Functional and Anatomical study of the Inferior Olive: From slice physiology to in vivo recordings

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From slice physiology to in vivo recordings

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Cover art: “The inferior olive is like an ingenious guitar” by Sebastián Loyola. It was inspired by that statement which was said by my supervisor, Chris De Zeeuw, during a data discussion meeting. It was also inspired for the love I have for the guitar, the music, and the inferior olive.

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Functional and Anatomical study of the Inferior Olive: 
From slice physiology to in vivo recordings

Functionele en anatomische studie van de onderste olijf: 
Van in vitro fysiologie tot in vivo opnames

Thesis

to obtain the degree of Doctor from the 
Erasmus University Rotterdam 
by command of the 
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Prof. dr. F.A. van der Duijn Schouten,

and in accordance with the decision of the Doctorate board.

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by

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For all the people who care about me and for the ones who care about our old and well conserved friend across evolution, the inferior olive....
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Chapter 1

General Introduction

This chapter is the thoroughly revised and updated second edition of the book chapter:


Authors of the first edition: De Gruijl JR, Bosman LWJ, De Zeeuw CI, De Jeu MTG

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Sebastián Loyola, Laurens Bosman, Jornt De Gruijl, Marcel De Jeu, Mario Negrello, Tycho Hoogland, Chris De Zeeuw
1.1 Abstract

The inferior (IO) is a structure located in the ventrolateral part of the brainstem that plays a fundamental role in motor learning and motor coordination by providing one of the two major excitatory inputs to the cerebellar cortex: the climbing fibers. These climbing fibers elicit an all or none response in Purkinje cells named the "complex spike". In addition, the olivary axons provide collaterals to the cerebellar nuclei, further modulating the cerebellar output.

IO neurons have several distinguishing features one of them is exhibiting complex dendritic arbor morphologies with particular spatial orientations. Another striking feature is the presence of dendro-dendritic gap junctions in which electrotonically coupled dendritic spines receive both excitatory and inhibitory inputs. Due to this unique synaptic arrangement of the glomerulus, which forms the core of the hallmark of the olivary neuropil (De Zeeuw, 1990), temporal interaction of both inputs would have a big impact on synaptic integration and spatiotemporal activity patterns (Segev and Parnas, 1983; De Zeeuw et al., 1995). Furthermore, IO neurons also show subthreshold oscillations (STOs) that may well play an important role in motor learning and motor coordination by controlling spike timing (Van der Giessen et al., 2008; Yarden-Rabinowitz and Yarom, 2019). Due the low spike frequency of individual IO neurons (~1Hz), coordination of complex behaviors, such as eating, grooming or digging, is mediated by ensembles of IO neurons that generate dynamic spatiotemporal patterns of complex spikes in the cerebellar cortex (Welsh et al., 1995).

Despite the multiple studies focused on olivary function, there is little knowledge about the morphology of IO neurons and the anatomical organization of their inputs. Likewise, the impact of the temporal interaction of excitatory and inhibitory inputs on STOs, spike output and motor learning remains to be elucidated. In this thesis I will address those topics using in vitro and in vivo techniques that will allow us to have a better understanding of this structure. Furthermore, I will provide new insights in the engagement of olivo-cerebellar activity during sleep.
1.2 Development of the inferior olive and climbing fibers

1.2.1 The origin of Inferior olivary neurons

Neurons of the inferior olive (IO) arise from the neural tube in the embryonic hindbrain and migrate subsequently to the ventral site of the brainstem. During embryonic development, the neural tube becomes increasingly partitioned, with each partition giving rise to a specific part of the developing brain (Fig. 1a). Such partitioning is under strict genetic control. The segregation of the hindbrain from the midbrain is largely controlled by two homeodomain transcription factors: Otx2 and Gbx2. Initially, the expression domains of Otx2 and Gbx2 overlap (Fig. 1b1), but (in mice) around embryonic day 7.5 (E7.5), Otx2 becomes restricted to the forebrain and midbrain and Gbx2 to the hindbrain (Fig. 1b2) (Joyner et al., 2000; Rubenstein et al., 1994; Simeone et al., 1992). Otx2 and Gbx2 act antagonistically. While Otx2 inhibits formation of the hindbrain, thus allowing the midbrain to be formed, Gbx2 inhibits formation of the rostral brain, permitting the hindbrain to develop (Acampora et al., 1995; Hidalgo-Sánchez et al., 1999, 2005; Kikuta et al., 2003; Millet et al., 1996, 1999; Sakurai et al., 2010; Wassarman et al., 1997). At the border of the Otx2 and Gbx2 expression domains, the isthmic organizer develops (Fig. 1b3) (Broccoli et al., 1999; Joyner et al., 2000; Leto et al., 2016; Martinez et al., 2013; Simeone, 2000).

Around E8, the hindbrain becomes segmented into eight compartments, the rhombomeres (Lumsden and Krumlauf, 1996; Osumi-Yamashita et al., 1996; Vaage, 1969). Due to an alternating expression pattern of Eph receptors and their membrane-bound ephrin ligands, which exert a repelling effect, cell migration between rhombomeres is no longer possible (Fig. 1b4) (Becker et al., 1994; Bergemann et al., 1995; Dahmann et al., 2011; Egea and Klein, 2007; Kania and Klein, 2016; Kemp et al., 2009; Lumsden and Krumlauf, 1996). This compartmentalization of the neural tube allows region-specific differentiation (Fraser et al., 1990). The anterior rhombomere, r1, will develop into the cerebellum (Altman and Bayer, 1978a; Larsell, 1947; Leto et al., 2016; ten Donkelaar and Lammens, 2009; Zervas et al., 2004). The caudal rhombomeres (r2–r8) give rise to the neurons of the hindbrain nuclei, including the IO (Altman and Bayer 1978b; Ray and Dymecki, 2009).

The fate of the individual rhombomeres is largely determined by the specific sets of Hox genes expressed. A complex signaling cascade leads to the correct spatial pattern of Hox gene expression. Already during the formation of the rhombomeres, the first Hox genes are expressed. Retinoic acid, which is formed in the mesoderm of the trunk and underlies a concentration gradient decreasing in the rostral direction, induces the expression of among others Hoxa1 and Hoxb1
Fig. 1 Development of the inferior olivary neurons. A – The neural tube of the hindbrain is transiently divided into eight segments (“rhombomeres”) (r1–r8). The neural tube does not close completely over the fourth ventricle (v4). The border of the neural tube lining the fourth ventricle is called the “rhombic lip” (red and green zones). The neurons of the IO originate from the rhombic lip in r7 and r8. At the border between the hindbrain and the midbrain, the isthmic organizer (IsO) develops. B1 – During early development, the expression domains of the homeodomain transcription factors Otx2 (red) and Gbx2 (blue) overlap. B2 – Later on they become segregated, and Otx2 and Gbx2 have a mutually repulsive action. B3 – The hindbrain transiently forms eight rhombomeres. Cell migration between the rhombomeres is prevented by the alternated expression pattern of membrane-bound ephrins (EphA4) and their receptors (EphB2a). The combination of EphA4 and EphB2a has a repulsive action, preventing mixture of neuronal progenitor cells from different rhombomeres. B4 – Retinoic acid is formed in the trunk and diffuses into the hindbrain. It stimulates directly the expression of the early Hox genes Hoxa1 and Hoxb1. Due to the higher retinoic acid concentration in the posterior hindbrain, the expression domains of Hoxa1 and Hoxb1 are predominantly posterior. B5 – Interactions of fibroblast growth factors, released by the IsO, and of retinoic acid, released by the trunk, with several transcription factors create an anteroposterior pattern, which serves as a template (B6) to induce the late Hox genes. B7 – Ultimately, a nested pattern of Hox genes is established. Concentration gradients are indicated by different color intensities: the darker, the higher the concentration. Altogether, each rhombomere has now a unique set of Hox genes, with the borders between the rhombomeres serving as anterior borders of the expression domains. C – Schematic drawing of a coronal transection of the embryonic hindbrain at the level of the posterior hindbrain (r7/r8). The neural tube is composed of two plates: the basal plate (BP) and the alar plate. The alar plate, in turn, is composed of the mantle zone (MZ), the ventricular zone (VZ), and the rhombic lip (RL). Following the expression pattern of several basic helix-loop-helix transcription factors, the rhombic lip can be subdivided into four compartments along the dorsoventral axis (inset). Upon completion of mitosis, the IO neurons start to migrate around E13 (in rat) from their site of origin (r7/r8, compartment 4 of the rhombic lip) to the site of the IO. The first neurons that complete the migration along the submarginal stream reach the IO at E15. Around E18, migration is complete, and the three main compartments of the IO can be recognized: the DAO (dorsal accessory olive), the PO (principal olive), and the MAO (medial accessory olive).
Next, the isthmic organizer starts, stimulated by the homeodomain transcription factors Lmx1b and Pax2, to secrete the signaling proteins Wnt1 and Fgf8 (Adams et al., 2000; Guo et al., 2007; Ye et al., 2001). Wnt1 and Fgf8 stimulate, directly or via En1, each other’s expression (Fig. 1b5) (Chi et al., 2003; Ciani and Salinas, 2005; Lee et al., 1997; Ye et al., 2001). Wnt1 and Fgf8 stimulate, directly or via En1, each other’s expression (Fig. 1b5) (Chi et al., 2003; Ciani and Salinas 2005; Lee et al., 1997; Ye et al., 2001). Wnt1 promotes the development of the midbrain and the cerebellum (Amoyel et al., 2005; Ciani and Salinas, 2005; Klaus and Birchmeier, 2008; Mastick et al., 1996; Mcmahon and Bradley, 1990). It is also secreted by the rhombomere borders, where it contributes to neurogenesis in the hindbrain (Amoyel et al., 2005). Fgf8 is also transiently produced by r4 (Maves et al., 2002; Walshe et al., 2002). In addition, Fgf3 is secreted, first by r4, and later also by the more posterior rhombomeres (Mahmood et al., 1996; Maves et al., 2002; Walshe et al., 2002). Fgf and retinoic acid together activate vhnf1 (Hernandez et al., 2004). The expression domain of vhnf1 is anteriorly limited by the suppressive action of Iroquois (Lecauday et al., 2004). vhnf1 promotes, together with the Fgf’s, the expression of Krox20 and kreisler (Aragón et al., 2005; Kim et al., 2005; Marin and Charnay, 2000; Sun and Hopkins, 2001; Wiellette and Sive, 2003). Krox20 is exclusively expressed in r3 and r5 (Oxtoby and Jowett, 1993) and kreisler in r5 and r6 (Frohman et al., 1993). In addition, Fgf8 stimulates the degradation of retinoic acid in the more anterior rhombomeres by Cyp26 enzymes (Duester 2007; White and Schilling, 2008). This has implications for the expression pattern of Hoxb1. While it is no longer induced by retinoic acid in the anterior rhombomeres, it is also suppressed by Krox20 in r3 and r5 (Garcia-Dominguez et al., 2006), limiting Hoxb1 expression to r4 (Fig. 1b5) (Wilkinson et al., 1989).

Taken together, there is now a spatial framework which imposes the nested expression pattern of Hox genes (Fig. 1b6). Hoxb1 remains restricted to r4 (Wilkinson et al., 1989). Hoxa2 spans the largest area, r2–r8, but is enriched in r3 and r5 due to the positive action of Krox20 (Nonchev et al. 1996; Sham et al. 1993). Under the influence of kreisler, Hoxa3, Hoxb3, and Hoxc3 are expressed from r5 onward (Manzanares et al., 1997, 1999). The neurons of the IO are generated in r7 and r8 (Ambrosiani et al. 1996; Yamada et al. 2007). These rhombomeres express Hoxa4, Hoxb4, and Hoxd4 under the influence of retinoic acid (Fig. 1b7) (Alexander et al., 2009; Packer et al., 1998). Either a deficiency or an excess of retinoic acid, a vitamin A derivative, leads to a malformed IO (Yamamoto et al., 2005). Once the Hox genes are activated, they can sustain their expression by auto- and cross-regulation (Alexander et al., 2009; Tümpel et al., 2009). Thus, there are complicated, often mutual, interactions between rhombomere-specific transcription factors and signaling molecules that impose a nested expression of Hox genes along the anteroposterior axis of the developing hindbrain, determining the fate of each rhombomere.
In addition to the anteroposterior patterning, partitioning also occurs along the dorsoventral axis of the neural tube. The dorsoventral patterning is largely imposed by two antagonistic gradients, including sonic hedgehog (SHH) secreted by the floor plate and Wnt and bone morphogenetic proteins (BMPs) by the root plate (Hernandez-Miranda et al., 2017; Ulloa and Martí, 2010). The dorsal part of the neural tube, the “alar plate,” is composed of an outer layer, the “mantle zone,” and an inner layer, the “ventricular zone.” The dorsal part of the alar plate, thus the part forming the borders of the fourth ventricle, is referred to as the “rhombic lip” (Essick, 1912; His, 1891; Ray and Dymecki, 2009). The rhombic lip extends over the whole hindbrain, spanning the eight rhombomeres, and is therefore also affected by the rhombomere-specific expression of Hox genes. Next to the Hox genes, several other transcription factors are expressed in the alar plate of the hindbrain neural tube. The whole rhombic lip is characterized by the expression of Wnt1, while several other transcription factors occur in restricted areas along the dorsoventral axis (Landsberg et al., 2005; Ray and Dymecki, 2009). The homeodomain transcription factor Lmx1a is found only in the dorsal layer (dA1) (Chizhikov et al., 2010; Landsberg et al., 2005). Ventral to the Lmx1a expression area are those of the basic helix-loop-helix (bHLH) transcription factors Math1, Ngn1, and Ptf1a, respectively (Landsberg et al., 2005; Liu et al., 2008; Ray and Dymecki, 2009; Wang et al., 2005). The expression domain of the bHLH transcription factor Olig3 overlaps with those of Math1, Ngn1, and Ptf1a (Storm et al., 2009; Takebayashi et al., 2002). The neurons of the IO develop in the Ptf1a-expressing region dA4 (Hernandez-Miranda et al., 2017; Iskusnykh et al., 2016; Storm et al., 2009; Yamada et al., 2007). Ptf1a expression is required for the formation of IO neurons, probably by the induction of the direct downstream targets Nephrin and Neph3 (Iskusnykh et al., 2016; Nishida et al., 2010; Yamada et al., 2007). During formation of the IO neurons, Ptf1a and Olig3 act synergistically. Deletion of one of these bHLH transcription factors prevents the formation of the IO (Storm et al., 2009). This complicated network of gene expression ultimately leads to the generation of IO neurons in dA4 of r7 and r8.

1.2.2 Migration of Inferior olivary neurons

IO neurons are the first neurons that originate from the rhombic lip. In rats, they are formed at embryonic day 12 (E12) and E13 (Altman and Bayer, 1978b; Bourrat and Sotelo, 1988). After their last mitosis, the newly formed neurons start to migrate tangentially to their destinations (Altman and Bayer, 1978b; Bourrat and Sotelo, 1988). This migration occurs via well-defined streams. While the streams leading to the other precerebellar nuclei at some point cross the
midline, the so-called submarginal strand that leads to the site of the IO does not (Fig. 1c) (Altman and Bayer, 1978b; Bourrat and Sotelo, 1988; Ellenberger et al., 1969; Harkmark, 1954; Sotelo and Chédotal, 2005).

Neuronal migration is under tight control of tissue-dependent cues. The actual effects of these chemotactic molecules, being either chemo-attractive or chemorepellant, depend on the specific receptors of the migrating neurons. Multiple mechanisms are at stake. Netrin-1, secreted by the floor plate, attracts the migrating IO neurons (Bloch-Gallego et al., 1999; Marcos et al., 2009; Sotelo and Chédotal, 2005; Yee et al., 1999). Once the IO neurons reach their destination between E15 and E19 (Altman and Bayer, 1978b; Bourrat and Sotelo, 1988; Ellenberger et al., 1969), further migration of their somata across the midline is prevented by repulsive action of Slit and Robo as well as of EphA4 and EphrinB3 (Fig. 3a) (Di Meglio et al., 2008; Ypsilanti et al., 2010). Upon arrival at the IO primordium, between E15 and E18, the somata become more rounded, and the dendritic trees develop (Bourrat and Sotelo, 1988).

1.2.2 Inferior olivary subdivisions and cell types

When the first neurons arrive at the site of the IO, no subdivisions can be recognized yet in the IO primordium (Bourrat and Sotelo, 1988). The medial accessory olive (MAO) is the first part of the IO to develop (Bourrat and Sotelo, 1988). Gradually, also the other two main divisions, the principal olive (PO) and dorsal accessory olive (DAO) become clearly discernible, and by E19 (in rats), upon completion of the migration, the IO has reached its adult shape (Altman and Bayer, 1987; Bourrat and Sotelo, 1988).

Both the PO and DAO are composed of two parts: a dorsal and a ventral leaf. The MAO can be subdivided into a rostral and a caudal halve. At its caudal margin, the MAO has three cell groups, termed “a,” “b,” and “c” (Bowman and Sladek, 1973; Gwyn et al., 1977; Martin et al., 1975). Next to the three main subdivisions, there are also a few minor subnuclei: the dorsal cap of Kooy (DCK), the ß nucleus, the ventrolateral outgrowth (VLO), and the dorsomedial cell column (DMCC). The basic organization of the IO is similar in all mammals, although the relative size of the nuclei differs across species (Bowman and , 1973; Kooy, 1917). In porpoises the MAO is dominant, whereas in primates the PO is highly expanded and gyrated (Cozzi et al., 2016; Glickstein et al., 2007). Elephants also have an expanded PO, but lack the gyrations seen in the human IO (Cozzi et al., 2016). It is likely that such differences in olivary organization reflect the behavioral repertoire dictated by the animal’s ecological niche and specialized sensory systems.
The cellular composition of the IO is homogeneous with the majority of cells being glutamatergic. GABAergic interneurons may account for less than 0.1% of all neurons in the IO (Nelson and Mugnaini, 1988; Walberg and Ottersen, 1989). IO neurons have been classified according to their dendritic morphology as being “straight” and “curly” (Fig. 2) (De Zeeuw et al., 1990b; Devor and Yarom, 2002a; Foster and Peterson, 1986; Ruigrok et al., 1990; Scheibel and Scheibel, 1955). Furthermore, some studies have suggested that neurons in the intermediary and caudal MAO have straighter dendritic morphologies, while cells with more curly morphologies are found predominantly in other regions of the IO (Foster and Peterson, 1986; Ruigrok et al., 1990). A typical IO neuron has a spherical soma with a diameter of about 15–30 μm and highly branched dendrites, curling back toward the soma, at times creating spirals (Foster and Peterson, 1986; Ruigrok et al., 1990; Scheibel and Scheibel, 1955). The axon of the “curly” neurons branches frequently off from a first-order dendrite (Ruigrok et al., 1990). Since the dendrites of “straight” neurons tend to grow away from the soma, their axons usually start directly at the soma (Fig. 2) (Ruigrok et al., 1990). Due to the subjective estimations used in these studies, it is important to have a more quantitative and robust approach in order to obtain a better objective classification of IO neurons based on their dendritic morphologies.

1.2.3 Climbing fiber outgrowth and elimination

In contrast to most of the somata (De Zeeuw et al., 1996), inferior olivary axons cross the midline, reaching out for the contralateral cerebellum. Axon growth starts before reaching the IO primordium, and midline crossing occurs around E15 (Bourrat and Sotelo, 1988) under control of Robo3 and its Slit receptor (Badura et al., 2013; Marillat et al., 2004; Renier et al., 2010). Around E18 the olivocerebellar fibers reach the caudal part of the cerebellar plate (Chédotal and Sotelo, 1996).
After passing through the brainstem, some axon collaterals innervate the cerebellar nuclei and some axons provide terminal branches in the cerebellar cortex (in addition to their climbing fiber terminals) (Fig. 3a) (Ruigrok and Voogd, 2000; Sugihara et al., 1999; Van der Want et al., 1989; Wiklund et al., 1984; Voogd et al., 2003). There is some evidence for the existence of direct synapses of olivary axons onto stellate cells (Scheibel and Scheibel, 1954; Sugihara et al., 1999), but this is contradicted by an electron-microscopic study (Hámori and Szentágothai, 1980). Nevertheless, stellate cells do respond to activity of olivocerebellar axons (Ohtsuki et al., 2004), possibly in response to glutamate spillover (Szapiro and Barbour, 2007). In addition, there is anatomical evidence for the formation of synapses onto Golgi cells (Chan-Palay and Palay, 1971; Hámori and Szentágothai, 1966; Sugihara et al., 1999), but electrophysiological studies showed inhibition, rather than excitation, of Golgi cells by olivocerebellar axons (Schulman and Bloom, 1981; Xu and Edgley, 2008).

The immature climbing fibers creep between the multilayered Purkinje cells (“creeper stage”) (Chédotal and Sotelo, 1993; Morara et al., 2001; Sugihara, 2005), establishing the first functional synaptic contacts with the postsynaptic Purkinje cells around postnatal day 3 (P3) in rats (Altman, 1972; Crepel, 1971; Woodward et al., 1971). The number of presynaptic climbing fibers increases to on average five per Purkinje cell at P5 (Fig. 3b) (Crepel et al., 1981; Mariani and Changeux, 1981).

These early climbing fiber synapses are formed at the somata or at the small dendritic protrusions of Purkinje cells (Altman, 1972; Hashimoto et al., 2009a; Mason et al., 1990; Morara et al., 2001). Around P7, some of the climbing fibers start to form nest-like structures around the somata (Fig. 3b) (Altman, 1972; Hashimoto et al., 2009a; Mason et al., 1990; O’Leary et al., 1971; Ramón y Cajal, 1911; Sugihara, 2005). Of about 100 “creeper” climbing fibers per IO axon, only about 10 form nest-like structures (Sugihara, 2005; Sugihara, 2006).

Simultaneously, the premature dendritic protrusions disappear, and the apical dendrite, which is so characteristic of the adult Purkinje cells, starts to grow (Altman, 1972; Bosman et al., 2008; Hashimoto et al., 2009a; Mason et al., 1990; McKay and Turner, 2005). The first climbing fibers start to expand their innervation area to the proximal part of the nascent dendritic tree (Hashimoto et al., 2009a; Scelfo et al., 2003). It is possible that more than one climbing fiber translocates to the dendritic tree, but in most instances, the “largest” (or “winner”) climbing fiber rapidly increases in size and synaptic strength, whereas the other climbing fibers become atrophic and degenerate (Bosman and Konnerth, 2009; Bosman et al., 2008; Hashimoto et al., 2009a; Hashimoto and Kano 2005; Scelfo et al., 2003; Sugihara, 2005, 2006). Around P15, perisomatic nests are no longer observed, although a few somatic release sites may persist until P20 (Fig. 3b) (Hashimoto
et al., 2009a). By P20, the large majority of Purkinje cells is innervated by a single climbing fiber only, with numerous release sites all over the thick branches of the Purkinje cell dendritic tree (Hashimoto and Kano, 2003; Ramón y Cajal, 1911). A single, mature olivocerebellar axon gives rise to, on average, six to seven climbing fibers. These climbing fibers are strictly organized in a single, parasagittal plane spanning multiple lobules (Sugihara et al., 2001).

Selective elimination of redundant synaptic contacts, in combination with strengthening of those that survive, is a common theme in neuronal development (Bleckert and Wong, 2011; Katz and Shatz, 1996; Purves and Lichtman, 1980). The developmental elimination of redundant climbing fiber synapses probably depends on a combination of a genetic blueprint and activity-dependent synaptic competition (Bosman and Konnerth, 2009; Kano and Hashimoto, 2009). At P7, when the first climbing fiber synapses translocate to the Purkinje cell dendritic tree (Hashimoto et al., 2009a; Sugihara, 2005), also the first parallel fiber synapses are formed (Scelfo and Strata, 2005). These early parallel fiber synapses are located at the thick branches of the Purkinje cell dendritic tree (Altman, 1972). Thus, at the onset of the second postnatal week, both the climbing fibers and the parallel fibers project to the same dendritic compartment.

A process of heterosynaptic axonal competition ensues between the parallel fibers and the climbing fibers (Hashimoto et al., 2009b). During normal development, the climbing fiber establishes contact sites all over the proximal, thick, and smooth branches of the dendritic tree, while the parallel fiber synapses translocate to the distal, spiny branches (Altman, 1972; Robain et al., 1981). When, following experimental manipulation, functional parallel fibers do not develop, or develop only partially, the climbing fiber territory is expanded to the distal spiny branches, which are normally the exclusive domain of the parallel fiber synapses (Altman and Anderson 1972; Crepel 1982; Hashimoto et al., 2001; Hirai et al., 2005; Ichikawa et al., 2002; Watanabe, 2008). The reverse is also true: when climbing fibers do not develop normally, parallel fiber synapses persist abnormally also on the thick smooth branches (Miyazaki et al., 2004). The adult situation, with the climbing fiber synapses on the thick branches and the parallel fibers on the thin branches, seems to be a dynamic equilibrium. Weakening of one of the two afferent systems leads to an increased innervation by the other, even in adults (Cesa et al., 2005, 2007; Kakizawa et al., 2005; Miyazaki et al. 2010; Sotelo et al., 1975).
Fig. 3 Development of the olivocerebellar axons. A – Schematic drawing of a coronal slice of a mature rat. As shown in Fig. 1c, the IO neurons migrate from the rhombic lip, bordering the fourth ventricle (v4) along the submarginal stream to the IO primordium. Before they reach the IO, the migrating neurons start to grow axons that pass the midline and a part of the contralateral brainstem at E15 (in rats). Around E18, the first olivocerebellar axons reach the cerebellar anlage. Please note that at that developmental stage, the cerebellum is much less developed as on the schematic drawing shown here. A few days later, the first contacts with the cerebellar Purkinje cells are formed. On its way to the Purkinje cells, olivocerebellar axons form collaterals at the level of the inferior cerebellar peduncle (ICP), the cerebellar nuclei (CN), the cerebellar white matter, and the cerebellar granule layer. In the absence of Robo3, olivocerebellar axons do not cross the midline, but form ipsilateral connections. B – The thick ramifications of the olivocerebellar axons are the climbing fibers that innervate Purkinje cells. At P5 (in rats), each postsynaptic Purkinje cell is innervated by several, on average five, climbing fibers, originating from different olivocerebellar axons. The synapses are formed at the perisomatic protrusions and on the soma. This is called the “creepers” stage. The Purkinje cells still lack their apical dendrite. The synaptic strength of these “creepers” climbing fiber synapses are approximately equal, as shown by their EPSCs (bottom). Later on, the perisomatic protrusions disappear, and the apical dendrite starts to grow. Some of the climbing fibers are already lost, and the remaining ones form nests (“nest stage”) around the soma. They may even start to translocate to the nascent dendritic tree. The synaptic strength of the presynaptic climbing fibers is no longer equal (note the different y-scale). Around P20, the adult situation has almost been reached. There is only one, surviving climbing fiber, making hundreds of contact sites with the thick branches of the Purkinje cell. The last somatic contact site is lost around this day. Horizontal scale bar: 20 ms.

Other forms of axonal competition also exist, such as between climbing fibers impinging on the same immature Purkinje cells (Bosman and Konnerth 2009; Bosman et al. 2008; Ohtsuki and Hirano 2008). Voltage-gated Ca\(^{2+}\) channels are primarily found at the growing apical dendrite (Llano et al. 1994; Llinás et al. 1989) to which the climbing fibers translocate (Hashimoto et al. 2009a; Sugihara 2005). As postsynaptic Ca\(^{2+}\) is required for normal climbing fiber development
and elimination (Kawamura et al. 2013; Mikuni et al. 2013; Miyazaki et al. 2004), the unequal
distribution of both voltage-gated Ca^{2+} channels in combination with the spatial distribution of
immature climbing fibers across the developing Purkinje cell is likely to be a strong driving factor
of climbing fiber development (Bosman and Konnerth 2009). BDNF and semaphorins provide
retrograde signals to the climbing fibers that are required for their proper development (Bosman
et al., 2006; Choo et al., 2017; Uesaka et al., 2014).

In addition, there are indications for the existence of heterosynaptic competition between
climbing fibers and cerebellar stellate cells (Bosman et al., 2006; Bosman and Konnerth, 2009).
One should note that, at that age, the input of the GABAergic stellate cells to the Purkinje cells is
excitatory (Eilers et al., 2001), while their synapses are still at the same dendritic compartment as
the climbing fiber and parallel fiber synapses (Ramón y Cajal, 1911; Smirnow, 1897).

1.3 Ultrastructure of the Inferior olivary neuropil

1.3.1 Glomeruli and gap junctions

The ultrastructure of the mammalian IO neuropil has been described in many studies of various
animals (De Zeeuw 1990). The segments of IO dendrites as well as the hillocks of IO axons bear
pedunculated club-shaped and/or racemose spiny appendages (De Zeeuw et al., 1990a, b, c;
Gwyn et al., 1977; Ruigrok et al., 1990; Sotelo et al., 1974). While it is clear that the dendritic
spines are frequently electrotonically coupled by gap junctions formed by connexin36 (Cx36; Fig.
4) (De Zeeuw et al., 1989a, b, 1990b, 1995; Sotelo et al., 1974), it remains to be demonstrated
whether this also holds true for the axonal spines (De Zeeuw et al., 1990c). Both the dendritic and
axonal spines have unusually long spine necks. Because of their long necks, the spine heads can
cluster together and form the core of what is the most characteristic feature of the IO neuropil: the
glomerulus (De Zeeuw et al., 1990b; Gwyn et al., 1977; Nemecek and Wolff, 1969; Sotelo et al.,
1974). In general, a glomerulus contains a core of five to ten dendritic and/or axonal spiny
appendages derived from different neurons (De Zeeuw, 1990; De Zeeuw et al., 1990b, c). This
core is surrounded by four or five terminals and several glial sheaths. Serial section analysis has
demonstrated that virtually all spines are located in glomeruli. When the expression of Cx36 is
blocked, the formation of gap junction plaques is disturbed (Fig. 4a–b), but the remnants of gap
junction-like structures are still visible in the center of the glomeruli (Fig. 4c–d) (De Zeeuw et al.,
2003).
Several attempts have been made to estimate the extent of electrotonic coupling of IO neurons via gap junctions. A number of groups have been able to demonstrate the existence of clusters of coupled neurons through intracellular injections of small molecules such as biocytin (see also section: “Subthreshold Oscillations and Spike Timing”) or Neurobiotin that readily cross gap junctions (Benardo and Foster, 1986; De Zeeuw et al., 2003; Hoge et al., 2011; Leznik and Llinás, 2005). Typical cluster sizes range from a few to more than ten coupled IO neurons in a slice of several hundred μm. Given that these studies have been performed in sectioned tissue and thus include neurons with severed dendrites, the actual number of connected neurons is likely...
Performing multiple unit recordings following application of harmaline and picrotoxin, Llinás and colleagues have demonstrated that in the intact IO, synchronous firing can be induced in coupled cellular aggregates of tens of neurons (Lang et al., 1996; Llinás and Volkind, 1973). In addition, population level imaging of neighboring Purkinje cell dendrites has revealed large-scale coherence of complex spikes in awake behaving rodents, suggesting that during certain types of behavior, synchrony may encompass tens of climbing fibers and thus many synchronized IO neurons (De Grujil et al., 2014a; Hoogland et al., 2015; Ozden et al., 2012). In fact, bilateral multiple unit recordings from the cerebellar cortex demonstrated that an ensemble of coupled IO neurons in the rat can even extend beyond the midline (De Zeeuw et al., 1996). In the same study, it was estimated that one IO neuron may have 500–1000 gap junctions and that two individual IO neurons may be coupled by 10–20 gap junctions (De Zeeuw et al., 1996). Estimates of the density of neuronal gap junctions with the use of antibodies against Cx36 are in line with these counts (Fig. 4a).

Usually the types of IO neurons that are coupled are of the same subtype (see section “Inferior Olivary Subdivisions and Cell Types”; Devor and Yarom 2002a). However, the coupling shows a striking level of heterogeneity and asymmetry (Lefler et al., 2014), which may serve to finely influence the synchronization of IO neurons (Hoge et al., 2011). This raises the possibility that variations in coupling could result from glomerulus-specific short- and long-term modulation of gap junctions, which is supported by studies investigating the impact of excitatory synaptic inputs to the IO (Mathy et al., 2014; Turecek et al., 2014). Another prominent, possibly related feature of the IO neuropil is the presence of dendritic lamellar bodies (De Zeeuw et al., 1995). This organelle consists of stacks of membranous cisternae with electron-dense deposits in between, and it occurs exclusively in the varicose dilatations that are abundant in the peripheral IO dendrites just outside the glomeruli. Although other possible functions cannot be excluded such as intracellular Ca\(^{2+}\) control or the exchange of excitable dendritic membranes (De Zeeuw et al., 1997a), various lines of evidence suggest that the dendritic lamellar bodies may serve to control the turnover or assembly of dendrodendritic gap junction channels. The fact that the density of dendritic lamellar bodies in the IO is higher than in any other area of the brain points to the importance of electrotonic coupling between IO neurons (De Zeeuw et al., 1995).
1.3.2 Inputs and origin

Although some of the IO subnuclei have some unique characteristic features, they all share the presence of glomeruli, dendrodendritic gap junctions, and dendritic lamellar bodies, and they all receive both excitatory and inhibitory inputs (De Zeeuw et al., 1989a, 1990b, c, d, 1993, 1994, 1996; Strata, 1989). The IO receives sensory and motor signals from nuclei such as the trigeminal nuclei, dorsal column nuclei, pretectal complexes, and red nuclei as well as direct feedback signals from the cerebellar nuclei and indirect feedback signals that are relayed via the mesodiencephalic junction (De Zeeuw and Ruigrok, 1994; Garden et al., 2017; Onodera and Hicks 1995; Swenson, 1983). Sensory and motor inputs are partially segregated, but also converge within parts of the IO (Berkley and Worden, 1978). For example, motor inputs deriving from the nucleus of Darkschewitsch and nucleus of Bechterew, part of the mesodiencephalic junction, as well as the primary motor cortex innervate the PO and rostral MAO (Onodera, 1984; De Zeeuw and Ruigrok, 1994; Garden et al., 2017; Berkley and Worden, 1978), whereas somatosensory inputs coming from the spinal cord, dorsal column nuclei, pretectum, and lateral cervical nucleus innervate the DAO and caudal MAO (Berkley and Worden, 1978; Bull et al., 1990; Boesten and Voogd, 1975). An overlap of motor and sensory fibers coming from the primary motor cortex, spinal cord, dorsal column nuclei, and lateral cervical nucleus, is observed in the medial part of the rostral DAO and a small area in the middle of the caudal MAO (Berkley and Worden, 1978). Supporting these latter findings, olivary cells located in the DAO respond to cerebral cortex and spinal cord stimulation, exhibiting a high degree of convergence of motor and sensory inputs in this subnucleus of the IO (Crill and Kennedy, 1967). Whether in this case the excitatory responses of IO cells vary depending on the type of input – motor or sensory - requires further investigation. However, it has been observed that excitatory synaptic responses to motor inputs coming from the primary motor cortex and MDJ differed in that synaptic responses to cortical afferents were smaller. This could reflect a scarce direct innervation from that cortical region (Garden et al., 2017). Regardless of whether the responses of IO neurons to convergent inputs are different or not, the location of the IO cells also dictates the way the information is processed, since a specific area of an IO subnucleus projects to a particular longitudinal microzone of the cerebellar cortex, which can be involved in either a motor or non-motor task (Apps and Hawkes, 2009; De Zeeuw, 2020). Likewise, a specific area of an IO subnucleus projects and receives afferents back from specific regions of the cerebellar nuclei (Apps and Hawkes, 2009; Ruigrok and Voogd, 1990, 2000). Therefore, the spatial distribution of sensory and motor inputs within the IO will determine which microzones of the cerebellar cortex and specific areas of the cerebellar nuclei are engaged
with particular functional consequences for motor program adjustment or cognitive processes. How IO neurons integrate sensory and motor information requires further investigation.

As mentioned above, in the PO and MAO, all terminals derived from the mesodiencephalic junction are excitatory and display the corresponding morphological features consisting of rounded vesicles and asymmetric synapses. In contrast, all the cerebellar terminals in the PO and MAO are GABAergic and have pleiomorphic vesicles and symmetric synapses (De Zeeuw et al., 1988). Approximately half of both types of terminals contact dendritic elements inside glomeruli (De Zeeuw, 1990; De Zeeuw et al., 1989a, 1990b, c, d; Strata, 1989). The large majority of the remaining terminals contact the proximal and intermediate dendrites, while relatively few terminate on the somata and axon hillock; presynaptic axo-axonal contacts have not been observed in the IO. The innervation of the IO by the non-GABAergic mesodiencephalic terminals and GABAergic cerebellar terminals is apparently random, because neither type of terminal has a preference for either the extra- or intra-glomerular neuropil and there is no obvious pattern in the distribution of the two types of terminals within the individual glomeruli (Strata, 1989). Every spine on the dendrites and axon hillock of all IO neurons in the PO and rostral MAO receives a synaptic input from both an excitatory mesodiencephalic terminal and an inhibitory cerebellar terminal. Since in most regions of the central nervous system the vast majority of dendritic spines are contacted solely by asymmetric synapses, the ubiquitous, combined excitatory and inhibitory input to the IO spines can also be considered as one of the characteristic features of its neuropil. The other subnuclei such as the DAO, β nucleus, DCK, VLO, and DMCC follow the same configuration, but with different origins of the inputs involved (De Zeeuw et al., 1993, 1994, 1996). Despite the fact that we have a reasonably detailed view on which afferents project to what subnuclei in the inferior olive, we still lack sufficient detailed insight as to how these afferents are distributed with respect to the different cell types in the inferior olive.

1.3.3 Neurotransmitters and receptors

Apart from the glutamatergic and GABAergic inputs and receptors present in the IO glomeruli (Garden et al., 2017; Hoge et al., 2011; Lefler et al., 2014; Mathy et al., 2014; Turecek et al., 2014), there is also a widespread indolaminergic and catecholaminergic innervation present in the IO (Bishop and Ho, 1984; Paré et al., 1987). These inputs generally serve as level setting systems, determining the membrane potential of the olivary neurons and thereby controlling ensemble oscillations and rhythmic activity (Barragan et al., 1983). Even though the signaling
through these pathways is more diffuse and generally takes place outside the olivary glomeruli through termi-
nals with abundant dense core vesicles, the distribution of the receptors involved appears to be strictly organized within the IO complex and usually at least partly overlaps with that of the inputs and transmitters. However, there are substantial differences among species. For instance, in cat, indolaminergic receptors appear to be most densely present in the MAO and DAO, whereas in rats these are restricted to the lateral DAO (Wiklund et al., 1977). Likewise, the catecholaminergic innervation (norepinephrine and dopamine) of the IO is generally also not spread evenly and also differs among species (Sladek and Hoffman, 1980). In rat, the dopaminergic projections from the mesodiencephalic junction target the VLO, which is involved in vertical compensatory eye movements (Toonen et al., 1998), whereas in cat, the dopaminergic nerve terminals are most prominent in the DAO, which may be involved in sensorimotor processing during locomotion (Horn et al., 2010, 2013; Maqbool et al., 1993). And again, differently, in nonhuman primates, catecholaminergic fibers are mostly seen in the MAO and lateral lamella of the PO (Kamei et al., 1981; Sladek and Bowman, 1975), whereas in humans the noradrenergic fibers in the IO are much more homogeneously distributed than in rat, cat, and monkey (Powers et al., 1990).

In many extraglomerular terminals with dense core vesicles, one can find a coexistence of a classical neurotransmitter like an indolamine or catecholamine with some neuropeptide. For example, in the DAO of the rat, many terminals have both serotonin and substance P, while the corresponding receptors can be found in adjacent dendrites (Bishop and Ho, 1984; Paré et al., 1987). However, some of the inferior olivary subnuclei appear to receive peptidergic inputs without concomitant indolaminergic or catecholaminergic substances in the same terminals. Indeed, in the DCK, the β nucleus, and the DMCC of the rat, there are many substance P fibers and varicosities as well as related receptors, but little trace of serotonin immunoreactive elements (Bishop and Ho, 1984). Possibly, neuropeptides like substance P can also serve relevant functions without co-release of one of the classical neurotransmitters. Substance P can exert its effects through tachykinin neurokinin-1 receptor (NK1R), which plays an integral role in the modulation of homeostatic functions in the medulla, including regulation of respiratory rhythm generation, integration of cardiovascular control, and modulation of the baroreceptor reflex and mediation of the chemoreceptor reflex in response to hypoxia (Bright et al., 2017).
1.4 Cell physiology of inferior olivary neurons

1.4.1 Subthreshold oscillations and spike timing

One of the distinguishing features of IO neurons is that they can exhibit prominent membrane potential subthreshold oscillations (Fig. 5a; Benardo and Foster 1986; Llinás et al. 1974; Llinás and Yarom 1981b, 1986). Such oscillations are generated through the sequential activation of various voltage-gated ionic conductances such as a dendritic high-threshold Ca$^{2+}$ conductance, a somatic low-threshold Ca$^{2+}$ conductance, a Ca$^{2+}$-activated K$^{+}$ conductance, and a hyperpolarization-activated cationic conductance (IH) (Bal and McCormick, 1997; Benardo and Foster, 1986; Llinás and Yarom, 1981b, 1986; Matsumoto-Makidono et al., 2016; Zhang et al., 2017). Electrophysiological experiments on IO neurons of mutant mice lacking either the CaV2.1 gene (P/Q-type) or the CaV3.1 gene (T-type Ca$^{2+}$ channel) have shown that these channels support the generation of IO membrane potential oscillations (Choi et al., 2010; Matsumoto-Makidono et al., 2016; Park et al., 2010). Indeed, immunohistochemical stainings confirmed that T-type Ca$^{2+}$ channels are only expressed in IO neurons displaying subthreshold oscillations (Bazzigaluppi and de Jeu, 2016). The Ca$^{2+}$-activated K$^{+}$ conductances are responsible for the after-hyperpolarization (AHP), and blocking these channels prevents the rhythmogenesis of the membrane potential in IO neurons (Bal and McCormick, 1997; Llinás and Yarom, 1981a, b). The IH conductance mainly determines the pacemaker potential and the oscillation frequency (Bal and McCormick, 1997; Matsumoto-Makidono et al., 2016). In rodents, IO neurons can exhibit two prevalent types of subthreshold oscillation (Fig. 5a): 3–10 Hz sinusoidal subthreshold oscillation (SSTO) and rhythmic 1–3 Hz low-threshold Ca$^{2+}$ oscillations (LTO). IO cells can express either one or a mixture of these oscillations (Khosrovani et al., 2007; Lampl and Yarom, 1997; Llinás and Yarom, 1986). Only IO neurons from the DCK and the VLO do not express subthreshold oscillations (Urbano et al., 2006). In non-human primates, SSTOs and LTOs are found in similar proportions, but the overall oscillation frequency is in general significantly slower (1–2 Hz), most likely due to the increased capacitive load of the more elaborate IO dendrites (Turecek et al., 2016). With such slow oscillations, phase differences in populations of IO neurons might be important to confer the temporal precision required for rapid motor adaptation. Frequency modulation of subthreshold oscillations has been observed within IO recordings, but it is more frequently seen in vitro than in vivo (Devor and Yarom, 2002b; Khosrovani et al., 2007). The origin of shifts activation of NMDA receptors in the IO can support and modulate subthreshold rhythmogenesis in IO neurons. In vivo IO recordings from animals anesthetized by ketamine
Fig. 5 Electrophysiological properties of inferior olivary neurons. A – Membrane potentials of IO neurons can express different spontaneous subthreshold oscillations. In the top trace, a spontaneous sinusoidal subthreshold oscillation (SSTO) was recorded from an IO neuron in vivo, whereas the bottom trace shows an IO neuron that expresses spontaneous low-threshold Ca²⁺ depolarizations (LTO). B – Enlargement of a typical IO action potential. The arrows indicate IO wavelets on top of the after depolarization (ADP). C – Electrotonic coupling between two adjacent IO neurons was measured using a dual whole-cell patch-clamp technique. Current injections into cell 2 (left panel) induced direct voltage responses in cell 2 and indirect responses in cell 1. This current flow was bidirectional (right panel). (Reprinted with permission of Bazzigaluppi et al. (2012a) and De Zeeuw et al. (2003)).

(blocker of NMDA receptors) show more often LTOs than SSTOs, whereas IO recordings of animals in which the anesthetics used does not block the NMDA receptors (medetomidine/midazolam/ fentanyl mixture) report more often SSTOs than LTOs (Bazzigaluppi et al., 2012a). By contrast, pharmacological stimulation of NMDA receptors can, through a concomitant increase in coupling between IO neurons, amplify or even initiate IO oscillations (Devor and Yarom, 2002b) (Turecek et al., 2014). Thus, in individual IO neurons, synaptic activation of NMDA receptors could provide a means to dynamically change a cell’s oscillatory behavior and the number of synchronized neighboring cells. The fact that freeze-fracture analysis of IO glomeruli shows close proximity of glutamatergic postsynaptic densities and Cx36-
containing gap junctions further supports this idea (Hoge et al., 2011). Several studies revealed that a number of neuroactive substances are able to modulate the amplitude of the sub-threshold oscillations. For example, serotonin suppresses the amplitude of the subthreshold oscillation (Placantonakis et al., 2000), whereas harmaline, like NMDA, can amplify oscillation amplitude (Llinás and Yarom, 1986).

Subthreshold oscillations determine the firing behavior of IO neurons in a temporal manner. During the peak of the oscillation, there is an enhanced spiking probability, which in turn is reduced at the trough. IO neurons do not generate an action potential at every depolarizing phase of the oscillation, but on average only spike once every ten cycles. Harmaline facilitates the spiking behavior of IO neurons by increasing the amplitude of the oscillation, resulting in the generation of a spike on every depolarizing phase of the oscillation (Llinás and Volkind, 1973; Llinás and Yarom, 1986). The harmaline-induced firing is, therefore, very rhythmic and brings the IO neurons up to their maximal firing rate. The interaction (phase locking of spikes) between the subthreshold oscillations and spiking is not unidirectional. The phase of the subthreshold oscillation influences the probability of spiking, but spiking also influences the phase of the following oscillation (Khosrovani et al., 2007; Leznik et al., 2002). IO spikes consistently lead to a shift in the subthreshold oscillation phase such that the spike would have occurred at the peak of the phase-shifted subthreshold oscillation (i.e., at 90°). Choi et al. (2010) showed that CaV2.1 and CaV3.1 are also required for the phase resetting of oscillations in IO neurons. Thus, IO neurons are equipped with a self-regulating temporal pattern generator.

1.4.2 Electrical synapses in the inferior olive

IO neurons are interconnected by dendrodendritic gap junctions formed by Cx36 proteins, while Cx45 and Cx47 are also found in oligodendrocytes in the IO (Fig. 6) (Condorelli et al. 1998; Weickert et al., 2005). Gap junctional coupling allows direct communication across multiple neurons. The electrophysiological properties of Cx36 gap junctions are characterized by a low unitary conductance of 10–15 pS, weak voltage sensitivity, and low-pass filter function (Srinivas et al. 1999). These properties ensure that low-frequency membrane oscillations are preferentially transmitted from one IO neuron to another (Fig. 5c) and that subthreshold oscillations can be synchronized among coupled IO neurons. Both dual whole-cell recordings and voltage-sensitive dye imaging in the IO have demonstrated that coupled IO neurons can synchronize their subthreshold oscillations (Devor and Yarom, 2002a, b; Leznik and Llinás, 2005). Optical imaging
has confirmed that such activity can propagate across the olivary nucleus (Devor and Yarom, 2002b).

It is important to note that the electrotonic coupling between IO neurons is not a prerequisite for the generation and maintenance of oscillations in IO neurons. Both genetic and pharmacological uncoupling of IO neurons do not abolish the generation of subthreshold oscillations (De Zeeuw et al., 2003; Leznik and Llinás, 2005; Long et al., 2002), suggesting that oscillations are generated by the intrinsic conductances of individual neurons. However, genetic uncoupling of IO neurons does abolish the synchrony of subthreshold oscillations and the synchrony of firing among IO neurons (Long et al., 2002). Synchronization of subthreshold oscillations and synchrony of firing among IO neurons are often considered the most critical function of gap junctional coupling. In fact, the ability to synchronize membrane oscillations across IO neurons through Cx36 gap junctions may have functional consequences, as the crispness of reflexive movements is impaired in Cx36 global knockout mice (De Gruijl et al., 2014a).

The uncoupling of IO neurons does affect one of the oscillatory properties; their oscillatory behavior is limited to a smaller range of membrane potential levels (De Zeeuw et al., 2003; Leznik and Llinás, 2005; Long et al., 2002). Therefore, the gap junctions (or coupled network) act to stabilize the subthreshold oscillatory activity in the olive cell by making the oscillations less sensitive to the membrane potential with respect to frequency and amplitude. The uncoupling of the IO network also increases the excitability of IO neurons at hyperpolarized levels (De Zeeuw et al., 2003; Leznik and Llinás, 2005), which results in an altered timing of climbing fiber activities in the cerebellar cortex (Van Der Giessen et al., 2008).

### 1.4.3 Synaptic modification of oscillations and coupling

Sensory and motor signals reach the IO via excitatory projections, which are most likely glutamatergic (Onodera and Hicks, 1995; Swenson, 1983). The feedback projection that connects the cerebellar nuclei via the mesodiencephalic junction with the IO is also excitatory (De Zeeuw and Ruigrok 1994), whereas the direct pathway is inhibitory and uses GABA as neurotransmitter (De Zeeuw et al., 1989a). The contribution of GABA and glutamate to the activity of IO neurons is subtle (Duggan et al. 1973; Lang 2002; Lang et al. 1996). Application of glutamate in the IO increased the spiking activity of IO neurons by only 2–3 Hz (Duggan et al. 1973), and blocking
GABAergic inhibition increased the spiking activity of IO neurons by only 1–2 Hz (Lang, 2002; Lang et al., 1996). This limited modulatory capacity of these neurotransmitters on IO activity can be attributed to the unique membrane properties of IO neurons (Llinás and Yarom 1981a; Llinás and Yarom, 1981b). Furthermore, depriving the IO from inputs does not abolish the intrinsic spiking activity of IO neurons. Thus, IO neurons are intrinsically active and do not require the glutamatergic or GABAergic inputs to spike (Lang 2001, 2002).

Stimulation of excitatory projections to the IO results in phase resetting of the subthreshold oscillation without affecting the amplitude or frequency of the oscillation (Leznik et al., 2002). Somatosensory stimulations in vivo reveal a similar phase resetting mechanism, except that under these conditions, no IO cell can be stimulated during the hyperpolarizing phase of their subthreshold oscillations (Khosrovani et al. 2007). This resetting mechanism allows IO neurons to adjust their spiking patterns in a temporal manner by using online sensory and motor ("reference") feedback.

Activation of the CN in vivo induces a biphasic response in IO neurons. First, a depolarization occurs which can evoke an action potential. Subsequently, a long-lasting GABAergic hyperpolarization is generated (Bazzigaluppi et al., 2012b). This hyperpolarization blocks the supra- and subthreshold activity of the IO neurons for the duration of one subthreshold oscillation.
cycle, and after this hyperpolarizing block has been raised, all subthreshold oscillations are restarted (inducing a 0◦ resetting point). Under these conditions, The input from the CN did not alter the amplitude or frequency of the reappearing subthreshold oscillations. Thus, the direct GABAergic, nucleo-olivary pathway gates information during specific time windows (i.e., approximately one subthreshold oscillation cycle) and resets very bluntly the subthreshold oscillation. Such resetting has also been demonstrated in optogenetic experiments where nucleo-olivary axons to the IO neurons were stimulated directly and repeated stimulation could transiently silence subthreshold oscillations (Lefler et al., 2014).

IO neurons receive GABA and glutamatergic inputs that terminate close to the electrical synapse (at IO glomeruli, see section “Glomeruli and Gap Junctions”), and these inputs may, therefore, also control the electrotonic coupling between IO neurons (Lang et al., 1996; Llinás and Sasaki, 1989). Imaging studies (Leznik and Llinás, 2005; Leznik et al., 2002) showed that blocking GABAA receptors in the IO increased the number of neurons oscillating inphase, indicating an expansion of the number of electrotonically coupled neurons. This increment of cluster size has also been observed in Purkinje cells after blocking the GABA receptors in the IO (Lang, 2002). By contrast, stimulation of GABAergic nucleo-olivary afferents can transiently reduce coupling between IO neurons (Lefler et al., 2014). The role of glutamatergic inputs on IO coupling is a bit less clear. Low-frequency electrical stimulation of glutamatergic projections was shown to reduce coupling (Mathy et al., 2014), whereas pharmacological activation of NMDA receptors was shown to increase coupling strength (Turecek et al., 2014). In another study, a pharmacological block of glutamatergic input resulted in an overall reduction in IO coupling (Lang, 2002). Gap junctional plasticity could be mediated by the PKA and CaMKII pathways, where activation of the PKA pathway most likely reduces the opening probability of Cx36 gap junctions, whereas the CaMKII pathway could increase the number of Cx36 gap junctions (Bazzigaluppi et al., 2017). Discrepancies in these results could in fact be explained by considering the Ca2+ levels in IO spine heads evoked by the different stimulus paradigms (De Grujil et al., 2014b, 2016). Overall, these results suggest that activity of the intra-glomerular chemical synapses dynamically regulates the efficacy of electrotonic coupling and therefore the patterns of synchronous activity in the olivocerebellar system (Llinás et al., 1974). Despite all the studies addressing these topics, it is still unknown how postsynaptic integration of both responses modulates STOs, electrical coupling and spike probability of IO neurons.
1.4.4 Action potential waveforms

IO neurons have action potentials with a characteristic waveform (Fig. 5b): a sharp spike is followed by a prolonged afterdepolarization (ADP) and a long-lasting AHP (Llinás and Yarom, 1981a, b). The discharge rate is low: only once or twice per second (Benardo and Foster 1986; Llinás and Yarom, 1981a, b). Although IO neurons are sensitive to sensory stimulation, their responsiveness is limited. Stimulating excitatory afferents to the IO either from sensory nuclei such as the trigeminal nucleus or from higher systems such as the mesodiencephalic junction evokes only a single action potential in IO neurons. Using a chemical excitant, such as harmaline, IO firing can be driven to a maximum of approximately 10 Hz (Llinás and Volkind, 1973; Llinás and Yarom, 1986). The low discharge rate and limited responsiveness of IO neurons are unique features in the generally very active olivocerebellar circuit.

The fast IO spike is mediated by Na⁺, whereas the ADP is generated by the activation of dendritic high-threshold Ca²⁺ conductances (Llinás and Yarom, 1981a, b). Choi et al. (2010) have shown the involvement of the P/Q-type Ca²⁺ channel CaV2.1 in this process. However, there also is evidence that CaV3.1 affects spike repolarization (Matsumoto-Makidono et al., 2016). The influx of Ca²⁺ activates dendritic Ca²⁺-activated K⁺ conductances inducing subsequently the slow AHP (Bal and McCormick, 1997; Llinás and Yarom, 1986). During this hyperpolarization, two processes are initiated: hyperpolarization-activated cationic conductances (IH) are activated, and somatic low-threshold Ca²⁺ conductances become de-inactivated (T-type; Bal and McCormick, 1997; Llinás and Yarom, 1986). It is likely that both processes are involved in the termination of the AHP and the generation of a rebound spike. The long duration of the AHP and the de-inactivation process of the T-type Ca²⁺ conductances are probably responsible for the low discharge rate and poor responsiveness following high-frequency stimulation.

One to seven small wavelets (<10 mV) are superimposed on the ADP of IO neurons (Fig. 5b, arrows) and occur at very high frequencies ranging from 200 to 500 Hz. They represent a high-frequency bursts of action potentials that are most likely generated in the axon of IO neurons by the ADP (Crill, 1970; Crill and Kennedy, 1967; Maruta et al., 2007; Mathy et al. 2009). Propagation of the ADP to the axon leads to the initiation of a burst of action potentials, which in turn propagate back to the soma. Here, they give rise to attenuated wavelets. Na⁺ spikes are not evoked because the Na⁺ channels are still inactivated at the soma (Mathy et al., 2009). IO burst firing is transmitted to the Purkinje cells via the climbing fibers, where they can modify the Purkinje cell complex spike itself or the synaptic transmission between the parallel fibers and Purkinje cells (Hansel, 2009; Mathy et al., 2009).
Information about spike timing relative to the phase of the subthreshold oscillation may be conveyed by axonal burst activity of IO neurons (Mathy et al., 2009). The timing of the IO activity is encoded by the number of spikes in the IO axonal burst. Such bursts might provide the olivocerebellar system with a mechanism that allows timing-dependent learning of parallel fiber input patterns in Purkinje neurons. However, in vivo studies (Bazzigaluppi et al., 2012a) suggest that the amplitude of the IO subthreshold oscillations also contributes to the size of the IO axonal burst. The bursts are reduced when a spike is evoked on top of a subthreshold oscillation with a large rather than a small amplitude. This amplitude coding might provide the olivocerebellar system with a mechanism to grade the expectancy (or saliency) of an event, gating only relevant information to Purkinje cells. There now is some evidence that the climbing fiber axons that originate from IO neurons indeed respond in a graded manner to sensory stimuli of different duration and strength (Najafi et al., 2014; Najafi and Medina, 2013).

1.5 Models of olivary neurons

1.5.1 Single-cell models

In addition to research conducted in vitro (Bleasel and Pettigrew, 1992; Leznik and Llinás, 2005; Leznik et al., 2002), in silico studies of the IO have investigated the spectrum of cellular and network behavior as a function of crucial parameters. A small number of dynamical system models of IO neurons exist, with each model being characterized by its level of detail, such as ionic conductances and number of compartments (Manor et al., 1997; Schweighofer et al., 1999; Van Der Giessen et al., 2008; Velarde et al., 2002). Based on its electrophysiological characteristics, the oscillating IO neuron can be classified as a so-called resonator: a cell that is highly sensitive to the frequency at which sequential inputs arrive (Izhikevic, 2007).

Among the most detailed is the compartmental model by Schweighofer et al. (1999), later modified by De Gruijl et al. (2012) to include an axonal compartment. The original Schweighofer IO cell model includes a somatic and a dendritic compartment, each with its own ionic currents (section “Subthreshold Oscillations and Spike Timing”) as illustrated in Fig. 7a. Across a large range of parameters, the single IO cell model neuron reproduces a number of properties of its biological counterpart, such as (1) that the cell shows SSTOs with a frequency range of 7 to 9 Hz and an amplitude of 10 to 20 mV, (2) that the IO cell basically does not generate a spike in the trough of the oscillation, (3) that the IO cell shows differentially damped oscillations after an
Fig. 7 Two-compartment model of two electrotonically coupled olivary neurons. A – Coupled cell pair with compartments separated by gray-shaded areas. For the red cell, the currents used in the model are depicted at the appropriate compartment. Somatic currents are T-type Ca$^{2+}$ (IT), sodium (INa), delayed rectifier potassium (IKdr), and a passive leak current (IL). Dendritic currents are P/Q-type Ca$^{2+}$ (IP/Q), cation h-current (Ih), a passive leak current (IL), and Ca$^{2+}$-dependent K$^+$ (IK). For IK internal Ca$^{2+}$ concentration ([Ca$^{2+}$]) is also modeled in the dendritic compartment. In the blue cell, the values for internal conductance (gint) and gap junctional conductance (gGJ) are indicated. B – Reaction of depicted coupled IO neurons to a stimulus. The red cell is stimulated and shows the distinct spike-shaped, long AHP and differentially damped SSTOs of its biological counterpart. The blue cell receives no input, but its state is changed due to the electrotonic coupling between the cells. As can be deduced from the gray reference trace, the action potential causes a shift in the phase of the SSTOs of both coupled cells after the event. C – Tendency of uncoupled cells (red) to fire doublets and respond rhythmically to repeated input. This is less apparent in coupled cells (black outline). Cells received temporally randomized input at 200 10 ms and 300 10 ms. Uncoupled cells often respond to one or both of the inputs but can also fire one or even two additional action potentials afterward. Coupled cells fire only on the first input and tend to synchronize their spikes, as is apparent from the narrower distribution. (Adapted from Van Der Giessen et al. 2008). D – Oscillations in cells at different injected currents. In coupled cells (left), oscillations in a cell can be sustained over a wider range of injected currents and resulting membrane potentials than in uncoupled cells (right). Depicted cell in the coupled network is the center cell of a 3 3 network, connected to all other cells. Values on the left side of the graphs are the average membrane potential in mV, taken from the last 500 ms of the simulation.

action potential, and (4) that the IO cell undergoes strong phase resetting following an action potential (Fig. 7). Dynamical systems analysis indicates that oscillatory characteristics are driven by the coupled interplay between dendritic and somatic conductances (Schweighofer et al., 1999; Van Der Giessen et al., 2008). Both the prevalence of SSTO and the SSTO frequency of the model cell are probably mainly controlled by the conductance of the low-threshold (T-type) somatic Ca$^{2+}$ channels and the somato-dendritic coupling. When IO model neurons are uncoupled, they exhibit an increased excitability, shorter firing windows, and an increased voltage...
dependency of the SSTOs (Fig. 7c–d). All these alterations can be explained by the removal of
the gap junctional leak current and the thwarting of the transmission of the oscillatory behavior of
coupled neighboring neurons (De Zeeuw et al., 2003; Van Der Giessen et al., 2008). In addition
to the properties above, the extended model including an axonal compartment was also able to
display high-threshold Ca\textsuperscript{2+} spikes, which produced bursts of axonal spikelets that were partly
dependent on the network state (De Gruijl et al., 2012).

### 1.5.2 Network models

The network properties of the IO can change dramatically depending on the conductance value
of the gap junctions, controlling both synchrony and the presence of network-wide oscillations. At
high gap junctional conductances, ensembles of IO neurons operate in synchrony (i.e., their
membrane potentials all oscillate in sync). The high gap junctional conductances also increase
the input resistance of the model cell by increasing the leak to neighboring cells, preventing the
IO neuron from generating a spike and making it more difficult to excite. At very low gap junctional
conductances, the ensembles start to desynchronize, and the individual neurons become more
excitable. Gap junctional coupling may bring a pair of non-oscillating cells to the oscillatory domain
(Lefler et al., 2013). The coupling coefficient between IO neurons in situ, generated by Cx36 gap
junctions, ranges from relatively small to significant (0.002–0.17; Devor and Yarom, (2002a).
Simulations of small networks in that range show that electrotonic coupling yields the most
versatile and interesting network properties (Schweighofer et al., 2004). In a low to moderately
coupled network, a volley of excitatory events sent to an ensemble of IO neurons does not need
to arrive in perfect synchrony for the IO network in order to generate a more synchronized output
due to multiple cell phase resetting (Fig. 7c; Van Der Giessen et al., 2008). In low to moderately
coupled networks, the synchrony is transient, as cells retain some of their abilities to operate
individually. Due to local electrotonic coupling, neighboring cells can also tangentially change the
state of other close-by cells. For instance, a network IO model demonstrated to produce quasi-
synchronous spatiotemporal wavefronts in SSTOs (Latorre et al., 2013). By applying noisy inputs
to a low to moderately electrotonically coupled IO network, Schweighofer et al. (2004) were able
to simulate low-firing IO network behavior. In a series of in silico experiments, they showed that
under certain assumptions, the low-frequency signals generated by the IO show a higher degree
of mutual information with the input (Schweighofer et al., 2004). Not only the low-frequency firing
can carry information, but also the high-frequency pattern of the climbing fiber bursts produced
by individual IO axons can contain important information. In that context, a network model by De
Gruijl et al. (2014b) showed that the number of spikelets in a climbing fiber burst (Mathy et al., 2009) transmits information about amplitude – more so than the phase – of the network subthreshold oscillation. Network state is subject to Ca²⁺-dependent modulation of gap junctional plasticity, which was experimentally demonstrated and subsequently modeled in De Gruijl et al. (2016).

1.6 Climbing fiber patterns and behavioral consequences

1.6.1 Spatiotemporal patterns

Purkinje cells produce two types of spikes: complex and simple spikes. Simple spikes are produced endogenously, but their firing pattern is modulated by excitatory and inhibitory synaptic input from parallel fibers and molecular layer interneurons (MLI), respectively (Häusser and Clark, 1997; Llinás and Sugimori, 1980; Miyashita and Nagao, 1984; Raman and Bean, 1999; Wada et al., 2007; Wulff et al., 2009). Simple spikes occur at a sustained, high frequency (10–200 Hz). Complex spikes are evoked by the climbing fiber input (Eccles et al., 1964). Climbing fibers form very large synapses on Purkinje cells, with each individual synapse having around 500 release sites (Silver et al. 1998). Complex spikes occur relatively infrequent (0.2–10 Hz) with an average around 1 Hz. Following a complex spike, a Purkinje cell cannot fire a simple spike during a period of about 20 ms (Bell and Grimm, 1969; Granit and Phillips, 1956; Simpson et al., 1996). The duration of this so-called climbing fiber pause can even be prolonged by optogenetic stimulation of the climbing fibers due to their impact on the molecular layer interneurons (Mathews et al., 2012), and dependent on the network properties as well as the intrinsic properties of the Purkinje cell involved, it can also be followed by a period of increased simple spike firing (Simpson et al., 1996). Thus, input from the IO does not only evoke complex spikes in Purkinje cells but also modulates simple spike firing (Badura et al., 2013; De Zeeuw et al., 2011).

Because of the exceptionally strong synaptic coupling between climbing fibers and Purkinje cells, complex spike firing follows in part the oscillations observed in the IO (Armstrong, 1974; Bell and Kawasaki, 1972; Blenkinsop and Lang, 2006; Khosrovani et al., 2007; Lang et al., 1996; Llinás and Sasaki, 1989; Marshall et al., 2007; Van Der Giessen et al., 2008). The subthreshold oscillations of the IO neurons may affect both the rate and the absolute timing of the complex spikes for short durations (Devor and Yarom, 2002a; Lampl and Yarom, 1993; Van Der Giessen et al., 2008). Input to the IO can reset the phase of the subthreshold oscillations (Khosrovani et al., 2007; Leznik et al., 2002; Van Der Giessen et al., 2008). Consequently, the complex spike
pattern could be “restarted” following a strong, unexpected sensory input, thus triggering a new motor program (Llinás, 2009). In addition to these acute effects of IO oscillations, they may also facilitate long-term plasticity by affecting the number of spikelets per complex spike, which in turn could modulate heterosynaptic plasticity at the parallel fiber to Purkinje cell synapse (Bazzigaluppi et al., 2012a; Mathy et al., 2009; Van Der Giessen et al., 2008).

In addition to the temporal patterning, climbing fiber input also organizes the spatial extent of complex spike firing. Because of the strong electrotonic coupling within the glomeruli, groups of IO neurons fire in synchrony (De Zeeuw et al., 2003; Long et al., 2002; Sotelo et al., 1974). The divergence of climbing fibers ensures that the synchrony within the IO is transmitted to large areas of the cerebellar cortex (Blenkinsop and Lang, 2006; Bosman et al., 2010; De Zeeuw et al., 1997b; Marshall et al., 2007; Ozden et al., 2009; Sasaki et al., 1989; Van Der Giessen et al., 2008). Coherent complex spike firing, which we define as co-occurrences during bins of 3–10 ms, can occur across larger parts of the cerebellar cortex (Fig. 8) (Bell and Kawasaki, 1972; Bosman et al., 2010; De Zeeuw et al., 1996; Kros et al., 2017; Welsh et al., 1995). Well-timed complex synchrony, occurring within 2 ms bins, is usually restricted to parasagittal zones (up to ca. 500 μm wide) that are defined by the pattern of climbing fiber innervation (Fig. 9) (Bosman et al., 2010; Lang et al., 1999; Llinás and Sasaki, 1989; Mukamel et al., 2009; Ozden et al., 2009; Schultz et al., 2009; Sugihara et al., 1993, 2007; Wylie et al., 1995). Two-photon Ca²⁺ imaging of Purkinje cell dendrites showed that the highest degree of complex spike synchrony is found in parasagittal “microzones,” which are 3–19 Purkinje cells wide (Mukamel et al., 2009; Ozden et al., 2009; Schultz et al., 2009). Microzones can act as ensembles of Purkinje cells encoding sensorimotor and cognitive processes (Mukamel et al., 2009; Ozden et al., 2012; Schultz et al., 2009; De Zeeuw 2021). Furthermore, two types of Purkinje cell microzones can be recognized based on changes in simple spike frequency during the initial stages of learning: ‘upbound’ and ‘downbound’ microzones. Purkinje cells of upbound microzones exhibit low frequency spontaneous simple spike firing, which can be increased upon learning, whereas Purkinje cells of downbound microzones show high frequency spontaneous simple spike firing, which can be decreased upon learning. These opposing changes in the activity exhibited by both types of microzones might allow them to operate in a complementary manner or a particular sequence during learning (De Zeeuw, 2021). Differences observed in simple spike firing frequency among these two groups have mainly been attributed to different expression patterns of proteins involved in controlling the membrane potential and cellular excitability (Perkins et al., 2018; Wu et al., 2019). However, it has been shown that the inferior olive, via climbing fiber activity, may also affect the baseline of the simple spike frequency. Specifically, climbing fiber terminals present in downbound
microzones exhibit a larger pool of vesicles ready to be released, increased multivesicular release, and larger synaptic glutamate transients. This results in longer-duration complex spikes containing an increased number of spikelets (Paukert et al., 2010), which would cause a drop in simple spike firing frequency by temporarily prolonging their interval (De Zeeuw, 2021).

The finding that complex spike synchrony is not restricted to microzones may reflect IO projections to different zones (Bosman et al., 2010; De Zeeuw et al., 1998; Welsh, 2002; Welsh et al., 1995). In addition, the extent of electrotonic coupling may be modified by afferent input to the IO (De Zeeuw et al., 1998; Llinás and Sasaki, 1989; Van Der Giessen et al., 2008; Welsh et al., 1995). Thus, complex spikes occur in specific spatiotemporal patterns, dictated by input to the IO, and dynamic regulation of the olivocerebellar pathway can have acute and long-term effects on the spiking pattern of the Purkinje cells and thereby on the output of the cerebellar cortex. Considering these facts, it would be very interesting to investigate the spatiotemporal footprints of complex spikes underlying different behaviors and how they change as motor learning occurs.

1.6.2 Behavioral consequences

Alterations in climbing fiber activity affect motor behavior. A number of studies have indicated that normal activity of the IO is essential for motor performance, motor learning, and memory. Moreover, recent studies are challenging the idea that the inferior olive is purely involved in motor functions. Indeed, it has been shown that complex spike activity is able to encode non-motor tasks such as reward expectation, delivery, and omission (Kostadinov et al., 2019). In these experiments, it was observed that complex activity was spatially and temporally heterogeneous, because while some cerebellar microzones show an increased complex activity upon reward delivery, other adjacent microzones show an inhibited complex spike activity. Furthermore, ramp-like activity was observed in some microzones previous to the reward delivery. Complex spike activity was also inversely scaled with reward predictability and enhanced when the reward was omitted. Finally, complex spike activity scales with reward size (Larry et al., 2019). Complex spike activity has also been associated with decision-making in that complex spike firing is increased when a wrong decision is made in a decision-making task (Deverett et al., 2018). Finally, the olivo-cerebellar loop has been involved in motor planning (Gao et al., 2018). In this latter study, ramping activity was observed seconds before the onset of the movement in the frontal cortex and the cerebellar nuclei (fastigial nucleus). Transient perturbations of this cerebellar region resulted in erroneous subsequent correct responses without affecting the motor performance.
The inferior olive must play a crucial role in this non-motor task, since it has a strong reciprocal connection with the cerebellar nuclei (Ruigrok and Voogd, 1990, 2000).

Lesions and (acute) inactivation of the IO result in a large variety of altered motor behaviors, including deficits in both acute motor performance and motor learning (McCormick et al., 1985; Rondi-Reig et al., 1997; Turker and Miles, 1986; Voneida et al., 1990; Welsh and Harvey, 1998; Yeo et al., 1986). Experiments that hyperactivate the IO, either by electrical or optogenetic means, also reveal interesting effects; under these conditions acute motor activity can be triggered, and motor learning can be either blocked or enhanced, dependent on the timing of the climbing fiber activity with respect to that of the mossy fiber system (Harvey and Romano, 1993; Kesner et al., 1995; Luebke and Robinson, 1994; Turker and Miles, 1984; Welsh, 1998). Under non-pathological conditions, the majority of climbing fibers are considered to signal general disturbances of the body (Gibson et al., 2004) or errors in motor performance (Simpson et al., 1996). The body disturbances relayed by the IO might provide the cerebellum with a representation of an unconditioned stimulus that can be used during associative motor learning, and together with conditioned stimuli, it enables the animal to (re)organize their motor behavior (Gibson et al., 2004). In order to process and correct errors in motor performance, the firing frequency of single climbing fibers is too low to evaluate and control fast motor movements. To accomplish motor control at this level, population coding is required. Synchronization of the climbing fibers signals will then be essential for the temporal organization of the population in order to establish an appropriate control of movement (Ten Brinke et al., 2017). Accordingly, simultaneous electrophysiological recordings of ensembles of Purkinje cells (located in parasagittal zones) have revealed correlated changes in complex spike synchrony in specific spatial domains during motor tasks (Lang et al., 2006; Welsh et al., 1995) as well as during sensory stimulation (Wise et al., 2010; Wylie et al., 1995). Moreover, these synchronizing effects of motor and sensory signals have also been confirmed by optical imaging in both wild-type animals and mouse mutants (Mukamel et al., 2009; Ozden et al., 2009, 2012).

Desynchronization of the climbing fiber signal can be accomplished genetically (Marshall et al. 2007) as well as pharmacologically (Blenkinsop and Lang, 2006; Ozden et al., 2009) by removing or blocking the Cx36 protein in the IO. Genetically altered mice that lack Cx36 have subtle, but significant, deficits in motor performance (Kistler et al., 2002), as well as more prominent problems during associative motor learning tasks that require timing (Van Der Giessen et al., 2008).
Fig. 8 Coherent complex spike firing. Two-photon Ca\(^{2+}\) imaging of ensembles of Purkinje cells in an awake mouse expressing GCaMP6f. Purkinje cells show dynamic complex spike coherence in awake animals. At times large numbers of complex spike-triggered Ca\(^{2+}\) transients are seen in many Purkinje cell dendrites (1), sometimes only a few cells are responsive (2), or a subset of cells that also participated during large-scale coherence (3). This suggests that the coherence of IO neurons is dynamically regulated. In the images responding PC dendrites are shown in red pseudo-color. All detected PC dendrites are shown as gray outlines.
Fig. 9 Spatial patterns of complex spike synchrony. A – In normal animals, the neurons of the IO are organized in glomeruli, in which they are functionally coupled by dendrodendritic gap junctions (brown lines). Due to their electrotonic coupling, the neurons within a glomerulus have an increased tendency to fire synchronously. Because of the strict anatomical organization of the climbing fiber projections, each glomerulus projects to a parasagittal band of Purkinje cells in the cerebellar cortex. The upper part shows a schematic section through the molecular layer parallel to the surface. Each Purkinje cell forms a line, the transected dendritic tree. The synchrony in the glomeruli results in increased complex spike synchrony within “microbands.” B – Disruption of the electrotonic coupling of the neurons in the IO abolishes synchrony within the glomeruli of the IO and thus also of complex spike firing by Purkinje cells. Each Purkinje cell now follows its own firing pattern, symbolized by different colors.

Indeed, eyeblink conditioning experiments revealed abnormal timing of the conditioned eyeblink responses in Cx36 / mice (Fig. 10a–b). The abnormal timing in these Cx36 -/- mice is probably due to an increased variety in the timing of climbing fiber responses, which in turn is probably the result of the desynchronization of the subthreshold oscillations in the IO (Van Der Giessen et al., 2008). In fact, this process will not only affect climbing fiber activity mediating the unconditioned stimulus but as the learning progresses also that of the conditioned stimuli (Ohmae and Medina 2015; Ten Brinke et al., 2015, 2017, 2019). Similar results in aberrant conditioned responses can be obtained when Cx36 gap junctions are pharmacologically blocked by mefloquine (Ozden et al., 2009). Interestingly, this impaired associative motor learning can also be observed in humans who use mefloquine as prophylaxis of malaria (van Essen et al., 2010). In silico simulations of the olivocerebellar module support the idea that unsynchronized climbing fiber feedback to Purkinje cells affects the plasticity of the parallel fiber – Purkinje cell synapse (Fig. 10c–d) – which ultimately results in an impaired timing of the conditioned behavior (see also De Zeeuw et al. 2011; Ten Brinke et al., 2015, 2017).

The IO receives feedback signals from the cerebellar and vestibular nuclei in the hindbrain. The inhibitory nucleo-olivary pathway gates the information relayed by the IO. Bengtsson et al.
(2007) showed, using eyeblink conditioning, that blocking the unconditioned stimuli by activating the nucleo-olivary pathway results in extinction of the conditioned blink response. Along the same line, blocking the inhibitory transmission of the nucleo-olivary pathway prevented the extinction of the conditioned response (Medina et al., 2002). Therefore, the inhibitory nucleo-olivary pathway might serve as a negative feedback loop to control motor learning (Bengtsson et al., 2007). In short, the behavioral data indicate that the output of the IO forms a population signal that contains a temporal code, which is able to adapt or reorganize the active motor system according to its needs and which can be controlled by feedback inputs from the hindbrain (De Zeeuw and Ten Brinke, 2015).

1.7 Functional models of the olivocerebellar system

1.7.1 Marr-Albus-Ito learning models

Important pioneering work on the theory of cerebellar function was made by Marr, Albus, and Ito (Albus 1971; Ito, 1984; Marr, 1969). Their theories combined are now widely known as the Marr-Albus-Ito hypothesis. Based on anatomical data, Marr initially put forward the idea of parallel fiber to Purkinje cell synapses as the main site of plasticity in the cerebellar cortex (Marr, 1969). Although plasticity was first proposed to potentiate synaptic transmission, it was Albus who proposed the opposite. From his point of view, climbing fiber activation served to signal an error that is able to cause depression of the synaptic transmission (Albus, 1971). Subsequently, Ito provided evidence that climbing fiber activation can indeed induce long-term depression at the parallel fiber to Purkinje cell synapse when it is conjunctively applied with parallel fiber activation (Ito and Kano, 1982; Ito et al. 1982). The size of such effects may even be graded, dependent on the duration of the burst in the IO axon involved (Mathy et al., 2009; Najafi and Medina, 2013).

The occurrence of a complex spike often correlates with some sort of unexpected event that can serve as an error signal. These include, for example, retinal slip signals during adaptation of the vestibulo-ocular reflex (Stone and Lisberger, 1986), sensory cornea signals during classical or Bayesian eyeblink conditioning with an air puff as an unconditioned stimulus (Anderson and Steinmetz, 1994), skin signals while triggering limb reflexes during locomotion (Armstrong et al., 1968; Ekerot and Jörntell, 2001), or whisker stimulation during a discrimination task (Bosman et al., 2010; Rahmati et al., 2014). All these conditions can be readily incorporated in the Marr-Albus-
Fig. 10 Inferior olivary gap junctions are essential for proper timing in eyeblink conditioning task. A – Representative examples of eyeblink traces in a wild type (blue), global Cx36 / mutant (red), and Cx36 del(LacZ)/floxCre control mouse (green). They all show conditioned eyeblink responses after four training sessions using a tone as the conditioned stimulus (CS) and an air puff as the unconditioned stimulus (US). However, while the timing of the learned response in wild-type and floxed-Cre control mice improves over the sessions, that in the global Cx36 / mutants gets worse (see arrows). B – While the latency to peak amplitude (left panel) in the global knockouts of Cx36 (red) got worse during the training sessions, that in the parvalbumin-Cre control mouse (green) was indistinguishable from that in their unaffected littermates (blue). Similarly, the average latency to peak velocity (right panel) in the mutants was also significantly reduced (p < 0.01; t test). C – A mathematical network simulation was used to analyze the impact of altered IO coupling on eyeblink conditioning. Panel C shows the schematic representation of the olivocerebellar model that is used for our network simulation. Purkinje cells (PK), neurons of the cerebellar nuclei (CN), and neurons of the IO (IO) form a close loop. A key feature is the strong convergence of Purkinje cells onto CN neurons. MF, PF, and BS indicate mossy fibers, parallel fibers, and basket/stellate cells, respectively. D – Conditioned eyeblink responses of wild types (blue) and Cx36 / mutants (red) resulting from the network simulation. More details can be found in Van der Giessen et al. (2008). (Reprinted with permission of Van der Giessen et al. 2008).
Ito hypothesis, as it states that learning takes place at the parallel fiber to Purkinje cell synapses when climbing fiber activation signals undesired events. Due to the simplicity and elegance of this hypothesis and its similarity to the simple neural networks used in the field of machine learning (e.g., perceptrons), many models simulating the olivocerebellar system have been built using the Marr-Albus-Ito framework (Albus, 1975; Fujita, 1982; Medina et al., 2000; Porrill et al., 2004; Schweighofer et al., 1998; Spoelstra et al., 2000; Van Der Giessen et al., 2008). Often, these models consist of an expansion filter for the input layer (granular layer), one layer of learning units (Purkinje cell layer), a layer to interpret the output (cerebellar nuclei), and a source for providing a corrective signal to guide learning (IO). The Golgi cells usually serve to prevent saturation and to ensure sparseness of encoding in the input layer, but under physiological circumstances, granule cells appear to be activated at relatively high numbers during sensorimotor learning (Badura and De Zeeuw, 2017; Giovannucci et al., 2017).

More recently, the Marr-Albus-Ito hypothesis has expanded into the concept of distributed synergistic plasticity (Gao et al., 2012). It involves three extra steps, all of which built upon the original theory. First, it is not only the presence of climbing fiber activity (i.e., complex spikes) but also the absence of it that can mediate motor learning. Indeed, parallel fiber activation without climbing fiber activation generates long-term potentiation (LTP) of the parallel fiber to Purkinje cell synapse (Coesmans et al., 2004), and this type of postsynaptic plasticity appears to be relevant, if not essential, for particular forms of motor learning, such as adaptation of the vestibulo-ocular reflex (Gutierrez-Castellanos et al., 2017; Schonewille et al., 2010; Voges et al., 2017) and reflex adaptation (Romano et al., 2018). Possibly, this form of plasticity is particularly relevant when the Purkinje cells involved fire their simple spikes at a relatively low baseline firing frequency, i.e., those of the zebrin-positive modules (De Zeeuw and Ten Brinke, 2015; Zhou et al., 2014). This is in contrast to the zebrin-negative modules, which fire at a high baseline firing frequency and which show decreases during other forms of motor learning, such as eyeblink conditioning (Halverson et al., 2015; Jirenhed et al., 2007; Ten Brinke et al., 2015). Second, climbing fibers facilitate and suppress many forms of plasticity, including both post- and presynaptic forms of plasticity at not only the parallel fiber to Purkinje cell Synapse, but also the parallel fiber to molecular layer interneuron synapses and the molecular layer interneuron to Purkinje Cell synapses (for review, see Gao et al., 2012; Boele et al., 2018). However, these synapses are not Modified in a random fashion; the climbing fibers make sure that they influence them in a synergistic manner. For example, enhanced climbing fiber activity will induce long-term depression (LTD) at the parallel fiber to Purkinje cell synapse but potentiation at the parallel fiber to molecular layer interneuron synapse and the molecular layer interneuron to Purkinje cell
synapse, ultimately all leading to a reduced simple spike activity of the Purkinje cells. Likewise, absent or reduced climbing fiber activity will not only result in LTP at the parallel fiber to Purkinje cell synapse but also to depression at the parallel fiber to molecular layer interneuron synapse and the molecular layer interneuron to Purkinje cell synapse, ultimately increasing the simple spike activity. Third, the climbing fibers do not only influence activity in the output layer, but by modifying the parallel fiber to Golgi cell synapse, they may also influence activity in the input layer, further strengthening the synergy in a distributed fashion (Gao et al. 2012).

1.7.2 Motor timing models

An increasing amount of evidence indicates that the IO serves to signal errors and consequently guide the process of motor learning. The question that remains is: how can a heterogeneously electrotonically coupled network of IO neurons, with all the properties described above, consistently convey errors in such a way that it is well-suited for motor learning? So far, there have been only a few large-scale learning models that take the specific quirks of the IO into account (e.g., Van Der Giessen et al., 2008), as it is computationally demanding. As a result, it remains as of yet rather unclear how such properties may or may not contribute to providing an error and/or teaching signal.

The outcome may well be that the IO is not just a “teaching structure.” In theory, if the IO only flags errors or body disturbances, learning could take a long time due to the low incidence of spikes. Attempts to solve this problem by increasing the amount by which synaptic weights are modified would lead to inaccurate or even unstable learning. The low-firing rate of IO neurons, the oscillatory properties of the IO, and the immediate short-term effect of climbing fiber activations on the cerebellar cortex led to alternative hypotheses and models, in which the IO is a timing device necessary for accurate motor execution or timing-dependent motor learning.

One such theory highlights the role that recurrent connections in the olivocerebellar system may have (Kistler and De Zeeuw, 2002). The IO receives direct inhibitory input from the cerebellar nuclei and excitatory input from the mesodiencephalic junction, which in turn receives excitatory input from the cerebellar nuclei (De Zeeuw et al., 1998). Since cerebellar nuclei neurons have a tendency to show rebound activity several tens of ms after inhibition by Purkinje cells (Aizenman and Linden 1999; Aizenman et al. 1998; Czubayko et al. 2001; Jahnsen 1986; Llinás and Mühlethaler 1988; Ruigrok 1997), and this inhibition is likely to be most pronounced as a result of complex spikes (Hoebeek et al., 2010; Ten Brinke et al., 2017; Witter et al., 2013), the input from cerebellar nuclei neurons to the IO can be interpreted as a representation of IO activity tens of
ms earlier. Using simulations with a randomly distributed connectivity of the cerebellar nuclei neurons to the IO, it has been suggested that the reverberating loops present in the system may produce particular olivary firing patterns at subsequent firing windows, effectively rendering the IO capable of representing past events (Kistler and De Zeeuw, 2002).

Rodolfo Llinás, Yosef Yarom, John Welsh, and Eric Lang are advocates of a more IO-centric view of the olivocerebellar system (Welsh et al., 1995; Tang et al., 2019). Their hypotheses are focused on IO subthreshold oscillations and their possibilities to impose temporal control on dynamically shaped and coupled IO networks. In their models, the mossy fiber pathway may function to communicate a requested pattern, upon which the cerebellum primes the IO by splitting up the network into functional ensembles. These ensembles may in turn fire a temporal pattern determined by the phase differences in oscillations between cell ensembles, in theory allowing the system to use finer timing than just the 10 Hz dictated by IO subthreshold oscillations and to adjust the speed of motor control without affecting the sequence (Jacobson et al., 2008a).

Indeed, the temporal patterns of IO activity may drive the beat of motor activity, similar to what can be seen following application of the tremorgenic drug harmaline (Llinás and Volkind, 1973). Similar motor-related activity can also be seen under more natural circumstances, such as at the onset of compensatory eye movements or of conditioned responses (Frens et al. 2001; Sato et al. 1993). Interestingly, the complex spikes and rebound activity highlighted above following eyeblink conditioning both emerge most prominently at the onset of these movements when the learning is fully completed (Ten Brinke et al., 2017).

A model based on the timing framework has been put forward as a working universal control system (Kazantsev et al., 2003). The system utilizes a hardware implementation of IO neurons and is reminiscent of the olivocerebellar system in architecture. In this model, oscillating units (i.e., IO cells) are connected by means of resistors (i.e., gap junctions) that can be altered by a controller (i.e., parts of the cerebellar nuclei and/or cerebellar cortex). The input consists of motor intention patterns sent to the coupling controller and the oscillators and external feedback sent to the oscillators only. The output of the oscillators is used to drive an actuator system and is also sent to the coupling controller for internal feedback. Despite some simplifications of the anatomy of the olivocerebellar system, it does show that alternative interpretations of the olivocerebellar system may be viable. Thus, most classical theories like the one propagated by Marr-Albus-Ito fail to fully explain some of the cellular features of the cerebellar nuclei and the IO, whereas the IO-centric motor timing theories leave questions to be answered about the necessity and abundance of parallel fibers. As is often the case when theories are not mutually exclusive, the actual truth is likely to be found in the combination of them (De Zeeuw et al., 2011).
1.8 Pathology of the inferior olive

Dysfunction of the IO may contribute to diseases including essential tremor, palatomyoclonus, and dystonia, as well as more rarely to disorders like some forms of ataxia, sudden infant death syndrome and other respiratory disorders, and autism spectrum disorders. Essential tremor is a common action tremor, starting most often in the arms, but it may also emerge in other parts of the body like the head, trunk, or vocal muscles (Deuschl and Elble, 2009; Louis and Ferreira, 2010). Patients with essential tremor share several symptoms with typical cerebellar patients, including overshoot and intention tremor during voluntary movements (Deuschl et al. 2000; Köster et al. 2002), impaired eyeblink conditioning (Kronenbuerger et al., 2007), and abnormal rhythm generation (Avanzino et al., 2009; Farkas et al., 2006). The characteristics of the 8–10 Hz tremor can be mimicked by application of harmaline, which hyperpolarizes neurons of the IO and presumably leads to burst firing by de-inactivating calcium currents (Bernard et al., 1984; Llinás and Volkind, 1973, 1986; Long et al., 2002; Louis et al., 2002; Martin et al., 2005). Indeed, blocking T-type Ca^{2+} channels in the IO abolishes a harmaline-induced tremor (Handforth et al. 2010; Park et al., 2010). Interestingly, silencing the synaptic output of the IO can also induce a tremor with a frequency of about 10 Hz (White and Sillitoe, 2017), which highlights the possibility that the rhythms in the IO are partly generated to dampen some of the oscillations that may occur within the motor system under natural circumstances. Thus, both abnormalities that overactivate the olivary output and those that inactivate it can cause, or contribute to, essential tremor, highlighting the importance of fine-regulating the olivary oscillations.

Other types of tremor have also been described in combination with IO pathology. Hypertrophy of the IO, which typically occurs following a stroke affecting the dentato-olivary pathway (Koeppen et al., 1980), can lead to contralateral palatomyoclonus, which is an involuntary tremor of the soft palate (Deuschl et al., 1994; Lim and Lim, 2009). Palatomyoclonus occurs relatively often, but not always, together with pendular nystagmus (Deuschl et al., 1994; Liao et al., 2008; Lim and Lim, 2009). Hypertrophy of the principal olive and rostral medial accessory olive, both of which receive input from the mesodiencephalic junction (see section “Inputs and Origin”), can be mimicked in cats following lesions of the brachium conjunctivum or its source in the cerebellar nuclei (De Zeeuw et al., 1990d; Ruigrok et al., 1990). The hypertrophic cells are characterized by extensive dendritic and somatic coupling as well as relatively many long-latency responses following stimulation from mesodiencephalic junction. Possibly, the extensive coupling at the proximal level of the IO cells allows them to survive and generate
prominent oscillations at relatively low-firing frequency (De Zeeuw et al., 1990d; Ruigrok et al., 1990).

In addition, a tremor induced by abnormal olivary activity can be part of a larger syndrome, such as dystonia, which is characterized by symptoms like early-onset twisting and stiff limbs. Indeed, in some patients suffering from basal ganglia-independent dystonia, the IO is infiltrated by microglia and macrophages and shows metabolic defects (Raike et al., 2005; Simonyan et al., 2010; Zhao et al., 2011). Likewise, olivary lesions and/or reduced olivary activity may also contribute to dystonic postures in animals (LeDoux, 2011; Llinás et al., 1975). But then again, in other rodent models, the dystonic symptoms can be rescued by inactivation of the IO (Hisatsune et al., 2013). Thus, here too it appears to be critical to fine-regulate IO activity in a well-balanced way to avoid symptoms of dystonia.

Finally, the IO is infrequently also implicated in specific disorders that are associated with general movement deficits, like spinocerebellar ataxia (SCA) type 6, 7, and 23 (Holmberg et al., 1998; Verbeek, 2009; Wang et al., 2010a, b), in specific disorders that are associated with inhalation or speech issues, like fatal insomnia (Ayuso Blanco et al., 2006; Montagna et al., 2003), Wolfram syndrome (Hilson et al., 2009), semantic dementia (Davies et al. 2005), acute respiratory defects (Harper et al., 2000; Matschke and Laas, 2007), or forms of sudden infant death syndrome that result from abnormalities in substance P neurokinin-1 receptor binding (Bright et al., 2017), or in more general disorders with both motor and cognitive dysfunctions, like fetal alcohol syndrome (Dikranian et al., 2005; Jacobson et al., 2008b; Swayze et al., 1997) and autism spectrum disorders (Bailey et al., 1998; Prandota, 2010; Welsh et al., 2005).

1.9 Inferior Olive and Sleep

Sleep is one of the most important, ubiquitous but still unknown animal behavior. It is crucial for cognition, memory, decision making, immunity and health in general (Scammel et al., 2017; Canto et al., 2017). Based on electroencephalogram (EEG) and electromyogram recordings (EMG) sleep can be divided in 4 stages: 1 stage of rapid eye movement (REM) and 3 stages of Non-REM (NREM 1, 2 and 3). As its name indicates, REM sleep is characterized by fast saccadic eye movement and cortical EEG recordings showing low amplitude waves in the theta range (4-7 Hz). The REM theta activity, which arises mostly from the dorsal hippocampus, is more abundant in rodents than in humans. In this sleep stage muscle activity is suppressed to avoid dream-enacting; indeed it is in this period during which one can experience vivid dreams or story-like dreams (Scammel et al., 2017).
Unlike REM sleep, during NREM sleep the animal is mainly unconscious or has bland thoughts and eyes movements are rather roving. NREM 1 and 2 are the light phases of sleep, which means that during these stages an animal can be awakened more easily. NREM1 is the lightest phase of sleep and shows theta activity and a loss of alpha activity (8 to 13 Hz), which was present previously during the drowsy wakefulness state (Carley and Farabi, 2016). NREM2 sleep is characterized by the presence of single slow waves called K-complexes, whereas NREM3 or slow-wave sleep shows high amplitude waves with a peak frequency of less than 1 Hz (Canto et al., 2017). These latter slow waves are probably generated by the cortical activity and reflect the synchronous hyperpolarization and depolarization with irregular firing of a big neuronal ensemble (Steriade and Amizca, 1993). These stages may be crucial for memory consolidation (Klinzing et al., 2019). NREM2 and NREM3 share in common the presence of spindles, short burst of activity in the range of 10 to 15 Hz, which may well facilitate memory formation and learning (Piantoni et al., 2016; Ulrich, 2016). They probably reflect the interaction between Gabaergic cells located in the reticular nucleus of the thalamus and glutamatergic thalamocortical cells (Piantoni et al., 2016).

Most of the sleep research has only focused on the interaction between cerebral cortex and subcortical structures ignoring the contribution of the olivo-cerebellar circuit. This is surprising, because there is a strong connectivity between the cerebellum and the cerebral cortex via the thalamus, the inferior olive (IO), mesodiencephalic junction - one of the main excitatory inputs of the IO - and the pons (De Zeeuw et al., 2008). Moreover, changes in the cerebellar structure such as decreased gray matter volume are related to chronic insomnia (Del Rosso and Hoque., 2014) and spinocerebellar ataxia can lead to sleep disorders (Pedroso at al., 2011; Sonni et al., 2014). Furthermore, cerebellar cortex activity of cats has also shown to be modulated by different sleep stages where simple and complex spikes activity are enhanced during NREM sleep and decreased during REM sleep (Hobson and McCarley., 1972). Similar observations apply to the cerebellar nuclei where firing frequency is increased during NREM sleep (Palmer, 1979). However, it is not known how spatiotemporal patterns of complex and simple spike activity in the cerebellar cortex change across the various sleep stages.
1.10 Conclusions

The inferior olive has evolved as a unique nucleus in the brain with neurons that show extensive electrotonic coupling and a propensity to oscillate. Most, if not all, their dendritic spines receive a combined excitatory and inhibitory input, by which the spatial configuration of ensembles of activated olivary neurons can be shaped and by which the onset of the oscillatory activity can be determined and reset. Through its climbing fibers, the inferior olive exerts a prominent impact on cerebellar output and thereby on motor behavior; it does so both acutely by pacing the motor commands and more long-term by modifying the weights of many different types of synapses in the cerebellar cortex in a synergistic fashion. As a consequence, deficits in olivary function can contribute to a plethora of syndromes, varying from simple tremors up to complex disorders like dystonia and autism.

1.11 Scopes of the thesis

In this thesis I investigate several aspects of the inferior olive (IO) that are crucial to understand how this brainstem structure processes information and controls motor learning. The topics studied in this thesis are the anatomical organization of the IO network, the temporal interactions of postsynaptic responses to excitatory and inhibitory inputs and their impact on subthreshold oscillations, spike output and motor learning, as well as the changes in the spatiotemporal patterns of complex spikes during sleep.

Previous studies have proposed that IO neurons can be divided into two types based on their dendritic morphology: straight neurons, with long dendrites that radiate away from the soma, and curly neurons, with dendrites coiling back to the soma with the appearance of spirals (Ruigrok et al.1990). These two types are supposed to be functionally distinct and spatially segregated in different IO subnuclei (Urbano et al., 2006). However, this classification is based on historical observations of a limited number of cells, making it difficult to judge if IO neurons really exist as two morphological cell types or whether perhaps there is a smooth continuum of morphologies. In Chapter 2, I study if the latter hypothesis holds by making 3D reconstructions of an extensive data set of labeled IO neurons spanning several IO subnuclei to obtain detailed and quantitative measures of IO dendrite morphology. One of these parameters, the directionality of their dendrites allow us to address a topic that is still under debate, i.e. whether IO neurons are grouped into anatomical clusters.
As a result of the close proximity of inhibitory and excitatory synaptic inputs on electrically coupled dendritic spines of IO neurons, their local temporal interaction is critical in affecting how IO neurons generate output (De Zeeuw et al., 1995; Segev and Parnas, 1983). A majority of IO neurons display subthreshold oscillations (STOs) that are modulated by both excitatory or inhibitory synaptic inputs (Turecek et al., 2014; Leefler et al., 2013). A subset of IO neurons do not generate STOs, but some of them, the so-called ‘conditional oscillators’ still respond to input with transient STOs following injection of a hyperpolarizing current pulse (Bazzigaluppi et al., 2016). Despite the unique features exhibited by IO neurons, there are no studies that have addressed how the presentation of the inhibitory and excitatory inputs they receive interact. For this reason, in Chapter 3 I investigate how inhibitory and excitatory inputs presented at different intervals affect the amplitude and phase of the STOs as well as the spike probability of IO neurons. Likewise, I investigate the impact of excitatory and inhibitory inputs on conditional oscillators. I use electrophysiological recordings in vitro along with optogenetic stimulation in order to selectively stimulate inhibitory and excitatory afferents coming from the CN and MDJ, respectively. Furthermore, I show how stimulating CN and MDJ afferents to the IO at specific intervals can have great consequences for motor learning using a paradigm of reflexive whisker protraction (Romano et al., 2018).

Despite the fact that the cerebellum and cerebral cortex are reciprocally connected (De Zeeuw et al., 2008) and that cerebellar loss-of-function can cause sleep disorders (Pedroso et al., 2011; Sonni et al., 2014), relatively little is known about the engagement of the olivo-cerebellar circuit during sleep. For this reason, in Chapter 4 I focus on the role of the inferior olive during sleep. By using miniscope recordings along with EEG and EMG recordings, I describe sleep stage specific modulation of complex spike activity in ensembles spanning many neighboring Purkinje cells.

1.13 References

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Chapter 2

Variability and directionality of inferior olive neuron dendrites revealed by detailed 3D characterization of an extensive morphological library


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Abstract

The inferior olive (IO) is an evolutionarily conserved brain stem structure and its output activity plays a major role in the cerebellar computation necessary for controlling the temporal accuracy of motor behavior. The precise timing and synchronization of IO network activity has been attributed to the dendro-dendritic gap junctions mediating electrical coupling within the IO nucleus. Thus, the dendritic morphology and spatial arrangement of IO neurons governs how synchronized activity emerges in this nucleus. To date, IO neuron structural properties have been characterized in few studies and with small numbers of neurons; these investigations have described IO neurons as belonging to two morphologically distinct types, “curly” and “straight”. In this work we collect a large number of individual IO neuron morphologies visualized using different labeling techniques and present a thorough examination of their morphological properties and spatial arrangement within the olivary neuropil. Our results show that the extensive heterogeneity in IO neuron dendritic morphologies occupies a continuous range between the classically described “curly” and “straight” types, and that this continuum is well represented by a relatively simple measure of “straightness”. Furthermore, we find that IO neuron dendritic trees are often directionally oriented. Combined with an examination of cell body density distributions and dendritic orientation of adjacent IO neurons, our results suggest that the IO network may be organized into groups of densely coupled neurons interspersed with areas of weaker coupling.
Introduction

The inferior olive (IO) provides the sole source of climbing fibers that evoke potent complex spikes in cerebellar Purkinje neurons (PNs), and thereby plays a critical role in controlling cerebellar function (Azizi, 2007; Jacobson et al., 2008; Ito, 2013; Llinás, 2014; Ten Brinke et al., 2018; Streng et al., 2018). The neurons within the IO are exclusively interconnected by dendro-dendritic gap-junctions (GJs; Sotelo et al., 1974; De Zeeuw et al., 1989; Placantonakis et al., 2004). The GJ-mediated signaling shapes sub-threshold oscillations (STOs) and spike timing among coupled IO neurons (De Zeeuw et al., 1998, 2003; Long et al., 2002; Blenkinsop and Lang, 2006; Jacobson et al., 2009; Kitazawa and Wolpert, 2005; Welsh et al., 1995; Lampl and Yarom 1997; Loewenstein et al., 2001; Manor et al., 1997; Placantonakis et al., 2006; Torben-Nielsen et al., 2012). Thus, the dendritic layout which determines connectivity within the nucleus is at the core of the spatiotemporal patterning of IO network activity.

Morphologically, IO neurons have historically been classified into “curly” and “straight” types (Ramón y Cajal, 1995; Scheibel and Scheibel, 1955; Foster and Peterson, 1986). The “curly” type is characterized by complex curled dendritic trees that branch and bend profusely within a very small volume of the neuropil around the soma. In contrast, the “straight” neurons have dendrites sparsely occupying a much larger volume. As GJs are overwhelmingly located on the IO neuron’s dendrites, the different dendritic shapes must lead to different connectivity profiles. Nevertheless, relatively little is known about the structural properties of IO neurons, and quantitative descriptions of different IO neuron morphologies are lacking. One reason for this is that anatomical investigations have long been limited to the examination of two-dimensional projections of neuronal structures. Using more advanced labeling techniques and detailed confocal imaging, we can now fully reconstruct and accurately quantify complex dendritic morphologies in 3D.

In this work we constructed an extensive library of IO neuron morphologies and give a detailed quantitative description of the variability in their morphological properties and the spatial arrangement of their dendritic arbors. Our results reveal that dendritic tree shapes span a continuum between the classically described “curly” and “straight” IO neuron morphologies and that dendritic trees are often directional. These findings have important implications for our understanding of connectivity in the IO network.
Materials and Methods

All animal experimental procedures were approved by the Hebrew University’s Animal Care and Use Committee, and the animal experiment committee of the Royal Netherlands Academy of Arts and Sciences (DEC-KNAW) which follows the European guidelines for the care and use of laboratory animals (Council Directive 86/6009/EEC).

Single neuron labeling

Sparse viral labeling of neurons was achieved by injecting a cre-dependent fluorophore-expressing virus mixed with a highly diluted cre-expression virus into the IO of juvenile or adult mice (6 weeks to 4 months old; all animals were at least 10 weeks old after the viral transfection period). The Cre-expression virus (AAV9.CamKIIa.cre, Penn Vector Core) was diluted (1:3000, 1:3500 or 1:4000) with saline in multiple steps, taking care to mix well at each step. The diluted viral suspension was then mixed 1:1 with a loxed GFP-expression virus (AAV9.CAG.flex.eGFP.bGH, Penn Vector Core). Mice were anaesthetized using a mixture of ketamine and xylazine (100 mg/kg and 20 mg/kg) and head-fixed into a stereotaxic device. The skull over the IO was exposed through a single incision into the skin and scraping away some of the soft tissue covering the area. A single craniotomy, ~ 2 mm wide was then drilled in the skull, centered around the midline just behind the posterior suture. ~500 nL of the mixture of Cre and lox viruses was then slowly injected at 6.5 mm depth, bilaterally to the midline using air pressure. After 4–6 weeks incubation time, mice were deeply anesthetized with pentobarbital and fixed through transcardiac perfusion with 4% para-formaldehyde (PFA) in phosphate-buffered saline (PBS), and brains were post-fixed overnight in the same solution. The brains were then washed in PBS and the brain stem cut into 150 µm-thick sections in coronal or sagittal plane using a Leica VT1000S or Leica VT1200S vibratome (Leica Biosystems, Germany) and subsequently mounted with prolong gold antifade mounting medium (RI 1.47; Thermo Fisher Scientific, MA) under #1.5 coverslip glass (Thermo Fisher).

Dye-filling of IO neurons was achieved during in vitro patch-clamp experiments on acute brainstem slices (performed by N.V. or S.L., for the purpose of other projects). Alexa-labeling of IO neurons was done in 200 µm-thick coronal brainstem slices prepared following the “hot” procedure (Huang and Uusisaari 2013; Ankri et al. 2014); in brief, adult mice (3–12 months old) of either sex were deeply anesthetized with pentobarbital, decapitated and their brain stem extracted from the skull while continuously kept in oxygenated artificial cerebrospinal fluid (ACSF) warmed
to a temperature of 30–35 °C. The ACSF was composed of (in mM) 126 NaCl, 3 KCl, 1.2 kH2PO4, 26 NaHCO3, 10 glucose, 2.4 CaCl2, 1.3 MgSO4 and continuously bubbled with carbogen (95% O2/5% CO2). Slices were then incubated at 35 °C for at least half an hour and then at room temperature. Fluorescent labeling of IO neurons was achieved by adding 20–50 µM Alexa-594 or Alexa-488 Hydrazide (Thermo Fisher Scientific, MA) to a patch pipette solution containing (in mM) 4 NaCl, 140 K-gluconate, 10 HEPES, 0.01 EGTA, 0.001 CaCl2 and 4 Mg-ATP (pH adjusted with KOH to 7.2–7.3, osmolality 290–310 mOsm) during whole-cell recordings performed at room temperature. Whole-cell configuration was maintained for at least half an hour and slices were incubated for an additional half hour after recordings were terminated to allow dye to spread through dendrites. Slices were subsequently preserved by fixation in 0.1 M PBS containing 1% PFA for 30 min and then washed and stored in PBS until mounted with Vectashield (RI 1.45; Vector laboratories, CA) and coverslipped. The biocytin-labeling experiments differed on several points: juvenile (4–8-week-old) mice of either sex were anesthetized with isoflurane, and their brain stem extracted and sliced in ice-cold ACSF. Slices were cut sagittally and then incubated at 35 °C for half an hour and at room temperature for at least half an hour, before being transferred into a recording chamber maintained at ~ 32 °C. The ACSF had the same composition as used in the Alexa-labeling experiments, as was the patch pipette solution except in that it contained 5 EGTA and 0.5 CaCl2, and 0.1–0.5% (w/v) biocytin (Sigma) was added. After recordings were completed, slices containing biocytin-filled neurons were fixed in 0.1 M PBS containing 4% PFA overnight at 4 °C. Slices were then washed three times (0.1 M PBS, 10 min at 4 °C), incubated with Alexa Fluor 594-conjugated streptavidin (Life technologies, 2 mg/ml) and 0.6% Triton X-100 (Sigma) in 0.1 M PBS (4 h at 4 °C), washed three times (0.1 M PBS, 10 min at 4 °C), mounted with Dako glycergel fluorescence mounting medium (RI 1.47–1.50; Dako) and coverslipped.

In our examinations of hundreds of IO neurons in both sagittal and coronal brain stem slices we noted no overt differences in the morphologies’ orientations relative to the confocal z-axis, or any tendency for “curlier” or “straighter” neurons to be more prevalent in juvenile or adult mice; regardless of the experimental conditions, labeled morphologies exhibited extensive heterogeneity covering the full range from “curly” to “straight”. We therefore chose to consider all the available material together and select only the most complete morphologies (see below) for inclusion in our library.
Recovery and reconstruction of morphologies

The labelled material was examined and imaged using confocal microscopy (Leica SP5 and SP8, Leica Microsystems, Germany; Zeiss LSM 510, 710, 780 and 880, Zeiss, Germany). Each mounted section was first scanned with low magnification (10×) and a maximal projection of the slice was created to record the position of the neurons within the IO volume and select candidates for high-resolution stack acquisition.

High-magnification confocal image stacks were obtained with either 40 or 63× plan-Apochromat objectives (NA 1.25–1.3) as were available at each confocal system, so that resolution ranged from 0.11 to 0.38 µm/pixel in XY plane. The sections were oversampled in z-dimension (ranging 0.1–0.3 µm/z-step) to support correction of the z-axis values due to shrinkage factor. The morphologies were manually reconstructed using the Vaa3D software (Peng et al., 2010), taking care that the reconstructions end up as sorted trees with a single root. The shrinkage was estimated from the thickness of the mounted section (as measured by confocal visualization) relative to the fresh section and the final reconstructions were expanded in z-dimension to account for the shrinkage (ranging 1.5–3×).

To ensure that the overall dendritic shape of the morphologies in our library was not distorted, morphologies were carefully selected for inclusion based on the completeness of their 3D reconstruction. Morphologies that appeared skewed, due to optical or physical distortions, were discarded from analysis. Distal and/or very thin dendrites were occasionally difficult to reconstruct in entirety due to decreasing signal/noise ratio, and reconstructions were discarded if multiple disconnected fine branches could be observed around a reconstruction’s dendrite tips in the confocal image stack. We also kept track of the number dendrite tips occurring at the slice surface counting these as “cut tips” and discarded any morphologies that had more than half of tips cut, or that had one or more proximal dendrites cut at < 50 µm path length. Out of the hundreds of neurons examined in confocal image stacks, ~ 150 morphologies were reconstructed, and a total of 36, 27 and 29 morphologies were selected for the viral, Alexa- and biocytin-labeled datasets, respectively. The selected morphologies will be submitted to NeuroMorpho.org.

Quantification of morphological properties

The included morphologies were first inspected by the authors and subjectively labeled as being either “straight”, “curly” or “ambiguous” (18, 44 and 30 out of 92 morphologies, respectively). Subsequently, the 25 parameters, covering both “within-tree” and “whole-tree” variables (Uylings
and van Pelt, 2002) were obtained as extracted by Vaa3D or by custom scripts in MatLab and btmorph (Torben-Nielsen, 2014). The complete list of measurements, together with their definition, is provided in Table 1.

Three of the measures obtained by custom scripts were defined as follows. Soma-border distance was defined as the shortest distance of the reconstruction root node to the extrapolated convex hull of the full reconstruction. Soma-center of mass distance was defined as the distance of the root node to the average location of all the nodes of the reconstruction. Stem directionality was defined as the length of the vector obtained by sum of all vectors formed from reconstruction root node to the first nodes of each dendrite, normalized by the number of stem vectors; in this way, a soma with one dendrite would have a directionality value of 1, whereas a soma with dendrites stemming evenly around the cell body would approach directionality of 0.

**Statistical analyses**

Statistical analyses were performed in R (R Core Team, 2018; Wickam et al., 2017, 2018; Fox and Weisberg, 2011; Wickam, 2016; Revelle, 2018; Ogle, 2018; Peterson and Carl, 2018; Kassambara and Mundt, 2017; Venables and Ripley, 2002), unless stated otherwise. The assumption that data are sampled from a normal distribution was rejected for almost all measures based on the Shapiro–Wilk normality test (p < 0.1 in each data set for all measures except number of stems and average local bifurcation angle). Therefore, correlation estimates and p values were calculated using Spearman’s rank correlation test, and group-level comparisons in mean and variance were calculated using Welch’s ANOVA and Levene’s test for equality of variance, respectively.

For performing PCA on the morphometric data per dataset, the values of all morphometric features were scaled and centered to have zero mean and unit variance. We then applied K-means clustering into two clusters to the data as represented along the first three components of the decomposition (which resulted in nearly identical clustering as applying K-means clustering to the data represented along the first two PCs only).

On individual morphologies, PCA was performed in MatLab and applied to the x-, y-, z-coordinates of each point on the reconstruction without re-scaling the data since variance has the same units in each dimension of 3D space.
Variability and directionality of inferior olive neurons dendrites revealed by detailed 3D characterization of an extensive morphological library

Table 1 The morphometric measures used in the study

<table>
<thead>
<tr>
<th>Measure #</th>
<th>Name Description</th>
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<tbody>
<tr>
<td>1</td>
<td>Number of stems</td>
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<tr>
<td>2</td>
<td>Stem diameter—mean</td>
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<tr>
<td>3</td>
<td>Stem diameter—sum</td>
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<tr>
<td>4</td>
<td>Stem diameter—maximum</td>
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<td>5</td>
<td>Stem directionality</td>
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<tr>
<td>6</td>
<td>Dendrites—total length</td>
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<tr>
<td>7</td>
<td>Dendrite diameter—mean</td>
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<tr>
<td>8</td>
<td>Dendrites—longest single path length</td>
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<tr>
<td>9</td>
<td>Number of bifurcations</td>
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<td>10</td>
<td>Local bifurcation angle—mean</td>
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<tr>
<td>11</td>
<td>Remote bifurcation angle—mean</td>
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<td>Number of branches</td>
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<td>13</td>
<td>Branch order—maximum</td>
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<td>Number of tips</td>
</tr>
<tr>
<td>15</td>
<td>Number of cut tips</td>
</tr>
<tr>
<td>16</td>
<td>Number of tips—total</td>
</tr>
<tr>
<td>17</td>
<td>Soma area</td>
</tr>
<tr>
<td>18</td>
<td>Hull volume</td>
</tr>
<tr>
<td>19</td>
<td>Soma-to-hull distance</td>
</tr>
<tr>
<td>20</td>
<td>Soma-to-center of gravity distance</td>
</tr>
<tr>
<td>21</td>
<td>Reach—maximum</td>
</tr>
<tr>
<td>22</td>
<td>Straightness</td>
</tr>
<tr>
<td>23</td>
<td>Mean contraction</td>
</tr>
<tr>
<td>24</td>
<td>Hausdorff dimension</td>
</tr>
<tr>
<td>25</td>
<td>Mean fragmentation</td>
</tr>
</tbody>
</table>

Numbers in the first column are used to reference to the measures in Fig. 3c. “Compartment” refers to the variable-length nodes of reconstruction within which the dendrite thickness and shape is uniform.

Reconstruction and analysis of IO soma distribution

For analyzing the spatial distribution and clustering of IO somata, we used two mice obtained from a PDX-cre (Song et al. 2010) X Ai9 (Madisen et al. 2010) mating, resulting in strong expression of tdTomato in IO neurons. The mice were perfusion-fixed and their brains sectioned and mounted as described above, and all somata on one hemisphere in both animals were reconstructed manually for each subnucleus using Fiji software (Schindelin et al. 2012).

The density distribution of IO somata was estimated by 3D binning the somata in voxels of 10 µm³, and subsequently applying an isotropic 3D-gaussian kernel to account for binning artifacts. The standard deviation parameter utilized for the 3D kernel was 4. To test the null-hypothesis that
the density of somata was isotropic, the distribution of voxel densities of the data was compared to a volume bootstrapping the somata densities assuming a uniform density. Thus, the bootstrap was constructed by drawing somata counts from a uniform distribution within the bounded volume formed by the non-zero somata voxels. The density per voxel in the uniform distribution is simply the total somata count in the actual subnucleus divided by the total volume included in non-zero voxels. A two-sample Kolmogorov–Smirnov test was used to compare the distributions.

Presence of local soma clusters was examined using the DBSCAN algorithm (Ram et al. 2010) implemented in MatLab. This algorithm assigns cluster membership to any group of at least N somata where any one soma within the cluster is at most D µm removed from another cluster member. Clustering was explored for values of D ranging from 15 to 100 µm and values of N ranging from 3 to 20.

Results

Variability of IO dendritic morphology

We used a sparse viral labeling technique to induce strong fluorescent labeling in a small number of neurons in a given IO (Fig. 1a, b) as well as IO neurons that were labeled using either Alexa (488 or 594) or Biocytin during in vitro patch-clamp experiments (see “Methods”). Confocal image stacks were acquired from the labeled tissue and a total of 90 manually reconstructed morphologies were analyzed (see “Methods”). Except for two neurons from the dorsal cap of Kooy (DCK)-subnucleus (which has been shown to be phenotypically distinct from the main IO subnuclei; Urbano et al., 2006), neurons from all IO subnuclei were included in our analyses. The different methods of neuronal labeling lead to some variability in the quality of the confocal image stacks; specifically, the viral-labeled material was of higher quality regarding the ratio between signal strength and background noise. Nonetheless, qualitative differences between morphologies reconstructed from the differently labeled materials were not immediately apparent (see Fig. 1c–f). To exemplify morphologies from each of the three data sets, Fig. 1c–e show maximal Z-projections of confocal stacks from viral-, Alexa- and biocytin-labeled data, respectively; the corresponding reconstructed morphologies are shown in the left column in Fig. 1f. Additional examples of morphologies reconstructed from the three datasets are shown in the middle and right columns of Fig. 1f, indicating a progression from “very curly” (left column) to “very straight” (right column) morphologies in each of the three data sets.
Examining the full morphological library, both “clearly curly” (30 out of 90; Fig. 1f, left column) and “clearly straight” morphologies (16 out of 90; Fig. 1f, right column) could be subjectively identified. However, categorical distinction was ambiguous, as a significant portion of the morphologies could not be easily classified (44 out of 90; examples are shown in Fig. 1f, middle column).

It has been previously considered that IO neurons with subjectively straight and curly appearance would be anatomically segregated into different parts of the olivary nucleus (Scheibel and Scheibel 1955; Ruigrok et al. 1990). However, we found that “curly” and “straight” neurons could be found within each of the main IO subnuclei. This is demonstrated in Fig. 1g, where morphologies from different sources are shown at their anatomical locations approximated at two different levels of the anterio-posterior axis (see “Methods”). These results demonstrate extensive morphological heterogeneity in IO neuron dendritic morphologies across all subdivisions of the nucleus.

**Quantitative analyses reveal a continuum in neuronal morphology**

While the ambiguity of dendritic morphologies seemed to rule out clear classification, we investigated whether features distinguishing between IO cell classes could be revealed using a quantitative approach. To this end we measured 25 morphometric parameters from each of the reconstructed neurons (see Table 1 and “Methods” for measurement definitions). The measured parameters included basic ones such as the number of dendrite stems, number of branches, dendritic path length and maximal reach (see Fig. 2a). We also measured a number of parameters aimed at describing the overall shape of the dendritic trees; most prominent among these (as explained below) is “straightness”, which was defined as maximal reach divided by the longest single dendrite path length.

Examining the distributions of morphological parameter values, we noted that there are some differences between the three groups of neurons reconstructed from material obtained using different labeling methods. The outcome of statistical tests performed to assess differences across groups are summarized in Table 2 and show that group means were significantly different on almost all measures. Thus, we performed quantitative analyses of morphometric measures on each of the three data sets separately.
Fig. 1 Fluorescent labeling of IO neurons reveals complex projection of a 100 µm-thick confocal image stack of a coronal brain stem slice labeled sparsely by viral transfection (1:3500 dilution of cre-expression virus). IO borders are marked with a white dotted line. Scale bar 100 µm. b as in a, but with 1:4000 dilution. exemplifying “very curly” IO c), Alexa-594 (d) or biocytin (e) staining. Scale bar 20 µm. f Examples of reconstructed morphologies from the three data sets (as in c–e) ranging from “very curly” (left column, same examples as shown in c–e) to “straight” (rightmost column). Note that the scale varies between subpanels and perspective; scale bars represent 20 µm in the xy plane. Encircled numbers denote reconstruction IDs as referred to in the text. g A composite drawing showing the shape and orientation of a selection of the morphologies within the volume of the IO. Note the presence of curly and straight neurons in all subnuclei (abbreviations: PO principal olive, DAO dorsal accessory olive, MAO medial accessory olive).
In an ideal and simple case, a distinction between groups is implied by a clear bi- or multimodal distribution in one or more features. However, we observed no immediately apparent groupings in the distributions of any of the measured parameters. To exemplify the variability in morphological parameter distributions, histograms of maximal reach, number of stems, number of branches, total dendrite length and straightness are shown in Fig. 2b–e for each data set as indicated in the legend, demonstrating that there are no clear multimodal distributions in any of the data sets. Nonetheless, it was evident that IO neurons do not form a single population with normally distributed inter-individual variability: as shown in Table 3, we found that in each data set, the null-hypothesis that data are sampled from a single normal distribution should be rejected for almost all measures except number of stems and local bifurcation angle. To enumerate the distributions of the measured morphological parameters, Table 4 displays the minimal, mean, median and maximal values of each parameter distribution in each data set. Taken together, while we should conclude that the observed morphological heterogeneity is unlikely to result from normally distributed inter-individual variability in a single neuronal population, we did not find any single morphometric parameter that would clearly distinguish between morphologically different classes of neurons.

We then asked which of the objectively defined morphological parameters could best be used to describe the subjectively perceived range of variability from “curly” to “straight” by calculating the Spearman correlation between the subjectively assigned categories (curly, ambiguous and straight) and each of the measured parameters (Table 5, left side). Of all the different measures describing dendritic tree shape, straightness best corresponded to our subjective categorization across all three datasets; therefore, we chose this measure as an objective representation of a neuron’s position along the curly–straight continuum. Notably, besides measures directly aimed at describing the dendritic tree shape we found that in each data set at least one other measure was also correlated with the subjectively assigned classes (see Table 5); for example, in all three datasets the number of dendrite tips was significantly correlated with subjective class such that the “straight” neurons had the fewest tips. Similarly, these correlations could be found with the straightness-parameter instead of subjective class; for example, the number of stems and branches are strongly correlated both to subjective class and straightness in the viral- and Alexa-labeled datasets (see Table 5). Correlation statistics between straightness and all other morphometric parameters are reported on the right side in Table 5, and as examples, correlations of straightness to the number of stems, branches and total length are shown in Fig. 2f–h for the data obtained from viral-labeled morphologies; correlations between these parameters in the other two data sets follow the same trends and are shown in Supplementary Fig. 1.
Taking another approach to assessing which properties might best distinguish “curly” from “straight” morphologies we performed principal component analysis (PCA) and K-means clustering on the quantified morphological data (see “Methods”). If distinct morphological classes could be defined based on a combination of parameters, then dimensionality reduction of the data through PCA would result in a gap between groups of data points belonging to different classes. Due to the previously mentioned quantitative differences between the three data sets, the PCA-decomposition and K-means clustering results also vary quantitatively across data sets; nonetheless, the obtained results were qualitatively similar in each case, and are shown for viral-labeled data in Fig. 3 while the results of the same analyses performed on the patch-filled data sets are provided in Supplementary Fig. 2.

As shown in Fig. 3a, we found that a clear gap between groups of data points did not become apparent in the distribution of the data along the first two principal components (PC1 and PC2) of the PCA-decomposed morphometric data. Groups was defined almost exclusively along PC1; this is shown in Fig. 3a by the almost vertical grey dashed line marking the border between the two clusters. More than that, PC1 appeared to follow our subjective classification of the IO neuron morphological types; this is apparent in Fig. 3a in that most morphologies that were subjectively classified as being “curly” are found on the left side, while subjectively “straight” morphologies are all found on the right and “ambiguous” morphologies are mostly in between. Thus, it seems that PC1 closely follows the curly-to-straight continuum, and that “curly” and “straight” are indeed relevant descriptors of the morphological variability among IO neurons, i.e., a classification based on features unrelated to morphological “straightness” was not found. This idea was also reflected in the strong and significant correlation between PC1 and straightness (Fig. 3b, Rho = 0.84, p < 0.0001).

Our quantified morphometric data set contains multiple parameters aimed at describing the overall shape of dendritic trees; such parameters are correlated with each other by definition, and this may artificially cause the main principal component to follow measures of dendritic tree shape. However, as depicted in Fig. 3c where the relative contribution of each measure to the first two PCs is displayed on a scale from 0 to 1 for the viral-labeled morphologies, measures such as the number of stems and branches also contributed strongly to the separation along PC1. This shows that properties not directly describing dendritic tree shape also vary systematically with the measured straightness of the morphologies, and further strengthens our confidence that the curly–straight axis is the most relevant descriptor of morphological variability in the IO neuron population.
Fig. 2 Morphological properties of IO neurons quantified. a Schematic illustration of some of the basic morphological parameters used to characterize the dendritic morphologies. Maximal dendritic reach is defined as the furthest reach of the dendritic tree away from the soma; the longest single path length is defined as the longest soma-to-tip path length on a dendritic tree; and straightness is defined as the maximal dendritic reach divided by the longest single dendrite path length. For a list of all morphometric parameters and their definitions, see Table 1. Distributions of maximal dendritic reach (b), number of dendrite stems emerging from the soma (c), number of branch points on the dendritic trees (d) and straightness (e) in each of the three data sets; shadings refer to morphologies recovered using different labeling method as indicated. Distributions of number of dendrite stems (f), number of branch points (g) and total dendritic length (h) with respect to straightness in the viral-labeled data; the same distributions in the Alexa- and biotin-labeled data sets are displayed in Supplementary Fig. 1. Reported correlation statistics represent the strength and direction (Rho) and significance level (p) calculated using Spearman’s rank correlation test (see “Methods”). Correlations between straightness and all other morphological measures are reported in the right half of Table 2 for each of the three data sets.
Table 2: Significance of group level differences in mean (left column) and variance (right column)

<table>
<thead>
<tr>
<th>Measure names</th>
<th>Welch’s ANOVA</th>
<th>Levene’s test for equality of variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of stems</td>
<td>&lt; 0.0001</td>
<td>0.2562</td>
</tr>
<tr>
<td>Stem diameter—mean</td>
<td>0.0129</td>
<td>0.7094</td>
</tr>
<tr>
<td>Stem diameter—sum</td>
<td>&lt; 0.0001</td>
<td>0.0071</td>
</tr>
<tr>
<td>Stem diameter—maximum</td>
<td>0.0004</td>
<td>0.4644</td>
</tr>
<tr>
<td>Stem directionality</td>
<td>0.5522</td>
<td>0.8356</td>
</tr>
<tr>
<td>Dendrites—total length</td>
<td>&lt; 0.0001</td>
<td>0.0743</td>
</tr>
<tr>
<td>Dendrite diameter—mean</td>
<td>0.0005</td>
<td>0.6540</td>
</tr>
<tr>
<td>Dendrites—longest single path length</td>
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<td>0.3177</td>
</tr>
<tr>
<td>Number of bifurcations</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Local bifurcation angle—mean</td>
<td>0.2153</td>
<td>0.1322</td>
</tr>
<tr>
<td>Remote bifurcation angle—mean</td>
<td>0.7173</td>
<td>0.1410</td>
</tr>
<tr>
<td>Number of branches</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Branch order—maximum</td>
<td>0.0002</td>
<td>0.0295</td>
</tr>
<tr>
<td>Number of tips</td>
<td>0.0065</td>
<td>0.0460</td>
</tr>
<tr>
<td>Number of cut tips</td>
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<td>0.1199</td>
</tr>
<tr>
<td>Number of tips—total</td>
<td>&lt; 0.0001</td>
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</tr>
<tr>
<td>Soma area</td>
<td>&lt; 0.0001</td>
<td>0.0279</td>
</tr>
<tr>
<td>Hull volume</td>
<td>0.0152</td>
<td>0.3412</td>
</tr>
<tr>
<td>Soma-to-hull distance</td>
<td>0.0076</td>
<td>0.0862</td>
</tr>
<tr>
<td>Soma-to-center of gravity distance</td>
<td>0.0138</td>
<td>0.2072</td>
</tr>
<tr>
<td>Reach—maximum</td>
<td>0.0522</td>
<td>0.3715</td>
</tr>
<tr>
<td>Straightness</td>
<td>0.0543</td>
<td>0.0755</td>
</tr>
<tr>
<td>Mean contraction</td>
<td>&lt; 0.0001</td>
<td>0.2088</td>
</tr>
<tr>
<td>Hausdorff dimension</td>
<td>0.0002</td>
<td>0.0123</td>
</tr>
<tr>
<td>Mean fragmentation</td>
<td>0.0016</td>
<td>0.3353</td>
</tr>
</tbody>
</table>

Values reflect p levels calculated using Welch’s ANOVA (left) and Levene’s test (right), for each of the morphological parameters as indicated in each row. Values highlighted in bold indicate measures on which morphological parameter distributions across the three data sets were significantly different in their mean/variance, respectively.

Taken together, the results described so far do not support the idea that IO neurons could or should be classified into subtypes based on their morphological appearance. Furthermore, these results indicate that a description of the morphological variability based on the simple straightness-measure is as informative as a description based on a decomposition of the quantified data.
Variability and directionality of inferior olive neurons dendrites revealed by detailed 3D characterization of an extensive morphological library

<table>
<thead>
<tr>
<th>Measure names</th>
<th>Viral-labeled</th>
<th>Alexa-labeled</th>
<th>Biotin-labeled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of stems</td>
<td>0.1355</td>
<td>0.1881</td>
<td>0.1440</td>
</tr>
<tr>
<td>Stem diameter—mean</td>
<td>0.1712</td>
<td><strong>0.0078</strong></td>
<td>0.0366</td>
</tr>
<tr>
<td>Stem diameter—sum</td>
<td>0.8794</td>
<td>0.5099</td>
<td><strong>0.0007</strong></td>
</tr>
<tr>
<td>Stem diameter—maximum</td>
<td><strong>0.0068</strong></td>
<td><strong>0.0097</strong></td>
<td>0.0298</td>
</tr>
<tr>
<td>Stem directionality</td>
<td>0.1491</td>
<td>0.0814</td>
<td>0.6311</td>
</tr>
<tr>
<td>Dendrites—total length</td>
<td><strong>0.0003</strong></td>
<td>0.0689</td>
<td>0.1586</td>
</tr>
<tr>
<td>Dendrite diameter—mean</td>
<td>0.2036</td>
<td>0.6204</td>
<td><strong>0.0056</strong></td>
</tr>
<tr>
<td>Dendrites—longest single path length</td>
<td><strong>0.0010</strong></td>
<td>0.2313</td>
<td><strong>0.0009</strong></td>
</tr>
<tr>
<td>Number of bifurcations</td>
<td><strong>0.0012</strong></td>
<td>0.1020</td>
<td><strong>0.0007</strong></td>
</tr>
<tr>
<td>Local bifurcation angle—mean</td>
<td>0.2128</td>
<td>0.7908</td>
<td>0.5166</td>
</tr>
<tr>
<td>Remote bifurcation angle—mean</td>
<td>0.2437</td>
<td><strong>0.0913</strong></td>
<td>0.7735</td>
</tr>
<tr>
<td>Number of branches</td>
<td><strong>0.0015</strong></td>
<td>0.1663</td>
<td><strong>0.0010</strong></td>
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<td>Branch order—maximum</td>
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<td>0.0066</td>
<td><strong>0.0052</strong></td>
</tr>
<tr>
<td>Number of tips</td>
<td>0.0892</td>
<td>0.0368</td>
<td><strong>0.0040</strong></td>
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<tr>
<td>Number of cut tips</td>
<td>0.0826</td>
<td>0.2140</td>
<td><strong>0.0004</strong></td>
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<tr>
<td>Number of tips—total</td>
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<td>0.1568</td>
<td><strong>0.0003</strong></td>
</tr>
<tr>
<td>Soma area</td>
<td>0.8722</td>
<td><strong>0.0006</strong></td>
<td><strong>0.0408</strong></td>
</tr>
<tr>
<td>Hull volume</td>
<td>&lt; <strong>0.0001</strong></td>
<td>0.0059</td>
<td>&lt; <strong>0.0001</strong></td>
</tr>
<tr>
<td>Soma-to-hull distance</td>
<td><strong>0.0043</strong></td>
<td><strong>0.0039</strong></td>
<td><strong>0.0011</strong></td>
</tr>
<tr>
<td>Soma-to-center of gravity distance</td>
<td>&lt; <strong>0.0001</strong></td>
<td>&lt; <strong>0.0001</strong></td>
<td>0.0613</td>
</tr>
<tr>
<td>Reach—maximum</td>
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<td><strong>0.0099</strong></td>
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<td>0.0892</td>
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<td>0.5955</td>
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<td>Mean fragmentation</td>
<td><strong>0.0101</strong></td>
<td>0.6274</td>
<td><strong>0.0132</strong></td>
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</table>

Table 3 Likelihoods of normality

Values reflect p levels calculated using the Shapiro–Wilk normality test for each measure as indicated in each row, for each of the three data sets as indicated on the top of each column. Distributions that are unlikely to reflect a normal distribution are highlighted in italics (p < 0.1) and bold (p < 0.05).
Table 4. Minimal, median, mean, and maximal values of each morphological parameter distribution as indicated in each row, for each of the three data sets as indicated above the columns.

<table>
<thead>
<tr>
<th>Subjective class</th>
<th>Virus</th>
<th>Alexa</th>
<th>Biocytin</th>
</tr>
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<tbody>
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<td>6.03</td>
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<td>2.16</td>
<td>3.32</td>
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<td>Stem diameter—sum</td>
<td>6.11</td>
<td>12.7</td>
<td>4.6</td>
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<td>Stem diameter—maximum</td>
<td>1.46</td>
<td>3.5</td>
<td>4.67</td>
</tr>
<tr>
<td>Stem directionality</td>
<td>0.12</td>
<td>0.3665</td>
<td>0.865</td>
</tr>
<tr>
<td>Dendrites—total length</td>
<td>1260</td>
<td>2311</td>
<td>7006</td>
</tr>
<tr>
<td>Dendrite diameter—mean</td>
<td>0.81</td>
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<td>3.45</td>
</tr>
<tr>
<td>Dendrites—longest single path length</td>
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<td>906</td>
</tr>
<tr>
<td>Number of bifurcations</td>
<td>6</td>
<td>16.5</td>
<td>43</td>
</tr>
<tr>
<td>Local bifurcation angle—mean</td>
<td>61.4</td>
<td>74.95</td>
<td>89.4</td>
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<td>Remote bifurcation angle—mean</td>
<td>58.5</td>
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<td>Number of branches</td>
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<td>90</td>
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<td>13</td>
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<td>Number of tips</td>
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<td>11</td>
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<tr>
<td>Number of tips—total</td>
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<td>188</td>
<td>304</td>
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<td>Hull volume (10^4 μm^3)</td>
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<td>Soma-to-hull distance</td>
<td>0</td>
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<td>51.332</td>
</tr>
<tr>
<td>Soma-to-center of gravity distance</td>
<td>8.445</td>
<td>30.697</td>
<td>133.65</td>
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<td>Reach—maximum</td>
<td>65</td>
<td>121</td>
<td>139.53</td>
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<tr>
<td>Straightness</td>
<td>0.129</td>
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<td>Mean contraction</td>
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<td>Hausdorff dimension</td>
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<tr>
<td>Mean fragmentation</td>
<td>43.8</td>
<td>86.75</td>
<td>92.12</td>
</tr>
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</table>
Variability and directionality of inferior olive neurons dendrites revealed by detailed 3D characterization of an extensive morphological library

Table 5 Correlations between morphometric measures and subjective classification (left) and straightness (right)

<table>
<thead>
<tr>
<th>Measure names</th>
<th>Correlations to subjective classification</th>
<th>Correlations to straightness</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Viral-labeled</td>
<td>Alexa-labeled</td>
</tr>
<tr>
<td>Subjective class</td>
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<td></td>
</tr>
<tr>
<td>Number of stems</td>
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<td>0.067</td>
</tr>
<tr>
<td>Stem diameter—mean</td>
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<tr>
<td>Stem diameter—sum</td>
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<td>0.661</td>
</tr>
<tr>
<td>Stem diameter—maximum</td>
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<td>0.210</td>
</tr>
<tr>
<td>Stem directionality</td>
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<td>0.027</td>
</tr>
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<td>Dendrites—total length</td>
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<td>0.001</td>
</tr>
<tr>
<td>Dendrite diameter—mean</td>
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<td>Dendrites—longest single path length</td>
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<td>0.051</td>
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<td>Number of bifurcations</td>
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<tr>
<td>Number of branches</td>
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<tr>
<td>Branch order—maximum</td>
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<tr>
<td>Number of tips</td>
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<tr>
<td>Number of tips—total</td>
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<tr>
<td>Soma area</td>
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<tr>
<td>Reach—maximum</td>
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</tr>
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<td>Straightness</td>
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<td>Mean contraction</td>
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<td>&lt;0.0001</td>
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<tr>
<td>Mean fragmentation</td>
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</table>

Values reflect Spearman correlation statistics with each of the morphological parameters as indicated in each row, for each data set as indicated at the top of each column. Rho reflects the strength and direction of the correlation; p values highlighted in bold indicate measures that were significantly correlated with the subjective classification/straightness, respectively.
Fig. 3 Algorithmic classification does not reveal clearly separated clusters. Algorithmic classification shown as a scatter along the first two principal components (PCs) of separation for the viral-labeled data set. The grey dashed line marks the division into “curly” and “straight” groups as determined by a K-means algorithm; fill color represents subjective classification, as indicated. Note that the separation along the first principal component (PC1) appears to correspond to the subjective classification into morphological subtypes: subjectively “straight” neurons occupy the far-right side of the distribution while “ambiguous” and “curly” neurons are found in the middle and to the left. The slight mismatch between the subjective and algorithmic classification into “curly” and “straight” morphological types is another indication that seeking a quantitative justification for the subjective typification is futile. b Correlation of the main axis of separation to “straightness” in the viral-labeled data set; fill color represents subjective classification as indicated. c Relative contributions of the 25 morphometric parameters to the principal component separation in the viral-labeled data set. Numbers in circles correspond to the measures as listed in Table 1. The closer a parameter is to 1, the more it contributed to the separation in the PC space, in the direction indicated by its position within the unit circle; a parameter located at the origin did not contribute to the PC separation.
Non-isomorphic IO dendrite fields

Non-isomorphic, or “pyriform” IO neuron dendritic fields have been described as early as the anatomical work of Ramón y Cajal (first published between 1905 and 1911); however, it has been assumed that such directionality arises only in the proximity of borders of the IO or its different subnuclei and that IO neurons residing within the main IO volume have roughly spherical shapes with somata surrounded by dendrites on all sides (Ramón y Cajal, 1995; Scheibel and Scheibel, 1955). Contrary to this description we found that neurons with directionally extended dendritic trees were also regularly encountered at distances far (> 75 µm) removed from boundaries of IO subnuclei (see Fig. 1a, g). In the following paragraphs we present two descriptors of dendritic directionality in IO neurons, one pertaining to the distribution of dendrites within the 3D volume occupied by the neuron (Fig. 4a), and one pertaining to the location of the soma within the dendritic volume (Fig. 4b). As no more correlation statistics will be presented, data acquired using different labeling methods are shown overlaid in the same panel, using different symbols to mark the different data sets as indicated.

There are two distinct ways in which the dendritic arrangement of an individual IO neuron can be non-homogeneous. First, the neuron’s dendrites are not distributed evenly within a spherical volume. We quantified this by performing PCA on the x-, y-, z-coordinates of the dendritic tree of each individual morphology. The relative proportions of variance explained along each of the three principal components (PCs) of a decomposed morphology represent the “stretchedness” of the dendritic tree along the axes of 3D space; if dendrites are distributed evenly within a spherical volume, each PC would explain 33% of the variance. Figure 4a shows that a large portion of neurons occupy a highly uneven volume with the first PC explaining more than 60% of their ‘variance in space’, whereas very few neurons are even roughly spherically shaped. As the examples shown in Fig. 4d illustrate, there is a continuum of dendritic tree shapes ranging from spherical (orange, red, and pink examples) to ellipsoid (green) to conical (blue) and even flat (cyan) morphologies. Notably, while the “straightest” morphologies were almost always highly elongated (see positions of examples 7–9 from Fig. 1 and the examples marked with blue and cyan in Fig. 4d), very “curly” morphologies also tended to have elongated shapes (see Fig. 1, example 3 and the example marked with green in Fig. 4d).

Second, IO neuron somata are usually not located in the center of mass (CoM) of the dendrites; instead, we found that in more than 90% of all neurons the shortest distance between the soma and the border of the volume they occupy (soma-border (SB) distance) is smaller than the soma-CoM distance (Fig. 4b). This means that IO neuron dendrites do not uniformly occupy the space around the soma, but instead extend into a preferred direction. In contrast to the
directionality of the overall dendritic mass, the directionality in the positioning of dendrite stems on the soma is distributed randomly (Fig. 4c), so that directionality arises because dendrites take a sharp turn as they emerge from the soma and branch profusely only in the main direction.

Taken together, these results show that IO neuron dendritic trees are directional and indicate that this directionality is a relevant feature of the network’s architecture.

Fig. 4 IO neuron morphologies with spherical dendritic fields and somata in the center are rare. a Scatter plot showing the percentage of variance explained by the first two principal components of the decomposition of IO neuron morphologies. The schematic line drawing insets in the plot illustrate the transition from “spherical” to “directional” dendritic field shapes. Colored points correspond to examples shown in panel d, while numbered points refer to examples shown in Fig. 1f. Symbols correspond to morphologies from the three datasets as indicated. b Scatter plot showing the distance from the soma to the extrapolated border of the neuron’s dendritic field (soma-border (SB) distance) relative to the distance from the soma to the center of mass of the dendritic arbor (S-CoM distance). Schematic line drawings illustrate the transition from “eccentric” to “centered” somata within an idealized, ovaloid dendritic field shape. Dotted line depicts unity, highlighting that the majority of neurons have somata much closer to the border than to the center of the volume they occupy. Numbers, symbols and colors used as in a. c Distribution of dendritic stem directionality with respect to number of stems. Insets in the plot schematically depict the variation from isomorphic (left) to directional (right). Note that the morphologies shown as examples in Figs. 1f and 4d have mostly isomorphically extending dendrite stems. Numbers, symbols and colors used as in a. d Additional examples of IO neuron morphologies. Colored circles denote morphologies from the viral-labeled data set; colored x’s denote morphologies from the biotin-labeled data. The orange and red morphologies are the only two examples in our library in which dendrites densely surround the soma on all sides. The morphologies marked with green and pink exemplify extreme (though not infrequent) examples of soma eccentricity. The morphologies marked with blue and cyan are examples of extremely extensive IO neuron morphologies with dendritic trees spreading far and wide in almost every direction around the soma. Note that the scale in the reconstructions varies according to viewing angle; somata are 15–18 µm in diameter.
Variability and directionality of inferior olive neurons dendrites revealed by detailed 3D characterization of an extensive morphological library

Influence of dendrite directionality on network connectivity

As a final step in this anatomical investigation, we examined how the morphological variability and dendritic directionality might interplay in determining connectivity in the IO network. To this end, we first examined the distribution of IO neuron somata within the volume of the nucleus by manually reconstructing all 11,800 somata from one side of an entire rostro-caudal extent of an IO (Fig. 5a, “Methods”). While we found that the distribution of IO neuron somata is less homogeneous than would be expected if they were distributed uniformly within the IO volume (Fig. 5b), the inhomogeneities in the somata distribution were too weak to define anatomically segregated groups of neurons based on inter-soma distances alone. In fact, distance-based algorithmic clustering of somata showed that anatomically, somata are all grouped together into a single large cluster for inter-soma distances as small as 40 µm (Fig. 5c). Since all reconstructed morphologies have a reach of at least 35 µm, and the majority reach beyond 100 µm (see Fig. 2b), this result would indicate that IO neurons form a single, large interconnected mesh network. However, this assessment does not take into consideration that IO neuron dendritic trees can be strongly directional, as described in the previous paragraphs.

What connectivity properties may be bestowed on the IO network by the directionality in dendritic trees? We obtained data indicative of an answer to this question in experiments where labeling was less sparse than described so far, allowing us to occasionally visualize pairs or groups of neighboring neurons. In this material we observed that pairs of neighboring, directional morphologies were arranged such that their dendritic fields either expressly overlapped (Fig. 6a) or avoided each other nearly entirely (Fig. 6b). Furthermore, in rare cases where many nearby neurons could be reconstructed, their dendrites extensively overlapped and somata were located at the outer rim of the group of neurons (Fig. 6c). Thus, an attractive possibility is that the directionality of IO neuron dendritic trees, as well as their varying dendritic tree shapes, delineate anatomically segregated areas of mostly dense or more sparse connectivity.

Discussion

In this study we provide a detailed, quantitative description of the morphological properties of a large sample of individual IO neurons and show that the heterogeneity in dendritic tree shapes spans a continuum between the “curly” and “straight” morphological types, defying this classical categorization. Furthermore, we find that IO neuron dendritic arbors are often clearly directional. In combination with our examination of their spatial distribution within the IO volume and their
orientation relative to each other this leads to new ideas regarding the layout of connectivity within the IO network.

In the following paragraphs, we will first discuss some issues related to the description and classification of IO neuron morphologies, before delving into questions related to the significance of our findings for IO network architecture.

Fig. 5  Anatomical clusters cannot be detected in the distribution of IO neuron somata. a Fluorescent labeling of all IO neuron somata. Left panel: coronal cross-section showing a full hemi-olive (scale bar 200 µm); right panel: magnification of the area delineated with a white square on the left (scale bar 25 µm). Detected somata are outlined in thin green lines; black holes are blood vessels. b Density distribution of somata. b1 Soma density shown in a caudal view projection for the principal olive (PO). b2 Same as b1 but for shuffled surrogate data. b3 Comparison of soma densities per 10µm³ voxel. Note that while the PO data has more high-density “hotspots” as well as “empty” regions (see “Methods”), density gradients are too weak to delineate anatomical clusters of somata. c Detection of clusters using the DBSCAN clustering algorithm (see “Methods”), in which cluster membership is defined as a group of points where each point is at most D µm removed from another point in the cluster. c1 3D-representation of clustering in the medial accessory olive (MAO) for different values of D as indicated (the minimal number of somata per cluster was set to 3). c2 Total number of distinct clusters for different minimal cluster sizes as indicated by color-code. Dashed line represents average soma diameter. Note that multiple clusters are only detected at very short (< 20 µm) inter-soma distances, while the entire IO becomes a single cluster at inter-soma distance as short as 40 µm.
Variability and directionality of inferior olive neurons dendrites revealed by detailed 3D characterization of an extensive morphological library

Morphological characterization of IO neurons

Defining cell types has always been a major undertaking in neuroscience, as the layout of neuronal structures is of direct consequence to the connectivity, and thereby the function, of neuronal systems (Mukamel and Ngai, 2018). Neurons in the IO network have classically been described as belonging either to the “curly” or “straight” subtype; however, this classification has always been subjective, and generalizable quantitative definitions of the classes are lacking. In this study, we give a detailed quantitative description of IO neuron morphological properties and find that the inter-individual variability is best described as encompassing a continuum along the curly-to-straight axis. To our knowledge, the included 90 morphologies form the most extensive collection of IO neuron reconstructions to date. Nonetheless, there are several issues pertaining to the labeling, sampling and statistical analysis of our library of IO neuron morphologies that need to be addressed.

First, it should be noted that the different methods for staining individual IO neurons lead to slightly differing data sets. Sparse viral transfection with fluorescent reporter proteins effectively reveals full individual neurons with minimal staining in the background; thus, even the most
densely twisting, extremely “curly” morphologies could be reconstructed in fine detail. In contrast, reconstructions made of neurons patch-filled with either Alexa or biocytin may often underestimate the full extent of the dendritic arborizations, as incomplete penetration of the dye can leave parts of dendrites invisible. In our library, this is reflected in the overall lower number of branches in both patch-filled data sets, and the relatively short over-all length of Alexa-filled morphologies (see Fig. 2b–e; Table 2). Furthermore, the different sample preparation methods used for viral-labeled and patch-filled cells may result in geometrical inconsistencies due to tissue shrinking and/or deformation during the experiment. Nevertheless, the general similarity of measurement distributions across the three datasets strongly suggests that even somewhat incomplete and deformed morphologies provide reliable information on the extent of a neuron’s “curliness”.

Another point requiring consideration is that we selected neuron morphologies for reconstruction based on the completeness of their being contained within the slice (see “Methods”); and since “curlier” neurons tend to occupy smaller volumes, they were less likely than their “straighter” counterparts to be excluded based on having multiple dendrites cut off at the slice surface. This issue is particularly prominent in the neurons labeled with biocytin which were often very close to the slice surface and selected for reconstruction only if their dendrites could be seen to extend down into the slice, while being much less apparent in the Alexa-labeled data because care was taken to patch neurons residing deeper (> 40 µm) in the slice. In addition, and in contrast to the more homogenous tissue sample set obtained from perfusion-fixed brains, the shape of post-fixed acute slices is affected by details of the in vitro experiment, making it difficult to ascertain uniform geometry especially in z-dimension.

Given the extensive morphological heterogeneity and the fact that the morphologies in our library were selected for inclusion based on the completeness of the reconstruction, it should be noted that our sample encompassing 90 morphologies does not necessarily reflect the distribution of morphological properties in the IO neuron population in an accurate and statistically representative manner. It is possible that overlapping, yet distinct morphological categories could be characterized in the full population encompassing more than 20,000 neurons in a single mouse IO (see Fig. 5). The reasons enumerated above also preclude us from making any claims about the relative proportions of “curlier” and “straighter” morphologies in the IO neuron population based on the samples included in our library. Nevertheless, as our investigation uncovered the same trend of continuity in morphological properties in each of three independently acquired data sets, we can confidently state that if a categorization of IO neuron types does exist, the type of an individual IO neuron cannot be deduced with certainty from its morphological properties alone.
Significance of morphological variability and directionality for network architecture

The IO network is often implicitly considered as a homogeneously coupled mesh of neurons. However, such an organization would be computationally inefficient, and possibilities for delineating functional neuronal subgroups through modulation of GJ coupling between IO neurons have been examined through theoretical and experimental approaches alike (Benardo and Foster, 1986; De Zeeuw et al., 1998; Tokuda et al., 2013; Pereda et al., 2013; Kazantsev et al., 2003; Blenkinsop and Lang, 2006; Chaumont et al., 2013; De Zeeuw et al., 2011). For example, functional sub-groups could be defined by inhibitory inputs shunting GJ currents between IO neurons, thereby effectively decoupling them (Llinás, 1974; Lefler et al., 2014). The results presented in this paper are relevant to our understanding of the mechanisms generating synchronized activity in groups of IO neurons because they suggest that alongside the dynamic modulation of electrical coupling, the layout of coupling in the IO network is also defined in the variable density of dendro-dendritic overlap between neighboring IO neurons. Specifically, our results show that IO neuron morphologies have directional shapes (see Fig. 4a) and that somata are most often found at an eccentric location within the dendritic volume (see Fig. 4b). Importantly, such directionality occurs regardless of the distance between an IO neuron’s soma and the border of the subnucleus it resides in (see Fig. 1a, g). Thus, it is evident that the distribution of IO neuron somata (see Fig. 5) by itself is not directly indicative of the layout of functional connectivity between individual IO neurons.

Further evidence for a structured layout of electrical coupling in the IO network comes from examining the orientation of IO neuron dendritic trees relative to those of their neighbors. Examples where nearby directional neurons are labeled imply that IO neurons with closely situated somata need not necessarily form electrical connections (see Fig. 6b), and that dendritic directionality can delineate small subsets of IO neurons whose dendrites overlap with each other (see Fig. 6c). Thus, it is likely that the dendritic directionality delineates boundaries between groups of neurons, such that neurons residing within the same group are coupled to each other more tightly than to other neurons in the network. A network architecture like this has been previously proposed (Torben-Nielsen et al., 2012) as a mechanistic explanation for experimental observations of synchronized activity in groups of nearby IO neurons and propagating waves of oscillatory activity in slices (Leznik et al., 2002; Rekling et al., 2012; Kølvraa et al., 2014). Furthermore, experiments using tracer-diffusion as a measure of GJ-connectivity between IO neurons have shown that the extent and strength of coupling is heterogeneous and that coupled neurons usually reside within the dendritic field of the primary labeled neuron (Hoge et al., 2011),
which is in line with the idea that there exist anatomical boundaries between groups of neurons in the IO network.

In the same way that dendritic directionality likely underlies functional clustering of IO neurons, dendritic curliness is likely to be the structural correlate of especially extensive dendro-dendritic coupling. Considering this, we propose that the straighter and less-directional neurons may function to provide weaker electrical coupling across different clusters in the network, effectively forming “bridges” between them. In this scenario, “cluster neurons” and “bridge neurons” form functionally distinct IO neuron subtypes whose morphological appearance may coarsely correspond to the “curly” and “straight” morphological subtypes. However, variability in the cluster sizes and in the strength and remoteness of bridge-connections results in considerable variability in “cluster” and “bridge” neuron shapes, giving rise to a continuum of morphological properties rather than clearly defined classes.

A tantalizing example in line with such “cluster-bridge connectivity” is shown in Fig. 7. In this sample, a single patched neuron (Fig. 7b, reconstructed in orange) is accompanied by a number of densely overlapping dendritic arbors forming a compact cluster of neurons in a volume spanning the extent of the primary neurons’ dendritic field. Additionally, two neurons located further away from the primary labeled cell (indicated by blue arrows in Fig. 7c)

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Fig. 7 Example suggesting tight within-cluster coupling and weak inter-cluster coupling. a Schematic illustration of a “bridge neuron” (blue) providing weak coupling between two clusters (orange). b Confocal image stack z-projection showing a single directly labeled IO neuron (marked with an orange star) and a dense cluster of indirectly labeled neighbors, as well as two indirectly labeled neurons with somata residing outside the cluster (marked with blue and cyan arrows). Scale bar 50 µm. c Reconstructions of the neurons marked in b, revealing a point of close proximity between their dendrites. d High-magnification confocal z-stack image showing the area marked with a white box in c. Green dot marks a putative GJ-connection between the primary labeled neuron (orange-colored dendrite) and a “bridge neuron” (blue-colored dendrite). Scale bar 5 µm.
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were also labeled and could be resolved well enough to be partially reconstructed (blue and cyan reconstructions in Fig. 7d), revealing a location where a dendrite passes close by that of the directly labeled neuron (marked with a green dot in Fig. 7c, d). This raises the possibility of GJ-mediated coupling between the dense cluster and the “bridge neurons”.

In summary, our anatomical investigation of IO neurons showed that a binary classification into the classically described “curly” and “straight” morphological types is not justified as morphological heterogeneity is better described as varying along a continuous straightness-axis. In addition, the prevalence of directional over isomorphic dendritic fields implies that connectivity in the IO network is structured to support functional clustering. We propose that borders between anatomical clusters are delineated in the dense electrical coupling within groups of “cluster neurons”, and that coupling across such clusters is mediated by dedicated “bridge neurons”. The specific morphology of individual neurons forming clusters and bridges can both vary considerably, resulting in an apparent continuum of morphological properties.

However, the density of IO neuropil and the limitations of the present random-sampling approach preclude strong conclusions to be drawn from anatomical evidence alone, and further electrophysiological and imaging experiments detailing the relationship between the structure and activity of IO neurons will be required to confirm and refine any hypothesis about the hard-wired connectivity of the IO network.

References


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Ogle DH (2018) FSA: fisheries stock analysis. R package version 0.8.20


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Supplementary Figures

**Supplementary Figure 1** (supplement to Figure 2). Morphological parameter correlations to straightness in the patch labeled data sets. Distributions of number of dendrite stems (top), number of branch points (middle) and total dendritic length (bottom) with respect to straightness in the Alexa-labeled data (left column) and the Biocytin-labeled data (right column); the same distributions in the viral-labeled data set are displayed in Fig. 2f–h. Reported correlation statistics represent the strength and direction (Rho) and significance level (p) calculated using Spearman's rank correlation test (see "Methods"). Correlations between straightness and all other morphological measures are reported in the right half of Table 2 for each of the three data sets.
Supplementary Figure 2 (supplement to Figure 3). Algorithmic classification does not reveal clearly separated clusters in two data sets of patch-filled morphologies. a Algorithmic classification shown as a scatter along the first two principal components (PCs) of separation for the Alexa-labeled (left) and Biocytin-labeled (right) data sets. The grey dashed line marks the algorithmically forced K-means grouping into “curly” and “straight”; fill color represents subjective classification, as indicated. In the Alexa-labeled data, as in the viral-labeled data, the separation into distinct groups (as determined by the K-means algorithm) spreads along PC1, whereas in the Biocytin-labeled data, the distinction is better represented along PC2. b Correlation between “straightness” and the first (top panel) and second (bottom panel) principal component of separation for the Alexa-labeled (left) and Biocytin-labeled (right) data sets. In the Alexa-labeled data the first two principal components of separation are both correlated with straightness. In contrast, only PC2 is significantly correlated with straightness in the Biocytin-labeled data. Nonetheless, the classification results are qualitatively similar in all three data sets in that the grouping assigned by the K-means algorithm draws a distinguishing line almost perpendicular to the principal component most strongly correlated to straightness. c Relative contributions of the 25 morphometric parameters to the principal component separation in the Alexa-labeled (left) and Biocytin-labeled (right) data sets. Numbers in circles correspond to the measures as listed in Table 1. The closer a parameter is to 1, the more it contributed to the separation in the PC space, in the direction indicated by its position within the unit circle; a parameter located at the origin did not contribute to the PC separation. Notably, the distribution of parameters contributing strongly to the separation along PC2 in the Biocytin-labeled data set is similar to the distribution of parameters contributing strongly to PC1 in the Alexa-labeled and viral-labeled data (see Fig. 3c).
Summary

In this thesis, I address fundamental aspects of the inferior olive nucleus (IO) that have previously not been studied in detail. These include how the anatomy of IO neurons define IO network organization, how the IO integrates multiple inputs, how such integration affects motor learning, and how IO activity changes during sleep.

In Chapter 1, I provide an introductory overview of the known functional and anatomical aspects of the IO and highlight the knowledge gaps, some of which have been addressed in this thesis.

In Chapter 2, I demonstrate that classifying IO neurons in two distinct groups based on their dendritic morphology (‘curly’ and ‘straight’) is inaccurate. Using 3D morphological reconstructions and quantitative measures, I show that IO neurons exhibit dendritic morphologies spanning a continuum of morphologies that lies between these two cellular archetypes. Moreover, I show that IO dendrites have a strong directionality which supports the idea that IO neurons form clusters. Using preliminary evidence I propose that ‘straight’ cells bridge clusters of IO neurons.

In Chapter 3, I investigate how the IO integrates inhibitory and excitatory synaptic afferents, originating respectively in the cerebellar nuclei (CN) and mesodiencephalic junction (MDJ). In a subset of non-oscillating neurons, or ‘conditional oscillators’, synaptic afferents can induce transient sub-threshold oscillations (STOs) whose amplitude and timing depend on the magnitude of the hyperpolarizing component (V_{hyp.}) of the evoked synaptic potential. I further show that synaptic responses triggered by CN and MDJ afferent stimulation modulate the STOs of intrinsically oscillating neurons affecting both their oscillation amplitude and phase. The degree to which STOs are affected depends strongly on whether inhibitory (CN) or excitatory (MDJ) afferents are stimulated as well as on whether the evoked responses are subthreshold or suprathreshold. I then look at how the timing of CN and MDJ synaptic responses modulates the amplitude and phase of STOs and the output of IO neurons. I find that there are specific intervals that either suppress or enhance the STO amplitude, amount of phase-locking and initiation of spikes. If excitatory input from the MDJ is triggered 50 ms after inhibitory input from the CN (+50 ms) there is a decreased boost of the oscillation amplitude as well as reduced phase-locking and the generation of spikes is suppressed. By contrast, when excitation is triggered 150 ms after inhibition (+150 ms), we see the inverse, with boosted post-stimulus STO amplitudes, increased phase-locking and an increase in the generation of spikes. These findings demonstrate for the
first time how the IO, through its unique oscillatory behavior, sets specific timing intervals for inhibitory and excitatory inputs to generate IO output. My data supports previous hypotheses that predicted an important role for the temporal interplay of these afferents in modulating IO activity. This is especially relevant in light of the close approximation of excitatory and inhibitory afferents on the gap-junction coupled dendritic spines of IO neurons.

Finally, using the knowledge gained on the specific time intervals that suppress or facilitate IO output, I turn to the impact of such interval timing on motor learning. For this purpose, I use a whisker reflex learning paradigm that relies on long-term potentiation (LTP) of parallel fiber to Purkinje Cell (PC) synapses and is associated with an increase of simple activity of Purkinje cells in lobule Crus I and Crus II. This increase in firing is associated with increased whisker protraction. Based on preliminary data, I find that stimulating CN and MDJ input at intervals that suppress IO output are indeed associated with larger sensory evoked whisker protractions and retractions. This suggests that the temporal interplay of CN and MDJ synaptic afferents that projects to the IO play an important role in motor learning.

In Chapter 4, I tackle an important topic that has been largely ignored in sleep research, which is the engagement of the olivo-cerebellar circuit during sleep. By using EEG and EMG along with miniscope recordings, I find that complex spike frequency, complex spike evoked calcium transient amplitude, co-activation and spatial correlations of complex spike firing change during sleep. I also show that, despite such changes, spontaneous twitches are still associated with complex spike activity.

In Chapter 5, I provide an overview of the main findings of my previous Chapters and discuss them in relation to earlier work and previously postulated hypotheses on olivary function. I highlight some of the limitations of the present work and propose topics of future investigation.
Samenvatting

In dit proefschrift behandel ik een aantal fundamentele vraagstukken over de functie van de inferieure olijfkkern (IO) die nog niet eerder in detail zijn bestudeerd. Ik laat zien hoe de anatomie van zenuwcellen de netwerk organisatie van de IO kunnen bepalen, hoe de IO excitatoire en inhibitoire inputs integreert en in welke mate deze integratie van invloed is op de output van de IO en motorisch leren. Daarnaast beschrijf ik hoe de activiteit van de IO verandert tijdens slaap.

In Hoofdstuk 1 geef ik een uitgebreid overzicht van de bekende functionele en anatomische karakteristieken van de IO. Ik geef ook aan waar kennis ontbreekt in aanloop naar het adresseren van de vraagstukken in dit proefschrift.

In Hoofdstuk 2 laat ik zien dat de traditionele manier van onderverdelen van IO neuronen in twee types met ‘gekrulde’ en ‘rechte’ dendrieten niet accuraat is. Door gebruik te maken van 3D morfologische reconstructies en kwantitatieve metingen laat ik zien dat de morfologie van IO neuronen op een continuüm ligt. Tevens laat ik zien dat de dendrieten van IO neuronen een sterke directionaliteit vertonen. Dit pleit voor het idee dat IO neuronen zijn gerangschikt in clusters. Ik lever hiervoor experimenteel bewijs aan en poneer de hypothese dat IO neuronen met ‘rechte’ dendrieten een brug vormen tussen clusters.

In Hoofdstuk 3 doe ik verslag hoe inhibitoire en excitatoire synaptische inputs, respectievelijk uit de cerebellaire nuclei (CN) en mesodiencephalische junction (MDJ) worden geïntegreerd in de IO. In een subset van cellen, zogenaamde ‘conditionele oscillatoren’, leidt stimulatie van synaptische afferenten tot de inductie van transiente oscillaties van het membraan potentaal, waarbij de hyperpolarizerende component van de synaptische response van invloed is op de amplitude en timing van de oscillatie. Ook in cellen die al intrinsiek oscillaties vertonen heeft stimulatie van CN en MDJ afferenten een grote impact op de amplitude en timing van de oscillaties. De mate waarin hangt voor een groot deel af van welke afferenten worden gestimuleerd en of deze onder of boven de vuurdrempel van de cel liggen. Na het in kaart brengen van de impact van de afzonderlijke afferenten laat ik zien hoe de gelijktijdige presentatie en timing ervan IO cellen beïnvloedt. Specifieke intervallen van CN en MDJ stimulatie hebben een tegengesteld effect op de amplitude van oscillaties in de IO, de fase en het vuurgedrag. Als MDJ stimulatie valt in een interval 50 ms na presentatie van CN stimulatie (+50ms) wordt de amplitude verhoging van de oscillatie onderdrukt, lopen de oscillaties minder in fase en wordt de
vuurdeempel verhoogt. Wordt de MDJ stimulatie gepresenteerd met 150 ms na presentatie van CN stimulatie (+150 ms) dan neemt de oscillatie amplitude toe, lopen de oscillaties meer in fase en wordt de vuurdrempel verlaagt. Deze resultaten laten voor het eerst zien dat de IO middels haar intrinsieke oscillaties specifieke tijdsintervallen dicteert die de output van de IO beïnvloeden. Mijn data onderschrijft eerdere hypothesen die een belangrijke rol toeschreven aan de temporele interactie tussen de CN en MDJ afferenten die beiden synaptische contacten maken op gap junction gekoppelde IO spines.

Gebruikmakend van de kennis over de onderdrukkende of faciliterende intervallen van deze contacten kijk ik vervolgens naar hoe deze in rol kunnen in spelen in motorisch leren. Hierbij maak ik gebruik van een snorhaar reflex paradigma waarbij versterkte protractie afhankelijk is van parallel vezel naar Purkinje cell synapse lange termijn potentiatie (LTP). In verkennende experimenten vind ik dat intervallen van CN en MDJ stimulatie die IO output onderdrukken snorhaar protractie en retractie faciliteren. Hieruit kan worden geconcludeerd dat motorisch leren sterk kan worden beïnvloed door de temporele interactie van CN en MDJ afferenten.

In Hoofdstuk 4, snijd ik een belangrijk thema aan dat onderbelicht is in het slaaponderzoek, de veranderingen die tijdens slaap plaatsvinden in het olivo-cerebellaire netwerk. Door middel van electroencephalogram (EEG), electromyograph (EMG) en calcium imaging met geminiaturiseerde fluorescentie microscopen (miniscopes) vind ik dat het vuren van complex spikes, de amplitude van complex spike gedreven calcium transienten, co-activatie van complex spikes in Purkinje cellen en spatiele correlaties veranderen. Ondanks deze veranderingen zijn kortstondige bewegingen tijdens slaap geassocieerd met complex spike activiteit.

In Hoofdstuk 5 geef ik een overzicht van al mijn bevindingen en plaats deze in de context van eerder werk en bestaande hypothesen over de functie van de olijfkern. Ik zet ook uiteen hoe de uitkomsten van de beschreven experimenten de basis kun.
Curriculum vitae

Personal Information

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Education


Research experience

December 2013 - Present. PhD student in Chris De Zeeuw lab (Cerebellar coordination and cognition group) at Erasmus MC and Netherlands Institute for Neuroscience. Thesis project: “Impact of excitatory and inhibitory inputs on inferior olive subthreshold oscillations (STOs), output, and motor learning”.


April 2013 –September 2013. Research assistant of Patricio Rojas, Ph.D. at University of Santiago, Chile. Research project: “Bumetanide as an antipsychotic and antiepileptic drug”.


April 2012 – October 2012. DAAD short term scholarship for young scientists in Rudolf Deisz’s lab at Charité-Universitätsmedizin Berlin. Research project: “Functional and molecular characterization of activity induced by KCC2 modulation”.

July 2011 – February 2012. Research assistant of Patricio Rojas, Ph.D. at University of Santiago, Chile. Research project: “GABAA characterization and chloride dynamics in the axon initial segment of pyramidal neurons”.

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April 2011 – June 2011. Short term internship in Alfredo Kirkwood lab at Johns Hopkins University. Research projects: “LTD in NARP mice in visual cortex” and “LTP independent on NMDA receptors in hippocampus”.

May 2010 – March 2011. Research assistant in Bernardo Morales lab at University of Santiago, Chile. Research projects: “Molecular and cellular basis of the effect of MDMA (extasis) on synaptic plasticity in visual cortex and hippocampus”, “Effects of Methylphenidate on synaptic plasticity in hippocampus and prefrontal cortex”, and “Effects of gold nanoparticles on synaptic plasticity in hippocampus”.

Honors and awards

2013. Erasmus Mundus's Ph.D fellowship for the Joint Doctorate “Neural Processing of Time”.
2012. DAAD short term scholarship for young scientists and doctoral students.
2010. IBRO scholarship to attend the course “Mechanisms of Neuroplasticity” at University of Valparaíso, Valparaíso, Chile

Publications


Curriculum vitae


Loyola S*, Rozas C*, Ugarte M, Zeise ML, Reyes-Parada M, Pancetti F, Morales B (2011). MDMA acutely applied enhances long term potentiation in the rat hippocampus involving D1 and 5HT-2 receptors through a polysynaptic mechanisms. Published in European Neuropsychopharmacology. 22(8):584-95. *These authors contributed equally to this work.


Meeting presentations


Curriculum vitae


Rozas C., Arias-Cavieres, Loyola S., Morales B (2009). 5-HT2 and D1 receptors participate in the modulatory effect of MDMA on synaptic plasticity. International workshop "Motivated behavior, stress and addiction: from molecules to behavior". Milenium Nucleus in stress and addiction, NEDA. Faculty of Biological Science, Catholic University of Chile.Santiago. Chile.
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