ENHANCEMENT OF ADULT BRAIN PLASTICITY

ENHANCING PLASTICITY WITH FLUOXETINE AND ENRICHMENT IN THE ADULT RAT CORTEX

By

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Lay Abstract

The brain's ability to change in response to new experiences, called neuroplasticity, is high during development and declines into adulthood. Remarkably, the adult brain still retains a considerable amount of plasticity, but generally requires some assistance in order to activate it. My thesis investigates two treatments that aim to boost adult brain plasticity and reinstate the increased flexibility of young brains in adulthood. The first treatment is the drug fluoxetine. The second treatment is environmental enrichment. In my thesis, I measure the expression levels of several proteins to study how these treatments enhance plasticity in adulthood. I found that fluoxetine and enrichment exert distinct effects on dendritic spines, which are small protrusions on neurons that play a key role in processing and transmitting information. My findings also indicate that myelin, a physical structure that has generally been thought to prevent plastic changes, may actually help support plasticity.

Abstract

Developing brains have a great capacity for plasticity, whereas plasticity in adulthood is much more limited. However, the mature brain still retains a considerable amount of plasticity that can be augmented by a variety of plasticity-enhancing therapeutics. Two approaches include the antidepressant fluoxetine and environmental enrichment. Both induce functional improvements in multiple brain regions and produce alterations in both inhibitory as well as excitatory synaptic mechanisms. Multiple gene expression changes induced by fluoxetine and enrichment have been demonstrated including the alteration of mRNAs and proteins implicated in synaptic plasticity, dendritic spine morphology, cell signalling, survival, metabolism, molecular transport, transcription, histone deacetylation, and protein degradation. However, how these treatments work at the molecular level to bring about enhanced plasticity in adulthood is still not completely understood. We investigated the effects of fluoxetine, alone or in combination with monocular deprivation (MD), in the adult rat visual cortex and examined the impact of short- and long-term enrichment in the adult rat somatomotor cortex. We studied a collection of synaptic and non-neuronal proteins implicated in experience-dependent plasticity, including excitatory markers (PSD-95, GluN1); spine markers (Drebrin A - mature isoform, Drebrin E - immature isoform, Ube3A); and a myelin marker (Classic MBP). Protein expression was quantified using Western blot analysis. Enrichment produced modest changes in PSD-95 and GluN1 levels, with short-

term enrichment favouring a decrease in both excitatory markers. Interestingly, fluoxetine and enrichment elicited distinct effects on dendritic spine markers. Fluoxetine resulted in an overall reduction of dendritic spine markers, whereas enrichment shifted Drebrin levels to favour more Drebrin A relative to Drebrin E. Additionally, fluoxetine appeared to substantially rescue Ube3A losses that resulted from MD while enrichment exerted modest effects on Ube3A levels. We also found that MD increased MBP in the ipsilateral hemisphere but decreased MBP in the contralateral hemisphere, changes that were independent of fluoxetine. MBP was also elevated with enrichment. These findings suggest that changes in MBP are predominantly driven by changes in experience. This research identifies several plasticity mechanisms, including alterations in dendritic spine dynamics, effects on Ube3A-related plasticity, and experience-dependent MBP fluctuations, that fluoxetine and enrichment use to enhance adult brain plasticity and reactivate heightened plasticity in adulthood.

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Chapter 2

List of Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AD	Alzheimer's Disease
ARC	activity-regulated cytoskeleton-associated protein
ASD	Autism Spectrum Disorder
ATP	adenosine triphospahte
β-tubulin	beta-tubulin
BCA	bicinchinchoic acid
BDNF	brain-derived neurotrophic factor
CNPase	2′,3′-cyclic nucleotide-3′-phosphodiesterase
CI	confidence interval
CP	critical period
CSPG	chondroitin sulfate proteoglycan
CONTRA	contralateral
cTBS	continuous theta burst stimulation
DTT	dithiothreitol
E/I	excitatory/inhibitory
EDTA	ethylenediaminetetraacetic acid
EE	environmental enrichment
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Epn1	epsin 1
Esr1	estrogen receptor 1
EPSC	excitatory post-synaptic current
FDA	Food and Drug Administration
FLX	fluoxetine
FLX.MD	fluoxetine combined with MD
fMRI	functional magnetic resonance imaging
Fos	fos proto-oncogene, AP-1 transcription factor subunit
GABA	gamma-aminobutyric acid
Gabra2	GABA(A) receptor subunit alpha-2
Gabrb2	GABA(A) receptor subunit beta-2
$GABA_A$	GABA type A
GABA/AMPA ratio	ratio of GABA receptor IPSCs to AMPA receptor EPSCs
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GluA	glutamate receptor ionotropic AMPA receptor
GluN	glutamate receptor ionotropic NMDA receptor
Grin2a	glutamate ionotropic receptor NMDA type subunit 2A

Grip1	Glutamate Receptor Interacting Protein 1
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Homer1	homer scaffold protein 1
IPSC	inhibitory post-synaptic current
IPSI	ipsilateral
L-EE	long-term enrichment (1.25 years)
MAG	myelin-associated glycoprotein
MBP	myelin basic protein
MCT1	monocarboxylate transporter 1
MCT2	monocarboxylate transporter 2
MD	monocular deprivation
MDD	Major Depressive Disorder
NgR	Nogo-66 Receptor
NMDA	N-methyl-D-aspartate
NT-3	neurotrophin-3
OCD	Obsessive Compulsive Disorder
OD	ocular dominance
OMgp	oligodendrocyte myelin glycoprotein
OPC	oligodendrocyte precursor cell
P()	postnatal day
PBS	phosphate-buffered saline
PCC	Pearson correlation coefficient
PMSF	phenylmethylsulfonyl fluoride
PLP	proteolipid protein
PNNs	perineuronal nets
Prkca	protein kinase C alpha
PSD-95	post-synaptic density protein 95
PV+	parvalbumin
PVDF-FL	polyvinylidene difluoride
S1/M1	somatomotor cortex
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
S-EE	short-term enrichment (2 weeks)
SEM	standard error of the mean
SSRI	selective serotonin reuptake inhibitor
SZ	Schizophrenia
TMS	transcranial magnetic stimulation
tDCS	transcranial direct current stimulation
TrKB	tropomyosin receptor kinase B
Ube3A	ubiquitin protein ligase E3A
V1	primary visual cortex

Vegfa	vascular endothelial growth factor A

Declaration of Academic Achievement

My MSc thesis includes a research article (Chapter 2) that I am the lead on and it is in preparation for submission to Neural Plasticity, a peer-reviewed open access journal. This chapter was a collaboration between myself and Dr. Kathryn Murphy. I participated in the study conception and design, acquisition of data, analysis and interpretation of data, and drafting of the manuscript. Dr. Simon Beshara, Dr. Brett Beston, and Dr. Joshua Pinto assisted in animal rearing, tissue collection, and running Western blots.

Chapter 1. General Introduction

1.1 Critical Period and Adult Plasticity

1.1.1 Critical Periods

The organization and function of neural circuits within the brain can be modified by experience, especially during early life. The window of time during development where experience is capable of exerting permanent effects on neural circuit connectivity and specific behaviours is defined as a "critical period" (Hensch, 2005). The behavioural work of Konrad Lorenz in 1935 provided the first evidence for critical periods in animal models. He found that newly hatched goslings would take any moving object to be their mother and proceed to follow the object around, a phenomenon known as "imprinting", provided the imprinting process occurred between 13-16 hours after hatching (Lorenz, 1935).

Since then, critical periods have been discovered in multiple primary sensory areas for a variety of species (Hensch, 2004). For example, the motor, somatosensory, visual, auditory, and olfactory systems all have critical periods (Hensch, 2004). With respect to animal lines, mice, rats, cats, ferrets, various bird species, and humans exhibit critical periods for one or more of the primary sensory areas (Hensch, 2004). In fact, a critical period for human language acquisition that spans birth to 12 years of age has been described (Newport, Bavelier, Mehler, 2001). Additionally, string musicians who begin playing before age 12 years have greater cortical responsiveness to stimulation of the fingers compared to those who begin later in life (Elbert, Pantev, Wienbruch, Rockstroh,

& Taub, 1995), which indicates that musically-driven somatosensory maps of the finger digits have a critical period.

1.1.2 Ocular Dominance Plasticity During The Critical Period

Although critical periods exist within several primary sensory regions, the visual system has long served as the gold standard for examining mechanisms of critical period plasticity. The pioneer work of Wiesel and Hubel in the 1960s provided the first physiological evidence of developmental critical periods and demonstrated that manipulations of visual experience could lead to circuit level changes in the brain, specifically the visual cortex (V1). To investigate whether or not visual experience early in life influenced the receptive field properties of V1 neurons, Wiesel and Hubel deprived newborn kittens of vision in one eye via monocular deprivation (MD), a manipulation of visual experience that involves suturing the eye shut (Wiesel & Hubel, 1963). When the sutured eye was opened after 2.5 months, nearly all neurons recorded in V1 responded preferentially to the open eye, whereas a negligible number of neurons responded to the deprived eye (Wiesel & Hubel, 1963). Visual experience for a short 1-2 month period lessened the magnitude of the ocular dominance (OD) shift with a moderate number of neurons exhibiting deprived eye responses, although abnormal OD was still clearly observed (Wiesel & Hubel, 1963). Monocular deprivation for 3 months in adult cats did not affect OD relative to normal, visually non-deprived animals, with the majority of V1 cortical neurons responding to the closed eye (Wiesel & Hubel, 1963).

Future work revealed that the critical period for susceptibly to the effects of monocular deprivation in cats is between 4 to 12 weeks after birth, peaking between 6 to 8 weeks (Hubel & Wiesel, 1970). Additionally, 5 years of either binocular vision or reverse occlusion following 3 months of MD at birth was unable to restore normal OD plasticity, and the vast majority of neurons only responded to the eye that remained open during MD (Hubel & Wiesel, 1970). Clearly, early life experiences have profound effects on circuit connectivity and behaviour that persist into adulthood.

1.1.3 Closure of the Critical Period

During development, various structural and extracellular changes occur that align with the close of the critical period and restrict plasticity in adulthood. Chondroitin sulfate proteoglycans (CSPGs) are molecular components of the extracellular matrix that inhibit axonal growth (Fitch & Silver, 1997) and CSPG expression increases throughout the critical period (Pizzorusso et al., 2002). Furthermore, removal of CSPGs with chondroitinase-ABC is sufficient to induce OD shifts following MD in adult animals (Pizzorusso et al., 2002).

Myelin also places a structural boundary on synaptic plasticity. Myelin contains various molecular components, including Nogo, which binds to the Nogo-66 receptor (NgR), eventually leading to inhibited neurite outgrowth (McGee & Strittmatter, 2003). Ablating the NgR from axonal membranes reinstates OD plasticity in adult mice and prevents the closure of the critical period (McGee, Yang, Fischer, Daw, & Strittmatter,

2005). Additionally, myelination is a protracted process that occurs for more than two years in the mouse somatosensory cortex, peaking at 1.75 years of age, well beyond the close of the critical period (Hill, Li, & Grutzendler, 2018). In humans, classic myelin basic protein, a myelin component that is necessary for myelin compaction, continues to increase its expression levels until 42 years of age (Siu, Balsor, Jones, & Murphy, 2015).

In addition to an increase of structural brakes, numerous functional changes advance the attenuation of critical period plasticity. For example, Lynx1, a protein that serves as an inhibitor of nicotinic acetylcholine receptor signalling, contributes to the restriction of critical period plasticity. Lynx1 expression significantly increases following the close of the critical period (Morishita et al., 2010). Lynx1 knockout mice demonstrate a shift in OD plasticity in favour of the open eye when short-term MD is applied during adulthood, and this shift was completely eliminated when nicotinic acetylcholine receptor antagonists were administered concurrently with MD (Morishita et al., 2010). Also, when long-term MD occurs throughout the entire critical period, Lynx1 knockouts experience a complete recovery of visual acuity of the deprived eye by simply opening the initially closed eye (Morishita et al., 2010). However, when the same application of long-term MD occurs in wild-type mice, deprived eye visual acuity is substantially reduced even after one month of eye-opening (Morishita et al., 2010).

The relative ratio of excitation: inhibition (E:I balance) within neurons also controls the closure of the critical period. Critical period opening requires an initial activation of GABAergic circuitry, as precocious critical periods and MD shifts result from the early

application of benzodiazepines (Fagiolini & Hensch, 2000; Iwai, Fagiolini, Obata, & Hensch, 2003). Also, GAD65 knock mice that have insufficient GABA levels demonstrate delays in critical period onset until diazepam is utilized to raise inhibition to a certain threshold (Fagiolini & Hensch, 2000; Iwai, Fagiolini, Obata, & Hensch, 2003). The overall GABAergic tone continues to undergo maturation throughout the critical period, and it is believed that this maturation of inhibitory signalling contributes to the close of the critical period (Sale, 2010). Transgenic mice overexpressing BDNF experienced an accelerated maturation of GABAergic signalling and accelerated closure of the critical period for MD (Huang et al., 1999). Pharmacological interventions and treatments that reduce GABAergic neurotransmission and return inhibition to juvenile-like levels have been shown to reactivate plasticity in adulthood (Maya Vetencourt et al., 2008, Sale et al., 2007, Greifzu et al., 2014). Collectively, this evidence demonstrates that the maturation of GABAergic circuits contributes to the termination of the critical period.

Additionally, several excitatory synaptic mechanisms coincide with the close of the critical period. Electron microscopy combined with immunostaining, specifically in layer IV of the ferret visual cortex, has demonstrated that synaptic GluN2B NMDA receptor subunits are highly expressed at the onset and peak of the critical period but substantially decrease towards the end of the critical period (Erisir & Harris, 2003). GluN2A subunits steadily increase throughout the critical period into adulthood (Erisir & Harris, 2003). During the peak of the critical period, GluN2B subunits dominate, however, at the time of critical period closure, and beyond, GluN2A subunits are the most prevalent (Erisir &

Harris, 2003). It is interesting to note that similar results were observed in mouse spinal cord synapses where losses of GluN2B were observed at the end of the critical period (Isoo et al., 2016). Preventing reductions of GluN2B in mice deficient for GluN2A extended the critical period, and increasing GluN2B levels via proBDNF in cell cultures reopened plasticity following the closure of the critical period (Isoo et al., 2016). Thus, the developmental shift in favour of more GluN2A subunits relative to GluN2B subunits appears to be a mediator of critical period closure.

Recent work by Huang and colleagues implicates PSD-95 in the closing of the critical period and curtailing plasticity in adulthood. PSD-95 levels increase throughout the critical period and reach approximately 80% of adult expression when the critical period ends (Huang et al., 2015). In PSD-95 knockout mice, short-term MD induces an OD shift at 1.5 years of age that does not occur in age-matched wild-type controls (Huang et al., 2015). Interestingly, silencing PSD-95 expression with short-hairpin RNA in adult mice enabled OD plasticity after short-term MD (Huang et al., 2015). While the number of silent synapses was comparable at eye-opening for both PSD-95 knockouts and wild-type controls, PSD-95 knockouts had significantly more silent synapses during and after the critical period compared to controls, indicating that PSD-95 assists in the maturation of excitatory synapses (Huang et al., 2015). An emerging perspective is that silent synapses are in a premium state to be sculpted by experience and the maturation of silent synapses places a restraint on plasticity.

1.1.4 Plasticity in Adulthood

Despite the limitations placed on plasticity that occur with age, mature brains retain a considerable amount of plasticity and remain partially susceptible to experience-dependent modification. Wiesel and Hubel's research on visual deprivation in kittens indicated that the window for experience-dependent changes was restricted to early life, however, the sensory deafferentation work of Merzenich and colleagues in the 1980s challenged this notion. Digit amputation in adult owl monkeys leads to somatosensory cortical map reorganization, as the receptive fields for skin surfaces adjacent to the amputated digits eventually mapped onto cortical neurons that previously innervated the amputated digits (Merzenich et al., 1984).

Additionally, frequency discrimination training leads to an increased cortical representation of a specific frequency in the adult auditory cortex (Recanzone, Schreiner, & Merzenich, 1993). Sensory map reorganization also occurs in the visual cortex after retinal lesions (Heinen & Skavenski, 1991). Retinal lesions of the fovea result in V1 neurons that are silent and no longer respond to visual stimuli. Eventually, these neurons develop new receptive fields that service retinal regions surrounding the lesion (Heinen & Skavenski, 1991). Subsequent research, primarily in mice, has shown that adult animals do in fact exhibit some OD plasticity after MD (Sato & Stryker, 2008). Collectively, these studies indicate that experience in adulthood can cause plastic changes in the brain.

Several molecular mechanisms have been described that underlie the functional changes in adult plasticity. Deafferentation alters the E:I balance which enables the

modification of receptive fields in the somatosensory cortex. An initial strengthening of excitatory synapses results as AMPA receptor levels increase and peak 3-5 days after deafferentation, returning to baseline within 2 weeks (He, Rasmusson, & Quinlan, 2004). This is followed by an elevation of inhibitory synaptic strength as GABA type A ($GABA_A$) receptor expression undergoes a prolonged increase, peaking 14-16 days post-deafferentation, and returns to baseline within 4 months (He, Rasmusson, & Quinlan, 2004). Interestingly, NMDA receptor levels remain unchanged following deafferentation (He, Rasmusson, & Quinlan, 2004).

However, NMDA receptor-mediated signalling is still important for cortical map reorganization. Concurrent nerve deafferentation and NMDA receptor blockage prevents complete sensory map reorganization as a substantial portion of the somatosensory region remains unresponsive to the remaining digits (Garraghty & Muja, 1996). Further research demonstrated that NMDA receptors are not involved in initial circuit reorganization as NMDA receptor antagonists administered prior to nerve transection do not occlude initial somatosensory map reorganization (Myers, Churchill, Muja, & Garraghty, 2000). Also, blocking NMDA receptors 30 or more days after deafferentation did not disrupt the newly reorganized sensory map (Myers, Churchill, Muja, & Garraghty, 2000). Collectively, this evidence indicates that immediate sensory map reorganization after deafferentation is NMDA-receptor independent. Protracted cortical reorganization requires NMDA-receptor signalling, however, NMDA-meditated mechanisms are not required for the maintenance of reorganized maps.

In the visual cortex, OD shifts in adult mice following MD are mediated through NMDA receptor-dependent mechanisms, similar to juvenile plasticity (Sato & Stryker, 2008). Blocking NMDA receptors before and during MD in both adult and juvenile mice prevents OD shifts in favour of the open eye that occur with MD (Sato & Stryker, 2008). Additionally, MD applied in adulthood decreases the ratio of $GABA_A$ receptor levels to AMPA receptor levels in the visual cortex (He, Hodos, & Quinlan, 2006). An increase in the ratio of GluN2B relative to GluN2A subunits is also observed compared to controls, a change driven predominantly by elevated GluN2B levels (He, Hodos, & Quinlan, 2006).

1.1.5 Differences Between Critical Period and Adult Plasticity

Interestingly, research investigating various characteristics of OD plasticity indicates that adult plasticity displays several unique differences from juvenile plasticity. Firstly, more time is required for manipulations of experience to induce plastic changes in adulthood. In juvenile mice, 1-2 days of MD produces a significant OD shift, whereas 7-8 days of MD is required in order to shift OD plasticity in adult mice (Sato & Stryker, 2008).

Secondly, the overall magnitude of adult plasticity is less than during development. OD shifts are larger during the critical period compared to adulthood (Sato & Stryker, 2008). Also, the magnitude of the OD shift increases with a longer MD duration during the critical period, whereas MD in adults has a saturating effect and no additional shifts are seen with an extended MD duration (Sato & Stryker, 2008).

Thirdly, adult plasticity appears to utilize strategies distinct from those strategies drawn upon during the critical period. In adults, OD shifts are achieved via protracted open eye responses (Frenkel & Bear, 2004; Mrsic-Flogel et al., 2007; Sato & Stryker, 2008). However, OD shifts during the critical period are mediated by an initial, rapid inhibition of closed eye responses followed by an increase in responses from the open eye (Hofer, Mrsic-Flogel, Bonhoeffer, & Hübener, 2006; Sato & Stryker, 2008; Sawtell et al., 2003).

Lastly, the persistence of experience-dependent changes in adulthood is more transient compared to the long-standing effects of experience in early life. Mice that received MD for 13 days during the critical period still showed aberrant OD plasticity 2 months after the eye was reopened, whereas adult mice that received MD for 18 days had normal OD plasticity 2 months after reopening the eye (Prusky & Douglas, 2003).

1.2 Approaches to Reinstate Juvenile Plasticity or Enhance Adult Plasticity

Following the closure of the critical period, the brain's sensitivity to external stimuli is markedly reduced, and experience does not as readily modify neural circuits and synapses in the adult brain (Engel, Morland, & Haak, 2015). However, the mature brain still harbours a substantial amount of plasticity as experience can, to some degree, still refine various neural networks and behaviours (Hübener & Bonhoeffer, 2014). Reinstating critical period-like plasticity or enhancing existing adult plasticity with genetic, pharmacological, or behavioural interventions has both profound and broad impacts for improving brain function beyond the critical period. Treatments and therapies that capitalize on the limited but persistent plasticity within the mature brain have the potential to enhance the learning and memory of healthy adults, mitigate age-related cognitive declines, and improve functional recovery from various neurological diseases.

1.2.1 Diversity of Plasticity-Enhancing Treatments

Several therapeutic interventions have been studied extensively within the adult visual cortex to investigate promising treatments that are capable of enhancing the plasticity of the adult brain. Such therapies include relieving the structural brakes on plasticity that accumulate following the close of the critical period, altering experience and neuronal activity levels, and applying or altering neuromodulators of plasticity.

As discussed previously in Section 1.1.3 “Closure of the Critical Period”, removing structural plasticity brakes, such as the CSPG component of the extracellular matrix as well as specific myelin-associated proteins that curtail axonal sprouting, reinstates OD plasticity in adulthood (Pizzorusso et al., 2002; McGee, Yang, Fischer, Daw, & Strittmatter, 2005). Using chondroitinases that degrade CSPGs allows rats to experience OD shifts after MD during adulthood, a time period where they are typically no longer susceptible to the effects of MD (Pizzorusso et al., 2002). Additionally, making neurons insensitive to Nogo, which is one of several myelin-associated proteins that inhibits neurite outgrowth, results in heightened OD plasticity in adulthood and prevents critical period closure (McGee, Yang, Fischer, Daw, & Strittmatter, 2005). Thus, one way to enhance adult plasticity is by removing physical extracellular features that play a role in restricting plastic changes during adulthood.

Interventions rooted in the specific manipulation of visual input have also been shown to enhance OD plasticity in the visual cortex of adults. For example, depriving adult animals of visual input in both eyes, a manipulation known as dark-rearing, for 10 days prior to MD recreates juvenile-like OD plasticity (He, Hodos, & Quinlan, 2006). In adults, small OD shifts are predominantly due to a prolonged increase of neural responses to the open eye (Sato & Stryker, 2008). However, when dark-rearing is applied in adults, a suppression of deprived eye responses and an increase of open eye responses are coincidently observed (He et al., 2006), which is the OD plasticity mechanism that occurs during the critical period (Mrsic-Flogel et al., 2007; Sato & Stryker, 2008; Sawtell et al.,

2003). Furthermore, dark-rearing creates a critical period-like synaptic environment, which is indicated by decreased intracortical inhibition and an NMDA receptor subunit balance that resembles a juvenile-like state with more GluN2B relative to GluN2A expression (He et al., 2006).

The use of specific visual stimuli is another approach to enhance adult plasticity. Adult mice that were stimulated with moving square gratings during MD experienced significant and saturated OD shifts that occurred after only 2 days of MD (Matthies, Balog, & Lehmann, 2013). The OD shift did not further increase with extended time (Matthies, Balog, & Lehmann, 2013). Typically, approximately 1 week of MD alone is required to observe noticeable changes in OD plasticity in adult mice (Sato & Stryker, 2008), demonstrating that visual stimulation via the drifting gratings improved the time course for plastic changes to occur in the adult cortex. Also, a reduction of closed eye responses was initially observed, which is characteristic of juvenile OD plasticity (Matthies, Balog, & Lehmann, 2013). This was followed by a subsequent increase both of closed eye and open eye responses (Matthies, Balog, & Lehmann, 2013).

In another study that evaluated recovery from MD, OD plasticity and closed eye responses returned to normal levels when the animals viewed visual stimuli while running on a spherical treadmill with their heads fixed (Kaneko, Elife, 2014). No recovery occurred when either visual stimulation or running was applied in isolation of one another (Kaneko, Elife, 2014).

It appears that either specific sensory or combined sensory-motor stimulation may elicit plastic changes within the adult cortex and this has been studied to some degree in humans, particularly in the form of video game interventions. Video games, which combine aspects of visual/sensory and motor stimulation, seem to enhance adult brain plasticity in humans. A dichoptic version of a first-person shooter game has been shown to improve visual acuity and stereopsis in adult amblyopes (Vedamurthy, Nahum, Bavelier, & Levi, 2015). Another adult human amblyopic study found that dichoptic training via a falling block video game improved contrast sensitivity across a series of spatial frequencies (Li et al., 2015). Interestingly, the same falling block video game did not improve visual acuity compared to a control video game, although improvements in contrast sensitivity were observed (Gao et al., 2018). Therefore, it is important to match the type of sensory stimulation with the desired functional outcome to enhance plasticity and visual improvements in adult amblyopes.

In addition, perceptual learning for treating visual deficits utilizes sensory stimulation and involves viewing visual stimuli repeatedly in order to improve performance on specific visual tasks. While improvements in vision resulting from visual perceptual training tend to be task-specific and do not translate well to other tasks (Sengpiel, 2014), the efficacy of perception training on select visual functions is well documented (Vagge & Nelson, 2016). For example, a recent study demonstrated that visual perception training effectively promoted increased visual acuity and contrast sensitivity in both children and adults with amblyopia compared to a control group that

only received traditional patching therapy (Balci & Yalcin, 2013). Perceptual training involved the repeated discrimination of a target visual stimulus from a variety of stimuli (Balci & Yalcin, 2013). Participants underwent a total of 45 30-minute sessions, and a personalized training regimen was adopted as a computer algorithm continuously evaluated individual responses during training, while the degree of visual discrimination difficulty was adjusted based on subject performance (Balci & Yalcin, 2013). Improvements in contrast sensitivity were greatest at lower spatial frequencies, and both contrast sensitivity and visual acuity gains tended to increase with more training (Balci & Yalcin, 2013). Thus, viewing specific visual stimuli can draw upon adult plasticity to improve select visual functions in humans.

Though not a means of sensory stimulation per say, it is interesting to note that transcranial magnetic stimulation (TMS) has been shown to induce functional improvements in the visual cortex of adult amblyopic patients. The first study investigating the effects of TMS on human amblyopia patients demonstrated that repetitive TMS at both 1 Hz and 10 Hz enhances contrast sensitivity of the amblyopic eye at high spatial frequencies in adult amblyopes (Thompson, Mansouri, Koski, & Hess, 2008). These improvements were significant both immediately and 30 minutes after 10 Hz TMS (Thompson, Mansouri, Koski, & Hess, 2008). 1 Hz TMS only resulted in significant improvements 30 minutes after stimulation, however, a trend towards improvement was observed immediately following stimulation (Thompson, Mansouri, Koski, & Hess, 2008). Another study found that anodal transcranial direct current

stimulation (tDCS) improved contrast sensitivity for 8 of 13 adult amblyopic participants for up to 30 minutes following initial stimulation (Spiegel, Byblow, Hess, & Thompson, 2013). fMRI analysis revealed that cortical activation, which is predominantly driven by the non-amblyopic eye for amblyopic patients, was substantially reduced following anodal tDCS (Spiegel, Byblow, Hess, & Thompson, 2013). Also, continuous theta burst stimulation (cTBS) improved contrast sensitivity for high spatial frequencies (Clavagnier, Thompson, & Hess, 2013). Astoundingly, these cTBS-induced improvements persisted for 78 days following the final stimulation session (Clavagnier, Thompson, & Hess, 2013).

It is apparent that numerous approaches exist to enhance adult plasticity. In my thesis, I focus specifically on further studying two types of plasticity-enhancing treatments. The first is the neuromodulator fluoxetine. The second is the behavioural paradigm environmental enrichment.

1.2.2 Fluoxetine

Fluoxetine, whose brand names include Prozac or Sarafem, is an antidepressant that has been approved by the Food and Drug Administration (FDA) to treat a variety of mental disorders, such as Major Depressive Disorder (MDD), Obsessive Compulsive Disorder (OCD), Bulimia Nervosa, and Panic Disorder. Fluoxetine is a selective serotonin reuptake inhibitor (SSRI), which means that it prevents the removal, and subsequent degradation, of serotonin from the synaptic cleft. Thus, an increased

concentration of serotonin remains active in the synaptic cleft and can bind to post-synaptic receptors to elicit downstream signalling effects.

A study by Maya Vetencourt and colleagues demonstrated chronic fluoxetine administration following one week of MD in adult rats induces a shift in OD plasticity in favour of the open eye (Maya Vetencourt et al., 2008). Fluoxetine also recovers visual acuity of the deprived eye following MD, based on behavioural and electrophysiological measures (Maya Vetencourt et al., 2008). These functional changes were accompanied by a reduction of GABAergic neurotransmission and an increase in the neurotrophin brain-derived neurotrophic factor (BDNF) (Maya Vetencourt et al., 2008). Furthermore, the reduced intracortical inhibition appears to drive fluoxetine's ability to reopen V1 plasticity in adulthood as the application of diazepam, a $GABA_A$ receptor agonist, during MD completely prevents the subsequent OD shift after fluoxetine administration (Maya Vetencourt et al., 2008).

In addition to altering BDNF and GABAergic signalling, fluoxetine affects excitatory and inhibitory synaptic proteins. When fluoxetine is combined with MD, expression levels of the excitatory and inhibitory receptor anchoring proteins, PSD-95 and Gephyrin, respectively, are substantially increased (Beshara, Beston, Pinto, & Murphy, 2015). Additionally, both pure fluoxetine and fluoxetine paired with MD cause the subunit composition of glutamatergic and GABAergic receptors to shift towards the more mature state (Beshara, Beston, Pinto, & Murphy, 2015). Specifically, NMDA receptors shift in favour of more GluN2A relative to GluN2B subunits and $GABA_A$

receptors shift in favour of more alpha-1 relative to alpha-3 subunits (Beshara, Beston, Pinto, & Murphy, 2015).

This recent evidence suggests that fluoxetine does not result in a perfect recapitulation of juvenile plasticity. While reduced inhibitory signalling is consistent with juvenile-like plasticity (Maya Vetencourt et al., 2008), the finding that fluoxetine results in a maturation of glutamatergic and GABAergic subunit balances indicates that fluoxetine does not solely act to reinstate critical period-like plasticity (Beshara, Beston, Pinto, & Murphy, 2015). At the level of the synapse, fluoxetine creates a distinct synaptic environment. Thus, fluoxetine appears to utilize both juvenile-like mechanisms as well as unique approaches to activate plasticity in adulthood.

Furthermore, a recent study by Ruiz-Perera and colleagues (2015) used mass spectrometry to expansively investigate how fluoxetine impacts protein composition within the adult mouse visual cortex. Fluoxetine treatment resulted in elevated expression of 16 proteins and increases in expression levels varied between approximately 17% to 90% for individual proteins (Ruiz-Perera et al., 2015). Conversely, 8 proteins had reduced expression with fluoxetine treatment, and the decreases for specific proteins ranged between approximately 13% to 30% (Ruiz-Perera et al., 2015). The identified proteins were organized according to biological function, and interestingly nearly 60% of all proteins have a role in either intracellular signalling or metabolism (Ruiz-Perera et al., 2015). Proteins were also classified based upon their cellular localization, and

approximately half of the differentially expressed proteins are located in the cytoplasm (Ruiz-Perera et al., 2015).

Examination of changes in mRNA expression levels has also helped reveal specific molecular mechanisms supporting fluoxetine's plasticity-enhancing effects. One month of fluoxetine treatment alters the mRNA expression of genes implicated in receptor trafficking (ARC), molecular transport (Epn1), transcription (Esr1), excitatory signalling (Grin2a), phosphorylation (Prkca), and immediate early genes (Fos) in the frontal cortex of adult rats (Waller et al., 2017). Fluoxetine has also been shown to shift the mRNA expression of multiple $GABA_A$ receptor subunits, such as Gabra2 and Gabrb2, towards reduced levels in the nucleus accumbens of adult hamsters (Shannonhouse et al., 2016). Additionally, a single dose of fluoxetine treatment in the frontal cortex of adult rats demonstrates that Vegfa mRNA is significantly reduced after 2 hours of drug administration but recovers to normal levels by 8 hours post-injection (Jardin, Müller, Sánchez, Wegener, & Elfving, 2016). Grip1 and Homer1 mRNA levels are significantly depleted following 12 hours of fluoxetine treatment but recover to normal levels within 27 hours of the initial dosing (Jardin, Müller, Sánchez, Wegener, & Elfving, 2016). This evidence indicates that even a small, temporary quantity of fluoxetine is capable of inducing neuroplastic changes within the brain.

It is worth noting that fluoxetine has been evaluated in healthy human populations. A recent study by Lagas and colleagues investigated whether or not fluoxetine could improve the visual perceptual learning of a motion discrimination task of

adults. Interestingly, no improvements were observed (Lagas et al., 2016), suggesting more work is required to characterize fluoxetine's role in enhancing plasticity in adulthood and reconcile barriers in translation between animal models and humans.

1.2.3 Environmental Enrichment

Environmental enrichment is a behavioural treatment paradigm that involves altering one's surrounding environment to stimulate the brain. This enhanced stimulation is achieved by manipulations of the animal's physical and social environment. Physical stimulation includes large, more spacious cages that usually contain multiple levels, a variety of toys, and a running wheel. Social stimulation takes the form of housing animals in groups with multiple cage mates.

The first experiments investigating the impact of environmental enrichment on the brain were conducted in the 1960s. Rosenzweig and colleagues showed that enriched environments improve animal behavioural performance on reversal discrimination problems and alter the neurochemistry of the brain, as indicated by increases in cortical weight and cholinesterase activity in the visual and somatosensory areas of the cortex (Krech, Rosenzweig, & Bennett, 1962; Rosenzweig, Krech, Bennett, & Diamond, 1962). Furthermore, enrichment induces anatomical changes, as histological analysis reveals that the visual and somatosensory cortices increase in volume but decrease in neuron density after exposure to enriched environments (Diamond, Krech, & Rosenzweig, 1964).

The effects of enrichment have been extensively studied in the visual cortex. A study by Sale et al. (2007) investigated whether or not environmental enrichment could promote recovery of vision and enhance V1 plasticity in adult amblyopic rats that had undergone MD followed by reverse suture. Enrichment improved visual acuity of the initially deprived eye and induced a shift in OD plasticity (Sale et al., 2007). These functional changes were mediated by a reduction of inhibition, elevated BDNF levels, and a loss of perineuronal nets (PNNs) (Sale et al., 2007). Reduced intracortical inhibition appears to play a key role in facilitating the functional improvements induced by enrichment as administration of diazepam in V1 prevented the improvement of deprived eye visual acuity and inhibited OD shifts (Sale et al., 2007).

These findings were replicated in another study by Greifzu and colleagues. Mice that were raised in standard conditions from birth to P110 and then given 3-7 months of enrichment demonstrated an OD shift following MD (Greifzu et al., 2014). Enrichment again reduced intracortical inhibition, as the ratio of GABA receptor inhibitory post-synaptic currents (IPSCs) to AMPA receptor excitatory post-synaptic currents (EPSCs) (GABA/AMPA ratio) was significantly reduced relative to adult mice raised in standard cages (Greifzu et al., 2014). The GABA/AMPA ratio of enriched animals did not differ from juvenile mice raised in standard conditions, indicating that enrichment brought about juvenile-like levels of inhibition in adults (Greifzu et al., 2014). However, contrary to the finding of Sale et al. (2007), Griefzu et al. (2014) reported no change in PNNs.

Interestingly, environmental enrichment-induced functional and mechanistic changes described in the study by Sale et al. (2007) are very similar to the effects of fluoxetine after MD in the visual cortex reported by Maya Vetencourt et al. (2008). Both enrichment and fluoxetine improve visual acuity, induce a shift in OD plasticity, reduce inhibition, and elevate BDNF expression.

In addition to triggering plastic changes in the visual cortex, environmental enrichment causes plastic changes in the somatosensory cortex, the area of the brain responsible for receiving, processing, and integrating the enhanced sensory experience provided by enriched conditions. Enrichment promotes an enlargement of the forepaw skin surface representations in the adult rat somatosensory cortex and refines the precision of forepaw cutaneous receptive fields (Xerri, Coq, Merzenich, & Jenkins, 1996). In a tactile discrimination task, rats housed in enriched environments were able to learn how to differentiate between textures of varying degrees of roughness faster than rats housed in impoverished environments, indicating that enhanced sensory experience aids in sensory-based tasks (Bourgeon, Xerri, & Coq, 2004).

Furthermore, electron microscopy has revealed that enrichment produces morphological and circuit changes in the adult somatosensory cortex. Increases in the number of excitatory and inhibitory synapses found on dendritic spines and the number of excitatory synapses on dendritic shafts were reported in layer IV neurons (Landers, Knott, Lipp, Poletaeva, & Welker, 2011). A more recent study by Jung and Herms (2014) used two-photon in vivo imaging to study the effects of enrichment on spine dynamics in

the apical dendrites of layer V neurons and the basal dendrites of layer II/III neurons. Enrichment caused a transient increase in spine density and spine turnover, with a proportion of existing spines being eliminated and a small quantity of newly created spines persisting for several weeks (Jung & Herms, 2014). Additionally, enrichment increases components of somatosensory-evoked potentials in adult rats (Devonshire, Dommett, Grandy, Halliday, & Greenfield, 2010).

Insights into the molecular mechanisms utilized by environmental enrichment have been further explained by transcriptomic studies across various regions of the brain. Elevated mRNA expression of spinophilin, a protein involved in dendritic spine plasticity and morphology, was found in the adult rat cortex and hippocampus after 7 days and 31 days of enrichment (Hu, Bergström, Brink, Rönnbäck, & Dahlqvist, 2010). Interestingly, the number of spinophilin immunoreactive punta was significantly elevated after 7 days but returned to baseline levels at 31 days (Hu et al., 2010). Thus, enrichment may induce initial synapse remodelling and generation followed by the elimination of redundant synapses in newly reconstructed circuits (Hu et al., 2010). In line with enrichment-induced synaptic modifications, the mRNA expression of neurotrophin-3 (NT-3), a protein regulating neurogenesis, was increased in the visual cortex and hippocampus of adult rats after 30 days of enrichment (Torasdotter, Metsis, Henriksson, Winblad, & Mohammed, 1996). Furthermore, 1 hour of enrichment each day for a 3-week period significantly increased ARC mRNA expression in the striatum, all hippocampal regions,

and layers III and V of the visual and somatosensory cortices (Pinaud, Penner, Robertson, & Currie, 2001).

Enrichment has been studied in various human trials, particularly in the context of promoting recovery from various pathological conditions, such as autism, cerebral palsy, and stroke. With respect to adult afflictions, such as stroke, a pilot study investigating how enhanced environments affect activity levels and engagement of stroke patients within a rehabilitation facility found that participants in the enriched group were significantly more likely to be engaged in cognitive and social activities compared to controls (Janssen et al., 2013). Patients in enriched environments were also substantially less likely to be inactive, alone, or asleep during waking hours (Janssen et al., 2013). The encouraging functional improvements and neuroanatomical changes induced by enrichment in animal models have pushed for enrichment-based rehabilitation interventions to be implemented in stroke clinics and inpatient units (Livingston-Thomas et al., 2016). Accordingly, two clinical trials registered on clinicaltrials.gov are being conducted to evaluate if environmental enrichment promotes cognitive and functional recovery after traumatic brain injuries and during late-phase stroke, respectively ("Search of: environmental enrichment, brain, adult - List Results - ClinicalTrials.gov", 2018).

1.3 Myelin, Oligodendrocytes, and Plasticity

1.3.1 Properties of Myelin

Myelin is a combination of lipids and proteins that forms an insulating layer around neurons. With respect to its molecular composition, myelin contains far more lipids relative to proteins, as the relative proportion of lipids to proteins is approximately 186:1 (O'Brien & Sampson, 1965). Among the protein components, Myelin Basic Protein (MBP) is an important protein involved in myelination and is composed of two isoforms. The first isoform, Golli MBP, is located within developing oligodendrocytes and facilitates oligodendrocyte proliferation and migration (Paez et al., 2012). The second isoform, Classic MBP, is expressed in mature oligodendrocytes and plays a crucial role in myelin compaction as Classic MBP prevents the accumulation of bulky proteins within the sheath, allowing for the enrichment of lipids in the membrane (Aggarwal et al., 2011).

Myelin sheaths, which surround the axons of neurons within the central nervous system, are formed from the plasma membrane of glial cells known as oligodendrocytes (Pérez-Cerdá, Sánchez-Gómez, & Matute, 2015). The most well-characterized role of myelin is that it enables rapid action potential propagation along axonal membranes (Huxley & Stämpeli, 1949). This fast, saltatory conduction is achieved because myelin increases the resistance and reduces the capacitance of the axonal membrane, thereby facilitating the efficient conduction of electric current and reducing the number of locations where depolarization must occur on the axon (Huxley & Stämpeli, 1949; Rasminsky & Sears, 1972).

1.3.2 Myelin is a Structural Brake on Plasticity

In addition to increasing the rate of neural impulse transmission, myelin is implicated in experience-dependent plasticity. Myelin is typically regarded as a structural brake that physically limits synaptic plasticity (Bavelier, Levi, Li, Dan, & Hensch, 2010). Myelin contains molecular components such as Nogo, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp) that are ligands for the Nogo-66 receptor (NgR), a neuronal membrane-bound receptor, whose downstream signalling leads to inhibited neurite outgrowth and axonal arborization (McGee & Strittmatter, 2003). Knocking out the NgR was found to recapitulate juvenile ocular dominance plasticity in adult mice and extend the duration of the critical period (McGee, Yang, Fischer, Daw, & Strittmatter, 2005). Additionally, treating neurons with OMgp was found to promote NgR-mediated growth cone collapse of embryonic neurons (Wang et al., 2002). Furthermore, increased MBP expression was found in the visual cortex following the close of the critical period, which suggests that increased myelination is associated with critical period closure (McGee, Yang, Fischer, Daw, & Strittmatter, 2005). The increased expression of myelin, and corresponding restriction on structural plasticity, have been proposed to increase neural circuit stability and efficiency in adulthood (Bavelier, Levi, Li, Dan, & Hensch, 2010).

1.3.3 Myelin Provides Neurons with Metabolic Support

While myelin does place a structural boundary on circuit formation and modification, myelin is more than merely a break on plasticity. Myelin and the associated oligodendrocytes provide metabolic support to the underlying neurons (Philips & Rothstein, 2017; Saab, Tzvetanova, & Nave, 2013). Neurons have substantial metabolic requirements, as maintenance of the Na+/K+ pumps on the internodal axonal membrane requires large quantities of ATP (Ohno et al., 2011; Young et al., 2008). However, the energy stores within neurons are not sufficient to meet their metabolic needs, especially during periods of heightened activity (Falkowska et al., 2015). Oligodendrocytes, which express monocarboxylate transporter 1 (MCT1), can provide lactate to axons (Lee et al., 2012). Lactate can move out of oligodendrocytes into the periaxonal space through MCT1. The lactate is then shuttled into the neuron via the monocarboxylate transporter 2 (MCT2) that is expressed on the axonal membrane (Rinholm et al., 2011) and can be metabolized by neuronal mitochondria. Additionally, lactate from astrocytes can enter oligodendrocytes via connexin-based gap junctions (Niu et al., 2016), which can then be transferred to neurons using the MCT1-MCT2 pathway.

Furthermore, several knockout and knockdown studies investigating the selective deletion of oligodendrocyte-specific proteins have provided evidence of a metabolic role for myelin in maintaining axons. When proteolipid protein (PLP) is removed from oligodendrocytes, myelination surrounding the neurons is normal, but axonal degradation occurs (Klugmann et al., 1997, Griffiths et al., 1998). Also, loss of 2′,3′-cyclic

nucleotide-3′-phosphodiesterase (CNPase), an enzyme that facilitates the movement of nutrients, metabolites, and signalling molecules, results in progressive axonal degeneration without hampering transmission of action potentials (Lappe-Siefke et al., 2003). Additionally, downregulation of MCT1 leads to neurodegeneration and axonal damage in the absence of myelination changes (Lee et al., 2012). In "shiverer" mice, MBP expression is ablated, therefore myelin cannot undergo compaction and aberrant neural conduction results (Heredity 1981). Interestingly, these "shiverer" mice exhibit relatively healthy axons despite their dysmyelination pathology (Loers, Aboul-Enein, Bartsch, Lassmann, & Schachner, 2004). Collectively, these findings indicate that disturbances in myelin and oligodendrocyte properties can lead to disruptions in the underlying axons, suggesting that myelin and oligodendrocytes take on a metabolic role in maintaining axonal health.

1.3.4 Myelination is Activated by Neuronal Activity

In addition to neuronal metabolic support, myelin may help neurons cope with the demands brought about by increased experience and heightened neuronal activity. Electrical activity of neurons is one stimulus that drives myelination (Demerens et al., 1996; Gibson et al., 2014). Decreased myelination results when neuronal firing is inhibited with the Na+ channel antagonist tetrodotoxin, however, elevated myelin levels arise when neuron electrical activity is increased with the alpha-scorpion toxin (Demerens et al., 1996). Additionally, optogenetic stimulation of the premotor cortex of

young adult mice increases oligodendrocyte precursor cell (OPC) proliferation, the subsequent differentiation of OPCs into oligodendrocytes, and myelin thickness (Gibson et al., 2014). These cellular effects were also associated with improved motor function of the corresponding forelimb (Gibson et al., 2014), demonstrating that oligodendrocyte and myelin changes can affect behaviour.

It is apparent that changes in myelin plasticity can be brought about by changes in neuronal activity levels. Interestingly, environmental enrichment in the form of sensory stimulation increases myelination via increased oligodendrocyte proliferation and subsequent integration in the mature cortex (Hughes, Orthmann-Murphy, Langseth, & Bergles, 2018). Enhanced myelination will alter conduction velocity (Seidl, 2014), therefore even slight alterations in neural activity due to experience-dependent changes can produce circuit level and behavioural effects.

1.3.5 Myelin and Inhibitory Parvalbumin Interneurons

Parvalbumin-positive (PV+) neurons may have a unique role in myelin-related cortical plasticity. PV+ cells are a subset of GABAergic inhibitory interneurons neurons that are fast-spiking (Kawaguchi & Kubota, 1997) and crucial in the establishment and precise refinement of neural circuits (Pfeffer, Xue, He, Huang, & Scanziani, 2013). A substantial proportion of cortical myelin is located on PV+ cells in both the mouse and human cortex (Micheva et al., 2016; Stedehouder et al., 2017). Myelination of PV+ cells occurs at the proximal axon segment and is characterized by short, internodal myelin

fragments (approximately 27um) that are dispersed along the axon (Micheva et al., 2016; Stedehouder et al., 2017). Additionally, PV+ cells experience increased myelination and more elaborate axonal arborization in an activity-dependent manner (Stedehouder, Brizee, Shpak, & Kushner, 2018). Thus, neuronal activity is capable of alerting the myelination patterns and axonal anatomy of PV+ cells, which suggests that changes in experience can modify PV+ cell plasticity and associated circuits. Interestingly, a fraction of OPCs, which differentiate into myelin-producing oligodendrocytes, have cell bodies in close proximity to PV+ cells (Boulanger & Messier, 2017). The spatial location of OPCs indicates that cells that support myelination surround PV+ cells.

Chapter 2. Enhancing Neuroplasticity with Fluoxetine and Enrichment in the Adult Rat Cortex

2.1 Introduction

The brain's ability to undergo functional and structural modifications in response to experience changes across the lifespan. During development, experience readily alters behaviour and neural circuit connectivity, but in adulthood, it becomes more difficult for experience to elicit plastic changes (Engel, Morland, & Haak, 2015). However, the mature brain still retains a considerable amount of plasticity and can be modified, to a certain extent, by experience (Hübener & Bonhoeffer, 2014). Augmenting this adult plasticity has the potential to enhance the learning and memory of healthy adults, mitigate age-related cognitive declines, and improve functional recovery from various neurological diseases. Two approaches for enhancing adult plasticity include fluoxetine for vision and environmental enrichment for somatosensory and motor behaviours. Recent studies have started to reveal the transcriptomic and proteomic changes that accompany these treatments, but the precise molecular mechanisms that fluoxetine and enrichment use to reactivate plasticity in adulthood remain to be elucidated.

Fluoxetine induces functional changes within the visual system, such as the reinstatement of critical period-like ocular dominance plasticity and recovery of visual acuity following monocular deprivation (MD) (Maya Vetencourt et al., 2008). At the physiological level, fluoxetine alters various components of the excitatory:inhibitory (E:I) balance (Maya Vetencourt et al., 2008), a ratio that must be exquisitely maintained in order to regulate synaptic plasticity (Hensch, 2005). For instance, fluoxetine decreases intracortical GABAergic signalling (Maya Vetencourt et al., 2008). Additionally, when

fluoxetine is combined with MD, the subunit composition of glutamatergic and GABAergic receptors shift towards a more mature state and expression of the excitatory and inhibitory receptor anchoring proteins, PSD-95 and Gephyrin, respectively, are greatly elevated (Beshara, Beston, Pinto, & Murphy, 2015). These findings suggest that a hemisphere deprived of visual input may amplify specific actions of fluoxetine in the visual cortex (V1), the cortical area receiving the altered experience, especially on receptor scaffolding proteins.

In addition to fluoxetine, environmental enrichment (EE) is also capable of improving the plasticity of the adult brain. The enhanced sensory experience and stimulation resulting from EE produces substantial changes within the somatomotor cortex (S1/M1). The pioneer EE studies demonstrated that enrichment increases brain weight and cholinesterase activity in sensory areas including the somatosensory and visual cortices, and improves animal performance on reversal discrimination problems (Diamond, Krech, & Rosenzweig, 1964; Krech, Rosenzweig, & Bennett, 1962; Rosenzweig, Krech, Bennett, & Diamond, 1962).

Furthermore, EE increases PSD-95 levels within superficial and deep cortical layers implicated in sensory processing and learning, such as the forebrain, hypothalamus, and hippocampus (Nithianantharajah, 2004). EE also influences the development of dendritic spines, structures that are continuously modified by experience (Oray, Majewska, & Sur, 2004), and has been shown to increase spine density and modulate the stability of different types of dendritic spines (Jung & Herms, 2014; L. Zhang, Huang, & Hu, 2016).

Interestingly, recent studies have found that EE enhances myelination in the adult somatosensory cortex by increased oligodendrocyte proliferation (Hughes, Orthmann-Murphy, Langseth, & Bergles, 2018). This finding is unexpected because myelin is typically viewed as a structural brake on plasticity (Bavelier, Levi, Li, Dan, & Hensch, 2010) since myelin contains molecular components, including Nogo, MAG, and OMgp, that limit axonal branching and neurite outgrowth (McGee, Yang, Fischer, Daw, & Strittmatter, 2005; Wang et al., 2002). This new finding suggests that increased myelination may serve as an adaptive means of EE-induced plasticity in the adult cortex and raises the possibility that myelin possesses both plasticity-enhancing and plasticity-restricting roles.

Both fluoxetine and EE can drive functional improvements in adulthood, a time when it is more challenging to elicit plastic changes. However, less is known about how these therapies alter synapses and surrounding neuronal components to enhance plasticity. To address this question, we studied the effects of fluoxetine and visual deprivation in V1 and the effects of enrichment in S1/M1. We matched the manipulation of experience to the corresponding brain region in order to characterize the molecular mechanisms that support plastic changes in the brain. We used Western blotting to study a collection of molecular markers and found that fluoxetine and EE alter spine dynamics to different extents and also uniquely impact a marker of myelin, Classic Myelin Basic Protein (MBP).

2.2 Materials and Methods

Animals and Surgical Procedures

To investigate how fluoxetine alters protein expression levels in V1, we used adult male Long-Evans rats (P98) and randomly assigned animals to one of four experimental groups. The first group was normally reared to P98 (n=6). The second group was treated with fluoxetine for 1 month. Drug administration consisted of dissolving fluoxetine into the rats' drinking water (P70-P98; 0.2mg/ml of drinking water; n=8). The third group received monocular deprivation (MD) for 1 week (P91-P98; n=6). The final group was given 1-month fluoxetine treatment (P70-P98) combined with 1 week of MD (P91-P98; n=8). All animals were housed individually in Plexiglas cages with a 12h light/dark cycle and were permitted to self-regulate their intake of food and water. MD, completed through monocular lid closure, was carried out by trimming and suturing the eyelid margins with 5-0 vicryl. The surgery was performed in an aseptic environment and anesthesia was induced and maintained with gaseous isoflurane (1.5-5%) in oxygen. Eyelids were monitored on a daily basis to check for any suture openings. All procedures received approval from the McMaster University Animal Research Ethics Board.

The effects of EE on protein expression levels in S1/M1 were studied using adult male and female Long-Evans rats (P490) that were weaned and housed individually in standard cage conditions from P0-P64. At P64, animals were randomly assigned to one of three experimental groups: a normally reared group that did not receive any enrichment and lived in standard cage conditions from P64-P490 (n=12); a short-term enrichment

(S-EE) group that was normally reared from P64-P476, followed by 2 weeks of EE from P476-P490 (n=6); and a long-term enrichment (L-EE) group that received EE from P64-P490 (n=5). In order to selectively study the outer bounds of enrichment duration, we compared a considerably short enrichment period (2 weeks) against an exceptionally long enrichment period (1.25 years). Enriched environments consisted of multiple forms of physical stimulation including large, spacious cages, which contained 4 levels that were interconnected by 3 ramps, and a running wheel. Also, toys and the location of food and water were changed on a weekly basis. Social stimulation was included in the enrichment conditions by housing rats in groups with at least 4 cage mates. Regardless of experimental condition, animals were housed in Plexiglas cages with a 12h light/dark cycle and had unrestricted access to food and water. The experimental protocol was approved by the McMaster University Animal Research Ethics Board.

Tissue Collection

All animals were euthanized with Euthanyl (sodium pentobarbital, 150 mg/kg) and perfused with cold 0.1 M phosphate buffered saline (PBS) (4 ºC; 4-5 mL/min) until the fluid flowing from the right atrium of the heart ran clear. The brain was removed from the skull and placed in cold PBS to persevere the integrity of the sample for subsequent tissue sectioning and collection. For animals in the Fluoxetine and V1 study arm, tissue samples were collected bilaterally from monocular and binocular regions of V1. For animals in the EE and S1/M1 study arm, bilateral samples were collected from S1/M1.

All sectioned tissue was immediately frozen on dry ice, and then stored in a -80 ºC freezer unit.

Sample Preparation

For the V1 tissue pieces, homogenate samples were made by adding cold homogenization buffer (1 mL buffer: 50 mg tissue, 0.5 mM DTT, 2 mM EDTA, 2 mM EGTA, 10 mM HEPES, 10 mg/L leupeptin, 100 nM microcystin, 0.1 mM PMSF, 50 mg/ L soybean trypsin inhibitor) to the frozen tissue pieces. The samples were then crushed in a glass-glass Dounce homogenizer (Kontes), and lastly, 10% sodium-dodecyl-sulfate (SDS) (1 uL SDS: 9 uL homogenate) was added to the lysate and heated for 10 min. An additional set of samples was prepared according to components of several synaptoneurosome protocols (Hollingsworth et al., 1985; Murphy, Balsor, Beshara, Siu, & Pinto, 2014; Quinlan, Olstein, & Bear, 1999) to study Drebrin in V1. After tissue homogenization, the lysate was pushed through a 5 um pore mesh filter (Millipore) into a 1.7 mL flat top microtube (Diamed) and centrifuged at a force of 1000 x g for 20 min. The supernatant was removed, leaving a synaptic pellet at the bottom of the tube, 1 mL of cold homogenization was added to resuspend the pellet, and each tube was then subjected to another centrifugation at 1000 x g for 10 min. The supernatant was again removed and 100 uL of boiling 1% SDS was added to each tube. Lastly, all tubes were heated for 10 min at 70 ºC.

For the S1/M1 tissue pieces, homogenate samples were prepared by adding cold homogenization buffer (1 mL buffer: 50 mg tissue, 0.5 mM DTT, 2 mM EDTA, 2 mM

EGTA, 10 mM HEPES, 10 mg/L leupeptin, 100 nM microcystin, 0.1 mM PMSF, 50 mg/L soybean trypsin inhibitor) to the frozen tissue pieces. Proteins were lysed from the resuspended tissue with a FastPrep 24 Tissue and Cell Homogenizer (MP Biomedicals) for 40 s at 6 m/s. 10% sodium-dodecyl-sulfate (SDS) (1 uL SDS: 9 uL homogenate) was added to the lysate, and the mixture was heated for 10 min.

Protein Measurement and Equating

Recent literature (Lee et al., 2016) and previous findings from our lab (Pinto, Jones, Williams, & Murphy, 2015) have shown that commonly used loading controls, including the housekeeping proteins Ɓ-tubulin and GAPDH, experience variable levels of expression under different experimental and biological conditions. Hence, normalizing protein expression levels with a variable loading control can produce inaccurate results and interpretations of the data. To quantify protein expression reliably and robustly without the use of a loading control, we adhered to a strict three-phase procedure to measure and equate protein concentrations and load an equal volume of sample into each lane for Western blotting.

Phase 1: A bicinchoninic acid (BCA) assay was performed according to a comprehensive set of guidelines (Pierce, Rockford, IL) to determine the total protein concentration for each sample. Briefly, 3 uL of 0.25, 0.5, 1.0, and 2.0 mg/mL of standard (BSA protein standards, Bio-Rad Laboratories) and 3 uL of each sample was pipetted into separate wells of a 96-well microplate. This process was repeated two more times for each standard and sample. Next, 300 uL of BCA solution (1:100) was added to each well,

and the plate was incubated at 45 ºC for 45 min to ensure the reduction reaction went to completion. The reaction produces a change in solution colour, which can be used to determine the concentration of a solution of unknown volume when the absorption values of the solution in question are compared to the absorption values of a solution with a known concentration. To measure the resulting colourimetric change, the plate was scanned in an iMark Microplate Absorbance Reader (Bio-Rad Laboratories). The absorbance values of the standards were graphed against their known concentration values, and the linear correlation was determined. The coefficient of determination (R^2) for the correlation had to be greater than 0.99; otherwise, the entire BCA procedure was completed again until an R^2 value of 0.99 was achieved. The absorbance value for each sample was calculated by taking the average value from the three aliquots of each sample on the plate, and the linear equation from the standards was used to determine the protein concentration that corresponded with each sample's average absorbance value.

Phase 2: Protein concentrations were brought to a final concentration of 1 mg/mL using a combination of 4X Laemmli Buffer (Armesco) and 1X sample buffer (Novex, Thermo Fisher Scientific). The samples were initially diluted with 4X Laemmli Buffer. We aimed to add an equivalent amount of 4X Laemmli Buffer to each sample for this initial dilution. Then a variable amount 1X sample buffer, whose volume was specific to each sample, was added to precisely dilute all samples to a final concentration of 1 mg/ mL. We calculated the appropriate amount of 4X and 1X buffer to add to each sample based on the protein concentration provided by the BCA assay in Phase 1. At this point, a

control sample was prepared for each of three sample preparations including the V1 homogenate, V1 synaptoneurosome, and S1/M1 homogenate samples. For both the V1 homogenate and V1 synaptoneurosome samples, a small, equivalent volume from each of the 28 homogenate samples and 28 synaptoneurosomes samples were combined to create a homogenate control sample and synaptoneurosome control sample, respectively. The control sample for the S1/M1 homogenate was created by mixing an equal volume of the S1/M1 homogenate samples.

Phase 3: A high quality and calibrated pipette (e.g., Transferpette electronic) was used to load equivalent sample volumes into each lane of the Western blot gel by an experienced experimenter.

Immunoblotting

V1 homogenate, V1 synaptoneurosome and their respective control samples (25 ugs) were separated on 4-20% SDS-PAGE gels (Precise Protein Gels, Pierce Biotechnology). S1/M1 homogenate and control samples (25 ugs) were separated on 4-20% Tris-Glycine gels (Novex, WedgeWell Gels, Thermo Fisher Scientific). Each sample was run between 2 to 4 times. All samples were transferred to polyvinylidene difluoride (PVDF-FL) membranes (Millipore) before the membranes were blocked with blocking buffer (Odyssey Blocking Buffer 1:1 with PBS) (LI-COR Biosciences) for 1 h to guard against non-specific antibody binding. Membranes were then incubated in primary antibody overnight at 4 ºC. For the prepared and equated tissue samples, primary antibody concentrations were: PSD-95, 1:1000 (Millipore); GluN1, 1:4000 (BD

Pharmingen); MBP, 1:4000 (Abcam); Ube3A, 1:1000 (Bethyl Laboratories); Drebrin, 1:500 (Fitzgerald). The membranes underwent three sets of 10 min washes with PBS containing 0.05% Tween (PBS-Tween) (Sigma), followed by incubation for 1 hr at 20 ºC with the correct IRDye labeled secondary antibody (anti-mouse, 1:8000; anti-rabbit, 1:10,000) (LI-COR Biosciences), and another three iterations of 10 min PBS-Tween washes. To visualize protein bands, an Odyssey scanner (LI-COR Biosciences) was used to scan the membranes. When employed together, the Odyssey scanner and IRDye secondary antibodies provide a wide linear dynamic range and allowed us to determine that the various primary antibody concentrations used on the membranes fell within the linear dynamic range of the Odyssey scanner. To study multiple proteins, we stripped membranes using a Blot Restore Membrane Rejuvenation Kit (Millipore), scanned to ensure successful stripping had taken place, and then reprobed the membranes with an additional antibody. We repeated this process for each new antibody used. V1 homogenate sample blots were used two times in order to probe for Ube3A and MBP. V1 synaptoneurosome sample blots were only used once to probe for Drebrin. S1/M1 homogenate samples were probed for PSD-95, GluN1, both Drebrin isoforms (Drebrin A and E), Ube3A, and MBP, meaning each enrichment blot was used a total of 5 times.

Band Analysis

After membranes were scanned on the Odyssey scanner, protein expression was measured using densitometry (Licor Odyssey Software version 3.0; LI-COR Biosciences). Density profiles were generated for each protein band by selecting the area

around the band, subtracting the background intensity, integrating the pixel intensity across the band area, and dividing the band intensity by the band width to normalize for differences in lane width. For the V1 and S1/M1 samples, the control sample corresponding to that particular tissue area and preparation was run on each gel, and the band density of each sample was normalized to the control sample by dividing the calculated sample density by the calculated control density.

Statistical Analysis

To examine expression levels of individual proteins, we plotted histograms of the mean expression of each protein, along with the associated SEM, for each condition in V1 and S1/M1. The means were graphed relative to the expression of the corresponding 'Normal' group. A bootstrap analysis was used to investigate between-group differences. Bootstrapping is a resampling method that uses a sample dataset to generate a large, normal distribution with the same mean and standard deviation as the sample dataset. This technique provides an accurate estimation of the population mean and standard error of the sampling statistic. Bootstrapping helps address the issue of small samples sizes that is a limitation of the majority of animal studies. The bootstrap analysis was completed through R, and a dataset of 1,000,000 points was created for the group being compared. The simulated distribution was used to make comparisons with the observed means of other groups. 100,000 Monte Carlo simulations, sampling from the simulated dataset N times for each simulation, were then run. N was set to the number of animals in the other groups (i.e., the groups that were not used to generate the initial simulated distribution via

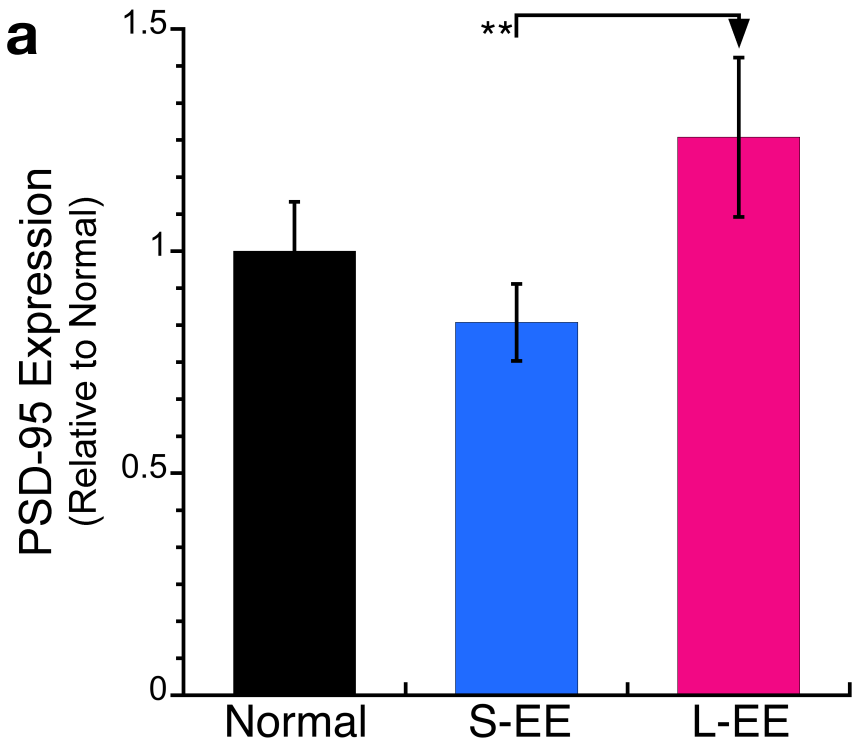
bootstrapping). After generating the estimated distribution for the N number of animals, the 95%, 99% and 99.9% confidence intervals (CI) for this distribution were calculated and compared to the observed means of the other groups. Differences between groups were considered significant (i.e., $p<0.05$) when the observed mean was outside the 95% CI of the simulated distribution. For each comparison between two groups, we ran the bootstrap analysis in both directions. That is, we simulated a distribution for one group and compared the simulated result to the observed mean of the other group. Then we simulated a distribution for the latter group and compared this result to the observed mean of the first group. The more conservative result of the significance test was always reported.

To analyze protein expression profiles on a systems level and how those profiles change across conditions, we used R to generate a Pearson correlation coefficient (PCC) matrix for all experimental groups in Contralateral V1 and S1/M1. For Contralateral V1, the dataset consisted of the average expression of each protein for each sample. This was because both homogenate and synaptoneurosome preparations were required to probe for all proteins studied. In addition, the raw data for PSD-95 and GluN1, previously published by Beshara et al., 2015, was added to the dataset in order to obtain a complete set of protein expression values that exactly matches the proteins studied in S1/M1 in this current study. For S1/M1, for which only homogenate samples were prepared, the dataset consisted of samples that had an expression value for each protein across at least one run.

This technique allowed us to increase the number of data points, allowing for more robust and reliable correlations.

2.3 Results

First, we studied how 2 weeks of EE (short-term enrichment (S-EE)) versus 1.25 years of EE (long-term enrichment (L-EE)) altered levels of excitatory synaptic signalling markers in S1/M1 of adult rats. We analyzed expression of PSD-95, an anchoring protein for postsynaptic glutamatergic receptors, and GluN1, the obligatory NMDA receptor subunit. We found S-EE trends towards decreased PSD-95 levels whereas L-EE trends towards increased PSD-95 expression (S-EE: -16%, SEM 8.6%, n.s.; L-EE: +26%, SEM 18%, n.s.; Fig. 1a). These PSD-95 changes with enrichment were not significantly different from normal. For GluN1, S-EE decreases expression (-21%, SEM 5.5%, $p<0.01$; Fig. 1b), but L-EE produces no change (-1.8%, SEM 8.3%, n.s.; Fig. 1a). It appears that enrichment elicits modest, temporary reductions in GluN1 that dissipates over time and recovers to normal levels with extended EE.


a
1.5
1
0.5
0
**
PSD-95 Expression
(Relative to Normal)
Normal
S-EE
L-EE

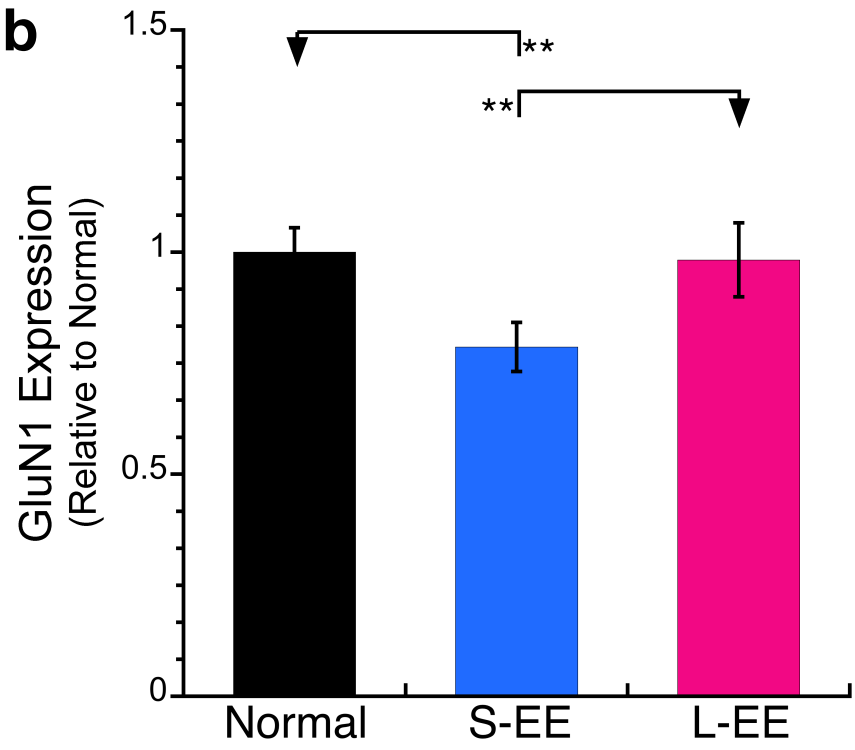

b
1.5
1
0.5
0
**
**
GluN1 Expression
(Relative to Normal)
Normal
S-EE
L-EE

Fig 1. Effects of enrichment on excitatory synaptic proteins. In S1/M1, PSD-95 **(a)** trends toward a decrease with S-EE, but favours elevated expression levels with L-EE. GluN1 **(b)** decreases with S-EE, but then recovers to levels comparable to normal with L-EE. The error bars represent the standard error of the mean (SEM) for each experimental group. S-EE = short-term (2 weeks) enrichment, L-EE = long-term (1.25 years) enrichment, *$p<0.05$, **$p<0.01$, ***$p<0.001$

We then examined the effects of fluoxetine and enrichment on the two Drebrin isoforms, the mature Drebrin A isoform and immature Drebrin E isoform, which are important molecular components for dendritic spine development and plasticity. As neurons mature, there is a shift from more Drebrin E to more Drebrin A, and it is this increase of the Drebrin A that facilitates spine maturation (Sekino, Koganezawa, Mizui, & Shirao, 2017). We analyzed Drebrin levels in V1 of 4 groups of adult rats that were either normally reared, had 1 month of fluoxetine treatment, had 1 week of MD, or had 1 month of fluoxetine treatment combined with 1 week of MD. For enrichment, the same durations of short-term and long-term enrichment were used again to investigate Drebrin levels in S1/M1. Fluoxetine alone results in a loss of Drebrin A relative to normal (-32%, SEM 3.2%, $p<0.001$; Fig. 2a), and fluoxetine combined with MD also decreases Drebrin A (-17%, SEM 4.4%, $p<0.05$; Fig. 2a). MD does not change Drebrin A levels significantly from normal (Fig. 2a). However, enrichment has the opposite effect on Drebrin A compared to the fluoxetine conditions. While S-EE does not significantly alter Drebrin A levels from normal (+15%, SEM 37%, n.s.; Fig. 2b), L-EE results in a substantial increase of Drebrin A (+76%, SEM 37%, $p<0.01$; Fig. 2b).

For Drebrin E, fluoxetine alone decreases Drebrin E expression levels relative to normal (-32%, SEM 6.0%, $p<0.001$; Fig. 2c), and fluoxetine combined with MD also results in a reduction of Drebrin E (-28%, SEM 7.4%, $p<0.001$; Fig. 2c). MD does not produce a significant change in Drebrin E, however, seems to trend toward increased Drebrin E (+30%, SEM 18%, n.s.; Fig. 2c). Thus, both the pure fluoxetine and fluoxetine

plus MD conditions result in a decrease of the immature spine marker Drebrin E. For enrichment, S-EE appears to reduce Drebrin E levels, although this result did not reach significance (-34%, SEM 8.2%, n.s.; Fig. 2d). This loss is much more pronounced and is magnified with L-EE (-41%, SEM 6.2%, $p<0.001$; Fig. 2d). Taken together, the loss of Drebrin A and Drebrin E that characterize fluoxetine suggest an overall elimination of dendritic spines. Extended enrichment, which robustly increases Drebrin A but reduces Drebrin E, indicates a greater presence of the more mature Drebrin isoform.

We calculated a Drebrin A:Drebrin E index to further analyze how fluoxetine and enrichment modify the relative balance and developmental shift of the two isoforms (Fig. 2e,f). Neither fluoxetine alone or combined with MD altered the A:E balance relative to normal. However, L-EE produced a significant shift toward the more mature Drebrin A isoform ($p<0.01$), and the index for S-EE also favoured more Drebrin A relative to normal ($p<0.05$). This substantial shift in favour of Drebrin A is driven by both the large increase of Drebrin A as well as the reduction of Drebrin E expression with extended enrichment. Hence, one plasticity mechanism that is specific to extended enrichment and not fluoxetine is the maturation of dendritic spines.

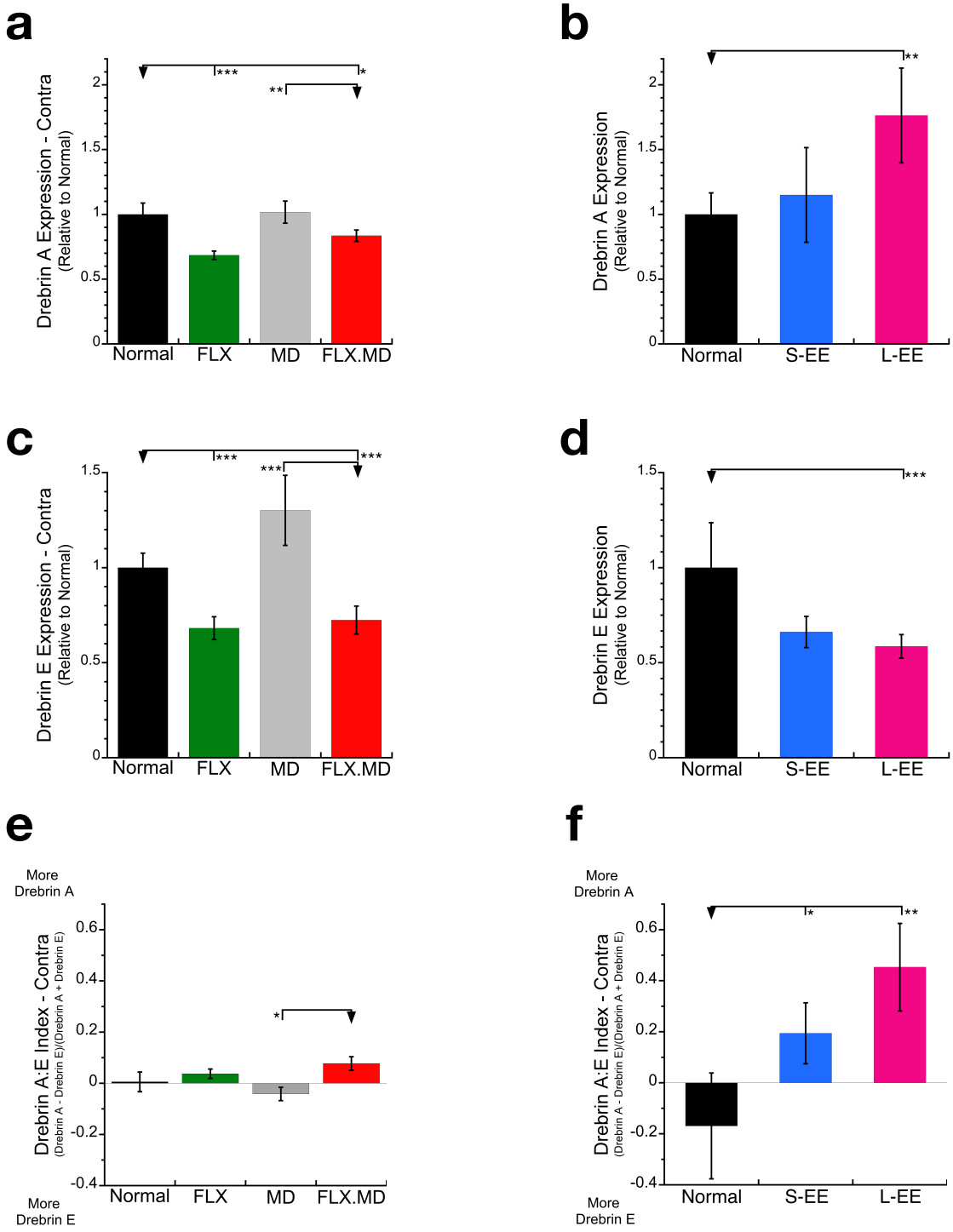

a
Drebrin A Expression - Contra
(Relative to Normal)
2
1.5
1
0.5
0
***
**
*
Normal
FLX
MD
FLX.MD
b
Drebrin A Expression
(Relative to Normal)
2
1.5
1
0.5
0
**
Normal
S-EE
L-EE
c
Drebrin E Expression - Contra
(Relative to Normal)
1.5
1
0.5
0
***
***
***
Normal
FLX
MD
FLX.MD
d
Drebrin E Expression
(Relative to Normal)
1.5
1
0.5
0
***
Normal
S-EE
L-EE
e
More
Drebrin A
Drebrin A:E Index - Contra
(Drebrin A - Drebrin E)/(Drebrin A + Drebrin E)
0.6
0.4
0.2
0
-0.2
-0.4
*
Normal
FLX
MD
FLX.MD
More
Drebrin E
f
More
Drebrin A
Drebrin A:E Index - Contra
(Drebrin A - Drebrin E)/(Drebrin A + Drebrin E)
0.6
0.4
0.2
0
-0.2
-0.4
*
**
Normal
S-EE
L-EE
More
Drebrin E

Fig 2. Effects of fluoxetine and enrichment on Drebrin isoforms. Drebrin A **(a,b)** decreases with FLX and FLX.MD, but substantially increases with L-EE. Drebrin E **(c,d)** decreases with FLX and FLX.MD, and is considerably reduced with L-EE. Drebrin A:E Index **(e,f)** did not differ from normal for any conditions in V1, but both S-EE and L-EE shifted the relative balance to favour the mature Drebrin A isoform. The error bars represent the standard error of the mean (SEM) for each experimental group.
FLX = 1 month Fluoxetine, MD = 1 week Monocular Deprivation, FLX.MD = 1 month Fluoxetine followed by 1 week Monocular Deprivation, S-EE = short-term (2 weeks) enrichment, L-EE = long-term (1.25 years) enrichment,*$p<0.05$, **$p<0.01$, ***$p<0.001$

Next, we studied how fluoxetine and enrichment alter the expression of a molecular plasticity regulator - Ube3A; and a molecular marker of myelin - Classic MBP. Myelination increases in response to neuronal activity (Demerens et al., 1996; Gibson et al., 2014), so we decided to look at expression levels within the ipsilateral hemisphere in addition to the contralateral hemisphere, as the ipsilateral hemisphere receives most of the open eye input, and therefore associated neuronal activity, in rat MD models (LeVere, 2013). After 1 week MD, Classic MBP expression increases in the ipsilateral hemisphere relative to normal (+72%, SEM 32%, $p<0.05$; Fig. 3a), and decreases in the contralateral hemisphere (-27%, SEM 13%, $p<0.05$; Fig. 3a). The same trends emerge both ipsilaterally and contralaterally when fluoxetine is combined with MD (IPSI +62%, SEM 18%, $p<0.01$; CONTRA -17%, SEM 4.8%, $p<0.01$; Fig. 3a). Fluoxetine alone causes no change in expression levels. In S1/M1, MBP increases substantially relative to normal with S-EE (+56%, SEM 30%, $p<0.05$; Fig. 3b). Although not significant, Classic MBP expression favours elevated levels also with L-EE (+27%, SEM 8.7%, n.s.; Fig. 3b).

For Ube3A, MD causes a loss in the contralateral hemisphere relative to normal (-45%, SEM 9.9%, $p<0.01$; Fig. 3c), however, no change occurs in the ipsilateral hemisphere. In addition, when fluoxetine is combined with MD, Ube3A levels trend towards recovery in the contralateral hemisphere, but no change occurs in the ipsilateral hemisphere (CONTRA +56%, SEM 33%, n.s.; IPSI n.s; Fig. 3c). These results indicate that MD creates a loss of Ube3A expression in the contralateral hemisphere, which can be rescued by the combination of fluoxetine and MD. Therefore, it seems that deprivation of

visual experience and fluoxetine are needed to induce plastic changes. Neither enrichment condition changes Ube3A levels relative to normal, however, L-EE trends toward increased Ube3A levels (+18%, SEM 13%, n.s.; Fig. 3d).

To assess whether the different experimental conditions created a molecular environment that enhances or stabilizes plasticity, we calculated an index of Ube3A:Classic MBP. In ipsilateral V1, MD and fluoxetine combined with MD favour more MBP (MD $p<0.01$; FLX+MD $p<0.001$; Fig. 3e). This change was driven by the substantial increase in MBP levels. Contralaterally, MD favours more MBP ($p<0.01$, Fig. 3e), which is primarily due to the substantial loss of Ube3A following MD. Fluoxetine combined with MD contralaterally is the only condition that favours more Ube3A ($p<0.01$, Fig. 3e), a change driven by the large increase of Ube3A. The balance is essentially equal for both enrichment conditions relative to normal (Fig. 3f).

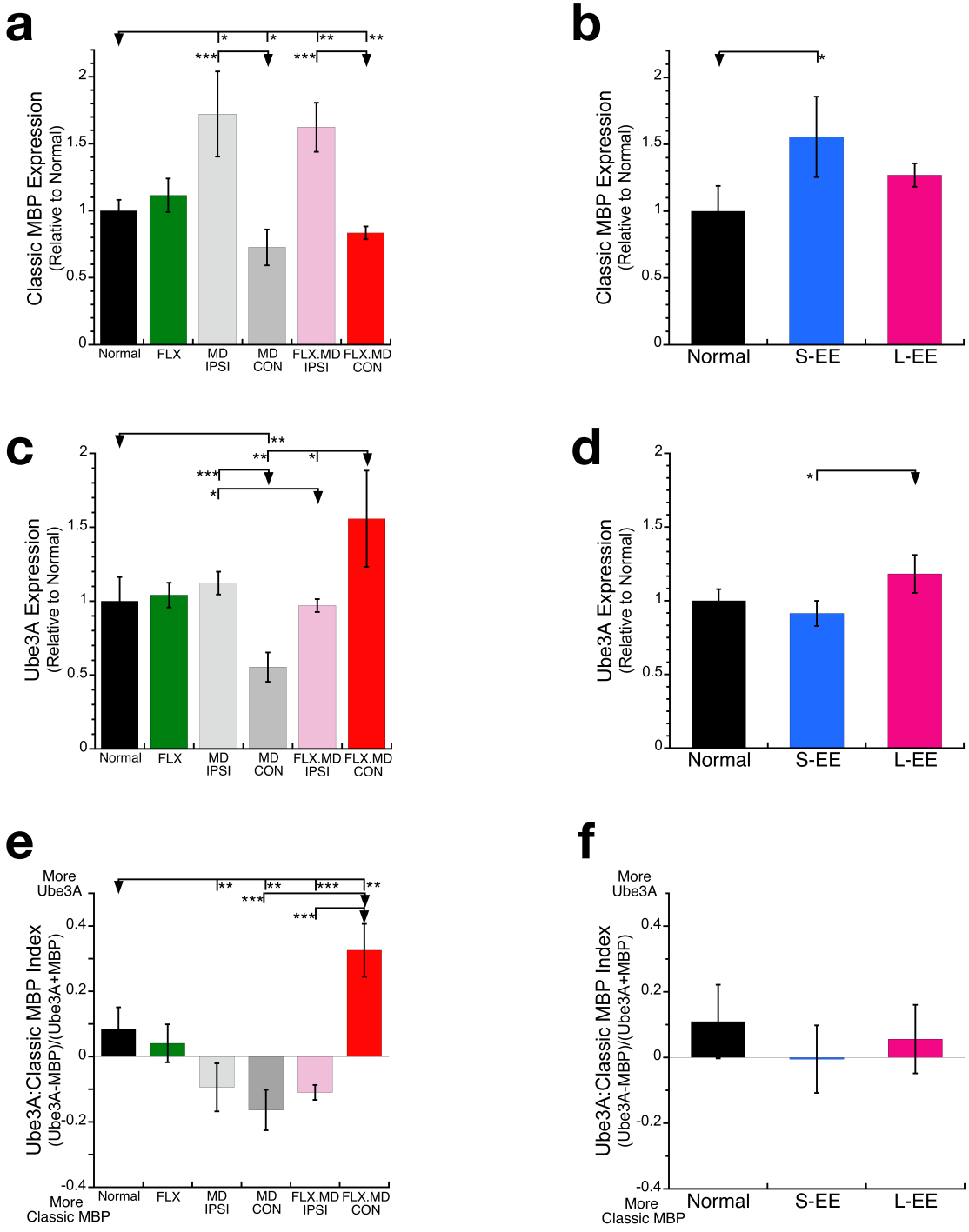

a
Classic MBP Expression
(Relative to Normal)
Normal
FLX
MD IPSI
MD CON
FLX.MD IPSI
FLX.MD CON
b
Classic MBP Expression
(Relative to Normal)
Normal
S-EE
L-EE
c
Ube3A Expression
(Relative to Normal)
Normal
FLX
MD IPSI
MD CON
FLX.MD IPSI
FLX.MD CON
d
Ube3A Expression
(Relative to Normal)
Normal
S-EE
L-EE
e
More Ube3A
Ube3A:Classic MBP Index
(Ube3A-MBP)/(Ube3A+MBP)
More Classic MBP
Normal
FLX
MD IPSI
MD CON
FLX.MD IPSI
FLX.MD CON
f
More Ube3A
Ube3A:Classic MBP Index
(Ube3A-MBP)/(Ube3A+MBP)
More Classic MBP
Normal
S-EE
L-EE

Fig 3. Effects of fluoxetine and enrichment on the plasticity regulators Ube3A and Classic MBP.

Classic MBP **(a,b)** increases in ipsilateral hemisphere with MD and FLX.MD, decreases in contralateral hemisphere with MD and FLX.MD, and increases with S-EE. Ube3A **(c,d)** decreases after MD in contralateral V1, but seems to recover substantially with FLX.MD; no significant changes are seen with enrichment Ube3A:Classic MBP Index **(e,f)** MD IPSI, MD CON, and FLX.MD.IPSI favour more Classic MBP relative to normal, but FLX.MD.CON is the only condition to favour more Ube3A; no changes with enrichment. The error bars represent the standard error of the mean (SEM) for each experimental group. FLX = 1 month Fluoxetine, MD = 1 week Monocular Deprivation, FLX.MD = 1 month Fluoxetine followed by 1 week Monocular Deprivation, S-EE = short-term (2 weeks) enrichment, L-EE = long-term (1.25 years) enrichment,*$p<0.05$, **$p<0.01$, ***$p<0.001$

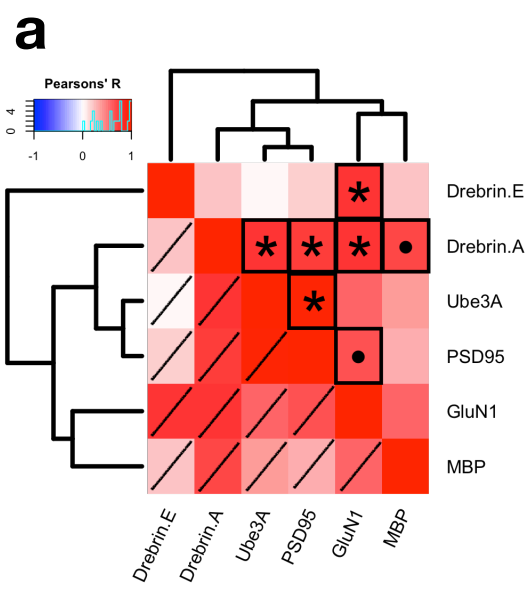
Finally, to create a molecular system profile for each condition, and investigate how the proteins change collectively across all conditions, we produced a Pearson correlation coefficient (PCC) matrix for the set of proteins studied for each experimental group in V1 and S1/M1.

The proteins comprising the PCC matrix axes are displayed using a dendrogram, which indicates the relative similarities and differences between a set of variables based on hierarchal clustering. Branch points or nodes that occur farther from the axes indicate a higher degree of difference between the proteins. Conversely, the shorter the distance between branch points, the greater the degree of similarity between the proteins.

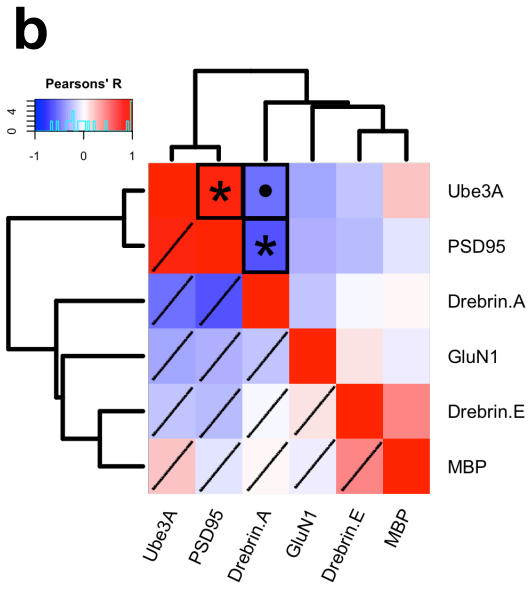
V1 (Fig 4): In the normal group, PCCs between Drebrin A and all other proteins expect Drebrin E, show strong positive correlations (e.g., Drebrin A versus Ube3A, PCC = 0.78; Drebrin A versus PSD-95, PCC = 0.73). Also, GluN1 has strong positive correlations with Drebrin E and PSD-95 (e.g., GluN1 versus Drebrin E, PCC = 0.76), in addition to Drebrin A. Hence, strong positive associations exist between multiple proteins, and the proteins making up the normal system seem to be reasonably well correlated with one another. Interestingly, the strong positive correlations between Drebrin A versus Ube3A, PSD-95, GluN1, and MBP, and GluN1 with Drebrin E and PSD-95, demonstrated in the normal condition, exhibit either a negative correlation or show no association in the fluoxetine group (e.g., Drebrin A versus Ube3A, PCC = -0.53; GluN1 versus Drebrin E, PCC = 0.08). The fluoxetine treatment appears to allow various proteins in the system to uncouple and become less or inversely correlated with each

other relative to normal. However, this uncoupling action produced by fluoxetine does not appear to characterize the MD system. With MD, various strong positive PCCs of normals shift to weaker PCCs or no correlation (e.g., Drebrin A versus PSD-95, PCC = 0.39). Also, several PCCs that were not strongly correlated in normals become more tightly correlated after MD (e.g., Drebrin E versus PSD-95, PCC = 0.66). When considered collectively, the findings suggest that MD functions to keep the system of proteins, at least partially, together after the insult. In the combination fluoxetine and MD group, the system exhibits a similar trend to the pure fluoxetine condition. Several proteins that showed a strong positive correlation in normals demonstrate no association or shift to a weak negative correlation (e.g., Drebrin A versus Ube3A, PCC = -0.14) in the fluoxetine and MD condition. The system decouples, but to a lesser extent than observed in the fluoxetine group.

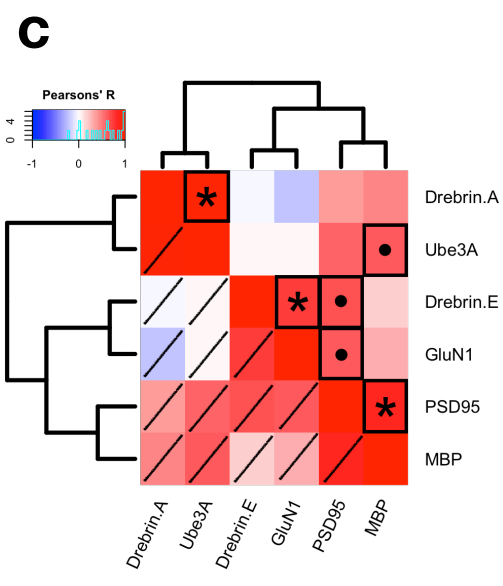
To summarize, MD produces a system that attempts, though ineffectively, to maintain the strong positive correlations between various proteins. In some cases, MD actually strengthens the correlations of certain protein pairs. The combination of fluoxetine and MD allows the system to decouple slightly, although this decoupling action is not as pronounced as in the pure fluoxetine condition.



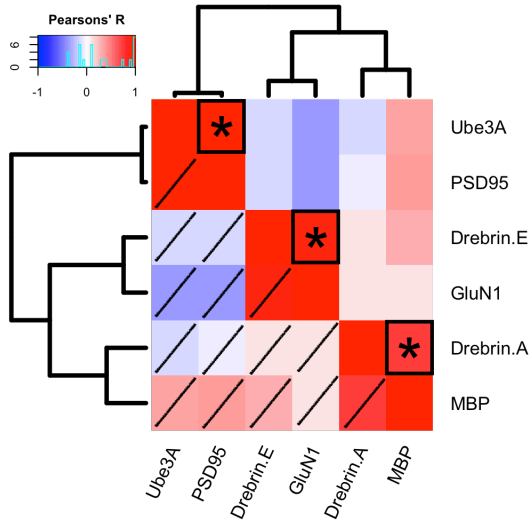
Normal



FLX



MD



FLX.MD

Fig 4. Pearson Correlation Coefficient Matrices for Fluoxetine and Visual Deprivation V1 conditions. Correlation Matrices based on the average expression value of each protein for each sample **(a)** Normal (n=6; PC critical value = 0.707) **(b)** FLX (n=8; PC critical value = 0.632) **(c)** MD (n=6; PC critical value = 0.707) **(d)** FLX.MD (n=8; PC critical value = 0.632)
FLX = 1 month Fluoxetine, MD = 1 week Monocular Deprivation, FLX.MD = 1 month Fluoxetine followed by 1 week Monocular Deprivation,

* = PCC below critical value of the Pearson Correlation Coefficient for 2 degrees of freedom (i.e., $p<0.05$)
- = PCC within 0.1 of critical value of the Pearson Correlation Coefficient for 2 degrees of freedom

S1/M1 (Fig 5): Across all conditions, strong positive PCCs exist between Ube3A and the excitatory markers PSD-95 and GluN1, as well as the spine markers Drebrin A and Drebrin E (e.g., Ube3A versus PSD-95, Normal PCC = 0.95; S-EE PCC = 0.93; L-EE PCC = 0.77). PSD-95 also exhibits a strong positive correlation with GluN1 regardless of experimental group (PSD-95 versus GluN1, Normal PCC = 0.96; S-EE PCC = 0.85; L-EE PCC = 0.78). Thus, neither S-EE or L-EE induce plastic changes by altering the associations of these select protein pairs. Furthermore, S-EE may create a small number of transient changes that dissipate with L-EE, as indicated by a few PCCs that gain strength with S-EE, but lose the strong association with L-EE (e.g., Drebrin A versus GluN1, Normal PCC = 0.46; S-EE PCC = 0.63; L-EE PCC = 0.18). S-EE demonstrates numerous similarities with normals and, at the systems level, appears remarkably similar to the normal group. Interestingly, with L-EE an uncoupling pattern emerges for several protein associations relative to the normal condition. The strong positive association between Drebrin E and Ube3A, and PSD-95 and Drebrin A, in normals weakens (e.g., Drebrin E versus Ube3A, Normal PCC = 0.67; S-EE PCC = 0.91; L-EE PCC = 0.52) and the positive correlation between Drebrin E and GluN1 is also reduced (Normal PCC = 0.75; S-EE PCC = 0.71; L-EE PCC = 0.58). The correlation between Drebrin A and MBP also moves from a moderately positive association in normals to a moderately negative association in L-EE (Normal PCC = 0.46; S-EE PCC = 0.36; L-EE PCC = -0.47). These findings demonstrate that extended enrichment produces either a decrease of positive

associations or an increase of negative associations for select proteins and allows the system to uncouple itself, similar to fluoxetine in V1.

In summary, S-EE closely resembles normals regarding their systemic profiles, with numerous positive correlations being conserved across both conditions. L-EE allows the system to uncouple and shares this similarity to fluoxetine treatment in V1. It appears that MD, a maladaptive form of experience, attempts to hold on to positive protein correlations relative to normal and keep the system moving together. However, both fluoxetine and L-EE, adaptive forms of experience, allow the system to uncouple relative to normal and seem to induce plastic changes by embracing the removal of positive protein correlations.

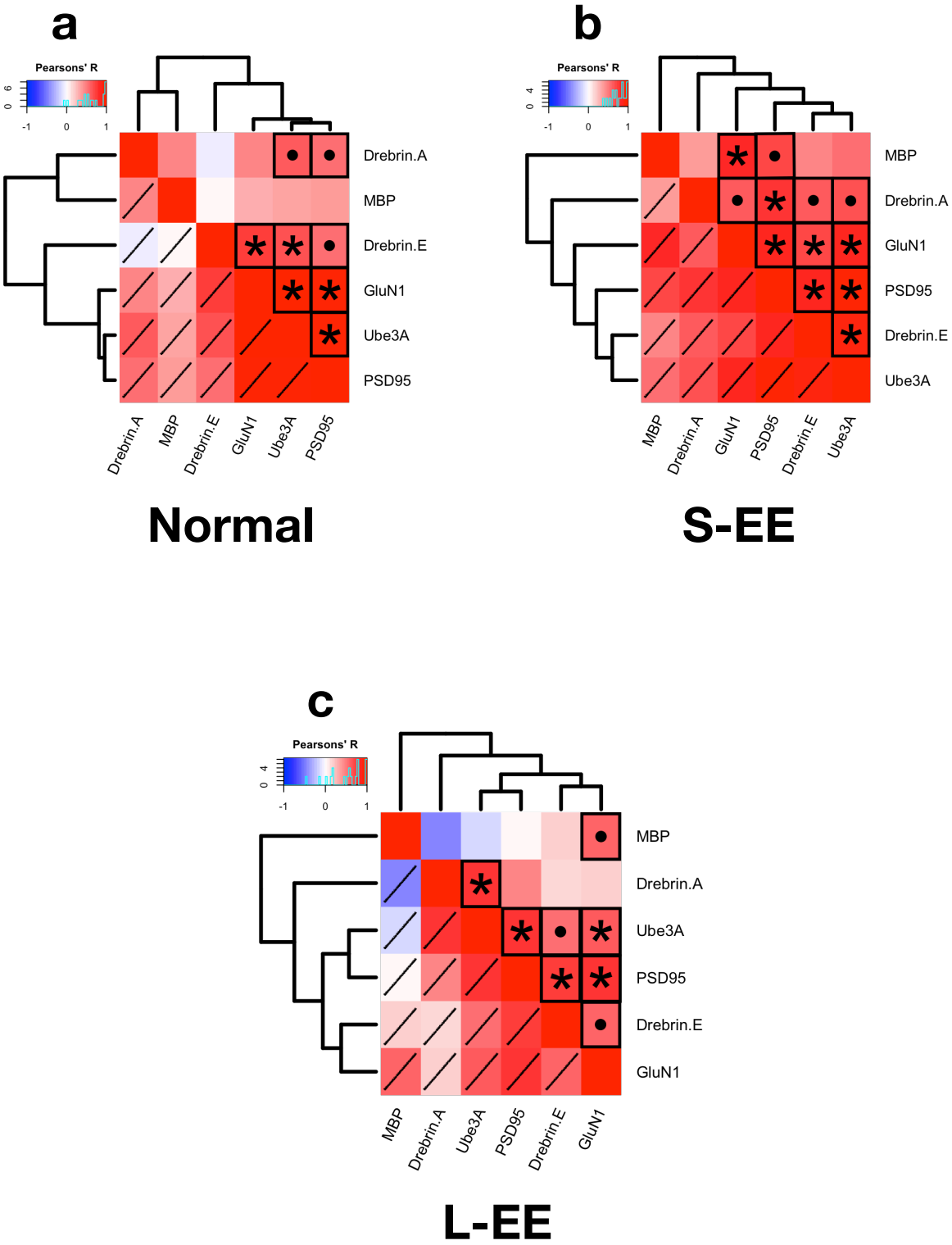

a
Pearsons' R
Drebrin.A
MBP
Drebrin.E
GluN1
Ube3A
PSD95
Normal
b
Pearsons' R
MBP
Drebrin.A
GluN1
PSD95
Drebrin.E
Ube3A
S-EE
c
Pearsons' R
MBP
Drebrin.A
Ube3A
PSD95
Drebrin.E
GluN1
L-EE

Fig 5. Pearson Correlation Coefficient Matrices for Enrichment S1/M1 conditions. Correlation Matrices based on number of runs that had a complete protein set (i.e., protein expression values for all 6 proteins) **(a)** Normal (n=7; PC critical value = 0.666) **(b)** S-EE (n=6; PC critical value = 0.707) **(c)** L-EE (n=9; PC critical value = 0.602) S-EE = short-term (2 weeks) enrichment, L-EE = long-term (1.25 years) enrichment,

* = PCC below critical value of the Pearson Correlation Coefficient for 2 degrees of freedom (i.e., $p<0.05$)
- = PCC within 0.1 of critical value of the Pearson Correlation Coefficient for 2 degrees of freedom

2.4 Discussion

In this study, we investigated how fluoxetine and environmental enrichment alter a collection of synaptic proteins and a marker for myelin as these treatments support enhanced plasticity in adulthood. We demonstrated that fluoxetine promotes an overall elimination of dendritic spines whereas EE fosters spine maturation. In ipsilateral V1, MD alone or in combination with fluoxetine, and short-term EE in S1/M1, increased MBP levels. At the systems level, adaptive forms of plasticity such as fluoxetine alone, fluoxetine combined with MD, and extended enrichment, are characterized by a decoupling of positive protein correlations. MD, a maladaptive plasticity manipulation, does not allow protein decoupling to occur, but instead tries to keep the system together by reducing, but not eliminating, positive associations.

The differential effects of fluoxetine and enrichment on Drebrin A and E levels have implications for rectifying diseases accompanied by alterations in spine dynamics or spine density. Several neuropsychiatric disorders including Autism Spectrum Disorder (ASD), Schizophrenia (SZ), and Alzheimer's Disease (AD) are accompanied by altered circuit connectivity and dendritic spine abnormalities (Penzes, Cahill, Jones, VanLeeuwen, & Woolfrey, 2011). Depending on the direction of the change in spine density, fluoxetine which eliminates spines, or enrichment which promotes spine maturation, has the potential to restore spine density to the appropriate equilibrium.

ASD is characterized by distal under-connectivity and local over-connectivity (Rane et al., 2015), and post-mortem analysis of human ASD brains reveals an increased

spine density on pyramidal neurons in the temporal, parietal, and frontal cortices (Hutsler & Zhang, 2010). Hence, the spine-eliminating action of fluoxetine may assist in rescuing pathological spine increases, provided spatial and cell-type considerations are taken into account. In SZ, spine loss occurs on pyramidal neurons in the dorsolateral prefrontal cortex (Glantz & Lewis, 2000), and spine and synapse losses have been reported in the hippocampus of AD patients (Davidsson & Blennow, 1999). Enrichment, which promotes spine maturation and therefore increased spine density, may serve as a potential treatment for the SZ and AD spine abnormalities.

The observed trend towards increased Ube3A with extended enrichment and fluoxetine combined with MD may have indirect effects on the composition of excitatory receptors at the synapse. Ube3A is an E3 ligase that targets various proteins, including ARC, for degradation (Greer et al., 2010). ARC functions as a molecular plasticity regulator by facilitating the endocytosis of AMPA receptors at the synapse. In Ube3A knockout mice, there is an increase in ARC expression, which leads to a decrease in postsynaptic AMPA receptors (Greer et al., 2010). Therefore, increased Ube3A would enhance the degradation of ARC, mitigate the endocytosis of AMPA receptors, and increase AMPA levels at the synapse. It is interesting to note that fluoxetine combined with MD in V1 slightly decreases expression of GluA2, the most pervasive AMPA receptor subunit (Beshara, Beston, Pinto, & Murphy, 2015). This reduction may occur because fluoxetine also elicits an increase in BDNF (Maya Vetencourt et al., 2008), which leads to increased ARC levels through TrKB receptor signalling (Yin, Edelman, &

Vanderklish, 2002). Increased BDNF is also a characteristic of EE (Sale et al., 2007). It is possible that increases in Ube3A with extended enrichment and fluoxetine combined with MD work coincidently with increases in BDNF levels to tightly regulate the amount of ARC intracellularly and precisely modulate excitatory receptors inserted into the membrane.

In this study, we observed MBP increases in ipsilateral V1 following MD and the combination of fluoxetine with MD. This myelination may occur selectively on a particular subset of GABAergic neurons: parvalbumin positive (PV+) cells. PV+ cells are fast-spiking, inhibitory interneurons (Kawaguchi & Kubota, 1997) that play an important role in establishing the precise connectivity of various neural circuits (Pfeffer, Xue, He, Huang, & Scanziani, 2013). In both mice and humans, a substantial volume of PV+ neurons are myelinated throughout the cortex, and this myelination is mainly confined to the proximal axonal segments (Stedehouder et al., 2017). Recently, neuronal activity has been shown to increase the myelination of PV+ interneurons in addition to modifying the structural complexity of PV+ axons (Stedehouder, Brizee, Shpak, & Kushner, 2018). It may follow that increased MBP in ipsilateral V1 occurs on PV+ cells and results from the increased neuronal activity driven by visual experience that the ipsilateral hemisphere receives from the open eye.

In the rat visual system, approximately 90% of the optic nerve crosses at the optic chiasm as the nerve travels backwards from the eye to the visual cortex, and approximately 10% of the nerve continues to extend back ipsilaterally (LeVere, 2013).

Therefore, when MD is applied to rat models, the hemisphere contralateral to the closed eye receives negligible visual input, whereas the hemisphere ipsilateral to the closed eye receives extensive visual input from the open eye. The direction of the change depends on the type of visual experience (deprived or non-deprived input) and can be observed in the different hemispheres. The hemisphere contralateral to the closed eye does not receive visual input, and MBP expression is reduced. The hemisphere ipsilateral to the closed eye receives visual input from the open eye, and MBP is elevated. It appears as though visual experience from the open eye drives the increase in MBP ipsilaterally, and the deprived vision from the closed eye results in a loss of MBP contralaterally. PV+ cells may serve as the prime target of this increased myelination as they exhibit a high degree of myelination throughout the cortex (Stedehouder et al., 2017).

We also found that short-term EE increases MBP levels, although this increase was no longer statistically significant with extended enrichment. Recent imaging literature demonstrates that enrichment increases myelination in the somatosensory cortex by enhancing the production of oligodendrocytes and subsequent axonal myelination (Hughes et al., 2018). While our results indicate that MBP does not differ significantly from normal with extended enrichment, the result trends toward an increase of MBP levels.

It is interesting that enrichment, a treatment capable of advancing plasticity in the adult brain, would also bring about increases in myelin, which is typically viewed as a structural brake on plasticity (Bavelier, Levi, Li, Dan, & Hensch, 2010). There is a

growing body of evidence indicating that myelin also functions to provide metabolic support to neurons (Philips & Rothstein, 2017; Saab, Tzvetanova, & Nave, 2013). Additionally, increased neuronal activity also increases myelination (Demerens et al., 1996; Gibson et al., 2014). Our findings in tandem with recent literature suggest that myelin may not only be a structural brake on plasticity. Myelin appears to serve an adaptive, plasticity-supporting role by allowing neurons and circuits to cope with heightened demands resulting from increased neuronal activity due to experience.

Accordingly, enriched environments may serve as a treatment or preventative measure for demyelinating disorders of the central nervous system, as well as mental disorders such as major depressive disorder (MDD) and SZ which have reduced myelin in the superficial layers of the prefrontal cortex (Lake et al., 2016). Enrichment may also be able to reduce, delay, or prevent myelin losses that occur as part of the normal aging process (Hill, Li, & Grutzendler, 2018; Siu, Balsor, Jones, & Murphy, 2015) and help limit the associated cognitive declines. Enrichment, which leads to improvements in learning and memory (Leal-Galicia, Castañeda-Bueno, Quiroz-Baez, & Arias, 2008; Sampedro-Piquero & Begega, 2017), may bring about these functional changes by increasing myelination or preventing myelin declines in the sensory and cortical areas responsible for memory and executive functions.

We used Western blot analysis to quantify how fluoxetine and environmental enrichment alter protein expression in adult rat V1 or S1/M1, respectively. Western blotting is a reliable, cost-effective approach to probe for many proteins, both synaptic

and extracellular, in a relatively short amount of time. However, this method does not provide spatial information regarding cell type or cortical layers, making it difficult to draw conclusions about the impacts of fluoxetine or enrichment on neural circuit organization. Furthermore, Western blotting does not allow for the examination of dendritic spine morphology, myelination, and transient spine or myelin changes. Neuroanatomical and neuroimaging studies are therefore essential to corroborate our findings and supplement our investigation of dendritic spine and myelin markers. Identifying how spine morphology and myelination patterns change in response to fluoxetine and environmental enrichment will allow us to reconcile the functional and structural effects of these two plasticity-enhancing treatments.

There is a need for longitudinal studies investigating the effects of fluoxetine and enrichment in order to determine how treatment duration influences protein expression. For example, fluoxetine and enrichment studies that include measurements taken on the time course of hours, days, months, or years can identify critical windows for changes to protein expression levels. Furthermore, longitudinal studies can determine if permanent changes take hold following treatment cessation. It would also be interesting to investigate if one treatment can augment the other and produce synergistic molecular and behavioural effects when fluoxetine and enrichment are used in combination. Lastly, it is important to tease apart the 'active ingredients' of each treatment. For fluoxetine, what relative proportion of the observed effects can be attributed to the drug itself versus increased serotonin at the synapse? For enrichment, how much of the effects can be

attributed to the individual components of the enriched environment paradigm, including social stimulation, exercise, and stimulation driven by interaction with inanimate objects?

Multiple treatments, including fluoxetine (Maya Vetencourt et al., 2008), enrichment (Sale et al., 2007), removal of perineuronal nets (Galtrey & Fawcett, 2007), and transplantation of immature neurons (Davis et al., 2015), that aim to enhance plasticity in adulthood have been extensively studied in animal models. However, translation of these results to humans has been a slow and arduous process. Translation is limited, in part, by the sparsity of information available about human neuroplasticity mechanisms.

In addition to identifying the functional and structural changes induced by different therapies, there is a need to characterize the proteomic profile of non-diseased animals and humans to gain an understanding of how the synaptic environments of different animal models align with those of humans at various developmental time points. Methodological approaches, including Western blotting, enzyme-linked immunosorbent assay (ELISA), and mass spectrometry, allowing for the high-throughput analysis of large quantities of proteins will be instrumental in characterizing the molecular mechanisms at play during different stages of development in a variety of animal species and humans.

Additionally, anatomical analysis of post-mortem human tissue samples via imaging techniques, such as immunostaining combined with microscopy, will assist in developing a comprehensive blueprint of the structural and circuit characteristics across multiple regions of the brain. Comparison of these results with anatomical findings in

animal species will assist in identifying neuroanatomical similarities and differences between animal models and humans and how structural differences may impact translation efforts.

By addressing these gaps in our knowledge base, we will be better able to facilitate translation between animal and human neurobiological mechanisms, design new effective treatments, and apply existing treatments with proven benefits in animal models to human patients.

Chapter 3. General Discussion

3.1 Summary of Main Findings

In my thesis, I studied two approaches to reactivate heightened plasticity levels in the adult brain by examining the effects of fluoxetine and visual deprivation in the visual cortex and the effects of environmental enrichment within the somatomotor cortex. I have further elucidated the molecular mechanisms used by fluoxetine and environmental enrichment as they work to increase the neuroplasticity of the adult brain.

Through my analysis of the dendritic spine marker Drebrin, whose two isoforms are developmentally regulated, I found that fluoxetine and enrichment exert distinct effects on dendritic spines. Drebrin A, the mature Drebrin isoform, was reduced when fluoxetine was applied alone or paired with MD but was substantially elevated with enrichment. Drebrin E, the immature Drebrin isoform, was decreased with pure fluoxetine and fluoxetine combined with MD and was also decreased with enrichment.

Collectively, my results suggest that fluoxetine, characterized by losses in both Drebin A and Drebrin E, facilitates an overall elimination of dendritic spines. However, enrichment, which increases Drebrin A and decreases Drebrin E, promotes spine maturation. Thus, my proteomic analysis indicates that fluoxetine and enrichment operate via distinct mechanisms at the level of dendritic spines.

I also found that expression of Classic MBP, a myelin protein that enables myelin compaction, was elevated with enrichment in the somatomotor cortex, and this increase was statistically significant with short-term enrichment. In the visual cortex, Classic MBP levels were increased in the hemisphere ipsilateral to the deprived eye in the MD and

fluoxetine paired with MD conditions. Classic MBP expression was decreased in the contralateral hemisphere for the MD and fluoxetine combined with MD conditions.

My work suggests that fluoxetine and enrichment have differences regarding myelin-associated plasticity since enrichment increases MBP levels while fluoxetine in the contralateral hemisphere deprived of vision produces no corresponding increase of MBP. If fluoxetine had a similar effect on myelin-related plasticity as enrichment, one would hypothesize that fluoxetine would rescue the MBP loss caused by MD. On the contrary, fluoxetine paired with MD was not different from the MD group, which suggests fundamental myelin-plasticity differences between enrichment and fluoxetine. Specifically, enrichment enhances myelination whereas fluoxetine does not impact myelin levels.

3.2 Methodological Considerations

I used Western blotting to study how fluoxetine and environmental enrichment alter the protein expression of five synaptic proteins and one extracellular, myelin-associated protein. The Western blotting methodology possesses several inherent strengths. First, Western blotting is a relatively cost-effective and time-efficient approach for generating large quantities of protein data from a cohort of samples. Second, the blot membranes can be reprobed to analyze the expression of multiple proteins. This feature allowed me to construct a comprehensive evaluation of the proteomic changes elicited by fluoxetine and enrichment. Lastly and most importantly, Western blotting produces reliable and reproducible data, meaning future research attempting to corroborate or replicate my work can be completed with ease.

As with any scientific methodology, there are limits to the type of information and conclusions that can be drawn using Western blot analysis. The technique does not reveal information regarding cell-type, cortical layer, or protein localization within the neuron. Therefore, additional studies that employ more elaborate neuroanatomical methods will be required to supplement findings from my Western blot analysis. Additionally, neuroanatomical studies are necessary to corroborate my analysis of proteins that serve as markers for dendritic spine and myelin dynamics. Imaging studies that provide a cellular view of how the actual spines and myelin change with fluoxetine or enrichment treatments will serve to validate and confirm the spine and myelin findings in my thesis.

A study limitation to acknowledge is that the Long Evans rats in the fluoxetine and visual deprivation experimental arm were only males. While this factor does not invalidate my results and conclusions, it is important to remain aware that my fluoxetine findings may be influenced by sex bias. Future studies examining the impact of fluoxetine in animal models should include both male and female animals. Replication studies, with large sample sizes, will be useful in order to bolster the external validity of my work and conclusively determine whether or not my findings are generalizable to both sexes.

3.3 Significance

Myelin Appears to Restrict AND Enhance Neuroplasticity

The elevated levels of Classic MBP that I found in the somatomotor cortex with enrichment support recent literature that suggests a novel function for myelin in promoting synaptic plasticity. Up to this point in time, myelin has typically been regarded as a structural brake on plasticity (Bavelier, Levi, Li, Dan, & Hensch, 2010). This view is primarily based upon studies that demonstrated myelin contains several proteins which activate an intracellular signalling pathway that leads to inhibited axonal outgrowth (McGee, Yang, Fischer, Daw, & Strittmatter, 2005; Wang et al., 2002) and ablating the neuronal receptor for these myelin-associated proteins extends the critical period (McGee, Yang, Fischer, Daw, & Strittmatter, 2005). Strategies to enhance plasticity after the close of the critical period or reinstate juvenile-like plasticity often discuss removing structural brakes on plasticity, such as myelin (Bavelier, Levi, Li, Dan, & Hensch, 2010). Based on this perspective, myelin serves as an impediment to plasticity: a structural boundary that must be removed to reactivate plasticity in the adult brain.

However, current literature along with my enrichment results indicate that myelin not only functions as a brake but also has an adaptive role in promoting plasticity, suggesting a change in perspective regarding myelin's role in experience-dependent plasticity. A very recent study by Hughes et al. (2018) published in the Nature Neuroscience shows that environmental enrichment, taking the form of whisker stimulation, increases the production of new oligodendrocytes and subsequent

myelination in the somatosensory cortex of adult rats (Hughes, Orthmann-Murphy, Langseth, & Bergles, 2018). My work corroborated the results of this imaging study at the molecular level, as I found that enrichment increases Classic MBP expression, which reached significance in the short-term enrichment condition.

Furthermore, neuronal activity promotes enhanced myelination and generation of myelin precursor cells (Demerens et al., 1996; Gibson et al., 2014). Recent evidence also indicates that myelin plays a key role in providing axons with metabolic support (Philips & Rothstein, 2017; Saab, Tzvetanova, & Nave, 2013). The enhanced experience from environmental enrichment translates into increased neuronal activity in the cortex. Increased myelination may play a plasticity-supporting role by providing metabolic support to neurons to sustain heightened activity levels driven by enhanced experience. Thus, we can think of myelin as having an adaptive, plasticity-enhancing role in addition to its plasticity-limiting functions. My work lends support to a potential paradigm shift where myelin is viewed both as an enhancer and brake on plasticity.

Enrichment to Treat Demyelinating Conditions

Since enhanced sensory experience increases myelination, environmental enrichment has the potential to serve as an intervention or preventative measure for demyelinating conditions. Enrichment may help restore myelin losses that characterize multiple sclerosis (Frohman, Racke, & Raine, 2006). Enrichment can also assist in the treatment of specific mental disorders, such as MDD and SZ, in which a loss of myelin

has been reported in the cortex (Lake et al., 2016). Additionally, enrichment has been shown to improve cognitive functioning (Leal-Galicia, Castañeda-Bueno, Quiroz-Baez, & Arias, 2008; Sampedro-Piquero & Begega, 2017), meaning it may serve as an effective approach to help limit cognitive declines and rescue age-related myelin losses that occur with aging (Hill, Li, & Grutzendler, 2018; Siu, Balsor, Jones, & Murphy, 2015).

Rectifying Dendritic Spine Abnormalities with Enrichment and Fluoxetine

My findings for dendritic spine markers indicate that enrichment and fluoxetine support plasticity via distinct spine dynamics, which has implications for treating neurological diseases that are accompanied by dendritic spine aberrations. For example, neuropsychiatric disorders including ASD, SZ, and AD exhibit dendritic spine pathologies (Penzes, Cahill, Jones, VanLeeuwen, & Woolfrey, 2011). Analysis of post-mortem ASD brains reveals that the dendritic spine density of pyramidal neurons in the frontal, temporal, and parietal regions is increased relative to age-matched controls (Hutsler & Zhang, 2010). Hence, fluoxetine, which produces an overall elimination of dendritic spines, can function as a treatment to restore ASD spine density to the appropriate equilibrium, although spatial precision and cell-type specificity must be considered. On the other hand, decreased spine density occurs in SZ on the deep layer III pyramidal neurons in the dorsolateral prefrontal cortex (Glantz & Lewis, 2000). Therefore, enrichment may serve as a potential approach to recover this spine loss. Spine and synapses losses also appear in the hippocampus in both human and animal models of

AD (Davidsson & Blennow, 1999; Lanz, Carter, & Merchant, 2003). Since most synapses occur on dendritic spines, the spine maturing effect of enrichment may help rescue the reduced spine and synapse quantities that result from AD.

Novel Approach for Applying Fluoxetine Treatments

In my thesis, I demonstrated that fluoxetine did not improve Classic MBP expression after MD in the contralateral V1 hemisphere, which is the hemisphere deprived of visual input. However, Classic MBP expression increased in the ipsilateral V1 hemisphere that received visual input from the open eye and increased in the somatomotor cortex that received enhanced sensory experience. Hence, enhanced experience and the elevated neuronal activity may help augment fluoxetine-induced plasticity.

Along this line of "optimal" experience, a study was completed by Huttunen and colleagues in May 2018 that evaluated the effects of fluoxetine paired with visual perceptual training in adult amblyopic patients. The fluoxetine treatment group did not differ from the control group in outcome measures indicative of improved vision, such as visual acuity and contrast sensitivity (Huttunen et al., 2018). However, the fluoxetine dosage may have been too small, and the duration of perceptual training may have been too short (30 min per day for 10 weeks) for group differences to emerge. Also, perceptual training occurred while one eye was patched. Thus, it is possible that the optimal

combination of fluoxetine, quality experience, and resulting neuronal activity may not have been achieved in this study.

Applying a combined pharmacological and experiential treatment paradigm may prove beneficial for enhancing adult brain plasticity. Pairing fluoxetine with a manipulation of experience that promotes neuronal activity in the cortex following MD, such as binocular vision or presentation of a specific visual stimulus to the deprived eye, may enhance the plastic changes elicited by fluoxetine.

3.4 Future Directions

My thesis examined how fluoxetine and environmental enrichment enhance adult brain plasticity by studying a collection of proteins in the adult rat cortex. Three of these proteins, Drebrin A, Drebrin E, and Classic MBP, are markers for neuronal structural components implicated in neuroplasticity. Drebin A and Drebrin E are proxies for dendritic spines, and Classic MBP is a marker for myelin. Future neuroanatomical and imaging studies will be useful in order to observe in real-time how fluoxetine and enrichment alter spine dynamics and myelination patterns. These anatomical studies will be essential as they will corroborate my proteomic findings and verify my conclusions that have been based on markers for structural plasticity mechanisms.

Investigating how fluoxetine and enrichment, as well as other therapeutics, impact PV+ neurons may prove an insightful area of study. PV+ neurons are a class of fast-spiking, inhibitory interneurons (Kawaguchi & Kubota, 1997) and assist in setting up precise circuit connectivity (Pfeffer, Xue, He, Huang, & Scanziani, 2013). Recent studies have shown that a substantial quantity of myelin within the mouse and human cortex is located on PV+ neurons (Stedehouder et al., 2017) and neuronal activity enhances the myelination of these neurons (Stedehouder, Brizee, Shpak, & Kushner, 2018). Additionally, these neurons exhibit a peculiar pattern of myelination as the myelin on PV+ cells is dispersed and mainly localized to the proximal axon segment (Stedehouder et al., 2017). If fluoxetine and enrichment alter PV+ cell function and conduction properties via changes to their myelination patterns, the effects could manifest as changes

in distal circuits as well as behavioural changes. It seems likely that PV+ cells would be affected by the enhanced myelination caused by enrichment, but the specific effects remain to be uncovered.

Another future direction is delineating the relative contribution of the multiple components of fluoxetine and enrichment therapies on enhancing adult brain plasticity. For example, fluoxetine is an SSRI, but are all of fluoxetine's plasticity-enhancing effects due to the global increase of serotonin or does fluoxetine produce unique effects independent of elevated serotonin levels? With respect to enrichment, what is the relative contribution of physical stimulation versus social stimulation and do these different components produce an additive or synergistic effect on plasticity? OD shifts have been reported in adult mice that were housed in pairs compared to mice housed in solidarity (Balog et al., 2014) and adult rats who engage in voluntary physical exercise (Kalogeraki, Greifzu, Haack, & Lowel, 2014). Interestingly, aerobic exercise in adult humans produced no effect on OD (Zhou, Reynaud, & Hess, 2017). Teasing apart the relative contributions of each treatment element will help us identify the "active ingredients" that are essential for improving plasticity in the adult brain and better understand the interaction between the different components.

Additionally, continuing to evaluate the effectiveness of fluoxetine in human populations should be undertaken in order to the determine the viability and potential plasticity benefits this therapeutic intervention offers human patients. As mentioned in Section 3.3 "Novel Approach for Applying Fluoxetine Treatments", a phase 2 clinical

trial conducted in adult human amblyopic patients found that fluoxetine augmented with perceptual training did not result in improvements in visual acuity beyond those improvements reported in the control group that only received perceptual training (Huttunen et al., 2018). However, the drug dosage and quality of visual experience may not have been appropriate to induce enhanced plasticity. Therefore, future research that evaluates how varying degrees of visual stimulation or other experiential manipulations affect patient responses to fluoxetine administration may help uncover the proper combination of fluoxetine dosage and experience to optimize plasticity in humans. Furthermore, investigating different fluoxetine dosages, administration schedules, and supplementing fluoxetine with other pharmaceuticals may help elucidate optimized fluoxetine treatments.

My results as well as findings from Beshara et al., 2015 indicate that fluoxetine has enhanced "neuroprotective" effects on plasticity when fluoxetine administration occurs before and coincidently with MD. Our findings suggest that fluoxetine causes plastic changes if administered prior to a maladaptive manipulation of experience. Thus, it would be interesting to investigate if safe dosages of fluoxetine in healthy human populations prevent the decline of visual acuity with aging using a longitudinal study design.

It may be useful to study drugs that have a similar mechanism of action as fluoxetine, such as other SSRIs, in human patients to determine the potential clinical efficacy of those drugs in promoting plasticity in adulthood. There may be species-

specific differences and nuances that hinder the immediate translation of benefits observed with fluoxetine in animal models to human patients. Perhaps other drugs exist that are more beneficial than fluoxetine for increasing plasticity in humans.

Also, additional insights may be gained by investigating whether or not fluoxetine and environmental enrichment can improve health outcomes of human patients afflicted by other neurological diseases that involve a component of restricted neuroplasticity. Along with this line of research, several clinical trials are investigating whether or not fluoxetine treatment can promote motor and visual recovery in human adult patients with motor or visual impairments following acute ischemic stroke ("Search of: fluoxetine, brain, adult - List Results - ClinicalTrials.gov", 2018). Interestingly, one clinical trial is actively recruiting patients to evaluate if fluoxetine is capable of slowing brain volume loss in individuals with multiple sclerosis ("Search of: fluoxetine, brain, adult - List Results - ClinicalTrials.gov", 2018). With respect to enrichment, two clinical trials evaluating the effects of enrichment on adults with late phase stroke or traumatic brain injury are registered on ClinicalTrials.gov ("Search of: environmental enrichment, brain, adult - List Results - ClinicalTrials.gov", 2018).

Finally, furthering translation efforts between human and animal models is necessary to be able to apply plasticity-enhancing treatments studied in animals to human patients. Characterizing the protein composition of the synaptic environment in humans and animal models before and after treatment is one approach. Methodologies like Western blotting, ELISA, and mass spectrometry as well as other techniques that enable

high-throughput analysis of numerous proteins are required to understand how the molecular components of synapses develop in both animal models and humans. It may also prove beneficial to investigate the degree of similarity in the transcriptome profiles and mRNA development across a variety of species. mRNA is the intermediate biomolecule between a gene and functional protein, so studying mRNA can provide unique insights into biological mechanisms early in the central dogma of gene expression. However, proteins are the key functional biomolecules within cells, and mRNA expression levels do not always accurately represent levels of the corresponding translated proteins (Vogel & Marcotte, 2012). Therefore, characterization of mRNA development across species should be conducted in tandem with proteomic analyses and differences in the mRNA and protein expression of individual genes should be noted.

Studying the neuroanatomy of animals and humans will also assist in improving translation efforts. Further characterizing the structural similarities as well as differences between various species may enable us to better understand how distinct anatomical features contribute to increased complexity and difficulty in translation between species. Also, investigating how neuroplasticity-improving therapies affect circuits and specific cell-types is another next step in the translation process. Spatiotemporal techniques like cell-type specific physiology and optogenetic manipulation will help identify how these plasticity-enhancing treatments precisely alter local neural circuits. These future lines of research will help us understand species-specific differences that we may need to take into account when trying to reactivate plasticity in the adult human brain.

Chapter 4. References

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