity between *BC1* RNA and *MAP1B*, α-*CamKII*, and *Arc* mRNAs, and these regions are located on the longer stem loop of the *BC1* RNA (Figures 5A and 5B). To verify our assumption, we interfered with the putative *BC1*/mRNA interaction using RNA oligos and demonstrated that the interaction of FMRP with its target mRNA is lost. Only *BC1* stem loop 1 is required for *BC1* RNA interaction with the neuronal mRNAs analyzed. In this respect, it is interesting to note that *BC1* stem loop 1 has been shown to be required for *BC1* localization to dendrites (Muslimov et al., 1997). Finally, we show that the association between *BC1* RNA and FMRP-regulated mRNAs can occur in vitro in absence of any protein. The binding is highly specific because it occurs in presence of a large population of mRNAs.

Very recently, it has been shown using the IRES system that *BC1* RNA can repress translation (Wang et al., 2002). This information supports the role of FMRP-*BC1* complex in the repression of synaptic mRNA translation.

A potential analog of *BC1* in primates is called *BC200* RNA. Distribution of the human *BC200* reveals a neuron specific expression and dendritic localization, suggesting a role in dendritic RNA transport and/or translation. Like *BC1* RNA, *BC200* RNA can be divided into three structural domains: a repetitive element, a central region, and a unique region. A possible secondary structure, using computer prediction (Zuker, 1989), revealed high structural homologies to *BC1* RNA (Figure 5D) and, more interestingly, we could detect a strong complementarity between *BC200* and human *Arc*, α-*CaMKII*, and *MAP1B* mRNAs. We have shown here that *BC200* is also able to form a complex with FMRP in neural tumor cells, so it is highly possible that the two *BC* RNAs have the same functional significance.

In conclusion, we suggest that the specificity of FMRP translational repression is defined through base-pairing interactions of the associated *BC1* RNA. In this model, one of the RNA binding domains of FMRP is most likely responsible for binding to *BC1* (Figure 7). Another model has recently been reported in which FMRP binds directly to its target mRNAs. In particular, it has been shown that FMRP binds in vitro to mRNAs that can form G quartet structures (Darnell et al., 2001; Schaeffer et al., 2001). We think that the two binding modes could both occur in the cell, and could possibly be linked to different functions, e.g., translation and transport of the mRNAs. This hypothesis is supported by our band shift experiments using brain extracts, since several complexes are formed on the *BC1* RNA and only one is clearly shifted by FMRP Ab. We find it interesting that the part of *BC1* RNA just before the poly(A) stretch is rather pyrimidine rich and could thus base-pair with a poly(G) stretch like the one identified as a possible G quartet. Therefore, the putative G quartet structures in the target mRNAs could be bound by *BC1* RNA.

The pathology in the brain of both FRAXA patients and *FMR1* knockout mice appears to be limited to abnormalities in the dendritic spines. Since local protein synthesis is required for synaptic development and function, the repressor role of FMRP likely underlies the behavioral and developmental symptoms of FRAXA patients. The fact that FMRP depletion does not have a lethal effect suggests that FMRP is a regulator only of a subset of mRNAs whose translation occurs in dendrites. We believe that these data offer new and important insights into the molecular mechanisms of the Fragile X syndrome and translational regulation at synapses.

Experimental Procedures

Antisera

Rabbit polyclonal antiserum rAM1 was raised and affinity purified against the hexahistidine-tagged C terminus of FMRP. The antibodies were checked by Western blotting, ELISA, and immunoprecipitation. Monkey COS-7 cells were transfected with full-length FMR1 (ISO7) or the isoform lacking the C terminus (ISO4) and extracts analyzed with rAM1 and m1C3 antibodies to validate the C terminus specificity.

Cell Culture

Neuroblastoma (SH-SY5Y), glioma (U373MG) and lymphoblast cell lines were grown at a concentration of 10^5 cells/ml. Lymphoblast cells were grown in RPMI 1640 supplemented with glutamax and 10% FCS (both GIBCO-Invitrogen) plus antibiotics. Neuroblastoma and Glioma cell lines were grown in 50% Dulbecco's modified Eagle's medium and 50% F-12HAM.

Protein and RNA Extraction

General procedures for protein and RNA preparation and analysis followed standard laboratory manuals. Proteins from total brain and synaptoneurosomes were resuspended in Laemli buffer, boiled, separated by polyacrylamide gel electrophoresis, and transferred to Immobilon-P membrane (Millipore), immunostained and visualized using the SuperSignal Chemioluminescent Substrate (Pierce) or, if accurate quantitation was required, ECF Western blotting reagent packs (Amersham Pharmacia Biotech). RNAs were prepared from total brain, synaptoneurosomes and gradient fractions by Proteinase K treatment, phenol/chloroform extraction, and ethanol precipitation.

Polysomes/mRNPs Distribution of mRNAs or Proteins

Animal care was conducted in conformity with the institutional guidelines that are in compliance with national (DL N116, GU, suppl 40, 18-2-1992) and international laws and policies (European Community Council Directive 86/609, OJa L 358, 1, December 12, 1987; National Institutes of Health Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). Synaptoneurosomes and total brain extract preparation, sucrose gradient sedimentation of polysomes, and analysis of the polysomes/mRNPs distribution of mRNAs were carried out as described (Bagni et al., 2000). Total brain or purified synaptosomes were homogenized in lysis buffer (100 mM NaCl, 10 mM $MgCl_2$, 30 mM Tris-HCl [pH 7.5], 1 mM dithiothreitol, 30 U/ml RNasin). After 5 min of incubation on ice, the lysates were centrifuged for 8 min at 12,000 $\times$ g at 4°C. The supernatants were sedimented in a 5%–70% (w/v) sucrose gradient by centrifugation for 135 min at 37,000 rpm in a Beckman SW41 rotor. Each gradient was collected in ten fractions. RNA was extracted from gradient fractions and analyzed by RT-PCR. For protein analysis, the supernatant was sedimented in a 15%–50% (w/v) sucrose gradient by centrifugation for 110 min at 37,000 rpm in a Beckman SW41 rotor.

RT-PCR for Polysome/mRNPs Analysis
RNA samples were extracted DNase treated and reverse transcribed into cDNA using 100 U of M-MLV RTase (RNaseH$^-$, Invitrogen). For quantitative RT-PCR analysis, an equal amount (10 pg) of an internal control RNA was added to each fraction before RNA extraction. This RNA was obtained by in vitro transcription (Ambion, Austin, TX) of the *Xenopus* ribosomal protein L22 sequence (a.n. X64207) and amplified with oligonucleotides annealing to the vector and the coding region. An aliquot of RT reaction was PCR amplified in a final volume of 50 μl, using 20 pmol of each primer, 100 μM of each dNTP and 0.5 U of Taq DNA Polymerase (Amersham Pharmacia Biotech). The amount of template and the number of amplification cycles were preliminarily optimized for each PCR reaction, so as to avoid saturation. For radioactive PCR, dCTP was reduced to 10 μM and 0.2 μCi of α-^{32}P-dCTP (Amersham Pharmacia Biotech; 3000 Ci/mmol) was added. Products were run on a 5% polyacrylamide gel and quantified by a PhosphoImager.

Mouse Brain Lysate Immunoprecipitation
Whole brain was washed in cold PBS and homogenized by 10 stroke dounce homogenization in 2 ml/brain ice cold lysis buffer (10 mM HEPES [pH7.4]; 200 mM NaCl, 0.5% TritonX-100, 30 mM EDTA) in presence of protease inhibitors (Sigma-Aldrich) and 30 U/ml RNasin. The following steps were performed according to Brown et al., 2001 with slight modifications. Briefly, nuclei and debris were pelletted at 8000 rpm in a Eppendorf centrifuge for 10 min at 4°C, the pellet was washed with 1 ml lysis buffer and pelletted again. Supernatants were pooled, raised to 400 mM NaCl, and clarified at 20,000 rpm in a Beckman SW41 rotor for 30 min. 300 μg of protein extract were immunoprecipitated for 1 hr at 4°C with 15 μg affinity-purified polyclonal antibodies rAM1 conjugated to 20 μl of protein A sepharose (Amersham Pharmacia Biotech). Beads were then washed in lysis buffer containing 150, 250, 350, 450, or 550 mM NaCl. The immunoprecipitate was analyzed by 10% SDS-PAGE and Western blotting.

Preparation of *BC1* RNA
Linearized plasmid containing the *BC1* sequence (Cheng et al., 1996) was used as a template to produce either ^{32}P-labeled *BC1* using in vitro transcription kit (Ambion) and 50 μCi of α-^{32}P-UTP (Amersham Pharmacia Biotech; 3000 Ci/mmol) or biotinylated *BC1* RNA in the presence of biotin-16-uridine-5′-triphosphate (Roche). RNA integrity was examined by agarose gel electrophoresis, and the biotin incorporation was verified by spotting the RNA onto a nitrocellulose membrane and detection with the streptavidin-alkaline phosphatase conjugate.

***BC1* Binding to Dynabeads Streptavidin and Duplex Assay**
Biotinylated *BC1* RNA (200 ng) was bound to 20 μl of streptavidin-conjugated magnetic beads (Dynal) for 20 min at RT in annealing buffer (10 mM Tris-HCl [pH 7.5], 2 mM $MgCl_2$, 400 mM NaCl, 0.2% SDS). Total RNA from mouse brain (500 ng) was incubated with *BC1*-magnetic beads in annealing buffer at 80°C for 5 min and at RT for 14 hr. After three washes with the same buffer, *BC1* RNA and the annealed RNAs were eluted by extraction with phenol/chloroform and ethanol precipitated.

5 μl of eluted RNA was reverse transcribed and an aliquot (1/4) was PCR-amplified as described above for the immunoprecipitated RNAs. For all the mRNAs, 30 cycles of amplification were performed.

Preparation of Cytoplasmic Extracts and RNA Binding Assay
Cytoplasmic extracts were prepared from brain and cell lines as previously described for immunoprecipitation experiments without clarification. For cell line extracts, (300 μg total proteins) were used for immunoprecipitation experiments.

RNA binding reaction was conducted as follows: purified FMRP, 0,4 μg, and ^{32}P-labeled *BC1*, 0,05 ng$=$2x10^4 cpm, were incubated on ice for 30 min in 15μl of RNA binding buffer containing 10 mM HEPES (pH 7.9), 3 mM $MgCl_2$, 10 mM DTT, 100 mM KCl, 750 mM NaCl, 5% glycerol, 7 mM β−Mercaptoethanol, 15 μg Albumine, 20 μg Heparin. For competition experiments or supershift assays, unlabeled BC1 (5 ng) or tRNA (5 ng) or rAM1 Ab were preincubated with the protein for 10 min before addition of RNA probe. For brain extracts, 10 μg of proteins were incubated in the same buffer containing 150 mM NaCl. Samples were separated on a non-denaturing 5% polyacrylamide gel. Gel was exposed to a PhosphoImager for quantification.

Blocking of *BC1* RNA by DNA or 2′O-Me-oligonucleotide Interference
A whole brain was homogenized as described for the immunoprecipitation experiments. 1/100 of a brain was incubated in presence of 300 pmol, estimated to be 150× excess compared to the endogenous BC1 RNA, or 1500 pmol (750×) of *BC1* DNA oligonucleotide (*BC1* sl-1) or O-Me-RNA oligo (MWG Biotech AG) at 37°C for 20 min. After the incubation, FMRP immunoprecipitation and RT-PCR was performed as described below.

RT-PCR for Immunoprecipitated RNAs
To coimmunoprecipitate RNA, brain lysates were precleared for 1 hr with 20 μl protein A sepharose (preblocked with 0.1 μg/ml each BSA, yeast tRNA, glycogen) and immunoprecipitated as described above. DNase I (50 U RNase-free, Amersham Pharmacia Biotech) was added during washes. The immunoprecipitate was treated with 50 μg proteinase K (Sigma-Aldrich) for 15 min at 37°C. RNA was phenol/chloroform extracted and ethanol precipitated.

First-strand synthesis was achieved using $p(dN)_6$ and 100 U of M-MLV RTase (Invitrogen). An aliquot (3 μl) was used in a PCR reaction with Taq polymerase using gene-specific primers for *BC1*, *Arc*, *MAP1B*, and α-*Tubulin*.

FMRP Recombinant Proteins
The DNA plasmid containing the human FMRP C terminus fragment (nt 1545-1899) was a generous gift of Salvatore Adinolfi (MRC, London). The construct was expressed in *E. coli* strain BL21 (DE3). Human FMRP was produced in baculovirus Sf21 cells using a His-TAT tag in front of the full-length FMRP protein sequence to purify the recombinant protein (S.R. and B.O., unpublished data).

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Chapter V

Transport kinetics of FMRP containing the I304N mutation of severe fragile X syndrome in neuritesof living rat PC12 cells

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Transport kinetics of FMRP containing the I304N mutation of severe fragile X syndrome in neurites of living rat PC12 cells

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Abstract

Lack of fragile X mental retardation protein (FMRP) causes the fragile X syndrome, a common form of inherited mental retardation. The syndrome usually results from the expansion of a CGG repeat in the *FMR1* gene with consequent transcriptional silencing of *FMR1*. However, one missense mutation (Ile304Asn) was reported in the second KH domain of the protein involved in RNA binding. The protein containing this mutation showed an impaired function, leading to an extremely severe phenotype. In the present report, we have studied the role of FMRP I304N in living PC12 cells to better understand the (dys) function of this mutant FMRP. We have generated an *FMR1* I304N-EGFP stably transfected PC12 cell line with an inducible expression system (Tet-On) for regulated expression of the FMRP I304N-EGFP fusion protein. After Dox-induction, FMRP I304N-EGFP was localized in the neurites of PC12 cells; however, no granules were formed as has been recently demonstrated for the normal FMRP. Time-lapse microscopy in combination with bleaching technology illustrated that although FMRP I304N-EGFP does not form visible granules, the transport into the neurites is microtubule dependent. Immunoprecipitation with antibodies against GFP demonstrates that FMRP I304N-EGFP coprecipitate with both the 60S ribosomal protein P0 and FXR1P, suggesting that the mutant FMRP is still able to form complexes, however, with different characteristics compared to normal FMRP.
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Keywords: Time-lapse microscopy; Dendritic mRNA transport; RNP particle; Microtubules; Fragile X syndrome

Introduction

Fragile X syndrome is the most common form of inherited mental retardation, with a prevalence of 1:4000 for males and 1:6000 for females (Kooy et al., 2000). In the majority of the cases, the syndrome is caused by an expansion of a CGG repeat in the 5′ UTR region of the *FMR1* gene (Fu et al., 1991; Oberlé et al., 1991; Verkerk et al., 1991). This expansion leads to methylation of the CpG island in the promoter region of the *FMR1* gene, thereby silencing the gene and subsequently this results in the absence of the gene product, the fragile X mental retardation protein (FMRP) (Sutcliffe et al., 1992). Clinical manifestations in males with the fragile X syndrome include mental retardation, mild abnormal facial features, and macroorchidism.

FMRP is highly conserved between vertebrates. The protein contains several important structures like a nuclear localization signal and a nuclear export signal, which suggest the shuttling of the protein between the nucleus and the cytoplasm (Eberhart et al., 1996). Three RNA binding motifs were identified: two KH-domains and an RGG box (Ashley et al., 1993; Siomi et al., 1993). KH domains are present in several proteins involved in RNA regulation (Gibson et al., 1993; Siomi et al., 1993), whereas the RGG box in FMRP mediates the more specific RNA interaction via G-quartets (Brown et al., 2001; Darnell et al., 2001; Ramos et al., 2003a,b; Schaeffer et al., 2001). It has been shown that FMRP binds to 4% of the total fetal mRNAs in vitro, which confirm that FMRP has RNA binding capacities.

FMRP is predominantly expressed in the central nervous system and in the testis (Bakker et al., 2000; De Diego

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Otero et al., 2000; Devys et al., 1993; Feng et al., 1997b; Tamanini et al., 1997). The subcellular distribution is largely cytoplasmic, although nuclear localization has occasionally been found (Bakker et al., 2000; Feng et al., 1997b; Willemsen et al., 1996). In nerve cells, most of the FMRP is found in the cell soma associated with ribosomes; however, small quantities are also found in dendrites in particular within dendritic spines (Feng et al., 1997b; Weiler et al., 1997). The association with ribosomes is mRNA dependent and is mediated by ribonucleoprotein particles (RNP) (Eberhart et al., 1996; Feng et al., 1997a; Khandjian et al., 1996; Tamanini et al., 1996). Two homologues have been identified, named FXR1P and FXR2P, and the three proteins form a small family of proteins, the FXR proteins. They are able to form homo- and heterotypic interactions with FMRP, and both FXR1P and FXR2P contain the same functional motifs as FMRP (Siomi et al., 1995; Zhang et al., 1995).

Although the precise physiological function of FMRP is not yet defined, it has been suggested that the protein plays a role in both transport and translational regulation of mRNAs at the synapses (Brown et al., 2001; Darnell et al., 2001; Miyashiro et al., 2003). Dendritic mRNA transport plays an important role in neuronal processes including modulation of synaptic plasticity (Kiebler and DesGroseillers, 2000). The absence of FMRP could lead to abnormalities in mRNA transport and efficiency of protein synthesis at the synapse, particularly after mGluR activation, and this is the proposed basis of the mental retardation in fragile X syndrome (Huber et al., 2002). Recently, we have shown that FMRP is transported in a microtubule-dependent way from the cytoplasm into the neurites via large granules, containing other proteins including the plus-end-directed motor protein kinesin, the ribosomal protein P0, and the FMRP-related protein FXR1P (De Diego Otero et al., 2002). The granules move with an average speed of 0.19 μm/s, which is in accordance with granular mRNA transport kinetics (Ainger et al., 1993; Knowles et al., 1996; Muslimov et al., 1997; Rook et al., 2000).

Although the vast majority of mutations in the *FMR1* gene lead to the loss of FMRP, one missense mutation has been described (Ile304Asn) in a patient with an extremely severe phenotype (De Boulle et al., 1993). The mutation, present in the second KH domain, concerns a highly conserved hydrophobic amino acid, which results in expression of mutant FMRP with an impaired RNA binding and folding instability (Musco et al., 1996, 1997; Ramos et al., 2003a,b; Siomi et al., 1993). Furthermore, it was shown that the mutant protein no longer associates with translating polyribosomes, is part of an abnormal RNP particle, and loses its function as a translational repressor due to the loss of homo-oligomerization (Feng et al., 1997a; Laggerbauer et al., 2001; Siomi et al., 1994; Verheij et al., 1995). Since the mutation leads to an unusual severe phenotype, studying the characteristics of this protein is of interest to understand the physiological function of normal FMRP.

Materials and methods

Cell culture, expression vectors, and transfection

Rat pheochromocytoma cells (PC12) with a Tet-On gene expression system were used; this cell system permits tightly regulated expression of the *FMR1* gene in response to doxycycline (Dox; doxycycline hydrochloride; Sigma #D9891: 1 μg/ml). PC12 Tet-On cells were obtained from Clontech and grown as specified by the manufacturer. Briefly, cells were cultured in DMEM medium supplemented with 10% horse serum, 5% fetal bovine serum, 125 μg/ml hygromycin, 100 μg/ml penicillin/streptomycin, and 100 μg/ml G418 in a 10% CO_2 incubator at 37°C. PC12 cells were differentiated into a neuronal phenotype on collagen-coated coverslips in medium supplemented with 100 ng/ml nerve growth factor (NGF-7S; Sigma) for 72 h (DMEM + G418 + hygromycin + 0.75% horse serum + 0.25% fetal bovine serum).

Recently, a PC12 cell line stably transfected with FMRP-EGFP with an inducible expression system (Tet-On) was generated (De Diego Otero et al., 2002). As a next step, we would like to generate a similar cell line for the I304N-mutated FMRP. Therefore, the pFMRP-I304N-EGFP construct (pFMRPmt-GFP)(Castren et al., 2001) was cloned into the pTRE response plasmid (Clontech) to generate a double stably transfected Tet-On cell line, using the lipofectamin procedure (30 μl lipofectamin from Gibco BRL and 20 μg plasmid DNA). The reading frame of the fusion plasmid was controlled by sequencing. A pHyg resistance vector was used in the cotransfection as a selection marker. Transfected cells were cultured in medium containing Hygromycin (0.125 mg/ml; Gibco BRL) and Dox (1 μg/ml), and subsequently individual double stable cell colonies were tested and further selected for the presence of the FMRP I304N-EGFP fusion protein using fluorescence microscopy. Several cell lines (n = 12) were selected both on the basis of the presence of FMRP I304N-EGFP fusion protein after Dox treatment and the absence of the FMRP I304N-EGFP fusion protein without Dox treatment (leakage) and further investigated by Western blotting and immunocytochemistry. PC12, PC12 Tet-On cells, and primary cultures of hippocampal neurons from both wild-type and *Fmr1* knockout mice were used for transiently transfection studies. For the transient transfection protocol, we used the lipofectamin procedure according to the manufacturer with 1 μg plasmid DNA. To obtain primary cultures, hippocampi were dissected from E18 old mouse brains and prepared and cultured as described before (Tamanini et al., 1997).

Antibodies and reagents

The following antibodies were used: monoclonal and rabbit polyclonal antibodies against human FMRP (clone 1C3 and KI, respectively) (Devys et al., 1993; Willemsen

et al., 2003), monoclonal anti-GFP antibody (Roche USA; #1814460), monoclonal anti-FXR1P (3FX) (Khandjian et al., 1998), monoclonal anti-tyrosine tubulin (clone TUB-1A2; Sigma), human anti-ribosomal P antigen (P0; Immunovision), and monoclonal anti-kinesin heavy chains (CAMPRO scientific). Texas red-labeled phallotoxins were used to visualize F-actin (T-7471; Molecular Probes). As secondary antibodies, we used the following: swine anti-rabbit Ig conjugated with HRP (P0217; DAKO), sheep anti-human Ig conjugated with HRP, rabbit anti-mouse Ig conjugated with HRP (P0260; DAKO), and rabbit anti-mouse Ig conjugated with FITC. All drug treatments were carried out in DMEM medium (complete) at 37°C. To disrupt microfilaments, cells were incubated with cytochalasin-D (5 μg/ml; Sigma) for 30 min. Depolymerization of microtubules was accomplished by incubation of the cells with nocodazole (10 μM; Sigma) for 30 min.

Selection of cell lines using Western blotting

PC12 Tet-On cells and 12 selected PC12 Tet-On cell lines stably transfected with pFMR1 I304N-EGFP (coded PC12/MT1-PC12/MT12) were grown with and without Dox. Cells were harvested after 24 h and lysates from the different cell lines were run on a 10% gel and blotted onto nitrocellulose. Immunodetection of the FMRP I304N-EGFP fusion protein was performed using monospecific antibodies against GFP followed by incubation with secondary antibodies conjugated with peroxidase allowing detection with the chemiluminescence method (ECL kit, Amersham). To compare the relative quantitative estimates between endogenous wild-type FMRP and the newly synthesized FMRP I304N-EGFP fusion protein, PC12 Tet-On cells, FMR7 cells and the selected MT7 cell line were harvested as described above and Western blot was performed using rabbit antibodies against FMRP (KI) (Willemsen et al., 2003). In each lane 25 μg of total protein was loaded. In the same experiment, anti-actin antibodies were used to show that equivalent amounts of protein from the different cell lines have been applied to each lane (data not shown). The intensity of the FMRP-EGFP fusion proteins and the endogenous FMRP was measured on the Kodak image station 440 CF. The intensity of the endogenous protein was set as 1. The intensity of the fusion proteins was compared to that of the endogenous FMRP protein.

Immunocytochemistry

FMRP-EGFP and FMRP I304N-EGFP distributions in transiently transfected PC12 and PC12 Tet-On cells were studied 1, 2, 3, and 4 days after transfection using either the pFMR1-EGFP or the pFMR1-I304N-EGFP expression plasmids (Castren et al., 2001). Before the transfection procedure, cells were seeded on collagen-coated coverslips and differentiated for 3 days with NGF. The fusion proteins were detected by direct immunofluorescence for GFP fluorescence signal (see below). Quantification of the percentage of neurites containing granules was determined by counting 100 transfected cells (GFP signal in cell soma). For the stably transfected cell lines, cells were cultured on collagen-coated coverslips and treated with NGF for 72 h followed by treatment with both Dox and NGF for times varying from 0.5 to 72 h. For direct visualization of GFP by immunofluorescence, cells were fixed with 95% ethanol for 30 min at room temperature (RT). Wash steps were carried out with 0.1 M phosphate-buffered saline (PBS) and coverslips were mounted with Vectashield mounting medium. Cells were examined with a Leitz fluorescence microscope using a 63× objective, standard FITC, and TRITC filters, a 100-W HBO mercury light source, and a Sony DXC-95OP 3CCD color video camera.

Immunoprecipitation

PC12 Tet-On cells and 12 selected PC12 Tet-On cell lines stably transfected with pFMRP I304N-EGFP (coded PC12/MT1-PC12/MT12) were grown with NGF in the presence or absence of Dox). Cells were harvested in IP buffer containing 20 mM Tris, pH 7.5, 2.5 mM $MgCl_2$, 100 mM KCl, 25 mM EDTA, 0.5% NP-40, and protease inhibitors and kept on ice in IP buffer for 30 min. Subsequently, cells were scraped from the dishes and centrifuged at 10,000 rpm at 4°C for 10 min. The supernatant was precleared with prot G sepharose beads for 2 h at RT after which anti-GFP antibodies were added followed by an incubation o/n at 4°C and 2 h incubation with prot G beads at RT. The pellet was washed three times with PBS and sample buffer was added before SDS-PAGE analysis.

Confocal microscopy

Stably transfected PC12 cells (PC12/MT7 clone) were grown on coverslips in the presence of both NGF and Dox. Time-lapse microscopy recording at physiological temperature (37°C) of living cells was performed using a Zeiss LSM510NLO microscope. The following setup was used: excitation 488 nm, 0.5% acoustic optical tunable filter, HFT 488, emission filter BP 500–550, and tube current 6.1A. Simultaneously with the fluorescent image, a Nomarsky image was made.

For photo bleaching, the growth cones of the neurites were exposed to a prolonged and extensive excitation (laser power 100%, 30 s), which causes an irreversible loss of fluorescence.

For quantification, stably transfected cells were chosen for positive signal of EGFP and images were recorded and evaluated for the intensity of fluorescence within the growth cone by the Zeiss advanced imaging microscopy (AIM) software package.

Results

A PC12 cell line was used for our studies since it is a commonly applied model for neuronal differentiation. Upon exposure to NGF, the PC12 cells form neurites but no axons; however, the differentiated cells do contain synaptic-like vesicles. A Tet-On expression system was used to allow a regulated expression of FMRP in response to Dox. FMRP I304N was fused to EGFP to follow the transport of the mutant protein (FMRP I304N-EGFP) in living cells using immunofluorescence and confocal microscopy (Fig. 1).

Several stably transfected Tet-On cell lines with pFMRP I304N-EGFP were selected for GFP expression by direct immunofluorescence microscopy. From those cell lines, one was selected that showed moderate FMRP I304N-EGFP expression and negligible leakage of FMRP I304N-EGFP without supplementation of Dox. The moderate FMRP I304N-EGFP expression was important because overexpression of the normal FMRP has not only been shown to be toxic for the cells (Ceman et al., 1999) but would also not reflect the endogenous expression of FMRP. Fig. 2A shows the FMRP I304N-EGFP expression of the selected cell line (MT7) on Western blot with and without Dox using antibodies against GFP. The observed molecular weight of approximately 110 kDa is in line with the expectation, that is, 70–80 kDa for FMRP I304N and 27 kDa for EGFP. Only a very slight leakage can be seen for MT7 cells without Dox; however, this was not significant compared to MT7 cells after induction with Dox. As a negative control, the original PC12 Tet-On cell line was included that was used to generate the stably transfectant cell line. To obtain relative quantitative estimates between the endogenous wild-type FMRP and the newly synthesized FMRP I304N-EGFP fusion protein, Western blot analysis was also performed using monospecific antibodies against FMRP. Fig. 2B illustrates the endogenous rat FMRP expression in PC12 Tet-On, FMR7, and MT7 cell lines. In PC12 Tet-On cells, only endogenous wild-type FMRP can be detected with a molecular mass of approximately 70–80 kDa; whereas in FMR7 and MT7 cells, two prominent bands of 70–80 kDa and 110 kDa are present, illustrating the presence of wild-type FMRP and the FMRP I304N-EGFP fusion protein, respectively. The intensity of the fusion protein band was 1.4 for wild-type FMRP-EGFP and 0.5 for FMRP I304N-EGFP compared to the endogenous FMRP protein band, illustrating almost endogenous expression levels of the fusion proteins.

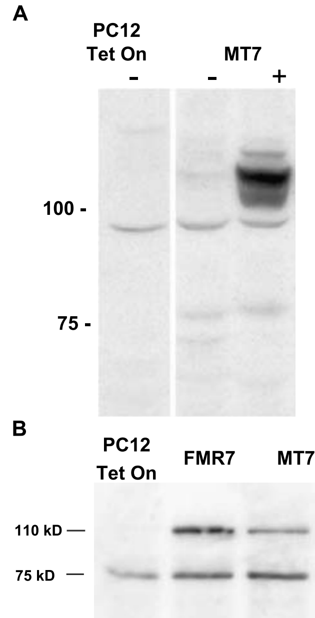

Fig. 2. Western blot of the stably transfected PC12/MT7 cell line. (A) PC12 Tet-On cells and PC12/MT7 cells were analyzed by Western blotting for the expression of the FMRP I304N-EGFP fusion protein. The blot was stained with anti-GFP antibodies. The PC12/MT7 cells treated with Dox showed expression of the fusion protein at the expected size of 110 kDa. The untreated cells showed a very weak band indicating that there is slight leakage. The PC12 Tet-On cells showed no expression, as was expected. (B) PC12 Tet-On cells, PC12/FMR7 cells, and PC12/MT7 cells were analyzed by Western blotting for the expression of the GFP fusion protein compared to the endogenous FMRP protein. The blot was stained with anti-FMRP antibodies. The PC12 Tet-On cells only show the endogenous FMRP protein, whereas the FMR7 and the MT7 cells express both the endogenous (lower band) and the GFP-fusion protein (upper band). The levels of the GFP fusion protein compared to the endogenous FRMP are comparable, which indicates that there is no significant overexpression of the fusion protein in both the FMR7 and MT7 cells.

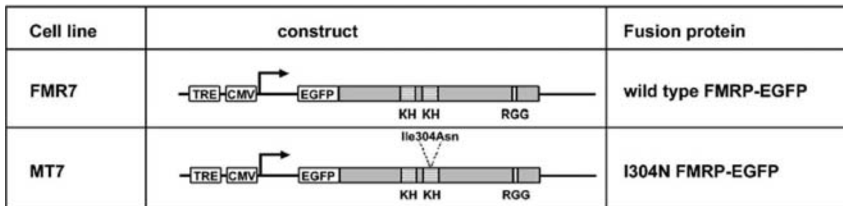

Fig. 1. Scheme showing the two different cell lines used in this study, the construct used for stable transfection, and the fusion protein that is expressed in these cell lines.

For further characterization of the MT7 cells, the expression pattern and cellular localization were followed in time after induction with Dox. Fig. 3 shows the expression of the fusion protein at time 0 (A), 30 min (B), and 2.5 h (C) after induction with Dox. After 30 min, FMRP I304N-EGFP is solely expressed in the cell soma. The expression pattern of FMRP-EGFP (FMR7 cell line) was compared with that of FMRP I304N-GFP (MT7 cell line) after 2.5 h of Dox treatment. As illustrated in Figs. 3C and 3D both FMRP-EGFP (Fig. 3D) and FMRP I304N-EGFP (Fig. 3C) expressions are present in neurites; however, in the FMR7 cell line granules are formed, whereas no visible granules were formed in the neurites from the MT7 cell line. Instead, FMRP I304N-EGFP was equally distributed within the neurites. To confirm the physiological relevance of this distribution in better-defined polarized neuronal cells, primary cultures of mouse hippocampal neurons from *Fmr1* knockout mice were transiently transfected with either CMV-FMR1 I304N-EGFP (Fig. 3E) or CMV-FMR1-EGFP (Fig. 3F). Although these primary cultures do not allow tightly regulated expression for both the mutant and the normal FMRP, a similar expression pattern was observed as for the MT7 cells. Only very occasionally a single granule could be detected in the proximal dendrites. In addition, the MT7 cells (without Dox treatment) were stained for endogenous wild-type FMRP using antibodies against FMRP. The expression pattern of the endogenous wild-type FMRP was similar to that of the FMR7 cell line and of wild-type primary neurons (data not shown).

In an earlier study, we have reported the subcellular distribution of FMRP and its I304N-mutated form in transiently transfected PC12 cells that overexpress the protein in high quantities (Castren et al., 2001). We described the presence of granules for both the wild-type and mutant

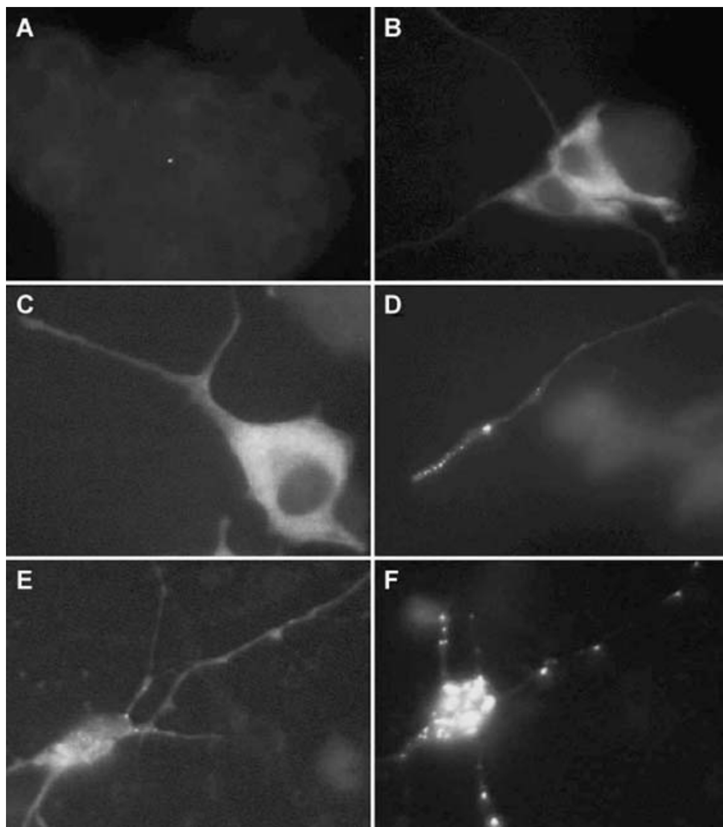

Fig. 3. Visualization of the FMRP I304N-EGFP fusion protein in stably transfectant cell line MT7. The FMRP I304N-EGFP protein was visualized by direct immunofluorescence. Expression of the FMRP I304N-EGFP fusion protein was followed in time after induction with Dox. (A) Time = 0; (B) time = 30 min; (C) time = 2.5 h; (D) normal FMRP-EGFP after 2.5 h Dox. (E) Mouse primary hippocampal neurons transiently transfected with FMR1 I304N-EGFP; (F) mouse primary hippocampal neurons transiently transfected with FMR1-EGFP. Magnifications: (A and B) 720×; (C and D) 960×; (E and F) 750×.

FMRP along the neuronal processes. In the present study, we use stably transfected PC12 Tet-On cells to allow tightly regulation of expression and expression levels at endogenous levels to ensure physiological conditions. To explain the discrepancy between these studies and to study the effect of overexpression on the formation of granules in more detail, transient transfection protocols were performed with both FMRP-EGFP and FMRP I304N-EGFP expression plasmids using PC12 and PC12 Tet-On cells. The formation of granules was studied at different time points (1, 2, 3, and 4 days) after transfection and the percentage of neurites containing granules was determined. Transiently transfected PC12 (data not shown) and PC12 Tet-On cells with high FMRP-EGFP expression showed a similar intense labeling of the cell soma and the presence of granules within the neurites (Fig. 4A for PC12 Tet-On cells at 4 days after transfection). The percentage of neurites containing granules gradually increased in time (Fig. 4C; wt). In contrast, transiently transfected PC12 (data not shown) and PC12 Tet-On cells with FMRP I304N-EGFP expression showed an intense labeling of the cell soma; however, neurites were mostly devoid of granules but instead an equally distributed fluorescence signal could be detected in neurites (Fig. 4B for PC12 Tet-On cells 4 days after transfection). A minority (<20%) of the transfected cells showed neurites with a few granules, however, only in those cells that expressed extremely high levels of FMRP I304N-EGFP (Fig. 4C; point mutation).

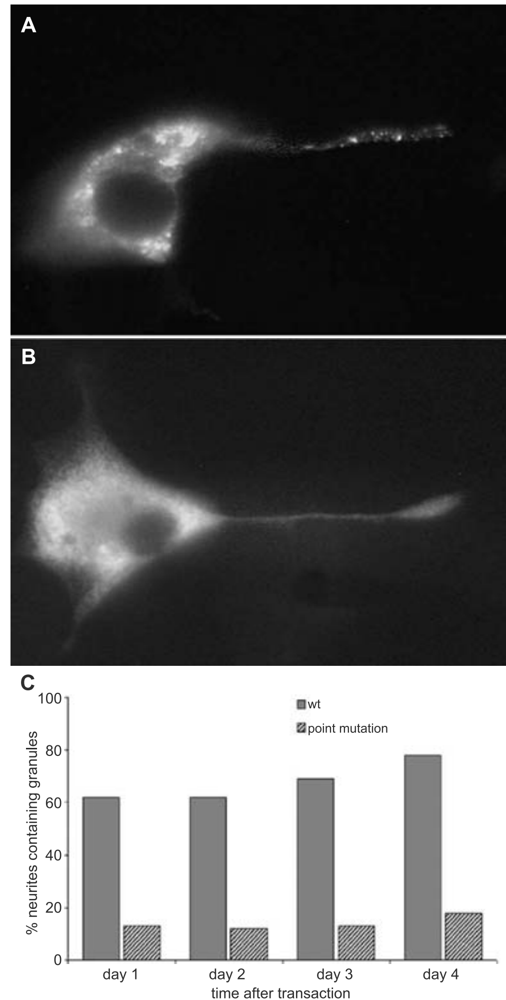

Fig. 4. Transient transfection of PC12 Tet-On cells. PC12 Tet-On cells were transiently transfected with *FMR1*-EGFP or *FMR1* I304N-EGFP. Pictures of wild-type FMRP (A) and FMRP with the point mutation (B) were taken 4 days after transfection. The transfected cells, illustrated by GFP expression in the cell soma, were checked for the presence of granules within the neurites. The neurites containing granules were counted as a percentage of total number of cells expressing GFP (C).

Transport of FMRP I304N

Since FMRP I304N-EGFP was visible in the neurites but did not form visible granules, the question was raised whether the protein is still actively transported into the neurites or entered by diffusion. We have previously shown that normal FMRP-EGFP is transported into the neurites via microtubules with kinesin as the likely motor protein. The FMRP I304N-EGFP fusion protein did not form granules; thus, the transport of individual granules could not be measured by time-lapse microscopy. Alternatively, transport kinetics can be studied by photo bleaching, a prolonged and extensive excitation, which causes an irreversible loss of fluorescence. In the present study, parts of the neurite and growth cone of several neurites were bleached and the intensity of the FMRP I304N-EGFP signal was measured in time. The results are depicted in Fig. 5A and show that in MT7 cells treated with Dox, the FMRP I304N-EGFP signal was returned to 80% of the starting level after 10 min indicating an active transport mechanism. To further investigate which active transport system was used (microtubules or microfilaments), cytoskeleton-disrupting drugs were added to the MT7 cells after treatment with Dox. Exposure of the cells to cytochalasin D (cytD), which disrupts the actin filaments, had no effect on the transport of FMRP I304N-EGFP (Figs. 5B and 5D). However, after treatment with nocodazole, which depolymerizes microtubules, the FMRP I304N-EGFP signal did not return (Figs. 5C and 5E), indicating that the microtubules are indeed involved in the transport of FMRP I304N-EGFP. The specificity of the drugs used in these experiments was demonstrated by double staining protocols with antibodies against both tubulin (Figs. 6A and 6C) and F-actin (Figs. 6B and 6D), either in the presence of nocodazole (Figs. 6C and 6D) or the presence

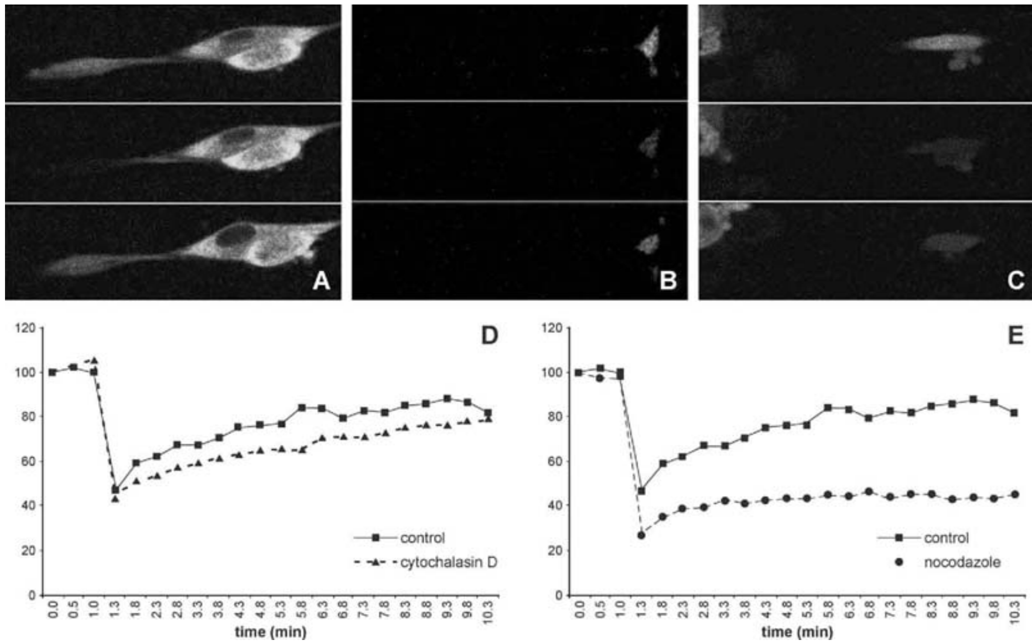

Fig. 5. Transport dynamics of FMRP I304N-EGFP in neurites of MT7 cells. Part of the neurite and the growth cone was photo bleached for 30 s. The intensity of the EGFP signal was followed in time. (A) Untreated MT7 cells; (B) MT7 cells treated with cytochalasin D; (C) MT7 cells treated with nocodazole. Pictures were taken before (top), directly after (middle), and 8 min (bottom) after bleaching. (D and E) The intensities of the EGFP signal were plotted. Scans were made every 30 s; the growth cone was bleached after 1 min of measuring.

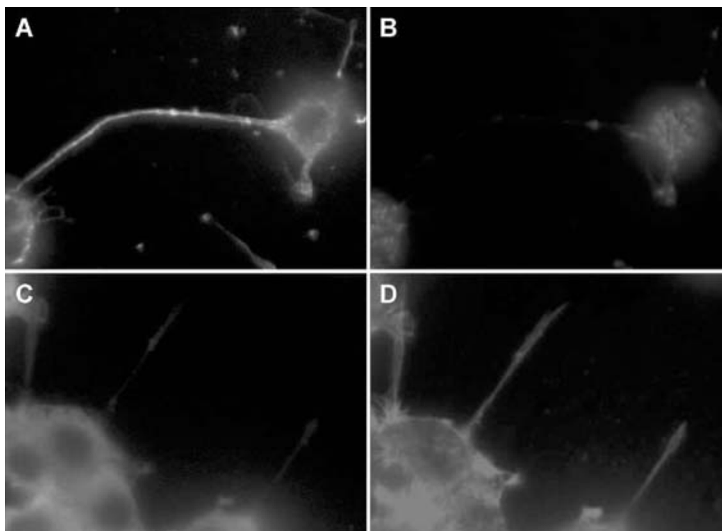

Fig. 6. Disruption of the cytoskeleton after treatment with either cytochalasin D or nocodazole. As a control to show that the inhibitors used in this study did not disrupt the complete cytoskeleton, MT7 cells were treated with either cytochalasin D (A and B) or nocodazole (C and D) for 30 min. After treatment, the cells were fixed in acetone and double stained for F-actin (B and D) and tubulin (A and C).

of cytD (Figs. 6A and 6B). Fig. 6B shows that treatment with cytD disrupted only the actin filaments, while the microtubules (Fig. 6A) were still intact. Nocodazole disrupted the microtubules (Fig. 6C), but not the actin filaments (Fig. 6D).

Immunoprecipitation of the FMRP I304N compared to wt FMRP

A next step would be to identify other proteins that interact with FMRP I304N-EGFP using an immunocytochemical approach. However, the lack of FMRP I304N-EGFP-positive granules in the neurites of MT7 cells made it difficult to perform colocalization studies at the light microscopic level using double labeling protocols. Nevertheless, proteins known to interact with FMRP and proteins already known to be present in RNP particles (e.g., FXR1P, kinesin, and P0) were localized in the MT7 cell line. These proteins were present in large granules in the neurites; thus, granule formation was not affected in the MT7 cells (data not shown).

Alternatively, immunoprecipitation studies were performed to identify FMRP I304N-EGFP-interacting proteins in MT7 cells after treatment with Dox for 72 h. Anti-GFP antibodies were used for the immunoprecipitation to circumvent the coprecipitation of endogenous FMRP. Fig. 7 (top) illustrates that in untreated MT7 cells, no FMRP I304N-EGFP could be detected; whereas after induction with Dox, an FMRP I403N-EGFP band at 110 kDa could be detected. FMR7 cells, expressing FMRP-EGFP, were used as a control and similar results were observed. After induction with Dox, both the normal FMRP-EGFP and FMRP I304N-EGFP showed coprecipitation with the ribosomal protein P0 and the FMRP-related protein FXR1P. The specificity of the coprecipitation was shown by the absence of interacting proteins in cells that were not treated by Dox.

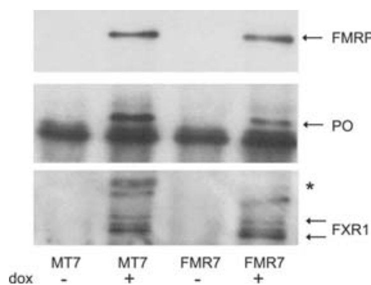

Fig. 7. Immunoprecipitation of normal and mutant FMRP. Antibodies against GFP were used to immunoprecipitate either the FMRP I304N-EGFP (MT7 cell line) or normal FMRP-EGFP (FMR7 cell line), untreated (−), or treated with Dox (+). The blots were stained for FMRP, the 60S ribosomal protein P0, and FXR1P. *Cross reactivity of the FXR1 antibody with FXR2 (Khandjian et al., 1998).

Discussion

A growing body of evidence supports a role of FMRP in both dendritic transport and local dendritic protein synthesis (reviewed Willemsen et al., 2004). As a first step to determine in vivo the role of FMRP in dendritic mRNA transport, a PC12 cell line stably transfected with human FMR1-EGFP fusion gene with an inducible expression system (Tet-On) has been generated (De Diego Otero et al., 2002). Using time-lapse microscopy, the movement of FMRP-EGFP-positive granules was demonstrated from the cell soma into the neurites of living PC12 cells. The movement of the granules containing RNA, ribosomal subunits, FXR1P, and kinesin within the neurites was microtubule dependent. To further delineate the precise role of FMRP in dendritic mRNA transport, we have investigated the I304N missense mutation in the FMRP protein, which is of particular interest since the patient carrying this mutation is the only known patient with a missense mutation who presented a more severe phenotype than fragile X patients lacking the protein (De Boulle et al., 1993). The I304N mutation is present in a highly conserved part of the protein involved in RNA binding. This mutation leads to normal expression levels of FMRP that is still able to interact with polyA-mRNA; however, its function in vitro as a translational repressor has disappeared due to loss of homo-oligomerization (Feng et al., 1997a; Laggerbauer et al., 2001). Oligomers cannot be formed between two FMRP molecules that both carry the missense mutation (Laggerbauer et al., 2001). Instead, the mutant FMRP is incorporated in abnormal RNP particles that do not associate with polyribosomes. Studies on the characteristics of the mutant FMRP, including transport kinetics and protein–protein interactions, in neurons are of special interest to understand the functional specificity of FMRP (protein–mRNA interactions).

We created a PC12 Tet-On cell line expressing an FMRP I304N-EGFP fusion protein, which made it possible to follow the protein in living cells using time-lapse microscopy. The inducible system was chosen to carefully control the expression levels of the protein. This inducible system leads to expression levels of the fusion protein in the range of wild-type FMRP levels, which was logically not toxic for the cells. Thus, our stably transfected cell line enable us to perform transport studies in living cells under physiological conditions.

The FMRP I304N-EGFP fusion protein was located predominantly in the cell soma; however, the protein was also present in the neurites. Interestingly, the mutant FMRP could not be detected in granules as seen for wild-type FMRP. This result is in contrast with earlier studies from Castren et al. (2001), who demonstrated granular labeling along the neuronal processes with the same pFMR1 I304N-EGFP plasmid using transient transfection protocols in PC12 cells. Therefore, we studied the formation of granules in more detail using transiently transfected PC12 and PC12

Tet-On cells. In the vast majority of transient transfected cells, the mutant FMRP showed a diffuse staining of the neurites, which was similar to our MT7 cells. Apparently, the very high overexpression of mutated FMRP in the earlier study results in artificial aggregate formation, a phenomenon that is often seen in overexpression studies. In the present study, we use 1 μg of plasmid DNA for the transient transfection; whereas in the earlier study from Castren et al. (2001), 3 μg of plasmid DNA was added to the transfection mixture, which may explain the very high overexpression and consequently the formation of aggregates. Alternatively, very high levels of mutated FMRP may influence granule formation in a positive way; however, this result does not reflect the characteristics of mutant FMRP under physiological conditions.

Thus, the missense mutation prevents or may delay the incorporation of mutant FMRP into granules for efficient dendritic transport, as described for normal FMRP and several other RNA binding proteins (Bassell et al., 1999; De Diego Otero et al., 2002; Ohashi et al., 2002; Tang et al., 2001). Despite of the lack of FMRP I304N-EGFP-positive granules in the neurites, the mutant FMRP could be detected in the neurites, suggesting that either the protein can be actively transported into neurites or local translation of *FMR1* mRNAs occurs. Photo-bleaching experiments in combination with the use of specific drugs that disrupt either the microfilaments or microtubules showed a microtubule-dependent transport of FMRP I403N-EGFP into neurites, as was reported for normal FMRP previously (De Diego Otero et al., 2002).

By using an antibody against GFP, we were able to immunoprecipitate the fusion protein specifically and not the endogenous FMRP. In PC12 cells stably transfected with normal FMR1-EGFP, a band at the height (70–80 kDa) of endogenous FMRP could sometimes be seen (data not shown); however, cells stably transfected with FMR1 I304N-EGFP never showed the presence of this band in the GFP-specific immunoprecipitation studies, indicating that mutant FMRP I304N-EGFP did not bind endogenous normal FMRP. Furthermore, these results strongly indicate that the FMRP I304N-EGFP-interacting proteins do not associate with the mutant protein via endogenous normal FMRP. On the other hand, we demonstrated that mutant FMRP efficiently co-immunoprecipitate with P0, a 60S ribosomal component, indicating the in vivo association of mutant FMRP, present in abnormal RNP particles, with the large ribosomal subunit. Apparently, the abnormal RNP particles containing mutant FMRP are still able to associate with the large ribosomal subunit but are missing critical constituents that are required for their further association with polyribosomes (Feng et al., 1997a). Its role in polysomal association has been further attributed by Tamanini et al. (1999), who demonstrated that mutant FMRP shows an increased shuttling to the nucleus, and this is thought to occur because the mutant protein does not bind to active polyribosomes present in the cytoplasm. The interaction with FXR1P is in accordance with the findings of Feng et al. (1997a).

The binding of the motor protein kinesin to RNP particles has been proposed to be indirect (Ohashi et al., 2002), suggesting that FMRP does not have to be part of an active polysome to be transported actively. Thus, in our case the abnormal RNP complex did not have to influence kinesin binding. Unfortunately, we were not able to demonstrate the binding of FMRP I304N-EGFP to the motor protein kinesin. Since kinesin is released from the protein complex when NP-40 is added to the buffer (Ohashi et al., 2002), several other protocols with milder detergents or with the omission of detergents have been applied, however, without success.

As a control experiment for the distribution of the FMRP I304N-EGFP in the absence of endogenous FMRP primary neurons of *Fmr1*, KO mice were transiently transfected. A similar distribution pattern of the mutant FMRP was found as for the stably transfected PC12 cells. This again indicates that FMRP I304N-EGFP is not transported via endogenous normal FMRP. Importantly, these results imply that oligomers are not formed between FMRP and mutant FMRP; thus, in our MT7 cell line homo-oligomerization of mutant FMRP does not occur, which mimics the actual situation in cells from the patient with the missense mutation (Laggerbauer et al., 2001).

We speculate that mutant FMRP shows impairment in polyribosomal binding due to loss of homo-oligomerization, and this is preventing the incorporation into the macromolecular structure of the granules. Thus, incorporation of FMRP in granules must be functionally important. Recent studies have shown the presence of components of the protein machinery within these RNP-positive granules, including clusters of polyribosomes (Knowles et al., 1996). Mutant FMRP is able to sequester target mRNAs; however, these mRNAs are incorporated in nontranslatable RNP particles (Feng et al., 1997a; Laggerbauer et al., 2001). The presence of the protein machinery at the postsynaptic site of dendrites is essential for neurons to rapidly respond on stimuli at particular synapses through local translation of specific mRNAs. Thus, the sequestration of specific mRNAs in nontranslatable RNP particles near synapses results in impairment of controlled efficient translation of those specific mRNAs. This might be the underlying cause of the more severe fragile X phenotype by the I304N missense mutation. Alternatively, the abnormal RNP particles may bind irreversible to large ribosomal subunits, and thereby blocking translation of other important mRNAs or mutant FMRP may bind to other proteins present within the RNP particle and consequently their function could also be disturbed leading to a more severe fragile X phenotype. Total absence of FMRP in neurons, as observed in almost all fragile X patients, may result in a milder phenotype because the target mRNAs of FMRP can be partially translated near synaptic connections within the dendrite via alternative RNP particles.

Studying mutations, particularly in conserved domains of FMRP, will lead to new insides into the function of this protein. In the case of FMRP, not only the biochemical properties are important but also the subcellular localization within neuronal cells since FMRP has an important function in both mRNA transport to the dendrites and regulation of local mRNA translation.

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Chapter VI

Isolation of mouse neuritic mRNAs by a two-step two-layer cell culture system

Chapter VI

Isolation of mouse neuritic mRNAs by a two-step two-layer cell culture system

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Abstract

Impaired local protein translation at postsynaptic sites has been hypothesized to be the cause of several neurological disorders such as fragile X syndrome, neurofibromatosis-1, Rett syndrome, and other syndromic and non-specific forms of mental retardation. Identification of which mRNAs are present in dendrites and revelation of the molecular pathways that they promote will be imperative to the understanding of the neuropathology of these diseases. Mouse models are the most widely used animal models of human diseases, since (a) the mouse genome has a high homology to the human genome; (b) it is relatively easy to create knockout models in order to mimic human diseases, and (c) it consequently allows comparison between the normal and the disease related situation. We therefore developed a cell culture based technique to isolate mRNA from mouse neurites. Neurites are the sum of the axon and the dendrites of a neuron, regardless of the developmental stage of the cell. Although mRNAs have been shown to be present also in axons, the majority of neuritic mRNA is located at postsynaptic sites. We therefore propose, that (a) our cell culture technique enables the comparison between the mRNA pool of wild-type cells versus knockout cells; (b) it will provide the means to identify which mRNAs are present in neurites; (c) it will permit valuable insight into the neuropathology of many diseases.

Introduction

Cognition comprises all the neuronal and intellectual pathways through which information is received, processed, stored, recalled, and handled by the brain. The capacity to form long-lasting memories and to retrieve these memories when needed lies at the basis of human cognition. Personal memories and experiences define the individual being. Fundamental learning, memory formation, and memory retrieval are highly conserved capacities and are essentially driven by the genetic make-up of the brain. Throughout life, the brain maintains its capability to shape and reshape, to learn and to remember, and to recover after injury. These abilities are summarised with the term *plasticity*. Activity-dependent plasticity may manifest itself in the size of a specific brain region, the number and nature of neuronal connections, and the architecture of dendritic branches. Synaptic plasticity in particular occurs through

means of changes in synaptic strength, long-term potentiation (LTP), long-term depression (LTD), changes in neurotransmission, and the number and architecture of glutamate receptors. Both, LTP and LTD, have been implied in memory formation. The presence of dendritic polyribosomes in close proximity to spines [1] and the characterisation of selective localisation of particular mRNAs within dendrites [2, 3] led to the hypothesis that protein synthesis may occur locally in dendrites and that it is a prerequisite for proper synaptogenesis [4]. Evidence has been provided that potentiation of hippocampal Schaeffer collateral synapses, long-term facilitation of *Aplysia* sensory-motor synapses, LTP, long-term spatial memory, associative fear conditioning, and object recognition memory all depend on dendritic protein translation [5-8]. The actual verification of active protein synthesis in dendrites has been provided by Torre and Steward, who physically isolated neurites from their cell bodies. Subsequently they demonstrated protein translation by pulse labelling neurites with ^{3}H-leucine. While dendrites (identified by immunocytochemistry with specific antibodies) were heavily labelled, axons were hardly if at all labelled [9]. The rationale of protein synthesis at the synapse is to enable a neuron to quickly respond to signalling events, rather than having to await a relatively slow protein delivery from the cell body. Spatial distribution of specific mRNAs to distinct cellular compartments has been shown in many cells. A dendrite specific expression of mRNAs has been confirmed for *MAP2, Arc, CaMKII*, and glutamate receptors [2, 10-12]. Asymmetric distribution and site restricted translation of specific mRNAs may be a means to reduce unwanted effects of a protein at inappropriate sites. Responsible for establishing an asymmetric localisation of mRNAs are ribonucleoprotein particles (RNPs). RNPs are high molecular weight complexes comprising mRNAs, RNA binding proteins, translational factors, and ribosomal subunits [13-15]. The RNPs fulfil three important functions for proper synaptic processes: they transport specific mRNAs from the cytoplasm to the synaptic sites, they provide the translational machinery, and they control the translation.

The significance of mRNA transport, asymmetric mRNA distribution, and signal-related initiation of local protein synthesis for proper synaptic processes is demonstrated by numerous neurological diseases. Genetic aberrations caused by a disruption of the capacity for memory formation manifest in individuals as pronounced cognitive deficits. The most common form of inherited mental retardation, the fragile X syndrome (FRAXA), has been related to a defect in synaptic plasticity. FRAXA is caused by the lack of the fragile X mental retardation protein 1 (FMRP). Fragile X patients as well as a null mutant mouse model of the fragile X mental retardation gene 1 (*FMR1)* show impaired spine architecture, impaired spine maturation, and deficits in hippocampal-related memory function [16-19]. Further evidence for a defect in synaptic plasticity to be causative to FRAXA is the finding of enhanced LTD in *Fmr1* knockout mice [20-22]. It has been hypothesized that FMRP normally binds to a specific subset of messenger RNAs, that it is involved in (or mediates) mRNA transport to dendrites, and that it acts as an inhibitor on the translation of its mRNA cargo at the synapses [23]. In absence of the FMRP protein, the mRNA targets of FMRP are thought to be improperly translated, as has been demonstrated for MAP1B-mRNA. MAP1B plays a leading role in synapse formation and neurite development. It has been shown that its expression is elevated

in a crucial time frame for the dynamic organisation of the neuronal cytoskeleton in *Fmr1* knockout mice. The impaired MAP1B regulation may be the origin of the abnormal spine morphologies observed in *Fmr1* knockout mice [24].

The example of fragile X syndrome demonstrates the importance of identifying the dendritic mRNA pool in order to decipher the molecular pathways involved in cognitive (dys)functions. Eberwine *et al* succeeded in providing a list of several hundred rat dendritic mRNAs by mechanically separating the cell soma from dendrites with a microelectrode of a micromanipulator. Subsequently dendrites were aspirated with another microelectrode, followed by isolation and amplification of RNA in order to perform microarray analysis [25]. However, since the mouse is the most commonly used animal model, we have developed a cell culture-based method to isolate the neuritic mRNA content of mice. The strategy was to provide a system that would allow the separation of neuronal cell bodies from neurites. Torre and Steward previously described a 'double-surface coverslip' method: the top surface (the plating surface) consisted of a porous membrane that allowed the passage of neurites, but not the cell bodies; the second surface (receiving surface) was attached to the first by a protein matrix; after 10-15 days of culturing, the plating surface was separated from the receiving one, leaving mainly neurites on the lower surface [9]. We modified this method in order to obtain sufficient amounts of pure mouse neurites to isolate mRNA.

Materials and Methods

Cells and culture conditions

Primary cultures of mouse neuronal cells were obtained upon isolation of the cortex of E16 embryos of wild-type and *Fmr1* knockout mice. Briefly, cortices of mice were dissected from the brains and treated with trypsine, trypsine inhibitor, and DNAse, 1 minute each in a 37°C warm waterbath. The tissue was washed in Dulbecco's modified Eagle's medium (DMEM; GIBCO laboratories, Grand Island, NY, USA), and dissociated in neurobasal medium (GIBCO) containing 2% B27 and penicillin/streptomycin by repeated passage through a P1000 and a P200 Gilson Pipette. 1 ml of cell suspension of 2-3x10^6 cells/ml was added to the plating surface. Cells were cultured at 37°C and 5% CO_2. Culture medium was replenished if necessary.

Design of the cell culture assembly

The system is based on a two-step two-layer-system (see figure 1).
Step one: Initiation of cell culture

The plating surface. Neurons were plated onto a porous polyethylene terephthalate (PET) track-etched membrane. We chose cell culture inserts for a 6 well plate (Becton Dickson Labware, Franklin Lakes, New York, USA), rather than nucleopore polycarbonate filters, since cell inserts significantly facilitated handling of the system (no need to seal each

individual set up with paraffin as described by Torre and Steward). To determine the pore size, we evaluated membranes with 1, 3, and 8µm pore diameters. Inserts were coated for 30 min with poly-L-lysine (Sigma, Chemical Co., St. Louis, USA), followed by a second 30 min coating with laminin (Sigma).

The removal surface. This surface was used to initiate growth of neurites through the pores of the plating surface as well as to 'capture' stray cell bodies and non-neuronal cells. Based on extensive experience with mouse neuronal cell cultures we found that in the first three post-plating days, glia cells are highly proliferous and motile. We have decided against inhibition of glia proliferation by means of cytosine arabinoside, because in our experience, survival and proliferation of neurons are heavily compromised in absence of glia cells. Therefore, we have decided to provide a thick layer of Matrigel (Becton Dickson Labware) on the lower side of the cell inserts in order to 'catch and remove' the glia cells, which initially may have migrated through the pores into the matrigel. Matrigel is an extract from Engelbreth-Holm-Swarm (EHS) mouse sarcoma, that is rich in laminin, collagen IV and other extracellular matrix proteins [26]. It is used for culturing many different cell types, including neurons [27]. The matrigel was diluted in the culture medium and a collagen solution (Sigma; 6:3:1). Upon double coating of the plating surface, the inserts were turned upside-down to apply 1ml of diluted thawed matrigel. Set up was left for 1 hour at room temperature to allow the removal surface to gel.

Initiation of cell culture. After gelling of the matrix, cell inserts were placed into 6 well plates. Immediately cells were added into the inserts and culture was allowed to develop for 3 days.

Step two: culturing of neurites

Elimination of removal surface. Three days after plating, culture medium was nearly completely removed (and kept for further use) from the plating surface in order to allow careful removal of the matrigel with a cell scraper.

The receiving surface. This is the surface onto which neurites grew after they had grown through the pores of the cell inserts. We used polyethylene naphthalate (PEN) membrane slides (PALM Microlaser Technologies AG, Bernried, Germany). The rationale of using these slides was to allow inspection of the neurite population after cell culturing and, if given, removal of non-neuritic specimens by laser dissection. The PEN membrane slides were double coated with poly-L-lysine and laminin.

Assembling the system. 100 µl of diluted thawed matrigel were applied onto the coated PEN membrane slide. Subsequently, the plating surface was firmly pressed for 30 seconds onto the matrigel, allowing attachment of the two surfaces. Subsequently a minimum of the kept culture medium was added into the cell insert, so that the cells were covered. The assembly was allowed to further attach for 1 hour in the incubator. After this hour, the culture medium on top of the cells was increased to 1ml. Cells were cultured for a further 7 days.

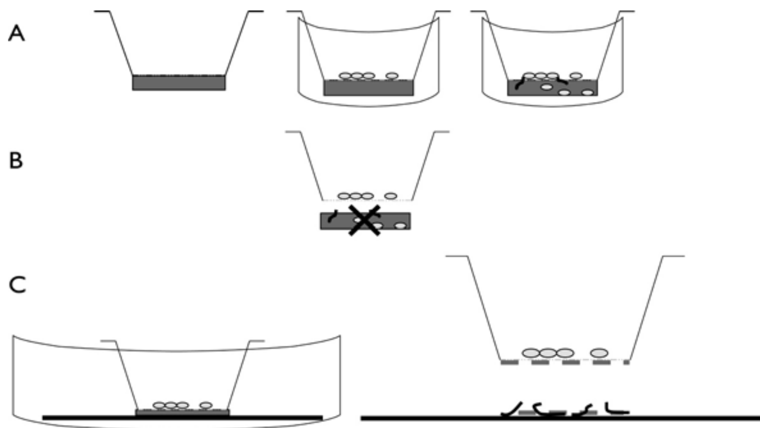

Figure 1: Set up of the two-step two-layer cell culture

A – Step 1:
- cell culture insert is coated and a thick layer of matrigel is applied on the lower side
- cells are cultured on the porous plating surface
- within 3 days, the cell culture starts to proliferate; glia, neurons + neurites extend through pores into the matrigel

B – removal of matrigel containing glia and other cells that have migrated through the pores

C – Step 2:
- plating surface is transferred to coated PEN membrane slide and attached by a thin layer of matrigel; culture is performed in 10cm dish
- after 7 more days of culturing, neurites have extended through the pores and have attached to the receiving surface; cell insert with most of the cell bodies can be removed

Immunocytochemistry, antibodies, staining for subsequent laser dissection

After culturing the cells for a total of 10 days, cell culture inserts were carefully lifted off the PEN membrane. For immunocytochemistry, cells on plating and receiving surface were fixed with 4% paraformaldehyde for 10 min, followed by 20 min of 100% methanol for permeabilization. Double incubation of cells with rabbit polyclonal GFAP antibody (Sigma) and mouse monoclonal MAP2 (Boehringer, Mannheim, Germany) was performed overnight at 4°C. One hour treatment with Cy3- and FITC-labelled secondary antibodies (Nordic Immunologic Laboratories, Tilburg, The Netherlands; Jackson, West Grove, USA, respectively) was used for visualization. For laser dissection, the PEN membrane slide was carefully rinsed in 1x PBS and fixed in 100% methanol for 20 min at room temperature. For visualisation, the membrane was treated for 15 min with a crystal violet solution, which was subsequently removed. Remaining solution was then eliminated with distilled water, and slides were left to dry at room temperature for 15 min. Slides for RNA isolation were stored at -80°C.

Laser dissection microscopy

In order to verify and warrant the purity of the neurite sample, laser dissection microscopy was applied (PALM). In a well defined area on the membrane slide all non-neuritic material was laser catapulted into a cap. After cleaning roughly 1 cm^2 of membrane, the area was excised with the laser, and the membrane was transferred with forceps into sufficient amounts of RNAlater (Qiagen GmbH, Hilden, Germany). The isolated membrane pieces with the neurite samples were stored at -80°C for RNA isolation.

RNA isolation, amplification, and verification

Total RNA isolation was performed using the RNeasy Micro kit (Qiagen) according to the manufacturer's protocol for microdissected cryosections. At this point, visualisation of RNA was not possible. Therefore, RNA amplification was performed. We used the pico version of the ExpressArt mRNA amplification kit (Artus GmbH, Hamburg, Germany), since this method allows 3 linear rounds of mRNA amplification, if necessary. With this method, the original mRNA is converted to cDNA with an anchored oligo(dT)-primer lacking a T7-promoter. In the following step, double stranded cDNA is generated with a special 'box/randomized primer mix'. Subsequently, double stranded cDNA with a functional T7 promoter at one end will be generated by priming the denaturated cDNA strand in reverse orientation with a T7-promoter/oligo(dT)primer. The dsDNA template is then used for generation of antisense oriented RNA. Second and third rounds of amplification make use of the added box sequences in order to minimize artefacts.
To visualize and quantify the amplified mRNA, the Agilent 2100 BioAnalyzer (Agilent Technologies, Deutschland GmbH, Waldbronn, Germany) was used.

Results

Culturing of neurites

In pilot experiments, we tested cell culture inserts with different pore sizes. Membranes with 1 µm pore sizes hardly produced any neurites (or any cells) on the receiving surface. In contrast, membranes with 8 µm pores allowed the passage of a high percentage of neuronal cell bodies and glia cells. However, the most desirable result in respect to the ratio of neurites versus non-neuritic material on the receiving surface was obtained with a pore size of 3 µm. In another set of pilot experiments, we compared a one-step versus a two-step culturing system. Culturing the primary neurons on the plating surface directly attached to the receiving surface (one-step approach) resulted in higher numbers of glia cells on the receiving surface than with the two-step approach. To verify an enrichment of dendrites on the receiving surface, we performed immunocytochemistry experiments (see figure 2). We used antibodies directed against MAP2, a protein that is highly expressed in dendrites, and antibodies directed against GFAP, a marker for glia cells, to identify the different cell types on both surfaces. On the plating surface, highly mixed cell populations of neurons and glia

cells were growing intertwined with each other. The MAP2 positive neurons displayed cell bodies as well as dendrites. However, under optimal conditions (3µm pore size + two-step approach), many dendrites without a cell body could be detected by MAP2 staining on the receiving surface, while hardly any GFAP positive glia cells were present.

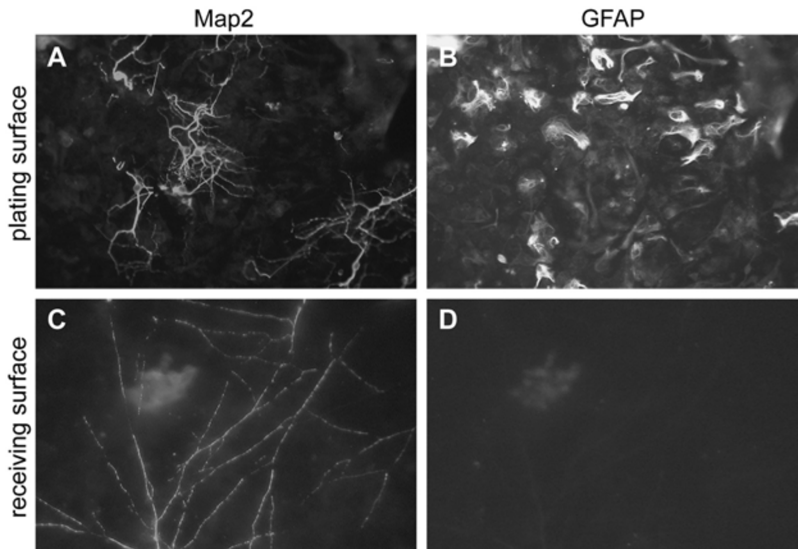

Figure 2: Assessing the enrichment of dendrites with double stainings

A – A high number of neurons (dendrites + cell bodies) can be detected in the plated cell culture
B – There are also many glia cells in the cell culture
C - On the receiving surface, most of the cell bodies have been removed
D – Hardly any glia cells are present on the receiving surface

Laser dissection

Although we could improve the neurite to cell body ratio towards the neurites, we did not succeed in eliminating cell bodies and glia cells completely from the receiving surface. Therefore we had to laser dissect any unwanted material from the PEN membrane (see figure 3). The combination of the cell culture system and laser dissection microscopy enabled us to obtain higher numbers of pure mouse neurites than with any other technique tested in the past. We chose to laser catapult the unsolicited material from the PEN membrane rather than the neurites, since (1) the number of neurites far exceeded the number of non-neuritic material, and (2) multiple reassessment of the cleaned area on the PEN membrane was possible. To guarantee that only neurites were collected for ensuing mRNA isolation, we used a more stringent definition of neurites at this stage: neurites were long, thin, multiply branched extensions, while everything else was defined as non-neuritic and was therefore removed from the slides. If ever in doubt, the site on the PEN membrane was removed.

Small cell bodies could be removed by high power impulses of the laser, so that holes in the PEN membrane would occur at the sites where the cell bodies were located earlier (compare figures 3A and 3C). Figure 3A gives examples of the neurite/non-neurite definition, while figure 3C displays the same field during the lasering. If a non-neurite cell was larger than the hole possibly created by a single laser impulse (see figure 3B), the laser was instructed to cut around the area in question. Excised pieces of the PEN membrane were then catapulted with a single laser impulse into a collection cap. In this fashion, approximately 1 cm^2 of the PEN membrane for a single mRNA isolation sample was cleared. The cleared area was then cut off by the laser from the remainder of the receiving surface, and it was subsequently lifted with forceps into an eppendorf containing RNAlater to minimise RNA degradation.

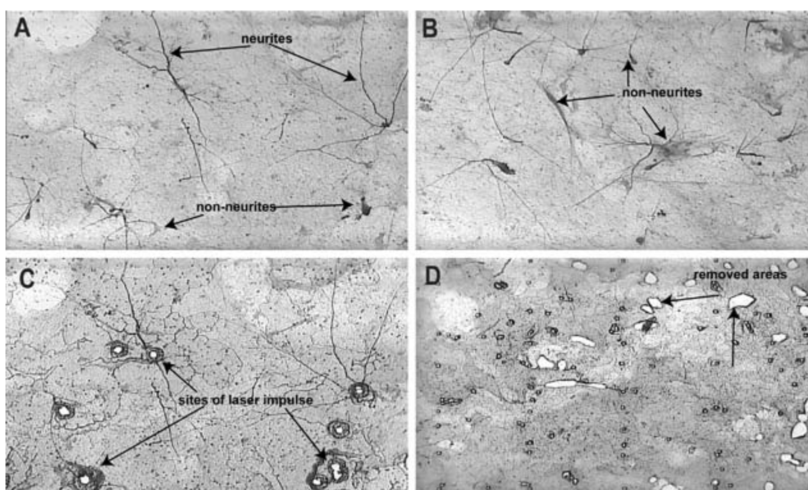

Figure 3: pictures of the receiving surface under the laser dissection microscope

A + B: examples of cells on the receiving surface after 10 days of culturing. For capturing purposes neurites are defined as long, thin, multiply branched extensions. Everything else is considered here as non-neuritic.
C: examples of single laser impulses having destroyed the PEN membrane at previously selected sites.
D: overview with a lower magnification of a 'cleaned'area. Very small sites may be destroyed by single laser impulses, while bigger sections are first cut and then catapulted into a cap.

RNA isolation, amplification, and verification

We did not succeed to visualise mRNA directly after RNA isolation. Therefore, we applied an RNA amplification method. Since we expected very low amounts of mRNA to be present in dendrites, we opted for an approach that would allow us multiple rounds of RNA amplification if necessary. A minimum of 2 rounds of amplification was necessary to visualise RNA on the BioAnalyzer. However, the amounts obtained would be too low to continue with downstream experiments such as microarray technologies. Therefore we amplified the RNA for 3 rounds. 3 rounds of amplification yielded roughly 120 µg of RNA (sample 1, see picture 4). However, the size of the RNA templates did not exceed 200 – 300bp.

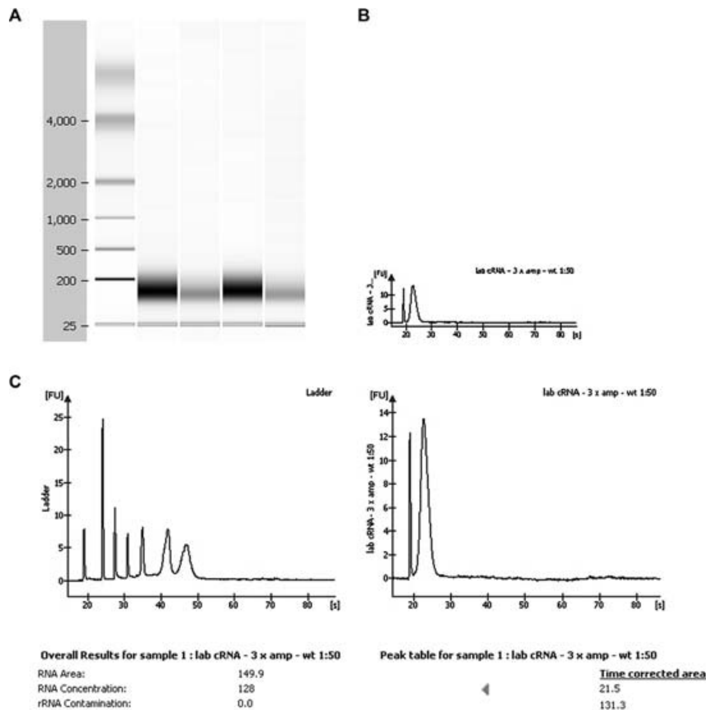

Figure 4: Results from the BioAnalyzer run.
A – Electrophoresis File Run Summary. Lane 2 different RNA-isolation batches, each run in dublo (lane 1+3 for sample 1; lane 2+4 for sample 2).
B - Electropherogram summary for sample 1
C – Electropherogram summary for sample 1 in comparison to ladder

Discussion

Many neuronal processes have been shown to rely on local protein translation at synaptic sites [5-8]. In concordance with protein synthesis, mRNA has to be transported from the cell bodies to the dendrites, in particular the postsynaptic density compartments. Local translation of asymmetrically distributed mRNAs at synapses is believed to facilitate and accelerate specific neuronal answers on the postsynaptic site. In order to learn more about the molecular pathways involved in cognitive functioning, it is essential to identify the mRNAs present in dendrites and their individual roles.

The method described by Torre and Steward [9], though providing means of producing neurites, did not warrant a cell body free sample. Therefore we adapted their system by combining a double-layer culture system with laser microdissection microscopy (LMD).

LMD has been shown to enable generation of small starting samples for microarray analysis [28-33].

We used a high cell density for the primary plating and a pore size that would allow some cell bodies and glia cells to grow towards the receiving surface. Radial glia cells have long been suggested to play a role in axon/neuron guidance in the developing brain [34]. We imagine that the glia cells that have migrated through the pores into the thick layer of matrigel in the first step of our culture system leave guiding cues in the matrigel. After removing this layer (the removal layer) 72 hours post plating, we expect some matrigel to be left in the pores, including the 'sign posts' left by the glia. Having performed some pilot experiments by playing with the thickness of the matrigel, we have observed that it is the neurites, which are scouting the 'unconquered' areas first, before cell bodies are following. Fine-tuning the exact thickness of the matrigel between the two surfaces with the scouting speed of the neurites before the cell bodies reach the receiving surface may be a possibility to further improve the efficiency of our proposed system. One draw back of using the matrigel is that it has been reported to influence the gene expression in adult rat hepatocytes [35]. Any identified mRNA pool may therefore be differing from an *in vivo* mRNA content of neurites.

In our set up we were not able to separate axons from dendrites. Although mRNAs have been described to be also present in axons, the number and amounts are significantly lower than in dendrites (for a review see [36]). We cultured the neurons for a total of 10 days, a time frame that should allow the development and maturation of dendrites to surpass axonal development (Dr. Ger Ramakers, pers. communication). Expanding the culture phase may improve the dendrite to axon ratio in favour of the former. Currently there is no means to physically separate axons and dendrites in mouse primary neuron culture. Even using a recently developed upgrade of the PALM software, which allows to automatically laser capture fluorescently labelled cells by means of colour recognition, will not be sufficient to exclude axons from dendrites due to the close proximity and the intertwined nature of the two subpopulations. However, use of this software could improve capture time and it could eliminate human error in the process of identifying (non-)neuritic material [37]. On the other hand, crystal violet stained slides refrain from fading [38]. Cell cultures and subsequently the harvestable amount of neurites on the receiving surface varied. Explanation for these variations may be day performance, differences in the embryonic material (we observe differences regularly in primary neurons), a high sensibility of the set up to minuscule differences, and changes in the properties of matrigel constituents after long storage etc.

We performed three rounds of amplification, based on a method that has been recently suggested for single cell mRNA isolation [39]. We obtained roughly 120 µg of amplified RNA, enough material to carry out microarray experiments (Affymetrix protocol). Although the size of the mRNA species did not exceed 300 bp, we have been able to isolate mRNA from mouse neurites. Additionally we have demonstrated that mRNA is transported to neurites in the absence of FMRP. We therefore conclude that the two-step two-layer cell culture system is useful in

obtaining pure fractions of neuritic mRNA. To certify if identified mRNAs are either axonal or dendritic, localisation experiments could be investigated by follow up experiments. Therefore, this method will enable the identification of the mRNAs targeted into the dendrites to the synapses and it will provide means to decipher the molecular pathways in cognitive processes.

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Appendix

Preliminary data on the identification of the mRNA content of mouse neurites

Having successfully isolated mRNA from mouse neurites, we decided to run microarray assays as a pilot experiment. The scientific questions that we wanted to address were (a) what kind of mRNAs are present in the neurites, especially the dendrites; (b) is it possible to detect differences in the mRNA content of dendrites between wild-type and *Fmr1* knockout neurons; if yes, what are the differences, and may they provide insights into the pathophysiology of FRAXA?

Total RNA isolation and 3 rounds of mRNA amplification were performed as previously described (chapter VI). After synthesis of the second strand cDNA in the 3rd round of the Artus'-protocol, materials and procedures were used according to the Affymetrix-protocol for Eukaryotic Target Preparation (Affymetrix, Santa Clara, USA). Briefly, double stranded cDNA was cleaned up; biotin-labeled aRNA was synthesized, cleaned up and quantified with the Agilent 2100 BioAnalyzer. Subsequently, 20µg of each sample were fragmented for 10 min, instead of the recommended 35 min due to the short nature of the labeled aRNA templates. For a second run, labeled aRNA was not fragmented at all. For the microarray assay, the GeneChip Mouse Genome 430 2.0 array was used. Hybridization, washing, staining, and scanning of the arrays was carried out according to the Affymetrix protocol. Microarray assays were performed for wildtype and *Fmr1* deficient neurite samples. Two different *Fmr1* knockout samples were used, while the same wild-type sample was used twice for the microarray analysis. Two samples (the wild-type and one *Fmr1* knockout) were run on 2 different days.

Data obtained from the microarray hybridizations were processed with the GCOS v1.1 (Affymetrix) Software. Intensity values for all genes were calculated using the default algorithms specified by Gene Chip Operating System (GCOS1.1). Further normalization was performed by Bioconductor quantile methods in R 2.01, following deletion of probe sets that were absent across all samples. Log2ratios were calculated with respective controls for all normalized probe sets.

Significant gene lists showing 1.5 linear fold change in log2ratios were submitted to Pubgene, a data-mining software that can search through the millions of biology-related papers published for the names of genes and proteins, sequence homologues, mutations, diseases, pathways and processes. Based on this information, a map of all associations is made.

All arrays displayed a moderate background, however, the overall signal intensity of the samples was even lower. Strikingly, the samples clustered according to the day of the assay performance rather than the genotype (see figure 1). This is especially conspicuous considering that the identical wild-type sample was run twice.

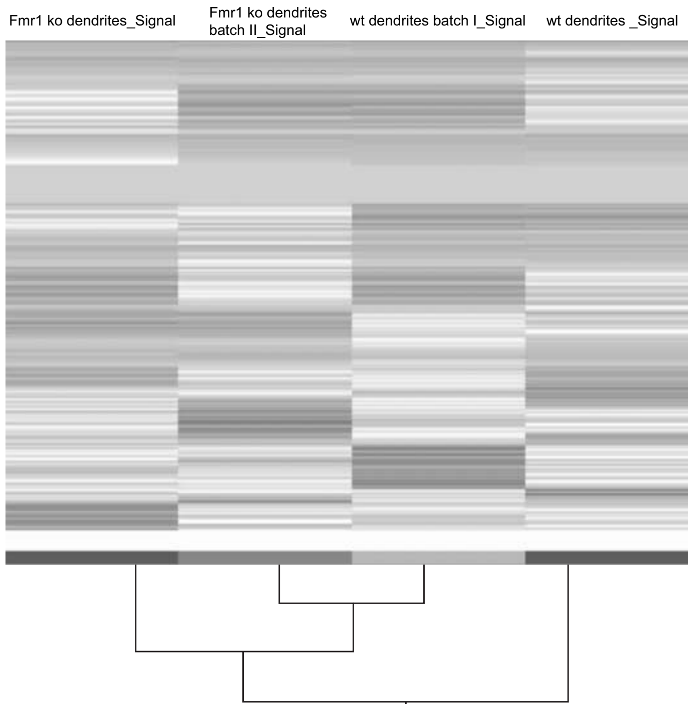


Figure 1: clustering of samples
Clustering of all the genes on the microarray, regardless if a gene is called present or absent for following analysis. The expression profiles cluster according to date when they were run instead of genotype.

This clustering problem most likely has been caused by in-house problems at the microarray facilities with the staining solutions. Based on the low signal intensities and the problem with the clustering, any conclusions from the data analysis have to be treated with caution. Considering that *Fmr1* mRNA has been shown to be present in dendrites, we used the average signal intensity of *Fmr1* over the 2 wild type samples as the cut-off signal to call a gene present or absent. The signal of a present-called gene had to be higher than this cut-off signal in at least one of the 4 arrays. On these preconditions, we could produce a list of 193 'significant' genes. However, we are not confident about the results and the experiment has to be repeated before the data can be analyzed further.

Based on information from other microarray users, who performed their assays in the same time frame (*i.e.* using the same batch of staining solution), we concluded that the first run could be considered trustworthier than the second. Therefore we restricted reflection on gene ontology on the first data set (see figure 2).

A

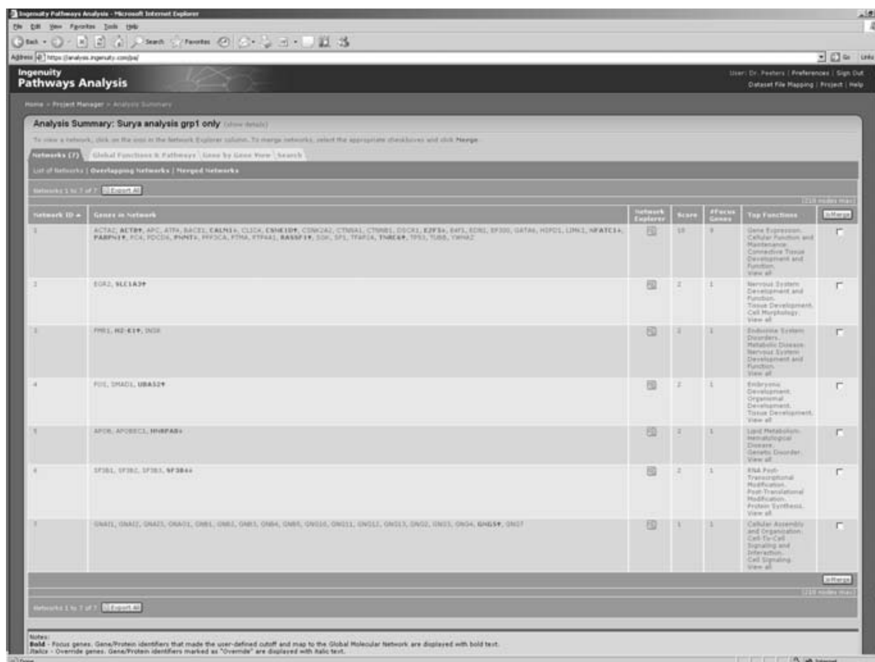
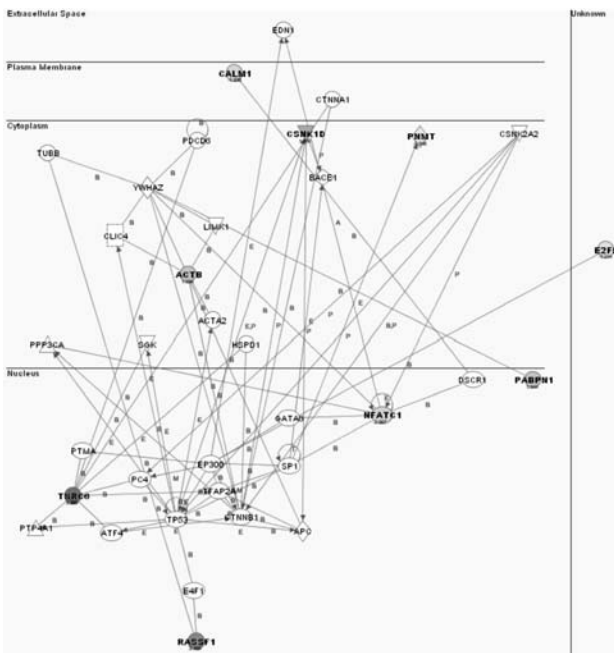


Figure 2: predictions of networks of genes in similar processes by Ingenuity Pathway analysis software.
A- the 7 most significant networks defined by the ingenuity software
B- relationships of genes within the first network

B



The Ingenuity and PubGene software predicted a number of networks, biological processes, and molecular functions for this list of 193 genes (for the most statistical relevant ones see table 1 and 2).

Category	Descriptions	Score
Biological processes	Neurite guidance	2.145e-56
	Dihydrofolate biosynthesis	2.046e-33
	Regulation of keratinocyte differentiation	4.737-19
	Cell cycle	1.334e-11
	Transcription initiation	6.789e-08
Molecular function	Polyubiquitin	1.5e-131
	Apoptosis regulator activity	5.052e-44
	Peptide antigen binding	1.599e-39
	Class I major histocompatibility	3.345e-17
	Transcription regulator activity	9.441e-17
Cellular components	neurites	0.0001496
	Nuclear pore	0.0496

Table 1: Gene/Protein to Gene Ontology Predictions by Pubgene software

Description	Score	Symbol
Ubiquitin A-52 residue ribosomal protein fusion product 1	2.47e-131	Uba 52
Ubiquitin specific protease 14	6.31e-45	Usp14
Cell division cycle 34 homolog (*S. cerevisiae*)	7.89e-39	Cdc34
Neural precursor cell expressed. Developmentally down-regulated gene 8	1.6e-31	Nedd8
Tnf receptor-associted factor 6	3.104e-23	Traf6
Ribophorin II	3.104e-23	Rpn2
Pseudodouridine synthase 1	8.695e-18	Pus1
Ribophirin I	5.753-16	Rpn1
MMTV LTR integration site 1	1.8083-11	Pad1
Ubiquitin C	4.503e-09	UbC
CDC28 protein kinase 1	6.656e-05	Cks1
Repeat sequence probe 5	6.656e-05	Rsp5
General transcription factor II, H polypeptide 1 (62kD subunit)	9.411e-05	P62
Friend leukemia integration 1	0.001676	Fli1
Cullin 1	0.002106	Cul1

Table 2: genes in the most relevant category (molecular functions: polyubiquitin) identified by PubGene software

A number of considerations have to be taken into account, when interpreting the data sets obtained from the initial microarray experiments described here: (a) although we successfully isolated mRNA from neurites, the sizes of the isolated templates were relatively short. Considering that *Fmr1* mRNA is located in the dendrites and that its size is considerably longer than 300 bp, we must conclude that the integrity of the mRNA templates has been compromised during the course of culturing, neurite isolation, RNA isolation and amplification. However, there are different options to improve the quality of the RNAs throughout sample collection, as discussed in chapter VI. Two of the easiest options to improve the data significance are the increase of the sample volumes and the sample numbers. (b) The optimization of the microarray assay conditions in a way, that an identical sample run on different occasions will produce similar results, is of the utmost importance!

We have provided (see table 1 and 2) a preliminary short list of biological processes and molecular functions for our gene list, as predicted by the different software tools. However, bearing in mind the discussed problems, an analysis of these predictions and their interpretations would be purely speculative at this moment. Despite these restrictions, we are convinced that we have developed a technology that will provide in due course insights into the molecular pathways involved in synaptic processes.

Chapter VII

Prospects of TAT-mediated protein therapy for fragile X syndrome

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Prospects of TAT-mediated protein therapy for fragile X syndrome

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Summary

Fragile X syndrome is due to the absence of the fragile X mental retardation protein (FMRP). Patients are mentally retarded and show physical as well as behavioural abnormalities. Loss of protein in the neurons results in changes of dendrite architecture, and impairment of the pruning process has been indicated. Apart from some minor differences, no severe morphological changes have been observed in the brain. Until now, no therapy is available for fragile X patients. Recently it has been reported, that a protein transduction domain (TAT) is able to deliver macromolecules into cells and even into the brain when fused to the protein in question. Upon production of a TAT–FMRP fusion protein in a baculovirus-expression system, we used immunohistochemistry to verify TAT-mediated uptake of FMRP in fibroblasts. However, uptake efficiency and velocity was lower than expected. Neuronal uptake was highly inefficient and the fusion protein demonstrated toxicity.

Introduction

One in 4 000 men and 1 in 6 000 women are affected by fragile X syndrome, a common genetic cause for mental retardation (de Vries *et al.* 1997). The molecular basis of the disease is the absence of the fragile X mental retardation protein (FMRP), product of a gene located at Xq27.3, the fragile X mental retardation gene 1 (*FMR1*) (Verkerk *et al.* 1991, Devys *et al.* 1993, Verheij *et al.* 1993). Characteristic for *FMR1* is a CGG repeat in its 5′-untranslated region (5′-UTR), which may demonstrate instability during transmission (Fu *et al.* 1991, Kremer *et al.* 1991, Yu *et al.* 1991). So-called premutation carriers have 50–200 CGG units, compared with 6–50 units in normal individuals. Individuals with repeat units exceeding 200 (full mutation) cannot produce FMRP since the expansion leads to methylation of both the promoter and the repeat and subsequent silencing of the gene. Clinical manifestations of the syndrome are mental retardation, facial abnormalities, macro-orchidism as well as some behavioural anomalies (for review (Hagerman 2002b)). Magnetic resonance imaging (MRI) studies have revealed that on one side fragile X patients have a reduced posterior cerebellar vermis and on the other side that their hippocampus, the caudate nucleus and the lateral ventricles are slightly increased in size (Reiss *et al.* 1991, 1994, 1995, Kates *et al.* 1997, Mostofsky *et al.* 1998). FMRP is a cytoplasmic RNA-binding protein that is highly expressed in the brain and the testis. It is hypothesized that FMRP acts as a translational repressor of mRNAs not only in the cytoplasm, but also in the dendrites at synaptic sites. Several studies indicate that the neuronal cell structure is impaired in fragile X patients. In particular, dendritic spines have been reported to be elongated, with immature shapes and a higher density, suggesting that arrest of normal spine maturation may be causing the phenotype involved in the syndrome (Hinton *et al.* 1991, Irwin *et al.* 2001). Other data supporting the hypothesis of delayed or arrested maturation is an impaired synaptic pruning process. Changes in dendritic spine morphology have also been observed in a knockout mouse model of the disease (Comery *et al.* 1997, Galvez *et al.* 2003).

Though the brain's morphology is slightly affected in fragile X patients and the knockout mouse, no gross impairment has been found. The phenotype seems to be due to failure of 'neuronal communication', thus suggesting that delivery of the missing FMRP to neuronal cells may be sufficient to correct the phenotype. Recently, it has been reported that it is possible to deliver macromolecules into living cells, but more strikingly, delivery of a TAT-β-galactosidase fusion protein across the blood–brain-barrier upon intraperitoneal injection into mice has been demonstrated (Schwarze *et al.* 1999). Delivery of the protein was mediated by a TAT domain, which is derived from the human immunodeficiency virus (HIV). Transduction of this TAT peptide has been shown to take place in a rapid, concentration-dependent manner, though the mechanism of this transduction is still unknown. We report here the production of TAT–FMRP fusion protein and its delivery into fibroblasts of a fragile X patient.

Materials and methods

Constructs and cloning

PTAT vector containing a His(6) tag and the TAT domain (kindly donated by Dr. Steven Dowdy, Washington

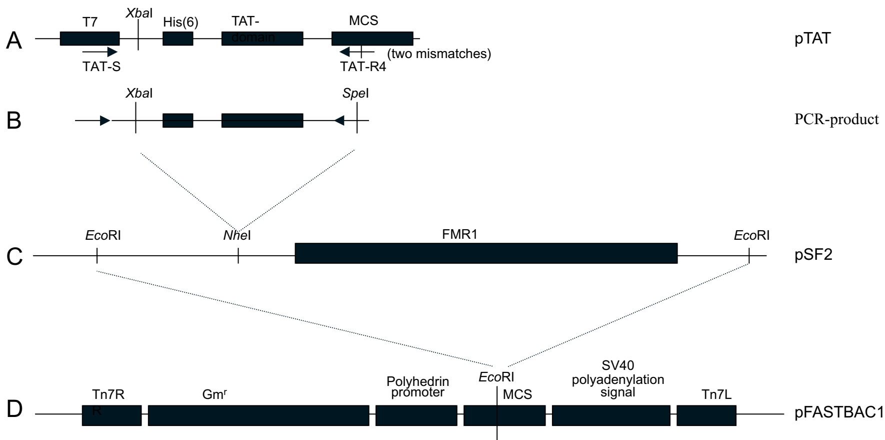

Figure 1. Cloning strategy. (A) Scheme of pTAT vector containing His(6) tag and TAT domain. TAT-S and TAT-R4 indicate position of the primers used to PCR-amplify insertion fragment. Primer TAT-R4 contains two mismatches to obtain a *Spe*I recognition site. (B) PCR-product indicating position of the restriction sites. (C) Scheme of vector pSF2 containing *FMR1* cDNA. PCR-product containing His(6) tag and TAT domain was cloned into the *Nhe*I site. (D) Scheme of pFASTBAC1 vector and indication of cloning site for His(6)-TAT-FMR1 insert.

University, St Louis, USA) was polymerase chain reaction (PCR)-amplified using primers TAT-S 5′-CGATCCCGCG-AAATTAATACGACT and TAT-R4 5′-CGTACTAGTCTC-GAGGTGCAT introducing a 3′ *Spe*I recognition site into the multiple cloning site (MCS) (see Figure 1). Upon double digestion with *Spe*I and *Xba*1, the fragment was ligated into the *Nhe*I recognition site of a modified version of pSG5 (Stratagene), named pSF2, into which the human *FMR1* cDNA had been cloned (Verheij *et al.* 1993). Insertion was verified by sequencing. Subsequently, the *Eco*R1 fragment of the pTAT-FMR1 plasmid was ligated into the *Eco*R1 recognition site of pFASTBAC-1 (Gibco BRL). PET21a-FMR1 vector to produce FMRP in *Escherichia coli* (Laggerbauer *et al.* 2001) was kindly provided by B. Laggerbauer (Max-Planck Institute for Biochemistry, Martinsried, Germany).

Cells and culture conditions

SF21 cells (Invitrogen) were maintained in Grace's Insect Medium (Gibco BRL) containing penicillin/streptomycin (PS) and 10% foetal calf serum (FCS) at 27 °C. COS cells, fibroblasts cell lines 86RD613 (fragile X cell line) and 86RD540 (control cell line) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with PS, 10% FCS at 37 °C and 5% CO_2. Primary cultures of mouse neuronal cells were obtained upon isolation of the cortex of E18 embryos of wild-type and *FMR1* knockout mice (Bakker *et al.* 1994). Cells were resuspended in Neural Basal Medium (Gibco BRL) containing 2% B27, plated onto coverslips coated with poly-D-lysine and cultured at 37 °C and 5% CO_2.

Transfection of COS cells

Cells were transfected according to manufacturer's protocol with 1 μg pSF2 and pTAT-FMR1, respectively, 6 μl Plus Reagent and 4 μl lipofectamine (Gibco BRL) for 3 h in a 35 mm well. The following day cells were harvested by trypsinization and seeded on glass coverslips. Immunocytochemistry was performed the day after (48 h post-transfection).

Immunocytochemistry and antibodies

Cells were fixed with 4% paraformaldehyde for 10 min followed by a 20 min permeabilization step in 100% methanol. Incubation with primary and secondary antibodies was performed at room temperature for 1.5 and 1 h, respectively. Primary antibodies for detection of FMRP in transfected COS cells were either rabbit polyclonal ab-734 directed against the N-terminal part of FMRP (Verheij *et al.* 1995) or ab-KI, a polyclonal antibody which we raised in rabbits against the C-terminus of FMRP (aa 516–632; Adinolfi *et al.* 1999). TRITC-labelled secondary antibodies (Sigma) were used for detection. Immunocytochemistry of fibroblasts, and SF21 cells (performed 0, 24, 48, 72 h post-transfection) included a 30 min inhibition step of endogenous peroxidase activity using a phosphate-buffered saline (PBS) solution containing 0.6% hydrogen peroxide and 1.25% sodium azide. Primary antibody used was mouse monoclonal antibody ab-IC3 (Devys *et al.* 1993). As secondary antibody histofine, a peroxidase labelled anti-mouse and -rabbit polymer (Nichirei Corporation) was used, followed by detection with 3′,3′-diaminobenzidine-tetrahydrochloride (DAB) (Bakker *et al.* 2000).

Production of baculovirus, amplification of virus, protein production

The BAC-TO-BAC Baculovirus-Expression System (Gibco BRL) was used to generate recombinant baculovirus.

Transposition and isolation of recombinant bacmid DNA were performed according to manufacturer's protocol. A 3-day-old culture of SF21 cells was transfected with 5 μl bacmid DNA, 75 μl Optimem, 20 μl Superfect in a 35 mm well. Primary virus was harvested 1 week post-transfection.

For amplification of virus, SF21 cells (T175, 90% confluent) were infected for 1 week with 60 μl (=multiplicity of infection (MOI) 0.08 for formula, see Instruction Manual of BAC-TO-BAC Expression Systems, Gibco BRL) of virus-stock in 30 ml Grace's Insect medium containing PS.

For each virus generation, cells were infected with MOI 0.08, 1, 2, 5 and 10 of virus for 2–7 days to determine optimal protein production conditions. Upon harvesting of cell pellets and media, protein production was monitored by SDS–PAGE and Western blotting.

SDS–PAGE, Western blotting and Coomassie staining

Protein samples were separated by SDS–PAGE and electroblotted subsequently onto nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Immunodetection was carried out using primary antibody ab-IC3 and a peroxidase labelled secondary antibody (Sigma), enabling chemiluminescence detection with ECL® kit (Amersham). The separated proteins were stained with Coomassie Brilliant Blue R250 (Bakker *et al.* 2000).

Protein purification and uptake of TAT–FMRP in fibroblasts

SF21 cell pellets were harvested 4 days post-infection with MOI 2 of TAT–FMR1-baculovirus, resuspended in lysis buffer, pH 7 (1 M NaCl, 50 mM Na_2PO_4, 10% glycerol, 5 mM β-mercaptoethanol) and sonicated 3×20 s. Crude lysates were added to TALON Cell Thru beads (Clontech) and purified according to manufacturer's protocol, including an intermediate washstep with lysis buffer containing 10 mM imidazol prior to elution. Elution was performed with 150 mM imidazol in lysis buffer. Eluted fractions were collected in eppendorfs containing bovine serum albumin (BSA) to obtain a final concentration of BSA of 1 mg/ml. Expression and purification of FMRP without TAT domain from *E. coli* was conducted as described by Laggerbauer *et al.* (2001).

About 200 or 300 ng of purified proteins (TAT–FMRP or FMRP) were added to 0.5 ml culture medium of fibroblasts in 24-well plates and immunocytochemistry was performed the following day as described above.

Results

Constructs and localization of the fusion protein

To study the possibility of therapy for fragile X syndrome, a construct, named pTAT-FMR1, was designed which contained an in-frame TAT domain N-terminally to the *FMR1* cDNA. This TAT sequence encodes for a protein transduction domain. For facilitation of protein purification, the construct also contained a His(6) tag upstream of the TAT domain (see Figure 1A–C). To test the fidelity of the construct and to study protein expression and localization of TAT–FMRP *versus* FMRP, COS cells were transfected with pTAT-FMR1 and pSF2. The proteins could be detected as well with antibodies directed against the N-terminal part (ab-734; Figure 2A, b + d) as well as antibodies directed against the C-terminal part (ab-KI; Figure 2A, a + c) by immunocytochemistry. TAT–FMRP was also detected by anti-His antibody (data not shown), indicating that full-length protein had been expressed. Production of full-length TAT–FMRP was also verified by Western blotting experiments of SF21 cells infected with the baculovirus (Figure 2C).

As FMRP, TAT–FMRP was localized in the cytoplasm of the COS cells. However, in a small percentage of cells, TAT–FMRP was also found in nucleoli with all three antibodies as illustrated in (Figure 2A-a). This may be due to the fact that the TAT domain can also function as a nucleolar targeting signal (Green & Loewenstein 1988). Despite this occasional nucleolar localization, FMRP as well as the fusion protein, presumably due to the presence of a nuclear export signal (NES) in FMRP, are largely targeted to the cytoplasm, the correct subcellular localization for core FMRP function.

Baculovirus and protein production

To produce the fusion protein, *TAT-FMR1* was cloned into a baculovirus-expression vector (see Figure 1D) for subsequent baculovirus production. To validate the virus, SF21 cells were infected with primary baculovirus (MOI 0.08) and harvested at different timepoints post-infection. Immunocytochemical experiments showed production of TAT–FMRP in SF21 cells 48 h after infection (Figure 2B-c). At 72 h post-infection, 80% of cells were producing the protein (Figure 2B-d), which was located not only in the cytoplasm, but also in the nucleus of the insect cells as detected by immunocytochemistry (Figure 2B), indicating that the baculovirus was inducing TAT–FMRP production.

Time experiments to determine culturing parameters for optimal protein production demonstrated the earliest detectable levels of TAT–FMRP at day 2 post-infection in cell pellets by Western blotting (Figure 2C). Maximum levels were reached at day 4. At day 5, no full-length protein was detected anylonger, which is probably due to cell death caused by overexpression of the protein as well as virus production. Expression levels varied insignificantly between the different amounts of virus (MOI 2/5/10) used for infection. In the medium, no TAT–FMRP was detectable by Western blotting. Therefore, SF21 cells were infected with MOI 2 of baculovirus and cells were pelleted 4 days post-infection for bulk production of TAT–FMRP.

Protein purification

In order to purify TAT–FMRP, we utilized the binding capability of the His(6) tag 5′ of the TAT domain to metal affinity

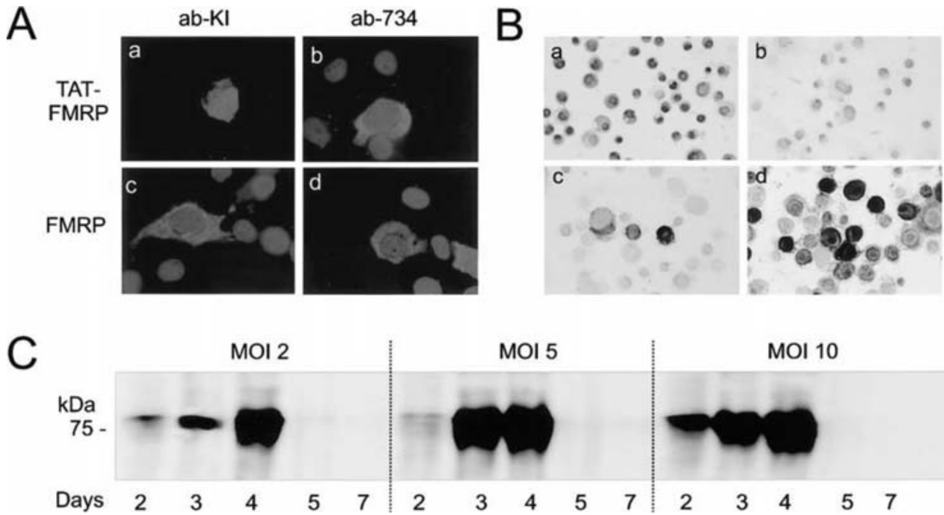

Figure 2. Validation of construct and virus. (A) Transfection of COS cells with pTAT-FMR1 (a + b) and pSF2 (c + d). Immunocytochemistry to study localization of the proteins. (a + c) – stained with ab-KI directed against the C-terminus of FMRP; (b + d) – stained with ab-734 directed against N-terminal part of FMRP. (B) Infection of SF21 cells with small amount (MOI 0.08) of baculovirus for virus amplification. Immunocytochemistry with ab-IC3 against FMRP at (a) 0, (b) 24, (c) 48, (d) 72 h post-infection. (C) Production of TAT–FMRP. Western blot with ab-IC3 against FMRP. SF21 cells were infected with different amounts of virus (MOI 2/5/10) for 2–7 days. Cells were pelleted at indicated times and cell lysates were separated on SDS–PAGE gel.

columns. Several problems arose during protein purification. Binding of solubilized protein to TALON-cobalt-column was relatively inefficient. Once protein was bound, it was difficult to elute it from the column. A possible cause for this could be that TAT–FMRP demonstrated a tendency to stick to most materials, to aggregate in time and to precipitate. Aggregated fusion protein was unable to bind to the affinity columns tested. However, when the protein was present as a dimer or a monomer, the binding was very strong and elution of the protein difficult. Increasing imidazol concentrations did not improve elution efficiency. Use of enterokinase to cut off the fusion protein from the His-tag as well as stripping the cobalt off the column resulted in the protein sticking to the beads via other forces. When purifying under denaturing conditions, hardly any protein could be eluted from the affinity columns. We experienced loss of material with every transfer of the protein to another vehicle. However, this could be partially counteracted with the addition of BSA to the elution fractions. Addition of BSA also partially worked against loss of material during storage caused by the aforementioned aggregation, precipitation and stickiness. Preincubating the beads with BSA neither improved binding capacity nor elution efficiency of the fusion protein (data not shown). Having tested numerous purification conditions, we succeeded in obtaining sufficient amounts of purified TAT–FMRP with the conditions described in the Materials and methods section, to carry out uptake studies, although recovery of purified protein was low. In lane 1 shown in Figure 3, 1/320th of the start material is depicted. For the elution fractions, 1/20th of each fraction was applied on the SDS gel.

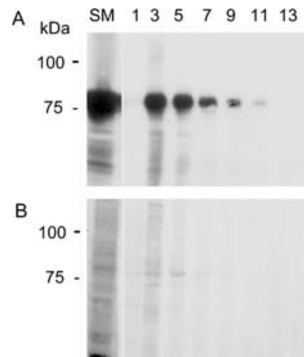

Figure 3. Protein purification. (A) Western blot of purified samples with ab-IC3 against FMRP. SM = start material; cell lysates added to TALON beads. Elution fractions 1, 3, . . . , 13. (B) Coomassie staining of the same fractions.

Despite the fact that intermediate washsteps with 10 mM imidazol were included in the purification procedure, elution fraction 3 still contained a number of aspecific proteins, as seen on the Coomassie staining (Figure 3B). Elution fraction 5, however, contained purified TAT–FMRP, coinciding with maximum concentration of the protein (Figure 3).

Cellular uptake of TAT–FMRP

To establish if cells would incorporate the purified TAT–FMRP, we administered the eluted fusion protein to cultured

Figure 4. Uptake of TAT–FMRP in fragile X fibroblasts. Immunocytochemistry after overnight exposure to protein. Staining with ab-IC3 against FMRP. (A) negative control, (B) addition of 200 ng and (C) addition of 300 ng TAT–FMRP in 0.5 ml of medium.

fibroblasts and performed immunocytochemical staining after overnight exposure to the protein with ab-IC3 against FMRP. Of the fibroblasts 50% had taken up TAT–FMRP (Figure 4). Background staining of negative fibroblasts was neglectable. The protein was mainly located in the cytoplasm and occasionally as well in the nucleus. Uptake took place in a concentration-dependent manner. Precise quantification of the incorporated protein amounts was not possible, since immunohistological methods are not sensitive enough and no activity assay is available for FMRP. However, the level of uptake usually did not equal concentration of endogenous FMRP in control cell lines. This might be due to the observation that quantities exceeding 300 ng of TAT–FMRP per 24-well lead to cell death (Figure 4C), suggesting that overexposure to the protein is toxic for the cells. We also monitored time-dependent uptake of TAT–FMRP. No uptake was observed 30 min after addition of the protein. At 5 h after addition, only a very small percentage, between 5% and 10% of cells had taken up detectable amounts of the protein. Maximum uptake (50% of cells) was detected after overnight exposure to the protein. No increase in uptake was detectable at 48 h post-protein addition.

To verify if the observed uptake was TAT-mediated, we also produced FMRP lacking the TAT domain and added it to the culture medium of fibroblasts. FMRP by itself was not taken up by fibroblasts, indicating that the observed uptake was due to the additional protein transduction domain in the fusion protein (data not shown).

Discussion

The foremost cause of fragile X syndrome is the absence of the FMRP protein. Morphological modifications in the nervous system of fragile X patients and *Fmr1* knockout mice seem to be confined to small volume changes of different brain regions as well as abnormalities in dendritic structures (Hinton *et al.* 1991, Comery *et al.* 1997). However, no significant neurological degeneration has been reported. FMRP is also expressed throughout adult life in normal individuals, suggesting a life-long function of FMRP. The rather mild symptoms in neuronal development as well as the temporal expression pattern suggest that postnatal therapy may be applicable. 'Proof-of-principle' that fragile X syndrome is a potentially correctable disorder comes from mouse model studies. Two independent rescue mice lines, where the intact *FMR1* gene was introduced into the knockout line, demonstrated partial restoration of the phenotype of fragile X syndrome (Peier *et al.* 2000, Gantois *et al.* 2001). It has to be taken into account, however, that in the case of these mouse-models, the transgene is also expressed embryonically. Hence the models do not provide insight into the potentiality of postnatal correction of the syndrome.

The objective for remedy for fragile X patients will be the presence of functional protein in the neurons. Different approaches towards cures for genetic disorders in general are currently being investigated worldwide. In the case of fragile X syndrome, the significance of the requirement of appropriate regulation of expression level is underlined by findings that overexpression of FMRP in transgenic mice leads to behavioural problems (Peier *et al.* 2000, Gantois *et al.* 2001). Currently, no vectors fulfilling all these requirements are available.

Another potential avenue of therapy is the direct administration of the protein itself. This method has been successfully employed for treatment of Pompe disease by injection of α-glucosidase (Van den Hout *et al.* 2001) or the treatment of type I diabetes. Conversely, an additional obstacle for therapy of fragile X syndrome and other neurological diseases has been the blood–brain barrier (For a review on 'drug and gene delivery to the brain', see Pardridge 2002). This hurdle may be overcome with the observation of two independent groups that the HIV contains a transactivator protein (TAT) that can cross cell membranes (Frankel & Pabo 1988, Green & Loewenstein 1988). The ability to cross cell membranes is due to a protein transduction domain consisting of 11 basic amino acid residues, which has been identified as a nucleolar targeting signal in rev proteins (Kubota *et al.* 1989). A promising fact of this TAT domain with future therapeutic relevance was established when Anderson *et al.* (1993) reported cellular uptake of a protein coupled with the domain. The ability to confer transducing capability to fusion proteins has been fortified by different groups (Hagerman 2002a). Not only has the uptake of fusion proteins been demonstrated in almost every cell type, organ and animal, but more strikingly, transport of a biologically active intra peritoneal injected TAT-β-gal fusion protein via the blood–brain barrier into the brain has been reported (Schwarze *et al.* 1999). The mechanism of protein transduction is still unknown. However, the arginine-rich stretch of the TAT domain is cationic. Cationic groups are reported to interact electrostatically with anionic groups on the cellular membranes. This interaction increases membrane permeability by inducing absorptive-mediated endocytosis into the cell (Pardridge 2001). The TAT-dependent uptake has been reported to be as rapid as 10 min after administration and to be highly efficient.

Though, protein therapy may also raise a set of problems (such as delivery exclusively to target cells, immunological reactions to protein, delivery of the correct amount of protein), the availability of protocols for the production of TAT fusion proteins (Nagahara *et al.* 1998), the effectiveness, the speed of the uptake and the transport of the fusion protein to the brain initiated the study reported on here to analyse if protein therapy with a TAT–FMRP fusion protein could be applicable for treatment of the fragile X syndrome.

Fusion of the TAT domain N-terminal to the FMRP protein did not influence the localization of the fusion protein within the cells, since it was predominantly cytoplasmic. Only a small percentage of transfected COS cells and the SF21 cells displayed nucleolar localization of the fusion protein. No considerable consequences for therapy are expected as a result of this, since low expression of FMRP in the nucleolus has been observed *in vivo* in hippocampal neurons (Bakker *et al.* 2000), suggesting an as yet unidentified minor nucleolar function of FMRP within the brain. Nevertheless, the majority of the fusion protein localizes in the chief subcellular target compartment, the cytoplasm, probably due to signals such as the nuclear export signal within FMRP.

Since TAT–FMRP was not released into the medium during production, cell pellets had to be used for attainment of the protein. Purification of TAT–FMRP was not straightforward. The fusion protein had a propensity to aggregate and precipitate and displayed a high affinity to all materials.

In contrast to the reported uptake of fusion proteins in 80–100% of cells, we only observed uptake in 50% of the fibroblasts. The uptake appears to be TAT mediated, since FMRP lacking the TAT domain was not taken up within the same timeframe (data not shown). TAT–FMRP protein, which was taken up partially, also formed aggregates. The observed uptake of TAT–FMRP, however, was neither as efficient as reported in the literature nor as rapid, since we could only detect it after a few hours, with a maximum after 24 h. The amount of protein internalized by cells was variable. Controlling the quantity of uptake was thus not possible. Cells, which had incorporated too much, died. Hence TAT–FMRP is toxic in excess. We believe that the efficiency of TAT-mediated uptake of FMRP was hindered due to the problems, which we already encountered during purification, namely, that the protein seems to aggregate and that it also sticks to any surface. Since neurons are the primary target for therapy, we also administered TAT–FMRP to cultures of primary cortical neurons from E18 *Fmr1* knockout mice. However, neuronal uptake was hardly detectable (in $<2\%$, data not shown). We suggest that protein therapy via a TAT-mediated cellular uptake is not applicable for treatment of the fragile X syndrome. The TAT fusion protein at hand was less efficiently taken up in fibroblasts than other fusion proteins have been reported to do so. Uptake also took place less rapidly than reported. Neuronal uptake, the principal aim for therapy, was so inefficient that the study of a potential morphological influence of the presence of FMRP in cells lacking the protein was impossible (data not shown). Due to the difficulties described, we believe that it is doubtful that fragile X syndrome can be treated by protein therapy. The problems encountered are most likely due to the chemical properties of FMRP itself, rather than the TAT domain, since successful delivery of TAT fusion proteins into the brain has been demonstrated by independent groups. It is unlikely that the use of a different protein transduction domain or cloning the TAT domain C-terminally of FMRP will be sufficient to overcome the observed aggregation and sticking properties of FMRP. Even if new and improved production and purification systems are developed, unless a means can be found which suppresses these aggregating and sticking 'qualities' of FMRP, a successful administration, delivery of the protein to the brain and neuronal internalization is questionable. In order to avoid protein-caused problems, *FMR1* DNA or RNA could be administered, possibly even TAT-mediated. Nonetheless, the most conspicuous observation that we and others have made is the toxicity of excessive FMRP, a key setback for any method of *FMR1* DNA, RNA or protein delivery to neurons. Consequently, the utmost importance for any future therapy has to be placed on finding a means of controlling the appropriate dosage of FMRP expression.

Acknowledgements

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General discussion

Für Daniel und David

General discussion

In recent years, we have seen a growing consideration of fragile X syndrome as a neurodevelopmental or paediatric disorder (for review see [1]). The discovery of enhanced LTD in *Fmr1* knockout mice has lead to the hypothesis that the neurological and behavioural phenotypes observed in patients and knockout mice may be caused by an impairment of synaptic plasticity [2, 3]. Plasticity plays especially a role in the developing brain, when neuronal networks are established and reinforced in an activity-dependent manner. We still do not know the exact function of the FMR1 protein. However, as more data accumulates, the model of FMRP's involvement in RNA shuttling between the nucleus and the cytoplasm, the involvement in transport of mRNAs into the dendrites, and the translational control over FMRP's target mRNAs at (poly)ribosomes in general and at synaptic sites in particular is strengthened. With the increased knowledge on the significance of local protein translation at synaptic sites, the understanding of the function of FMRP in neurons, especially the dendrites, becomes more pressing, if we want to identify and eventually cure the impaired molecular pathways leading to compromised cognitive abilities of patients.

Target recognition of FMRP

The cognitive deficits in fragile X patients and the equivalent mouse model are hypothesised to be caused by an exaggerated mRNA translation, which has significant effects on synaptic structure and function. Instead of trying to identify potential mRNA targets by any of the methods described in chapter III, another means of predicting these targets may be to first identify the mechanisms that FMRP employs to select its mRNA partners. One model has been proposed so far. Darnell and colleagues have identified a number of candidate mRNA targets that possess so-called G-quartet motifs in their sequences. Interestingly, *Fmr1's* own messenger contains this structure as well. The G-quartet has been proposed as a model of a structurally based target recognition mechanism. Although a lot of emphasize has been put onto these findings, it has to be stressed, that only very few mRNAs identified to date as FMRP targets have a G-quartet motif [4]. We have shown that FMRP acts as a regulator of translation at the synapses (see chapter IV). FMRP not only interacts with the described target mRNAs, but also with the small non-translatable RNA *BC1*. We propose that FMRP directly binds to this RNA, and that in return, *BC1* base pairs with target mRNAs. *BC1* RNA has been reported to be a specific repressor of translation initiation in the dendrites [5]. It has been reported that this RNA is also expressed in the basal layers of seminiferous tubules [6]. This finding suggests that the RNA-targeting mechanism that we identified for FMRP, may not be restricted to the brain. Since expression of BC1 in Sertoli cells has not been excluded, it can be hypothesised that an upregulation of Sertoli cell mRNAs, which are translationally controlled by an FMRP-*BC1* interaction, may be the cause of the observed macro-orchidism in *FMR1* ko mice [7, 8]. The human homologue of *BC1*, *BC200*, is generally quoted as 'a brain

specific RNA'. However, when the expression specificity of BC200 was investigated, only lung and kidney samples were included in the analysis [9]. Therefore, it seems possible, that *BC200*, like the rodent *BC1*, may be expressed in the human testes. Another striking feature of *BC1* is its developmentally regulated expression pattern. In neurons [10], as well as in testes [6], *BC1* is expressed at developmentally early stages. This allows the hypothesis that FMRP may find its appropriate mRNA targets via mediation of spatiotemporally regulated non-coding RNAs. The site- restricted expression of *BC1* (testes and brain) may be an explanation why lack of FMRP most severely affects those two tissues.
We have not identified a specific sequence domain within the *BC1* RNA to be responsible for target recognition, so that blasting this putative domain in a sequence database could be used to predict potential mRNA targets of FMRP. Since both G-quartet structures and *BC1* sequence homologies have been demonstrated in relatively few mRNAs (so far), it is reasonable to expect more recognition mechanisms. Only recently, a third model has been proposed. The Darnell group has identified a sequence within the KH2 domain of FMRP that interacts with a so-called kissing complex RNA. This kissing complex RNA mediates the interaction of FMRP with brain polyribosomes [11]. The authors suggest that this interaction is crucial for proper neuronal function, since a rare mutation in the KH2 domain, the previously mentioned I304N missense mutation, was identified in a severely affected male fragile X patient [12].

Transport of mRNAs into the dendrites

In order to establish an asymmetric distribution of mRNAs, it is necessary to transport these messengers from the nucleus to the desired locations. Based on the presence of both a nuclear localisation signal and a nuclear export signal, FMRP has long been suggested to play a role in mRNA transport. Blocking the NES function results in accumulation of FMRP in the nucleus [13]. Our laboratory has previously demonstrated that FMRP (as part of an RNP complex) is transported via microtubules into neurites of cultured PC12 cells [14]. Although FMRP is generally quoted in literature as being responsible for mRNA transport into dendrites, no direct proof has been forwarded yet to corroborate this notion. On the contrary, based on the study described in chapter V, it seems more reasonable to conclude that FMRP and its associated mRNA targets are not the driving motors for an active transport into the dendrites. We have observed that although the above-mentioned missense mutation has an effect on the granule formation properties of the mutated protein, FMRP-I304N is still transported to the dendrites in a microtubule-dependent way (chapter V). If FMRP would be the active motor behind localisation of specific mRNAs into dendrites, these mRNAs should be absent in the dendritic mRNA pool of FMRP deficient neurons. The role of FMRP might be more to prevent inappropriate mRNA translation during the transport in the dendrite. However, FMRP is only one of many RNA binding proteins. It is quite likely that in absence of FMRP, the assumed transport activity of FMRP may be (partially) compensated by other proteins, among them FXR1P and FXR2P. The presence of mRNA in the FMRP deficient neurite sample used in chapter VI, demonstrates that mRNA transport has not subsided in absence

of FMRP. The cell culturing system described (chapter VI), may enable us in the future to identify mRNA species that are located in dendrites of wild-type mice, while absent in FMRP deficient dendrites.

Target mRNAs of FMRP

FMRP has been reported to bind 4% of the total mRNAs expressed in the brain [15]. However, few of these have been identified so far. The most remarkable of the known targets is MAP1b mRNA. Not only has it been shown to be differentially expressed in *Fmr1* knockout mice in comparison to wild-type animals, but also a model for the involvement of FMRP, MAP1b, and LTD in the FRAXA phenotype has been forwarded [16, 17]. This hypothesis is the first model that directly links lack of FMRP to decreased cognitive functioning. In order to fully understand all the different phenotypes observed in FRAXA, it is necessary to identify all the target mRNAs of FMRP. Different laboratories have employed a number of different strategies to address this issue [4, 18-20]. In summary, the studies undertaken so far relied either on experiments in brain synaptoneurosomes lysates, polysomal fractions, or immunoprecipiations. Taking into account the significance of local protein translation at the synapses for proper cognitive functioning, we attempted to develop a method to identify the mRNA content of particular subcellular compartments of neurons, the neurites (see chapter VII). In our opinion, it is only a matter of time till we can provide a list of mRNAs located in neurites, as well as a list of mRNAs that are differentially expressed in the absence of FMRP. If differences in the level of mRNA expression are significant enough to be detected, remains to be seen. Possibly, the lack of FMRP at synaptic sites is only influencing the translation of certain mRNAs while the transport might not be affected. Another option might be that the other members of the FXR family of genes are taking over this specific role. If differences in the mRNA pool between normal and FMRP-deprived neurites may be detected, it has to be taken into account that these differences, though providing insight into the impaired molecular pathways in neurites, do not reveal, which specific mRNAs are directly targeted by FMRP. However, a combination of our proposed technique with immunoprecipitation may soon resolve this concern.

Finding means of possibly curing fragile X syndrome and its associated pathologies

The objective for remedy for fragile X patients will be the absence of the phenotypes. How this goal may be accomplished (absence of the phenotype or elimination of the cause) is only secondary for patients. At the moment, no treatment seems likely that will eliminate the molecular cause of the syndrome. Studies concerning the reactivation of the *FMR1* gene are still in their infancy [21-23]. Administration of the protein triggers problems concerning the dosage control, since we (chapter VII) and others ([24, 25]) have observed a toxic effect of

overexpressed FMRP. A similar concern is valid for DNA or RNA-based approaches. Hence, treatment will be confined to treatment of the different phenotypes, as has been suggested by the administration of MPEP as a glutamate receptor antagonist. In this context, the identification of the diverse molecular pathways controlled by FMRP is crucial. The studies described in chapter III referring to the role of FMRP in glutamate receptor dependent synaptic plasticity, propose another avenue to improve a patient's disposition: these events are activity-dependent. Hence early diagnosis and an adjusted lifestyle may improve the cognitive functioning.

Concluding remarks: *cogito ergo sum, non cogito non sum*?

The absence of FMRP causes rather mild morphological modifications in the nervous system of fragile X patients and *Fmr1* knockout mice. Changes seem to be confined to small volume changes in different brain areas and abnormal structures of a specific component of the dendrites, named the spines. No significant neurological degeneration has been reported. However, impaired cognitive abilities are still considered a stigma in society. What may be seen as a mild phenotype from a scientific point of view may have severe consequences for patients and their families in every day life. In order to provide a safe and effective cure for fragile X syndrome in the future, it is essential to fully understand the molecular pathways involved in cognition. Although it might be questionable whether a full therapy for the mental retardation is realistic, we expect that treatment of certain phenotypic elements of the syndrome such as hyperactivity, autistiform behavior, social avoidance and epilepsy may be feasible.

Many questions still remain unanswered in respect to the role of FMRP in synaptic plasticity. Identification of the precise mRNA content of dendrites, the mechanisms of mRNA targeting, mRNA transport, and translation, as well as the exact involvement of these mRNA species in neuronal processes at the synapses will be a big step in comprehending cognitive functions such as learning and memory. Genetic research surely will not be sufficient to provide answers to philosophical questions concerning the definition of life, what life may be worth living, or what type of behaviour should be considered normal. However, a profound knowledge of the molecular processes of the brain may provide means to cure or eliminate neurological diseases; it may reveal possibilities to reinforce or avoid certain activity-dependent behaviours; and it may create a greater understanding of general human nature.

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Summaries

Summary

Cogito ergo sum, I think therefore I am. Since humanity has developed consciousness, philosophers and scientists were interested to find out how and with what means we think. Another long-standing debate has been whether the mind and the body are separate entities. In support of an interaction between these two, are individuals with diminished mental capabilities, which are caused by genetic mutations. The study of congenital disorders correlated with impairment of cognitive functions will therefore provide insight into the physical nature of the human mind. The most common form of inherited mental retardation is the fragile X syndrome. This disorder, with a prevalence of 1 in 4000 men and 1 in 6000 women, belongs to a group of approximately 200 conditions related to mental retardation, which are caused by mutations on the X chromosome. Affected individuals display (among others) a varying severity of cognitive impairment, a number of behavioural problems, as well as some facial abnormalities. It has been shown in different brain regions that the cellular anatomy is affected in fragile X patients: they have an increased number of immature spines, while the number of mature spines is decreased. Spine abnormalities are a general phenomenon in disorders related to mental retardation. Predominantly, dendritic spines provide the sites for synaptic connections. Spines also have been suggested to play a central part in lasting forms of synaptic weight changes. These events of *synaptic plasticity* are hypothesized to be imperative for learning and memory formation. The intellectual disability in fragile X syndrome has therefore been suggested to derive from an impairment of brain plasticity, synaptic pruning, or maturation process. The molecular basis of the syndrome is the absence of the fragile X mental retardation protein (FMRP). Due to an expansion of an unstable CGG repeat in the 5'-untranslated region of the fragile X mental retardation gene 1 (*FMR1*), the repeat, as well as the promoter region of the gene become hypermethylated, which in turn suppresses the transcription and consequently the translation of the *FMR1* gene. However, the correlation between absence of the protein, change in the architecture of spines, and a cognitive disability in fragile X patients is not fully understood yet. This thesis aims to contribute to the unravelling of molecular pathways, which are involved in cognitive functions, such as learning and memory, by studying the function of FMRP in dendrites.

The first part of this thesis (general introduction) reviews the brain, fragile X syndrome, and the role of FMRP in dendrites. In **chapter I**, a historical, evolutionary, developmental, architectural, and functional overview of the brain is given. Essential for the complexity of the functions that the brain orchestrates, is the hierarchical order of different networks of cells. Therefore neurons, their means to communicate, and the 'communication intersection points', the synapses, are described in more detail. Furthermore, the ability of the brain to change its shape, the brain plasticity, is discussed. In **chapter II**, the clinical and molecular genetic aspects of fragile X syndrome are summarized. Additionally, the suitability of a mouse model of the syndrome is reflected upon. **Chapter III** reviews in more detail the function of FMRP in neurons, and in particular in dendrites.

The neuronal mRNA partners of FMRP, the trafficking of these to the dendrites, and the FMRP-mediated translational regulation of the messengers at the synaptic sites is debated.

The research, on which this thesis is based, is presented in the second part. It relates to the molecular mechanisms involved in neuronal processes, in particular establishing an asymmetric distribution and a spatially restricted translation of mRNAs at the synapses. **Chapter IV** proposes a model mechanism how FMRP succeeds in recruiting the appropriate mRNA cargo. The results confirm that FMRP acts as a repressor of the translation of specific mRNAs at synapses. Another important finding of the study is that not only FMRP, but also a non-coding RNA, *BC1*, is present in an RNP complex. *BC1* has been implied to play a role in neuronal development as well as learning and memory function. This research discloses that *BC1* can bind directly to FMRP, as well as to specific target mRNAs of FMRP via base-pairing. In the absence of *BC1,* the interaction of FMRP with its messengers is interrupted. These data suggest that target recognition of mRNAs and the translation thereof is *BC1*-mediated.
A rare mutation in the second KH domain has been identified in a patient with a severe fragile X phenotype. This mutation (I304N) compromises the RNA binding capacity, the folding capacity, the homo-oligomerisation, the function as a translational regulator, and the ability to form a normal RNP particle. Since locally regulated protein expression has been implied to play an important role in neuronal processes, the significance of FMRP's part in the asymmetric distribution of mRNAs in dendrites was investigated by examining the transport kinetics of a mutated FMRP-I304N-EGFP protein in a PC12 cell line (**chapter V**). The mutated protein is transported to the dendrites. However, the formation of RNP granules is heavily compromised. These findings allow speculation that erroneous distribution and translation of mRNAs due to mutated FMRP protein may have a more critical effect on neuronal processes than the absence of FMRP.
In order to identify the mRNAs, which are asymmetrically distributed to the dendrites, a cell culture system has been developed that allows the physical separation of neuronal cell bodies from their neurites (**chapter VI**). To ensure the absence of cell bodies and non-neuronal cells from the samples, laser microdissection microscopy was applied. The described methodology permitted sufficient material for the successful isolation of mRNA from mouse neurites. An application for the developed system has been tested, and the preliminary results are discussed in the **appendix**.
In addition to the above described work, we considered a therapy for fragile X syndrome based on protein replacement (**chapter VII**). However, cells demonstrated to be highly susceptible to the expression level of FMRP. Since the toxic effect resulting from overexpression is difficult to prevent, protein replacement therapy may be inappropriate to treat fragile X patients.

Samenvatting

Dutch translation by Esther de Graaff and Jeannette Lokker.

Cogito ergo sum, ik denk dus ik besta. Vanaf het moment dat het bewustzijn zich in de mensheid ontwikkelde, wilden filosofen en wetenschapppers ontdekken hoe en met welke middelen wij denken. Een tweede historisch debat handelt over de vraag of geest en lichaam twee verschillende eenheden zijn. Een argument voor een interactie tussen deze twee, zijn individuen met verminderde mentale capaciteiten, die veroorzaakt worden door genetische mutaties. Studies naar congenitale afwijkingen, die gecorreleerd zijn met een deficiëntie van de cognitieve functies, zullen derhalve inzicht verschaffen in het fysieke karakter van de menselijke geest.
De meest voorkomende vorm van een erfelijke verstandelijke handicap is het fragiele X syndroom. Deze aandoening, met een prevalentie van 1 op 4000 mannen en 1 op 6000 vrouwen, behoort tot een groep van ongeveer 200 aandoeningen die gerelateerd is aan een verstandelijke handicap veroorzaakt door een mutatie op het X chromosoom. Aangedane individuen vertonen (onder andere) een variërende ernst van een cognitief defect, een aantal gedragsstoornissen alsmede abnormaliteiten aan het aangezicht. In verschillende hersengebieden is aangetoond dat de cellulaire anatomie in fragiele X patiënten afwijkend is: er is een toename van het aantal onrijpe spines, terwijl het aantal rijpe spines verlaagd is. Afwijkingen van de spines worden vaker gevonden bij aandoeningen die gerelateerd zijn aan een verstandelijke handicap. Het zijn hoofdzakelijk de dentritische spines die de locatie van synaptische connecties bepalen. Spines lijken ook een centrale rol te spelen bij de blijvende vormen van synaptische veranderingen. Deze vormen van synaptische plasticiteit worden verondersteld essentieel te zijn voor het leren en de geheugenvorming. Er is daarom gesuggereerd dat de intellectuele handicap in het fragiele X syndroom veroorzaakt wordt door een verminderde hersenplasticiteit, synaptische inkorting of het rijpingsproces.
De moleculaire basis van het syndroom is de afwezigheid van het fragiele X mentale retardatie eiwit (FMRP). Door een verlenging van een instabiele CGG repeat in het 5' onvertaalde deel van het fragiele X mentale retardatie gen 1 (*FMR1*) vindt hypermethylering plaats van de repeat en de promoter van het gen, wat ertoe leidt dat de transcriptie en derhalve ook de translatie van het *FMR1* gen onderdrukt wordt. Echter, de correlatie tussen de afwezigheid van het FMRP, de veranderingen in de spine architecteur en het verminderde cognitieve vermogen in fragiele X patiënten, wordt nog niet volledig begrepen. Dit proefschrift is erop gericht, door de functie van FMRP in dendrieten te bestuderen, een bijdrage te leveren aan het oplossen van de moleculaire paden die betrokken zijn bij cognitieve functies, zoals het leren en het geheugen.

Het eerste deel van dit proefschrift (de algemene introductie) geeft een overzicht over de hersenen, het fragiele X syndroom en de rol van FMRP in dendrieten. In **hoofdstuk I** wordt een historische, evolutionaire, ontwikkelingsbiologische, architectonische en functionele samenvatting van het brein gegeven. De hierarchische structuur van de verschillende cellulaire netwerken is essentieel voor de complexiteit van de functies die door de hersenen georganiseerd

worden. Neuronen, hun middelen waarmee ze kunnen communiceren, en de 'communicati ekruispunten', de synapsen, worden derhalve gedetailleerder beschreven. Verder wordt het vermogen van de hersenen om van vorm te veranderen, de zogenaamde hersenplasticiteit, bediscussieerd. In **hoofdstuk II** worden de klinische en moleculair genetische aspecten van het fragiele X syndroom samengevat. Tevens wordt gereflecteerd op de geschiktheid van een muismodel van het syndroom. **Hoofdstuk III** geeft een gedetailleerder overzicht van de functie van FMRP in neuronen en in het bijzonder in de dendrieten. De neuronale mRNA partners van FMRP, het transport van deze naar de dendrieten en de FMRP-gemedieerde translationele regulatie van de mRNA's in de synaptische locaties wordt besproken.

Het onderzoek waarop dit proefschrift gebaseerd is, wordt gepresenteerd in deel twee van dit proefschrift. Dit onderzoek heeft betrekking op de moleculaire mechanismen die betrokken zijn in de neuronale processen, in het bijzonder in het ontstaan van een asymmetrische verdeling en een ruimtelijk beperkte translatie van mRNA's in de synapsen. **Hoofdstuk IV** beschrijft een modelmechanisme hoe FMRP erin slaagt de juiste mRNA lading aan te trekken. De resultaten bevestigen dat FMRP de translatie van specifieke mRNA's in de synapsen onderdrukt. Een andere belangrijke bevinding van dit onderzoek is, dat niet alleen FMRP maar ook niet-coderend RNA, *BC1*, aanwezig is in een RNP complex. Van *BC1* wordt geïmpliceerd dat het betrokken is bij zowel neuronale ontwikkeling, als het leren en de geheugenfunctie. Dit onderzoek toont aan dat *BC1* via base-paring zowel direct aan FMRP kan binden, als aan specifieke mRNA targets van het FMRP. Bij afwezigheid van *BC1* is de interactie tussen FMRP en de targets verstoord. Deze resultaten suggereren dat de target herkenning van de mRNA's en de translatie ervan door *BC1* gemedieerd wordt.
In het tweede KH domein is een zeldzame mutatie gevonden in een patiënt met een ernstig fragiele X fenotype. Deze mutatie (I304N) verstoort de RNA-bindende capaciteit, de vouwingscapaciteit, de homo-oligomerisatie, de functie als translationele onderdrukker en het vermogen om een normaal RNP partikel te vormen. Aangezien aangenomen wordt dat lokaal gereguleerde eiwitexpressie een belangrijke rol speelt in neuronale processen, is de significantie van de functie van FMRP in de asymmetrische verdeling van mRNA's in dendrieten onderzocht door de transportkinitiek van een gemuteerd FMRP-I304N-EGFP eiwit in een PC12 cellijn te bestuderen (**hoofdstuk V**). Het gemuteerde eiwit wordt getransporteerd naar de dendrieten, maar de vorming van RNP partikels is ernstig verstoord. Deze bevindingen leiden tot een hypothese dat de verkeerde distributie en translatie van mRNA's als gevolg van het gemuteerde FMRP eiwit een ernstiger effect kan hebben op neuronale processen dan de afwezigheid van FMRP.
Om mRNA's te identificeren die asymmetrisch naar de dendrieten verdeeld zijn, is een celkweeksysteem ontwikkeld dat de fysieke scheiding van neuronale cellichamen en hun neurieten mogelijk maakt (**hoofdstuk VI**). Laser microdissectie microscopie werd toegepast om er zeker van te zijn dat cellichamen en niet-neuronale cellen ontbraken in de verkregen monsters. Met de beschreven methode werd voldoende materiaal verkregen voor de succesvolle isolatie van mRNA uit muisneurieten. Een toepassing voor het ontwikkelde systeem is getest en wordt beschreven in de **bijlage**.

Naast het eerder beschreven onderzoek, hebben we een behandeling van fragiele X patiënten op basis van eiwitvervanging nauwkeurig overwogen (**hoofdstuk VII**). Helaas vertoonden cellen een hoge gevoeligheid voor de expressieniveaus van FMRP. Aangezien deze toxische effecten als gevolg van overexpressie moelijk te voorkomen zijn, lijkt therapie gebaseerd op eiwitvervanging niet geschikt voor het behandelen van fragiele X patiënten.

Zusammenfassung

Cogito ergo sum. Ich denke, also bin ich. Seit die Menschheit ein Bewusstsein entwickelt hat, waren Philosophen und Wissenschaftler daran interessiert, wie und mit welchen Mitteln wir denken. Eine weitere lang anhaltende Debatte beschäftigt sich damit, ob der Geist und der Körper zwei unterschiedliche Einheiten sind. Die Idee, daß diese beiden Einheiten miteinander agieren, wird unterstütz, da es Personen mit verminderten mentalen Fähigkeiten gibt, die von genetischen Mutationen verursacht werden. Das Erforschen von Erbkrankheiten, die Beeinträchtigungen der kognitiven Funktionen nach sich ziehen, wird daher Einsicht in die physikalische Natur des menschlichen Geistes gewähren. Die häufigste Form mentaler Retardation ist das fragile X-Syndrom. Diese Krankheit, mit einer Prävalenz von 1:4000 Männern und 1:6000 Frauen, gehört zu einer Gruppe von ca. 200 Konditionen von geistiger Behinderung, die durch Mutationen auf dem X Chromosom verursacht werden. Betroffene Individuen leiden (unter anderem) an variierend starker Beeinträchtigung der mentalen Fähigkeiten, einer Anzahl von Verhaltensproblemen und einiger Gesichtsanomalien. In verschiedenen Gehirnregionen wurde nachgewiesen, daß die zelluläre Anatomie in fragilen X-Patienten verändert ist: sie weisen eine erhöhte Anzahl von unterentwickelten Dendritenspinen auf, während die Anzahl der vollentwickelten Dendritenspinen verringert ist. Anomale Dendritenspine sind ein generelles Phänomen in Krankheiten mentaler Retardationen. Hauptsächlich liefern diese Dendritenspine den Ort für synaptische Verbindungen. Es wird angenommen, daß Dendritenspine eine zentrale Rolle in Langzeitformen synaptischer Gewichtsveränderungen spielen. Es wird weiterhin angenommen, daß diese *synaptischen Plastizitätsereignisse* unabkömmlich für Lernfunktionen und Gedächtnisbildung sind. Es wird daher vorgeschlagen, daß die intellektuelle Behinderung im fragilen X-Syndrom auf einer Beeinträchtigung von Gehirnplastizität, synaptischem Prunen oder Maturierungsprozessen beruht. Die molekulare Grundlage des Syndroms ist die Abwesenheit des „fragile X mental retardation protein" (FMRP). Aufgrund der Expansion eines unstabilen CGG-Repeats im 5'-untransliertem Gebiet des „fragile X mental retardation gene 1" (*FMR1*), wird sowohl der Repeat als auch der Promoter des Genes hypermethyliert, so daß die Transkription und folglich die Translation des *FMR1* Genes unterdrückt wird. Jedoch ist der Zusammenhang zwischen fehlendem Protein, veränderter Dendritenarchitektur und kognitiver Behinderung in fragilen X-Patienten noch nicht bekannt. Diese Doktorarbeit versucht, zum Aufschlüsseln der molekularen Prozesse beizutragen, die eine Rolle in kognitiven Funktionen, wie Lernen und Gedächtnis, spielen, indem sie die Funktion von FMRP in Dendriten studiert.

Der erste Teil dieser Arbeit (allgemeine Einführung) beschreibt das Gehirn, fragiles X-Syndrom und die Rolle von FMRP in Dendriten. In **Kapitel I** wird Überblick über die Geschichte, die Evolution, die Entwicklung, die Architektur und die Funktion des Gehirns gegeben. Essentiell für die Komplexität der Gehirnfunktionen ist die hierarchische Anordnung verschiedener Zellnetzwerke. Daher werden Neuronen, ihre Kommunikationsmittel und die 'Kommunikationspunkte', die Synapsen detaillierter beschrieben. Außerdem wird die Fähigkeit des Gehirns, sich zu verändern, die *Gehirnplastizität*, diskutiert. **In Kapitel II** werden die klinischen und molekulargenetischen Aspekte von fragilem X-Syndrom

zusammengefaßt. Zusätzlich wird über die Angemessenheit eines Mausmodelles für das Syndrom reflektiert. **Kapitel III** beschreibt im Detail die Funktion von FMRP in Neuronen, vor allem in den Dendriten. Die neuronalen mRNS Partner von FMRP, der Transport dieser zu den Dendriten und die FMRP regulierte Translation der messenger an den Synapsen wird debattiert.

Die Forschung, auf der diese Arbeit basiert, wird im zweiten Teil präsentiert. Sie bezieht sich auf die molekularen Mechanismen neuronaler Prozesse, vor allem das Herstellen einer asymmetrischen Distribution und lokal restriktiver Translation von mRNS in den Synapsen. **Kapitel IV** schlägt einen Modelmechanismus vor, wie es FMRP gelingt, die angemessene mRNS Ladung zu rekrutieren. Die Ergebnisse bestätigen, daß FMRP als Repressor der Translation bestimmter mRNS in den Synapsen agiert. Ein weiterer wichtiger Fund dieser Studie ist, daß nicht nur FMRP, sondern auch eine nicht kodierende RNS, *BC1*, sich in einem RNP-Komplex befindet. *BC1* wurde eine Rolle sowohl in neuronaler Entwicklung, als auch in Lern- und Gedächtnisfunktionen nachgesagt. Die vorliegende Studie offenbart, daß sich *BC1* sowohl direkt an FMRP, als auch an spezifische Ziel-mRNS von FMRP mittels Basenpaarung binden kann. In Abwesenheit von *BC1* ist die Interaktion von FMRP mit seinen messengern unterbrochen. Diese Daten suggerieren, daß Zielerkennung der mRNS und deren Translation *BC1* abhängig ist.
Eine seltene Mutation wurde in der zweiten KH-Domäne in einem Patienten mit einem extremen fragilen X-Phänotyp identifiziert. Diese Mutation (I304N) beeinträchtigt die RNS Bindungskapazität, die Faltkapazität, die Homo-Oligomerisation, die Funktion als Translationsregulator und die Fähigkeit einen normalen RNP-Partikel zu formen. Da lokal regulierte Proteinexpression eine wichtige Rolle in neuronalen Prozessen spielen soll, wurde die Signifikanz von FMRP in der asymmetrischen Verteilung von mRNS in den Dendriten erforscht, indem die Transportkinetik eines mutierten FMRP-I304N-EGFP Proteins in einer PC12 Zellinie untersucht wurde (**Kapitel V**). Das mutierte Protein wird zu den Dendriten transportiert. Jedoch ist das Formen von RNP Granulen schwerwiegend beeinträchtigt. Diese Ergebnisse erlauben zu spekulieren, daß fehlerhafte Distribution und Translation von mRNS aufgrund mutierten FMRP Proteins gravierendere Folgen auf neuronale Prozesse hat, als die Abwesenheit von FMRP.
Um die mRNS zu identifizieren, die asymmetrisch in den Dendriten verteilt sind, wurde ein Zellkultursystem entwickelt, das die physikalische Separation neuronaler Zellkörper von ihren Neuriten erlaubt (**Kapitel VI**). Um die Abwesenheit von Zellkörpern und nicht neuronaler Zellen zu versichern, wurde Lasermikrodissektionsmikroskopie angewandt. Die beschriebene Methode ermöglichte es, genügend Material für eine erfolgreiche Isolation von mausneuritischer mRNS zu liefern. Eine Anwendung des entwickelten Systems wurde getestet, deren vorläufige Ergebnisse im **Appendix** diskutiert werden.
Zusätzlich zu den oben beschriebenen Arbeiten, zogen wir eine Proteinersatztherapie für fragiles X-Syndrom in Betracht (**Kapitel VII**). Jedoch demonstrierten die Zellen eine hohe Empfindlichkeit für das Expressionsniveau von FMRP. Da der durch Überexpression hervorgerufene toxische Effekt schwer zu unterbinden ist, ist Proteinersatztherapie möglicherweise nicht angemessen, um fragiles X-Syndrom zu behandeln.

Resumé
Translated version by Iona Condacci.

Cogito ergo sum, je pense donc je suis. Depuis que l´humanité a développé la notion de conscience, les philosophes et les scientifiques furent intéressés de découvrir comment et par quels moyens nous pensons. Un autre débat a été, de longue date, de savoir si l´esprit et le corps sont des entités distinctes. A l´appui d´une interaction entre les deux entités, on trouve des individus dont les capacités mentales sont diminuées par des mutations génétiques. L´étude des maladies génétiques en corrélation avec les troubles de la fonction cognitive peuvent ainsi nous éclairer sur la nature physique de l´esprit humain. La forme la plus rependue de retard mental héréditaire est le syndrome fragile X. Cette maladie, dont l´occurrence est de 1 a 4000 chez les hommes et de1 a 6000 chez les femmes, appartient à un groupe d´environ 200 pathologies liées au retard mental, lesquelles sont causées par des mutations du chromosome X. Les individus touchés présentent, entre autres, divers degrés de troubles cognitifs, certains problèmes comportementaux de même que des anomalies faciales. Il a été démontré que l´anatomie cellulaire est affectée dans différente régions du cerveau chez les patients touchés par le syndrome fragile X : ils disposent d´un nombre supérieur d'épines des dendrites non maturées alors que le nombre d'épines des dendrites maturées est inférieur. Les anomalies constatées sur les épines des dendrites sont un phénomène rependu dans les troubles liés au retard mental. Généralement, les connections synaptiques se produisent au niveau des épines des dendrites. Il a aussi été avancé que ces épines jouent un rôle central dans les transformations durables du poids synaptique et que ces occurrences de « plasticité synaptique» sont nécessaires à la formation de la faculté d´apprendre et de mémoriser. C´est pourquoi, les causes des disfonctionnement intellectuels identifiés dans le syndrome fragile X ont été recherchées dans un défaut, soit de plasticité du cerveau, soit de formation synaptique ou encore de processus de maturation. La base moléculaire du syndrome fragile X consiste dans une absence de la protéine appelée fragile X mental retardation protéine (FMRP). En raison d´une expansion de la répétition de CGG instables dans la région 5 non traduite du gêne, appelé fragile X mental retardation gêne 1 (*FMR1*), non seulement la répétition mais encore la zone promoteur du gêne deviennent hypermethilés, ce qui cause la suppression de la transcription et partant de la traduction du gêne *FMR1.* Quoiqu´il en soit, la corrélation entre l´absence de protéine, le changement de l´architecture des branches et les troubles de la fonction cognitive chez les patients atteints du syndrome fragile X n´est pas totalement compris à ce jour. Cette thèse a pour but de contribuer à la découverte des chemins moléculaires qui sont impliqués dans les fonctions cognitives telle que la faculté d'apprendre et de mémoriser, en étudiant le rôle de la protéine FMRP dans les dendrites.

La première partie de cette thèse (introduction générale) passe en revue le cerveau, le syndrome fragile X et le rôle de la protéine FMRP dans les dendrites. Au **chapitre I** se trouve un bref aperçu historique, évolutif, architectural et fonctionnel du cerveau. Un des éléments essentiels de la complexité des fonctions du cerveau se trouve être l'ordre hiérarchique des

différents réseaux. C'est pourquoi les neurones, leurs moyens de communication, les points d'intersection de cette communication, les synapses, sont décrits de façon plus détaillée. De plus, la faculté du cerveau de changer de forme, soit la plasticité du cerveau, est discutée. Dans le **chapitre II**, les aspects cliniques et génétique moléculaires du syndrome fragile X sont résumés. De plus, l´adéquation d'un modèle du syndrome chez la souri est traité. Le **chapitre III** passe en revu avec plus de détails la fonction de la protéine FMRP dans les neurones et en particulier dans les dendrites. Le partenaire neuronal de la protéine FMRP, soit le mRNA, le transport de celui-ci aux dendrites et la traduction régulée par la protéine FMRP des messager dans les synapses est débattu.

La recherche sur laquelle se base cette thèse est présentée dans sa deuxième partie et à trait au mécanisme moléculaire impliqué dans le fonctionnement neuronal, en particulier l´établissement d´une distribution asymétrique et la traduction restreinte spatialement du mRNA au niveau des synapses. Le **chapitre IV** propose un modèle mécanique expliquant comment la protéine FMRP trouve le mRNA appropriée. Les résultats confirment que la protéine FMRP agit comme un répresseur de la traduction des mRNA spécifiques au niveau des synapses. Une autre découverte importante faite dans le cadre de cette étude a été de constater que non seulement la protéine FMRP mais également un RNA non codé, soit *BC1*, est présent dans un complexe RNP. Il a été avancé que *BC1* joue un rôle dans le développement neuronal ainsi que dans la fonction d´apprentissage et de mémorisation. Cette recherche a permis de découvrir que *BC1* peut se lier directement à la protéine FMRP et du de même au mRNA spécifique au moyen d´un base paring. Dans l´absence de *BC1* l´interaction de la protéine FMRP avec ses messagers est interrompue. Cette donnée laisse à penser que la reconnaissance du mRNA et la traduction de cet mRNA est causée par *BC1*.
Une rare mutation dans le second domaine KH a été identifié chez un patient avec un sévère phénotype de fragile X. Cette mutation (I304N) compromet la faculté du RNA de se lier, de sa formation ainsi que son homo-oligomerisation, sa fonction comme régulateur de traduction et sa capacité de former une particule normale de RNP. Dès lors que l´expression de la protéine régulée localement a été identifiée comme jouant un rôle important dans le fonctionnement neuronal, la signification du rôle de la protéine FMRP dans la distribution asymétrique de mRNA dans les dendrites a été étudiée à travers l´examen des transports cinétiques d´une protéine mutée, FMRP-I304N-EGFP, dans des cellules PC12 (**chapitre V**). La protéine mutée est transportée dans les dendrites, mais la formation des granules de RNP est fortement compromise. Ces découvertes permettent d´avancer l´hypothèse selon laquelle la distribution et la traduction erronée de mRNA causée par la protéine FMRP mutée peut avoir un effet plus critique sur le fonctionnement neuronal que l´absence de FMRP.
Afin d´identifier les mRNA qui sont distribués asymétriquement au niveau des dendrites, un système de culture cellulaire a été développé lequel permet la séparation physique des neuronales corps cellulairs de leur neurites (**chapitre VI**). Pour s´assurer de l´absence des corps célullaires et de cellules non neuronales dans l´échantillon, nous avons eu recours à une microdissection laser sous microscope. La méthodologie décrite a permis de créer la matière suffisante pour isoler le mRNA des neurites de souris. Une application du système développé

a été testé et les résultats préliminaires sont discutés dans **l´annexe**.
En sus du travail décrit ci-dessus, nous avons considérer une thérapie du syndrome fragile X basé sur un remplacement protidique (**chapitre VII**). Toutefois, les cellules ont démontré être fortement sensible au niveau d´expression de la protéine FMRP. Dès lors que l´effet toxique causé par la surexpression est difficile à prévenir, la thérapie de remplacement protidique pourrait ne pas être appropriée pour traiter les patients atteints du syndrome fragile X.

Riassunto

Translated version by Alessio di Fonzo and Filippo Tamanini.

Cogito ergo sum, penso dunque sono. Poiche' l'umanita' si e' sviluppata sulla base della conoscenza, filosofi e scienziati si sono dedicati allo studio di "come e con quali mezzi" siamo in grado di pensare. Un altro dibattito di lunga data ha riguardato l'ipotesi che il corpo e la mente fossero due entita' separate. In supporto all'interazione tra le due, vi sono soggetti con ridotte capacita' mentali dovute a mutazioni genetiche.
La forma piu' commune di ritardo mentale e' la sindrome dell' X fragile. Questa malattia, con una prevalenza di 1/4000 maschi e 1/6000 femmine, appartiene al gruppo di circa 200 condizioni cliniche di ritardo mentale causate da mutazioni sul cromosoma X. Gli individui affetti presentano una variabile gravita' del quadro cognitivo, una serie di problemi comportamentali e dismorfismi facciali. E' noto che in diverse aree dell'encefalo la disposizione anatomica dei neuroni e' sovvertita nei pazienti con sindrome dell'X fragile: il numero di spine neurali immature e' maggiore, mentre il numero di quelle mature e' inferiore. Anormalita' delle spine neurali sono un fenomeno comune nei ritardi mentali. In particolare, le spine dendritiche rappresentano il sito di connesione sinaptica. E' stato anche ipotizzato un ruolo delle spine neurali nelle forme durature di cambiamento synaptico. Questi eventi di plasticita' sinaptica sono stati ipotizzati essere importanti per la memoria e l'apprendimento. L'inabilita' intellettuale nella syndrome dell'X fragile e' quindi stata suggerita derivare da alterazioni della plasticita' neurale, dell'attivita' sinaptica o del processo di maturazione.
Dal punto di vista molecolare la sindrome e' causata dall'assenza della proteina FMRP (fragile X mental retardation protein) dovuta ad una espansione della tripletta CGG, altamente instabile, nella regione 5' non tradotto del gene *FMR1.* La tripletta, come anche la regione promoter, risulta ipermetilata, il che abolisce la trascrizione e di conseguenza la traduzione del gene *FMR1.* Comunque, la correlazione tra assenza della proteina, alterazione nell'architettura delle spine, e disturbi cognitive nei pazienti con X fragile non e' ancora chiara. Questa tesi ha lo scopo di contribuire nell'indagine dei meccanismi molecolari che sono interessati nelle funzioni cognitive, come l'apprendimento e la memoria, studiando la funzione della proteina FMRP nei dendriti.

La prima parte di questa tesi riassume il cervello, la sindrome dell'X fragile ed il ruolo della proteina FMRP nei dendriti. Nel **capitolo I** e' presentata una overview sul cervello a livello storico, evolutivo, di sviluppo, architettativo e funzionale. Essenziale per la complessita' delle funzioni orchestrate dal cervello e' l'ordine gerarchico delle diverse reti neurali. Quindi i neuroni, la loro capacita' di comunicare, e "i punti di intersezione comunicativa" (cioe' le sinapsi) sono descritte in dettaglio. Inoltre, e' discussa la capacita' del cervello di modificarsi: la plasticita' cerebrale.
Nel **capitolo II** sono esposti gli aspetti clinici e molecolari della sindrome dell'X fragile, con considerazioni riguardo anche al modello murino di questa sindrome. Il **capitolo III** riassume in dettaglio la funzione della proteina FMRP nel cervello, il trasporto di questa nei dendriti

e la regolazione della transizione dele RNA messaggeri a livello sinaptico mediata da FMRP. La ricerca, sulla quale si basa questa tesi, e' presentata nella seconda parte. Questa riguarda i meccanismi molecolari coinvolti nei processi neuronali, in particolare la distribuzione asimmetrica e la traduzione spazio correlata del mRNA a livello sinaptico. Il **capitolo IV** propone un meccanismo esemplificativo di come FMRP riesca a recrutare il proprio carico di mRNA. I risultati confermano che FMRP agisce come repressore della traduzione di specifici mRNA a livello sinaptico. Altri importanti risultati di questo studio riguardano non solo FMRP ma anche l'RNA non codificante, *BC1*, che e' presente nel complesso RNP. Si ritiene che *BC1* sia implicato nello sviluppo neuronale, memoria e apprendimento. Questa ricerca mostra che *BC1* puo' legare direttamente FMRP allo stesso modo di altri specifici mRNA target di FMRP attraverso il meccanismo di "base-pairing". In assenza di *BC1* l'interazione di FMRP con i messaggeri e' interrotta. Questi dati suggeriscono che il riconoscimento degli mRNA e di conseguenza la loro traduzione e' mediata da *BC1*.
Una rara mutazione nel secondo KH dominio e' stata individuata in un paziente con un grave fenotipo di X fragile. Questa mutazione (I304N) compromette la capacita' di legame dell'RNA, la capacita' di assumere la corretta conformazione, l'omo-oligomerizzazione, la funzione come regolatore della traduzione e la capacita' di formare normali RNP. Poiche' l'espressione proteica, regolata a livello della synapsi, si pensa giocha un ruolo importante nei processi neuronali, il significato di FMRP nella distribuzione asimmetrica di mRNA nei dendriti e' stata studiata esaminando la cinetica di trasporto della proteina aberrante FMRP-I304N-EGFP nelle linee cellulari PC12 (**capitolo V**). La proteina mutata e' trasportata nei dendriti. Tuttavia la formazione di RNP e' gravemente compromessa. Questi risultati permettono di speculare che l'erronea distribuzione e traduzione degli RNA messaggeri dovuta alla proteina mutata FMRP potrebbe avere un effetto piu' grave sui processi neuronali che la mancanza della proteina FMRP. Al fine di individuare gli RNA messaggeri che sono asimmetricamente distribuiti nei dendriti e' stato sviluppato un sistema di colture cellulari che permetta la separazione fisica dei corpi neuronali dai neuriti (**capitolo VI**). Per accertarsi dell'assenza di corpi cellulari e cellule non neuronali, e' stata utilizzata la microdissezione microscopica. La metodologia descritta permette di ottenere materiale sufficiente per una adeguata estrazione di mRNA dai neuroni murini. Un' applicazione di questa tecnica e' stata testata, e' i risultati preliminari sono discussi nell' **appendice**.
Oltre al lavoro descritto sinora, abbiamo preso in considerazione una terapia per la sindrome dell'X fragile basata sulla sostituzione della proteina (**capitolo VII**). Le cellule hanno dimostrato di essere altamente suscettibili al livello di FMRP. Poiche' e' difficile prevenire l'effetto tossico dovuto alla sua overespressione, la sostituzione della proteina potrebbe essere una terapia inappropriate per i pazienti con sindrome dell'X fragile.

Resumen

Translated version by Aida Bertoli Avella.

Cogito ergo sum. Pienso, luego existo. Desde que la humanidad ha sido consciente, filósofos y científicos han estado interesados en descubrir cómo y con qué medios los seres humanos pensamos. Por otro lado, el hecho de si la mente y el cuerpo son entidades separadas, ha sido objeto de debate durante muchos años. Un ejemplo de interacción cuerpo-mente lo constituyen ciertos individuos con discapacidades mentales, que son causadas por mutaciones genéticas. El estudio de las enfermedades congénitas con disfunciones cognitivas proporcionará por lo tanto conocimientos sobre la naturaleza física de la mente humana.

La forma más común de retraso mental de causa genética es el síndrome del cromosoma X Frágil. Esta enfermad, con una prevalencia de 1 en 4000 hombres y 1 en 6000 mujeres, pertenece a un grupo de aproximadamente 200 patologías que presentan retraso mental y que son causadas por mutaciones en el cromosoma X. Los individuos afectados exhiben (entre otros) discapacidad cognitiva de severidad variable, ciertos problemas del comportamiento, así como algunas anormalidades faciales. En pacientes con el síndrome del cromosoma X Frágil, se ha demostrado que la anatomía celular está afectada en diversas regiones del cerebro: las neuronas muestran un número creciente de espinas dendríticas inmaduras, y a la vez una disminución del número de espinas dendríticas maduras. Estas anormalidades de las espinas dendríticas son un fenómeno general en las enfermedades relacionadas con el retraso mental. De forma predominante las espinas dendríticas proporcionan los sitios para las conexiones sinápticas. Se ha sugerido que estas espinas también tienen un rol central en las formas duraderas de cambios sinápticos. Se presume que estos eventos de plasticidad sináptica son imprescindibles para el aprendizaje y la memoria y que la discapacidad intelectual en el síndrome del cromosoma X Frágil es el resultado de alteraciones en la plasticidad cerebral, del acortamiento de las sinapsis o de su proceso de maduración. La base molecular del síndrome es la ausencia de "fragile X mental retardation protein" (FMRP, según las siglas en inglés). La causa es la expansión o "crecimiento" de una región del ADN que contiene una repetición inestable de los nucleótidos CGG en la región 5'-no codificante del gen del X Frágil1 ("fragile X mental retardation gene", *FMR1*, según las siglas en inglés). Esto conlleva a una hipermetilación de la región del ADN que contiene los nucleótidos CGG repetidos y del promoter del gen, que resulta finalmente en una supresión de la transcripción y por lo tanto de la traducción del gen *FMR1.* Sin embargo, la correlación entre la ausencia de la proteína, el cambio en la arquitectura de espinas neuronales, y la discapacidad cognitiva en pacientes X frágil, no es completamente evidente. Esta tesis tiene como objetivo contribuir al conocimiento de los mecanismos moleculares que están implicados en funciones cognitivas, tales como aprendizaje y memoria, mediante el estudio de la función de la proteína FMRP en las dendritas.

La primera parte de esta tesis (introducción general) hace una revisión sobre el cerebro, el síndrome del cromosoma X Frágil, y el papel de la proteína FMRP en las dendritas. En el **capítulo I**, se describen desde una perspectiva histórica el desarrollo, evolución, y

función del cerebro. El matenimiento jerárquico de diversas redes celulares es esencial para mantener la complejidad de las funciones dirigidas por el cerebro. Por lo tanto en este capítulo describimos en detalle las neuronas, los medios por los cuales ellas se comunican y los puntos de intersección de esta comunicación, las sinapsis. Además, se describe la capacidad del cerebro de cambiar su forma, la llamada plasticidad cerebral. El **capítulo II**, resume los aspectos genéticos, clínicos y moleculares del síndrome del cromosoma X Frágil y aborda el tema de los modelos animales del síndrome, específicamente modelos murinos. El **capítulo III** repasa más detalladamente la función de la FMRP en neuronas, y en detalle en dendritas. En este capítulo se discuten también los RNA mensajeros (mRNA) que actuan como "colaboradores" neuronales de la proteína FMRP, la movilización de estos colaboradores en las dendritas, y de su regulación (a través de la FMRP) en los sitios de las sinapsis.

La segunda parte de esta tesis presenta el trabajo experimental realizado, el cual está relacionado con los mecanismos moleculares implicados en procesos neuronales, en particular aquellos que establecen una distribución asimétrica y una traducción espacial restringida de mRNAs en las sinapsis. El **capítulo IV** propone un mecanismo modelo para explicar cómo la proteina FMRP cumplimenta la función de reclutamiento del mRNA apropiado. Los resultados confirman que la proteína FMRP actúa como represor de la traducción de mRNAs específicos en las sinapsis. Otro hallazgo importante del estudio sugiere que además de FMRP, en el complejo ribonucloproteico (RNP) se localiza *BC1*, un RNA no-codificador. *BC1* desempeña una función en el desarrollo neuronal así como en el aprendizaje y la memoria. Esta investigación revela que *BC1* puede unirse directamente a FMRP, así como a mRNAs específicos relacionados con la proteína FMRP por medio de "apareamiento" de bases. En ausencia de *BC1*, la interacción de FMRP con sus mensajeros se interrumpe. Estos datos sugieren que el reconocimiento de mRNAs específos y la traducción de estos es mediada por *BC1*.
La identificación en un paciente con fenotipo severo de X Frágil, de una mutación rara localizada en el segundo dominio KH de la proteína (I304N), permitió el reconocimiento de que esta mutación compromete varias funciones de la proteína como la capacidad de enlace del RNA, la capacidad de plegamiento, la homo-oligomerización, la función como regulador de traducción, y la capacidad de formar una partícula RNP normal. Debido a que la expresión de la proteína (regulada localmente) desempeña un papel importante en procesos neuronales, el rol de FMRP en la distribución asimétrica del RNA mensajero en las dendritas fue investigada examinando la cinética del transporte de una proteína mutante, FMRP-I304N-EGFP en una línea celular PC12 (**capítulo V**). La proteína mutada es transportada a las dendritas, sin embargo, la formación de los gránulos RNP resulta completamente alterada. Estos resultados permiten especular que la distribución anormal y la traducción de los mRNAs debido a una proteína FMRP mutada pueden tener un efecto más crítico en procesos neuronales que la ausencia de FMRP.
Para identificar los mRNAs que se distribuyen asimetricamente a las dendritas, se ha desarrollado un sistema de cultivo celular que permite la separación física de los cuerpos neuronales y las neuritas (**capítulo VI**). Se aplicó microscopía de microdisección por láser

para asegurar la ausencia de cuerpos celulares y de células no-neuronales de las muestras. La metodología descrita permitió obtener el material suficiente para el aislamiento adecuado de mRNA en neuritas murinas. El sistema desarrollado ha sido puesto a prueba, los resultados preliminares se discuten en el **Apéndice**.
Además del trabajo anteriormente descrito, en el **capítulo VII** se evaluó un método basado en el reemplazo de la proteína como posible terapia para el síndrome del cromosoma X Frágil. Sin embargo, las células resultaron ser altamente susceptibles al nivel de expresión de FMRP. Puesto que el efecto tóxico resultante de la sobre-expresión es difícil de prevenir, la terapia del reemplazo de la proteína puede ser inadecuada para el tratamiento de pacientes con el síndrome del cromosoma X Frágil.

Curriculum Vitae

Name:	Surya Anne Reis
Nationality:	German
Date of Birth:	7th March 1975, in Bombay, India

Education

1980-1984	Elementary School
1984-1993	Gymnasium (High School); Nikolaus-Kistner Gymnasium, Mosbach, Germany
1994-1996	Grundstudium in Biology; Johannes Gutenberg University, Mainz, Germany
1996-1999	Bachelor of Science in Human Genetics; University College London, United Kingdom
1999-2005	Doctorate in Clinical Genetics; Erasmus MC Rotterdam, The Netherlands
2005-present	Postdoctoral Fellow in Neurology, Harvard Medical School, Boston, USA

Research Experiences

1997 (Jun-Aug) Medical Research Council, Department of Gnetics, Wolfson House, London, United Kingdom.
Staining methods for mucins. Supervisor Prof.Dr. D. Swalow.

1998 (Jun-Aug) Charity Wing of Genetic Research, University of Arizona, Tucson, USA.
RNA expression in Zfy knockout mice. Supervisor Prof.Dr. R. Erickson.

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Der Lebenslauf unserer Tochter Surya Anne

Am 20. September 1978 rannte auf einem Gartenweg in Isny im Allgäu ein Mädchen auf uns zu und rief: „Mama, Papa".

Nach langer Planung, Überwindung bürokratischer Hürden, Hilfe von Freunden und Organisationen war es umso überraschender, als wir erfuhren, dass nun endlich unsere Tochter Surya ankommen wird.
Der Haushalt stand Kopf! Auch Daniel, ihr gleichaltriger Bruder, wusste mit der großen Freude, dem entsprechenden Übermut und der Aufregung gar nicht wohin damit. Dazu konnte er noch nicht einmal die Sprache seiner Schwester verstehen. Das änderte sich rasch. Innerhalb von sechs Wochen konnte Surya deutsch sprechen, und zwar fehlerfrei! Ihre Wissbegier, die Auffassungsgabe und das schnelle Einleben gaben uns einen ersten Eindruck ihrer außergewöhnlichen Begabung. Scheinbar spielerisch gewann sie die Zuneigung aller im Kindergarten und wurde sozusagen der „Star". Keine leichte Situation für Bruder Daniel. Beeindruckend war neben der intellektuellen Fähigkeit ihre sehr stark entwickelte Hilfsbereitschaft und ihr soziales Verhalten.

Eine Episode ging in die Familiengeschichte ein, die gleichsam charakteristisch für Surya geblieben ist:

> Das deutsche Adoptionsverfahren zog sich über einen langen Zeitraum hin. In der Zwischenzeit bekam Surya ihr kleines Brüderchen David. Als dann im Dezember 1979, durch den Richter Eisele im Amtsgericht Wangen im Allgäu, die Adoption unserer in Bombay geborenen Tochter offiziell vollzogen wurde, fragte der hohe, aber bereits betagte Herr das vierjährige Mädchen: „Hast du auch noch Geschwister?" worauf Surya stolz antwortete, dass sie einen großen Bruder und ein kleines Brüderlein habe. Als der Richter dann Surya fragte:" Woher ist denn das Baby gekommen?" antwortete sie - selbstverständlich durch ihre Neugier bereits von der Mutter aufgeklärt - dabei etwas ratlos fragend sich zu den Eltern umdrehend:"Aus der Vagina natürlich".Richter Eisele indigniert, mit hochrotem Kopfe, murmelte nur noch: „Natürlich aus der Vagina."

Die natürliche und wissensdurstige Neugier begleitete Surya bis zum heutigen Tag. Wir Eltern erkannten schnell, dass sie im Kindergarten unterfordert war und schulten sie deshalb mit dem fünften Lebensjahr ein. Dadurch blieb sie bis zum Ende ihrer Schulzeit das „Nesthäkchen".

Dass sie ein Einser-Abitur mit Talent, Fleiß und Akribie ablegte und trotzdem ihr soziales Leben nicht vernachlässigte, zeigte sich darin, dass sie nebenher Gesangs- und Klavierstunden besuchte, sich aktiv in einem Fechtclub sportlich betätigte und außerdem ihre ebenso fechtenden Brüder mit anspornenden Eifer erfolgreich motivierte. Darüber hinaus zeigte sie soziales Engagement für behinderte Menschen durch Mitarbeit in einer Behinderteneinrichtung.

Mit Leichtigkeit lernte sie lateinisch, französisch, englisch, italienisch (während ihrer Doktorandenzeit zusätzlich niederländisch) und besuchte sowohl in den USA als auch in Frankreich die Schule. Aber ihre wirkliche Hingabe gehörte der Mathematik. Sie nahm ab dem 13. Lebensjahr an den Landeswettbewerben für Mathematik und später an den Bundeswettbewerben teil, wobei der 2. Preis ein krönender Abschluss war.

Im Anschluss an das Abitur studierte Surya zunächst Biologie an der Johannes-Guttenberg-Universität in Mainz. Dabei stellte sie fest, dass Genetik sie am meisten interessierte. Deshalb ging sie mutig nach London, immatrikulierte sich am University College für Humangenetik und verbrachte dort drei glückliche Jahre, wobei sie weiterhin ihre vielfältigen Talente außerhalb der Universiät praktizieren konnte, wie z.B. als Mitglied des UCL-Opernchores, beim Aufbau und der Organisation des UCL-Fechtclubs sowie bei vielen anderen gesellschaftlichen Aktivitäten. In dieser Ausbildungsphase verbrachte sie ebenso einen dreimonatigen Gastaufenthalt an einer amerikanischen Universität, um auch praktische Erfahrung zu sammeln. Dabei realisierte sie, welche Unterstützung die Wissenschaft in Amerika genießt.

Deshalb entschied sie sich nach ihrer Graduierung, an der Erasmus MC in Rotterdam in klinischer Genetik zu promovieren. Damit konnte sie ihr Interesse an der Neurologie mit ihrem Anspruch „Anderen zu helfen“ verwirklichen.

Ihre bisherige „Forschungsreise“, die Höhen und Tiefen beinhaltete, krönt sie nun mit dem Erhalt des Doktortitels.

Surya - diesen Namen hat unsere Tochter mitgebracht – bedeutet Sonne. Eine Sonne war und ist Surya immer für uns gewesen. Ihre Strahlen haben unsere Familie gewärmt.

Mit ihrer Promotion und mit der Entscheidung auf dem Gebiet der Forschung in Harvard/ USA weiter voranzugehen, erfüllt uns mit Freude und Stolz.

The life of our daughter Surya Anne

On September 20th 1978 a girl ran towards us on a garden path in Isny in the region Allgaeu and called out: "Mama, Papa!"

After a long planning phase, the overcoming of bureaucratic obstacles, help from friends and organizations we were more than surprised to learn that our daughter Surya had finally arrived.
The household was overjoyed! Daniel, her brother of the same age, could hardly cope with his joy, his high spirits and his excitement. Moreover, he could not even understand the language of his sister. This changed rapidly. Within six weeks Surya was able to speak German, without any mistakes! Her intellectual curiosity, her quick grasp and easy adaptation to our family life were the first hints of her extraordinary abilities. Without any effort she won the affection of everyone at the kindergarten and became, so to speak, its 'star'. This was not an easy situation for her brother Daniel. Apart from Surya's intellectual abilities we were impressed by her highly developed cooperativeness and her social behaviour.

One episode, that could be called characteristic for Surya, has entered family history:

> The German adoption procedure was a long affair. In the meantime Surya's little brother David had been born. When in December 1979 the official adoption of our daughter, who was born in Bombay, took place in the presence of Judge Eisele at the County Court in Wangen/Allgaeu, the dignified and rather elderly man asked the four-year-old girl: "Do you also have brothers or sisters?" Surya proudly answered that she had an elder brother and a baby brother. The judge asked Surya: "And where did your baby brother come from?" Already told by her mother because of her curiousness, she was a little bit perplexed and looking towards her parents replied: "Of course out of the vagina." Judge Eisele, indignant and with a very red face, only mumbled: "Of course out of the vagina."

Natural curiosity and a hunger for knowledge has always belonged to Surya, up until this very day. We as her parents quickly realized that the kindergarten was not a big enough challenge for Surya. Thus, she entered school one year early, at the age of five, which always made her the youngest through to the end of her school days.

Having passed her school leaving examination with the highest marks, which she achieved through talent, hard work and meticulousness, she also made sure she did not neglect her social life. She took up singing and piano lessons and joined a fencing club where she not only fenced herself but also successfully motivated her fencing brothers. Furthermore, her social dedication showed in her work at a facility for the disabled. She easily mastered Latin, French, English, Italian (and, during her studies for a doctorate, also Dutch) and went to school in the United States as well as in France. Her true devotion, however, was mathematics. From

the age of 13 she took part in state-regional and later national mathematics competitions. Receiving a second prize in the national competition was a real highlight.

After school Surya first studied biology at the Johannes-Guttenberg University in Mainz. Realizing that her interests lay in genetics she boldly went to London to enrol in a bachelors degree in Human Genetics at the University College. There she passed three happy years, still managing to practise her multiple talents next to her course work, as a member of the UCL Opera Society, or by giving new life to the fencing-club and many other social activities. During this time she spent three months at an American university to gain practical experience and realized the high degree of support the sciences enjoy in the United States.

After graduating she embarked on a doctorate in Clinical Genetics at the ErasmusMC in Rotterdam. This gave her the opportunity to combine her interest in neurology with her commitment ‘to help others’.

She is now topping off her journey through the sciences, one comprised of many ups and downs, by receiving her PhD.

Surya - our daughter already had this name, which means ‘sun’ - has always been and still is our sun. Her rays have warmed our family.

We are proud and happy about her promotion and her decision to continue her research at Harvard/USA.

Ad finum

Think where mans glory most begins and ends, and say my glory was I had such friends (William Butler Yeats).

First of all I'd like to thank my promoter Prof.Dr. B.A. Oostra. Ben, thank you very much for giving me the opportunity to 'plunge myself into the depth of research'. I arrived in Rotterdam with little practical experience. This changed very quickly - you gave me the chance to work on many different projects. If I have been called a 'theoretical' person in the past, these last 6 years gave me ample opportunity to activate my technical synapses.

Further, I would like to thank my 'two other supervisors': Dr. A.T. Hoogeveen and Dr. R. Willemsen. Andre, most of my time in Rotterdam I spent in your lab. Thank you very much for helping me through the difficult and highly technical ordeal of the 'TAT-work'. But, you have always been more than just a supervisor. I won't forget the beach walks (together with Stefano), the golf, the many dinners, or how you rushed over to 'safe me from the gas leak'. Dankje wel! Rob, although you were the last 'ingredient in my supervisor-cocktail', most of the work described in this thesis was done under your guidance. Thank you very much for your expert advice in introducing me to neuronal cell cultures. I especially appreciated your fair and unbiased judgment of experimental results.

My thanks also go to Prof.Dr. C. Bagni and Dr. F. Zalfa in Rome. Francesca, thank you for all the wonderful work that you did for the BC1-paper! It was a real pleasure meeting you and collaborating with you for this project. Claudia, thank you so much for the excellent time in Rome. Your personality is an inspiration to me. I hope I can develop the same passion for science, people, and life as you have.

Prof.Dr. C. de Zeeuw, Dr.ir. N. Galjart, and Dr. A. Reuser thank you very much for reading my thesis, the helpful comments, and for accepting an 'unorthodox' thesis!

My two paranimfs have been wonderful! Leontine, I am so happy that you agreed to be my paranimf. Nobody has given me as much experimental support as you have. While we were still a real group (with Stefano and Violetta), you always managed to support us with your efficiency, your cool headedness, and your smile. Thank you for everything. Esther has been my 'harbor' whenever I had any kind of problems. Esther thank you very much for always having an open door and ear, for all the support especially now with the finishing of my thesis, and for all the fun organizing parties, games, pantomimes etc.

Stefano, my dear, I can't even tell you how much I missed you ever since you left. For such a long and intense time you were my 'comrade in arms'. Whenever I worked late or on the weekends you were there. You would never cease to be interested in my work; you shared all the steps forward (and the many ones backward) with me. Not only inside the lab, but also

outside (yes, there is life outside!), I never had to feel alone, since you were there. I can't name it all, so just thank you for being such a wonderful friend.

I had the privilege to work in close proximity with all the FraX groups. Since at times it has been unclear, who is in which group, I would like to thank all my colleagues from the fragile X research at once: Cathy, Ingeborg, Marianne, Edwin, Lau, Mariette, Sandra, Judith, Bart, Lies-Anne, Maria, Femke, Violetta, my student Thessa (thanks for running all those Western blots and columns), formerly also Ngan, Yolanda and Pietro. Guys, if I didn't learn enough from you in respect to fragile X, I have learned a lot about group dynamics. Pietro, a special thanks to you, since you've been also a friend outside the lab. Thank you for the marvelous tours through Rome and your general support.

Many thanks also to all the colleagues from the rest of the Clinical Genetics research group: Guido, Aida, Onno, Herma, Bianca, Erik, Annemieke, Ozgur, Rachel, Vincenzo, Elly, Marian, formerly also Miriam, Joep, and Claudia. Thank you also to the people who have moved to Amsterdam: Peter, Patrizia, Iraad, Burcu, Maria, and Esther. Thanks for helping me over the last 6 years to become a scientist.

Three people have earned a special thank you: Jeltje, Mark, and Robert. From day one you all made me feel welcome and at home in Rotterdam. Robert I will never forget that you gave me your car my first weekend in R'dam and then you came to pick me up in A'dam, since the car had broken down. Mark, you are a wonderful cook and a great squash coach. You made me miss London less. Jeltje, since you left I haven't had anyone for a 'sex and the city' session. I hope we'll see each other more often now.

Imperative to succeeding with this thesis was the excellent technical support from some true experts. Dr. Clive Svendson, thank you for giving me the opportunity to stay in your lab at Madisson for one week to learn the neurosphere culturing. Dr. Ger Ramakers, thank you for your expert advice for neuronal cell culturing. Justine Peeters, thank you very much for your help with the analysis of the microarray data, even if we cannot publish it yet. Gert van Capellen, thanks a lot for introducing me to convocal microscopy. Sjozef, Ton, Pim bedankt voor de computer ondersteuning. Melle en Arthur v.d. Kamp dankje wel voor jullie bijdrage aan het degelijks functioneren in het lab. Jeannette heel erg bedankt voor al je hulp vooral in de laatste tijd. Tom de Vries Lentsch, je hebt iets geweldigs van mijn boekje gemaakt! Bedankt dat alles nog voor mijn vertrek klaar was.

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So. Finally. I have succeeded with my PhD.

q.e.d.