Structural plasticity at the input stage of the adult cerebellar cortex

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

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aus Italien

Basel, 2008

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LIST OF ABBREVIATIONS

AP	anteroposterior
BS	brainstem
CF	climbing fiber
CS	conditioned stimulus
CS	conditioned response
DCN	deep cerebellar nucleus
DV	dorso-ventral
EE	enriched environment
EXT	extinction
FC	fear conditioning
GC	granule cell
GCL	granule cell layer
GoC	Golgi cell
IN	interneuron
LTD	long-tem depression
LTP	long-tem potentiation
MF	mossy fiber
MFT	mossy fiber terminal
MIP	maximum intensity projection
ML	molecular layer
NMR	nictating membrane response
PC	Purkinje cell
PF	parallel fiber
SC	spinal cord
US	unconditioned stimulus
VOR	vestibular-ocular reflex

ABSTRACT

In the last decades, a growing amount of evidence has showed that the adult brain is capable of undergoing dramatic reorganisation: learning new skills or acquiring novel experiences determine brain responses in order to reflect the changing circumstances. Changes in behaviour and brain function are probably accompanied by structural alterations in neuronal cells: while extensive descriptions of local rearrangements driven by experience during postnatal development have been provided, the adult brain was classically described to be resistant to such structural modifications. Nevertheless, recent studies convincingly showed that the adult brain is able not only to adapt synaptic transmission, but also to undergo remodelling of pre- and postsynaptic compartments in response to experience.

Plasticity phenomena have been generally described for either pre- or postsynaptic compartments within small cortical fields, thus not fully clarifying the influence of structural remodelling on the synaptic transmission, on the neuronal behaviour (*e.g.* global *versus* local behaviour of a given axonal projection) and, lastly, on the circuit connectivity. In addition, considerable effort is currently made in order to establish a causal relationship between synaptic, structural and representational or topographic map plasticity.

In order to address, at least in part, these questions, we exploited the simple and well-characterized cerebellar circuitry: this system has long been known as a centre for fine motor control and sensorymotor integration; moreover, in the past decade, new results have suggested an involvement of the cerebellum in cognitive and emotional functions. In addition, the cerebellum is a brain region endowed with a high degree of structural plasticity during development, as well as in the adulthood, not only following damage, but also in order to maintain its normal architecture under the influence of activity. The cerebellar anatomy is characterized by a simple and stereotyped connectivity between readily identifiable neurons, and therefore allows the investigation of synaptic rearrangements on a comprehensive scale.

We focused our attention on the input stage of the vermal lobule V, namely at the synapse between mossy fiber (MF) terminals and granule cell (GC) dendrites. MF axons convey multimodal sensory information from distinct sources, such as spinal cord, vestibular system and cerebral cortex. The physiological properties and the

anatomical arrangement at this stage, make the MF-GC synapse an advantageous system to investigate pre- and postsynaptic structural plasticity induced by experience.

Here, we used two distinct behavioural paradigms: in one case, mice were housed in an enriched environment (EE), in order to provide social interactions and extensive sensorymotor stimulation. In a second set of experiments, mice were trained to associate a neutral stimulus with an aversive one, exploiting a pavlovian learning paradigm that relies on fear emotion (fear conditioning, FC). We combined these tasks with large-scale confocal imaging in transgenic mouse lines that express membrane-targeted GFP in few neuronal cells, thus revealing their morphology in crisp details. This approach allowed us to solve and analyze entire MF projections, and related presynaptic terminals, as well as GC dendritic compartments.

In general, we found that distinct experiences elicit substantially different remodelling events, in terms of structural outcome and time course. Upon EE, the MF-GC connectivity is profoundly altered in the properties of the afferent projections and in the putative amplification and transmission of the sensory information. The refinement of the connectivity (*i.e.* the number of presynaptic inputs per cell) is particularly altered in animals that were reared since birth in the enriched context; but a similar robust remodelling of single synaptic units (*i.e.* the dendritic endings) also occured in animals that experienced EE only in the adulthood.

We further found that FC triggers a stepwise remodelling of the local connectivity at the MF-GC synapse, which proceeds for several days after the training and affects the whole population; the extent and quality of remodelling is lobule-specific and can be further modulated according to subsequent experience.

The structural rearrangements we observed upon distinct experiences show that the adult cerebellar system is able to undergo extensive reshaping of its connectivity and synaptic organization. Complementary evidence about synaptic transmission at the MF-GC synapse upon EE and FC would help to assess a correlation between anatomical and functional properties.

CHAPTER 1

GENERAL INTRODUCTION

1.1 EXPERIENCE-DEPENDENT PLASTICITY

The word "plasticity" comes from the greek adjective *plastikós*, in turn derived by the verb *plássein*. Ancient Greeks used this verb referring to soil, wax, waves; since then, this term has been used to generally refer about something's capability of being mould and shaped.

In the last decades, a growing amount of evidences showed that the brain is capable of dramatic reorganisation: learning new skills or acquiring novel experiences determine brain responses in order to reflect the changing circumstances. This is why today we refer to this brain's ability in terms of "neuroplasticity".

Behaviour and brain function adaptations probably involve alterations in the structure of neurons and networks. Indeed, in studies on animal brains, it has been possible to visualise very localised structural changes, which would allow new, or remodelled, synaptic contacts to be formed between cells (Johansen-Berg, 2007). The capacity for reorganization could, at least partly, serve for certain forms of sensorial and motor learning. Currently, considerable effort is made in order to unravel the causal relationship between synaptic, structural and representational or topographic map plasticity phenomenology (Buonomano and Merzenich, 1998).

Here, we will briefly discuss the three mentioned "levels" of plasticity, and how they possibly correlate each other.

1.1.1 About neuronal physiology upon experience: synaptic plasticity

Most of the excitatory and many inhibitory synapses in the adult brain exhibit various forms of use- and activity-dependent synaptic plasticity. These are defined as

changes in the amplitude of synaptic potentials in response to an otherwise unchanged input, as a result of synapse modifications. Especially long-lasting forms of such synaptic plasticity are thought to be a cellular basis for the encoding of experience and storing of information in neuronal networks (Buonomano and Merzenich, 1998).

Several forms of long-term synaptic plasticity have been described, affecting both excitatory and inhibitory synapses in the CNS. They usually occur after repetitive trains of synaptic activity or upon specific pairings of pre- and postsynaptic firings. Collectively, these events are called long-term potentiation (LTP) and long-term depression (LTD).

The first LTP description came from Bliss and his colleagues (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973) and, since then, LTP has been intensely studied because of its presumed role in learning and memory (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999; Bennett, 2000). LTP can be induced by a single high-frequency stimulus train as well as by short, repetitive trains, such as in theta-burst stimulations, which are more reminiscent of the activity patterns recorded in awake-behaving animals. On the other hand, LTD can be induced by low frequency stimulation (Mulkey and Malenka, 1992; Dudek and Bear, 1993). In addition to the temporal proximity, the sequence of pre- and postsynaptic spiking also plays a key role in synaptic modifications. In spike timing-dependent plasticity, presynaptic spiking shortly before postsynaptic spiking leads to LTP, whereas the opposite order leads to LTD (Levy and Steward, 1983; Markram et al., 1997; Dan and Poo, 2004). The effects of spike timing-dependent plasticity were predicted by Donald Hebb in his famous postulate (Hebb, 1949):

"Let us assume that the persistence or repetition of a reverberatory activity (or "trace") tends to induce lasting cellular changes that add to its stability [...] When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased".

Evidence that mechanisms of the so-called *hebbian* plasticity play a crucial role in experience-dependent plasticity *in vivo* comes from studies in sensory cortices that show that correlated neuronal firing can induce receptive field and map plasticity

(Clark et al., 1988; Maffei and Galli-Resta, 1990; Schuett et al., 2001; Fu et al., 2002; Allen et al., 2003; Dan and Poo, 2004).

1.1.2 About neuronal connectivity: structural plasticity

In addition to changes in synaptic strength, structural alterations of cellular connectivity provide another, or a complementary, mechanism to encode experience in the brain. Through the specific loss and gain of synapses, or the remodelling of existing ones, alterations in connectivity change the properties of neuronal networks and their functional output.

I. Developmental structural plasticity

In the attempt to determine how visual information was processed in the brain, Hubel and Wiesel discovered a developmentally restricted competition between the eyes for control of cortical space (Wiesel, 1982) and, subsequently, led others to discover that seemingly analogous competitive events occur in other parts of the developing nervous system (Purves and Lichtman, 1980; Lichtman and Colman, 2000).

Many studies in both vertebrates and invertebrates suggest that the early events of axon outgrowth, pathfinding and target selection are relatively accurate and do not require electrical activity and synaptic transmission, as they rely on intrinsic cellular and molecular mechanisms that guide axons and facilitate correct synaptic partnership. Once the outlines of brain wiring are established, however, patterns of neural activity gradually increase the precision of synaptic connectivity by the selective addition or removal of connections throughout the developing brain (Crowley and Katz, 2000). This remodelling relies on competition with surrounding inputs and is capable of transforming an overlapping projection into a refined and highly tuned wiring. One hallmark of these developmental refinements in synaptic circuitry is the elimination of axonal connections. In the visual system, thalamocortical axons disconnect from cortical layer IV cells (Hubel et al., 1977); in the cerebellum, climbing fibers disconnect from Purkinje cells (Crepel et al., 1976; Lohof et al., 1996; see chapter 1.3.1); in

autonomic ganglia, preganglionic inputs disconnect from ganglion cells (Lichtman, 1977); at the neuromuscular junction, motor axons disconnect from muscle fibers. In each of these areas, elaboration of synapses by the remaining axon or axons also occurs. Thus, while some inputs are being eliminated, others are becoming stronger. After the phase of synapse elimination, each axon has its own unique circuit. Thus, synapse elimination can be viewed as a mechanism that creates large numbers of specific circuits out of initially more diffuse and redundant connections.

In the chapter 1.3.1, developmental plasticity in the cerebellum will be further described.

II. Structural plasticity in the adult

The so-called activity-dependent plasticity is more robust during development, however, neuronal circuits remain plastic in the adult brain: the ability to adapt to the environment is never lost throughout life. Although the extent and the readiness at which adaptations take place are reduced later on, similar adjustments as during critical periods can be induced in adults, though they seem to require either longer, repeated or incremental presentation of stimuli (Linkenhoker and Knudsen, 2002; Sawtell et al., 2003; Hofer et al., 2006). Therefore, critical period research has led to important insights into adult experience-dependent plasticity by the direct comparison of adult and juvenile reactions towards the same stimuli.

Although the adult plasticity is classically thought to depend on changes in the strength of established synaptic connections, many studies demonstrated that average spine densities and morphologies can be altered upon learning, age or other changes in experience, such as sensory stimulation, deprivation, stress or enriched environment (Parnavelas et al., 1973; Moser et al., 1994; Kozorovitskiy et al., 2005; Stewart et al., 2005; Tailby et al., 2005).

It is generally believed that adult neurons are morphologically much more stable than during the critical period. Several recent *in vivo* imaging studies demonstrated that dendritic arbors and spines are much more constant in adults than in juveniles and that spine motility decreases with age (Lendvai et al., 2000; Gan et al., 2003; Majewska and Sur, 2003; Konur and Yuste, 2004). However, *in vivo* time

lapse imaging revealed that the adult brain undergoes to a certain degree of physiological and experience-driven structural rearrangements (reviewed in Gogolla et al., 2007). For instance, defined dendritic spines as well as presynaptic boutons have been found to remodel extensively (Trachtenberg et al., 2002; De Paola et al., 2006; Majewska et al., 2006; Stettler et al., 2006), and it has been observed that spine growth and loss *in vivo* can be modified by experience (Holtmaat et al., 2006). Although the formation of a new spine does not automatically mean the formation of a functional synapse, recent evidence suggests that only about 4% of cortical spines do not bear synapses, and that spine growth precedes synapse formation *in vivo* (Knott et al., 2006; Arellano et al., 2007). Therefore, the assumption that the formation of new spines equals the appearance of new synapses seems generally valid.

Notably, several studies have demonstrated a clear correlation between alterations of synaptic strength and the turnover of dendritic spines (Yuste and Bonhoeffer, 2001, 2004). Likewise, electrical stimulations that induce LTP and LTD lead to dendritic spine formation and elimination, respectively (Toni et al., 1999; Nagerl et al., 2004). Furthermore, many forms of dendritic structural plasticity as well as LTP induction require NMDA receptor activation (Datwani et al., 2002; Sin et al., 2002; Nagerl et al., 2004).

1.1.3 About cortical representations: functional plasticity

Merzenich and colleagues showed in the early 1980's that peripheral nerve lesions in a monkey can lead to changes in the so-called cortical maps, for instance the topographic cortical representation of the hand skin: cortical areas corresponding to the denervated skin were reoccupied by the nerves serving the neighbouring hand areas within a few months (Merzenich et al., 1983a; Merzenich et al., 1983b; Merzenich et al., 1984). A variety of studies demonstrated such changes in cortical mapping upon lesions of sensory organs or amputation of digits. Similarly, changes in the receptive field properties of sensory neurons (reviewed in Buonomano and Merzenich, 1998; Johansen-Berg, 2007), as well as structural rearrangements of axons and dendrites, have been reported (Darian-Smith and Gilbert, 1994). These

modifications represent the attempts of the brain to recover functionality and demonstrate the immense capacity of the adult brain to adapt.

Importantly, changes upon experience not only occur upon insults and large-scale alterations of sensory input, but also underlie many physiological brain functions such as learning and memory. Many studies exploiting functional magnetic resonance imaging demonstrated that perceptual learning boosts specific activity in the involved brain regions (Furmanski et al., 2004; Li et al., 2006). Alterations underlying such changes in broad network activity on the cellular level have been identified. They can consist of specific potentiation of the individual neurons involved in the processing of the trained stimulus (Schoups et al., 2001; Frenkel et al., 2006) or in an increase in the number of neurons representing the trained input (Recanzone et al., 1992; Recanzone et al., 1993). Interestingly, significant changes in neuronal responses were not always detected in the classical receptive field for a given stimulus but often involved neurons neighbouring the representation of the trained stimulus, through a phenomenon called *contextual modulation* (Crist et al., 2001; Li et al., 2004).

1.1.4 The capacity to adapt, or plasticity, is influenced by lifestyle

Interestingly, not only the age of an animal but also lifestyle and environment have major impact on the plastic abilities of the nervous system.

A large variety of life conditions have been implicated in influencing experience-dependent plasticity. Among other factors, the animal's gender, hormone status, stress level, social behaviour, hibernation, parental care, and rearing conditions have been reported to influence plasticity (Rollenhagen and Bischof, 1994; Kozorovitskiy et al., 2006; Vyas et al., 2006; Gogolla et al., 2007).

Likewise, housing animals in environments that are "enriched" in comparison to their regular housing conditions induces a large variety of cellular, molecular and behavioural changes (van Praag et al., 2000; Nithianantharajah and Hannan, 2006).

Experimental enriched environment (EE) conditions consist of the possibility for social interaction, increased motor and exploratory activities as well as enhanced sensory stimulation given by toys of different colours, materials and odours. EE

produces consistent and robust effects on learning and memory performance and thus is a useful model to study experience-dependent plasticity. Early studies investigating the effects of differential housing showed that enrichment altered cortical weight and thickness (Bennett et al., 1969; Diamond et al., 1972, 1976). Subsequent and more detailed studies have shown that, in some neuronal populations, enrichment can increase dendritic branching, length and spine numbers, as well as synapse numbers and size (Greenough and Volkmar, 1973; Connor et al., 1982; Turner and Greenough, 1985; Faherty et al., 2003; Leggio et al., 2005). Furthermore, genes involved in synaptic function and cellular plasticity are altered upon enriched environment (Rampon et al., 2000). For instance, EE induces alterations in the expression of NMDA and AMPA receptor subunits. These findings are in line with evidence for increased synaptic strength, including specific forms of synaptic plasticity such as LTP, upon EE (Foster et al., 1996; Duffy et al., 2001; Foster and Dumas, 2001; Artola et al., 2006).

Notably, it was recently described that transferring adult animals in a "naturalistic environment" induces a large-scale functional refining of sensory maps in the barrel cortex (Polley et al., 2004). This plasticity was accompanied by massive anatomical contraction and weakening of whisker functional representations in such a way that each whisker had a smaller representation containing smaller and sharper receptive fields, thus enhancing high-resolution discrimination abilities (Frostig, 2006).

Considerable knowledge about plasticity mechanisms has been reached along the last decades. Further efforts in this field will most likely reveal and clarify how synaptic plasticity can lead to structural and finally functional remodelling in the neuronal networks.

1.2 CEREBELLUM: ANATOMY AND GROSS ORGANIZATION

The cerebellum (from Latin, *little brain*) constitutes only 10% of the total volume of the brain but contains more than three quarters of all its neurons (Andersen et al., 1992). These neurons are arranged in a highly regular manner as repeating units, each of which is a basic circuit module. Despite its structural regularity, the cerebellum is divided into several distinct regions, which receive projections from different portions of the brain and spinal cord (SC), and project to different motor systems. These features suggest that regions of the cerebellum perform similar computational operations but on different inputs.

The cerebellum influences the motor systems by evaluating disparities between intention and action and adjusting the operation of motor centres in the cortex and BS while a movement is in progress, as well as during repetitions of the same movement. Three aspects of the cerebellar organization underlie this function. First, the cerebellum is provided with extensive information about the goals, commands, and feedback signals associated with the programming and execution of movement. The importance of this input is evident in the fact that 40 times more axons project into the cerebellum than exit from it. Second, the output projections of the cerebellum are focused mainly on the premotor and motor systems of the cerebral cortex and brainstem (BS), systems that control spinal interneurons (INs) and motoneurons directly. Third, synaptic transmission in the circuit modules can be modified, a feature that is crucial for motor adaptation and learning (see chapter 1.4).

Damage to the cerebellum disrupts the spatial accuracy and temporal coordination of movement. It impairs balance and reduces muscle tone. It also markedly impairs motor learning and certain cognitive functions.

1.2.1 The cerebellum: gross anatomical divisions

The cerebellum occupies most of the posterior cranial *fossa*. It is composed of an outer mantle of gray matter (the cerebellar cortex), internal white matter, and three pairs of deep nuclei: the fastigial, the interposed and the dentate. The cerebellum is connected to the dorsal aspect of the BS by three symmetrical pairs of tracts: the inferior cerebellar peduncle (also referred as restiform body), the middle cerebellar peduncle (or *brachium pontis*) and the superior cerebellar peduncle (or *brachium conjunctivum*). The superior cerebellar peduncle contains most of the efferent projections. The cerebellar output originates from neurons in the deep nuclei, with the exception of the flocculonodular lobe, whose cells project to the lateral and medial vestibular nuclei in the BS (Figure 1).

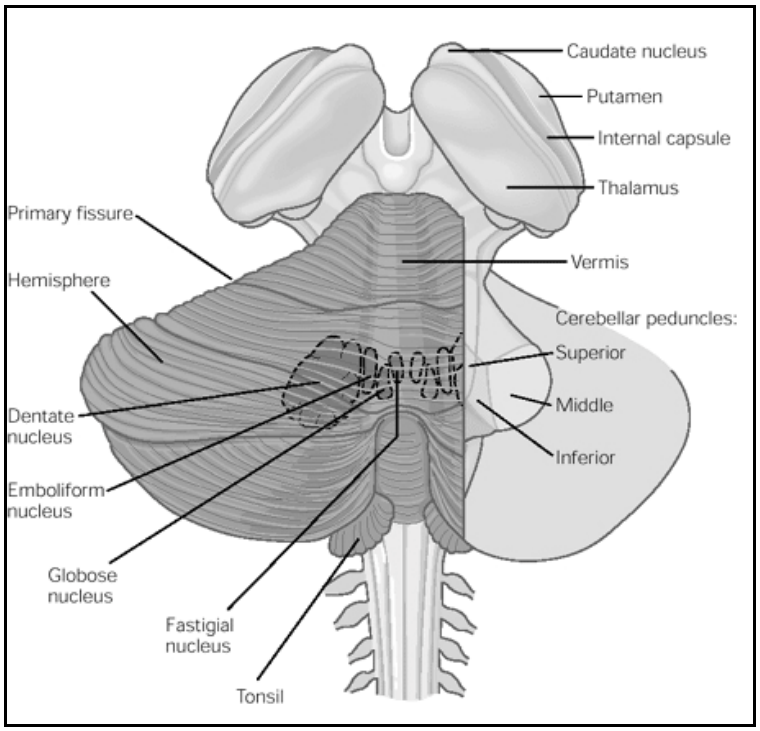


Figure 1 Gross features of the cerebellum
Dorsal view. Part of the right hemisphere has been cut out to show the underlying cerebellar peduncles. (from Kandel, Principles of Neuroscience, IVth ed.)

A striking feature of the cerebellar surface is the presence of many parallel convolutions, called *folia*, that run from side to side. Two deep transverse fissures divide the cerebellum into three lobes. The primary fissure on the dorsal surface separates the anterior and posterior lobes; in turn, the posterolateral fissure on the

ventral surface separates them from the flocculonodular lobe. Parasagittal sections reveal that shallower fissures further subdivide each lobe into several lobules comprising a variable number of folia.

1.2.2 Phylogenetic and functional divisions

Two longitudinal furrows distinguish functionally important regions: the vermis and, on either side of the vermis, the cerebellar hemispheres, each of them divided into intermediate and lateral regions. The vermis and the intermediate and lateral parts of the hemispheres receive different afferent inputs, project to different parts of the motor systems, and represent distinct functional subdivisions respect to the flocculonodular node (Figure 2).

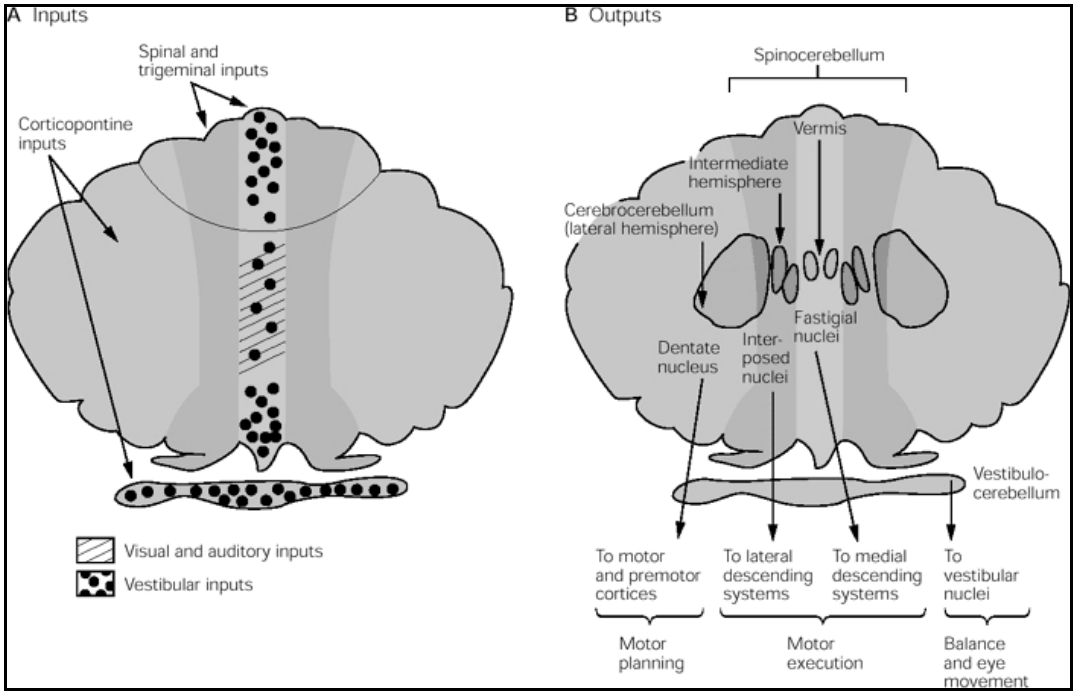


Figure 2 The three functional regions of the cerebellum have different inputs and outputs (from Kandel, Principles of Neuroscience, IVth ed.)

I. The vestibulocerebellum (*archicerebellum*) regulates balance and eye movements

The flocculonodular lobe (or *vestibulocerebellum*) is the most primitive part of the cerebellum, appearing first in fishes. In higher vertebrates its function is limited to controlling balance and eye movements. The vestibulocerebellum receives information

from the semicircular canals and the otolith organs, which sense motion of the head and its position relative to gravity. Mossy fibers that terminate in the vestibulocerebellar cortex arise from neurons in the vestibular nuclei. The vestibulocerebellar cortex also receives visual input via mossy fibers from the superior colliculi and from the striate cortex, the latter relayed through the pontine nuclei. Purkinje cells in the vestibulocerebellum inhibit neurons in the lateral vestibular nucleus, assuring balance during stance and gait, and in the medial vestibular nucleus, controlling eye movements and coordinating movements of the head and eyes.

II. The spinocerebellum (*paleocerebellum*) regulates body and limb movements

The vermis and hemispheres develop later in phylogeny. Cerebellar afferents from the SC, mainly from somatosensory receptors, are distributed exclusively to the vermis and to the intermediate hemispheres (also referred as *spinocerebellum*). Somatosensory information is conveyed to the spinocerebellum through several direct and indirect pathways. Direct pathways originate from neurons in the spinal gray matter and terminate as mossy fibers in the vermis or intermediate cortex. Two important pathways are the ventral and dorsal spinocerebellar tracts. These pathways provide the cerebellum with somatic sensory information from the limbs, notably from muscle and joint proprioceptors, and with information about descending commands.

Indirect pathways from the SC to the cerebellum synapse first with neurons in one of several so-called precerebellar nuclei in the BS reticular formation (the lateral reticular nucleus, *reticularis tegmenti pontis*, and paramedian reticular nucleus).

Direct and indirect inputs provide the cerebellum with different versions of the changing state of the organism and its environment and permit comparisons between such signals. Similar monitoring of outgoing commands is as crucial for perception as for movement, since the internal sensory signals resulting from movement must be distinguished from the external sensory signals in the environment. PC neurons in the spinocerebellum project somatotopically to different deep nuclei that control various components of the descending motor pathways.

III. The cerebrocerebellum (*neocerebellum*) is involved in planning movement and evaluating sensory information for action

The lateral parts of the hemispheres (or *cerebrocerebellum*) receive input exclusively from the cerebral cortex. They are phylogenetically more recent and much larger in humans and apes than in monkeys or cats. Clinical observations initially suggested that, like the rest of the cerebellum, the lateral hemispheres were primarily concerned with motor function. However, recent studies of patients with lesions of the lateral hemisphere and experiments using functional brain imaging indicate that the lateral hemispheres, or cerebrocerebellum, also have a variety of perceptual and cognitive functions (see chapter 1.5).

This cortical input originates mainly in the pontine nuclei and projects through the middle cerebellar peduncle to the contralateral dentate nucleus and terminate as mossy fibers in the lateral cerebellar cortex. Purkinje neurons in the lateral cerebellar cortex project to the dentate nucleus. Most dentate axons exit the cerebellum via the superior cerebellar peduncle and have two main terminations: the first one in the thalamus, in turn projecting to premotor and primary motor areas of the cerebral cortex; the second main termination of dentate neurons is in the red nucleus, projecting to the inferior olivary nucleus, which in turn projects back to the contralateral cerebellum in the climbing fibers, thus forming a feedback loop. The intriguing suggestion has been made, based on brain imaging, that this premotor-cerebello-rubrocerebellar loop is involved in the mental rehearsal of movements and perhaps in motor learning (Lacourse et al., 2005).

In the first half of the twentieth century, Gordon Holmes and Jean Babinski identified two characteristic motor disturbances in patients with localized damage in the cerebrocerebellum, namely variable delays in initiating movements and irregularities in the timing of movement components. The same defect is seen in primates with lesions of the dentate nucleus.

1.2.3 The cerebellar circuitry: a main excitatory loop and an inhibitory side-loop

The cerebellar cortex is a simple three-layered structure consisting of five major classes of neurons: the inhibitory stellate-, basket-, Purkinje- and Golgi neurons, and the excitatory granule cells (Figure 3). The cerebellar cortex also contains other, less numerous neuronal types, including the Lugaro and unipolar brush cells (Ambrosi et al., 2007).

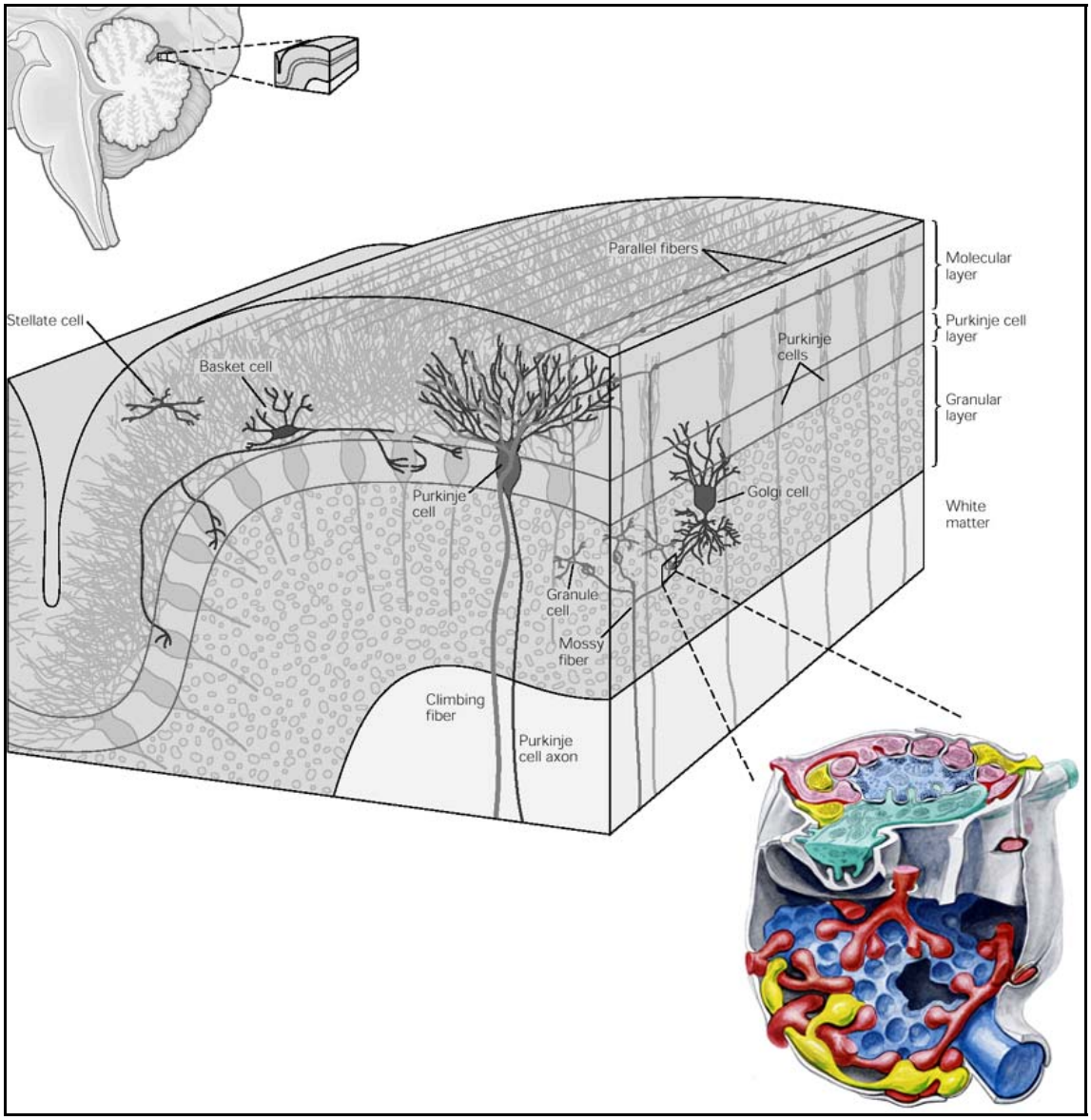


Figure 3 The cerebellar cortex is organized into three layers and contains five major types of neurons
A vertical section of a single cerebellar folium, in both longitudinal and transverse planes, illustrates the general organization of the cerebellar cortex. The detail of a cerebellar glomerulus in the granular layer is shown in the lower right corner: blue, mossy fiber rosette; red, granule cell dendrite; yellow, Golgi axon; green, Golgi dendrite; grey, glial capsule. (grey schema modified from Kandel, Principles of Neuroscience, IVth ed.; glomerulus sterodiagram from Ito, The Cerebellum and Neural Control)

The outermost, or molecular layer (ML), of the cerebellar cortex contains the cell bodies of two types of inhibitory INs, the stellate and basket cells, dispersed among the excitatory axons of granule cells (GCs) and the dendrites of inhibitory Purkinje cells (PCs), whose cell bodies lie in the underlying layer. GC axons run parallel to the long axis of the folia. The dendrites of PCs are oriented perpendicular to these axons.

Beneath the ML is the PC layer, consisting of a single layer of PC bodies. PCs have large cell bodies (about 40 μm) and fan-like dendritic arborizations that extend upward into the ML. Their axons project into the underlying white matter to the deep cerebellar or vestibular nuclei and provide the output of the cerebellar cortex. This output is entirely inhibitory and mediated by GABA neurotransmitter.

The innermost, or granular layer (GL), contains a large number (estimated at 10^{11}) of GCs and a few larger Golgi INs (GoCs). The mossy fibers (MFs), the major source of afferent input to the cerebellum, terminate in this layer, where their bulbous terminals contact GC dendrites. Additionally, GC dendrites also receive the GoC axon inhibitory input; direct contacts between MF rosettes and GoC dendrites have been observed as well (Eccles et al., 1967). The whole synaptic complex here described is organized in a structure called cerebellar *glomerulus*. (Figure 3, lower right inset).

1.2.4 Purkinje cells: excitatory afferent fiber systems and local inhibitory interneurons

The cerebellum receives two main types of afferent inputs, MFs and climbing fibers (CFs). Both groups of fibers form excitatory synapses with cerebellar neurons, but the two groups terminate differently in the cerebellar cortex and produce different patterns of firing in the PCs (Figure 4).

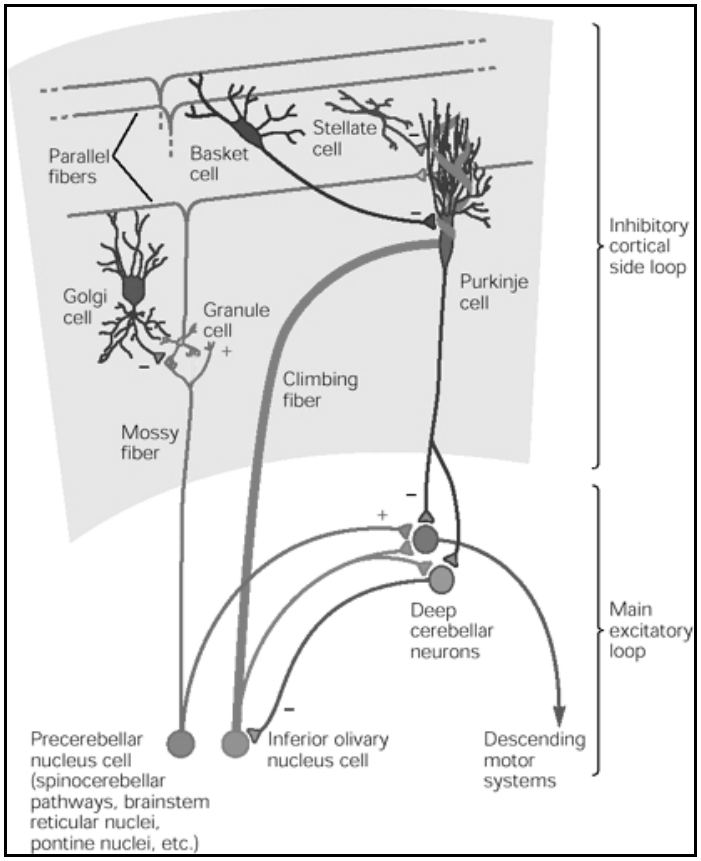


Figure 4 Synaptic organization of the basic cerebellar circuit module
MFs and CFs convey output from the cerebellum via a main excitatory loop through the deep nuclei. This loop is modulated by an inhibitory side-loop passing through the cerebellar cortex. This figure shows the excitatory (+) and inhibitory (-) connections among the cell types. (modified from Kandel, Principles of Neuroscience, IVth ed.)

MFs originate from nuclei in the SC and BS and carry sensory information from the periphery, as well as information from the cerebral cortex. They terminate as excitatory synapses on the dendrites of GCs in the GL. The axons of the GCs (the parallel fibers, PFs) travel long distances along the long axis of the cerebellar folia in the ML, thus exciting large numbers of PCs in the same transverse plane (Mugnaini, 1983). In humans, each PC receives input from as many as 10^6 GCs, each of which collects input from many MFs.

CFs are the terminal arbours of neurons located in the inferior olivary nucleus and convey somatosensory, visual peripheral, or cerebral cortical information. CFs wrap around the cell bodies and proximal dendrites of PCs, making numerous synaptic contacts. Individual PCs receive synaptic input from only a single CF, whereas each inferior olive neuron contacts up to 15 PCs, according to the species. The terminals of the olivocerebellar neurons in the cerebellar cortex are arranged topographically, the axons of clusters of olivary neurons terminating in thin parasagittal strips that extend across several folia (Figure 6). In turn, the PCs within

each strip project to common groups of deep nuclear neurons. This highly specific connectivity of the CF system contrasts markedly with the massive convergence and divergence of the MFs and PFs.

CFs have unusually powerful synaptic effects on PCs. Each action potential in a CF generates a prolonged voltage-gated Ca^{2+} conductance in the soma and dendrites of the postsynaptic PC. This results in prolonged depolarization that produces a complex spike: an initial large-amplitude spike followed by a high-frequency burst of smaller amplitude action potentials. In contrast, PFs produce a brief excitatory postsynaptic potential that generates a simple spike: consequently, spatial and temporal summation of inputs from several PFs are needed before the PC will fire.

The activity of the PCs is inhibited by the stellate- and basket cells and by their recurrent collaterals. The short axons of stellate cells contact the nearby dendrites of PCs, and the long axons of basket cells run perpendicular to the PFs and form synapses with PCs and at each side of the PF beam. Stellate and basket cells are facilitated by PFs. This arrangement, facilitation of a central array of neurons and inhibition of surrounding cells by local input, resembles the center-surround antagonism in visual and somatosensory pathways (Jacobs and Werblin, 1998).

The GoC has an elaborate dendritic tree in the overlying ML. The GABAergic terminals of GoCs form synapses with the GCs in the glomeruli. GoC firing, initiated by PFs, suppresses MF excitation of the GCs and thus tends to shorten the duration of bursts in the PFs.

1.2.5 Different encoding of peripheral and descending information by MF and CF systems

MFs and CFs respond very differently to sensory stimulation and during motor activity. Spontaneous activity in MFs produces a steady stream of simple spikes in PCs (Figure 5). Somatosensory, vestibular, or other sensory stimuli change the frequency of the simple spikes, which may reach several hundred spikes per second. Voluntary eye or limb movements are also associated with a marked change in frequency. Thus, the

frequency of simple spikes can readily encode the magnitude and duration of peripheral stimuli or centrally generated behaviours. In contrast, CFs fire spontaneously at very low rates, and these spontaneous rates are changed only modestly by sensory stimuli or during active movement. The frequency of complex spikes in PCs is rarely more than 1-3 per second. A maximal frequency of 8-9 per sec has been obtained following a prolonged stimulation of the vestibular receptors (Ferin et al., 1970, 1971).

Such low frequencies cannot by themselves carry substantial information about the magnitude of natural stimuli or behaviour. It is possible that complex spikes might signal the timing of peripheral events or act as triggers for behaviour. Rodolfo Llinás has suggested that a form of timing signal might be provided by the synchronous firing of multiple PCs (Llinás, 1981). Neurons in the inferior olivary nucleus are often electrotonically connected each other through dendrodendritic synapses and therefore can fire in synchrony. The synchronous inputs of olivary neurons in CFs produces complex spikes in many PCs at almost the same time.

Despite the low frequency of their discharge, CFs may alter cerebellar output by modulating the synaptic effect of PFs input in different ways. First, each complex spike is followed by a pause due to a membrane hyperpolarization induced by a Ca^{2+}-dependent K^+ permeability, thus changing the pattern of firing. Second, following inferior olive inactivation by cooling, PCs fire at high rate (Montarolo et al., 1982; Benedetti et al., 1983); this effect is not due to the suppression of the post-CF pause, but to the suppression of a strong tonic inhibition exerted by the inferior olive on the PCs through CF collaterals to the inhibitory INs. Recently, Szapiro and Barbour (2007) showed that such a tonic inhibition is mediated, at least in part, by glutamate spillover from CFs to INs. Finally, activity in CFs can induce selective LTD in the synaptic strength of PFs that are active concurrently. In the 80's, it was found that concurrent stimulation of CFs and one set of PFs *in vitro* depresses the effect of later stimulation of the same PFs but has no effect on the stimulation of another set of PFs (Ito and Kano, 1982; Ito et al., 1982; Ekerot and Kano, 1985; Ito, 1989). For this depression to occur, however, the PF simple spike must occur within 100-200 ms of the CF complex spike. The resulting depression can last minutes to hours and depends critically on the prolonged depolarization and large influx of Ca^{2+} produced by the CF in PC dendrites.

This long-term effect of the CF on the transmission of signals from the MFs, GCs, and PFs through to the PCs may be important for the cerebellar role in motor learning (Ito, 2000, 2002).

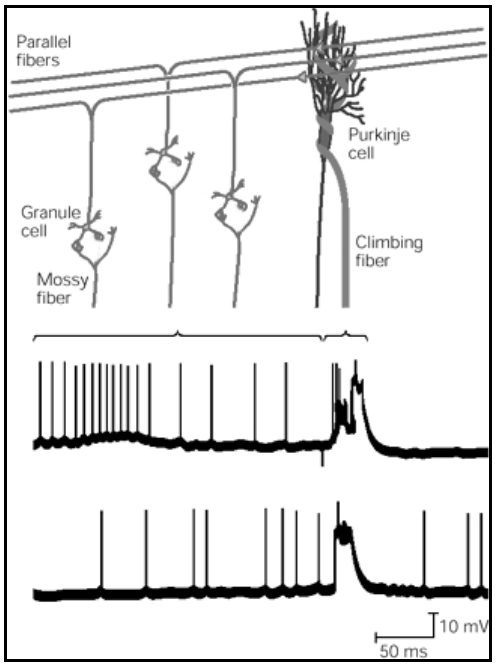


Figure 5 Simple and complex spikes recorded intracellularly from cerebellar Purkinje cells
Complex spikes (right bracket) are evoked by CF synapses, while simple spikes (left bracket) are produced by MF input or by intrinsic spontaneous activity. (modified from Kandel, Principles of Neuroscience, IVth ed.)

1.2.6 Cerebellar information processing: functional microanatomy of cerebellar circuitry

Fundamental to the operation of any CNS structure is the information processing that it accomplishes. The cerebellum receives a wide variety of sensory inputs and generates motor-related outputs according to internal rules of computation. These rules are determined by the internal connectivity of cerebellar neuronal networks and the intrinsic properties of cerebellar neurons (Apps and Garwicz, 2005). The importance of the cerebellum in the coordination of movement is undisputed and a growing body of evidence indicates that it might also be involved in certain cognitive processes (Stoodley and Schmahmann, 2009; see chapter 1.5). Cerebellar networks show long-term synaptic plasticity (Ekerot and Kano, 1985; Ito 1989; Hansel et al., 2001), which indicates that experience-dependent adaptive and learning processes are also a salient feature of cerebellar function (Ito et al., 1974; Robinson, 1976; Thach, 1998a,b).

Given the uniform structure of the cerebellar cortex, the basic neural computation performed is assumed to be similar throughout, whether used for the control of autonomic functions, limb movements or higher functions such as language. Notwithstanding regional differences in chemoarchitecture (for example, in the cerebellar cortical distribution of molecular markers such as zebrin (Hawkes 1997; Herrup and Kuemerle 1997), it follows that functional differences between various parts of the cerebellar cortex must arise primarily, if not exclusively, from local differences in input and output connectivity.

I. Microcomplexes are fundamental processing units

The modern key organizing principle, on the basis of detailed studies mainly in cats and rats, is a division of the cerebellar cortex into a series of longitudinally oriented strips or "sagittal zones" (Figure 6). Individual zones are typically 1-2 mm in width, running across the cerebellar lobules for many millimeters in the rostrocaudal plane (Voogd and Glickstein, 1998). The PCs in each zone receive CF input from a circumscribed region of the inferior olive and, in turn, send output to a circumscribed region in the cerebellar nuclei, thereby forming discrete olivo-cortico-nuclear complexes. For the olivocerebellar CF input to each cerebellar cortical zone, there is a corresponding detailed topography. In brief, rostral and caudal subdivisions of the contralateral inferior olive map onto zones located in the lateral (paravermal and hemispheral) and medial (vermal) parts of the ipsilateral cerebellar cortex. CFs therefore impose a very precise order on cerebellar cortical organization, which presumably has important implications for function (Apps and Garwicz, 2005). In terms of functional organization, different parts of the olive convey information from one or several specific spino-olivo-cerebellar pathways and, consequently, each zone can be readily identified with electrophysiological mapping techniques. Within each zone, smaller units known as "microzones" can also be readily identified electrophysiologically (Ekerot et al., 1991). Therefore, a microzone is a narrow longitudinal strip of cerebellar cortex within which all PCs receive CF-mediated input with similar receptive field identity. Microzones are also the defining components of

olivo-cortico-nuclear microcomplexes (Ekerot et al., 1979), which might be thought of as the cerebellar counterparts of cerebral cortical columns.

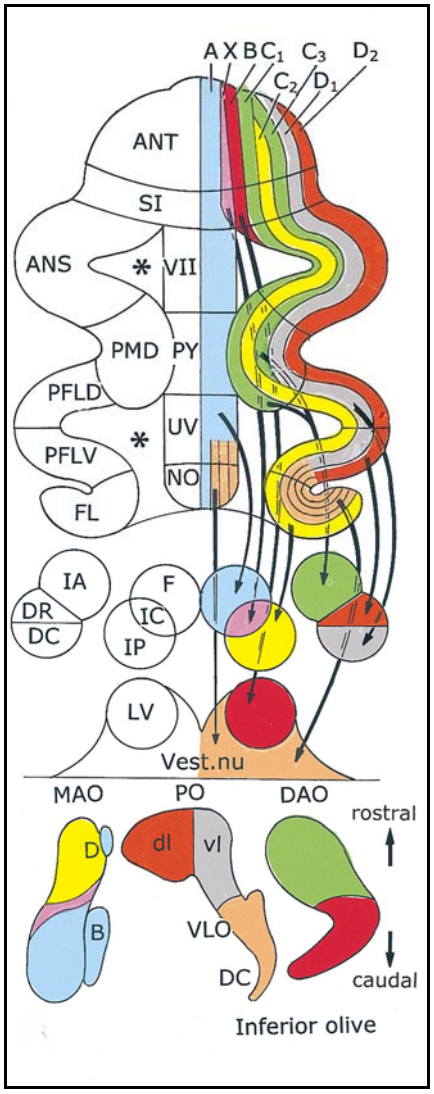


Figure 6 Longitudinal zones
The zonal arrangement in the corticonuclear and the olivocerebellar projections is illustrated in a diagram of the flattened cerebellar cortex of the cat. Three groups of cerebellar nuclei with their corticonuclear projection zones can be distinguished: 1. fastigial nucleus (F); 2. anterior interposed nucleus (IA) and dentate nucleus, (DC and DR); 3. lateral vestibular nucleus of Deiters (LV). The inferior olive is shown in the lower half of the figure, in a horizontal projection introduced by Brodal. The zonal projections of the individual subnuclei are indicated with the same colours (from Voogd and Glickstein, 1998).

II. MFs define a fractured somatotopy on the cerebellar cortex

MFs arise from many different sources in the BS and the SC. The initial mapping studies of the spinocerebellum by Edgar Adrian and Ray Snider in the 1940's revealed two inverted somatotopic maps in the cat vermis (Snider and Stowell, 1944) In both maps, the head was represented in the posterior vermis, and the representations of the neck and trunk extended on either side along the dorsal and ventral portions of the vermis. Arms and legs were represented adjacent to the vermis over the intermediate cortex of the hemispheres. Visual input from the superior colliculi and visual cortex were distributed to both vermal and paravermal portions of the posterior lobe. This

early mapping was based on recordings of surface potentials, which reflect the predominant input and provide only a coarse representation of somatotopic connections. The fine grain in the somatotopical localization of the face was studied by Welker with electrophysiological mapping of MF responses in the posterior lobe of the rat and other species (Shambes et al., 1978). They found a mosaic-like pattern with multiple representations of the same receptive fields. In this type of "fractured somatotopy", the precise topographical relations between adjacent receptive fields do not appear to be preserved (Figure 7). More refined mapping studies of the cerebellar cortex based on single-cell recordings revealed that input from a given peripheral site, such as a local area of skin, diverges to multiple discrete patches of GCs in lobule-specific patterns (Arends, 1997; Shumway et al., 2005).

The patches are usually discontinuous at the apex or at the base of a lobule (Voogd et al. 1996; Voogd and Glickstein 1998). In the central region of the cerebellum the distribution of the main MF systems is concentric, with vestibular fibers terminating ventrally and centrally in the base of the fissures (Voogd et al., 1996), pontocerebellar fibers in the apex of the lobules and spinocerebellar fibres occupying an intermediate position. The lateral parts of the cerebellum, with the exception of the flocculus, which receives secondary vestibular and reticular projections, receive an overwhelming input from the pons (Glickstein et al., 1994).

The relation of these MF patches to the microzonal organization in the somatosensory innervated CF zones is yet not fully understood, but recently it was shown that highly specific topography exists within cerebropontocerebellar projections with corresponding peripheral inputs relayed by the CF system, suggesting that functionally related information is conveyed to the same part of the cerebellar cortex via direct (olivocerebellar) and indirect (cerebropontocerebellar) pathways (Odeh et al., 2005).

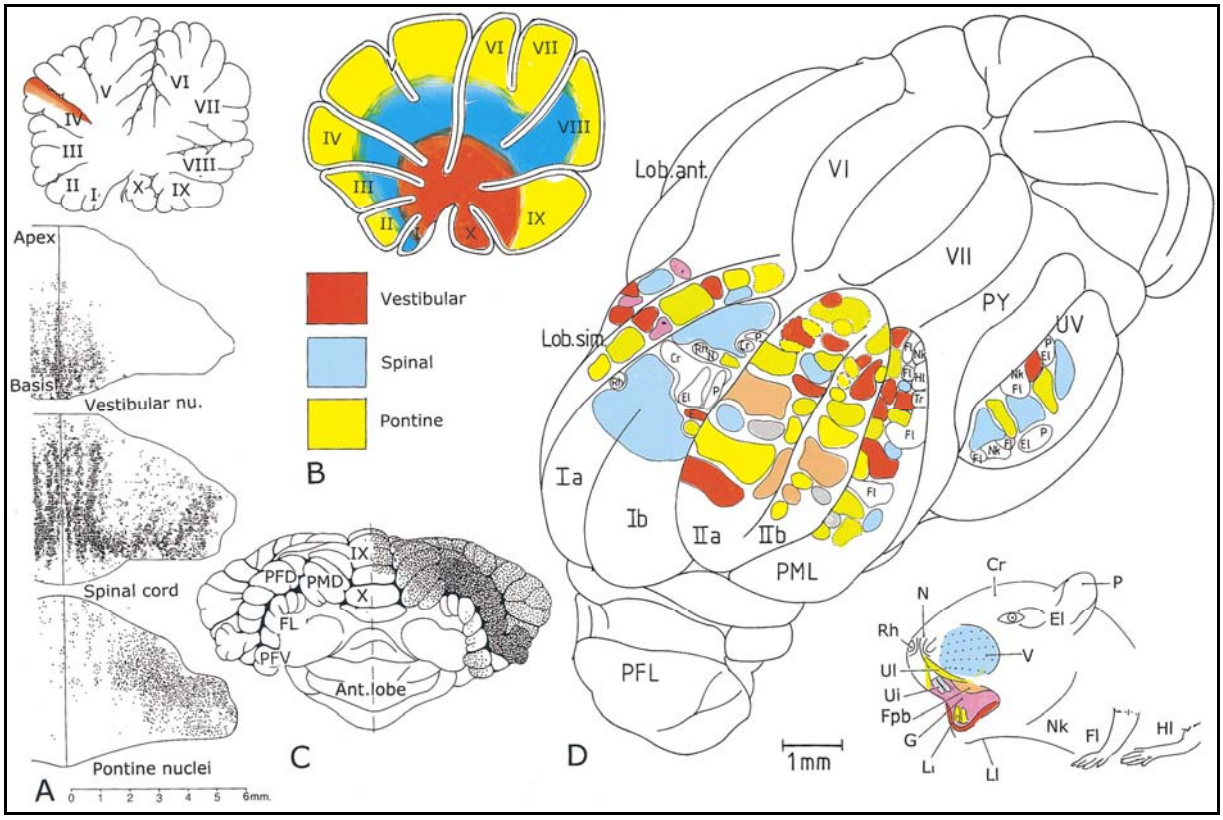


Figure 7 Mossy fibers

A. The lobule-specific, patchy and zonal distributions of different MF systems are exemplified in chartings of their distribution on the dorsal surface of lobule IV in the cat. **B.** The distribution of vestibulocerebellar, spinocerebellar and pontocerebellar MFs in medial cerebellum can best be described as a concentric one. **C**. Pontocerebellar fibres terminate heavily in the hemisphere, but spare the flocculus and the ventral paraflocculus. **D**. MFs in the posterior lobe of the rat terminate in a fractured somatotopical pattern of multiple patches. (from Voogd and Glickstein, 1998)

1.3 CEREBELLUM AND STRUCTURAL PLASTICITY

Several examples of activity-dependent plasticity of synaptic strength and intrinsic excitability have been reported in the cerebellum: plasticity of synaptic excitation and inhibition occurs at multiple synapses, and intrinsic plasticity has been reported in several cell types. In addition, the cerebellum is a brain region endowed with a high degree of structural plasticity during development, as well as in the adulthood: extensive work has been done to describe structural rearrangements taking place after damage or alteration in the patterns of activity.

Here, we will describe the state of the art in the field, focusing in particular on the plasticity reported for the adult cerebellar cortex.

1.3.1 Climbing fiber to Purkinje cell synapse: a model for synapse elimination

The CF-PC synapse in the cerebellum provides a good model to study the cellular and molecular mechanisms of synapse elimination in the developing CNS. Immature neurons initially make redundant synaptic connections; then, functionally important synapses are strengthened and kept, while less important synapses are weakened and finally eliminated morphologically, in an activity-dependent refinement process (Changeux and Danchin, 1976; Purves and Lichtman, 1980; Katz and Shatz, 1996; Lohof et al., 1996).

PCs in adult cerebellum receive two major excitatory inputs, namely PFs and CFs (see chapter 1.2.4). Each PF-to-PC contact is weak, but 100-200.000 PFs form synaptic contacts on a single PC. In contrast, only one CF innervates each PC in the adult cerebellum (*mono-innervation*), making strong synaptic contacts on PC proximal

dendrites (Eccles et al., 1966; Palay and Chan-Palay, 1974). However, in the early postnatal days PCs are innervated by multiple CFs (*multiple innervation*) (Crepel et al., 1976; Crepel, 1982; Lohof et al., 1996). These *surplus* CFs are eventually eliminated with the progress of postnatal development, and mono-innervation is complete by the end of the third postnatal week in mice (Kano et al., 1995; Kano et al., 1997; Kano et al., 1998).

Multiple CFs initially form synapses on the perisomatic processes of PCs in newborn mice (Chedotal and Sotelo, 1993; Morando et al., 2001). The stimulation of multiply-innervating CFs elicits excitatory postsynaptic currents that are much smaller than those elicited by mature CFs (Mariani and Changeux, 1981; Hashimoto and Kano, 2003): consequently, this indicates that CF inputs become stronger during postnatal development, and that weak, or *surplus*, CFs must be eliminated along the same time span. Indeed, several reports indicate that synaptic strength of multiply-innervating CFs (3-6 per PC) are relatively similar at P3, and that one CF is strengthened during P3–P7, while elimination of redundant CF proceeds in parallel. Thus, one CF is strengthened, namely selected, among multiple CFs innervating the same PC, over the first postnatal week (Hashimoto and Kano, 2003, 2005; Scelfo and Strata, 2005). Developmental synaptic refinement is made possible by multivesicular glutamate release from CF terminals, which allows high reliability of transmission by the strongest CF synapses, and by the expansion of innervation territory on PC dendrites, so that the strongest CF input can induce large Ca^{2+} transients over the whole PC dendritic tree (Hashimoto and Kano, 2005).

Several reports indicated the existence of competition between CFs and PFs for the innervation territory in the adult (Bravin et al., 1999; Morando et al., 2001; Cesa et al., 2003; Cesa and Strata, 2004; Miyazaki et al., 2004): a current developmental model indeed suggests that PF synapses confine the CF innervation site to the proximal dendrites of PCs; afterwards, PF activity, involving NMDA receptor at MF–GC synapse, drives molecular cascades in the PCs to elicit the elimination of redundant CF synapses (Kakizawa et al., 2000). Thus, the ultimate phase of CF synapse elimination would be driven through at least two distinct heterosynaptic interactions between PF and CF synapses (Hashimoto and Kano, 2005).

1.3.2 Axonal and synaptic plasticity in the adult cerebellum

The development of CF-PC synapse is an example of how a patterned neural activity can lead to a gradual increase in the precision of synaptic connectivity, transforming a partially overlapping projection into a refined and highly tuned wiring. However, subtle reorganization of terminal arbors and synaptic remodelling can occur throughout life in the intact brain and these changes are dependent on activity (Buonomano and Merzenich, 1998).

The cerebellar cortex provides a suitable model to study how activity controls the axonal and synaptic remodelling in the adult brain. Mature PC dendrites are characterized by a proximal compartment which receives a single CF input, and a distal one, on which many PFs impinge.

Following pharmacologically induced PC degeneration (target deprivation), the CF arbor undergoes a remarkable atrophy in terms of branching and axon diameter (Strata and Rossi, 1998). Conversely, a subtotal lesion of the inferior olive leads to a powerful expansion of the CF terminal arbor under the form of collateral sprouting; recordings of PC spontaneous activity 2–3 months after the lesion show that the newly formed synapses are functional (Benedetti et al., 1983). It is likely that diffusible factors, most likely released from the CF-deprived PCs, elicit the formation of collateral branches of the CF terminal arbors (Strata and Rossi, 1998).

The high structural target-dependent plasticity of the CF arbour is heavily influenced by activity (Morando et al., 2001): local blocking of electrical activity determine severe reduction of CF varicosity size and of CF-PC synapse number; consequently, PFs are able to take over the CF territory and to form synapses on the proximal dendrites (Morando et al., 2001).

Interestingly, both a subtotal lesion of the inferior olivary neurons (Strata and Rossi, 1998) and local chronic activity blockade (Bravin et al., 1999) are able to induce spinogenesis in the proximal dendritic domain, and newly formed spines, independently of the presynaptic partner (PFs, CFs or GABAergic neurons), express GluRδ2, whose presence is normally confined to the distal dendrites at the PF-PC synapse (Morando et al., 2001). GluRδ2 seems to play a major role in the competitive advantage of the PFs over the CFs, acting on the stabilization and strengthening of the synapse (Yuzaki, 2003). These findings suggest that CFs exert an activity-dependent

spine-pruning action; Cesa and colleagues reported that the CF exerts its repressive action on PC spinogenesis through ionotropic AMPA/kainate receptors (Cesa et al., 2007).

In conclusion, the mature cerebellar circuitry has a remarkable structural plastic potential not only following damage, but also in order to maintain its normal architecture under the influence of activity. Both excitatory inputs to the PC express several genes related to plasticity throughout the life span conferring the ability to remodel their synapses (Cesa and Strata, 2007). CFs and PCs show remarkable reciprocal trophic interactions, that are required for the maintenance of the correct synaptic connectivity.

1.4 CEREBELLUM AND MOTOR LEARNING

It is well established that the olivocerebellar system is crucial for motor learning. Recently, many forms of synaptic and non-synaptic plasticity have been described that could be involved in motor learning processes and an overall picture is slowly emerging about the mechanisms related to different stages of aspects of the learning processes (reviewed in Zeeuw and Yeo, 2005; Hansel et al., 2001). Pioneering description and dissection of the cerebellar circuitry and its operational mechanisms were brought starting from the 60's (Eccles et al., 1967) and later revised and extended by Ito (Ito, 1984).

The cerebellar circuitry is essentially composed of a relay station in the deep cerebellar nuclei (DCN) and a cortical "side-loop". Cerebellar output to premotor centers originates in the DCN and is additionally driven by direct excitatory input from the MFs. A further modulation is given by the inhibitory input from the PC axons, which convey computations and integrations taking place at the higher cortical synapse: computation is performed "upon a matrix of subtle and informationally rich excitatory PF input (~200,000 axons), massive and synchronous excitation produced by the one CF axon innervating each mature PC, and input from inhibitory INs" (De Zeeuw and Yeo, 2005). This unusual anatomical configuration inspired a model of motor learning by Marr (1969), who proposed that the PF-PC synapses could provide contextual information, that CF-PC synapses could signal an "error" in motor performance that required alteration of subsequent behaviour, and that the conjunction of these two signals could strengthen the PF-PC synapse to create a memory trace for motor learning. This model was modified by Albus (1971), who realized that a decrease in synaptic strength would be more appropriate given the sign-reversing function of the PC inhibitory output.

We will first introduce two cerebellar-dependent learning models and then describe cellular forms of LTP and LTD that could represent the underlying mechanisms.

1.4.1 Adaptation of the vestibulo-ocular reflex (VOR)

The vestibulo-ocular reflex (VOR) is the neural system by which rotations of the head, detected by means of the vestibular organs, determine an equal and opposite eye rotation. This reflex is a compensatory eye movement needed in order to stabilize retinal images during head movements. Its circuitry includes afferents from the vestibular apparatus that converge upon the vestibular nuclei, which relay via INs to the oculomotor nuclei that control eye movements. The role of the cerebellum in VOR adaptation was first described by Ito (Ito, 1986). In laboratory, VOR adaptation is readily induced by providing visual stimuli in conflict with the vestibular stimulus (De Zeeuw et al., 1998; Faulstich et al., 2004). Such visuo-vestibular training can modify both the amplitude and the timing of the VOR (De Zeeuw and Yeo, 2005). These parameters can increase or decrease after short-term or long-term training periods, the changes induced can persist for short or long periods, and the adaptations can depend on the history of eye movement behaviour before the training period (Boyden et al., 2004). During the past decade, many studies unravelled the role of LTD at the PF-PC synapse, but they clearly revealed that other sites of plasticity must also contribute to VOR adaptation.

1.4.2 Classical conditioning of the eyeblink and nictitating membrane response

One of the most studied forms of learning is the classical conditioned reflex described by Thompson (1986). Repeated pairing of a behaviourally neutral conditioned stimulus (CS), such as an auditory tone, with an unconditioned stimulus (US) that reliably elicits a blink response will lead to the gradual development of eyeblink conditioned responses (CRs) to the CS (De Zeeuw and Yeo, 2005). Hallmark

features are that the CR is accurately timed, so that its peak coincides with the impact of the US, its amplitude is scaled according to CS and US intensities and their contiguity and, with sufficient training, it is CS-specific. Thus, although the movement is simple, it is identifiably as motor learning. Two blink response systems have been studied. In rabbits, the third eyelid or nictitating membrane response (NMR) is a nerve VI response, with little or no voluntary component and near silent baseline, and is strongly dependent by the cerebellar system (Krupa et al., 1993; Hesslow and Yeo, 1998). In most of the other species, including mice, external eyelid blink is a nerve VII response, with a strong voluntary component. Baseline levels of eyelid responses are high in freely moving mice and the extent to which eyeblink CRs depend upon the cerebellum is less clear (Koekkoek et al., 2003; De Zeeuw and Yeo, 2005).

CS and US information are conveyed by the MF and CF systems, respectively, as supported by the following evidences: after normal conditioning with a peripherally applied CS, direct stimulation of MF can substitute directly for the CS; manipulation of olivary activity through the nucleo-olivary pathway affects behavioural blocking and extinction (De Zeeuw and Yeo, 2005). In particular, extinction learning involves the gradual decline of previously established CRs when the CS is repeatedly presented without the US.

The precise mechanism of eyeblink learning has been extensively studied and different models have been proposed for the memory trace localization (Thompson, 2005; Yeo and Hesslow, 2007). In 2007, Wada and colleagues, beautifully showed that the conditioned eyeblink learning is formed and stored without GC transmission, thus taking advantage of the direct MF-*nucleus interpositus* pathways, yet the PF-to-PC transmission is necessary for the expression of the learning (Wada et al., 2007).

1.4.3 Do VOR adaptation and eyeblink conditioning depend upon similar mechanisms?

VOR adaptation and eyeblink conditioning have some obvious similarities, so a common set of mechanisms, perhaps involving cortical and vestibular or nuclear plasticity, is appealing (Raymond et al., 1996), but, as learning model systems, they also have important differences. For the VOR, it is the vestibular signal that initially

drives the reflex and is conveyed as a "context" signal to the cerebellum by the MF-PF pathway modification, under control of the retinal slip-related CF signal. In eyeblink conditioning, the reverse applies. The reflex is initially driven by the face somatosensory information that also supplies the olive for the CF signal that controls learning. The auditory CS pathway does not initially drive the reflex but it might come to do so via changes in the MF-PF afferent system. Furthermore, the tasks have very different demands. The VOR must be rapid and accurate, although it only needs to deal with a vestibular signal. In eyeblink conditioning, CRs are well timed but not rapid and they are available to a wide range of context stimuli that include auditory, visual and somatosensory. For the VOR, retinal slip provides an unambiguous error signal; in eyeblink conditioning it is unclear whether there is a similar error signal (De Zeeuw and Yeo, 2005).

Other forms of motor learning could require plasticity in the cerebellar circuit: however, it should be stressed that there are also forms of motor learning (such as improvement in the performance of rotating rod and thin rod balancing tasks) that can persist even when some forms of cerebellar plasticity are blocked (De Zeeuw et al., 1998).

1.4.5 Diversity of synaptic and non-synaptic plasticity in the cerebellum

It is now interesting to analyze mechanisms of cerebellar synaptic plasticity and how they could subserve motor learning, in particular taking eyelid conditioning as reference example. Plasticity at each cerebellar synapse in relation to motor behaviour has been brilliantly and extensively reviewed by Hansel and colleagues (Hansel et al., 2001).

I. PF LTD

Cellular mechanisms that might underlie acquisition of associative eyelid conditioning would be expected to be located at points in the cerebellar circuit where CS and US signals converge and where their repeated co-occurrence results in an increase in DCN firing. As suggested by Marr–Albus models, PF LTD is one such

cellular mechanism. In PF LTD, first reported by Ito and collaborators, a persistent attenuation of the PF-PC synapse is produced when PF and CF inputs to a PC are stimulated together at low frequency (Ito et al., 1982). The requirements for PF LTD induction, like those for eyelid conditioning, are typically associative: PF synaptic strength is not attenuated by CF- or PF stimulation alone, as well as by stimulation of both in an unpaired manner. CFs contribute to LTD induction via Ca^{2+} influx through voltage-gated channels, occurring during the complex spike. The PFs release glutamate, which acts upon both mGluR1 and AMPA receptors. After these initial signals, there is a requirement for transient protein kinase C activation (Wang and Linden, 2000). PF LTD is expressed postsynaptically, as a reduction in the number of functional AMPA receptors produced by clathrin-mediated endocytosis.

II. PF LTP

One way in which repeated application of the CS alone in a trained animal could be made to result in CR extinction would be to produce homosynaptic LTP of PF-PC synapses. Indeed, this form of LTP may be observed after low-frequency (2–8 Hz) PF stimulation in the absence of CF activation (Hansel et al., 2001). In contrast to LTD, LTP at the PF synapse seems to be triggered by a presynaptic Ca^{2+} influx. Several lines of evidence suggest that PF LTP is mediated by the presynaptic activation of Ca^{2+}-sensitive adenylyl cyclase, leading to a rise in the concentration of cAMP and the consequent activation of protein kinase A (Hansel et al., 2001). The expression of PF LTP also seems to be presynaptic. It is associated with a decrease in the rate of synaptic failures and the extent of paired-pulse facilitation. Thus, whereas LTD at the PF-PC synapse is postsynaptically induced and expressed, LTP is presynaptically induced and expressed. In such a scheme, cerebellar LTP and LTD would not truly reverse each other, but would rather be additive, independent phenomena, a suggestion that has recently been confirmed experimentally (Wang and Linden, 2000). This is in striking contrast to results obtained at excitatory synapses received by pyramidal cells in the hippocampal CA1 subfield, in which both LTP and LTD can be postsynaptically expressed and where the synapses are endowed with reversible bidirectional modification.

III. CF LTD

Stimulation of the CF axon that innervates a mature PC results in a massive excitation of the PC, generally indicated as a complex spike. The classic Marr–Albus–Ito models of cerebellar function are generally silent regarding changes in the strength of this CF-PC synapse. However, CFs show a form of developmental plasticity during the first three weeks of postnatal life (see chapter 1.3.1); moreover, even in adult rats, CFs show some degree of morphological plasticity, depending on the availability of target PCs and the overall electrical activity of the cerebellar cortex (Strata and Rossi, 1998). Furthermore, the molecular machinery required for LTD at the PF synapse (mGluR1, P/Q- type Ca^{2+} channels, GluR2-containing AMPA receptors and PKC) is also present at the CF input. *In vitro* CF LTD was observed and characterized for the first time by Hansel and Linden (2000). Preliminary evidences suggest a postsynaptic locus of expression. This synaptic depression, even though the ~ 20% attenuation of EPSC amplitude produced is of no relevance for cerebellar function, could represent a way to modify PC complex spikes (Hansel et al., 2001).

IV. GABAergic IN–PC LTP

LTP of both spontaneous and evoked $GABA_A$ receptor-mediated IPSCs may be induced by repetitive CF activation. Alterations of IN–PC synaptic strength are likely to have a major influence on PC throughput. Indeed, it has been demonstrated that single action potentials evoked in inhibitory INs can generate delays in PC action potential firing and that tonic inhibitory input can drastically modulate the spike firing pattern in PC (Hausser and Clark, 1997).

V. MF-GC LTP

MFs constitute a large glutamatergic system of the brain and have GCs as their primary target (10^{11} granule cells and 4 times as many MF-GC synapses), providing a large potential substrate for information storage. Activation of MFs using either a theta-burst pattern (100 Hz for 100 ms repeated 8 times at 250 ms intervals), or a single tetanus (100 Hz for 1 s) that mimics endogenous activity, paired with

postsynaptic depolarization, results in LTP of MF EPSCs (D'Angelo et al., 1999; Armano et al., 2000), whereas protracted low-frequency stimulation (2 Hz for 5 min) causes LTD. LTP is similar to that commonly recorded in hippocampal area CA1 in that it requires postsynaptic depolarization, NMDA receptor activation and consequent Ca^{2+} influx: it is then quite sensitive to the level of inhibitory input (Armano et al., 2000), which is provided here by GoCs. Indeed, it has been assessed that LTP or LTD can be generated depending on the local excitatory/inhibitory balance: repeated high-frequency MF discharges (theta-bursts) induce LTP when synaptic inhibition is weak, whereas the same pattern induce LTD when synaptic inhibition is strong (Mapelli and D'Angelo, 2007).

Recent works contributed to the understanding of spatiotemporal processing of information in the GL, showing that theta-burst stimulation induces LTP and LTP in patches organized in such a way that, on average, LTP is surrounded by LTD (namely, inhibition is stronger in areas of the GL surrounding the GCs that were strongly activated by MF stimulation), a process referred as *lateral inhibition* (Mapelli and D'Angelo, 2007). The authors suggest that this activation pattern could be instrumental in regulating the contrast between GL fields, thus extending the original concept of pattern separation by Marr (1969), integrating the local activity and the topography of the afferent sensory inputs. Contrast enhancement in the GL could take part to cerebellar receptive field reshaping after sensory stimulation (Jorntell and Ekerot, 2002). In addition, Rancz and colleagues suggested that, at the physiological firing frequencies (more than 700 Hz), the MF-GC synapse exhibits remarkable sensitivity and high-fidelity transmission (Rancz et al., 2007); moreover, given the substantial anatomical divergence of MFs (at the level of axonal collaterals and at the synaptic terminal organization), a sensory-evoked burst in a single MF could lead to the activation of a substantial number of neighbouring GCs, thus ensuring the reliable activation of the downstream PCs (Barbour, 1993).

MF LTP can also be blocked by an mGluR antagonist or a PKC inhibitor. During MF LTP expression, amplitudes of both the AMPA and the NMDA receptor-mediated synaptic currents are increased. MF LTP expression is also associated with a change in the kinetics of the NMDA-EPSC such that deactivation is protracted. This could constitute an extension of the window for EPSP temporal summation and, thereby, coincidence detection (Hansel et al., 2001).

VI. Plasticity of intrinsic neuronal excitability

Memory storage in the brain is generally assumed to be mediated by long-term modifications in the strength of synaptic transmission, but activity-dependent changes of the intrinsic excitability of neurons could also regulate the information storage process in the mammalian brain. Evidences for persistent changes in intrinsic neuronal excitability induced by behavioural training have been reported (Hansel et al., 2001).

Interestingly, when theta-burst stimulation plus postsynaptic depolarization was applied to MF-GC synapses, a persistent increase in the intrinsic excitability of the postsynaptic GCs was observed (Armano et al., 2000). The increased excitability was dependent upon postsynaptic depolarization, NMDA receptor activation and a postsynaptic Ca^{2+} transient. GoC inhibition appears to be is critical for fine tuning plasticity at the MF-GC relay. Potentiation of intrinsic excitability may be relevant in conditions of low synaptic excitation, restoring GC readiness and regulating the number of GCs effectively activated by discharging MFs.

1.4.6 Motor learning and structural plasticity

Motor learning paradigms provide the opportunity to examine specifically the development of a set of behaviours in relation to the anatomical changes presumed to support them (Kleim et al., 1997b). To this point, it has been reported that animals trained on complex motor learning tasks have increased number of synapses per PC, compared to active and inactive controls (Black et al., 1990; Kleim, 1994). Interestingly, it has been reported that synaptic changes persist for at least four weeks in the absence of continued training (Kleim et al., 1997b). These data indicate that PC morphology may underlie skill acquisition and retention.

1.5 CEREBELLUM AND COGNITION

From the beginning of 90's, the cerebellum contribution to cognitive processing and emotional control, in addition to its role in motor coordination, is subject of systematic consideration (Andreasen and Pierson, 2008; Ito, 2008).

In 1996, it was convincingly reported in a fMRI study on humans that the lateral cerebellar output nucleus was not activated by the control of movement *per se*, but was strongly engaged during the acquisition and discrimination of sensory information (Gao et al., 1996). More recently, another study found no cerebellar activation associated with the learning phase, despite extensive involvement of other cortical and subcortical regions; cerebellar activation was rather significant during the expression of the learning (Seidler et al., 2002). Both reports indicate that the cerebellum does not contribute to the learning of the motor skill itself, but is engaged primarily in the modification of performance.

Further anatomical and physiological studies reveal that there is a primary sensorimotor region of the cerebellum in the anterior lobe, and a secondary sensorimotor region in the medial aspect of the posterior lobe. In contrast, cerebral association areas that subserve higher order behaviour are linked preferentially with the lateral hemispheres of the cerebellar posterior lobe. Reciprocal connections between the cerebellum and hypothalamus facilitate cerebellar incorporation into the distributed neural circuits governing intellect, emotion and autonomic function, in addition to sensorimotor control (Schmahmann and Caplan, 2006). These findings have been recently integrated in a meta-analysis of neuroimaging studies reporting cerebellar activation in several task categories (motor-, somatosensory-, language-, verbal working memory-, spatial- and executive function, and emotional processing). For instance, sensorimotor tasks are reported to activate the anterior lobe (lobule V)

and adjacent lobule VI, with additional foci in lobule VIII, while motor activation is in VIIIA/B; posterior lobe is involved in higher-level tasks (Schmahmann and Caplan, 2006).

A *cerebellar cognitive affective syndrome* has been described in patients with lesions confined to the cerebellum (Schmahmann and Sherman, 1997), as well as in several pathological conditions that involved the cerebellum. These neurobehavioural deficits may occur in the absence of the cerebellar motor syndrome, and are characterized by impairments in executive function, spatial cognition and linguistic processing when the lesions involve the hemispheric regions of the cerebellar posterior lobes.

Listed works provide evidence for an anterior sensorimotor *versus* posterior cognitive/emotional dichotomy in the human cerebellum. However, this field is undergoing a lively debate in order to establish the validity of fMRI studies on the cerebellum. For instance, Glickstein recently pointed out that the folding of the cerebellar cortex makes it a difficult structure for imaging studies and that activated areas may be related to actual or planned movement of the eyes, vocal apparatus or fingers (Glickstein, 2007).

In a comprehensive review about cerebellar involvement in motor learning and cognition, Thach identifies distinct input and output connections for the so-called "motor" (inputs from vestibular and spinocerebellar pathways, and from sensorimotor cortex via the pons) and "cognitive" (inputs from non-primary frontal-, parietal- and occipital cortex via the pons and output back to them via the thalamus) regions of the cerebellum, consistently with their differing specializations. The uniformity of the cerebellar circuitry suggests indeed that some common computation is performed in the two areas on the different sets of input information (Thach, 1998b). Current models suggest that corticocerebellar projections convey efference copies of information from cortical areas to modular cerebellar "internal models" (namely, a neural representation of the external world). During the acquisition of motor skills, movements would then become increasingly controlled by cerebellar internal models rather than by the cerebral cortical circuitry, and eventually be executed automatically in a feed-forward manner (Ramnani, 2006): Ito recently proposed that such internal modules are formed in each cerebellar microcomplex (Ito, 2008).

1.6 CEREBELLUM, EMOTIONAL BEHAVIOUR AND FEAR CONDITIONING

Much information about the brain circuitry underlying emotional processes is obtained by studying fear-related processes. In the laboratory procedure employed to investigate the neurobiological basis of fear learning (fear conditioning, FC), an innocuous stimulus (conditioned stimulus, CS) is repeatedly associated with a noxious one (unconditioned stimulus, US). As a result of this pairing, the CS comes to elicit spontaneous defensive responses that include autonomic (heart rate and blood pressure variations, pupillary dilation), endocrine (pituitary-adrenal hormone) and behavioural (increased startle reflex, escape, immobility or freezing) responses. The latter can be measured in order to gat a reliable quantification of the fear learning.

A large body of evidence from lesion, pharmacological and neurophysiological studies point to the amygdala as the key neural system subserving FC (LeDoux, 1996). The amygdala is one of the principal structures of the limbic system and has long been implicated as a crucial emotive brain centre. Anatomically, it receives sensory inputs from different brain areas, such as thalamus, neo- and olfactory cortex, hippocampus) and sends projections to several autonomic and somato-motor structures that mediate specific fear responses (*e.g.* bed nucleus of *stria terminalis* to activate the release of stress hormones, or the periacqueductal gray matter for freezing) (LeDoux, 1996). However, it is not clear whether the amygdala is the permanent storage site for long-term memories; more likely, the amygdala is the crucial component of multiple fear systems, including structures such as the insular cortex, the hippocampus, the perirhinal cortex, the cerebellum (Kim and Jung, 2006).

Here, we will briefly report the current knowledge about the cerebellar system and the fear-related processes, both innate and learned. Generally, the cerebellar vermis could serve to associate sensory stimuli, their emotional significance and the correct motor responses.

1.6.1 Cerebellum and learned fear

The cerebellum could, at first approssimation, be involved in the motor functions related to emotional learning. However, its motor role can be differentiated by its associative, non-motor processes coordination.

The expression of CS-US association in terms of heart rate changes requires the integrity of the vermis, but not of the hemispheres (Supple and Leaton, 1990; Sebastiani et al., 1992; Supple and Kapp, 1993; Yoshida et al., 2004). Further studies showed that cerebellar lesions hamper several types of aversive CRs, like the passive avoidance of a dark chamber (Guillaumin et al., 1991), shuttle box learning (Dahhaoui et al., 1990) and the association between a conditioned sound, a bar press response and shock avoidance (Steinmetz et al., 1993). The capacity to learn and to retain fear CRs was investigated in *hotfoot* mutant mice, which are characterized by a primary deficiency of the PF-PC synapses (Morando et al., 2001; Yuzaki, 2003) and show severe motor impairment. In *hotfoot* mice, the acquisition of the fear memory is normal, indicating that cerebellar dysfunction does not affect basal fear motor responses; however, the retention of the CR (as soon as 10 minutes after conditioning) is significantly reduced (Sacchetti et al., 2004). This reveals that the cerebellum, and, in particular, the PF-PC synapse is necessary for associative processing and is involved in fear memory. In line with this result, it has been reported that mice with a deficiency of PF-PC synapses exhibited severe memory impairment in the eyeblink conditioning (Kishimoto et al., 2001).

In addition, the reversible inactivation of the vermis after CS-US association was reported to elicit amnesic effects (Sacchetti et al., 2002), indicating an important role of the vermis in emotional memory, independently of its participation to sensory or motor processes. When amnesic agents are applied immediately after memory recall,

fear memories can be altered (Nader et al., 2000). These kind of post-retrieval manipulations can help to locate neural sites involved in the maintenance of long-term memories, independent of their involvement in motor responses or in innate fear behaviour: it has been reported that the reversible inactivation of the vermis strongly affects fear memories established several days before the manipulation (Sacchetti et al., 2007), implying a vermal involvement in the long-term maintenance of aversive memories.

1.6.2 Human cerebellum and fear memories

Recent functional neuroimaging studies revealed different activation patterns in the cerebellar regions during acute pain perception and during learning sensory cues that anticipate pain experience (Ploghaus et al., 1999; Ploghaus et al., 2000). The cerebellar regions activated by the administration of painful stimulus were restricted to the anterior cerebellum around the vermis, while learning that a sensory cue precedes and anticipates the painful stimulation led to an activation of posterior cerebellar vermis (Ploghaus et al., 1999; Ploghaus et al., 2000). These experiments indicate that in the cerebellum close, but separate, regions are engaged during fear experience and during associative learning processes. This difference reveals that during fear experience in human cerebellum some regions are activated by associative processes independent of the regulation of motor/autonomic processes.

Damasio and colleagues (2000), in a fMRI study aimed at investigating the neural basis of subjective processing of feeling, reported a marked activation of cerebellum, largely in midline structures, during mental recall of emotional- *versus* neutral personal episodes. The authors state that the evolutionarily older components of the cerebellum could contribute to the coordination of emotional responses, and to the learned adjustment of those responses in a social setting. More recently, Singer and colleagues (2004) observed brain activity when the subjects experienced a painful stimulus, or when they were observing their loved partner receiving a similar pain stimulus: among other structures, anterior cerebellum around the vermis was activated in subjects receiving the painful stimulation, while posterior cerebellar vermis was activated in the same subjects when observing others' pain. Both conditions included a

concomitant activation of the lateral cerebellum. These results suggest that cerebellum is involved also in the empathic experience related to pain. According to the authors, "such decoupled representations -which are independent of the sensory inputs of the outside world- have been postulated to be necessary for our ability to understand the thoughts, beliefs, and intentions of others" (Singer et al., 2004).

Imaging studies on healthy subjects do not allow to infer about the necessity of a brain structure for emotional learning. Conversely, studies on cerebellar patients suggest that the vermis is necessary to learn a new association between sensory stimuli and aversive ones, while it is not required in the regulation of baseline fear responses and in the cognitive evaluation of a dangerous situation (Maschke et al., 2002; Turner et al., 2007). In line with this observation, animals with vermal lesion are able to remember the context in which a painful stimulation is administered (Supple et al., 1988).

Collectively, all these data show that under physiological conditions the vermis is involved in the formation of fear memory traces. On the basis of the present knowledge, the cerebellum could be not only involved in regulating the autonomic/motor responses, but also participating in the formation of new CS-US associations and in learning to set the most appropriate responses to new stimuli and/or situations.

1.6.3 Neural basis of the cerebellar involvement in learned fear

Learning-induced synaptic plasticity has been described in amygdala (McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997) and hippocampus (Sacchetti et al., 2001; Sacchetti et al., 2002; Whitlock et al., 2006). However, fear learning may be associated with similar neural mechanisms in several brain structures. It was shown that, upon FC, the PF-mediated postsynaptic current, but not the climbing fiber one, is significantly increased compared to controls and pseudo-conditioned mice (Sacchetti et al., 2004): this provided evidence that a long-term potentiation is present in the vermis after fear learning. Synaptic changes were localized to vermal lobuli V and VI, consistently with sensory representations in that area (Snider and Stowell, 1944; Saab and Willis, 2003). A further study showed that fear learning is specifically restricted to

synaptic efficacy in PCs (Zhu et al., 2006); furthermore, learning-induced PF-PC LTP resembles *in vitro* LTP, which at these synapses is induced by repetitive PFs stimulation (Lev-Ram et al., 2002; Zhu et al., 2007): this suggests that learning-induced LTP is the result of conjunctive activation of two separate PF channels conveying CS and US stimuli. In this respect, it has to be underlined that PFs are known to carry, together with acoustic inputs, also nociceptive information (Saab and Willis, 2003). Additionally, it was recently reported that the frequency, but not the amplitude, of spontaneous and miniature GABAergic events onto the PCs, extensively innervated by inhibitory INs, is significantly increased 24 hours after conditioning (Scelfo et al., 2008), implying a presynaptic form of inhibitory LTP. Theories of timing concerning the cerebellar system require that the output of the cerebellar cortex, encoded in the axons of PCs, is precisely tuned in response to sensory stimulation, indeed excitatory LTP ensures a more effective detection, while inhibitory potentiation serves to keep the coincidence detection unchanged, thus ensuring that the temporal fidelity of the network is maintained (Scelfo et al., 2008).

The presence in the vermis of synaptic changes related to learned fear, as well as the behavioural data showing the involvement of this site in the formation of a fear memory trace, raises the question of what anatomical circuitry underlies the involvement by the vermis. Interestingly, studies in the 80's showed that the vermis is connected to the SC, BS and the hypothalamus by way of the fastigial nucleus, thus it can regulate the cardiovascular tone, respiration, gastrointestinal functions, as well as other autonomic processes (Berntson and Torello, 1982). In addition, the vermis is functionally connected with brain sites that are associated not with motor function but with affective and learning processes, such as basolateral amygdala and hippocampus (Snider and Maiti, 1976). In turn, the vermis and fastigium project to the *locus cœruleus* and the ventral tegmental area, crucial nuclei of the catecholaminergic systems (Berntson and Torello, 1982). Therefore, given its anatomical and functional connections with several structures involved either in somatosensory perception (as the brainstem, thalamus), in emotional state (amygdala, septum and *locus cœruleus*) and in the control of motor responses, the vermis may represent an interface between the sensory stimuli, the emotional state of the subject and the motor responses.

CHAPTER 2

RESULTS 2.1

Structural plasticity at the input stage of the adult cerebellar cortex upon enriched environment

Claudia Vittori, Nadine Gogolla and Pico Caroni

unpublished results

2.1.1 INTRODUCTION

Sustained rearrangements of synaptic connections can provide mechanisms to alter connectivity in neuronal circuits and encode experience in the brain (Lichtman and Colman, 2000; Poirazi and Mel, 2001; Chklovskii et al., 2004). Local rearrangements of the circuit connectivity have been widely described to occur in the early postnatal period (Lichtman and Colman, 2000; Linkenhoker and Knudsen, 2002; Linkenhoker et al., 2005), while adult brain is generally more resistant to wide reorganizations of the system. However, recent *in vivo* time-lapse imaging studies in neocortex revealed physiologic turn over spines, and further modulation according to sensory experience (Chen et al., 2000; Lendvai et al., 2000; Trachtenberg et al., 2002; Holtmaat et al., 2005). Liwewise, presynaptic plasticity has also been reported *in vivo* (De Paola et al., 2006; Stettler et al., 2006).

However, most published studies describe groups of either pre- or postsynaptic elements within small cortical regions, thus not fully clarifying the impact of plasticity events at the axonal level (global *versus* local behaviour of a given axonal projection) and at the circuitry level, namely on synaptic transmission and amplification. In particular, it has still to be unraveled how local changes in synaptic contacts can influence neuronal transmission and functional representation in the brain.

To address these questions, we exploited a simple and well-characterized neuronal circuitry, that had been implicated in functional and anatomical plasticity, namely the cerebellar system. The cerebellum has long been known as a centre for fine motor control and sensory-motor integration; its topographic and functional organization, as well as its essential role, have been investigated extensively. Moreover, in the past decade, a growing body of evidences has suggested that the

cerebellum may be involved in cognitive and emotional functions (Schmahmann and Sherman, 1997; Thach, 1998a).

Several examples of activity-dependent plasticity of synaptic strength and intrinsic excitability have been reported in the cerebellum (Hansel et al., 2001). In particular, plasticity of synaptic excitation and inhibition occurs at five different sites (reviewed by Hansel et al., 2001) and intrinsic plasticity has been reported in at least three cell types (Schreurs et al., 1997; Aizenman and Linden, 2000; Armano et al., 2000).

In addition, the cerebellum is a brain region endowed with a high degree of structural plasticity during development (Sugihara, 2006), as well as in the adulthood: extensive work has been done to describe structural rearrangements taking place after damage or alteration in the patterns of activity. Referring in particular to the olivocerebellar system, climbing fibers arising from the inferior olivary nucleus are able to form collateral sprouting and reinnervate their territory upon massive olivary lesions (Benedetti et al., 1983). Furthermore, some studies showed that motor experience induces the increase of spine density on the PC tree (Black et al., 1990; Kleim et al., 1998a; Lee et al., 2007).

We focused our attention on the input stage of the lobule V cerebellar cortex, namely the synapse between mossy fiber (MF) terminals (MFTs) and granule cell (GC) dendrites. MF axons convey multimodal sensory information from distinct sources, such as numerous brainstem nuclei, spinal cord and pontine nuclei, which in turn are innervated by massive projections from the cerebral cortex. MF axons build a large and complex presynaptic terminal, whose postsynaptic elements are individual dendrites from GCs; each GC has 3-5 short dendrites ending in a claw-like structure that receives excitatory synaptic input from one MFT (Voogd and Glickstein, 1998) or from inhibitory Golgi axon varicosities. Quantitative analysis assessed that the presynaptic terminal is contacted in average by 30-50 GC dendrites in the rat (Palkovits et al., 1972; Jakab and Hamori, 1988), each of them coming from separate GCs. A GC dendrite emits in average 3-5 digits, each of them forming one or more synaptic contact with the MFT (75%) or with a Golgi cell axon (Jakab and Hamori, 1988). The physiological properties and the anatomical arrangement at the cerebellar

glomerulus, make the MF-GC synapse an advantageous system to investigate pre- and postsynaptic structural plasticity induced by experience.

To study experience-dependent structural plasticity events, adult mice were housed in an enriched environment in (EE) order to provide extensive sensorymotor stimulation (Rosenzweig and Bennett, 1996; van Praag et al., 2000; Mohammed et al., 2002); we combined this experimental paradigm with large-scale confocal imaging in transgenic mouse lines, previously generated in our laboratory (De Paola et al., 2003), that express membrane-targeted GFP in few neuronal cells, thus revealing their morphology in crisp details.

We found that the MF to GC connectivity considerably rearranges pre- and postsynaptically upon EE experience both at the synaptic and circuitry level; furthermore, we found that some aspects of the rearrangements we observed can be further modulated according to the intensity of the experience.

2.1.2 RESULTS

Transgenic mouse lines that express membrane-targeted GFP in few cells reveal neuronal morphology in the cerebellar cortex

In order to investigate anatomical plasticity in the cerebellar cortex, we first analyzed patterns and distribution of neuronal labelling in the cerebellum of different *Thy1-mGFP** mouse lines expressing membrane-targeted GFP in only a few neurons (De Paola et al., 2003), as well as the morphology of the labelled structures. We will refer to the particular transgenic lines we used in this study as *Lsi1* and *Lsi2*; both of them are characterized by a sparse labelling pattern of specific cell types, described later in this section. Most of the experiments were carried on *Lsi2*, and additional data will be presented concerning *Lsi1*, in order to show that our results are not dependent by a specific cell subset labelling. We observed that, in the listed transgenic lines, mGFP expression in the cerebellar cortex was mostly attributable to a small subset of afferent mossy axons and sparse GCs (Figure 8A-C).

Occasionally, we could observe labelled Bergman glia cells, Golgi cells and neuromodulatory fibers (Figure 8D). The pattern and number of labelled cells in the cerebellum was variable according to the mouse line (Figure 8B-C), and an additional inter-individual variability in the number of labelled cells had to be accounted for each particular subject; nevertheless, common features were shared by mice belonging to the same line.

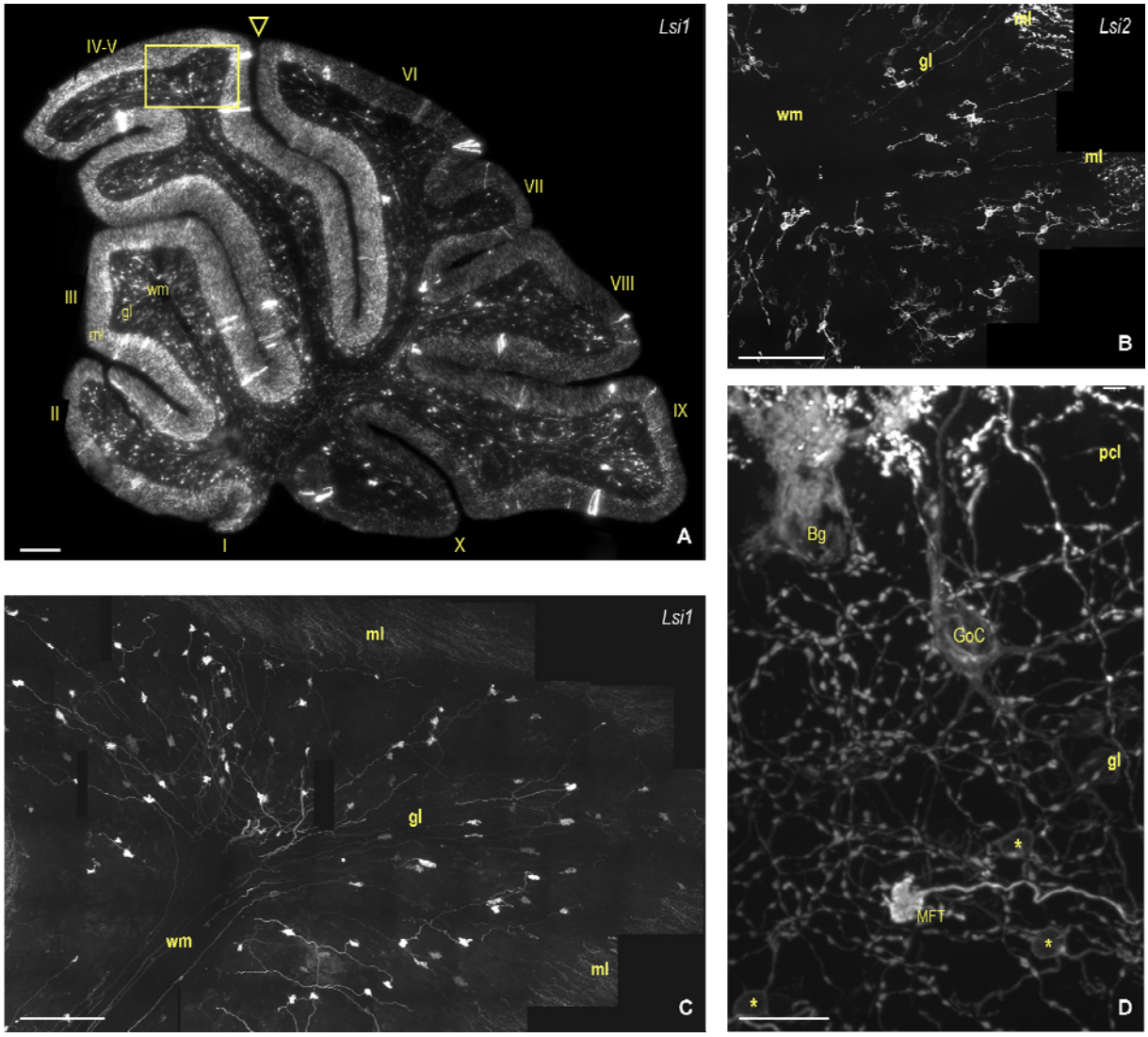


Figure 8 Transgenic mouse lines that express membrane-targeted GFP in few cells reveal neuronal morphology in the cerebellar cortex

A: sagittal mid-vermal section from an adult Lsi1 mouse (epifluorescence). Cerebellar cytoarchitecture is revealed by mGFP sparse labelling. Roman numbers indicate the lobule classification. The arrowhead indicates the primary fissure, interposed between anterior and posterior cerebellum. ML, where GC axons project, clearly reveals the anteroposterior gradient in the GC labelling; in lobule III an example of the preferential projection of GC axons to the deeper ML is evident. Sparse bright spots mostly represent MFTs. Intense bright signal spanning tangentially the ML is given by labelled Bg cells. **B**: Mid-vermal apical region of lobule V (see representative inset in A) of an adult Lsi2 mouse. Several MIPs (collapsed 40-µm confocal stacks) are tiled to represent the whole region. Sparse GC are labelled; labelling in the ML is given by GC axons. **C**: Tiled 40 µm-MIPs spanning the mid-vermal apical region of lobule V (see representative inset in A) of an adult Lsi2 mouse. Fibers running in the wm are MF afferents projecting to the distal lobule V; bright signal represents individual MFTs; dim signal in the ML is given by GC axons. **D**: 40 µm-MIP where a small cortical region displays labelling of different cell types. Bg arborisation develops in the ML (not visible here); an isolated GoC and part of its axonal compartment are included in the MIP; asterisks mark three GCs; the extensive neurite network decorated with varicosities represent a neuromodulatory axon innervating a defined cortical region. Bars in A, B, C: 300 µm; D: 20 µm.

Lsi1 and Lsi2 labelled sparse GCs in all cerebellar lobules, but a clear antero-posterior gradient in the number of labelled cells was evident in cerebellar parasagittal sections: denser expression was detected in the anterior cerebellum (lobules I-V), while few scattered cells were found in lobule IX and X (Figure 8A). GC morphology revealed by mGFP labelling was fully compatible with classic findings (Figure 12A). GCs are characterized by a small soma (6-8 µm diameter) and 2-6 dendritic processes

emerging from the soma (~40-50% of the cells exhibited 4 dendrites) of variable length (20-30 µm in average; rarely, processes reached 40-50 µm of length). Every dendrite ending is characterized by a terminal "claw" made of several short digits (5-6 µm in average). GC axon is a thin projection ascending towards the molecular layer. In the mouse line chosen for this study, namely *Lsi2*, very rarely labelled GC dendrites were contacted by labelled MFs, or GC claws belonging to distinct cells converged onto the same glomerulus. Interestingly, despite of their sparse position on the granular layer, labelled GC axons, the parallel fibers, were often observed to be enriched at specific molecular layer levels, in particular close to the Purkinje cell layer (see for example lobule III in Figure 8A).

Mossy afferents were detected in all the lobules, but their presence in lobule X was particularly poor (Figure 8A).

Labelled MFs were heterogeneous with respect to the axonal caliber, branching frequency, MFT density and presynaptic terminal morphologies. In particular, MFT were very diverse based on the following properties: 1) surface convolution, as smooth terminals coexisted with extremely lobulated ones; 2) number and length of filopodia/processes: a fraction of MFTs did not have any process, while the others ranged from 1 to 20 processes of 2-10 µm length in average; 3) size. Given the variety of extracerebellar sources for the MF axons, most likely, many of these differences were due to their different anatomical origin.

A further effort was made in order to characterize the anatomical origin of the labelled MFs in the *Lsi* lines object of our studies. *Lsi* pattern was analyzed in parallel with *Lmu* pattern, whose massive labelling allowed easier identification of the precerebellar nuclei and axon bundles. Analyzing the anatomical sites of the inferior and middle cerebellar peduncles, known to be the afferent ways to the cerebellar cortex, we were able to observe labelled corticocerebellar fibers in *Lsi* lines (Figure 9A-B), while none or few labelled spinocerebellar projections were found (Figure 9D, compare with 9C).

We could further exclude the labelling of primary vestibular afferences to the flocculonodular lobe, as these axons are known to run through the inferior peduncle, which in our hands did not present GFP-labelled fibers (Figure 9D). A more detailed analysis of the expression pattern in the brainstem revealed the presence of few

labelled somata representing several different nuclei. In particular, sparse GFP positive neurons were found in the pontine (Figure 9E) and vestibular nuclei (Figure 9F), as well as in other minor brainstem nuclei (Figure 9G-H), in some cases projecting to the cerebellum (*e.g.* cuneate nucleus, not shown). It is worth noting that the inferior olivary nucleus, where the CFs arise from, was found to be GFP negative. This is compatible with the absence of labelled climbing fibers in the cerebellar molecular layer (Figure 8A-C).

Thus, our analysis revealed that the MFs that are labelled in the cerebellar cortex of our *Lsi* lines widely represent the afferent populations, with a prevalence of pontine and vestibular projections. In addition, a patchy expression within several brainstem nuclei was observed, thus increasing the heterogeneity of the MFs object of our study in the cerebellar cortex.

The scattered mGFP expression pattern in the cerebellar cortex, together with large scale confocal imaging, allowed us to reconstruct in crisp details the three-dimensional morphology of neuronal cells and to collect quantitative data about the morphological features of a significant number of neuronal cells per animal.

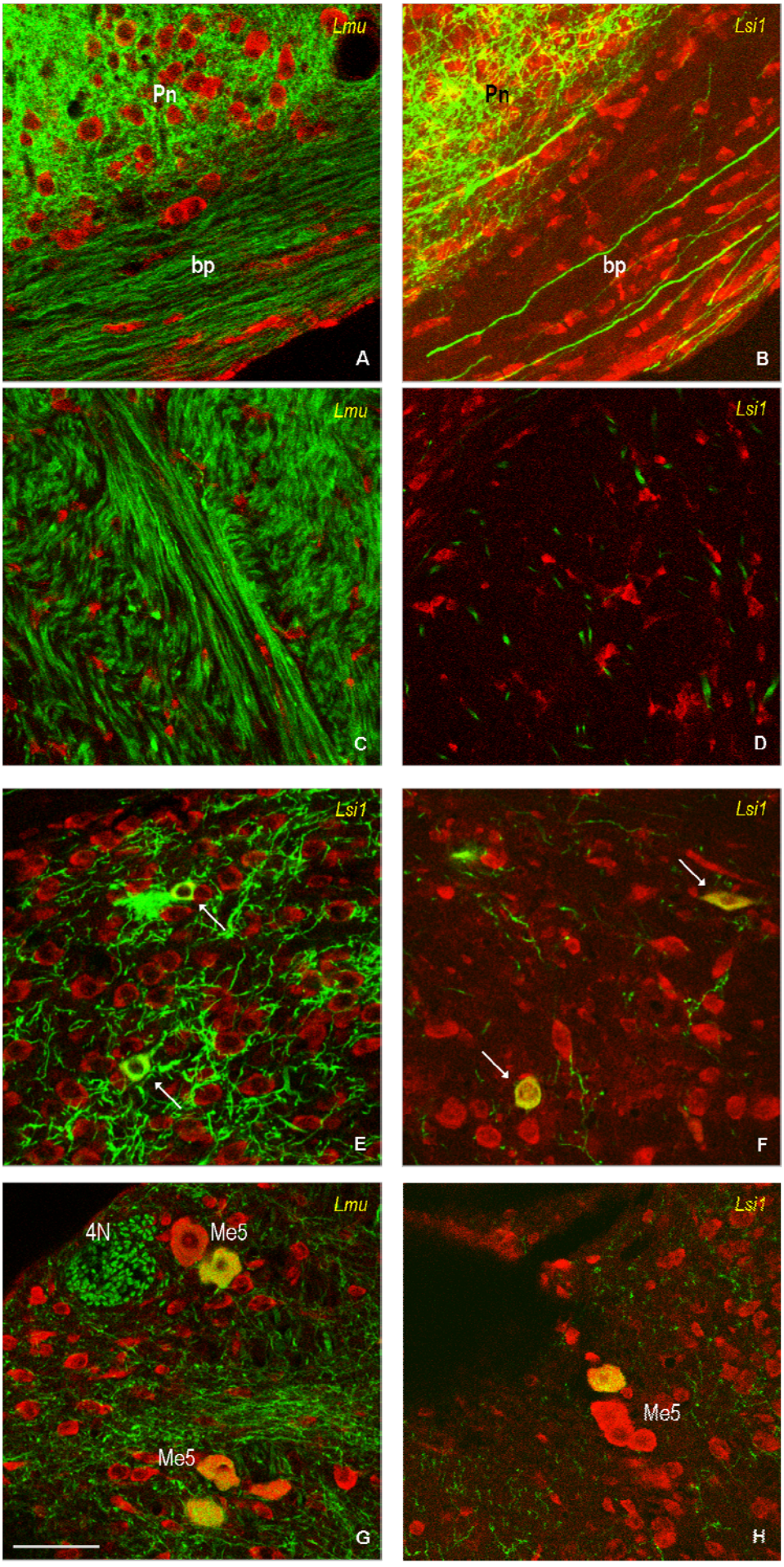

Lmu
Pn
bp
A
Lsi1
Pn
bp
B
Lmu
C
Lsi1
D
Lsi1
E
Lsi1
F
Lmu
4N
Me5
Me5
G
Lsi1
Me5
H

Figure 9

Figure 9 Labelled projections to the cerebellar cortex represent a heterogeneous MF population
Lsi1 **or** ***Lmu*** **brainstem slices from adult mice were fixed and counterstained with fluorescent Nissl to visualize neuronal cell bodies. All the panels are single confocal planes, unless otherwise stated. Green: mGFP; red: Nissl. A-B**: ***brachium pontis*** **(middle cerebellar peduncle) made by corticocerebellar projections.** ***Lmu*** **(A) shows a massive labelling of these afferents, while in** ***Lsi1*** **(B, 20-μm MIP) scattered axonal projections are running from pontine relay to cerebellar cortex. C-D: restiform body (inferior cerebellar peduncle) shows massive presence of spinocerebellar projections in** ***Lmu*** **(C), while the same projections are extremely few, if not absent at all, in** ***Lsi1*** **(D). E: red stained cells represent pontine nucleus neurons; two double-labelled cells are pointed by arrows. F: Nissl stained vestibulocerebellar nucleus; two double-labelled neurons are pointed by arrows. G-H: mesencephalic trigeminal nucleus (Me5) shows patchy mGFP expression both in Lmu (G) and Lsi1 (H). Note trochlear nerve fascicles (4N) running to innervate the superior oblique muscle of the eye. Bar: 50 μm.**

Experience is able to induce sustained morphological plasticity at the input stage of the adult cerebellar cortex

We aimed at describing the anatomical plasticity events triggered by enriched environment (EE) experience in the adult mouse. To this purpose, we carried two sets of experiments:

i. *"regular EE"*: mice were housed with parents and littermates from birth date to P30; then 3-4 female mice were moved together to an enrichment cage (see Experimental Procedures) and sacrificed at P50 (20 days of EE, "regular EE"). Control mice ("CTRL") were kept in the same conditions of the EE group until P30 and then moved to standard single cage until P50.

ii. *"EE colony"*: a breeding was set in an EE cage; newborn mice were housed in the EE cage with parents and littermates since birth to P30. At P30, a first group of animals was sacrificed ("EE baseline"); a second group of 3-6 female mice was moved to another EE cage. Animals were then sacrificed at P50 ("EE colony", 50 days of EE). A parallel control breeding was set simultaneously in standard cages (control parent mice were littermates of EE parents). Newborn control mice were kept with parents and littermates until P30. At P30 a set of control mice was sacrificed ("CTRL baseline"); another set was moved to standard single cages until P50 and then sacrificed ("CTRL").

It is worth noting that P50 control animals undergo the same experimental conditions in the "regular EE" and in the "EE colony" experiments.

We focused our analysis on the input stage of the cerebellar cortex in lobule V, namely the synapse between MFTs and GC dendrites. MF axons build a large and

complex presynaptic terminal, whose postsynaptic elements are individual GC dendrites, each of them ending in a claw that receives synaptic input from one MFT. The arrangement at cerebellar glomeruli has advantageous properties to investigate pre- and postsynaptic morphologies.

EE experience affects MFT density and axonal branching

We wondered whether enrichment in the adulthood (P30-P50, "regular EE") is able to affect the general connectivity properties of the mossy axons, such as the number of MFTs or the number of branching points within one lobule. To this purpose, we traced stretches of individual MF axonal arbors innervating lobule V on reconstructed 3D data sets and compared their branching patterns as well as the density of MFTs on these individual axons (Figure 10).

The mossy axons revealed very heterogeneous properties: in particular, we found that MFT density relatively to the axonal length was extremely variable between different axons (Figure 10B; see also Figure 11C); in addition, MFTs could be regularly placed along the projection, evenly spaced along the axon, or rather concentrated in short stretches despite of long solved axonal tracts (Figure 10B). Branching points were found at *en passant* MFTs (when processes arising from one MFT were able to build other MFTs) or as bifurcations along the axonal projection (Figure 10A-B). We observed that mGFP labelled axons were particularly enriched in branching points in the apical and medial region of lobule V, what we believe to be related to the cerebellar anatomy of projections (Figure 10A).

This analysis revealed that MF axons of EE mice exhibited a higher branch point- and MFT frequency than those of CTRL animals (Figure 11).

The trend was verified in two different transgenic mouse lines, *Lsi1* (1 animal per condition) and *Lsi2* (3 animals per condition). Despite of different absolute values, both lines displayed changes of very similar magnitude (Figure 11D). In particular, we found that the increase in the MFT density was 15% in *Lsi1* (not shown) and 24% in *Lsi2* (Figure 11A). Branching properties were dramatically affected upon EE, being the branching points increased by 52% in *Lsi1* (not shown) and 64% in *Lsi2* (Figure 11B), relatively to axonal length.

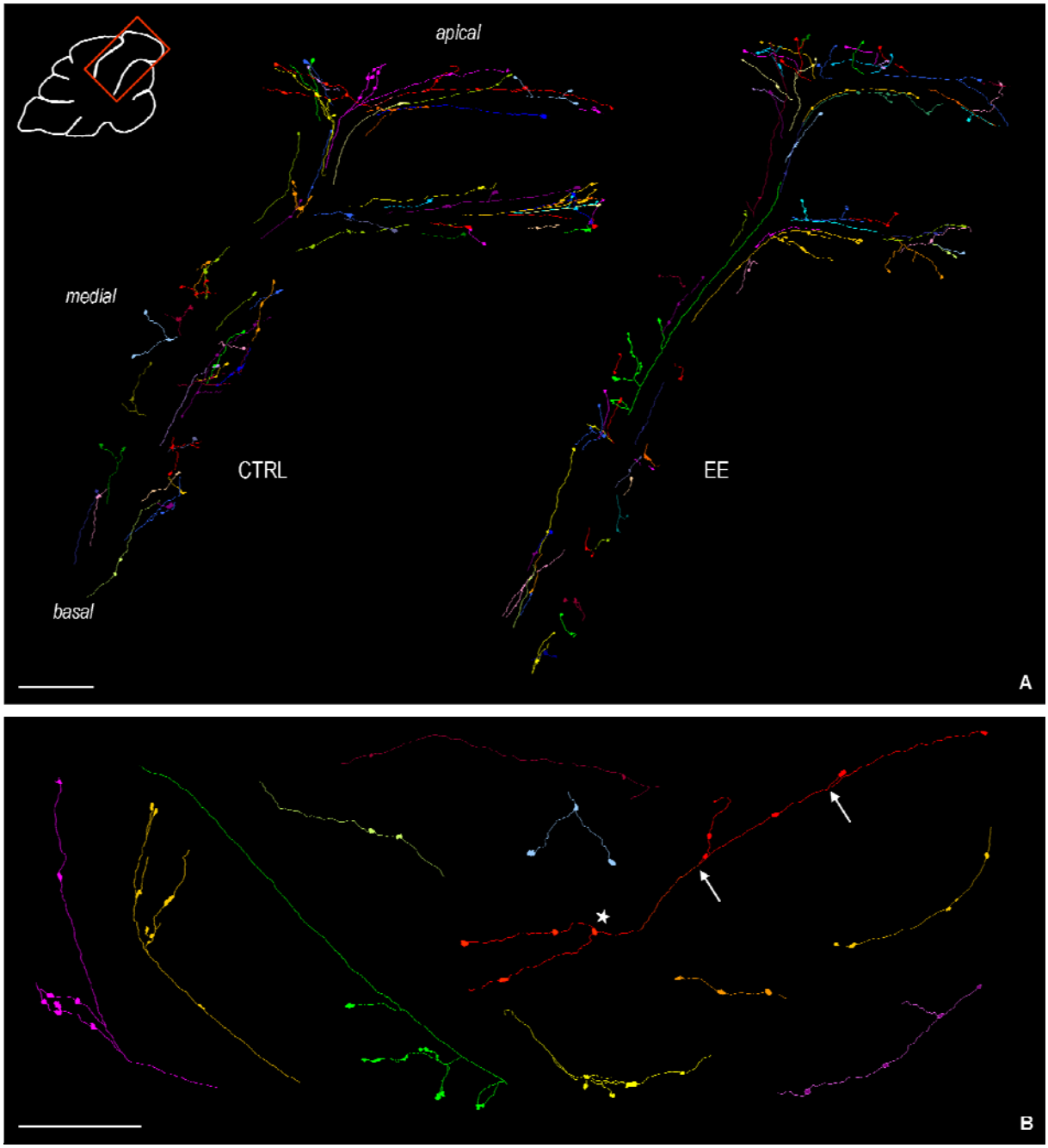


Figure 10 Experience induces rearrangements of MF connectivity in the adult
Camera lucida of traced axons obtained from 3D data sets. **A**: in the upper corner, a red inset shows the cerebellar lobule V that was reconstructed and analyzed; a representative set of solved axons is shown per each experimental condition. **B**: representative axons from the two data sets in A are illustrate the variety in terminal density and branching frequency between different axons. The green axon shows accumulation of MFTs and branching at one subregion, while the axon red presents distributed MFTs and branching points. The latter axon presents two branch points from the main axon (arrows) and one arising from an *en passant* MFT (star). Bars: 250 μm.

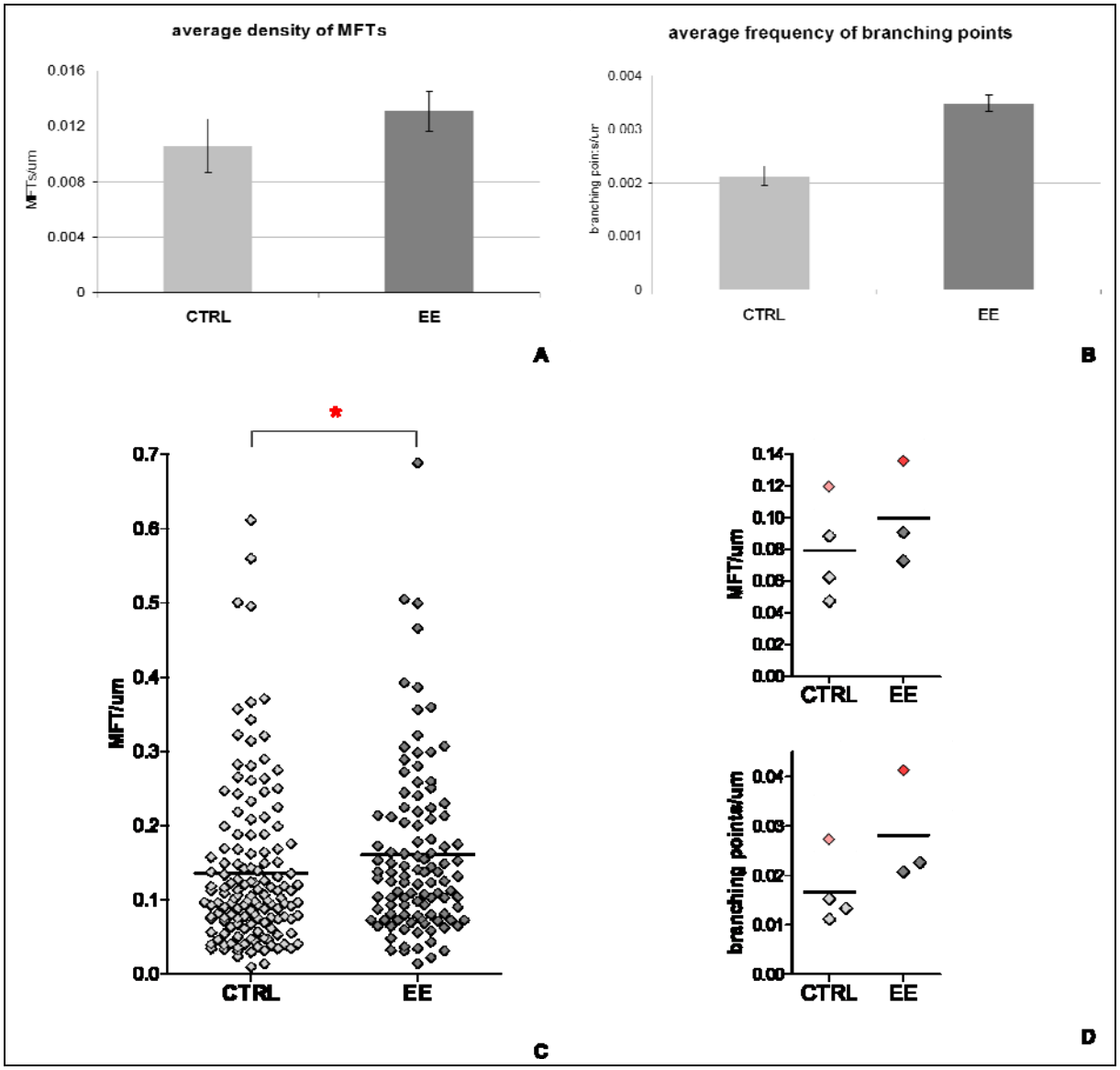


Figure 11 Experience influences axonal connectivity in the adult cerebellar cortex
A: quantitative analysis of MFT density in *Lsi2* line. The number of MFT is normalized to the total axonal length traced per each subject; n = 3 mice per condition; columns represent mean ± SEM. **B**: quantitative analysis of branching point frequency in *Lsi2* line. The number of branching points is normalized to the total axonal length traced per each subject; n = 3 mice per condition; columns represent mean ± SEM. **C**: quantitative analysis of MFT density in *Lsi1* and *Lsi2* line showing the number of MFT per axonal length; N_{CTRL} = 141 axons; N_{EE} = 101 axons; n = 4 mice per condition; bars represent mean. Post hoc Student's t-test: *p < 0.05. **D**: quantitative analysis of MFT density (upper graph) and MFT branching (lower graph), every diamond represents one subject; red dots are *Lsi1*, grey diamonds are *Lsi2*. Bars represent mean.

Additional observations made in our laboratory, confirmed the same results about MFT density and branching pattern in animals that experienced EE later in the adulthood, from P60 to P90 (N. G., unpublished data).

We compared the single axon values for the two parameters we took in exam between control and EE: we found that the range of MFT densities and axonal branching was similar in the two conditions (Figure 11C). This suggests that only a subset of the axonal population is responding to the EE condition with structural

rearrangements: in fact, if all the axons would respond similarly to the EE, we would rather found a general shift in the density distribution of the population.

When considering the average MFT density or the average branching frequency per each experimental subject, we found that EE condition clearly triggered an increase in the axonal density and branching points (Figure 11D). Interestingly, the inter-individual variation and the distribution range of the average values was kept similar upon EE. This argues for a response of similar amplitude by all the experimental subjects involved.

These findings provide first evidence that MF to GC connectivity considerably rearrange upon EE as mossy axons are able to build up a higher number of terminals and to increase their branching behaviour.

EE affects GC connectivity properties in their dendritic compartment

We next aimed at describing plasticity events taking place at the postsynaptic counterpart of the MF-GC synapse. To this purpose, we focused most of our analysis on *Lsi2* mouse line, whose scattered expression pattern in the granular layer allowed us to reconstruct 3D morphology of a representative number of GC dendrites in the cerebellar cortex (Figure 12B). We carried our analysis on the apical region of mid-vermal lobule V, taking advantage of large-scale confocal imaging of the region of our interest (spanning about 2-2.5 cm) and then built a 40-µm three-dimensional dataset. Cells that were entirely included in the stack and sufficiently isolated to be completely solved were included in the analysis (Figure 12B). This approach, made possible by the peculiar GC morphology, allowed us not only to study the plasticity of single synaptic endings, but also to study the global cell structural rearrangements taking place upon experience.

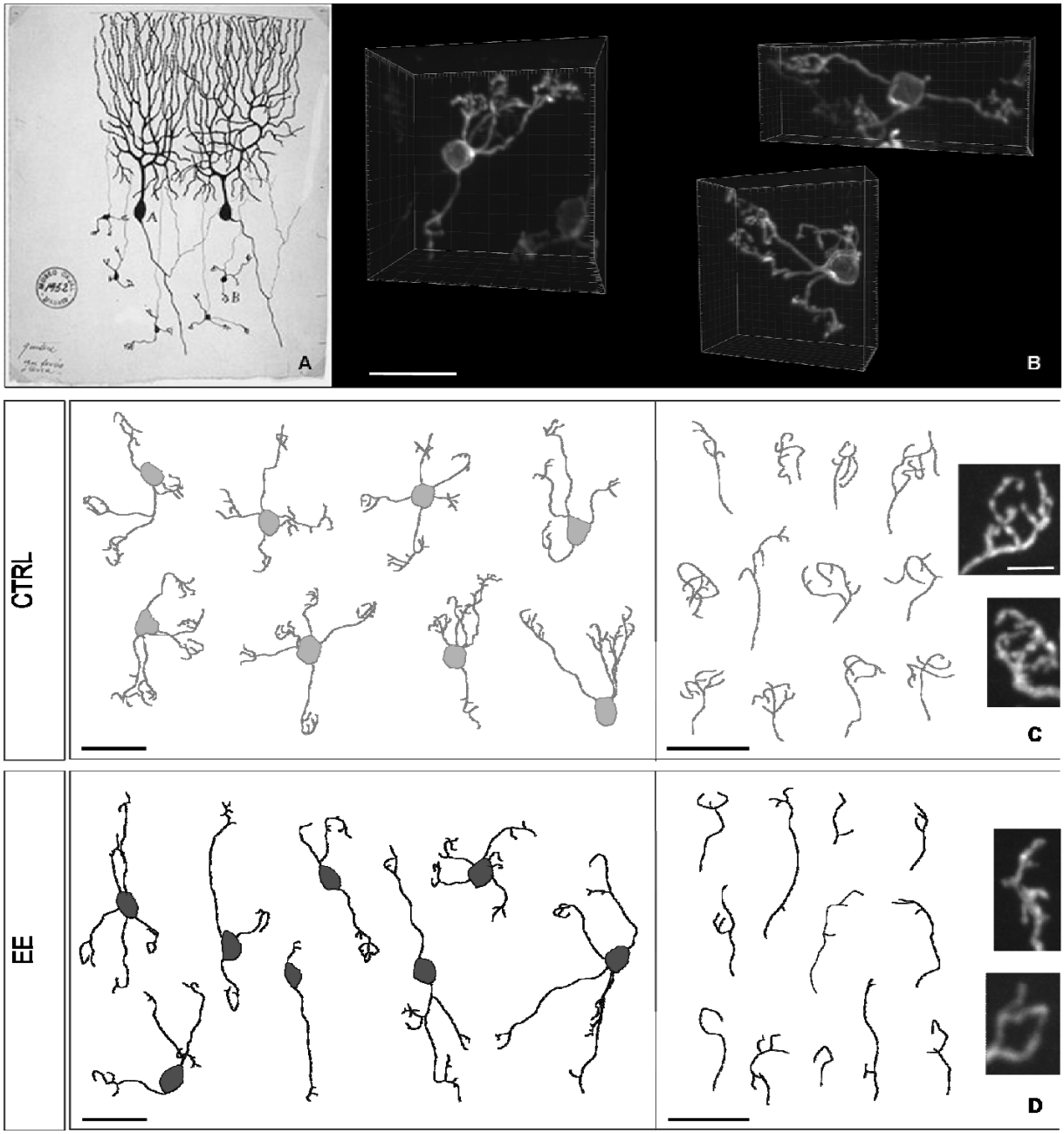


Figure 12 **Experience affects GC connectivity in their dendritic compartment**
A: Drawing of PCs and GCs ("B" in the drawing) from pigeon cerebellum by Santiago Ramón y Cajal, 1899. Instituto Santiago Ramón y Cajal, Madrid, Spain. **B**: Volume projections of individual mGFP labelled GCs. **C-D**: representative gallery of GC morphologies in CTRL or EE condition (left); representative dendritic endings are shown on the right. Insets: MIPs showing isolated claws. Bars in B, C, D: 20 μm, insets: 5 μm

We took in exam the GC dendritic number and the dendritic ending (claw) morphology.

First, we compared the number of dendrites characterizing mGFP-labelled GCs. In the control population (see Figure 12C for some representative examples), 39% of the GCs was characterized by 4 dendrites, being the most represented group, while 33% displayed 5 dendrites and 23% had 3 dendrites (Figure 13A).

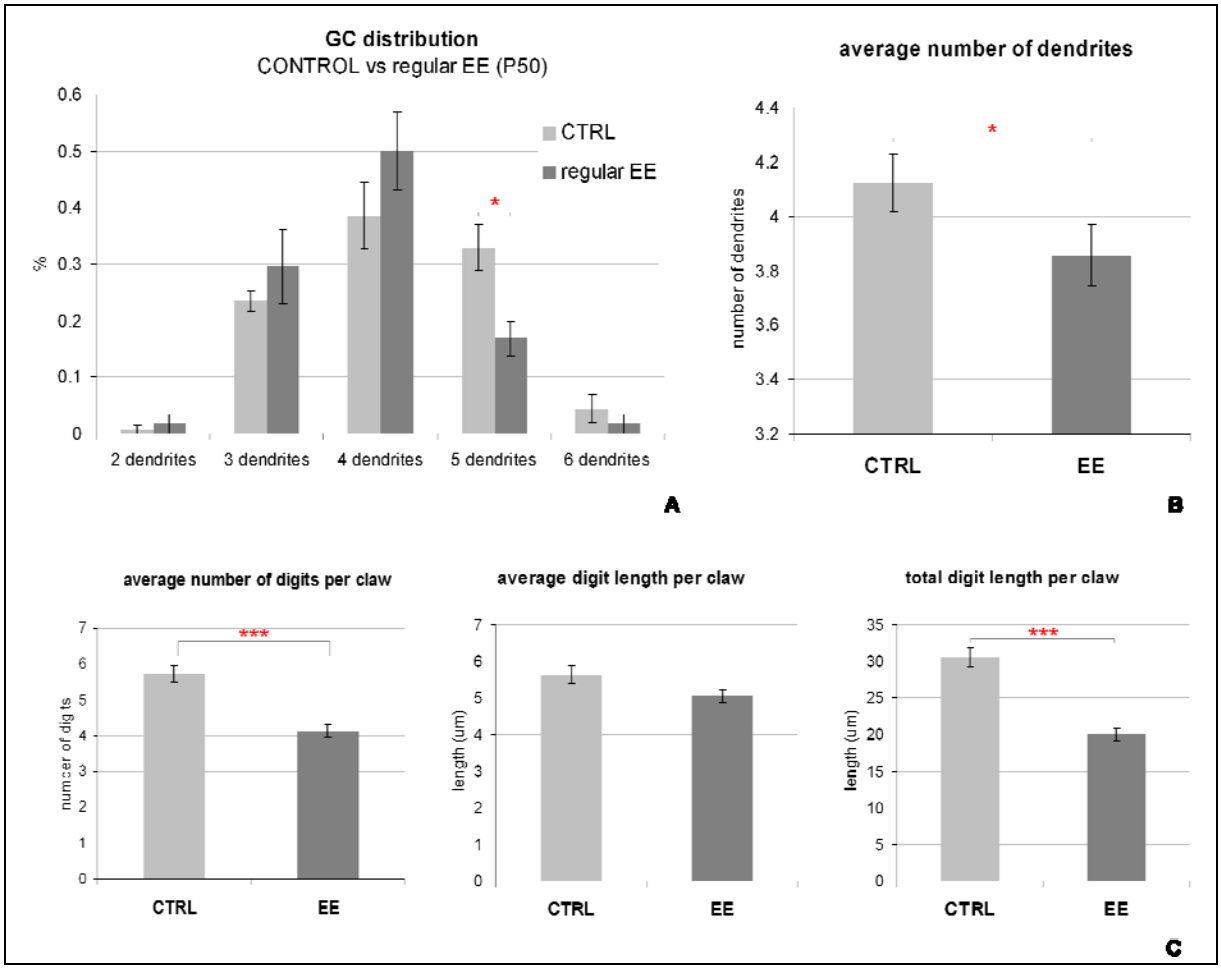


Figure 13 Experience induces rearrangements of GC dendritic properties
A: analysis of GC population distribution according to the number of dendrites; N_{CTRL} = 8 mice; N_{EE} = 4 mice. **B**: average number of dendrites per GC; N_{CTRL} = 119 cells (8 mice); N_{EE} = 49 cells (3 mice); columns represent mean ± SEM. Post hoc Student's t-test: *p = 0.0431. **C**: quantitative analysis of the claw morphology (P50) according to digit number, average- and total length within one claw; N_{CTRL} = 74 claws (3 mice), N_{EE} = 83 claws (3 mice); columns represent mean ± SEM. Post hoc Student's t-test: digit number, ***p < 0.0001; average digit length, p = 0.18 (n.s.); total digit length, ***p = 0.0006.

Conversely, we found that in EE mice (see Figure 12D) this distribution was shifted towards a minor number of dendrites. In particular, 50% of GCs was characterized by 4 dendritic processes, still representing the biggest group as in the control population, but only 17% of the GC population had 5 dendrites. Consequently, upon EE about 31% of the GCs was characterized by 2 or 3 dendrites, while this group represented only the 24% of the control population (Figure 13A). This shift in the GC distribution consequently leads to an effect on their connectivity properties: this change is revealed when looking at the average number of GC dendrites in the different conditions. GC control population displays in average 4.17 ± 0.1 dendrites, while, upon "regular EE", GCs have in average 3.86 ± 0.1 dendrites (p = 0.0431 according to Student's t-test; Figure 13B). As, in general, every claw is able to contact one MFT, this shift means that the GC population is contacting a lower

number of MFTs; at the same time, fewer postsynaptic counterparts are available to MFTs for synaptic transmission.

Second, we analyzed the ending claw morphology within entirely solved GCs. It is worth noting that we generally found that claws belonging to the same cell displayed a very similar morphology in terms of covered presynaptic area and digit length/number (Figure 12). We noticed that the terminal claws appeared to cover a smaller presynaptic territory in the EE animals, being their complexity and the number of digits reduced. This was made evident when measuring the average number of digits making a claw (from 5.7 ± 0.3 to 4.1 ± 0.2 digits, namely a reduction of the 28%) and the average digit length within one claw (from 5.63 ± 1.39 μm to 5.05 ± 0.90 μm); the total reduction in the digit length was by 34% (Student's t-test $p < 0.0006$; Figure 13C).

Taken together, these observations suggest that GCs respond to EE experience in the adulthood rearranging the balance of the synaptic inputs they get from the presynaptic MFTs in two coexisting ways:

- the number of presynaptic terminals, and thus cells, that each GC is contacting is in average reduced;
- the synaptic transmission at each MF-GC synapse is affected by changes in morphology.

EE starting in the early postnatal period enhances postsynaptic structural rearrangements

We next wondered if the environmental enrichment would differentially influence synaptic connectivity if experienced during the adulthood or starting from early postnatal development. To this purpose, we set up parallel breedings, one held in a standard cage and the other in an EE cage (see Experimental Methods).

Experimental mice aged of 30 days were first analyzed ("baseline CTRL" and "baseline EE"). Both groups lived from birth with their parents and littermates; control mice were housed in a standard cage, while EE colony mice were born and kept in the EE cage.

First, we analyzed the GC distribution according the number of their dendrites in the "CTRL baseline" mice. We found that 70% of the GCs displayed 4 dendrites and consequently constituted the predominant population; 20% of the cells was characterized by 2 or 3 dendrites and 10% of the cells had 5 dendrites (Figure 14A). This distribution resulted to be extremely preserved across mice. Interestingly "CTRL baseline" distribution (P30) looked very different from the later distribution at P50 (Figure 14C). This indicates that physiological GC connectivity is still rearranging from P30 to P50.

Comparing the two experimental conditions at P30, "EE baseline" mice also showed a peak in their distribution at 4 dendrites, but the distribution curve resulted to be much flatter than in control animals, being 41% the fraction of cells displaying 4 dendrites, 32% the fraction characterized by 3 dendrites and about 25% the cells with 5 or 6 dendrites (Figure 14A). Interestingly, when comparing the two chosen time points (P30 and P50), we found that the distribution pattern for "EE baseline" GCs rather resembled the distribution of CTRL GCs at P50 (Figure 14A). The average number of dendrites for the GC population was not significantly different between CTRL and "EE baseline (3.85 ± 0.15 dendrites for the control population versus 4.05 ± 0.22 in the EE population, $p = 0.7873$ according to Student's t-test, not shown).

From these evidences, we conclude that, despite "baseline CTRL" and "baseline EE" GCs are distributed differently at P30 when considering the number of dendrites, yet the trend we previously described upon 20 days of "standard EE" during adulthood (P30-P50) is not established after enrichment during the first 30 days of life.

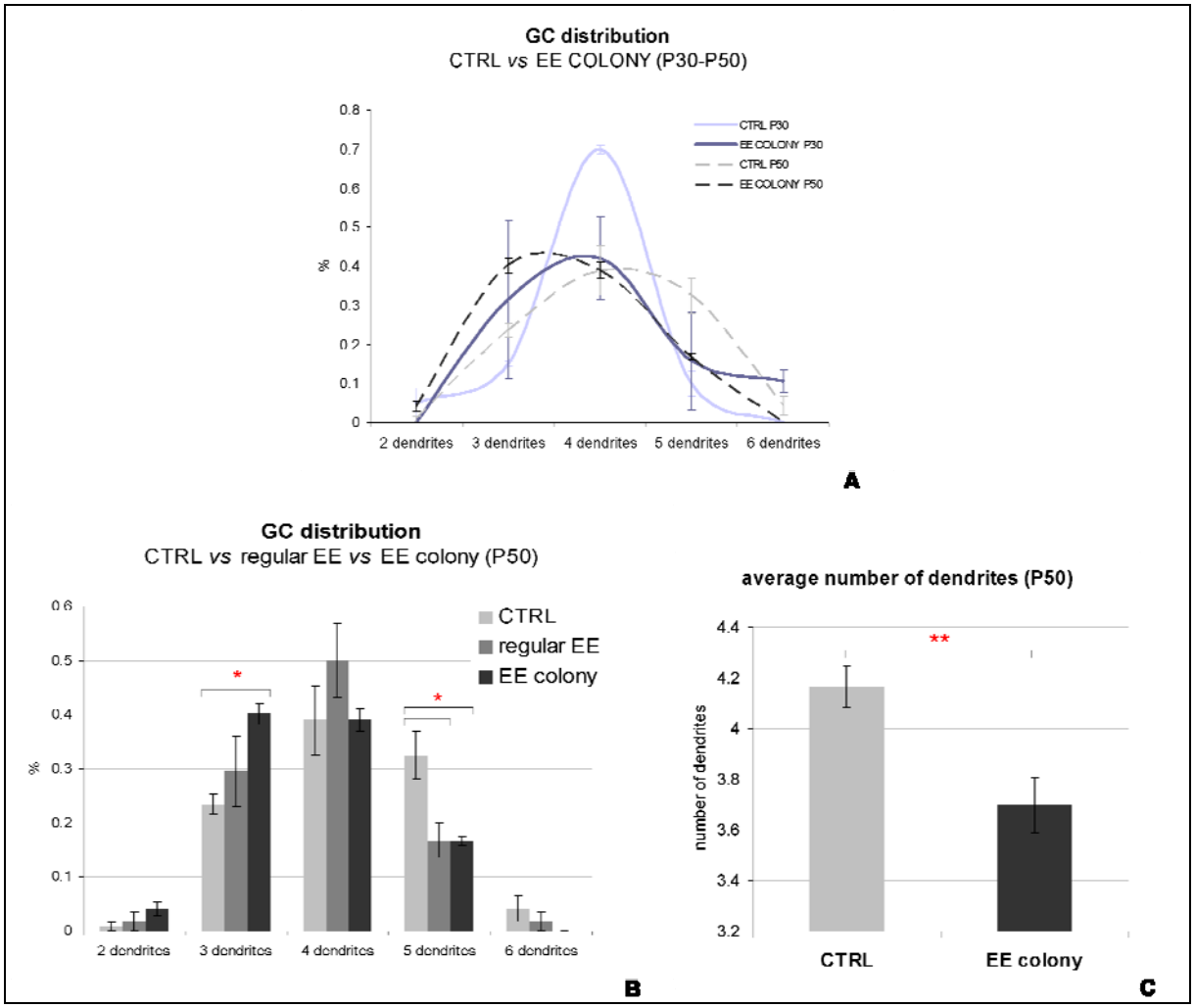


Figure 14 Early EE dramatically affects postsynaptic structural rearrangements
A: analysis of GC population distribution according to the number of the dendrites at P30; N_{CTRL} = 2 mice; N_{EE} = 2 mice; GC population distribution (CTRL and EE) according to the number of the dendrites at P30 and P50 is shown for a comparison. **B**: analysis of GC population distribution according to the number of the dendrites for CTRL (n = 8 mice), regular EE (n = 3 mice) and EE colony (n = 5 mice); columns represent mean ± SEM. Post hoc Student's t-test: CTRL *vs* EE colony (3 dendrites, 5 dendrites) *p < 0.05. **C**: average number of dendrites per GC; N_{CTRL} = 119 cells (8 mice); N_{EE} = 53 cells (5 mice); columns represent mean ± SEM. Student's t-test: **p = 0.0028.

We previously described that EE from P30 to P50 ("regular EE") is able to induce a shift of the GC population towards a smaller number of dendrites (Figure 13A). We performed the same distribution analysis on "EE colony" mice that were sacrificed at P50, and compared with proper controls. The same redistribution was found in animals that were enriched since birth (Figure 14B, C); furthermore, the "EE colony" protocol, compared to the "regular EE" could additionally enhance the redistribution of GCs: in the "EE colony" cerebellar cortex, 3- and 4- dendrite GC populations were equally represented, each group constituting about 40% of the population. Only 17% of the GCs had 5 dendrites, while in control condition this group represented about 37% of the population (Figure 14B). When looking at the average number of dendrites per GCs, we found a decrease from 4.17 ± 0.1 dendrites

per cell to 3.69 ± 0.1 (Figure 14C). Despite the apparent small difference in the connectivity properties triggered by EE (average reduction of 0.48 dendrites per cell), these changes yield to dramatic effects on the connectivity at the input stage of the cerebellar cortex, as we will discuss later.

We therefore conclude from these observations that:

- GC distribution based on the dendrite number is undergoing physiological rearrangements from P30 to P50;
- "EE baseline" population differs from the proper control population and rather distributes similarly to P50 CTRL population;
- at P50, animals that experienced EE since birth date ("EE colony") further redistribute their GC population towards fewer dendrites when compared to animals that were enriched only from P30 to P50 ("regular EE").

We next analyzed the synaptic claw morphology by comparing the average digit length within one claw, the total digit length per claw and the number of digit per claw.

We found that generally P30 control claws were characterized by smaller values than P50 control claws, meaning that, in physiological conditions, claws are dynamically rearranging their morphology from P30 to P50 (Figure 15A). Our data show that, from P30 to P50, the average digit length of the control population is increased from 5.07 ± 0.15 µm to 5.63 ± 0.25 µm, the number of digits goes from 4.8 ± 0.2 to 5.7 ± 0.2 and the total digit length per claw goes from 23.89 ± 1.06 to 30.56 ± 1.39 µm (Figure 15A).

Despite of the ongoing remodelling processes concerning physiological development/aging, we found that the rearrangements induced by experience on the synaptic ending morphology were already established by P30 in the mice grown in EE, with a very modest rearrangement still ongoing from P30 to P50 in the EE condition: in particular, the average digit length was 4.73 ± 0.18 µm at P30 and 4.54 ± 0.10 µm at P50; the digit number per claw was found to be 4.2 ± 0.2 at P30 and 4.4 ± 0.1 at P50; the total digit length was 19.60 ± 1.20 µm at P30 and 19.28 ± 0.91 at P50 (Figure 15A, B).

In conclusion, when comparing data from two different time points in the "EE colony" experiment, we found that synaptic claw morphology is physiologically rearranging from P30 to P50; nevertheless, EE induced claw plasticity is soon established, as the rearrangements were present at P30 with minor effects added until P50. In addition, when comparing the two different experimental sets, namely "regular EE" and "EE colony", we found that the latter paradigm induced structural rearrangements similar to the "regular EE", but the amplitude of the rearrangements (in particular, concerning the length of digits building a single claw) was further increased (Figure 15A).

EE experience affects MFT morphology

Animals that were grown in an EE, and sacrificed at the age of 50 days, displayed dramatic structural rearrangements in the GC dendritic compartment, and particularly in the number of dendrites (Figure 14) and in their terminal ending morphology (Figure 15). In addition, we showed that EE is able to affect mossy axon properties, such as branching behaviour and presynaptic terminal density (Figure 11).

We wondered if this complex series of events implied rearrangements at the level of the single MFT morphology. We then aimed at describing the structural rearrangements occurring presynaptically at the MF-GC synapse, namely at the MFTs, placed in GL by afferent fibers entering the cerebellar cortex. We carried our analysis on the apical region of mid-vermal lobule V: in order to make conclusions about the population behaviour, we included in our analysis all the MFTs which were completely included in our 3D datasets. Our *Lsi2* line labels few mossy axons, still we were able to reconstruct the morphology of 50-60 MFTs per condition (3 animals per group, Figure 16A-B). Measurements were pooled according to the experimental group.

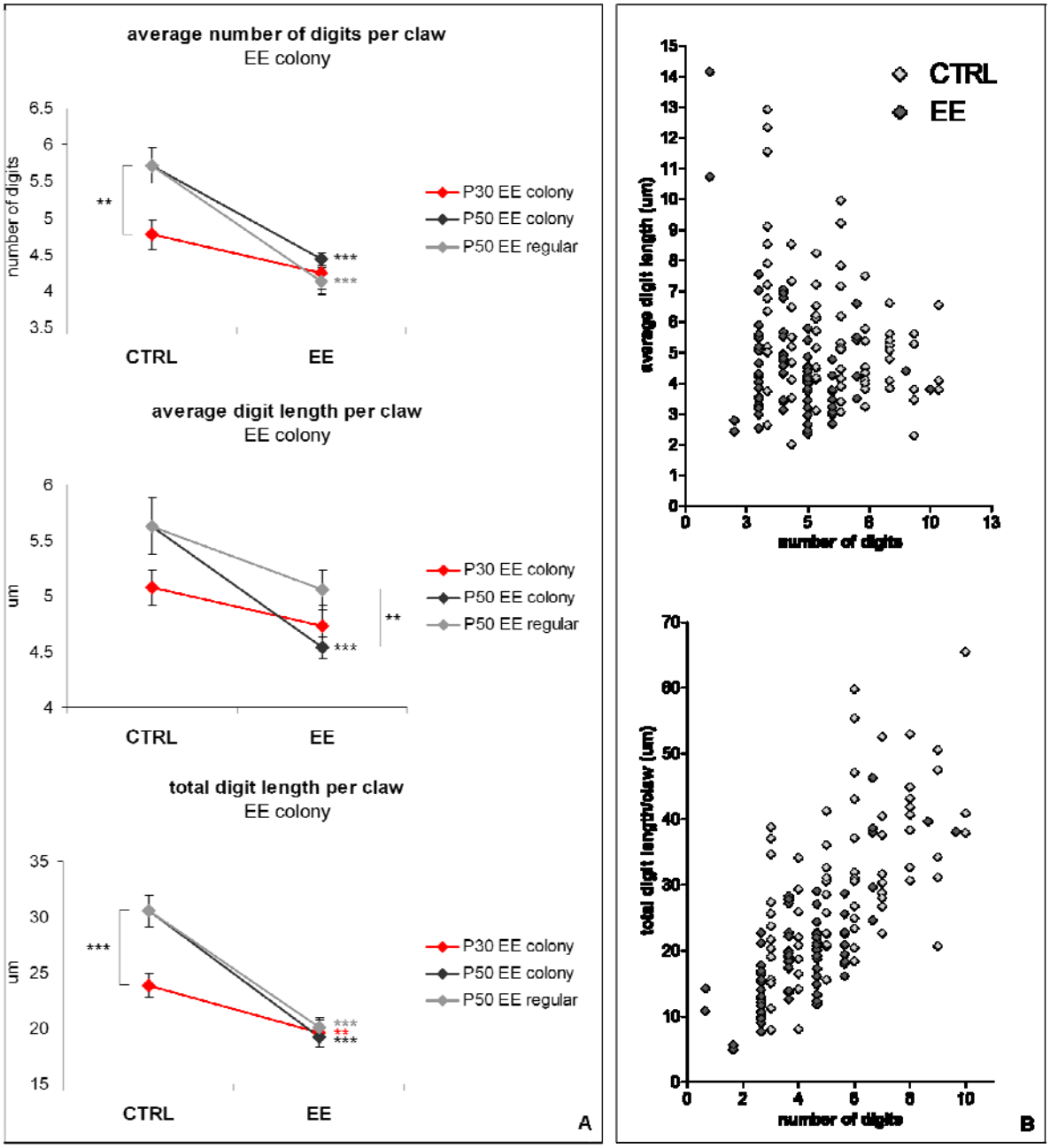


Figure 15 Early EE affects claw morphology similarly to adult experience
A: quantitative analysis of the claw morphology (P30 and P50) according to digit number, average- and total length within one claw; N_{CTRL_30} = 91 claws (2 mice), N_{EE_30} = 77claws (2 mice); N_{CTRL_50} = 74 claws (3 mice), N_{EE_50} = 83 claws (3 mice), N_{EECOL_50} = 77 claws (3 mice); diamonds represent mean ± SEM. Post hoc Student's t-test: **p < 0.005, ***p < 0.0001. **B**: correlation between the number of digits and average- or total digit length compared between control (N = 74) and "EE colony" (N = 77) at P50.

We took in exam three parameters describing MFT morphology:

- size (calculated on the maximum intensity projection, MIP, area, as described in the Experimental Procedures);
- complexity, namely the convolution and irregularity of the terminal surface (see Experimental procedures);
- process number and length.

First, MFT size was calculated in control and in "EE colony" animals. We noticed that housing in an EE from birthday to P50 is able to induce a decrease of the MFT size by 25% in average (Figure 16C-D).

Second, MFT complexity was carefully analyzed. The general appearance of the MFTs did not look affected by the EE (compare Figure 16A and B), observation that was confirmed upon quantification in term of a "complexity index" (Figure 16F). Nevertheless, we noticed that a higher fraction of MFTs in the EE condition displayed a sort of "fragmented" morphology, possibly indicating dynamic structural processes taking place (Figure 16E).

Last, process properties were taken into account. Process length and number did not show a clear trend when comparing control subjects with EE subjects; however, in average the pooled EE population was characterized by fewer and shorter processes than the control condition (not shown).

In conclusion, "EE colony" induces a reduction of 25% of the average MFT size and a general shortening of processes; MFT surface complexity was not affected by EE.

EE effects on the synaptic connectivity at the input stage of the cerebellar cortex

We have shown that housing adult mice in an EE for 20 days (P30-P50) determines structural changes at the MF-GC synapse of the cerebellar cortex, both at the pre- and postsynaptic level. In addition to plasticity affecting cell morphology (in particular for what concerns the postsynaptic ending claws of the GCs), sustained reorganization of the connectivity is taking place. We noticed that EE determines an increase in the presynaptic terminal density by 24% in the Lsi2 (Figure 11A); at the same time, we have shown that the average size of the MFTs decreases by 25% (Figure 16D), which is fully compatible with the previous data. In addition, we have shown that GCs reduce their number of dendrites by ~13% when grown in EE (P0-P50, Figure 14C). Even though MF axon properties were measured for the "regular EE", we assume that the "EE colony" experience triggers similar events, based on the postsynaptic data (this assumption that most likely constitutes an underestimation of the "EE colony" effects on the presynaptic part). It is possible to explore the general effects of EE on the circuit connectivity by combining the data together.

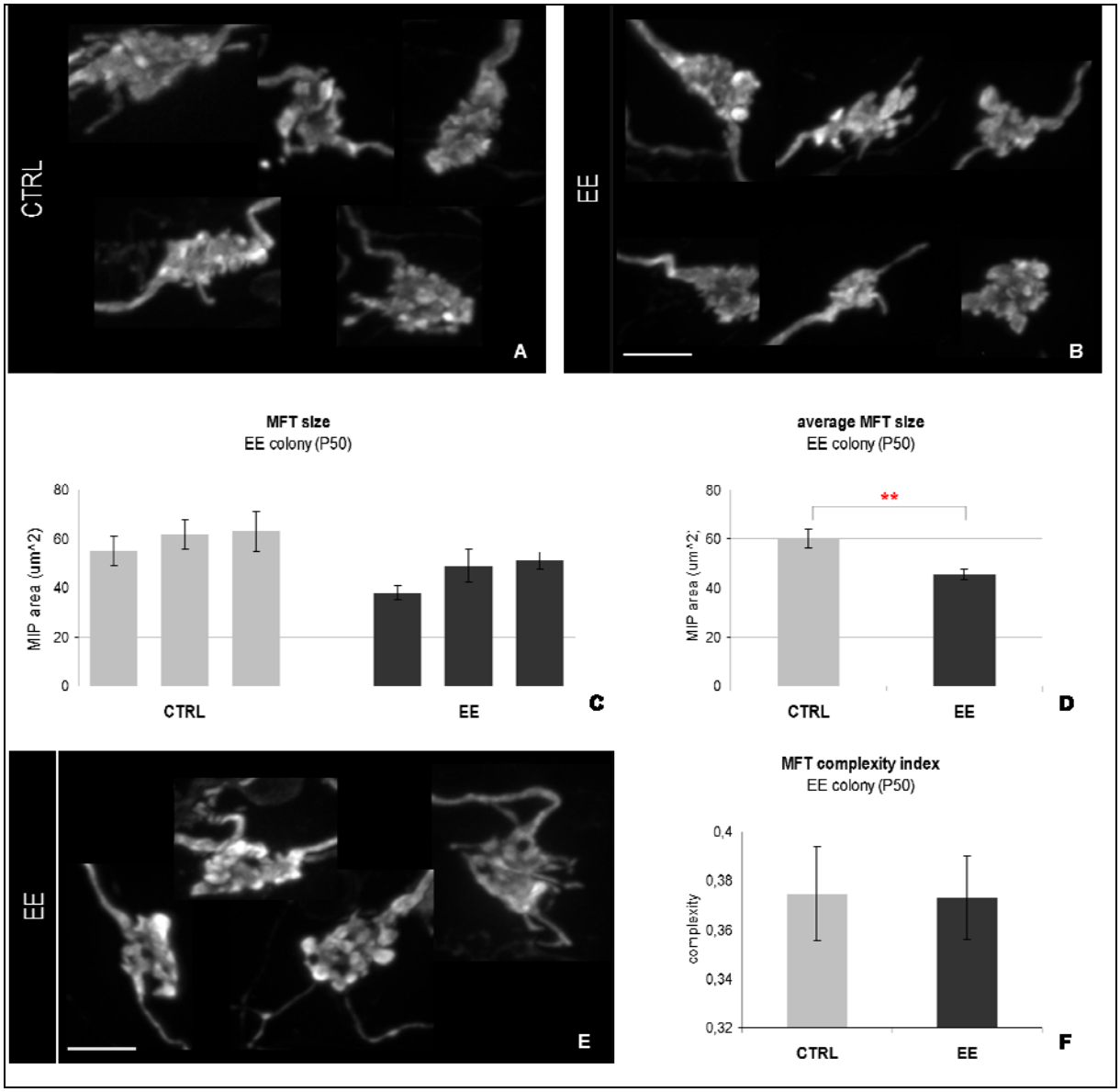


Figure 16 EE experience affects MFT morphology
A-B: representative MFTs cropped from 40 µm-MIPs. CTRL and EE morphologies are compared. **C**: quantitative analysis of MFT size comparing average values (±SEM) for 3 CTRL and 3EE subjects. **D**: quantitative analysis of MFT size; N_{CTRL} = 48 MFTs (3 mice); N_{EE} = 51 MFTs (5 mice); columns represent mean ± SEM. Post hoc Student's t-test: **p=0.0064. **E**: representative MFTs from EE conditions where a fragmented morphology is shown. F: quantitative analysis of MFT complexity; N_{CTRL} = 48 MFTs (3 mice); N_{EE} = 51 MFTs (5 mice); columns represent mean ± SEM. Post hoc Student's t-test: p = 0.81 (n.s.). Bars: 10 µm.

Considering that a single MFT is known to be in average contacted by 30 claws, or 30 GCs (Palkovits et al., 1972), we would like to quantify the effects on the synaptic connectivity determined by EE experience. We consider a representative ensemble of 100 MFTs (pre_{tot}) populating an ideal region. This MFT population, according to classic knowledge, "requires" a total number of claws, or a total number of GCs ($post_{tot}$), which is given by the following calculation:

$$pre_{tot} = (100 \cdot 30) = 3000 \text{ claws}$$

In the control condition, being 4.17 the average number of dendrites (or claws) per GC, the above calculated required number of claws is provided by

$$post_{tot} = (3000/4.17) \cong 720 \text{ GCs}$$

Upon EE, we have found an increase in the number of MFTs of the 24%; meaning that the MFT population is now consisting of

$$EEpre_{tot} = (pre_{tot} + 0.24 \cdot pre_{tot}) = 124 \text{ MFTs}$$

We have now to consider the reduction in the number of dendrites/claws per cell from 4.17 to 3.70. This means that the previously defined number of GCs, that we consider to be constant (as suggested by the anatomy of the granular layer, where the GCs are closely packed), provides upon EE

$$EE\ post_{tot} = 720 \cdot 3.70 \cong 2664 \text{ claws}$$

Thus, upon EE, 124 MFTs can arrange contacts with 2664 claws, namely each MFT is able to elicit synaptic transmission to

$$EE\ post_{MFT} = (2664/124) \cong 21 \text{ GCs}$$

In summary, in control condition every MFT is able to trigger synaptic transmission, and thus to amplify its signal, in 30 postsynaptic cells, while, upon EE, the same MFT is able to contact only 21 GCs (-30%). Additionally, and not considered in this paragraph, the total digit length per claws is reduced by 37% upon EE, further affecting the transmission (Figure 17).

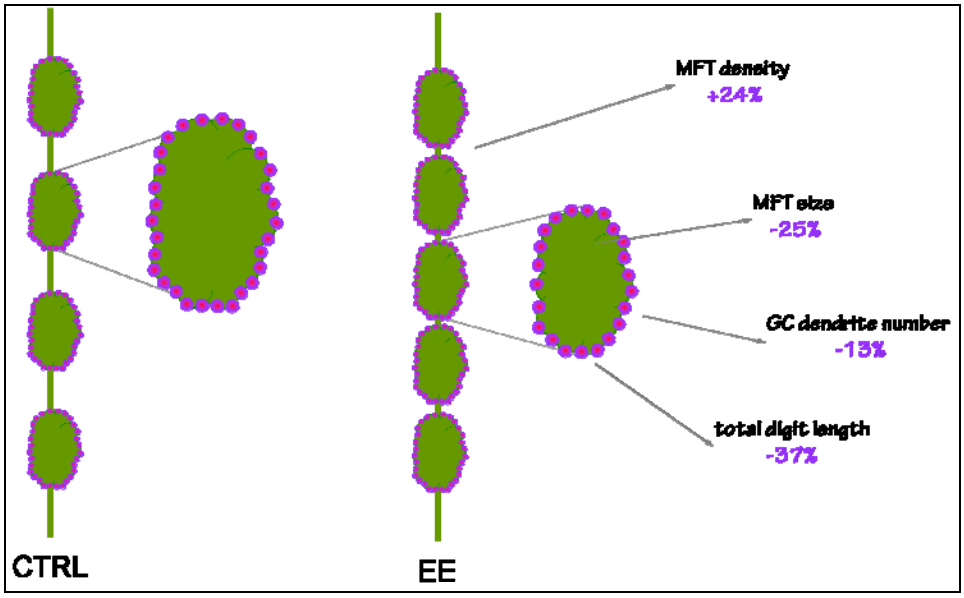


Figure 17 Experience affects synaptic connectivity at the input stage of the cerebellar cortex

A representative control axon is shown on the left. Green clouds represent MFTs, red dots represent GC claws. One MFT is contacted by 30 claws. Upon EE, MFT density increases by 24% (for the same axonal length), average MFT size decreases by 25%; GC claws are reduced by 10%, thus leaving only 22 GC to contact each MFT. In addition, each claw field is reduced by 37%.

2.1.3 DISCUSSION

We aimed at describing the experience-dependent structural rearrangements taking place at the input stage of the cerebellar cortex in the adult mouse. We showed that MF afferents respond to EE experience reshaping their connectivity pattern in terms of terminal density and branching frequency, as well as affecting single MFT size. Similarly, the postsynaptic compartment runs into modifications of the cell connectivity and of the single synaptic ending morphological properties. In addition, we showed that the described rearrangements of the connectivity can be modulated according to experience. Taken together, these observations provide evidence that housing mice in EE produces a long-term reshaping of the connectivity at the input stage of the adult cerebellar cortex.

Below, possible implications of these findings will be discussed and future directions will be suggested.

Experience affects presynaptic terminal morphology and axonal connectivity

We traced long stretches of axons running along lobule V of the mouse cerebellar cortex, and compared axon connectivity properties between control and EE animals (Figures 10 and 11), as well as the morphology of single MFTs found in the apical subregion of lobule V (Figure 9). This approach allowed us to study the global behaviour of a mossy afferent entering the cerebellar cortex. Nevertheless, the transgenic lines we used for this study allowed us to study in detail the single terminal morphology, so that we could combine the two approaches and describe both general and local presynaptic changes. We found that EE triggers conspicuous remodelling of

the axonal connectivity, increase the number of terminals along the axons, as well as the branching points (Figure 11A, B). It is likely that the increased number of MFTs is due to the collaterals emerging from the new branching points.

Extensive axonal remodelling occurring in the adulthood was only recently reported to occur (Gogolla et al., 2007). One inferred function of the axonal remodelling is the search for appropriate synaptic partners within a given region, eventually leading to the modification of the system connectivity. It is likely that such remodelling further affects the whole system in the topographical and functional aspect.

From an anatomical point of view, axons can sample the territory at different scales (Figure 18). For instance, new branch sprouting allows to extensively explore a territory and could possibly heavily impact system connectivity: this could represent the underlying mechanism for learning a new skill or sensory deprivation experience (Das and Gilbert, 1995; Kleim et al., 2002). Presynaptic structures have the additional opportunity of local plasticity by remodelling terminal boutons or by turn over of *en-passant* boutons (DePaola et al., 2006; Stettler et al., 2006; Figure 18).

In particular, the cerebellar granular layer, given its peculiar anatomical organization, is potentially prone to functional plasticity through the above mentioned axonal structural rearrangements: classical works stablished that MF projections in the posterior lobe of the rat and other species are organized in a mosaic-like pattern with multiple representations of the same receptive field (Shambes et al., 1978; Bower et al., 1981; Voogd and Glickstein, 1998), referred as "fractured somatotopy". Comparison in the patch localization between different subjects by means of fMRI revealed a degree of variability in the activation pattern (Peeters et al., 1999). Technical issues could partly accounting for these differences, but it is likely that genuine differences were present between individual rats. It is then tempting to speculate that experience can remodel the size and number of the somatotopic representations in the cerebellar cortex, and that our observations would constitute the structural basis for this functional plasticity to occur.

Nevertheless, we should consider that our study describes a mixed axonal population, as mGFP labelled MF axons come from distinct sources (Figure 9), so that each subpopulation most likely exhibits different structural plasticity properties. For instance, it was shown that in thalamocortical afferents 15% of EPBs are not stable

over one month, while in layer VI axons the unstable EPB population increased to 50% in the same period length (De Paola et al., 2006; Stettler et al., 2006). With regard to this aspect, we noticed that, both in control and EE subjects, the axonal population was characterized by very different branching frequency and MFT density properties (Figure 10). Interestingly, the range of values for the two experimental conditions was similar, thus suggesting that not all the axons respond to the EE in the same way or with the same amplitude of remodelling (Figure 11C). This could possibly reflect the selective response of a subset of MF afferents, whose stimulation was stronger or preferential during the EE experience.

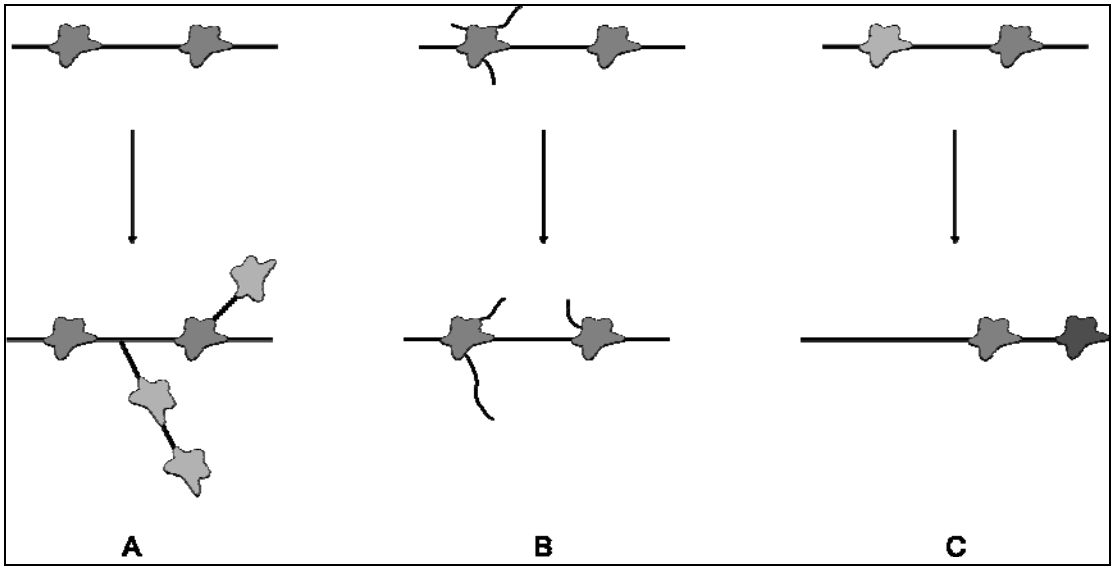


Figure 18 Sampling scales of axonal structural plasticity
The schematic indicates the state of an axonal segment at two successive time points. Side-branch sprouting mediates sampling of postsynaptic territories ranging tens and hundreds of micrometers away from the original axon branch (A). At a local scale, axons can exhibit remodelling of pre-existing terminals (B) or turn-over of en passant terminals (C). Terminal plasticity in B and C can sample many tens of micrometers but might not lead to the establishment of stable new circuitry. (modified by Gogolla et al., 2007)

Experience affects GC connectivity properties in their dendritic compartment

GC dendritic compartment provides an optimal model to study rearrangements of connectivity occurring at isolated synaptic complexes: each dendritic branch exhibits a claw-like ending, made by few and short processes (digits), which is suitable for quantitative analysis. In addition, each GC has in average 3-5 short dendrites, whose morphological parameters are convenient to solve and analyze.

We found that, upon EE experience, each GC contacts in average less presynaptic counterparts, thus altering MF-GC connectivity (Figures 13B and 14C). This

conclusion is based on the assumption that each dendritic branch emerging from the cell body is contacting only one MFT. It is worth noting that, in the GC population included in our studies, we found that ~40% of the cells at P30 and ~33% of the cells at P50 displayed at least one dendrite ending in two or more terminal claws (not shown): we could not directly visualize that presynaptic counterparts, but in most cases it was evident that two claws belonging to the same dendritic branch could not share the presynaptic terminal because of the reciprocal distance. However, at P30 the proportion of multi-claw dendrites was similar between control and EE population, while at P50 this proportion was slightly increased in EE animals (from 33% to 38%).

In addition to the remodelling of GC dendrite number, we found that ending claws were smaller and simpler upon EE, in terms of number and length of the digits (Figure 15A). Since each digit is known to make at least one synaptic contact with the presynaptic counterpart, we infer that the number of potential contacts is reduced. In this regard, we compared the total number of bassoon-positive puncta (a marker for presynaptic active zones) in defined cortical regions and found no differences between control and EE subjects (not shown); however, it has to be taken into account that MFTs decrease their average size, thus counterbalancing the decrease in available postsynaptic sites and virtually letting the overall density of synaptic contacts unchanged. In a further step, we will try to correlate the bassoon puncta on a given MFT with its volume or surface.

Furthermore, we generally found that the morphology of different claws belonging to the same cell was comparable in terms of size and complexity (Figure 12C-D). In our opinion, this is likely to support the current view of one GC receiving different inputs (one per claw) of the same sensory modality (Jorntell and Ekerot, 2006). In addition, this probably argues for the interpretation that the remodelling involves MF-GC, rather than GoC-GC synapse: in the latter case, parallel rearrangements of every GC claw would be difficult to achieve given the extensive and intermingled GoC axonal branching.

EE starting in the early postnatal period enhances postsynaptic structural rearrangements

In a second set of experiments, mice were subjected to EE experience since birth. This implies unusual sensorymotor stimulation in the early postnatal period, known to be critical for the shaping of later connectivity, as it was described in classical sensory deprivation experiments (Hooks and Chen, 2007). Recent studies have expanded current knowledge of the precise changes that can be induced by sensory experience in the final stages of neuronal development. In the auditory system, for example, changing patterns of sensory input during postnatal development can have important effects on the response properties of individual neurons (Chang and Merzenich, 2003). Interestingly, it has been shown that early mechanosensory patterned stimulation leads to changes in adult behaviour and gene expression in *C. elegans* (Ebrahimi and Rankin, 2007).

Our experimental design allows to get insights into structural rearrangements from different points of view:

i. age dependent physiological plasticity (P30 control *vs* P50 control)
ii. plasticity along the time course (P30 EE *vs* P50 EE)
iii. plasticity triggered by different experiences ("regular EE" *vs* "EE colony")

Comparing the two different time points, we first observed that the GC control population at P30 differs substantially from P50 population. In particular, we found that 70% of GCs were characterized by 4 dendrites. Conversely, at P50, the 4-dendrite population, which still constitutes the most abundant group, represents only 41% of the cells (Figure 14A). As at P30 the postnatal development in the mouse is complete, this shift probably represents an age dependent physiological reshaping of the connectivity in the GL; the increased divergence in GC connectivity properties could reflect peculiarity in sensory transmission. On the same perspective, our studies on the claw morphology show that GC synaptic ending are in the process of refining their connectivity at examined time points (Figure 15A). This is like to be a continuous process throughout life, as previous studies in our laboratory brought evidences for the claw morphology being dependent on aging. In particular, we found that aged

mice displayed complex claws, characterized by long digits covering extensively a limited presynaptic territory (N.G., unpublished data).

Nevertheless, 30 days of EE starting from birth influence GC connectivity, so that the distribution according to dendrite number differs substantially from the control population, rather resembling to P50 control distribution (Figure 14A). As also indicated by the average dendrite number, 30 days of EE experience were still not enough to induce the reduction in the GC dendrite number that was observed at P50. In our understanding, this finding most likely indicates that early postnatal EE triggers a faster GC maturation in terms of connectivity and, therefore, early pronounced divergence of the GC population. Conversely, the claw remodelling in the EE colony animals was fully achieved already at P30 (Figure 15A).

Finally direct comparison between "EE colony" and "regular EE" revealed that the two protocols trigger similar connectivity remodelling, even though the distribution shift of the GC based on the dendritic number was more dramatic in the animals that experienced early EE (Figure 14B). Again, the claw reshaping was similarly triggered by the two experimental condition, with an additional significant reduction in the digit length in EE colony animals (Figure 15A).

Taken together, these last observations indicate that single MF-GC connectivity reshaping is easier and faster to achieve, being basically observed to occur at a similar extent in all the experimental conditions and time points; conversely, the global reshaping of the cell connectivity is a process strongly influenced by the time and length of the experience, on top of being an ongoing physiological process in the adult animals.

EE effects on the synaptic connectivity at the input stage of the cerebellar cortex

MF-GC synapse organizes the sensorimotor representation in the cerebellar cortex. The reshaping that we observed in pre- and postsynaptic connectivity indicates that EE experience modulate system connectivity in the cerebellar cortex, acting both

on the spread (determined by axonal connectivity) and amplification (determined by pre- and postsynaptic properties) of a given sensory or motor information.

Our data indicate that, upon EE, an axon is able to place a higher number of MFT, probably accounting for higher specificity and refining of the carried information. In addition, fewer GCs carry and amplify the signal coming from each MFT, thus causing the sharpening of synaptic transmission (Figure 17). This could likely result in the remodelling of the somatotopic representation in the cerebellar cortex (as discussed in the chapter 1.2.6) leading to its refinement and to increased discrimination of sensory inputs (Figure 19).

This is a particularly attractive hypothesis as it was described that transferring adult animals in a "naturalistic environment" induces a large-scale functional refining of sensory maps in the barrel cortex (Polley et al., 2004). This plasticity was underlay by massive anatomical contraction and weakening of whisker functional representation in such a way that each whisker had a smaller representation containing smaller and sharper receptive fields allowing high-resolution discrimination abilities (Frostig, 2006). The structural rearrangements could serve as an anatomical substrate in order to refine sensory representations in the cerebellar cortex.

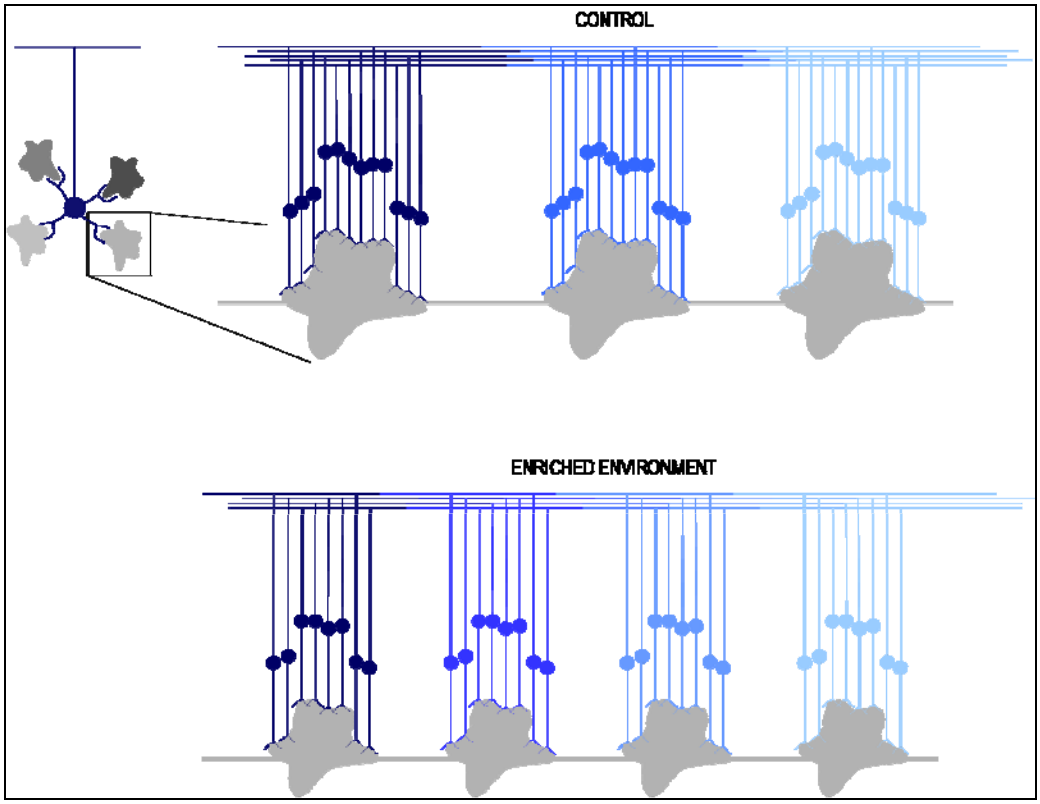


Figure 19 A model of refined sensory-motor transmission
Experience influences MF-GC connectivity by increasing MFT density per axon and decreasing the available postsynaptic sites. This could serve to convey a wider repertoire of sensory-motor information (more *nuances* in this schema) and to sharpen to sensory transmission.

2.1.4 CONCLUSIONS AND FUTURE DIRECTIONS

So far, we have provided evidences that a long-term reshaping of the connectivity at the input stage of the adult cerebellar cortex is induced by experience in the adulthood. From an anatomical point of view, we found that the circuitry connectivity is profoundly altered in the properties of the afferent projections and in the amplification and transmission of the sensory information. We think that these rearrangements have an impact on the sensory representation in the cerebellar cortex. Nevertheless, functional consequences of the connectivity reshaping still have to be proved. Evidences about synaptic transmission at the MF-GC synapse should be brought in order to make final statements about the correlation between anatomical and functional properties. *In vivo* recordings on enriched animals in order to test the refinement of somatotopic representation in the cerebellar cortex would constitute the ultimate test to verify the functional consequences underlay by structural rearrangements.

In addition, we are currently investigating the effects of a longer period in EE. From preliminary observations, animals aged of 90 days that were born in EE, present extreme rearrangements in GC dendrites, those being polarized and elongated, spanning up to 50 μm in some cases. Again, confirming these observations would support the idea of the reshaping of sensory representation, being the GC dendritic field strongly affected by experience. Moreover, we are in the process of studying the effects of reverting the animal to housing in standard conditions, after having experienced environmental enrichement.

Finally, we have shown the rearrangements of neuronal circuit connectivity that occur upon a complex paradigm including many factors, such as increased social and sensorymotor activities, able to influence hormonal, metabolic, neuromodulatory

physiology. Thus, it is not clear whether the observed changes are a direct effect of the training and learning occurred during EE, or whether they reflect general changes induced by the above mentioned systemic influences. Data generated in our laboratory (Galimberti et al., 2006; Gogolla et al., submitted; C.V., unpublished observations) indicate so far the predominance of region- and synapse-specific effects, as well as peculiar rearrangements of the connectivity according to the type, the time and the intensity of the experience. In order to further dissect EE effects, we propose to carry out a similar study on animals specifically trained for a cerebellar-dependent motor task.

CHAPTER 2

RESULTS 2.2

Structural plasticity at the input stage of the adult cerebellar cortex upon fear conditioning

Claudia Vittori, Benedetto Sacchetti and Pico Caroni

unpublished results

2.2.1 INTRODUCTION

The mature brain needs to constantly update its function to past experience and to the actual context. Changes can occur at several levels and increasing evidences describe that sustained structural rearrangements occur physiologically in the adult, as well as triggered by particular experience (Lendvai et al., 2000; Trachtenberg et al., 2002; Holmaat et al., 2005, 2006).

Concerning learning-related structural plasticity, changes in synapse number within the mammalian brain have been reported in specific training paradigms, such as maze learning (Greenough et al., 1979; Chang and Greenough, 1982; Moser et al., 1994) and motor skill training (Black et al., 1990; Kleim et al., 1996; 1997a; 1998b). Importantly, studies showed a correlation between perceptual learning in the reorganization of representations in sensory cortical areas (Recanzone et al., 1992; 1993). In an example, monkeys trained to discriminate between auditory stimuli of different frequencies show an expansion in the area of auditory cortex responsive to the trained frequency and a subsequent reduction in the area of non-trained frequencies (Recanzone et al., 1993). Finally, colocalization of learning-dependent synaptogenesis and functional reorganization in specific cortical regions has been showed (Kleim et al., 2002; 2004).

Fear conditioning (FC) is a pavlovian learning paradigm that relies on fear emotion, a basic evolutionarily conserved mechanism that is essential for survival because it triggers a set of defensive reactions aimed at dealing with a threatening environment. Fear is accompanied by a set of autonomic (heart rate and blood pressure variations, pupillary dilation), endocrine (pituitary-adrenal hormone), and behavioural (increased startle reflex, escape, immobility, or freezing) responses (LeDoux, 2000; Maren, 2001). In experimental conditions, a neutral stimulus serves as

a conditioned stimulus (CS) and is repeatedly associated with an aversive one, usually a foot-shock (unconditioned stimulus, US). As a result of this pairing, the CS comes to elicit a set of spontaneous defensive behavioural responses that include freezing, increased heart rate, and startle (LeDoux, 2000; Maren, 2001). Evidences demonstrate that amygdala is critically involved in innate and learned emotional behaviour, both for contextual and cued fear learning (LeDoux, 2000; Maren, 2001; McGaugh, 2004), while the dorsal hippocampus is necessary for contextual, but not cued, learning (LeDoux, 2000; Maren, 2001; Maren and Holt, 2004). An increasing amount of evidences has proved that other areas, cerebellum included, are also involved in fear-related processes (Sacchetti et al., 2005).

There are currently few reports that show structural remodelling occurring upon FC. Radley and colleagues reported that FC triggers an increase by 35% of spinophilin-immunoreactive spines in the dorsal subnucleus of the lateral amygdala upon FC (Radley et al., 2006). More recently, an up-regulation of PSA-NCAM, known to decrease cell adhesion and promote structural remodelling, was reported specifically in the dorsal dentate gyrus of the hippocampus, 24 hours after contextual FC. Additionally, specific removal of PSA led to a decrease in freezing responses to the conditioned context (Lopez-Fernandez et al., 2007).

However, existing studies are not exhaustive at clarifying the impact of fear-related plasticity at the synaptic level (namely, the rearrangement of contacts between identified synaptic partners within one synaptic complex), at the axonal level (global *versus* local behaviour of a given axonal projection), on synaptic transmission and amplification and, lastly, on functional representation in the cortex.

To address part of these questions, we exploited a simple and well-characterized neuronal circuitry, that had been implicated in functional and anatomical plasticity, namely the cerebellar system. The cerebellum has long been known as a centre for fine motor control and sensory-motor integration; its topographic and functional organization, as well as its essential role, have been investigated extensively. Moreover, in the past decade, a growing body of evidences has suggested that the cerebellum may be involved in cognitive and emotional functions, particularly coordinating autonomic (Supple and Leaton, 1990; Sebastiani et al., 1992; Supple and Kapp, 1993; Yoshida et al., 2004) and cognitive (Ploghaus et al.,

1999; Ploghaus et al., 2000) responses, as well as in aversive memory maintenance (Sacchetti et al., 2004; Sacchetti et al., 2007).

The cerebellum is a brain region endowed with a high degree of structural plasticity during development (Sugihara, 2006), as well as in the adulthood: extensive work has been done to describe structural rearrangements taking place after damage or alteration in the patterns of activity (Benedetti et al., 1983; Strata and Rossi, 1998). Furthermore, studies showed that motor experience induces the increase spine density on the PC tree (Black et al., 1990; Kleim et al., 1998b; Lee et al., 2007). At the synaptic level, activity-dependent plasticity in terms of strength and intrinsic excitability have been reported in the cerebellum (Hansel et al., 2001). In particular, plasticity of synaptic excitation and inhibition occurs at five different sites (Hansel et al., 2001) and intrinsic plasticity has been reported in at least three cell types (Schreurs et al., 1997; Aizenman and Linden, 2000; Armano et al., 2000). Importantly, the synapse between parallel fibers (PF) and Purkinje cells (PCs) has been shown to be potentiated upon FC in lobule V and VI, a region where acoustic and nociceptive stimuli converge (Snider and Stowell, 1944; Huang et al., 1982) and is related to emotional behaviour (Sebastiani et al., 1992; Supple and Kapp, 1993).

These findings suggest that the cerebellar system has the potential capacity of undergoing experience-dependent structural and functional plasticity during adulthood, but still little is known about physiological mechanisms of plasticity taking place upon experience and learning.

We focused our attention on lobule V of the cerebellar cortex, a region known to be involved in fear-related learning (Sacchetti et al., 2004), and in particular at the input synapse between mossy fiber terminals (MFTs) and granule cell (GC) dendrites. Mossy fiber (MF) axons convey multimodal sensory information from distinct sources (brainstem nuclei, spinal cord and pontine nuclei), which in turn are innervated by massive projections from the cerebral cortex (Brodal, 1972). MF axons build a large and complex presynaptic terminal, whose postsynaptic elements are individual dendrites from GCs; each GC has 3-5 short dendrites ending in a claw-like structure that receives excitatory synaptic input from one MFT (Voogd and Glickstein, 1998) or from inhibitory Golgi axon varicosities.

Here, in order to study long term experience-dependent structural plasticity events, we combined a fear learning experimental paradigm with large-scale confocal imaging in transgenic mouse lines, previously generated in our laboratory (De Paola et al., 2003), that express membrane-targeted GFP in few neuronal cells, thus revealing their morphology in crisp details.

We found that the lobule V MF-GC synapse considerably rearranges pre- and postsynaptically upon FC, according to a defined sequence of events taking place along the investigated time course (2-15 days); the maximal amplitude of remodelling is observed 5 days after the training and then gradually decreases. Furthermore, FC-related structural rearrangements can be modulated by subsequent experience following the FC training.

2.2.2 RESULTS

Transgenic mouse lines that express membrane-targeted GFP in few cells reveal neuronal morphology in the cerebellar cortex

In order to investigate anatomical plasticity in the cerebellar cortex, we took advantage of a *Thy1-mGFP*^{s} mouse line, here referred as *Lsi1*, expressing membrane-targeted GFP only in few neurons (De Paola et al., 2003); this line is characterized by a sparse labelling pattern of specific cell types, that was described in detail in the previous section of the Results (see Figure 8 and 9, chapter 2.1.2). Briefly, *Lsi1* mGFP expression in the cerebellar cortex is mostly attributable to a subset of afferent ponto-/spinocerebellar axons and sparse GCs.

The scattered mGFP expression pattern in the cerebellar cortex, together with large scale confocal imaging, allowed us to reconstruct the fine three-dimensional morphology of neuronal cells and to collect quantitative data about the morphological features of a significant number of neuronal cells per animal.

Fear conditioning induces long-term morphological plasticity at the input stage of the adult cerebellar cortex

We aimed at describing the anatomical plasticity events triggered by the FC paradigm in the adult mouse. To this purpose, male mice aged P50-P60 received a repeated pairing of a tone (CS) with an electric shock (US), and were returned to their cage and left undisturbed until they were sacrificed (see Experimental Procedures for a detailed description of the experiment); survival times were chosen between 2 and 15 days after the FC paradigm ("COND" group, where the suffix number indicates the

days of survival after conditioning). Before sacrifice, animals were tested for memory retention and included in the analysis according to the behavioural readout. Control mice underwent the same procedures, but the tone was not associated with the shock.

FC affects MFT morphology in a process spanning several days

Lobule V and VI of the cerebellar vermis is the dominant region of convergence of acoustic and nociceptive stimuli (Snider and Stowell, 1944; Huang et al., 1982) and is related to emotional behaviour (Supple and Kapp, 1993; Sebastiani et al., 1992). In addition, lobule V-VI PF-PC synapse was shown to be potentiated upon FC (Sacchetti et al., 2004): thus, it is a logical site to look for structural rearrangements triggered by FC. We carried our analysis on the apical region of mid-vermal lobule V, taking advantage of large-scale confocal imaging of the region of our interest (spanning about 2-2.5 mm) and then built a 40-µm three-dimensional dataset. We focused at the MF-GC synapse, the input stage of the cerebellar cortex; we first wanted to describe the remodelling events occurring at the MFTs, namely the synaptic endings placed in GL by the mossy axons. In order to describe the population behaviour, we considered in our analysis all the MFTs which were completely included in our 3D datasets and unambiguously solvable, thus collecting 30-50 MFTs per animal. We took in exam three parameters describing MFT morphology:

- size (expressed as the MFT area calculated on the maximum intensity projection, MIP, as described in the Experimental Procedures);
- complexity, namely the convolution and irregularity of the terminal surface (see Experimental procedures);
- process length.

Proper control animals were checked for all the time points and protocols used: despite of the sacrifice time point (namely, the time included between the training and the death), they appeared to be homogenous in terms of the MFT population morphology, and thus they will be treated as a unique control group in the rest of the section.

In general, obvious remodelling of MFT morphology was observed in the days following FC (Figure 20), with a maximal intensity 5 days after FC (Figure 21). We will then describe a reproducible patterned sequence of events taking place along the investigated time course.

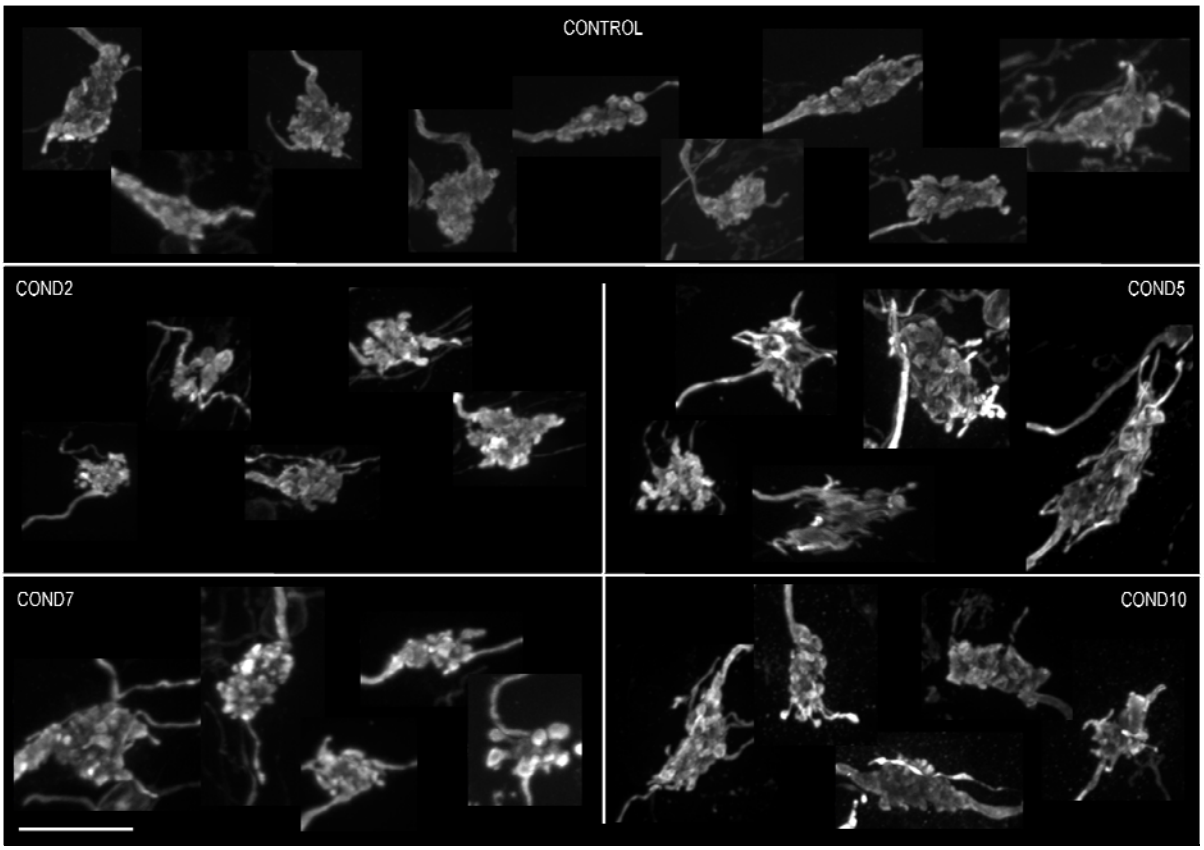


Figure 20 FC affects MFT morphology in a process spanning several days
Representative MFTs cropped from 40-μm MIPs are shown. MFTs rearrange according to a defined series of events along the time course. Parameters that define MFT morphology are surface complexity, size and process number and length. Bar: 20 μm.

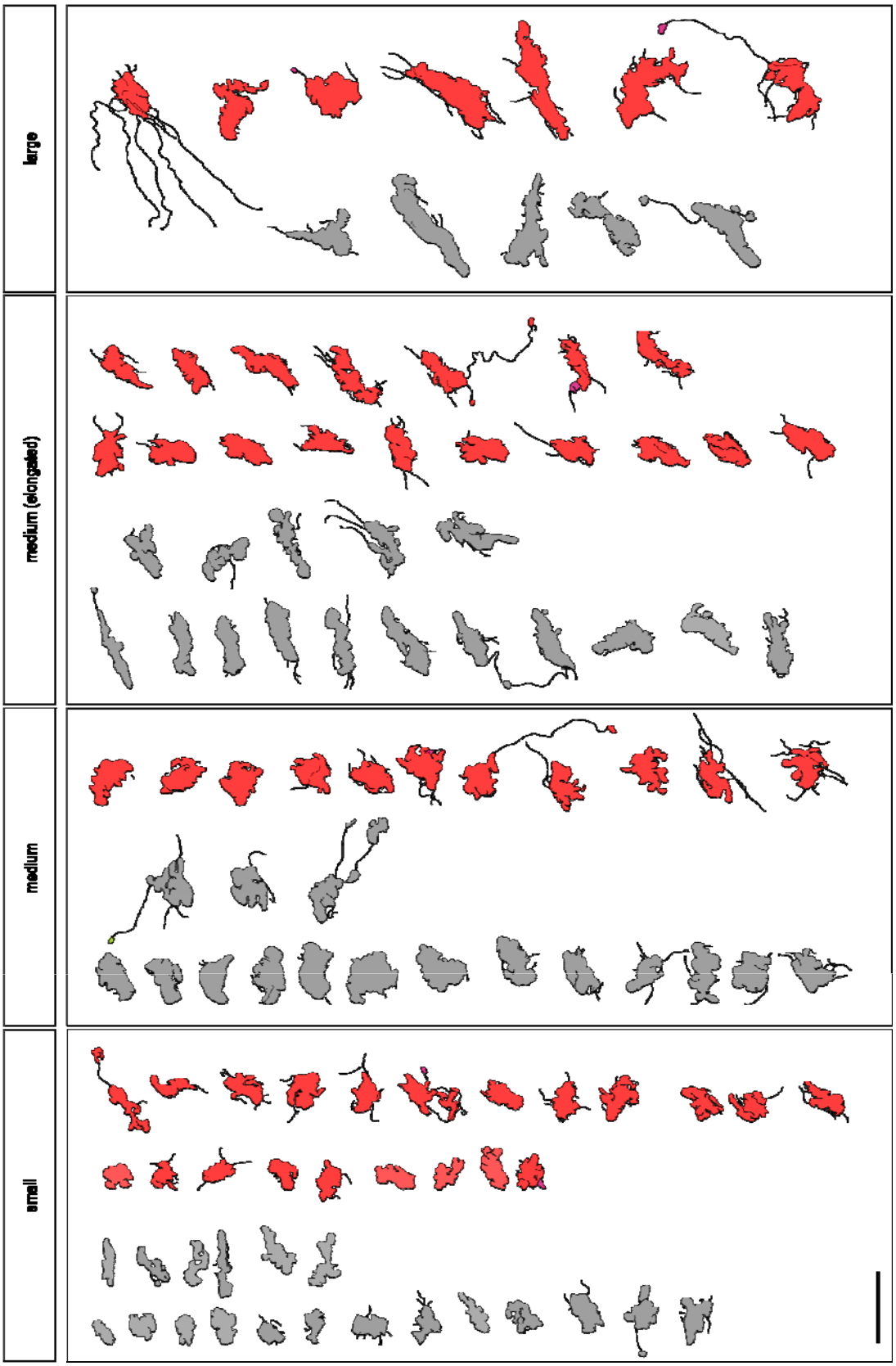


Figure 21 Remodelling of MFT morphology is maximal 5 days after FC
Camera lucida of MFTs population from one control (grey, N = 56 MFTs) and one COND5 (pink, N = 56 MFTs). Morphology of all the MFTs populating the apical region of lobule V was analyzed in 3D and then drawn on the corresponding MIP; the population was then arbitrarily divided in four categories based on size and shape. COND5 MFTs show a more convoluted (complex), profile, irregular shapes and a fraction of very large MFTs. Bar: 20 µm

2 days: MFTs respond to FC with considerable changes in size, complexity and process length

Two days after FC (COND2), we observed that the MFT surface was significantly more convoluted than in control conditions (Figure 20), in particular showing more pronounced outward swellings, or bulges. We thus defined a complexity index that took into account this particular feature (see Experimental Procedures) and compared with control values. We confirmed quantitatively that the complexity of the MFTs was increased in COND2 (Figure 22A, B). Interestingly, the increase in MFT complexity was a whole-population tract, as evident in the scatter diagram (Figure 22A): the global interquartile range of values shifted from 0.25-0.37 to 0.32-0.48, while the standard deviation of the population was preserved across the two conditions (0.12 both for control and COND2). The described increase in complexity was accompanied by a decrease in the average size of the MFTs (Figure 22C, D), again due to a global population shift, in this case mostly attributable to larger terminal shift (interquartile range 38-64 μm^2 in control and 32-53 μm^2 in COND2, Figure 22C). In addition, the summed length of processes belonging to the same terminal (here referred as "total process length") was decreased in COND2 from 13.2 ± 1.2 µm (control) to 10.0 ± 1.3 µm (Figure 23A); conversely, average process length was kept constant at all the investigated time points (Figure 23B). In summary, two days after FC, MFTs show a convoluted surface, accompanied by a decrease of the size and of the total process length.

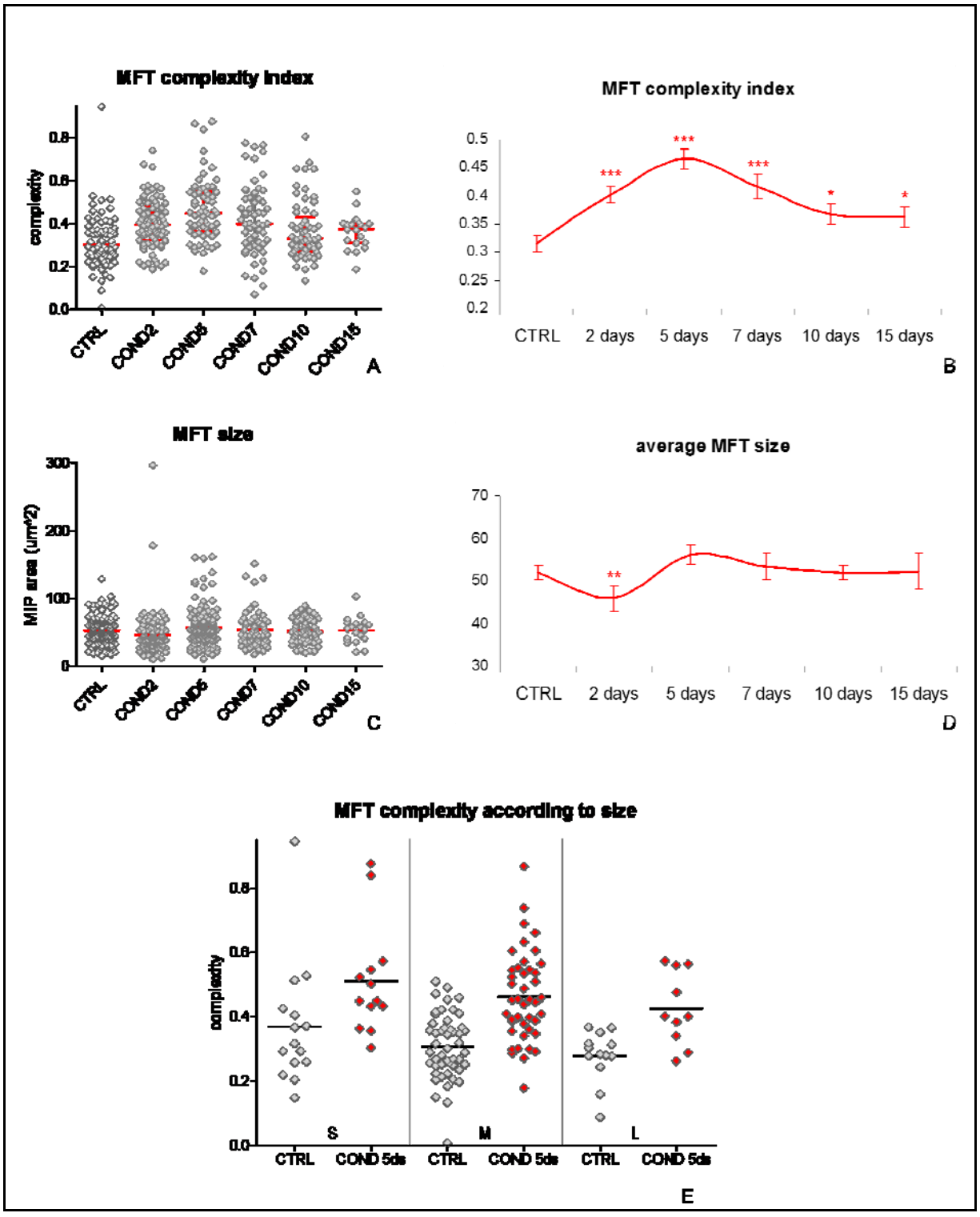


Figure 22 MFT complexity and size are affected in response to FC

Quantitative analysis of MFT morphology along the time course. Complexity index and average area were calculated according to the criteria presented in the Experimental Procedures. **A-B**: Quantitative analysis of MFT complexity. **A**: every diamond represents one MFT; red lines represent *median* with interquartile range; N_{CTRL} = 77 MFTs (2 mice); N_{COND2} = 77 (2 mice); N_{COND5} = 66 (2 mice); N_{COND7} = 58 (2 mice); N_{COND10} = 61 (2 mice); N_{COND15} = 20 (1 mouse). **B**: Red line connects mean values ± SEM; Control population is plotted in the graphs as starting point. Two-way ANOVA (*vs* control): ***p < 0.0001; *p(COND10) = 0.02235. Post hoc Student's t-test: *p(COND15) = 0.0193. **C-D**: Quantitative analysis of MFT size. **C**: every diamond represents one MFT; red lines represent mean; N_{CTRL} = 147 MFTs (3 mice); N_{COND2} = 111 (2 mice); N_{COND5} = 151 (3 mice); N_{COND7} = 70 (2 mice); N_{COND10} = 61 (2 mice). **D**: Red line connects mean ± SEM. Two-way ANOVA (*vs* control): **p = 0.002873. **E**: MFT complexity distribution according to the size. The population was divided in 3 groups: small (S, area < 35 µm^2), medium (M, area 35-70 µm^2); large (L, area > 70 µm^2). Black lines represent mean.

5 days: MFTs dramatically rearrange their morphology, expanding in size and processes

Five days after FC (COND5), we observed the largest scale rearrangements for all the evaluated parameters. COND5 MFTs showed dramatic rearrangements of their complexity, not only in terms of surface convolution, but also for the irregularity of the shape, being elongated, bended, asymmetric terminals more frequent in this time point (Figure 20 and 21). We found the average complexity to be increased by ~48% (from 0.32 ± 0.01 in control to 0.47 ± 0.01 in COND5; Figure 22A, B). Consistently with the observations for COND2, we observed that the response in terms of complexity was attributable to the global population, with an additional expansion towards higher complexity values, as inferred from the larger interquartile range (0.36-0.55) and the standard deviation (0.14; Figure 22A). The described increase in MFT complexity was independent on MFT size, as it was preserved across the whole population (Figure 22E). In addition, we observed a modest increase in the average MFT size, which was actually attributable to a subset of MFTs (Figure 21 and Figure 22C). Differently from COND2, COND5 MFTs showed a conspicuous increase in the total process length per MFT (Figure 23A), while the length a single processes was unvaried at this time point (Figure 23B), meaning an increase in the number of processes per MFT. In summary, 5 days after conditioning, the MFT population exhibits a general dramatic increase of the complexity and process number together with an increase of the average size strongly affecting a subgroup of terminals.

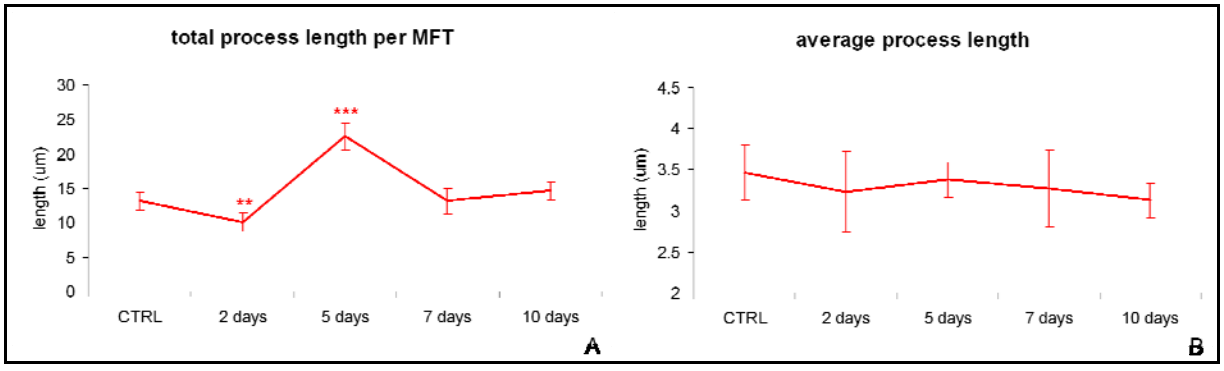


Figure 23 MFT process length is affected by FC
Quantitative analysis of MFT process length along the time course. A: Quantitative analysis of total process length per MFT; red line connects mean values ± SEM. N are listed in Figure 3C. Post hoc Student's t-test: **p=0.0046, ***p<0.0001. B: Quantitative analysis of average process length per MFT; red line connects mean values ± SEM. No significant changes were found according to post hoc Student's t-test. One-way-ANOVA: p = 0.0615 (n.s.).

7 days: MFT rearrangements decrease the overall amplitude

Seven days after FC (COND7), we observed that amplitude of the rearrangements decreased compared to COND5. The general appearance of the MFTs looked to some extent similar to COND2, being many terminals characterized by a bulgy aspect (Figure 20). The average complexity index was still sustained compared to control population (0.42 ± 0.02; Figure 22A, B), but, interestingly, the population distribution was found to be less compact compared to all the previous time points (interquartile range 0.30-0.52, standard deviation 0.167; Figure 22A); it is in particular worth noting that the 75%-percentile was similar to COND5, but a part of the population dropped back to a range that was absent in the two time points previously analyzed (Figure 22A). The average size was slightly decreased with respect to COND5 and moving back to control average values; in addition, the MFT population did not show remarkable features in its distribution compared to control. Finally, COND7 total process length was also decreased compared to the previous time point, getting closer to control values (Figure 23B).

10 days and 15 days: MFT morphology reverts to control values

Ten days after FC (COND10), the general aspect of the MFT population was closer to baseline values with respect to many morphological aspects (Figure 20). First of all, the average complexity was 0.37 ± 0.02 (Figure 22A, B), thus decreased compared to COND5 and COND7; additionally, the population exhibited a more compact distribution compared to COND7 (standard deviation 0.14), yet still characterized by a high-complexity MFT fraction (Figure 22A). Fifteen days after FC, the average complexity was very similar to COND10 and the population further compacting around average values (Figure 22A). The COND10 and COND15 average MFT size and the distribution across the population was in the control range (Figure 22C, D), as well as the total process length (Figure 23B). In summary, COND10 and COND15 MFTs showed a reversion to control range values for the investigated parameters, even though the complexity index was still significantly higher than the baseline.

In summary, we observed that FC induces long-term rearrangements in a process spanning several days and involving globally the MFT population; peak remodelling events are found 5 days after conditioning; afterwards, MFT morphology average parameters get back, or closer, to the control range.

FC effects are played out at the axonal level

We next wondered whether: 1) FC is able to affect the general connectivity properties of the mossy axons, such as the number of MFTs or the number of branching points within one lobule; 2) the remodelling events we observed upon FC are played at the single terminal level, or rather a whole axon response is following the behaviour paradigm. To this purpose, we traced stretches of individual MF axonal arbors innervating lobule V on reconstructed 3D datasets and compared their branching patterns as well as the density of MFTs on these individual axons (Figure 24).

The mossy axons that we were able to trace revealed very heterogeneous properties: for instance, we found that MFT density relatively to the axonal length could vary from 1 to 20 MFTs per 100 µm and the frequency of branching points was roughly between 1 and 10 for the same distance. In addition, MFTs could be regularly placed along the projection, evenly spaced along the axon, or rather concentrated in short stretches despite of long solved axonal tracts (Figure 24 and 25). Branching points were found at *en passant* MFTs (when processes arising from one MFT were able to build other MFTs) or as bifurcations along the axonal projection. As the MF axons run mostly parallel to the medial line, the length of traced axons varied according to the slice cut. We observed that mGFP labelled axons were particularly enriched in branching points in the apical and medial region of lobule V, what we believe to be related to the cerebellar anatomy of projections.

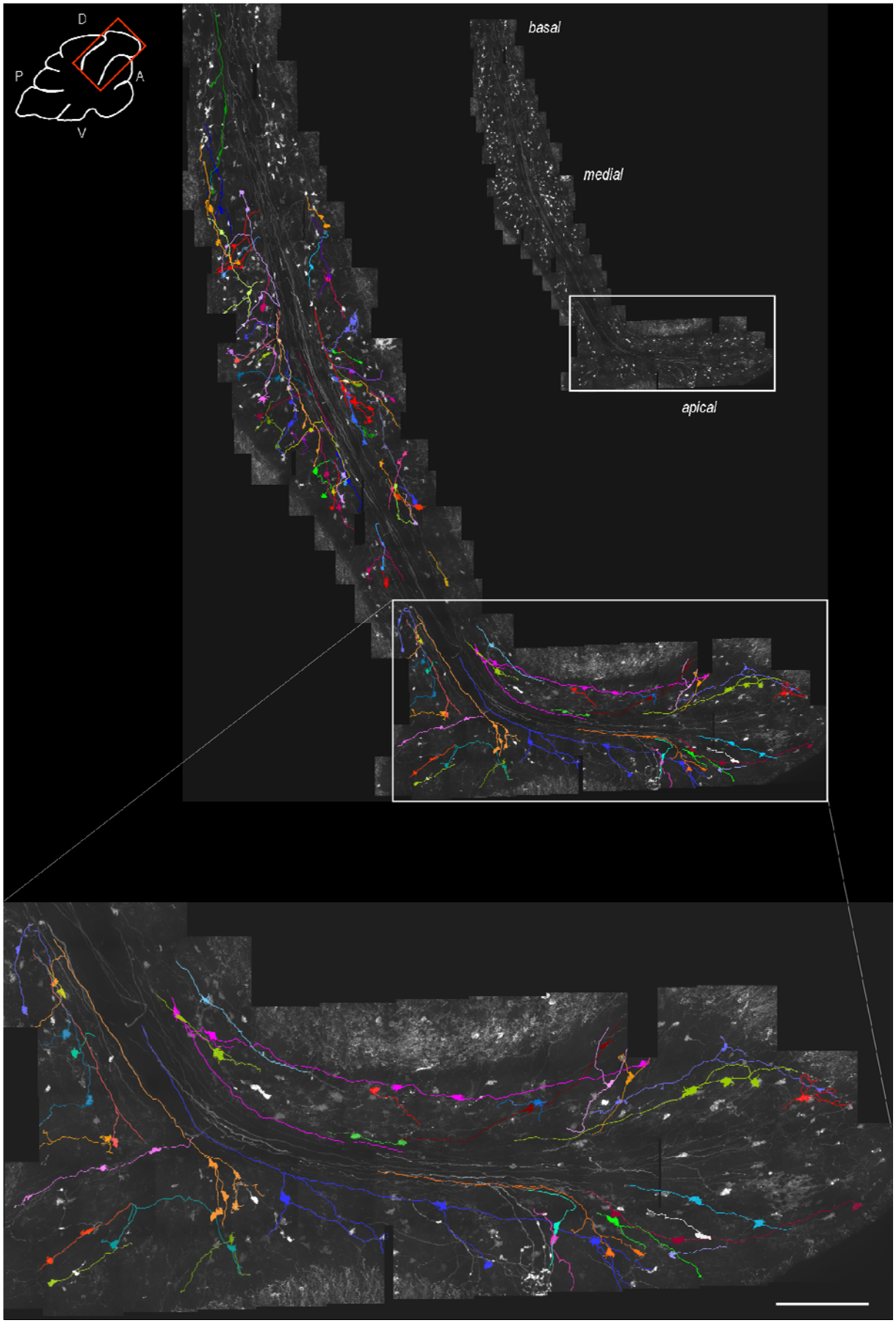


Figure 24

Figure 24 FC effects are played out at the axonal level
Tiled MIPs (collapsed 100-µm confocal stacks) spanning the lobule V (mid-vermis) of an adult *Lsi1* mouse. A cartoon of the cerebellum is representing the lobule V region and indicating the DV and AP axis. In the middle panel, several traced mossy axons are superimposed on the stitched dataset. The region included in the white boxes is magnified in the lower part of the panel. Bar: 100 µm

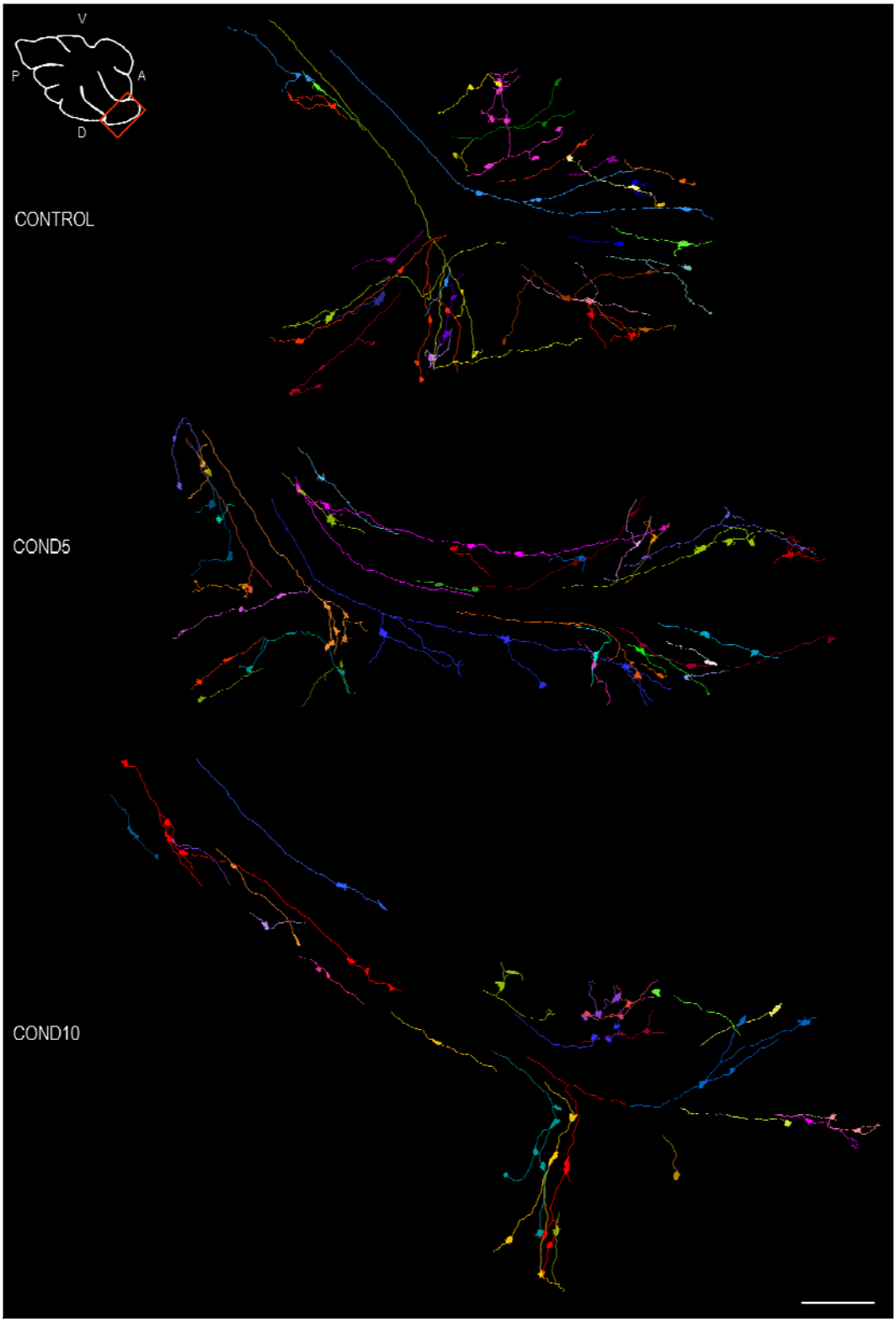


Figure 25 FC tracing analysis reveals axonal features
Camera lucida of traced axons obtained from 3D data sets. A cartoon of the cerebellum indicates the orientation of the analyzed region. Only the apical region (magnified in Fig. 5) is shown. Tracing analysis was yield on one control, one COND5, one COND10. Bar: 100 µm

We carried out the whole-lobule tracing on one control animal, one COND5 and one COND10 (Figure 25). We first analyzed MFT density and axonal branching and found no significant differences in these axonal properties between control and the two COND time points, thus indicating that mossy axons connectivity properties were not affected by FC training (Figure 26A, B).

Second, we asked if the rearrangements in the MFT morphology that we described in the previous section were specific to single terminals within one axon or rather common to all the terminals belonging to the same axon. Thus, we analyzed the MFT morphology in the axons where we could trace at least two terminals, and compared their morphology according to size, number/length of processes, shape and size (Figure 26C). We found that about 80% of the control axons were characterized by MFTs sharing similar morphology, and this proportion was preserved across the different experimental conditions (Figure 26D). Thus, being the morphology of single terminals dramatically affected by FC, as demonstrated in the previous section, we infer that all the MFT belonging to the same axon must be equally affected. We therefore conclude that the FC effect on MFT remodelling is played out at the axonal level.

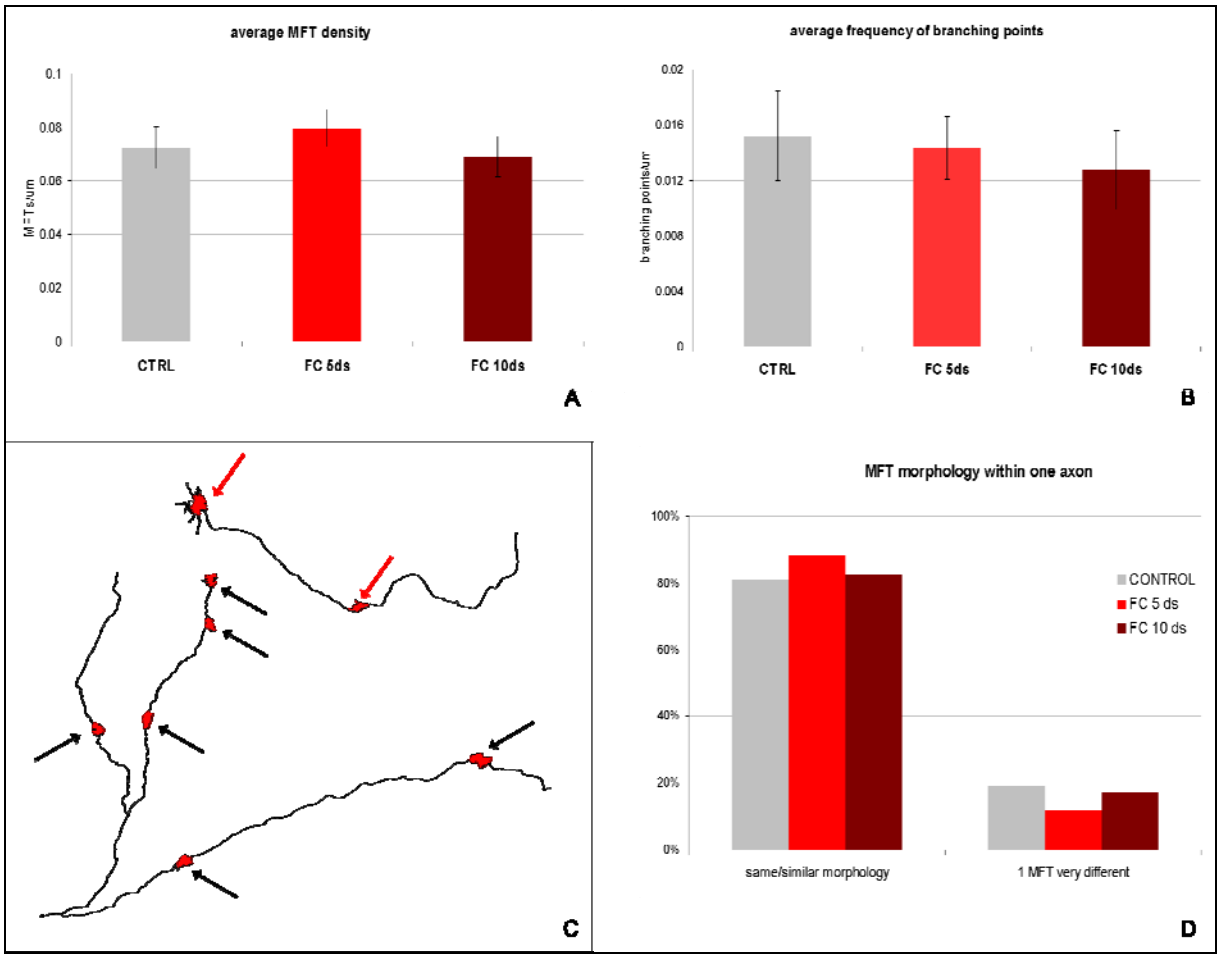


Figure 26 FC does not affect general mossy axon connectivity properties
A: quantitative analysis of MFT density in control, COND5, COND10. The number of MFT was normalized to the corresponding axon stretch length; N_{CTRL} = 48 axons; N_{COND5} = 87 axons; N_{COND10} = 39 axons; columns represent mean ± SEM. One-way ANOVA: p = 0.8167 (n.s.). **B**: quantitative analysis of branching point frequency in control, COND5, COND10. The number of branching points was normalized to the corresponding axon stretch length; One-way ANOVA: p = 0.9706 (n.s.). **C**: two representative axons exhibiting different properties: the axon pointed by black arrows has 6 MFTs with comparable size/shape/processes (category "same morphology" in D); the axon pointed in red has two MFTs very different for size and processes (category "1 MFT very different" in D). **D**: Subdivision of traced axons in two qualitative population, one bringing homogenous MFTs, the other bringing *at least* one MFT with very different morphology. The proportion of this subdivision is not affected by FC.

FC effects on MFT morphology are lobule-specific

We described extensively presynaptic structural rearrangements taking place in the mid-vermis lobule V, a region previously shown to be functionally affected by FC (Sacchetti et al., 2004). We then decided to investigate MFT morphology in another cerebellar region, lobule IX, known to be not involved in aversive behaviour (Sebastiani et al., 1992; Supple and Kapp, 1993) and dominated by vestibular inputs; in addition, PF-PC transmission is left undisturbed upon FC (Sacchetti et al., 2004).

Lobule IX MFT population was analyzed in control and COND5 in the same samples analyzed for lobule V. First, we noticed that, as expected from the anatomy

of mossy projections, the features of the labelled MFTs differed to some extent from lobule V, as suggested by unusual MFT morphology found for a subset of the population (Figure 27A). We found that lobule IX MFTs underwent a modest increase in the average size (Figure 27B, C upper graph), which, differently from lobule V, did not imply a redistribution of sizes in the population, but was rather given by a shift in the median value (not shown). Nevertheless, unlike lobule V MFTs, we did not find changes in the complexity of the terminals, as shown by the complexity index (Figure 27B, C middle graph), as well as in the population distribution for this parameter, exception made for a slight increase in the spread of the population (standard deviation 0.11 for control and 0.14 for COND5; not shown). Again, concerning the process features, there was no significant effect on COND5 lobule IX MFTs (Figure 27C, lower graph).

We observed that the dramatic remodelling of MFT morphology described in lobule V, particularly in terms of shift in the complexity, process behaviour and size distribution, is not taking place in lobule IX. Therefore, we conclude that FC effects are not generally affecting the whole cerebellar cortex, rather showing a regional specificity.

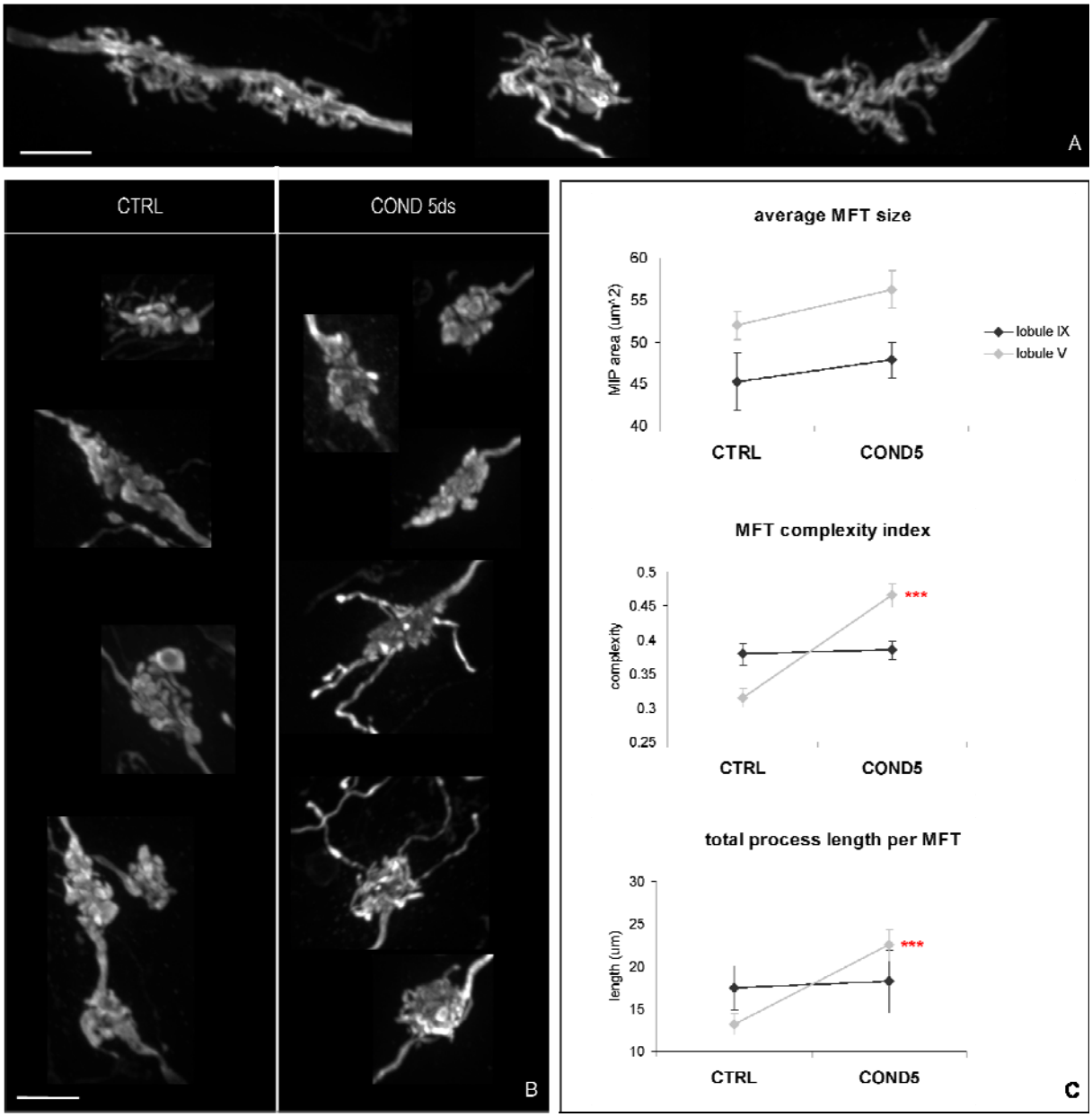


Figure 27 FC effects on MFT morphology are lobule-specific
A: representative examples from cropped MIPs showing peculiar morphology of lobule IX MFTs. **B**: gallery of representative MFTs cropped from 40-µm MIPs. CTRL and COND5 morphologies in lobule V are compared. **C**: comparative quantitative analysis of MFT size, complexity and processes between lobule V and IX of the same animals. Diamonds represent mean ± SEM; lobule V: N_{CTRL} = 147 MFTs (3 mice); N_{COND5} = 151 MFTs (3 mice); lobule IX: N_{CTRL} = 51 MFTs (2 mice); N_{COND5} = 109 (3 mice); Post hoc Student's t-test: ***p<0.0001. None of the parameters measured in lobule IX was found to change significantly (area p=0.25; complexity p=0.31; total process length p=0.46). Bar: 10 µm.

Extinction modulates structural plasticity triggered by fear conditioning

Next, we tried to modulate the structural plasticity outcome by acting on the administered experimental paradigm. Therefore, we decided to apply an extinction protocol following the FC training: this procedure results in a decline in conditioned fear responses. A body of evidences showed that this is due to a form of inhibitory learning, rather than an erasure of acquired fear (Myers and Davis, 2007). We asked

whether the structural remodelling we described to occur upon FC was still taking place upon extinction, or was specifically related to behavioural output; moreover, we aimed at describing whether the time course and amplitude of plasticity events were left undisturbed, or whether the extinction induced different series of rearrangements. We applied our standard FC protocol; 5 hours from the training, the mouse was subjected to a first extinction session, where only the CS was played several times; a second extinction session was repeated 24 hours after the FC training. The mouse was then left undisturbed until the sacrifice time (see Experimental Procedures for a detailed description of the experiment); survival times were chosen between 2 and 10 days after the FC paradigm ("EXT" group, where the suffix number indicates the days of survival after conditioning). In a further series of experiments, extinction sessions were played at day 2 and 3 after training ("late-EXT" group). Before the sacrifice, animals were tested for memory retention and included in the analysis according to the behavioural readout. Control mice underwent the same procedures, but the tone was never associated with the shock.

2 days after EXT: morphology is remarkably different between EXT2 and COND2

MFT population morphology was examined in lobule V according to the previously listed criteria. EXT2 group showed a sustained increased complexity of the terminal surface, which was very similar qualitatively to rearrangements described for COND2 (Figure 28; compare with Figure 20), as MFTs were generally characterized by a bulgy surface. This was expresses by an average increase of the complexity index by ~43% compared to the control population, while COND2 index was increased by ~28% in average (Figure 29A, B). With respect to COND2, EXT2 population showed a comparable shift of the lower-quartile value, but the MFT complexity distribution "stretched" towards high values (interquartile range 0.33-0.57, doubled compared to the control range; Figure 29A). Differently from COND2, no shift in the size distribution was observed in EXT2 (Figure 29C, D). Finally, a decrease in the total process length was observed (Figure 30B), mostly attributable to the decrease of the average process length (Figure 30A). We thus observed that two days after extinction the remodelling processes that take place are basically enhancing the sign of

rearrangements observed two days after conditioning, excepting the size distribution, not affected at this time point.

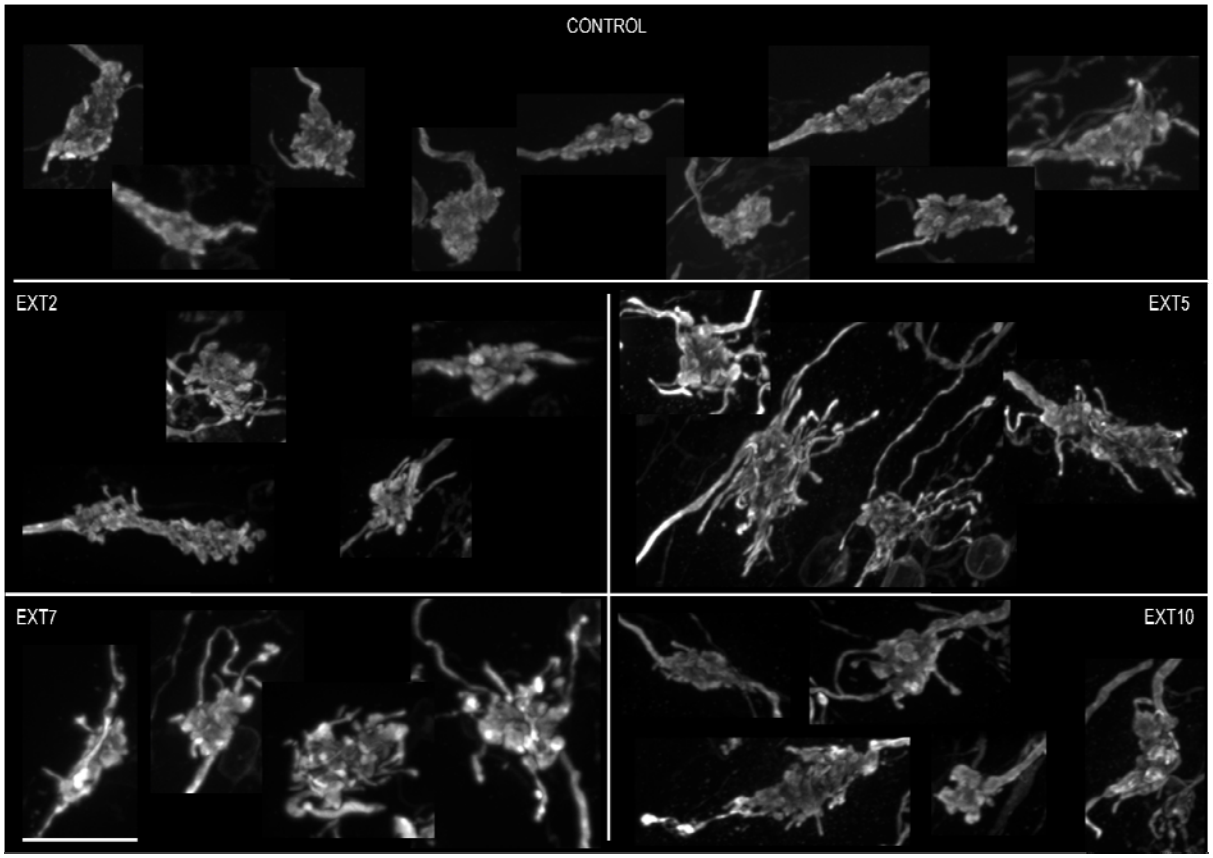


Figure 28 Extinction modulates structural plasticity triggered by fear conditioning
Representative MFTs cropped from 40 μm-MIPs are shown. MFTs rearrange upon EXT in a time scale comparable to FC-induced remodelling. Bar: 20 μm.

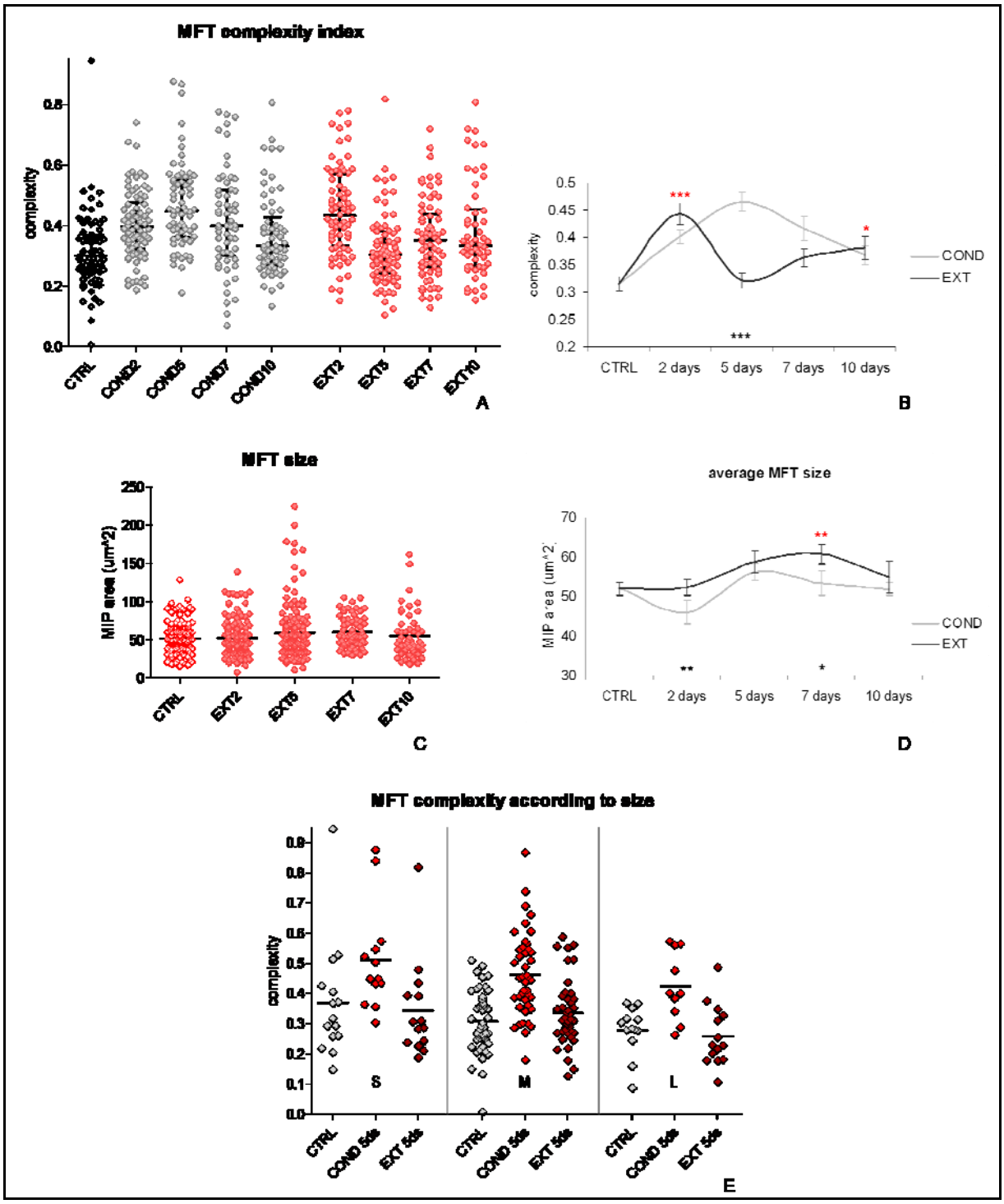


Figure 29 MFT complexity and size are affected in response to EXT

Quantitative analysis of MFT morphology along the time course. **A-B**: Quantitative analysis of MFT complexity. **A**: every diamond represents one MFT; red lines represent *median* with interquartile range; N_{CTRL} = 77 MFTs (2 mice); N_{EXT2} = 69 (2 mice); N_{EXT5} = 71 (2 mice); N_{EXT7} = 68 (2 mice); N_{EXT10} = 54 (2 mice). **B**: COND (grey) and EXT (black) time courses are represented. Lines connect mean values ± SEM. Two-way ANOVA (*vs* control): ***p < 0.0001 (red); *p = 0.02172; ***p(COND5 *vs* EXT5) < 0.0001 (black). C-D: Quantitative analysis of MFT size. **C**: every diamond represents one MFT; black lines represent mean values ± SEM ; N_{CTRL} = 147 MFTs (3 mice); N_{EXT2} = 135 (2 mice); N_{EXT5} = 153 (3 mice); N_{EXT7} = 68 (2 mice); N_{EXT10} = 54 (2 mice). **D**: Lines connect mean ± SEM. Two-way ANOVA (*vs* control): **p = 0.001789 (red); **p(COND2 *vs* EXT2) = 0.0097 (black); *p(COND7 *vs* EXT7) = 0.01029 (black). **E**: MFT complexity distribution according to the size, comparing control, COND5, EXT5. The population was divided in small (S, area < 35 μm^2), medium (M, area 35-70 μm^2); large (L, area > 70 μm^2) terminals. Black lines represent mean.

5 days after EXT: the largest divergence between COND and EXT time course

Five days after extinction, the general MFT population aspect was dramatically remodelled compared to the previous time point, EXT2 (Figure 28), and the corresponding time point COND5 (Figure 20). First, the MFT complexity properties reverted back to control range (Figure 29A, B), both for the average values and the population distribution. The decrease in average complexity compared to COND5 was common to the whole MFT population, while, when compared to control population, the effect was enhanced for very small or very large terminals (Figure 29E). Nevertheless, this remodelling was accompanied by a conspicuous increase in the average MFT size of the population upper quartile (Figure 29C, D) and by a dramatic increase of the average process length (Figure 30A, B). Thus, 5 days after extinction the overall structural rearrangements have partially distinct properties compared to COND5, being the MFT complexity values reverting to control range, but the effect on size and processes following the same kinetics as in the COND time course.

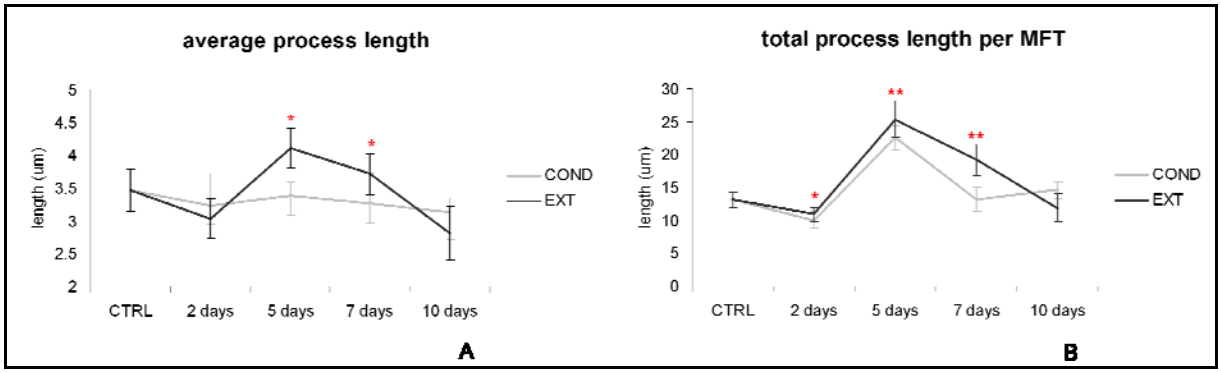


Figure 10 MFT processes are strongly a affected by EXT
Quantitative analysis of MFT length along the time course. A: Quantitative analysis of average process length per MFT; lines connect mean values ± SEM. N are listed in Figure 11C. Post hoc Student's t-test: *p(EXT5) = 0.0153, *p(EXT7) = 0.0265. B: Quantitative analysis of total process length per MFT; lines connect mean values ± SEM. Post hoc Student's t-test: *p(EXT2) = 0.0441; **p(EXT5) = 0.0062; **p(EXT7) = 0.0044.

7 and 10 days after EXT: MFT morphology converge again

After pronounced divergence 5 days post FC training, the general aspect of EXT7 and EXT10 reminded COND7 and COND10 MFT morphology (Figure 28; compare with Figure 20). This was confirmed by looking at the MFT complexity value distribution, which was found to be basically overlapping between the corresponding

time points, particularly observing the median values (Figure 29A, B). Size distribution and total process length were observed to convergence to corresponding COND7 and COND10 values, and to control ranges in general (Figure 29D and Figure 30A, B).

In summary, we found that extinction experience profoundly affects structural rearrangements imposed by FC training. In particular, MFT surface complexity reacts differently along the time course when compared to COND time points; on the other end, effects on the MFT size and process length properties followed the COND time course but rearrangements generally took place with an increased amplitude.

Structural plasticity is affected by the interval occurring between FC training and extinction

We next asked whether the interval occurring between FC training and the first extinction session was critical in order to observe the structural rearrangements described in the previous paragraph. In order to answer this question, we decided to delay the time of the two extinction sessions to day 2 and 3 after FC and to sacrifice the mice 7 days after FC training (see Experimental Procedures for details). In this way, we could directly compare late-EXT7 with EXT7 and COND7, being 7 days the survival time after FC for all the groups, and late-EXT7 with EXT5, being 3 days the time interval between the last extinction session and the sacrifice time for the two groups. Note that, if the EXT timing we previously showed is critical for the structural outcome, the terminals should fall in the COND7 range; conversely, if extinction triggers a series of rearrangements independently of its timing respect to the FC training, MFTs should fall in EXT5.

A schematic representation of the experiment outcome is shown in Figure 31A. From the surface complexity aspect, we observed that the late-EXT7 terminals were in average falling in the COND7 group, though the population deviation was smaller (Figure 31B). We then analyzed the MFT distribution in terms of size: at 7-day survival time, the discrepancy between COND and EXT was significant: the average values for late-EXT7 fell in between of the other two groups, with a distribution range overlapping with the other population, thus not allowing clear interpretation of these

data. Finally, the process properties clearly followed the EXT time course, being the average process length between EXT5 and EXT7 values and the total length coinciding to EXT7 average values (Figure 31D-E).

From this complex pattern, we inferred that the timing of the extinction session is critical to determine the effect on MFT complexity, being extinction not able to affect this parameter when delayed respect to the initial training; conversely, the extinction experience *per se* induces sustained outgrowth of processes, independently of its timing. It is worth noting that any of the parameters reached the EXT5 amplitude in the late-EXT7, that represents the proper time point to compare in terms of time length from the training (3 days of survival time for both conditions), thus pointing out the global differential effect induced by this protocol compared to COND and EXT experience.

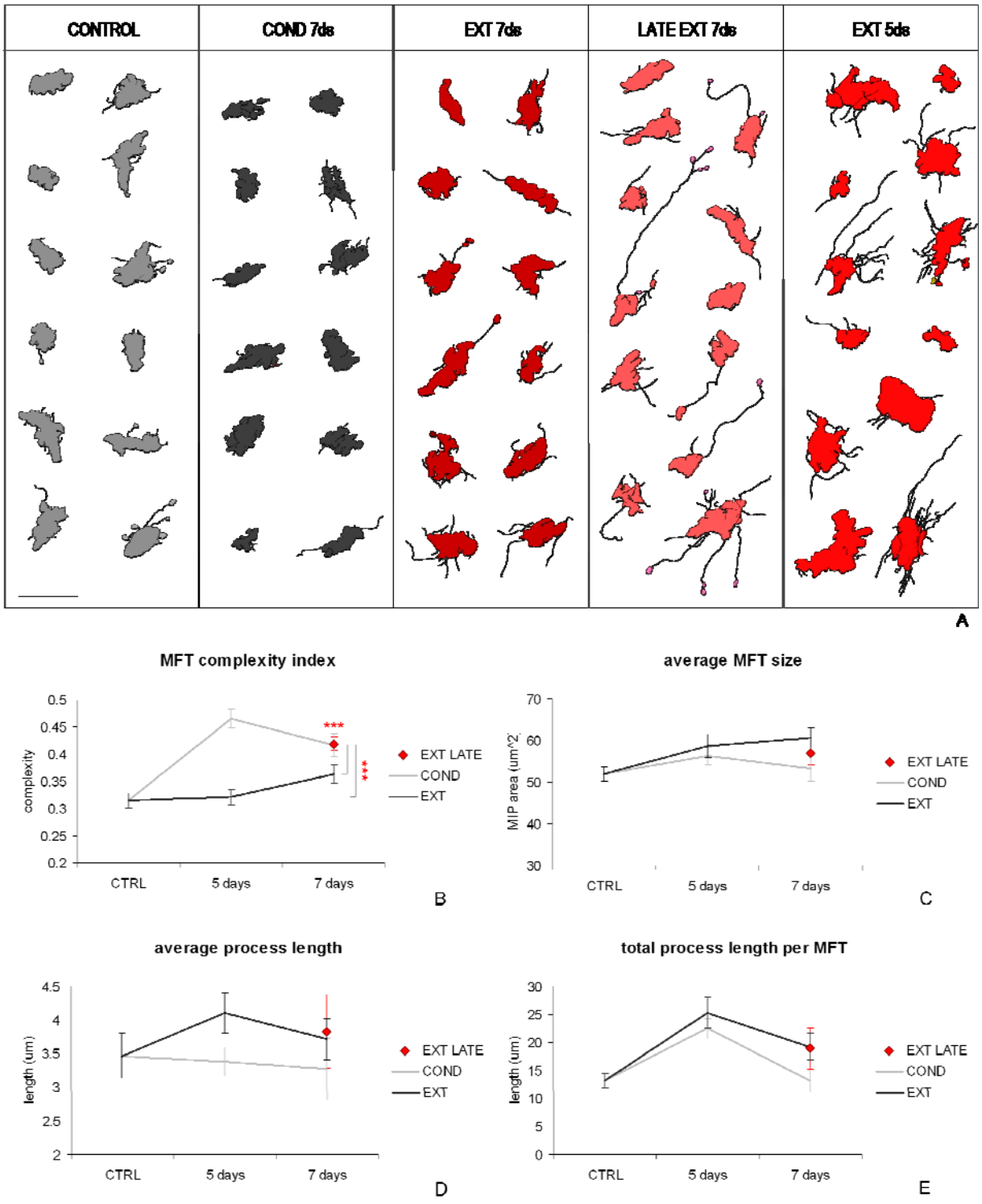


Figure 11 Plasticity is differentially affected by late extinction

A: *camera lucida* of single MFTs from control, COND7, EXT5, EXT7 and late-EXT7 samples. 12 MFTs were arbitrarily chosen for every condition in order to highlight experimental effects. **B**: Quantitative analysis of MFT complexity; Lines connect mean values ± SEM; red diamond represents late-EXT7 mean ± SEM. N_{EXT7_LATE} = 60 (2 mice). Two-way ANOVA (*vs* control): ***p < 0.0001 (red); ***p(EXT5 *vs* late EXT7) < 0.0001 (black); ***p(EXT7 *vs* late EXT7) < 0.0001 (black). **C**: Quantitative analysis of MFT size; N_{EXT7_LATE} = 60 (2 mice). No significative relations according to 2-way ANOVA were found (late-EXT7 *vs* COND7, EXT5, EXT7). **D-E**: Quantitative analysis of average (**D**) and total (**E**) process length. Late-EXT7 fell on EXT7 range. No significant relations according to 2-way ANOVA were found (late EXT7 *vs* COND7, EXT5, EXT7). Bar: 20 µm

Postsynaptic remodelling is differentially triggered by FC and EXT

Last, we aimed at describing postsynaptic morphology of the MF-GC synapse 5 days after COND or EXT. *Lsi1* mouse line labels GCs in the granular layer, thus allowing to reconstruct 3D morphology of a representative number of GC dendrites in the cerebellar cortex (Figure 32). Given the high number of MFTs and GCs labelled in Lsi1 GL, we were not able to solve completely an adequate number of GCs in order to comment on general GC connectivity properties; our focus will be on the morphology of single dendritic endings, or claws (Figure 33). Treated samples showed an increased divergence in the morphology of claws belonging to the same cell when compared to control population (Figure 32; not further investigated yet).

We analyzed claw morphology by considering the number and the average- and total length of digits building up a single claw. Qualitatively, we noticed that both COND5 and EXT5 population displayed an increased fraction of claws characterized by numerous very short digits, despite of simpler morphology in COND5; conversely, in EXT5 population complex claws were found less frequently (Figure 33). We looked at the correlation between the listed parameters within the same claw: we found that the correlative relation between the digit number and length was preserved across the samples and that the population distribution varied considerably according to the condition (Figure 34). In particular, we observed that, compared to the control population distribution, COND5 and EXT5 population were more spread: EXT5 population frequently showed few-/short digit claws, as evident from Figure 33; on the other hand, COND5 displayed a fraction of medium-/long and many-/long digit claws that was absent from control distribution, thus confirming our initial observations.

We therefore conclude that 5 days after the initial training, COND and EXT differentially act on postsynaptic morphologies increasing the deviation of the population, and, more specifically, expanding the distribution of the population towards simpler claw morphology (EXT) or to more complex claw organization (COND).

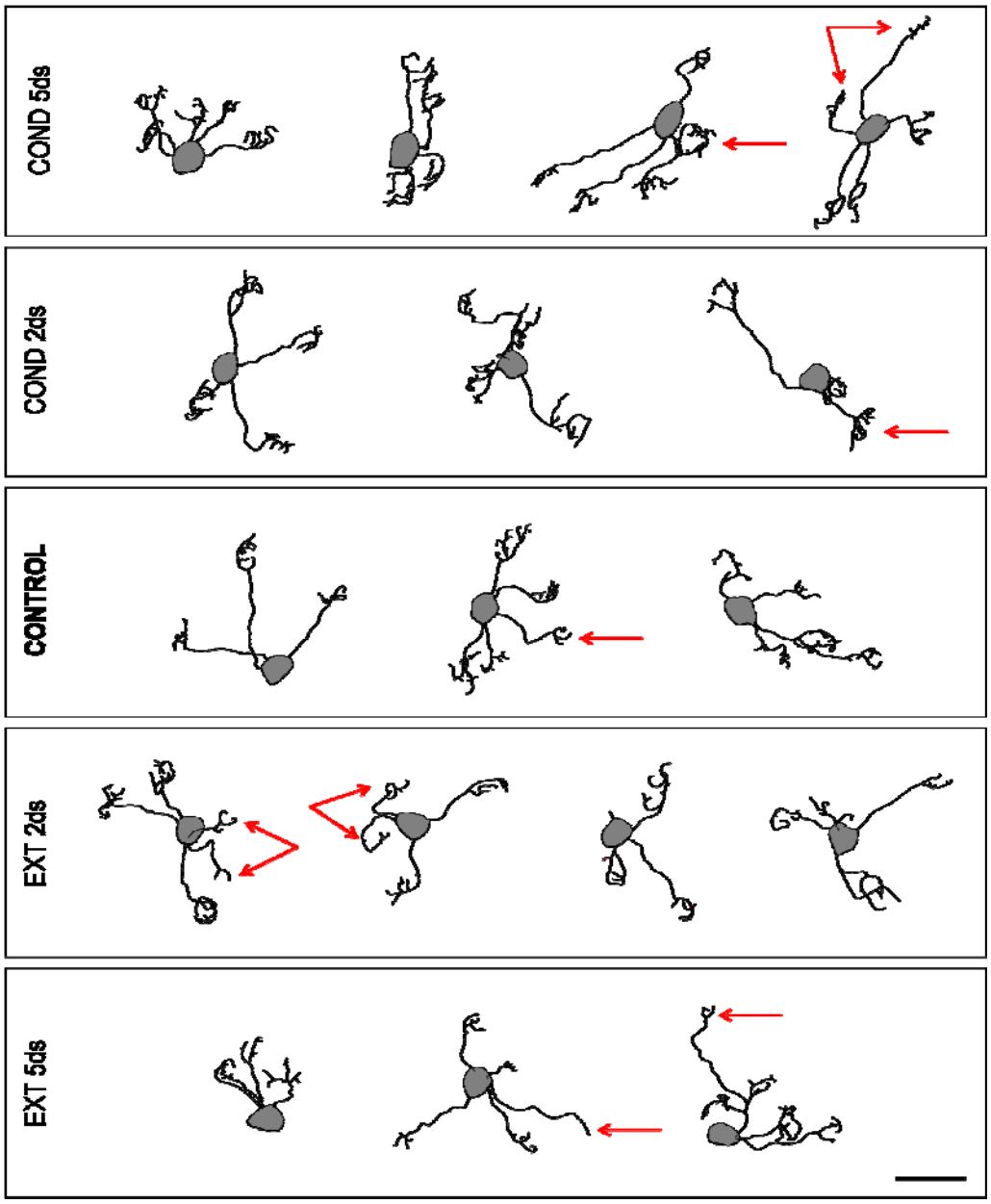


Figure 12 Postsynaptic remodelling is differentially triggered by FC and EXT
Camera lucida of isolated GCs chosen from different samples. When comparing global cell morphology between control and treated samples, a higher divergence in claw morphology belonging to the same cell was evident. Examples are pointed with red arrows; double arrows indicate pairwise similar claws.

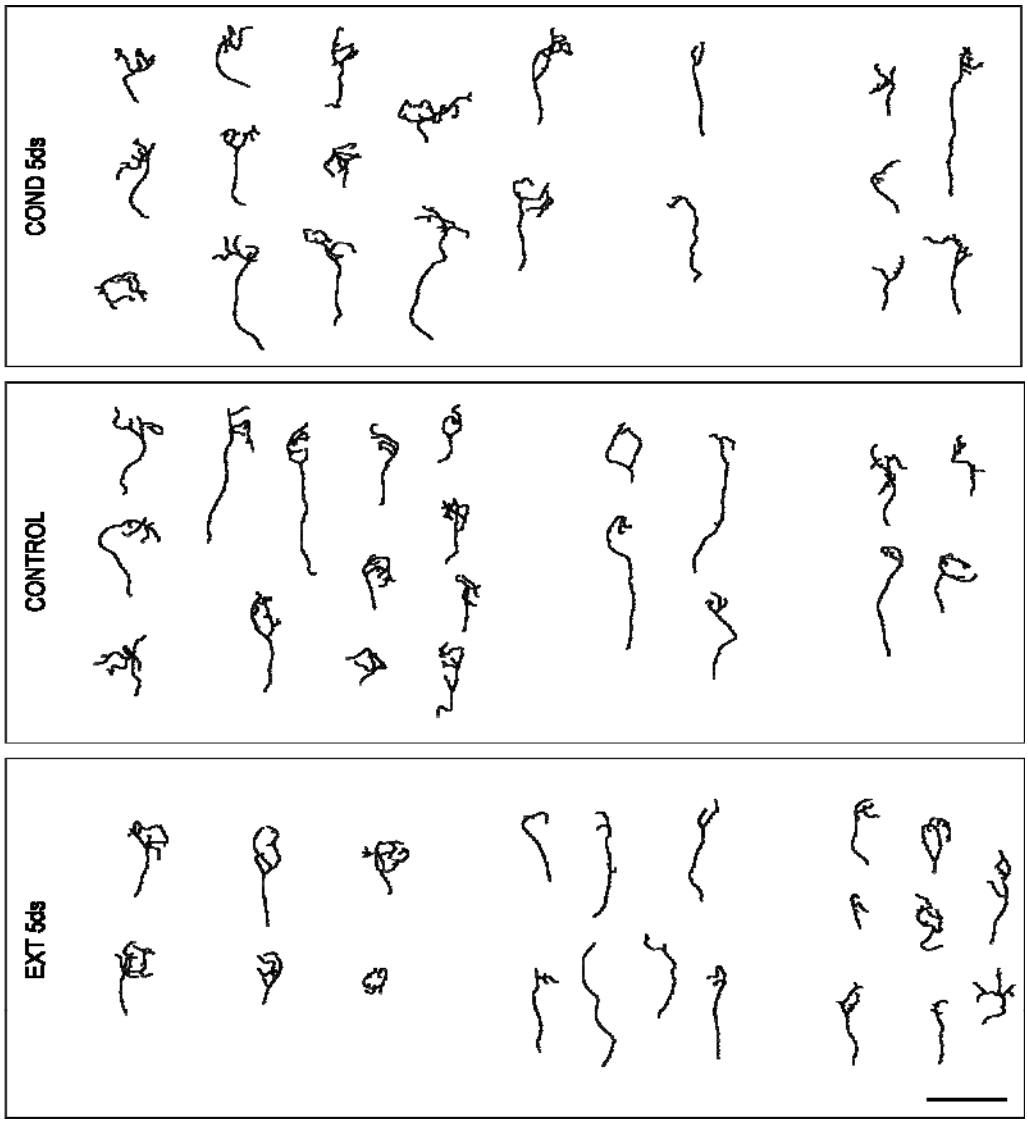


Figure 33 Postsynaptic remodelling acts on single claws morphology
Camera lucida of isolated dendritic claws chosen from sparse cells in different samples. COND5 and EXT5 population displayed an increased fraction of claws characterized by numerous very short digits, despite of simpler morphology in COND5; conversely, in EXT5 population complex claws were found less frequently. More complex claws are grouped on the left, simpler claws in the middle, claws decorated with very short digits on the right. Bar: 10 μm.

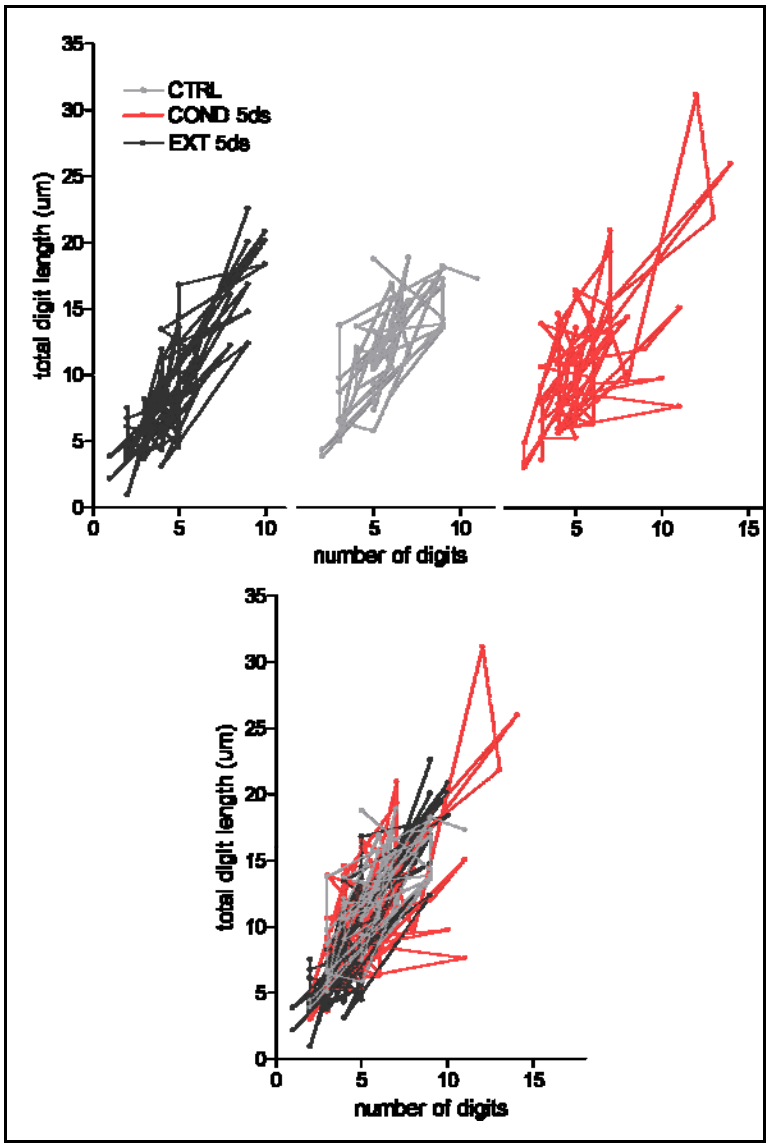


Figure 34 Postsynaptic remodelling affects digit number within one claw

Correlative analysis of the claw morphology according to digit number and total digit length within one claw. In the upper graph, the correlation between the number of digits and the total digit length is shown for control, COND5 and EXT5 (same y axis, shifted x axis; dots represent single claws and are randomly connected by lines to better visualize the population spread; in the lower graph, distributions are merged. The uniformity of the control claw population is evident in the compact gray shape; black (EXT5) and red (COND5) shapes show higher deviation and increased presence of few-/short digits claws (EXT5) or many-/medium-long digits (COND5).

2.2.3 DISCUSSION

We have shown that FC profoundly affects MFT morphology in a process spanning several days and peaking 5 days after conditioning (Figure 22 and 23), without altering general connectivity properties of the mossy axons (Figure 26). Similarly, the postsynaptic compartment exhibits modifications of synaptic ending morphological properties (Figure 34). In addition, we showed that the structural rearrangements can be modulated according to subsequent experience (Figure 29 and 30). Taken together, these observations provide evidence that FC triggers a long-term reshaping of synaptic connections at the input stage of the adult cerebellar cortex.

Below, possible implications of these findings will be discussed and future directions will be suggested.

FC affects synaptic morphology, leaving system connectivity properties intact

We described morphological rearrangements involving mossy axon terminals in the apical region of cerebellar lobule V over the 15 days following a FC training: we found that these events were axon-related, affecting the morphology of all the terminals placed by a given axon in lobule V, yet not affecting its general connectivity properties, such as MFT density or branching frequency. This kind of axonal structural plasticity requires a re-sampling process of potential synaptic partners and leads to the modification of local connectivity in neuronal circuits (Chklovskii et al., 2004; Gogolla et al., 2007). Instances of these phenomena were shown in repeated *in vivo* imaging studies in adult animals: axons physiologically exhibited local target exploration, consisting in particular in the appearance and disappearance of *en-passant* boutons

along pre-existing axon branches, or remodelling of terminal boutons (De Paola et al., 2006; Stettler et al., 2006) (Figure 18, chapter 2.1.3).

These observations give a readout to interpret the strong terminal remodelling we observe upon FC as a mechanism underlying changes in local connectivity. This is additionally supported by the evidence that MFT density and branching behaviour of mossy axons are let unvaried 5 and 10 days after FC (Figure 26), indicating that the system connectivity properties are essentially not targeted by the FC training. Similarly, the postsynaptic compartment rearrangements are concentrated at the single dendrite level and do not involve globally GC connectivity. With this respect, we speculate that most of the structural remodelling triggered by FC could underlie "weight" changes between previously connected neurons. Chklovskii and colleagues (2004) suggest that this mode of plasticity could be expressed in terms of changes in the efficacies of existing synapses, or by structural changes that lead to the addition or subtraction of synaptic contacts between previously connected pre- and postsynaptic units. In this *scenario*, the encoding of learned information exploits the ability of the system to increase and decrease the "weights" on existing connections (McClelland, 1985). In fact, several types of experiments have provided evidence that synapse formation and elimination, as well as differential turn over rates, can occur in the adult brain in response to sensory experience (Turner and Greenough, 1985; Knott et al., 2002; Trachtenberg et al., 2002; Holtmaat et al., 2006). Nevertheless, in our experiments, a wide remodelling of the whole examined region is still achieved: we found that all the terminals belonging to a given axon are equally affected by the structural remodelling, independently of their distribution or position along the axon. In addition, we found that MFT distribution according to the surface complexity involves a global response of the population (Figure 22A), indicating that a large fraction of the axons projecting to lobule V of the cerebellar cortex reacts to FC experience.

It is worth noting that the time scale on which the events are played (5 days are needed to reach the maximal amplitude of the remodelling events, Figure 35) is compatible with phenomena taking place at a local scale: in fact, experience-related shifts in functional representation would take at least 7 days to appear (Kleim et al., 2002). Interestingly, when we made our criteria to classify MFT morphology within one axon more strict, we found in COND10 an increased fraction of axons showing subtle differences between their terminal morphologies (not shown). This could

indicate that the remodelling back to control range morphologies, which we know is happening by that time point, can proceed with slightly different speed across MFTs belonging to the same axons, or could indicate that not all the terminals will fall back in that range.

Finally, MF projections in the cerebellar granular layer are organized, in the rat and other species, in a mosaic-like pattern with multiple representations of the same receptive field (Shambes et al., 1978; Bower et al., 1981; reviewed in Voogd and Glickstein, 1998): it is then tempting to speculate that, upon FC experience, a single axon is able to coordinate weight or strength remodelling in distinct somatotopic representations by means of terminals projecting to different patches, thus constituting the structural basis for functional plasticity to occur.

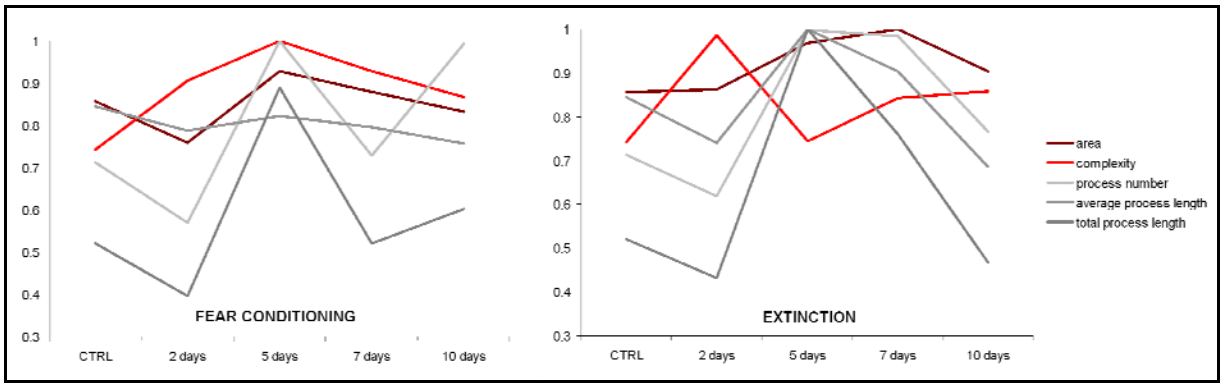


Figure 35 Remodelling events peak 5 days after initial training
Parameters describing MFT morphology at each time point are here normalized to their maximum amplitude value (considering both experiments) and plotted in two separate graphs, one for COND and the other for EXT experiment. Convergence of maximal amplitude of the rearrangements 5 days post COND is evident. In EXT time course, the complexity peak is moved forward.

From structure to function

We noticed that, upon FC, the MFT surface showed gradual rearrangement in terms of convolution, with a deviation from the average control population aspect, peaking 5 days after FC, gradually decreasing until 15 days and involving the overall population. This feature was already evident in COND2, being this time point characterized by the presence of local swelling of the surface ("bulges") that made the MFT surface more convoluted. In COND5 we could still detect bulges on the surface,

in addition to a pronounced irregularity of the surface and of the terminal shape (Figure 20 and 21). Geometrically, the convolution of the surface brings to an increased ratio between MFT surface and volume, thus meaning that, in the same volume, a MFT has an increased surface available for potential synaptic contacts. This interpretation is supported by ultrastructural data about glomerulus morphology during postnatal development: in fact, it was shown that MFT convolution, or complexity, increased proportionally to the number of digits insisting on its surface (Hamori and Somogyi, 1983). Second, we found that COND5 GCs display a wide fraction of very complex claws (Figure 33), characterized by many and long digits (Figure 34), thus providing a postsynaptic counterpart consistent with our interpretation. Our ultrastructural data, finally, indicate that, within comparable areas, COND5 MFTs are contacted by a higher number of digits than controls (Figure 36). Under these circumstances, we propose that the functional consequence of the increased complexity could be the enhancement of synaptic transmission, at least in terms of signal amplification, of a given MFT. So far, we have no data about the strength of the synaptic transmission, which could also be affected at this synapse by FC (as briefly described in the introduction). Furthermore, it is possible that the increase of digits contacting a single MFT is attributable to more GCs contacting the same presynaptic units, thus increasing the divergence of the transmission; however, given the body of our observations, we consider more likely that the increase in complexity is given by higher number of contacts between a pre- and a postsynaptic unit previously connected.

As a complementary aspect of the surface convolution, the size distribution in COND5 population shows an increased fraction of very large terminals (Figure 21 and 22C), which shifts the average size of the population, though not reaching significant deviation from the regular population. We infer from this that only a subgroup of the population might react to FC by increasing its size: it is possible that this group represents an axonal population whose sensory information is further privileged in term of transmission compared to the rest of the afferences.

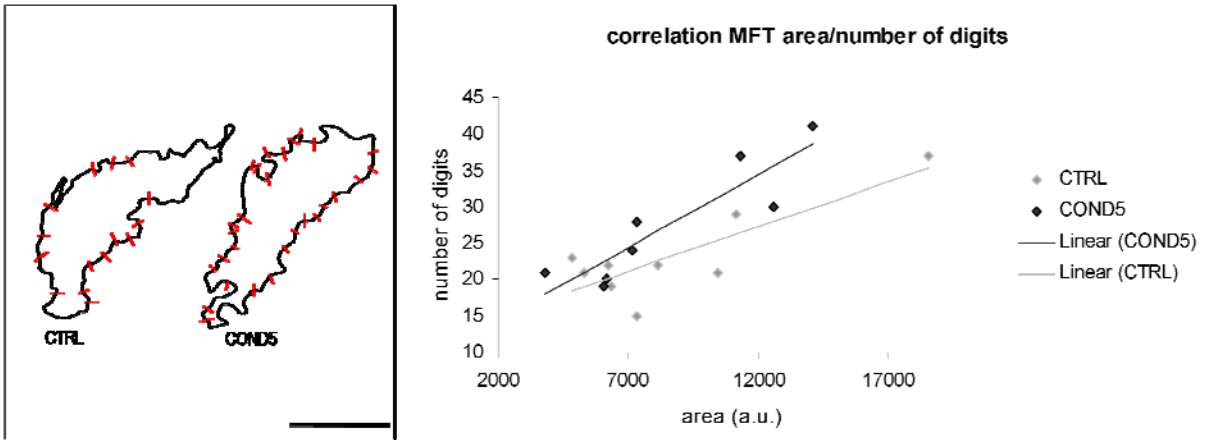


Figure 36 Ultrastructure reveals a higher number of digits contacting COND MFTs
Camera lucida **drawing of two representative MFTs from CTRL and COND5 samples. The black shape represents MFT profile; red lines correspond to different digits contacting the MFT surface. Regions devoid of digits are generally touched by glial cells or other MFT subunits. The graphs shows the correlation between size and number of digits contacting a MFT: in the same area, COND5 MFTs form a higher number of contacts with distinct postsynaptic partners. Bar: 1 μm**

We found that the total process length for one MFT was significantly increased both 5 days after FC, being the average length kept constant, both 5 days after EXT, being in the latter case also the average length increased (Figure 23 and 30). This means that COND mainly triggers an increase in the process number, while EXT acts both on the process number and average length. Process (or filopodia) structure lets suppose an explorative and dynamic behaviour (Mattila and Lappalainen, 2008) but, despite having been classically reported (Mason and Gregory, 1984), there is currently no hypothesis explaining their role in the adult cerebellum. However, the mGFP line used in this study allowed us to make some interesting observations (Figure 37).

Active zone staining revealed that MFT processes can have release sites on them (Figure 37A), thus possibly participating in synaptic transmission. In one case we were able to observe an isolated GFP positive process emerging from the MFT core and ending at the basis of a dendritic claw placed at several tens of micrometers away from the terminal itself (Figure 37B). These observations suggest roles for MFT processes in system connectivity. One possibility is that a process is able to leave its original glomerulus and to make contacts outside of it, possibly with GCs placed in another islet. Filopodia emerging from large mossy terminals in the hippocampus are known to excite GABAergic inhibitory interneurons (Danzer et al., 2008): thus, an intriguing hypothesis is that processes are responsible for direct contacts between MFTs and Golgi cells (Eccles et al., 1967), thus promoting Golgi cell inhibitory action on postsynaptic claws, and consequently affecting claws connectivity within the

glomerulus: in the EXT experiment, the increase in length and number of processes would then promote inhibitory transmission on GCs, thus weakening MF-GCs contacts, consistently with the presence of simple- and short digit claws in EXT5 (Figure 33 and 34).

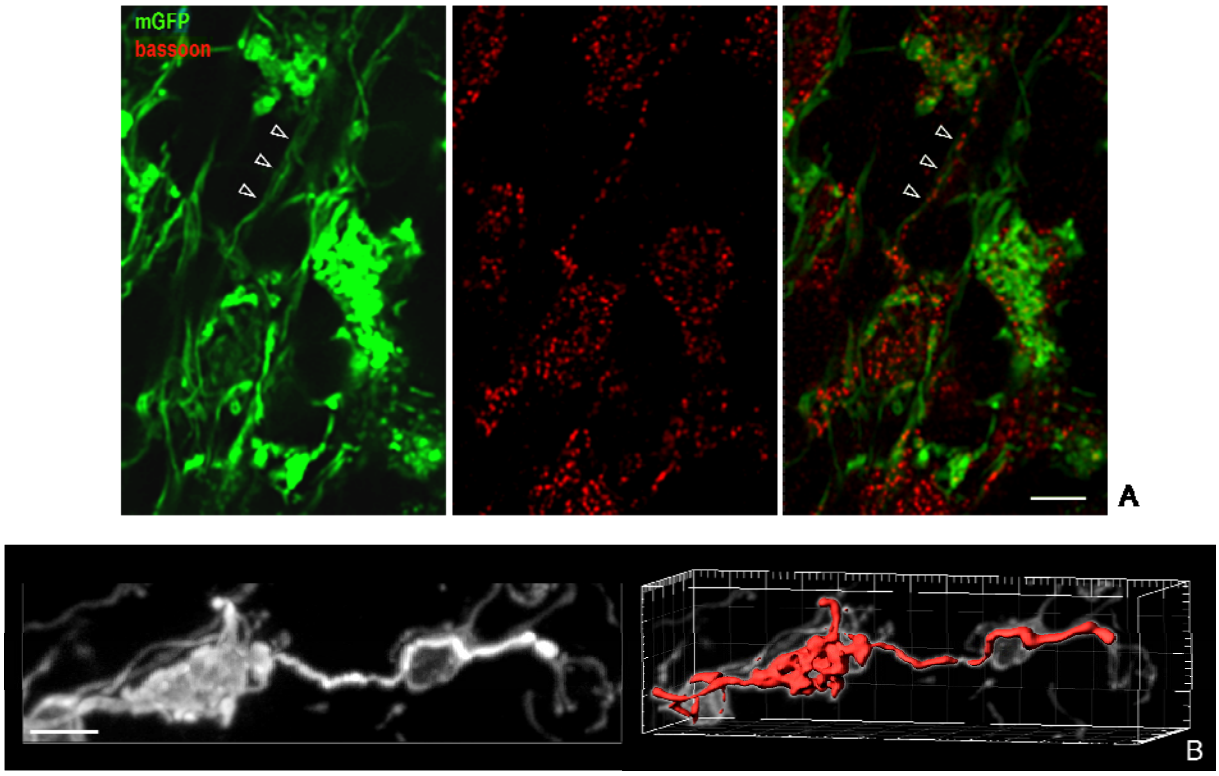


Figure 37 MFT processes actively participate in the connectivity
A: bassoon labelling (red) shows active zone distribution in mGFP-positive cells (green): arrowheads indicate bassoon puncta decorating processes that depart from a MFT. Single GFP channel (left) has been adjusted in order to better visualize thin processes. **B**: on the left, a collapsed confocal stack represents one process departing from the core terminal, stepping over a GC body and terminating on its claw. On the right, the volume projection of the same stack is shown: different fluorescent intensities between the two cells allowed to build an isosurface isolating the MFT profile (in red). Bar: 10 μm

Finally, observing the time course of the events we described, we believe that the structural remodelling we found cannot account for autonomic responses, immediately triggered as part of the freezing response (Supple and Leaton, 1990; Sebastiani et al., 1992; Supple and Kapp, 1993; Yoshida et al., 2004), or synaptic potentiation, which is present already 10 minutes after FC training (Sacchetti et al., 2004), but could rather participate to later processes, such as in the long-term maintenance of aversive memories (Sacchetti et al., 2007) and behavioural output (from a motor and emotional point of view, see Damasio et al., 2000). To this point, in our experiments, we found no obvious correlation between the amplitude of

structural remodelling and the behavioural output in term of freezing response (not shown).

Taking these considerations together, we observe that structural remodelling that occur upon FC are compatible with an increase of local synaptic transmission, in terms of active contact sites and amplification of the signal; growth of processes could further contribute to the enhanced transmission within or, possibly, outside the original glomerulus; alternatively, they could rather serve the inhibitory loop in the cerebellar cortex.

FC-related structural rearrangements are lobule-specific

We investigated structural rearrangement of the MFT morphology occurring 5 days after COND in two distinct cerebellar lobules in the mid-vermis, namely lobule V and lobule IX. We found a profound alteration of the regular morphology in lobule V in terms of complexity, process outgrowth and size distribution; in lobule IX none of these events took place, except for an increase in the average MFT size, which was mainly due to redistribution of sizes in the physiological range (Figure 27B-C). Thus, FC acts differentially according to the cerebellar regions. This is particularly interesting with respect to the cerebellar anatomical organization: lobule V and VI of the cerebellar vermis represent the region of dominant convergence of acoustic and nociceptive stimuli (Snider and Stowell, 1944; Huang et al., 1982) and is related to emotional behaviour (Sebastiani et al., 1992; Supple and Kapp, 1993). In addition, lobule V-VI PF-PC synapse was shown to be potentiated upon FC (Sacchetti et al., 2004). Conversely, lobule IX is part of the vestibulocerebellar system, thus receiving mainly primary and secondary vestibular projection, sensory ways which are not involved in the FC experience. In addition, lobule IX, as part of the posterior cerebellum, is known not to be involved in aversive behaviour (Sebastiani et al., 1992; Supple and Kapp, 1993). Furthermore, it was shown that the PF-PC synapse is specifically potentiated after FC in lobule V and not in lobule IX. These anatomical and functional notes are consistent with our observations and suggest that the structural remodelling that we observe is strictly related to specific sensory modalities

that are involved in the FC task, together with cognitive aspects that could be coded in the cerebellar cortex.

Modulation of FC-related structural plasticity by experience

Fear extinction is defined as a decline in conditioned fear response following non-reinforced exposure to a feared CS. Crucial sites for extinction are basolateral amygdala, prefrontal cortex and hippocampus, which are specifically involved in different phases of extinction learning (Quirk and Mueller, 2008). Behavioural evidences indicates that extinction is a form of inhibitory learning, as supported by the evidence that extinguished fear responses reappear with the passage of time, a shift of the context or an unsignaled presentation of the US (Myers and Davis, 2007). Moreover, specific extinction circuits have recently been described in the amygdale (Herry et al., 2008). Notably, it has been found that rats extinguished 24-72 hours following fear acquisition exhibit a behaviour compatible with a form of inhibitory learning, whereas rats extinguished 10-60 minutes after acquisition rather exhibited an "unlearning" of FC training. This report supports the idea that different neural mechanisms are recruited depending on the temporal delay of fear extinction (Myers and Davis, 2007).

In our experiments, we found that two extinction sessions performed 5 and 24 hours after the FC training were able to modulate the structural remodelling induced by the mere FC experience. In particular, MFT complexity was considerably increased after two days (which could possibly represent an acceleration of the COND processes, see Figure 35), to then drop back to control values 5 and 7 days post initial FC and finally reach COND10 complexity range. In addition, average size as well as process outgrowth were kept higher throughout the time course when compared to COND remodelling (Figure 29 and 30). It is difficult to reconcile this active and dynamic remodelling process with a mere "erasure" of the previous experience, as suggested for early fear extinction: on the other side, our first extinction session was played 5 hours after the training, which is rather thought to fall out of the short-term plasticity time range (Cain et al., 2005). However, we found that delayed extinction sessions (53 and 72 hours post FC) were not able to modulate the effects of sole FC

on MFT complexity, while the extinction experience *per* se, independently of its timing, still induced sustained outgrowth of processes. We speculate that the process outgrowth could be related to the recruitment of inhibitory components of the circuitry, as previously discussed.

2.2.4 CONCLUSIONS AND FUTURE DIRECTIONS

So far, we have provided evidences that a reshaping of the connectivity at the input stage of the adult cerebellar cortex is induced by FC in the adulthood. From an anatomical point of view, we found that the local connectivity at the MF-GC synapse is step-wise remodelled for several days following the FC training; the extent and quality of remodelling is lobule-specific and can be further modulated according to subsequent experience. We think that these structural rearrangements could mostly underlie weight changes between previously connected neurons, as well as differential recruitment of inhibitory components of the circuitry. However, the remodelling globally affected the region in exam, thus accounting for massive reshaping of connectivity and transmission. Evidences about synaptic transmission at the MF-GC synapse upon FC would help in order to clarify the correlation between anatomical and functional properties.

It is not clear yet if the structural rearrangements we observe are transitory or an anatomical "trace" is left by such an experience in the cerebellar cortex. Our studies were carried until 15 days post FC: longer time points will help in order to generally address this question. However, our study was made on *ex vivo* samples, meaning that we could not follow rearrangements at a particular synaptic unit: thus, it is not clear whether these massive plasticity events lead to a rewiring of the circuitry, or the decline in the amplitude of rearrangements rather means a reversion to the baseline connectivity. An *in vivo* time lapse imaging approach would allow to solve these questions; with this respect, lobule V of the vermis is a suitable site, being easily accessible for these kind of studies. However, technical issues still make this approach not applicable.

As a further direction, we are interested in investigating how these phenomena are regulated: for instance, neuromodulatory afferents could be crucial in triggering these events. Additionally, specific signalling pathways could be involved in FC-related structural plasticity. Data from our laboratory (N.G., submitted) indicate that the Wnt-7b pathway is crucially involved in experience-dependent structural remodelling. Thus, we propose to test these hypotheses by *in vivo* local injection of mimicking/blocking compounds.

Finally, FC is a complex paradigm that involves sensory stimulation, learning and cognitive/emotional aspects. In order to further dissect its effects, we propose to carry out a similar study on animals specifically trained for a cerebellar-dependent motor task, and specifically investigate regions known to be involved in the learning.

CHAPTER 3

GENERAL DISCUSSION

3.1 DISCUSSION

This thesis work aimed at describing the structural rearrangements that occur in the adult cerebellar cortex upon distinct experiences, such as enriched environment (EE) and fear conditioning (FC). Our large-scale imaging approach, together with the use of a transgenic mouse line that labels sparse neuronal structures (De Paola et al., 2003), allowed us to approach this question at several levels: indeed, we described changes occurring at single synaptic units, both pre- and postsynaptically, as well as global changes occurring in one neuronal cell (*i.e.* complete axonal projections or dendritic arborizations); in addition, we described the general behaviour of a population of synaptic complexes in a wide anatomical region.

The MF-GC synapse of the cerebellar cortex turned out to be extremely suitable for this study, for two important aspects: first, MF projections represent sensory inputs whose topography has been extensively described, so that there is deep knowledge about the sensory information conveyed in a particular region; second, pre- and postsynaptic morphologies easily allow to study structural rearrangements, being both counterparts large and complex synapses, whose connectivity in terms of divergence/convergence is well defined (Ito, 2006).

The work we describe here provides evidence that distinct long-term reshaping of the connectivity at the input stage of the adult cerebellar cortex is induced by different experiences.

We found that, upon EE, the circuit connectivity is profoundly altered in the properties of the afferent projections and in the putative amplification and transmission of the sensory information. The refinement of the connectivity (for example, the number of presynaptic inputs per cell) is particularly enhanced in animals

that were reared in the enriched context; nevertheless, the remodelling of single synaptic units (for example, the dendritic endings) occurs in a comparable extent in animals that experienced EE only in the adulthood.

In another way, we found that FC triggers a stepwise remodelling of the local connectivity at the MF-GC synapse, which proceeds for several days following the training and affects the whole population; the extent and quality of remodelling is lobule-specific and can be further modulated by subsequent experience.

Implications of our data have been discussed in the specific sections. Here I would like to discuss the body of the results in an integrated fashion, in particular examining common and distinct features of the remodelling events observed in the different sets of experiments, and relating them to the particular experience that initiated them. Intriguingly, the structural events triggered by different experiences not only implied distinct remodelling outputs, but also distinct timelines for the plasticity progression, suggesting a correlation with the length of the experience.

A further aspect resides in the maintenance of the structural rearrangements for long periods, eventually leading to everlasting structural "traces" of the experience. This issue was not solved in our studies, but preliminary observations we made could contribute to some general observations.

Lastly, I would like to comment our results in light of the body of knowledge in the field: experience-triggered responses have been previously analyzed from the structural and functional point of view, in our laboratory among others, thus I will integrate previous reported findings with novel aspects that came from our data.

3.1.1 Experience determines the structural remodelling outcome

I would like here to report and directly compare rearrangements that were specifically triggered by EE or FC. To this purpose, I will describe separately pre- and postsynaptic events.

I. Presynaptic remodelling

When we examined terminal density as well as branching frequency of long axonal tracts projecting to the vermal lobule V, we found that these properties were affected by 20 days of EE conditions during adulthood, being both evaluated parameters increased upon the experience. When we looked at the morphology of the MFTs placed by the axons in the apical region of lobule V, we found that their shape and surface complexity were not affected; instead, the average MFT size was heavily decreased, thus well correlating, from an anatomical point of view, with the increased number of MFTs we observed.

Conversely, FC experience did not influence the above mentioned axonal connectivity properties, but rather focused its striking effect on the MFTs, which collectively responded with dramatic remodelling of surface complexity, shape, size and process outgrowth. Based on our data, it appears correct to state that all the axons responded equally and homogenously, in the examined lobular subregion, in terms of surface complexity, whereas shifts in size and process properties affected only a subset of the population.

II. Postsynaptic remodelling

GCs connectivity properties determine the divergence of the information carried by a single presynaptic terminal (Ito, 2006). This aspect was found to be profoundly altered upon EE experience, particularly in animals that experienced EE since birth; furthermore, the postsynaptic units, namely the GC claws, were also affected, being their morphology simpler and poorer.

Differently, even though we were not able to analyze extensively GC connectivity properties upon FC training, it is our impression that no redistribution of the dendrite number was triggered by FC training: however, single claw morphology exhibited a larger variation, with a peculiar increase in the large/complex claw population.

Here I suggest that EE experience is able to remodel extensively, but not only, system connectivity, whereas FC might trigger plasticity of synaptic transmission.

Chklovskii and Svoboda (2004) interestingly commented on the synaptic changes that underlie memory storage, or, more generally, encode experience, by defining two, possibly co-existing, strategies: *weight* changes or *wiring* changes. Weight-plasticity implies changes in the efficacy of existing synapses, and/or structural changes that lead to the addition or subtraction of synapses between previously connected pre- and postsynaptic units; in the second case (wiring-plasticity), the storage capacity lies in the system's flexibility to choose which presynaptic units provide input to each postsynaptic unit.

With this respect, EE experience is able to determine extensive wiring changes, basically reshaping the system connectivity, and possibly reverberating on cortical functional representations. Additionally, alterations in GC dendrite number in enriched animals contribute to regulate the divergence of the MF signal (Ito, 2006); on the other hand, the simplified claw morphology upon EE is likely to limit the synaptic transmission (weight-plasticity), as ultrastructural data show that each digit makes at least one synaptic contact (Jakab and Hamori, 1988). Notably, the degree of MF-GC divergence could be also functionally regulated by LTP (D'Angelo et al., 1999) or GoC inhibition (Chadderton et al., 2004).

Conversely, FC-related remodelling gives little evidence for extensive rewiring of the circuitry, while, most likely, changes in pre- and postsynaptic morphology underlie the so-called weight changes. Interestingly, the amplitude of the weight changes could be, to a certain degree, differently regulated at each GC dendrite, as suggested by larger population deviations when looking both at pre- and postsynaptic (between- and within GC) morphology. Structural rearrangements observed 5 days after FC are compatible with an increased synaptic transmission at each MF-GC synapse: this interpretation would be consistent with the finding that FC elicits LTP at the PF-PC synapse (Sacchetti et al., 2004), namely one stage further in the cerebellar circuitry. MF-GC synapse is able to undergo LTP (D'Angelo et al., 1999), however, we did not verify the possibility that the MF-GC synapse is potentiated upon FC.

3.1.2 Experience determines the timeline for structural rearrangements

We used as experimental models of experience two radically different paradigms. EE is a positive, "chronic" (lasting at least 20 days in our protocol) experience consisting in a substantial change of lifestyle compared to control conditions: groups of animals are kept in large cages containing toys, running wheels, stairs, thus potentiating social interactions and sensorymotor stimulation. Specifically, rats and mice housed in these conditions, improve learning and memory, enhance neurogenesis in the dentate gyrus of the hippocampus, increase brain weight and size, enhance gliogenesis and structural remodelling in several areas of the brain, such as the cerebral cortex and the basal ganglia (reviewed in Mora et al., 2007). *Ex vivo* recordings of EE hippocampal slices exhibit changes in excitatory postsynaptic potentials and LTP. Furthermore, EE elicits the increase of NGF, BDNF and GDNF gene expression (Mora et al., 2007).

Remarkably different from EE, FC is an "acute" experience, lasting about 5 minutes in our experimental procedure, that leads to the formation of an aversive learning given by the repeated association of a neutral CS to a noxious US. This experience implies a painful stimulation and a fearful emotion that elicits spontaneous defensive responses, including autonomic, endocrine and behavioural components. Though definitive proof is still lacking, there is strong evidence that FC is mediated at the synaptic level by potentiation of glutamatergic synapses at the different brain sites (amygdala and hippocampus being the two major systems involved). At present, scarce evidences have been brought about FC-related structural plasticity: recent reports describe an increase of spines in the lateral amygdala (Radley et al., 2006), and the up-regulation of PSA-NCAM, a molecule known to promote structural remodelling, in the dorsal dentate gyrus of the hippocampus upon FC (Lopez-Fernandez et al., 2007).

It is evident from this brief collection of reports from the literature that the two paradigms elicit very different responses: EE represents a lasting change in lifestyle, thus affecting systemically the organism and leading to significant changes at the cellular, molecular and behavioural level; FC is a single episode that mainly triggers long-lasting potentiation of transmission at certain synapses. As a consequence, also the timing of the specific responses are different in the two paradigms.

Few reports investigated the time scale under which experience-related structural rearrangements take place (Kleim et al., 1997b; 2002; 2004). Kleim and colleagues reported that a 10-day motor learning task triggers an increase in spine density in layer V of the rat motor cortex. More specifically, they described that the motor learning rapidly improves in the first 5 days of training, and then stays constant in the late phase; spinogenesis was found to occur from day 7, namely during the late phase of learning, while cortical map reshaping was observed only after 10 days of training (Kleim et al., 2004). These observations suggest that first motor learning takes place, followed by spinogenesis in specific cortical areas, and only afterwards functional reorganization occurs. Differently, in a FC paradigm, the learning, as well as the synaptic potentiation, takes place immediately, being present already 10 minutes after the training (see for example (Sacchetti et al., 2004); FC-related structural remodelling were observed as soon as 24 hours after the FC (Radley et al., 2006; Lopez-Fernandez et al., 2007).

From our experiments, and from previous observations made in our laboratory (N.G. and C.V., unpublished observations), it appears that the structural rearrangements elicited by EE are not yet there after one week of enrichment, and rather take at least two weeks to appear. This is compatible with a slow adaptation to the new lifestyle and with the longer time period that the large-scale reshaping of the connectivity probably requires to occur. Conversely, FC-elicited effects are already present two days after the training, and peak 5 days post-conditioning: indeed, the rearrangements we observe mostly affect synaptic complex morphology, and do not involve circuitry remodelling, thus being potentially accomplished faster. It is tempting to speculate that this structural plasticity could be involved in synaptic potentiation mechanisms.

One could argue that we cannot exclude FC effects on the system connectivity, similarly to what was found upon 20 days of EE, as we analyzed axonal properties only 5 and 10 days post FC. However, it appears quite evident from our data that the body of FC-elicited events is taking place in the first week after the training, to then gradually decrease: it appears unlikely that massive reshaping of connectivity would take place only afterwards. Further supporting a different remodelling timing between EE and FC, claw remodelling was not evident after 7 days of EE (not shown in this

thesis), suggesting that the process still had to take place; differently, 7 days after FC, remodelling events were remarkable.

3.1.3 Plasticity or elasticity?

Following a learning event, two possible *scenarios* appear: the system can keep a measurable trace of the past experience (namely, the system reaches a new state, implying a *plastic* remodelling); alternatively, the system can revert back to the baseline situation (meaning a sort of *elasticity* of the system). Notably, the latter situation could represent a true reversion to the situation preceding the event, or that the trace that is left cannot be measured as the average values virtually return back to the control range.

It is generally believed that memory of past events (learning or general experience) is regulated by cellular traces. These molecular, physiological, or structural changes, induced for example by a CS-US association, regulate behaviour by altering neuronal response to the learned environment. This is initially triggered by elevation in intracellular Ca^{2+} and consequent activation of second messenger signalling pathways in the postsynaptic neuron; subsequent cytoskeletal and adhesion remodelling are able to lead to new synaptic connections. For example, increase in spine density was detected in the hippocampus, as well as in the cerebellum, after eyeblink conditioning, or in the piriform cortex after olfactory learning (Lamprecht and LeDoux, 2004). Long-term changes in spine morphology could contribute to the modulation of synaptic transmission that occurs after learning or LTP. Even though FC traces have not being fully characterized yet, there is general agreement that protein synthesis and BDNF signalling are required in order to stabilize synaptic modifications (McGaugh, 2000).

However, it still has to be unravelled if, and how long, a structural trace can be maintained after an episodic learning, or after a general event occurred. With this regard, Kleim and colleagues reported that increased spine density on cerebellar PC was kept at least for 4 weeks after a 10-day motor training (Kleim et al., 1998b).

Concerning our data, we have shown that an acute episode of FC triggers a series of remodelling events at the MF-GC synapse; the amplitude of the events is

maximal 5 days after the training and then gradually decreases. According to the complexity parameter we took into account, which in our hands was the best index of the time course progression, 15 days after FC MFT morphology is still significantly different from the control; in addition, preliminary observations (not presented here) suggest that the baseline is still not reached 20 days post FC. As we did not investigate further, we cannot make final statements about this issue.

Concerning the EE set of experiments, we are currently in the process of studying the effects of reverting from the EE lifestyle to control conditions. However, work done in our laboratory (N.G., submitted) suggests that, following 20-30 days in EE, several months of housing in control conditions are required before the EE-related structural effects in the hippocampus revert to the baseline. Nevertheless, these observations were made on fixed samples, meaning that we can just infer the trend of the remodelling by looking at different time points: one cannot exclude that remodelling events compatible with the reversion to control range were kept in the system (*e.g.* local rewiring). This point could definitely be answered with time lapse *in vivo* imaging.

Additionally, the maintenance of a structural trace upon learning raises the important point of how several or subsequent memories are stored and integrated in a brain circuit. In a recent report, it was demonstrated that fear learning and extinction learning activate two distinct neuronal populations within the basal amygdala, differently connected with hippocampus and medial prefrontal cortex (Herry et al., 2008): this indicates that specific subsets of cells are selectively involved in different tasks; in the same way, distinct microcircuits could then keep and integrate the traces for distinct kind of events.

3.1.4 Contribution of our work to the state of the art

Structural plasticity upon EE has been previously described. In particular, increase of dendritic length and/or arborization has been reported at the cortical level (Connor et al., 1982; Faherty et al., 2003; Leggio et al., 2005), as well as increase of the spine density (Turner and Greenough, 1985; Leggio et al., 2005). Work done previously in our laboratory described presynaptic structural rearrangements in the

large mossy terminals of the CA3 hippocampal projection upon EE (Galimberti et al., 2006): EE specifically increased the frequency of satellites made by the mossy terminals, together with the length and complexity of postsynaptic thorny excrescences. These remodellings were likely to reflect an increase in local divergence and convergence driven by experience. A step further, motor skill learning has been shown to induce adaptation of the cortical maps, generally causing an expansion of the movement representation (Nudo et al., 1996; Kleim et al., 2004).

To some extent, it is difficult to read our results in light of the data previously reported, as we used a novel approach to study the structural rearrangements triggered by experience: most of the published works describe groups of either pre- or postsynaptic elements within small cortical regions, thus not fully clarifying consequences of plasticity events for the synaptic transmission, for the cell behaviour (in terms of connectivity, for example) and for the circuitry. Differently, we chose to describe the behaviour of the whole axonal projection within one region, or the whole dendritic compartment. To our knowledge, no description was found in the literature about extensive axonal remodelling triggered by experience.

A second distinctive aspect of our work is that we chose to study the MF-GC synapse, which is at the input stage of the cerebellar cortex. MF axons convey sensorial information to GCs, which reliably transmit the information to higher stations, and particularly to the PF-PC synapse (Rancz et al., 2007); no multisensory integration is occurring at the GL, as information of the same sensory modality, yet coming from distinct mossy axons, are converging onto a single GC (Jorntell and Ekerot, 2006). Differently, previous works describe changes occurring at higher cortical stations (e.g. cerebellar PCs, see Kleim et al., 1997b). This notation could account for an apparent discrepancy between our findings and previous reports, namely that in our system dendritic branches, as well as number and length of terminal digits, decrease upon EE.

Finally, we reported data about reshaping of the connectivity upon EE, displaying both a pre- and a postsynaptic component. This novel observation would be theoretically compatible with a weakening of synaptic transmission and a refinement of the sensory representation in the cerebellar cortex: this would be an opposite effect compared to the expansion of cortical representation that is generally reported after motor skill learning; nevertheless, sharpening of the cortical

representation upon EE has been reported for the barrel cortex (Polley et al., 2004); as the cerebellum was described to be activated during sensory acquisition and discrimination rather than in mere motor control (Gao et al., 1996), this differential direction of plasticity compared to motor cortex could be due to distinct functional output.

Finally, I would like to comment about the FC-related plasticity we described. Generally, the cerebellar system does not get a great interest in the learning & memory field, however we concentrated our attention onto a synapse that is recruited during the FC training because of the sensory information it conveys; we were then able to describe lobule-specific rearrangement events, which were consistent with the cerebellar anatomical organization. The sign of plasticity we found is compatible with the upstream PF-PC synapse's potentiation, previously reported to occur upon FC (Sacchetti et al., 2004), as MF-GC remodelling could underlie a potentiation of the synaptic transmission. In addition, spinogenesis (Radley et al., 2006) or up-regulation of plasticity-permissive molecules (Lopez-Fernandez et al., 2007) were described to occur as soon as 24 hours after the FC training: this would be consistent with the time course we observed, as wide structural rearrangements were already detectable at the MF-GC synapse 48 hours post FC training.

An additional novel aspect that came out from our work is that experience following the training (namely, extinction sessions in our experiments) can modulate the quality and amplitude of structural plasticity triggered by FC training.

3.2 CONCLUSIONS

In this work, I have provided evidence that the adult mouse cerebellar system is able to undergo extensive reshaping of its connectivity and synaptic organization in response to changes in the lifestyle or an acute experience: as a consequence of the structural remodelling, changes in the synaptic transmission between previously connected neurons, and extensive rewiring of the circuit connectivity are likely to occur.

Our experimental approach allowed us to reveal several novel aspects of experience-related plasticity: first, we described that a brain circuit is able to respond to an experience remodelling at several levels, for instance reshaping system connectivity, as well as synaptic organization. In addition, the structural rearrangements that occur are likely to affect synaptic transmission and amplification.

Second, we described the local as well as the global behaviour of a given neuronal cell compartment, and we found that generally the whole cell compartment responds homogenously to a given experience.

Third, we found that the presynaptic terminals, belonging to distinct cells but populating the same anatomical region, are able to undergo, under certain circumstances, orchestrated rearrangements that globally involve the population.

Fourth, we described that structural rearrangements can be modulated by events that follow the event that originally elicited the plasticity.

Future directions of investigation were discussed in the previous sections; however, I would like to bring here some points that I consider fundamental for future investigations.

It has still to be assessed if, and how, the anatomical rearrangements we described are able to affect synaptic transmission at the MF-GC synapse (*e.g.* potentiating the synapse), in order to establish a correlation between anatomical and functional properties. A contribution to this issue could come from further ultrastructural studies of the cerebellar glomerulus, particularly for the active zone organization. A step further, *in vivo* recordings in the cerebellar cortex would constitute the ultimate test to verify the functional consequences underlay by structural rearrangements, and particularly in order to test the possibility of the refinement of somatotopic representation induced by EE housing.

In this work, we did not investigate the regulation of the plasticity phenomena we observed. For instance, neuromodulatory innervation could be crucial in triggering these events. Additionally, specific signalling pathways could be involved in FC-related structural plasticity. Data from our laboratory indicate that the Wnt-7b pathway is crucially involved in experience-dependent structural remodelling. Thus, we propose to test these hypotheses by *in vivo* local injection of mimicking/blocking compounds.

Finally, it is still not clear if the structural rearrangements we observed are transitory or an anatomical "trace" is permanently left by such an experience in the cerebellar cortex. Longer FC time points and reversible EE experiments will help in order to generally address this question. However, an *in vivo* time lapse imaging approach would allow to definitely answer these questions; with this respect, lobule V of the vermis is a suitable site, being easily accessible for these kind of studies.

CHAPTER 4

EXPERIMENTAL PROCEDURES

Mice

Transgenic mice expressing membrane-targeted GFP in only few (*Thy1-mGFP*[s], *Lsi1* and *Lsi2*) or most neurons (*Thy1*-mGFP[mu], *Lmu*) were generated as previously described (De Paola et al., 2003). Transgenic males were crossed over more than 10 generations with non-transgenic F1 offsprings from C57Bl6 x BalbC crosses, so that the genetic background of the mice was 50% each of C57Bl6 and BalbC.

Behavioural procedures

1. Enriched environment

regular EE: *Lsi1* or *Lsi2* mice were housed with parents and littermates from birth date to P30; then 3-4 female mice were moved together to an enrichment cage: this was a larger cage including toys, running wheels, stairs. Mice were sacrificed at P50 (20 days of EE, "regular EE"). Control mice were kept in the same conditions of the EE group until P30 and then the control mice were moved to standard single cage until P50.

EE colony: A *Lsi2* breeding was set in an EE cage; newborn mice were housed in the EE cage with parents and littermates since birth to P30. At P30, a first group of animals was sacrificed ("EE baseline"); a second group of 3-6 female mice was moved to another EE cage. Animals were then sacrificed at P50 ("EE colony", 50 days of EE). A parallel control breeding was set simultaneously in standard cages (control parent mice were littermates of EE parents). Newborn control mice were kept with parents and littermates until P30. At P30 a set of control mice was sacrificed ("CTRL baseline"); another set was moved to standard single cages until P50 and then sacrificed ("CTRL").

Note that CTRL mice undergo the same protocol in both experiments, so we finally considered them as a unique group.

II. Fear conditioning (FC)

Lsi1 male mice were housed in single cages 3-5 days prior the conditioning training. When aged between P50 and P60, mice were randomly divided into two behavioural groups. The first group (fear conditioned, COND) was trained in a basic Skinner box module (Coulbourn Instruments, Whitehall, PA, USA). Once placed inside the conditioning apparatus, the mice were left undisturbed for 2 minutes. After this time, the CS (a 1000 Hz tone amplified to 70 dB lasting 10 s) was administered 5 times at 30 s intervals. The last 1 s of each CS was paired with the US, consisting of an electric foot shock (0.8 mA). The second group of mice (control) were subjected to the same protocol, but the CS was never paired with the US. After the conditioning training, mice were placed back in their single cages and sacrificed at different time ponts (2, 5, 7, 10 or 15 days post conditioning). Fear retention was tested 20-60 minutes before the sacrifice. Retention test consisted in a 2-minute exploration and subsequent administration of 5 CS; the test was generally performed in the conditioning context, in order to assess both the context- and the CS-dependent freezing. A set of mice was retested in a different context, in order to exclude that our conditioning protocol could induce generalized fear (mice should not freeze upon mere exploration of a novel environment). In all the experiments, the acquisition and retention sessions were digitally recorded, and the freezing response measured as a fear index. Freezing was defined as the complete absence of somatic motility, except for respiratory movements. Mice that showed a significant freezing response were included in the subsequent analysis.

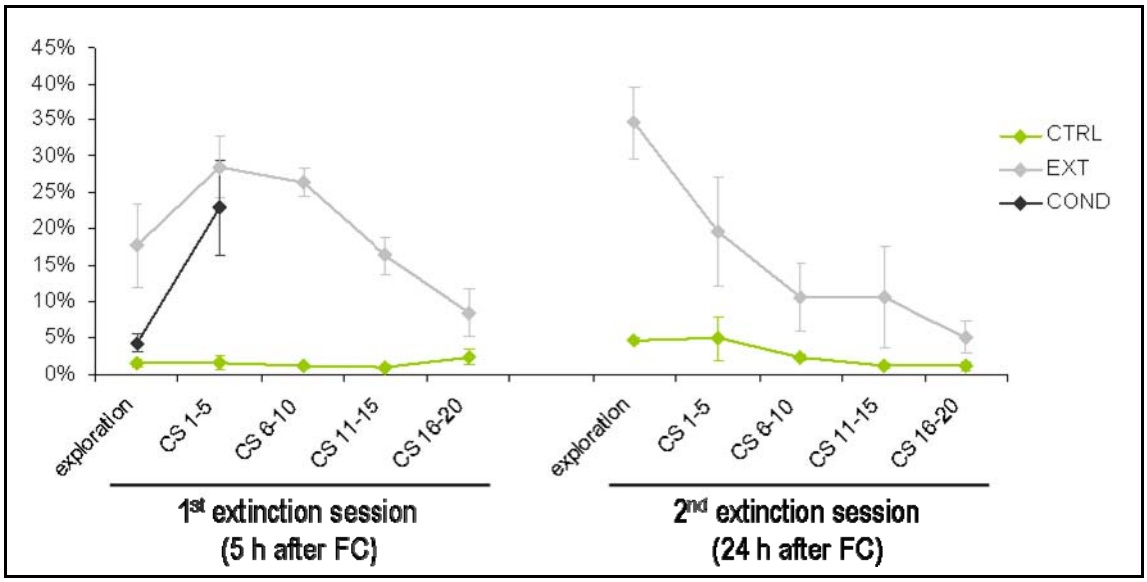
III. Extinction (EXT)

Mice were randomly divided into three behavioural groups (control, COND, EXT). COND and EXT animals underwent the described FC procedure (5CS/US, day 0). Five hours after the conditioning, only EXT mice underwent the first extinction session, consisting in a 2-minute exploration and subsequent administration of 20 CS

at the usual time intervals. A second extinction session was administered 24 hours (day 1) after the conditioning training. Extinction sessions were done in the conditioning context, in order to extinguish both the context and the tone component. Generally, mice were left undisturbed until sacrifice time (2, 5, 7 o 10 days post conditioning); a group of mice was additionally retested just before sacrifice to verify the freezing extinction. Control mice underwent the conditioning and the two extinction sessions but the US was never associated to the CS.

IV. Late extinction (late-EXT)

In a further set of experiments, extinction sessions were performed on day 2 (53 hours) and 3 (72 hours) after FC.



Typical freezing responses measured during the extinction sessions. COND retention was measured just before sacrifice (day 2 or day 5) and is plotted here to compare COND and EXT curves. N_{CTRL} = 2 mice, N_{COND} = 3 mice, N_{EXT} = 5 mice.

Histological procedures and imaging

Mice were perfused transcardially with 100 ml ice-chilled 4% paraformaldehyde in PBS, and brains kept in fixation solution overnight at 4ºC. Afterwards, dissected cerebella were moved in 30% sucrose and kept there until they

sank. Floating 40-µm or 100-µm sagittal sections were cut using a Microm HM500 microtome (GMI, Ramsey, MN, USA) and collected in PBS.

I. Immunofluorescence

mGFP signal was always amplified in sections used for morphological studies. This step allowed to obtain an optimal and wider dynamic range in the imaging sessions and to avoid saturated signal for the brightest structures.

Sections were washed in PBS, permeabilized in 0.25% Triton X-100 PBS (PBS+) and then incubated with the proper primary (overnight, 4ºC) and secondary (30-60 min, RT) antibody in 3% BSA. Primary antibodies were used as follows: rabbit anti-GFP (Molecular probes, Eugene, OR, USA), 1:1000; mouse anti-Bassoon (StressGen, Ann Arbor, MI, USA), 1:200; rabbit anti-VGluT1 (SySy, Göttingen, Germany), 1:1000; secondary antibodies: AlexaFluor 568 or Cy5 (Molecular Probes), 1:1000. Neuronal cell bodies were counterstained with Nissl fluorescent staining Neurotrace 530/615 (Molecular Probes).

Sections were mounted with ProLong Antifade medium (Molecular Probes), coverslipped (0.17±0.01 mm, Assistent, Sondheim, Germany), sealed with nailpolish 24 hours after mounting and stored at 4ºC.

II. Electron microscopy

Mice were perfused with buffered 2.5% glutaraldehyde, followed by fixation in buffered 2.5% glutaraldehyde (2 hours), post-fixation in buffered 2% Osmium tetroxide (2 hours), and dehydration through alcohol, followed by propylene oxide. Fixed brain material (apical region of the vermis lobule V) was embedded in Docupon, stained with uranyl acetate and lead hydroxide, and sectioned with a diamond knife. Complete serial sections (75-85 nm each; total of 8-10 µm) were deposited on slot grids with formvar. Sections were recorded on Kodak electron image plates using a Zizze EM900 at 100 kV.

Confocal imaging

Routine large-scale imaging was done using a 1.3NA63X Plan-Apochromat glycerol immersion lens on a custom designed parallel confocal imager, based on a Zeiss AxioImager M1 microscope, a Yokogawa CSU23 scanhead and a Photometrics CascadeII:512B EMCCD camera; Metamorph 6.2 acquisition software (Molecular Devices, Sunnyvale, CA, USA) was used. Standard settings were 50 ms exposition time, gain 1500, 561 laser at 50% intensity; (final resolution 0.18 µm/px; 0.5 µm step). 1 to 3 slices per animal were acquired, typically in a 40-50 multistage-position acquisition.

High resolution images were acquired on an inverted Zeiss Axioplan2 LSM510 confocal microscope, using a 1.4NA100X Plan-Neofluar oil immersion objective; settings were adjusted for each set of staining and channel in order not to saturate the signal; 1024x1024 pixels.

Image analysis

When necessary, deconvolution was performed with Huygens Deconvolution Software (SVI, Hilversum, Netherlands). The iterative Maximum Likelihood Estimation (MLE) algorithm was used with the computed Point Spread Function (PSF).

Stack series were opened with Metamorph software, maximum intensity projections (MIPs) of each stack were calculated and stitched together by means of a Metamorph journal or XuvTools (FMI and LMB, Universität Freiburg). For 3D analysis, stacks were opened in Imaris 6.1.5 and volume projections created and analyzed.

Data quantification

Objects were solved in 3D and then the contour shape/processes were carefully drawn on the corresponding MIPs. When objects were spanning across several stacks, several Imaris windows were analyzed in parallel to carefully reconstruct the

morphology; alternatively, a 3D stitching was run on XuvTools. Object length, area and perimeter were measured with the ImageJ opensource software.

Complexity index for MFTs was defined as follows: the detailed contour shape for each MFT (A_{EXT}) was drawn and then a "core" region (A_{INT}) was defined, based on the 3D structure, and drawn on the corresponding MIP. A_{INT} was corresponding with good approximation to the area connecting A_{EXT} concavity points. Both contours' areas where measured and then the following formula was applied:

$$1-(A_{INT}/ A_{EXT})$$

being the complexity index included between 0 (low complexity) and 1 (high complexity).

All the objects present in our region of interest, if completely included in the confocal stack and sufficiently isolated to be solved, were included in our analysis.

Statistical analysis

Average values in the text are expressed as mean ± S.E.M. Statistical differences were assessed by Student's *t*-test (GraphPad Prism4, GraphPad Software, La Jolla, CA, USA) or by two-way ANOVA (R 2.7.0 freeware).

Acknowledgements

I am grateful to Pico Caroni, who welcomed me in his laboratory at the "Friedrich Miescher Institute" in Basel (CH), where the work described in this thesis was done, and offered precious supervision and attention; I am grateful to Piergiorgio Strata, who followed my studies and formation in Italy and gave me the opportunity of completing my PhD project at the FMI.

Benedetto Sacchetti (Dpt Neuroscience, Physiology, Università di Torino) gave an essential contribution to the FC project, helping me to set up the experiments and to interpret the data, on top of being always available for discussion and suggestions.

I had fruitful collaboration with Nadine Gogolla, who introduced me to imaging techniques and to the EE project, and Sarah Rüdiger, who carried parallel studies to mine on FC.

I had qualified, daily support from Jens Rietdorf, Patrick Schwarb, Aaron Ponti, Laurent Gelman (Facility of advanced Imaging and Microscopy, FMI) and Sjörd Van Eeden (Information Technology, FMI): their assistance is at the core of this work.

I thank current and former members of Caroni's lab for valuable and constant comments and suggestions on my work.

Finally, I thank Federica Premoselli and Annalisa Buffo (Università di Torino), whose technical and scientific teaching made my stay in Basel successful and enjoyable.

This work was supported by "Fondazione CRT – Progetto Lagrange" (prof. Strata) and the "Friedrich Miescher Institute for Biomedical Research a branch of the Novartis Research Foundation" (prof. Caroni).

APPENDIX

adapted from http://en.wikipedia.org/

Plasticity is a fascinating word of Greek origin. As I mentioned at the beginning of this thesis, the term comes from the adjective *plastikós*, in turn derived by the verb *plássein*, to model, form, shape.

Nowadays, plasticity generally means the ability to permanently change or deform, in contrast with *elasticity*, which refers to ability to change temporarily and revert back to original form.

Interestingly, a broad variety of scientific disciplines takes advantage of this word:

- in *physics* and *engineering*, plasticity is the propensity of a material to undergo permanent deformation under load;
- in *psychology*, plasticity is an intelligence factor that determines the ease of changing one's perception of a situation for finding a new solution to a problem (in contrast to *rigidity)*;
- in *genetics*, plasticity describes the degree to which an organism's phenotype is determined by its genotype;
- in *histology*, the plasticity of a body tissue refers to the ability of differentiated cells to undergo transdifferentiation.

Most notably, this word makes a link between science and art: the *plastic arts* are those in which a material is formed or deformed into a new, permanent shape, such as sculpture. A magnificent example is shown in this page.

G.L. BERNINI, Apollo and Daphne (1622-1625)
Galleria Borghese, Roma

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Ich erkläre, dass ich die Dissertation, **Structural plasticity at the input stage of the adult cerebellar cortex**, nur mit der darin angegebenen Hilfe verfasst und bei keiner anderen Fakultät eingereicht habe.