

# MECHANISMS OF REINSTATED PLASTICITY

PLASTICITY MECHANISMS IN VISUAL CORTEX:  
ANIMAL MODELS AND HUMAN CORTEX

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## **Lay Abstract**

Neurons change to rewire, adapt, and recover. This plasticity is greatest early in development, so much research has focused on bringing it back in adults. There has been amazing progress in animal models, but this has not translated to humans. Two reasons for this are that we do not fully understand the mechanisms of these treatments in animals or whether those mechanisms are relevant for humans. My thesis addresses this by studying how 2 treatments, fluoxetine and D-serine, affect proteins that are important for plasticity, and how those proteins develop in the humans.

I found that these treatments are neuroprotective, but do not recreate a younger state. One interesting standout is an increase in Ube3A, which is essential for juvenile plasticity. I also found that much of human development is similar to animals, but the time course for some proteins is uniquely prolonged in humans. These findings have implications for the use of plasticity-enhancing treatments at different ages.

## Abstract

A holy grail in neuroscience is being able to control plasticity to facilitate recovery from insult in the adult brain. Despite success in animal models, few therapies have translated from bench to bedside. This thesis is aimed at addressing 2 major stumbling blocks in translation. The first gap is in our understanding of the mechanisms of plasticity-enhancing therapies, and the second is in our understanding the relevance of those mechanisms for human development.

In chapters 2 and 3, I address the first gap by asking whether fluoxetine, a selective serotonin reuptake inhibitor, which reinstates juvenile-like plasticity in adult animals, reinstates a juvenile-like synaptic environment. We found evidence to suggest that fluoxetine is neuroprotective, as it rescued all of the MD-driven changes, but surprisingly we found no evidence that fluoxetine recreated a juvenile-like synaptic environment, with the exception of Ube3A. Ube3A is necessary for critical period plasticity, indicating that Ube3A may play a crucial in enhancing plasticity in the adult cortex.

In chapter 4, I address whether D-serine, an amino acid that has similar effects to fluoxetine in terms of both plasticity and anti-depression, shares a common neurobiological signature with fluoxetine. I found that D-serine's effects were strikingly similar to fluoxetine, with respect to markers of the E/I balance, indicating that it may be an effective alternative to fluoxetine.

In chapter 5, I address the second gap by studying the development of 5 glutamatergic proteins in human V1. Some changes occurred early, as would be predicted from animals studies, while other changes were protracted, lasting into the 4th decade. These results will help guide the use of treatments, like fluoxetine, which effect glutamatergic proteins.

Together the findings in this thesis significantly advances our understanding of the mechanisms involved in restating plasticity in the adult cortex, and their relevance to humans.

## Acknowledgements

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## List of Abbreviations

AMPA	$\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
$\beta$ -tubulin	beta-tubulin
BDNF	brain derived neurotrophic factor
CB1R	cannabinoid receptor type 1
CP	critical period
Contra	contralateral
DTT	dithiothreitol
E/I	excitatory/inhibitory
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
GABA	gamma-aminobutyric acid
GAD	glutamate decarboxylase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GRIP1	glutamate receptor interacting protein 1
GluA	glutamate receptor ionotropic AMPA receptor
GluN	glutamate receptor ionotropic NMDA receptor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Ipsi	ipsilateral
KCC2	potassium chloride cotransporter
LTD	long term potentiation

LTP	long term depression
MD	monocular deprivation
mTOR	mechanistic target of rapamycin
NMDAR	N-methyl-D-aspartate receptor
P()	postnatal day
p	phospho-
PBS	phosphate-buffered saline
PICK1	protein interacting with C-kinase 1
PMSF	phenylmethylsulfonyl fluoride
PSD-95	post-synaptic density protein 95
PVDF-FL	polyvinylidene difluoride
Ras/ERK	rat sarcoma viral oncogene homolog/extracellular signal-regulated kinase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
Ube3A	ubiquitin protein ligase E3A
V1	primary visual cortex
VGAT	vesicular gamma-aminobutyric acid transporter
VGluT1	vesicular glutamate transporter 1

## **Declaration of Academic Achievement**

Chapter 2 is a paper that was published in eNeuro. This chapter was a collaboration between myself, Dr. Brett Beston, Dr. Joshua Pinto, and Dr. Kathryn Murphy. I was the lead on analyzing and interpreting the data, and writing the paper.

Chapter 3 is a manuscript that will be submitted to eLife. This chapter was a collaboration between myself, Lilia Tcharnaia, Dr. Brett Beston, Dr. Joshua Pinto, and Dr. Kathryn Murphy. I was the lead on analyzing and interpreting the data, and writing the manuscript.

Chapter 4 is a manuscript that is being prepared for submission. This chapter was a collaboration between myself and Dr. Kathryn Murphy. I was the lead on designing and performing the experiments, analyzing and interpreting the data, and writing the manuscript.

Chapter 5 is a manuscript that has been submitted to the Journal of Neuroscience and is currently in revision. This Chapter was a collaboration between myself, Caitlin Siu, Dr. David Jones, and Dr. Kathryn Murphy. Along with Caitlin Siu, I co-lead analyzing and interpreting the data, and writing the manuscript.

### **Chapter 2 citation**

Beshara S, Beston BR, Pinto JGA, Murphy KM (2015) Effects of Fluoxetine and Visual Experience on Glutamatergic and GABAergic Synaptic Proteins in Adult Rat Visual Cortex. *eneuro* 2:ENEURO.0126–15.2015–15.2015 Available at: <http://eneuro.sfn.org/cgi/doi/10.1523/ENEURO.0126-15.2015>.

# **Chapter 1. General Introduction**

## **1.1 Developmental Plasticity**

### **Critical periods**

The question of nature versus nurture is often answered with “both.” In no organ is this principle more apparent than the brain. Neurons in the brain form complex networks to process complex stimuli and direct complex behavior. Establishment of these networks is partly guided by genetics (Rubenstein and Rakic, 1999). Yet despite strict genetic programs, not even the brains of monozygotic twins are identical (Thompson et al., 2001) because many neural networks are subject to experience-dependent refinement which can significantly alter their functional characteristics. Often, the impact of experience is greatest during a brief, well-defined periods early in development, referred to as "critical periods" (Hensch, 2004). The power of these periods for shaping neural networks has led to much interest in both understanding and controlling critical period plasticity.

Among the earliest systematic explorations of critical periods are the classic behavioral observations of Konrad Lorenz. He noted that geese chicks would imprint any moving object as their mother, but only during a specific period after hatching (Lorenz, 1958). In the decades since, our understanding of how critical periods shape behavior has been expanded to many other species and behaviors. Song acquisition in birds involves critical periods during which the bird hears and practices a song, after which it becomes stereotyped (Doupe and Kuhl, 2003). Similarly, language acquisition in humans involves a period when infants learn to selectively discriminate native phonemes, after which learning new languages becomes progressively more difficult (Kuhl et al., 2005). As data from a recent New York Times poll suggests that even

complex and nuanced behavior such as selecting your favorite sports team may have a critical period (Stephens-Davidowitz, 2014)!

### **Ocular dominance model of critical period plasticity**

While critical periods shape complex behaviors like singing and speaking, the characteristics and mechanisms of critical periods are more easily studied in primary sensory systems. The premier model of critical period plasticity, first characterized in the classic work of Wiesel and Hubel (1963), is the developing visual system. Early in development, monocular deprivation (MD) reduces the responsiveness of the primary visual cortex (V1) to the deprived eye. MD is most potent during the peak of the critical period when only a few days of MD can cause this shift (Gordon and Stryker, 1996). This ocular dominance shift involves 2 phases (for review see Frenkel and Bear, 2004). The first is a rapid physiological reduction in the cortical response to the deprived eye (Flom and Neumaier, 1966). The second is a slow increase in responsiveness to the non-deprived eye. These physiological shifts are accompanied by anatomical changes in the primary visual cortex, including a loss of horizontal connections and thalamocortical afferents serving the deprived eye, leading to the shrinking of columns serving the deprived eye, and the expansion of columns serving the non-deprived eye (Hubel et al., 1977; Shatz and Stryker, 1978; Trachtenberg and Stryker, 2001).

The developing visual system has remained the premier model for studying experience-dependent plasticity *in-vivo* for several reasons: it is one of the most well-characterized developmental phenomena in terms of physiological, anatomical, and molecular changes; the experimental manipulation is relatively simple; and the outcomes are robust and readily measured. Also, MD is an excellent animal model of human amblyopia, which is the loss of

acuity due to abnormal visual experience early in development. Importantly, the loss of acuity is not due to defects in the eye, but rather due to rewiring of V1 during a critical period.

## 1.2 Mechanisms of Ocular Dominance Plasticity

### Synaptic Mechanisms

The 2 phases of ocular dominance plasticity have long been attributed to classic Hebbian homosynaptic plasticity and homeostatic synaptic scaling. An attractive hypothesis is that the initial loss of deprived-eye responses is due to long term depression (LTD) of thalamocortical synapses serving the deprived eye (Heynen et al., 2003; Yoon et al., 2009), while a combination of long term potentiation (LTP) and homeostatic synaptic scaling contribute to the later potentiation (Mrsic-Flogel et al., 2007; Smith et al., 2009). Some studies, however, have found a mechanistic dissociation between homosynaptic plasticity and ocular dominance shifts. For example, brain-derived neurotrophic factor (BDNF) prevents LTD in V1 (Jiang et al., 2003) but BDNF over-expression does not prevent the loss of deprived-eye responsiveness after MD (Huang et al., 1999). Another example is that inhibiting the protein synthesis necessary to sustain LTP and LTD does not prevent ocular dominance shifts (Frey et al., 1993; Taha and Stryker, 2002). Thus, while homosynaptic Hebbian mechanisms play a major role in ocular dominance plasticity, their contribution is likely part of a broader picture that includes heterosynaptic mechanisms and the balance between excitation and inhibition (Hensch and Fagiolini, 2005; Levelt and Hübener, 2012). Importantly, both Hebbian mechanisms and the excitatory/inhibitory (E/I) balance are determined by the presence and function of proteins at glutamatergic and gamma-Aminobutyric acidergic (GABAergic) synapses. There are many mechanisms involved in regulating critical period plasticity that are beyond the scope of this thesis, so in the next section, I focus on the E/I proteins studied in my experimental chapters.

## **Glutamatergic mechanisms**

AMPA receptors (AMPA receptors) mediate the fast component of excitatory transmission (Kleppe and Robinson, 1999). They are tetrameric structures composed of four homologous subunits (AMPA-type glutamate receptor subunit 1-4 [GluA1-4]). Different combinations of subunits give AMPARs different properties. For example, GluA2-containing AMPARs are calcium impermeable (Hollmann and Heinemann, 1994) and have a linear current-voltage relationship (Hollmann and Heinemann, 1994; Dingledine et al., 1999). The presence of AMPARs also affects synaptic plasticity. Nascent or “silent” glutamatergic synapses are typically NMDA receptor (NMDAR)-dominant, but progressively accumulate AMPARs in an experience-dependent manner as they become more active and less plastic (Isaac et al., 1997; Huang et al., 2015).

GluA2-containing AMPARs play an important role in both the depression and potentiation phases of ocular dominance shifts. In V1, MD causes phosphorylation of GluA2 subunits (forming pGluA2) and the subsequent rapid internalization of GluA2-containing AMPARs (Heynen et al., 2003; Yoon et al., 2009; Lambo and Turrigiano, 2013), which is necessary for depression of deprived-eye responses (Yoon et al., 2009). In addition, GluA2-containing AMPARs are necessary for the homeostatic synaptic scaling that allows neurons to maintain relatively constant activity levels despite fluctuations in sensory input: MD causes a significant loss of feedforward activity, to which neurons respond by increasing their sensitivity to glutamate by increasing the density of GluA2-containing AMPARs (Gainey et al., 2009; Lambo and Turrigiano, 2013).

The movement of GluA2-containing AMPARs into and out of synapses is tightly regulated by AMPAR interacting proteins (Anggono and Huganir, 2012). Two of these proteins are glutamate receptor-interacting protein 1 (GRIP1) and protein interacting with C-kinase 1 (PICK1). GRIP1 helps stabilize AMPARs at synapse (Dong et al., 1997), and interfering with GRIP1 reduces synaptic accumulation of GluA2 (Osten et al., 2000). PICK1 helps stabilize AMPARs in intracellular pools (Gardner et al., 2005; Liu and Cull-Candy, 2005) and remove AMPARs from the synapse (Perez et al., 2001; Terashima et al., 2004). Thus, GRIP1 and PICK1 are an important components of AMPAR regulation.

In addition to AMPARs, NMDARs also play a central role in experience-dependent plasticity. NMDARs are tetrameric proteins with 3 families of subunits and a total of 7 known subunits (NMDA-type glutamate receptor subunit 2A-D, 3A-B [GluN2A-D, GluN3A-B]) (Monyer and Sprengel, 1992). Functional NMDARs contain 2 obligatory GluN1 subunits and 2 regulatory subunits. The GluN2 subunit affects the functional properties of the NMDARs (Kutsuwada et al., 1992), and 2 of the GluN2 subunits in particular, GluN2B and GluN2A, undergo a well-characterized developmental switch that affects synapse function. GluN2B subunits are abundant in the young cortex, but GluN2A levels rise with development (Flint et al., 1997; Quinlan et al., 1999; Roberts and Ramoa, 1999) in an activity-dependent manner (Yashiro and Philpot, 2008). This developmental trajectory is important because different subunits confer different receptor properties. GluN2A reduces the NMDARs' binding affinity for glutamate and speeds up receptor kinetics (Flint et al., 1997). The different subunits also differentially contribute to perception and plasticity. For example, selective deletion of GluN2A reduces the degree of ocular dominance plasticity during the critical period and prevents maturation of

orientation selectivity (Fagiolini et al., 2003), whereas GluN2B reduces the trafficking and overall expression of AMPARs (Hall et al., 2007). Furthermore, the relative expression of GluN2A and GluN2B is an important regulator of the threshold for synaptic modification, termed metaplasticity (for review see Yashiro and Philpot, 2008). The developmental shift to GluN2A increases the threshold for synaptic modification, facilitating the weakening of synapses and making LTP more difficult. This synaptic modification threshold model explains the timing and order of the events of critical period ocular dominance plasticity (Cooper and Bear, 2012): First, MD causes the activity from the deprived eye to fall below base-line, leading to LTD. Next, the threshold adjusts to the loss of activity by shifting towards GluN2B, thereby lowering the minimal activity level necessary for LTP. Third, activity from non-deprived eye inputs is now above the newly-lowered threshold, resulting in potentiation of the non-deprived eye.

A unique aspect of NMDARs is that they require 2 ligands for activation, glutamate and a so-called “glycine”-site agonist. The co-agonist binding site is called the glycine site because glycine was the first endogenous co-agonist discovered. Over the last 20 years, however, it has become clear that D-serine is the main endogenous co-agonist. D-serine is more abundant, has a higher binding affinity, and shares more anatomical overlap with NMDARs, than glycine (for review see Henneberger et al., 2012). In fact, synaptic D-serine is necessary for canonical LTP (Henneberger et al., 2010), and recently (Meunier et al., 2016) found that D-serine is the endogenous co-agonist required for LTP of both excitatory and inhibitory synapses during the critical period for ocular dominance plasticity. Thus it’s clear that D-serine plays an important role in regulating NMDAR-dependent plasticity in V1.

NMDARs are also unique in that activation requires pre-existing membrane depolarization to remove a magnesium ion from blocking the channel (Nowak et al., 1984). The dual requirement of pre-synaptically released ligands and post-synaptic depolarization means that NMDARs function as coincidence detectors that report when both pre- and post-synapses are simultaneously depolarized. This property allows NMDARs to contribute to classical Hebbian “fire together wire together” plasticity by reporting when the pre- and post-synapse are indeed firing together (Malenka and Nicoll, 1993). The wiring together aspect also depends on NMDARs. NMDARs are Ca<sup>2+</sup> permeable which allows them to trigger Ca<sup>2+</sup> dependent intracellular pathways. These pathways (i.e., Ras/ERK pathway) affect transcription and translation to either strengthen or weaken the synapse (Kim et al., 2005; Wang et al., 2011). It is, therefore, no surprise the NMDARs are a necessary component of critical period plasticity (Sawtell et al., 2003), and that blocking NMDARs prevents ocular dominance plasticity (Bear and Rittenhouse, 1999).

### **GABAergic mechanisms**

GABA<sub>A</sub> receptors (GABA<sub>A</sub>Rs) are pentameric ionotropic receptors that mediate the majority of fast inhibitory synaptic transmission (Semyanov et al., 2004). There are at least 19 GABA<sub>A</sub> R subunits ( $\alpha$ 1-6,  $\beta$ 1-3,  $\gamma$ 1-3,  $\delta$ ,  $\rho$ 1-3,  $\epsilon$ ,  $\pi$ , and  $\theta$ ), which confer unique receptor properties (Lüscher and Keller, 2004). For example, early in development GABA<sub>A</sub>Rs are composed largely of  $\alpha$ 3 subunits, which have a lower binding affinity for GABA (Böhme et al., 2004), and slower decay time (Bosman et al., 2002) than  $\alpha$ 1. Through the course of development GABA<sub>A</sub>Rs shift to incorporate more  $\alpha$ 1 subunits (Fritschy et al., 1994; Chen et al., 2001; Murphy et al., 2005) which results in a greater binding affinity and faster decay time (Bosman et

al., 2002). Pharmacological and gene studies have lead to the conclusion that development of  $\alpha 1$  in particular drives critical period plasticity: pre-critical period activation of GABA<sub>A</sub>Rs triggers a precocious critical period, but not in  $\alpha 1$  KO mice (Fagiolini et al., 2004). The role of  $\alpha 1$  in critical period plasticity is linked to its contribution to fast-spiking inhibition that shapes sensory input.  $\alpha 1$  is enriched at the proximal dendritic and perisomatic synapses formed by fast-spiking parvalbumin positive (PV+) inhibitory interneurons (Klausberger et al., 2002). The fast decay properties combined with the influential synaptic positioning allows  $\alpha 1$ -enriched synapses to regulate back-propagation of signals into dendrites (Tsubokawa and Ross, 1996). This function makes them ideal for controlling spike time dependent plasticity, which depends on the precise timing of inhibition (Dan and Poo, 2004). During the critical period, the increased perisomatic inhibition due to a developmental rise in  $\alpha 1$  may make spike time dependent plasticity more discerning between inputs, thereby facilitating the depression of deprived-eye synapses.

Early in development, activation of GABA<sub>A</sub>Rs is depolarizing (Cherubini et al., 1991). This is because immature neurons lack an efficient mechanisms for chloride extrusion, and since GABA<sub>A</sub>Rs are not direction selective, chloride moves along its gradient and out of the neuron (Ben-Ari, 2002). The early maturation of potassium chloride cotransporter 2 (KCC2) expression, the main chloride extruder, renders GABA hyperpolarizing (Rivera et al., 1999; Ben-Ari, 2002). Thus KCC2 regulates the efficacy of GABAergic inhibition, and its development is necessary for the maturation of fast-spiking inhibition that shapes critical period plasticity.

An important integrator of excitatory and inhibitory signaling is the cannabinoid receptor type 1 (CB1R) receptor and the endocannabinoid system, which shapes activity-dependent plasticity through retrograde signaling, and itself is subject to experience-dependent plasticity

(Xu and Chen, 2005). One important function of CB1R is to reduce GABAergic transmission by providing negative feedback inhibition at GABAergic synapses (Hájos and Freund, 2002). In addition, normal maturation of GABAergic transmission requires activation of CB1R during the critical period (Jiang et al., 2010).

### **E/I balance**

Hebbian and homeostatic plasticity underlie much of the phenomena in ocular dominance shifts; however those forms of plasticity are present throughout the lifespan, raising the question of what makes critical period plasticity unique? One hypothesis is that the effect of these mechanisms depends the E/I balance, which reaches an optimal balance during the critical period (Hensch, 2004; Hensch and Fagiolini, 2005; Levelt and Hübener, 2012). Support for this hypothesis comes from the many studies that alter the E/I balance to enhance or abolish critical period plasticity. For example, the developmental increase of GABA is necessary for critical period onset: reducing GABA by knocking out one of its synthesis enzymes (glutamate decarboxylase 65 [GAD65]) prevents the normal critical period from occurring (Hensch et al., 1998). This can be rescued by activating GABA<sub>A</sub>Rs with the exogenous agonist diazepam (Fagiolini and Hensch, 2000). In addition, increasing GABAergic activity earlier in development through the application of diazepam (Fagiolini and Hensch, 2000) or speeding up the development of GABAergic synapses (Hanover et al., 1999; Huang et al., 1999), can trigger a precocious critical period. Thus the development of E/I synapses has a profound effect on the potential for critical period plasticity.

In this thesis, I studied the E/I balance using synaptic markers that provide an indication of the quantity and quality of E/I synapses. On the pre-synaptic side, the E/I balance was analyzed

through the expression of vesicular glutamate transporter 1 (VGluT1) and the vesicular GABA transporter (VGAT). On the post-synaptic side I measured the excitatory and inhibitory receptor scaffolding proteins, post-synaptic density protein 95 (PSD-95) and gephyrin, respectively, because interactions between PSD-95 and gephyrin regulate the number of excitatory and inhibitory synapses, thus affecting the physiological E/I balance (Prange et al., 2004; Lardi-Studler et al., 2007; Keith and El-Husseini, 2008).

## 1.3 Reinstating Plasticity in Adults

### Critical period vs. adult plasticity

Synapses remain dynamic even after developmental critical periods, as organisms continue to develop, acquire, and modify behaviors. Thus, some degree of synaptic plasticity must persist to permit adaptation and refinement of neural networks across the lifespan. In V1, ocular dominance plasticity has been observed in adult mice (Sawtell et al., 2003), cats (Jones et al., 1984), and monkeys (Blakemore et al., 1978). However, there are important differences in quantity and quality of plasticity present during the critical period and adulthood. First, ocular dominance plasticity in adults requires longer periods of deprivation (Sato and Stryker, 2008). Second, the magnitude of the shift is smaller (Sato and Stryker, 2008). Third, the shift is less permanent (Prusky and Douglas, 2003; Pham et al., 2004). Fourth, the underlying physiological changes are different: during the critical period, MD causes a rapid loss of deprived-eye responses followed by a delayed increase in non-deprived-eye responses (Frenkel and Bear, 2004; Mrsic-Flogel et al., 2007), whereas adults primarily experience the delayed increase in non-deprived-eye responses (Sawtell et al., 2003; Hofer et al., 2006). Fifth, ocular dominance plasticity in adults is not observed in experiments which use anesthetics that act on the GABAergic system (Fagiolini and Hensch, 2000; Pham et al., 2004; Sato and Stryker, 2008), suggesting that there are at least some unique synaptic mechanisms in adulthood. Thus, while adults naturally retain some plasticity, the quantity and quality differs significantly from the critical period.

## **Why reinstate critical period-like plasticity?**

A hallmark of neurological disorders is abnormal cortical networks. Rewiring of these networks is difficult in the adult cortex, partly due to the reduced synaptic plasticity. For this reason, many researchers have sought to reinstate critical period-like plasticity in adult animals. The demand for plasticity-based therapies is exemplified by amblyopia, one of the most common neurodevelopmental disorders, which affects 1-4% of the general population (Flom and Neumaier, 1966; Noorden, 1990; Eibschitz-Tsimhoni et al., 2000). The gold standard treatment for amblyopia, patching the non-amblyopic eye, is often unsuccessful: 15-50% of treated children do not achieve normal acuity and more than 60% of those who achieve normal acuity require re-treatment within 5 years (Birch, 2013). In addition, most diagnoses are delayed until the patient reaches school age (Wu and Hunter, 2006). Together the failure in treating some patients and the delay in treating others has resulted in a large population of older amblyopes (Attebo et al., 1998; Brown et al., 2009). Prognosis for these older patients is poor because they lack the plasticity necessary to repair the abnormal networks that were set during the critical period.

The therapeutic need for enhancing synaptic plasticity expands far beyond amblyopia. From neurodevelopmental disorders that are characterized by abnormal networks (like autism and down syndrome), to acute injuries like stroke, traumatic brain injury, and spinal cord injury. For these conditions, controlling synaptic plasticity may provide the flexibility necessary to repair damaged networks, or to optimize function with the structure that is left. Thus there is a great need to control and manipulate synaptic plasticity in adults.

## **Models of reinstating plasticity**

This need to control synaptic plasticity has fostered a hunt for interventions that reinstate the juvenile-like plasticity in the adult cortex. Many experimental manipulations used in animal models have been very successful. Among others, these included transplanting embryonic astrocytes into mature animals (Müller and Best, 1989), genetic and pharmacologic manipulation of GABAergic circuitry (Fagiolini and Hensch, 2000; Iwai et al., 2003; Fagiolini et al., 2004; Harauzov et al., 2010), degradation of extracellular matrix (Pizzorusso et al., 2002), genetic reduction of myelin-mediated inhibition of neurite outgrowth (McGee et al., 2005), infusion of histone deacetylase inhibitors (Putignano et al., 2007) and acetylcholinesterase inhibitor (Morishita et al., 2010), dark exposure (He et al., 2006), food restriction (Spolidoro et al., 2011), environmental enrichment (Sale et al., 2007), chronic administration of fluoxetine (Vetencourt et al., 2008) and D-serine (Yang et al., 2011).

That great success in animal models, however, has not translated to human application. The goal of this thesis is to help bridge human and animal findings. For that reason, I chose to study 2 treatments that would be amenable to translation: chronic administration of fluoxetine and D-serine. Both fluoxetine and D-serine reinstate critical-period like plasticity in mature animals (Vetencourt et al., 2008; Yang et al., 2011), and although the mechanisms of action remain unknown, both have lead to improvements in human patients for a wide range of disorders that may benefit from enhanced plasticity, including depression (Montgomery et al., 1988; Emslie et al., 2002), schizophrenia (Goff et al., 1995; Tsai et al., 1998), stroke (Dam et al., 1996; Chollet et al., 2011), and traumatic brain injury (Horsfield et al., 2002).

## **Difficulty in translation**

Despite the extensive literature on the mechanisms of critical period plasticity, the numerous successes of reinstating critical period-like plasticity in animal models, and the great need to manipulate plasticity in humans, there has been a dearth of translation from bench to bedside. There are 2 main gaps in the literature that have made translation more difficult. First, the molecular consequences targeted by many of the animal interventions are poorly understood, making it difficult to know which mechanisms should be targeted in humans. Second, very little is known about normal development in humans, so it is difficult to determine whether the candidate mechanisms discovered in animals are even relevant to humans. My thesis addresses the dearth in translation by addressing these 2 gaps. I address the first gap by investigating how 2 interventions, fluoxetine and D-serine treatment, alter an important subset of the glutamatergic and GABAergic synaptic proteins that regulate critical period plasticity through the E/I balance, Hebbian plasticity, synaptic scaling, and metaplasticity. I address the second gap by studying the normal development of glutamatergic synaptic proteins in human V1 and comparing them to previously published GABAergic development.

## 1.4 Methodological Approach

My thesis aims to address the gap in translation between the rich animal literature on reinstating critical period-like plasticity, and human application. A fundamental problem for this gap is the ability to compare results between animals and humans. One way to address this is by using similar techniques in both species, but relatively few techniques are truly amenable to this goal. In animal models, especially rodents, there are a plethora of sophisticated anatomical, physiological, and molecular techniques that allow exquisite experimental control and rich data sets. Among others, these include *in-vivo* physiology, pharmacological, and genetic and optogenetic manipulation. Many of these techniques, however, are either too invasive or are too impractical to be used frequently in studies human brains.

For example, *in-vivo* single cell physiology provides unparalleled resolution for measuring the properties of individual neurons and their contribution to perception and behavior. In animals, *in-vivo* physiology was fundamental to the classic studies that defined the critical period for ocular dominance plasticity (Wiesel and Hubel, 1963), and functional architecture of V1 (Hubel and Wiesel, 1968). In humans *in-vivo* physiology has been used, for example, to study mirror-neurons (Mukamel et al., 2010), but is largely restricted to patients who are undergoing operations for pathological conditions such as epilepsy. Thus, it is too invasive for common use and is subject to significant sampling bias. Another important tool in animal literature is pharmacological manipulation of individual receptors or cell types. Application of GABA<sub>A</sub>R agonists revealed the role of that receptor in controlling the timing of the critical period (Fagiolini and Hensch, 2000; Hensch, 2005). In humans, pharmaceuticals have been used to separate the contribution of different receptors to receptive field properties (Meuwese et al.,

2013), but the use of drugs in humans is restricted in terms of the drug, dose, delivery method, and participants, thereby severely limiting its usefulness. Next, finer titration of cellular function was achieved through genetic manipulation in animals, revealing in greater detail the GABAergic circuits regulating the critical period (Hensch, 2005; Levelt and Hübener, 2012). Gene technology eventually evolved into optogenetics, which allowed control over the membrane potential of specific subcellular locations, in specific cell types, with millisecond precision (Fenno et al., 2011). This unprecedented spatial, temporal, and cell-type specificity accelerated our understanding of cellular and network contributions to plasticity and function. For example, optogenetic control of different inhibitory interneurons revealed their unique contributions to receptive field properties (Atallah et al., 2012; Lee et al., 2012; Wilson et al., 2012). In humans, genetic manipulation for experimentation is simply not ethical or technologically feasible (though CRISPER technology may soon change that (Doudna and Charpentier, 2014)). Thus, while genetic and optogenetic techniques may provide the most precise experimentation, some of their findings are not immediately translatable to humans.

On the other hand, several techniques, like fMRI and EEG, have been designed and optimized for human experiments. These have yielded incredible insight into the structure (Engel et al., 1994; 1997) and function of V1, and its correlates to perception and behavior (Polonsky et al., 2000; Haynes and Rees, 2005; Lunghi et al., 2015a). Yet despite significant advances in developing these techniques for animals (Mirsattari et al., 2007), they remain relatively uncommon in the animal literature. This is because these tools are designed to be non-invasive and are therefore often more complex, expensive, or less accessible ways to answer questions that may be addressed with cheaper, but more invasive, techniques in animals.

Taken together, many of the most powerful techniques used in animal and human research are not amenable to direct comparisons. This has made translation more difficult. One approach to addressing this is to study tissue samples ex-vivo using convenient molecular techniques. To this end, our lab has chosen western blotting as a rapid, reliable, and relatively inexpensive way to measure protein expression across species. I chose to study protein expression over gene expression because, while common tools allow large-scale analysis of mRNA that can yield great amounts of data, proteins ultimately define the characteristics and actions of a cell, and the correlation between gene and protein expression is often weak (Maier et al., 2009). I chose immunoblotting over anatomical techniques as it is an easier, faster, and more robust way to reprobe for larger sets of proteins. Another benefit of this approach is the availability of special preparations that isolate particular subcellular compartments. Much of this thesis focuses on pre and post-synaptic proteins, and for those I made use of the synaptosome preparation that isolates the pre- and post-synaptic membrane and adjacent intracellular compartments (Hollingsworth et al., 1985).

An important question for any technique used in translational research is: is it truly comparable between species? There are many important differences between the animal and human tissue used in our studies. While experimental animals were kept in standard conditions, human tissue comes from subjects with variable experiences and life history. In addition, processing of tissues was slightly different between species. Chief among these differences are the postmortem interval, which was uniformly short in animals (minutes), but variable in humans (hours); and the removal of blood via perfusion in animals versus special filtration for humans. Despite these differences, there were interesting similarities that make the results comparable.

For example, I was able to use the same primary and secondary antibodies, in similar concentrations on both species. In addition, the synaptosome preparation yielded similar enrichment in both species. Together these suggest that our immunoblotting is comparable despite differences in species, experimental control, and tissue processing.

As with all techniques, however, this approach has limitations. These include the lack of cell-type information and laminar and subcellular localization. In addition, functional information is inferred from protein expression rather than direct observation. Still, our approach is an important step in the translation process, and will guide future studies that use less accessible but more powerful techniques.

## Preamble for Chapter 2

In chapter 2, I studied the effects of fluoxetine on glutamatergic and GABAergic synaptic proteins. Four weeks of fluoxetine treatment reinstates critical-period like ocular dominance plasticity in adult rats (Vetencourt et al., 2008; 2011; Ruiz-Perera et al., 2015). It is still a bit of a mystery, however, how this SSRI effects a form plasticity that is heavily regulated by glutamatergic and GABAergic synapses (Hensch and Fagiolini, 2005; Levelt and Hübener, 2012). To answer this mystery, I investigated how fluoxetine alone, or combined with a change in experience (MD), altered glutamatergic and GABAergic synaptic proteins that are essential for experience-dependent plasticity.

Using western blotting, I began by quantifying markers of synaptic maturity to test if fluoxetine caused a juvenile-like shift. On the glutamatergic side, immature “silent” synapses are dominated by NMDARs, and progressively accumulate AMPARs as they become more active and less plasticity (Isaac et al., 1997; Huang et al., 2015). On the GABAergic side, the GABA<sub>A</sub>R subunit composition changes during development from more  $\alpha 3$  to more  $\alpha 1$  (Fritschy et al., 1994; Chen et al., 2001; Murphy et al., 2005). I was surprised to find that fluoxetine had no effect of on the AMPAR:NMDAR index, and that it shifted the GABA<sub>A</sub>R index towards the mature,  $\alpha 1$ , subunit. Next, I studied markers of metaplasticity. Juvenile synapses are dominated by NMDARs containing the GluN2B subunit, but experience promotes a shift to GluN2A (Quinlan et al., 1999), which raises the threshold for synaptic modification (Yashiro and Philpot, 2008). Again I was surprised to find that fluoxetine shifted the balance in favor of the mature, GluN2A, subunit. Lastly, I turned to the E/I balance because the maturation of inhibition regulates the critical period (Hensch, 2005), and many plasticity-enhancing treatments are aimed

at relieving inhibition (Bavelier et al., 2010). I measured the E/I balance using pre- and post-synaptic markers of excitatory and inhibitory synapses, and found that fluoxetine did not shift either measure in favor of excitation.

I hypothesized that fluoxetine might reinstate an immature synaptic composition, but instead I found that fluoxetine favored expression of mature subunits. Interestingly, the shift to both mature glutamatergic and GABAergic receptor subunits supports faster receptor kinetics, which may enhance spike time dependent plasticity. Thus, it is possible that fluoxetine enhances mature forms of plasticity rather than reinstating juvenile ones. Ruling out the reinstatement of juvenile-like plasticity mechanisms, however, will require studying a wider set of proteins associated with critical period plasticity. In addition, future studies using electrophysiological, anatomical, cell-type identification, and subcellular localization techniques will be needed to confirm the types of plasticity and the specific circuits that hold the keys to enhancing plasticity in the adult cortex.

### **Preamble for Chapter 3**

A puzzling finding from Chapter 2 was that while fluoxetine consistently normalized MD-driven changes in expression of glutamatergic proteins and markers of the E/I balance, it did not shift any of the proteins or indices in favor of a juvenile-like state (Beshara et al., 2015). While our results suggest that fluoxetine is neuroprotective, the question remains: how does this SSRI affect plasticity that depends on glutamatergic and GABAergic synapses? To explore this I extended the analysis to proteins involved in regulation of glutamatergic and GABAergic transmission, including those that regulate AMPAR trafficking (GluA2, pGluA2, GRIP1, PICK1), inhibitory post-synaptic potentials (KCC2), heterosynaptic integration of E/I transmission (CB1R), and ocular dominance plasticity (ubiquitin protein ligase E3A, Ube3A).

Consistent with the glutamatergic gain reported in chapter 2, I found that MD increased the expression of all 4 AMPAR-associated proteins, and decreased expression of KCC2. In each case, fluoxetine combined with MD reversed the changes caused by MD alone. While these findings provide more evidence that fluoxetine is neuroprotective, none of them are consistent with a shift to juvenile-like synaptic environment. In contrast, the change in Ube3A expression may reflect a juvenile-like mechanism. Ube3A expression is necessary for critical period-like ocular dominance plasticity (Yashiro et al., 2009; Sato and Stryker, 2010), is highly expressed in juvenile animals, and is significantly lost in aging animals (Williams et al., 2010). I found that MD halved the expression Ube3A, but fluoxetine combined with MD increased it by ~50%. Interestingly the increase in Ube3A only occurred when fluoxetine was combined with MD, suggesting that the change requires a combination of drug and experience. These findings answer an important question about how fluoxetine reinstates plasticity in the adult cortex, and helps to

connect research on plasticity with that on neurodevelopment disorders associated with Ube3A expression, like Angelman syndrome.

## Preamble for Chapter 4

Fluoxetine is an FDA approved antidepressant which may enhance plasticity and neuroprotection, but it has undesirable physiological and psychological side effects (Riddle et al., 2009). There are, however, alternatives that enhance plasticity with potentially fewer side effects. One of these is D-serine, the main endogenous co-agonist of the NMDAR (Mothet et al., 2000; Shleper et al., 2005).

D-serine is released by astrocytes (Mothet et al., 2005; Henneberger et al., 2012) to regulate synaptic plasticity (Panatier et al., 2006), and is necessary for induction of LTP (Henneberger et al., 2010). During the critical period for ocular dominance plasticity, D-serine is required for LTP at both excitatory and inhibitory synapses (Meunier et al., 2016). Like fluoxetine, exogenous D-serine facilitates critical period-like plasticity and recovery of visual acuity in adult animals (Yang et al., 2011), and has antidepressant effects (Otte et al., 2013). Together this lead us to believe that D-serine may have similar effects as fluoxetine on the proteins that regulate experience-dependent plasticity. In chapter 4, I explore this using an experimental design similar to that used in chapters 2 and 3.

I compared the expression of post-synaptic markers of the E/I balance, PSD-95 and gephyrin, on animals treated with either MD, D-serine, or D-serine combined with MD. The results were strikingly similar to fluoxetine: D-serine alone did not alter the expression or balance of PSD-95 or gephyrin, but combining D-serine with MD normalized the MD-driven shift in favor of excitatory synapses. These results suggest that D-serine may share a common mechanisms with fluoxetine. Future studies will need to compare the full range of proteins tested

on fluoxetine to determine how similar their mechanisms are, and especially to test whether D-serine affects Ube3A, similarly to fluoxetine.

## Preamble for Chapter 5

There have been very few human applications of the knowledge gained from animal studies on enhancing plasticity. For this reason, translational research is necessary. The first part to translating experimental results is to establish the normal baseline across both species. In terms of the glutamatergic and GABAergic synaptic proteins studied in this thesis, development in animals is relatively well known (Carmignoto and Vicini, 1992; Laurie et al., 1992; Monyer et al., 1994; Roberts and Ramoa, 1999; Beston et al., 2010; Murphy et al., 2012; Huang et al., 2015; Pinto et al., 2015), whereas development in human V1 is much less studied (Murphy et al., 2005; Pinto et al., 2010; 2015).

Early anatomical studies suggested that the anatomy of human V1 matured within the first few years of life (Huttenlocher et al., 1982; Burkhalter, 1993; Burkhalter et al., 1993), but behavioral studies revealed that functional development of vision continued into adulthood (Kovács et al., 1999; Lewis and Maurer, 2005; Germine et al., 2011). This discrepancy is usually attributed to later development of extrastriate areas, but recent evidence suggests that there is considerable development in V1 across the lifespan (Pinto et al., 2010; Williams et al., 2010; Pinto et al., 2015; Siu et al., 2015). For example, our lab found that development of some GABAergic synaptic proteins continues well into adulthood (Pinto et al., 2015). In V1, however, over 80% of synapses are glutamatergic (Beaulieu et al., 1992), thus it remains unknown whether the majority of V1 synapses mature during childhood, or if they develop later into adulthood.

In Chapter 5, I address this question by studying the development of 5 glutamatergic synaptic proteins that have crucial roles in both plasticity and perception (PSD-95, GluA2, GluN1, GluN2A, GluN2B). I found that maturation of some glutamatergic proteins is as long as

GABAergic proteins, with the GluN2A:GluN2B index continuing to increase into the fourth decade, before significantly decreasing in old adulthood. I also found 5 stages of development that map onto life-long changes in human visual perception. These results suggest that the apparent discrepancy between the development of the anatomical structure of V1 and development of vision may be explained by life-long synaptic changes. These findings also suggest that fluoxetine treatment may be beneficial in old adulthood, when there is a substantial loss of GluN2A.

**Chapter 2. Effects of fluoxetine and visual experience on  
glutamatergic and GABAergic synaptic proteins in adult rat  
visual cortex**

## Abstract

Fluoxetine has emerged as a novel treatment for persistent amblyopia because in adult animals it reinstates critical period-like ocular dominance plasticity and promotes recovery of visual acuity. Translation of these results from animal models to the clinic, however, has been challenging because of the lack of understanding of how this SSRI affects glutamatergic and GABAergic synaptic mechanisms that are essential for experience-dependent plasticity. An appealing hypothesis is that fluoxetine recreates a CP-like state by shifting synaptic mechanisms to be more juvenile. To test this we studied the effect of fluoxetine treatment in adult rats, alone or in combination with visual deprivation (monocular deprivation, MD), on a set of highly conserved pre- and post-synaptic proteins (Synapsin, Synaptophysin, VGluT1, VGAT, PSD-95, gephyrin, GluN1, GluA2, GluN2B, GluN2A, GABA<sub>A</sub>α1, GABA<sub>A</sub>α3). We did not find evidence that fluoxetine shifted the protein amounts or balances to a CP-like state. Instead, it drove the balances in favor of the more mature subunits (GluN2A, GABA<sub>A</sub>α1). In addition, when fluoxetine was paired with MD it created a neuroprotective-like environment by normalizing the glutamatergic gain found in adult MDs. Together our results suggest that fluoxetine treatment creates a novel synaptic environment dominated by GluN2A- and GABA<sub>A</sub>α1-dependent plasticity.

## Significance

Patching therapy is the most common treatment for children with amblyopia. For many, the acuity recovered during patching is lost when the treatment stops leaving the child with persistent amblyopia. Fluoxetine has emerged as an interesting treatment option because it reinstates critical period-like ocular dominance plasticity and promotes acuity recovery in adult animals. It remains unclear, however, how this selective serotonin reuptake inhibitor affects visual cortex plasticity, which relies heavily on glutamatergic and GABAergic synapses. Here we report the effects of fluoxetine and visual manipulation on the visual cortex of adult rats. Surprisingly we found that fluoxetine did not reinstate a critical period-like state, but rather created a novel synaptic environment that favors mature NMDA and GABA<sub>A</sub> receptor subunits.

## 2.1 Introduction

Amblyopia is commonly treated with patching but in some cases the recovered acuity is lost when patching stops, leaving the child with persistent amblyopia (Birch, 2013). A variety of therapeutics have been proposed to treat persistent amblyopia in adolescents or young adults. Fluoxetine has emerged as a treatment option because it reinstates critical period-like (CP) ocular dominance plasticity and promotes acuity recovery in adult rats (Vetencourt et al., 2008). It is unclear, however, what effects this selective serotonin reuptake inhibitor (SSRI) has on visual cortex (V1) plasticity that relies heavily on maturation of glutamatergic and GABAergic synapses (Levelt and Hübener, 2012). An appealing idea is that fluoxetine shifts the synaptic environment in V1 to a CP-like state that supports heightened experience-dependent plasticity.

During the CP, experience-dependent plasticity is driven by visually evoked responses that depend upon maturation of pre- and post-synaptic mechanisms. Development of pre-synaptic vesicle cycling proteins (e.g. Synapsin, Synaptophysin) and transporters (e.g. VGluT1, VGAT) are necessary for reliable neurotransmitter release (Hopf et al., 2002; Conti et al., 2004; Wojcik et al., 2004; 2006) that drives strong visually evoked activity. Also, shifts in the excitation:inhibition (E/I) balance set up the physiological environment needed for heightened plasticity, triggering the CP (Hensch, 2005; Hensch and Fagiolini, 2005). That E/I balance is mediated by post-synaptic scaffolding proteins PSD-95 and gephyrin that regulate the number of excitatory and inhibitory synapses (Prange et al., 2004; Lardi-Studler et al., 2007; Keith and El-Husseini, 2008). Furthermore, the start of the CP in rat and human V1 coincides with a rapid switch from much more gephyrin to an equal balance with PSD-95 (Pinto et al., 2013; 2015).

A host of glutamatergic and GABAergic receptor mechanisms affect the threshold for CP plasticity. These include addition of AMPA receptors (AMPA) that end the period of NMDA receptor (NMDAR)-dominated silent synapses (Huang et al., 2015) and add the fast component to excitatory post-synaptic currents (EPSCs) (Kleppe and Robinson, 1999). Furthermore, the addition of GluN2A containing NMDARs (Flint et al., 1997; Stocca and Vicini, 1998) speeds up receptor kinetics (Cull-Candy et al., 2001) and affects signalling pathways such as GluN2B activation of Ras/ERK or alpha calcium-calmodulin kinase II and mTOR pathways (Kim et al., 2005; Wang et al., 2011). The shift to GluN2A also affects functional maturation by mediating sharpening of orientation selectivity (Fagiolini et al., 2003). Finally, activation of GABA<sub>A</sub> receptors (GABA<sub>A</sub>R) triggers the start of the CP (Hensch, 2005) and GABA<sub>A</sub>α1 subunits in particular regulate patterns of activity needed for development of ocular dominance (Fagiolini et al., 2004).

Despite our understanding of the influence of fluoxetine treatment on adult plasticity, there is little evidence to identify *how* fluoxetine affects the expression profile of synaptic mechanism that are critical in the initiation of CP plasticity. To address this, we treated animals with fluoxetine and quantified a set of glutamatergic and GABAergic synaptic proteins to assess if they changed to a CP-like state. We then determined the effects of monocular deprivation (MD) alone, or in combination with fluoxetine on these synaptic proteins. Surprisingly, fluoxetine alone shifted both NMDAR and GABA<sub>A</sub>R subunits to a more mature composition. Furthermore, when fluoxetine was combined with MD, the treatment normalized the increase in glutamatergic proteins found in adult MD rats. These results show that fluoxetine treatment does not recreate a CP-like synaptic environment but instead shifts plasticity mechanisms to a new state.

## **2.2 Materials and Methods**

### **Animals and surgical procedures**

We studied changes in expression of 12 synaptic proteins in V1 of young adult male Long Evans rats (P98). Rats were individually housed in plexiglas cages with food and water ad libitum, and a 12:12 light/dark cycle. Animals were randomly assigned to 1 of 4 groups: normally reared to P98 (n=6), animals given 4 weeks of fluoxetine (from P70-P98) (0.2mg/ml of drinking water) (n=8), animals monocularly deprived (MDed) (P91-P98) (n=6), or animals that received both fluoxetine (P70-P98) and MD (P91-P98)(n=8). Eyelids were closed by trimming the lid margins and suturing them together with 5-0 vicryl using aseptic surgical techniques. The surgery was done using gaseous anesthetic [isoflurane (1.5–5%) in oxygen] for induction and maintenance of anesthesia. Eyelids were inspected daily for openings. All experimental procedures were approved by the [Authors' University] Animal Research Ethics Board.

### **Tissue collection**

Animals were euthanized (sodium pentobarbital, 150 mg/kg), and transcardially perfused with cold 0.1 M phosphate buffered saline (PBS) (4°C; 4-5 ml/min) until circulating fluid was clear. The brain was quickly removed from the skull and immersed in cold PBS. Bilateral samples of V1 including monocular and binocular regions, quickly frozen on dry ice, and stored at -80°C.

### **Sample preparation**

To study high-abundance vesicle cycling proteins and receptor scaffolding proteins (Synapsin, Synaptophysin, PSD-95, gephyrin) we prepared homogenate samples. The frozen tissue was added to cold homogenization buffer (1 ml buffer:50 mg tissue – 0.5 mM

dithiothreitol (DTT), 1mM EDTA, 2 mM EGTA, 10 mM HEPES, 10 mg/L leupeptin, 100 nM microcystin, 0.1 mM PMSF, 50 mg/L soybean trypsin inhibitor) and homogenized in a glass-glass Dounce homogenizer (Kontes, Vineland, NJ, USA). The sample was then combined with 10% sodium-dodecylsulfate (SDS). To study lower abundance receptor subunits (GluA2, GluN1, GluN2A, GluN2B, GABA<sub>A</sub>α1, GABA<sub>A</sub>α3) and transporters (VGluT1, VGAT) we enriched the samples following a synaptoneurosome protocol (Hollingsworth et al., 1985; Quinlan et al., 1999; Murphy et al., 2014). Following the homogenization step each sample was passed through a 5µm pore hydrophilic mesh filter (Millipore, Bedford, MA), then centrifuged at 1,000 g for 10 min. Both the synaptic pellet and the whole homogenate samples were resuspended in boiling 1% SDS. Protein concentrations for each sample was determined using the bicinchoninic acid assay guidelines (Pierce, Rockford, IL, USA) and final concentrations were adjusted to 1mg/ml using Laemmli sample buffer. A control sample was made by combining a small amount of each of the 28 samples.

### **Immunoblotting**

Samples (25 µg) were resolved on 4-20% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) gels (Precise Protein Gels, Pierce Biotechnology Inc., Rockford, IL, USA) and transferred to polyvinylidene difluoride (PVDF-FL) membranes (Millipore, Billerica, MA, USA). Blots were incubated with blocking buffer (Odyssey Blocking Buffer 1:1 with PBS) for 1 hour (LI-COR Biosciences; Lincoln, NE, USA), then with primary antibody overnight at 4°C using the following concentrations: GAPDH, 1:4000 (Imgenex, San Diego, CA); Synapsin 1, 1:8000 (Invitrogen, Carlsbad, CA); Synaptophysin, 1:2000 (Sigma-Aldrich, St. Louis, MO); PSD-95, 1:32000 (Millipore, Billerica, MA); gephyrin, 1:2000 (Millipore, Billerica, MA);

VGluT1 , 1:2000 (Synaptic Systems, Goettingen, Germany); VGAT, 1:2000 (Synaptic Systems, Goettingen, Germany); GluA2, 1:2000 (Invitrogen, Carlsbad, CA); GluN1, 1:8000 (Chemicon International, Temecula, CA); GluN2B, 1:2000 (Chemicon International, Temecula, CA); GluN2A, 1:2000 (PhosphoSolutions, Aurora, CO); GABA<sub>A</sub>α1 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); GABA<sub>A</sub>α3 1:2000 (Chemicon International, Temecula, CA, USA). The blots were washed with PBS containing 0.05% Tween (Sigma, St. Louis, MO, USA) (PBS-T; 3 × 10 min), incubated for 1 hour at room temperature with the appropriate IRDye labeled secondary antibody, (Anti-Mouse, 1:8000, Anti-Rabbit, 1:10,000; LI-COR Biosciences; Lincoln, NE, USA), and washed in PBS-T (3 × 10 min). The blots were visualized using an Odyssey scanner (LI-COR Biosciences; Lincoln, NE, USA). The combination of IRDye secondary antibodies and Odyssey scanner provides a wide linear dynamic range so that both strong and weak bands could be accurately quantified on the same blot. Blots were stripped and re-probed with additional antibodies (Blot Restore Membrane Rejuvenation kit, Millipore, Billerica, MA, USA).

### **Analyses**

To analyze the bands, we scanned the blots (Odyssey infrared scanner) and quantified the bands using densitometry (LI-COR Odyssey Software version 3.0; LI-COR Biosciences; Lincoln, NE, USA). Density profiles were determined by performing a subtraction of the background, integrating the pixel intensity across the area of the band, and dividing the intensity by the width of the band to control for variations in lane width. Protein loading was checked using GAPDH as a control for sample concentration and volume loaded in each well. Each band was normalized to the average for the set of blots run at the same time and the control sample on the individual blot.

To quantify the relationship between functional pairs of proteins we calculated contrast indices that are commonly used in signal processing to determine the quality of the signal. AMPAR-NMDAR Index --  $(\text{GluA2}-\text{GluN1})/(\text{GluA2}+\text{GluN1})$ . NMDAR Index --  $(\text{GluN2A}-\text{GluN2B})/(\text{GluN2B}+\text{GluN2A})$ . GABA<sub>A</sub>R Index --  $(\text{GABA}_{\text{A}\alpha 1}-\text{GABA}_{\text{A}\alpha 3})/(\text{GABA}_{\text{A}\alpha 1}+\text{GABA}_{\text{A}\alpha 3})$ . Pre-Synaptic E/I Index --  $(\text{VGluT1}-\text{VGAT})/(\text{VGluT1}+\text{VGAT})$ . Post-Synaptic E/I Index --  $(\text{PSD-95}-\text{gephyrin})/(\text{PSD-95}+\text{gephyrin})$ .

To compare levels of protein expression among the groups we made histograms showing the mean and standard error of the mean for each group. All results were plotted normalized to the normal young adult group. To make statistical comparisons between groups we used bootstrapping, a modern resampling statistical method that provides robust estimates of standard error and confidence intervals, that is especially useful for animal studies such as ours constrained to smaller sample sizes. We used R to simulate a normally distributed data set with 1,000,000 points and the same mean and standard deviation as the group being compared. To determine differences between groups we compared the simulation data set with average protein expression with each of the other groups. We ran a Monte Carlo simulation which randomly samples from the simulation data set N time, where N was the number of animals in each of the other groups (N=6 or 8). This simulation was repeated 10,000 times to create the normal distribution expected for the N sample sizes. We calculated confidence intervals for the simulated distribution and compared those with the observed means for the other groups. Groups were identified as significantly different (e.g.  $p < 0.05$ ) when the observed average expression was either greater or less than 95% of the simulated distribution and thus outside its confidence interval.

## **Image Manipulation**

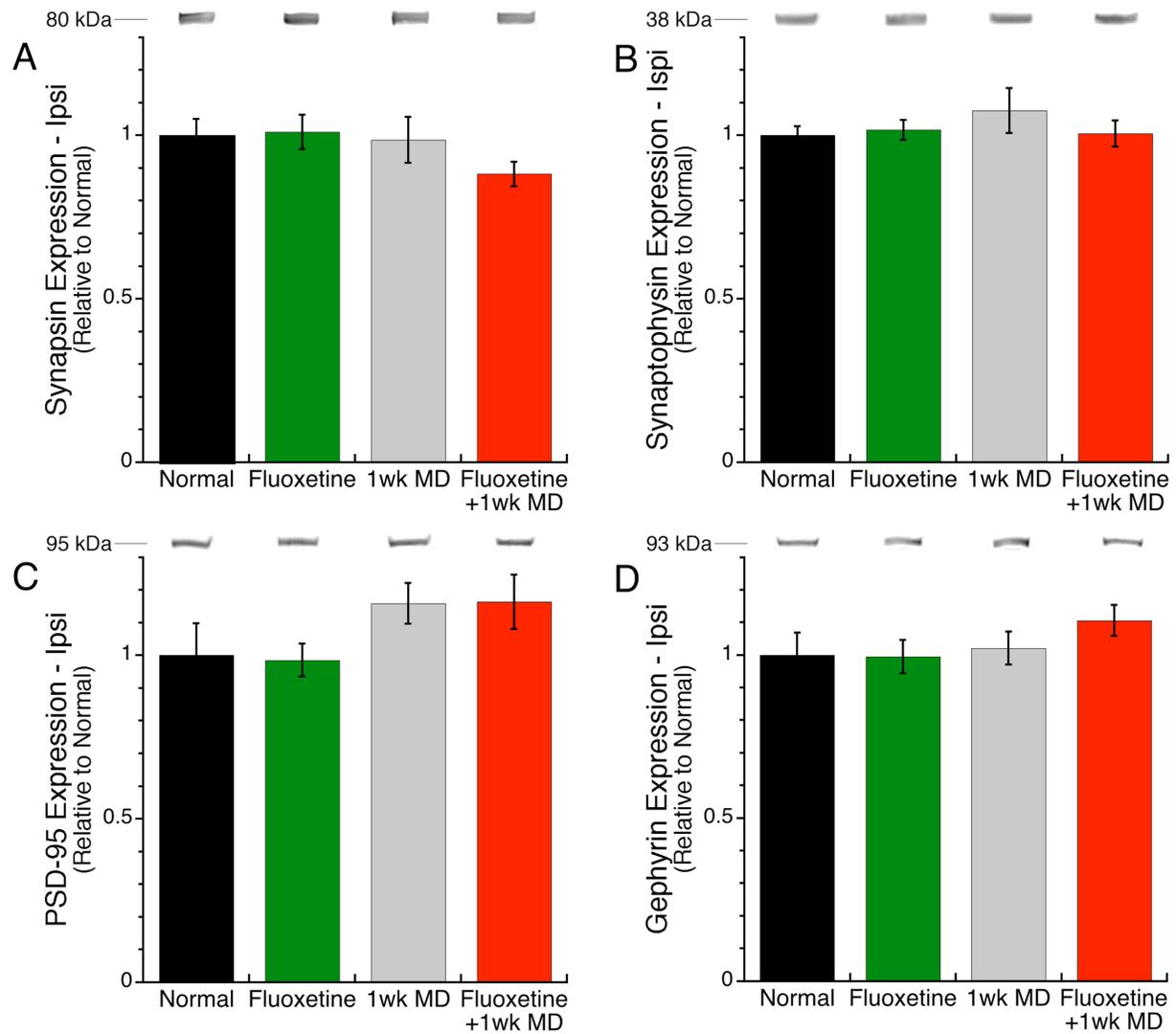
Bands are representative samples taken from different parts of the same gel or different gels. Horizontal and vertical transformations were uniformly applied to size bands appropriately for each figure. A linear adjustment layer was applied uniformly to all bands of each protein, preserving the relative intensities between groups.

## 2.3 Results

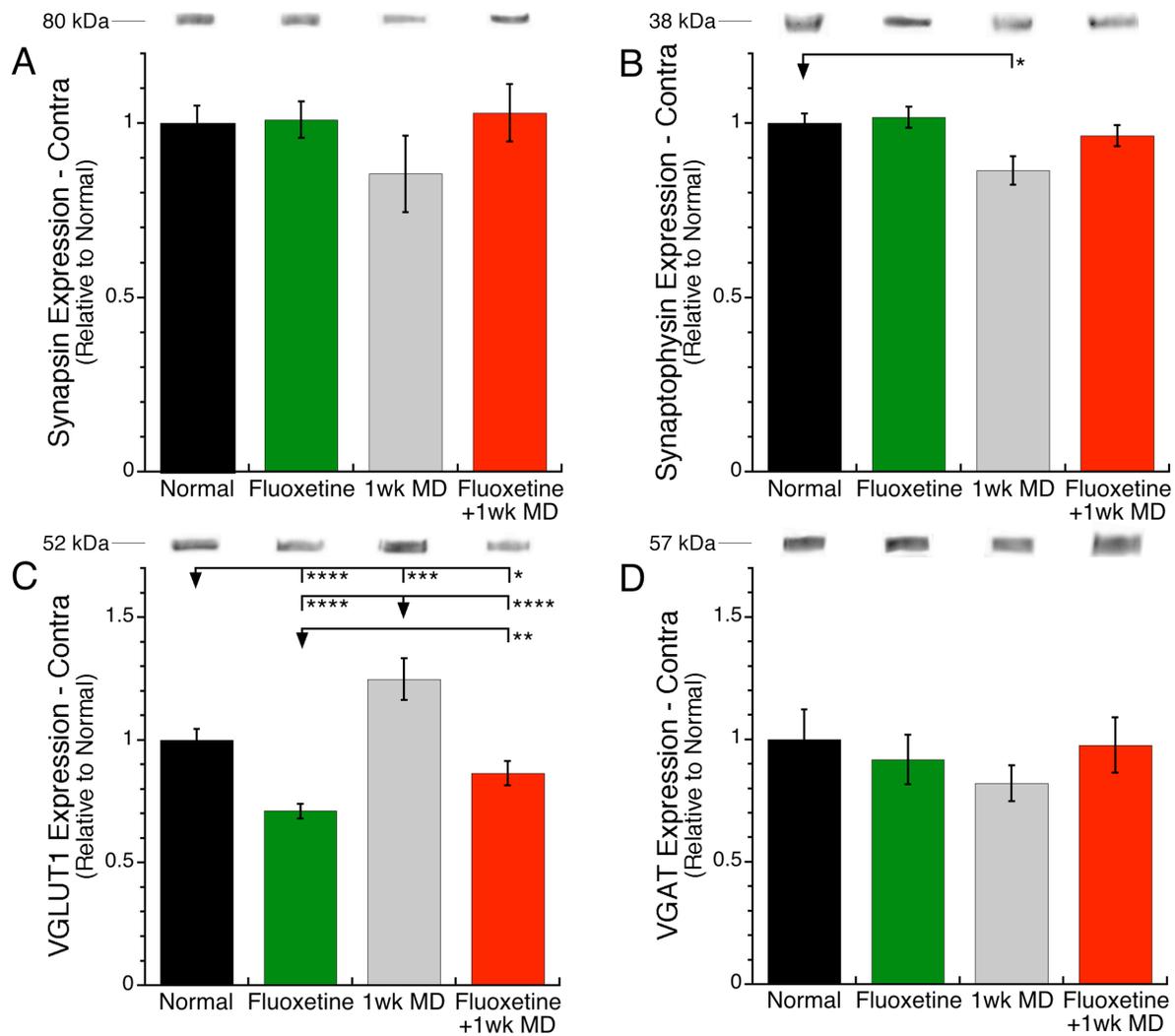
We verified that GAPDH was an appropriate loading control by comparing expression of it among the 4 groups. We found no significant differences from normals demonstrating that GAPDH is an appropriate loading control. We began by examining expression of Synapsin, Synaptophysin, PSD-95 and gephyrin in V1 ipsilateral to the deprived eye. MD effects are much weaker in the ipsilateral hemisphere (Sawtell et al., 2003) and we did not find any significant differences among the groups for those synaptic proteins (Fig. 1). Thus, all of the following analyses are for V1 contralateral to the deprived eye.

### Pre-Synaptic changes

We analyzed how fluoxetine changed the pre-synaptic environment by quantifying a set of proteins involved in cycling, transport and loading of glutamatergic and GABAergic vesicles. We compared expression of Synapsin, Synaptophysin, VGluT1 and VGAT in V1 of normally reared adult rats, rats given 1 month of fluoxetine, 1 week of MD, or the combination of fluoxetine and MD. We found no differences among the groups for Synapsin (n.s.) (Fig. 2A) or the GABAergic transporter VGAT (n.s.) (Fig. 2D) and only a modest loss of Synaptophysin for the MDed animals (-13% SEM 4.1%,  $p < 0.05$ ) (Fig. 2B). The glutamate transporter VGluT1, however, had more changes. MDed animals had an increase in VGluT1 (+25% SEM 8.4%,  $p < 0.001$ ) while both groups of fluoxetine treated animals had less VGluT1 than normal (fluoxetine alone -29% SEM 3.0%,  $p < 0.0001$ ; fluoxetine+MD -13% SEM 4.9%,  $p < 0.05$ ) (Fig. 2C).



**Figure 1.** Pre- and post-synaptic proteins in ipsilateral V1. In V1 ipsilateral to the deprived eye, there was no effect of experimental condition on the expression of Synapsin (A), Synaptophysin (B), PSD-95 (C), or Gephyrin (D) (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ ).

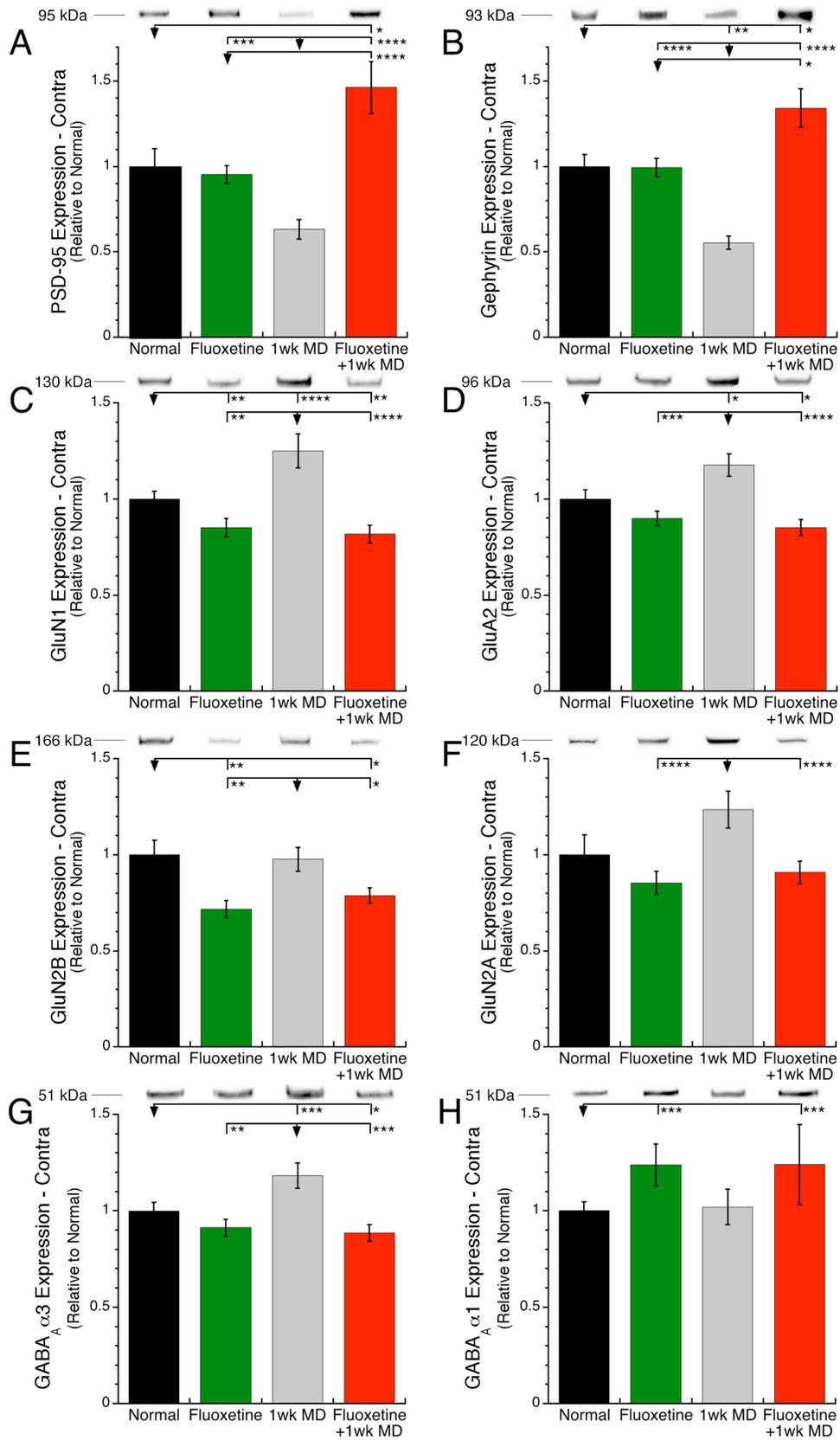


**Figure 2.** Presynaptic vesicle cycling and transporter proteins. In contralateral V1, Synapsin (A) was not affected by experimental condition. For Synaptophysin (B) fluoxetine alone had no effect, MD alone caused a loss of expression, but combining fluoxetine with MD prevented the MD-induced loss. For VGLUT1 (C) fluoxetine alone or with MD caused a loss of expression, but MD alone increased expression. VGAT (D) was not affected by experimental condition. (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ ).

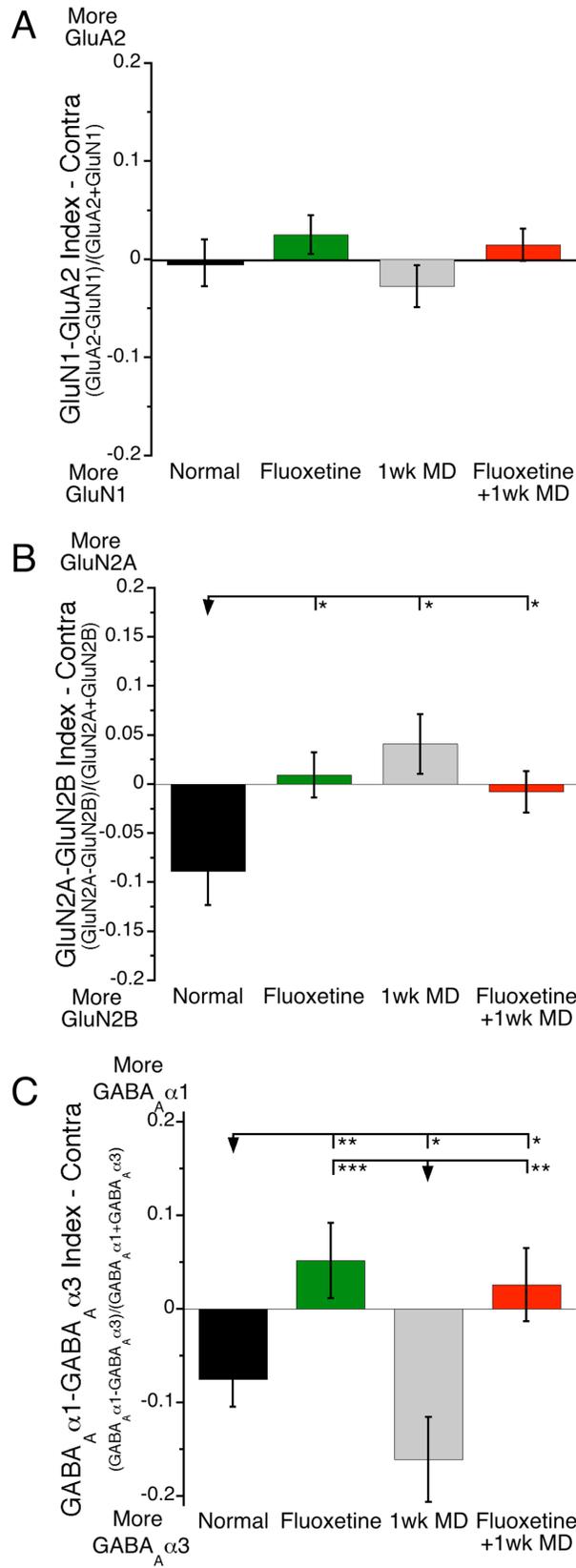
### Post-Synaptic changes

Next we examined how fluoxetine changed the expression of a set of post-synaptic scaffolding proteins and receptor subunits for glutamatergic and GABAergic receptors. Changes among the groups were very similar for PSD-95 and gephyrin. Fluoxetine alone did not change the level of expression relative to normal animals, but MD caused loss of expression (PSD-95 -37% SEM 5.6%,  $p \sim 0.06$ ; gephyrin -45% SEM 4.0%,  $p < 0.01$ ) and fluoxetine combined with MD increased expression (PSD-95 +46% SEM 15%,  $p < 0.05$ ; gephyrin +34% SEM 11%,  $p < 0.05$ ) (Fig. 3A-B).

The pattern of changes for the receptor subunits was almost opposite to the scaffolding proteins. For the glutamatergic receptor subunits (GluN1, GluA2, GluN2B, GluN2A) fluoxetine alone caused losses for GluN1 and GluN2B (GluN1 -15% SEM 4.8%,  $p < 0.01$ ; GluN2B -28% SEM 4.5 %,  $p < 0.01$ ) and when combined with MD caused a loss of GluA2, as well as losses for GluN1 and GluN2B (GluA2 -15% SEM 4.2%,  $p < 0.0001$ ; GluN1 -18% SEM 4.5%,  $p < 0.0001$ ; GluN2B -21% SEM 4 %,  $p < 0.05$ ) (Fig. 3C-F). But MD alone caused either an increase (GluN1 +25% SEM 8.8%,  $p < 0.0001$ ; GluA2 +18% SEM 5.9%,  $p < 0.05$ ) or no significant change from normal (GluN2B, GluN2A, n.s.). Thus, MD alone caused gains for these glutamatergic subunits that were reduced when MD was combined with fluoxetine. MD also increased GABA<sub>A</sub>α3 (+18% SEM 6.6%,  $p < 0.001$ ) (Fig. 3G) but did not change GABA<sub>A</sub>α1 (Fig. 3H). In contrast, GABA<sub>A</sub>α1 was increased in both fluoxetine treated groups (fluoxetine alone +24% SEM 11%,  $p < 0.001$ ; fluoxetine+MD +24% SEM 20%,  $p < 0.001$ ) (Fig. 3H).



**Figure 3. Postsynaptic receptor scaffolding proteins and subunits.** In contralateral V1, PSD-95 (A) and Gephyrin (B) had a similar pattern of changes: fluoxetine alone had no effect, MD alone caused a loss of expression, but combining fluoxetine with MD prevented the MD-induced loss and caused super-compensation above normal levels. GluN1 (C) was reduced by fluoxetine regardless of visual experience, while MD alone caused an increase. GluA2 (D) was unaffected by fluoxetine alone, MD caused an increase, but combining fluoxetine with MD caused a decrease. GluN2B (E) was reduced by fluoxetine regardless of visual experience, while MD had no effect. GluN2A (F) expression of each experimental group was not different from normal animals, but the MDed group had higher expression than either fluoxetine alone or fluoxetine combined with MD. GABAA $\alpha$ 3 (G) was unaffected by fluoxetine alone, MD caused an increase, but combining fluoxetine with MD prevented the MD-induced increase. GABAA $\alpha$ 1 (H) was increased by fluoxetine regardless of visual experience, while MD alone had no effect. (\*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001; \*\*\*\*p < 0.0001).



**Figure 4.** Postsynaptic receptor subunit balances. Neither fluoxetine, MD, nor fluoxetine combined with MD affected the relative abundance of GluN1-containing NMDARs and GluA2-containing AMPARs in contralateral V1 (A). Fluoxetine shifted the relative abundance of NMDAR subunits in favour of the more mature GluN2A subunit, regardless of visual experience. MD caused a shift in favour of the more immature GluN2B (B). Fluoxetine shifted the relative abundance of GABAAR subunits in favour of the more mature  $\alpha 1$  subunit, regardless of visual experience. MD caused a shift in favour of the more  $\alpha 3$  subunit (C). (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ ).

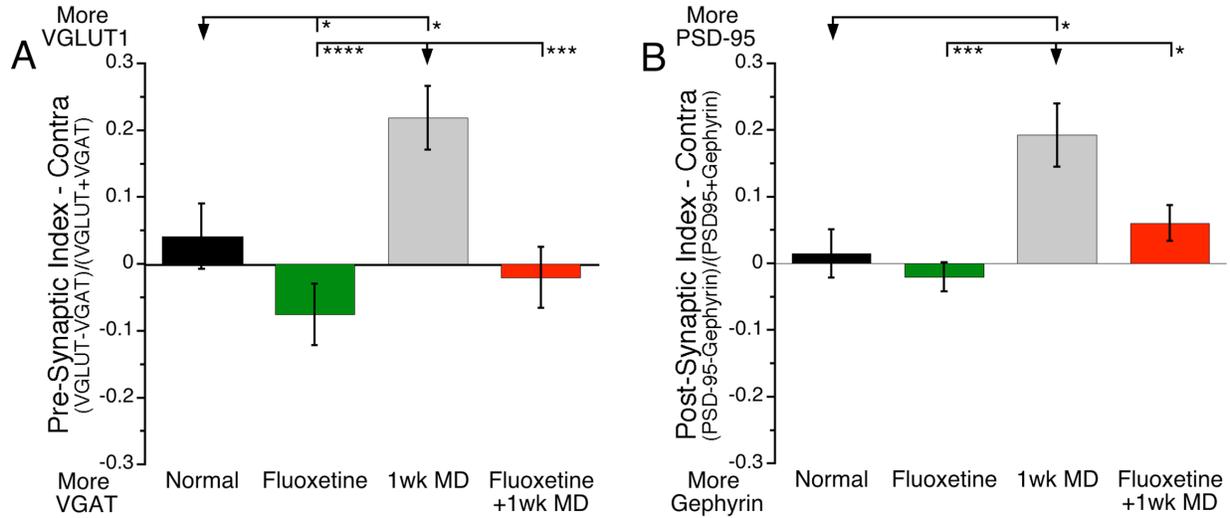
## **Receptor subunit balances**

During development there are a series of maturational shifts in expression of glutamatergic and GABAergic receptor subunits. One of the shifts is the change from NMDAR-dominated silent synapses to AMPAR activated synapses. We studied if fluoxetine created a CP-like state by shifting the GluA2:GluN1 balance in favour of GluN1 but found no changes from the normal adult balance (n.s.) (Fig. 4A). Different results were found when the GluN2A:GluN2B and GABA<sub>A</sub>α1: GABA<sub>A</sub>α3 balances were examined. During normal development there is an increase in GluN2A, shifting the balance from much more GluN2B to slightly in favour of GluN2B in young adult rats (Fig. 4B). But all of the experimental groups changed beyond that level towards even more GluN2A ( $p < 0.05$ ). There were differences, however, in what drove the changes in the GluN2A:GluN2B balance with the fluoxetine groups shift being caused by less GluN2B while the MD shift was caused by more GluN2A. The GABA<sub>A</sub>α1: GABA<sub>A</sub>α3 balance revealed another dissociation among the experimental groups (Fig. 4C). Here the MD shift was caused by a 20% increase in GABA<sub>A</sub>α3 ( $p < 0.05$ ), while the shift for the fluoxetine groups was caused by a 20% increase in GABA<sub>A</sub>α1 (fluoxetine alone,  $p < 0.01$ ; fluoxetine+MD,  $p < 0.05$ ) (Fig. 4C). This series of subunit balances unpacks subtle effect of fluoxetine treatment showing that it does not cause a shift to a CP-like state, instead it maintains subunit balances that are like normal adults (GluA2:GluN1) or shifted to more of the mature subunits (GluN2A, GABA<sub>A</sub>α1).

## **E/I balances**

The final analyses examined pre- and post-synaptic proteins that regulate the E/I balance. First, we calculated a pre-synaptic E/I balance using the vesicular transporters VGluT1 and VGAT. MD caused a large shift towards VGluT1 ( $p < 0.05$ ) (Fig. 5A) but when combined with

fluoxetine there was no change in the pre-synaptic E/I balance. The same pattern was seen on the post-synaptic side, here MD also caused a large shift towards the excitatory side (more PSD-95) ( $p < 0.05$ ) (Fig. 5B) but when MD was paired with fluoxetine there was no change from the normal adult E/I balance.



**Figure 5.** Pre- and Post-synaptic E/I balance. Presynaptic Index in contralateral V1 (A): (VGLUT1-VGAT)/(VGLUT1+VGAT). Postsynaptic Index in contralateral V1 (B): (PSD-95-Gephyrin)/(PSD-95+Gephyrin). We found strikingly similar patterns in the pre- and post-synaptic indexes of E/I synapses. Fluoxetine caused a slight shift towards inhibition in the presynaptic index and had no effect on the postsynaptic index. MD caused a strong shift to excitatory markers. Combining fluoxetine and MD kept the balance at normal levels. (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ ).

Data point	Data structure	Type of test	95% Confidence interval vs Normal	95% Confidence interval vs Fluoxetine	95% Confidence interval vs 1wk MD	95% Confidence interval vs Fluoxetine + 1wk MD
V1 Ipsi Synapsin - Normal	Normal	Bootstrapping Monte Carlo Simulation	+0.8112-1.1888	0.7825-1.2380	1.1441-0.8279	0.7813-0.9824
V1 Ipsi Synapsin-Fluoxetine	Normal	Bootstrapping Monte Carlo Simulation	+0.8388-1.1612	0.8131-1.2074	1.1275-0.8445	0.7945-0.9692
V1 Ipsi Synapsin - 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.8094-1.1906	0.7820-1.2384	1.1457-0.8263	0.7798-0.9839
V1 Ipsi Synapsin - Fluoxetine + 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.8378-1.1622	0.8126-1.2079	1.1258-0.8462	0.7951-0.9686
V1 Ipsi Synaptophysin - Normal	Normal	Bootstrapping Monte Carlo Simulation	+0.8812-1.1188	0.8583-1.1757	0.9164-1.2347	0.9007-1.1095
V1 Ipsi Synaptophysin - Fluoxetine	Normal	Bootstrapping Monte Carlo Simulation	+0.8957-1.1043	0.8817-1.1523	0.9400-1.2110	0.9152-1.0950
V1 Ipsi Synaptophysin - 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.8783-1.1217	0.8604-1.1735	0.9147-1.2364	0.9011-1.1091
V1 Ipsi Synaptophysin - Fluoxetine + 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.8977-1.1023	0.8782-1.1558	0.9352-1.2159	0.9150-1.0953
V1 Ipsi PSD-95 - Normal	Normal	Bootstrapping Monte Carlo Simulation	+0.6143-1.3857	0.7437-1.2262	1.0125-1.3042	0.9502-1.3776
V1 Ipsi PSD-95 - Fluoxetine	Normal	Bootstrapping Monte Carlo Simulation	+0.6678-1.3322	0.7799-1.1900	1.0345-1.2823	0.9780-1.3498
V1 Ipsi PSD-95 - 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.6117-1.3883	0.7469-1.2230	1.0125-1.3043	0.9499-1.3779
V1 Ipsi PSD-95 - Fluoxetine + 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.6678-1.3322	0.7799-1.1900	1.0325-1.2843	0.9765-1.3513
V1 Ipsi Gephyrin - Normal	Normal	Bootstrapping Monte Carlo Simulation	+0.7124-1.2876	0.7326-1.2570	0.9003-1.1418	0.9795-1.2321
V1 Ipsi Gephyrin - Fluoxetine	Normal	Bootstrapping Monte Carlo Simulation	+0.7491-1.2509	0.7669-1.2228	0.9169-1.1253	0.9974-1.2142
V1 Ipsi Gephyrin - 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.7048-1.2952	0.7297-1.2599	0.9035-1.1386	0.9795-1.2320
V1 Ipsi Gephyrin - Fluoxetine + 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.7533-1.2467	0.7677-1.2219	0.9182-1.1239	0.9961-1.2155
V1 Contra Synapsin- Normal	Normal	Bootstrapping Monte Carlo Simulation	+0.8105-1.1895	0.7781-1.2424	0.5763-1.1335	0.7372-1.3212

Data point	Data structure	Type of test	95% Confidence interval vs Normal	95% Confidence interval vs Fluoxetine	95% Confidence interval vs 1wk MD	95% Confidence interval vs Fluoxetine + 1wk MD
V1 Ipsi Synapsin - Normal	Normal	Bootstrapping Monte Carlo Simulation	+0.8112-1.1888	0.7825-1.2380	1.1441-0.8279	0.7813-0.9824
V1 Ipsi Synapsin-Fluoxetine	Normal	Bootstrapping Monte Carlo Simulation	+0.8388-1.1612	0.8131-1.2074	1.1275-0.8445	0.7945-0.9692
V1 Ipsi Synapsin - 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.8094-1.1906	0.7820-1.2384	1.1457-0.8263	0.7798-0.9839
V1 Ipsi Synapsin - Fluoxetine + 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.8378-1.1622	0.8126-1.2079	1.1258-0.8462	0.7951-0.9686
V1 Contra Synapsin-Fluoxetine	Normal	Bootstrapping Monte Carlo Simulation	+0.8384-1.1616	0.8055-1.2150	0.6118-1.0979	0.7764-1.2820
V1 Contra Synapsin - 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.8138-1.1862	0.7726-1.2478	0.5732-1.1365	0.7322-1.3262
V1 Contra Synapsin - Fluoxetine + 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.8381-1.1619	0.8103-1.2102	0.6112-1.0986	0.7680-1.2904
V1 Contra Synaptophysin - Normal	Normal	Bootstrapping Monte Carlo Simulation	+0.8801-1.1199	0.8473-1.1555	0.7132-1.0231	0.8204-1.0900
V1 Contra Synaptophysin - Fluoxetine	Normal	Bootstrapping Monte Carlo Simulation	+0.8777-1.1223	0.8663-1.1366	0.7315-1.0048	0.8387-1.0717
V1 Contra Synaptophysin - 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.8959-1.1041	0.8459-1.1569	0.7144-1.0219	0.8202-1.0902
V1 Contra Synaptophysin - Fluoxetine + 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.8930-1.1070	0.8693-1.1336	0.7350-1.0013	0.8389-1.0715
V1 Contra VGLUT1 - Normal	Normal	Bootstrapping Monte Carlo Simulation	+0.8693-1.1307	0.6128-0.8079	1.0116-1.4828	0.7065-1.0228
V1 Contra VGLUT1 - Fluoxetine	Normal	Bootstrapping Monte Carlo Simulation	+0.8872-1.1128	0.6250-0.7957	1.0435-1.4509	0.7247-1.0046
V1 Contra VGLUT1 - 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.8685-1.1315	0.6107-0.8100	1.0034-1.4910	0.7044-1.0249
V1 Contra VGLUT1 - Fluoxetine + 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.8876-1.1124	0.6227-0.7980	1.0387-1.4557	0.7228-1.0065
V1 Contra VGAT - Normal	Normal	Bootstrapping Monte Carlo Simulation	+0.6458-1.3542	0.5777-1.2580	0.6151-1.0278	0.6073-1.3463
V1 Contra VGAT - Fluoxetine	Normal	Bootstrapping Monte Carlo Simulation	+0.6993-1.3007	0.6330-1.2027	0.6390-1.0039	0.6511-1.3025

Data point	Data structure	Type of test	95% Confidence interval vs Normal	95% Confidence interval vs Fluoxetine	95% Confidence interval vs 1wk MD	95% Confidence interval vs Fluoxetine + 1wk MD
V1 Ipsi Synapsin - Normal	Normal	Bootstrapping Monte Carlo Simulation	+0.8112-1.1888	0.7825-1.2380	1.1441-0.8279	0.7813-0.9824
V1 Ipsi Synapsin-Fluoxetine	Normal	Bootstrapping Monte Carlo Simulation	+0.8388-1.1612	0.8131-1.2074	1.1275-0.8445	0.7945-0.9692
V1 Ipsi Synapsin - 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.8094-1.1906	0.7820-1.2384	1.1457-0.8263	0.7798-0.9839
V1 Ipsi Synapsin - Fluoxetine + 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.8378-1.1622	0.8126-1.2079	1.1258-0.8462	0.7951-0.9686
V1 Contra VGAT - 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.6512-1.3488	0.5808-1.2549	0.6160-1.0269	0.6015-1.3521
V1 Contra VGAT - Fluoxetine + 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.6956-1.3044	0.6339-1.2019	0.6414-1.0015	0.6515-1.3020
V1 Contra PSD-95 - Normal	Normal	Bootstrapping Monte Carlo Simulation	+0.6038-1.3962	0.7218-1.1861	0.4462-0.8174	0.9084-2.0180
V1 Contra PSD-95 - Fluoxetine	Normal	Bootstrapping Monte Carlo Simulation	+0.6683-1.3317	0.7503-1.1575	0.4720-0.7916	0.9719-1.9545
V1 Contra PSD-95 - 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.6097-1.3903	0.7204-1.1875	0.4505-0.8131	0.9037-2.0227
V1 Contra PSD-95 - Fluoxetine + 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.6679-1.3321	0.7493-1.1585	0.4700-0.7937	0.9767-1.9497
V1 Contra Gephyrin - Normal	Normal	Bootstrapping Monte Carlo Simulation	+0.7050-1.2950	0.7343-1.3432	0.4036-0.7036	0.8690-1.8151
V1 Contra Gephyrin - Fluoxetine	Normal	Bootstrapping Monte Carlo Simulation	+0.7480-1.2520	0.7847-1.2928	0.4257-0.6815	0.9298-1.7543
V1 Contra Gephyrin - 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.7089-1.2911	0.7444-1.3331	0.4053-0.7019	0.8845-1.7996
V1 Contra Gephyrin - Fluoxetine + 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.7515-1.2485	0.7858-1.2916	0.4304-0.6768	0.9435-1.7406
V1 Contra GluN1 - Normal	Normal	Bootstrapping Monte Carlo Simulation	+0.8909-1.1092	0.6978-1.0043	1.0128-1.4852	0.6713-0.9632
V1 Contra GluN1 - Fluoxetine	Normal	Bootstrapping Monte Carlo Simulation	+0.9037-1.0963	0.7187-0.9834	1.0445-1.4536	0.6910-0.9434
V1 Contra GluN1 - 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.8910-1.1090	0.6980-1.0042	1.0159-1.4822	0.6696-0.9648

Data point	Data structure	Type of test	95% Confidence interval vs Normal	95% Confidence interval vs Fluoxetine	95% Confidence interval vs 1wk MD	95% Confidence interval vs Fluoxetine + 1wk MD
V1 Ipsi Synapsin - Normal	Normal	Bootstrapping Monte Carlo Simulation	+0.8112-1.1888	0.7825-1.2380	1.1441-0.8279	0.7813-0.9824
V1 Ipsi Synapsin-Fluoxetine	Normal	Bootstrapping Monte Carlo Simulation	+0.8388-1.1612	0.8131-1.2074	1.1275-0.8445	0.7945-0.9692
V1 Ipsi Synapsin - 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.8094-1.1906	0.7820-1.2384	1.1457-0.8263	0.7798-0.9839
V1 Ipsi Synapsin - Fluoxetine + 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.8378-1.1622	0.8126-1.2079	1.1258-0.8462	0.7951-0.9686
V1 Contra GluN1 - Fluoxetine + 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.9053-1.0947	0.7206-0.9815	1.0457-1.4523	0.6897-0.9447
V1 Contra GluA2 - Normal	Normal	Bootstrapping Monte Carlo Simulation	+0.8632-1.1368	0.7766-1.0205	1.0076-1.3460	0.7128-0.9906
V1 Contra GluA2 - Fluoxetine	Normal	Bootstrapping Monte Carlo Simulation	+0.8824-1.1176	0.7943-1.0028	1.0326-1.3210	0.7128-0.9906
V1 Contra GluA2 - 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.8605-1.1395	0.7774-1.0197	1.0017-1.3519	0.7368-0.9667
V1 Contra GluA2 - Fluoxetine + 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.8790-1.1210	0.7940-1.0030	1.0316-1.3220	0.7310-0.9724
V1 Contra GluN2A - Normal	Normal	Bootstrapping Monte Carlo Simulation	+0.7104-1.2896	0.6612-1.0471	0.9664-1.5040	0.7161-1.1003
V1 Contra GluN2A - Fluoxetine	Normal	Bootstrapping Monte Carlo Simulation	+0.7063-1.2937	0.6880-1.0203	1.0035-1.4669	0.7431-1.0733
V1 Contra GluN2A - 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.7430-1.2569	0.6628-1.0455	0.9607-1.5097	0.7190-1.0974
V1 Contra GluN2A - Fluoxetine + 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.7418-1.2582	0.6832-1.0251	1.0056-1.4648	0.7427-1.0737
V1 Contra GluN2B - Normal	Normal	Bootstrapping Monte Carlo Simulation	+0.7772-1.2228	0.5712-0.8636	0.8007-1.1522	0.6562-0.9201
V1 Contra GluN2B - Fluoxetine	Normal	Bootstrapping Monte Carlo Simulation	+0.7812-1.2188	0.5881-0.8466	0.8229-1.1300	0.6740-0.9022
V1 Contra GluN2B - 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.8074-1.1926	0.5659-0.8688	0.8029-1.1500	0.6584-0.9179
V1 Contra GluN2B - Fluoxetine + 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.8120-1.1880	0.5862-0.8485	0.8239-1.1289	0.6728-0.9034

Data point	Data structure	Type of test	95% Confidence interval vs Normal	95% Confidence interval vs Fluoxetine	95% Confidence interval vs 1wk MD	95% Confidence interval vs Fluoxetine + 1wk MD
V1 Ipsi Synapsin - Normal	Normal	Bootstrapping Monte Carlo Simulation	+0.8112-1.1888	0.7825-1.2380	1.1441-0.8279	0.7813-0.9824
V1 Ipsi Synapsin-Fluoxetine	Normal	Bootstrapping Monte Carlo Simulation	+0.8388-1.1612	0.8131-1.2074	1.1275-0.8445	0.7945-0.9692
V1 Ipsi Synapsin - 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.8094-1.1906	0.7820-1.2384	1.1457-0.8263	0.7798-0.9839
V1 Ipsi Synapsin - Fluoxetine + 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.8378-1.1622	0.8126-1.2079	1.1258-0.8462	0.7951-0.9686
V1 Contra GABAA3 - Normal	Normal	Bootstrapping Monte Carlo Simulation	+0.8712-1.1288	0.7659-1.0577	0.9939-1.3721	0.7512-1.0196
V1 Contra GABAA3 - Fluoxetine	Normal	Bootstrapping Monte Carlo Simulation	+0.8908-1.1092	0.7856-1.0380	1.0139-1.3520	0.7645-1.0063
V1 Contra GABAA3 - 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.8729-1.1271	0.7641-1.0596	0.9921-1.3738	0.7447-1.0261
V1 Contra GABAA3 - Fluoxetine + 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.8894-1.1106	0.7883-1.0353	1.0207-1.3452	0.7655-1.0053
V1 Contra GABAA1 - Normal	Normal	Bootstrapping Monte Carlo Simulation	+0.8751-1.1249	0.8854-1.5893	0.7594-1.2798	0.5585-1.9208
V1 Contra GABAA1 - Fluoxetine	Normal	Bootstrapping Monte Carlo Simulation	+0.8898-1.1102	0.9302-1.5445	0.7971-1.2422	0.6434-1.8359
V1 Contra GABAA1 - 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.8713-1.1287	0.8863-1.5883	0.7642-1.2751	0.5339-1.9454
V1 Contra GABAA1 - Fluoxetine + 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.8883-1.1117	0.9312-1.5435	0.7932-1.2461	0.6465-1.8328
V1 Contra GluA2:GluN1 - Normal	Normal	Bootstrapping Monte Carlo Simulation	+/-0.0675-0.0603	-0.0351-0.0859	-0.0838-0.0295	-0.0379-0.0676
V1 Contra GluA2:GluN1 - Fluoxetine	Normal	Bootstrapping Monte Carlo Simulation	+/-0.0595-0.0523	-0.0279-0.0787	-0.0766-0.0223	-0.0304-0.0601
V1 Contra GluA2:GluN1 - 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+/-0.0675-0.0603	-0.0360-0.0868	-0.0834-0.0291	-0.0370-0.0667
V1 Contra GluA2:GluN1 - Fluoxetine + 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+/-0.0596-0.0525	-0.0270-0.0778	-0.0774-0.0231	-0.0317-0.0614

Data point	Data structure	Type of test	95% Confidence interval vs Normal	95% Confidence interval vs Fluoxetine	95% Confidence interval vs 1wk MD	95% Confidence interval vs Fluoxetine + 1wk MD
V1 Ipsi Synapsin - Normal	Normal	Bootstrapping Monte Carlo Simulation	+0.8112-1.1888	0.7825-1.2380	1.1441-0.8279	0.7813-0.9824
V1 Ipsi Synapsin-Fluoxetine	Normal	Bootstrapping Monte Carlo Simulation	+0.8388-1.1612	0.8131-1.2074	1.1275-0.8445	0.7945-0.9692
V1 Ipsi Synapsin - 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.8094-1.1906	0.7820-1.2384	1.1457-0.8263	0.7798-0.9839
V1 Ipsi Synapsin - Fluoxetine + 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.8378-1.1622	0.8126-1.2079	1.1258-0.8462	0.7951-0.9686
V1 Contra GluN2A:GluN2B - Normal	Normal	Bootstrapping Monte Carlo Simulation	+ -0.1879-0.0107	-0.0659-0.0841	-0.0451-0.1269	-0.0775-0.0616
V1 Contra GluN2A:GluN2B - Fluoxetine	Normal	Bootstrapping Monte Carlo Simulation	+ -0.1755-(-0.0018)	-0.0553-0.0735	-0.0331-0.1149	-0.0694-0.0536
V1 Contra GluN2A:GluN2B - 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+ -0.1862-0.0090	-0.0679-0.0862	-0.0456-0.1274	-0.0767-0.0608
V1 Contra GluN2A:GluN2B - Fluoxetine + 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+ -0.1738-(-0.0034)	-0.0569-0.0752	-0.0347-0.1165	-0.0684-0.0526
V1 Contra GABAA1:GABAA3 - Normal	Normal	Bootstrapping Monte Carlo Simulation	+ -0.1582-0.0079	-0.0817-0.1848	-0.2900-(-0.0325)	-0.1058-0.1577
V1 Contra GABAA1:GABAA3 - Fluoxetine	Normal	Bootstrapping Monte Carlo Simulation	+ -0.1463-(-0.0039)	-0.0619-0.1650	-0.2744-(-0.0481)	-0.0873-0.1392
V1 Contra GABAA1:GABAA3 - 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+ -0.1594-0.0092	-0.0804-0.1835	-0.2919-(-0.0306)	-0.1062-0.1582
V1 Contra GABAA1:GABAA3 - Fluoxetine + 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+ -0.1472-(-0.0031)	-0.0610-0.1641	-0.2729-(-0.0496)	-0.0866-0.1385
V1 Contra Presynaptic E/I - Normal	Normal	Bootstrapping Monte Carlo Simulation	+ -0.0981-0.1807	-0.2242-0.0731	0.0853-0.3517	-0.1710-0.1301
V1 Contra Presynaptic E/I - Fluoxetine	Normal	Bootstrapping Monte Carlo Simulation	+ -0.0809-0.1635	-0.2033-0.0523	0.1032-0.3338	-0.1525-0.1116
V1 Contra Presynaptic E/I - 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+ -0.0983-0.1808	-0.2288-0.0777	0.0850-0.3520	-0.1725-0.1316
V1 Contra Presynaptic E/I - Fluoxetine + 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+ -0.0770-0.1595	-0.2074-0.0563	0.0987-0.3383	-0.1521-0.1112

Data point	Data structure	Type of test	95% Confidence interval vs Normal	95% Confidence interval vs Fluoxetine	95% Confidence interval vs 1wk MD	95% Confidence interval vs Fluoxetine + 1wk MD
V1 Ipsi Synapsin - Normal	Normal	Bootstrapping Monte Carlo Simulation	+0.8112-1.1888	0.7825-1.2380	1.1441-0.8279	0.7813-0.9824
V1 Ipsi Synapsin-Fluoxetine	Normal	Bootstrapping Monte Carlo Simulation	+0.8388-1.1612	0.8131-1.2074	1.1275-0.8445	0.7945-0.9692
V1 Ipsi Synapsin - 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.8094-1.1906	0.7820-1.2384	1.1457-0.8263	0.7798-0.9839
V1 Ipsi Synapsin - Fluoxetine + 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+0.8378-1.1622	0.8126-1.2079	1.1258-0.8462	0.7951-0.9686
V1 Contra Postsynaptic E/I - Normal	Normal	Bootstrapping Monte Carlo Simulation	+/-0.1202-0.1499	-0.1150-0.0745	0.0653-0.3197	-0.0334-0.1542
V1 Contra Postsynaptic E/I - Fluoxetine	Normal	Bootstrapping Monte Carlo Simulation	+/-0.0999-0.1295	-0.1009-0.0604	0.0834-0.3016	-0.0208-0.1417
V1 Contra Postsynaptic E/I - 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+/-0.1199-0.1495	-0.1155-0.0750	0.0629-0.3221	-0.0324-0.1532
V1 Contra Postsynaptic E/I - Fluoxetine + 1wk MD	Normal	Bootstrapping Monte Carlo Simulation	+/-0.1008-0.1304	-0.1021-0.0616	0.0818-0.3032	-0.0210-0.1418

**Table 1.** Statistical table. Statistical comparisons were made by using Bootstrapping and Monte Carlo Simulations to generate expected 95% confidence intervals for each group being tested.

## 2.4 Discussion

In this study, we quantified the effect of fluoxetine treatment on 12 glutamatergic and GABAergic markers linked with visual experience-dependent plasticity in V1. Fluoxetine caused a pattern of change in those markers that provides new insights into how this drug affects plasticity in adult V1. We compared normal adult rats with ones treated with either fluoxetine alone, MD, or fluoxetine paired with MD. The main findings are that fluoxetine treatment in adult rats does not shift these markers to a younger pattern but instead rebalances MD driven glutamatergic gain and promotes a novel synaptic environment.

In this study, we used Western blotting to quantify the effects of fluoxetine treatment on a collection of synaptic proteins in adult V1. A strength of this approach is that a large number of synaptic proteins were analyzed. Western blotting, however, does not provide laminar or cell specific information that is needed to identify the neural circuits in V1 affected by fluoxetine. Future neuroanatomical studies are needed to address that question and those studies may be guided by the current results.

### **Fluoxetine does not recreate a younger synaptic environment**

An appealing hypothesis about drug treatments, such as fluoxetine, is that they re-instate ocular dominance plasticity in adult V1 by changing the synaptic environment to a CP-like state. During the CP there are increases in amount of proteins and shifts in balances between functional pairs of synaptic proteins. Our results do not support the idea that fluoxetine in adult rats dials back synaptic age. For example, we found that fluoxetine combined with MD caused greater expression of PSD-95 and gephyrin. These levels were higher than found during the CP (Pinto et al., 2015) and are consistent with a spike in PSD-95 that ends the CP (Huang et al., 2015).

Furthermore, fluoxetine alone did not reduce expression of either scaffolding protein and only MD caused a loss of PSD-95 and gephyrin. The modest losses for VGluT1 and receptor subunits caused by fluoxetine suggest a shift to a more immature stage, but the balances among the subunits do not support that conclusion. If a younger synaptic environment was recreated then it should favour NMDARs over AMPARs (Wu et al., 1996), GluN2B over GluN2A (Carmignoto and Vicini, 1992; Flint et al., 1997; Stocca and Vicini, 1998) and GABA<sub>A</sub>α3 over GABA<sub>A</sub>α1 (Laurie et al., 1992). Instead, the NMDAR to AMPAR ratios were balanced for both fluoxetine groups (GluN1~GluA2), while subunit balances jumped past age matched adults towards even more of the mature subunits (GluN2A, GABA<sub>A</sub> α1). Finally, E/I balances for pre- and post-synaptic markers were similar to the normal adults in both fluoxetine groups. Together these findings illustrate that fluoxetine treatment did not simply recreate a CP-like synaptic environment in V1.

It is important to note that we examined synaptic proteins after 1 month of fluoxetine treatment and 1wk of MD. We know from previous studies (Williams et al., 2015) that there are dynamic changes in synaptic proteins during a period of MD and it seems reasonable to propose that fluoxetine treatment may cause similarly dynamic changes. Thus the findings here provide a snapshot of long-term effects of fluoxetine treatment. It will be important for future studies to probe other time points to understand the full landscape of synaptic changes and how transient changes caused by fluoxetine (Vetencourt et al., 2011) impact long-term plasticity in V1.

### **Fluoxetine triggers a novel synaptic environment in adult V1**

The original study showing that fluoxetine reinstates ocular dominance plasticity also found improvement of visual function, reduced intracortical inhibition, and increased BDNF

expression (Vetencourt et al., 2008). All of those changes occurred without significantly altering neuronal responsiveness or orientation selectivity in V1. Here we found normal pre- and post-synaptic E/I balances, and adult levels of GABA<sub>A</sub>α1 that could support normal responsiveness and orientation selectivity. A previous study of gene expression found reduced VGAT after fluoxetine treatment but no changes in other genes associated with E/I mechanisms (Tiraboschi et al., 2013). We, however, did not find that fluoxetine caused a loss of VGAT protein expression. Some forms of GABAergic plasticity involve changes in VGAT protein expression associated with the amount of neurotransmitter in vesicles (Hartman et al., 2006), and the lack of change in VGAT makes it unlikely that fluoxetine altered this type of plasticity.

A recent proteomic analysis found that fluoxetine caused alterations in cytoskeleton organization, endocytosis, molecular transport, intracellular signaling, redox cellular state, metabolism and protein degradation (Ruiz-Perera et al., 2015). Those changes included proteins that regulate AMPAR and GABA<sub>A</sub>R, and may affect the E/I balance. Nonetheless, our quantification of synaptic proteins, along with the gene and proteomic studies, show that fluoxetine affects mechanisms that regulate experience-dependent plasticity.

The GluN2A:GluN2B and GABA<sub>A</sub>α1: GABA<sub>A</sub>α3 balances were both affected by fluoxetine and importantly the GABA<sub>A</sub> balance differentiated fluoxetine treatments from the effects of MD. The changes in these functional pairs of glutamatergic and GABAergic receptor subunits suggest that fluoxetine creates a novel synaptic environment in adult V1. An environment that is dominated by GluN2A and GABA<sub>A</sub>α1 but also has balanced levels of pre- and post-synaptic E/I markers. Both GluN2A and GABA<sub>A</sub>α1 subunits are described as mature components because they gradually increase during development and affect plasticity. For

example, the developmental shift from GluN2B to more GluN2A speeds up receptor kinetics (Cull-Candy et al., 2001), changes cellular signalling (Kim et al., 2005; Wang et al., 2011), relieves GluN2B negative regulation of AMPARs (Hall and Ghosh, 2008), and controls metaplasticity in V1 (Philpot et al., 2007). GABA<sub>A</sub>α1 is necessary for normal development of orientation tuning (Fagiolini et al., 2004) and gamma rhythms (Cardin et al., 2009; Sohal et al., 2009). The prevalence of GABA<sub>A</sub>α1 positive synapses on pyramidal cell bodies makes them important components in GABAergic regulation of experience-dependent plasticity (Hensch, 2005; Griffen and Maffei, 2014). The different roles of these subunits in experience-dependent plasticity suggests that fluoxetine creates a unique synaptic environment in adult V1 that can support both GluN2A-dependent metaplasticity and GABAergic regulation of ocular dominance plasticity.

### **How might fluoxetine trigger adult plasticity?**

Reduced intracortical GABA and GABAergic transmission have been found after fluoxetine treatment in adult rats (Vetencourt et al., 2008; Baroncelli et al., 2011). In contrast, we found a small increase in GABA<sub>A</sub>α1 expression and no loss of GABA<sub>A</sub>α3 or VGAT in rats treated with fluoxetine. Previous studies have shown that fluoxetine positively modulates GABA<sub>A</sub>Rs and one way is by increasing receptor sensitivity to small amounts of GABA (Robinson, 2002). The α1 subunit is one of the subtypes that confers that increased sensitivity and perhaps more GABA<sub>A</sub>α1 expression modulates GABAergic drive when the amount of neurotransmitter is reduced by fluoxetine. Interestingly, during the CP a brief exposure to vision after deprivation causes a rapid rebound potentiation in mini inhibitory post-synaptic currents (mIPSCs) that is correlated with an increase in GABA<sub>A</sub>Rs (Gao et al., 2014). Perhaps the

increase in GABA<sub>A</sub>α1 expression found here supports a similar potentiation of mIPSCs and since GABA<sub>A</sub>α1 containing synapses form a key part of the neural circuitry involved in ocular dominance plasticity (Hensch, 2005) fluoxetine may drive a compensatory mechanisms where sensitized GABA<sub>A</sub>Rs enhance adult plasticity.

We also found that fluoxetine caused changes to glutamatergic receptor subunit expression. Fluoxetine is known to inhibit NMDA receptors and may provide neuroprotective effects by regulating glutamatergic involvement in excitotoxicity (Szasz et al., 2007). We found that fluoxetine paired with MD ameliorated glutamatergic gain driven by MD alone, suggesting that one of fluoxetine's effects in adult V1 may be neuroprotective. Fluoxetine acts by inhibiting GluN2B containing NMDARs (Kiss et al., 2012) and that may trigger increases in both BDNF and AMPARs. GluN2B-mediated signalling inhibits AMPAR trafficking and the amount of GluA2 containing AMPARs (Kim et al., 2005; Derkach et al., 2007; Hall et al., 2007; Hall and Ghosh, 2008) through unique cellular processes that include Ras/ERK, αCamKII, and mTor pathways (Kim et al., 2005; Wang et al., 2011). One way that fluoxetine could affect adult ocular dominance plasticity is if the loss of GluN2B changes the length of GluN2B-mediated Ras/ERK activation (Kim et al., 2005) thereby increasing insertion of AMPAR into synapses and supporting long-term potentiation (LTP). ERK activation is necessary for ocular dominance plasticity in developing V1 (Di Cristo et al., 2001) and fluoxetine in adult animals may enhance ERK-dependent plasticity through the loss of GluN2B.

During the CP ocular dominance plasticity reflects the depression of deprived-eye responses but in adults MD leads to enhancement of open eye responses in V1 (Sawtell et al., 2003). That adult plasticity is dependent on activation of NMDARs and may use hebbian (LTP,

long-term depression LTD, spike time dependent plasticity), homeostatic, or metaplasticity (synaptic modification threshold) mechanisms (for review, see Hofer et al., 2006). Visual experience driven changes to LTP and LTD during the CP depend on GluN2A and previous studies have identified shifts in the GluN2A:GluN2B balance as the mechanism underlying an adjustable synaptic modification threshold in V1 (Philpot et al., 2007). Perhaps the shift to balanced GluN2A:GluN2B expression after fluoxetine treatment is an indication that metaplasticity plays a dominant role in fluoxetine driven adult plasticity. Interestingly, in auditory cortex fluoxetine reduces the potential for LTP (Dringenberg et al., 2014) raising the possibility that the effects of fluoxetine might not be uniform across the cortex.

Fluoxetine could also trigger events similar to those promoted by other NMDAR antagonists that cause a transient burst of glutamate, followed by BDNF release and synapse formation (Duman and Aghajanian, 2014). BDNF plays a key role in fluoxetine's reactivation of plasticity (Castrén and Rantamäki, 2010) suggesting that a fluoxetine induced loss of GluN2B signalling may enhance BDNF and AMPAR involvement in experience-dependent plasticity in adult V1. Thus, fluoxetine appears to enhance glutamatergic and GABAergic mechanisms that support experience-dependent plasticity in adult V1.

### **Implications for other therapies**

A variety of other methods are being explored to promote adult recovery from persistent amblyopia, such as dark rearing in animals (He et al., 2006; 2007; Montey and Quinlan, 2011; Duffy and Mitchell, 2013), manipulation of the brakes on plasticity including PirB (Bochner et al., 2014) and chondroitin sulphate proteoglycans (CSPGs) (Pizzorusso et al., 2002; Morishita et al., 2010; Bukhari et al., 2015), environmental enrichment (Sale et al., 2007), patterned visual

stimulation (Montey et al., 2013), or perceptual learning (Levi and Li, 2009; Baroncelli et al., 2011; Bonaccorsi et al., 2014; Tsirlin et al., 2015). All of these appear to reactivate a certain degree of plasticity that can support ocular dominance plasticity and even visual recovery. The cellular mechanisms typically include LTP of cortical synapses, and although some molecular changes have been identified (He et al., 2006), the full extent has yet to be explored. Do these other techniques mimic the novel pattern of fluoxetine driven glutamatergic and GABAergic changes or do they create different synaptic environments? These are important questions to answer to determine if these adult manipulations activate one or many different forms of experience-dependent plasticity in V1.

Future studies will need to determine the long-term consequences of fluoxetine-induced changes in adult V1. It is not clear if stopping drug treatment will allow the synaptic environment to shift back to a normal adult state. And if not what effects that new synaptic environment may have on neural function in the long-term. Finally, it will be important to determine how much of these effects are driven by the increase in serotonin, as opposed to unique effects of fluoxetine. Each of these are important questions to answer that will help to understand plasticity in adult V1 and translate that knowledge into effective treatments for persistent amblyopia.

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# **Chapter 3. Fluoxetine normalizes MD-driven glutamatergic gain and rescues Ube3A expression**

## Abstract

Fluoxetine treatment reinstates critical period-like ocular dominance plasticity and promotes recovery of acuity in adult rats. We previously asked how this selective serotonin reuptake inhibitor affects glutamatergic and GABAergic synaptic proteins that are essential for experience-dependent plasticity. We found that rather than reverting to a juvenile-like synaptic environment, fluoxetine rebalanced monocular deprivation-driven glutamatergic gain, and shifted some subunit proteins to a more mature composition (GluN2A, GABA<sub>A</sub>α1). These proteins, however, are only part of a complex system of cellular and synaptic mechanisms that shape experience-dependent plasticity. Here we used Western blotting to quantify synaptic proteins involved in regulating glutamate receptor trafficking (pGluA2, PICK1, GRIP1), inhibitory post-synaptic potentials (KCC2), heterosynaptic integration of excitatory and inhibitory transmission (CB1R), and ocular dominance plasticity (Ube3A) to determine if fluoxetine treatment shifts these regulators to a younger state.

We again found evidence to suggest that fluoxetine is neuroprotective, as it rescued all of the MD-driven changes in both glutamatergic and GABAergic proteins. All but one of these changes, however, were not consistent with a juvenile synaptic environment. In contrast, the change in Ube3A expression may reflect a juvenile-like mechanism. Ube3A expression is necessary for critical period ocular dominance plasticity, and is highly expressed in juvenile animals and humans. We found that fluoxetine combined with MD increased Ube3A expression. Together our findings indicate although fluoxetine may be neuroprotective, it does not simply reinstate a juvenile-like synaptic environment, and that Ube3A may be a crucial part of reinstating plasticity in the adult cortex.

### 3.1 Introduction

An important question in neuroscience is how to promote plasticity and recovery of function in the adult brain after insult or disease. In the visual cortex (V1), the drug fluoxetine causes critical period-like ocular dominance plasticity and facilitates recovery from monocular deprivation (MD) in adult animals (Vetencourt et al., 2008). That increased plasticity is thought to occur because fluoxetine has reinstated a juvenile-like synaptic environment. In our recent study, however, we did not find evidence that fluoxetine recreates a younger synaptic environment (Beshara et al., 2015). Instead, combining fluoxetine and MD in adult rats rebalanced MD-driven glutamatergic gain and promoted a novel pattern of synaptic proteins. Ameliorating the glutamatergic gain suggests that fluoxetine is neuroprotective, but the new pattern of synaptic proteins is shifted to more mature receptor subunits, GluN2A and GABA<sub>A</sub>α1, away from those found during critical period plasticity (Hensch, 2005; Levelt and Hübener, 2012). Although we studied 12 glutamatergic and GABAergic proteins, they are only part of a complex system of cellular and synaptic mechanisms that shape experience-dependent plasticity. Here, we studied the effect of combining fluoxetine treatment with adult MD on expression of synaptic proteins involved in regulating glutamate receptor trafficking (pGluA2, PICK1, GRIP1) (Chung et al., 2000; Heynen et al., 2003), inhibitory post-synaptic potentials (KCC2) (Ben-Ari, 2002), heterosynaptic integration of excitatory and inhibitory transmission (CB1R) (Xu and Chen, 2005), and ocular dominance plasticity (Ube3A) (Yashiro et al., 2009; Sato and Stryker, 2010) to determine if fluoxetine treatment shifts these regulators to a younger state.

Reduced inhibitory tone is a key mechanism supporting enhanced plasticity in adult V1 (Baroncelli et al., 2011). Fluoxetine treatment reduces levels of GABA in V1 (Vetencourt et al.,

2008), but on the receptor side it increases GABA<sub>A</sub>α1 expression (Beshara et al., 2015) and sensitivity (Robinson, 2002). The different directions of these changes suggest that fluoxetine may dysregulate GABAergic neurotransmission rather than simply return it to a younger state. The efficacy and polarity of GABA<sub>A</sub>α1 Rs, however, changes during development as KCC2 expression increases to modulate intracellular chloride homeostasis and dynamically regulate inhibitory post-synaptic potentials (Ben-Ari, 2002). Furthermore, the overall E/I balance is crucial for controlling critical period plasticity. Here, endocannabinoid receptor 1 (CB1R) participates in regulating plasticity (Castillo, 2012) that includes signaling heterosynaptic integration of excitatory and inhibitory synaptic transmission (Xu and Chen, 2005). Thus, examining KCC2 and CB1R expression may help identify fluoxetine-induced changes in E/I balance regulators.

During development, MD produces transient losses of GluA2-containing AMPARs, the AMPAR interacting protein PICK1, and the E3 ubiquitin ligase Ube3A (Williams et al., 2015), and the amount and timing of the being similar among the proteins. The loss of AMPAR proteins reflects the rapid depression of the deprived-eye that is associated with the phosphorylation of GluA2 (pGluA2) (Heynen et al., 2003) to trigger removal of AMPARs from the synapse. These AMPAR trafficking mechanisms are tightly regulated even during development when increases in GluA2, pGluA2, GRIP1 and PICK1 proceed in unison (Murphy et al., 2012). Ube3A is necessary for critical-period plasticity (Yashiro et al., 2009; Sato and Stryker, 2010). The defects in the maternal copy causes the neurodevelopmental disorder Angelman's Syndrome (Kishino et al., 1997), and animals with deletion of the maternal gene have significantly reduced ocular dominance plasticity (Yashiro et al., 2009; Sato and Stryker, 2010). Moreover, the loss of

plasticity depends on visual experience, as dark rearing rescues plasticity, but exposure to visual experience rapidly depletes it (Yashiro et al., 2009). This shows a relationship between visually-driven experience and Ube3A-dependent plasticity (Yashiro et al., 2009). Although these AMPAR mechanisms and Ube3A expression are necessary for critical period plasticity in V1, the effect of fluoxetine treatment on their expression in adults has not been examined. That leaves a gap for determining similarities and differences between developmental plasticity and fluoxetine-induced adult plasticity.

Here we found that MD in adult rats produced increases in all 4 AMPAR proteins (GluA2, pGluA2, PICK1, GRIP1), a reduction of KCC2 and no change to CB1R expression. Surprisingly, MD caused a reduction of Ube3A expression. However, when fluoxetine was combine with MD, it rescued all of the MD-driven changes to normal adult levels, except for Ube3A, which fluoxetine pushed expression to higher levels similar to those found in juveniles (Williams et al., 2010).

## **3.2 Materials and Methods**

### **Animals**

In this study, we used the same rearing conditions tissue preparation, and immunoblotting procedures published previously (Beshara et al., 2015). Here we studied the expression of 7 proteins in V1 of 28 adult male Long Evans rats. Animals were either normally reared to P98 (n=6), treated with fluoxetine for 4 weeks (P70-P98, 0.2 mg/ml of drinking water; n=8), MD for 1 week (P91-P98; n=6), or fluoxetine (P70-P98) combined with MD (P91-P98; n=8). MD, euthanasia, and perfusions were done according to procedures described previously (Beshara et al., 2015).

### **Tissue sample collection and preparation**

Samples of V1 including monocular and binocular regions were taken from both hemispheres. Homogenate samples were prepared to study higher-abundance proteins (KCC2 and Ube3A), and synaptosomes were prepared to study lower-abundance synaptic proteins (GluA2, pGluA2, GRIP1, PICK1, and CB1R). Both synaptosome and homogenate samples were prepared following procedures described previously (Beshara et al., 2015). A control sample was made by combining a small amount of each sample, and the control was run on every gel.

### **Immunoblotting**

Immunoblotting procedures were the same as described previously (Beshara et al., 2015). Briefly, 25 µg of each sample was resolved on a SDS-PAGE gel and transferred to a PVDF-FL membrane blot. Blots were incubated with blocking buffer, followed by primary antibody using concentrations that were previously optimized (Pinto et al., 2010; Beshara et al., 2015; Murphy et al., 2015). β-tubulin, 1:4000 (Invitrogen, Camarillo, CA); GAPDH, 1:4000 (Imgenex); GluA2,

1:2000 (Invitrogen, Camarillo, CA); pGluA2 (GluA2-ser880); 1:200 (PhosphoSolutions, Aurora, CO); GRIP1, 1:250 (BD Biosciences, San Diego, CA); PICK1, 1:200 (NeuroMab, Davis, CA).

The blots were washed, incubated for 1 hour with the appropriate IRDye labeled secondary antibody (LI-COR Biosciences), and washed again before being visualized with an Odyssey scanner (LI-COR Biosciences). Blots were then stripped and re-probed with the next primary antibody.

### **Analyses**

Bands were quantified using densitometry. Density profiles were determined by subtracting the background, integrating the pixel intensity across the area of the band, and dividing the intensity of the band by its width. Volume loading was checked with the loading controls, GAPDH or  $\beta$ -tubulin then each band was normalized to the control sample on the blot, and the average of all samples run at the same time with the same antibody. To ensure that the experimental conditions did not affect the overall amount of protein expression we compared expression of the loading controls among the 4 groups and found no significant differences.

To analyze the relationship between AMPAR surface and internalization-associated proteins, we calculated contrast indices between subunits (GluA2 and pGluA2) and interacting proteins (GRIP1 and PICK1). Subunit index:  $(\text{GluA2-pGluA2}/\text{GluA2+pGluA2})$ . Interacting protein index:  $(\text{GRIP1-PICK1})/(\text{GRIP1+PICK1})$ .

We plotted histograms with each groups' mean and standard error, relative to the normal controls. To compare groups we used bootstrapping, a modern resampling technique that provides robust estimates of standard error and confidence intervals, which is especially useful for smaller sample sizes. We used R to simulate a normally distributed data set with 1,000,000

points and the same mean and standard deviation as the group being compared. Next, we ran a Monte Carlo simulation to randomly sample from the simulated data set N times, where N was the sample size of the group that was being tested (N=6 or 8). This simulation was repeated 10,000 times to create a normal distribution of expected means. We then repeated the test but reversed which group was being compared and which was being tested. Groups were identified as significantly different (e.g.  $p < 0.05$ ) if their observed mean was outside the 95% confidence interval of expected means, in both directions.

### **Image Manipulation**

Bands are representative samples taken from different parts of the same gel or different gels. Uniform transformations and linear adjustments were applied to display bands appropriately for each figure and preserve the relative size and intensities between groups.

### 3.3 Results

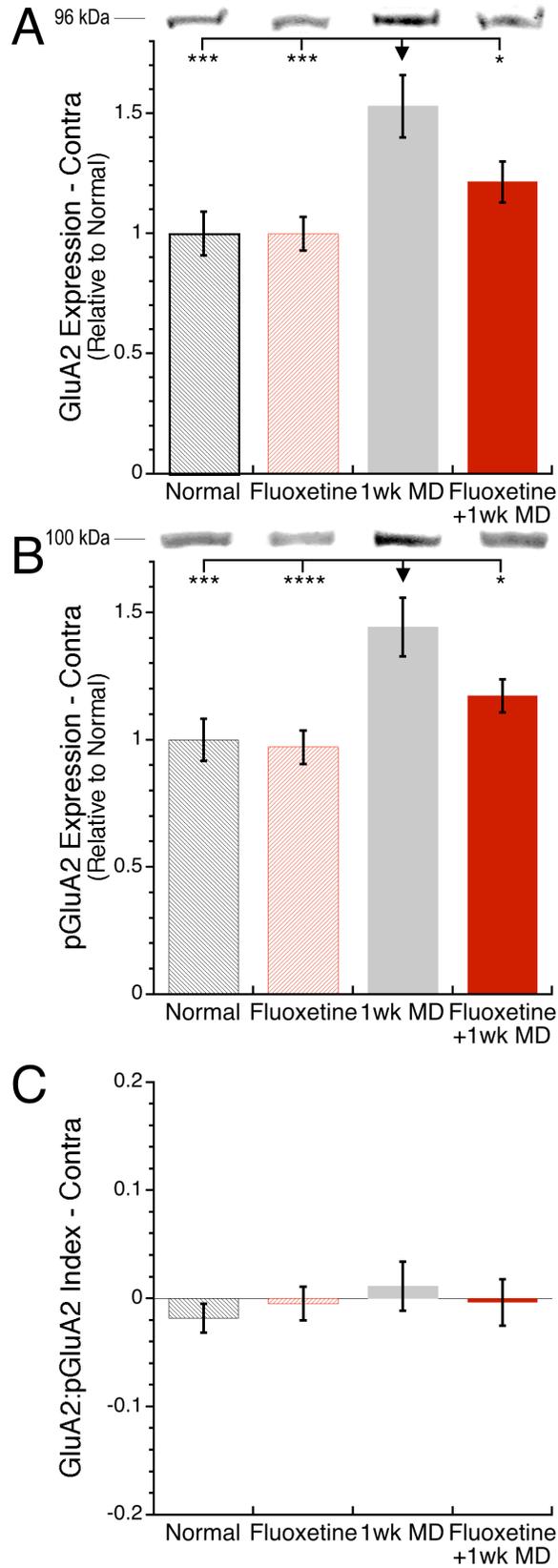
#### **No changes in the hemisphere ipsilateral to the deprived eye**

We quantified expression levels for each of the proteins, except CB1R, in the hemisphere ipsilateral to the deprived eye and used bootstrapping to make statistical comparisons among the 4 groups. Similar to our previous study (Beshara et al., 2015), we did not find any differences among the groups for the ipsilateral hemisphere. The following results focus on the changes found in the hemisphere contralateral to the deprived eye.

#### **MD causes increases in GluA2 and pGluA2, adding fluoxetine normalizes those increases**

First, we measured GluA2 and pGluA2 expression in contralateral V1 because both proteins increase during development, and peak after the critical period (Murphy et al., 2012). Furthermore, MD during the critical period causes a transient loss of GluA2 (Williams et al., 2015), but an increase of pGluA2 leading to the removal of AMPARs from the synapse (Heynen et al., 2003). We found that fluoxetine did not change expression of GluA2 or pGluA2 relative to normal adults, but MD increased both (GluA2, +53%, SEM 13%,  $p < 0.001$ ; pGluA2, +44%, SEM 11%,  $p < 0.001$ ; Fig. 1A, B). When fluoxetine was combined with MD, the MD-driven gains in GluA2 and pGluA2 were reduced (both  $p < 0.05$ ) to normal adult levels (n.s.; Fig. 1A, B).

Since GluA2 expression represents the total pool in the synaptosome preparation, and pGluA2 the fraction tagged for internalization (Kim et al., 2001), we calculated a GluA2:pGluA2 balance to analyze the relationship between the total pool and the amount in the synapse. We found roughly equal balances for GluA2:pGluA2 among the 4 groups (n.s.; Fig. 1C) which is similar to our previous finding of a tight relationship during cortical development (Murphy et al., 2012).

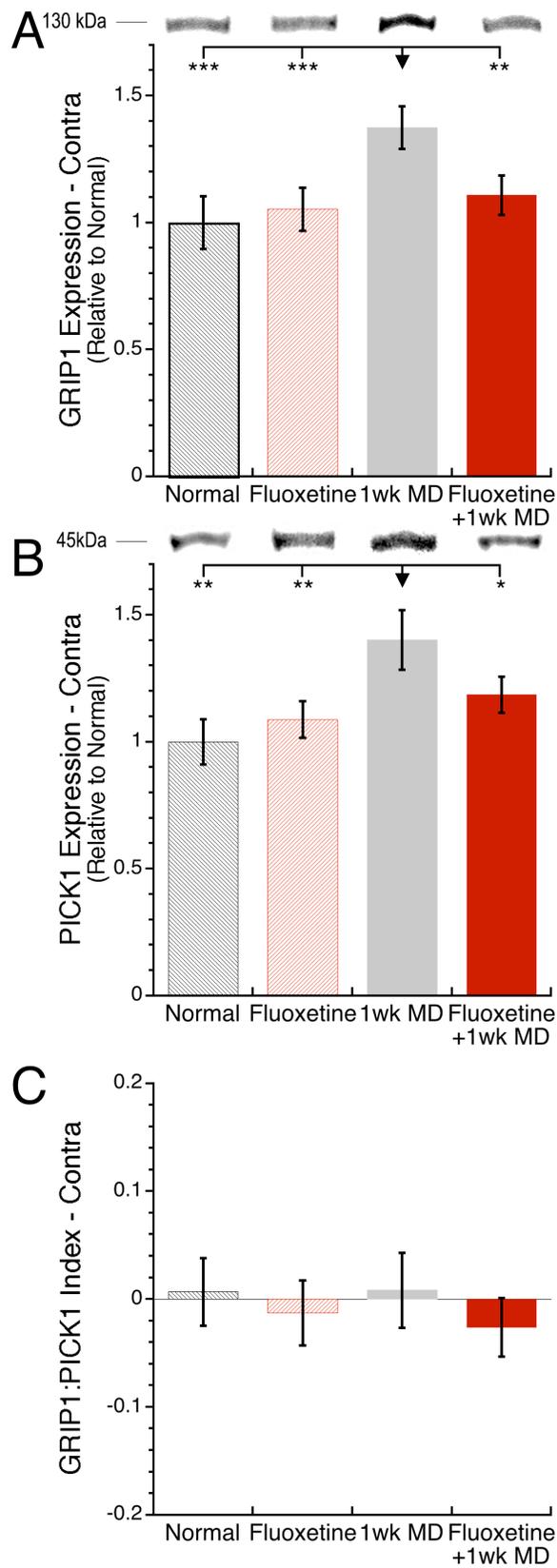


**Figure 1.** Expression of AMPAR subunits in contralateral V1. In contralateral V1, expression of both GluA2 (A) and pGluA2 (B) were unaffected by fluoxetine, increased by MD, and normalized by fluoxetine combined with MD. The GluA2:pGluA2 index remained balanced for all experimental conditions (C). Histograms show the mean and SEM for each condition, normalized to normal animals. Representative bands are shown above each condition. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

### **MD causes increases in GRIP1 and PICK1, adding fluoxetine normalizes those increases**

In addition to phosphorylation, AMPAR trafficking is regulated by interacting proteins like GRIP1 and PICK1, which move GluA2-containing AMPARs in and out of the synapses (Chung et al., 2000). Both GRIP1 and PICK1 expression increase during development, peaking after the critical period (Murphy et al., 2012). Furthermore, when only PICK1 was studied, we found that MD during the critical period causes a transient loss (Williams et al., 2015). Here we asked if fluoxetine shifts GRIP1 or PICK1 to a juvenile level of expression. We found adult MD caused an increase in both GRIP1 and PICK1 (GRIP1 +38%, SEM 8.4%,  $p < 0.001$ ; PICK1 +40%, SEM 12%,  $p < 0.01$ ; Fig. 2A, B), but fluoxetine alone or in combination with MD caused no change from normal adult levels (n.s.; Fig. 2A, B). These changes in the trafficking proteins are very similar to the GluA2 and pGluA2 changes and do not provide evidence that fluoxetine shifts expression levels back to a critical period-like state. Instead, they point to a homeostatic response to adult MD that is ameliorated by a neuroprotective effect of fluoxetine.

Since GRIP1 is involved in trafficking AMPARs into the synapse, and PICK1 out of the synapse, we calculated a GRIP1:PICK1 index to analyze if there were any changes in this trafficking balance. Previously, we showed that this balance remains tightly regulated during cortical development (Murphy et al., 2012). Here we found that GRIP1:PICK1 expression is roughly balanced for normal adults, as well as animals treated with fluoxetine, MD and fluoxetine combined with MD (n.s.; Fig. 2C), providing additional support for a tight balance between these trafficking proteins.



**Figure 2.** Expression of AMPAR interacting proteins in contralateral V1. In contralateral V1, expression of both GRIP1 (A) and PICK1 (B) were unaffected by fluoxetine, increased by MD, and normalized by fluoxetine combined with MD. The GRIP1:PICK1 index remained balanced for all experimental conditions (C). Histograms show the mean and SEM for each condition, normalized to normal animals. Histograms plotted using the conventions described in Figure 1.

### **MD causes a loss of KCC2, adding fluoxetine normalizes that loss**

Next, we measured KCC2 expression because it increases during development to make GABAergic transmission hyperpolarizing, thus increasing inhibitory tone (Ben-Ari, 2002). Since a change in inhibition regulates the critical period (Hensch, 2005) we asked if fluoxetine would reduce KCC2 expression to a critical period-like environment but found no change when compared to normal adults (n.s.; Fig 3A). Interestingly, adult MD reduced KCC2 expression (-37%, SEM 11.0%,  $p < 0.05$ ; Fig. 3A), but when fluoxetine was combined with MD the KCC2 levels were increased well above MD animals ( $p < 0.0001$ ; Fig 3A). Once again we did not find evidence for fluoxetine driving a shift to a critical period-like environment. Instead, these findings are consistent with a homeostatic response to MD where inhibition is turned down while excitation is turned up. They also suggest that one effect of combining fluoxetine with MD is to engage neuroprotective mechanisms that reduce excitatory gain.

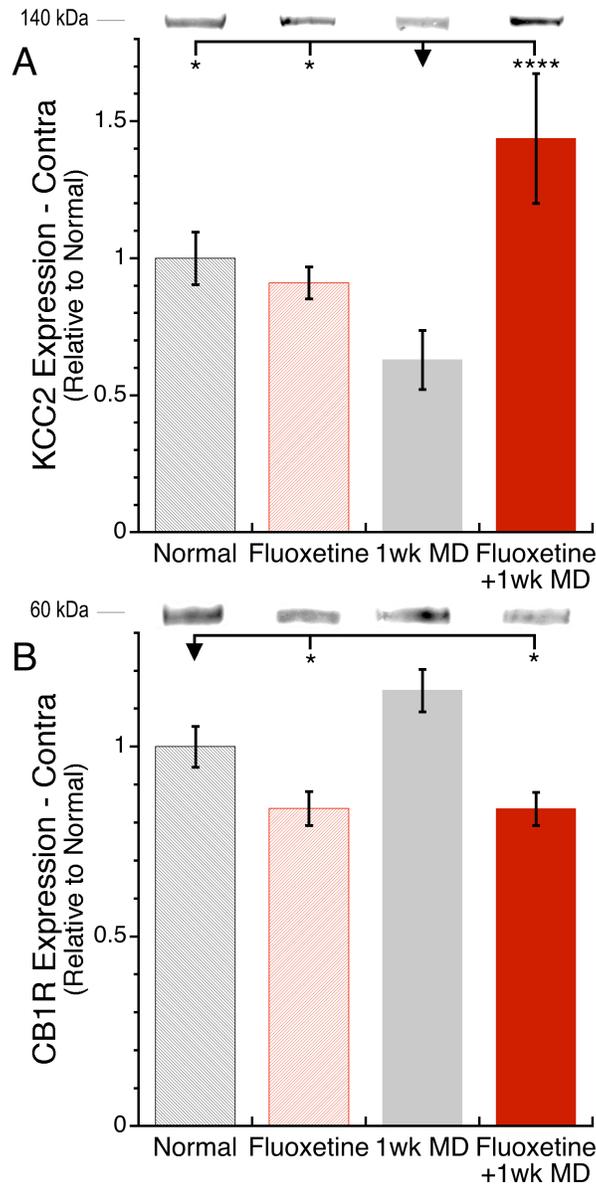
### **Fluoxetine reduces CB1R**

CB1R is the primary receptor for the endocannabinoid system and one of its functions is integration of E/I transmission that modulates both activity-dependent plasticity and metaplasticity (Xu and Chen, 2005). CB1R expression is high in juveniles and rapidly lost after the critical period (Pinto et al., 2010). CB1R activation during the critical period also mediates reduced GABA release probability, short-term depression, and variability that are central to maturation of GABAergic transmission (Jiang et al., 2010). We anticipated that fluoxetine might increase CB1R expression to a juvenile level, but found that it reduced CB1R expression below adult levels regardless of visual experience (fluoxetine -16%, SEM 4.5%,  $p < 0.05$ ; fluoxetine +

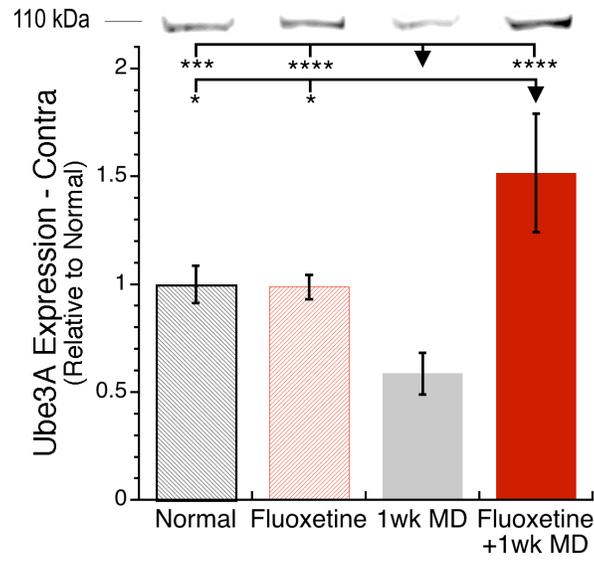
MD -16%, SEM 4.3%,  $p < 0.05$ ; Fig. 3B). Thus, the effect of fluoxetine on CB1R expression did not shift it to a critical period-like level.

### **MD causes a loss of Ube3A, adding fluoxetine increases Ube3A to juvenile levels**

To this point, we have studied the effect of fluoxetine on 17 glutamatergic and GABAergic proteins, between the current and our previous study (Beshara et al., 2015), and found no evidence of a shift to critical period-like expression levels. A different result was found when we quantified expression of the E3 ubiquitin ligase, Ube3A. Ube3A is part of Arc controlled AMPAR trafficking (Greer et al., 2010) and is necessary for critical period ocular dominance plasticity (Yashiro et al., 2009; Sato and Stryker, 2010). It is highly expressed in juveniles and declines in aging (Williams et al., 2010). Here we found that MD in adult rats caused a substantial loss of Ube3A expression (-41%, SEM 9.6%,  $p < 0.001$ ; Fig. 4), to levels similar to those found in aging animal and humans (Williams et al., 2010). In contrast, fluoxetine combined with MD significantly increased Ube3A expression well above normal adult levels (+52%, SEM 28%,  $p < 0.05$ ; Fig. 4), and to levels typically found in critical period-aged animals and humans (Williams et al., 2010). This is the first finding where combining fluoxetine with MD caused a change towards critical period levels. This fluoxetine-driven increase in Ube3A expression completely ameliorated the loss caused by MD, suggesting that fluoxetine may correct an MD-induced shift to an older synaptic state.



**Figure 3.** Expression of KCC2 and CB1R in contralateral V1. In contralateral V1 expression of KCC2 (A) was unaffected by fluoxetine. MD greatly reduced expression, and fluoxetine combined with MD brought expression within normal range. Expression of CB1R (B) was slightly reduced by fluoxetine and fluoxetine combined with MD, but unaffected by MD alone. Histograms plotted using the conventions described in Figure 1.



**Figure 4.** Expression of Ube3A in contralateral V1. In contralateral V1, expression of Ube3A (A) was unaffected by fluoxetine, reduced by MD, and greatly increased by fluoxetine combined with MD. Histograms plotted using the conventions described in Figure 1.

### **3.4 Discussion**

For the majority of proteins studied here and in our previous paper (Beshara et al., 2015), fluoxetine treatment did not simply shift expression to a younger synaptic environment. The results in this paper extend our previous finding, that adult MD causes a homeostatic response that involves a gain in glutamatergic proteins and a shift of E/I balances towards excitatory mechanisms. The addition of fluoxetine normalizes those changes to adult levels, suggesting that fluoxetine has a neuroprotective effect. The new findings here, are that Ube3A is involved in experience-dependent plasticity in adults. MD causes persistent loss of Ube3A expression, but adding fluoxetine recuses Ube3A and shifts it to critical period-like levels. Ube3A ubiquitinates many substrates involved in a wide range of neuronal functions, so the finding that fluoxetine alters the effect of MD on Ube3A suggests that fluoxetine could engage various mechanisms involved in both adult plasticity and neuroprotection (Mabb et al., 2011).

Here, and in our previous study (Beshara et al., 2015), we used Western blotting to identify MD combined with fluoxetine-induced changes in a large number of glutamatergic and GABAergic proteins in adult V1. Together, the results reveal new aspects of adult plasticity but leave unanswered which synapses are affected and how circuit function is altered. To answer those questions, future studies can build on our findings to uncover anatomical and functional consequences of fluoxetine-induced plasticity, especially those that are Ube3A-dependent.

#### **Adult MD-driven plasticity in V1**

The changes caused by MD and fluoxetine combined with MD provide new insights into normal plasticity in the adult cortex, and how reinstating critical period-like ocular dominance plasticity (Vetencourt et al., 2008) can change the synaptic environment.

MD alone caused increases in glutamatergic mechanisms (GluA2, pGluA2, GRIP1, PICK1, GluA2, GluN1, GluN2A, VGlut1), and decreased KCC2 which regulates inhibitory post-synaptic potentials. These are consistent with a homeostatic response that involves greater EPSCs and an expansion of spines to accommodate more AMPARs (Keck 2013) and a simultaneous decrease in IPSCs, both of which occur during the homeostatic response to MD in adult mice (Keck et al., 2011; 2013). An important question, however, is how these changes compare with a juvenile synaptic environment. On one hand, MD shifted the NMDAR index in favor of GluN2B (Beshara et al., 2015), which is traditionally associated with younger, more plastic, synapses (Quinlan et al., 1999). On the other hand, here we showed that MD significantly reduced Ube3A expression to levels similar to those found in aging animals and humans (Williams et al., 2010). Ube3A is necessary for critical-period plasticity and animals with deletion of the maternal UBE3A gene have significant deficits in visual plasticity (Yashiro et al., 2009; Sato and Stryker, 2010). This can be rescued by dark rearing, but rapidly disappears when vision is restored (Yashiro et al., 2009). This illustrates an integral relationship between visual experience and Ube3A expression (Yashiro et al., 2009).

Furthermore, during development MD drives a transient loss of AMPAR proteins that is mirrored by Ube3A (Williams et al., 2015). In contrast, we found that adult MD caused a disassociation where the AMPAR proteins increased, but Ube3A decreased. Ube3A, however, has many functions (Mabb et al., 2011, Table 2), so perhaps the loss of Ube3A in adult MD reflects its role in the function of other substrates. For example, ubiquitination of alpha-synuclein prevents neurodegeneration (Mulherkar et al., 2009). This raises the possibility that the MD-induced loss of Ube3A could drive accelerated aging. The shift to GluN2B may also be

associated with accelerated ageing since we have recently found a shift back to GluN2B in aging human V1. (Siu et al., submitted Aug 2016 to The Journal of Neuroscience). It is tempting to speculate, therefore, that the changes in both GluN2B and Ube3A reflect an experience-driven shift to a much older synaptic environment. Since Ube3A is necessary to support experience-dependent remodeling of spines (Mabb et al., 2011), this hypothesis leads to predictions about the effect of MD. For example, the persistent loss of Ube3A and increase in glutamatergic proteins suggest that adult MD reduces the potential for remodeling while increasing the gain at existing glutamatergic synapses.

### **Fluoxetine normalizes MD-driven gain, and increases Ube3A**

Fluoxetine combined with MD caused a very different pattern of changes that are characterized by normalizing MD-driven effects, which included returning glutamatergic receptor subunits, vesicular transporters, the E/I balances back to normal adult levels (Beshara et al., 2015). These effects suggest that fluoxetine is neuroprotective, an interpretation that is supported by many in-vitro and in-vivo studies which demonstrate that fluoxetine is neuroprotective against excitotoxicity due to NMDA, kainic acid, MDMA, inflammation, and stroke (Lim et al., 2009; Li et al., 2010; Zhang et al., 2012; Vizi et al., 2013). Although combining fluoxetine with MD causes critical period-like-ocular dominance plasticity (Vetencourt et al., 2008), it does not reinstate a juvenile-like synaptic environment. Only one of the 18 proteins studied here and previously (Beshara et al., 2015), Ube3A, shows a shift to juvenile-like expression (Williams et al., 2010). Since Ube3A is necessary for critical period plasticity, perhaps the fluoxetine-induced increase facilitates Ube3A-dependent plasticity mechanisms. Ube3A has many functions, but the one mostly commonly linked with experience-

dependent plasticity is regulation of AMPAR endocytosis through ubiquitination of Arc (Greer et al., 2010).

### **Conclusions and future directions**

This study unveils a connection between Ube3A and plasticity in the adult brain. Furthermore, the upregulation of Ube3A when fluoxetine was combined with MD identifies a mechanism capable of restoring critical period-like ocular dominance plasticity in adult V1. Since Ube3A ubiquitinates a wide range of proteins, these findings open the door to studying Ube3A-dependent pathways for reinstating plasticity in the adult cortex. Importantly these findings may lead to synergies between treatments being developed for the neurodevelopmental disorder Angelman Syndrome, and plasticity based therapies for treating adult brain disorders.

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**Chapter 4. D-serine has similar effects to fluoxetine on postsynaptic markers of the E/I balance**

## 4.1 Introduction

Neuroplasticity plays an essential part of both normal development and disease. The ability to refine neural networks allows efficient processing, but too much, too little, mistimed, or abnormal plasticity contributes to neurodevelopmental disorders including schizophrenia (Do et al., 2015), autism (LeBlanc and Fagiolini, 2011), and amblyopia (Wiesel and Hubel, 1963). In addition, the ability to enhance plasticity may significantly improve recovery after insults like stroke and traumatic brain injury. For these reasons, there has been growing interest in developing therapies that enhance plasticity in the adult cortex. Fluoxetine has emerged as a novel treatment because it reinstates critical period-like ocular dominance plasticity and promotes recovery of visual acuity in adult animals (Vetencourt et al., 2008; 2011; Ruiz-Perera et al., 2015). Unfortunately, fluoxetine has undesirable physiological and psychological side effects (Riddle et al., 2009), leading some to search for safer alternatives.

D-serine is the main endogenous NMDA receptor (NMDAR) co-agonist, and thus plays a major role in experience-dependent plasticity (Henneberger et al., 2012). D-serine is released by astrocytes (Mothet et al., 2005; Henneberger et al., 2012) to regulate synaptic plasticity (Panatier et al., 2006; Henneberger et al., 2010), and is the NMDAR co-agonist required for LTP at both excitatory and inhibitory synapses during the critical period (Meunier et al., 2016). Interestingly, like fluoxetine, exogenous D-serine facilitates critical period-like plasticity and recovery of visual acuity in adult animals (Yang et al., 2011), and has antidepressant effects (Otte et al., 2013). Together this lead us to hypothesize that D-serine may have similar effects as fluoxetine on the synaptic proteins that regulate experience-dependent plasticity, but this has not been studied.

To test this hypothesis this we treated animals with either D-serine, MD, or D-serine combined with MD. We measured the expression of the excitatory and inhibitory receptor scaffolding proteins, PSD-95 and gephyrin, because their interactions regulate the number of E/I synapses and thus affect the physiological E/I balance (Prange et al., 2004; Lardi-Studler et al., 2007; Keith and El-Husseini, 2008) which regulates critical period plasticity (Hensch and Fagiolini, 2005). The results were strikingly similar to our previous findings with fluoxetine (Beshara et al., 2015). We found that D-serine alone did not alter the expression or balance of PSD-95 or gephyrin. MD caused a loss of both proteins and a shift in favour of PSD-95, which is consistent with the homeostatic glutamatergic gain reported previously (Keck et al., 2013; Beshara et al., 2015). Combining D-serine with MD restored the expression of both proteins, and their balance, to normal. Our results suggest that D-serine's effects may be neuroprotective, and may share at least some neurobiological mechanisms with fluoxetine.

## **4.2 Materials and Methods**

### **Animals and surgical procedures**

We studied the expression of post-synaptic E/I markers in V1 of adult male Long Evans rats. Rats were individually housed with food and water ad libitum, in a 12:12 light/dark cycle. The rats were randomly assigned to 1 of 4 groups: normally reared to P98 (n=6), 2 weeks of D-serine (P91-P105) (n=7), 4 weeks of MD (P91-P98) (n=6), or both D-serine (P91-P105) and MD (P98-105)(n=7). D-serine dosage was tailored for each animal to reach 32mg/kg per day. During a baseline period of 2 weeks, water consumption and body weight were recorded daily. Based on these values, individual D-serine dosages were administered in the drinking water. During the experiment, water consumption and body weight were recorded every 2 days to adjust the D-serine content in the drinking water. Eyelids were closed by trimming the lid margins and suturing them together with 5-0 vicryl using aseptic surgical techniques. The surgery was done under gaseous anesthetic (isoflurane [1.5–5%] in oxygen) for induction and maintenance of anesthesia. Eyelids were inspected daily for openings. All experimental procedures were approved by the McMaster University Animal Research Ethics Board.

### **Tissue collection**

Animals were euthanized using 150 mg/kg sodium pentobarbital, and then transcardially perfused with cold 0.1 M phosphate buffered saline (PBS) (4°C; 4-5 ml/min) until the circulating fluid was clear. The brain was quickly removed from and immersed in cold PBS. Bilateral samples of V1 were frozen on dry ice and stored at -80°C.

## **Sample preparation**

Frozen tissue and cold homogenization buffer (1 ml buffer:50 mg tissue – 0.5 mM DTT, 1mM EDTA, 2 mM EGTA, 10 mM HEPES, 10 mg/L leupeptin, 100 nM microcystin, 0.1 mM PMSF, 50 mg/L soybean trypsin inhibitor) were added to Lysing Matrix D tubes (MP Biomedicals, Solon, OH,USA) and homogenized using a FastPrep-24 Tissue and Cell Homogenizer (MP Biomedicals, Solon, OH,USA) at 6m/s for 40s. The homogenate sample was then combined with 10% sodium-dodecylsulfate. Protein concentrations for each sample were determined using the bicinchoninic acid (BCA) assay guidelines (Pierce, Rockford, IL, USA) and final concentrations were adjusted to 1mg/ml using laemmli sample buffer. A control sample was made by combining a small amount of each of the 26 samples.

## **Immunoblotting**

Samples (25 µg) were resolved on 4-20% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) gels (Precise Protein Gels, Pierce Biotechnology Inc., Rockford, IL, USA) and transferred to polyvinylidene difluoride (PVDF-FL) membranes (Millipore, Billerica, MA, USA). Blots were incubated with blocking buffer (Odyssey Blocking Buffer 1:1 with PBS) for 1 hour (LI-COR Biosciences; Lincoln, NE, USA), then with primary antibody overnight at 4°C using the following concentrations: GAPDH, 1:4000 (Invitrogen); PSD-95, 1:32000 (Millipore); gephyrin, 1:2000 (Millipore). The blots were washed with PBS containing 0.05% Tween (Sigma, St. Louis, MO, USA) (PBS-T; 3 × 10 min), incubated for 1 hour at room temperature with the appropriate IRDye labeled secondary antibody, (Anti-Mouse, 1:8000, Anti-Rabbit, 1:10,000; LI-COR Biosciences; Lincoln, NE, USA), and washed in PBS-T (3 × 10 min). Blots were visualized using an Odyssey scanner (LI-COR Biosciences; Lincoln, NE, USA). The combination of IRDye

secondary antibodies and Odyssey scanner provides a wide linear dynamic range so that both strong and weak bands could be accurately quantified on the same blot. Blots were then stripped and re-probed with additional antibodies (Blot Restore Membrane Rejuvenation kit, Millipore, Billerica, MA, USA).

## **Analyses**

We quantified the bands using densitometry (LI-COR Odyssey Software version 3.0; LI-COR Biosciences; Lincoln, NE, USA). Density profiles were determined by performing a subtraction of the background, integrating the pixel intensity across the area of the band, and dividing the intensity by the width of the band to control for variations in lane width. Protein loading was checked with GAPDH as a loading control, and each band was normalized to the control sample on the blot, and the average of all samples run at the same time with the same antibody. To ensure that the experimental conditions did not effect overall protein expression, we compared expression of the loading control (GAPDH) among the 4 groups and found no significant differences normal.

To analyze the post-synaptic E/I balance, we calculated a contrast index that is commonly used in signal processing to determine the quality of the signal. Postsynaptic E/I Index: (PSD-95-gephyrin)/(PSD-95+gephyrin).

To compare protein expression among the groups we plotted histograms with the mean and standard error for each group. All results are normalized to the normal group. Statistical analysis was done with bootstrapping, a modern resampling statistical method that provides robust estimates of standard error and confidence intervals, that is especially useful for animal studies such as ours constrained to smaller sample sizes. First, we used R to simulate a normally

distributed data set with 1,000,000 points and the same mean and standard deviation as the group being compared. Then, we used a Monte Carlo simulation to determine differences between groups by comparing the simulated data set with average protein expression of the other groups. We used R to randomly sample from the simulated data set N time, where N was the number of animals in the group being compared (N=6 or 7). This simulation was repeated 10,000 times to create a normal distribution of expected means for that group. We calculated confidence intervals for the expected distribution, and groups were identified as significantly different (e.g.  $p < 0.05$ ) when the group's observed mean fell outside the 95% confidence interval.

### **Image Manipulation**

Bands are representative samples taken from different parts of the same gel or different gels. Transformations were uniformly applied to size bands appropriately for each figure. A linear adjustment layer was applied uniformly to all bands of each protein, preserving the relative intensities between groups.

## 4.3 Results

### **No changes in the hemisphere ipsilateral to the deprived eye**

We quantified expression levels for both proteins, in the hemisphere ipsilateral to the deprived eye and used bootstrapping to make statistical comparisons among the 4 groups. Similar to our previous studies with fluoxetine (Beshara et al., 2015), we did not find any differences among the groups in the ipsilateral hemisphere. Here we present the changes found in the hemisphere contralateral to the deprived eye.

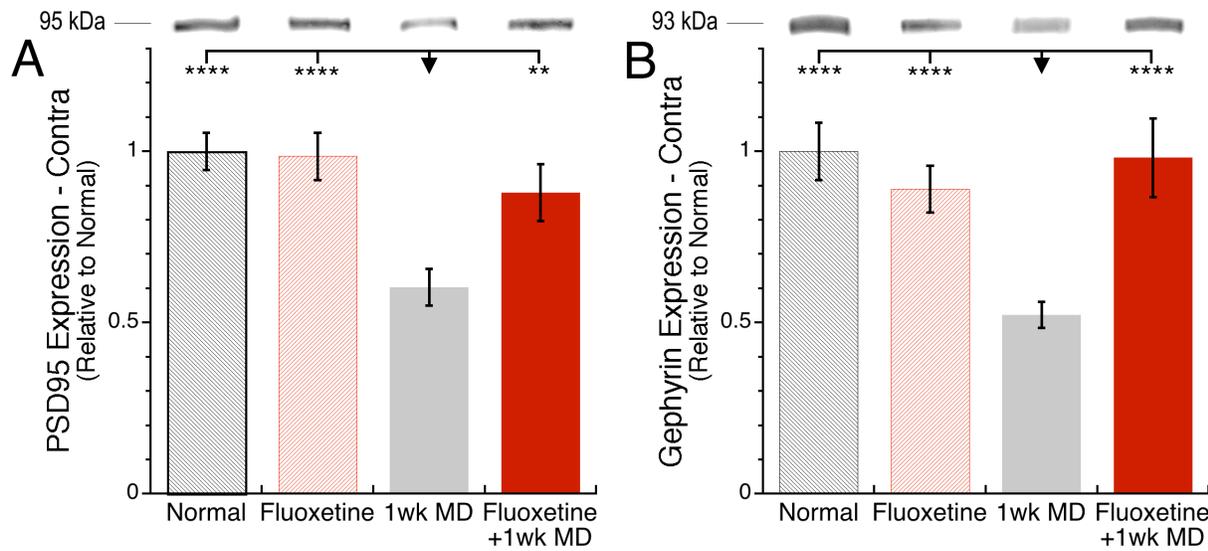
### **D-serine rescues post-synaptic receptor scaffolding protein expression**

We compared expression of PSD-95 and gephyrin in contralateral V1 of normally reared adult rats, rats given D-serine, MD, or the combination of D-serine and MD. The pattern of change between groups was very similar for PSD-95 and gephyrin (Fig. 1A, B). D-serine alone did not change expression relative to normal animals, but MD caused a major loss of both PSD-95 and gephyrin (PSD-95 -42%, SEM 5.2%,  $p < 0.0001$ ; gephyrin -50%, SEM 3.6%,  $p < 0.0001$ ). D-serine combined with MD rescued both PSD-95 and gephyrin expression, although PSD-95 expression remained slightly below normal PSD-95 -12% SEM 7.8%,  $p < 0.01$ ; gephyrin n.s.; Fig. 1A, B).

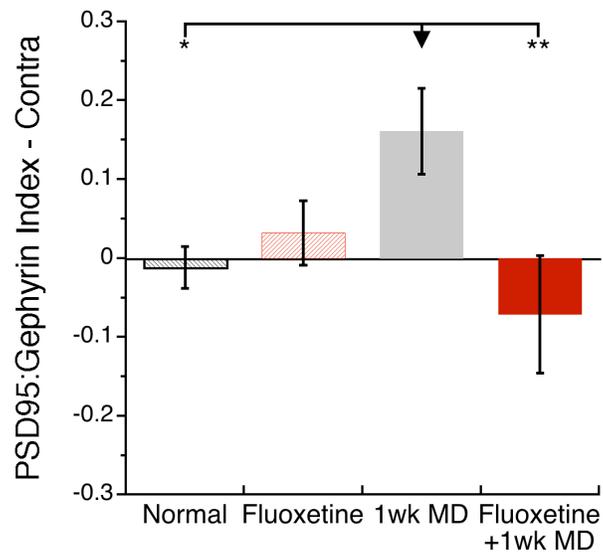
### **D-serine normalizes the post-synaptic E/I balance**

Next we analyzed the postsynaptic E/I balance using a contrast index because interactions between PSD-95 and gephyrin regulate the number of excitatory and inhibitory synapses, thus affecting the physiological E/I balance (Prange et al., 2004; Lardi-Studler et al., 2007; Keith and El-Husseini, 2008). In contralateral V1, D-serine had no effect on the balance. MD caused a

large shift toward excitatory synapses ( $p > 0.0001$ ; Fig. 4), but when D-serine was combined with MD, the balance returned to normal adult levels (n.s.; Fig 2).



**Figure 1.** Expression glutamatergic and GABAergic post-synaptic receptor scaffolding proteins in contralateral V1. In contralateral V1, expression of both PSD-95 (A) and gephyrin (B) were unaffected by fluoxetine, significantly reduced by MD, and rescued by fluoxetine combined with MD, although PSD-95 remained slightly below normal. Histograms show the mean and SEM for each condition, normalized to normal animals. Representative bands are shown above each condition. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .



**Figure 2.** Post-synaptic E/I balance in contralateral V1. Post-synaptic Index: (PSD-95-gephyrin)/(PSD-95+gephyrin). In contralateral V1, D-serine alone did not change the E/I balance, MD alone caused a strong shift in favor of excitatory synapses, but combining D-serine with MD returned the balance to normal. There were no significant changes in ipsilateral V1 (B). The histogram is plotted using the conventions described in Figure 1.

## 4.4 Discussion

We studied the effect of D-serine on the expression of the excitatory and inhibitory receptor scaffolding proteins, PSD-95 and gephyrin. We compared 4 groups of animals: normal control, 2 weeks of D-serine, 1 week of MD, or D-serine combined with MD. Our results support 3 main conclusions. First, D-serine may share synaptic mechanisms with other plasticity-enhancing and neuroprotective drugs, like fluoxetine. Second, D-serine protects against the shift towards excitatory synapses caused by MD. Third, D-serine's effect on the E/I balance is latent and only revealed with a change in experience.

We used Western blotting to quantify changes in protein expression, which allowed us to reprobe the same samples for PSD-95 and gephyrin. Western blotting, however, does not provide information about laminar, cell-type, or subcellular-specific changes. Follow-up studies using anatomical, cell-type identification, and subcellular localization techniques will be needed to identify the circuits that underlie D-serine's effects. In addition, we measured expression at a single time point, but changes caused by MD can be transient (Williams et al., 2015), and it is possible that the effect of D-serine on E/I synapses is also transient. Future studies should address this by studying expression at multiple time points.

### **D-serine has neuroprotective effects**

The MD-induced shift to excitatory synapses agrees with a homeostatic model where neurons respond to reduced input from the deprived eye by turning up their glutamatergic gain (Keck et al., 2013). An excessive increase in glutamatergic gain, however, can cause excitotoxic cell death, as it does after stroke or traumatic brain injury (Hazell, 2007; Obrenovitch and Urenjak, 2009). D-serine's normalization of the MD-induced shift suggests that chronic D-serine

may be neuroprotective. This seems counter-intuitive since several studies have found that D-serine is an important component of NMDAR-dependent excitotoxicity (Katsuki et al., 2004; Shleper et al., 2005). Those experiments, however, were conducted on acute cortical slices with artificially induced excitotoxicity, and only demonstrated that D-serine was necessary, not sufficient, for excitotoxicity. This is very different from chronic D-serine supplementation which can lead to changes in neural circuits that support long term depression (LTD) (Yang et al., 2011).

### **D-serine's effects are latent and revealed by a change in experience**

D-serine alone has no effect on the expression or balance of post-synaptic markers of the E/I balance. Instead, D-serine's effects were latent and only revealed in animals that combined D-serine with a change in experience. Interestingly, the fact that D-serine's effect was latent until combined with a change in experience supports the hypothesis that neuroplasticity-based therapies for psychiatric disorders work best when drugs are combined with rigorous behavioural therapy (see Krystal et al., 2009).

### **Towards a common mechanism for reinstating plasticity in the adult cortex**

Many treatments that reinstate juvenile-like plasticity in the adult cortex been discovered (Müller and Best, 1989; Pizzorusso et al., 2002; He et al., 2006; Sale et al., 2007; Vetencourt et al., 2008; Yang et al., 2011). This raises the question: do they share a common mechanism? While there will undoubtedly be differences, there is likely some overlap, particularly at the level of glutamatergic and GABAergic synapses. To that end, we have previously studied the effect of fluoxetine, a drug that reinstates critical period-like plasticity (Vetencourt et al., 2008; 2011; Ruiz-Perera et al., 2015), on a wide range glutamatergic and GABAergic synaptic proteins

(Beshara et al., 2015). The effect of fluoxetine on PSD-95 and gephyrin very similar to D-serine: fluoxetine alone had no effect, but when combined with MD it prevented the MD-induced loss of both PSD-95 and gephyrin, and normalized their balance. To fully assess the degree of overlap, future studies will need to compare the expression of a wider range of synaptic proteins. Finding common mechanisms between different plasticity-enhancing therapies may point to novel targets that provide better control over plasticity with fewer side effects.

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**Chapter 5. Development of post-synaptic glutamatergic  
proteins in human visual cortex across the lifespan**

## Abstract

Traditionally, human primary visual cortex has been thought to mature within the first few years of life, based on anatomical studies of synapse formation, and establishment of intra- and inter-cortical connections. Human vision, however, develops well beyond the first few years. Previously, we found prolonged development of some GABAergic proteins in human V1 (Pinto et al., 2010). Yet as over 80% of synapses in V1 are excitatory, it remains unanswered if the majority of synapses regulating experience-dependent plasticity and receptive field properties develop late like their inhibitory counterparts. To address this question, we used Western blotting of post-mortem tissue from human V1 covering a range of ages. Then quantified a set of post-synaptic glutamatergic proteins (PSD-95, GluA2, GluN1, GluN2A, GluN2B), calculated indices for functional pairs that are developmentally regulated (GluA2:GluN1; GluN2A:GluN2B), and determined inter-individual variability. We found early loss of GluN1, prolonged development of PSD-95 and GluA2 into late childhood, protracted development of GluN2A until ~40 years and dramatic loss of GluN2A in aging. The GluA2:GluN1 index switched at ~1 year but the GluN2A:GluN2B index continued to shift until ~40 year before changing back to GluN2B in aging. We also identified young childhood as a stage of heightened inter-individual variability. The changes show that human V1 develops gradually through a series of 5 orchestrated stages, making it likely that V1 participates in visual development and plasticity across the lifespan.

## **Significance**

Anatomical structure of human V1 appears to mature early, but vision changes across the lifespan. This discrepancy has fostered 2 hypotheses: either other aspects of V1 continue changing, or later changes in visual perception depend on extrastriate areas. Previously, we showed that some GABAergic synaptic proteins change across the lifespan but most synapses in V1 are excitatory leaving unanswered how they change. So we studied expression of glutamatergic proteins in human V1 to determine their development. Here we report prolonged maturation of glutamatergic proteins, with 5 stages that map onto life-long changes in human visual perception. Thus, the apparent discrepancy between development of structure and function may be explained by life-long synaptic changes in human V1.

## 5.1 Introduction

Anatomical development of human V1 in the first few years includes differentiation of layers (Zilles et al., 1986), rapid formation and elimination of synapses (Huttenlocher et al., 1982), and establishment of intra- and inter-cortical connections (Burkhalter, 1993; Burkhalter et al., 1993). Maturation of vision, however, continues much longer through childhood, adolescence, adulthood and aging (Kovács et al., 1999; Lewis and Maurer, 2005; Germine et al., 2011; Owsley, 2011). This discrepancy between development of structure and function has led to the suggestion that maturation of visual perception beyond infancy depends heavily on extrastriate areas (Lewis and Maurer, 2005) or other aspects of V1 circuits not captured by traditional anatomical studies (Taylor et al., 2014). We have found that development of some GABAergic synaptic proteins in human V1 continues into adulthood (Pinto et al., 2010), however, over 80% of V1 synapses are excitatory (Beaulieu et al., 1992), leaving unanswered whether the majority of synapses develop in young children or if some aspects of V1 development are prolonged. Here we address this question by studying development of glutamatergic proteins that are essential components of excitatory circuits regulating experience-dependent plasticity (Hensch, 2004; Turrigiano and Nelson, 2004; Cooper and Bear, 2012; Levelt and Hübener, 2012) and receptive field properties (Fagiolini et al., 2003; Self et al., 2012).

Animals studies have shown that maturation of ocular dominance plasticity and orientation tuning depend on activation of ionotropic glutamate receptors AMPAR ( $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and NMDAR (N-methyl-D-aspartate) (Kleinschmidt et al., 1987; Daw et al., 1992; Fagiolini et al., 2003). The developmental switch in NMDAR subunits from GluN2B to GluN2A regulates the effect of visual experience on ocular dominance plasticity

(Quinlan et al., 1999a; Philpot et al., 2007; Cho et al., 2009; Smith et al., 2009), while the loss of GluN2B is linked with ending the critical period (CP) (Erisir and Harris, 2003) and the increase in GluN2A controls maturation of orientation selectivity (Fagiolini et al., 2003). In addition, the glutamate receptor scaffolding protein PSD-95 participates in activity-dependent regulation of AMPARs through distinct mechanisms that mediate homeostatic plasticity (Sun and Turrigiano, 2011) and help to end the CP (Huang et al., 2015). Furthermore, visual experience modulates expression of NMDARs and AMPARs in ways that can inhibit or support plasticity (He et al., 2006; Beston et al., 2010). Thus, glutamate receptors support a variety of mechanisms that regulate experience-dependent plasticity in V1 (Turrigiano, 2008; Yashiro and Philpot, 2008; Cooke and Bear, 2014).

AMPARs dominate feed-forward drive to monkey V1 while NMDARs dominate recurrent connections that control figure-ground modulation (Self et al., 2012). NMDAR involvement in feedback processing is also found in human V1 (van Loon et al., 2015). Neurons expressing AMPARs and NMDARs in primate V1 are densest in layers 2/3 and 4, including GluA2-containing AMPARs (Huntley et al., 1994; Kooijmans et al., 2014) and GluN1/GluN2A- or GluN2B-containing NMDARs (Huntley et al., 1994). Those circuits contribute to spatial integration by recruiting glutamatergic synapses onto parvalbumin-positive interneurons (PV+) to support functions such as contrast integration (Nienborg et al., 2013; Vaiceliunaite et al., 2013). Thus, glutamate receptors in V1 provide a key link between receptive field processing and developmental plasticity.

Here we characterize changes in post-synaptic glutamatergic proteins in human V1 across the lifespan by quantifying expression of a receptor scaffolding protein (PSD-95), and NMDAR

(GluN1, GluN2A, GluN2B) and AMPAR (GluA2) subunits. We determined developmental trajectories for each protein, two indices representing functional pairs between receptor subunits (GluA2:GluN1, GluN2A:GluN2B), and waves of inter-individual variability. We found a pattern of changes that includes development of PSD-95 and GluA2 into late childhood and protracted increase of GluN2A into the fourth decade before significant loss in aging. These life-long changes in glutamate receptor expression in human V1 must contribute to visual processing and plasticity throughout the lifespan.

## **5.2 Materials and Methods**

### **Samples**

The post-mortem tissue samples from human V1 used in this study were obtained from the Brain and Tissue Bank for Developmental Disorders at the University of Maryland (Baltimore, MD, USA) and the study was approved by the McMaster University Research Ethics Board. Cortical samples were from individuals with no history of brain disorders, and all causes of death were with minimal trauma. Samples were collected within 23 hours post-mortem, sectioned coronally in 1cm intervals, flash frozen at the Brain and Tissue bank, and stored at -80°C. V1 samples were taken from the posterior pole of the left hemisphere and included both superior and inferior portions of the calcarine fissure. A total of 30 cases were used and ranged in age from 20 days to 79 years (Table 1).

### **Sample preparation**

A small piece of tissue (50-100mg) was cut from the calcarine fissure of each frozen block of human V1, suspended in cold homogenization buffer (1ml buffer: 50mg tissue, 0.5mM DTT, 2mM EDTA, 2mM EGTA, 10mM HEPES, 10mg/L leupeptin, 100nM microcystin, 0.1mM PMSF, 50mg/L soybean trypsin inhibitor), and homogenized in a glass-glass Dounce hand homogenizer (Kontes, Vineland, NJ, USA). To enrich for synaptic proteins we used a synaptosome preparation. Homogenate samples were filtered through coarse (100 µg) and fine (5 µg) pore hydrophilic mesh filters (Millipore, Bedford, MA, USA), and then centrifuged at 1000 x g for 10min to obtain the synaptic fraction. The synaptosome pellet was resuspended in boiling 1% sodium-dodecyl-sulfate (SDS), heated for 10 minutes and stored at -80°C. Protein concentration was determined using the bichinchonic acid assay protocol (Pierce, Rockford, IL,

USA) and samples were diluted with sample loading buffer (M260 Next Gel® Sample loading buffer 4x, Amresco LLC, Solon, OH, USA), and Laemmli buffer (Cayman Chemical Company, Ann Arbor, MI, USA) to attain a standard concentration of 1 µg protein/ml. A control sample was made by combining a small amount of the synaptosome preparation from each of the 30 cases.

### **Immunoblotting**

Synaptosome samples (20 µg) were separated on 4-20% SDS polyacrylamide gels (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF-FL) membranes (EMD Millipore, Billerica, MA, USA). Each sample was run multiple times. Blots were pre-incubated in blocking buffer for 1h (Odyssey Blocking Buffer 1:1 with phosphate buffer saline (PBS)) (Li-Cor Biosciences; Lincoln, NE, USA), then incubated in primary antibody overnight at 4°C using these primary antibodies: Anti-NMDAR1, 1:4000 (BD Pharmingen, San Jose, CA); Anti-NR2A, 1:1000 (EMD Millipore, Billerica, MA, USA); Anti-NMDAR2B, 1:1000 (EMD Millipore, Billerica, MA, USA); Anti-GluA2, 1:1000 (Invitrogen, Waltham, MA, USA); Anti-PSD-95, 1:16000 (EMD Millipore, Billerica, MA, USA). The blots were washed with PBS-Tween (0.05% PBS-T, Sigma, St. Louis, MO, USA) (3x10min) and incubated for 1 hour at room temperature with the appropriate IRDye labeled secondary antibody, (Anti-Mouse, 1:8000; Anti-Rabbit, 1:10,000; Li-Cor Biosciences, Lincoln, NE, USA), and washed again in PBS-T (3x10min). The bands were visualized using the Odyssey scanner (Li-Cor Biosciences; Lincoln, NE, USA), then stripped using a Blot Restore Membrane Rejuvenation kit (EMD Millipore, Billerica, MA, USA) and re-probed with another antibody. Following this protocol blots were probed with each antibody.

## **Band analysis**

To analyze the bands, blots were scanned on an Odyssey infrared scanner and quantified using densitometry (LI-COR Odyssey Software version 3.0; LI-COR Biosciences; Lincoln, NE, USA). A density profile for each band was calculated by performing a subtraction of the background, integrating the pixel intensity across the area of the band, and dividing the intensity by the width of the band to control for variations in lane width. A control sample, made by combining a small amount from each sample, was run on each gel and the density of each sample was quantified relative to the control (sample density/control density). To ensure that protein loading was not affected by age we analyzed expression of a loading control, GAPDH, across the lifespan and found it to remain relatively constant. Furthermore, none of the age bins differed in expression of GAPDH.

## **Band image manipulation**

Bands shown on figures are representative samples taken from the same gel or different gels and were added to the figures in Photoshop (Adobe Systems Inc, San Jose, CA, USA). Horizontal and vertical transformations were applied to size and orient the bands for each figure. A linear adjustment layer was applied uniformly to all bands for each protein, preserving the relative intensities among bands.

## **Receptor subunit index**

To quantify the balance between functional pairs of proteins we calculated a difference ratio, often called a contrast index, that is commonly used in signal processing to determine the quality of a signal. We calculated 2 indices that reflect the balance between pairs of proteins that are developmentally regulated: AMPA:NMDA index --  $(\text{GluA2}-\text{GluN1})/(\text{GluA2}+\text{GluN1})$ ; and

NMDAR subunit 2A:2B index --  $(\text{GluN2A}-\text{GluN2B})/(\text{GluN2B}+\text{GluN2A})$ . These indices can have values between -1 and +1.

### **Curve-fitting and statistical analyses**

The results were plotted in two ways to visualize and analyze changes in expression across the lifespan. First, to describe the time course of changes in protein expression, scatterplots were made for each protein showing the expression level from each run (grey dots) and the average of the runs (black dots). To determine the trajectory of changes across the lifespan we used a model-fitting approach (Christopoulos and Lew, 2008) and found the best curve-fit to the data using Matlab (The MathWorks, Inc, Natick, MA). A single-exponential decay function ( $Y=A*\exp(-(x/\tau))+B$ ) was fit to the data for GluN1. A Gaussian function ( $Y=A*\exp(-((\log(x/\mu))^2/(2*(\sigma^2))))+B$ ) was fit to the data for PSD-95, GluA2, GluN2B, and the 2A:2B index. A quadratic function was fit to the AMPA:NMDA balance ( $Y=A+B*\log(x)+C*\log(x)^2$ ). Finally, a weighted average was used to describe the trajectory for GluN2A. The fits were found by least squares and the goodness-of-fit ( $R^2$ ) and statistical significance of the fit ( $p$ ) were determined. For the decay function, we calculated the time constants ( $\tau$ ) and defined  $3\tau$  (when 87.5% of the change in expression had occurred) as the age when mature expression was reached with the 95% confidence interval (95% CI) around that age. For Gaussian functions, the age at the peak was calculated and the 95% CI determined.

Second, to compare changes among different stages across the lifespan, samples were binned into age groups (<0.3 years, Neonates; 0.3-1 year, Infants; 1-4 years, Young Children; 5-11 years, Older Children; 12-20 years, Teens; 21-55 years, Young Adults; >55 years, Older Adults) and histograms were plotted showing the mean and standard error of the mean (SEM) for

each group. We used bootstrapping to make statistical comparisons among the groups since this method provides robust estimates of standard error and CI, that are especially useful for human studies constrained to smaller sample sizes. The statistical software R was used for the bootstrapping and we began by simulating a normally distributed dataset (1,000,000 points) with the same mean and standard deviation as the group being compared. We used this normally distributed dataset to determine if the observed means for the other age groups were significantly different. A Monte Carlo simulation was used to randomly sample from the simulated dataset N times, where N was the number of cases in the other age groups. This simulation was run 10,000 times to generate an expected distribution for the N number of cases. Confidence intervals (CI) were calculated for that simulated distribution (i.e. 95%, 99% CI) and compared with the observed group means. The age groups were considered to be significantly different (i.e.  $p < 0.05$ ) when the observed mean was outside the 95% CI.

### **Analysis of Inter-individual variability**

Previously we identified ages during infancy and childhood with waves of high inter-individual variability (Pinto et al., 2015; Siu et al., 2015). To analyze if the glutamatergic proteins studied here have similar waves of inter-individual variability we calculated the Fano-Factor (Variance-to-Mean Ratio - VMR) for each protein and examined how it changed across the lifespan. The VMR around each case was determined by calculating the mean and variance for the protein expression within a moving box that included 3 adjacent ages and then dividing the variance by the mean. Scatter plots were made to show how the VMRs changed across the life span and functions were fit to those data to identify ages when there was high inter-individual variability. The VMRs were fit with the same Gaussian function described above and

a wave of higher inter-individual variability was identified when 4 or more points at the peak fell above the 95% CI for lower bound of the curve.

<b>Age</b>	<b>Age Group</b>	<b>Sex</b>	<b>PMI (Hours)</b>
20 days	Neonate	M	9
86 days	Neonate	F	23
96 days	Neonate	M	12
98 days	Neonate	M	16
119 days	Neonate	M	22
120 days	Neonate	M	23
133 days	Infant	M	16
136 days	Infant	F	11
273 days	Infant	M	10
1 year 123 days	Young Children	M	21
2 years 57 days	Young Children	F	21
2 years 75 days	Young Children	F	11
3 years 123 days	Young Children	F	11
4 years 203 days	Young Children	M	15
4 years 258 days	Young Children	M	17
5 years 144 days	Older Children	M	17
8 years 50 days	Older Children	F	20
8 years 214 days	Older Children	F	20
9 years 46 days	Older Children	F	20
12 years 164 days	Teens	M	22
13 years 99 days	Teens	M	5
15 years 81 days	Teens	M	16
19 years 76 days	Teens	F	16
22 years 359 days	Young Adults	M	4
32 years 223 days	Young Adults	M	13
50 years 156 days	Young Adults	M	8

53 years 330 days	Young Adults	F	5
69 years 110 days	Older Adults	M	12
71 years 333 days	Older Adults	F	9
79 years 181 days	Older Adults	F	14

**Table 1.** Human V1 tissue samples used in this study. Each case is identified by their age in years and days, age group assignment, sex, and post-mortem interval (PMI).

## 5.3 Results

### Postmortem interval

We examined whether glutamate protein expression levels were affected by post-mortem interval (PMI). We first verified that immunoreactivity was present and then we analyzed the correlation between PMI and protein expression. We did not find any significant correlations between PMI and expression of the 5 post-synaptic glutamatergic proteins (PSD-95:  $r=0.05$ ,  $p=0.66$ ; GluA2:  $r=0.17$ ,  $p=0.13$ ; GluN1:  $r=0.26$ ,  $p=0.11$ ; GluN2A:  $r=0.17$ ,  $p=0.41$ ; GluN2B:  $r=0.16$ ,  $p=0.24$ ) so all of the data was included in the following analyses.

### Slow development of PSD-95, earlier development for GluA2 and GluN1

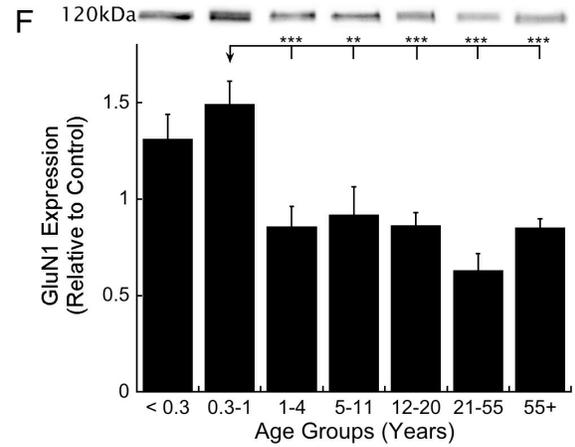
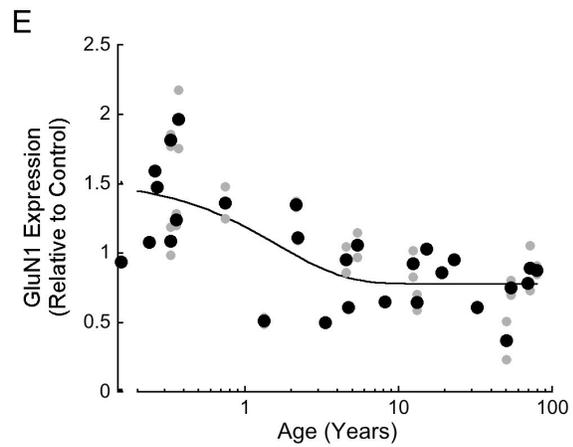
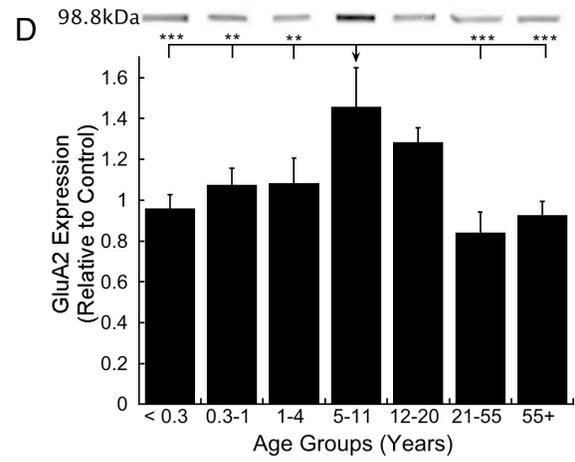
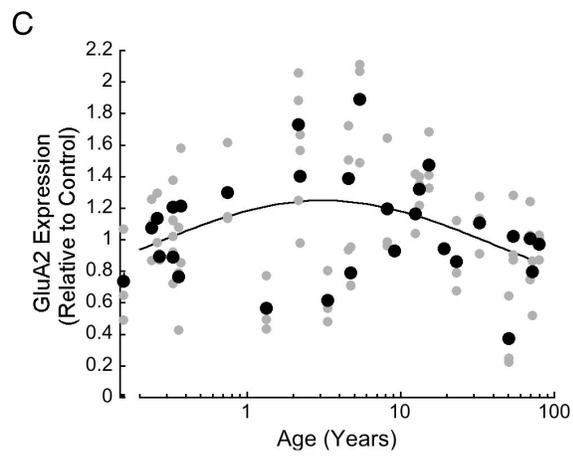
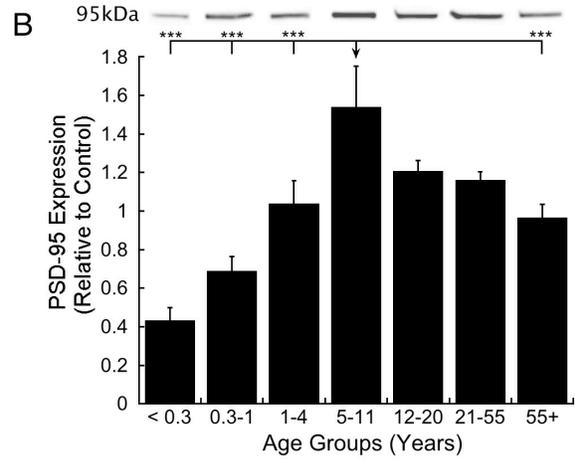
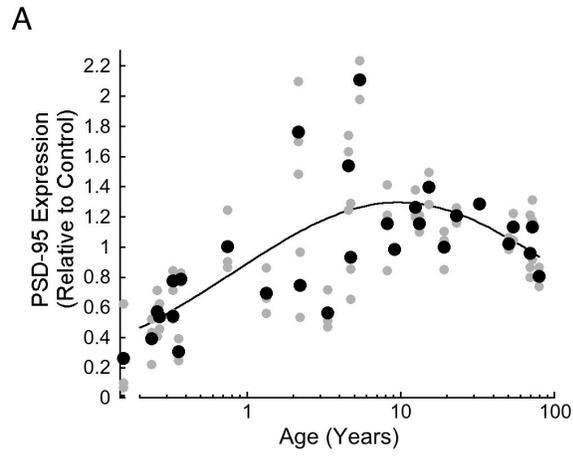
We began analyzing development of post-synaptic glutamate proteins in human V1 by measuring expression of PSD-95, a scaffolding protein involved in anchoring AMPA and NMDA receptors (Kim and Sheng, 2004), controlling visual developmental plasticity (Yoshii et al., 2003), and ending the critical period for ocular dominance plasticity (Huang et al., 2015). We found a steady increase in expression of PSD-95 in the synaptosome preparation used in this study and analyzed the results in two ways (Fig. 1). First, by model-fitting to all the data to determine the best curve to capture changes across the lifespan, and second, by binning the data into age groups and using bootstrapping for statistical comparisons between groups.

Development of PSD-95 peaked at 9.6 years ( $\pm 4.1$  years;  $R^2=0.457$ ,  $p<0.0001$ ) (Fig. 1A). This result is similar to our previous findings using whole homogenate samples (Pinto et al., 2015). The magnitude of the peak, however, was about half as much in the synaptosome samples compared with that found using homogenate (Pinto et al., 2015, Figure 3), suggesting there could be a large mobile pool of PSD-95 during late childhood. Comparing the age-binned results

showed a 3-fold increase in PSD-95 expression during development that reached a peak in older children (5-11 years,  $p < 0.001$ ) before dropping about 30% into aging ( $p < 0.001$ ) (Fig. 1B). The PSD-95 peak corresponds with the age when children are no longer susceptible to amblyopia (Lewis and Maurer, 2005) and may signify that PSD-95 contributes to ending the CP for ocular dominance plasticity in humans similar to its role in rat V1 (Huang et al., 2015).

Next, we quantified development of GluA2 and GluN1, which identify the 2 main classes of ionotropic glutamate receptors AMPARs and NMDARs, respectively. Development of these subunits followed a similar pattern to that found in animal studies, where GluA2 increased, while GluN1 decreased during development (Fig. 1C-F). GluA2 expression increased about 40% during childhood and then declined a similar amount into adulthood and aging. The GluA2 developmental trajectory peaked at 3.1 years ( $\pm 1.8$  years,  $R^2 = 0.131$ ,  $p < 0.01$ ) (Fig. 1C). Comparison among the age groups, however, identified a slightly later peak for GluA2 expression during late childhood (5-11 years) (Fig. 1D). The uncertainty about the peak for GluA2 probably reflects variability in expression during childhood and the modest increase between neonates and older children.

The trajectory of GluN1 expression started high under 1 year of age, then rapidly decreased to a relatively constant level for the rest of the lifespan (Fig. 1E,F). The change in GluN1 expression was fit with an exponential decay function ( $R^2 = 0.482$ ,  $p < 0.0001$ ) that fell to mature levels ( $3\tau$ ) by 4.2 years ( $\pm 1.7$  years) (Fig. 1E). The same pattern was found when we compared age groups showing that GluN1 levels were higher under 1 year and dropped by almost half during young childhood (1-4 years) ( $p < 0.001$ ) where it remained for the rest of the lifespan (Fig. 1F).

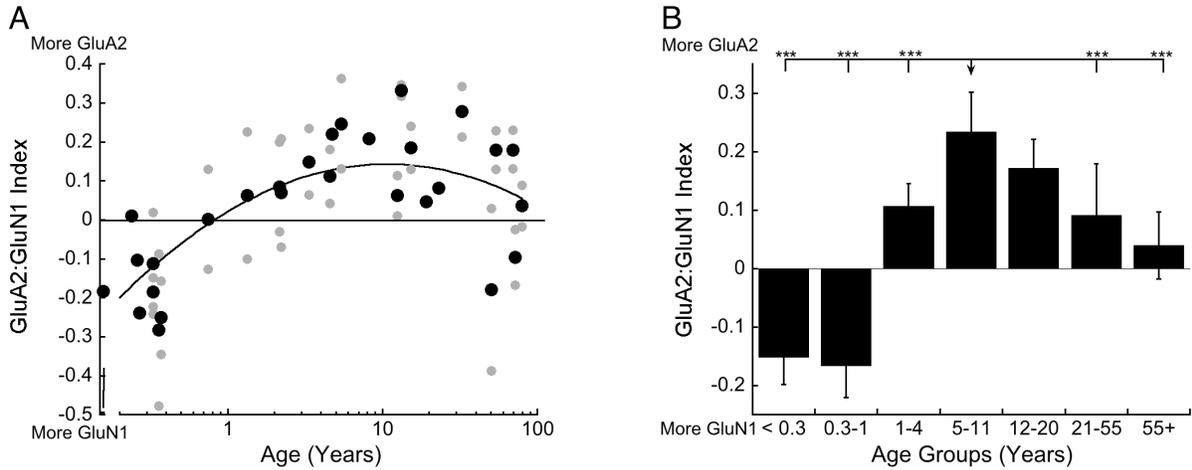


**Figure 1.** Development of PSD-95, GluA2 , and GluN1 expression in human V1. (A) A scatterplot of PSD-95 expression across the lifespan fit with a Gaussian function ( $R^2=0.457$ ,  $p<0.0001$ ) with peak expression at 9.6 years ( $\pm 4.1$  years). (B) Age-binned results for PSD-95 expression. (C) A scatterplot of GluA2 expression across the lifespan fit with a Gaussian function ( $R^2=0.131$ ,  $p<0.01$ ), with peak expression at 3.1 years ( $\pm 1.8$  years). (D) Age-binned results for GluA2 expression. (E) A scatterplot of GluN1 expression across the lifespan fit with an exponential decay function ( $R^2=0.482$ ,  $p<0.0001$ ), and fell to mature levels ( $3\tau$ ) at 4.2 years ( $\pm 1.7$  years). (F) Age-Binned results for GluN1 expression. For the scatterplots, grey dots represent each run, black dots represent the average for each case and age was plotted on a logarithmic scale. For the histograms, protein expression was binned into age groups (< 0.3 years, Neonates; 0.3-1 year, Infants; 1-4 years, Young Children; 5-11 years, Older Children; 12-20 years, Teens; 21-55 years, Young Adults; >55 years, Older Adults) showing the mean and SEM. Representative bands are shown above each age group. (\* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ ).

Comparing the changes across the lifespan for PSD-95, GluA2, and GluN1 we found different timing (GluA2 and GluN1 matured before PSD-95), different directions (PSD-95 and GluA2 increased while GluN1 decreased), and different amounts of protein change. Thus, even these 3 tightly associated proteins had different developmental trajectories.

### **Early shift from more NMDA to more AMPA in human V1**

Animal studies have shown that there is an early developmental shift from NMDAR-dominated silent synapses to functional synapses with AMPARs (Isaac et al., 1997; Rumpel et al., 1998). Here we examined development of the AMPA:NMDA balance in human V1 as an indication of functional maturation of glutamatergic transmission. We calculated an AMPA:NMDA index where a value of -1 indicated only GluN1 expression, 0 indicated equal expression, and +1 indicated only GluA2 expression. We found an early switch from more GluN1 under 1 year of age to more GluA2 after 1 year (Fig. 2). The AMPA:NMDA balance was fit with a quadratic function ( $R^2=0.406$ ,  $p<0.0001$ ) that captured the shift in favor of GluA2 and peaked at 10.7 years (95%CI 4.8-23.7 years) before slowly returning to equal expression during aging (Fig. 2A). The age-binned results showed the same pattern of a significant switch at 1 year, GluA2 peaking during late childhood, and returning to balanced expression in older adults (Fig. 2B). The changes in this AMPA:NMDA balance suggest an early stage of human V1 development during infancy (<1 year) that may characterize unsilencing of glutamate synapses followed by AMPAR dominated excitatory drive during childhood and young adults before regressing to balanced AMPAR and NMDAR expression in aging.

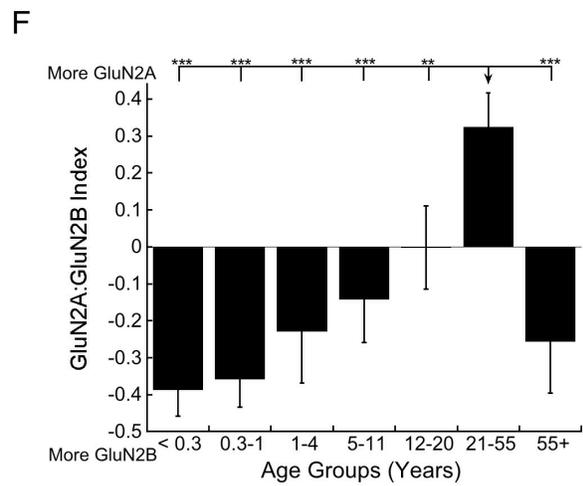
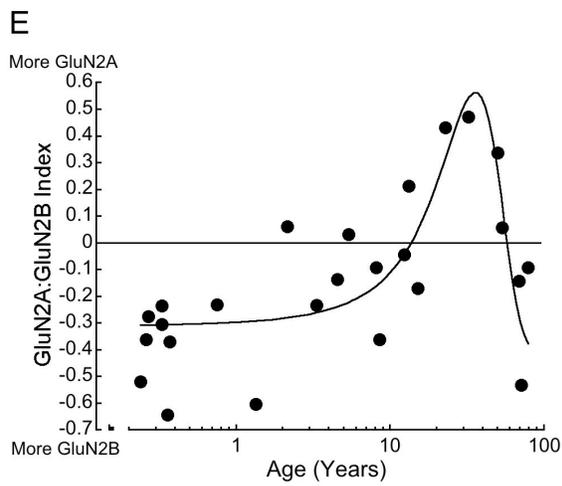
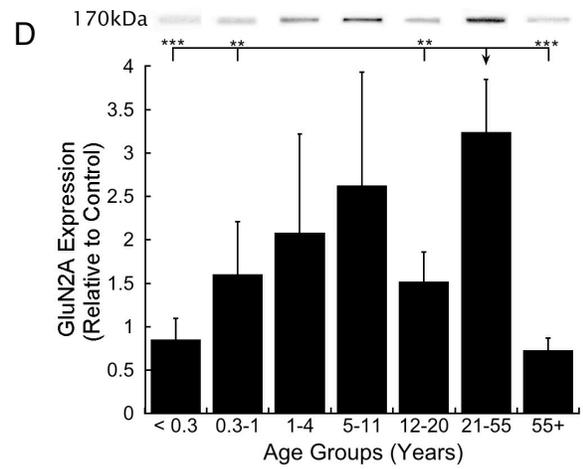
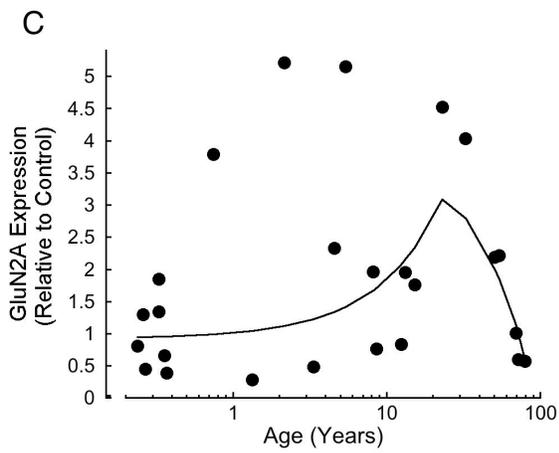
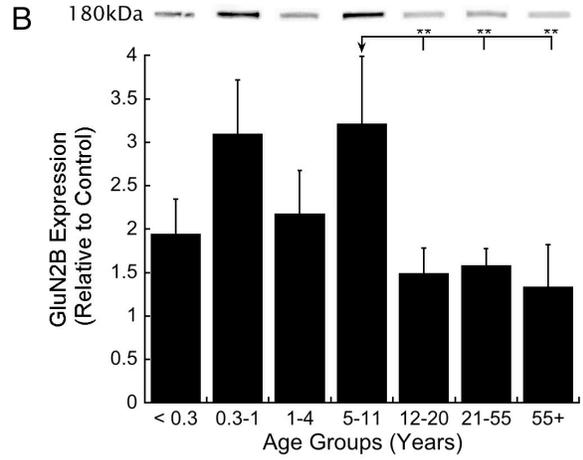
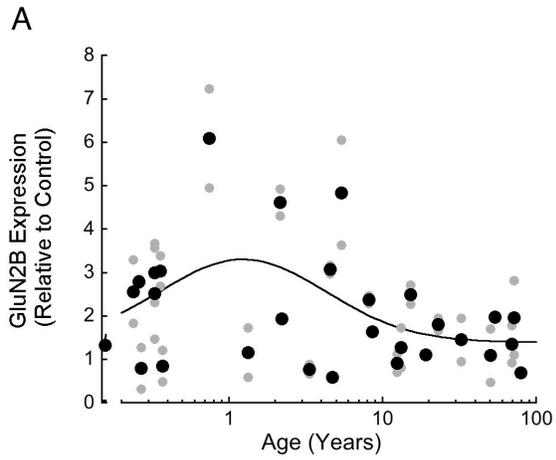


**Figure 2.** Development of the AMPA:NMDA balance ((GluA2-GluN1)/(GluA2+GluN1)) in human V1. (A) A scatterplot of the AMPA:NMDA balance across the lifespan fit with a quadratic function ( $R^2=0.406$ ,  $p<0.0001$ ), which peaked at 10.7 years (95% CI 4.8-23.7 years). (B) Age-Binned results for the AMPA:NMDA balance. Scatterplot, histogram and significance levels plotted using the conventions described in Figure 1.

## **GluN2A and GluN2B subunit expression in human V1**

We examined developmental changes in expression of 2 NMDAR subunits, GluN2A and GluN2B because they affect development of receptive field tuning and ocular dominance plasticity. In particular, the rise in GluN2A and concomitant loss of GluN2B during the CP is one mechanism that causes reduced ocular dominance plasticity in adult cortex (Philpot et al., 2007). The scatterplot of GluN2B expression showed a modest peak during childhood and relatively constant expression through teens, young adults, and older adults (Fig. 3A&B). The GluN2B trajectory was fit by a Gaussian function ( $R^2=0.176$ ,  $p<0.01$ ) that peaked at 1.2 years ( $\pm 0.7$  years) (Fig. 3A). We compared GluN2B expression among the age groups and found higher levels during childhood (5-11 years) relative to teens, young adults, and older adults (Fig. 3B) ( $p<0.01$ ).

The developmental trajectory for GluN2A was different from GluN2B. Initially, GluN2A expression was low, then during childhood and teenage years was variable (8 cases with low and 3 cases with high GluN2A expression) followed by high expression in young adults and ending with a ~75% decline into aging. The variability during childhood reduced the goodness-of-fit for a Gaussian function so instead we plotted a simple descriptive weighted curve (Fig. 3C). Interestingly, the 3 childhood cases with high GluN2A expression also had high GluN2B expression. Binning the results into age groups showed that young adults had more GluN2A expression than infants ( $p<0.001$ ), young children ( $p<0.01$ ), teens ( $p<0.01$ ), and older adults ( $p<0.001$ ) (Fig 3. D).



**Figure 3.** Development of GluN2B, GluN2A, and the 2A:2B balance in human V1. (A) A scatterplot of GluN2B expression across the lifespan fit with a Gaussian function ( $R^2=0.176$ ,  $p<0.01$ ), with peak expression at 1.2 years ( $\pm 0.7$  years). (B) Age-Binned results for GluN2B expression. (C) A scatterplot of GluN2A expression across the lifespan fit with a weighted curve. (D) Age-Binned results for GluN2A expression. (E) A scatterplot of the 2A:2B balance across the lifespan fit with a Gaussian function ( $R^2=0.633$ ,  $p<0.0001$ ), which peaked at 35.9 years ( $\pm 4.6$  years). (F) Age-Binned results for the 2A:2B balance. Scatterplots, histograms and significance levels plotted using the conventions described in Figure 1.

## **2A:2B balance: protracted change across the lifespan**

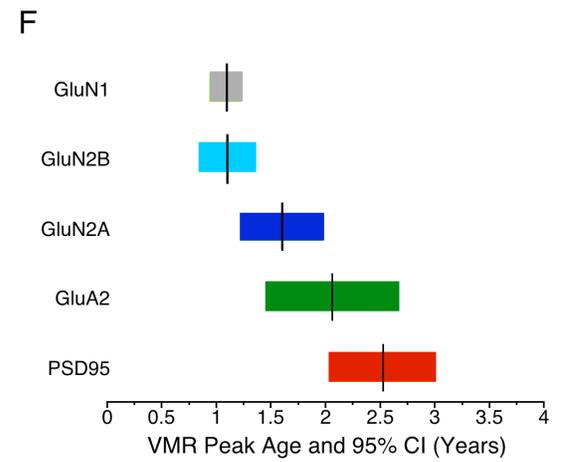
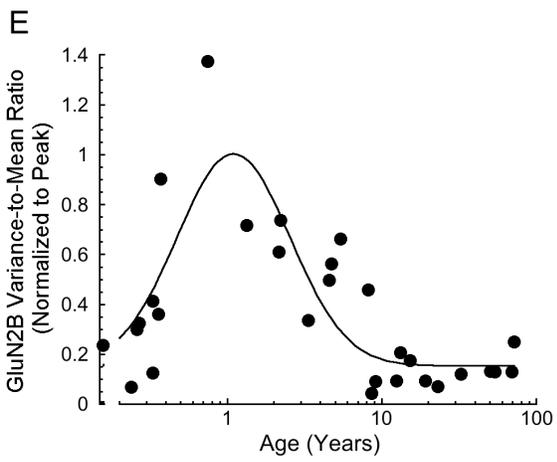
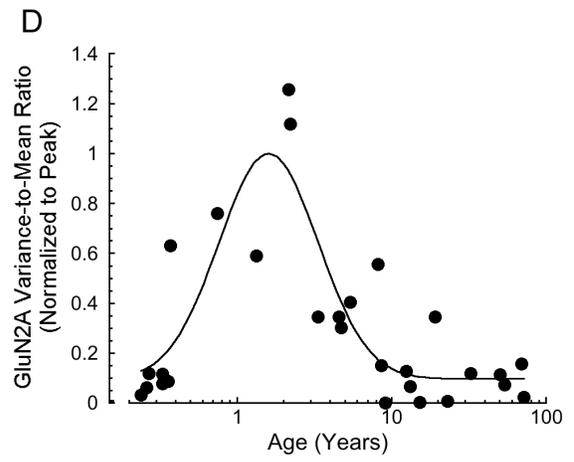
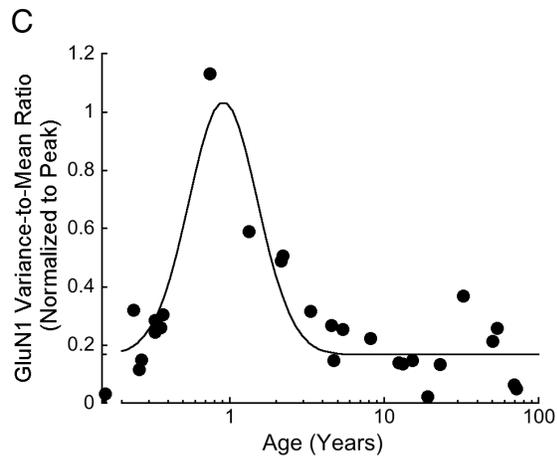
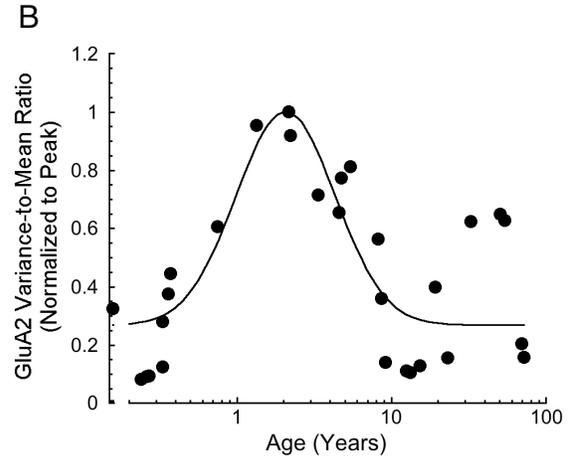
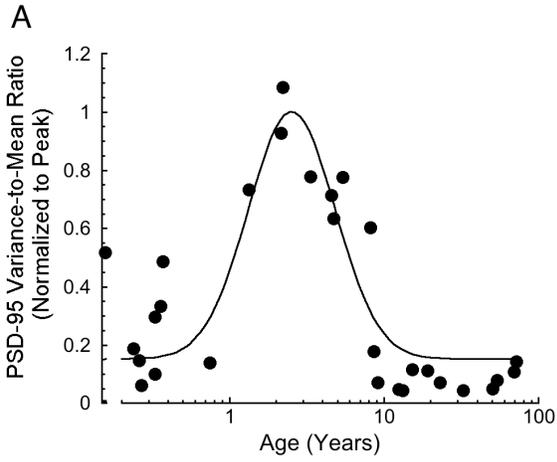
Visual experience drives the 2A:2B balance in favour of GluN2A (Quinlan et al., 1999a) and that shift regulates the synaptic modification threshold for engaging long-term potentiation (LTP) versus long-term depression (LTD) (Philpot et al., 2007). Since the 2A:2B balance is a key mechanism regulating visual experience-dependent metaplasticity, we analyzed it for human V1 by calculating an index of relative 2A:2B expression for each case. Here we found an orderly progression from more GluN2B under 5 years of age, to roughly balanced GluN2B and GluN2A during childhood and teen years, to a peak with more GluN2A during adulthood, followed by a drop back to more GluN2B in aging (Fig. 3 E,F). These changes in the 2A:2B balance were fit by a Gaussian function ( $R^2=0.633$ ,  $p<0.0001$ ) that peaked at 35.9 years ( $\pm 4.6$  years) (Fig. 3E). The binned results illustrate the progressive shift towards significantly more GluN2A in adulthood and shift back to GluN2B in aging (Fig. 3F). The orderly shift of this 2A:2B balance, especially through childhood, was somewhat surprising since the individual subunits showed a lot of variability at that stage. The lack of variability in the 2A:2B index suggests that the balance between this pair of subunits, rather than the absolute amount of each, is a critical component for GluN2A and GluN2B regulation of developmental plasticity.

## **Waves of inter-individual variability during childhood**

Many studies of human brain development and function have found large inter-individual variations including our studies of synaptic and non-synaptic proteins in human V1 where we analyzed inter-individual variability and found waves of higher variability in childhood (Pinto et al., 2015; Siu et al., 2015). Here we applied the same approach and calculated the Fano factor to

determine how the variance-to-mean ratio (VMR) changed across the lifespan for the current set of glutamatergic proteins.

We found that each glutamatergic protein had a wave of higher inter-individual variability during childhood that was well fit by a Gaussian function (Fig 4A-E). There was a progression in the peak age of inter-individual variability (VMRs) that began with GluN1 and GluN2B at 1.1 years (GluN1, +/- 0.2 years,  $R^2= 0.8$ ,  $p < 0.0001$ )(GluN2B, +/- 0.3 years,  $R^2= 0.618$ ,  $p < 0.0001$ ), to GluN2A at 1.6 years (+/- 0.4 years,  $R^2= 0.694$ ,  $p < 0.0001$ ), to GluA2 at 2.1 years (+/- 0.6 years,  $R^2= 0.641$ ,  $p < 0.0001$ ), to PSD-95 at 2.5 years (+/- 0.5 years,  $R^2= 0.778$ ,  $p < 0.0001$ ) (Fig 4A-E). We plotted the progression of peak ages in inter-individual variability with the 95% CIs to show that the waves of variability occurred between 1-3 years of age and the peaks for GluA2 and PSD-95 were later than the peaks for GluN1 and GluN2B while the timing of GluN2A variability was intermediate (Fig. 4F).



**Figure 4.** Development of the VMR for PSD-95, GluA2, GluN1, GluN2A, and GluN2B in human V1. Black dots are the VMR for a moving window of 3 cases. Each protein's scatterplot were fit with a Gaussian function, and the data were normalized to the peak of the function. (A) PSD-95 VMR peaked at 2.5 years ( $\pm 0.5$  years) ( $R^2=0.778$ ,  $p<0.0001$ ). (B) GluA2 VMR peaked at 2.1 years ( $\pm 0.6$  years) ( $R^2=0.641$ ,  $p<0.0001$ ). (C) GluN1 VMR peaked at 1.1 years ( $\pm 0.2$  years) ( $R^2=0.8$ ,  $p<0.0001$ ). (D) GluN2A VMR peaked at 1.6 years ( $\pm 0.4$  years) ( $R^2=0.694$ ,  $p<0.0001$ ). (E) GluN2B VMR peaked at 1.1 years ( $\pm 0.3$  years) ( $R^2=0.618$ ,  $p<0.0001$ ). (F) A summary chart showing the progression of peaks of inter-individual variability (vertical black line) and the 95% CI (colored bar) for each protein.

## 5.4 Discussion

Our results show that development of glutamatergic synaptic proteins in human V1 mirror changes in visual perception across the lifespan. Human visual perception matures in stages, beginning with the onset of basic visual functions (Braddick et al., 2005), to maturation of acuity and contrast sensitivity (Elleberg et al., 1999), to peak performance on higher-level tasks such as object or face perception (Germine et al., 2011; Hartshorne and Germine, 2015), and finally to loss of visual functions during aging (Owsley, 2011). The glutamate receptor proteins (PSD-95, GluA2, GluN1, GluN2A, GluN2B) and balances (AMPA:NMDA, 2A:2B) studied here showed a pattern of 5 stages, similar to maturation of GABAergic proteins in human V1 (Pinto et al., 2010). This leads to an interesting conclusion that synaptic changes in human V1 are likely to be significant factors underlying changes in visual perception and plasticity across the lifespan.

It is important to consider the strengths and limitations of quantifying expression of glutamatergic synaptic proteins in human V1 to address questions about the role of V1 in maturation of visual perception. Here, we used a synaptosome preparation to concentrate synaptic proteins; this preparation aids reliable quantification of low abundance synaptic proteins using Western blotting. The proteins studied here regulate fundamental aspects of excitatory neurotransmission (Cull-Candy et al., 1998), visual plasticity (Turrigiano, 2008; Yashiro and Philpot, 2008; Cooke and Bear, 2014), and receptive field properties in V1 (Fagiolini et al., 2003; Self et al., 2012). Furthermore, excitatory synapses make up more than 80% of the synapses in primate V1 (Beaulieu et al., 1992). The number and location of synapses expressing these proteins, or which cell types and circuits are changing across the lifespan, however, remain

unstudied. The current findings provide a blueprint to focus anatomical studies on key stages of synaptic change and identify potential age-appropriate targets for plasticity based therapies.

### **Five stages of post-synaptic glutamatergic protein development in human V1**

#### **Stage 1: the first year**

The first stage of human V1 development had a high level of GluN1 expression then a rapid loss at ~1 year causing a switch in the AMPA:NMDA balance to relatively more GluA2. That pattern suggests initial dominance by NMDAR-containing silent synapses that are rapidly replaced by AMPAR-containing active synapses (Flint et al., 1997; Rumpel et al., 1998). The loss of GluN1 at ~1 year coincides with a loss of the endocannabinoid receptor CB1R in human V1 (Pinto et al., 2010). CB1R plays a central role in establishing intra-cortical and inter-cortical patterning of nascent excitatory connections (Harkany et al., 2008). Those high levels of CB1R and GluN1 align with anatomical development of intra-cortical horizontal connections under 1 year (Burkhalter et al., 1993) and foreshadow maturation of inter-cortical connections at ~2 years (Burkhalter, 1993).

Previously, we found a rapid shift in a measure of the excitatory:inhibitory balance (PSD-95:gephyrin) to more PSD-95 at 4-6 months (Pinto et al., 2015). Experience-dependent plasticity is also quick during this stage, since just 1 hour of visual experience is enough to improve acuity of an infant treated for congenital cataracts (Maurer et al., 1999). Perhaps the first year of life reflects the system establishing a networks of nascent excitatory synapses to support continued development and refinement of V1 circuits.

## **Stage 2: young children (1-4 years)**

The second stage of V1 development had a modest peak for GluN2B that contributed to keeping the 2A:2B balance in favor of GluN2B. The AMPA:NMDA balance switched to favor GluA2-containing AMPARs and there was a series of waves of inter-individual variability.

Many animal studies of V1 have shown that the 2A:2B balance contributes to developmental plasticity and visual function (Quinlan et al., 1999a; Erisir and Harris, 2003; Philpot et al., 2007; Cho et al., 2009; Smith et al., 2009; Durand et al., 2012). The modest bump of GluN2B expression in young children may reflect maturation of layer 4 (Erisir and Harris, 2003), while the dominance of GluN2B in the 2A:2B balance suggests a synaptic modification threshold that favors LTP (Philpot et al., 2007; Yashiro and Philpot, 2008), making V1 neurons more receptive to potentiation of an open eye's inputs (Cho et al., 2009).

A shift to more GluN2A in V1 is driven by visual experience (Quinlan et al., 1999b) and we found here and previously (Murphy et al., 2005) that the 2A:2B balance begins to shift in young children. GluN2B, however, is necessary for sustained development of visual acuity (Durand et al., 2012) and GluN2A is necessary for development of orientation selectivity (Fagiolini et al., 2003) highlighting the importance of this stage for visual development. Furthermore, the increase in GluA2-containing AMPARs in young children could enhance action potential firing (Savtchouk and Liu, 2011) and support strong feedforward input to V1 (Self et al., 2012; Kooijmans et al., 2014). Those changes would transform responses from weak and sluggish to strong and sustained firing that can support a shift to efficient coding of visual information (Rust et al., 2002). GluA2-containing AMPARs are also required for homeostatic synaptic scaling in developing V1 (Lambo and Turrigiano, 2013). Thus, the combination of more

GluA2 and a 2A:2B balance that favors 2B could support firing patterns and plasticity mechanisms needed for experience-dependent refinement of V1 circuits.

During this stage of development we found waves of inter-individual variability in expression of the glutamatergic proteins. These waves are similar to variability we found previously for pre- (Synapsin, Synaptophysin) and post-synaptic (gephyrin) proteins as well as a non-neuronal protein (Golli myelin basic protein, MBP) (Pinto et al., 2015; Siu et al., 2015). Each protein, however, has different timing for the wave of variability. Here we found a progression of peaks starting with GluN1 and GluN2B at ~1 year, GluN2A at ~1.5 years, GluA2 at ~2 years, and ending with PSD-95 at ~2.5 years. Those waves may indicate true inter-individual variability in young children with cortical development taking off at different ages and with different trajectories. The waves may also represent high levels of intra-individual variability driven by dynamics of network states occurring within each child where expression of each synaptic protein could be high one day and low the next. Since the data here are cross-sectional, we cannot differentiate between these 2 ideas, but the implications of them for cortical development are different. For example, if the waves reflect on-going dynamics of synapses then they could function similar to how feedback from network states shift the processing of olfactory circuits in *C. elegans* (Gordus et al., 2015). In that model, the V1 waves could be driven by environmental or other factors that affect the state of synaptic plasticity and how visual experience interacts with different plasticity mechanisms to fine tune V1 circuits. Rather than thinking of the waves as random or unpredictable, they may reveal a fundamental aspect for developing adaptive circuits that support normal visual processing.

### **Stage 3: older children (5-11 years)**

The third stage of V1 development was characterized by peak expression for PSD-95, GluA2 and the AMPA:NMDA balance. Peak expression for each of those points to their involvement in ending the CP for ocular dominance plasticity (Erisir and Harris, 2003; Huang et al., 2015). In V1 of rats (Huang et al., 2015) and cats (Beston et al., 2010) peak expression for PSD-95 occurs at the end of the CP when PSD-95 consolidates AMPA-containing synapses (Huang et al., 2015). That role for PSD-95 may be important for increasing excitatory drive that reduces internal noise in V1 and improves visual processing in older children (Jeon et al., 2014). The 3 peaks also coincide with maturation of low-level visual perception (Elleberg et al., 1999) and the end of susceptibility for developing amblyopia (Epelbaum et al., 1993; Keech and Kutschke, 1995; Lewis and Maurer, 2005).

### **Stage 4: teens and young adults (12-55 years)**

The fourth stage was characterized by protracted development of GluN2A and the related late switch of the 2A:2B balance. Peak expression of GluN2A did not occur until ~40 years of age which may seem like surprisingly slow development for human V1, but it is comparable to development of the GABAergic proteins (GAD65 and GABA<sub>A</sub>α1) (Pinto et al., 2010) as well as cortical myelin (classic-MBP) (Siu et al., 2015). Protracted development of the 2A:2B balance is long enough for NMDAR-mediated surround modulation (Self et al., 2012) to contribute to late development of high-level visual perceptions (Hartshorne and Germine, 2015).

The shift to more GluN2A expression in young adults suggests a synaptic modification threshold that makes it more difficult to engage LTP (Yashiro and Philpot, 2008). GluN2A-containing NMDARs are more stable (Groc et al., 2006) and their activation promotes cell

survival(Liu et al., 2007). These suggest a time of synaptic stability in human V1 and perhaps why visual training can take 1000's of trials to improve amblyopic vision (Levi and Li, 2009). The protracted 2A:2B shift may reflect maturation of NMDARs on PV+ inhibitory interneurons because, as found in mouse V1, the developmental shift to more GluN2A is slower for PV+ cells than pyramidal neurons (Mierau et al., 2016). Fast-spiking PV+ cells also have GluA2-containing AMPARs (Kooijmans et al., 2014), so they are a site where changes in visual experience might activate inhibitory and excitatory aspects of short-term plasticity in human V1 (Lunghi et al., 2015a; 2015b).

#### **Stage 5: aging (>55 years)**

The last stage of human V1 changes saw a dramatic ~75% loss of GluN2A expression, bringing it back to levels found in infants (<1 year of age). In contrast, there was no change in GluN2B expression. As a result, the 2A:2B balance in older adults switched back in favor of GluN2B, which may affect processing of visual information, synaptic plasticity, and age-related degeneration in human V1.

Age-related changes in human vision (Bennett et al., 2007; Betts et al., 2007) and monkey receptive field properties (Wang et al., 2005; Zhang et al., 2008) have been described as resulting from reduced signal-to-noise involving loss of inhibition. GluN2A-containing NMDARs are dense on PV+ inhibitory interneurons in young mice (Mierau et al., 2016) and the loss of GluN2A may link excitatory and inhibitory changes in aging. In addition, the shift in aging to relatively more GluN2B could contribute to poor signal-to-noise because those receptors have slower decay time and weaker conductances (Cull-Candy et al., 1998; Vicini et al., 1998). It could also facilitate synaptic plasticity by adjusting the synaptic modification threshold to more

readily engage LTP. That plasticity, however, may come at a cost of higher metabolic stress and GluN2B-activated excitotoxicity (Liu et al., 2007). Thus, the specific loss of GluN2A in aging could be a harbinger of degeneration in human V1.

### **Summary**

The current results and our other investigations of human V1 show that synaptic and non-synaptic proteins develop gradually through a series of orchestrated stages that extend across the lifespan (Murphy et al., 2005; Pinto et al., 2010; Williams et al., 2010; Pinto et al., 2015; Siu et al., 2015). The glutamatergic proteins studied here are central players in visually-driven plasticity, receptive field properties, and visual function making it likely that on-going changes in human V1 participate in all stages of visual development and plasticity. Finally, these findings may guide selection of age-appropriate plasticity-based therapies that facilitate translation to clinical applications.

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## **Chapter 6. General Discussion**

## 6.1 Summary of Main Findings

In this thesis, I have advanced the translation of plasticity- and neuroprotection-enhancing therapies by addressing 2 main gaps. The first is a gap in understanding the molecular mechanisms targeted by therapies that have been successful in animal models. The second is a gap in our understanding of how those molecular mechanisms normally develop in humans.

In chapter 2, I address the first gap by investigating the effects of fluoxetine, alone or in combination with MD, on glutamatergic and GABAergic synaptic proteins. I hypothesized that fluoxetine might reinstate juvenile-like synaptic composition in terms of synaptic maturity, metaplasticity, or the E/I balance. Instead, fluoxetine either had no effect or favored expression of mature subunits (GluN2A and GABA<sub>A</sub>α1). Also, MD increased the expression of glutamatergic synaptic proteins (GluA2, GluN1, GluN2A, VGluT1) and shifted both pre- and post-synaptic E/I balances in favor of excitatory mechanisms. Fluoxetine combined with MD normalized expression of glutamatergic proteins and restored the E/I balance, indicating that it may be neuroprotective. Taken together the findings from chapter 2 suggest that fluoxetine does not reinstate an immature synaptic environment, but instead may enhance mature forms of plasticity and promote neuroprotection from glutamatergic gain.

In chapter 3, I expanded upon the neuroprotective findings from chapter 2 by examining the effects of fluoxetine and experience on regulators of receptor trafficking, post-synaptic potentials, integration of excitatory and inhibitory transmission, and ocular dominance plasticity. I confirmed my previous finding that that MD in adult animals increases GluA2 expression, and found similar increases in 3 other AMPAR proteins: pGluA2, GRIP1, and PICK1. MD also reduced the expression of the inhibitory post-synaptic potential regulator KCC2, which is

consistent with a homeostatic response. Fluoxetine alone had no effect except a minor reduction of CB1R, but when combined with MD, fluoxetine brought each of the MD-driven changes back to normal. Although this suggests that fluoxetine is neuroprotective, none of these effects are consistent with a juvenile-like synaptic environment. Next, we studied the expression of Ube3A, a protein that is necessary for experience-dependent plasticity during the critical period (Yashiro et al., 2009; Sato and Stryker, 2010). Interestingly, MD caused a loss Ube3A, but combining fluoxetine with MD increased expression above normal. These changes are similar to changes we have previously found in development: Ube3A expression is highest in juvenile animals and is reduced in aging (Williams et al., 2010). Together chapter 2 and 3 suggests that while the behavioral plasticity observed after fluoxetine treatment is juvenile-like, the synaptic environment is not, and that Ube3A-dependent mechanisms may play a key role for reinstating juvenile-like plasticity in the adult cortex.

In chapter 4, I began to address whether D-serine, an amino acid with similar behavioral effects as fluoxetine, has similar neurobiological effects on the glutamatergic and GABAergic synaptic proteins that regulate experience-dependent plasticity. I compared the expression of post-synaptic markers of the E/I balance, PSD-95 and gephyrin, and the results were strikingly similar to those of fluoxetine. D-serine alone did not alter the expression or balance of PSD-95 or gephyrin. MD shifted the balance in favor of excitation, but combining D-serine with MD normalized that balance. Although future studies will need to compare more proteins to determine how similar the neurobiological effects are, these results suggest that D-serine and fluoxetine may share common mechanisms.

Despite the progress in reinstating plasticity in the adult cortex with treatments like fluoxetine and D-serine, there have been very few human applications of the knowledge gained from animal studies. This is partly because the development of plasticity-related molecules in human cortex is unknown. In chapter 5, I addressed this by studying the development of 5 glutamatergic synaptic proteins, which have crucial roles in synaptic plasticity, in human V1. I found an early loss of GluN1 and prolonged development of PSD-95 and GluA2 into late childhood. GluN2A development continued until ~40 years, followed by a dramatic loss in aging. The silent synapse index (GluA2:GluN1) switched at ~1 year but the metaplasticity index (GluN2A:GluN2B) continued to shift until ~40 years before changing back to GluN2B in aging. I also found a unique period of heightened inter-individual variability in young childhood. The results demonstrate that human V1 develops gradually through a series of 5 orchestrated stages, with some aspects developing early, as would be predicted from animal studies, while others (i.e., GluN2A and the metaplasticity index) are unique in that they continue to develop across the lifespan. In addition to establishing the normal development of these proteins, the finding of prolonged development of synaptic proteins in V1 may help reconcile the discrepancy between the timelines of early structural development of V1 and later functional development of vision.

## **6.2 Methodological Considerations**

### **Western blotting**

The principal technique used in my thesis is Western blotting, which has inherent strengths and weakness. Western blotting is a rapid, reliable, and relatively inexpensive method for probing a large set of proteins. While this approach does not provide information about localization to specific layers, cell-types, or sub-cellular compartments, it does facilitate analysis of a large set of proteins which provides a more complete snapshot of the molecules affected by each experimental condition. Follow-up studies using more advanced anatomical techniques will be needed to confirm the specific location and functional consequences of the changes reported in this thesis. Another strength is that our Western blotting protocol allowed reprobing of blots, which meant that many comparisons of protein expression could be done within a sample, minimizing experimental error. Reprobing was especially beneficial for making the most of valuable and rare fresh frozen human tissue. Furthermore, this technique worked robustly across species, which is an important step in facilitating translation.

For some synaptic proteins, I used a synaptosome preparation, which aids in the reliable quantification of low abundance synaptic proteins by enriching them in the sample. Importantly, using this preparation reduces the information about extrasynaptic and non-neuronal changes, and thus is only appropriate for testing predictions about synaptic changes.

### **Species and translation**

To translate the molecular effects of treatments like fluoxetine and D-serine to humans, we need to understand the baseline development of these molecules in the human brain. Although new techniques are being developed, this line of research is largely limited to postmortem

analysis, so I studied a collection of post-mortem human V1 that spanned across the lifespan, from 20 days to 80 years. Post-mortem human studies like this, however, are often constrained to smaller samples sizes. Larger studies with access to more brains will be necessary to determine more precise trajectories, but our findings provide an important map to guide future studies and attempts at translation.

An important question for research that aims to facilitate translation is whether results are truly meaningful when compared across species. In this thesis, both rat and human samples were probed with the same primary and secondary antibodies, in similar concentration, and showed similar expression levels. In the past, our lab has shown that synaptic proteins provide a good method for translating synaptic development between rats and humans (Pinto et al., 2015). In addition, rodents and humans share 75% 1:1 gene orthologs (Church et al., 2009), but slight differences in the genome make translation difficult and indicate that animal models are incomplete. Several advancements will allow future studies to bridge that gap in translation. These include the use of more intermediary species, especially primates; the development of techniques that allow detailed neurobiological measurements *in-vivo* in humans; and clinical trials. Some of these are already being undertaken. For example magnetic resonance spectroscopy has been used to quantify neurotransmitter changes in humans (Lunghi et al., 2015b), exciting new techniques are being developed to image synaptic density in live humans (Finnema et al., 2016), and a clinical trial for the use of fluoxetine with amblyopes is currently underway (Li et al., 2011).

## 6.3 Significance

### Reinstating juvenile-like plasticity

While many techniques have reinstated behavioral plasticity in the adult cortex (Müller and Best, 1989; Pizzorusso et al., 2002; He et al., 2006; Sale et al., 2007; Vetencourt et al., 2008; Yang et al., 2011), it remains unknown whether that plasticity is truly “juvenile-like”. The findings in chapters 2 and 3 indicate that fluoxetine does not reinstate a completely juvenile-like synaptic environment. In fact, it shifted both the NMDAR and GABA<sub>A</sub>R composition in favor of more mature subunits. Interestingly, both of these shifts support faster receptor kinetics (Laurie et al., 1992; Cull-Candy et al., 2001), which may improve spike time dependent plasticity. Of the 18 proteins studied in this thesis, the only change that was consistent with a juvenile synaptic environment was an increase in Ube3A when fluoxetine was combined with MD. Ube3A is highly expressed in juvenile animals (Williams et al., 2010) and is necessary for critical period plasticity (Yashiro et al., 2009; Sato and Stryker, 2010), and the findings in chapter 2 and 3 points to it as an important part of reinstating plasticity in the adult cortex. Despite Ube3A’s role in critical period plasticity, this is the first indication that it may also be crucial for plasticity in the adult cortex.

### Neuroprotection

Many adverse events are associated with excitotoxicity. For example, in stroke and traumatic brain injury, the insult is followed by increased activation glutamate receptors leading to excitotoxic neuron death (Hazell 2007, Obernovitch and Urenjank, 2009). *In-vitro* and *in-vivo* studies show that fluoxetine can reduce glutamatergic excitotoxicity caused by NMDA, kainic acid, MDMA, inflammation, and stroke (Lim et al., 2009; Li et al., 2010; Zhang et al., 2012; Vizi

et al., 2013). In chapters 2 and 3, I provide *in-vivo* evidence to support that hypothesis by showing that fluoxetine ameliorated the MD-driven increases in glutamatergic proteins, the shift in E/I balances, and the loss of KCC2. Interestingly, fluoxetine has already been used to treat both stroke (Dam et al., 1996; Chollet et al., 2011) and traumatic brain injury (Horsfield et al., 2002), but its mechanisms were unknown. This thesis provides evidence that fluoxetine mechanisms involve neuroprotection against up regulation of glutamatergic synaptic proteins, and may advance the understanding and application of fluoxetine as a neuroprotective therapy.

### **Safer alternatives**

Although fluoxetine shows great potential as a plasticity-enhancing therapy, it has undesirable physiological and neuropsychiatric side effects (Riddle et al., 2009). This has encouraged a search for an alternative with fewer side effects. D-serine is a non-essential amino acid which, like fluoxetine, reinstates ocular dominance plasticity (Yang et al., 2011) and has antidepressant effects (Otte et al., 2013). These findings show similarities in behavioral effects of these drugs, but whether they share common molecular mechanisms is unknown. Chapters 2 and 4 begin to address this and suggest that fluoxetine and D-serine overlap at the level of synaptic markers for the E/I balance. These results are encouraging and warrant further investigation into the effects of D-serine on the full array of proteins tested with fluoxetine, especially Ube3A, and into the viability of D-serine as an alternative to fluoxetine.

### **Moving translation forward**

More than 25 years ago, Muller et al. (1989) reinstated critical period-like ocular dominance plasticity in adult cats and since then a variety of genetic, molecular, and behavioral techniques have been used to successfully treat amblyopic animals (Müller and Best, 1989;

Pizzorusso et al., 2002; He et al., 2006; Sale et al., 2007; Vetencourt et al., 2008; Yang et al., 2011), yet none of these have translated into effective clinical treatments for amblyopia or other disorders that would benefit from enhanced plasticity. A roadblock in translating has been a lack of understanding about the molecular effects of treatments. Chapters 2, 3, and 4 address this by characterizing the effect of fluoxetine on glutamatergic and GABAergic synaptic proteins involved in experience-dependent plasticity. With that knowledge, the next step in translation is to determine the relevance of those molecular effects for humans. This requires knowledge of the baseline development of these molecules in human cortex. Our lab has quantified the normal development of a wide range of GABAergic synaptic proteins in human V1, but the development of glutamatergic synaptic proteins was unknown. In chapter 5, I address this by describing, for the first time, normal development of 5 major glutamatergic proteins in human V1.

An important finding from this chapter is that some aspects develop early, as in animal models, while others like the GluN2A:GluN2B balance, have a much longer development than would be predicted. These findings suggest caution, and point to opportunities, for plasticity-enhancing interventions like fluoxetine. Since fluoxetine shifts the GluN2A:GluN2B balance in favor of the mature GluN2A subunit, using it in childhood or even young adulthood may inadvertently accelerate maturation of that balance before development is complete.

### **Reconciling structure and function: challenging early development of V1**

The traditional view of human cortical development is that primary sensory areas like V1 mature early in the first few years of life. Since many complex behaviors like object recognition emerge over decades (Kovács et al., 1999; Lewis and Maurer, 2005; Germine et al., 2011), their maturation is typically ascribed to later-developing higher-order cortical areas. Recent studies of

human V1, however, have challenged the notion that primary sensory areas like V1 develop quickly (Gogtay et al., 2004; Sowell et al., 2004; Murphy et al., 2005; Shaw et al., 2008; Pinto et al., 2010; Williams et al., 2010; Pinto et al., 2015). The findings in Chapter 5 indicate that some aspects glutamatergic development also continue into adulthood. These findings add to the literature challenging the view that V1 matures early in life, and suggests primary sensory areas like V1 may indeed develop late enough to influence the later maturation of complex vision.

## 6.4 Future Directions

### Potential for amblyopia

Amblyopia is one of the most common neurodevelopmental disorders, affecting 1-4% of the population (Flom and Neumaier, 1966; Noorden, 1990; Eibschitz-Tsimhoni et al., 2000), yet treatments have hardly progressed since patching was first described in the 1700s (Barrett et al., 2004). Moreover, patching has low patient compliance, is ineffective for many patients over the short and long term (Birch, 2013), and is of little use for patients older than 12 years (Epelbaum et al., 1993). Understanding the molecular mechanisms behind drugs that treat amblyopia in animal models is an important step towards developing effective therapies for human amblyopes. The next step is to begin applying this knowledge in the clinic, and to that end clinical trials to treat amblyopia with fluoxetine are already underway in Finland, Estonia, India and New Zealand (personal communication with L Maffei cited in Bavelier et al., 2010). The work in this thesis raises several interesting questions for these trials. First, one of the most consistent findings in this thesis is that fluoxetine's effect on protein expression was mostly latent and only revealed when fluoxetine was combined with a change in experience. This raises the question: to what extent is a change in the patient's sensory experience necessary for recovery? Do they require normal visual experience, binocular training, patching, or some combination? Second, the effect of fluoxetine on inhibition remains unclear: it reduces intracortical GABA (Vetencourt et al., 2008) but also increases the expression (Beshara et al., 2015) and sensitivity (Robinson, 2002) of GABA<sub>A</sub>α1 subunits. This raises the question: what effect does treatment have on intracortical inhibition? MRS measurements of neurotransmitters, combined with functional measures of inhibition in V1 (e.g., centre-surround antagonism, Tadin et al., 2003) may help to

answer that question. Third, the GluN2A:GluN2B index normally peaks in favor of GluN2A in the fourth decade (chapter 5), but fluoxetine shifts the index in favor of GluN2A (chapter 2), raising the question: could fluoxetine interfere with normal development? An excessive shift in favor of GluN2A during development has been linked to Rett syndrome (Durand et al., 2012), so it will be important to determine whether fluoxetine-treated patients exhibit any signs of altered development.

### **Potential for Angelman syndrome**

Despite studying the effect of fluoxetine on 18 proteins (chapters 2 and 3), only a single marker was consistent with a shift to a juvenile-like synaptic environment: Ube3A. This intriguing result opens the door between research on reinstating plasticity and neurodevelopmental disorders affected by Ube3A, like Angelman syndrome. Angelman is a genetic neurodevelopmental disorder characterized by developmental delay, language, cognitive, and motor dysfunction, seizures, and autistic behavior (for review see Clayton-Smith and Laan, 2003). It is caused by deficient expression of the maternal UBE3A gene (Kishino et al., 1997). In mouse models, the loss of Ube3A protein impairs critical period plasticity (Yashiro et al., 2009; Sato and Stryker, 2010). In chapter 3, I found that MD reduced Ube3A expression, whereas combining fluoxetine with MD increased it. To the extent that fluoxetine can increase Ube3A expression in the low-Ube3A condition of Angelman syndrome, as it did in the low-Ube3A condition of adult MD, fluoxetine may have therapeutic uses for Angelman. Interestingly fluoxetine has already been shown to improve the loss of neurogenesis and parvalbumin in animal models of Angelman (Godavarthi et al., 2014; 2015). My thesis provides a potential mechanistic explanation, but raises important questions for moving forward. First, does

fluoxetine ameliorate classic symptoms of Angelman syndrome in animal models? Second, does it increase expression of the paternal UBE3A gene in animal models? Third, does it need to be administered early in development before symptoms appear, or can it treat symptoms in mature animals? All of these are important and interesting questions that may lead to synergies between research on plasticity and neurodevelopmental disorders.

### **Potential for Age-related vision loss**

Age-related vision changes have been linked to a loss of inhibition (Leventhal et al., 2003; Owsley, 2011). Interestingly, the GluN2A:GluN2B balance is fivefold greater on PV+ inhibitory interneurons than on excitatory pyramidal neurons, and PV+ interneuron function is impaired when that ratio is reduced (Kinney, 2006). I found a ~75% loss of GluN2A, and switch back to GluN2B, in old adults (chapter 5). If that loss affects PV+ inhibitory interneurons, it may contribute to reduced inhibition in aging. In chapter 2, I found that fluoxetine switched the GluN2A:GluN2B balance in favor of GluN2A. If fluoxetine can do the same in the V1 of old adults, it may help treat age-related vision loss. Before testing in humans, however, there are important questions to address. First, in old adults, is GluN2A specifically lost from PV+ interneurons? Second, can fluoxetine shift the balance in favor of GluN2A in old animals, like it does in young animals? It seems likely that the loss GluN2A in old adults effects V1 function, but the precise consequences on the neurobiology underlying vision, and whether it can be reversed with fluoxetine, remains to be seen.

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