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Mapping Cortical Plasticity Induced by Noise Exposure Using C-Fos Immunoreactivity

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MAPPING CORTICAL PLASTICITY INDUCED BY NOISE EXPOSURE USING
C-FOS IMMUNOREACTIVITY

by

Paul Sirek

Graduate Program in Clinical Anatomy

A thesis submitted in partial fulfillment
of the requirements for the degree of
Masters of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

Using in vivo electrophysiological recordings in rats, our lab has recently observed that high-intensity noise exposure causes an increase in the number of neurons in the auditory and multisensory cortices that are responsive to visual stimuli (i.e., cortical crossmodal plasticity). To extend this work, the present study evaluated our hypothesis that this noise-induced crossmodal plasticity can also be assessed by mapping the expression of the activity marker, c-Fos, across multiple cortical areas in response to visual stimuli.

Adult male rats were exposed to a 120dB noise (0.8-20kHz) for two hours, and the level of hearing loss was assessed with an auditory brainstem response (average hearing loss $\sim 22 \pm 5$ dB). Fourteen days later, noise-exposed rats (and age-matched shams) were subjected to a visual stimulation protocol known to induce c-Fos activation (200 light flashes; 1-3s ITI), followed by transcardial perfusion two hours post-stimulation.

Visually-responsive neurons in the noise-exposed and sham rats were confirmed with immunohistochemistry and fluorescent microscopy. Inconsistent with our previous electrophysiological studies, the molecular mapping of c-Fos did not demonstrate an increased responsiveness to visual stimulation in the auditory and multisensory cortices following noise exposure. However, these results may be confounded by the short duration (6 min) of the visual stimulation protocol, as it evoked lower levels of c-Fos than previously reported in the literature. Future work will continue to investigate whether molecular mapping represents a useful tool for studying crossmodal plasticity.

Keywords

Crossmodal Plasticity, Molecular Mapping, c-Fos, Noise-Exposure

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Chapter 1

1. Literature Review

1.1 Overview of Cortical Plasticity and Development

The visual, auditory and somatosensory cortices of many mammals are both anatomically and physiologically immature at birth (Hubel & Wiesel, 1963; Boothe, Dobson & Teller, 1985; Fagiolini, Pizzorusso, Berardi, Domenici & Maffei, 1994; Rosen, Semple & Sanes, 2010; Killackey, Rhoades & Bennett-Clarke, 1995). Throughout development, cortical neurons rapidly alter connections and begin to function similarly to adults. A great deal of research starting in the late 1950s focused on early periods of plasticity in almost all species called “critical periods” (Barth, Hirsch, Meinertzhagen & Heisenberg, 1997; Berardi, Pizzorusso & Maffei, 2000; Scott 1962; Fagiolini, Pizzorusso, Berardi, Domenici & Maffei, 1994; Denenberg, 1964). Critical periods are important in many aspects of development including song acquisition in birds and language in humans (Doupe & Kuhl, 1999).

Sensory deprivation in critical periods leads to drastic changes in cortical organization, whereas similar deprivations before or after the critical periods have little to no effect (Issa, Trachtenberg, Chapman, Zahs & Stryker, 1999; Fagiolini, Pizzorusso, Berardi, Domenici & Maffei, 1994; Berardi, Pizzorusso & Maffei, 2000). Issa and colleagues (1999) monocularly deprived ferrets by suturing one eye shut, for 2, 7 or 14 days at several different time periods throughout development ranging from postnatal day 19 to postnatal day 110. They demonstrated that monocular deprivation occurring for 7

days, beginning at approximately postnatal day 42, caused the most drastic changes in the cortex. The deprived eye showed weak orientation tuning compared to the non-deprived eye measured by microelectrodes placed in the respective visual cortices. If monocular deprivation ended before postnatal day 32 or began after postnatal day 100, the orientation tuning of the respective eyes did not significantly change. Moreover, if the deprivation occurred between postnatal day 50 and 65, it was half as effective as if deprivation occurred beginning on day 42. These results demonstrate that visual orientation tuning in ferrets develops around postnatal day 42. If sensory deprivation occurs before or after this critical period of development, the orientation tuning in the cortex is much less affected. Fagiolini and colleagues presented another example of the importance of critical periods for the development of the visual system in 1994. Rats were dark reared until postnatal day 60 and visual acuity was then tested. The dark reared rats showed impaired orientation and movement direction detection of visual stimuli. Therefore, these results indicate that organization of the visual system is dependent on stimulation arising during the critical period.

The auditory cortex also changes drastically during development and modified stimulation during the critical period can alter normal cortical organization. Zhang and colleagues in 2001 exposed rat pups to pulsed white noise at moderate intensity during postnatal day 9 to postnatal day 28. They demonstrated that in the primary auditory cortex, the exposure to white noise disrupted tonotopicity and the frequency-response selectivity of neurons in the primary auditory cortex. If the same white noise was given to rats over the age of postnatal day 30, no disruption was indicated. Thus, there is a critical

period in the development of both the visual and auditory pathways where the correct stimulation must be present for normal cortical organization.

Although the capacity for change is significantly larger during the critical period, neuroplasticity can still occur in adulthood. A breakthrough experiment conducted by Merzenich and colleagues in 1984 demonstrated somatosensory cortical map alterations after amputating the middle finger in adult monkeys. Cortical representations of the hand were mapped using microelectrodes in area 3b [Figure 1.1 A]. The monkeys were then subjected to bilateral middle digit amputations, and area 3b was remapped after 2-8 months [Figure 1.1 B]. In all monkeys studied, the representation of the surrounding digits (D2 and D4) and palm region were topographically mapped in the same area that originally responded to the middle finger. Thus, cortical maps do not only change in the critical period during development, they are dynamic throughout life.

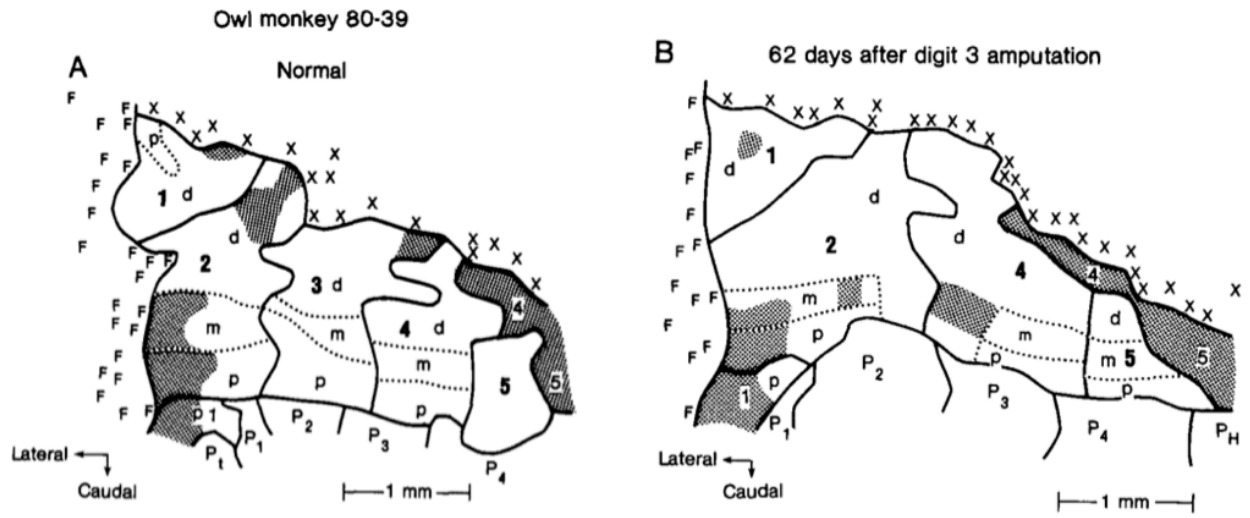


Figure 1.1: Representation of hand surfaces in area 3b of normal (A) and 62 days after the third digit was amputated (B) of the same adult owl monkey. The map was derived from 244 penetrations. The numbers represent areas in the cortex that are activated in response to digits 1 (thumb) to 5. d,m and p are representations from the distal, middle and proximal phalanges. P1-P4 are palmar pads from the base of the digits (Image from: Merzenich, Nelson, Stryker, Cynader, Schoppmann & Zook 1984, with permission).

1.2 Unimodal Plasticity

Adult-onset plasticity occurs when there is decreased or altered input to cortical sensory areas, such as the somatosensory, visual or auditory cortices. Two well-studied causes of plasticity occur when animals are subjected to lesions (elimination of a set of normal inputs), or experience-dependent plasticity, where normal inputs are altered. Several days, weeks or months after inputs are changed, sensory cortices will show significant differences, where the representation of still-intact inputs expands to surrounding unresponsive cortical areas.

Several experiments have documented the change that occurs after lesioning somatosensory nerves (Merzenich, Kaas, Wall, Nelson, & Felleman, D.J; Merzenich et al, 1984; Rasmusson, 1982; Kelahan & Doetsch, 1984; Calford & Tweedale, 1988; Kalaska & Pomeranz, 1979; Wall & Cusic, 1984; Pons et al, 1991). Merzenich et al conducted one of the original experiments demonstrating plasticity following a lesion in 1983. They topographically mapped the somatosensory areas 3b and 1 in adult monkeys. Later, they transected the median nerve, which removes the normal sensory inputs on the ventral side of digits D1-D3. Immediately after the transection, areas of the cortex originally representing the median nerve were unresponsive. As the weeks progressed, the unresponsive region of the cortex began to be activated by surrounding areas of skin (palm, posterior skin areas, and adjacent digits). This activation was thought to occur due to existing horizontal cortical projections that were initially inhibited. As time passed, an increasingly larger area of the unresponsive cortex became re-activated. Therefore, it is evident that the somatosensory cortex of adult monkeys drastically changes following a sensory nerve lesion.

The somatosensory cortex of smaller rodents, more specifically rats, also changes after lesioning somatosensory nerves. In 1984, Wall & Cusick severed the sciatic nerve of several rats. The hindpaw of rats is topographically mapped and innervated by two nerves: the sciatic nerve represents 85% of the cortical area S1, and the saphenous nerve represents 15%. As soon as 1-2 days after transection, the area of S1 responsive to the saphenous nerve expanded three times the size compared to unlesioned controls. Thus, both higher and lower-order animals show drastic changes in their somatosensory cortices following sensory nerve transection.

The visual cortex is also susceptible to remodeling after loss of sensory inputs (Kaas et al, 1990; Schmid et al, 1996; Heinen & Skavenski, 1981; Gilbert & Wiesel, 1992; Sugita, 1996; Chino et al, 1992; Buonomano & Merzenich, 1998). Kaas et al in 1990 lesioned a small area on one retina and enucleated the other retina in cats. Several weeks later, they mapped the visual cortex and discovered that regions surrounding the lesion now activated the previously unresponsive area. In contrast, Schmid et al in 1996 lesioned only one eye and left the other intact. A few weeks later, they reported larger receptive fields in areas surrounding the lesion of one eye, and normal cortical organization in the normal unlesioned eye. Cortical reinnervation is thought to be due to new cortical-cortical horizontal projections rather than thalamocortical projections (Darian-Smith & Gilbert in 1995). Several weeks following focal binocular retinal lesions, Darian-Smith and Gilbert injected a retrogradely transported fluorescent tracer into the reorganized cortex. They showed cortical-cortical projections accounted for the reorganization and there were no thalamocortical connections beyond their normal lateral territory. Thus, cortical plasticity occurs in the visual cortex in adult animals following

retinal lesions of one or both eyes and this plasticity is due to altered cortical-cortical projections.

The adult-onset plastic changes in the auditory cortex have also been well documented (Robertson & Irvine, 1989; Rajan et al, 1993; Schwaber et al, 1993; Buonomano & Merzenich, 1998). Schwaber et al in 1993 recorded changes in A1 following bilateral cochlear lesions in monkeys. They demonstrated that neighboring frequencies increased representation in the area that was initially destroyed by the cochlear lesions. Robertson and Irvine in 1989 studied how the auditory cortex changed following restricted monaural lesions of the cochlea in guinea pigs. They showed that one month following the lesion, neurons in the deprived cortical area responded to neighboring frequencies, and normal recordings were found in the unlesioned ear. Thus, somatosensory, visual and auditory areas all demonstrate unimodal plastic change following partial or full sensory deprivation, such that unaffected neighboring areas will respond to regions in the cortex that were unresponsive following the lesion.

Adult-onset neuroplasticity can also occur when normal peripheral information is altered in the absence of a lesion. The cortex can dynamically change in a use-dependent manner (Buonomano & Merzenich, 1998). For example, nipple-bearing skin in female lactating rats showed a two-fold increase in cortical representation compared to non-lactating controls (Xerri et al, 1994). Also, monkeys performing an intensive small-object retrieval task demanding fine-touch discrimination showed almost a two-fold increase in size of cortical representation of digit-tips compared to controls (Xerri et al, 1994). Therefore, significant changes to the somatosensory cortex arise, called experience-dependent plasticity, due to altered peripheral stimulation.

Experience-dependent plasticity has not only been documented in the somatosensory cortex, but also in the auditory cortex. For example, Recanone et al subjected monkeys to a behavioural frequency discrimination task and mapped the auditory cortex after several weeks of training. The results showed that the representation of the frequency band was several times larger in experimental animals than in controls (Recanzone et al, 1993). Another experiment used a classical conditioning paradigm, where a tone was paired with an electric shock. A1 was mapped before and after several weeks of training, and it was shown that the frequency band corresponding to the conditioned stimulus tone was much larger in experimental animals versus controls. These experiments together indicate that cortical organization can change due to altered peripheral input in a use-dependent manner. In conclusion, cortical organization changes dramatically following sensory deprivation due to either a lesion or altered peripheral information.

1.3 Crossmodal Plasticity

Historically, crossmodal plasticity has been described in individuals with early-onset deprivation. Following deprivation of one modality, the brain can compensate with supranormal performance of another intact modality. For example, visual sensory deprivation alters the primary visual area and surrounding areas such as the auditory cortex. (Bavelier and Neville, 2002; Ryugu et al, 1975; Gyllensten et al, 1965; Vidyasagar, 1978; Hyvarinen et al, 1981; Raushchecker & Kniepert, 1994). Researchers have demonstrated that following early visual deafferentiation in rats (Ryugu et al, 1975) and mice (Gyllensten et al, 1965) there was increased spine and neuronal density in the auditory cortex. This hypertrophy may be explained by the abnormal maturation of the

visual cortex, resulting in stabilization of usually transient signals from the auditory cortex (Kato et al, 1993; Innocenti & Clarke, 1984; Negyessy et al, 2000). Rauschecker and colleagues in 1992 explained the increased neuronal and spine density due to the reliance of the individual on non-deprived senses. Thus, significant changes were observed in the primary auditory area following early visual sensory deprivation.

Multimodal brain areas also show enhanced input from the non-deprived modality following early unimodal visual deprivation. Both Vidyasagar (1978) and Hyvarinen and colleagues (1981), showed that after visual deprivation, there was an increase in auditory and somatosensory activation in polymodal association areas, such as the superior colliculus and parietal cortex. Moreover, cats that were visually deprived from birth show improved auditory localization and greater auditory spatial tuning in the ectosylvian cortex compared to sighted controls (Rauschecker & Kniepert, 1994). Therefore, following early visual sensory deprivation, both multimodal brain areas and primary sensory cortices from other modalities will undergo significant changes.

Early-onset auditory sensory deprivation also leads to significant changes in visual representation. For example, several experiments have demonstrated that deaf individuals process events in the peripheral visual field more effectively than hearing individuals (Bavelier et al, 2000; Proksch & Bavelier, 2002; Neville & Lawson, 1987; Loke & Song, 1991). Bavelier et al in 2000 showed that deaf subjects presented with moving stimuli in the peripheral visual field had greater recruitment of the motion-selective area MT/MST compared to hearing controls, as measured by functional magnetic resonance imaging. These findings indicate that deaf individuals give more visual attention to the peripheral visual space compared to hearing controls.

The primary auditory cortex of deaf individuals can also be activated in response to visual stimulation. Rebillard and colleagues (1977) demonstrated that 2-8 months following early cochlear destruction, the primary auditory cortex responded to visual stimulation. Moreover, several experiments have shown that the auditory cortex of congenitally deaf human subjects is activated when viewing sign language (Nishimura et al, 1999; Petitto, Zatorre, Gauna, Nikelski, Dostie & Evans, 2000) or other visual stimulation (Finney, Fine & Dobkins, 2001). These results indicate that there is significant cross-modal plasticity following unimodal deprivation. However, these experiments were all conducted on individuals deprived early in development.

Recently, Allman and colleagues demonstrated significant crossmodal plasticity in adult ferrets following a profound hearing loss (Allman et al, 2009). Adult ferrets were deafened by coadministration of kanamycin and ethacrynic acid. An auditory brainstem response was conducted after several weeks to confirm the animals were profoundly deaf. Approximately 76 days following administration of the drugs, no neurons in the primary auditory cortex responded to acoustic stimulation, however, 84% of neurons responded to somatosensory cues. Thus it is evident that adult ferrets experienced significant crossmodal plasticity following a profound hearing loss, where neurons that previously responded to auditory stimulation now responded to somatosensory stimulation.

Although Allman et al (2009) showed crossmodal neuronal replacement in the primary auditory cortex of adult ferrets with a profound hearing loss, few studies have documented the cortical reorganization following adult-onset partial hearing loss. In 2012, Meredith et al demonstrated significant multisensory changes in the auditory cortex of ferrets following a partial hearing loss. Almost all neurons (99%) in the primary auditory

cortex of animals with a partial hearing loss still responded to auditory stimulation. However, 68% (versus 34% in normal hearing controls) of neurons in the primary auditory cortex were also influenced by another modality including visual or tactile cues. Therefore, following a partial hearing loss there appears to be a significant increase in crossmodal convergence in the primary auditory cortex, compared to crossmodal neuronal replacement that occurs in response to profound hearing loss.

Furthermore, a recent study in our laboratory not only examined changes following a partial hearing loss in the primary auditory cortex, but also the surrounding cortical regions such as the multisensory and visual areas. Experimental rats were exposed to a 120 dB SPL noise (0.8-20kHz), for two hours. Fourteen days later, they were given a visual stimulus and electrodes were placed in the visual, multisensory and auditory cortices. Following a noise exposure, rats showed an increased number of neurons responding to a visual stimulus in the multisensory and auditory cortices compared to controls. There appeared to be a shift of visual responsiveness in the multisensory and auditory areas as shown in Figure 1.2. Thus, significant changes to visually responsive neurons can be observed in adults following a partial hearing loss in both the primary auditory cortex and the multisensory audiovisual areas.

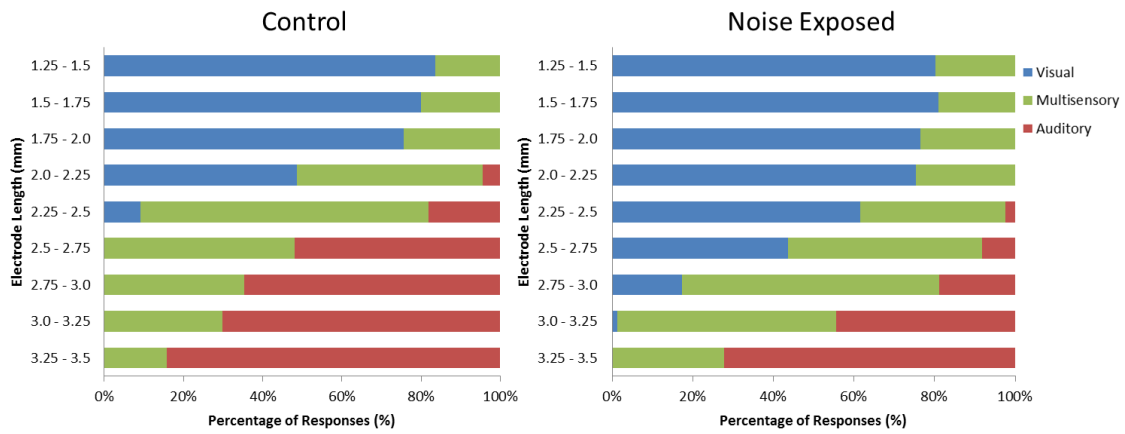


Figure 1.2: Electrophysiological recordings in visual, multisensory and auditory cortical areas in controls versus noise exposed rats. Following noise exposure, there are more visually responsive neurons in the visual, multisensory and auditory cortical areas. (Personal Communication with A. Schormans, March 2015).

1.4 Molecular Mapping

Upon stimulation, neurons must organize a variety of different cellular functions, which allows them change, add or modify existing synaptic connections (Farviar et al, 2004). An important aspect of neuronal integration is the activation of a specific transcription factor, AP-1, which is thought to regulate processes such as proliferation, differentiation and apoptosis (Hess et al, 2004). AP-1 is composed of several different immediate early genes, specifically members of the Fos and Jun families (Kaminska et al, 1999). Immediate early genes (IEGs) are a class of genes that are rapidly and transiently up regulated upon neuronal stimulation. Jun proteins can create either homodimers or heterodimers with members of the Fos family to form the AP-1 complex (Kaminska et al, 1999).

In particular, c-Fos is an immediate early gene that has been well characterized and extensively used as a neuronal activity marker (Kaczmarek & Chaudhuri 1996; Farivar et al, 2004). Basal expression of c-Fos is low, and it is highly up regulated after auditory (Zangenehpour & Chaudhuri, 2001; Zuschratter et al, 1995) or visual (Zangenehpour & Chaudhuri, 2001; Zangenehpour & Chaudhuri, 2002; Yamada et al, 1999) stimulation. Zuschratter et al in 1995 described increased c-Fos expression one hour after auditory tone burst stimulation in the primary auditory cortex. In 2002, Zangenehpour and Chaudhuri demonstrated that peak c-Fos protein levels occurred 120 minutes after the onset of light stimulation [Figure 1.3]. They later described its down-regulation 240 minutes after the onset of light stimulation (or 120 minutes after peak activation time). Thus c-Fos is highly ordered, showing specific peaks of up- and down-

regulation after neuronal stimulation, and proven to be an effective neuronal activity marker.

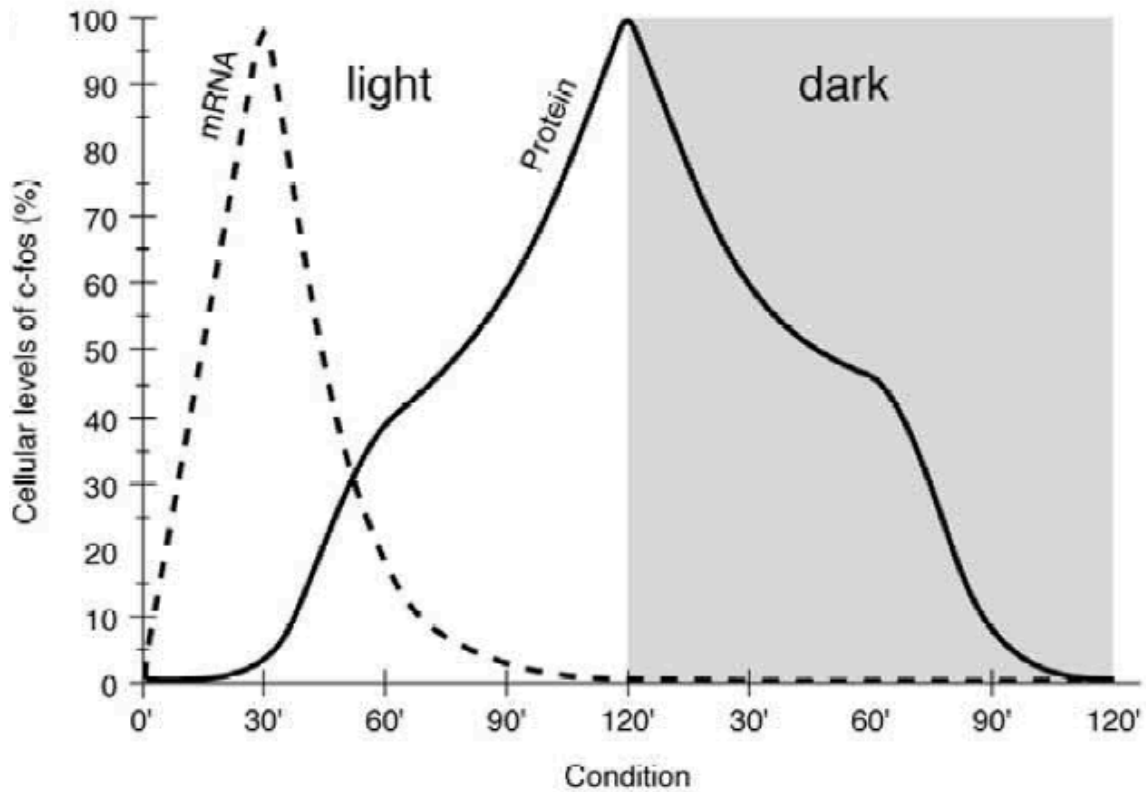


Figure 1.3: The temporal up- and down-regulation profile of c-Fos mRNA and protein levels in the rat visual cortex. Light stimulation lasted for 0,30, 60, 90 or 120 minutes. Dark adaptation occurred 30, 60, 90 and 120 minutes after 120 minutes of light stimulation (Image from: Zangenehpour & Chaudhuri, 2002, with permission).

An interesting experiment using zif268 as a molecular mapping tool identified crossmodal visual neurons following an auditory stimulus presentation (Hirst et al, 2012). Experimental monkeys were subjected to a pairing of auditory and visual stimuli for 45 minutes to establish implicit association between the auditory and visual modalities with the given stimuli. When experimental animals were presented with sounds alone 24 hours later, they showed an increased number of zif268 active neurons in the visual cortex compared to controls. Therefore, these results indicate that molecular mapping can be used as an effective tool in analyzing cortical crossmodal plasticity.

A significant amount of research has been conducted on adult-onset unimodal plasticity, however, relatively little is known about adult-onset crossmodal plasticity. More specifically, the changes that occur in the primary auditory cortex and surrounding areas following adult-onset partial hearing loss are not well understood. This is surprising given that it is estimated that approximately 16% of adults in the USA show some form of mild hearing loss (Mitchell, 2006; Agrawal et al, 2008). To our knowledge, however, no previous study has used molecular mapping of c-Fos to map adult-onset cortical crossmodal plasticity following a partial hearing loss. This experiment extends the electrophysiological work observed recently in our lab showing an increase in the number of neurons in the auditory and multisensory cortices that are responsive to visual stimuli after a noise exposure. If, as hypothesized, we observe an increase in the number of c-Fos-immunoreactive neurons in noise-exposed rats, this would establish that molecular mapping represents a useful tool for studying cortical crossmodal plasticity.

Chapter 2

2. Hypotheses and Objectives

This study was conducted to test the overall hypothesis that noise-induced crossmodal plasticity can be assessed by mapping the activation of the immediate early gene, c-Fos, across multiple cortical areas in response to visual stimuli.

There was one main objective for this work:

1. Using an established rat model of noise-induced crossmodal plasticity, we sought to evaluate whether c-Fos expression could serve as a complimentary technique to electrophysiological recordings to map the increased responsiveness to visual stimuli that occurs in the auditory and multisensory cortices.

Chapter 3

3. Materials and Methods

3.1 Animal Care and Handling

All experiments were conducted in accordance with the University Council on Animal Care and approved by the animal research committee at the University of Western Ontario. All measures were taken to minimize animal suffering and reduce the number of animals used in the present study.

3.2 Overview of Experimental Series

The goal of the present experiment was to visually activate c-Fos to map cortical plasticity following partial hearing loss. Experimental animals were subjected to a noise exposure and the level of hearing loss was assessed with an auditory brainstem response. Fourteen days later, noise-exposed rats (and age-matched shams) were subjected to a visual stimulation protocol known to induce c-Fos expression, discovered in a pilot study followed by transcardial perfusion. Control animals were given only one ABR and subjected to the visual stimulation protocol followed by transcardial perfusion. Visually-responsive neurons in the noise-exposed, sham and control rats were confirmed with immunohistochemistry and fluorescent microscopy. If, as hypothesized, we observe an increase in the number of c-Fos-immunoreactive neurons in noise-exposed rats, this would establish that molecular mapping represents a useful tool for studying cortical crossmodal plasticity.

3.3 Animals and Stimuli

Adult male Sprague Dawley rats (N=10; Charles River, Quebec, Canada) were used for immunohistochemistry analysis. All rats were anesthetized with a combination of Ketamine (dose = 80mg/kg body; concentration = 100mg/mL) and Xylazine (dose = 5mg/kg body; concentration = 20mg/mL) and given an initial auditory brain stem response (ABR) on day 0. Immediately following the initial ABR, experimental animals (N=5) were subjected to a previously established noise exposure protocol (120 dB SPL noise, 0.8-20 kHz, 2 hours). Both groups of animals were then returned to the animal care facility (usual 12-h/12-h light/dark cycle). According to Yamada et al in 1999, c-fos expression following a visual stimulation with preceding dark rearing did not induce significantly higher mRNA levels in the visual areas than without dark rearing. Thus it was not necessary for the rats to be kept in the dark before the experimental procedure to evoke visually responsive c-fos. Fourteen days later, rats were anesthetized with Ketamine and Xylazine and subjected to a final ABR to assess hearing levels, and as expected, noise exposed rats had a significantly higher click threshold. Control rats were only given an initial ABR. Two hours after the ABR, rats were subjected to a visual stimulation protocol known to induce c-Fos expression (200 light flashes; 1-3 s ITI), followed by transcardial perfusion two hours post-stimulation. Visually-responsive neurons in the noise-exposed, sham and control rats were confirmed with immunohistochemistry and fluorescent microscopy.

3.4 Auditory Brainstem Response

An initial and final ABR were performed on each noise-exposed and sham animal to assess overall hearing loss. The initial ABR was conducted to measure the incoming hearing levels of all animals. Since the original hearing levels of the rats are unknown, it is crucial to confirm that they do not initially have a partial hearing loss before beginning the experiment. The final ABR was performed to confirm a partial hearing loss in the experimental group, and no change in the sham group.

Prior to performing the ABRs, the speaker was calibrated using a Larson Davis Model 2530 microphone. The microphone was initially calibrated with a Larson Davis precision acoustic calibrator piston phone that generated a tone at 251.2 Hz at 114 dB. Finally, the MF1 speaker was calibrated with the microphone.

For the ABR, three subdural needle electrodes were inserted in: the vertex, posterior the right pinna directed at the mastoid process and a ground placed in the rats back midway between front and hind limbs. A speaker (MF1) was placed 10cm from the base of the right pinna. To ensure a monaurally presented stimulation, an earplug was positioned in the left ear and held in place by tape. Click stimulations (0.1 ms duration, presented at a rate of 21 clicks/second) were presented using BioSigRZ delivered by a Tucker Davis RZ6 Multi I/O Processor, at varying sound pressure levels (ranging from 10dB-90dB). Electrophysiological activity was filtered by highpass and lowpass filters set to 300 Hz and 3 kHz respectively and amplified by 20 times. Averaging was done during a 10-ms window over 1000 stimulus presentations. Hearing threshold (in dB) was

defined as the lowest threshold that produced repeatable waveform morphology as confirmed by two judges.

3.5 Noise Exposure

Prior to performing the noise exposures, the speaker was calibrated using a Larson Davis Model 2530 microphone. The microphone was initially calibrated with a Larson Davis precision acoustic calibrator piston phone that generated a tone at 251.2 Hz at 114 dB. Finally, the T90A speaker was calibrated with the microphone.

In accordance with a previously established noise exposure paradigm, the five noise-exposed animals received a 120 dB SPL broadband noise (0.8-20 kHz) binaurally for 2 hours in a foam-lined Whisper Room inc. soundproof booth (Tennessee, USA). The noise was generated using RpvdsEx and delivered by a Tucker Davis RZ6 Multi I/O Processor, to a T90A speaker.

3.6 Visual Stimulation

A light was placed above the animal and angled at 45 degrees to the right eye. The experimental animals were presented with light flashes at 3.0V, which were previously shown in our lab electrophysiologically to be appropriate to generate visual responses in the cortex. A total of 200 trials were presented with varying intertrial times of 1-3s. Thus visual stimulus was presented for a total of approximately 6.6 minutes. Control animals were not presented with the visual stimulation.

3.7 Perfusion

Prior to perfusion, all animals were administered a lethal dose of pentobarbital (dose=100 mg/kg body; concentration = 54.7 mg/mL) and a level of deep anesthesia was confirmed. They were then administered 0.1 mL of Heparin (1000 USP Units/mL) to prevent blood clotting. Rats were perfused transcardially with 300 mL of 0.09% saline (with 0.85 mL heparin/L), and fixed with 400mL of a 4% paraformaldehyde in 0.1M phosphate buffer. The brains were removed and placed in a solution of 4% paraformaldehyde for 24 hours. They were then switched to a 15% sucrose solution until the brain sank (approximately 24 hours), and then placed in a 30% sucrose solution until sectioned.

3.8 Immunohistochemistry

Using a sliding microtome, 40 μ m sections were taken from bregma -4 to -7 mm and allowed to free float in 0.1 M phosphate buffer (PB). Five sections were chosen at bregma -4.5, -5.0, -5.5, -6.0 and -6.5 and were washed and then blocked with 10% non-specific goat serum, 10% BSA and 80% buffer to achieve a final volume of 1 mL/well and spun for 1 hour. The washing and blocking steps were taken to remove any impurities and allow the primary antibody to bind more accurately to the antigen. The sections were then incubated with rabbit anti-c-fos primary antibody (Santa Cruz Biotechnology, Texas, USA) at a concentration of 1:2000 for 24 hours. Sections were then washed and incubated with a goat anti rabbit IgG conjugated to biotin (Vector Laboratories, California, USA) at a concentration of 1:000 in a solution containing 20% triton-x and 80% PB for 1 hour. Triton-x allows for improved antibody penetration. The

sections were then washed and placed in a solution containing avidin-biotin complex (Vector Laboratories, California, USA) for 1 hour to amplify the signal from the secondary antibody. They were then washed and incubated for 10 minutes in a biotin conjugated tyramide solution (1:250; Perkin Elmer, Massachusetts, USA) containing 3% H₂O₂ (1:333 concentration) and PB. Sections were immediately washed and placed in streptavidin AF 568 (Molecular Probes Invitrogen Detection Technologies, Oregon, USA). Lastly sections were washed and plated on gelatin-coated slides and kept in the dark for 24 hours to dry. A coverslip with vectashield (vector laboratories) was placed on each slide to prevent photobleaching.

3.9 Data Acquisition and Analysis

One picture of each of the five sections (bregma -4.5, -5.0, -5.5, -6.0 and -6.5) was taken with an Axioplan 2 imaging light microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) under 2.5x magnification. The picture was taken by measuring from the rhinal fissure to the primary auditory cortex. c-Fos expression was assessed in the auditory (Au1), multisensory (V2L) and visual (V1) cortices of animals in each condition (noise-exposed, sham and control) according to the Paxinos and Watson (2007) rat brain atlas. Five coronal sections (bregma -4.5, -5.0, -5.5, -6.0 and -6.5 mm) were taken from each animal and c-Fos labeled cells were counted in all three cortical areas by two counters [Figure 3.1]. Statistical comparisons were made using a mixed-repeated measures ANOVA and Bonferroni post-hoc tests were used for comparison when significance was indicated by the ANOVA.

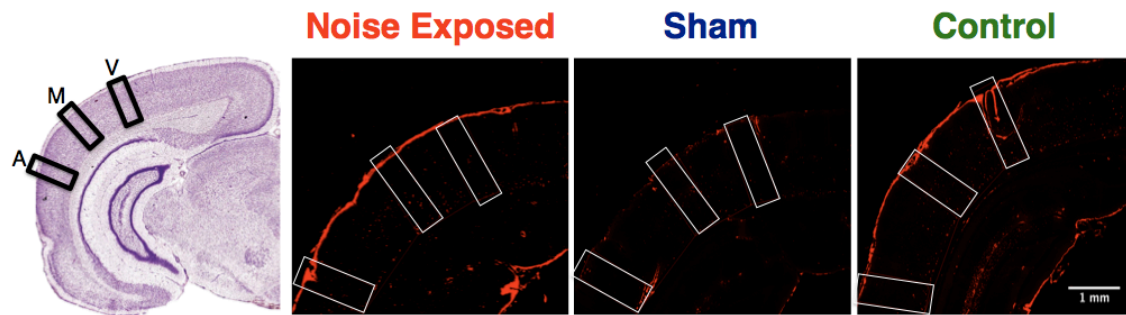


Figure 2.1: c-Fos expression in noise-exposed, sham and control animals. A 0.50 x 2.00 mm area was taken to count c-Fos labelled cells in the auditory (A), multisensory (M) and visual (V) cortical areas.

Chapter 4

4. Results

4.1 Auditory Brainstem Response Results

The adult, noise-exposed rats ($n=5$) revealed an average click threshold increase of 22 ± 5 dB indicating an effective partial hearing loss [Figure 4.1]. A one-way ANOVA was conducted on the initial click thresholds and revealed no significant main effect for condition ($F(2,12)=2.38, p=0.135$). To determine if there was a significant difference in initial and final click thresholds of both noise-exposed and sham animals, a mixed repeated measures ANOVA was conducted and revealed a significant main effect of time ($F(1,8)=9.966, p=0.013$), and an interaction between time and condition ($F(1,8)=25.138, p=0.001$). Further analysis using a paired t-test at a corrected 97.5% confidence interval indicated a significant difference between the initial and final click threshold of noise-exposed animals ($t(4)=-4.745, p=0.009$), but not shams ($t(4)=1.826, p=0.142$).

ABR Thresholds

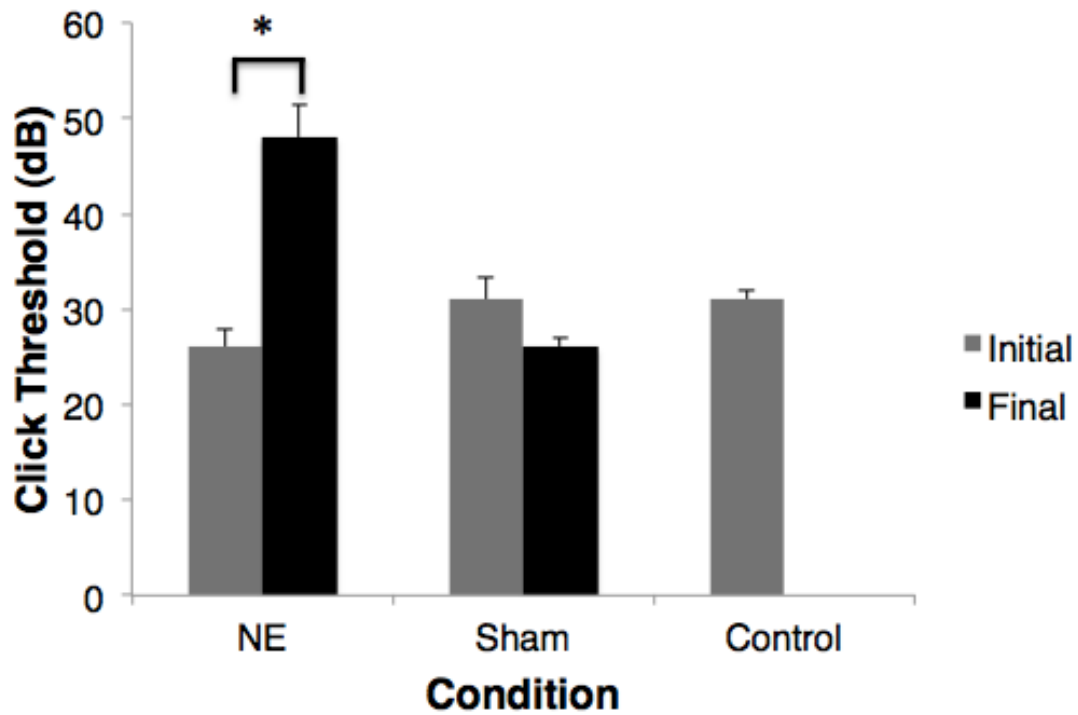


Figure 4.1: Initial and final ABR thresholds from noise-exposed (NE), sham and control animals. Noise exposure caused a significant increase in threshold ($p=0.009$). Error bars represent standard error of the mean.

The ABR wave I amplitude was also analyzed to provide further evidence that the partial hearing loss was effective. Previous studies have demonstrated that even without a rise in ABR threshold, a decrease in wave I amplitude indicates significant damage to the auditory system (Kujawa and Liberman, 2009). The noise-exposed rats revealed on average a more than two-fold decrease in wave I amplitude (average decrease = $0.82 \pm 0.16 \mu\text{V}$; Figure 4.2). A one-way ANOVA was conducted on the initial wave I amplitudes with no significant main effect between groups ($F(2, 12) = 0.503, p = 0.617$). To determine if there was a significant difference in initial and final wave I amplitudes of noise-exposed and sham animals, a mixed repeated measures ANOVA was conducted and revealed a significant main effect of time ($F(1, 8) = 14.935, p = 0.005$), and an interaction between time and condition ($F(1, 8) = 20.622, p = 0.002$). Further analysis using a paired t-test at a corrected 97.5% confidence interval indicated a significant difference between the initial and final wave I amplitude of noise-exposed animals ($t(4) = 4.918, p = 0.008$), but not shams ($t(4) = -0.651, p = 0.550$). Therefore, the noise-exposed animals had significantly higher hearing thresholds and decreased wave I amplitudes compared to age-matched shams. In conclusion, noise-exposed animals had a significant partial hearing loss compared to sham and control animals.

ABR Wave I Amplitudes

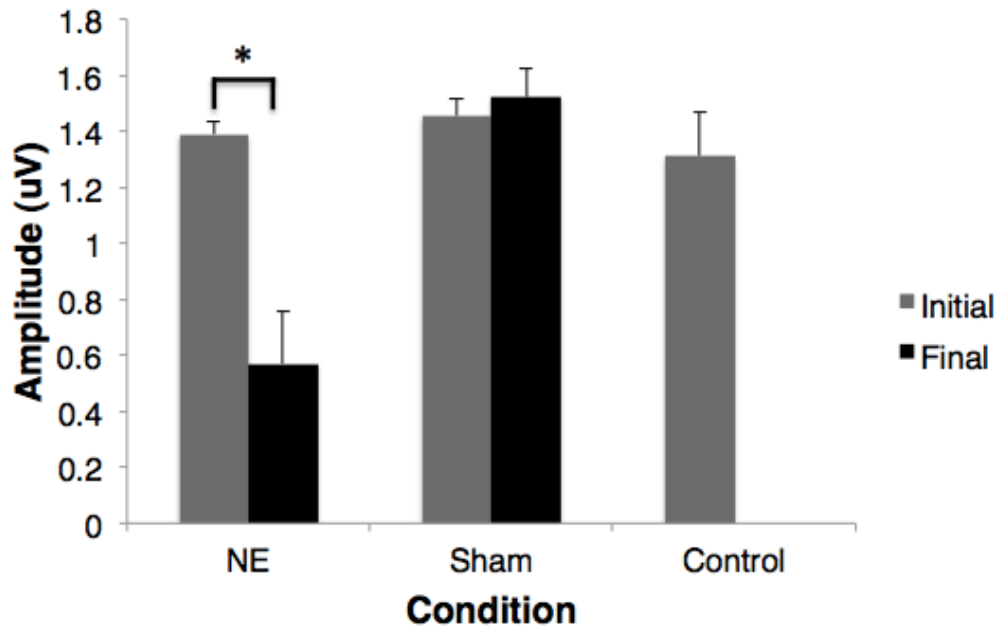


Figure 3.2: Initial and final wave I amplitudes from noise-exposed (NE), sham and control animals. Noise exposure caused a significant decrease in wave I amplitudes ($p=0.008$). Error bars represent standard error of the mean.

4.2 c-Fos Expression Across Cortical Areas

4.2.1 c-Fos Expression Across Cortical Areas in Sham and Control Animals

To determine if there was a difference in c-Fos expression between shams and controls, a mixed repeated measures ANOVA was conducted. c-Fos labeled cells in the auditory, multisensory and visual areas were averaged across all coronal sections (bregma -4.5, -5.0, -5.5, -6.0 and -6.5) to achieve a total c-Fos expression in each area [Figure 4.3].

There was a significant main effect of area ($F(2,16)=11.643$, $p=0.001$) but no interaction between area and condition ($F(2,16)=1.832$, $p=0.192$). Post-hoc tests using a Bonferroni correction revealed a significant difference between c-Fos expression in the auditory and multisensory cortices ($p=0.025$) and the auditory and visual cortices ($p=0.008$), but no difference between the multisensory and visual cortices ($p=0.974$). More importantly, there was no significant difference in c-Fos expression between sham and control animals ($p=0.224$). Therefore, for the remaining discussion control animals will be disregarded.

c-Fos Expression of Sham and Control Animals

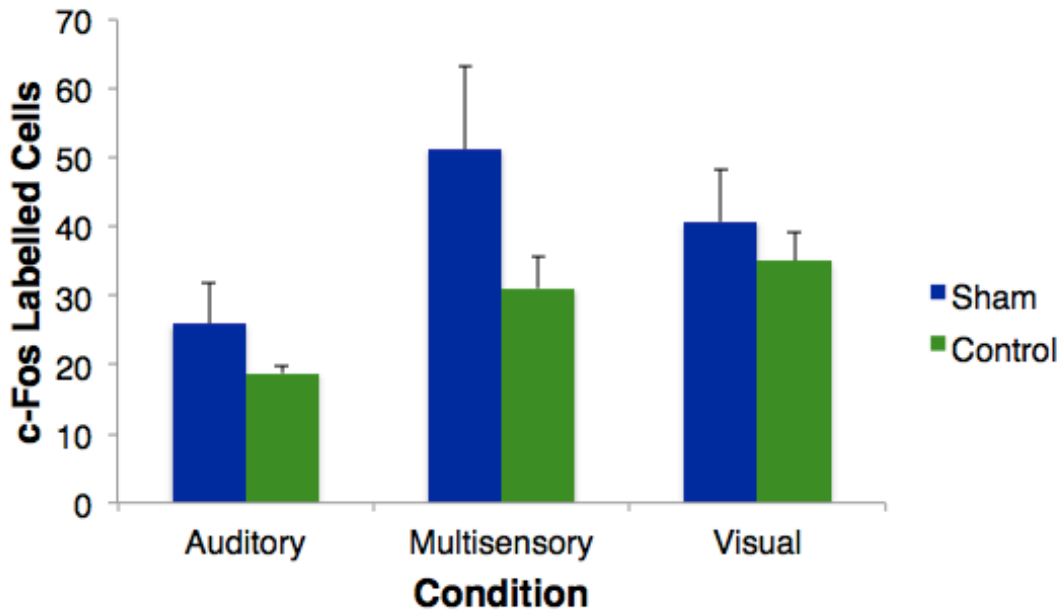


Figure 4.3: c-Fos expression across all coronal sections (bregma -4.5 to -6.5) in the auditory, multisensory and visual cortices of sham and control animals. A mixed repeated measures ANOVA revealed a significant main effect of area between sham and control animals ($p=0.001$). Post-hoc analysis revealed no difference between sham and control animals or noise-exposed and sham animals in any area. Error bars represent standard error of the mean.

4.2.2 c-Fos Expression at Varying Distances from Bregma

c-Fos expression was analyzed between sham and noise-exposed animals at varying distances from bregma in the auditory, multisensory and visual cortices individually. Using a mixed repeated measures ANOVA in the auditory cortex (Figure 4.4 A), there was no significant main effect of distance from bregma ($F(4,32)=1.575, p=0.205$) and no interaction between distance from bregma and condition ($F(4,32)=1.276, p=0.300$). Similarly in the multisensory cortex (Figure 4.4 B), there was no main effect of distance from bregma ($F(4,32)=1.088, p=0.379$) or interaction between distance from bregma and condition ($F(4,32)=0.456, p=0.767$). Lastly in the visual cortex (Figure 4.4 C), there was no significant main effect of distance from bregma ($F(4,32)=1.363, p=0.269$), or interaction between distance from bregma and condition ($F(4,32)=0.355, p=0.839$). In conclusion, there was no significant difference in c-Fos expression at varying distances from bregma, therefore it is possible to collapse across distances from bregma and compare overall c-Fos expression between the auditory, multisensory and visual cortices in both sham and noise-exposed animals.

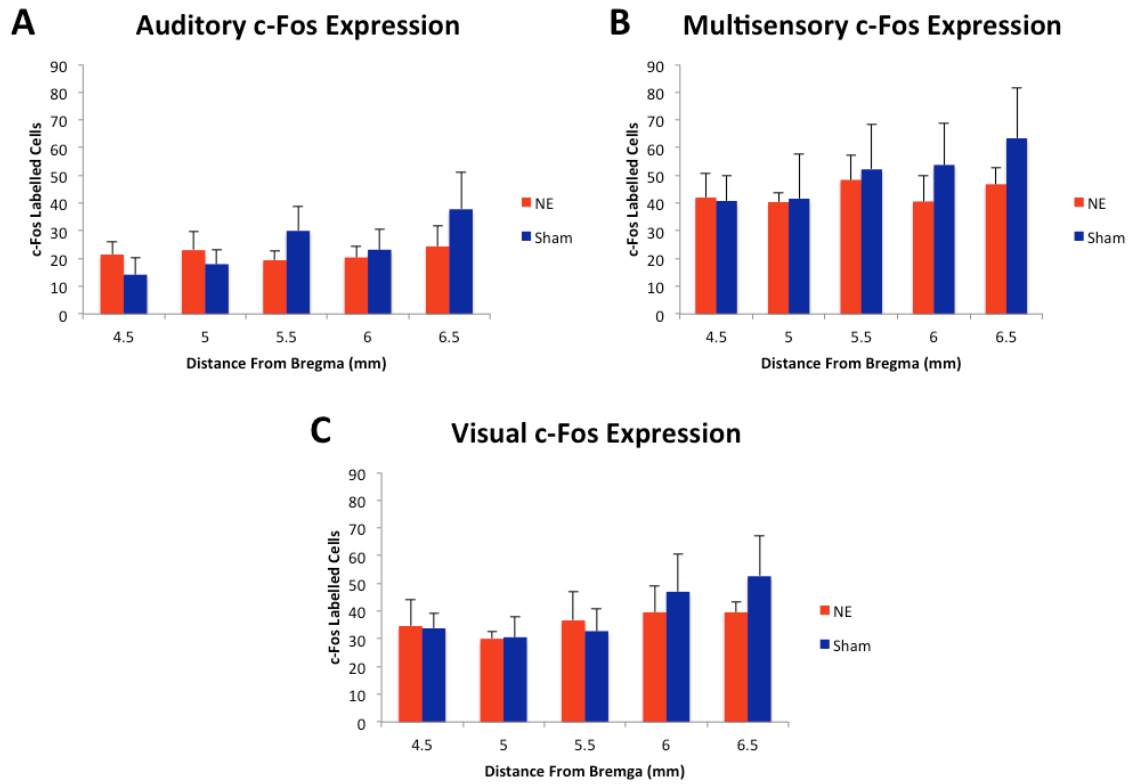


Figure 4.4 : c-Fos expression in the auditory (Panel A), multisensory (Panel B) and visual (Panel C) cortices at varying distances from bregma (-4.5, -5.0 -5.5, -6.0 and -6.5).

Error bars represent standard error of the mean.

4.2.3 c-Fos Expression Across Cortical Areas of Sham and Noise-Exposed Animals

c-Fos labeled cells in the auditory, multisensory and visual areas were averaged across all coronal sections (bregma -4.5, -5.0, -5.5, -6.0 and -6.5) to achieve a total c-Fos expression in each area for noise-exposed and sham animals [Figure 4.5]. Using a mixed repeated measures ANOVA, there was a significant main effect of area ($F(2,16)=14.409$, $p<0.001$), but no interaction between area and condition ($F(2,16)=8.892$, $p=0.090$). Post-hoc analysis using a Bonferroni correction indicated a significant difference between c-Fos expression in the auditory and multisensory cortices ($p=0.006$) and the auditory and visual cortices ($p=0.009$), but no difference between the multisensory and visual cortices ($p=0.232$). Although there was no significant difference in c-Fos expression in sham and noise-exposed animals in any of the three areas, there is a trend where sham animals had consistently higher c-Fos expression levels compared to noise exposed animals.

c-Fos Expression of Noise-Exposed and Sham Animals

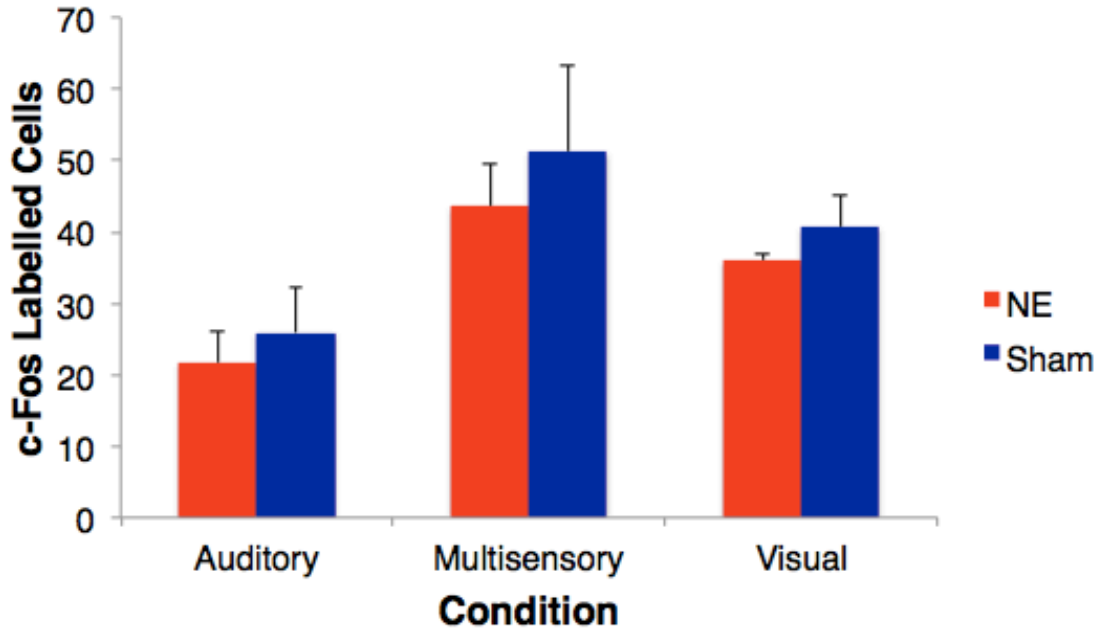


Figure 4.5: c-Fos expression across all coronal sections (bregma -4.5 to -6.5) in the auditory, multisensory and visual cortices of sham and noise-exposed animals. A mixed repeated measures ANOVA revealed a significant main effect of area between sham and noise-exposed animals ($p < 0.001$). Post-hoc analysis revealed no difference between noise-exposed and sham animals in any area. Error bars represent standard error of the mean.

Chapter 5

5. Discussion

The present study attempted to extend the findings of crossmodal plasticity in the auditory and multisensory cortices following hearing loss in adult animals. In 2009, Allman et al. reported significant crossmodal plasticity in adult ferrets, showing 84% of neurons in the primary auditory cortex responded to tactile stimulation following a profound hearing loss. In 2012, Meredith et al. subjected animals to a moderate hearing loss and also revealed a profound increase in the proportions of neurons in the auditory cortex responding to non-auditory cues (such as visual and tactile). Recent findings in our laboratory extended the findings to surrounding multisensory cortical areas, demonstrating that after a moderate hearing impairment, animals have an increased visual responsiveness in the auditory and multisensory cortices compared to control animals. The present experiment sought to evaluate whether c-Fos expression could serve as a complimentary technique to electrophysiological recordings recently shown in our laboratory and effectively map the increased responsiveness to visual stimuli that occurs in the auditory and multisensory cortices following noise exposure.

Adult Sprague-Dawley rats were subjected to an established noise-exposure procedure developed in the Allman laboratory. To confirm the noise-exposed animals had a significant hearing impairment, auditory brainstem response thresholds and wave I amplitudes were measured before and fourteen days following the noise-exposure. Indeed, noise-exposed animals had a significant increase in threshold of 22 ± 5 dB, and decrease in wave I amplitude of 0.82 ± 0.16 μ V indicating an effective partial hearing loss. Kujawa

and Liberman in 2009 demonstrated that even without an increase in hearing threshold, a decrease in wave I amplitude indicated significant damage to the auditory system. Thus both wave I amplitudes and thresholds were measured and confirmed the effectiveness of the established noise-exposure protocol.

c-Fos expression was assessed in the auditory, multisensory and visual cortices of noise-exposed, sham and control animals. Three groups were initially chosen because the sham and noise-exposed animals were retained in the animal facility for fourteen days during the experimental procedure. To rule out the possibility that animals contained in the animal facility were not exposed to certain confounding stimuli such as stress or noise that would affect the results, control animals were used. Control animals were taken from the animal facility, subjected to an initial ABR and visual stimulation procedure and immediately perfused. On the other hand, sham animals were given an initial ABR, maintained in the animal facility for fourteen days, subjected a final ABR then a visual stimulus and perfused. As expected, there was no difference in c-Fos expression between sham and noise-exposed animals in any area; thus, it was not necessary to analyze control animals farther and we concluded that there was no confounding variable in the animal facility that significantly altered the results.

When comparing sham and noise-exposed animals, there was a significant main effect of area, indicating that the visual stimulation appeared to have induced more c-Fos expression in the visual and multisensory cortical areas compared to the primary auditory cortex. However, there was no difference between sham and noise-exposed animals in any area. Previous electrophysiological results in our laboratory indicated that animals subjected to a partial hearing loss had more visually responsive neurons in the primary

auditory and multisensory cortex compared to controls. Therefore, we expected to see an increase c-Fos labeled cells in noise-exposed animals compared to shams in the auditory and multisensory areas, and not in the visual area, which was not shown. It appears as if the visual stimulation was sufficient to evoke more c-Fos labeled cells in the visual and multisensory cortical areas compared to auditory areas in both animal groups, but not accurate enough to differentiate between noise-exposed and sham animals. Inconsistent with our previous electrophysiological studies, the molecular mapping of c-Fos was unable to demonstrate an increased responsiveness to visual stimulation that occurs in the auditory and multisensory cortices following noise exposure.

Compared to a previous study (Yamada et al., 1999), our visual stimulation protocol did not evoke sufficient levels of c-Fos to accurately differentiate between noise-exposed and sham animals, which may have been due to its short duration (6 min). Yamada et al. found on average 100-150 c-Fos labeled cells over a cortical area of approximately 0.064 mm², whereas the present study found on average 40-60 cells expressing c-Fos over a much larger area of approximately 0.85 mm² [Figure 5.1]. A stimulation length of 6 minutes was chosen because pilot studies were focused on dual-labeling audiovisual cortical neurons using both c-Fos and another molecular marker, pERK. In order to effectively dual label, it was necessary to shorten the stimulation length for c-Fos. Thus future studies will use the same experimental procedure, except increase the visual stimulation time from 6 minutes to 45 minutes.

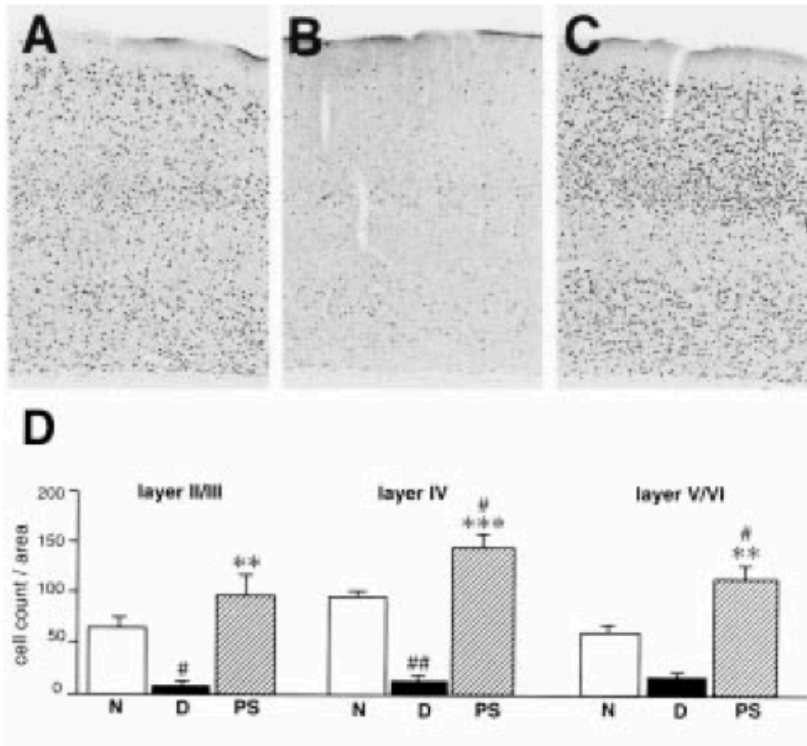


Figure 5.1: c-Fos expression in animals without a photic stimulation that were either naïve (panel A) or dark reared for one week (panel B), and animals subjected to a 45 minute photic stimulation after one week of dark rearing (panel C). Panel D shows c-Fos labeled cells in a 0.064 mm² area in naïve (N) dark reared (D) and photic stimulated (PS) animals in several cortical layers. (Image from: Yamada et al. 1999, with permission).

Another possible explanation for the inconsistent results between molecular mapping of c-Fos and previous electrophysiological studies demonstrating an increased visual responsiveness in noise-exposed animals versus controls, is that molecular mapping may not be reliable in demonstrating cortical crossmodal plasticity. To our knowledge, no previous experiments have attempted to use this technique to study adult onset cortical plasticity following a partial hearing loss. Previously, it has been shown that basal expression of c-Fos is low, and it is highly up regulated after auditory (Zangenehpour & Chaudhuri, 2001; Zuschratter et al, 1995) or visual (Zangenehpour & Chaudhuri, 2001; Zangenehpour & Chaudhuri, 2002; Yamada et al, 1999) stimulation. Zuschratter et al in 1995 described increased c-Fos expression one hour after auditory tone burst stimulation in the primary auditory cortex in experimental animals compared to non-exposed controls. Furthermore, Zangenehpour and Chaudhuri in 2001 demonstrated that expression of another immediate early gene, zif268, was elevated in the primary visual cortex and not in the primary auditory cortex following a visual stimulation. The present study is consistent with these results showing that c-Fos expression is in fact significantly elevated in the visual cortex compared to the auditory cortex following visual stimulation. Therefore, c-Fos expression might be sensitive enough to show increased expression in the core sensory areas following a stimulation procedure, but not to describe cortical crossmodal plasticity.

The discrepancies between molecular mapping and electrophysiology could also be explained due to counting c-Fos in all cortical layers. Previous experiments have described that early after sensory deprivation (approximately 10 days) cortical-cortical horizontal projections lead to plasticity rather than altered thalamocortical projections.

However, 30 days following deprivation, there are changes to thalamocortical projections (Buonomano & Merzenich, 1998; Darian-Smith & Gilbert, 1995; Armstrong-James et al, 1994). Thus it would be more accurate to only analyze c-Fos labeled cells in cortical areas I-III where the majority of cortical-cortical fibers are located rather than all layers including those with thalamocortical projections.

In conclusion, molecular mapping of c-Fos was unable to demonstrate an increased responsiveness to visual stimulation that occurs in the auditory and multisensory cortices following noise exposure, which is inconsistent with our previous electrophysiological studies. Several explanations for the discrepancies are the short visual stimulation time, the sensitivity of molecular mapping and the cortical layers analyzed. Research in this field is necessary because it has previously been shown that when crossmodal plasticity in the auditory cortex is the most extensive, an individual is least likely to benefit from a cochlear implant (Lee et al, 2001). Also, the longer the delay to implantation, the less likely the cochlear implant will be successful (Proops et al 1999). More research must be conducted to assess the usefulness of molecular mapping as a tool for studying cortical crossmodal plasticity because it would allow a much wider area of the cortex to be analyzed compared to electrophysiology.

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APPENDIX A

Curriculum Vitae

Name: Paul Sirek

Post-secondary Western University

Education and London, Ontario, Canada

Degrees: 2008-2012 B.Sc. Honours Specialization

Honours and Western Graduate Research Scholarship (WGRS)

Awards: 2013-2015

Anatomy and Cell Biology (ACB) Graduate Entrance Scholarship

2013-2014

Related Work Teaching Assistant for Dentistry – Head and Neck Anatomy

Experience: Western University

2014-2015

Teaching Assistant for Medicine – Gross Anatomy

Western University

2014-2015

Teaching Assistant – Histology 3309

Western University

2013-2014

Teaching Assistant – Human Systemic Anatomy 3319

Western University

2013-2014

Publications:

Published abstract for American Association of Anatomists (2015)

Published abstract for Vision Sciences Symposium (2012)

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