Experience-Dependent Loss of Cross-Modal Plasticity in Mouse Visual Cortex

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Abstract

We perceive the world through sensory experience. Sensory information is registered and processed by our brain in a modality specific fashion. Interestingly, studies have shown that the visual cortex of early but not late blind subjects is able to respond to touch or sound (Sadato et al., 1996; Buchel et al., 1998; Weeks et al., 2000; Gougoux et al., 2009). Here, we investigated whether sensory parcellation in adult cortex is innate or is acquired during early postnatal life in an experience-dependent manner. Furthermore, we studied the anatomical substrates and molecular pathways possibly involved in cross-modal activation and its plasticity.

First, mice were reared from birth in total darkness until adulthood (DR) to replicate the human blind condition. Cross-modal activity and the underlying circuitry were analyzed. We found that DR visual cortex was strongly activated by sound stimulation using functional imaging, single-unit recording, and *c-Fos* immunohistochemistry. Functional analysis was followed by anatomical tracing studies, which showed ectopic projections from the auditory thalamus and auditory cortex into the secondary visual area in DR animals.

The second half of our study looked at how visual experience affects cross-modal plasticity. We found that cross-modal activity and ectopic connectivity is present in normally reared young mice (25 postnatal days: P25). Normal sensory experience through the first two months of postnatal life was sufficient to decrease the number of ectopic inputs. Interestingly, exposing DR mice to visual experience as adults established transient functional sensory specificity in the visual cortex without eliminating the ectopic anatomical inputs.

Lastly, we tested several molecular pathways that can potentially regulate cross-modal plasticity. We found that myelin signaling and cholinergic modulation controls the duration of cross-modal plasticity and consolidates sensory modularization.

Overall, our work proposes a model of how cross-modal inputs into early sensory areas are pruned or retained depending on early life experience. This study provides insight into how the cortex develops functional specificity, and help approach disorders that exhibit abnormal sensory integration and disrupted neuronal connectivity such as Autism Spectrum Disorder.

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Acknowledgement

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I hope this thesis makes all of you proud!

Part I: Background

Chapter 1

Brain Plasticity

Plastic young brain

It is common wisdom that children are better at learning than adults. It seems natural to absorb different skills and make them intrinsic to oneself during early life. We gradually lose this ability as we grow older, requiring many hours of practice and hard work to gain new skills. Scientific studies have confirmed that there is indeed a fundamental difference in the way information is stored in our brain depending on the time point when learning occurs. For instance, second language acquired during adolescence (about 11 years old) is encoded in a spatially separated region from native languages in the brain, while both are represented in a common area for those exposed to second language during infancy (Kim et al., 1997). Early life experience also affects the way we sense our surroundings. Early exposure to different languages allows auditory discrimination of greater number of phonemes (Kuhl, 2004), and early training in musical instruments have been shown to influence the cortical representation of fingers and sounds (Elbert et al., 1995; Pantev et al., 1998 and 2001). As these examples demonstrate, the young brain dynamically responds to experience and environment by modifying its neural circuitry.

The extent of early brain plasticity is demonstrated through many studies. In particular, Sur et al. have shown how altering the input to primary sensory cortices early in life is sufficient to change the function of the targeted sensory cortex. By removing the superior colliculus,

which is normally targeted by retinal inputs, and severing major auditory inputs into thalamus, they were able to direct retinal inputs to the auditory thalamus in neonatal ferrets and hamsters (Sur et al., 1990). In a follow-up study, they showed that rewired visual inputs to the auditory cortex are functional and are interpreted as visual sensations (von Melchner et al, 2000; Mao et al., 2011). This study strongly suggests that early sensory cortex may have the potential to process inputs from different modalities. Such observation of sensory cortex responding to inputs other than its specific modality is termed 'cross-modal plasticity.'

Critical period regulation in visual cortex

The degree of brain plasticity rapidly decreases with age. However, research shows that there are specific windows of opportunity for change during development, termed "critical period." During critical period, sensitivity to external environment is increased and promotes experience-dependent modification in various sensory properties, such as tonotopy, binocular vision, and whisker mapping (Chang and Merzenich, 2003; de Villers-Sidani et al., 2007; Hubel et al, 1977; Wiesel, 1982; Holmes and Clarke, 2006; Van der Loose and Woolsey, 1973; Fox, 1992). Having a critical period allows customization of neuronal circuits to suit the individual beyond what can be encoded by the genome. Considering how the surrounding environment and physical characteristics vary across individuals, this ability to customize neuronal circuits based on experience is crucial for proper brain functioning (Hensch, 2004). The wide range of possible implications of understanding critical period plasticity, from education to recovery from injury, has motivated much research. The most rigorous studies took place in the visual system. With the first description of functional loss in visual cortex of kittens with monocular deprivation almost half a century ago (Wiesel and Hubel, 1963), much progress has been made in

understanding the physiological, anatomical and molecular mechanisms underlying critical period plasticity in visual cortex.

Upon monocular deprivation (MD), primary visual cortex shows physiological loss of responsiveness to the deprived eye (Wiesel and Hubel, 1963). This functional loss is accompanied by anatomical changes (Antonini and Stryker, 1993; Antonini et al., 1999; Treachtenberg and Stryker, 2001), as well as behavioral consequences (Daw, 1995; Prusky et al., 2000). Weakening of inputs coming from the deprived eye shifts the ocular dominance towards the open eye, and thus causing amblyopia in the deprived eye (Daw, 1995; Maurer et al., 1999). Interestingly, this happens only during a transient developmental critical period (Hubel and Wiesel, 1970; Prusky and Douglas, 2003). It has been shown that plasticity in visual cortex of rodents and cats is low at the time of eye opening, increases to reach its peak around four weeks of age, and slowly declines (Fagiolini et al., 1994; Daw, 1995; Gordon and Stryker, 1996).

The critical period for ocular dominance plasticity can be delayed through dark-rearing or altering the local excitation/inhibition balance (Mower, 1991; Morales et al., 2002; Hensch and Fagiolini, 2005) (Fig. 1.1). Delaying the maturation of inhibitory circuits by knocking out one of the isoforms of GABA-synthesizing enzymes (GAD65) prevented ocular dominance shift by MD (Hensch et al., 1998). Further studies showed that the potential for plasticity remains open throughout life until the inhibitory threshold is attained (Fagiolini and Hensch, 2000). Delay of excitatory circuit maturation through targeted gene-disruption of NR2A, which prevents the activity-dependent NR2B to NR2A subunit switch of NMDA receptor (Nase et al., 1999; Fagiolini et al., 2003), caused a decrease in magnitude of MD-driven ocular dominance shift during critical period (Fagiolini et al., 2003). Interestingly, orientation selectivity also failed to develop in NR2A KO mice. Taken together, these studies show how maturation of inhibitory and excitatory

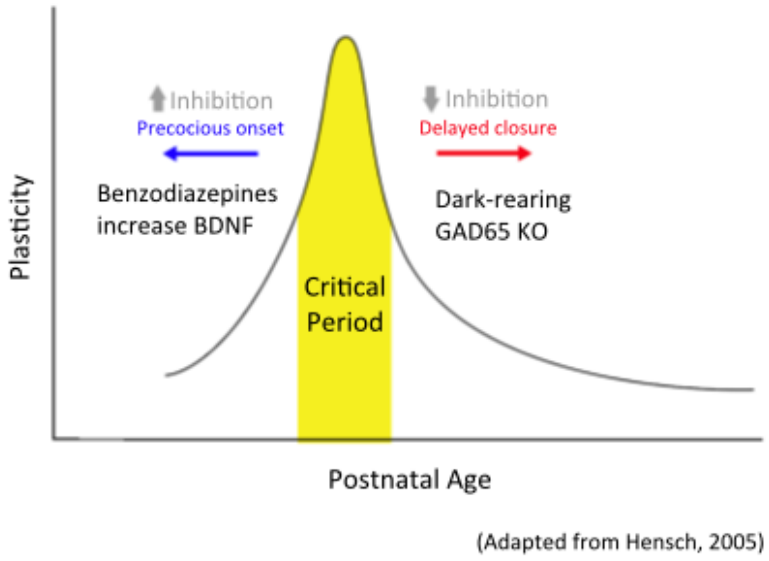


Figure 1.1. GABA-mediated control of critical period

Sensitivity to monocular deprivation is restricted to a critical period that begins, in mice, about 1 week after the eyes open (around P13) and peaks 1 month after birth. The onset of critical period can be delayed by preventing the maturation of GABA-medicated transmission through gene-targeted deletion of *Gad65*, which encodes a GABA-synthesizing enzyme, or by dark-rearing from birth. Conversely, the critical period can be brought forward by enhancing GABA transmission with benzodiazepines application just after eye-opening, or by promoting the rapid maturation of interneuron's through excess brain-deprived neurotrophic factor (BDNF) expression.

circuits control separate features of visual cortex plasticity, and regulate critical period plasticity (Hensch, 2005).

While maturation and balance of excitatory and inhibitory circuits initiate and regulate critical period, mechanisms that decrease neuronal plasticity close the critical period to consolidate the structural changes. Myelin-associated inhibitors such as myelin-associated glycoprotein (McKerracher et al., 1994; Tang et al., 2001), Nogo-A (Caroni and Schwab, 1988; Schwab and Caroni, 1988), and Oligodendrocyte-myelin glycoprotein (Wang et al., 2002; Kottis et al., 2002) have been shown to restrict neurite outgrowth and axon regeneration in the central nervous system (Schwab, 1990). Nogo-receptors (NgR) that are expressed in adult cerebral cortex and hippocampus (Josephson, 2002) bind to myelin-associated inhibitors and send inhibitory signals (McGee and Strittmatter, 2003). Increase in myelin-associated inhibitory signals consolidates the functional circuits and reduce plasticity (McGee et al., 2005; Syken et al., 2006). In mice that lack NgR, ocular dominance plasticity failed to cease and retained its plasticity through adulthood (McGee et al., 2005).

In addition to myelin signaling, modulatory networks of norepinephrine, acetylcholine, serotonin, and dopamine are known to regulate functional plasticity (Bear and Singer, 1986; Kasamatsu, 1991; Kilgard and Merzenich, 1998; Bao et al., 2001; Weinberger, 2007; Maya Vetencourt et al., 2008; Goard and Dan, 2009). In adult brains, brake-like molecules have been found to inhibit neuromodulatory networks and limit functional plasticity in adults. One of these molecules, Lynx1, is up-regulated during development and is shown to limit visual cortex plasticity in adults by restricting cholinergic modulation (Morishita et al., 2010).

In this study, we apply our knowledge about these molecular mechanisms that regulate critical period plasticity to dissect the mechanism underlying cross-modal plasticity.

Chapter 2

Cross-Modal Plasticity in Sensory Cortex

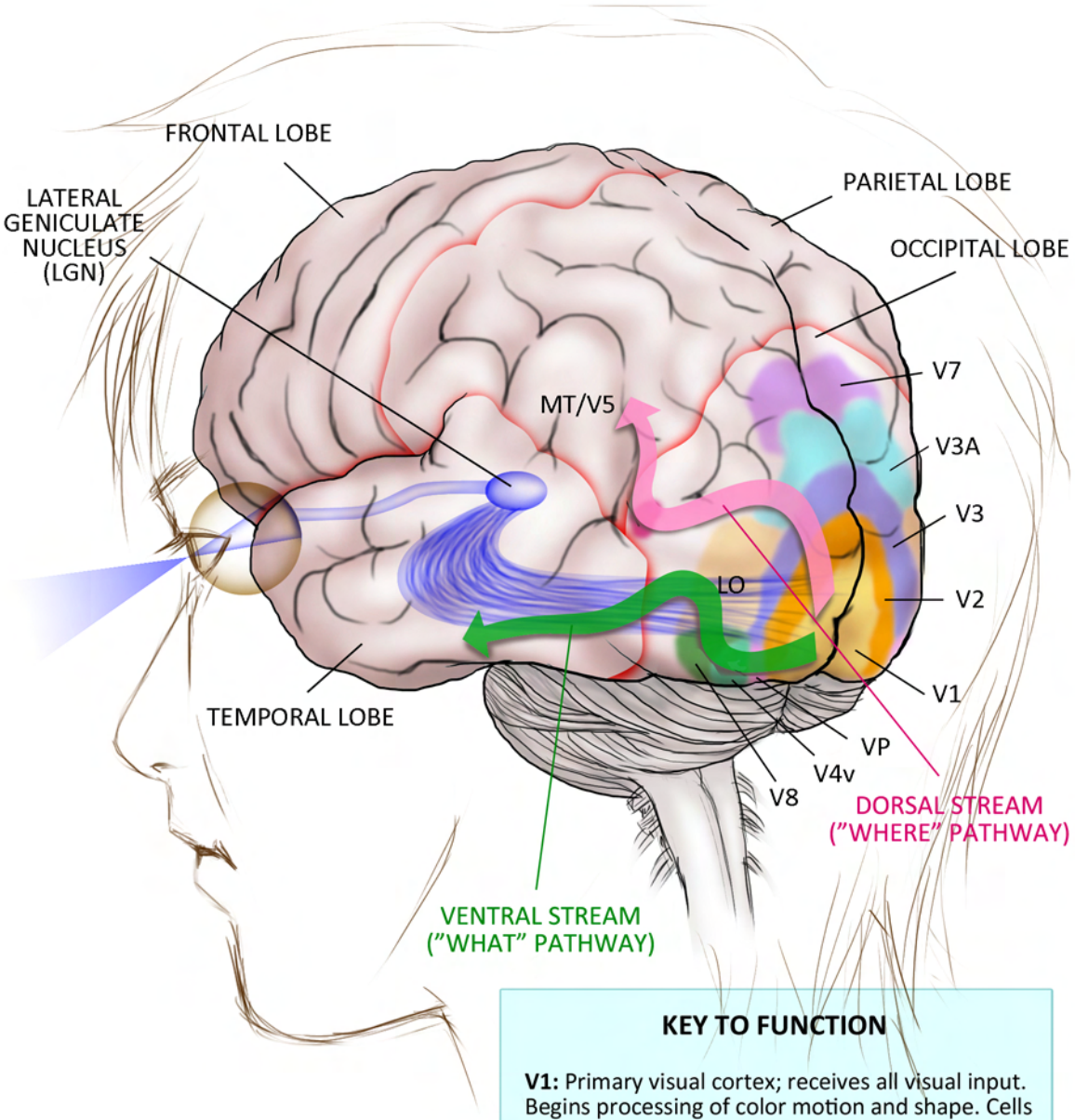
Arealization and developmental exuberance

One of the most fundamental properties of our brain is its function-specific parcellation (Brodmann, 1909; von Economo, 1929; see Jones, 2008 for review). The cortex, the outermost sheet of neural tissue of the mammalian brain, is described as largely comprising of three parts: sensory, motor, and association areas. Each of these parts is further organized into functionally specific regions. The sensory area for example, is composed of distinct regions devoted to processing inputs of different modalities, such as somatosensory, vision and audition. Different functional regions can be distinguished from one another by their cytoarchitecture and chemoarchitecture, and their input and output connections. Each area is connected to each other and to sub cortical structures through distinct hierarchical patterns of neural networks, which allow for the proper flow of information through the brain (Felleman & Van Essen, 1991). Cortical areas of lower functional hierarchy have relatively simple receptive fields. Information collected in lower functional areas then makes connections with dominant, driving influences to areas of higher functional order (Callaway, 1998a; Crick & Koch, 1998; Sherman & Guillery, 1998). In contrast to the relatively simple receptive fields of lower areas, high functional areas respond to complex combination of features, such as object recognition, or spatial localization (Riesenhuber & Poggio, 1999; Haxby et al., 1991). An example of hierarchical functional organization for visual input is illustrated in Fig. 2.1.

Development of such cortical areas with sharp boundaries in the adult brain involves interaction between intrinsic and extrinsic mechanisms. During embryonic development,

Figure 2.1. Visual Pathway

Human visual pathway begins with the eyes and extends through several interior brain structures before ascending to the various regions of the visual cortex. Visual information travel from the retinal ganglion cells, along the optic nerve and optic tract, to the thalamus and midbrain, and through to primary visual cortex (V1), and on into different extra-striate parietal, temporal, and frontal regions. Each cortical region specializes in processing different properties of visual stimulus such as color, shape, depth and movement. These different aspects of vision are subsequently integrated as it flows into higher brain regions. It is widely accepted that as visual information exits the occipital lobe, it follows two different pathways. The dorsal stream is thought to be involved in guidance of actions and spatial awareness. The ventral stream is associated with object recognition and form representation.



KEY TO FUNCTION

V1: Primary visual cortex; receives all visual input. Begins processing of color motion and shape. Cells in this area have the smallest receptive fields.

V2, V3 and VP: Continue processing; cells of each area have progressively larger receptive fields.

V3A: Biased for perceiving motion.

V4v: Function unknown.

MT/V5: Detects motion.

V7: Functiona unknown.

V8: Processes color vision.

LO: Plays a role in recognizing large-scale objects.

Figure 2.1. (Continued)

graded expressions of transcription factors initiate the process of arealization by guiding thalamocortical axons (TCA) to the different areas of the neocortex (Muzio and Mallamaci, 2003; Hamasaki et al., 2004; Armentano et al., 2007; O'Leary and Sahara, 2008; Little et al., 2009). Upon reaching the cortex, TCA plays a significant role in differentiating cortical areas (Rakic, 1988; Chenn et al., 1997; Dehay et al., 2001). Prior to sensory experience, spontaneous neural activities through TCA projections drive cortical differentiation and functional refinement (Molnar et al., 2003). Patterned activities driven by sensory input further refines the functional properties in each area (Grubb and Thompson, 2004; Sur and Rubenstein, 2005).

During this initial phase of cortical arealization, there is ample evidence of exuberant thalamocortical and cortico-cortical projections coming in and out of regions that they will not persistently innervate (Dehay et al., 1984 & 1988; Huttenlocher, 1994). These extra connections start to decrease after reaching a certain stage of development. It has been shown that synaptic density within human visual cortex increases to maximum during the first postnatal year, and gradually decreases to the adult level by the age of 11 (Huttenlocher and de Courten, 1987). Studies have also shown that while the initial increase in synaptic density does not depend on vision, subsequent pruning depend on visual experience (Winfield, 1981; Bourgeois et al., 1989; Bourgeois and Rakic, 1996). These suggest that experience-dependent patterned activity through TCA may affect functional specialization of cortical areas by guiding the pruning process of weak exuberant projections while condensing the strong functional sensory specific connections. However, most studies have focused on a single modality to examine changes in developmental exuberance. In this study, we looked for connections that cross over to different sensory modalities and characterized the developmental process of such cross-modal exuberance.

Experience-dependent reorganization

With the exception of the olfactory area, each primary sensory region receives its input directly from a corresponding portion of the thalamic nuclei (Jones, 1998). For instance, visual inputs that enter our system through the retina travel to the lateral geniculate thalamus (LGN), which projects to the primary visual cortex. Adjacent to primary sensory cortex are hierarchically organized higher sensory regions. Modality specific sensory information relayed from each sensory organ remains largely independent from sensory inputs of other modalities until it reaches higher functional areas that serve as the center of sensory integration (Dubner and Rutledge, 1964; Jones and Powell, 1970; Pandya and Seltzer, 1982; Wallace et al., 1992). This modular organization and development of sensory system has historically dominated the view on neurobiology of perception. As a consequence, majority of studies have focused on dissecting one sensory modality as independent from others. However, functional studies of the blind and the deaf show that even the primary sensory cortex have the ability to process cross-modal inputs (reviewed by Merabet and Pascual-Leone, 2010). In blind or deaf subjects, the sensory cortex associated with the deprived modality become colonized by the remaining modalities. Occipital cortex in the blind has been reported to respond to Braille reading (Sadato et al., 1996), auditory motion perception (Poirer et al., 2006), verbal language processing (Bedny et al., 2011), and olfactory discrimination (Kupers et al., 2011). Similarly, visual activity in auditory cortex has been reported in deaf subjects (Nishimura et al., 1999; Lomber et al., 2010).

Animal models of sensory deprivation provide further support for cross-modal activation in sensory cortex of the deprived modality. Early bilateral eyelid suture in macaque monkeys replicates the observation made in blind humans (Hyvarinen et al., 1981). Similarly,

auditory activation in visual cortex was observed in cats with neonatal bilateral eyelid sutures or bilateral enucleation (Rauschecker and Korte, 1993; Rauschecker, 1995 & 1996; Yaka et al., 1999), as well as in functionally blind mice (Larsen et al., 2009). In addition, early enucleation expands the cortical space dedicated to processing auditory and somatosensory information in opossums, particularly at the borders of visual cortex (Kahn & Krubitzer, 2002; Brussel et al., 2011). Also, removing the eye in neonatal hamsters and rats caused the occipital cortex to become cross-modally responsive (Toldi et al., 1994; Izraeli et al., 2002).

It is most interesting to find in these studies that cross-modal reorganization is dependent on the age when sensory deprivation begins. Studies show the strongest cross-modal activity in visual cortex of congenitally and early blind subjects (Sadato et al., 2002; Burton et al., 2002a & 2002b; Li et al., 2012). Such age dependency indicates that there might be a time window early in life when cortical specificity can be modified in an experience-dependent manner.

Although this phenomenon of cross-modal reorganization in sensory cortex has been known for some time, the anatomical and molecular background underlying this phenomenon remains unclear. Comparison between the early and late blind MRI scans revealed an increase in thickness in the visual cortex of the early blind, which may indicate increased number of connections within the visual cortex (Jiang et al., 2009). Many studies have attempted to identify the source of cross-modal inputs into visual cortex of the blind. However, there are large discrepancies among what has been reported. Studies that use dynamical causal modeling of fMRI, which measures activity linkage across brain regions as an indirect measure of cortical connectivity, provided conflicting evidence for direct cross-modal corticocortical networks involving visual cortex of the blind, and inconsistent results for subcortical networks (Fujii et al.,

2009; Klinge et al., 2010). Animal models also show wide range of different anatomical origins for cross-modal inputs. In blind mole rats and neonatally enucleated hamsters, inferior colliculus (IC) projection to lateral geniculate nucleus (LGN) was shown to be the origin of the auditory activity in the occipital cortex (Bronchti et al., 1989; Doron and Wollberg, 1994; Izraeli et al., 2002). In contrast, such subcortical rewiring was not detected in mice enucleated at birth (Chabot et al., 2007). Chabot et al. have compared neonatally enucleated mice and mutant anophthalmic mouse stains, and showed that the anatomy underlying cross-modal activity in each group is different (Chabot et al., 2007). This suggests that there is anatomical variance among different models of blindness, which is likely due to the developmental time point of visual deprivation. However, the circuitry that gives rise to auditory activation in visual cortex in enucleated mice was not identified in this study, and the mechanism that leads to such anatomical changes remains unanswered.

In this study, we wanted to know whether sensory parcellation in adult cortex is innate or is acquired during early postnatal life through experience. We aimed to have a clear anatomical understanding of the cross-modal activity in the blind. Furthermore, we wanted to learn whether we could alter sensory specificity in adults through adjusting molecular pathways that regulate cortical plasticity. The first half of our experiments consisted of characterizing the sensory modularization in mice that are dark-reared from birth (DR). We analyzed cross-modal activity and the underlying circuitry in DR visual cortex. The second half of our study examined cross-modal plasticity in normally raised young mice, and how it is affected by visual experience. Lastly, we looked at genetically engineered mice to uncover the role of specific signaling pathways controlling cross-modal plasticity during development.

Part II:

Experience-dependent Loss of

Cross-Modal Plasticity in Visual Cortex

Chapter 3

Materials and Methods

Animals and Rearing Conditions

Mice Experiments were performed on the C57Bl/6 inbred strain of house mouse (*Mus musculus*) born and raised in standard mouse cages with food and water *ad libitum*. Mice used in each experiment were inspected for any physical malformations and used only when they fell within an appropriate weight range for their age (juvenile >9 g, adults 18~35 g). Light-reared mice (LR) were housed in normal mouse facility on a 12-hour light/dark cycle. Mice at postnatal day 25 (P25), when visual receptive field properties develop functional maturity (Hensch, 2004), were used as our juvenile sample. Our adult sample consisted of mice over P60, when mice are fully sexually mature (Fig.3.1).

Dark-rearing Visual deprivation was achieved by placing mice in a specially designed room under 24-hours of complete darkness. Dark-rearing from birth (DR) is a well-characterized paradigm of non-invasive visual deprivation that delays visual cortex maturation (Benevento et al., 1992; Fagiolini et al., 1994; Gianfranceschi et al., 2003; Tropea et al., 2010). Mice litters were born and raised in complete darkness and examined after reaching adulthood at >P60. In

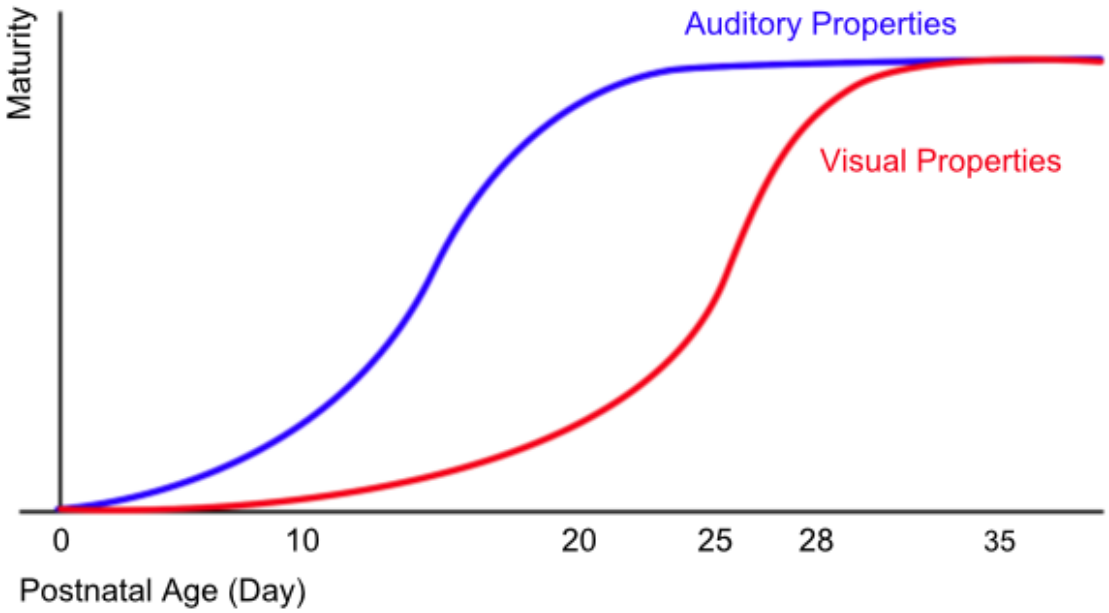


Figure 3.1. Sensory development in mice

The timeline for audition and vision maturation in mice. At birth, mice are considered to be deaf. At postnatal day 10 (P10), audition is restricted to frequency ranging from 5-40 kHz. Auditory maturity is reached by the 4th postnatal week, and the upper frequency limit shifts up to 100 kHz (see Rubsamen, 1992 for review). However, establishment of adult-like tonotopy in the inferior colliculus is achieved by P20 (Romand and Ehret, 1990). Visual maturation comes later than auditory maturation; reaching adult-like visual acuity levels at P35 (Berardi et al., 2000). Mice have their eyes closed from birth until P13. In the following week, vision develops at a slow rate and at around P25, it goes through a rapid maturation process. This is when the critical period for ocular dominance plasticity peaks, indicating that experience-dependent plasticity also occurs during that time. The end of the critical period roughly coincides with the completion of development at around P35 (Berardi et al., 2000).

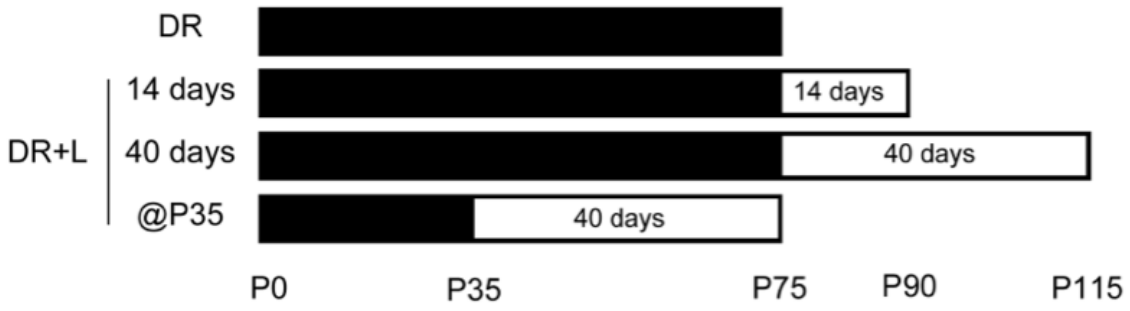


Figure 3.2. Dark-rearing protocol

Mice are reared in complete darkness from birth until adulthood (DR, top row). All experiments involving DR mice were carried out with extra caution to insure they were not exposed to light during experiment procedures. For some experiments, mice were taken out of the darkness and placed in normal lighting of 12 hrs light/dark cycle (DR+Light: DR+L, bottom three rows).

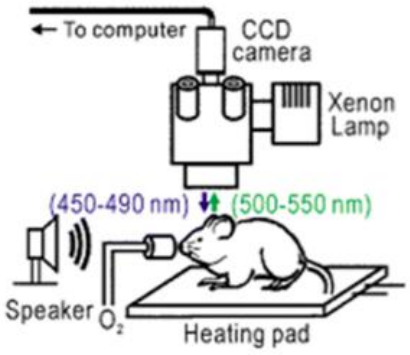
addition to DR from birth, mice were taken out of the darkness at various ages for light exposure experiments (Fig. 3.2).

All procedures and protocols were approved by the Children's Hospital Boston animal care committee and were in accordance with the guidelines of the National Institute of Health and the Society of Neuroscience.

Riboflavin Imaging (by Daniel Brady and Ryoma Hattori)

Surgical Preparation Mice were transiently anesthetized with isoflurane gas (3.5% with O_2), their weight was measured, and appropriate amounts of Nembutal (50 mg/kg, intraperitoneal injection), Chlorprothixene (0.2 mg, intramuscular injection of the left thigh), and Atropine (0.3 mg, subcutaneous injection) were administered. A tube providing supplemental Nembutal (1mg/ml for juveniles, 5 mg/ml for adults) was inserted into the peritoneal cavity to maintain anesthesia during recording. To maintain proper respiration, a custom L-shaped borosilicate glass tube (1.0 mm outer diameter, 0.75 mm inner diameter) was inserted into the trachea. The head was fixed using a standard mouse stereotaxic frame, modified to prevent occlusion of ears. Under sterile conditions, the skin on the top of the head and skull was removed to expose the visual cortex. The dura was removed and the surface of the brain kept moist by a combination of saline and 3% agar. Glass coverslip was fixed to provide a clear window over visual cortex (Fig. 3.3a). Throughout recording, the animal's temperature was maintained at 38°C using a heating pad and rectal thermometer. The eyelids were trimmed and the corneas were covered with silicone oil to prevent drying. In cases when the eyes became dry to form a thin opaque film over the cornea, the eyes were flushed with saline until the film dissolved. At the end of the experiment, mice were euthanized with an overdose of Nembutal and cervical dislocation.

a



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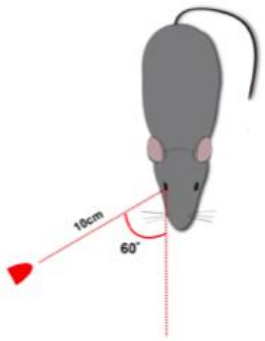


(adopted from Takahashi et al., 2006)

Figure 3.3. Riboflavin imaging setup

(a) Schema of recording setup for riboflavin imaging of mouse visual cortex

(b) Visual stimulation setup. 5 mm red light-emitting diode is placed 10 cm away at monocular location of 60 degree azimuth and 30 degree elevation.

Sensory stimulation The visual stimulus was a 5 mm red light-emitting diode (λ = 630 nm, luminance = 12 cd/m^2). The auditory stimulus was a free field 4.4 kHz piezo buzzer at 70dB. Both stimuli were placed 10 cm away at monocular location of 60° azimuth and 30° elevation towards the contralateral eye and turned on for one second during each trial (Fig. 3.3b).

Trials Cortical images of endogenous green fluorescence (λ = 500-550 nm) in blue light (λ = 470-490 nm) were recorded at eight frames/second using a cooled CCD camera system attached to a dissecting microscope. Each trial lasted for 10s and was composed of 3s pre-stimulation, 1s stimulation, and 6s post-stimulation. 10s resting period followed each trial to allow processing of images. 3 types of stimuli, visual / auditory / visuoauditory, were presented alternately. One run consisted of 10 visual trials, 10 auditory trials, and 10 visuoauditory trials in total (Fig 3.4a). 10 trials (one run) for each type of stimulus were added together and pixel values were divided by the number of trials (Fig 3.4b). Images were normalized with respect to a reference (average of first twenty frames) and passed through a low-pass square filter to improve image quality (Fig. 3.4c). Finally, the normalized images were transformed to a pseudocolor scale (Fig.3.4d).

Analysis Several 10-trial runs of each stimulus (visual, auditory, and blank) were averaged together to reduce noise. To correct photo bleaching over the length of a trial, average visual and auditory movies were divided by the average blank. Regions of interest (primary and secondary visual cortices as determined by stereotaxic coordinates) were demarcated by circular windows (1 mm in diameter) and compared to a reference region outside the area of neuronal activity. Timelines of relative fluorescence changes in ROIs were calculated by subtracting ROI values with the reference. The peak amplitude was calculated by taking the ROI timeline and averaging the frames 0.5 second around the peak. Animals with V1

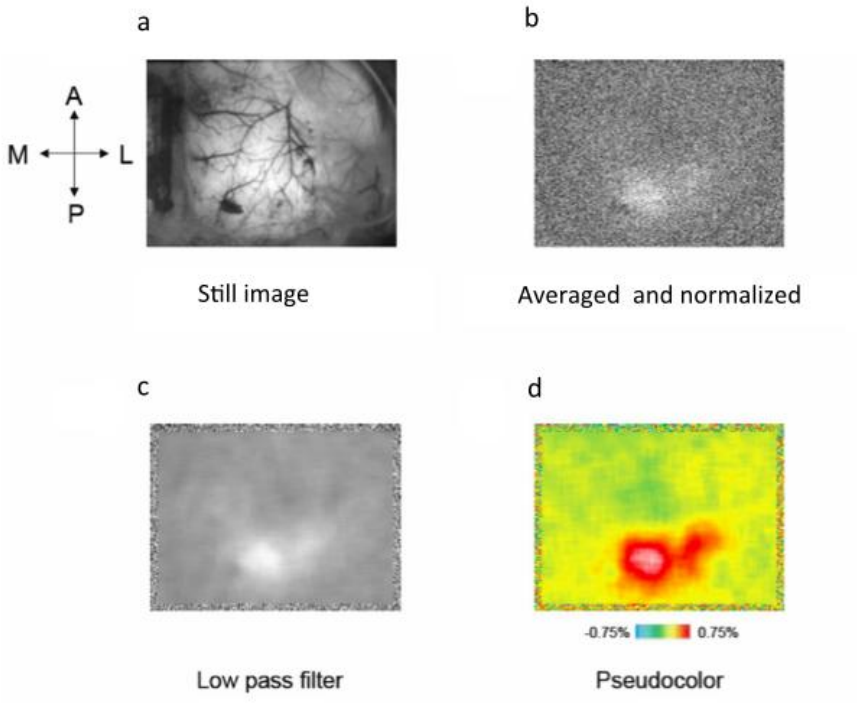


Figure 3.4. Riboflavin imaging process and analysis (images in courtesy of Daniel Brady)

(a) Still images from an original riboflavin fluorescence movie after 30 trials (1 run) in an adult anesthetized C57BL/6 mouse (A: anterior, P: posterior, L: lateral, M: medial).

(b) Resultant image after averaging.

(c) Smoothed image of part (b) after low-pass square filtering (10x10 pixels).

(d) Pseudo coloring of part (c) gives the final image ready for analysis.

visual peak amplitudes less than 0.5% $\Delta F/F_0$ were excluded from analysis because it was likely that its visual system was compromised.

Immunohistochemistry

Sensory stimulation Mice were transferred into a soundproof box, equipped with fluorescent light fixture and speakers. For DR mice, this transfer process was carried out with extra caution to ensure that there was no light penetration using double sealed transfer boxes. Prior to sensory stimulation, mice were kept in complete silence with no light for more than 6 hours to minimize the background level of immediate early gene expression in the visual and auditory brain regions (Fig. 3.5). After >6 hours of adaptation in silent darkness, mice were exposed to either ambient light (generated from 8-watt F8T5 fluorescent bulb) or sound (series of 90dB 4.4 kHz tones at 0.2 Hz frequency). The stimulation lasted for about an hour, enough time for c-Fos to be expressed.

Perfusion Mice were anesthetized with isofluorane gas (3.5% with O_2) immediately after sensory stimulation. Overdose of Nembutal (5 mg/ml, intraperitoneal injection) was administered and mice were taken out of the soundproof box and set up for perfusion. Mice were perfused transcardially with 0.9% saline followed by chilled 4% paraformaldehyde (PFA). Perfusion process lasted no more than 15 minutes per mouse. Brains were removed from the carcass and post-fixed in 4% PFA for 2 hours at 4°C. 4% PFA solution was then replaced with 30% sucrose solution and kept overnight at 4°C for cryoprotection. Fixed and cryoprotected brains were then embedded into O.C.T. compound and frozen into blocks over acetone/dry ice for cryostat sectioning. Brains were stored at -80°C until cutting sections.

Immunohistochemistry 40μm thick coronal sections covering the entire visual and auditory region (ranging from Bregma -5.0 mm to -0.8 mm in adults) were cut on the cryostat and

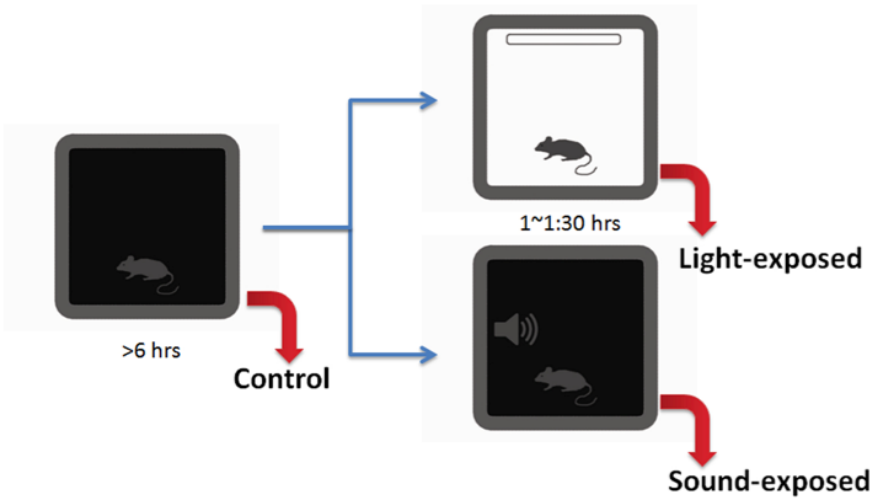


Figure 3.5. Exposure to sensory stimuli for c-Fos analysis

LR or DR mice are placed in soundproof box with no light for more than 6 hours prior to sensory stimulus. Mice are taken out and immediately perfused at the end of this habituation period for non-exposed controls. Mice are exposed to either light or sound stimulus for about an hour and immediately perfused.

systematically collected into wells filled with 0.1M phosphate buffered saline (PBS). Every fourth section were washed in fresh 0.1M PBS and kept overnight at 4°C in blocking solution (0.3% Triton X-100 and 10% normal goat serum in 0.1M PBS). Rest of the brain sections were stored in PBS and kept in -80°C for future use. Blocking solution was switched to primary antibody solution (1:1000 dilution in blocking solution) and incubated at 4°C for two days. Sections were washed in 0.1M PBS three times, each lasting about 10 minutes, and incubated in secondary antibody solution overnight at 4°C (1:500 dilution in 0.3% Trito X-100 and 3% normal goat serum in 0.1M PBS). Finally, sections were washed three times in 0.1M PBS for 10 minutes each and systematically mounted on glass slides. Mounted slides were air-dried and coverslipped with DAPI Fluoromount-G (SouthernBiotech®). Slides were kept at room temperature overnight and stored at 4°C.

Table 3.1. List of antibody used for immunohistochemistry

1º antibody	2º antibody
Anti-c-Fos (Sigma-Aldrich, F7799)	Alexa Fluoro® 488 (Invitrogen, A11008)
Anti-Arc (Synaptic Systems, 156005)	Alexa Fluoro® 594 (Invitrogen, A11076)
Anti-VGlut2 (Millipore, AB2251)	Alexa Fluoro® 546 (Invitrogen, A11074)
Anti-GABA (Millipore, AB175)	Alexa Fluoro® 647 (Invitrogen, A21450)

Image Acquisition Digital stacks that go through the thickness of each region of interest were collected from each brain section using an Olympus laser-scanning confocal imaging system (FV1000^{TM}) on an upright microscope. Primary and secondary visual cortex and auditory cortex, and different thalamic nuclei were identified by comparing morphology of the surrounding structures, such as the hippocampus and white matter to the Mouse Brain Atlas (Paxinos and Franklin, 2001). Step sizes were optimized for each optical section (5μm for 10x,

and 1.14μm for 20x respectively). Image acquisition was accomplished in blind condition, with the samples coded to conceal the type or condition of the brain section from the experimenter.

Retrograde Labeling

Surgical Preparation Mice were anesthetized with isofluorane gas (2.0% in O_2) and body temperature was maintained with a heating pad during surgery. The head was held in place by standard mouse stereotaxic frame. After disinfection of the surgical area, a slit was made into the skin covering the top of the head, exposing the skull that covers visual cortex. The opening was cleaned of any connective tissue using a cotton tip applicator. A small hole was drilled into the skull at the point of injection guided by stereotaxic coordinates and blood vessel patterns (Fig. 3.6). Dura was punctured using a needle. A 28-gauge Hamilton® syringe (701RN) was filled with the tracer solution (1.0 mg/ml Cholera toxin subunit B conjugated to Alexa 488 in PBS, Invitrogen®; reviewed by Conte et al., 2009) and connected to a motorized microinjector (Narishige® IMS10). Syringe was lowered into about 0.35 mm from the surface of the cortex and 300 nl of tracer solution was injected at the rate of 50 ml/min. For juvenile mice at postnatal day 25, 250 nl was injected at 0.3 mm below the surface. The syringe was not pulled out until 10 minutes after the injection completed to prevent spillage of tracer solution. Once the syringe was slowly pulled out, the area was cleaned with 0.9% saline and skin sutured shut. Mice were given Meloxicam every 24 hours for two days after the surgery. For injection into DR mice, extra caution was taken to ensure that the mice were exposed to minimum light. DR mice were anesthetized in darkness, went through surgery with eyes covered with cotton pads bound with tape, and immediately returned to darkness following the surgery. Also, it has been shown previously that plasticity mechanisms are precluded under anesthesia (Imamura and Kasamatsu, 1991).

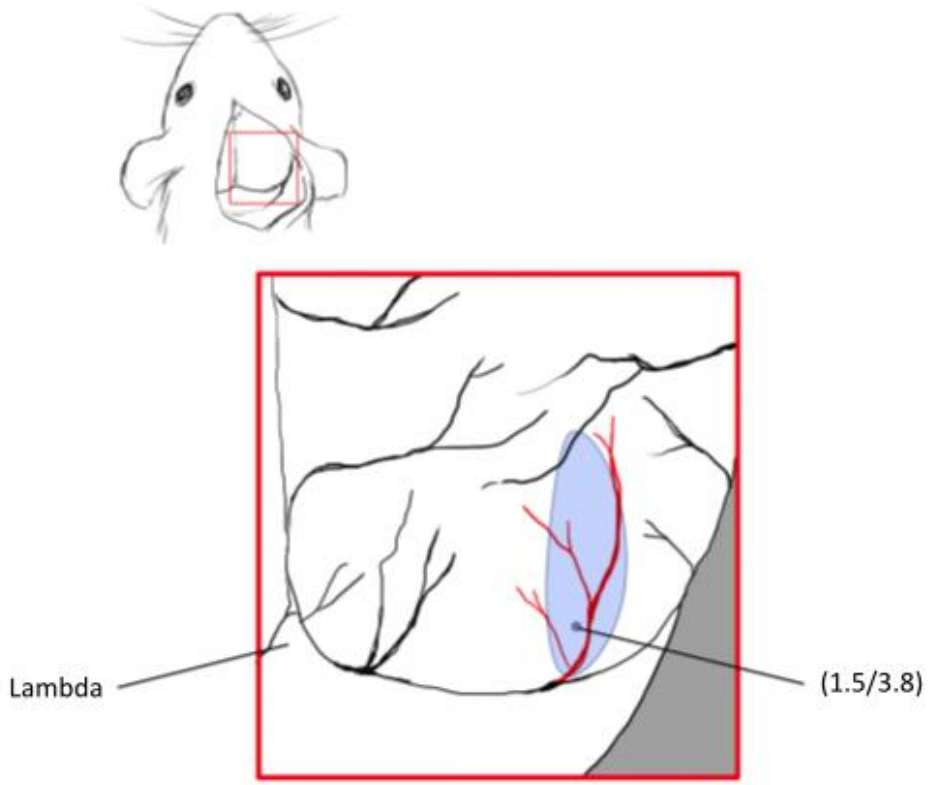


Figure 3.6. V2L injection site

Schema of injection sites for secondary visual cortex (V2L). The region of interest, initially identified through riboflavin imaging, was noted to be along the blood vessel that runs from dorsal to ventral (red). Stereotaxic coordinates (3.8 mm lateral to the midline and 1.5 mm anterior to the lambda) and blood vessel patterns guided further injections. In adults, injections were made along the blood vessel (blue). Because stereotaxic coordinates are different for young brain, injections in juvenile animals relied mostly on blood vessel patterns. Coronal sections of all brains were retrospectively examined to ensure that injection is made in V2L.

Perfusion Mice were perfused three days after the injection. Perfusion protocol was identical to that of immunohistochemistry.

Confocal Imaging Sections were prepared and processed to generate digital stacks from the injected brains. Protocol was identical to that of immunohistochemistry. Brain samples that did not fit the criteria as good injections were not imaged (Fig. 3.7).

Quantification Methods

Image J® (Cell Counter) Digital stacks of each ROI were imported into Image J®. Cell Counter, a plug-in analysis module, was used to manually count the labeled cell bodies in each stack. DAPI and VGlut2 staining (highly expressed in thalamocortical terminals) was used to identify different layers for laminar analysis (Fig. 3.8). Total volume of analysis was calculated by multiplying area of ROI by the thickness of each digital stack. Quantifications were shown as a measure of density (number of counted cells over volume of analysis). Three to six digital stacks were analyzed per brain, and experimenter was blind to the origin of each brain during image acquisition and counting process. Experimenter was blind to the origin of the brain undergoing analysis.

NIS-Elements® (Object Count) Images of each section were digitalized on a Nikon® Eclipse 80i fluorescent microscope. NIS-Elements® Object Count software automatically detects and counts the number of objects (labeled cell bodies) based on user-defined parameters (Fig. 3.9). Threshold for luminance, roundness, and size were slightly modified for each section due to variance in staining, background fluorescence, and tissue condition. Five to six digital sections from each brain covering structurally defined regions of interests (visual cortex, auditory cortex, and the thalamus) were analyzed. Experimenter was blind to the origin of the brain undergoing analysis.

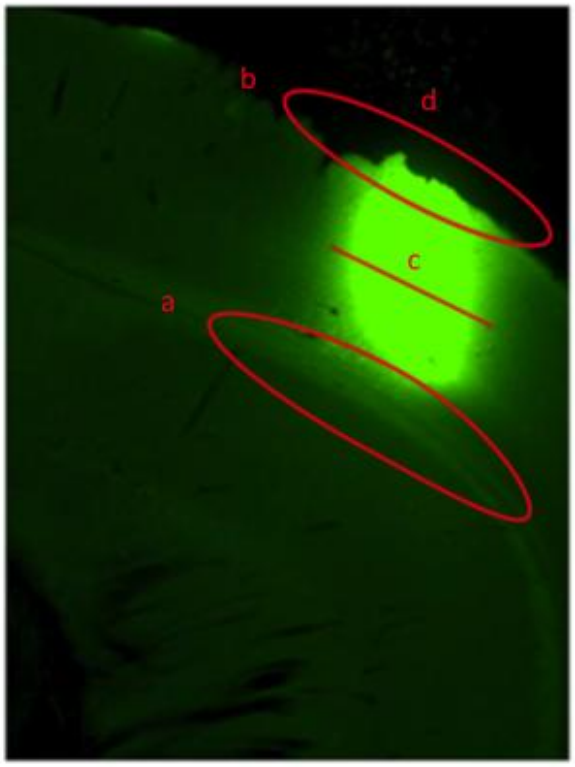


Figure 3.7. Criteria of a successful injection

Four different parameters based on the physical appearance of the injection site were used to determine a successful injection.

(a) First, there should be no tracer contamination in the white matter.

(b) Second, there should be no overspill of tracer on the surface of the cortex.

(c) Third, the halo diameter for injected tracer should be between 200~400 µm.

(d) Lastly, there should be minimum tissue damage at the injection site.

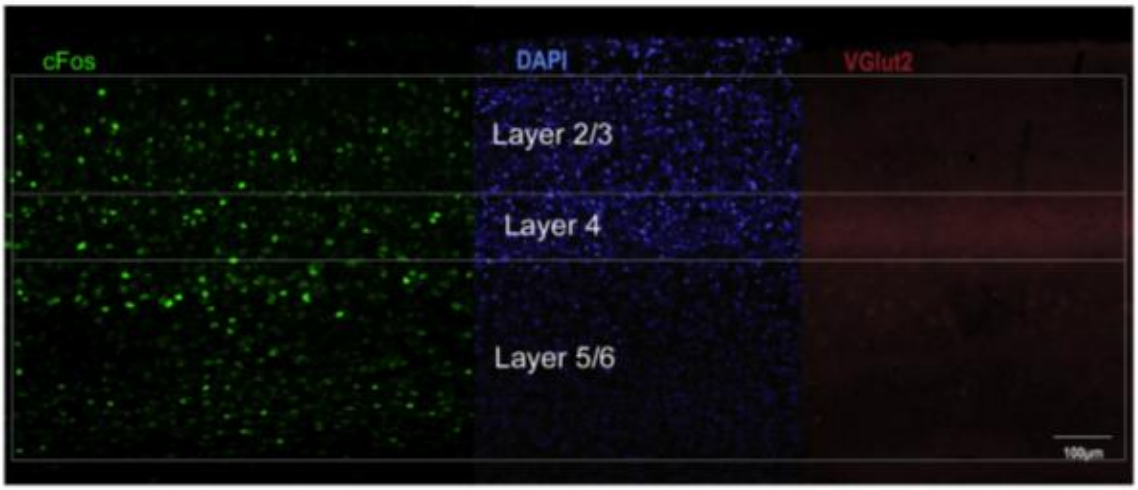


Figure 3.8. Laminar identification in cortical slices

DAPI (blue) and VGlut2 (red) staining were used to identify different cortical layers in each tissue section during laminar analysis. DAPI staining reveals the highly packed cells in layer IV. VGlut2 is expressed in thalamocortical terminals, which terminates in layer IV.

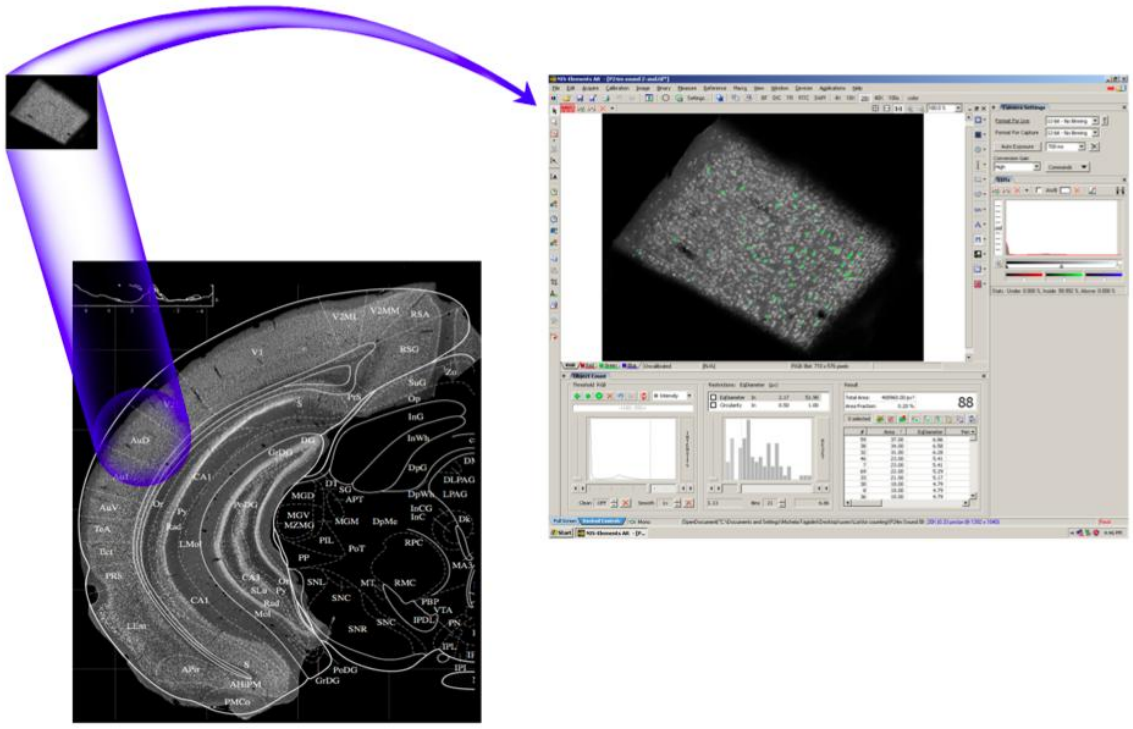


Figure 3.9. NIS-Elements (Object Count)

Region of interest from each section (AuD in this example) is identified using the brain atlas as a reference. Image of the selected area is brought into NIS-Elements software for cell quantification using Object Count. User defines the threshold values for luminance, roundness, and size in the bottom panels, and the software automatically detects and counts the number of objects that fall into these parameters.

Stereo Investigator® Twelve to fifteen consecutive sections from a subset of brains quantified using *Object Count* was re-analyzed using the image analysis program Stereo Investigator® (Version 8.10, Microbrightfield®). ROIs were identified in each section and cortical layers were identified by DAPI and VGlut2 staining. Labeled cell bodies within each ROI were counted using the optical fractionator method (Fig. 3.10). Counting frames (30 x 30 µm) were placed in a virtual grid (150 x 150 µm) randomly generated by the software over the ROI. Manual counting was performed with a 40x objective (n.a. 0.75) for each counting frame. Finally, cell density was calculated by the software, which divided the estimated number of cells within each ROI by the total area of ROI. Experimenter was blind to the origin of the brain undergoing analysis.

Analysis Data collected using Image J®, NIS-Elements®, and Stereo Investigator® were processed in Prism® (from GraphPad Software®). Within group distribution and variability were analyzed through column statistics, and cross group analysis was carried out with unpaired t-test and one-way analysis of variance (ANOVA) with Kruskal Wallis test (statistical methods used for each data set are indicated in the figure legends).

Single-Unit Recording (by Daniel Brady)

Surgical Preparation Mice preparation for Single-Unit Recording was identical to that of riboflavin imaging.

Sensory Stimulation Visual and auditory stimulus was identical to that of riboflavin imaging. However, prior to the experiment, moving bars of light were used to determine the receptive field location of a given penetration. The LED and buzzer were then placed 10 cm away at the appropriate angle.

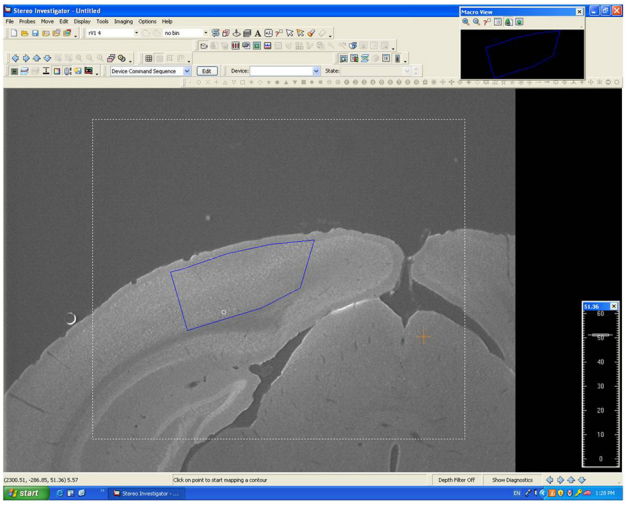
Figure 3.10. ***Stereo Investigator***

(a) Live image of a cortical section to be analyzed is opened in the *Stereo Investigator* software and the user outlines the region of interest.

(b) Counting frames (small squares colored red and green) are placed in a virtual grid randomly generated by the software.

(c) User manually counts the number of labeled cell bodies within each counting frame.

a



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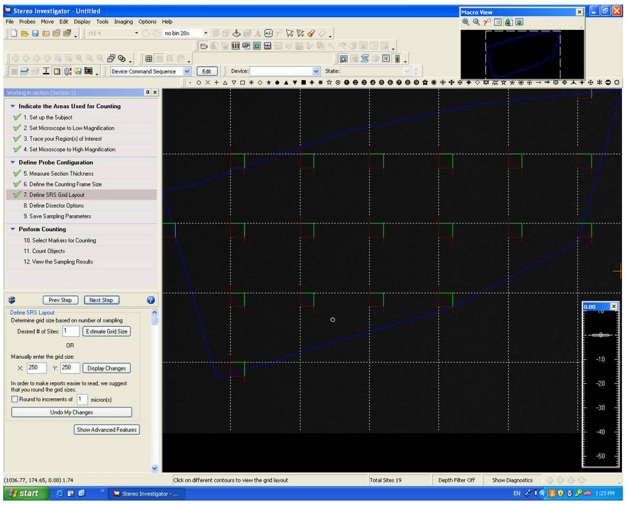

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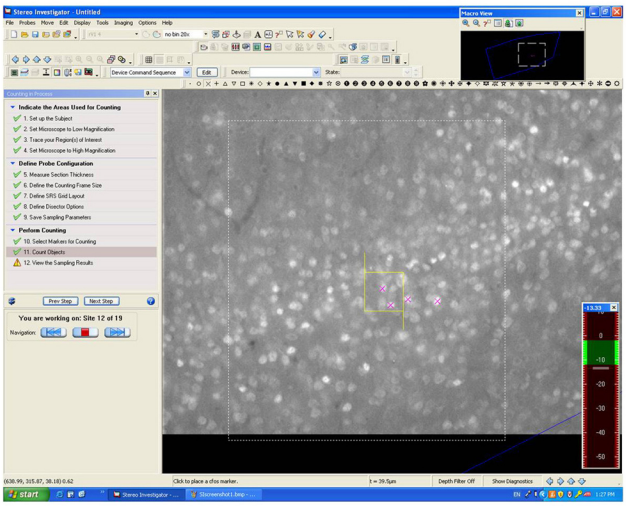


Figure 3.10. (Continued)

Recording A silicone Neuronexus Technologies® probe (linear probe with 16 sites spaced at 50μm intervals, model a1x16-3mm50-177) was inserted at multiple sites along visual cortex to a depth of >800 μm below the pial surface to record cells from all layers. Recordings began in the binocular zone of V1 and progressed anterior-laterally through V2L to the borders of auditory cortex. At all recording sites, each stimulus condition (visual, auditory, visual + auditory, blank) was presented 20 times. Two to eight penetrations were made in each mouse, with each penetration lasting about 30 minutes. The signal was amplified and processed with a threshold, band-pass filter, and discrimination (SciWorks, DataWave or SortClient from Plexon Technologies®). The waveforms of recorded units were further examined offline (Offline Sorter from Plexon Technologies®) and discriminated on the basis of their waveforms to ensure single unit isolation.

Analysis Data from individual cell were processed with customized software designed in Matlab® (from MathWorks®). This involved generating a raster plot, and a peristimulus time histogram (PSTH) in a sliding 25 ms bin with a 2 ms step. Classification of each cell were done by comparing their stimulus evoked firing rate to the blank firing rate using one-way analysis of variance (ANOVA) and Tukey's HSD post-hoc comparisons.

Chapter 4

Cross-modal Activity in Dark-Reared Mice

Introduction

Sensory perception is a vital part of our lives. We experience the world, form thoughts, and interact with one another on the basis of sensory experiences. The mechanism of perception has intrigued many, and has inspired scholarly debates over the years. In the recent decades, thanks to the advent of physiological and molecular techniques, scientists were able to get a more detailed look at how sensory information is received and processed by the brain. It is widely known that sensory information, picked up by sensory organs that are each designed for a specific sensory modality, travels through the circuits of our brain in a modality specific fashion. For instance, visual information that enters our system through the retina is separated from other sensory inputs until it reaches a region in our brain whose function is to integrate sensory information of multiple modalities. The mechanism underlying the development of modality-specific functionality in the cortex, whether it is based on genetic coding or environmental factors or both, continues to intrigue researchers. Interestingly, there are studies that report cross-modal activity in lower visual areas (Sadato et al., 1996; Macaluso et al., 2000; Burton, 2003). Large number of these studies comes from looking at people with visual impairment, which presents a unique opportunity to understand the role of visual experience in cortical development.

Early studies that look at changes in cortical activity found that the occipital region of the blind shows an increase in response during non-visual tasks (Wanet-Defalque et al., 1988; Uhl et al., 1991). Further investigation unveiled different response patterns depending on the type of discrimination task that the participants performed and whether the stimuli were passively or

actively perceived (Kujala et al., 1995; Sadato et al., 1996; Cohen et al., 1997; Merabet et al., 2004). Inactivation of the occipital region in the blind resulted in decrease in performance of other modalities, showing that cross-modal responses seen in visual region are functionally relevant (Cohen et al., 1997; Hamilton et al., 2000). One of the most interesting findings was the effect of age of onset for blindness on cross-modal responses (Sadato et al., 2002; Burton et al., 2002a, b, and 2004; Li et al., 2012). In these studies, the greatest degree of cross-modal activity was seen in the visual cortex of the early but not late blind subjects. This suggests that there is a time window early in life when sensory cortex goes through experience-dependent cross-modal plasticity. Here, we used an animal model of early blindness to characterize cross-modal activity in visual cortex and examined the underlying anatomical substrates.

Animal models with visual deprivation have been useful in exploring the mechanism behind cross-modal plasticity in the blind. Early bilateral eyelid suture in macaque monkeys replicates the observation made in blind humans by showing multimodal response driven by touch in visual cortex (Hyvarinen et al., 1981). Auditory activation in cat visual cortex was observed with bilateral eyelid sutures or bilateral enucleation shortly after birth (Yaka et al., 1999). Similarly, removing the eye in neonatal hamsters and rats caused the occipital cortex to be cross-modally activated (Toldi et al., 1994; Izraeli et al., 2002). However, studies that use large mammals are fundamentally impaired in their ability to perform physiological, anatomical, and genetic manipulations that are necessary. We decided to use mice for our study to take advantage of the genetic tools, which allow us to dissect the molecular mechanism behind cross-modal plasticity. In addition, we have a detailed understanding of the anatomical and functional changes in mouse visual cortex during development through previous studies (Gordon and Stryker, 1996; Antonini et al, 1999; Hensch, 2005; Hubener, 2006; Wang et al., 2007; Marshal et

al., 2011). Such prolific knowledge and availability of various techniques makes the mouse a good animal model for this study.

Here, we used mice reared in darkness from birth (DR) as a model of human blindness in order to elucidate the cellular and molecular substrates underlying cross-modal plasticity with minimal interference. First, we identified a region in visual cortex that responds to sound in DR mice. Second, we characterized neuronal circuits that underlie cross-modal activity.

Results

Cross-modal activity in visual cortex of dark-reared mice (DR)

First, we examined visual cortical response to auditory or visual stimuli in adult mice reared in complete darkness from birth (DR) or in normal 12 hours light/dark cycle (LR). Changes in autofluorescence of mitochondrial flavoproteins in synapses were used as a measure of neuronal activity (Shibuki et al., 2003; Reinert et al., 2004; Tohmi et al., 2006), and found that the primary visual cortex (V1) and a adjacent region responded much more strongly to sound in DR than in LR (Fig. 4.1a). Injecting a fluorescence marker into the area of maximum sound response in DR identified the region as the lateral secondary visual cortex (V2L) (Paxinos and Franklin, 2001) (Fig. 4.1b). Comparison to recent description of areal organization in mouse visual cortex using stereological coordinates suggests that this strong sound response occurs in the anterolateral extrastriate area (AL) and covers the lateromedial extrastriate area (LM) as well (Wang and Burkhalter, 2007).

Since riboflavin imaging only measures population activity in superficial layers (Tohmi et al., 2006), sensory activity in DR and LR was given a closer look by examining c-Fos expression patterns in visual and auditory cortical areas after light or sound exposure in awake behaving animals. *c-fos* is an immediate early gene that is transcribed when neurons fire action potentials, and is widely used as a marker of neuronal activity (Sagar et al., 1988; Sheng and Greenberg, 1990). Baseline levels of c-Fos expression for each regions of interest were measured from animals that were kept in silence and darkness for more than 6 hours (Fig. 4.2, red line). Immunohistochemical analysis showed significant increase of c-Fos expression after 1 hour-long light exposure compared to the baseline levels in both LR and DR visual cortex (Fig. 4.2a). In contrast, cross-modal sound stimulus elicited increase in c-Fos expression only in DR

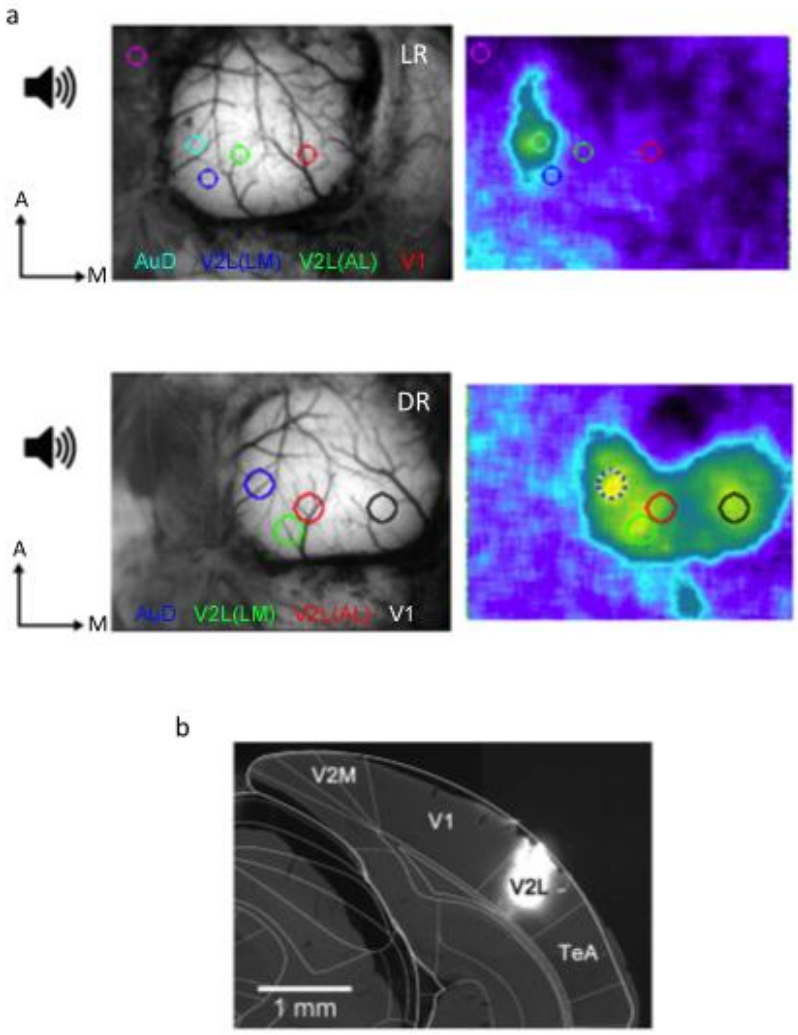


Figure 4.1. Auditory response in visual cortex of dark-reared mice (DR)

(a) Original riboflavin fluorescence and pseudo color images after auditory stimulation in visual cortex of anesthetized C57BL/6 adult mice (>P60) raised in normal 12-hour light/dark cycle (LR, top row) or 24-hour darkness (DR, bottom row). *Data generated and analyzed by Ryoma Hattori.*

(b) Alignment of retrograde tracer injection (Cholera toxin subunit B conjugated to Alexa 488) over the region of maximum auditory response (green circle in bottom row of part (a)) with the Mouse Brain Atlas (Paxinos and Watson, 2001) confirms the area as lateral secondary visual cortex (V2L).

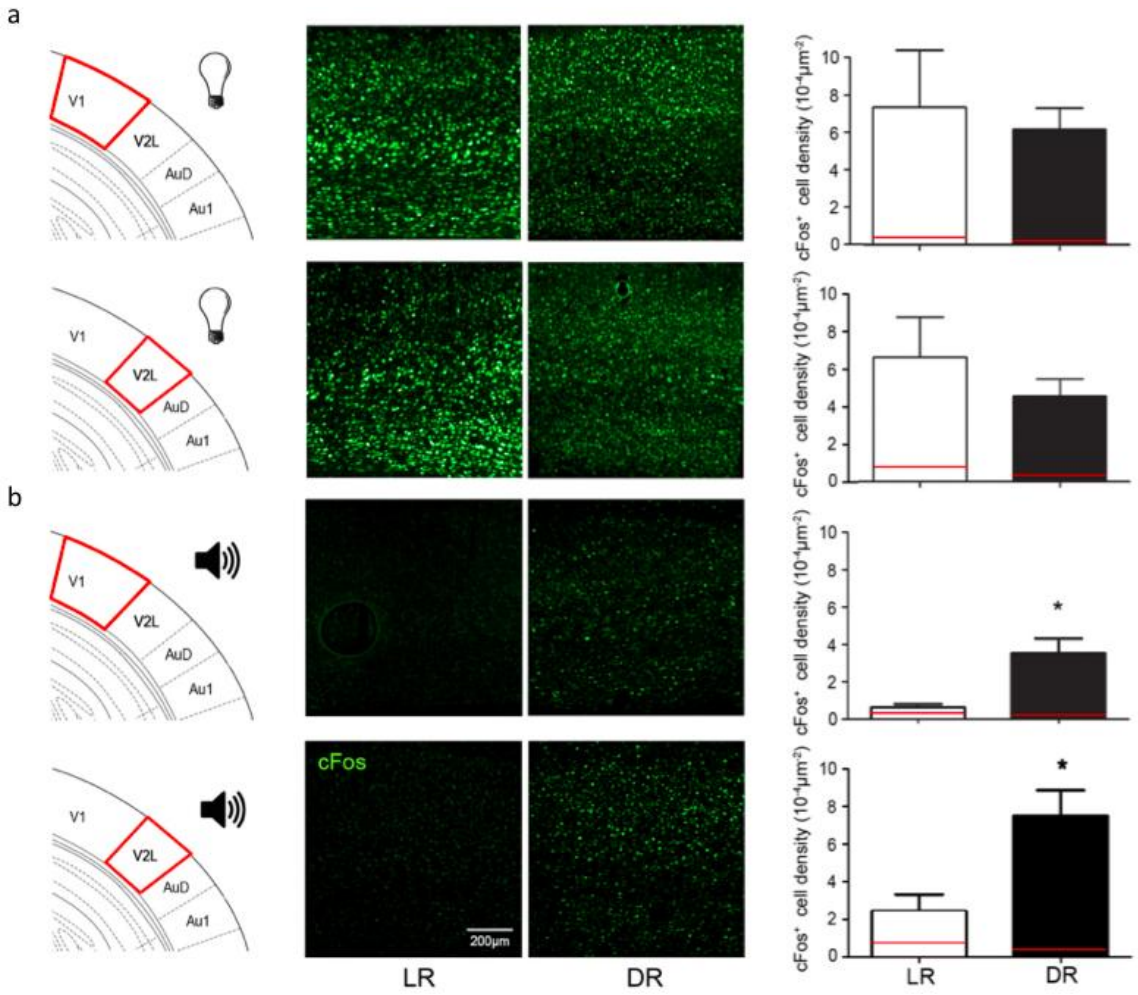


Figure 4.2. Neuronal activity in visual cortex after exposure to light or sound

(a) c-Fos expression level in visual cortex of LR and DR mice were examined after being exposed to ambient light for an hour (n=4 for LR, n=5 for DR). Both V1 (top) and V2L (bottom) show significantly increased number of c-Fos$^+$ cells compared to the controls (n=4 for LR, n=5 for DR; red line=control mean; $P<0.05$, unpaired t-test with Welch's correction). There is no significant difference between LR and DR visual cortex response to light ($P=0.74$, unpaired t-test with Welch's correction).

(b) c-Fos expression in LR and DR visual cortex after exposure to 1s long 5 kHz tone repeated every 5s for an hour (n=6 for LR, n=7 for DR). c-Fos expression is dramatically increased above control level in DR visual cortex in response to sound (V1: $P=0.0057$; V2L: $P<0.0001$), and are significantly different from LR (*$P<0.05$, unpaired t-test with Welch's correction). LR V2L also shows an increase in c-Fos expression following sound exposure ($P<0.05$, unpaired t-test with Welch's correction).

c-Fos expression levels (mean ± s.e.m.) are quantified using *NIS-Elements® Object Count* and normalized for the area of analysis in each animal.

visual cortex (Fig. 4.2b). Examination of c-Fos expression in auditory cortex showed no difference between LR and DR in response to light or sound stimulus (Fig. 4.3). The absence of visual response in the auditory cortex of DR strongly suggests that cross-modal response is exclusive to the sensory cortex specific to the deprived sensory modality (Fig. 4.3 left column). Thus, c-Fos results show that cross-modal activity occurs specifically in the cortex of the deprived modality. It is interesting to note that V2L and AuD of both LR and DR showed an increase in c-Fos expression in response to cross-modal stimuli (Fig. 4.2b bottom row; Fig. 4.3 left column, top row). Such cases of cross-modal responses in regions that are at the border of sensory areas of different modalities have been previously observed, and suggest cross-modal modulation of the sensory cortex (Kayer & Logothetis, 2007; Musacchia & Schroeder, 2009).

We next obtained laminar information of the cross-modal response to better understand the pattern of activity. It is known that each cortical layer is composed of different neuronal subtypes with distinct input and output circuitry (Lund, 1988; DeFelipe, 1993). For instance, the cortex receives perceptive information from the thalamus from thalamus at layer 4. Supragranular layer 2/3 makes corticocortical connections from other areas of the cortex, and infrogranular layer 5/6 sends projections to extra-cortical structures. Detailed quantitative laminar analysis of c-Fos expression was carried out in a subset of brains from sound exposed LR and DR using Stereo Investigator®. Each layer was identified with DAPI staining, which visualizes the dense population of cell bodies in layer 4, and VGlut2 staining, which labels the thalamocortical terminals in layer 4 (Nahmani and Erisir, 2005) (Fig. 4.4a). Our result showed cross-modal activation across all layers in the visual cortex of DR (Fig. 4.4b). This may be due to prolonged duration of exposure, which lasted an hour: enough time to engage the local circuitry across layers and activating neurons beyond the population that receives direct input.

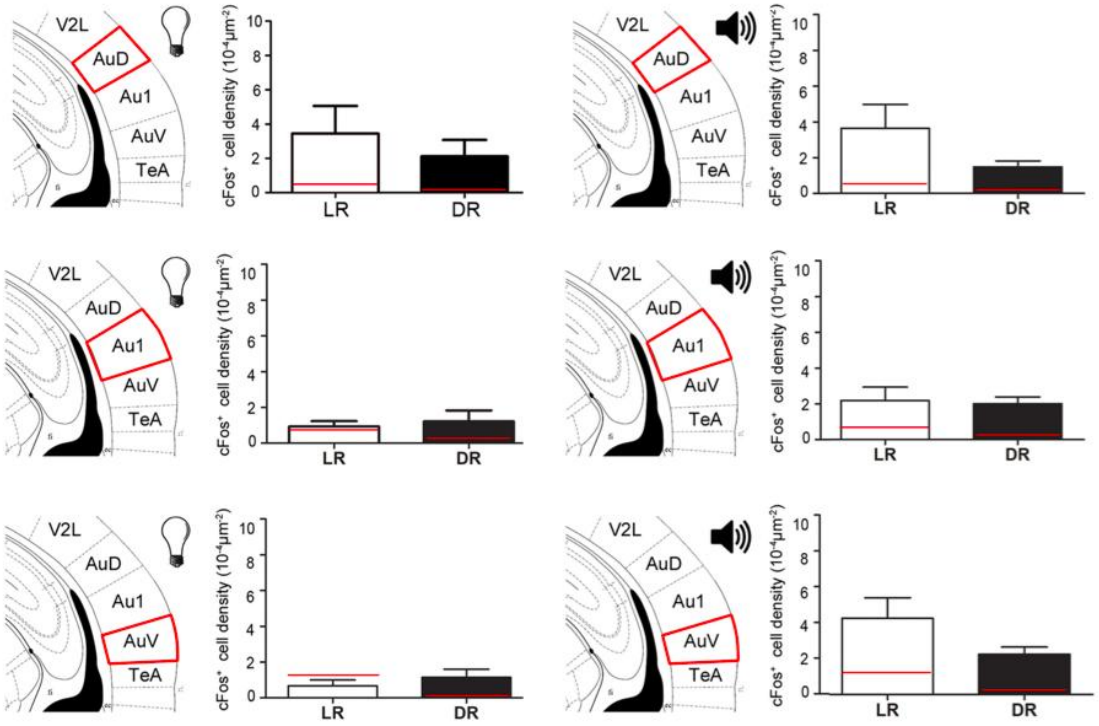


Figure 4.3. Neuronal activity in auditory cortex after exposure to light or sound

c-Fos expression analysis is shown for dorsal secondary auditory cortex (AuD, top row), primary auditory cortex (Au1, middle row), and ventral secondary auditory cortex (AuV, bottom row) of the same animals analyzed in figure 4.2. Cortical activities in LR and DR do not differ significantly regardless of the stimulus type ($P>0.05$, unpaired t-test with Welch's correction). Auditory cortex of LR and DR is activated by tone (right column) but not by light (left column) with the exception of AuD of both LR and DR where an increase above control levels is observed ($P<0.05$, top left).

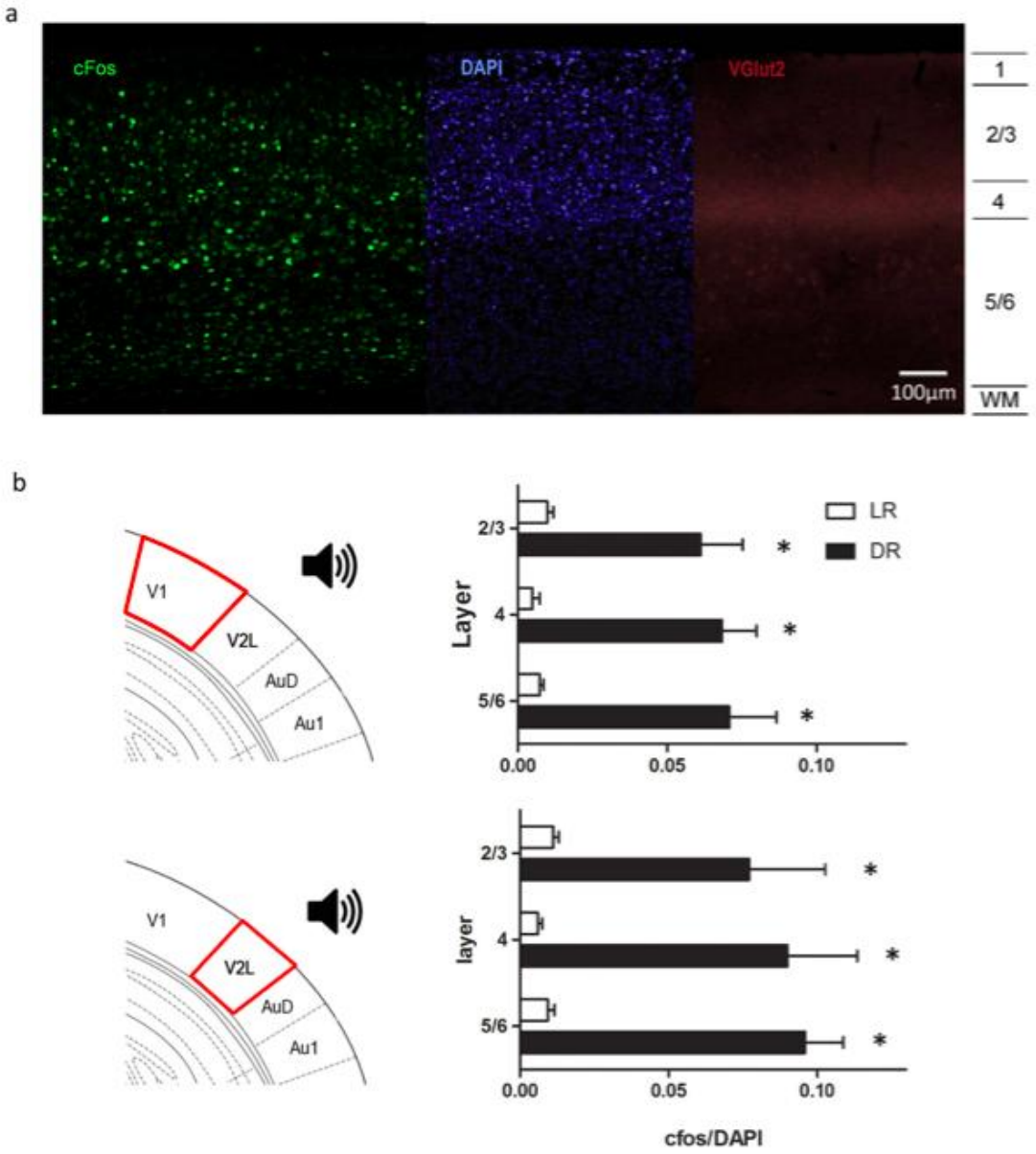


Figure 4.4. Cross-modal activity in DR visual cortex activates all cortical layers

(a) Sections are co-stained for c-Fos, DAPI, and VGlut2 for laminar analysis. Cortical layers are separated according to DAPI and VGlut2 labeling, and c-Fos$^+$ cells are quantified for each layer.

(b) Quantification shows that both V1 (top) and V2L (bottom) of DR mice (n=5) are activated across all layers by tone stimuli compared to LR (n=5, *P<0.005, unpaired t-test with Welch's correction).

Laminar c-Fos expression levels (mean ± s.e.m.) are shown as a ratio of c-Fos$^+$ cells over total number of neurons within each region of interest. *Stereo Investigator®* was used to quantify the c-Fos$^+$ cells and DAPI-stained nucleus for each region of interest.

Majority of DR V2L neurons respond to cross-modal input

To determine whether cross-modal activity arose from a distinct population of auditory neurons in DR visual cortex or from population of multimodal cells, we recorded from individual neurons with extracellular single-unit electrodes. Multiple recordings were made in each animal covering the entire visual region and some auditory region as well. We found that the majority of cells in DR V1 are visual cells that are strongly modulated by auditory input (Fig. 4.5a). On the other hand, in DR V2L, the majority of cells responded to both light and sound (Fig. 4.5b). In DR V1, 30% of responsive cells recorded were multimodal, with 69% classified as visual only, and 1% auditory only (Fig. 4.5c). In DR V2L, 40% were multimodal, with 47% classified as visual only, and 13% auditory only. Overall, the percentage of auditory responsive neurons in V1 and V2L of DR was significantly higher than in LR V2L, where 5% of cells were multimodal, 95% visual only, and <1% auditory only. Our single-unit recording results shows that cross-modal response in DR V2L involves activation of neurons that respond specifically to auditory inputs and multimodal neurons that respond to both auditory and visual inputs. Around 30% of the total auditory responsive population in DR V2L exhibited a short response latency to sound (Fig. 4.5d). The average auditory peak latency in DR V2L were faster than visual peak latency from the same region, with some showing the same timescale as been recorded in primary auditory cortex (Linden et al., 2003).

For experiments that followed, we decided to focus on the DR V2L, rather than V1. We aimed to investigate the anatomical source of the auditory input that could lead to the short peak latency and large number of auditory-only responsive neurons in DR V2L.

Figure 4.5. Majority of DR V2L neurons respond to cross-modal input (data generated and analyzed by Daniel Brady)

(a) Raster plot and peristimulus time histogram (PSTH, 25 ms bins) for a multimodal cell in DR V1. Trials are divided into the different stimulus conditions: visual (yellow), auditory (blue), visual+auditory (red), and blank (white). Bold and dotted black lines indicate when the stimulus turned on and off respectively. The blue dotted line in the PSTH is the blank response. The multimodal response is much stronger than to just vision alone.

(b) Raster plot and PSTH for a multimodal cell in DR V2L. This cell shows strongest response to sound stimulus.

(c) The distribution of visual (yellow), auditory (blue), and multimodal (red) cells in DR V1 (n=95), DR V2L (n=147), and LR V2L (n=131). A significant larger portion of cells respond to auditory stimuli in DR than in LR V2L (*P<0.0001, χ^2 test).

(d) Cumulative distribution function of peak latencies in DR V2L shows faster auditory (blue, n=50) than visual (black, n=88) responses (**P<0.004, two-sample Kolmogorov-Smirnov test)

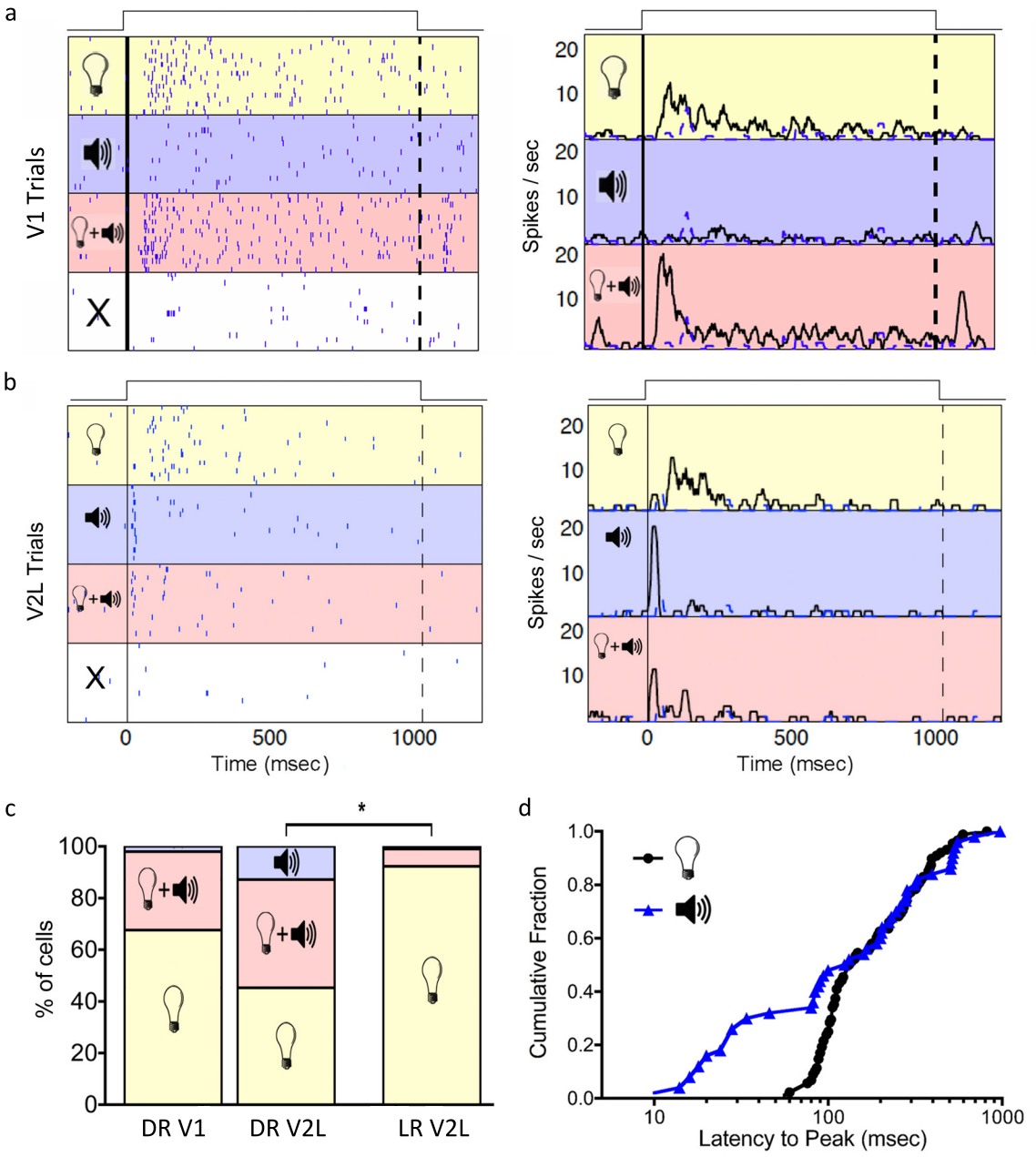


Figure 4.5. (Continued)

Cross modal response include both excitatory and inhibitory neuronal activities

There are multiple types of neuron in the cortex that can be classified by functional properties and molecular composition. Understanding the types of neuron involved during cross-modal activity can help uncover the functional significance of this response. As the first step, activation of inhibitory neurons in response to sensory stimuli was examined by co-staining c-Fos with γ-Aminobutyric acid (GABA), an inhibitory neurotransmitter (Fig. 4.6a). Comparison between LR and DR showed no significant difference in the ratio of inhibitory cells activated in response to either specific or non-specific stimuli (Fig. 4.6b). However, there are numerous subtypes of inhibitory neurons that cannot be classified with GABA staining alone (Gonchar and Burkhalter, 1997) as well as excitatory neuronal subtypes that serve distinct functions (Lodato et al., 2011). Thus, it is possible that further analysis looking at different neuronal subtypes will show a difference in the composition of cross-modal versus specific sensory responses.

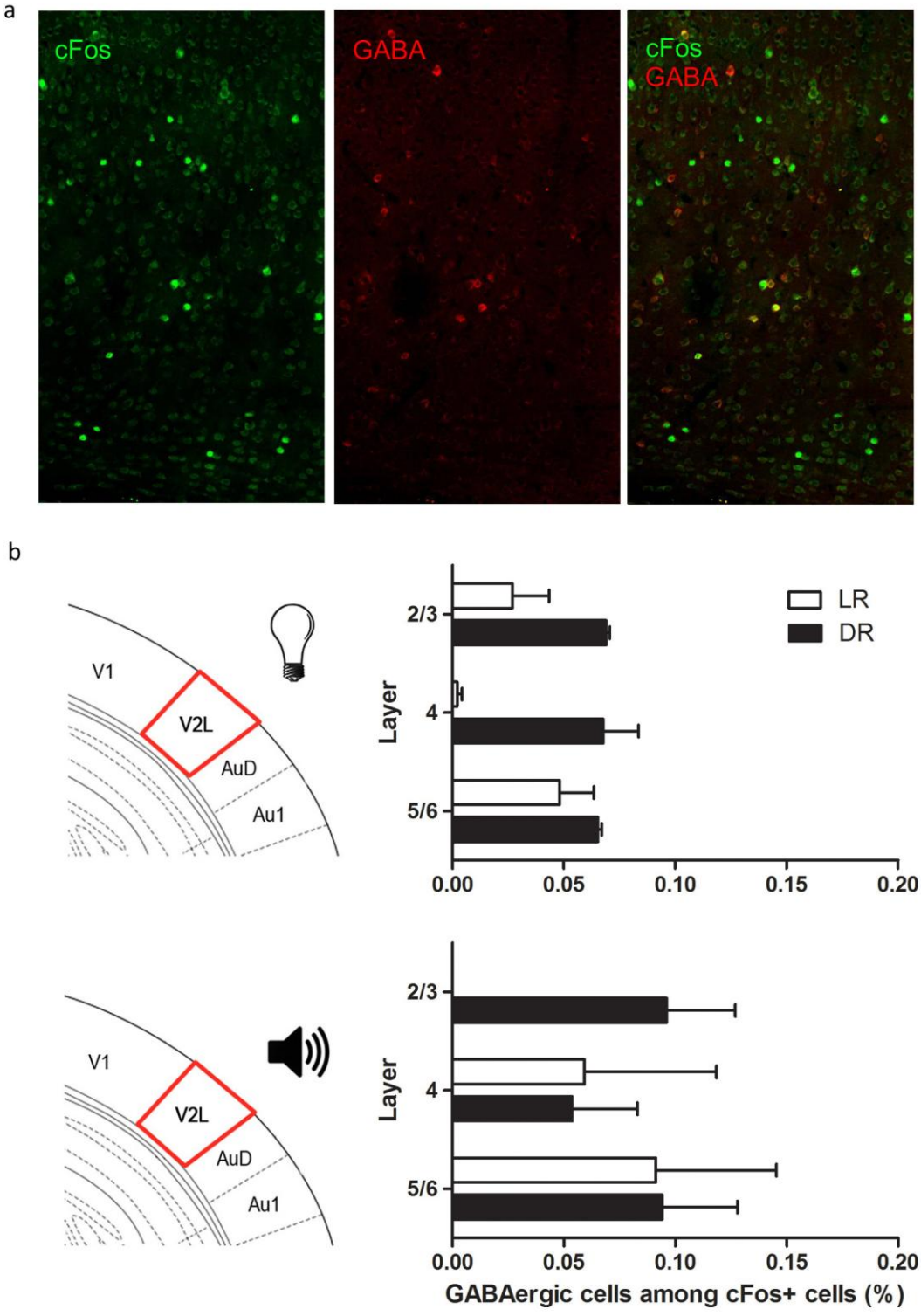
Interestingly, although not in direct relation to the cross-modal response in DR V2L, there was an incidental finding during the process of quantifying GABAergic cells. Abnormally high proportion of GABAergic cells was found to be activated in a subset of DR brains exposed to light. Retrospective examination revealed that DR brains with high inhibitory response have been exposed to light using the old protocol (Fig. 4.7a), where light exposure was carried out in mice that had gone through sound exposure the day before (Fig. 4.7a lower half). In these mice with previous sound exposure (DR Light w/ Pre-S), about half of the V2L neuronal population activated by light was inhibitory, despite the fact that more than 6 hours of darkness and silence separated the two exposure sessions (Fig. 4.7b). In contrast, for V2L of DR exposed only to light, about 10% of c-Fos^{+} cells were inhibitory, similar to that in LR. It is also interesting to note that the number of vision activated neurons in DR V2L decrease with previous sound exposure. We currently have no clear understanding of how this cross-modal modulation occurred with

Figure 4.6. Ratio of inhibitory cells among c-Fos$^+$ cells in V2L

(a) V2L from a DR exposed to sound co-stained with c-Fos and GABA.

(b) Ratio of GABAergic cells among c-Fos$^+$ cell in V2L. Among neurons that express c-Fos in response to light (top), about 5% are GABAergic in both DR (n=3) and LR (n=5). No significant difference is found between LR and DR (n>0.05, unpaired t-test with Welch's correction).

Laminar GABAergic activation (mean $\pm$ s.e.m.) is quantified by diving the number of c-Fos$^+$ cells by the number of cells co-expressing c-Fos and GABA. *Image-J®* was used to count the number of c-Fos$^+$ cells and cells that express both GABA and c-Fos within each region of interest.



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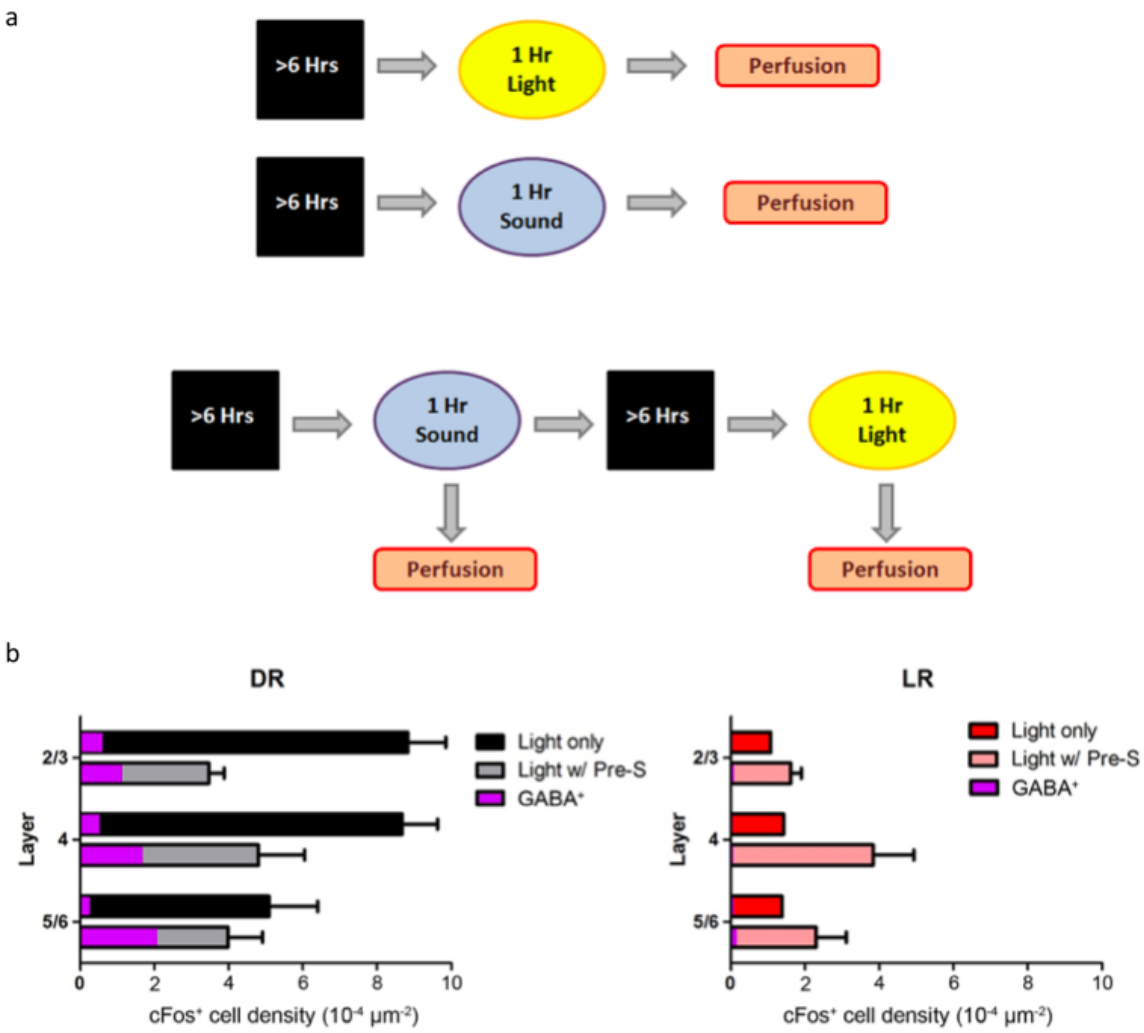


Figure 4.7. Increased inhibitory response in DR during light stimulation with previous sound stimulation

(a) Two different protocols used for sensory stimulation for c-Fos experiments. The new protocol (above) carries out the light and sound exposure separately at different times (Light only; Sound only). The old protocol (below) takes out half of the mice to be perfused after exposing all the mice in the soundproof box to sound stimulus, and leave the rest in the soundproof box for more than six hours. The remaining half are exposed to light stimulus and perfused (Light w/ Pre-S).

(b) Number of GABAergic neurons among c-Fos$^+$ cells. In the DR, close to half of all c-Fos$^+$ cells during light exposure are inhibitory for mice exposed to sound stimulus more than six hours ago (DR Light w/ Pre-S; n=3). In DR with light exposure only, about 10% of c-Fos$^+$ cells are inhibitory (DR Light only; n=3). Previous sound exposure in LR did not affect the number of GABAergic cells activated by light (LR Light only; n=1, LR Light w/ Pre-S; n=4).

c-Fos$^+$ cell and c-Fos$^+$/GABA$^+$ cell density were quantified using *ImageJ*.

sensory stimulation from more than 6 hours ago. Functional impact and underlying mechanism of this long lasting cross-modal modulation in DR visual cortex is a subject of future investigation.

Ectopic cross-modal inputs from auditory pathway into DR V2L

It is unclear from previous studies how auditory signals reach occipital cortex in the visually deprived. Dynamic causal modeling of human cross-modal responses suggests intracortical connections as the primary pathway (Klinge et al., 2010), while non-human anatomical experiments have led to various results that involve heteromodal corticocortical pathways and sub-cortical structures (Falchier et al., 2002; Cappe and Barone, 2005; Laemle et al., 2006; Chabot et al., 2007). In order to clarify the anatomical source of cross-modal response in visual cortex of DR mice, we looked at sensory response in the thalamic nuclei using c-Fos immunohistochemistry and conducted retrograde tracing to compare regions that project to V2L in DR and LR mice.

Based on previous studies that show subcortical reorganization (Pallas et al., 1990; Zeng et al., 2009), we first examined whether or not the visual thalamic nucleus (lateral geniculate nucleus: LGN) could be the source of auditory input. Using VGlut2 as a marker for different thalamic nuclei, levels of c-Fos expression in response to light or sound stimuli were analyzed for visual LGN and auditory medial geniculate body (MGB) (Fig. 4.8a). Quantification showed that DR LGN responds specifically to light, and not to cross-modal sound stimuli (Fig. 4.8b). MGB also showed specific response to sound stimuli. Similar to the auditory cortex response to sound, MGB activity was not so robust. This may be due to the pure tone sound stimulus, which activates only a small population of neurons that are specific to that tone frequency.

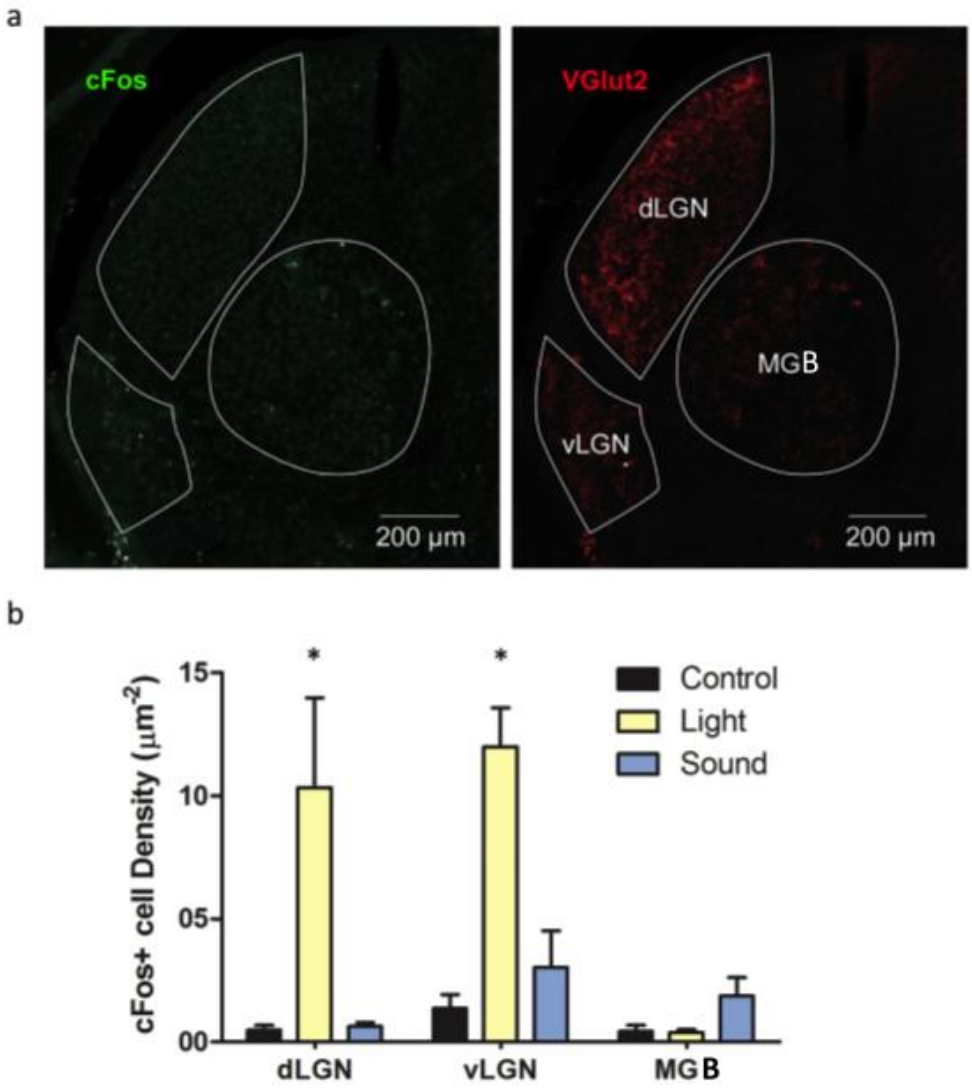


Figure 4.8. DR Thalamic nuclei are sensory-specific

(a) Dorsal lateral geniculate nucleus (dLGN), ventral lateral geniculate nucleus (vLGN), and medial geniculate body (MGB) are identified based on pattern of VGlut2 staining (right). c-Fos expression in each nucleus is examined in DR mice exposed to either light or sound.

(b) Quantification of c-Fos expression shows that LGN responds specifically to light, and not to cross-modal stimuli (n=3 for control, n=4 for light and sound exposure, *P<0.05, ANOVA). MGB also shows that it responds only to auditory stimuli although statistically insignificant (P=0.11, ANOVA Kruskal-Wallis test).

c-Fos expression levels (mean ± s.e.m.) are quantified using *NIS-Elements® Object Count* and normalized for the area of analysis in each animal.

Nevertheless, our data show that LGN responds specifically to vision, and is unlikely the source of auditory input into V2L of DR mice.

To identify any ectopic connections that reach visual cortex in DR mice, we injected retrograde tracer (cholera toxin subunit-B conjugated to Alexa 488: CTB-488) into V2L (Fig. 4.9a). The relatively large injections (300nl per animal) were targeted to V2L through stereotaxic coordinates and blood vessel patterns based on flavoprotein imaging. However, when cross sections of each brain were retroactively examined, about half of the attempted injections did not hit the V2L as defined by the Mouse Brain Atlas (Paxinos and Franklin, 2001) and were not used for analysis. Analysis was carried out with the experimenter blind to the rearing condition of each mouse. Quantification of CTB^{+} cells showed labeled cell bodies in the dorsal secondary auditory cortex (AuD) in both LR and DR (Fig. 4.9b). It was not surprising to find such corticocortical connections, since such intracortical networks have been observed even in normal LR animals (Budinger et al., 2000; Campi et al., 2010). Also, the large number of CTB labeled cells in AuD of DR mice concurred with previous studies that showed intracortical connections to be the primary source of cross-modal responses in the blind (Klinge et al., 2010; Laramee et al., 2011). More importantly, we identified direct projections coming from the auditory thalamus (medial geniculate body: MGB) into V2L in DR (Fig. 4.9c). These cross-modal thalamocortical connections were absent in LR. This result suggests two different auditory pathways that reach V2L in DR mice. The cross-modal thalamocortical input explains the single-unit data that shows fast auditory responses that comprise around 30% of total auditory responsive population in DR V2L (Figure 4.5d).

Because the site of injection was adjacent to the auditory and association cortex there is the possibility that cortical maps in DR mice shifted, and that the injections were actually made in the auditory region rather than the intended V2L. Also, with the large injections it is hard to

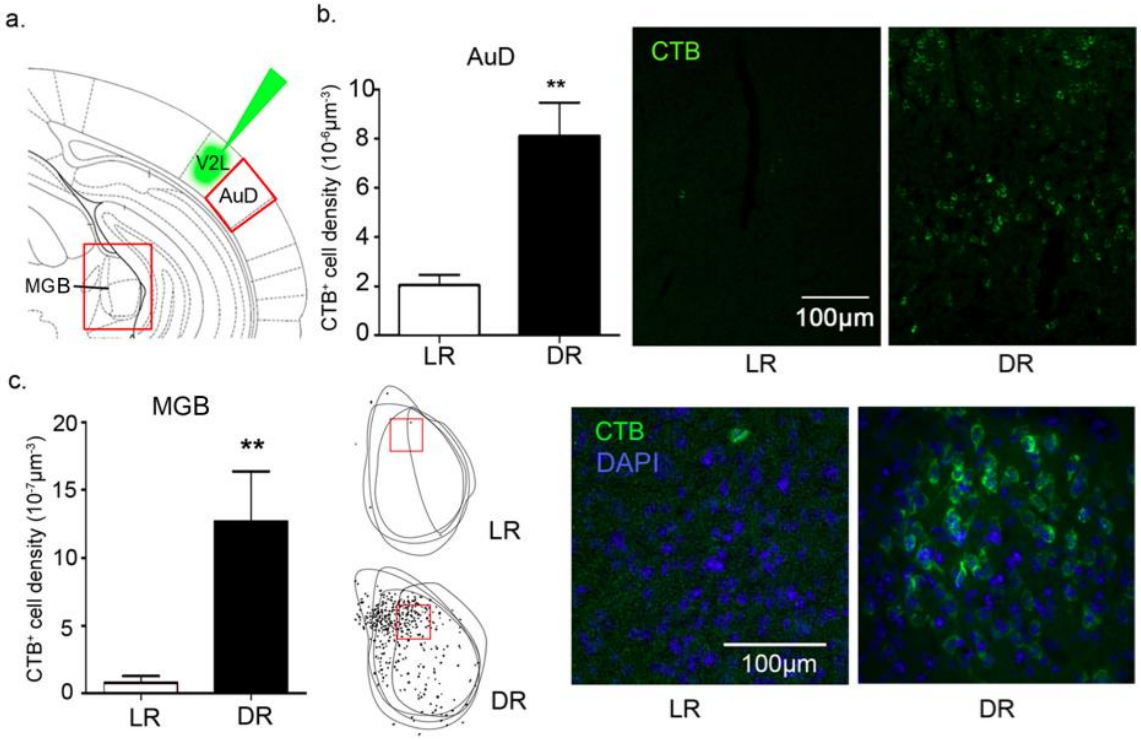


Figure 4.9. Ectopic cross-modal connections in DR V2L

(a) Schema of retrograde tracing experiment. Retrograde tracer (CTB-488) was injected into V2L of DR and LR animals. Labeled cell bodies were found in AuD and MGB, and quantification was carried out in the two regions.

(b) Representative images of AuD in LR and DR after injection (right). Quantification of CTB+ cell bodies illustrates greater connectivity between V2L and AuD in DR (n=6) than in LR (n=5, **P=0.0036, unpaired t-test).

(c) Representative images (right), camera lucida reconstruction (middle), and quantification (left) in MGB. This shows direct projections from auditory thalamus to V2L in DR mice (n=6), which is absent in LR (n=6, **P=0.0098, unpaired t-test).

CTB^+ cell density was calculated using *ImageJ.*

carry out any mapping analysis in detail. We can argue that the injections were indeed made in V2L based on incidental injections made in auditory regions lateral to V2L (dorsal secondary auditory cortex: AuD), which showed projections from associative thalamic regions rather than MGB for both LR and DR (data not shown). However, this study should be complemented with a clear way to identify borders of different cortical regions through techniques such as callosal connection labeling or m2AChR expression (Wang et al., 2011).

Projection pattern of cross-modal thalamocortical inputs in DR mice

It was noted that CTB-488 injection at different sites of V2L resulted in uneven distribution of CTB^+ cells within DR MGB across different animals. Color-coding the site of injection within V2L and matching it to the corresponding MGB produced a rough picture of projection pattern (Fig. 4.10). The auditory thalamus is organized into primary sensory thalamic nuclei (ventral division of MGB: MGBv) and non-primary sensory nuclei (dorsal division of MGB: MGBd). Each is known to be involved in different functions and to project to distinct auditory regions in non-deprived animals (Arnault and Roger, 1990; Hackett et al., 2011). Each subdivision can be delineated by differential distribution of calcium-binding proteins (Lu et al., 2009). Staining the tissues from CTB-488 injected DR for calbindin (CB), present in MGBd, and parvalbumin (PV), present in MGBv, showed that the distribution pattern of CTB^+ cells corresponds to these subdivisions of MGB (Fig. 4.11). From our preliminary observation, it seems like MGBd, which sends projections to secondary auditory cortex in normal LR mice (Llano and Sherman, 2008), projects to LM extrastriate area in DR mice. In contrast, MGBv, which normally projects to primary auditory cortex, projects to AL extrastriate area in DR. The significance of this discovery is unclear at this point, but some thoughts will be discussed in chapter 6.

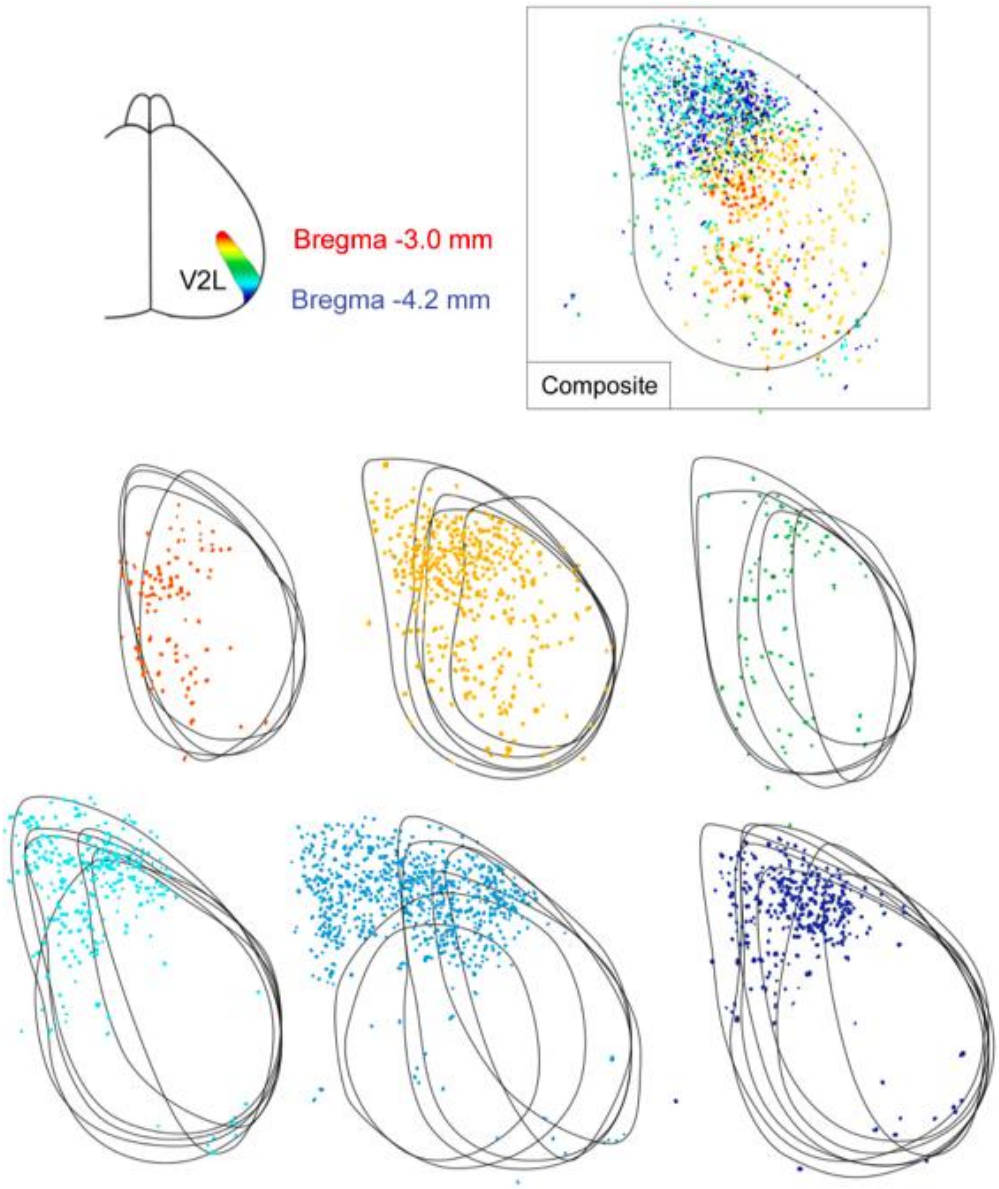


Figure 4.10. Location of V2L injection and distribution of CTB^{+} cells in MGB of DR

The distribution of CTB+ cells in DR MGB is color-coded to match their site of injection in V2L (top left). Five to eight MGB sections from total of six DR mice were analyzed (bottom). The resulting composite image (top left) indicates that ectopic inputs coming from dorsal part of MGB projects to the posterior V2L, while inputs originating in the ventral part of MGB terminates in the anterior V2L.

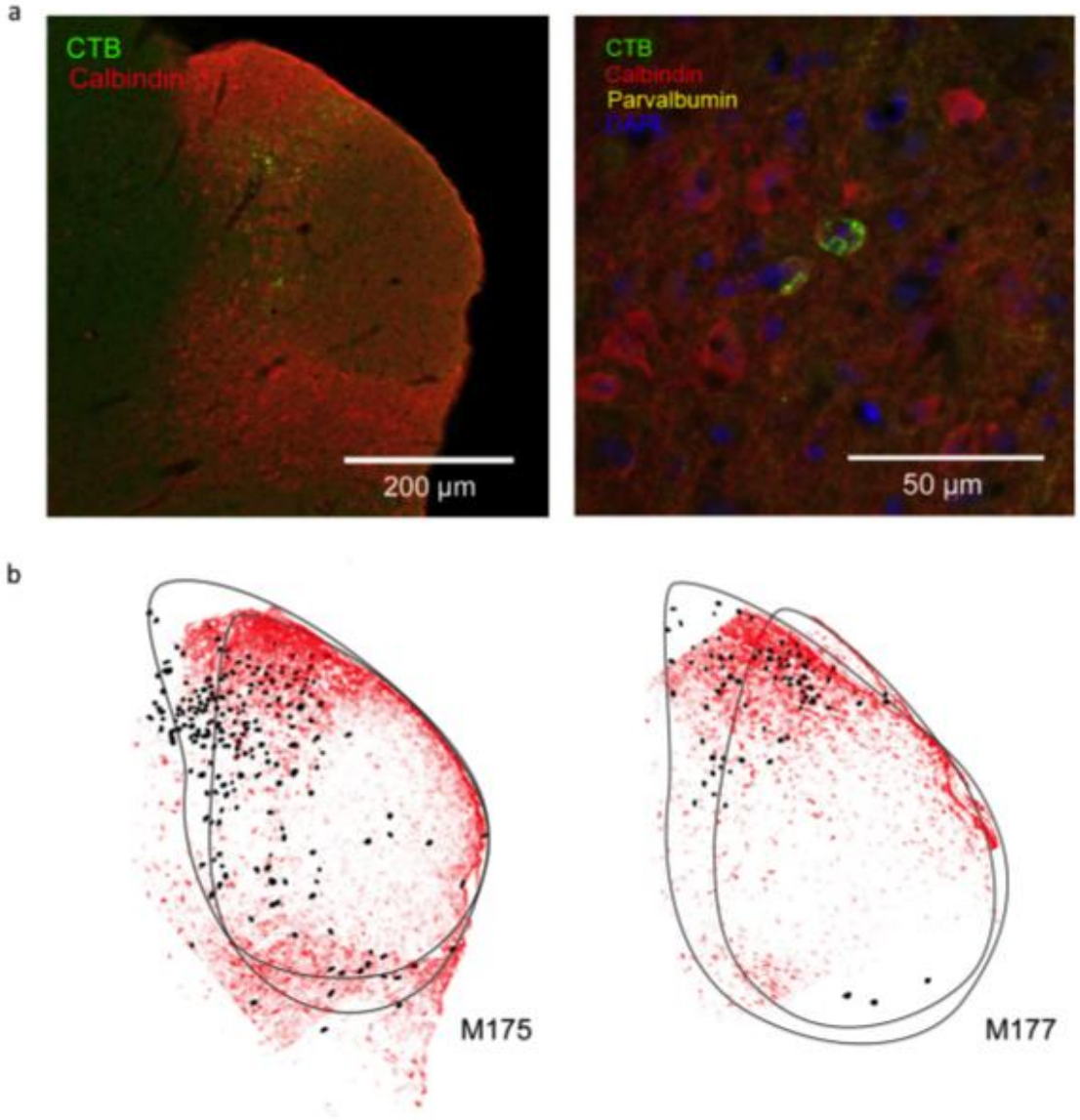


Figure 4.11. Calbindin staining of DR MGB with retrograde labeling

(a) Few sections from DR mice injected with CTB-488 were stained with calbindin, which is strongly expressed in the MGd region (left). However, no co-labeling of CTB and calbindin was found (right).

(b) Overlapping calbindin staining with camera lucida drawings of the same section showing CTB^+ cells. The region of strong calbindin staining contains high number of CTB^+ cells. Both brains were injected in LM.

In addition, we found greater number of projection from the lateral posterior thalamic nuclei (LP), which is part of the associative visual pathway (Kamishina et al., 2008), to DR V2L than LR (Fig. 4.12). This exuberant connectivity suggests how lack of visual experience in DR negatively affects the natural developmental process that involves potentiation of stronger thalamocortical projections and pruning of the weak ones.

Figure 4.12. LP projection to V2L

(a) Injection of retrograde tracer (CTB) into V2L labeled cell bodies in the lateral posterior nucleus of thalamus (LP) in both LR and DR.

(b) Quantification of CTB^+ cells in LP thalamus from P25, adult LR and adult DR mice. Although statistically not significant, there is a clear trend towards increased projection from LP to visual cortex in the P25 (n=5) and adult DR (n=7) when compared to adult LR (n=5) (P=0.15, unpaired t-test with Welch's correction).

CTB^+ cell density was calculated using *ImageJ.*

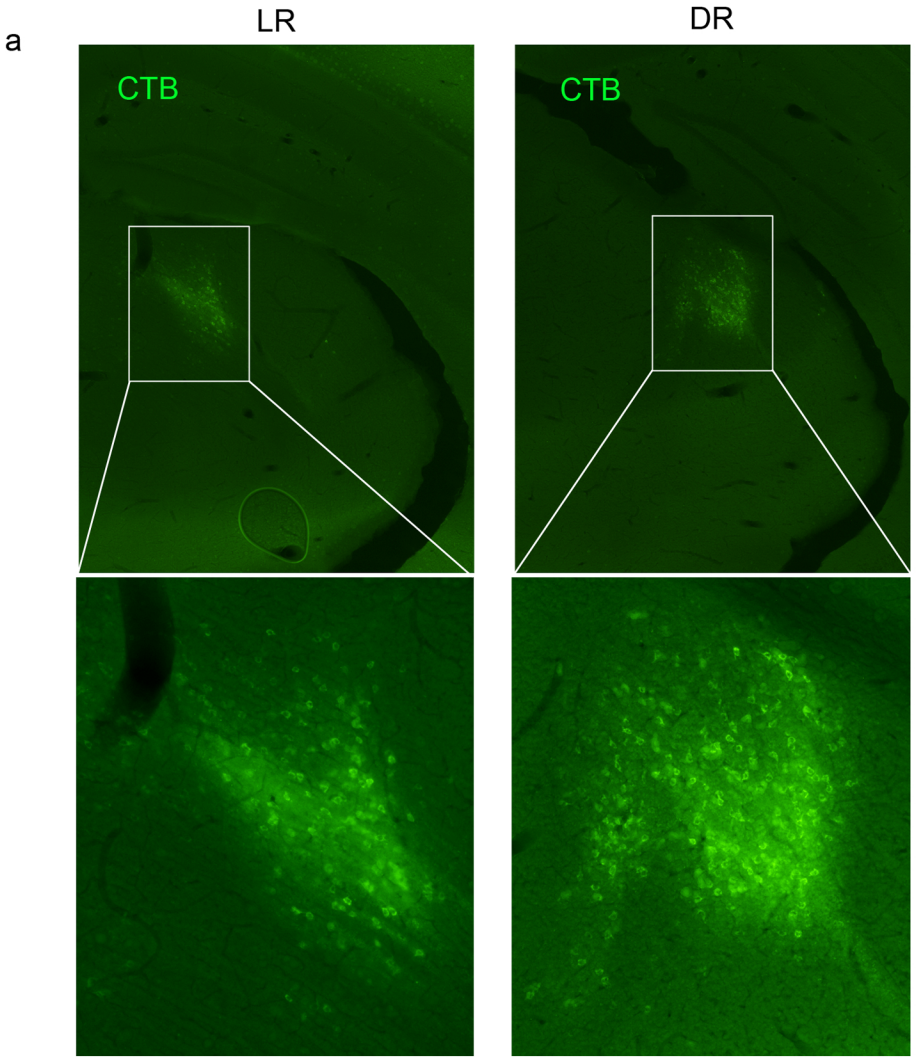


Figure 4.12. (Continued)

Chapter 5

Loss of Cross-Modal Plasticity during Normal Development and its Proposed Molecular Mechanism

Introduction

Functional studies have discovered enhancement of perceptual abilities in the spared sensory modalities, which could be reduced upon disrupting occipital cortical activity through transcraniel magnetic stimulation (Collingnon et al., 2009; Cohen et al., 1997; Ptito et al., 2008). Thus, cross-modality seen in the visual cortex of blind individuals is widely believed to be a result of compensatory mechanism. It has been thought that cross-modal compensation is acquired in the early blind through gross reorganization of neuronal circuits. However, we propose that cross-modality in occipital cortex of the blind represents arrestment in development rather than formation of novel connections through reorganization of neuronal circuitry.

Studies that look at the neural network in immature animals describe presence of transient cross-modal connections in kittens (Innocenti and Clarke, 1984; Innocenti, 1995; Dehay et al., 1984) as well as in infant macaque monkeys (Webster et al., 1991; Callaway, 1998). Transient ectopic inputs from auditory thalamus into somatosensory cortex were also found in neonatal rats, and could be stabilized with whisker removal (Nicolelis et al., 1991). Also, neuroimaging and EEG recording in human infants has revealed activation in occipital cortex by sound (Hoffman, 1978; Neville, 1995; reviewed in Maurer and Mondloch, 2005). Such evidence support the hypothesis that multimodal sensory cortex represents a normal developmental phase that all juvenile animals go though. From the studies conducted so far, it is unclear whether the cross-modal activity seen in the blind represents a form of immaturity –its

development arrested by lack of sensory input, or abnormality –absence of sensory input causing creation of novel connections not seen in the course of normal development. Here we followed mice through its normal development and characterized the functional and anatomical changes in the secondary visual area that may account for cross-modal plasticity.

Although cross-modal plasticity has long been recognized and has been the subject of many studies, none was able to come up with the underlying molecular mechanism that governs the process. One of the advantages of using a mouse as a model organism is the genetic technique that allows us to understand the molecular mechanism underlying cross-modal plasticity. Through previous studies we know of few molecular mechanisms that limit adult brain plasticity. Here, we identified two that are essential in the consolidation of cross-modal plasticity. One is the myelin-associated inhibitory signaling. As myelination takes place during cortical maturation, myelin signaling consolidates functional circuits and reduces plasticity (McGee et al., 2005; Syken et al., 2006) via the Nogo receptors (NgR) and/or paired immunoglobulin-like receptor B (PirB) (Atwal et al., 2008; Fournier et al., 2001; Cafferty and Strittmatter, 2006). The other is the cholinergic modulation. Suppression of cholinergic neurotransmission by inhibiting nicotinic acetylcholine receptors (nAChRs) through Lynx1 has also been shown to limit plasticity in adult brains (Morishita et al., 2010). Here, we examined cross-modal activity in mice that lack one of these plasticity limiting mechanisms and characterized their neuronal connectivity.

Results

Cross-modal activity and ectopic networks in juvenile mice

Increased cross-modal activity and ectopic inputs in DR visual cortex can arise from two different possibilities. One possibility is that cross-modality is acquired in the V2L as ectopic inputs from the neighboring auditory cortex and auditory thalamus invades the deprived cortex through competition (Merabet and Pascual-Leone, 2010; Mao et al., 2011). The other possibility is that V2L is initially innervated by cross-modal inputs, which gradually decrease through experience-dependent refinement during development (Innocenti and Price, 2005). In order to discriminate between these two hypotheses, we first examined functional sensory specificity in visual cortex of juvenile mice raised in normal 12 hours light/dark cycle (postnatal 25 days: P25). Since c-Fos expression in young mice turned out to be unreliable (data not shown; Sakurai-Yamashita et al., 1991; Yamada et al., 1999), we looked at Arc expression level. Arc is another member of the immediate-early gene family, and although it shows different pattern of expression from c-Fos, it has been shown to successfully measure cortical activity in young animals (Ons et al., 2004; Tagawa et al., 2005). It was shown that the visual cortex of juvenile mice responds to both light and sound stimuli (Fig. 5.1a left). In contrast to DR mice, the auditory cortex of P25 also showed an increase in Arc expression to either stimulus (Fig. 5.1a right). Laminar analysis showed that cross-modal Arc expression in P25 tends to concentrate around layer 4 (Fig. 5.1b). One hypothesis based on this observation would be that cross-modal inputs arriving in layer 4 have weak connections to local circuits so that cross-modal signals don't spread to other layers. Additional physiological experiments that look at laminar profile of visual cortex to cross-modal stimuli during development would be helpful in determining whether this hypothesis is true or not.

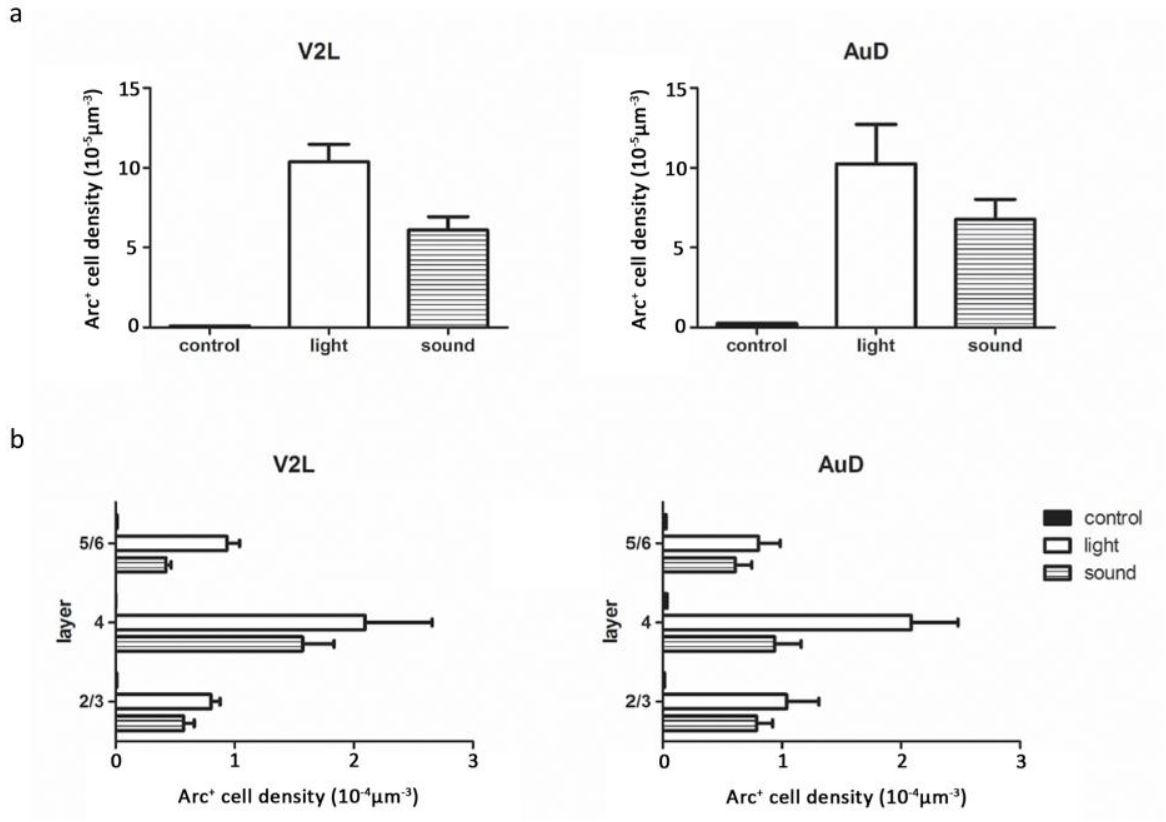


Figure 5.1. Cross-modal activity in juvenile mice

(a) Arc expression levels in visual cortex (lateral secondary visual cortex: V2L) and auditory cortex (dorsal secondary auditory cortex: AuD) of juvenile mice (postnatal 25 days: P25) was examined after 1-hour exposure to ambient light (n=3) or 1s bursts of 5 kHz tone repeated every 5s (n=4). Both V2L and AuD show increased Arc expression in response to either stimulus when compared to the non-exposed control (n=1).

(b) Laminar analysis show Arc expression is increased especially in layer 4 following cross-modal stimulation.

Arc expression level (mean ± s.e.m.) is quantified using *ImageJ.*

The source of ectopic auditory inputs in juvenile mice was examined by retrograde tracer injection into V2L. Similar to DR, we saw exuberant ectopic projections into V2L originating from the MGB and AuD in P25 mice (Fig. 5.2). These ectopic connections gradually decrease through normal maturation. *Camera lucida* drawings of MGB with CTB labeled cells from individual animal are shown in Fig. 5.3. However, it is hard to recognize any pattern of distribution like what we saw in the DR. This is likely due to the small number of samples and the location of injection, which was kept constant since it was harder to target different areas within V2L in the small brains of young mice.

In order to clarify the developmental pattern of ectopic thalamocortical inputs, visualization of cross-modal axonal terminals in visual cortex would be the next experiment. Since different type of inputs arrive at different cortical layer (Larkum et al., 1999), it will be especially helpful to see whether there are any layer specific changes in number of ectopic thalamocortical terminals during development in understanding the underlying mechanism of cross-modal plasticity.

Visual experience in adult DR leads to transient functional loss of cross-modal activity

To see if decrease in cross-modal plasticity over development were due to age-dependent maturation or prolonged visual experience, we looked at how vision affects cross-modal activity in DR V2L. Dark-rearing from birth is believed to maintain the visual cortex in its immature state, and subsequent exposure to normal 12 hours light/dark cycle for 2-3 weeks in adulthood is shown to drive rapid maturation of visual field properties (Fagiolini et al., 2003; Miyamoto et al., 2003; Lee et al., 2006). Thus, we tested whether visual experience in DR mice also leads to maturation of cross-modal plasticity, and develops visual cortex that is specific to visual inputs only.

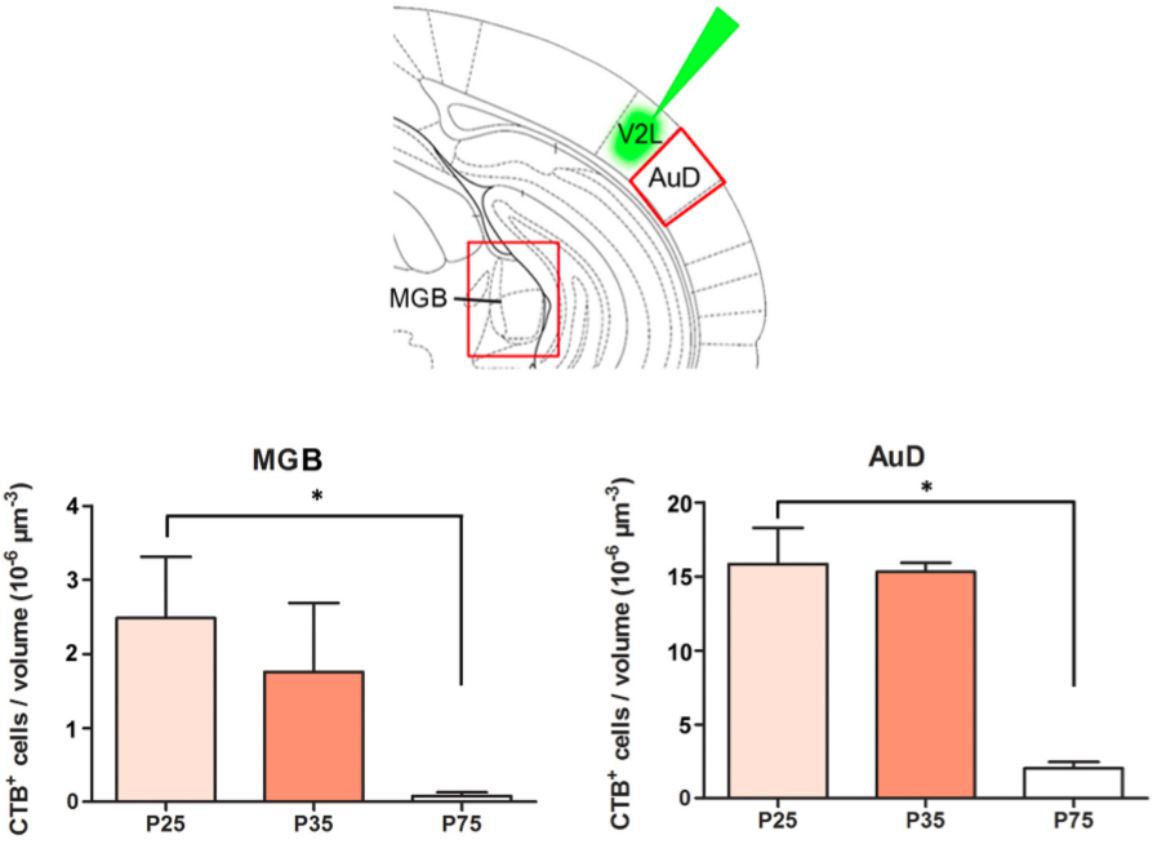


Figure 5.2. Ectopic connections to V2L in young mice

Retrograde tracer injection into V2L of LR P25 mice (n=4) reveals exuberant ectopic projections originating from both MGB and AuD (*P<0.02, ANOVA with Kruskal Wallis test). These ectopic connections decrease as mice mature into adulthood (n=3 for P35, n=5 for P75).

CTB^+ cell density was calculated using *ImageJ.*

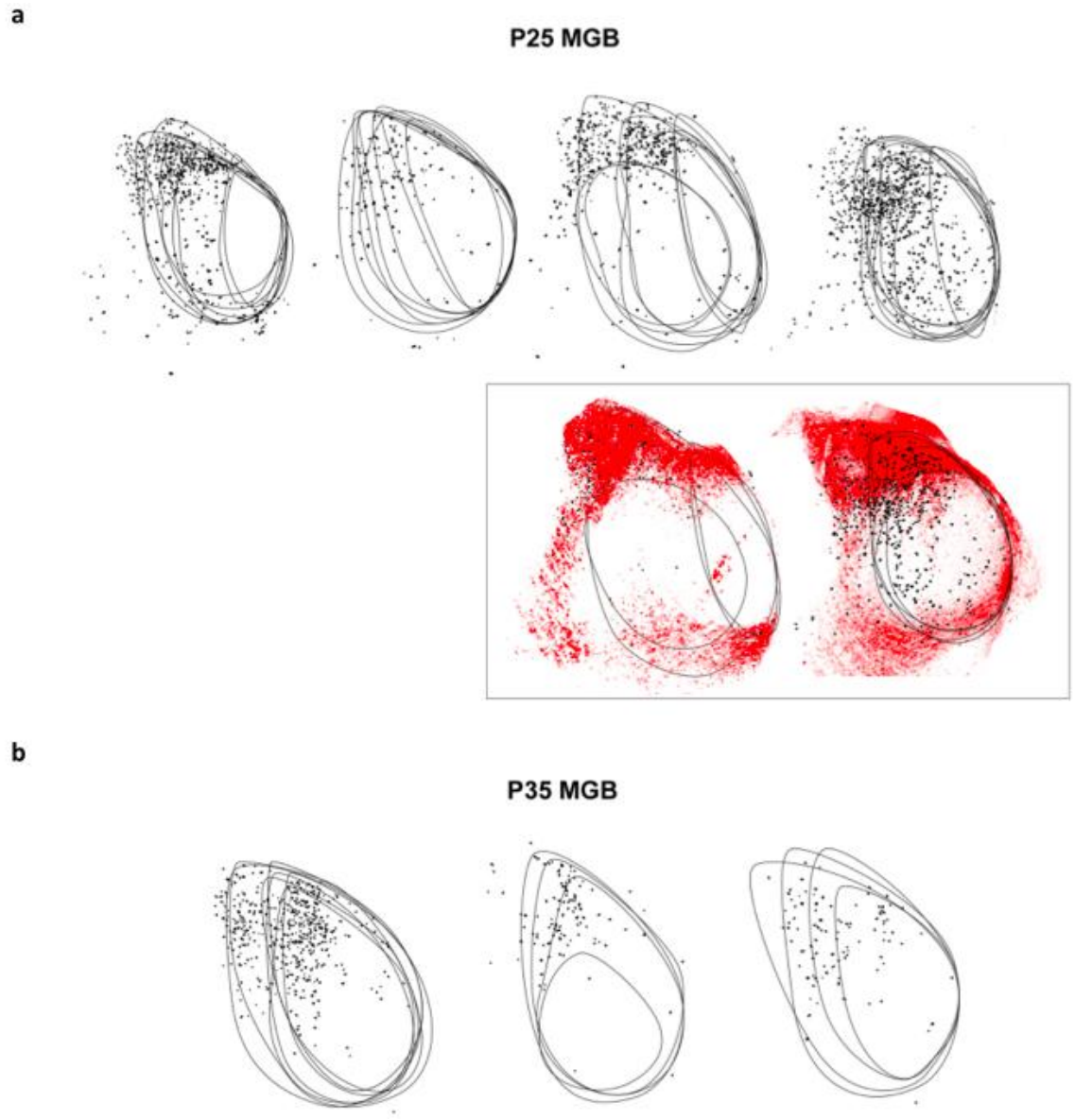


Figure 5.3. Distribution of CTB+ cells in MGB of young mice

(a) The distribution pattern of CTB^{+} cells (above) and calbindin staining (inset) in P25 is shown for P25 MGB.

(b) The distribution pattern of CTB^{+} cells in P35 MGB.

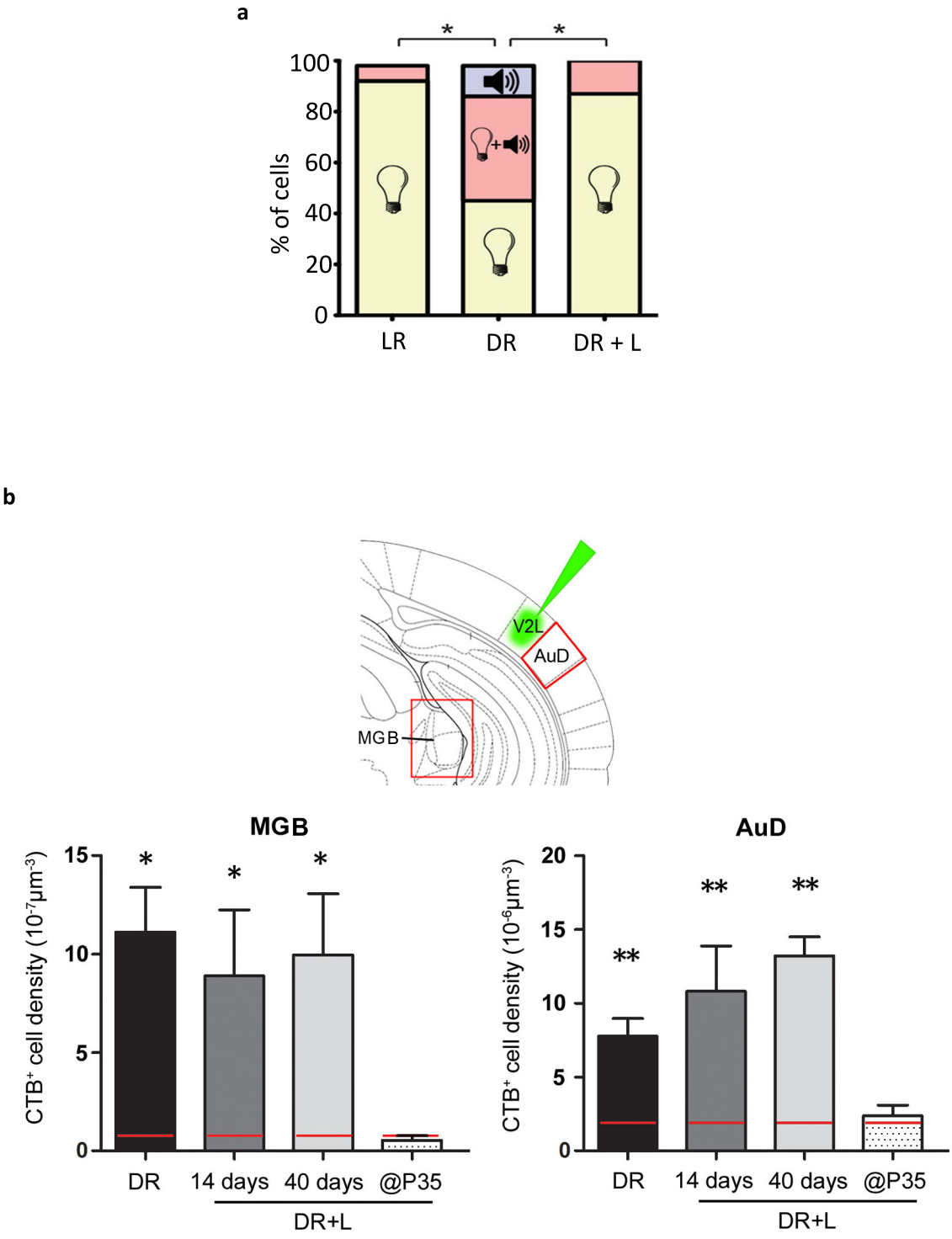
Functional observation using single-unit recordings showed that distribution of cells that respond to sound stimulus in DR V2L is drastically reduced to resemble that of LR V2L with two-week long exposure to light (DR+Light) (Fig. 5.4a). However, retrograde tracing analysis revealed that ectopic connections are present in DR+Light even when the mice are put in normal light environment for 40 days (Fig. 5.4b). Interestingly, these ectopic connections could not be found in DR mice that were taken out of the darkness at P35. These results strongly indicate that there is a time point after P35 when visual experience can no longer prune the non-specific connections that enter the visual cortex. In other words, the functional specificity observed in DR+Light V2L does not involve retraction of ectopic inputs, but likely involves some type of inhibitory mechanism to inactivate the cross-modal auditory inputs.

Interestingly, these ectopic connections in DR+Light regained function upon re-entry into complete darkness (DR+Light -dark) (Fig. 5.5). c-Fos expression analysis was carried out at the end of the light exposure and after subsequent re-emission to darkness. Quantification of c-Fos^{+} neurons in response to sound showed that cross-modal activity remarkably returns to V2L after two weeks of re-emission to complete darkness (Fig. 5.5b). Previous studies have shown that one mechanism of functionally suppressing an inappropriate response is through inhibition mediated by $GABA_A$ receptors (Zheng and Knudsen, 1999). Here we hypothesize that activation of local inhibitory circuit by vision leads to suppression of auditory inputs into V2L in DR+Light. However, the number of sound activated inhibitory neurons in DR+Light was not different from DR+Light – dark (Fig. 5.5c). Although the number of activated GABAergic cells did not differ, the strength of inhibition cannot be measured using c-Fos expression as there could be variation in number of active synapses by inhibitory neuron or their location on excitatory neuron could differ. A direct way to test this hypothesis would be to disinhibit DR+Light visual cortex with GABA receptor

Figure 5.4. Visual experience in DR mice causes loss in cross-modal activity but not in cross-modal connections

(a) The distribution of visual (yellow), auditory (blue), and multomodal (red) cells in DR+Light resembles that of LR (n=133). In contrast to DR V2L (n=147), where more than half of the recorded neurons respond to auditory stimuli, the majority of cells in DR+Light V2L are specific to visual inputs (*P<0.0001, χ^2 test).

(b) Retrograde tracer was injected into V2L of DR+Light and AuD and MGB were examined for CTB^+ cells. Exuberant ectopic connections from MGB (left) and AuD (right) are shown in mice exposed to LR environment after they reach adulthood regardless of the extension of exposure (n=7 for DR, n=4 for DR+Light for 14 days, n=6 for DR+Light for 40 days). However, ectopic connections in V2L are at the same level of LR (red line, n=5) in DR+Light that were placed in normal lighting at P35 (n=5) (**P=0.0005, ANOVA Kruskal-Wallis test).



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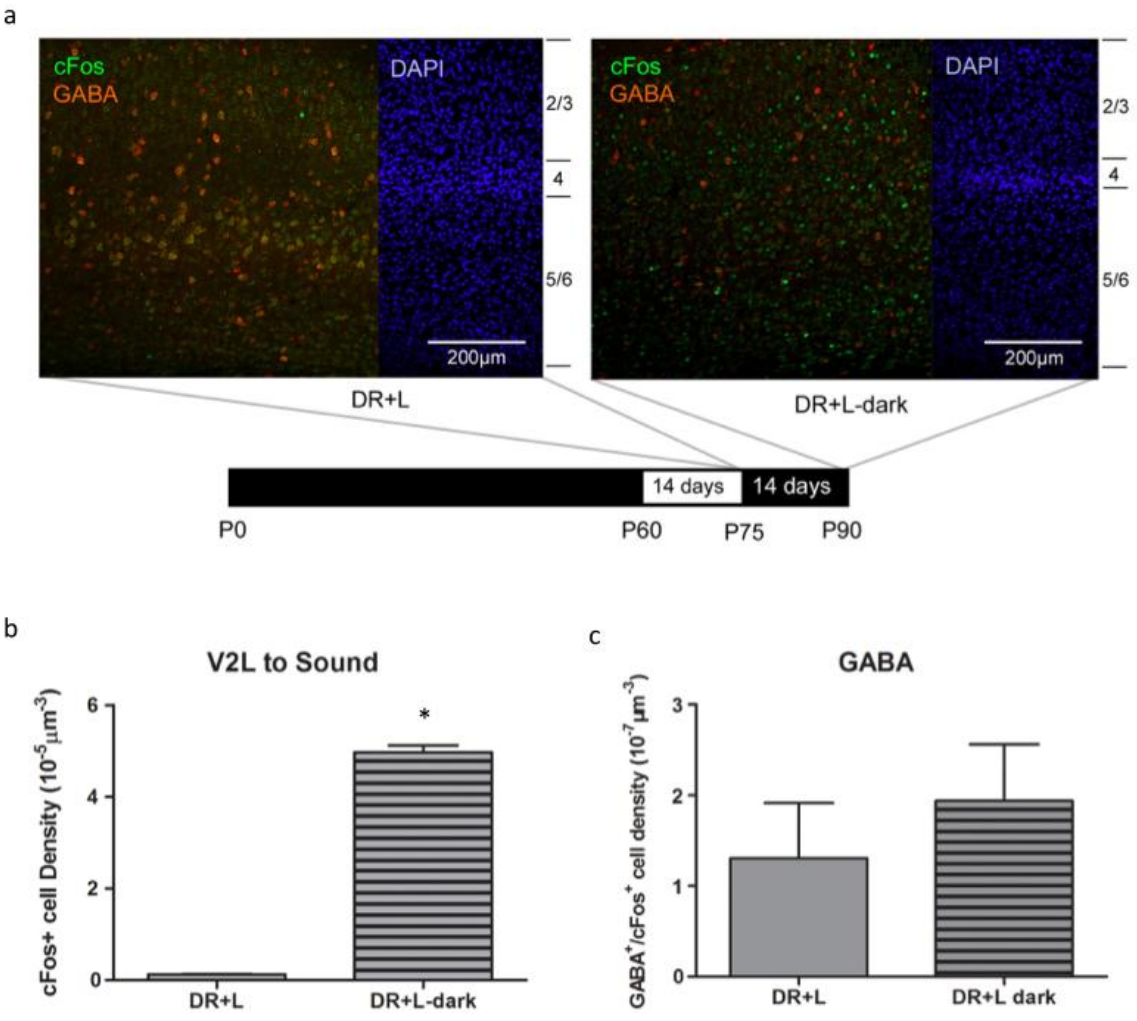

Figure 5.5. Cross-modal activity returns in DR+Light mice without visual experience

(a) c-Fos and GABA staining of V2L from DR+Light and DR+Light returned to darkness. DR mice exposed to normal 12-hour light/dark cycle for two weeks show decreased level of c-Fos expression in response to cross-modal stimuli. However, cross-modal activity in V2L returns upon putting DR+Light mice back into 24-hour darkness for two weeks.

(b) Quantification of c-Fos$^+$ cells in V2L shows a dramatic decrease upon two week long light exposure in DR mice (n=4). Returning DR+Light mice back into 24-hour darkness for another two weeks causes V2L to become cross-modal again (n=5, *P=0.0093, unpaired t-test with Welch's correction).

(c) Quantification of c-Fos$^+$/GABA$^+$ cells in V2L shows that there is no significant difference in activation of GABA cells in response to sound between DR+Light and DR+Light –dark V2L (P=0.5, unpaired t-test with Welch's correction).

c-Fos$^+$ cells and GABAergic cells were quantified using *ImageJ.*

antagonist such as GABAzine and to see if the visual cortex starts responding to cross-modal stimulation.

Our data shows cross-modal activity in DR visual cortex being transiently suppressed without changing the gross cross-modal anatomical projections in adults. This demonstrates an apparent discrepancy between functional and anatomical cross-modal plasticity in DR mice. Anatomical plasticity decreases with age even without visual experience. We show that cross-modal projections decrease with visual experience only during a certain period early in life. For animals that reach maturity in darkness, cross-modal connections are maintained regardless of the duration of visual experience as adults. Functional plasticity, on the other hand, remains open through adulthood in DR mice and develops sensory specificity in DR visual cortex as adults. For future studies on cross-modal plasticity, it should be noted that functional specificity does not infer anatomical specificity and vice versa.

Cross-modality is retained in adult NgR -/- and Lynx1 -/- mice

Riboflavin imaging in normally reared adult NgR -/- found auditory activation in visual cortex (Fig. 5.6a). In addition, single-unit recording in V2L of NgR -/- showed similar distribution of cellular responses to DR V2L (Fig. 5.6b). However, in contrast to the fast auditory cells in DR V2L, NgR -/- V2L showed much slower auditory responses (Fig. 5.6c). We also performed c-Fos expression analysis in NgR -/-. As in the DR, V2L of NgR -/- showed similar degree of activity in response to sound and light (Fig. 5.7). However, c-Fos expression in NgR -/- was very weak overall compared to DR or LR. Such difference in response strength and latency may reflect disruption in the regulation of synaptic strength driven by Nogo (Lee et al., 2008; Raiker et al., 2010).

Exuberant ectopic connections were observed in NgR -/- and Lynx1 -/- through retrograde tracer injection (Fig. 5.8). Interestingly, the level of neuronal projections from MGB and AuD to V2L

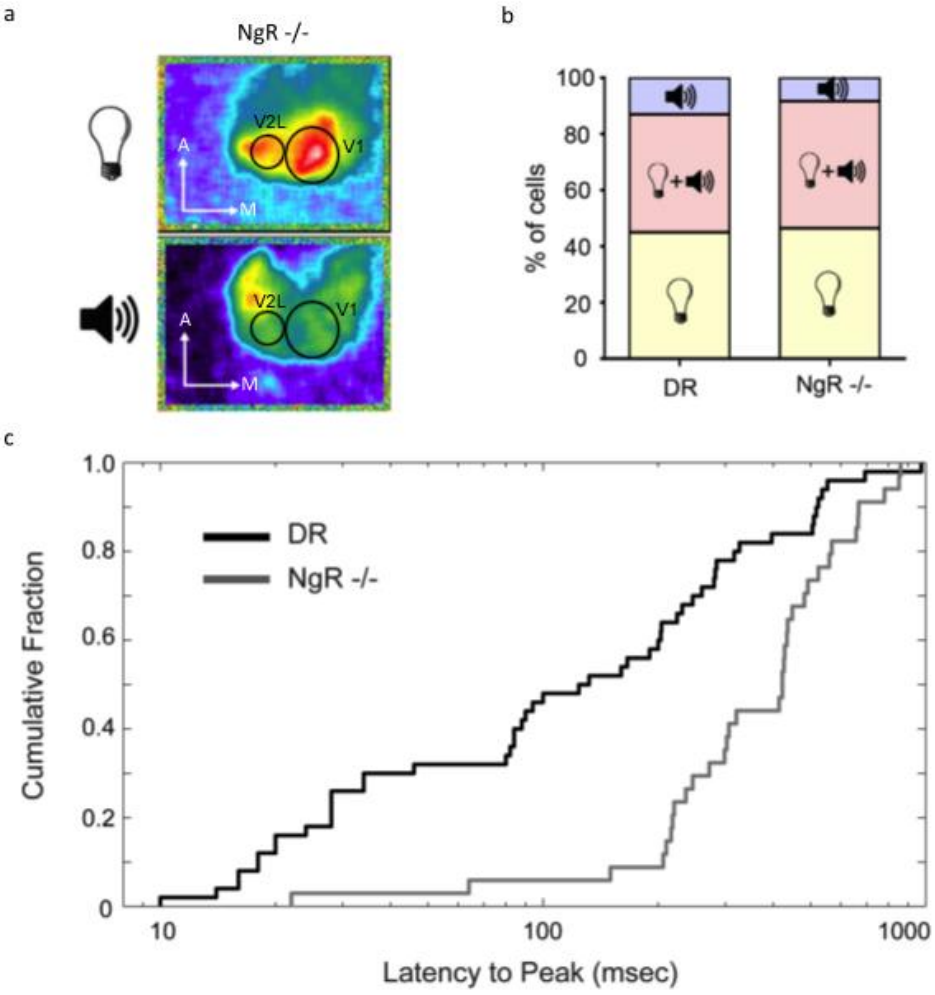

Figure 5.6. Cross-modal response in normally reared adult NgR -/-
(data generated and analyzed by Daniel Brady and Ryoma Hattori)

(a) Pseudo color riboflavin fluorescence images of light-reared NgR -/- mice with visual (above) and auditory (below) stimulus.

(b) NgR -/- (n=101) have a similar distribution of visual, auditory, and multimodal cells in V2L to DR mice (n=147, P=0.67, χ^2 test) as measured by single-unit recordings.

(c) Cumulative distribution function of auditory latencies in V2L shows faster responses in DR (n=50) than NgR -/- mice (n=34, **P<0.01, two-sample Kolmogorov-Smirnoff test). The fast auditory component is nearly absent in NgR -/- mice.

Figure 5.7. cFos expression in NgR -/- mice

Level of cFos expression in V2L in response to light or sound stimuli is shown for LR +/+ (top), DR +/+ (middle), and NgR -/- (bottom) for comparison. LR +/+ V2L shows significant increase in activity when exposed to light, but no to sound (n=5 for sound, n=3 for light; *P=0.02, unpaired t-test). DR +/+ V2L is activated by both light and sound stimuli (n=5 for sound, n=3 for light). In NgR -/- (n=4 for both sound and light;), DR c-Fos expression is significantly increased above the baseline level in response to both sound and light (control: red line, n=4, $P<0.05$, unpaired t-test with Welch's correction), but overall neuronal activity is significantly smaller when compared to LR or DR.

c-Fos expression levels (mean $\pm$ s.e.m.) are quantified with *NIS-Elements® Object Count.*

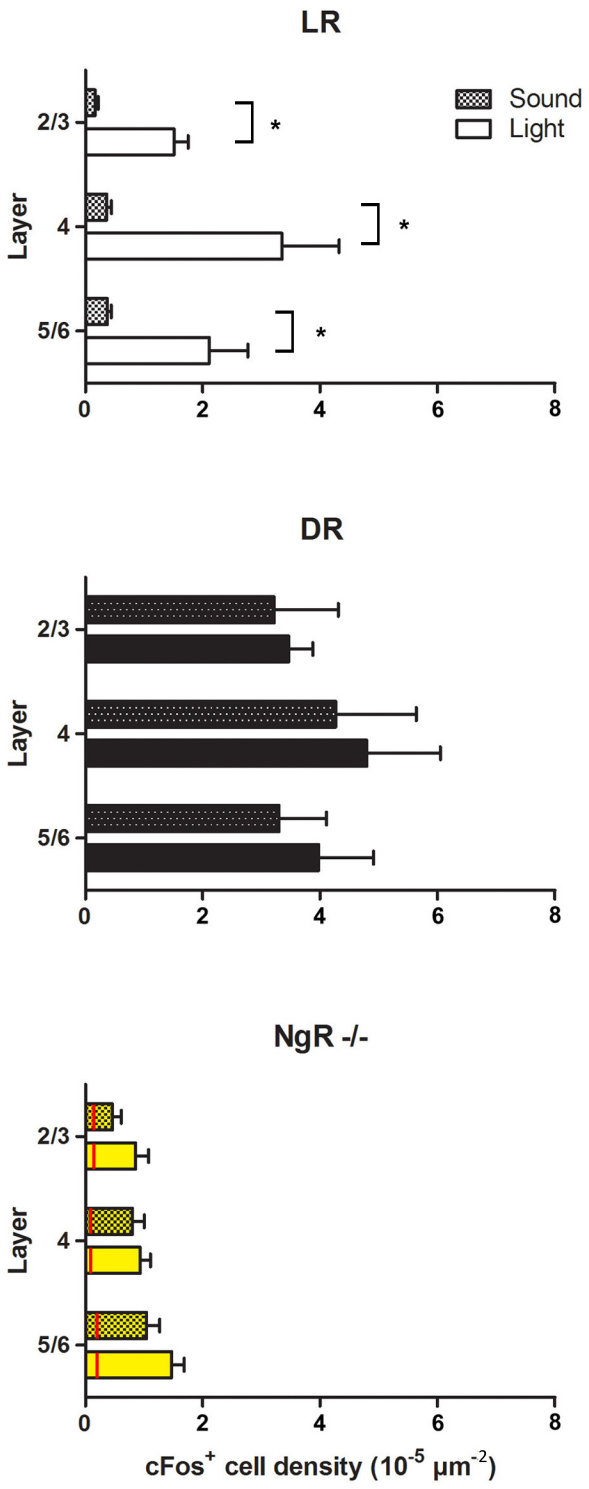

Figure 5.7. (Continued)

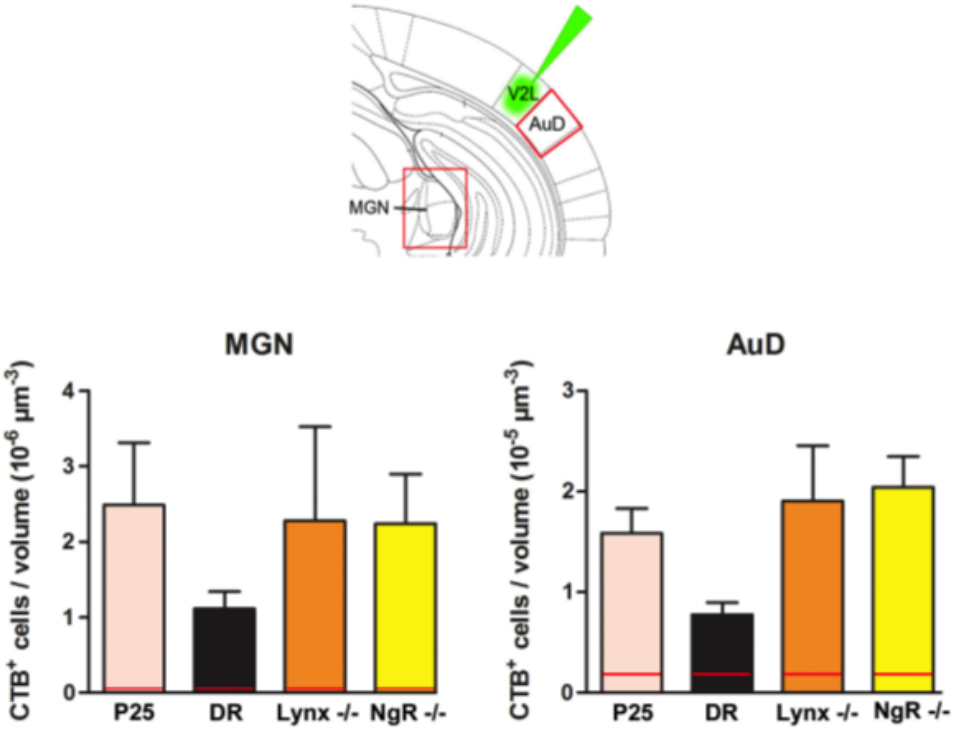

Figure 5.8. Ectopic auditory inputs into V2L of NgR -/- and Lynx1 -/-

Injection of retrograde tracer into V2L labels many cells in MGB (left) and AuD (right) of NgR -/- (n=5) and Lynx1 -/- mice (n=4). The extent of neuronal projection from MGB and AuD to V2L in these mice is similar to that of P25 mice (n=4), while the DR mice (n=10) has an intermediate level of ectopic connections (red line: LR +/+, n=6).

CTB^+ cell densities (mean ± s.e.m.) were quantified using *ImageJ*.

in both NgR -/- and Lynx1 -/- were similar to that of wild type P25 mice, which has more ectopic connections than the DR. This may reflect the fact that dark-rearing disrupts the development of visual cortex selectively, while the effect of genetic knockout is ubiquitous. In DR cortex, cortical areas other than the visual cortex undergo normal maturation, and this may indirectly affect the amount of ectopic inputs coming into the visual cortex.

Our results showed that V2L is multimodal in young mice, and identified two possible mechanisms that drive cross-modal plasticity in adults. Disruption in myelin signaling leads to active neurite outgrowth through adulthood, prolonging the period of visual cortex plasticity (McGee et al, 2005; Syken et al., 2006). Similarly absence of Lynx1 molecule, which acts as a brake to cholinergic modulation, drives visual cortex to remain plastic through adulthood (Morishita et al., 2010). This suggests that cross-modal plasticity occurs early in life as part of experience-dependent refinement of sensory cortex, and its consolidation is dependent upon closure of the critical period.

Chapter 6

Summary and Discussion

Sensory specificity in visual cortex develops in an age-dependent and experience-dependent manner

In this study, we first established dark-reared mice (DR) as a model to study cross-modal plasticity in the visual cortex. Our functional data showed strong auditory response in the visual cortex of DR, and anatomical analysis revealed cross-modal projections from the auditory thalamus (medial geniculate body: MGB) and the secondary auditory cortex (dorsal secondary auditory cortex: AuD) to the secondary visual cortex (lateral secondary visual cortex: V2L). Since dark-rearing delays many aspects of visual cortex maturation, we also carried out our study in young mice that have yet not reached maturity. Presence of ectopic exuberance in juvenile mice (postnatal 25 days: P25) strongly suggests cross-modality is part of the normal development process, and that multimodality in DR mice reflects a delay in visual cortex maturation rather than a compensatory anatomical reorganization of the non-deprived sensory systems. In summary, our result shows evidence for transient cross-modal thalamocortical and corticocortical auditory projections into visual cortex that are pruned out during development in an experience-dependent manner (Fig. 6.1). Failures to provide adequate sensory experience during a certain period early in life disrupts this process and is likely to cause the visual cortex to retain multimodality as in the early blind patients.

Another interesting point that was not approached by this study is whether this cross-modal plasticity exhibits a true 'critical period,' in a sense that there is a time point of entering the plastic phase. Knowing whether this phenomenon has a critical period or not will help us understand how sensory information is processed in infants and how it changes through

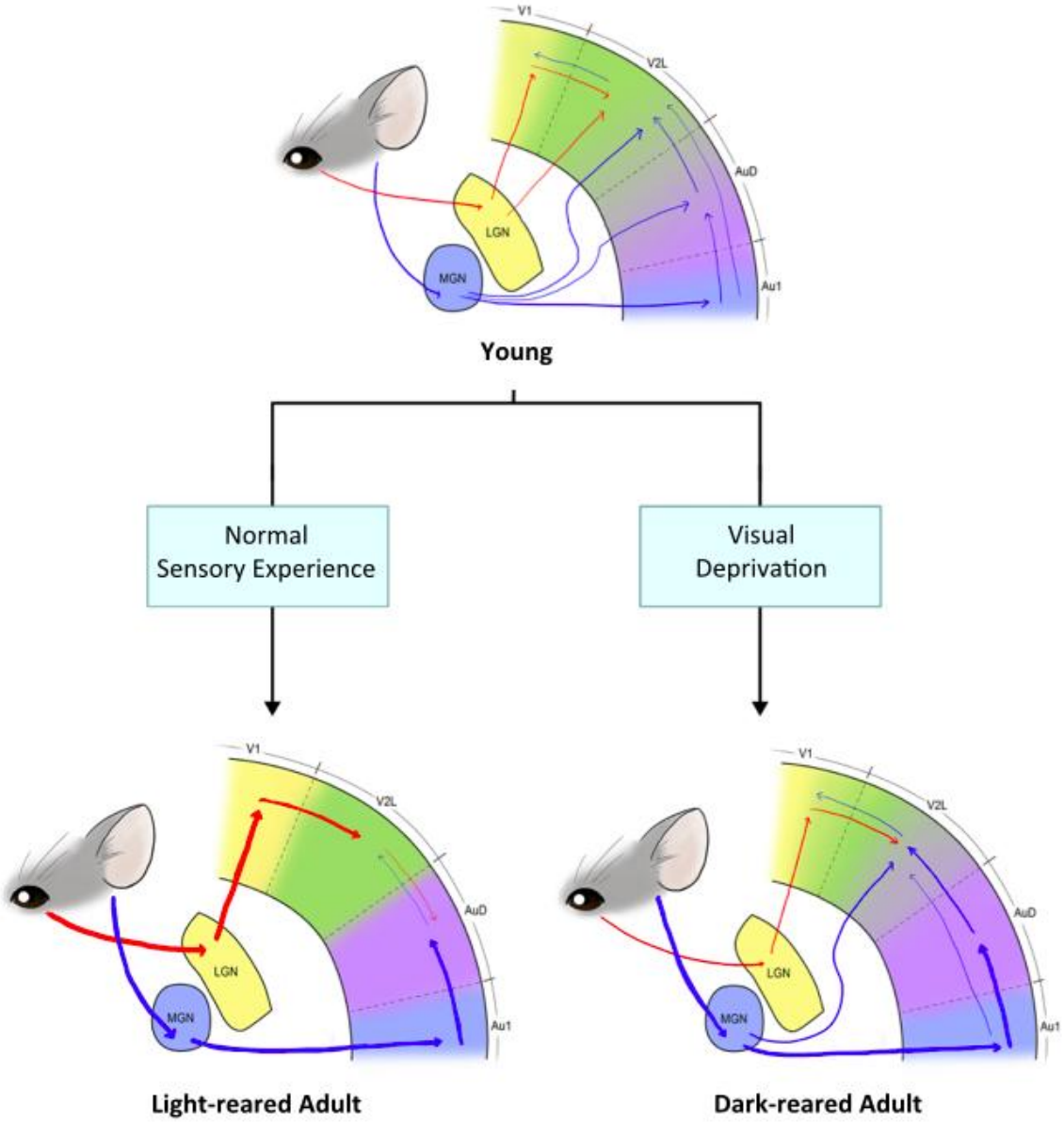

Figure 6.1. Summary of cross-modal refinement by visual experience in V2L

Exuberant cross-modal ectopic projections are initially present in young mice (P25, top panel). V2L in young mice receives auditory projections from the auditory thalamus (medial geniculate body: MGB) and the neighboring secondary auditory cortex (dorsal secondary auditory cortex: AuD). Under normal rearing conditions, visual input strengthens modality-specific thalamocortical connections while weak cross-modal projections are pruned out, and further refines the functional segregation between cortical areas (lower left panel). In the absence of visual input through dark-rearing, cross-modal projections to V2L is maintained and visual cortex fails to develop proper sensory specificity as adults.

adolescent and adulthood. Presence of developmental exuberance alone is not enough to show how cross-modal plasticity is functionally expressed. Ectopic networks in early stages of sensory cortex development may lead to continuous multimodality until reaching maturity, or may remain non-functional until reaching a critical period. Upon entering the critical period, the less utilized cross-modal connections will be pruned out, while strengthening the sensory-specific networks in an experience-dependent manner. In support of the second point, non-functional synaptic connections have recently been found to innervate the neuromuscular junctions at birth, which go through massive pruning during the first postnatal days in mice (Tapia et al., 2012). The next step in our experiment would be to follow changes in cross-modal activity through development, and to identify whether or not there is a critical period when dark-rearing leads to cross-modal activity in visual cortex.

Each sensory modality reaches maturity at different developmental time points (Alison et al., 1983; Eggermont, 1988). Thus, development of the nervous system might have series of critical periods when pruning of ectopic connections occur in each sensory and motor system based on their distinct timelines. Although our study has focused on the visual cortex and the ectopic inputs coming from the auditory pathway, early transient cross-modal exuberance in other functional areas have also been reported previously (Dehay et al., 1984; Naegele et al., 1988; Nicolelis et al., 1991; Catalano et al., 1996). Thus, deprivation models of other modalities, such as white noise rearing to delay maturation of auditory cortex (Chang and Merzenich, 2003; Chang et al., 2005) or whisker deprivation to disrupt barrel cortex formation (Fox, 1992; Fox, 2002), should be examined to see if cross-modal plasticity is a general phenomenon across different sensory areas.

V2L of adult DR mice becomes specific to vision without elimination of ectopic inputs upon long-term visual experience

Independent from the question regarding functional cross-modal plasticity during development as discussed above, we show that exposure to normal light environment from P35 can dramatically decrease ectopic auditory inputs into V2L. However, once the mice reach adulthood (~P75) in complete darkness, the ectopic connections do not change even with prolonged exposure to normal light environment. This indicates that the anatomical cross-modal plasticity is open at P35, but closes by P75. When looking at functional cross-modal plasticity on the other hand, we found that transient sensory specificity is established in the visual cortex upon bringing DR adults into light environment despite presence of cross-modal connections.

Molecular mechanisms that regulate cortical plasticity affects establishment of sensory specificity

Ectopic exuberance in young animals and functional cross-modal activity in cortex of sensory deprived subjects has been observed by previous studies but the mechanism behind the refinement process was left unanswered. Here, we provide the first glimpse into changes that occur at the molecular level to drive experience-driven pruning of ectopic inputs in sensory cortex.

We have examined the anatomical connections in mice lacking mechanisms for suppressing adult brain plasticity. Our data show exuberant cross-modal connections in both NgR -/- and Lynx1 -/- mice. This suggests that the Lynx1 dependent modulation of cholinergic input and NgR dependent regulation of neurite outgrowth play an essential role in the refinement modality-specific inputs into V2L (Fig. 6.2). In addition, previous studies show that the development of myelin sheaths and Lynx1 expression are paralleled by formation of perineuronal

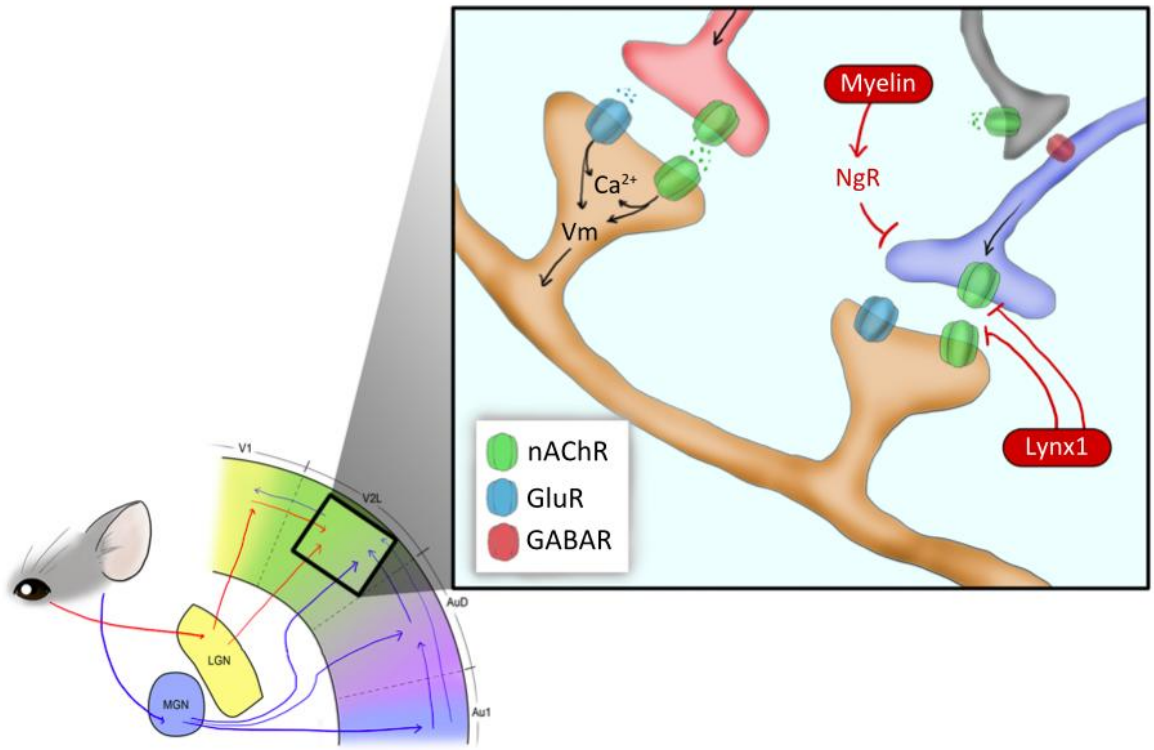

Figure 6.2. Molecular mechanism of cross-modal refinement during development

During development, ectopic auditory inputs (blue) that project to extrastriate visual cortex are lost in an experience-dependent manner, leaving the stronger visual afferents (red). This process is dependent on molecular mechanisms that function as brakes on adult plasticity. Lynx1 inhibits nAChRs, which influences the balance of synaptic excitation and inhibition in addition to regulating calcium influx. Myelin inhibitory signals from NgR prevent neurite formation and consolidate the active afferent fibers. Both molecular pathways lead to destabilization of weak cross-modal inputs. In mice with disruption in either of these pathways, cross-modal inputs are maintained independent of visual experience.

nets that surround inhibitory interneurons. These nets are rich in chondroitin sulfate proteoglycans (CSPGs), which suppress ocular dominance plasticity in adult brains (Pizzorousso et al., 2002). It has also been shown that preventing condensation of CSPG into perineuronal nets cuases the cortical plasticity to persist through adulthood (Miyata et al., 2012). The next step would be to see if CSPG-dependent pathway of preventing plasticity also plays a role in establishment of refined sensory specificity in secondary sensory cortex.

In this study, we have identified mechanisms of consolidation, or 'closing' the plasticity, to regulate cross-modal plasticity. However, we are yet to look at the effects of mechanisms that 'open' plasticity during critical period. Based on previous research that characterized the functional and anatomical modulation in DR visual cortex by Dr. Fagiolini and Dr. Hensch as well as many others, the molecular changes underlying opening of the critical period are well-understood. Dark-rearing, which delays functional maturation, is coupled with delayed maturation of GABAergic inhibitory cells, and arrested NMDA receptor subunit switch from NR2B to NR2A (Fagiolini et al., 1994; Benevento et al., 1995; Hensch et al., 1998; Carmingnoto and Vicini 1992; Quianlan et al., 1999; Morales et al., 2002). Mice with genetic manipulation that recapitulate this imbalance of excitation/inhibition ratio have been shown to alter the expression of visual cortex plasticity similar to that of DR mice (Fagiolini et al., 1994; Fagiolini and Hensch, 2000; Fagiolini et al., 2003; Hensch and Fagiolini, 2005). Since the balance of excitation/inhibition ratio plays a crucial role regulating cortical plasticity during development, it is our interest to see if this mechanism also affects developmental cross-modal plasticity.

Thalamocortical projection patterns with and without vision

An interesting observation was made during our tracing study that examines MGB to V2L ectopic projection in DR mice. Our preliminary analysis indicated a distinct projection pattern that could be delineated based on sub-regions within MGB. In normal non-deprived animals, the auditory thalamus is organized into primary sensory thalamic nuclei (ventral division of MGB: MGBv) and non-primary sensory nuclei (dorsal division of MGB: MGBd), and each are known to be involved in different functions and to project to distinct auditory regions (Arnault and Roger, 1990; Hackett et al., 2011). It was noted that in the DR, MGBv and MGBd, which normally projects to the primary and secondary auditory cortex sends ectopic inputs into the lateral medial extrastriate area (LM) and anterior lateral extrastriate area (AL) (Fig. 6.3). It was recently shown that LM and AL are spatially and functionally distinct regions that serve as gateways for ventral and dorsal stream respectively (Wang and Burkhalter, 2007; Marshel et al., 2011; Wang et al., 2011; Wang et al., 2012). Our observation of projection patterns from MGB in DR mice suggests that segregation of AL and LM are innate and does not require visual inputs. This demonstrates how thalamocortical axon (TCA) guidance involves multiple levels of mechanisms that combine intrinsic molecular cues with extrinsic experience-driven inputs. Cortical arealization is first initiated by graded expression of transcription factors that guide TCA to different areas of the neocortex. Upon reaching the cortex, TCA activities drive further cortical differentiation and functional refinement. Then refined expression of molecular cues that distinguish sub regions such as AL and LM may appear, which in turn help TCA to make region specific connections. As the last step, sensory experience may drive the pruning process of weak cross-modal connections as we have shown in this study. Much more extensive research is needed to test this proposal, but our study provides the first glimpse into the complexity of the interaction between intrinsic and extrinsic factors that govern cortical arealization and sensory modularization.

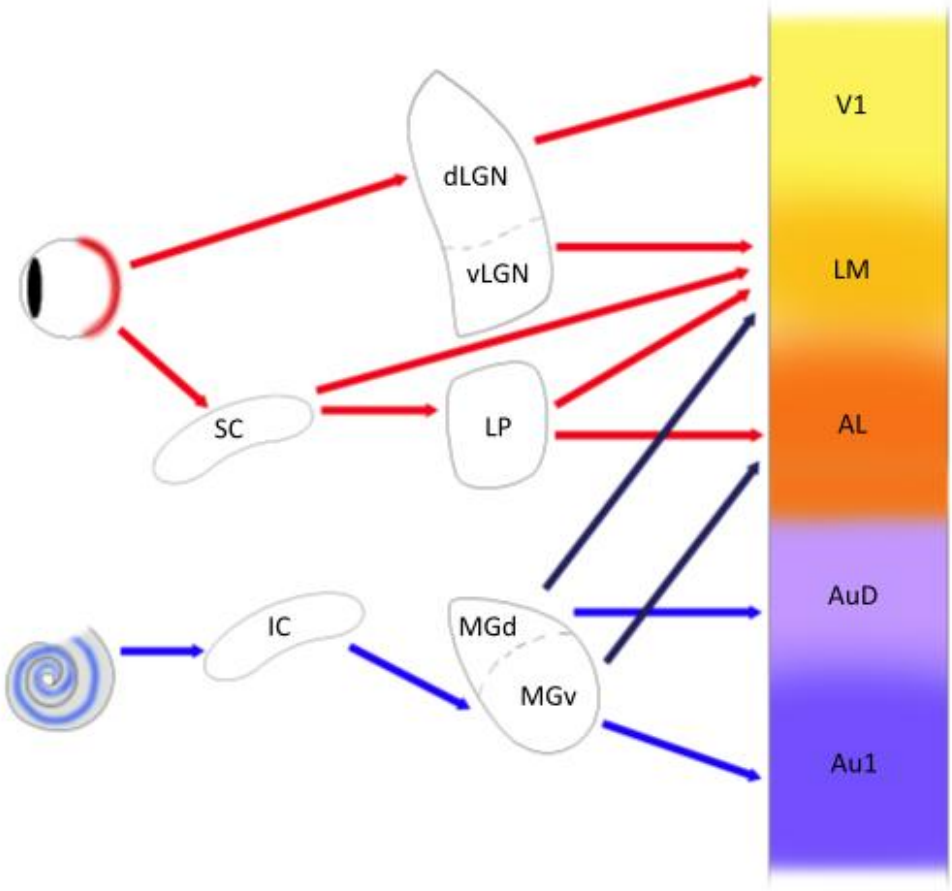

Figure 6.3. Summary schematic of thalamocortical projection pattern

Simplified schematic showing visual (red arrows) and auditory (blue arrows) feed forward connections. In normally reared mice, sub regions of the extrastriate visual cortex show distinct connectivity and carry out different functions. The lateromedial field (LM) receives input from the lateral posterior thalamus (LP), superior colliculus (SC), and the lateral geniculate nucleus of the thalamus (LGN). On the other hand, the anterolateral field (AL) receives the only sub cortical input from LP. We see that such pattern is conserved even for the ectopic projections coming from the auditory thalamus (medial geniculate body: MGB) into extrastriate visual cortex in DR mice (dark blue arrows).

Part III: Perspectives

Chapter 7

Specification of Sensory Cortex and Further Implications

Two different circuit models for early visual deprivation

Searching through the literature, one can find many discrepancies among what is being reported about the function and anatomy in the visual cortex of the visually deprived. Here, we combine our findings with earlier reports to show that these variations may not be mutually exclusive, but represent two different mechanisms at play (Fig. 7.1). In animal models that have undergone visual deprivation at early stages of development sub-cortical rewiring is evident (Fig. 7.1 left). This includes the blind mole rat, anophthalmic mice, and neonatal enucleation in hamsters and ferrets (Dehay et al., 1984; Rehkamper et al., 1995; Bronchti et al., 2002; Izraeli et al., 2002; Laemle et al., 2006;), and we term these as models of 'premature' blindness. In this model, failure to establish functional connection between the retina and the visual thalamus (LGN) leads to cross-modal invasion of LGN by sub-cortical structures, such as the inferior colliculus. The visual cortex consequently becomes multimodal by receiving cross-modal input from the rewired LGN in these animals, similar to how visual information is rerouted to somatosensory or auditory thalamus by the destruction of lower somatosensory or auditory areas (Frost & Metin, 1985; Sur et al., 1988; Pallas et al., 1990; Sharma et al., 2000). On the other hand, visual deprivation delivered in later stages of development, which we will call the 'early' blind, does not show sub-cortical rewiring (Chabot et al., 2007). Instead, studies have suggested intracortical connections to be the source of ectopic inputs, and our study shows that

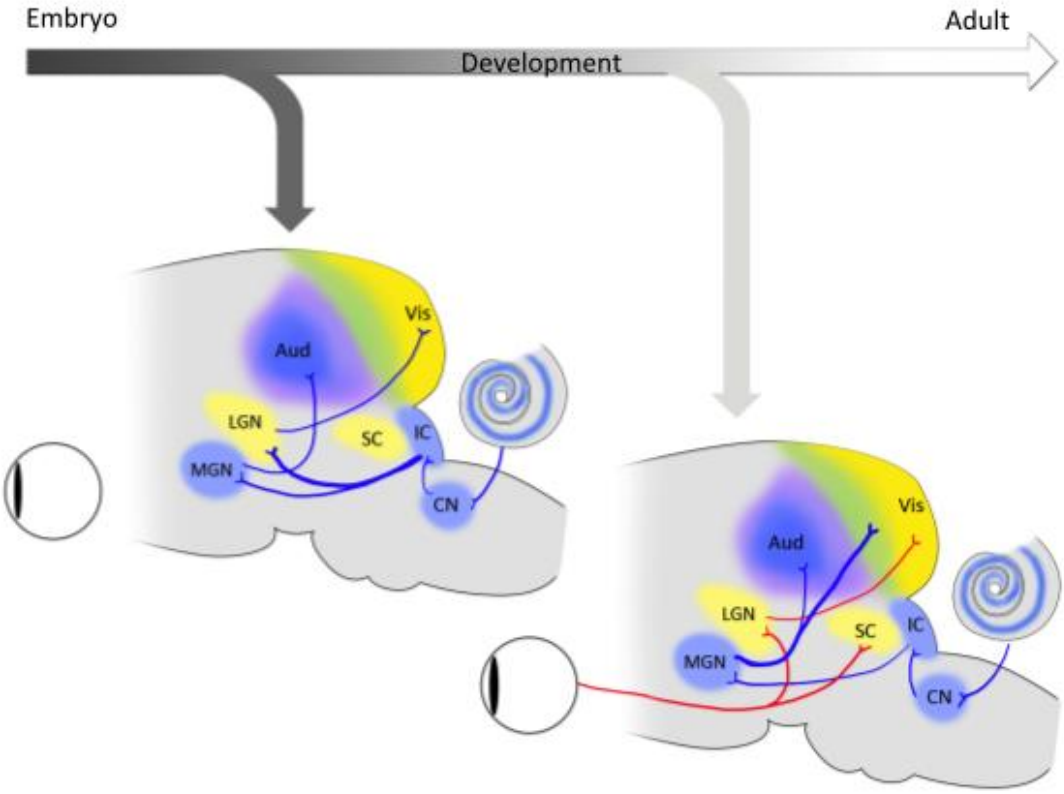

Figure 7.1. Two different models of visual deprivation and their subcortical circuitry

Two different cross-modal circuitries are proposed for different time points for the onset of blindness (bold blue projection highlights the difference between the two). In animal models of 'premature' blindness (left), there is no functional connection between the retina and the visual thalamus (LGN), which is subsequently invaded by cross-modal inputs from subcortical structures such as the auditory inferior colliculus (IC). In these models, the primary visual cortex is the main target of cross-modal inputs. 'Early' blind model (right) show stabilization of ectopic thalamocortical input that usually goes away with experience during development.

in addition, cross-modal thalamocortical inputs into V2L give rise to the multimodality in visual cortex (Fig. 7.1 right). This 'early' blind model reflects developmental ectopic exuberance that usually goes away with experience during development. Without visual experience, these transient ectopic connections are stabilized to express multimodality in visual cortex. The 'early' blind model includes binocularly sutured animals (un-patterned visual input is present), enucleated rats and mice (both are non-altricial species (Clancy et al., 2001)), and dark-reared kittens and mice (spontaneous retinal activity is present).

These two different types of cross-modal circuitry raise the possibility that different sets of molecular mechanisms could control the plasticity at different stages of development. Our work describes postnatal refinement of ectopic exuberance through sensory input. Signaling pathways for axon guidance and arealization may function during refinement of sub-cortical cross-modal circuitry much earlier in life.

I believe that this work has successfully laid a framework in understanding the mechanisms underlying cross-modal plasticity. The anatomical and molecular targets outlined in this work can help facilitate neuronal circuit rehabilitation after loss of a sensory system. In addition, as our understanding of how experience modifies brain circuitry advance, it will provide new perspectives on the etiology and treatment of neurodevelopmental disorders that exhibit abnormal sensory integration and neuronal connectivity patterns.

Functional significance of cross-modal plasticity

Based on our findings, we propose that cross-modal plasticity is part of the normal developmental process and that ectopic exuberance is eliminated with sensory experience. We can think of several hypotheses about the significance of having such flexibility to process different modalities in the sensory cortex. One is to allow maximum efficiency for the neural

system to accommodate to the way the individual functions. We all have individual variance in the degree we utilize each sensory modality. Blindness or deafness presents extreme cases of underutilization of one sensory modality. As we have discussed in previous chapters, the sensory cortex of the deprived modality can be activated by other sensory inputs. But for most of us, the variation in sensory experience is very subtle. Some may rely on vision a little more than sound to understand the world, and others may rather use touch to get a sense of something. It is not hard to imagine how cross-modal plasticity can help nudge our sensory circuits to better utilize the varying degrees of sensory inputs among individuals.

Cross-modal activity during early life may also play a role in sensory integration. In adults, integration across different sensory modalities occurs in regions designated as sensory integration centers such as the superior colliculus (SC) and anterior ectosylvian sulcus (AES) in cats (Wallace et al., 1992; Wallace and Stein, 2000). However, studies in young animals showed that multisensory neurons do not appear until 12 weeks of age in AES (Wallace et al., 2006). Kittens at 12 weeks engage in play fights and actively explore their surroundings and are old enough to be weaned. It is hard to think that animals lacking the ability to combine different sensory inputs in a meaningful way could display such behaviors. Interestingly, we found that there is greater degree of sensory integration in primary visual cortex in DR mice than normal LR mice (Brady, data not shown). This could indicate that multisensory process takes place in lower sensory areas prior to establishing a proper sensory integration center.

This inevitably raises question about the way environment is perceived by an infant. For an adult, it seems quite unusual to experience a mixture of sensation triggered by a sensation in another modality. But our findings and other studies that show transient connections between neural structures of different modalities suggest that the world to an infant may seem as a mass of sensory confusion. This hypothesis, termed the Neonatal

Synaesthesia (NS) hypothesis, was originally proposed by Maurer, who argued that all babies go through an early initial phase of synaesthesia and different sensory modalities become increasingly modular at some point in their lives (Maurer, 1993). Our data is the first to provide direct evidence that immature sensory cortex is cross-modally activated, and develops sensory specificity with age and experience. According to this hypothesis, adult synaesthesia represents a failure in this modularization process.

Synesthesia and Autism Spectrum Disorder (ASD)

For this study, we have focused on expression of multimodality in visual cortex of the blind. However, in some people, a stimulus is naturally perceived through two or more sensory modalities that are not usually associated with each other. This, unlike the condition of blind people, seems so natural that most people with synesthesia don't even realize it. This interesting phenomenon of having unusual integration of senses is called Synesthesia. In synesthesia, sensory regions that are normally dedicated to processing a single type of input are activated by another modality. For instance, functional imaging study revealed that the visual cortical area that represents colors (V4/V8) is activated by spoken words in 'colored-hearing' synesthesia (Nunn et al., 2002). Synesthesia is thought to be a heritable trait, but the underlying genetic or neuronal mechanism is yet to be identified. Recently, hyper- connectivity between two or more sensory regions has been proposed to underlie synesthesia (for review see Hubbard, 2007; Bargary and Mitchell, 2008). Although it is hypothetical, mechanisms from our study may underlie this fascinating phenomenon. Based on the results of our study, cross-modal projections persist and remain plastic well into adolescence (P25 in mice is considered a juvenile). If it were true cross-modal connections are modified through late development, possibly through adolescence or early adulthood, we can imagine how small variations in the

molecular composition or the way a child experience and interacts with the world could leave a lasting impression on the sensory circuitry to result in sensory integration on slightly different levels.

On a different note, disruption of connectivity in higher brain regions has also been associated with detrimental neurodevelopmental disorders such as schizophrenia (Zikopoulos & Barbas, 2007) and autism (Müller et al., 2011). It is interesting to note that there recently has been increasing evidence for disruption in Nogo receptor signaling in schizophrenia (Schwab, 2010). The Nogo receptor gene is located in a key genetic locus associated with schizophrenia (Hsu et al., 2007), and several rare sequence variants of NgR in patients with schizophrenia has been found to cause failure in transduction of myelin signals of axonal inhibition (Budel et al., 2008). Patients with Williams Syndrome, a rare genetic form of mental retardation, exhibit visual cortical activity in response to music (Thorton-Wells et al., 2010) and have aberrant levels of white and grey matter in the temporal and frontal cortices (Campbell et al., 2009). In Autism Spectrum Disorders (ASD), abnormal sensory integration patterns and exuberance in local circuitry were observed (Courchesne and Pierce, 2005; Andreasen et al., 1996; Harrison et al., 2001; Muller et al., 2011; for review see Lewis and Lieberman, 2000; Geschwind and Levitt, 2007). Recent studies in ASD have revealed mutations in genetic pathways, many of which are involved in synapse regulation (Jamain et al., 2003; Durand et al., 2006; Garber, 2007). Synesthesia in ASD patients has also been documented by case studies and anecdotes (Baron-Cohen et al., 2007; Bor et al., 2008). It is not hard to imagine how disruption in sensory integration could lead to difficulties in learning very complex skills such as language, and social cues. It is possible that genes associated with ASD predispose the individual to become more or less sensitive to environmental inputs and leads to permanent changes in the neuronal network. Also, since communication and social skills require harmony across so many different brain

regions, slight changes in connectivity pattern may lead to expression of ASD symptoms. Thus, it will be interesting to adapt the methods used in this study to look for cross-modal connections and/or aberrant networks in mouse model of ASD with an emphasis on the interaction between intrinsic genetic mutations and environmental factors.

Our work provides a model of how sensory modularization develops in an experience-dependent manner. We show anatomical insight into cross-modal inputs that are pruned out or retained depending on early life sensory experience. Lastly, we propose molecular targets that regulate this process. We hope that this research help construct better understanding of the lasting influences of early life experience, which could lead to individual differences in perspectives or to even detrimental result in a developing child.

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