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Top-down processing and perceptual learning: role of the lateromedial secondary visual cortex in a mouse model of visual perceptual learning

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Introduction

The nervous system senses environmental stimuli creating an internal representation of the world. This representation, however, is far from depending only on the physical properties of the environment. Prior experiences, stored by the nervous system as neural networks, shape both perception and reaction to incoming external stimulations. With practice and experience, the nervous system's abilities to perceive a specific stimulus are progressively increased, leading to the discrimination of previously undetectable stimulus characteristics. The unconscious acquisition of such improved perceptual discrimination falls into a form of implicit learning known as perceptual learning (Prettyman, 2019). Different experimental strategies have revealed that practice improves perceptual abilities in all sensory modalities. However, visual perceptual learning is currently the most studied form of perceptual learning. Accumulating studies have investigated visual perceptual learning to understand the neuronal substrates mediating the effects of this implicit form of learning. The emerging view suggests that perceptual improvements result from plastic cortical rearrangements (Sale et al., 2011). Moreover, recent finding supports the notion that perceptual learning involves global reorganization across cortical areas relying on the complex interaction between bottom-up and top-down processes that ultimately sharpen internal responses to changing environmental demands (Crist et al., 2001, 1997; Li, 2016; Li et al., 2008, 2004). Nevertheless, the current knowledge of this phenomenon is scant, despite more than a century of multidisciplinary studies.

1. The plastic structure of the visual system

The neuronal property of modifying the strength and efficacy of synaptic transmission is a fundamental process for learning and memory acquisition. This phenomenon is known as neural plasticity or neuroplasticity and can be defined as the intrinsic property of the nervous system to change functionally and structurally in response to internal or external stimuli by reorganizing its physiology, structure, or connections.

The most dramatic plastic changes are confined to specific temporal window called critical periods (CPs), occurring early during development. Developing circuits are indeed extremely adaptive, and experience severely sculpts their mature structure and functionality.

The existence of CPs has been reported in all the species studied so far, from Drosophila (Barth et al., 1997) to primates, with a time extension that is closely correlated with the average life expectancy (Berardi et al., 2000). Experimental evidence points toward the existence of different CPs for each specific sensory system (visual, auditory, and somatosensory representation), and even for more complex neuronal functions, such as social behavior and song or language acquisition in birds and humans, respectively (Harlow et al., 1965; Doupe and Kuhl, 1999; Werker and Hensch, 2015).

Over the course of these specific time windows, which are different for distinct sensory systems, a tight interaction is established between genetic patterns and experience-dependent changes, leading to the maturation of an adaptive individual nervous system (Lewis and Maurer, 2009; Weliky, 2000). Among sensory systems, visual cortex emerges as the prime brain region to study how external stimulations sculpt the internal representation of the environment. Employing the visual system as a paradigmatic model, several studies have indeed proven the crucial role of external stimulations for developing a properly functional nervous system. For example, it is now widely proven that kittens reared in an artificial visual environment

consisting of stripes of one of tree orientations- horizontal, right oblique, or left oblique- develop solid visual deficits (Blasdel et al., 1977; Hirsch, 1985). Moreover, animals reared in complete darkness from birth (a protocol commonly known as dark rearing, DR) display an abnormal functional and anatomical maturation of the primary visual cortex, with a cortical structure appearing immature far beyond the closure of the CP (Crair et al., 1998; Sherman and Spear, 1982). DR animals, indeed, present several neurophysiological deficits including abnormally large receptive fields (Fagiolini et al., 1994), and anatomically immature ocular dominance columns (Crair et al., 1998; Sherman and Spear, 1982). Numerous alterations can also be found in the fine morphology of visual cortical neurons; cells depleted of visual stimulations exhibit alterations in dendritic arborizations and in the size, morphology, and density of dendritic spines (Valverde, 1971; Wallace and Bear, 2004). A total lack of visual experience also affects several parameters of visual response: visual neurons undergo fast habituation, and they have a lower orientation selectivity and responsiveness. Furthermore, both latency and spontaneous activity of visual response are increased (Gianfranceschi et al., 2003; Pizzorusso et al., 1997). Consistently, DR animals show extremely low visual spatial resolution (visual acuity), as measured by electrophysiological and behavioral techniques (Fagiolini et al., 1994; Gianfranceschi et al., 2003). Remarkably, however, a brief light exposure can reinstate both normal electrical properties and anatomical features, setting the path for a late regular developmental process (Buisseret et al., 1988; Wallace and Bear, 2004). Notably, it has been more recently demonstrated that DR delays maturation of higher visual cortical areas, affecting in particular a specific subnetwork of visual areas projecting to dorsal brain regions (Smith et al., 2017).

Even more subtle manipulations of visual experience can affect visual system maturation, as shown by the seminal work of Torsten Wiesel and David Hubel. These Nobel laureates demonstrated that depriving one eye from visual experience, a manipulation usually

referred as monocular deprivation (MD), strongly affects the binocular properties of cortical neurons. Such a manipulation is indeed reflected in a considerable loss of neurons driven by the deprived eye and an almost simultaneous gain of neurons driven by the open eye. In normal binocular conditions, visual cortical neurons show different degrees of preference for one eye, despite a large number of these cells receive inputs from both eyes. When one eye is depleted of visual experience, a marked electrical response shift occurs in favor of the open one inside the primary visual cortex. As a consequence of that shift, the deprived eye shows a strongly reduced visual acuity and its contrast sensitivity is blunted (Hubel and Wiesel, 1970, 1963; Movshon and Dursteler, 1977; Olson and Freeman, 1980, 1975). Hubel and Wiesel observed that the susceptibility to MD changes with age, peaking during CP and then declining. The effects of MD can notably be reversed by removing the eye occlusion within CP progression. At the closure of CP, however, these effects become irreversible (Antonini et al., 1998; Berardi et al., 2000; Blakemore et al., 1981; Movshon, 1976; van Sluyters, 1978; Wiesel and Hubel, 1965). Consistently, no detectable visual alterations can be found when MD is started during adulthood (Hubel and Wiesel, 1970; Olson and Freeman, 1980).

Neural plasticity drastically decays as development proceeds, likely as a result of evolutionary pressures toward a final stabilization and maintenance of the mature neuronal structure, and of the ensuing sensory functions emerged from developmental events. Yet, there is no doubt that learning as a result of practice occurs in adulthood; even though adult learning appears quantitatively and qualitatively different compared to juvenile learning (Bavelier et al., 2010). Recent studies have indeed revealed that the nervous system is intrinsically plastic even in adulthood, albeit in a more local and specific manner, likely to retain a certain degree of adaptability to an ever-changing environment, while ensuring a hard-wired neuronal structure for known environmental conditions. Targeted pharmacological interventions and environmental manipulations can increase cortical plasticity in adulthood inducing functional

and structural recovery in MD subjects far beyond the CP closure (Baroncelli et al., 2012; Consorti et al., 2021, 2019; Fagiolini and Hensch, 2000; Sale et al., 2014, 2007). Among these experimental procedures, visual perceptual learning has been proven extremely successful in locally reinstating neural plasticity in both humans and rodents (Baroncelli et al., 2012; Consorti et al., 2022; Levi and Li, 2009). Thus, perceptual learning's ability to exploit unspoiled reservoir of cortical plasticity in the adult brain might represent the neurophysiological mechanism leading to perceptual improvements. Top-down connections emerging from higher-lever circuits may exert a pivotal role in this process by their ability to precisely and locally drive the coding efficacy of lower-order circuits.

2. Perceptual Learning

Perceptual learning (PL) refers to "an increase in the ability to extract information from the environment, as a result of experience and practice" (Gibson, 1969). PL involves long-lasting uncharacterized rearrangements in brain circuity that change how a stimulus is perceived as a result of practice with that same stimulus. PL is an implicit form of memory (Prettyman, 2019) and it is supposed to rely on the acquisition of improved abilities after repeated experience. It is hypothesized that the neural changes underlying PL take place in the sensory cortices recruited during the learning task, without relying on the medial temporal lobe (Manns and Squire, 2001). PL differs from another form of implicit memory linked to repeated stimulus exposure, sensory adaptation, for its long-lasting nature (Schacter, 1992, 1987) and for involving a task specific practice rather than passive stimulus exposure (Bedford, 1993; Dodwell and Keith Humphrey, 1990). Perceptual priming is the form of implicit memory most closely related to PL. Perceptual priming consists in any effect of a stimulus on the perception

of a second stimulus that is presented shortly after (Weingarten et al., 2016). Nevertheless, the priming effect is not the result of practice, and it does not imply any perceptual improvement.

The improvement of perceptual processes allows an organism to respond quickly and effectively to external stimuli. On this account, Robert Goldstone wrote: “Perceptual learning involves relatively long-lasting changes to an organism's perceptual system that improves its ability to respond to its environment” (Goldstone, 1998). PL may then be an adaptive process enabling an organism to be efficiently responsive to unique environmental demands.

Even though the great majority of studies on PL has focused on adult subjects, PL should be considered a life-long process. PL can promote the acquisition of perceptual expertise during development (Kovács, 2000; Kovács et al., 1999), and it can also restrain the physiological deterioration occurring during aging (Deveau et al., 2013).

To date, PL has been documented in all sensory modalities and in response to a variety of perceptual tasks. These tasks range from simple discriminations to complex categorizations. Simple tasks include discrimination of visual orientation (e.g., Schoups et al., 1995; L P Shiu and Pashler, 1992; Vogels and Orban, 1985), auditory pitch (e.g., Recanzone et al., 1993; Wright and Fitzgerald, 2001), and tactile frequency (e.g., Jenkins et al., 1990; Recanzone et al., 1992). Complex tasks include discrimination of complex forms, objects, and faces (e.g., Fine and Jacobs, 2000; Furmanski and Engel, 2000; Hussain et al., 2009; Mclaren, 1997).

2.1 Visual Perceptual Learning

In the visual system, practice improves performance in various sensory tasks including grating, texture, vernier or stereoscopic discriminations. Visual perceptual learning (vPL) has been indeed documented in response to a wide range of visual tasks: discrimination of orientation

(Karni and Sagi, 1993; Matthews et al., 1999; Matthews and Welch, 1997; Schoups et al., 1995; L P Shiu and Pashler, 1992; Vogels and Orban, 1985) of motion direction (Ball and Sekuler, 1987, 1982), of texture (Ahissar and Hochstein, 1996; Karni and Sagi, 1991) and of differences in the waveform between two sinusoidal stimuli (Berardi and Fiorentini, 1987; Fiorentini and Berardi, 1981, 1980); detection of visual gratings (De Valois, 1977; Mayer, 1983); changes in spatial frequency within simple or complex plaid pattern (Fine and Jacobs, 2000); ability to detect small depth differences between two targets (Fendick and Westheimer, 1983; Westheimer and Truong, 1988) or to perceive depth in random-dot stereograms (Ramachandran and Braddick, 1973), object (Furmanski and Engel, 2000) and face recognition (Gold et al., 1999).

The effects of vPL are long-lasting; the acquired expertise persists from months to years (Ball and Sekuler, 1987; Fiorentini and Berardi, 1981; Karni and Sagi, 1993). Once learned, the improved discriminability can be retrieved after years without the need of further training, even after a prolonged period of inactivity (Karni and Sagi, 1993). However, the time course required to acquire such expertise seems to be task-specific. For some tasks, vPL occurs within one or two hours of training, showing a fast saturation after few hundred of trials (Fahle et al., 1995; Fiorentini and Berardi, 1981, 1980; Liu and Vaina, 1998; Ling Po Shiu and Pashler, 1992). For other tasks, vPL is characterized by a fast phase of saturation followed by a slow phase during which the performance improves from one daily session to the next until a stable plateau is reached (Karni and Sagi, 1991). For some other tasks, instead, vPL occurs after thousands of trials or even after years, displaying the characteristics of a long-term learning process (Karni and Sagi, 1993).

When simple stimuli are involved, vPL displays high specificity for the features of the trained stimulus, i.e., the perceptual improvement obtained by practicing the discrimination task

is usually lost when the trained stimulus parameters are changed. Specificity of vPL has been currently documented for orientation (Fahle and Edelman, 1993; Fiorentini and Berardi, 1980, 1981, 1980; Karni and Sagi, 1991; McKee and Westheimer, 1978; Poggio et al., 1992; Ramachandran and Braddick, 1973; Schoups et al., 1995), spatial frequency (Fiorentini and Berardi, 1981, 1980), motion direction (Ball and Sekuler, 1987, 1982), chromatic contrast, and for the location of the stimulus in the visual field (Ball and Sekuler, 1987; Fiorentini and Berardi, 1981; Karni and Sagi, 1991; Schoups et al., 1995; Ling Po Shiu and Pashler, 1992). Indeed, several studies have reported a drop in performance in trials using stimuli different from the trained ones. For example, the perceptual improvement in discriminating two sinusoidal gratings did not transfer to stimuli rotated by 90° (Fiorentini and Berardi, 1980). The specificity of vPL for basic features of the stimulus has led to hypothesize the involvement of neuronal circuitries as early as the primary visual cortex, where neurons have relatively small receptive fields and are selective for specific features (Hubel and Wiesel, 1959).

It is worth noticing, however, that transfer of vPL can occur under several conditions, especially when more complex stimuli are involved (McGovern et al., 2012). Specificity versus learning transfer can depend on multiple factors: level of the trained task (Fine and Jacobs, 2000), the task difficulty (Ahissar and Hochstein, 1997), precision of the transfer task (Jeter et al., 2009), extent of training (Jeter et al., 2010), state of adaptation induced by training (Censor et al., 2006), and the exact training and transfer procedures (Hung and Seitz, 2014; Xiao et al., 2008).

2.1.1 Neuronal mechanisms underlying vPL

Studies on PL have strongly contributed to challenge the traditional dogma of an adult immutable brain. Several studies have indeed revealed unexploited reservoir of neuronal plasticity in the anatomical structure of the adult brain (Gilbert et al., 2001; Gilbert and Li, 2012). During adulthood, the precisely organized retinotopic map may undergo considerable reorganization. For instance, neurons within a cortical scotoma retain their responsiveness by shifting their tuning outside the injured region (Giannikopoulos and Eysel, 2006; Gilbert et al., 1990; Gilbert and Li, 2012; Schmid et al., 1996). To date, however, the precise neuronal mechanisms underlying PL remain unclarified. Learning is supposed to rely on plastic changes in specific brain areas leading to long-lasting modifications in neural circuit output (Kandel, 2009). Sensory areas undergo different plastic changes in response to PL. Several studies have reported different PL-dependent changes in adult cortical structure and function, including cortical reorganization, changes in neuronal populational code, enhancement in neuronal selectivity, and global cortical reorganization (Li, 2016). The heterogeneity of these changes may depend on the nature of perceptual tasks and may reflect different cortical mechanisms of PL.

Some of the plastic changes associated with PL involve topographic reorganization of sensory cortices: a region of the sensory area coding for the trained stimulus parameter expands by recruiting adjacent untrained regions. Classic examples are cortical changes observed in the monkey somatosensory and auditory cortices in response to perceptual practice (Recanzone et al., 1993, 1992). For instance, training in a vibration frequency discrimination task leads to cortical expansion of the cortical representation of the trained skin area within the primary somatosensory cortex (Recanzone et al., 1992). Similarly, training in tone discrimination enlarges the cortical area representing trained frequencies within the primary auditory cortex

(Recanzone et al., 1993). Other evidence supports the notion that learned information may be encoded by temporal firing pattern of neurons (Gilbert et al., 2001), i.e., the synchrony of the response in a neuronal population. Learning can indeed synchronize cell firing to the trained stimulus (Mountcastle et al., 1990). For example, neuronal subpopulations acquire a high temporal coherence with the stimulus when trained in a frequency discrimination task (Recanzone et al., 1993). Cortical recruitment and temporal changes in firing pattern, however, have been mainly observed in somatosensory and auditory cortices.

vPL, instead, does not seem to rely on topographic reorganization: for instance, in monkeys practicing an orientation discrimination task, improved behavioral performance correlated with changes in orientation tuning in the primary visual cortex (Schoups et al., 2001). Only a neuronal subset within the retinotopic trained region increased selectivity for orientational changes around the trained orientation. No significant increases were observed in the proportion of neurons tuned to the trained orientation, a result that seems to suggest that vPL could correlate with changes in the tuning curves of specific group of neurons coding for the trained orientation (Schoups et al., 2001). Subsequently, similar findings were reported for neurons lying in the higher-order visual area 4 (V4) in correlation with an orientation discrimination task (Raiguel et al., 2006). Another study has reported narrowing of orientation tuning curves of V4 neurons in rhesus monkeys trained to do an orientation match-to sample task (Yang and Maunsell, 2004).

2.1.2 Cortical localization of vPL

One of the most controversial issues in vPL research is the stage at which the visual processing pathway can account for the perceptual improvement in response to practice. The unexpected degree of plasticity observed in adult early visual stages has led to hypothesize that the neuronal

changes underlying vPL could localize in the lower stages of the visual system. In agreement with this hypothesis, neuronal changes in response to vPL have been found as early as the primary visual cortex. An observation corroborated by the high specificity of vPL for orientation and position. In spite of the high vPL specificity for stimulus parameters, however, it has been argued that the perceptual improvement associated with vPL could not be entirely induced by the stream of connections that convey information to the primary visual cortex from the periphery (feedforward or bottom-up connections). More recent studies have indeed observed neuronal changes occurring into higher stages of the visual pathway. Therefore, it is more likely that perceptual improvement emerges from the complex interplay between bottom-up connections and the stream of connections that return to the primary visual cortex from higher visual areas (feedback or top-down connections).

2.1.2.1 Changes in lower-order visual areas

Classical physiological and psychological observations have led to the assumption of a dominant primary visual cortex in vPL. A number of studies have shown that vPL is highly specific for the trained location, feature, and eye. On the basis of such specificity, it has been argued that vPL occurs at early visual stages. According to neurophysiological findings, neurons in the primary visual area possess smaller receptive fields than those in higher visual cortices (Goldman-Rakic and Rakic, 1991). Furthermore, few studies reported that an external feedback is not necessary for vPL to occur (Fahle et al., 1995; Poggio et al., 1992). Consistently with this view, multiple and sound experiments reported that vPL correlates with structural changes in the primary visual cortex in both human and non-human primates.

In humans, a pivotal study by Fahle and Skrandies (1994) showed that improvements in a motion-detection task correlate with changes in the visual representation of the primary visual

cortex. Using multichannel evoked-potential, they indeed recorded significant difference in potential distributions with a latency of 100 ms over the occipital lobe, a result that clearly suggest the involvement of human primary visual cortex in vPL (Fahle and Skrandies, 1994). More recently, supportive evidence comes from electrophysiological and functional imaging studies. For example, changes in an early visual area response have been found after practice of different tasks measuring C1, the earliest component of the visual evoked potential (Bao et al., 2010; Pourtois et al., 2008). These electrophysiological changes have been also associated with retinotopic variations occurring in the primary visual cortex (Furmanski et al., 2004; Hua et al., 2010; Jehee et al., 2012). In addition, an increase in blood-oxygenation-level- dependent (BOLD) signal in the trained region of the primary visual cortex has been associated with vPL training. No changes were instead observed in other areas but only in the trained location inside the primary visual cortex, ruling out the possibility that the observed activation changes were mediated by top-down or bottom-up influences (Yotsumoto et al., 2008). Lastly, vPL has been shown to affect even pre-cortical circuits: Yu et al. (2016) have indeed reported that neuronal response changes in the M-layers of the Lateral Geniculate Nucleus (LGN) correlate with a contrast detection vPL task.

In non-human primates, electrophysiological studies have shown that vPL for simple discrimination tasks or contour-detection task alter neuronal response properties in primary visual cortex (Crist et al., 2001; Li et al., 2008; Schoups et al., 2001). Using chronically implanted multielectrode arrays, Yan et al. (2014) were able to capture the dynamic changes in response properties of neurons in the primary visual cortex over the course of a contour detection task. These authors detected activity changes in the trained neuronal populations inside the primary visual cortex. The results showed a progressive strengthening in the facilitation of the neurons encoding the contour elements, and suppression of neurons responding to the background components (Yan et al., 2014). Moreover, practice in a contour

detection task can also lead to anatomical reorganization of the primary visual cortex circuitry, shaping the axonal arbors of neurons representing the trained part of the visual field (Van Kerkoerle et al., 2018).

2.1.2.2 Changes in higher-order visual areas

Visual information is encoded, at least in part, by plastic rearrangements that shape the circuitry of the primary visual cortex to cope with changing environmental demands. However, learning appears to rely not only on changes in single visual areas but also on distributed neural processes and plastic reorganizations. Accumulating studies have indeed started questioning the classic notion of a vPL process totally relying on neuronal rearrangements in early visual stages. Indeed, these neuronal changes have been proved insufficient to entirely account for the behavioral improvements observed over vPL sessions. In 2004, Yang and Maunsell provided the first evidence of vPL-induced changes in basic neuronal response properties occurring in monkey middle-order visual areas (Yang and Maunsell, 2004). After orientation discrimination training, V4 neurons with receptive fields overlapping the trained location had stronger responses and narrower orientation tuning curves than neurons with receptive fields in the untrained hemifield. Remarkably, V4 neurons with preferred orientations close to the trained range of orientations underwent most prominent changes (Yang and Maunsell, 2004). Since this pioneering study, several empirical evidence has reported changes in the response neuronal properties in the monkey visual cortex V4. More recently, changes in V4 neurons have been also dynamically followed during vPL through chronically implanted electrodes (Sanayei et al., 2018). In response to a fine categorization task, V4 neurons increased their ability to represent small contrast differences. Moreover, learning also altered the relationship between signal and noise correlations facilitating downstream decoding (Sanayei et al., 2018).

Changes in neuronal response properties associated with vPL training have been now documented in several high-order visual areas, including the middle temporal area (Zohary et al., 1994), the lateral intraparietal area (Gold et al., 2008), the inferior temporal area (Adab et al., 2014), the lateral occipital cortex (Kuai et al., 2013), and the fusiform face area (Bi et al., 2014), among others. The fusiform face area (FFA) is a small visual cortical region specialized in face recognition that lies inside the fusiform gyrus. In human subjects trained to perform a face recognition tasks, behavioral improvements correlate with the stability improvement of spatial activity pattern in the left FFA. Interestingly, the thickness of the left FFA before training can also predict subjects' behavioral learning effects (Bi et al., 2014). It is worth noticing that extensive practice with simple discrimination tasks not only affects early visual cortices but can also change neuronal response properties in higher visual areas. Training with simple grating stimuli changes the orientation preference of neurons located in the inferior temporal area (PIT), a higher-order visual cortex recruited for visual object recognition. Single unit recordings have proven that PIT neurons discriminated the trained orientations better than the untrained orientations after practice, suggesting a crucial involvement of higher-order visual areas even in simple vPL discrimination tasks (Adab et al., 2014).

The effects of vPL on global cortical organization are evident in task-related shifts of stimulus representation from one visual cortex to another. In humans, interfering with the left posterior parietal cortex (PPC) by repetitive transcranial magnetic stimulation (TMS) impairs feature-difference and signal-in noise discrimination tasks before training. On the contrary, TMS impairments of the lateral occipital cortex (LO) activity has little effects. After vPL practice, however, TMS interference has opposite effect: TMS of the LO impairs discrimination task, TMS of the PPC, instead, no longer affects the behavior (Chang et al., 2014). These findings suggest that practice can shift the task representation from PPC to other neurons located in a different cortical area. Similarly, another TMS study has revealed that the cortical

locus for processing noisy motion signals is shifted from middle temporal area to the visual area 3 accessory (V3A) after motion direction discrimination (Chen et al., 2016). Therefore, neuronal mechanisms underlying vPL perceptual improvement may be ascribed to the global reorganization of the visual system mediated by top-down influence, and not just to an improved stimulus representation in early visual areas in response to repeated practice.

2.1.3 Top-down processing and vPL: toward a unified brain model

The functional properties of visual neurons are now considered more dynamic than what postulated by the classical notion of a visual cortical hierarchy, in which visual scene is analyzed in a feedforward manner from simple to complex attributes moving from one cortical stage to the next. Computed information at one cortical stage is indeed sent back to the initial stage to effectively adapt neuronal response according to the behavioral task, expectations, or stored memories (Gilbert and Li, 2013). Anatomical and functional studies have consistently observed that, in the primate visual cortex, top-down connections re-enter the primary visual cortex as parallel projections resembling feedforward streams (Federer et al., 2013) to locally modulate receptive field size, surround suppression, and response amplitude (Nurminen et al., 2018).

In the contest of perceptual tasks, top-down connections are shown to change neuronal tuning allowing the system to engage stimulus components relevant for the task being performed and simultaneously discard those components irrelevant to the same task (Gilbert and Li, 2013). For example, neurophysiological studies suggest that the frontal eye field, a prefrontal cortical region, serves as a source of top-down signals to area V4 that are necessary for attentional selection of a target among distractors (Monosov et al., 2008; Zhou and Desimone, 2011). Employing transcranial magnetic stimulation, it has been consistently shown

that backpropagating signal from this prefrontal region to visual areas is dependent on the task being performed (Morishima et al., 2009).

Nowadays, several lines of research suggest that top-down processing plays a fundamental role also in vPL. Indeed, vPL mechanisms are currently considered more complex than the simple processes of plasticity in early sensory areas. In support of this view, recent studies have shown that generalization of learning may occur when multiple tasks (Szpiro and Carrasco, 2015; Zhang et al., 2010; Zhang and Yang, 2014) or more stimulus categories (Green et al., 2015) are involved, and when exogenous attention is directed towards the trained stimuli (Astle et al., 2014). Top-down influence in vPL is further demonstrated considering the effects that both attention and internal or external feedbacks can exert on vPL performance.

Selective attention to trained stimulus features is usually necessary for proper vPL, being required not only to enhance vPL stimulus features but also to suppress irrelevant ones (Vidnyánszky and Sohn, 2005). Mental imagery (internal feedback) can specifically improve orientation discriminability, provided that it can repeatedly induce activation patterns in early visual stages that are similar to those activated by real grating discrimination (Shibata et al., 2011). Repeatedly imagining different vPL tasks (such as three-line bisection discrimination, low contrast grating detection, or motion direction discrimination) can improve behavioral performance without actual physical stimulation (Tartaglia et al., 2012, 2009). Regarding the external feedback, it has been proved that informing human observers of their behavioral performance during training can facilitate learning (Herzog and Fahle, 1997; Ling Po Shiu and Pashler, 1992), whereas random feedback uncorrelated with the behavioral responses can render the learning process impossible (Herzog and Fahle, 1997). In addition, a number of data has shown that C1 (i.e., the early component of the event evoked response) can also be top-town modulated (Kelly et al., 2008; Rauss et al., 2009). C1 changes have been traditionally

regarded as strong evidence for neural plasticity at early visual stages in response to vPL (Bao et al., 2010; Pourtois et al., 2008). More recently, however, C1 changes have been associated with learning transfer, highlighting the hypothesis that these changes could at least partially result from top-down modulation of high-order visual cortices (Zhang et al., 2015).

In a series of studies, the research group of Charles D. Gilbert has investigated top-down processing in vPL by the means of bisection tasks (Crist et al., 2001, 1997; Li et al., 2008, 2004). Practice with this visual discrimination task produces behavioral improvement and alters neuronal response properties in the monkey primary visual cortex (Crist et al., 2001, 1997). Despite the involvement of a simple discrimination, contextual interactions can modify neuronal responses throughout this bisectional task, which was believed to be computed by early visual stages (Crist et al., 2001 see also Ito and Gilbert, 1999). Remarkably, striking context-related effects were found in monkeys trained to do two different discrimination tasks with the same visual stimulus at the same visual field location; primary visual neurons responded very differently to the same visual stimulus under different experimental conditions (Li et al., 2004). This body of empirical data led the authors to postulate a critical contribution of top-down processing even for simple vPL discriminations. To specifically dissect the role of top-down influences, in a subsequent study, they recorded neuronal response after vPL practice in anesthetized monkeys. Inhalation anesthetics indeed appeared to preferentially reduce top-down connections (Jordan et al., 2013; Ku et al., 2011; Lamme et al., 1998; Raz et al., 2014). Previously recorded learning-induced neuronal changes in the primary visual cortex disappeared completely under anesthesia (Li et al., 2008). Similarly, transient chemical inhibition of the monkey middle temporal area impaired perceptual performance on a coarse-depth discrimination task (Chowdhury and DeAngelis, 2008). In humans, a transient interference of the middle temporal area or of the posterior parietal cortex affects the performance in different discrimination tasks (Chang et al., 2014; Chen et al., 2016).

The evidence presented so far suggests that vPL gives rise to structural and functional changes at different stages of visual processing. Learning a vPL task may thereby rely upon a distribution of plastic rearrangements across the brain (Maniglia and Seitz, 2018). To date, there is a wide acceptance of the notion that perceptual improvement, as well as the associated brain changes, could result from specific interactions between stimulus-driven bottom-up processes and goal-directed top-down influences (Li, 2016; Maniglia and Seitz, 2018; Watanabe and Sasaki, 2015). In agreement with this view, simultaneous recordings from the monkey primary visual cortex and V4 have unraveled interdependent bottom-up and top-down processes that operate synergistically to enhance the internal representation of the practiced stimulus feature, resulting in parallel increments of coded information in both visual areas (Chen et al., 2014).

To conclude, vPL dependence on different top-down factors seems to suggest that perceptual improvements, just like perception per se, might be mediated by several processes engaging cortical areas specialized for sensory processing, attentional deployment, and decision making (Maniglia and Seitz, 2018).

2.1.3.1 Top-down integration in vPL

Several models for vPL have been proposed, including the reverse hierarchy theory (Ahissar and Hochstein, 2004, 1993), the reweighting model of perceptual learning (Dosher and Lu, 1999), the dual plasticity model (Watanabe and Sasaki, 2015), and the dynamic performance-monitoring model (Sotiropoulos et al., 2018). All these computational models are reweighting models (Dosher and Lu, 2017), in which, essentially, learning is accomplished by weight changes in the readout connections from lower to higher visual units (Weiss et al., 1993).

A highly influential model in vPL research is the reweighting model of perceptual learning (Dosher et al., 2013; Dosher and Lu, 1999; Lu et al., 2010; Petrov et al., 2005). According to this model, vPL emerges from two independent mechanisms: improved filtering of external noise and removal of internal noise. The reductions of these two noises are accomplished by the selective reweighting of connections between lower and higher visual areas (Dosher and Lu, 1999). Consistently, top-down connections entering the primary visual cortex can selectively enhance the contour signals and suppress background elements over the course of contour detection tasks (Chen et al., 2014; Piëch et al., 2013). Training may also refine perceptual representation in higher-order visual stages, which in turn could refine sensory processing in lower visual areas (Kuai et al., 2013).

Another model dealing with top-down integration in vPL is the reverse hierarchy theory postulated by Ahissar and Hochstein (Ahissar and Hochstein, 2004, 1993). According to this model, vPL is a top-down guided process; perceptual improvements stem from a top-down-guided progressive shift in task-relevant information usability from high-order areas to lower-order areas, which have a better signal-to-noise ratio. The learning process is therefore mediated by a cascade of top-to-bottom modifications that enhance task-relevant and prune irrelevant information, leading to a reduction in the signal-to-noise ratio. The reverse hierarchy theory received strong support from fMRI studies in human subjects (Chen et al., 2016; Furmanski et al., 2004; Mukai et al., 2007; Sigman et al., 2005). Learning in shape identification, for example, lead to global activation changes across the entire visual pathway, with earlier stages becoming more active with training and later stages becoming less active with training (Sigman et al., 2005).

2.1.3.2 Anatomical interaction between top-down connections and local cortical circuits

Little is known about the anatomical framework mediating top-down control of local cortical circuits in early visual stages during vPL encoding.

One possibility is that functional changes associated with vPL are conveyed by top-down projections re-entering anterior visual areas and synapsing on horizontal connections (Gilbert et al., 2001; Gilbert and Li, 2013, 2012). These connections extend from pyramidal neurons within the primary visual cortex, whose axons travel for long distances parallel to the cortical surface, linking neurons with separated receptive fields but with similar orientation preference (Gilbert and Wiesel, 1989, 1983, 1979; Rockland and Lund, 1982). These long-range horizontal connections enable neurons to integrate information over large parts of the visual field and give neurons selectivity for stimulus context. Their extent and high specificity make horizontal connections ideally suited conveyors of top-down integration. In this regard, numerous studies have proposed that these hard-wired connections are essential for input selection over the course of vPL. By selecting relevant components of the trained stimulus, horizontal connections may allow neurons located in early stage of visual stage processing to encode high-order information relevant to the vPL task being performed (Piëch et al., 2013). The selectivity of the horizontal connections is indeed dynamic and can be modulated according to task demands, providing a framework for integrating complex stimulus attributes re-entering anterior visual stages through top-down connections. In agreement with this view, sprouting and pruning of horizontal connections have been detected in the trained part of the monkey primary visual cortex over the course of vPL (Van Kerkoerle et al., 2018).

Top-down integration may also require changes in inhibitory cortical connections (Gilbert and Li, 2012). Inhibitory neurons, indeed, show experience-dependent structural changes, both in their dendrites (Chen et al., 2011) and in their axon (Marik et al., 2014), and

recalibration of inhibitory and excitatory balance correlates with vPL practice (Baroncelli et al., 2012). Consistently, activation of specific interneurons is sufficient to improve perceptual discrimination (Lee et al., 2012).

2.2 vPL in rodents

To date, few studies involving vPL have been performed in rodents. A long-studied form of experience-induced changes is the stimulus-selective response potentiation (SRP). SRP is the long-lasting response enhancement occurring in the primary visual cortex of awake mice repeatedly exposed to visual stimuli (Frenkel et al., 2006). Alike vPL, this phenomenon is selective for trained stimulus features (such as orientation, spatial frequency, and contrast), shows a progressive onset over training sessions, and it can occur in both juvenile and adult mice (Cooke and Bear, 2012; Montgomery et al., 2022). SRP acquisition is tightly linked to the expression of a specific class of ionotropic glutamate receptors (NMDAR) in the primary visual cortex: local infusions of NMDAR antagonists or the genetical ablation of NMDAR subunits, indeed, prevent SRP acquisition (Frenkel et al., 2006; Montgomery et al., 2022). Notably, several studies have now demonstrated that inhibitory neurons are crucially involved in SRP generation (Heynen and Bear, 2001; Kaplan et al., 2016; Montgomery et al., 2022). For example, chemogenetic inactivation of parvalbumin cells occludes SRP expression. Moreover, the selective ablation of NMDARs in these inhibitory neurons is sufficient to impair SRP (Kaplan et al., 2016). Even though SRP is reminiscent of perceptual improvements, SRP cannot be used to model this type of learning, as it lacks the incremental component of vPL being a passive view of visual stimuli.

A rat model of vPL was developed by Sale et al. (2011), adapting a discrimination task developed by Fiorentini and Berardi (1980). Adult animals were trained in a two-alternative

forced choice discrimination inside a water maze tank. Rats were required to distinguish between two vertical gratings differing only for their spatial frequency; then, the two stimuli were made progressively more similar to each other until the animal performance reached a steady plateau. In parallel, a group of control animals were subjected to a purely associative version of the same test, i.e., they were only required to discriminate between a grating and a homogeneous gray panel. Exploiting this perceptual task, the authors were able to associate perceptual improvement with long-term potentiation (LTP), a classical cellular mechanism of cortical plasticity. Within one hour from the last trial, LTP from layer II-III of cortical slices appeared occluded in trained animals compared to controls, both in vertical (stimulating electrode placed in layer IV) and horizontal configuration (stimulating electrode placed in layer II/III). Moreover, a significant shift toward increased amplitude of field evoked response was found in the input/output curves of trained compared to control rats. Thus, electrical recordings displayed occlusion, mimicry, and saturation: three of the most commonly accepted criteria used to relate LTP with learning. This finding showed that perceptual improvements in spatial frequency discrimination can be explained in terms of long-term increments of synaptic efficacy occurring early in visual stimulus processing (Sale et al., 2011).

2.2.1 Mouse higher-order visual areas and top-down integration

Decades of anatomical studies in primates and carnivores have revealed a complex hierarchical cortical structure by which the visual system generates meaningful percepts from a heterogenous array of signals streamed from the retina. Once generated, the central nervous system engages these perceptual outputs to guide decisions and actions, ultimately leading to complex behavioral responses (Glickfeld et al., 2014; Glickfeld and Olsen, 2017). Sprouting from the optic tract, feedforward projections define a complex hierarchy into the visual system

reaching the primary visual cortex and then ascending as two main reciprocally interconnected streams: a ventral stream, mainly devoted to object recognition and memory consolidation, and a dorsal stream, mainly devoted to visually-guided movements and attentional controls (Milner and Goodale, 1992).

The high degree of complexity emerging from this system has threatened a clear understanding of system mechanisms that compute complex perceptual representations from visual inputs. As a consequence of such limitation, the mouse has become an invaluable model to study visual processing over the last two decades. Recent anatomical studies have observed multiple analogies between mouse and primate visual systems (Glickfeld and Olsen, 2017). Moreover, accumulating behavioral evidence has revealed that mice display a great richness of visually-guided complex behaviors (i.e., Dickson et al., 2017; Morcos and Harvey, 2016; Prusky et al., 2000) that can be driven by an elaborate network of higher-order visual areas (HVAs) (Goldbach et al., 2021; Jin and Glickfeld, 2020). Moreover, it is worth mentioning that genetic and molecular tools afforded in the mouse have brought to this research field the possibly to precisely investigate cellular, circuity and inter-cortical interactions.

Like other mammals, the mouse visual system lies in the posterior part of the brain (Rosa and Krubitzer, 1999). In contrast with primate visual neuroanatomy, where the primary visual cortex is surrounded by a single secondary visual area (V2), the mouse primary visual cortex is adjoined by multiple distinct areas. In the last 60 years, however, the number and boundaries of these areas have been redefined and drawn several times in the light of new evidence emerging from cytoarchitectonic, electrophysiological, or tracer injection experiments (Caviness, 1975; Olavarria and Montero, 1989; Wagor et al., 1980). The current classification of HVAs is defined by the turning point research of Quanxin Wang and Andreas Burkhalter (Wang and Burkhalter, 2007). By means of triple tracer anterograde injections, these authors

have generated a comprehensive map of the mouse visual cortex revealing the presence of nine retinotopically organized areas adjoined to the primary visual cortex (Wang and Burkhalter, 2007). The anatomical map generated by Wang & Burkhalter have been largely replicated, in its core features, using electrophysiological and imaging experiments, and it thereby represents the basis of our current understanding and investigations of HVAs (Andermann et al., 2011; Marshel et al., 2011). Despite the presence of multiple adjoined areas, the mouse primary visual cortex shares its vertical meridian only with one of these HVAs, the lateromedial cortex (LM), resembling the border between primary and secondary visual areas in primates and carnivores. On the basis of this and other observations, LM is currently regarded as the only homologous of the primate V2, discarding the hypothesis of a single mouse area V2 parcellated in nine modules (Wang and Burkhalter, 2007). The great majority of feedback projections emerging from the primary visual cortex indeed terminates in LM, and this area is the only one, among HVAs, receiving few direct inputs from the geniculate nucleus (Oh et al., 2014). In addition, the paucity of connections that from LM reaches the superior colliculus (corticotectal projections) closely resemble that of V2 in primates (Wang and Burkhalter, 2013).

Recent attempts to understand the functional and structural organization of HVAs support the notion of a mouse visual system clustered in two internally connected subnetworks that are reminiscent of the dorsal and ventral streams seen in primates (Smith et al., 2017; Wang et al., 2012). One subnetwork, constituted by four lateral HVAs, is mainly devoted to process visual stimulus attributes with high spatial frequency (Murakami et al., 2017; Smith et al., 2017), linking the visual system to ventral brain regions implicated in memory and object identification, including temporal association areas and the entorhinal cortex (ventral stream) (Wang et al., 2012). The other subnetwork, constituted by five anteromedial HVAs, is mainly devoted to process visual stimulus attributes with high temporal frequency (Smith et al., 2017), linking the visual system to dorsal brain regions implicated in spatial movement and navigation,

including the retrosplenial, anterior cingulate, and second motor cortices (dorsal stream) (Wang et al., 2012). These two subnetworks also differ for their subcortical projections. Corticotectal projections from the ventral stream strongly innervate superficial/sensory layers of the superior colliculus, whereas those from the dorsal stream innervate deeper/ motor layers (Wang and Burkhalter, 2013). Areas belonging to the ventral, but not dorsal stream strongly target the amygdala (Burgess et al., 2016). HVA projections to the lateral posterior nucleus of the thalamic cluster in different zone in a stream-specific manner (Tohmi et al., 2014).

The transition between ventral and dorsal streams is set by the anatomical border dividing LM from the anterolateral cortex (AL) (Wang et al., 2011), which are the first and second HVAs in terms of connections with the primary visual cortex (Wang et al., 2012). Moving across this border, one can appreciate several chemoarchitectonic and cytoarchitectonic differences, including changes in layer IV expression of type 2 muscarinic acetylcholine receptor and changes in the representation of the lower visual field periphery. For this reason, LM and AL are currently considered the gateways for ventral and dorsal streams, respectively (Wang et al., 2011). The division of the mouse visual system in two parallel streams is also supported by brain connectivity mapping and developmental trajectories (Smith et al., 2017; Wang et al., 2012). For instance, connectivity clustering has revealed that two among several cortical subnetworks closely map into the ventral and dorsal streams (Wang et al., 2012; Zingg et al., 2014). These two subnetworks also comply with the neurodevelopmental tenet that ‘areas that work together develop together’; indeed, it has been recently observed that neurons in the ventral and dorsal streams follow two distinct developmental patterns. After eye opening, neurons in dorsal stream HVAs have a slower visual response maturation compared to neurons in ventral stream HVAs, displaying a high degree of vulnerability to a lack of visual experience. On the other hand, neurons in ventral stream HVAs sharp their orientation tuning and increase

their receptive field size during development with modest changes in their overall response strength (Smith et al., 2017).

The current knowledge on the anatomical organization of feedback projections to the primary visual cortex is incomplete. According to a very recent retrograde tracer study, these projections originate from at least 24 brain areas, but the great majority of these fibers re-enter the primary visual cortex emerging from HVAs and mostly from LM (Morimoto et al., 2021). It is currently held that the primary visual cortex and HVAs form closed-loop circuits that are topographically organized, with primary visual neurons receiving re-entrant projections from those higher-order neurons to which they project feedforward inputs (Gonchar and Burkhalter, 1999; Johnson and Burkhalter, 1997). Nevertheless, the laminar pattern between feedforward and feedback projections is asymmetric, with feedforward inputs ramifying across all cortical layers, and feedback inputs showing high density in layer I and low density in layer IV (Dong et al., 2004; Gonchar and Burkhalter, 1999; Yang et al., 2013).

Feedback projections mainly originate from pyramidal neurons and primarily form excitatory synapses with pyramidal neurons (~80%) and, only to a smaller degree, with GABAergic neurons (~20%) (Gonchar and Burkhalter, 2003, 1999). In compliance with the massive recruitment of excitatory cells, some studies have supported the notion of a top-down integration mainly mediated by an excitatory drive (Glickfeld and Olsen, 2017; Yang et al., 2013) that may be engaged to amplify tuned responses in downstream regions (Pafundo et al., 2016). Nevertheless, accumulating results are pointing toward a non-negligible suppressive role for top-down integration (Bastos et al., 2012). For instance, optogenetic inactivation of LM in awake mice decreases surround suppression in the primary visual cortex leading to increased responses to large visual stimuli in primary visual neurons (Vangeneugden et al., 2019). It is not directly intuitive and currently under debate how feedback projections can locally increase

the inhibitory rather than the excitatory gain since they are mainly glutamatergic and do not preferentially target inhibitory neurons. However, there are several hypotheses that may account for this counter-intuitive suppressive effect. Among others: inhibitory neurons may respond supra-linearly to HVAs feedback, while excitatory neurons respond sub-linearly; feedback projections may target inhibitory neurons that are widely connected to pyramidal neurons or that are widely distributed in the recipient cortex (Vangeneugden et al., 2019); feedback projections may indirectly increase cortical suppression targeting excitatory neurons that locally recruit inhibitory neurons (Angelucci and Bressloff, 2006).

HVAs compute the integration between environmental and internal signals to guide the execution of complex behavior according to the task demands. Complex behaviors indeed require the integration of information across several sensory modalities and the transition from decisions to actions. Consistently, higher-order neurons can code for multiple stimulus, cognitive, or motor variables (Glickfeld and Olsen, 2017). The multidimensional functional properties of HVAs have been initially investigated by lesions study in rats, which share the basic features of their visual cortex organization with mice. Thus far, functional studies have supported the anatomical division in ventral and dorsal stream. Lesions to ventral area HVAs are consistent with traditional notions of a ventral stream involved in processes related with memory and identification. For example, lesions to the postrhinal cortex (POR) selectively impair object recognition memory and cross-modal object recognition but has no effect on radial maze navigation (Winters et al., 2004; Winters and Reid, 2010). On the contrary, lesions to anteromedial HVAs are consistent with traditional notions of a dorsal stream involved in spatial processes. For example, lesions to the anteromedial cortex (AM) erase spatial learning without any effect on brightness discrimination task (Sánchez et al., 1997).

More recently, HVAs have been involved in representing the perceptual features of learned visual categories. Using long-term two-photon calcium imaging, it has been shown that visual category learning resulted in changes in stimulus and category tuning of HVA neurons (Goltstein et al., 2021). Some studies have started applying modern molecular techniques, like opto- or chemogenetic approaches, to precisely investigate the involvement of HVAs in perception. It has been recently shown, for example, that LM and AL are needed to perceive even simple visual features as contrast and orientations (Jin and Glickfeld, 2020). The optogenetic suppression of these two HVAs, indeed, decreased sensitivity for both orientation discrimination and contrast detection in mice trained to perform a go/no go discrimination task, revealing a crucial involvement of HVAs in visual perception. Notably, suppression of another HVA, the posteromedial cortex (PM), selectively increased false alarm rate without any significant effect on visual perception (Jin and Glickfeld, 2020). This last result seems to confirm that different HVAs provide specific visual computations to the primary visual cortex concurring to the elaboration of different visual percepts. Consistently, another study has demonstrated that several HVAs -but not PM- are required for the correct execution of a contrast-change discrimination task. Selective inactivation of HVAs is indeed sufficient to decrease perceptual performance in trained mice. This behavioral impairment, remarkably, can sometimes be greater than the effects provoked by the direct inhibition of the primary visual cortex (Goldbach et al., 2021).

Nonetheless, nothing is currently known regarding the involvement of HVAs in vPL. The results of the present thesis, however, point toward a key involvement of top-down processing in vPL.

Aims of the thesis and experimental design

In more than one century of research, numerous studies have been published on PL, and particularly, on vPL. Yet, several fundamental questions remain to be addressed.

Despite it is widely accepted that PL relies on plastic changes occurring in brain regions coding for the trained stimulus, the precise nature of these changes is still debated. Even less is known about brain mechanisms leading to these plastic rearrangements. Although several studies have proven that vPL is associated with morphological and functional changes in primary visual neurons, it is currently under debate whether, and to what extent, these changes are mediated by top-down projections that re-enter the primary visual cortex from higher-order areas. Indeed, plastic rearrangements have been also detected in high-order visual areas over the course of vPL. Accumulating models and evidence coming from primate studies, moreover, point toward a crucial involvement of top-down integration in vPL. However, a clear experimental proof is still missing.

The aim of the present Thesis is to investigate the role of top-down processing in PL employing a mouse model of vPL.

To address this issue, I have implemented a simple discrimination task developed by Fiorentini and Berardi (1980) to induce vPL in human subjects, and then adapted by Sale et al. (2011) to test Long Evans rats. Mice were tested in a forced-choice visual discrimination task consisting in the distinction of two vertical gratings, differing only for their spatial frequency. The training continued until the animals reached a plateau in performance. To test specificity of learning effects, the two gratings were then rotated by 90°.

In order to dissect the involvement of top-down processing in vPL improvements, I have exploited a chemogenetic approach to suppress the stream of information re-entering the primary visual cortex from HVAs. To this end, I used the inhibitory muscarinic-based DREADDs (namely, designer receptors exclusively activated by designer drugs) hM4D(Gi) that have a high sensitivity to the exogenous ligand CNO (namely, Clozapine N-oxide) and are instead insensitive to the native endogenous ligand (Armbruster et al., 2007). Two weeks before vPL, mice were subjected to intracortical viral injection in one of the nine higher-order mouse visual area, the latero-medial cortex (LM). LM is indeed considered the homologous of the primate V2 and it is currently regarded as the gateway of the mouse visual ventral stream, which is involved in percept formation and memory consolidation. Moreover, the great majority of feedback projections to the mouse primary visual cortex sprout from LM. The precision of the viral injection has been tested by electrophysiological and histological techniques. A group of injected mice was then engaged in the vPL task while LM activity was exogenously suppressed. Then, I have used a classical behavioral procedure to measure visual acuity, the Prusky water maze test, to rule out the possibility that the observed worsening of mouse performance was due to visual deficits and not to perceptual learning impairments. The obtained results seem to suggest that LM is required for both the acquisition and maintenance of vPL improvements.

Materials and Methods

3.1 Animal Treatment

C57BLK6/J mice of 2-3 months of age were used in this study, in accordance with the approved guidelines and regulations of Italian Ministry of Public Health. All the experiments have been designed to minimize the number of animals used and their discomfort or distress. Animals were housed in a room with a temperature of 22°C and a 12 h light/dark cycle; food and water were provided ad libitum.

3.2 Visual perceptual learning task

I used a modified version of the visual water box task (Prusky et al., 2000; Sale et al., 2011). The apparatus consisted of a trapezoidal-shaped pool with two computer-controlled monitors (diagonal screen size 40 cm) placed side-by-side at one end of the pool. The pool (140 cm long) was made of 6 mm Plexiglas, with 55 cm high walls, and was wider at one end (80 cm) than the other (25 cm). A midline divider extended between the monitors into the pool, bisecting its longitudinal axis. The length of the divider set the animal choice point and the visual angle at which the spatial frequency (SF) of the stimulus was that calculated by the computer software that generated the grating. An escape platform was placed below one of the two monitors and the pool was filled with tepid (25°C) water to a depth of 15 cm. White paint rendered the platform invisible from water level. Visual stimuli were presented on the two monitors visible through two glass windows.

A group of mice was first trained to distinguish a test grating with a spatial frequency (SF) of 0.522 c/deg from a reference grating of 0.116 c/deg until they achieved a performance level of at least 80% of accuracy in three subsequent sessions. The two gratings had the same luminance (40.06 cd/m^2) and the same contrast (100%). A custom-made software presented the two stimuli on the monitors, alternating the position of the test and the reference grating in a pseudorandom schedule. The submerged platform was always positioned in correspondence with the test grating. After animals achieved a level ≥80% of accuracy in at least three subsequent sessions, for a first group of mice (vPL mice) the vPL task was started by gradually reducing the SF of the test grating. If the animal made a correct choice, the SF of the test grating was decreased by one step and another trial was executed. This procedure continued until an error was made. Once an error occurred, the SF was increased by one step and another block of trials was run. After trials covering approximately half of the animal's projected threshold were completed, the minimum number of trials in a block was increased to four. For the last three SFs of the test grating, the required performance to decrease the SF was always 7/10 correct choices. A discrimination threshold (DT) was measured until the mouse performance reached a steady plateau (minimum discrimination threshold, MDT). In parallel, a different group of control mice (first-step (FS) mice) was allowed to only discriminate the reference grating from a test grating whose SF was always maintained at the starting value of 0.522 c/deg. Control mice were matched to vPL mice in terms of overall swim time and training sessions in the water tank (Fig.1).

When the performance plateau was reached, an experiment of stimulus orientation shift was performed: the orientation of the two gratings was rotated by 90°. For each mouse, the vPL task restarted from the last DT perceived before the orientation shift and new trials were applied in order to measure the MDT for horizontal gratings.

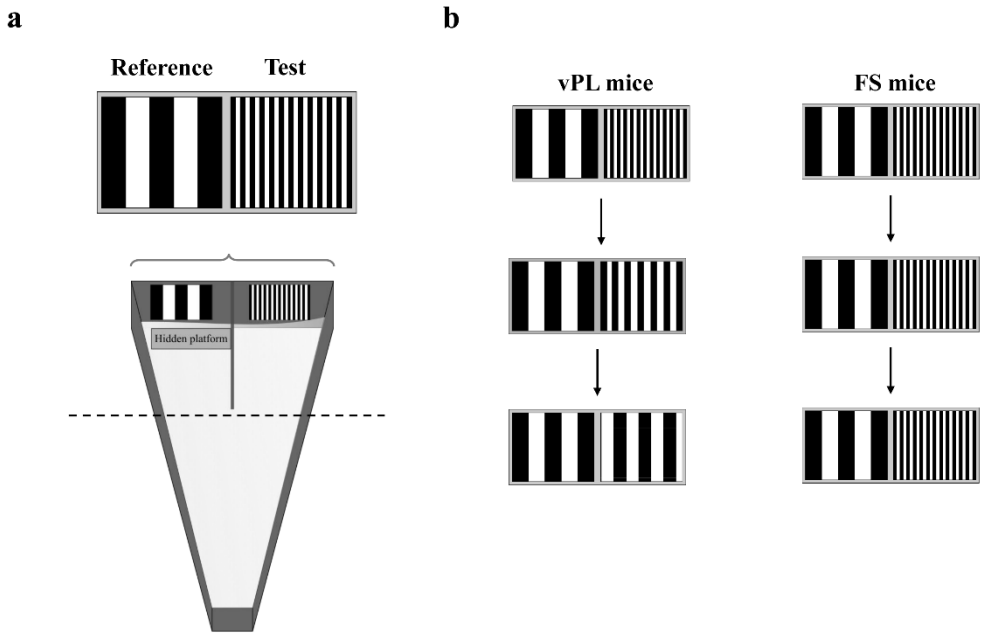

Figure 1 Schematic representation of the vPL task. **a**) A modified version of the visual water box task was used to perform vPL. **b**) Examples of stimulus discrimination for each group (vPL and FS mice). For vPL mice the SF of the test grating was gradually decreased from 0.522 c/deg to 0.129 c/deg. FS mice was allowed to distinguish the reference grating from a test grating whose SF was always maintained at the starting value of 0.522 c/deg.

3.3 In vivo electrophysiology

Electrophysiological recordings were performed as previously described (Consorti et al., 2022; Mazziotti et al., 2017; Porciatti et al., 1999; Sansevero et al., 2020). Mice were anesthetized with intraperitoneal (i.p.) injection of urethane (0.7 g/kg, 20% in saline; Sigma-Aldrich) and placed on a stereotaxic frame, with the body temperature maintained at 37°C. Animals were also ventilated through an oxygen mask. During surgery, eyes were protected applying a dexamethasone-based ointment (Tobradex, tobramycin 0.3% and dexamethasone 0.1%). A craniotomy was performed over the lateromedial visual cortex (3.6–4.1 mm lateral to lambda) leaving the *dura mater* intact. An electrode (2 × 2-tet-3 mm-150–150-121-A16-15, Neuronexus Technologies) was slowly lowered into LM cortex to record local field potential and single-unit activity. Signals were acquired using a 16 channels Neuralynx device and data analysis was

performed using a custom MATLAB software. Visual stimuli were generated in MATLAB using Psychophysics Toolbox extension and displayed, with gamma correction, on a monitor (Sony Trinitron G500, 60 Hz refresh rate, 32 cd m−2 mean luminance) placed 20 cm in front of the animal.

For visual evoked potentials (VEPs), extracellular signal was filtered from 0.1 to 275 Hz and sampled at 20 kHz. VEPs in response to sinusoidal wave patterns with a spatial frequency of 0.06 c/deg and abrupt phase inversion (2 Hz temporal period), were evaluated in the time domain by measuring the peak-to-baseline amplitude and latency. VEPs were acquired using the responses coming only from the tetrode in the upright position inside the inserted probe (responses from the four contact points were averaged together), at 200 µm of cortical depth. Computer controlled mechanical shutters were used to alternatively close the two eyes. Visual acuity and contrast threshold were obtained by extrapolation to zero amplitude of the linear regression through the data points in a curve where VEP amplitude was plotted against log spatial frequency or contrast percentage, respectively. Ocular dominance (OD) was assessed by calculating the contralateral to ipsilateral VEP ratio (C/I ratio), i.e., the ratio of VEP amplitudes recorded by stimulating the eye contralateral and ipsilateral, respectively, to the visual cortex where the recording is performed.

Extracellular signal was filtered from 0.6 to 9 kHz and sampled at 30.3 kHz. To improve single unit isolation, recordings from groups of four neighboring sites (tetrode) were linked, so that each spike was composed by four waveforms. Data were loaded on the Offline Sorter software (Plexon), and a principal component analysis was performed to score spikes with a high degree of similarity in a 3D feature space. Waveforms from each electrode of the tetrodes were processed together to improve isolation. Clusters were progressively defined using convex hulls and then recalculating principal component analysis. Quality of separation was

determined based on the following criteria: (1) during manual clusterization with convex hulls, raw waveforms in the clusters were visually inspected to check the uniformity of single waveforms; (2) clusters contained < 0.1% of spikes within a 1.0 ms interspike interval; (3) auto- and cross-correlograms of the clusters were also inspected to reveal if the cluster contained more than a single unit or if several clusters contained spikes of the same unit; and (4) the peak amplitude of a unit remained stable over the entire recording session. Drifting sinusoidal gratings were used as visual stimuli (1.5 s duration, temporal frequency of 2 Hz, 12 different orientations with a step of 30°, 6 spatial frequencies: 0.01, 0.02, 0.04, 0.08, 0.16 and 0.32 c/deg). Stimulation was repeated five times per eye, with stimulus conditions randomly interleaved, and two grey blank conditions (mean luminance) were included in all stimuli sets to estimate the spontaneous firing rate. The average spontaneous rate for each unit was calculated by averaging the rate over all blank condition presentations. Responses at each orientation and spatial frequency were calculated by averaging the spike rate during the 1.5 s presentation and subtracting the spontaneous rate. The preferred stimulus was determined finding the combination of spatial frequency and orientation that maximize the response, independently for each eye. Orientation tuning curves were constructed for the spatial frequency that gave maximal response at this orientation. Given this fixed preferred orientation (OPref), the tuning curve was fitted as the sum of two Gaussians centered on OPref and OPref + π, of different amplitudes but equal width, with a constant baseline. From this fit, I calculated an orientation selectivity index (OSI) representing the ratio of the tuned versus untuned components of the response, and the width of the tuned component. OSI was calculated as follows: (respOPrefresp - OOrtho)/(respOPref + respOOrtho), where resp is the maximal response evoked by visual stimulation and OOrtho is the orientation orthogonal to the preferred one. Tuning width is the half-width at half-maximum of the principal gaussian. In addition, a direction selectivity index (DSI) was calculated as follows: (respOPref-respOOppo)/(respPref + respOppo). In order to

assess OD, sorted units were clustered in OD classes. OD classes were evaluated on the basis of the ratio of contralateral to ipsilateral peak response. More specifically, neurons in ocular dominance class 1 were driven only by stimulation of the contralateral eye; neurons in ocular dominance classes 2 (ratio of contralateral to ipsilateral peak response, ≥2) and 3 (ratio of contralateral to ipsilateral peak response, between 1.2 and 2) were binocular and preferentially driven by the contralateral eye; neurons in ocular dominance class 4 were equally driven by the two eyes (ratio of contralateral to ipsilateral peak response, between 0.83 and 1.2); neurons in ocular dominance classes 5 (ratio of contralateral to ipsilateral peak response, between 0.5 and 0.83) and 6 (ratio of contralateral to ipsilateral peak response, ≤0.5) were binocular and preferentially driven by the ipsilateral eye; and neurons in ocular dominance class 7 were driven only by the ipsilateral eye.

3.4 Intracortical viral injection in LM

Adult mice were anesthetized with isoflurane and mounted on a stereotaxic apparatus. An incision was made on the scalp, the skin above the skull was retracted and a small craniotomy was made 3.6 mm lateral to lambda. A viral vector, AAV8-hSyn-HA-hM4D(Gi)-mCherry (a gift from Bryan Roth (Addgene viral prep # 50475-AAV8); http://n2t.net/addgene:50475; RRID: Addgene_50475), was delivered using a 10 µL Hamilton syringe with a nanoliter syringe pump (Kd Scienti, speed 0.05 µL/min). Two injections were made at different cortical depths (220 and 450 µm below the pial surface) into LM (200 nL per injection site). For the vPL task and visual acuity assessment, animals were bilaterally injected. The scalp was then sutured, and an antibacterial pomade was applied along the sutures. During surgery, body temperature was maintained using feedback regulated heating pad.

To probe AAV expression, a group of mice were perfused intracardially with PBS followed by 4% paraformaldehyde (PFA) in phosphate buffer. Brains were post-fixed overnight at 4 °C and then impregnated with 30% sucrose in phosphate buffer. Coronal brain sections (50 µm) were cut on a freezing microtome and collected in PBS. All reactions were performed on free-floating sections. After a washing step PBS-T (PBS with 0.3% Triton), sections were incubated for 10 min at RT in Hoechst and mounted on glass slides and covered with VectaShield mounting medium. mCherry fluorescence was acquired on a fluorescent microscope empowered with an Apotome 2.0 slit. LM was identified using the Allen Mouse Brain Atlas (https://atlas.brain-map.org/).

3.5 Chemogenetic experiments

For all chemogenetic experiments, CNO (Tocris Bioscience, Cat.No. 4936) was dissolved in sterile 0.9% NaCl, which was administer as vehicle.

Electrophysiological assessment of LM suppression

In order to assess the chemogenetic suppression of LM, single units were recorded from a separated group of injected mice as previously described. The probe was lowered of 400 µm and single-unit responses were recorder before and 15 minutes after i.p. CNO administration (2 mg/kg) without changing the recording parameters. As control, a group of injected mice was instead recoded before and 15 minutes after i.p. saline administration (2 mg/kg). The electrical responses were compared to single units sorted from naïve animals at the same cortical depth.

Visual perceptual learning

A separated group of injected mice was subjected to the vPL task previously described two weeks after surgery. Once the 80% criterion was reached, mice were subject to the incremental phase of the vPL task 30 minutes after the i.p. administration of CNO (2 mg/kg, CNO mice) or saline (2 mg/kg, SAL mice). Sessions were interleaved by a 4-hour gap to minimize animal discomfort and stress.

When the performance plateau was reached, an experiment of administration shift was performed, i.e., CNO mice were subjected to i.p. administration of saline (CNO_SAL mice), and vice-versa, SAL mice were subjected to i.p. administration of CNO (SAL_CNO mice). For each mouse, the vPL task restarted from the last DT perceived before the administration shift and additional vPL training was then applied to measure MDT under these experimental conditions.

Behavioral assessment of visual acuity

Visual acuity (VA) was behaviorally measured in a separated group of injected mice two weeks after AAV intracortical injection. VA was assessed through the behavioral method of the Prusky water maze task. The apparatus consists of a trapezoidal-shaped pool with two panels placed side by side at one end. A midline divider is extended from the wide end of the pool into the middle, creating a maze with a stem and two arms. The length of the divider sets the choice point and effective SF. An escape platform is placed below the grating. Animals are released from the center at the end of the pool opposite the panels. The position of the grating and the platform is alternated in a pseudorandom sequence over training trials. Mice are shaped to swim towards the grating in one of the maze arms. A trial is recorded as incorrect if an animal enters

the arm without the platform. Mice were first trained to distinguish a low (0.116 c/deg) SF vertical grating from grey. After animals achieved a level ≥80% of accuracy in at least three subsequent sessions, mice were subjected to this task 30 minutes after i.p. saline administration (2 mg/kg), and the VA limit is estimated by increasing the SF of the grating. VA has been taken as the SF corresponding to 70% of correct choices on the sigmoidal function fitting the psychometric function. Then, starting from the last SF at which animal performance was ≥70% of correct choices, mice were subjected to the task 30 minutes after i.p. CNO administration (2 mg/kg), and VA was re-measured (Fig.2).

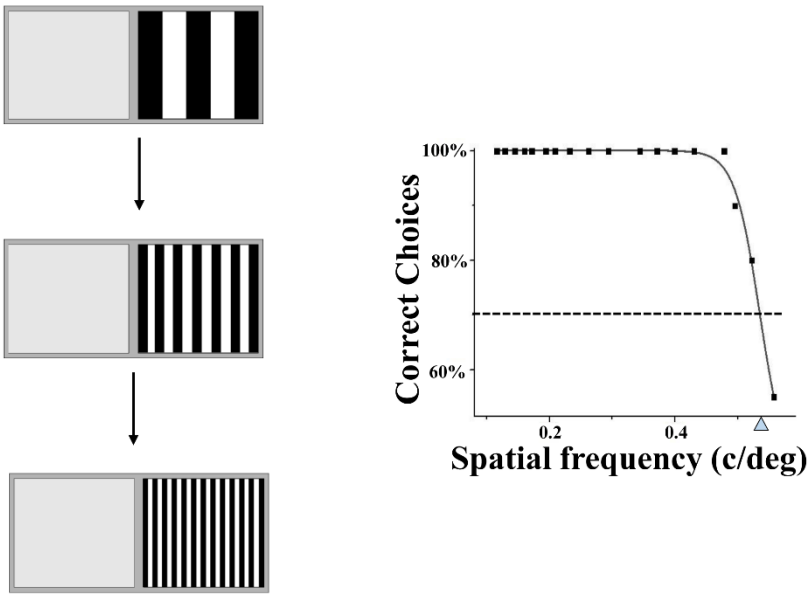

Figure 2. Examples of steps for the assessment of VA. Incremental changes in the SF of the visual stimulus are made among successive blocks of trials until the ability of animals to distinguish a grating from grey falls to chance. VA has been taken as the SF corresponding to 70% of correct choices on the sigmoidal function fitting the psychometric function.

3.6 Statistical analysis

Statistical analysis was done using SigmaStat Software. Data were tested for normality before running statistical tests; parametric tests were run on normally distributed data and, in case normality test failed, non-parametric tests were performed as appropriate. Differences between two independent groups were assessed with a two-tailed t-test; differences between two dependent groups were assessed with a two-tailed paired t-test. One-way ANOVA, One-way RM ANOVA, and Two-way RM ANOVA were used to compare normally distributed data belonging to more groups. One-way ANOVA or Two-way on ranks were performed to compare not normally distributed data belonging to more than two groups. Level of significance was $p<0.05$, unless otherwise specified. The size of biological replicates is indicated by the n numbers in the various experimental sections.

Results

4.1 vPL in mice

A group of mice (n=14) was subjected to a simple grating discrimination task; mice were asked to discriminate two vertical (0°) gratings with equal contrast, but different SF. During an initial training phase, mice were required to distinguish a test grating with a SF of 0.522 c/deg from a reference grating of 0.116 c/deg until they achieved a level of at least 80% of accuracy in three subsequent sessions. A significant increase was measured in the percentage of correct choices over the course of training sessions (One-way RM ANOVA, Holm-Sidak method, $F=24.276$, $DF=1$, $p<0.001$, Fig. 3a).

Then, for a group of mice (vPL mice, n=7) the SF of the test grating was made progressively more similar to that of the reference grating, starting from the SF difference of the training phase (0.406 c/deg, 0.522 vs 0.116 c/deg). Therefore, vPL consisted in the improvement of visual discrimination abilities in distinguishing the two gratings when they became more and more similar to each other. A discrimination threshold (DT) was measured until the mouse performance reached a steady plateau (minimum discrimination threshold, MDT). Trained mice displayed robust vPL, as shown by the progressive reduction in the DT between the reference and test gratings across sessions. On the first session, the mean DT was 0.282 ± 0.016 c/deg, while this value reached 0.043 ± 0.004 c/deg at the end of vPL training (One-way RM ANOVA on ranks, $p<0.001$, Fig. 3b). The minimum SF difference between the test and reference gratings achieved by each vPL mouse was of 0.056, 0.044 or 0.029 c/deg (Fig. 3c).

In parallel, a separated group of control mice (first-step (FS) mice, n=7) learned the vPL task but was allowed to only distinguish the reference grating from a test grating whose SF was

always maintained at the starting value of 0.522 c/deg. After few sessions, the performance of control FS mice remained stable (Fig. 3d). Control mice were matched to vPL mice in terms of overall swim time and training sessions in the water tank. Notably, no differences were found in visual discrimination abilities between FS and vPL mice during the training phase (Future FS and Future vPL, respectively) (Two-way RM ANOVA, Holm-Sidak method, $F=1.334$, $DF=1$ $p=0.346$, Fig. 3e).

4.2 vPL and transfer of learning: an experiment of orientation shift

When the performance plateau was reached, the two vertical gratings were rotated by 90°, and new trials were applied to assess the MDT for horizontal gratings. I found that the grating discrimination vPL task was selective for the orientation of the stimulus, as demonstrated by the marked impairment in the discrimination abilities displayed by vPL mice after the stimuli were rotated (n=7, MDT before shift: 0.040 ± 0.004 c/deg, MDT after shift: 0.327 ± 0.021 c/deg; paired t-test, $t = -13.084$, $DF=6$, $p<0.001$, Fig.4a). Consistently with previous studies (Fiorentini and Berardi, 1981; Sale et al., 2011) vPL mice were totally unable to discriminate the newly oriented stimuli when the two gratings were maintained at the same SFs reached before the orientation shift, with mice being able to only discriminate a difference between the SFs of the two gratings much higher than that recorded before the orientation shift. The percent of correct choices fell below the 70% criterion for two consecutive vPL sessions when the SFs were rotated by 90° (gray bars, Fig.4c and 6a), proving that vPL improvements did not transfer between stimuli of orthogonal orientation. Starting from this point, the vPL procedure was repeated, until the animal performance reached a new perceptual plateau. The MDT for horizontal grating was not different from that achieved before the stimulus orientational change

(n=7, MDT before shift: 0.040 ± 0.004 c/deg, MDT after shift and vPL training: 0.040 ± 0.004 c/deg; paired t-test on ranks, p=1.000, Fig.4b and 6a).

To test whether learning transfer was correlated with vPL complexity, FS mice were subjected to the protocol of orientation shift. FS mice were still able to perform the task after the orientation shift without a significant change in their performance (n=7, paired t-test on ranks, p=0.125, Fig. 5a), retaining the effects of previous practice with the newly oriented stimuli (Fig.5b and 6b).

4.3 Electrophysiological characterization of LM activity

To characterize LM electrical activity, visual evoked potentials (VEPs) and single-units were analyzed in a group of anesthetized naïve mice through multichannel electrophysiological recordings.

VEP recording represent a well-established method to link cortical electrophysiology with sensory perception (Pizzorusso et al., 1997; Porciatti et al., 1999). To assess visual acuity, VEP responses were recorded from a silicon electrode inserted 3.6 mm lateral to lambda and advanced 200 µm within the cortex, in response to a horizontal grating of different spatial frequencies and 90% contrast. VEP acuity was obtained extrapolating VEP amplitude to 0 V. The average acuity found in different mice (n=5) was 0.402 ± 0.056 c/deg (Fig.7a). To measure contrast sensitivity, VEPs were recorded in response to a grating of 0.06 c/deg at different contrasts. Contrast sensitivity was obtained extrapolating to 0 V the recorded VEP amplitudes. Contrast threshold was measured in different mice (n=5) and the average value was of 9.768% ± 2.865% (Fig.7c). To determine ocular dominance (OD), I measured the contralateral to ipsilateral (C/I) VEP ratio in response to a grating of 0.06 c/deg. The obtained C/I VEP ratio in recorded animals (n=5) was of 2.504 ± 0.708 (Fig.7b).

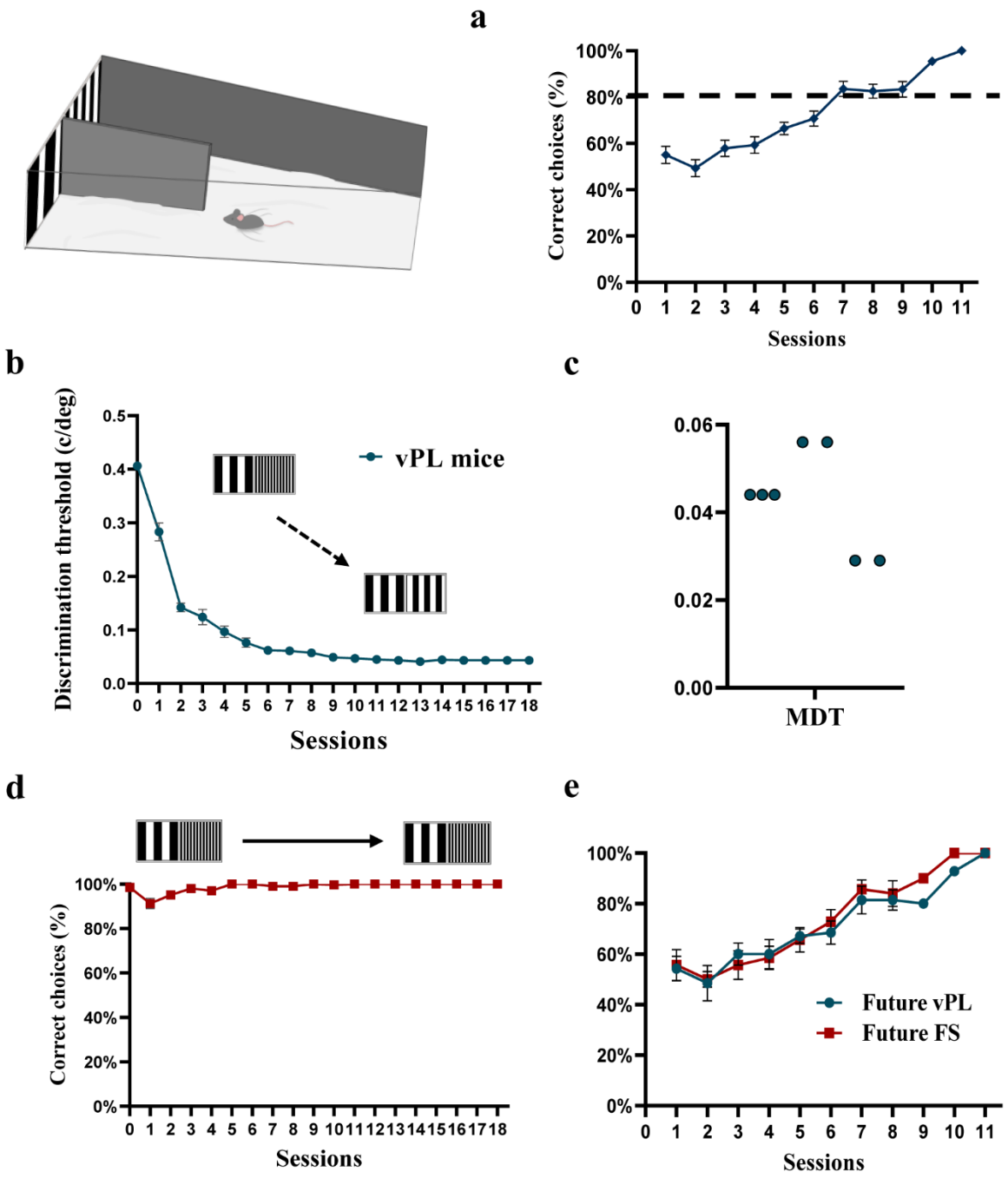

Figure 3. A mouse model of vPL. A modified version of the visual water box task was used to perform vPL. **a)** Mean performance in distinguishing a test grating of 0.522 c/deg from the reference grating of 0.116 c/deg across the training sessions. All animals (n=14, vPL and FS mice) have been pulled together. The increase in the percentage of correct choices with sessions was significant (One-way RM ANOVA, Holm-Sidak method, $p < 0.001$). **b)** Improvement of discrimination threshold in vPL mice engaged in the vPL task. The threshold, calculated as the minimum spatial frequency difference between the reference and the test gratings, decreased significantly across training days (One-way RM ANOVA on ranks $p < 0.001$). **c)** MDT achieved by vPL mice (n=7) once the vPL task was completed. **d)** After few sessions, the performance of FS mice involved in a discrimination task lacking the incremental component remained stable across the training days. **e)** No difference could be found in the percentage performance of mice that have been subsequently required to distinguish between these two gratings even during the test phase (future FS mice) and mice that have been subsequently subjected to the vPL task (future vPL mice) (Two-way RM ANOVA, Holm-Sidak method, $p=0.346$). *Statistical significance. Error bars, s.e.m.

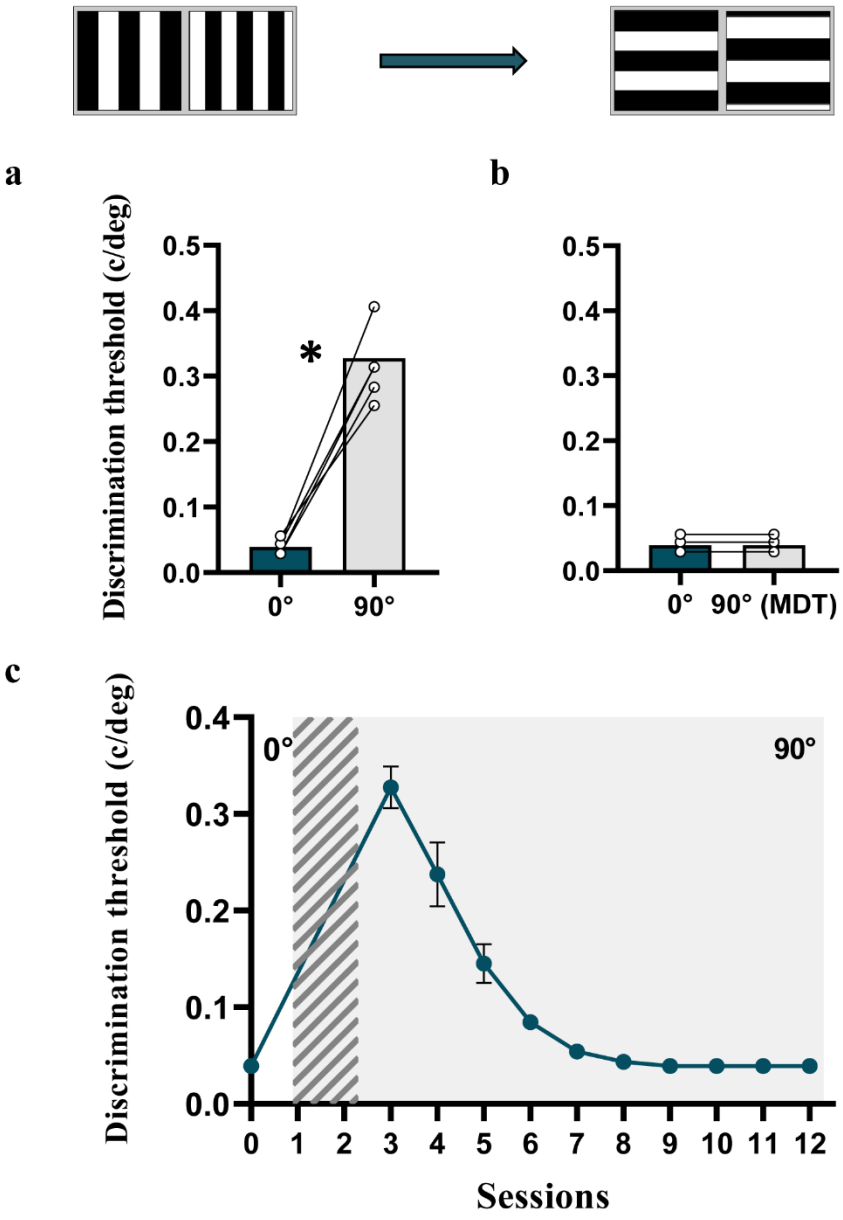

Figure 4. Orientation shift in vPL mice. After vPL mice achieved their MDT, the refence and test gratings were shifted by 90° keeping fixed the spatial frequency difference. **a)** vPL mice were totally unable to discriminate the newly oriented stimuli (MDT before shift: 0.040 ± 0.004 c/deg, MDT after shift: 0.327 ± 0.021 c/deg; paired t-test, $p<0.001$). **b,c**) Starting from this DT, additional training was applied. The new performance plateau was not significantly different from that achieved before the stimulus orientational change (n=7, MDT before shift: 0.040 ± 0.004 c/deg, MDT after shift and vPL training: 0.040±0.004 c/deg; paired t-test, $p=1.000$. *Statistical significance. Error bars, s.e.m.

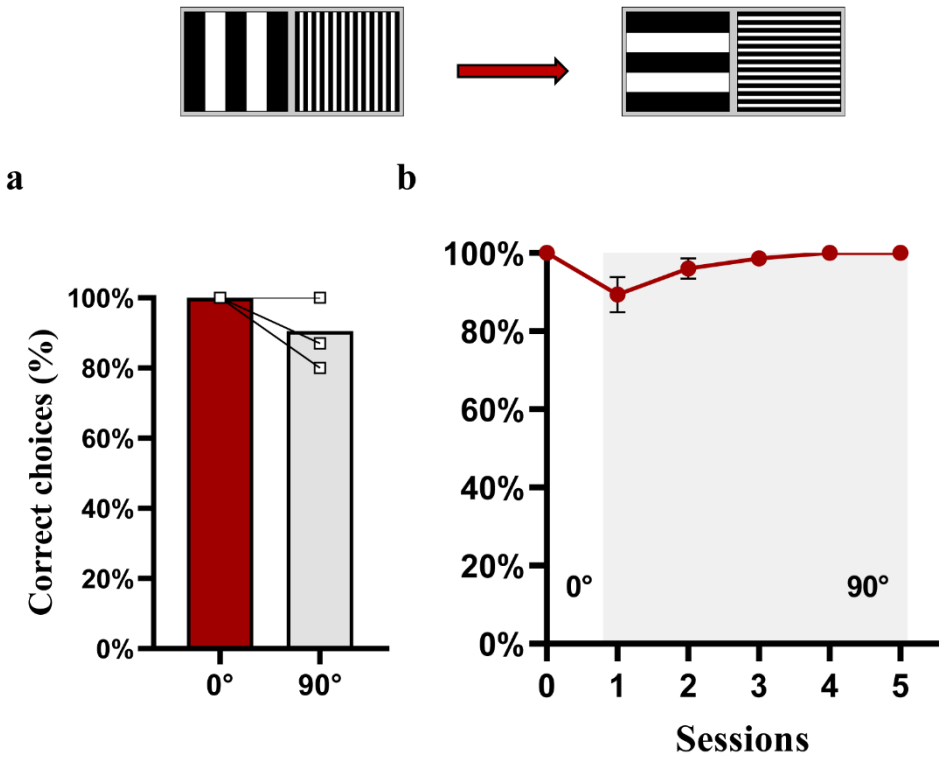

Figure 5. Orientation shift in FS mice. When the stimuli were shifted, FS mice were still able to distinguish the reference grating (0.116 c/deg) from the initial test grating (0.522 c/deg) **a,b)** No significant changes in the performance could be detected after the orientation shift (before: 100%±0.0% of correct choices, after: 91%±0.035%, paired t-test, p=0.125). *Statistical significance. Error bars, s.e.m

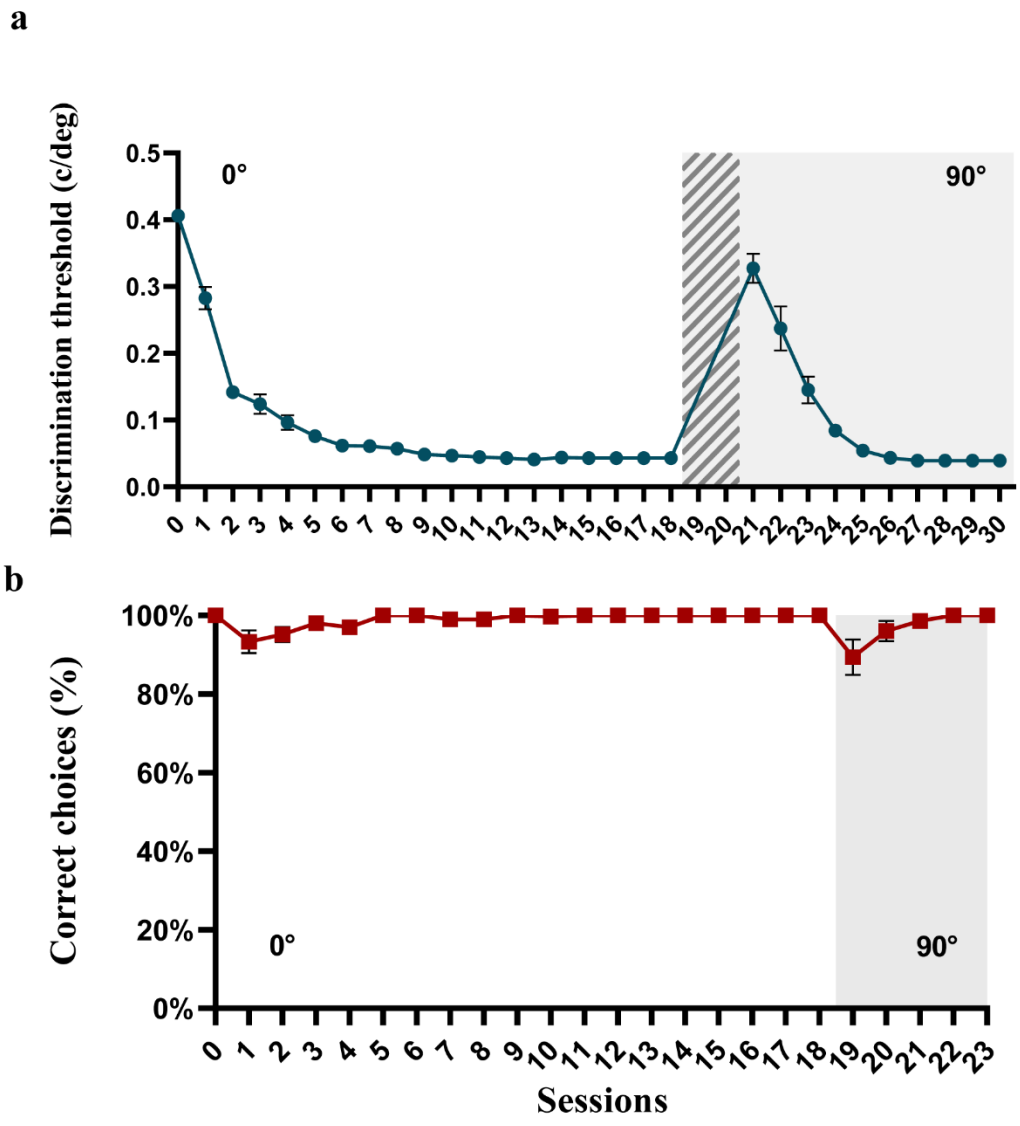

Figure 6. vPL task. a) vPL mice were subjected to a simple incremental discrimination task until an MDT was achieved. The stimuli were then rotated by 90°. vPL mice were totally unable to discriminate the newly oriented stimuli. However, when additional vPL training was applied, vPL reached the same MDT achieved before the orientation shift. **b)** A group of control mice was required to discriminate the initial spatial frequency difference between the two gratings and was matched with vPL mice in terms of swimming time in the water tank. When the visual stimuli were rotated by 90°, FS mice retained the learned discrimination ability.

Single units were recorded in anesthetized animals in response to drifting sinusoidal gratings that varied in orientation and spatial frequency, at multiple depths spanning all cortical layers. Monocular tuning properties were analyzed in sorted units that were recorded by sampling from layers II/III to VI in a group of anesthetized mice (n=6). To better analyze LM electrophysiology, single-units were clustered in 7 OD classes on the basis of the ratio of contralateral to ipsilateral peak response according to the Hubel & Wiesel's classification. The great majorities of all recorded cells (n=117) fell into the intermediate OD classes, with no units exclusively driven by the contralateral (class 1) or ipsilateral (class 7) eye (Fig. 8a). In contrast with the C/I VEP ratio, the electrical activity of the recorded single-units was not shifted towards the contralateral eye stimulation. This difference could be ascribed to the possibility of sampling exclusively the electrical activity of local neurons by single-unit recordings. Indeed, single-unit recordings were most likely not affected by the close proximity of the larger primary visual cortex, whose electrical activity is mainly driven by neurons tuned to contralateral eye stimulation (Porciatti et al., 1999).

To characterize LM properties, spontaneous and evoked responses were measured (Fig.8b, c). Furthermore, orientation and direction selectivity were evaluated calculating the orientation selectivity index (OSI) and direction selectivity index (DSI) (Fig. 8d, e).

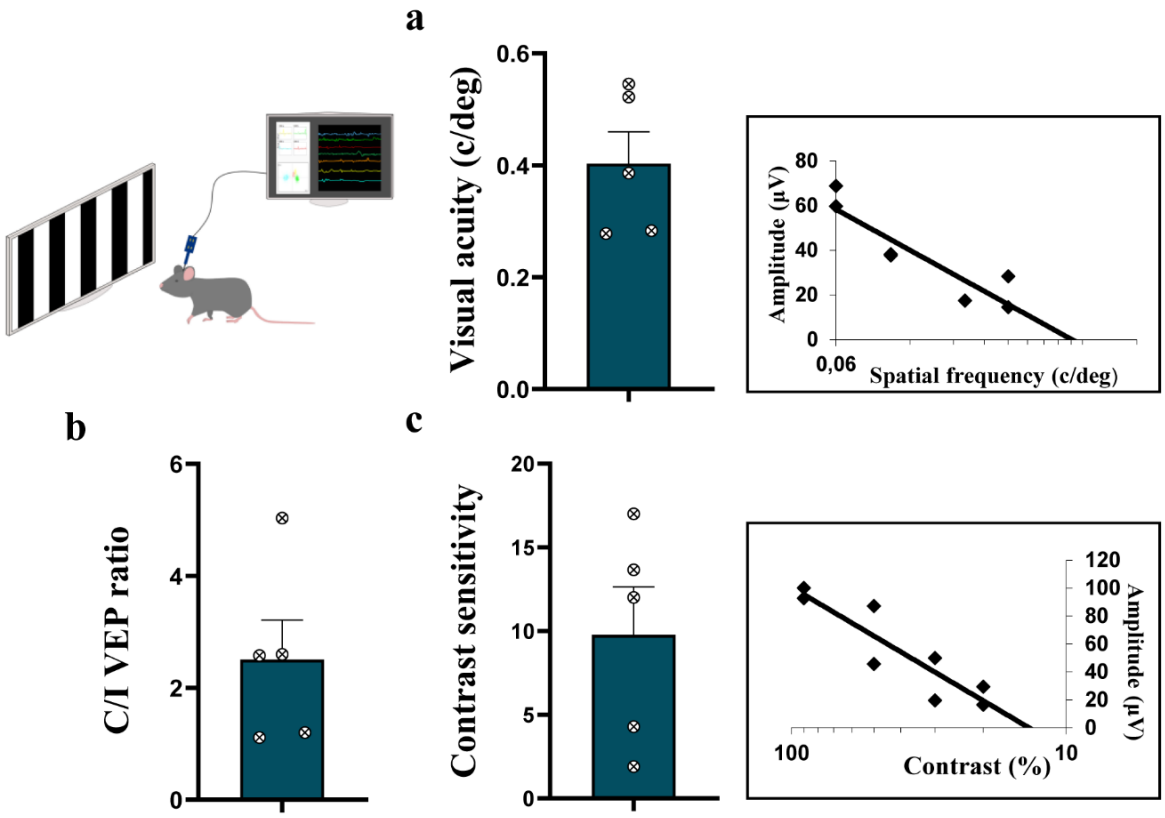

Figure 7. Field recordings into LM. Field potentials were recorded in the LM of naïve mice (n=5) through a multichannel Neuronexus probe. **a,c)** Visual acuity and contrast sensitivity were obtained extrapolating VEP amplitude to 0 V. **b)** Binocularity was measured calculating the contralateral to ipsilateral (C/V) VEP ratio**.**

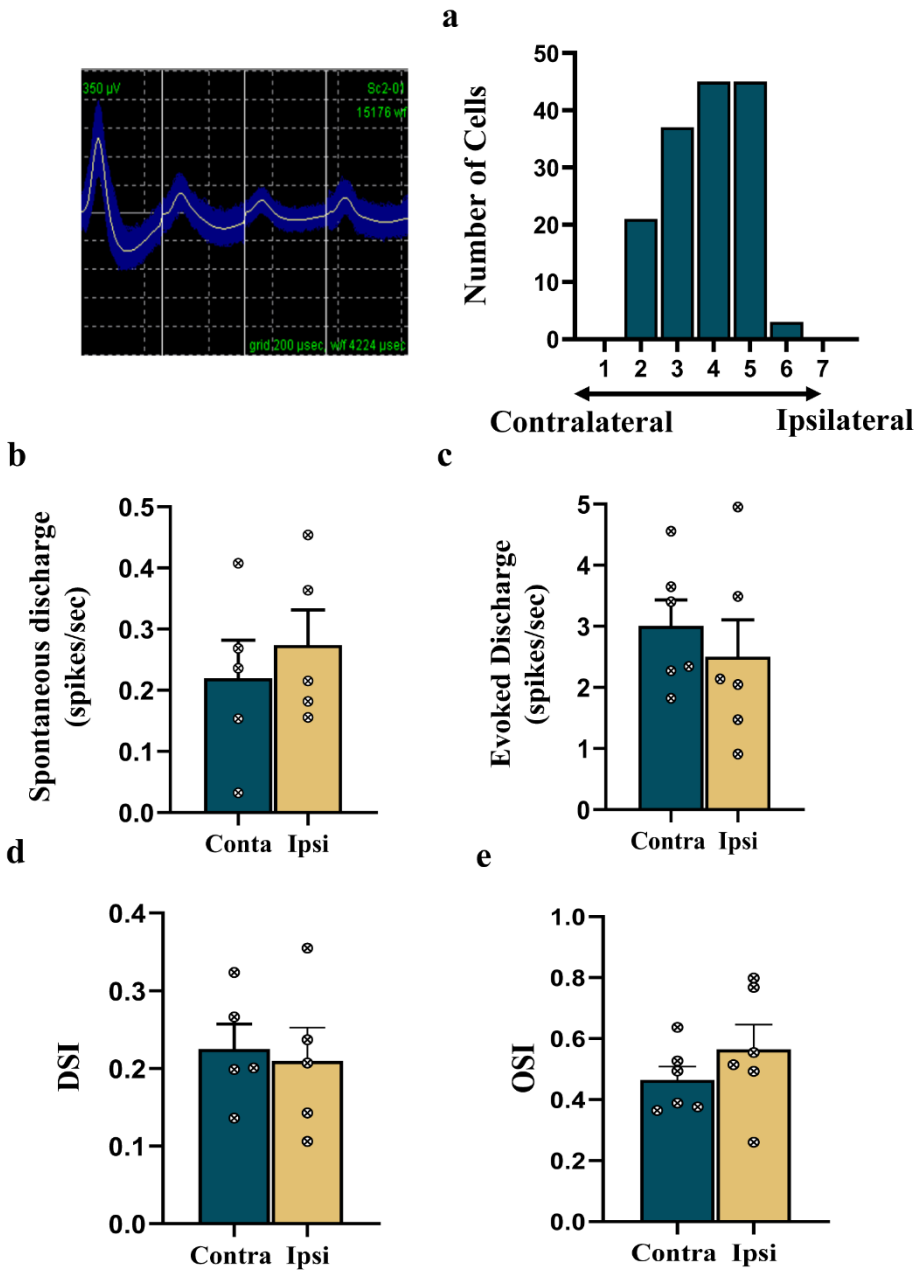

Figure 8. Single unit recordings from the LM. Single units were recorded in a group of naïve animals (n=6) sampling at different cortical depths. **a)** In order to assess LM binocularity, sorted cells were clustered in 7 ocular dominance classes according to their contralateral to ipsilateral response. Then, to characterize LM activity, the spontaneous and evoked activities (**b** and **c**, respectively), the direction selectivity and the orientation selectivity indices (**d** and **e**, respectively) were measured in all sorted units. Single dots were obtained averaging all the single-units recorded in each animal.

4.4 Chemogenetic inhibition of LM activity

A constitutive viral vector was injected into the LM of naïve mice, in order to induce the expression of hM4D(Gi)-mCherry in all transfected neurons. Histological analysis showed that the expression of hM4D(Gi)-mCherry did not intrude much into the primary visual cortex and was mostly confined to LM, without crossing the border between LM and AL (Figs. 9, 10).

To probe the chemogenetic inhibition of LM activity, single units were recorded in a group of injected mice (n=9) inserting the probe at 400 µm of cortical depth, two weeks after surgery. For some mice (n=5), monocular electrical signals were recorded before (pre Clozapine N-oxide (CNO)) and 15 minutes after an i.p. administration of CNO (post CNO). The exogenous hM4D(Gi) activation led to significant suppression of evoked activity in response to both the contralateral (pre CNO = 2.473 ± 0.690 Hz, post CNO = 0.789 ± 0.112 Hz; paired t-test, t = 3.039, DF=4, p=0.038, Fig.11a) and ipsilateral (pre CNO =2.559 ± 0.597 Hz, post CNO = 0.415 ± 0.188 Hz; paired t-test, t = 4.180, DF=4, p = 0.014, Fig.11a) stimulation. On the contrary, no reduction in LM activity could be found in mice (n=4) recorded before (pre SAL; contra = 1.708 ± 0.325 Hz, ipsi =1.681 ± 0.294 Hz) and 15 minutes after an i.p. administration of saline (post SAL; contra = 1.87 ± 0.358 Hz, ipsi =1.274 ± 0.288 Hz) (paired t-test, contra: on ranks, p = 0.375 and ipsi: t = 0.734, p = 0.516, Fig.11b).

To evaluate the effect of the viral injection on LM physiology, the electrical activity recorded from CNO and SAL mice was compared to sorted single units sampled from naïve animals (n=6), at 400 µm of cortical depth. Only the evoked responses recorded from the contralateral and ipsilateral eyes of animals treated with CNO (post CNO) was significantly different compared to the electrical responses recorded from naïve mice (Naïve, contra = 2.659

± 0.354 Hz ipsi = 2.421 ± 0.603 Hz) (One-way ANOVA vs control, Holm-Sidak method, DF=4, contra: F= 3.791, p = 0.02 and ispsi: F= 3.919, p = 0.031, Fig.11c).

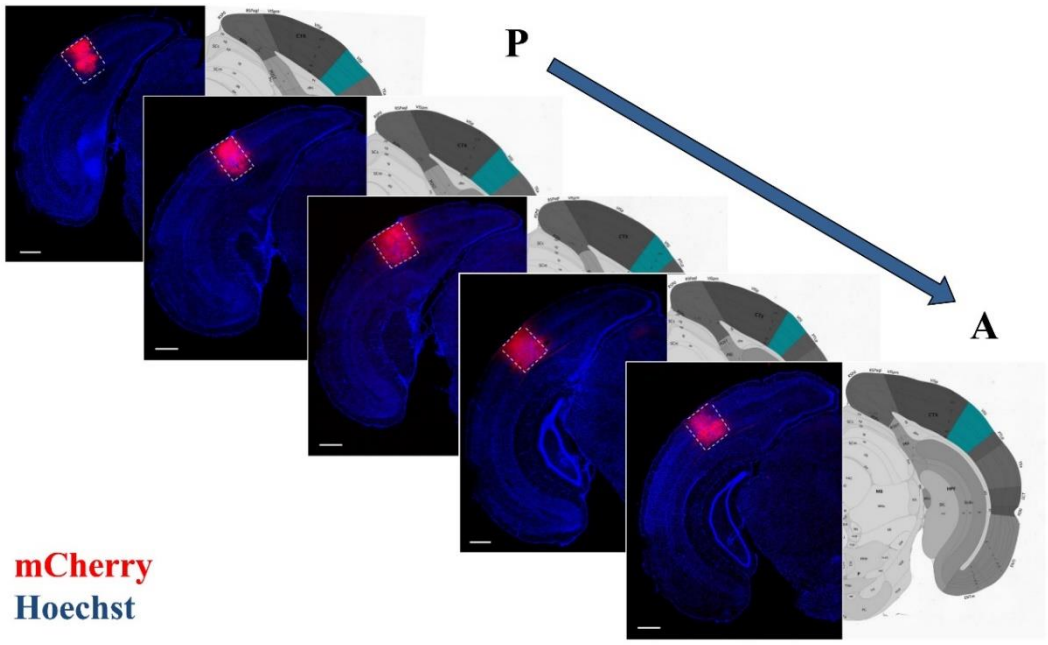

Figure 9. Histological evaluation of mCherry expression. To indirectly assess hM4D(Gi) expression, mCherry expression was analyzed along the postero (P) anterior (A) axis. Brain slices (50 µm) were acquired using a fluorescent microscopy and analyzed using ImageJ. LM borders were identified according to the Allen Brain Atlas. mCherry expression was mostly confined into LM. Scale bar 500 µm.

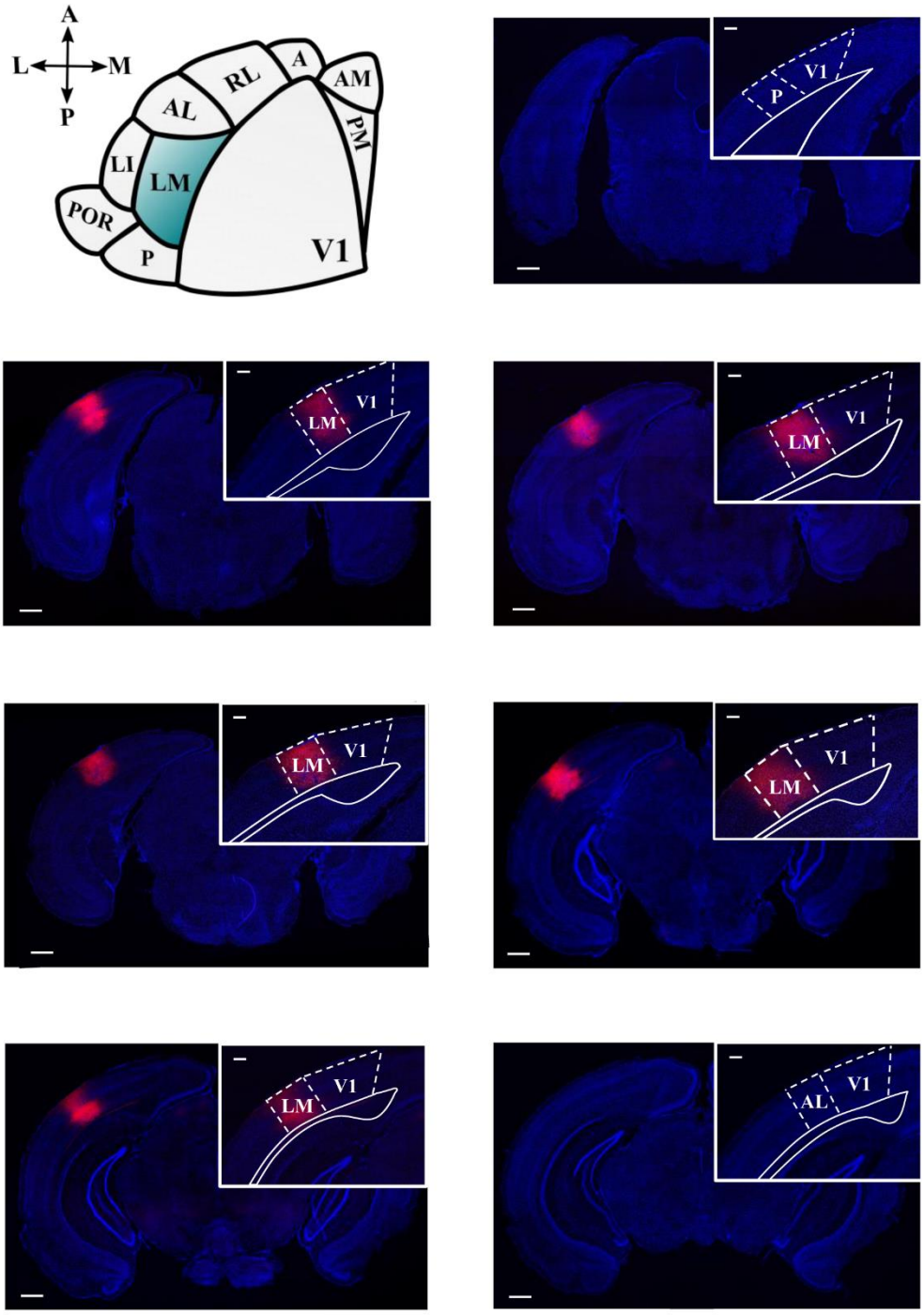

Figure 10. mCherry expression and HVAs. Representation of the HVAs along the postero (P) anterior (A) and latero (L) medial (M) axes. The anatomical transition between the LM and AL cortex is considered the physiological border between the ventral and dorsal visual streams. mCherry expression was entirely confined into the ventral stream. No fluorescence could be detected within AL borders. Scale bar 500 µm; inset 200 µm. Abbreviations for visual areas: V1, primary visual cortex; A, anterior; AL, anterolateral; AM, anteromedial; LI, laterointermediate; LM, lateromedial; P, posterior; PM, posteromedial; POR, postrhinal; RL, rostrolateral.

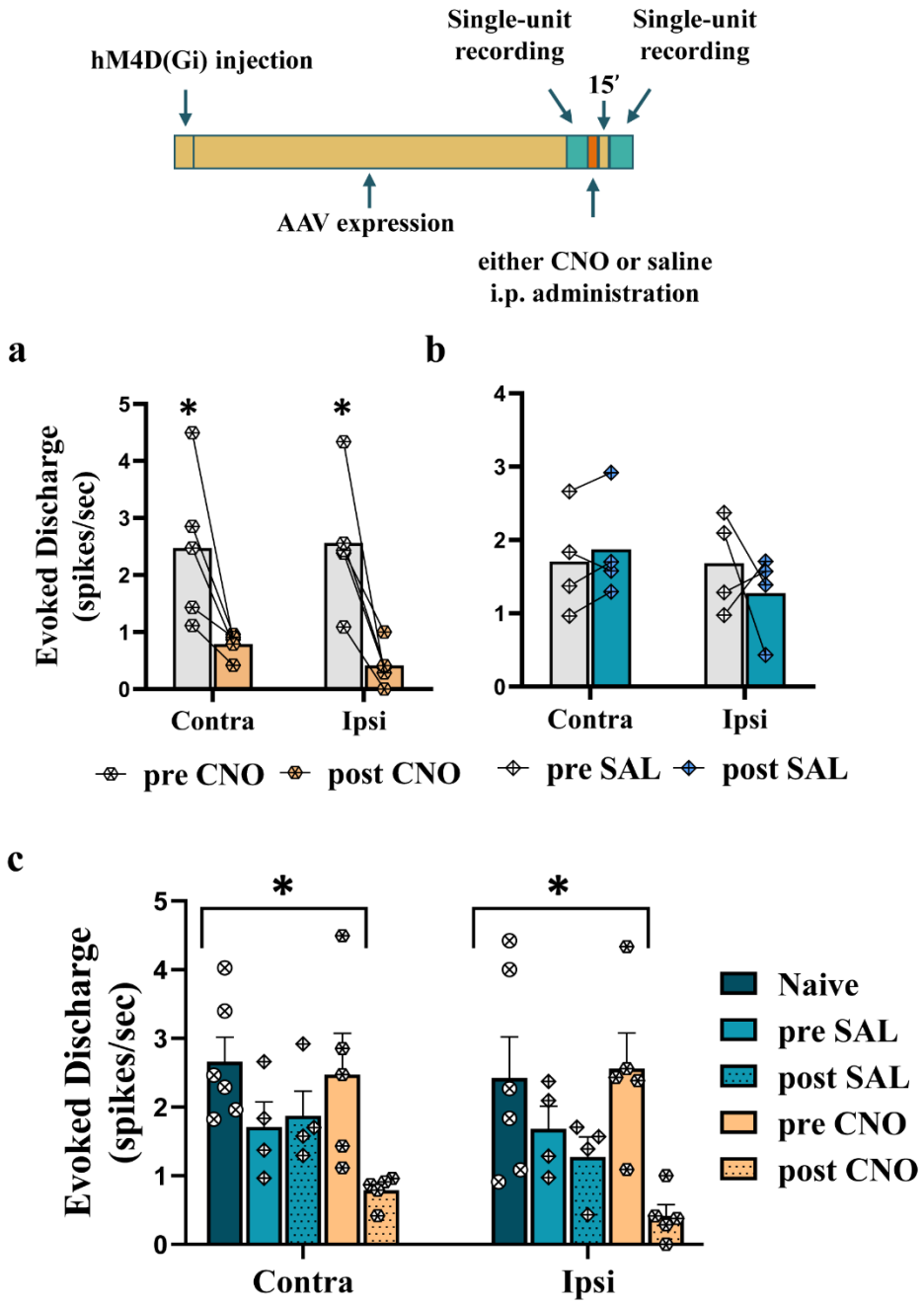

Figure 11. Electrophysiological assessment of LM inhibition. Single units were recorded around 400 µm of cortical depth in a group of injected mice before and 15 minutes after the i.p. injection of CNO or saline, keeping all the recording parameters fixed. **a)** A significant decrease in LM activity was detected after CNO administration (contralateral eye: paired t-test, p=0.038; ipsilateral eye: paired t-test, p = 0.014). **b)** On the contrary, no changes in LM activity were detected after saline administration in a separated group of injected mice (contralateral eye: paired t-test, p = 0.375; ipsilateral eye: paired t-test, p = 0.516). **c)** After the i.p. administration, the LM activity recorded in CNO administered mice, but not saline administered mice, was significantly different from that of naïve mice (One-way ANOVA, contralateral eye: p = 0.02 and ipsilateral eye: p = 0.031). Single dots were obtained averaging all the single-units recorded in each animal. *Statistical significance. Error bars, s.e.m.

4.5 LM is required for the acquisition of perceptual improvements

In order to test whether top-down processing is engaged in the acquisition of perceptual improvements, I manipulated LM activity during the vPL task. To this end, LM neurons were inhibited by expressing hM4D(Gi) via bilateral stereotaxic injections.

A group of mice (n=14) was required to learn the vPL task two weeks after hM4D(Gi) injection, an inhibitory DREADD. Once the 80% criterion was achieved, some mice were subjected to the incremental phase of the discrimination task 30 minutes after i.p. administration of CNO (n=7, CNO mice), while other mice were tested on the same task 30 minutes after i.p. administration of saline (n=7, SAL mice). The performance plateau reached by CNO mice (0.185 ± 0.033 c/deg) was significantly different from that achieved by SAL mice (0.04 ± 0.005), with the former group of animals displaying a robust learning impairment throughout the vPL task (Two-way RM ANOVA on ranks, Holm-Sidak method, $p < 0.001$, Fig.12a). The perceptual learning performance of CNO mice, but not of SAL mice, was consistently different from the performance displayed by naïve animals subjected to the same vPL task (Two-way RM ANOVA on ranks, Holm-Sidak method, vPL mice vs CNO mice: $p < 0.001$; vPL mice vs SAL mice $p = 0.535$; SAL mice vs CNO mice: $p < 0.001$; Fig. 12b); with CNO mice reaching a significantly higher MDT comparted to SAL and vPL mice (One way ANOVA on ranks, Dunn's method, $p < 0.05$). Notably, no differences could be found between mice that were administered with CNO (future CNO mice) or saline (future SAL mice) during the learning phase of the discrimination task (Two-way RM ANOVA, Holm-Sidak method, $F=0.158$, $DF=1$, $p = 0.696$, Fig13a).

Lastly, to rule out the possibility that the observed impairment could be ascribed to a learning deficit due to the surgical procedure, the learning capabilities of injected mice (n=14) were compared to those of naïve mice (n=14). No difference could be found between these two

groups of mice over the course of the learning phase of the vPL task (Two-way RM ANOVA, Holm-Sidak method, F=0.997, DF=1, p = 0.324, Fig. 13b).

Taken together, these results show that interfering with the neurophysiological activity of LM leads to a marked impairment in vPL abilities.

4.6 LM inhibition causes perceptual learning but not visual impairments

To rule out the possibility that the deficit observed in CNO mice was due to a visual impairment caused by interrupting the interaction between LM and the primary visual cortex, visual acuity (VA) was measured while inactivating LM. VA was assessed through the Prusky water maze test, employing the exact same apparatus used for the vPL task.

A separated group of mice (n=8) was required to distinguish sinusoidal gratings from a homogeneous grey two weeks after the bilateral injection of hM4D(Gi). Mice were first trained with a low SF and then tested for their capability to distinguish higher SFs 30 minutes after i.p. administration of saline. VA was calculated as the SF corresponding to 70% of correct choices on the sigmoidal function fitting the psychometric function. At the end of this first part of the procedure, the averaged VA was of 0.514 ± 0.005 c/deg. Then, mice were retested in the water task 30 minutes after i.p. administration of CNO, starting from the last achieved SF (the last SF at which the animal performance fulfilled the criterion of at least 70% of correct choices). The VA measured in the same group of mice under this new experimental condition was of 0.516 ± 0.005 c/deg. No VA differences could therefore be found in mice required to perform the exact same visual task when suppressing LM activity (paired t-test, DF=7, t =-0.678, p = 0.519, Fig.14). Thus, this result suggests that the chemogenetic inhibition of LM activity has no impact on visual abilities.

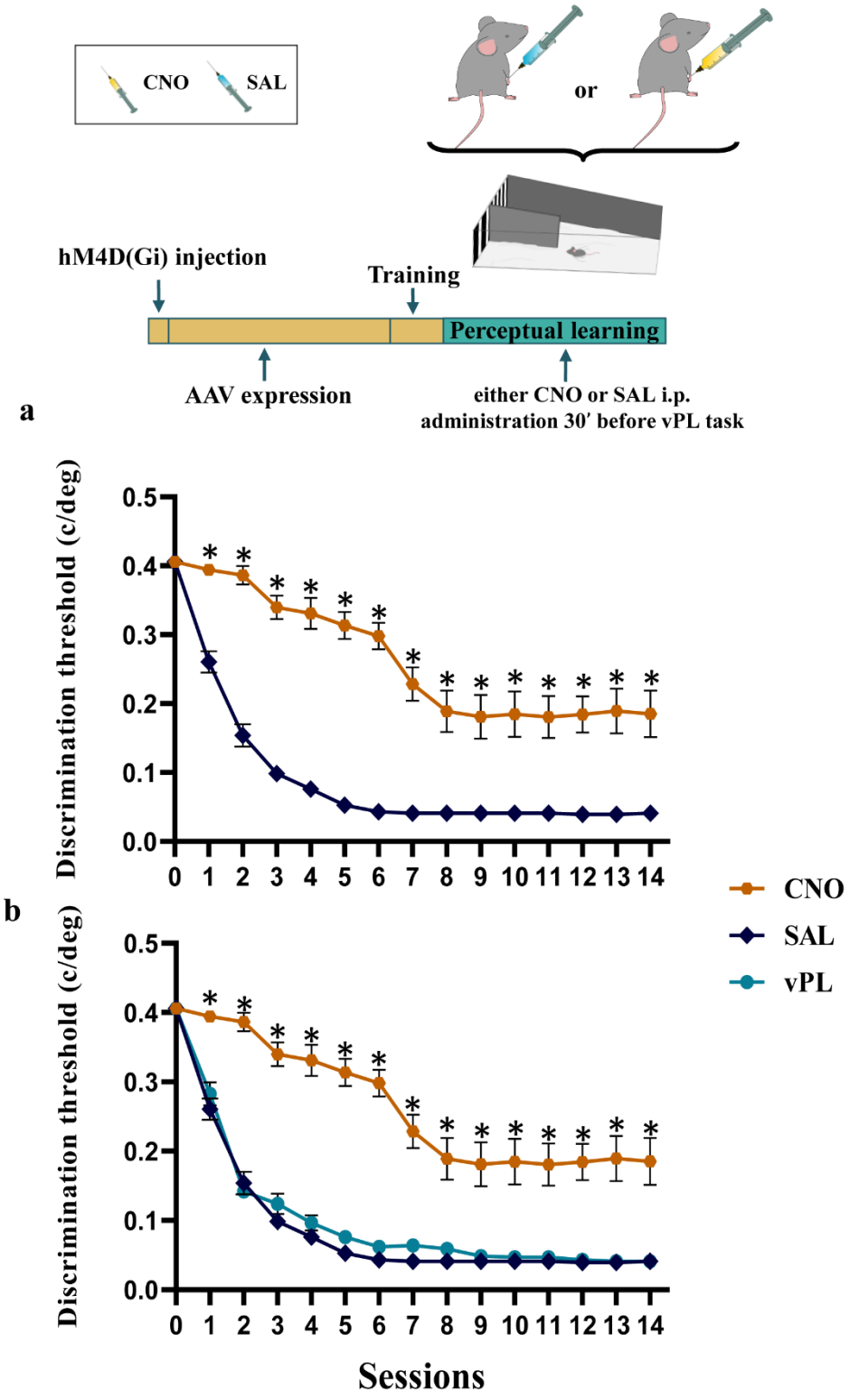

Figure 12. vPL and LM inhibition. A group of injected mice was subjected to the vPL task 30 minutes after the i.p. administration of either CNO (CNO mice) or saline (SAL mice) before each session. **a)** CNO mice showed a strong vPL impairment during the perceptual task compared to SAL mice (Two-way RM ANOVA on ranks, Holm-Sidak method, $p < 0.001$). **b)** Compared to vPL and SAL mice, the perceptual performance of CNO mice was altered throughout the vPL task (Two-way RM ANOVA on ranks, Holm-Sidak method, $p < 0.001$). CNO mice indeed displayed a different vPL progression and achieved a higher MDT. *Statistical significance. Error bars, s.e.m.

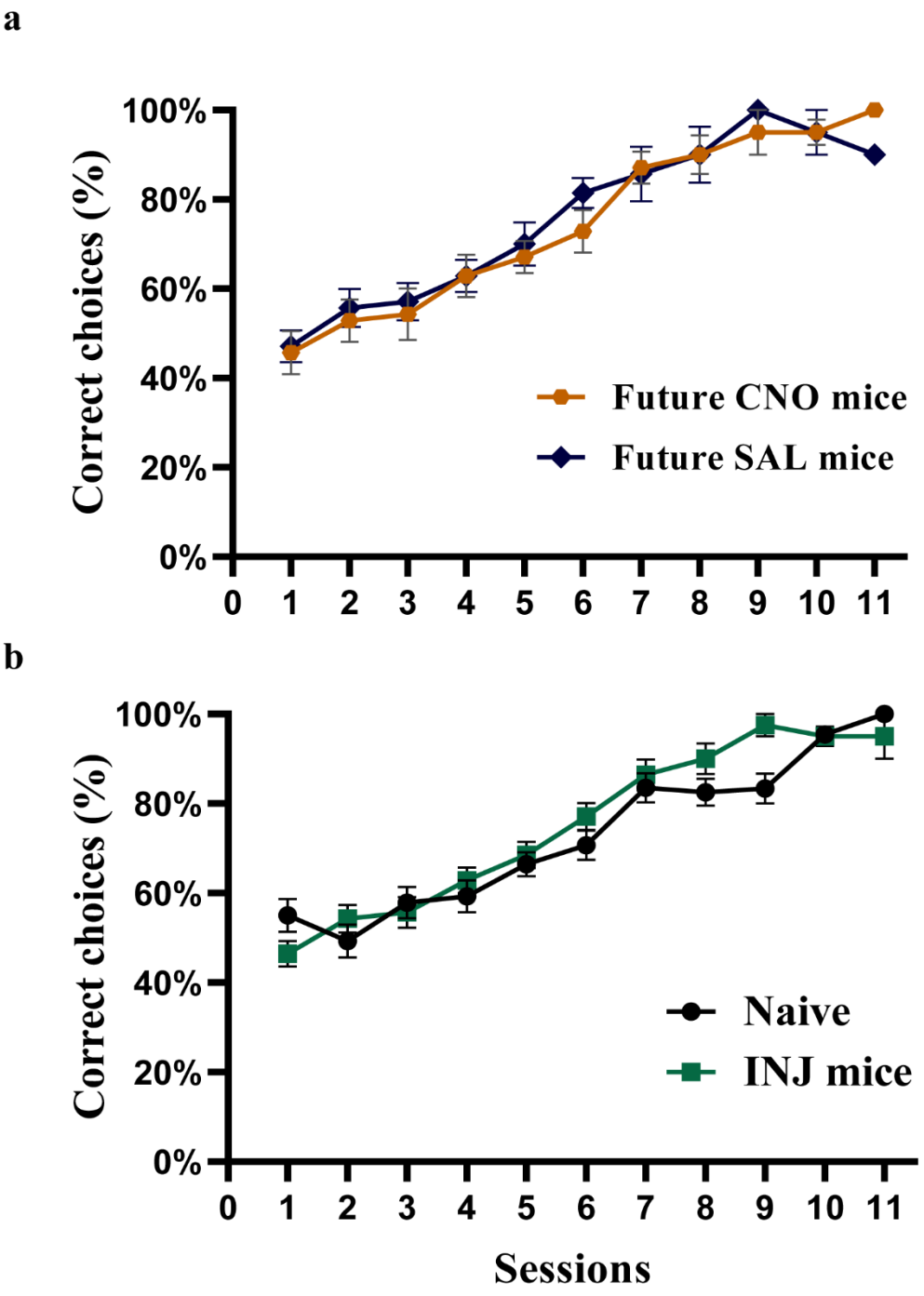

Figure 13. Learning ability in injected mice. a) The discrimination abilities of CNO and SAL mice were different during the incremental phase of the vPL task, but not during the training phase (Two-way RM ANOVA, Holm-Sidak method, p = 0.696. **b)** To evaluate the effects of the surgical procedure on learning capability, the learning performance during the training phase of injected mice (n=14, CNO and SAL mice pulled together) was compared to that of naïve mice (n=14, vPL and FS mice pulled together). No difference could be found between these two groups of animals (Two-way RM ANOVA, Holm-Sidak method, p=0.324). *Statistical significance. Error bars, s.e.m.

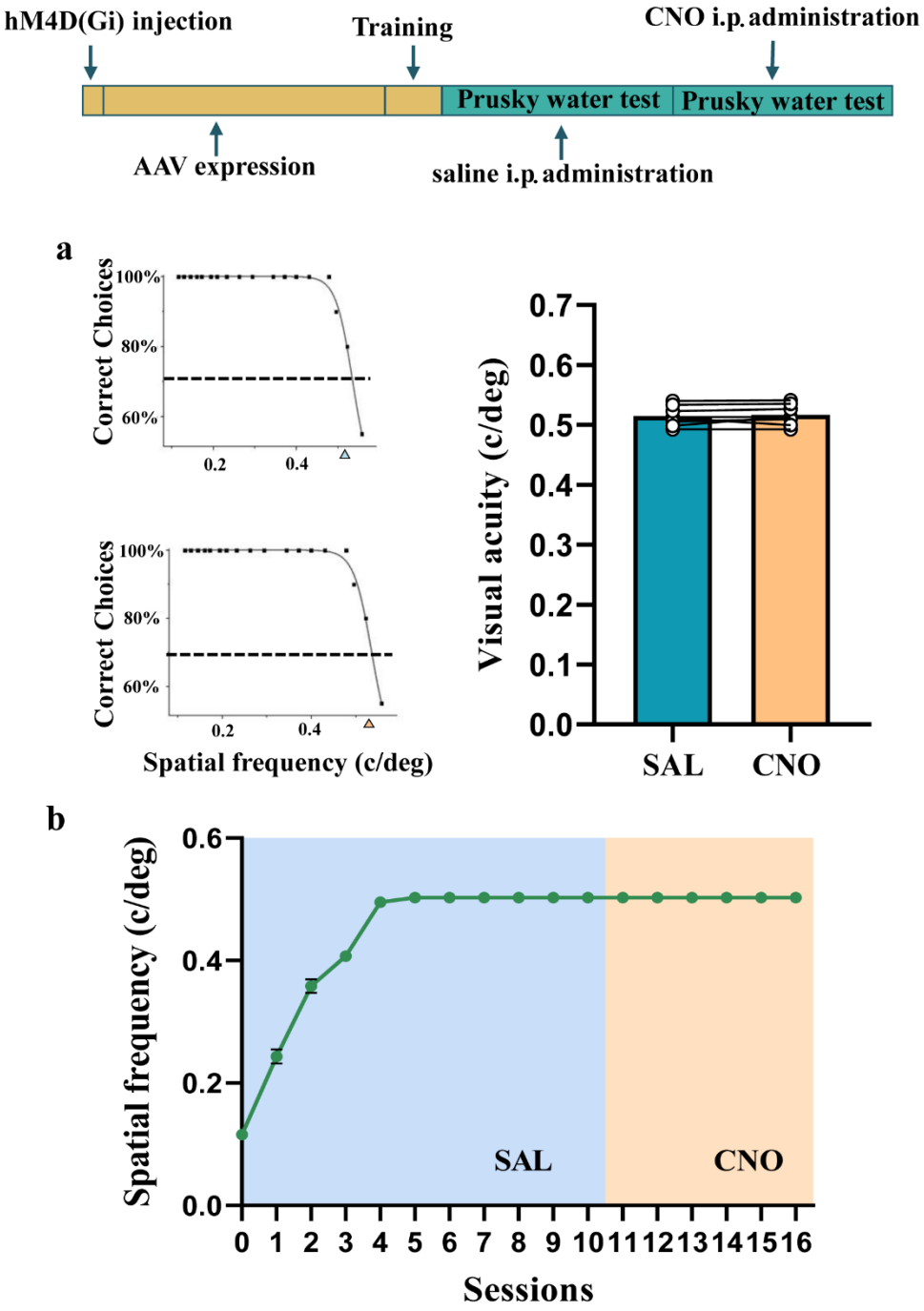

Figure 14. Assessment of visual acuity in a group of injected mice. Visual acuity (VA) was measured in a group of injected mice to evaluate whether the LM inhibition caused visual impairment. **a)** VA was measured using the water maze test. Example of sigmoidal extrapolations of psychometric curves used to calculate VA are reported on the left panel. No significant differences could be found in the VAs measured before and after the chemogenetic inhibition of LM (paired t-test, p = 0.519). **b)** Prusky water task progression during the i.p. administration of saline (light blue panel) or CNO (orange panel). *Statistical significance. Error bars, s.e.m.

4.7 LM is required for the maintenance of perceptual improvements

To get further insight into the role of LM in vPL, CNO and SAL mice were subjected to an experiment of administration shift (AS). After they reached their perceptual plateau, CNO mice were subjected to i.p. administration of saline before additional perceptual practice (CNO_SAL mice), and vice-versa, SAL mice were subjected to i.p. administration of CNO before additional perceptual practice (SAL_CNO mice) (Fig.15). CNO_SAL and SAL_CNO mice were both asked to perform additional vPL training 30 minutes after the administration of the new treatment. When LM was released from chemogenetic inhibition, CNO_SAL mice displayed a progressive improvement in the vPL task (Fig. 16a and 18a). Immediately after the AS, the DT of CNO_SAL mice was not different then the last DT reached after the last i.p. administration of CNO (paired t-test, DF=6, t = 2.071, p = 0.084, Fig. 16b); afterwards, CNO_SAL mice reached a new MDT (0.047 ± 0.003 c/deg) that was significantly lower than the MDT reached before the AS (paired t-test, DF=6, t = 4.046, p = 0.007, Fig. 16c) and no longer different with respect to that achieved by vPL and SAL mice (One-way ANOVA, DF=2, F=0.523, p = 0.601). On the other hand, SAL_CNO mice were totally unable to perform the vPL task for two consecutive sessions when LM activity was suppressed by the AS (gray bars, Fig. 17a and 18b). SAL_CNO mice were able to only discriminate a difference between the SFs of the two gratings much higher than that recorded before the AS (0.406 ± 0.0 c/deg, paired-t test, DF=6, t = -78.849, p < 0.001, Fig. 17b). When additional vPL practice was applied starting from this point, SAL_CNO mice reached a new MDT (0.323±0.029 c/deg) (Fig.17a, 18b). This MDT was significantly higher than the MDT achieved before the AS (paired-t test on ranks, p = 0.016, Fig.17c) and was no longer similar to the MDT achieved by vPL mice (t-test on ranks, p <0.001).

To sum up, the MDT of CNO but not that of SAL mice was significantly different compared to the MDT of vPL before the AS (One-way ANOVA on ranks, Dunn's Method, $p < 0.001$, Fig. 19b); after the AS, instead, the MDT of SAL_CNO but not that of CNO_SAL mice was significantly different compared to the MDT of vPL (One-way ANOVA on ranks, Dunn's Method, $p < 0.001$, Fig. 19c). Notably, the MDT achieved by SAL_CNO mice was also significantly higher than the MDT achieved by CNO mice (0.185 ± 0.033 c/deg) (t-test on ranks, $p = 0.026$, Fig. 19d). This last result strongly suggests that major perceptual impairments can be elicited when LM activity is impaired after vPL practice (Fig. 19a).

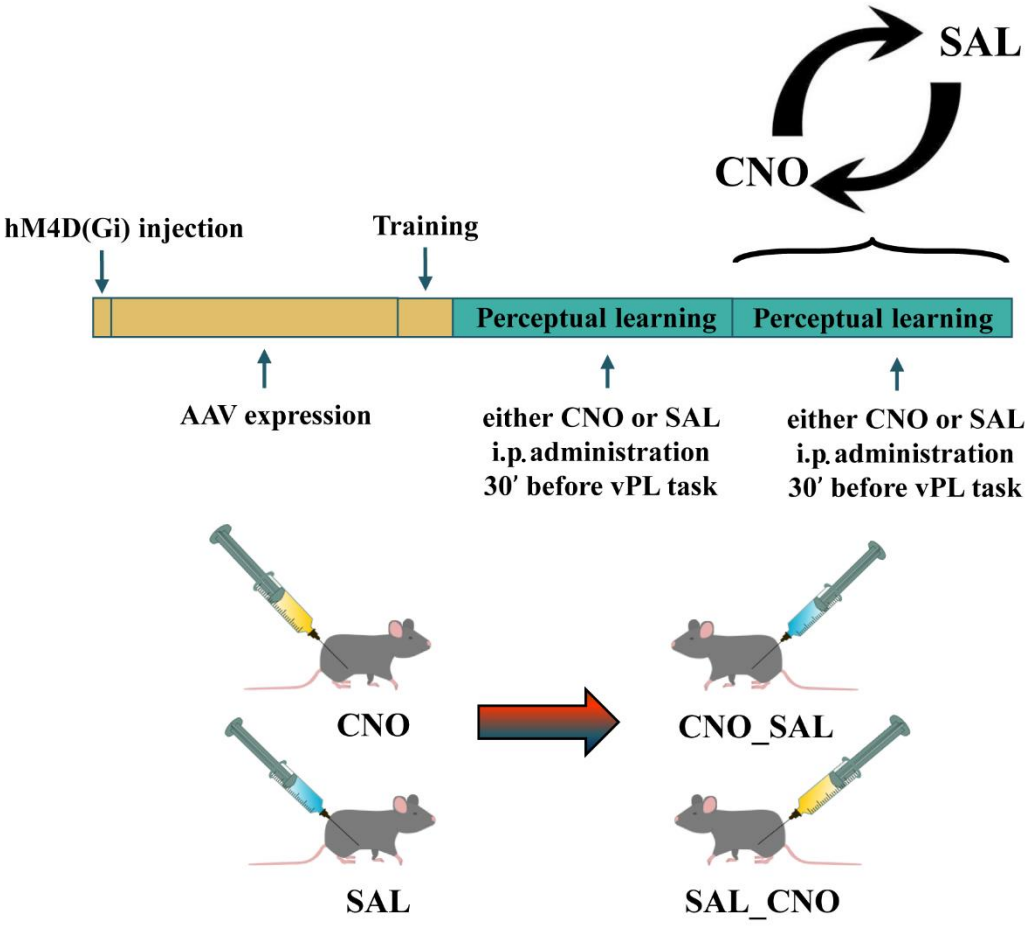

Figure 15. Administration shift. Schematic representation of the administration shift procedure.

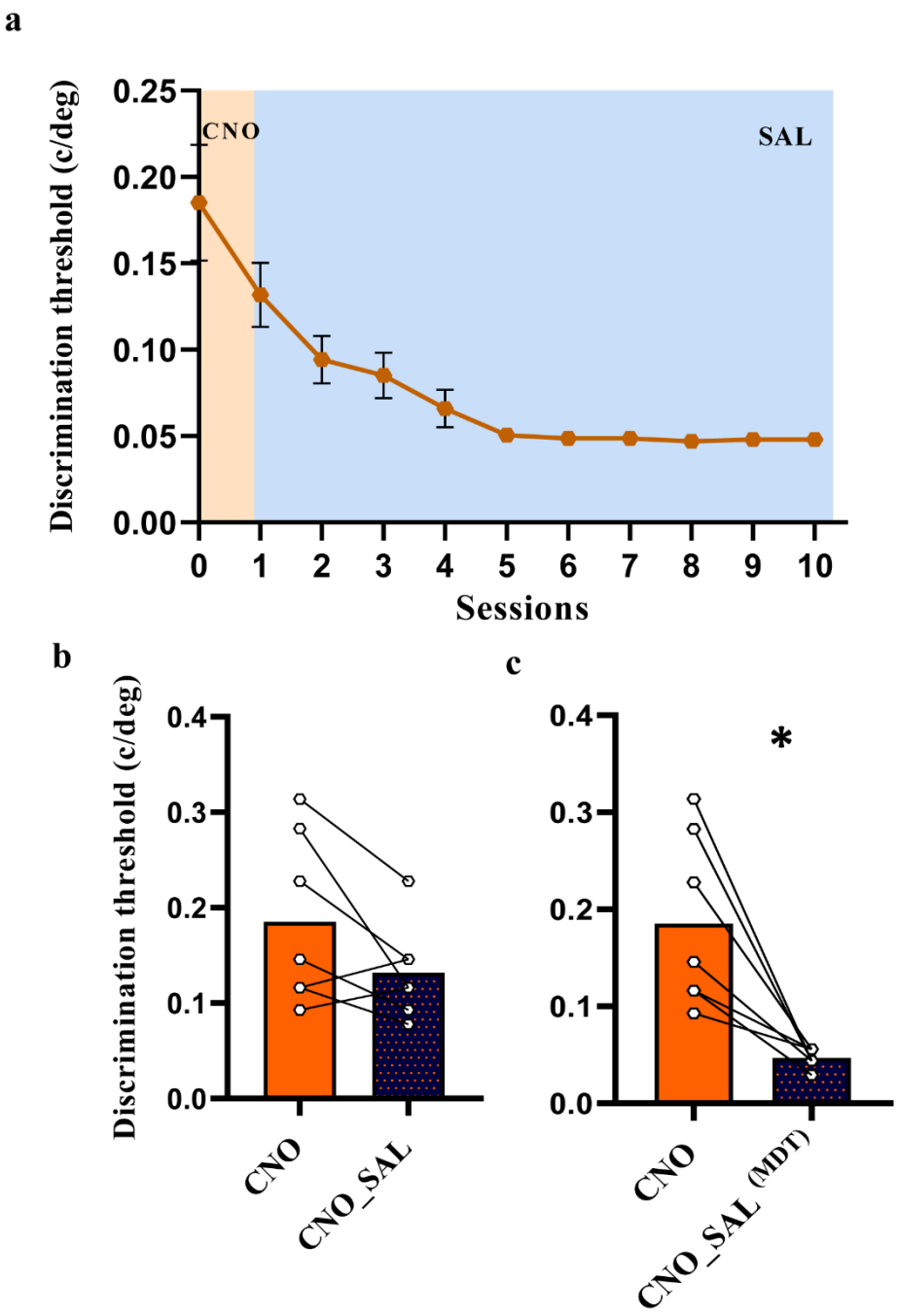

Figure 16. Administration shift in CNO mice. a) When LM was released from the chemogenetic inhibition, the group of former CNO mice (now CNO_SAL mice) underwent a progressive vPL improvement. **b)** Immediately after the administration shift no statistically significant changes could be detected in the performance of CNO_SAL mice (paired t-test, p = 0.084). **c)** When additional vPL practice was applied, CNO_SAL mice showed a significant improvement in the achieved MDT (paired t-test, p = 0.007). *Statistical significance. Error bars, s.e.m.

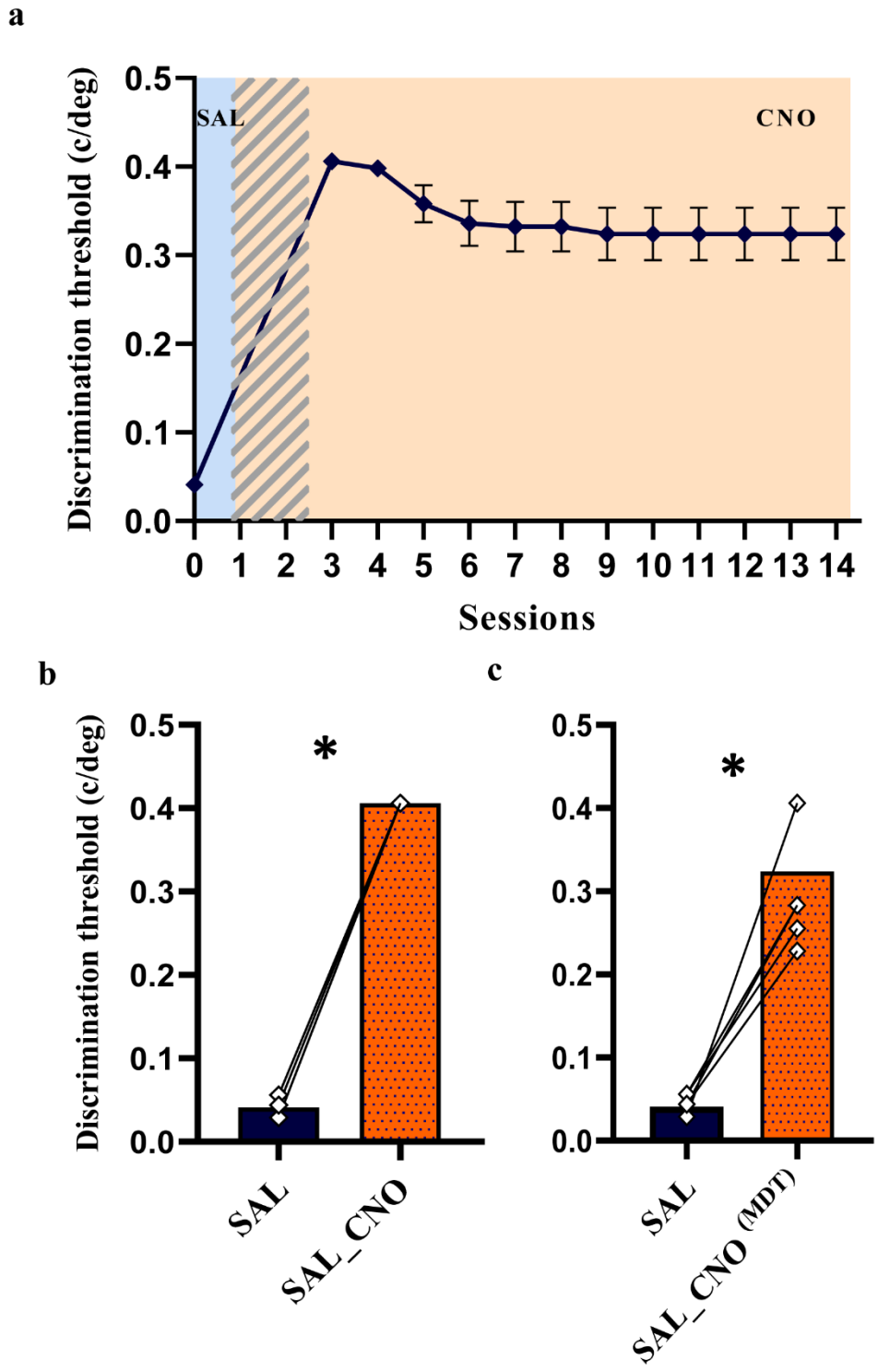

Figure 17. Administration shift in SAL mice. a) Marked perceptual impairment was detected in the vPL performance in the group of former SAL mice (now SAL_CNO mice) when LM was inhibited, with mice being completely unable to perform the vPL task for two consecutive sessions. **b)** After the administration shift, SAL_CNO mice could only discriminate a significantly higher DT (paired-t test, $p < 0.001$) (**c)** When additional vPL practice was applied, CNO_SAL mice achieved a new MDT that was still higher than the MDT measured during saline administration (paired-t test, $p=0.016$). *Statistical significance. Error bars, s.e.m.

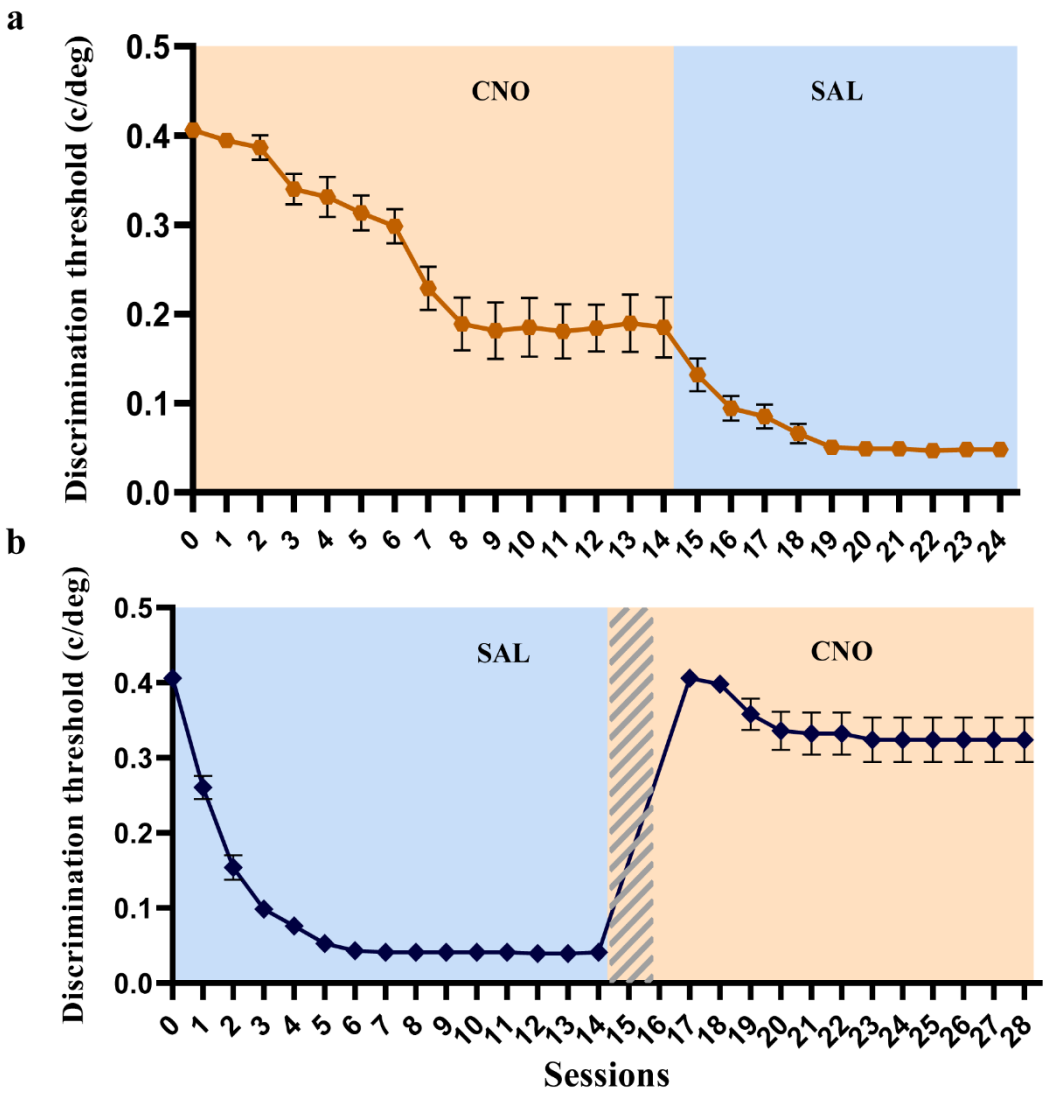

Figure 18. Roles of LM in vPL. a) The neurophysiological activity of LM is required for the proper acquisition of perceptual improvements. **b)** LM is also required after the emergence of perceptual improvement for a proper vPL execution. These two results suggest that LM is required for both the acquisition and maintenance of vPL improvements.

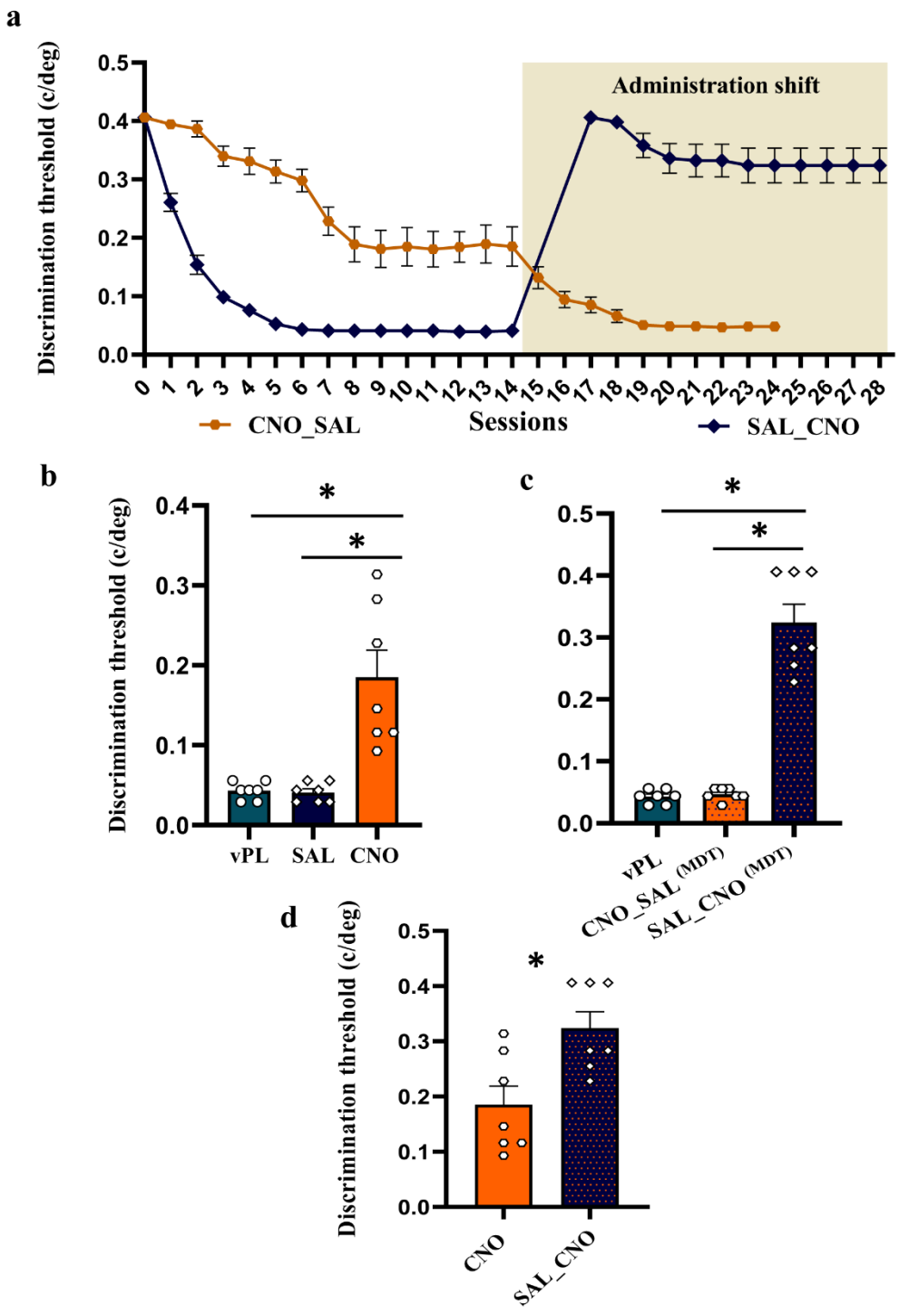

Figure 19. LM inhibition and perceptual impairments. a) vPL learning curves in CNO and SAL mice before and after the administration shift. **b)** Before the administration shift, the MDT of CNO mice was different compared to those of vPL and SAL mice (One-way ANOVA on ranks, Dunn's Method, $p < 0.001$). **c)** After the administration shift, the MDT of SAL_CNO mice was different compared to those of vPL and CNO_SAL mice (One-way ANOVA on ranks, Dunn's Method, $p < 0.001$). **d)** Surprisingly, the MDT achieved by SAL_CNO mice was significantly higher than that achieved by CNO mice (t-test, $p = 0.026$), suggesting that major perceptual impairments can be elicited interfering with LM activity after the acquisition of perceptual improvements. *Statistical significance. Error bars, s.e.m.

Discussion

Recent evidence supports the notion that HVAs are crucially involved in visual perception (Goldbach et al., 2021; Jin and Glickfeld, 2020). In the contest of vPL research, a previous study has shown that, in monkeys, when top-down projections are non-selectively inactivated by inhaled anesthesia the perceptual improvements emerged from vPL practice are severely compromised, a result that clearly suggests the involvement of higher area computations in this implicit form of learning (Li et al., 2008). Nonetheless, a direct experimental analysis probing the role of top-down processing in vPL was still missing. Indeed, the use of anesthesia does not allow a rigorous spatial control of top-down connections or chronic interference with higher-order computations throughout the vPL task. Here, I investigated the involvement of top-down processing in perceptual learning by directly manipulating the neurophysiological activity of the higher-order area LM over the course of a simple discrimination vPL task employing the mouse as experimental model.

With the aim of providing an answer to this open issue, I studied vPL in a mouse model, adapting a visual discrimination task that has been previously described (Fiorentini and Berardi, 1980; Sale et al., 2011) and extensively studied in humans and rats (Baroncelli et al., 2012; Bonaccorsi et al., 2014; Consorti et al., 2022; Fiorentini and Berardi, 1981, 1980; Sale et al., 2011). I showed that a robust and reliable vPL can be induced in the visual cortex of adult mice, with all trained animals reaching a steady perceptual plateau after few days of practice. The observed mean MDT achieved by trained mice was higher than the perceptual threshold previously measured in Long Evans rats (Sale et al., 2011), likely reflecting the differences in term of visual abilities between these two species of rodents (Prusky et al., 2000).

I demonstrated that, in adult mice trained with simple sinusoidal gratings, the visual perceptual improvements acquired as a result of practice with vertical gratings do not transfer to horizontal stimuli. Consistently, previous studies have already proven that perceptual improvements in visual discrimination return to baseline levels when the stimulus parameters used during vPL practice were altered (Ball and Sekuler, 1987; Fahle et al., 1995; Fahle and Edelman, 1993; Fiorentini and Berardi, 1981; Sale et al., 2011). It has been indeed shown that the effects of practice in discriminating the SF difference between simple sinusoidal gratings do not transfer when the visual stimuli are rotated by 90° (Fiorentini and Berardi, 1981; Sale et al., 2011), but only when the sinusoidal gratings are rotated by an angle of less than 45° from the orientation used in vPL trials (Fiorentini and Berardi, 1981). I also demonstrated that transfer of learning to orthogonal stimuli could instead be observed in mice trained with an easy version of the employed vPL task (i.e., animals were required to only distinguish between the two visual stimuli used for the training phase (first-step training)). The obtained results provide support to the hypothesis that vPL specificity depends on the complexity of the perceptual task (Ahissar and Hochstein, 1997). In humans, it has been consistently shown that with easy task conditions, learning generalizes across orientation and retinal position, but as task difficulty increases, vPL becomes more specific with respect to both orientation and position (Ahissar and Hochstein, 1997).

The perceptual improvements in distinguishing visual gratings of progressively close spatial frequencies have been shown to rely on long-term potentiation of synaptic efficacy within the primary visual cortex (Sale et al., 2011). The notion of a primary visual cortex crucially involved in this vPL task is also supported by the behavioral evidence that vPL effects do not transfer to orthogonal stimuli. However, the emergence of local plastic rearrangements inside the circuitry of the primary visual cortex might be driven by, or might at least require, the activity of higher stages in visual cortical processing. Indeed, in order to solve the vPL task

inside the water tank, trained mice most probably need to rely on an integrated information that is elaborated from the neuronal computations occurring in higher-order visual stages, in which information concerning the context and reward components of the vPL task can be computed. In particular, trained mice probably need to retrieve cortical representations of prior information regarding the vPL task, a type of information that is encoded in higher stage circuitries (Glickfeld and Olsen, 2017).

To test this hypothesis, I interfered with LM activity by an exogenous supply of CNO before each vPL session. Trained mice showed substantial perceptual learning impairments throughout the vPL task. Indeed, when LM activity was functionally severed from the primary visual cortex, mice displayed a slower progression in the discrimination task and a significant deficit in the achieved MDT compared to controls with functional LM. Notably, the perceptual impairments can be completely reversed by releasing LM from the chemogenetic inhibition. These results strongly suggest that LM plays a fundamental role in the acquisition of vPL.

In a separate group of mice, I interfered with LM activity when the vPL task was already completed. I showed that major perceptual learning impairments can be induced manipulating the LM activity at the end of the vPL task, with all trained mice being able to only distinguish the difference in spatial frequencies of the stimuli previously used in the training trails. Even when additional vPL practice was applied, the previously detected perceptual improvements in terms of discrimination threshold could no longer be restored. These results indicate that LM exerts a key role also in the maintenance of vPL.

It is worth noticing that the observed impairments induced by blockade of LM activity are likely not dependent on visual deficits. Indeed, I proved that treated mice were perfectly capable of discriminating a grating of progressively high SF from a gray stimulus when the LM activity was remotely inhibited, with no effect on visual acuity. Notably, the obtained values of

visual acuity were in line with previous measurements of naïve mice engaged in the same behavioral task (Prusky et al., 2000). This evidence strongly suggests that the activity of the primary visual cortex was not significantly affected by the cortical spreading of the inhibitory DREADD, despite the close proximity of this area to LM borders. The unfeasibility of precisely constrain the AAV spatial expression is indeed notoriously considered the major flaw of the chemogenetic technique.

The mouse primary visual cortex is surrounded by at least nine retinotopically organized HVAs. I decided to target the chemogenetic inhibition to LM activity based on previously obtained anatomical and physiological results. Primarily, the great majority of feedback projections enter the primary visual cortex sprouting from LM (Morimoto et al., 2021). Thus, the neuronal computations elaborated from this specific HVA could represent the major source of top-down information able to drive the activity of primary visual neurons. Additionally, LM is currently considered the gateway of the mouse ventral stream (Wang et al., 2011). The ventral stream represents a highly interconnected network of four lateral HVAs whose projections find their prime target in a cluster of ventral structures implicated in learning and memory processes (Wang et al., 2012). Previous studies have shown that anatomical lesions to visual areas belonging to this pathway can severely impair perception and object identification (Tees, 1999; Winters et al., 2004; Winters and Reid, 2010). Lastly, anatomical evidence suggests that LM is the homologous of the primate V2 in the mouse visual system (Wang and Burkhalter, 2007). As mouse homologous of V2, LM receives the large majority of projections from the primary visual cortex (Wang et al., 2012) and might therefore represents the first higher cortical stage in which visual associations start to emerge.

Even though I targeted the chemogenetic inhibition on this visual area, the possibility that other HVAs could have a role in vPL cannot be excluded and certainly deserve future

investigation. In particular, I cannot exclude that similar results might be obtained interfering with the physiological activity of other visual areas along the ventral streams. For example, several studies have demonstrated that the postrhinal cortex (POR) is required for contextual scene representation (Furtak et al., 2012), encodes the association between visual cue and reward (Burgess et al., 2016) and the internal representation linking objects to places (Furtak et al., 2012). To solve the task, trained mice have to orient their movement and navigate inside the arena toward the reference grating in order to reach the hidden platform completing a task trial. Therefore, a substantial role of dorsal stream areas for the correct execution of this task could not be discarded. On the other hand, it is equally plausible that LM might act as a central hub in the visual network with the great majority of information that are integrated by different HVAs streaming toward the primary visual cortex via the LM projections. LM is indeed the first HVA in terms of reciprocal interconnections with the primary visual cortex (Wang et al., 2012).

Further experiments are also needed to clarify how LM can modulate neuronal firing within the primary visual cortex in response to vPL. According to anatomical studies, feedback projections are mostly excitatory by nature (Gonchar and Burkhalter, 2003, 1999). Therefore, it is possible that the plastic changes occurring inside the primary visual cortex emerge under the influence of top-down inputs that directly potentiate the response of selected primary visual neurons probably engaging the horizontal connections (Gilbert et al., 2001; Gilbert and Li, 2013).

Recent studies support the notion of a not negligible and anatomically counter-intuitive role of the inhibitory system in top-down integration (Bastos et al., 2012; Nurminen et al., 2018; Vangeneugden et al., 2019). A prime role of the GABAergic system in vPL finds also support in biochemical assays showing a substantial reduction in the inhibitory tone after vPL

(Baroncelli et al., 2012). Specific GABAergic interneurons are targeted by feedback projections from LM (Gonchar and Burkhalter, 2003). However, the potential implication of given inhibitory interneurons in top-down processing remains to be clarified. The main GABAergic target of feedback projections is the class of parvalbumin (PV) interneurons, which constitute the largest proportion of inhibitory neurons (Tremblay et al., 2016; van Versendaal and Levelt, 2016). Most PV neurons have wide dendritic tree and predominantly innervate pyramidal neurons providing the main source of intracortical somata inhibition (Somogyi et al., 1983). PV interneurons are known for the precise control that they can exert on the spike timing of neighboring excitatory neurons (Pouille and Scanziani, 2001; Wehr and Zador, 2003). Therefore, this class of interneurons might be recruited during vPL to regulate the firing of the specific cluster of pyramidal neurons trained during the perceptual task, improving the evoked response of selected excitatory neurons within the electrical noise of the primary visual cortex. However, some evidence suggests that the synapses between feedback projections and PV neurons are small and located on thin dendrites, at least in the rodent visual cortex (Gonchar and Burkhalter, 1999; Yamashita et al., 2003). The excitation provided by feedback projection onto PV neurons might therefore be insufficient to precisely drive the activity of primary visual neurons in response to top-down information (Shao and Burkhalter, 1999). Feedback projections are also strongly connected with inhibitory cells located in the superficial layers of the primary visual cortex. Thus, the potential involvement of GABAergic neurons that are most numerous in these cortical regions, like vasoactive intestinal peptide (VIP) interneurons, cannot be excluded. This hypothesis could be in line with the emerging notion that sees the superficial cortical layers crucially involved in mediating the integration of top-down inputs (Ibrahim et al., 2021; Schuman et al., 2021). Differently from PV cells, VIP interneurons are typically bipolar and are specialized in inhibiting other interneuron subtypes (Tremblay et al., 2016; van Versendaal and Levelt, 2016). In this scenario, top-down integration might be mediated by

specific rearrangements within the GABAergic circuitry that eventually regulate the activity of selected pyramidal neurons. However, an experiment of selective chemogenetic inhibition targeting the main classes of GABAergic neurons should be afforded to dissect the role of specific inhibitory neurons in the top-down control of vPL.

The results obtained in this thesis suggest that that vPL deficits might be attributed to functional deficits of the top-down connections between LM and the primary visual cortex. Even though primary visual neurons are critically engaged in perceptual learning as proven by the high specificity for the stimulus orientation observed in vPL trained mice, LM processing is indeed required for the proper execution of the vPL task and is likewise necessary to retain the perceptual improvements acquired as a result of vPL practice.

I showed that LM, the first visual area along the ventral stream, guides the brain response to perceptual practice, providing the first direct evidence of the substantial involvement of a HVA in vPL. Further efforts are needed to unravel the role that other HVAs could have in this learning process. In particular, subsequent studies should address whether HVAs belonging to the ventral or dorsal stream modulate different features of the perceptual performance in this vPL task.

Much remains to be clarified about the nature of the interactions between top-down inputs and lower-stage circuitries that lead to perceptual improvements. It is worth noticing that top-down excitatory and inhibitory drives are not mutually exclusive processes and they could both concur to precisely regulate the signal to noise ratio within the primary visual cortex, ultimately leading to these improvements in perceptual discrimination. The demonstration that top-down projections from LM play a necessary role in the learning process which leads to perceptual improvement is an important step towards unraveling the neural networks responsible for the neural underpinnings of vPL.

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Appendix A:

vPL induces long-lasting recovery of visual acuity, visual depth perception abilities and binocular matching in adult amblyopic rats

In recent years, there has been significant processes in applying vPL-based treatments to improve general human conditions (Deveau et al., 2013). In older subjects, PL can be used to improve general perceptual performance or to counteract vision reduction due to age-related macular degeneration. Consistently, five days of training on feature search task can elicit long-lasting visual improvements in subjects with severe to profound low visual impairments spanning from glaucoma to retinitis pigmentosa (Liu et al., 2007).

Known that PL impacts on neuronal plasticity potentiating those cortical connections that are active during practice, this implicit form of learning has been exploited to promote recovery from neuropathological conditions correlated to defective brain connectivity. A number of vPL-based treatments can indeed lead to better visual outcomes in individuals affected by cortical blindness following tumors, stroke, trauma, or infections (Deveau et al., 2013). In vision research, the potential therapeutic application of vPL has been extensively investigated in amblyopic subjects (Bonaccorsi et al., 2014). Amblyopia is the prevalent monocular impairment with an incidence of 1–5% in the world human population (Holmes and Clarke, 2006). A central physiological mechanism in amblyopia is considered the inter-ocular suppression, which is the empowering of the inputs coming from the apparently healthy eye at the expense of those coming from the amblyopic eye (Antonini et al., 1999). This suppression is evident at the level of the primary visual cortex and appears to be mediated by the inhibitory circuitry (Sengpiel et al., 2005). Amblyopia, if not promptly treated during childhood, leads to severe visual impairments including loss of visual acuity and defective stereopsis. Occlusion

therapy, i.e., the temporary exclusion of the healthy eye from visual activity by means of an eye patch, completely reverses amblyopia when performed during the critical period for binocular vision (Loudon and Simonsz, 2005). Nevertheless, amblyopia is an almost untreatable disease in adulthood. The occlusion therapy is indeed completely ineffective in adult subjects due to the dramatic decline in cortical plasticity caused by the maturation of different plasticity-limiting factors as the GABAergic and the cholinergic systems (Hensch et al., 1998; Morishita et al., 2010). Remarkably, engagement in subtle visual discrimination tasks such as those associated with vPL can promote visual function recovery in adult amblyopic subjects (Ding et al., 2006; Huang et al., 2008; Levi et al., 1997; Levi and Li, 2009; Levi and Polat, 1996; Li et al., 2007; Polat et al., 2004; Zhou et al., 2006). In the same context, engagement in active videogames has been shown to promote plasticity in adult subjects with amblyopia. Nonetheless, this promising vPL protocol has apparently limited effects in children (Holmes et al., 2016; Kelly et al., 2016; Li et al., 2011).

Few studies have investigated the therapeutic effects of vPL in animal models of amblyopia. Amblyopia can indeed be artificially induced in animals depriving one eye of visual stimuli through a long-term lid suture. Notably, vPL practice with the amblyopic eye can promote recovery of visual functions in adult amblyopic rats. Despite the increasing evidence showing beneficial effects elicited by vPL practice, many open questions remain to be addressed. In particular, the experimental paradigms applied so far were based on a reverse occlusion approach in which the subjects are required to perform the vPL task with the amblyopic eye open and with the concomitant occlusion of the fellow eye (Baroncelli et al., 2012). Monocular approaches are not easily applicable to adult human subjects; moreover, in contrast to conventional occlusion therapies, binocular training procedures are particularly promising in adult amblyopic patients (Hess et al., 2014, 2010; Li et al., 2013). Additionally, very little is known about the effects of vPL on visual depth perception abilities. Finally, the

long-term maintenance of visual function recovery after the end of vPL, has not been examined so far.

Here, I analyzed these still unsolved issues in adult amblyopic rats with binocular viewing conditions.

Materials and Methods

Animal Treatment

The experiments were conducted on Long-Evans black hooded rats, in accordance with the approved guidelines and regulations of Italian Ministry of Public Health. Animals were housed in a room with a temperature of 21◦C and a 12-h light–dark cycle, with food and water available ad libitum.

Surgery and Experimental Procedures

Rats were anesthetized with zolazepam + tiletamine (Zoletil, 1 mg/kg) and monocular deprivation (MD) was performed through eyelid suture at postnatal day (P) 21. Lid margins were trimmed and sutured with 6-0 silk. A post-operative health check control was performed daily until complete cicatrization; subjects with spontaneous re-opening were excluded. At P70, the long-term deprived eye was re-opened under anesthesia using thin scissors. After reopening of the deprived eye, rats were divided in two groups according to the experimental protocol: vPL and control animals. A third group of naïve, age-matched animals was added as control group. In all conditions, the animals were maintained in standard housing conditions, consisting of 40 × 25 × 20 cm cages (three animals per cage).

vPL Task

I used a modified version of the visual water box task (Prusky et al., 2000; Sale et al., 2011). Animals were trained to distinguish a low spatial frequency grating (reference grating, 0.116 c/deg) from a higher spatial frequency grating (test grating, 0.592 c/deg). When the animals achieved a level of ≥80% of accuracy in at least three subsequent sessions (criterion level), the vPL protocol was started by gradually reducing the spatial frequency of the test grating. Thus, the test grating became progressively more similar to the reference grating. For each animal, a daily threshold was calculated as the average spatial frequency of the test grating that the rat was able to distinguish (at least 70% correct performance) from the reference grating within the different sessions. The vPL task ended when the animal performance reached a plateau (performance at a given spatial frequency of the test grating oscillating around 70% of correct choices for three consecutive days). A group of control animals (CTRL) was trained to distinguish the reference grating from a test grating whose spatial frequency was always maintained at the starting value of 0.592 c/deg (associative test). CTRL rats were matched to vPL animals in terms of overall swim time and training days in the water maze.

Assessment of Visual Functions

Visual Acuity

I used n = 7 vPL and n = 8 CTRL rats. I first measured visual acuity of the fellow eye (not deprived). Then, I measured visual acuity of the amblyopic eye (by temporary occlusion of the fellow eye) five times, i.e., immediately after eyelid reopening (at P70), immediately at the end of the perceptual learning or control task, and then at 30, 90, and 180 days past the end of the procedure. Visual acuity was measured using the visual water task (Prusky et al., 2000), which trains animals to first distinguish a low (0.1 c/deg) spatial frequency vertical grating from grey,

and then tests the limit of this ability at higher spatial frequencies. Once 80% accuracy was achieved, the limit of the discrimination was estimated by increasing the spatial frequency of the grating. Animals were trained 60 trials per day until the achievement of discrimination limit criteria (Prusky et al., 2000). Visual acuity was calculated as the spatial frequency corresponding to 70% of correct choices on the sigmoidal function fitting the psychometric function. During each session, the experimenter was blind to the experimental group.

Visual Depth Perception

To assess visual depth perception, I used n= 8 vPL and n= 6 CTRL rats. Visual depth perception was assessed as spontaneous exploration in the visual cliff apparatus, as previously described (Baroncelli et al., 2013; Sansevero et al., 2020, 2019). Briefly, the arena was divided into a shallow and a deep side. On the shallow side, a patterned floor was positioned immediately below the glass plate, while on the deep side the checked platform was moved to 29 cm below the glass plate. Each animal was placed on the shallow side, and the total time the rat spent exploring each side of the arena was automatically recorded by the Noldus EthoVision system. The trial ended after 5 minutes. The arena was cleaned between trials with an alcohol solution. An exploration index was calculated as follows: (ts-td)/ttot, where ts and td are, respectively, the time spent exploring the shallow side and the deep side of the arena, and ttot is the total time of the test procedure. Each animal was tested only once.

In vivo Electrophysiology

Electrophysiological recordings were performed as previously described (Baroncelli et al., 2016; Mazziotti et al., 2017; Sansevero et al., 2020). Rats were anesthetized with i.p. injection of urethane (1.4 g/ kg, i.p., 20% in saline; Sigma-Aldrich) and placed on a stereotaxic frame, with the body temperature maintained at 37°C. A craniotomy was performed over the binocular visual cortex (4.8–5.2 mm lateral to lambda) and the dura mater was removed. The two eyes

were fixed and kept open by means of adjustable metal rings surrounding the external portion of the eye bulb. An electrode (2×2-tet-3 mm-150–150-121-A16- 15, Neuronexus Technologies) was lowered into the cortex to record local field potentials and single-unit activity. Signals were acquired using a 16 channels Neuralynx device and data analysis was performed using a custom MATLAB software. Visual stimuli were generated in MATLAB using Psychophysics Toolbox extension and displayed, with gamma correction, on a monitor (Sony Trinitron G500, 60 Hz refresh rate, 32 cd m^{-2} mean luminance) placed 20 cm in front of the animal. For visual evoked potentials (VEPs), extracellular signal was filtered from 0.1 to 275 Hz and sampled at 20 kHz. VEPs in response to sinusoidal wave patterns with a spatial frequency of 0.03 c/deg and abrupt phase inversion (0.5 Hz temporal period), were evaluated in the time domain by measuring the peak-to-baseline amplitude and latency. VEPs were acquired using the responses coming only from the tetrode in the upright position inside the inserted probe (responses from the four contact points were averaged together), at 100 µm of depth first, and then at 400 µm, corresponding to layers I, III, and IV, respectively. Computer controlled mechanical shutters were used to alternatively close the two eyes. Visual acuity was obtained by extrapolation to zero amplitude of the linear regression through the data points in a curve where VEP amplitude was plotted against log spatial frequency. Ocular dominance was measured by calculating the contralateral to ipsilateral VEP ratio (C/I ratio), i.e., the ratio of VEP amplitudes recorded by stimulating the eye contralateral and ipsilateral, respectively, to the visual cortex where the recording is performed. Recordings were performed only from the visual cortex contralateral to the previously deprived eye (amblyopic eye). During recording through one eye, the other was occluded by an automatic black shutter. The C/I VEP ratios obtained at the two depths did not differ between each other and have been averaged together. For single-unit recordings, extracellular signal was filtered from 0.6 to 9 kHz and sampled at 30.3 kHz. Only cells with receptive fields within 20° from the vertical meridian were included in our sample. Spiking

events were detected online by voltage threshold crossing and waveforms of 1 ms were acquired around the time of threshold crossing. To improve single unit isolation, recordings from groups of four neighboring sites (tetrode) were linked, so that each spike was composed by four waveforms. Data were loaded on the Offline Sorter software (Plexon), and a principal component analysis was performed to score spikes with a high degree of similarity in a 3D feature space. Waveforms from each electrode of the tetrodes were processed together to improve isolation. Clusters were progressively defined using convex hulls and then recalculating principal component analysis. Quality of separation was determined based on the following criteria: (1) during manual clusterization with convex hulls, raw waveforms in the clusters were visually inspected to check the uniformity of single waveforms; (2) clusters contained < 0.1% of spikes within a 1.0 ms interspike interval; (3) auto- and cross-correlograms of the clusters were also inspected to reveal if the cluster contained more than a single unit or if several clusters contained spikes of the same unit; and (4) the peak amplitude of a unit remained stable over the entire recording session. Units were included in the sample for analysis of tuning properties when they had an average peak firing rate, across trials of the optimal stimulus for the dominant eye, of > 0.5 Hz. Drifting sinusoidal gratings were used as visual stimuli (1.5 s duration, temporal frequency of 2 Hz, 12 different orientations with a step of 30°, 3 spatial frequencies: 0.02, 0.04, 0.08 c/deg). Stimulation was repeated five times per eye, with stimulus conditions randomly interleaved, and two grey blank conditions (mean luminance) were included in all stimuli sets to estimate the spontaneous firing rate. The average spontaneous rate for each unit was calculated by averaging the rate over all blank condition presentations. Responses at each orientation and spatial frequency were calculated by averaging the spike rate during the 1.5 s presentation and subtracting the spontaneous rate. The preferred stimulus was determined finding the combination of spatial frequency and orientation that maximize the response, independently for each eye. Orientation tuning curves were constructed

for the spatial frequency that gave maximal response at this orientation. Given this fixed preferred orientation (OPref), the tuning curve was fitted as the sum of two Gaussians centered on OPref and OPref + π, of different amplitudes but equal width, with a constant baseline. From this fit, I calculated an orientation selectivity index (OSI) representing the ratio of the tuned versus untuned components of the response, and the width of the tuned component. OSI was calculated as follows: (respOPref - respOOrtho)/(respOPref + respOOrtho), where resp is the maximal response evoked by visual stimulation and OOrtho is the orientation orthogonal to the preferred one. Tuning width is the half-width at half-maximum of the principal gaussian. In addition, I also calculated a direction selectivity index (DSI), as follows: (respOPref-respOOppo)/(respPref + respOppo). The difference in preferred orientation between the two eyes (binocular matching, ΔO) was calculated by subtracting ipsilateral OPref from contralateral OPref along the 180° cycle.

Statistical Analyses

Statistical analysis was done using SigmaStat Software. Data were tested for normality before running statistical tests; parametric tests were run on normally distributed data and, in case normality test failed, non-parametric tests were performed as appropriate. Differences between two independent groups were assessed with a two-tailed t-test; differences between two dependent groups (e.g., visual acuity of the deprived and fellow eyes in the same animals) were assessed with a two-tailed paired t-test. One-way ANOVA, One-way RM ANOVA, and Two-way RM ANOVA, followed by Holm-Sidak multiple comparison procedure, were used to compare normally distributed data belonging to more groups. One-way ANOVA on ranks, followed by Dunn's method or Tukey test, were performed to compare not normally distributed data belonging to more than two groups. Level of significance was $p < 0.05$, unless otherwise

specified. The size of biological replicates is indicated by the n numbers in the various experimental sections.

Results

The effects of vPL on visual functions were investigating by assessing visual abilities in adult rats subjected to long-term monocular visual deprivation started at the peak of the CP for plasticity (Postnatal day 21, P21), and then exposed, after reopening of the previously deprived eye at 2 months of age, to a forced-choice vPL task performed in binocular sight conditions (vPL rats). Animals were required to distinguish between two vertical gratings differing only for their spatial frequency (Fig. A1a, b), with a fixed low spatial frequency reference grating (0.116 c/deg), and a higher spatial frequency test grating (0.592 c/deg). When the animals achieved a level of ≥80% of accuracy in at least three subsequent sessions (criterion level), the vPL protocol was started by gradually reducing the spatial frequency of the test grating (incremental phase). The performance of vPL rats was compared with that of control (CTRL) rats exposed to a simplified version of the same task, in which the incremental phase was lacking. vPL rats displayed a robust perceptual improvement, as evidenced by the progressive reduction in the perceived spatial frequency difference between the two gratings across the learning days (Fig. A1c, d) (One-way RM ANOVA on ranks $p < 0.001$).

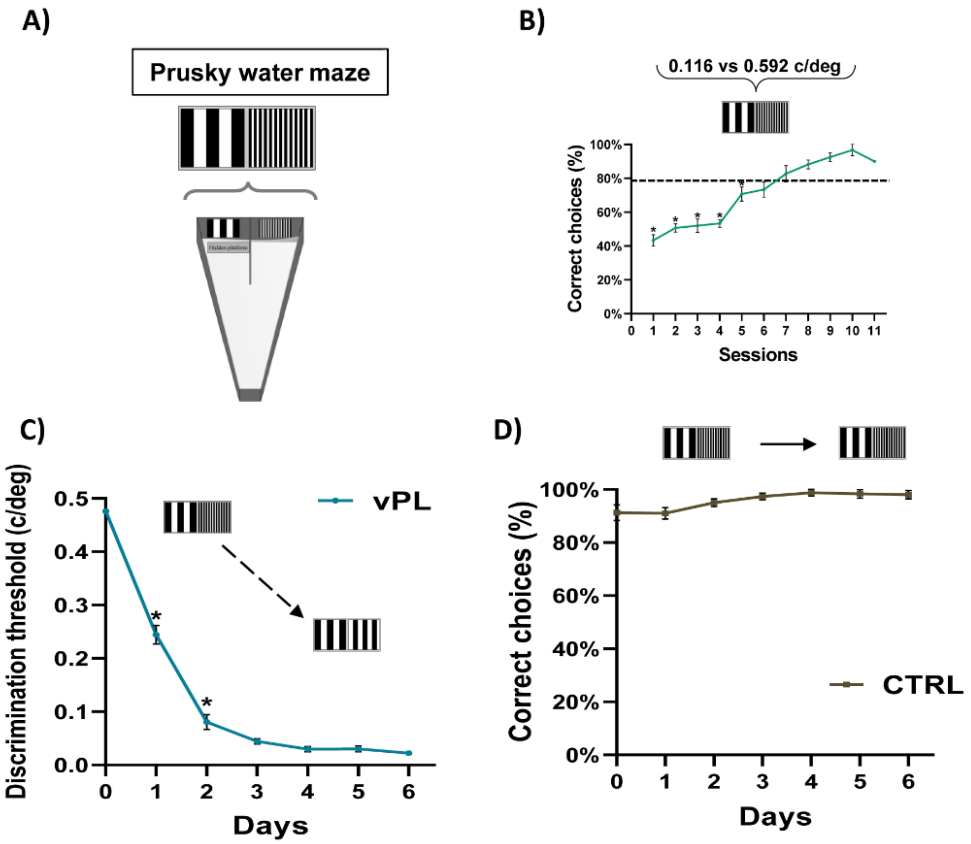

Figure A1. Visual perceptual learning in adult amblyopic rats. (**A**) A modified version of the visual water box task was used to perform vPL. (**B**) Mean performance in distinguishing a test grating of 0.592 c/deg from the standard grating (0.116 c/deg) across the training sessions. All animals (both vPL and CTRL rats) have been pulled together. The increase in the percentage of correct choices with sessions is significant (One-way RM ANOVA on ranks $p < 0.001$). (**C**) Improvement of discrimination threshold in vPL rats involved in the visual discrimination task. The threshold, calculated as the minimum spatial frequency difference between the reference and the test gratings discriminated, decreases significantly with the training days (One-way RM ANOVA on ranks $p < 0.001$). (**D**) The performance of CTRL animals involved in a discrimination task lacking the incremental component remained stable across the training days*Statistical significance. Error bars, s.e.m.

The impact of vPL on visual function recovery was initially assessed by measuring visual acuity (Fig. A2a), using the visual water box task (Prusky et al., 2000). Using this task longitudinally in the same individuals, I measured visual acuity through the previously deprived eye five times: immediately before treatment, immediately at the end of vPL, and at three additional follow-ups (1, 3, and 6 months past the end of the treatment). A complete visual acuity recovery was evident in vPL animals (n = 7) (amblyopic eye: 1.03 ± 0.06 c/deg; fellow eye: 1.00 ± 0.05 c/deg), but not in CTRL rats (n = 8; amblyopic eye: 0.66 ± 0.01 c/deg; fellow eye: 0.99 ± 0.02 c/deg) (Two-way RM ANOVA, treatment × time F = 78,526, DF = 1, Holm-Sidak method, p = 0. 297 for vPL rats, p < 0.001 for CTR rats) (Fig. A2a). Notably, visual acuity recovery was long-lasting being maintained at all the time points tested beyond the end of the vPL procedure (Two-way RM ANOVA on ranks, Holm-Sidak method, post-treatment vs. 1 month after treatment, p = 0.817; post-treatment vs. 3 months, p = 0.095; post-treatment vs. 6 months, p = 0.958) (Fig. A2a). After vPL, the visual acuity measured in the formerly amblyopic eye of vPL animals was not significantly different from that of an additional group of non-deprived and untreated rats (naïve animals), while both were significantly higher than visual acuity values measured in CTRL rats (naïve, 0.95 ± 0.03 c/deg; One-way ANOVA, F = 32.783, DF = 2, Holm-Sidak method, p = 0.143, p < 0.001, p < 0.001, respectively).

Visual depth perception abilities were then assessed using the visual cliff task, which exploits the spontaneous tendency of rodents to avoid the deep side of a visual cliff (Fig. A2b). Discrimination between the deep and the shallow side of the arena requires an intact binocular vision. Rats with either acute monocular occlusion or amblyopia are known to display no preference for the shallow side, as rats with binocular vision do (Baroncelli et al., 2013). Accordingly, CTRL rats were impaired in their visual depth perception, and showed no preference for the shallow side of the arena (n = 6; exploration index = 0.26 ± 0.07). In contrast,

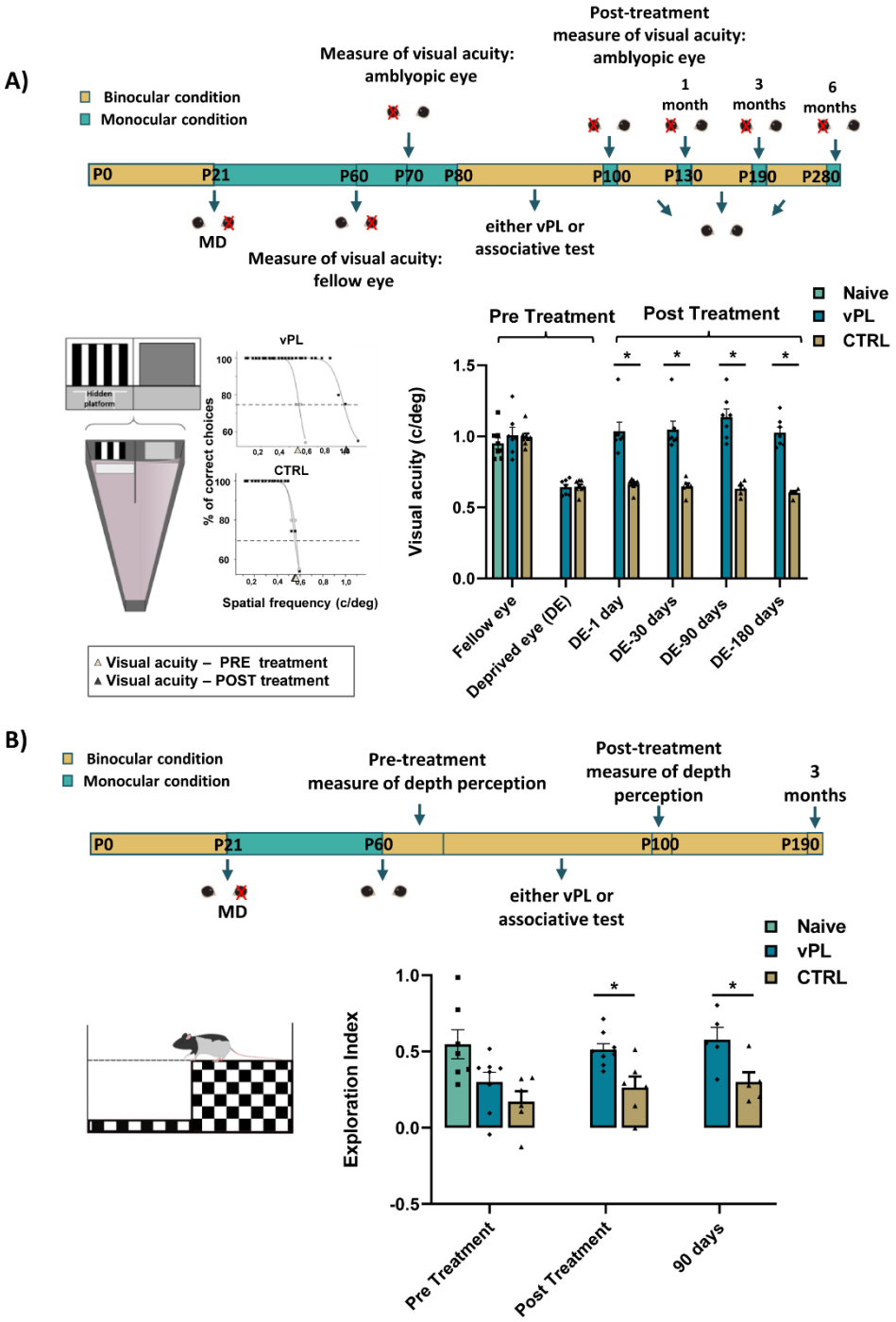

Figure A2. vPL induces recovery of visual functions. (**A**) Top panel: schematic diagram of the protocol. Left panel: visual acuity through the long-term deprived and the fellow eye was measured using the visual water box task; example of sigmoidal extrapolations of psychometric curves used to calculate visual acuity are also reported. Right panel: visual acuity of the previously deprived eye was significantly different from that of the fellow eye in CTRL rats (Two-way RM ANOVA, $p<0.001$), but not in vPL animals ($p=0.297$). Two-way RM ANOVA on ranks revealed that, in vPL rats, visual acuity of the previously deprived eye immediately after the end of the perceptual learning task was significantly increased with respect to that measured before treatment ($p < 0.001$), and remained unaltered 30, 90, and 180 days after the end of the treatment ($p =0.817$, $p=0.095$ and $p=0.958$, respectively). In contrast, the visual acuity of the long-term deprived eye did not change throughout the study in CTRL rats (Two-way RM ANOVA on ranks with Holm-Sidak method, pre-treatment vs. post-treatment, $p=0.999$; pre-treatment vs. 1 month after treatment, $p=0.982$; pre-treatment vs. 3 months, $p=0.964$ and pre-treatment vs. 6 months, $p=0.997$). (**B**) Top panel: schematic diagram of the protocol. Left panel: visual depth perception was assessed using the visual cliff task, as the exploration preference for the shallow and depth side of the arena. Right panel: one-way ANOVA showed a significant preference for the shallow side in vPL animals, which exhibited an exploration index statistically higher than that of CTRL rats at all time-points (Holm-Sidak method, $p<0.05$). All animals were tested after restoration of binocular vision. *Statistical significance. Error bars, s.e.m..

vPL rats (n = 8) showed a clear preference for the shallow side, with an exploration index (0.51 ± 0.03) significantly higher than that of CTRL rats (Fig. A2b), but not significantly different from that of naïve rats (n = 7; 0.54 ± 0.09) (One-way ANOVA, between groups F = 4.329, DF = 2, Holm-Sidak method, p = 0.041, p = 0.046, p = 0.728, respectively). Consistently with the results of visual acuity, recovery of visual depth perception abilities in vPL rats were long-lasting, persisting for the entire 3-month period of follow-up analysis (Fig. A2b). (Two-way repeated measure ANOVA, F = 1.035, DF = 2, Holm-Sidak method, time among vPL, pre-treatment vs. post-treatment, p = 0.021; pre-treatment vs. 3 months, p = 0.025; post-treatment vs. 3 months, p = 0.801).

In separate groups of animals, in vivo electrophysiology was used to monitor visual evoked potentials (VEPs) within the primary visual cortex immediately after the end of the vPL procedure (Figure 3A). In CTRL rats (n = 5), visual acuity of the deprived eye remained significantly lower (0.63 ± 0.02 c/deg) with respect to the fellow eye (0.91 ± 0.03 c/deg) (Two-way RM ANOVA, Holm-Sidak method, F = 23.468, DF = 2, p < 0.001) (Fig. A3b). In contrast, no difference could be found between visual acuity values of the previously deprived vs. the fellow eye in both vPL rats (n = 5, contralateral visual acuity: 0.84 ± 0.02 c/deg; ipsilateral visual acuity: 0.84 ± 0.03 c/deg; p = 0.370; Fig. A3b), and in naïve animals (n = 5, contralateral visual acuity: 0.88 ± 0.02 c/deg; ipsilateral visual acuity: 0.94 ± 0.03 c/deg; p = 0.118). Moreover, visual acuity in the formerly amblyopic eye of vPL rats did not significantly differ from that of naïve animals, while it was significantly higher than that of CTRL rats (Fig. A3b) (p = 0.433 and p < 0.001, respectively). Ocular dominance was measured by computing the contralateral to ipsilateral (C/I) VEP ratio in response to a low spatial frequency grating (0.1 c/deg). In CTRL rats (n = 5), the C/I VEP ratio remained significantly lower than in naïve rats (n = 5) C/I VEP ratio in CTRL rats = 1.14 ± 0.2, in naïve rats 2.4 ± 0.17, (One-way ANOVA, Holm-Sidak method, p = 0.026), indicating the occurrence of a severe OD deficit (Fig. A3c).

In contrast, vPL rats (n = 5) had a C/I VEP ratio of 2.32 ± 0.41, significantly higher than that of CTRL rats (One-way ANOVA, Holm-Sidak method, p = 0.025), but not significantly different from that of naïve rats (n = 5; C/I VEP ratio = 2.4 ± 0.17; p = 0.847). Accordingly, VEPs average amplitude was higher for the contralateral eye with respect of the ipsilateral one in both naïve and vPL rats (Fig. A3d,e) (Two-way RM ANOVA, Holm-Sidak method, F = 27.261, DF = 2; Naïve: contra = 231.22 ± 44.11 μV, ipsi = 100.38 ± 19.32 μV; vPL: contra = 235.89 ± 64.1 μV, ipsi = 132.96 ± 45.97 ± V; p < 0.01 and p = 0.004, respectively); no difference was instead observed between the potentials of contralateral and ipsilateral eye in CTRL animals (contra = 179.43 ± 48.33 μV, ipsi = 152.49 ± 30.99 μV, p = 0.369). No difference was instead observed for VEPs latencies across the different experimental groups (Two-way RM ANOVA, F = 0.609, DF = 2, p = 0.560 for the factor treatment and p = 0.230 for the factor eye, without any interaction, p = 0.881).

Visual deprivation during the CP may permanently compromise the matching of orientation preferences through the two eyes in individual cortical neurons; a fundamental step for the development of binocular vision (Levine et al., 2017). Single units were recorded (Mazziotti et al., 2017) to investigate whether the binocular properties of visual cortical neurons were affected in amblyopic animals (CTRL rats), and whether the recovery of visual depth perception abilities induced by vPL was accompanied by a rescue of these binocular properties. Single units were recorded in anesthetized animals in response to drifting sinusoidal gratings that varied in orientation and spatial frequencies. The distributions of single unit binocular matching of orientation preference (ΔO) were then compared in vPL or CTRL rats, using the same animals previously subjected to the behavioral visual cliff analysis. In naïve animals, the majority of cells displayed a ΔO around 0 deg (central bin is −12.8°< 1O < 12.8°; kurtosis = 0.63, Fig. A4a). CTRL rats instead displayed a decreased kurtosis (kurtosis = −1.08) with

respect to naïve animals. Kurtosis levels were higher in vPL rats with respect to CTRL animals (kurtosis = −0.77) (Fig. A4a), with a ΔO distribution very similar to that of naïve animals. Moreover, the averages of ΔO absolute values (|ΔO|) were compared among the three experimental groups (Fig. A4b). CTRL rats displayed a higher |ΔO| (46.70° ± 2.16°) than both naïve (32.68° ± 2.1°) and vPL animals (30.77° ± 1°) (One way ANOVA, Holm-Sidak method, $F = 22.298$, $p < 0.001$ and $p < 0.001$, respectively). No difference was observed between Naïve and vPL rats ($p = 0.498$). Equal variance of |ΔO| did not change across the three groups, either when computed on the average values of the subjects (F-test, $p = 0.422$), or when computed for all cells in the three different groups (Levene test, $p = 0.115$). To rule out the possibility that these effects at the level of ΔO were due to any change in terms of monocular selectivity to distinct features of the visual stimulus (orientation, direction), or to changes in terms of spontaneous activity, the orientation selectivity index (OSI), the direction selectivity index (DSI) and levels of spontaneous activity in the two eyes were measured in all three experimental groups. No difference was found among the three groups of animals in either OSI, DSI or spontaneous activity (Fig. A4c) (Two-way RM ANOVA, Holm Sidak method, DF = 2, $F = 1.075$, $p = 0.357$; $F = 0.682$, $p = 0.515$; $F = 0.199$, $p = 0.821$, respectively). Having shown both a behavioral effect on visual depth perception abilities using the visual cliff task and a rescue of ΔO distribution in single unit recordings from the primary visual cortex, I measured the relationship between these changes. For each vPL or CTRL subject used to record binocular matching, the mean |ΔO| between the two eyes for all single units analyzed at the preferred grating orientation was correlated with the exploration index in the visual cliff past the end of the behavioral procedure. The mean |ΔO| values correlated significantly with the visual cliff exploration index (Pearson $r = −0.651$, 95% confidence intervals −0.9080 to −0.03466, R squared = 0.4226, $p = 0.0419$, Fig. A5). This correlation indicates that the better the binocular

matching of orientation preference, the greater the visual depth perception abilities, suggesting a causal link between the two measures.

Taken together, these results show that vPL performed under binocular vision can induce a marked and enduring recovery of visual acuity, visual depth perception abilities and binocular matching in adult amblyopic rats.

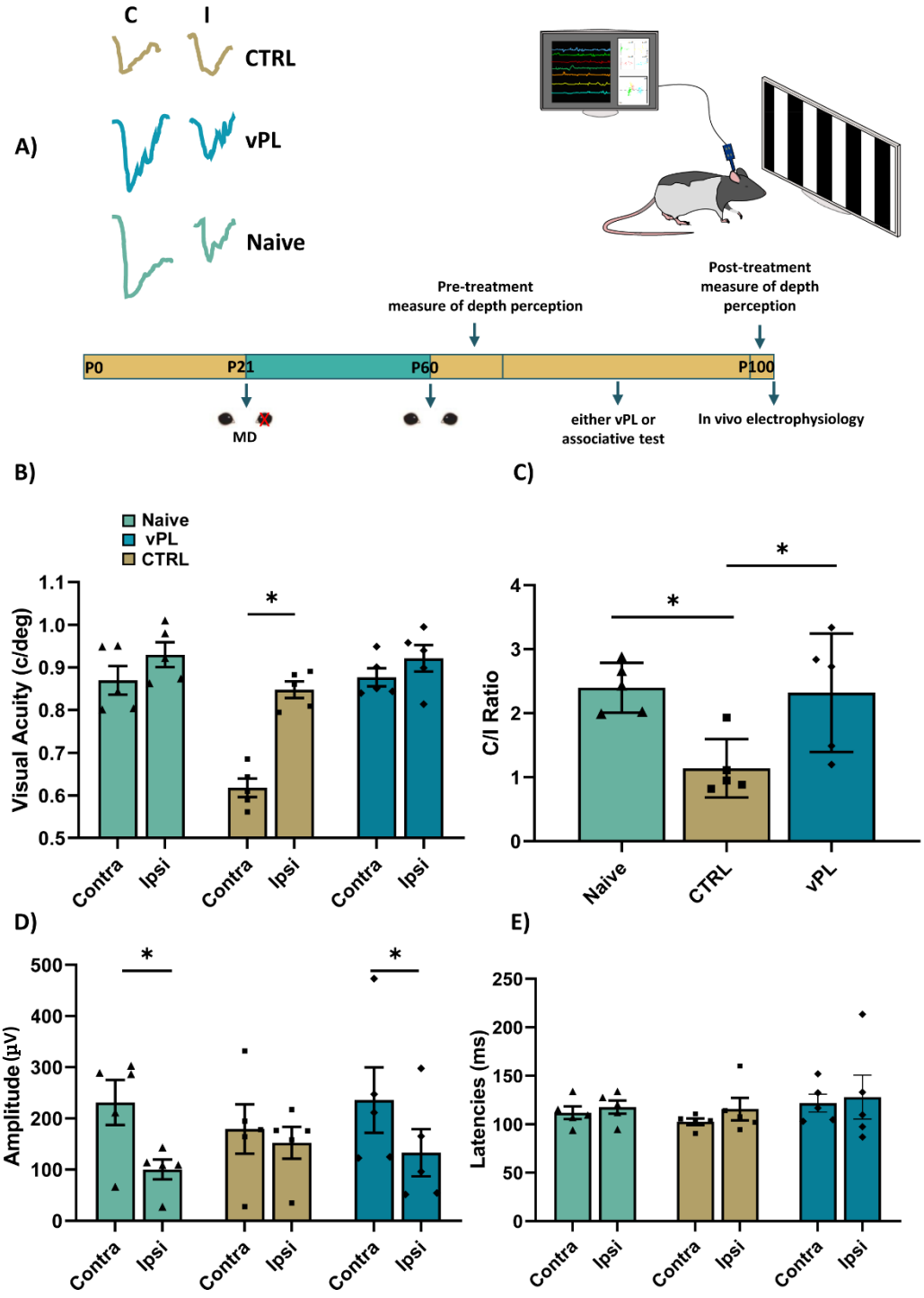

Figure A3. (**A**) Schematic diagram of the protocol. (**B**) Electrophysiological recordings of visual evoked potentials form the primary visual cortex. In CTRL rats, visual acuity of the deprived eye remained significantly lower with respect to the other eye (One-way RM ANOVA, Holm-Sidak method, $p < 0.001$); in contrast, a full visual acuity recovery was achieved by vPL rats ($p < 0.001$) with values not different from those of naïve animals ($p = 0.433$). (**C**) Ocular dominance was assessed through the C/I VEP ratio in response to a low spatial frequency grating. The C/I VEP ratio was significantly higher in vPL than in CTRL rats (One-way ANOVA, Holm-Sidak method, $p = 0.025$), but not different from that of naïve rats ($p = 0.847$). Error bars indicate s.e.m.; $*$ indicates statistical significance. (**D**) Accordingly with the C/I ratio, the average amplitude of VEPs was higher for the contralateral eye with respect of the ipsilateral one, in both Naïve and vPL rats (Two-way RM ANOVA, $p < 0.01$ and $p = 0.004$, respectively); no difference was instead observed between the potentials of contralateral and ipsilateral eye in CTRL animals ($p = 0.369$). (E) No difference was observed for VEPs latencies across the different experimental groups (Two-way RM ANOVA, $p = 0.560$ for the factor treatment and $p = 0.230$ for the factor eye, without any interaction, $p = 0.881$). *Statistical significance. Error bars, s.e.m.

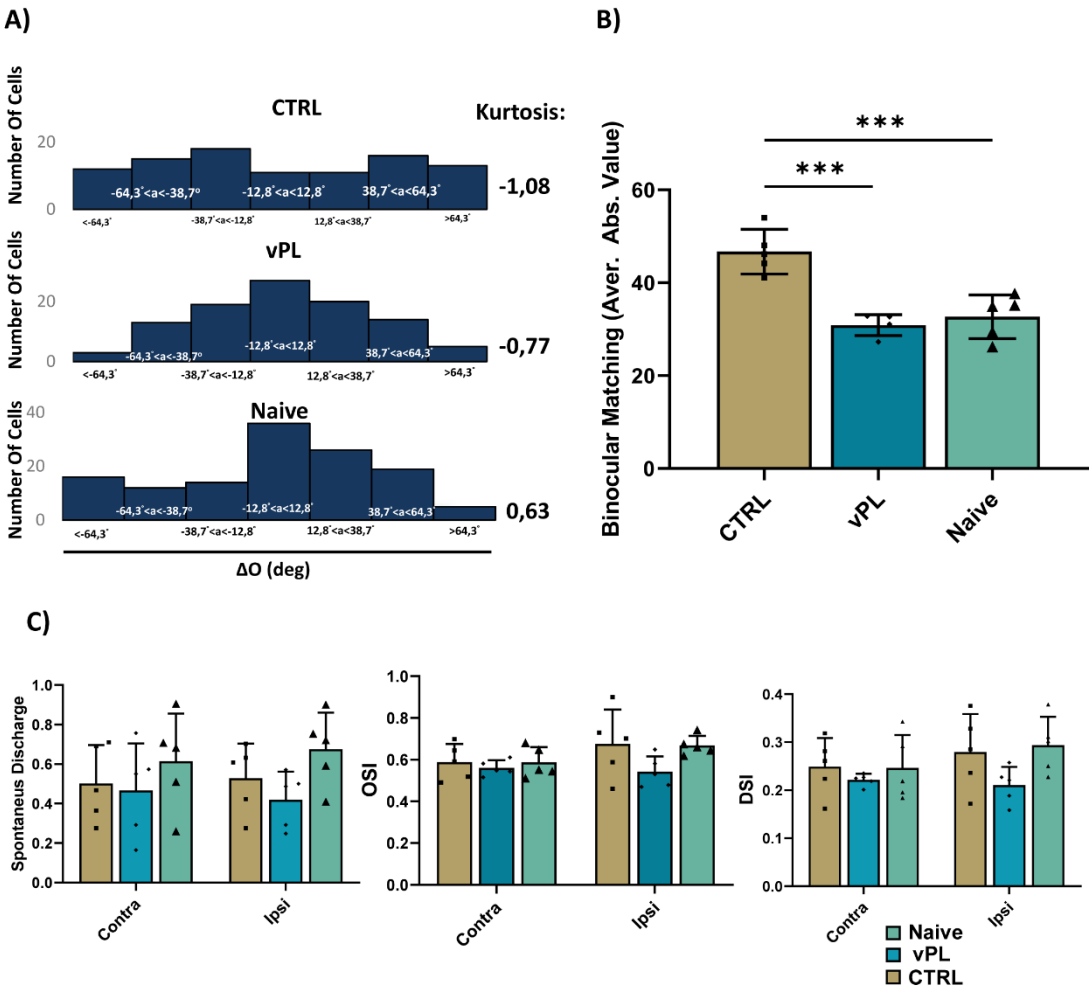

Figure A4. (**A**) Distributions of ΔO within the three experimental groups. (**B**) Comparison between average |ΔO| for the different groups; CRTL rats displayed higher |ΔO| in comparison with both vPL and Naïve animals (One-way ANOVA, Holm-Sidak method, $p < 0.001$ and $p < 0.001$, in both cases). Interestingly, no difference was observed between naïve and vPL rats ($p = 0.498$). (**C**) Assessment of spontaneous discharge, direction, and orientation selectivity in V1. No difference was found in either the spontaneous discharge, the orientation selectivity index (OSI), or the direction selectivity index DSI among the three groups of animals (Two-way RM ANOVA, Holm-Sidak method, DF = 2, $F = 1.075$, $p = 0.357$; $F = 0.682$, $p = 0.515$; $F = 0.199$, $p = 0.821$, respectively). *Statistical significance. Error bars, s.e.m.

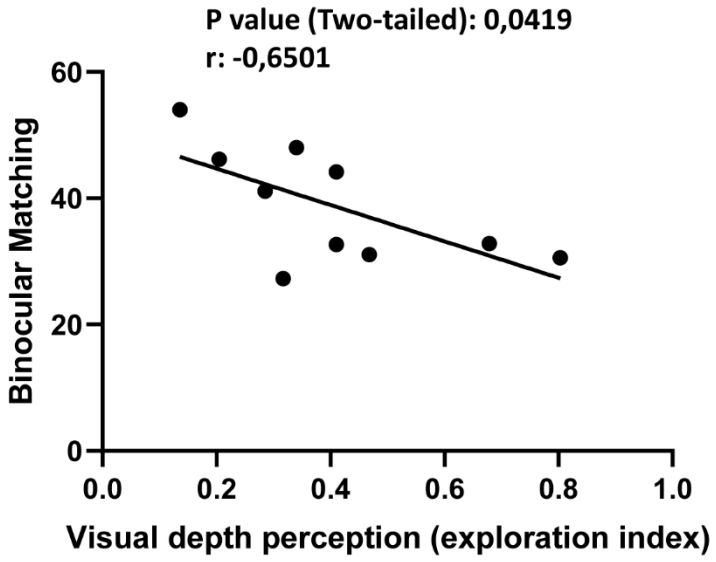

Figure A5. Significative correlation between | ΔO| and visual cliff scores suggests a causal link between binocular matching of orientation preference and depth perception abilities (Pearson r = –0.651, 95% confidence intervals –0.9080 to –0.03466, R squared = 0.4226, p = 0.0419).

Discussion

Here, I reported that adult amblyopic rats subjected to vPL show a marked long-term recovery of visual acuity Indeed, the visual acuity recovery effect induced by vPL persisted for at least 6 months. This result appears particularly promising for clinical application to human patients, given that it has been estimated that a day of a rat's life is equivalent to 34.8 human days (Sengupta, 2013). No recovery was instead found in control rats that only learned to discriminate a reference grating from a test grating that was always maintained at its initial spatial frequency, suggesting that the incremental practice is essential for the capability of visual learning to promote plasticity and visual function recovery.

Previously, similar effects have been reported in rats subjected to the vPL task under a monocular condition (Baroncelli et al., 2012). The monocular protocol clearly maximizes the use of the amblyopic eye, but it is limited in terms of application to human patients due to potentially relevant risks (Hess and Thompson, 2015; Levi et al., 2015). Indeed, an increasing consensus is raising about the need to understand the cellular bases of visual improvements promoted by dichoptic therapies targeting binocular functions, such as videogames (Hess et al., 2011; Li et al., 2013) or passive dichoptic video viewing (Li et al., 2015b, 2015a). In this field, the results here reported indicate a strong beneficial effect of vPL when performed without eye occlusion procedures encouraging further studies based on binocular stimulation in adult amblyopic subjects. As a note of caution, however, it should be noted that the monocular deprivation approach used in this study simulates only deprivation human amblyopia, but not strabismic or refractive amblyopia, for which further studies in animal models are needed to assess the impact of vPL.

Notably, the results obtained at the behavioral levels were fully confirmed by the electrophysiological analysis of VEPs, the gold standard procedure for visual acuity assessment (Campbell et al., 1973; Campbell and Maffei, 1970; Hamilton et al., 2021; Ridder and Nusinowitz, 2006). VEPs recordings were also used to measure ocular dominance by computing the C/I VEP ratio, a fundamental parameter strongly affected in amblyopic subjects (Frenkel and Bear, 2004).

I could not find any effect of vPL on monocular OSI or DSI. This may be dependent on the difference between visual acuity and orientation/direction selectivity in terms of their dependence on visual experience during development. While selectivity of the primary visual cortex for spatial frequencies strongly depends on postnatal visual experience (Prusky et al., 2000), the orientation/direction preference is established just after eye-opening, independently of visual experience (Buisseret and Imbert, 1976; Fregnac and Imbert, 1984; Gödecke and Bonhoeffer, 1996; Thompson et al., 2008; Wiesel, 1982; Wiesel and Hubel, 1974). Thus, long-term monocular deprivation started at the peak of the critical period was likely to strongly affect visual acuity, without exerting any effect on monocular OSI and DSI. Therefore, exposure of amblyopic animals to vPL might have improved visual acuity, leaving unaltered OSI and DSI.

vPL also induced long-term recovery of visual depth perception abilities. This could have remarkable repercussion on the potential translational interest of this research. Deficits in stereopsis indeed constitute a relevant impediment for everyday life activities in amblyopic subjects (Levi et al., 2015; Webber and Wood, 2005). The improvement in the visual cliff test induced by vPL was unlikely to be dependent on an improvement of monocular functions, like visual acuity. It has been previously shown, indeed, that naïve adult rats with one eye occluded but with normal vision are severely impaired in performing the same visual cliff task used in our manuscript (Baroncelli et al., 2013). Thus, monocular cues are not sufficient for a proper

performance in the visual cliff task, even when the used eye is completely normal. Moreover, I did not find any difference in terms of monocular selectivity for direction or orientation among the three groups of animals. Lastly, the visual stimuli presented in the visual cliff were of very low spatial frequency, thus being fully perceivable also by amblyopic animals.

I also provide evidence that long-term monocular deprivation was associated with altered binocular matching properties for primary visual neurons, and that vPL, which is able to favor recovery of visual depth perception, was also accompanied by normalization of binocular matching in the primary visual cortex of amblyopic animals. Thus, the obtained results in favor of a direct link between the recovery of visual depth perception abilities assessed at the behavioral level, and normalization of binocular matching of orientation preference. The current study therefore suggests that rescue of binocular matching might be a critical mechanism underlying the beneficial effects of vPL on visual depth perception.

At the visual cortical circuit level, other active training procedure, like voluntary physical exercise, are known to have an impact on GABAergic neural connections, with a selective and differential regulation in the activity of distinct sub-populations of interneurons (Fu et al., 2014; Sansevero et al., 2020). Specifically, amblyopia has been linked to increased numbers of active somatostatin positive interneurons (SOM +), without any detectable effect on either vasointestinal protein (VIP +) or parvalbumin positive cells. In contrast, physical exercise was associated with both a specific increase of active VIP + cells, and a restoration to basal numbers of active SOM + interneurons, in agreement with the model put forward by Stryker and colleagues (Stryker, 2014). Further efforts are however needed to dissect the possible changes at the GABAergic circuit level induced by vPL in amblyopic animals.

To conclude, the obtained results demonstrate that vPL under binocular sight conditions is an effective non-invasive procedure able to elicit long-lasting recovery from amblyopia far

beyond the closure of the critical period. These results could have a direct therapeutic impact on human health, promoting new treatment strategies for amblyopia and other still cureless neurodevelopmental disorders.

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Appendix B:

Scientific production

-Articles in peer-reviewed journals

Sansevero G, **Consorti A**, Di Marco I, Terzibasi Tozzini E, Cellerino A, Sale A. Antioxidants prevent the effects of physical exercise on visual cortical plasticity. Cells 2023, 12(1), 48; https://doi.org/10.3390/cells12010048

Consorti A, Sansevero G, Torelli C, Di Marco I, Berardi N and Sale A. Visual Perceptual Learning Induces Long-Lasting Recovery of Visual Acuity, Visual Depth Perception Abilities and Binocular Matching in Adult Amblyopic Rats. Front. Cell. Neurosci. 16:840708, (2022) https://doi.org/10.3389/fncel.2022.840708

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-Posters

Consorti A, Sansevero G, Di Marco I, Berardi N, Sale A. Visual perceptual learning in mice. Neuroscience 2022, November 12th-16th, San Diego. Poster Presentation.

Di Marco I, Sansevero G, **Consorti A**, Terzibasi Tozzini E, Cellerino A, Sale A. Antioxidants counteract the plastic effect of physical exercise in the adult primary visual cortex.
BraYn 5th Edition, September 28th-30th 2022, Rome.

Consorti A, Sansevero G, Di Marco I, Capecchi V, Novelli E, Berardi N, Sale A. Top-down influences and visual perceptual learning.
BraYn 4th Edition, October 20th- 22nd 2021, Pisa. Poster Presentation.

Consorti A, Sansevero G, Torelli C, Di Marco I, Berardi N, Sale A. Active training promotes visual function recovery in adult amblyopic rats.
BraYn 3rd Edition, November 25th-26th 2020, Web Conference. Poster Presentation.

Sansevero G, **Consorti A**, Di Marco I, Cellerino A, Sale A. Antioxidant supplementation and physical exercise: beyond the ordinary notion.
BraYn 3rd Edition, November 25th-26th 2020, Web Conference.

Torelli C, Sansevero G, Mazziotti R, **Consorti A**, Cenni MC, Berardi N, Sale A. Active training promotes recovery of visual functions in adult amblyopic rats.
Annual Retreat, Institute of Neuroscience, October 2nd-4th, Pisa