ANALYSIS OF AMPA RECEPTOR TRAFFICKING

AMPA RECEPTOR TRAFFICKING: A MECHANISM OF EXCITATORY SYNAPTIC PLASTICITY

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TITLE: AMPA Receptor Trafficking: A Mechanism of Excitatory Synaptic Plasticity

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Abstract

Glutamate is the main excitatory neurotransmitter in the cortex and the tetrameric AMPA receptors (AMPARs) mediate the fast excitatory component of postsynaptic potential. Trafficking of AMPARs has been implicated in synaptic plasticity regulation, including long-term potentiation, long-term depression, and synaptic scaling. Two proteins, GRIP for stabilization at the synapse and PICK for internalization, are involved in trafficking GluR2-containing AMPARs in and out of the synapse. During development, GluR2 expression increases substantially, but we do not know the developmental trajectories of GRIP and PICK expression or if the balance between these proteins changes. In Chapter 2, using quantitative Western Blot analysis, I addressed the changes in the mechanisms of AMPAR trafficking by characterizing the developmental trajectories of GluR2, the phosphorylated form pGluR2, GRIP, and PICK and comparing expression in visual vs. frontal cortex. I found significant differences between cortical areas in the developmental trajectories of GluR2 and pGluR2. In visual cortex, expression levels exhibited smooth developmental increases. In frontal cortex, GluR2 and pGluR2 rose to an exuberant expression between P18 and P35. Developmental trajectories for GRIP and PICK showed smooth increases that were consistent across cortical areas. Furthermore, looking at the correlation between the surface components (GluR2 and GRIP) and internalized components (pGluR2 and PICK), I found that the development of AMPAR trafficking components is tightly regulated across the cortex.

In Chapter 3, I looked at AMPAR expression in adult cortex, which is believed to be relatively rigid. Fluoxetine has previously been reported to induce a juvenile like state of plasticity in visual cortex and this plasticity was assessed through monocular deprivation. My results indicated that fluoxetine administration was not associated with significant changes in AMPAR expression levels. However, monocular deprivation induced significant upregulation in expression levels of all four proteins. These results imply the presence of AMPAR-mediated plasticity in the adult brain.

Preface

Chapter 2 of this thesis is a paper in preparation for submission to Frontiers in Neuroscience. In Chapter 3, I present my part from a lab-wide collaboration in defining synaptic markers of adult plasticity.

Acknowledgements

In the completion of this thesis, I received a lot of help and support from people around me. Most importantly, I would like to thank my supervisor, Dr. Kathy Murphy, who was incredibly helpful and provided her expertise and guidance every step of the way. I would also like to thank Dr. Jones for his help in data analysis. Thanks to Dr. Beston for providing tissue for the fluoxetine experiment. Deepest gratitude is due to the members of my supervisory committee, Dr. Patrick Bennett and Dr. Joseph Gabriele for their understanding and assistance in the completion of this thesis. Finally, many thanks to the Murphy lab members for making this whole experience an enjoyable one.

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Declaration of Academic Achievement

For the experiment presented in Chapter 2, I was extensively involved in all aspects of the work, including formulation of the experiment, tissue collection, Western Blotting, and analysis.

For the experiment presented in Chapter 3, cortical tissue was provided by Dr. Brett Beston. I was involved in tissue preparation, Western Blot, and analysis.

Chapter 1

General Introduction

Cortical Development

The developing brain is very different from the adult brain. As opposed to humans, a considerable amount of central nervous system development in rodents occurs post natally (Rice & Barone, 2000). Critical periods of development refer to times when the brain is especially vulnerable to changes. Exposure to various forms of abnormal experience is more likely to cause permanent changes if it occurs during these critical periods of development.

The precursors for the brain and spinal cord, which comprise the central nervous system, develop early in embryogenesis during a process called neurulation. Neurulation in the rodent is complete by gestational day (GD) 11. During the second gestational week (~GD 9.5 in rodents), parts of the brain begin to form with the neurogenesis and migration of cells in the forebrain, midbrain, and hindbrain (Angevine & Sidman, 1961). The posterior structures of the brain develop prior to the anterior and the medial aspects develop before the lateral ones. Brain formation follows a specific sequence of events, which include proliferation, migration, differentiation, synaptogenesis, apoptosis, and myelination. The extent and length of neurogenesis vary within the regions of the brain (Rice & Barone, 2000).

The mature cortex has six histologically distinct layers, where layers VI is the deepest layer and layer I is the most superficial. By GD 16 and 17, the recently proliferated cells are arriving in the deepest layer of the brain, which forms first (Angevine & Sidman, 1961). Through the remainder of gestation and

continuing post natally, cells proliferate and migrate to the superficial layers. As neurons migrate, the combination of intracellular and extracellular cues guide the expression of genes, which further determine the specific niche of the neurons (Rice & Barone, 2000).

Synapse maturation occurs over the first three postnatal weeks in rats (Jacobson et al., 1991), but is more prolonged in other species, including humans (Uylings & van Eden, 1990). Synaptogenesis requires complex biochemical and morphological interactions between the pre-and post-synaptic elements. The correct timing of the pre-synaptic signal and post-synaptic response is crucial in establishing a synaptic connection.

Apoptosis, programmed cell death, is an important part of both prenatal and postnatal CNS development. At the level of the synapse, lots of connections are pruned during development, leaving the more efficient synaptic configurations (Rice & Barone, 2000).

Mechanisms of Cortical Plasticity

The developing brain is highly plastic and possesses the fundamental property of responding to experience. The response of the visual cortex to changes in sensory experience serves as one of the most profound examples of cortical remodeling. Work by David Hubel and Torsten Wiesel demonstrated a significant remodeling of the visual system in the kitten in response to a surgical occlusion of one eye (Wiesel & Hubel, 1965). In response to monocular

deprivation, the visual system was modified such that visual cortex consisted primarily of representation from the unoccluded eye (Wiesel & Hubel, 1965).

Other cortical areas respond readily to changes in the external environment. Comparing the development of rodents reared in enriched environments versus typical laboratory settings, demonstrated that those exposed to higher levels of sensory stimulation had significantly increased cortical thickness (Diamond et al., 1975), synaptic density (Diamond et al., 1976), and dendritic branching (Greenough & Volkmar, 1973).

The remodeling and refining of connections in the CNS continues throughout the entire period of development and into adulthood and it is a complex process involving multiple synaptic and cellular mechanisms (Bliss & Cooke, 2011). These mechanisms have been proposed to include both physiological and structural changes (Feldman, 2009).

Early models of physiological mechanisms leading to plasticity changes focused on rapid excitatory NMDA receptor dependent changes in synaptic efficacy, followed by slower structural cortical network remodeling (Bear et al., 1987; Katz & Shatz, 1996). The physiological changes were explained by Hebbian forms of synaptic plasticity include long-term potentiation (LTP) and long-term depression (LTD). These are enduring changes in synaptic strength and serve as cellular models of information storage in the central nervous system (Hebb, 1949; Stent, 1973). LTP is a form of activity-dependent plasticity that results in a persistent enhancement of synaptic transmission. In classical

examples of LTP, calcium from postsynaptic NMDA receptors activates specific kinases, which interact with AMPA receptors, leading to their insertion at the synapse (Malinow & Malenka, 2002). In LTD, the efficacy of synaptic transmission is reduced (Hebb, 1949). Instances where NMDA receptor activation leading to AMPA receptor internalization have been observed in various regions of the cortex (Feldman et al., 1998; Crozier et al., 2007; Deng & Lei, 2007).

Others proposed that rapid structural changes were the means to plasticity in the developing brain (Berardi et al., 2003). Recently, with the development of new sensitive methods for looking at the cortex, many new forms of plasticity have been described and include Hebbian as well as non-Hebbian forms of plasticity (Feldman, 2009).

In the cortex, there persists a delicate balance between excitatory and inhibitory networks that is critical for proper sensory processing (Beston et al., 2010). Changes in the excitatory (AMPA and NMDA) (Kim & Linden, 2007) and inhibitory (GABA) (Hensch, 2005; Foeller & Feldman 2004) networks have been found to contribute to cortical remodeling. The maturation of the inhibitory GABA network is believed to regulate the onset of the critical period in primary visual cortex (Hensch, 2005).

In a non-Hebbian form of plasticity, increases or decreases in sensory drive induce compensatory homeostatic changes in an attempt to restore cortical activity to a set point (Turrigiano & Nelson, 2004). Classic examples of this

include the strengthening of visual responses in the deprived cortex after monocular deprivation (Mrsic-Flogel et al., 2007) as well as the weakening of response in somatosensory cortex following continuous whisker stimulation (Knott et al., 2002).

Visual Perception

In mammals, light from an image enters the eye at the cornea and is projected onto the retina, where photoreceptors, the rods and cones, act to convert the light energy into action potentials, the primary means of communication in the nervous system. Inputs from photoreceptors relay to bipolar cells, signals from which further converge on a single retinal ganglion cell. The information from retinal ganglion cells is sent to the optic nerve, through the optic chiasm, and up the optic tract, where the axons from the retinal ganglion cells terminate in the lateral geniculate nucleus (LGN) of the thalamus. Within the LGN, inputs from each eye are segregated and further project to the primary visual cortex (V1). Within the different layers of V1, there are cells that respond primarily to monocular inputs and neurons that respond to binocular inputs (Kandel et al., 2000). The early experiments by Hubel and Wiesel (1959) first showed that, within the binocular regions, most neurons do not respond to inputs from both eyes equally. The preference of the cells of the visual cortex to one eye or the other is called ocular dominance (OD). Furthermore, within the cortex, inputs from the eyes are organized in alternating columns with left eye/right eye

inputs (Hubel and Wiesel, 1965). The establishment of OD columns happens early in development and is highly dependent on sensory experience. In a series of experiments by David Hubel and Torsten Wiesel (1959, 1962, 1965), it was shown that sensory deprivation in the form of monocular deprivation early in life results in profound effects on the development of the visual system, where the neurons in the visual cortex lose responsiveness to the deprived eye and there is a shift in OD columns towards the updeprived eye.

Ocular Dominance plasticity has been the main model of experience-dependent plasticity in the mammalian CNS and the amblyopic brain serves as a hallmark of measuring cortical plasticity.

AMPA Receptors

Excitatory neurotransmission early in development is mediated primarily by NMDA receptors and the ratio of AMPA:NMDA in the cortex is initially low (Wu et al., 1996; Petralia et al., 1999). These synapses are called silent because, at resting potential, most NMDA ion channels are blocked by Magnesium ions; thus, little response is possible. Petralia and colleagues (1999) used post-embedding immunogold electron microscopy to examine glutamate receptors in the CA1 region of the hippocampus at different times during post-natal development. Synapses showed immunoreactivity for NMDA, which was consistent over the ages investigated, P2, P10 and P35. AMPAR immunoreactivity, however, was

low at P2 and P10, and high at P35. This study was one of the first to show that synapses acquire AMPARs during development (Petralia et al., 1999).

AMPARs are synthesized in the endoplasmic reticulum and Golgi and are then inserted into the plasma membrane at the soma or at synapses (Hollmann & Heinemann, 1994). AMPA receptors exhibit extremely fast kinetics (Jonas, 2000) and are responsible for the majority of excitatory neurotransmission in the central nervous system (Cull-Candy et al., 2006). AMPARs are composed of four subunits, GLUR1-4 or A-D, which assemble to form homo- or heterotetramers with functional differences that depend on specific subunit composition as well as the absence or presence of certain transmembrane AMPA receptor regulatory proteins (TARPS) (Cull-Candy et al., 2006). Furthermore, AMPARs undergo post-translational modifications, chemical alterations to protein structure, which further regulate their function (Santos et al., 2009). Phosphorylation of the AMPAR subunits by protein kinases is an important post-translational modification, one that can ultimately affect multiple receptor properties (Santos et al., 2009). Phosphorylation occurs at different places on the four GluR subunits (Song & Huganir, 2002).

The GluR2 subunit undergoes phosphorylation by Protein Kinase C at the C-terminal sequence within the postsynaptic density 95 (PDZ) domain binding region, an area through which GluR2 binds two important proteins, glutamate receptor interacting protein (GRIP) and protein interacting with C-kinase-1 (PICK1) (Chung et al., 2000; Matsuda et al., 2000; Seidenman et al., 2003;

Santos et al., 2009). Phosphorylation at residue serine 880 causes a change in GluR2's interaction with GRIP and PICK1, where the receptor binding to GRIP1 is decreased and PICK1 is recruited to excitatory synapses, causing rapid internalization of surface receptors (Chung et al., 2000). Osten and colleagues (2000) have shown that mutations of the GluR2 PDZ binding site that selectively block the interaction of the subunit with GRIP and PICK accelerate GluR2 endocytosis at synapses. Therefore, these two proteins act as anchors and the interaction of GluR2 with GRIP and PICK appears to be critical for AMPAR regulation.

Summary – Objectives and Hypotheses

Throughout this introduction, I have shown that AMPA receptors have a primary role in development and plasticity of the cortex. Normal visual system development requires the presence of binocular vision, and this is especially important during the critical period. During development, there is a lot of potential for plastic changes to take place in response to experience; however, with maturation, that capacity decreases and adult brains are considered to be relatively rigid.

In my thesis, my objectives are two fold: first, to look at the development of AMPA receptor trafficking proteins by quantifying trajectories of GluR2, pGluR2, GRIP, and PICK and comparing development in rat visual and frontal cortex (Chapter 2). My second aim is to investigate adult plasticity by quantifying expression of the four proteins involved in AMPA receptor trafficking following fluoxetine administration and monocular deprivation (Chapter 3). I predict that, during development, expression of AMPA receptor trafficking proteins will be highest between P19-P45, the critical period for ocular dominance plasticity (Fagiolini et al., 1994). Furthermore, I expect that developmental trajectories for visual and frontal cortex will be very similar. For the second experiment, I predict that monocularly depriving adult rats following fluoxetine administration will be associated with increases in expression of AMPA receptor trafficking proteins in visual cortex.

Chapter 2

Development of AMPA Receptor Trafficking in Visual and Frontal Cortex

Glutamate is the main excitatory neurotransmitter in the central nervous system and its action is mediated by three ionotropic receptors, N-Methyl-D-aspartic acid (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate (Hollmann & Heinemann, 1994). During cortical development, dynamic changes in excitatory synaptic transmission lead to plasticity. At the level of the synapse, many neurotransmitter receptors are mobile and undergo trafficking between the plasma membrane and intracellular compartments (Song & Huganir, 2002). Changes in the number of glutamate receptors and the time spent at the synaptic surface is an important component of plasticity that directly affect long-term potentiation (LTP) (Malenka & Bear, 2004), long-term depression (LTD) (Heynen et al., 2003), synaptic scaling (Turrigiano & Nelson, 2004), and the excitatory-inhibitory (E/I) balance (Beston et al., 2010) in the developing cortex.

AMPARs are concentrated at synapses, where they mediate the fast component (2ms) of excitatory post-synaptic current (EPSC) (Kleppe & Robinson, 1999). Studies of silent synapses using immunogold electron microscopy have revealed that, early in postnatal development, glutamatergic neurotransmission occurs primarily through NMDA receptors and AMPA receptors are progressively recruited to the synapse during development (Petralia et al., 1999). Structurally, AMPARs are composed of four homologous subunits, GluR1-GluR4, which combine in varying stoichiometries to form ion channels with distinct functional properties (Hollman & Heinemann, 1994; Dingledine, 1999).

Most AMPARs contain the GluR2 subunit, which gives the receptor the characteristic qualities of calcium impermeability (Hollmann & Heinemann, 1994) and a linear current-voltage relationship (Hollmann & Heinemnn, 1994; Dingledine, 1999). The expression of GluR2 increases with development in visual cortex (Hermann, 1996) and is affected by monocular deprivation, leading to subunit phosphorylation and LTD in the deprived cortex (Heynen et al., 2003). Furthermore, blocking neural activity with tetrodotoxin (TTX) has been found to induce synaptic scaling in cultured visual cortical neurons leading to the insertion of GluR2 AMPARs at the synaptic surface (Gainey et al., 2009).

AMPARs undergo multiple transcriptional and translational modifications which further regulate and establish receptor function. Receptor phosphorylation is a major event in synaptic plasticity regulation (Raymond, 1993). Generally, the addition of a phosphate group leads to LTD and removal of phosphate leads to LTP (Song & Huganir, 2002). The inhibition of Phosphate Kinase C (PKC) and CAMKII blocks the induction of LTP in CA1 region of rat hippocampus. Phosphorylation of GluR2 at ser 880 is associated with LTD induction in the hippocampus (Kim, 2001). Furthermore, monocular deprivation results in AMPA subunit phosphorylation and LTD as measured by immunohistomechitry and electrophysiology(Heynen et al., 2003).

Results of electrophysiogical studies of silent synapses in hippocampus first suggested AMPAR levels are regulated dynamically (Isaac et al., 1995; Liao et al., 1995; Durand et al., 1996). This trafficking enhanced or reduced synaptic

function by changing the numbers of AMPARs and mEPSC (O'Brien et al., 1998; Lissin et al., 1998). The endocytosis, exocytosis, and recycling of AMPARs are highly regulated processes requiring specific AMPAR-interacting proteins. GRIP and PICK are involved in trafficking GluR2 containing AMPARs in and out of the synapse (Chung et al., 2000), with GRIP stabilizing GluR2 at the synaptic surface (Dong et al., 1997) and PICK pulling pGluR2 into stable intracellular pools (Gardner et al., 2005; Liu, Cull-Candy et al., 2005). To maintain physiological homeostasis, AMPAR levels at the synaptic surface are regulated dynamically to compensate for variations in input (Turrigiano & Nelson, 2004). A mutation interfering with GRIP function results in a decreased GluR2 accumulation at the synaptic surface (Osten et al., 2000) and a loss of PICK function occludes synaptic scaling (Anggono et al., 2011).

It is well established that AMPARs play a key role in synaptic plasticity regulation and mechanisms by which AMPAR trafficking leads to LTP, LTD, and synaptic scaling are slowly emerging. The developing brain is highly plastic and although a few studies have addressed development of AMPAR subunits (Herrmann, 1996; Beston et al., 2010), none have addressed the developmental changes in phosphorylated GluR2 (pGluR2) or the trafficking proteins GRIP and PICK. Furthermore, none have addressed whether the developmental program for AMPAR trafficking is consistent across sensory and non-sensory cortical areas. Variations in balance of the trafficking mechanism will surely have downstream effects on the dynamic nature of AMPARs and their roles plasticity

(Song & Huganir, 2002). In the current study, we have addressed these questions by quantifying the developmental trajectories and balances for GluR2, pGluR2, GRIP, and PICK and comparing them between visual and frontal cortex. We saw changes in individual proteins and balances, including striking differences for GluR2 expression across the cortical areas. However, the trafficking system remained balanced in visual and frontal cortex. These results imply that the trafficking system for GluR2 develops as an integrated network in the cortex.

Materials & Methods

Samples and Tissue

We studied changes in expression of a set of proteins involved in AMPA receptor trafficking in visual and frontal cortex of 28 Long Evans rats (age 0-105 days) reared with normal visual experience in standard conditions.
All experimental procedures were approved by the McMaster University Animal Research Ethics Board.

Samples were collected from visual cortex (V1) and frontal cortex using stereotaxic coordinates (Shwarz et al., 2006). Rats were euthanized with Euthanol (sodium pentobarbital, 0.165mg/g) and were transcardially perfused with cold 0.1M Phosphate Buffered Saline (PBS, 4°C; pup: 1-2ml/min; adult: 4-5ml/min) until the circulating fluid was clear. The brain was removed from the skull and immersed in cold PBS. Small tissue samples (approx. 2 X 2mm) of presumptive V1 and frontal cortex were sectioned out of the cortex, rapidly frozen on dry ice, and stored at -80°C.

Tissue Sample Preparation

The frozen tissue sample was suspended in cold tissue homogenization buffer (1 ml buffer:50mg tissue, 10 mM HEPES, 1 mM EDTA, 2 mM EGTA, 0.5 mM DTT, 10 mg/L leupeptin, 50 mg/L soybean trypsin inhibitor, 100 nM microcystin, 0.1 mM PMSF,) and homogenized using a glass-glass Dounce homogenizer (Kontes, Vineland, NJ). A subcellular fractionation procedure

(synaptoneurosomes) (Hollingsworth et al., 1985; Titulaer & Ghijsen, 1997; Quinlan et al., 1999) was used to obtain protein samples that were enriched for synaptic proteins. The homogenate was passed through a 5-µm pore hydrophillic mesh filter (Millipore, Billerica, MA, USA) then centrifuged at 4°C and 1000 × *g* for 20 min to obtain the synaptic fraction of the membrane. The supernatant was removed, leaving a pellet enriched for synaptic proteins, the synaptoneurosome. The synaptic pellet was resuspended in boiling 1% sodium-dodecyl-sulfate (SDS) and stored at −80°C. Protein concentrations were determined using the bicinchonic acid (BCA) assay (Pierce, Rockford, IL, USA). Using antibodies for well characterized synaptic markers, the synaptoneurosome samples were compared with whole homogenate to verify that there was a 2-3 fold enrichment for synaptic proteins. A control sample was made by combining a small amount of the prepared tissue sample from each of the cases.

Samples prepared from P0 visual cortex had very low protein levels, too low to be used for immunoblotting. Therefore, for comparison purposes, frontal cortex samples at P0 were excluded from analysis.

Immunoblotting

A Western Blot analysis was performed on the synaptoneurosome samples to quantify protein expression. Samples (30 ug) were separated on 4-20% sodium-dodecyl-sulfate polyacrylamide (SDS-PAGE) gels (Pierce, Rockford, IL) in running buffer (100 mM Tris, 100 mM HEPES, 3 mM SDS; Pierce

Biotechnology Inc, Rockford, IL) and were further transferred to polyvinylidene difluoride (PVDF-FL) membranes (Millipore, Billerice, MA, USA). Each sample was run at least two times in an attempt to obtain the most accurate quantification of the levels of expression. The membranes were incubated in blocking buffer (Odyssey Blocking Buffer 1:1 with PBS) for one hour (LI-COR Biosciences, Lincoln, NE), followed by incubation in primary antibody overnight at 4C at the following concentrations: GluR2, 1:2000, (Imgenex Corporation, San Diego, CA); pGluR2 (GluR2-ser880), 1:200 (PhosphoSolutions, Aurora, CO); GRIP, 1:250, (BD Biosciences, Franklin Lakes, NJ); PICK, 1:200, (NeuroMab, Davis, CA); b-tubulin, 1:4000, (Invitrogen, Carlsbad, CA). Blots were washed (3 X 10 minutes) in PBS containing 0.05% Tween (PBS-T, SIgma, St. Louis, MO), incubated (1h, room temperature) in appropriate IRDye labeled secondary antibody (Anti-Mouse, 1:8000, Anti-Rabbit, 1:10,000) (Li-cor Biosciences, Lincoln, NE), and washed in PBS-T (3 x 10 minuntes). Blots were scanned and fluorescence was quantified using the Odyssey Scanner infrared-imager (LI-COR Biosciences, Lincoln, NE, USA). Blots were stripped using a two-step blot restore kit (Blot Restore Membrane Rejuvenation kit, Chemicon International, Temecula, CA) and further reprobed with additional antibodies.

Analysis

Blots were scanned (Odyssey Infrared Scanner) and band fluorescence was quantified using densitometry (Li-cor Odyssey Software version 3.0; Li-cor

Biosciences; Lincoln, NE, USA). To determine the density profile, a subtraction of the background was performed, intregrating the pixel intensity across the area of the band, and dividing the intensity by the width of the band to control for variations in band size. B-tubulin is a component of microtubules and is expressed in neurons. As such, its expression should not be affected by age of animals. We verified the absence of any age-related correlations in b-Tubulin expression in both cortical areas (both p values > 0.28) and b-Tubulin normalization was used as a loading control. A control sample (a mixture of all the samples) was run on each gel and the density of each sample was measured relative to that control (sample density/control density).

Scattergrams of protein expression were plotted and included the average expression level for each case (dark symbols) and every point from all runs (light symbols). For the purposes of curve fitting, the trends in protein expression through development were classified as either being smooth or exuberant. Smooth changes were fit using the least squares method with linear or exponential decay fits, as appropriate. The goodness of each fit was determined (R) and the time constant (tau) for the change in expression level was calculated from the exponential decay function. The attainment of adult levels of expression was defined as 3 tau, which provided an objective measure of the age at which expression reached 87.5% of its asymptotic level. A membrane transport curve was fit to the data that showed a developmental over expression. When quantitatively establishing a time frame to describe the period of over expression,

the ages between which the expression was half way between maximum expression and adult levels were used; for the purposes of this, adult values were chosen to be at 80 days of age.

Index calculations were carried out to quantify the relative changes in expression levels of the AMPA receptor components during development: changes in trafficking: GRIP:PICK = [(GRIP-PICK)/(GRIP+PICK)]; changes in AMPA receptor subunit state: GluR2:pGluR2 = [(GluR2-pGluR2)/(GluR2+pGluR2)]; changes at the synaptic surface: GluR2:GRIP = [(GluR2-GRIP)/(GluR2+GRIP)]; and changes in the internalized components: pGluR2:PICK = [(pGluR2-PICK)/(pGluR2+PICK)]. These calculations were carried out for each data point of every sample.

To assess global developmental changes in the mechanisms of AMPAR trafficking, correlations between the surface (GluR2 and GRIP) and internalized components (pGluR2 and PICK) were calculated. Also, the difference between the development of surface and internalized components was calculated: (GluR2:GRIP)-(pGluR2:PICK).

Results

In this study, we characterized the expression of four proteins involved in the trafficking of the AMPA receptor. Using Western Blot analysis, we quantified the developmental trajectories of the proteins in visual and frontal cortex in the rat. Comparing cortical areas allowed us to observe developmental differences in expression that occur in V1, a primary sensory area and frontal cortex, a non-sensory area used in higher-order cognitive processes. Furthermore, the establishment of normal developmental trajectories is a critical part of identifying developmental deviations from the norm.

Developmental Expression of AMPAR subunit GluR2 and pGluR2 in V1 and Frontal Cortex

To examine the developmental expression of AMPAR, expression levels of AMPAR subunit GluR2 and its phosphorylated form, pGluR2, were assessed between P4 and P105 using Western Blot analysis of tissue samples from V1 and frontal cortex. GluR2 is one of the subunits of the AMPA receptor and when it is phosphorylated at serine 880 by PKC the relative affinity of the receptor for anchoring proteins is altered (Chung et al., 2000), and it gets internalized into the synapse (Seidennman et al., 2003). Measuring the expression of both GluR2 and pGluR2 gave an indication of the developmental levels of surface expressed GluR2 and internalized pGluR2.

Protein expression relative to control was quantified and graphed as a function of age to show the changes in expression during development between postnatal day (P) 4 and P 105 in V1 and frontal cortex. The band for the GluR2 protein is a single band at 100 kD. The expression of GluR2 in V1 (**Figure 2.1A**) started low and increased progressively until reaching a plateau. A tau function was fit to the data ($R = 0.72$, $p<0.0001$). Expression levels increased 5-fold from P4 to P38, at which point adult levels were reached (3 tau).

The band for pGluR2 band is at 100kD. The trajectory for the phosphorylated form of the subunit, pGluR2 (**Figure 2.1B**), in visual cortex was virtually identical to GluR2 (**Figure 2.2A:** correlation, $R = 0.88$, $p<0.0001$), there was an initial progressive increase in expression and then leveling off. This trend was also well described by the tau function ($R = 0.68$, $p<0.0001$). Between P4 and P36, expression levels increased 6-fold and after P36, levels remained relatively constant.

In frontal cortex, there were obvious developmental changes and the trajectories for GluR2 and pGluR2 were very different from that of V1. For both proteins, there was a rapid increase in expression followed by decrease and eventually leveling off. For GluR2 in frontal cortex (**Figure 2.1C**), the data was fit with a membrane transport curve ($R = 0.77$, $p<0.0001$). The maximum levels of GluR2 expression were reached at P24 and there was an exuberant over expression between P18 and P35, followed by a decrease in protein levels.

The developmental trajectory of pGluR2 (**Figure 2.1D**) paralleled that of GluR2 in frontal cortex (**Figure 2.2C:** correlation, r = 0.94, p<0.0001): over expression followed by a plateau. The membrane transport curve described that data well (R = 0.79, p<0.0001). Maximum levels of pGluR2 expression were attained at P24 and the period of over expression was between P19 and P32.The expression levels of GluR2 and pGluR2 were highly correlated in visual (**FIgure 2.2A,** r = 0.88, p<0.0001) and frontal (**Figure 2.2C,** r =0.94, p<0.0001) cortex.

In order to quantify the developmental changes in surface AMPAR expression relative to the internalized receptor expression, we calculated an index of GluR2 expression relative to pGluR2. For this index, positive values indicate relatively more GluR2 and negative values indicate relatively more pGluR2. In V1, there was relatively more GluR2 early in development, but eventually a balance was reached (**Figure 2.2B**). Tau function was fit to the data (R = 0.37, p<0.0005). The index was in favour of GluR2 at P4 and up until P32, at which point GluR2 and pGluR2 came into balance that persisted throughout development. A different pattern was found for the frontal cortex, where the index remained in favour of more GluR2 throughout development (**Figure 2.2D**). An inverted membrane transport curve was fit to the data (R = 0.30, p = 0.04). There was relatively more GluR2 compared to pGluR2 throughout development, except for a brief period between P15 and 35 when the two proteins came into balance. After P35, GluR2 development surpassed that of pGluR2.

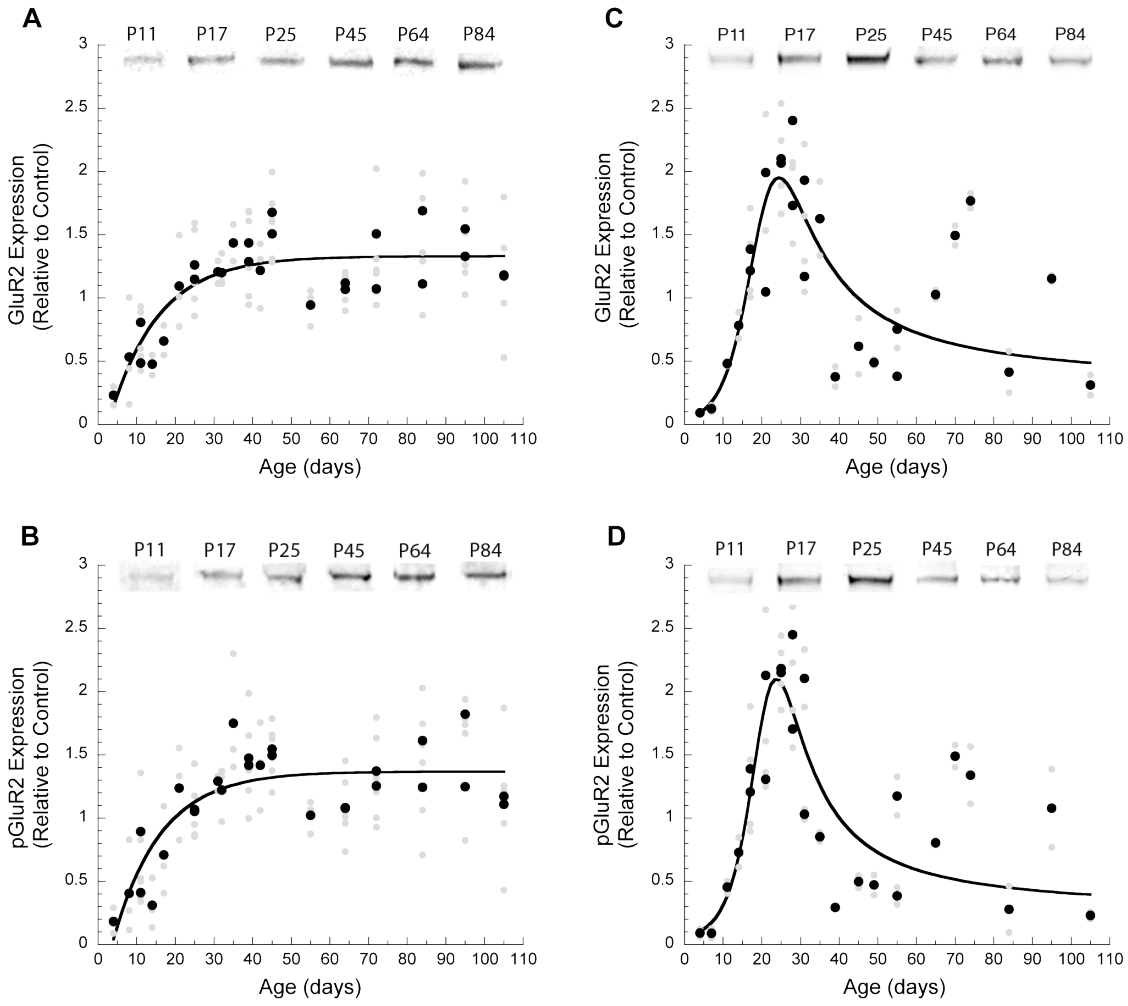

Figure 2.1. Development of AMPA receptor subunits GluR2 and pGluR2 in visual and frontal cortex. There are significant differences in the developmental trajectories of GluR2 and pGluR2 in visual versus frontal cortex. Data plotted is for each run of every sample (light symbols) as well as the averages for each run (dark symbols). Exponential decay curves were fit to V1 data for GluR2 expression (**A**) $R = 0.72$, $p<0.0001$, adult expression levels reached by 38 days of age (3τ); pGluR2 expression (**B**) $R = 0.68$, $p<0.0001$, adult levels of expression attained by 36 postnatal days. Data for frontal cortex was fit with a membrane transport curves. GluR2 expression (**C**) $R = 0.77$, $p<0.0001$, maximum expression levels reached at P24, over expression between P18 – P35. pGluR2 expression (**D**) $R = 0.79$, $p<0.0001$, maximum levels of expression at P24, over expression between P19 and P32.

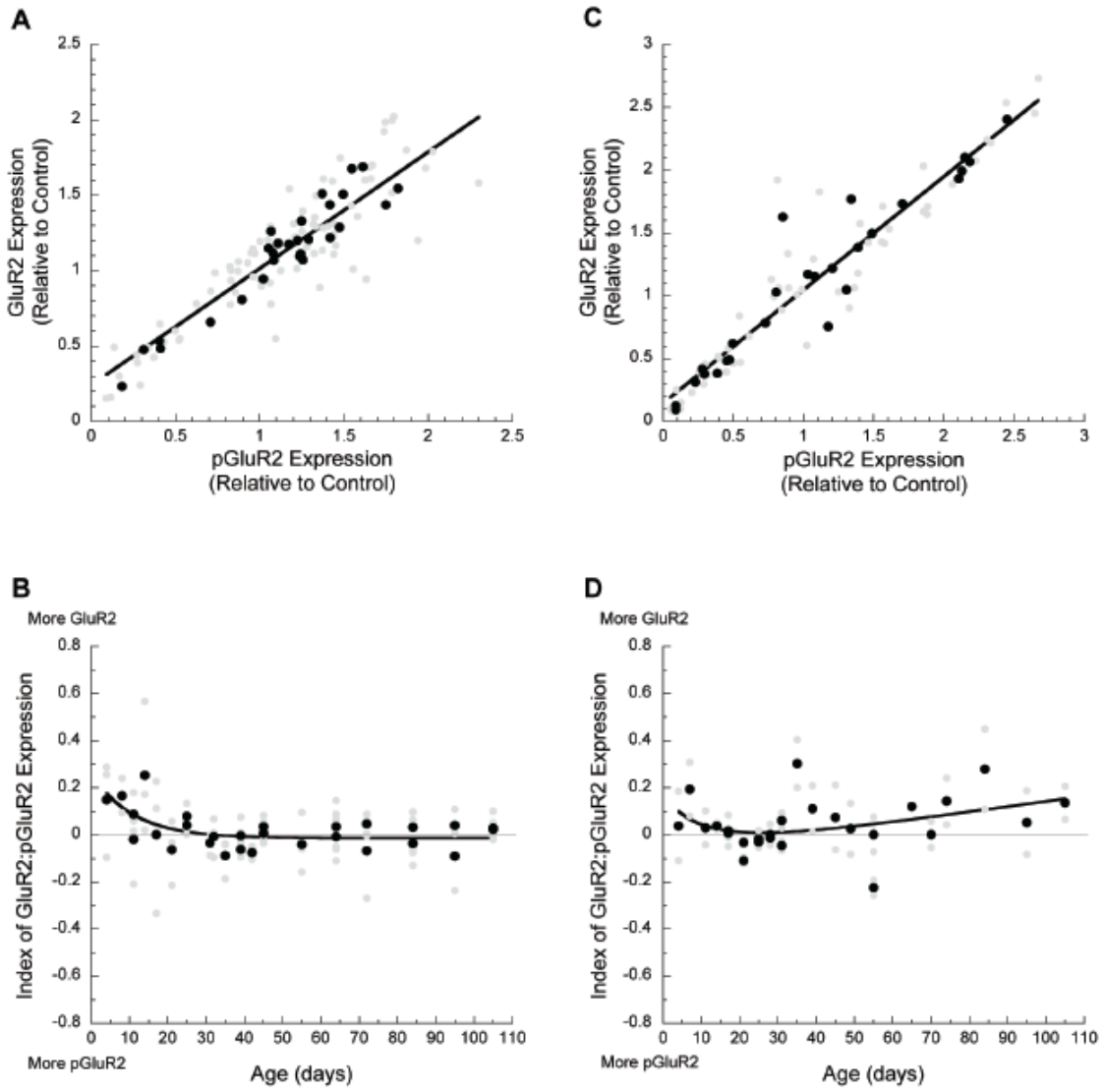

Figure 2.2. Developmental changes in AMPA receptor subunit expression in visual and frontal cortex. GluR2 and pGluR2 expression in both visual (**A**) ($r = 0.88$, $p<0.0001$) and frontal (**C**) ($r = 0.94$, $p<0.0001$) cortex is highly correlated during development. Index of GluR2:pGluR2 expression during development. In visual cortex (**B**), there is a higher GluR2 expression initially, but a balance is reached by P33 (tau function, $R = 0.37$, $p<0.0005$). In frontal cortex (**D**), the index remained in favour of relatively more GluR2 throughout development (membrane transport curve, $R = 0.30$, $p = 0.04$)

Developmental Expression of GRIP, PICK – Machinery Necessary for AMPAR Trafficking

At the synaptic surface, GluR2 subunit of the AMPAR is stabilized at the synaptic surface by GRIP (Dong, 1997). However, when GluR2 subunit is phosphorylated, it loses the interaction with GRIP and is bound by PICK and gets endocytosed (Seidenman et al., 2003). Measuring the developmental expression of GRIP and PICK allowed us to assess how much of the anchoring proteins are available for AMPAR trafficking.

The GRIP protein was visible as a single band at 130 kD. In V1, GRIP expression showed a slight developmental increase followed by leveling off (**Figure 2.3A**); this trend was fit with a tau function ($R = 0.33$, $p = 0.002$). During development, expression levels increased by 1.7 times reaching adult levels by 60 days of age. A similar pattern (**Figure 2.4A:** correlation, $r = 0.72$, $p<0.0001$) of development was seen with PICK (**Figure 2.3B**), which showed up as a single band at 45 kD. The slight developmental increase was fit with a tau function ($R = 0.32$, $p = 0.002$). Starting at P4, PICK expression increased 2 fold, reaching adult levels by 69 days of age.

In frontal cortex, while the developmental patterns of the two trafficking proteins exhibited a similar trend to V1, the expression levels of GRIP and PICK had more pronounced increases. GRIP (**Figure 2.3C**) data was fit with a tau function ($R = 0.62$, $p < 0.0001$) and underwent a 3-fold increase in expression starting at P4, reaching adult levels by 56 days of age. Expression levels of PICK

(**Figure 2.3D**) (tau fit: $R = 0.62$, $p < 0.0001$) exhibited a 3-fold increase during early development, attaining adult levels by P58. The pattern of PICK expression in frontal cortex during development paralleled that of GRIP (**Figure 2.4C:** correlation, $r = 0.48$; $p<0.0001$). Also, interesting to note was the considerably lower level of variability in frontal cortex protein expression when compared to that of V1.

We calculated an index of GRIP:PICK expression to quantify the developmental changes in the trafficking mechanism for the GluR2 subunit (Seidennman et al., 2003), which will ultimately allude to the state of the AMPAR at any given time. For this index, positive values indicate relatively more GRIP and negative values indicate relatively more PICK. In V1, GRIP and PICK expression was balanced throughout development (**Figure 2.4B**). In frontal cortex, while no clear trend was seen, the proteins appeared to develop in balance with one another (**Figure 2.4D**).

Development of Synaptic Surface and Internalized Components

AMPAR subunit GluR2 is held at the synaptic surface by GRIP (Dong, 1997). The phosphorylated form, pGluR2, associates with PICK as it is internalized into the synapse (Seidennman, 2003). We calculated indices for GluR2:GRIP expression and pGluR2:PICK expression to see how the surface and internalization components develop in visual and frontal cortex. For the GluR2:GRIP index, positive values indicate relatively more GluR2 and negative

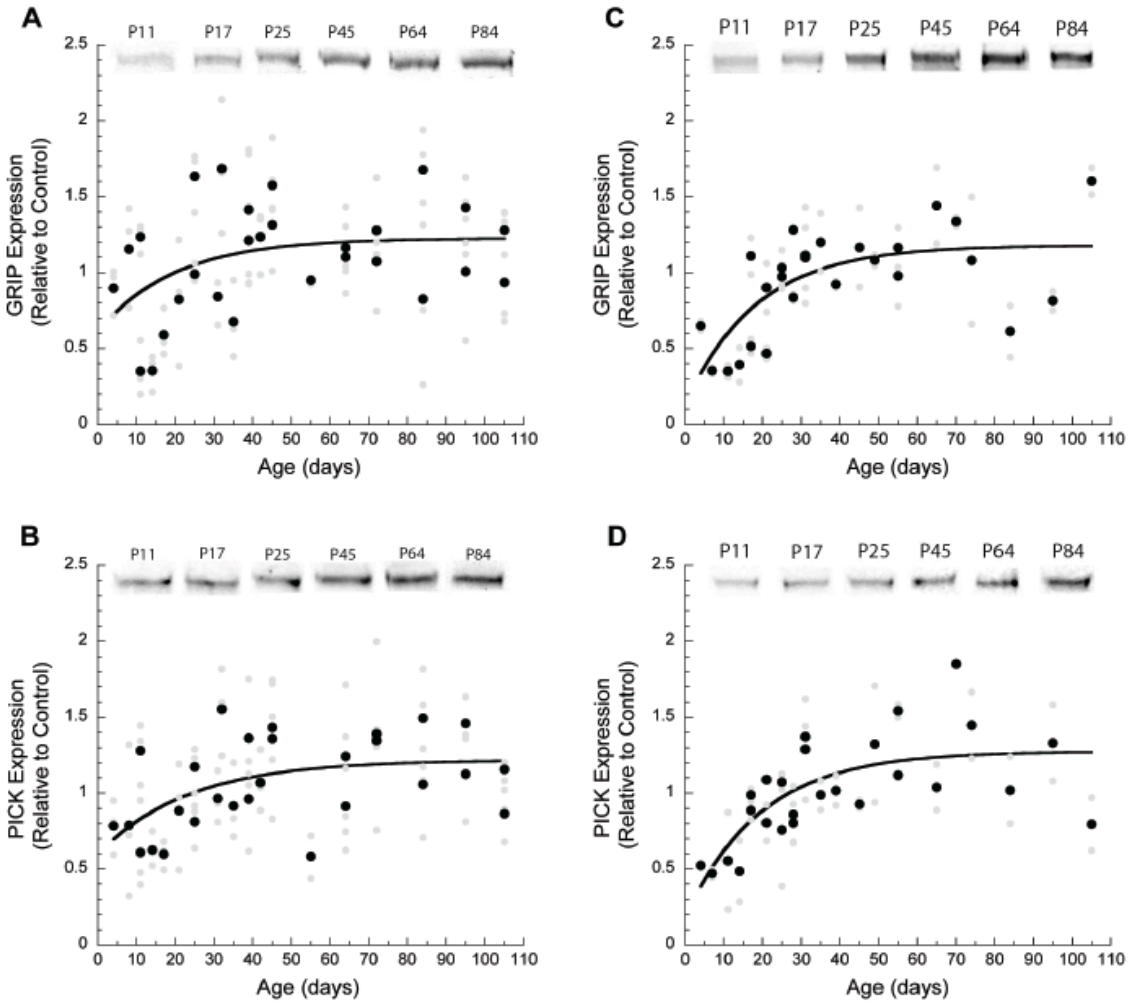

Figure 2.3. Development of GRIP and PICK in visual and frontal cortex. The developmental trajectories for GRIP and PICK are similar in both cortical areas. Tau curves were fit to all the data. In V1, GRIP (**A**) expression levels increased by 1.7 times and reached adult levels by 60 days of age (tau function: R = 0.33, p = 0.002). PICK (**B**) expression levels increased 2 fold and reached adult values by P69 (tau function: R = 0.32, p = 0.002). In frontal cortex, GRIP (**C**) levels increased 3 fold and reached adult levels by 56 days of age (tau function: R = 0.61, p < 0.0001). Similarly, PICK (**D**) expression increased 3 fold during development and adult level were attained by P58 (tau function: R = 0.63, p <0.0001)

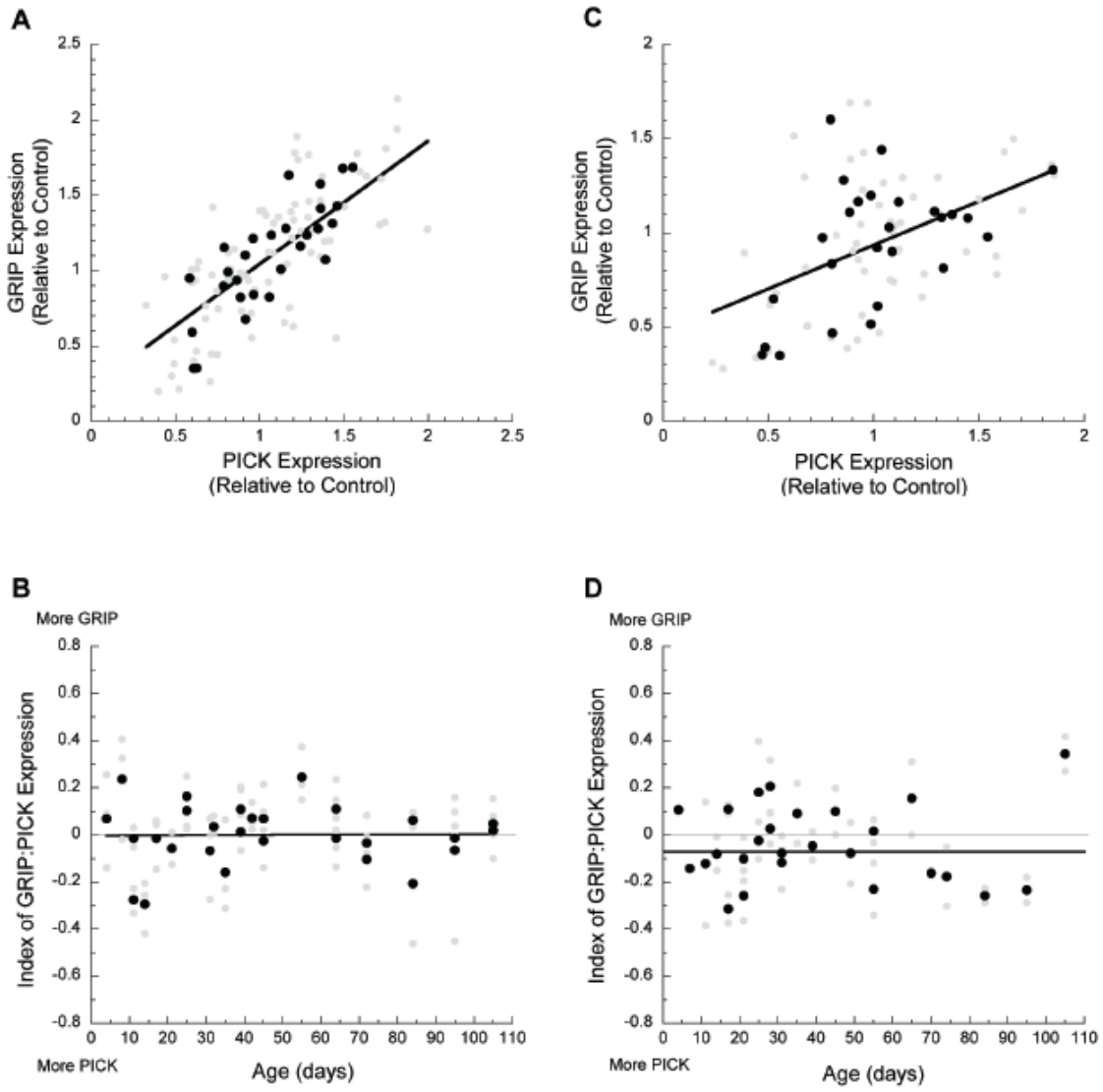

Figure 2.4. Developmental changes in GRIP and PICK in visual and frontal cortex. GRIP and PICK expression in visual cortex (**A**) is highly correlated during development ($r = 0.72$, $p<0.0001$). The index of GRIP:PICK expression (**B**) shows that the two proteins are balanced in post natal development.
GRIP and PICK expression in frontal cortex (**C**) is correlated in development ($r = 0.48$, $p<0.0001$). Index shows that GRIP and PICK develop in balance in frontal cortex (**D**).

values indicate relatively more GRIP. GluR2:GRIP balance in visual cortex (**Figure 2.5A**) was in favour of more GRIP early in development; however, it quickly changed to being in favour of slightly more GluR2. A tau function was fit to describe the trend ($R = 0.62$, $p<0.0001$). An adult balance where there was slightly more GluR2 was reached by 13 days of age and persisted throughout development. For the pGluR2:PICK index, positive values indicate relatively more pGluR2 and negative values indicate relatively more PICK. pGluR2:PICK index in V1 exhibited a similar developmental profile, starting off with relatively more PICK, followed by a fast increase it pGluR2 expression to reach a balance of slightly more pGluR2 (**Figure 2.5B**). A tau function was fit to describe this trend ($R = 0.69$, $p < 0.0001$). At 4 days of age, there was significantly more PICK; however the balance quickly shifted the being slightly more pGluR2 by 22 days and this balance persisted throughout development.

In frontal cortex, the initial trends are similar to those observed in V1, however the overall developmental trajectories of the balances of surface and internalization components were significantly different. Both GluR2:GRIP (**Figure 2.5C**) and pGluR2: PICK (**Figure 2.5D**) were fit with membrane transport curves (GluR2:GRIP: $R = 0.74$, $p<0.0001$; pGluR2:PICK: $R = 0.77$, $p < 0.0001$). The developmental balance for GluR2 and the surface anchoring protein was in favour of more GRIP very early on (<P10), followed by a brief shift in balance in favour of more GluR2 (P10-P40), and then another shift to relatively more GRIP, which persisted throughout development. Similarly, the balance for the

internalization components, pGluR2:PICK, was in favour of relatively more PICK early in development (<P13), then in favour of more pGluR2 between P13 and P34, followed by another shift to more PICK, which persisted throughout development.

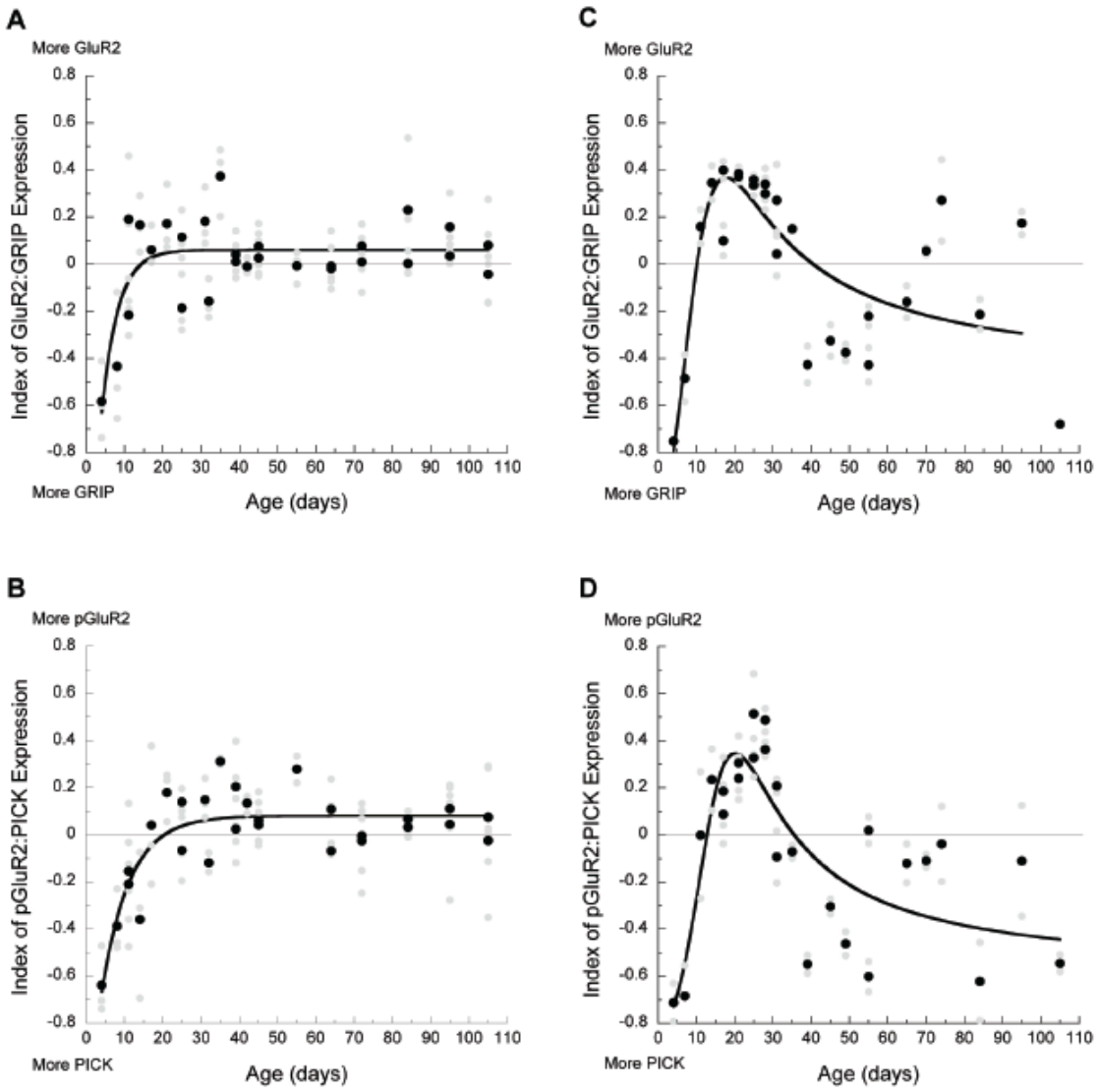

Figure 2.5. Development of surface and internalized components in visual and frontal cortex. Index of GluR2:GRIP during development in visual cortex (**A**) shows that initially more GRIP is present, but by P13, a balance on the order of slightly more GluR2 is reached (tau function: R = 0.62, p<0.0001). Index of pGluR2:PICK in visual cortex (**B**) shows that more PICK is present early in development, but a balance is reached by P22 (tau function: R = 0.69, p<0.0001). In frontal cortex, index of GluR2:GRIP (**C**) is in favour of GRIP before P10, followed by an increase in GluR2 expression between P10-P40, and then another shift to relatively more GRIP (membrane transport curve: R = 0.74, p<0.0001). Index of pGluR2:PICK (**D**) is in favour of more PICK before P13, more pGluR2 between P13 and P34, and then another shift to relatively more PICK that persists throughout development.

AMPAR Trafficking Development is Tightly Controlled

Because we saw very similar developmental trajectories in the synaptic surface and the internalization components within both cortical areas, we measured the correlation between the balances of GluR2:GRIP and pGluR2:PICK in visual and frontal cortex. **Figure 2.6A** shows pGluR2:PICK depicted on the x axis and GluR2:GRIP on the y axis; data for both cortical areas is shown on the same graph. Linear curves were fit to the data. Synaptic surface and internalized components were highly correlated in both visual ($r = 0.61$, $p<0.0001$) and frontal cortex ($r = 0.78$, $p < 0.0001$). This trend showed that the system for AMPAR trafficking is tighly controlled in both a sensory and a non-sensory areas.

We wanted to see the level of regulation that persists throughout development in the surface and internalization components in visual and frontal cortex. We took the difference between the indices for GluR2:GRIP and pGluR2:PICK and graphed the values as a function of age for both cortical areas (**Figure 2.6B**). For this graph, positive values indicate higher expression in surface proteins, GluR2 and GRIP; negative values indicate higher expression of internalized components, pGluR2 and PICK. Resultant values were generally centered around the 0 mark, indicating a tight balance in the expression of surface and internalized components. An ANOVA showed no significant differences between the cortical areas ($p = 0.40$).

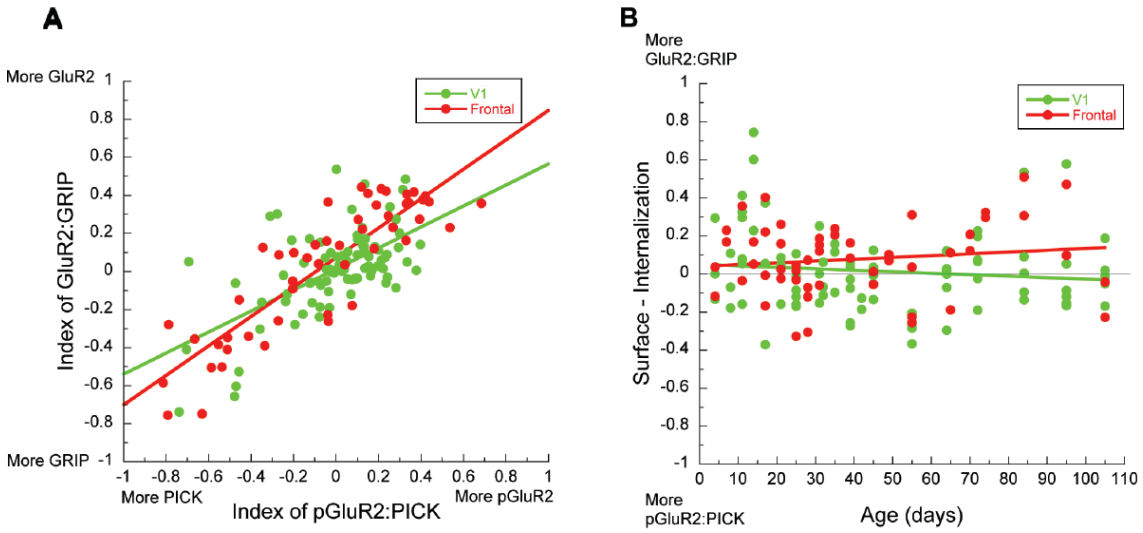

Figure 2.6. Development of AMPA receptor in visual and frontal cortex. There is a high correlation between the index for GluR2:GRIP and pGluR2 PICK in both cortical areas (**A**) visual cortex ($r = 0.61$, $p<0.0001$) frontal cortex ($r = 0.81$, $p<0.0001$). Differences between the index for GluR2:GRIP and pGluR2:PICK as a function of age shows that the development of the surface and internalized components is balanced (**B**) in both visual and frontal cortex. No significant differences were observed between the cortical areas (ANOVA: $p = 0.40$)

Discussion

We found significant differences in the developmental trajectories of AMPAR proteins between V1 and frontal cortex. Using Western Blot analysis, we found that expression levels of GluR2 and its phosphorylated form pGluR2 showed progressive developmental increases in V1, while the developmental trajectories of both exhibited an over expression in frontal cortex. The proteins involved in AMPAR trafficking, GRIP and PICK, demonstrated smooth progressive developmental increases – a trend that was similar in V1 and frontal cortex. The development of all four components was balanced and tightly correlated throughout the time period investigated. Taken together, our findings imply that normal cortical development relies on a tight balance in AMPAR trafficking components.

Although prior experiments looked at the developmental expression of AMPARs and specifically GluR2 (Beston et al., 2010; Herrmann, 1996), this is the first study to quantify developmental trajectories of pGluR2, GRIP, and PICK and compare across a primary sensory and a non-sensory area. The developmental increase we saw in GluR2 is consistent with previous findings in visual cortex (Hermann, 1996) as well as research on silent synapses in hippocampus, suggesting that AMPA reactivity is low early in development, but increases during maturation (Petralia et al., 1999). Furthermore, overexpression of GluR2 is associated with increases in spine density and size in hippocampal neurons (Passafaro et al., 2003), which is consistent with cortical development. The

similar developmental trajectories of pGluR2, GRIP, and PICK suggest that the components of AMPAR trafficking in visual cortex develop concurrently. Thus, the increasing levels of GRIP and PICK during development may allow for more AMPAR trafficking to and from the synapse in response to a higher expression of GluR2.

Measuring the developmental balance between the surface components, GluR2 and GRIP, we found that expression levels of GRIP were higher very early in development in both V1 and frontal cortex. In hippocampal neurons, a mutant form of GluR2 that does not bind GRIP targets appropriately to the surface, but its accumulation at synapses is significantly reduced when compared to wild-type GluR2 (Osten et al., 2000; Shi et al., 2001). Thus, the presence of GRIP early on may ensure the stabilization of GluR2 containing AMPARs at the synapse (Shepherd & Huganir, 2007) once AMPARs begin to be expressed. Similarly, the expression levels of PICK were initially higher than pGluR2, suggesting that the presence of PICK may ensure the method for the internalization of GluR2-containing AMPARs is in place prior to receptor development.

Glutamate receptors mediate the majority of excitatory neurotransmission and AMPARs have been heavily investigated in experiments of cortical plasticity (Shepherd & Huganir, 2007). AMPAR trafficking has been investigated in both visual and frontal cortex, however the contexts of previous investigations were very different. The visual cortex is a model for studying experience dependent plasticity and the critical period for its development has been defined (Fagiolini et

al., 1994) in terms of ocular dominance plasticity. In V1, AMPA receptor trafficking is involved in mechanisms of developmental plasticity including LTP (Shi et al,. 2001), LTD (Beattie et al., 2000; Lissin et al., 1999), and synaptic scaling (Nelson & Turrigiano, 2004; Gainey et al., 2009). Furthermore, AMPAR endocytosis plays a critical role in loss of visual responsiveness following monocular deprivation (Yoon et al., 2009), leading to LTD in the deprived cortex (Heynen et al., 2003). In the development of frontal cortex, however, the critical period is not as easily definable and manipulations analogous to monocular deprivation have not yet been developed. Majority of the studies looking at frontal cortex has focused on the period of adolescence as the sensitive period of time when stress, addiction, and alcohol and drug exposure are likely to have serious long lasting effects. All these imply plasticity; therefore, establishing synaptic markers for defining a window of time when the frontal cortex is especially sensitive to abnormal experience is critical. Our results showed a striking over expression in GluR2 and pGluR2, the timing of which overlapped with the defined critical period of ocular dominance plasticity within visual cortex (Fagiolini et al., 1994). This may imply the presence of a developmental vulnerability and period of GluR2 dependent plasticity in frontal cortex that is not present in visual cortex.

Chapter 3

Effects of Fluoxetine Administration and Monocular Deprivation on AMPAR Trafficking

The central nervous system in early life is very plastic and responsive to environmental stimuli, however the potential for plasticity decreases with postnatal development (Hensch, 2005). Such is the case with visual cortex: early in development, sensory deprivation by eyelid suture during the critical period causes a suppression of inputs from the deprived eye, a loss of cortical connectivity, and a decrease in binocular convergence (Wiesel & Hubel, 1963; Hubel & Wiesel, 1970; Gordon & Stryker, 1996). Furthermore, early sensory deprivation is associated with amblyopia, the hallmarks of which include an impairment of spatial acuity and contrast sensitivity (Fagiolini et al., 1994; Prusky et al., 2000; Dews & Wiesel, 1970). Thus, early manipulations have profound effects on the cortex. In the adult, however, there is a decline in plasticity, and sensory deprivation does not lead to amblyopia. This change in plasticity has largely been attributed to the maturation of intracortical inhibition (Huang et al., 1999; Fagiolini & Hensch, 2000).

Pharmacological interventions such as antidepressants function through the activation of certain molecular pathways that have been shown to promote neurogenesis and synaptogenesis in the adult hippocampus (Malberg et al., 2000; Hajszan et al., 2005). Other changes in the cortex promoted by repeated antidepressant medication include changes in neurotrophin signaling (Castren et al., 2007), chromatic structure (Krishman & Nestler, 2008) and gene expression (McClung & Nestler, 2006). These events are characteristic of neuronal plasticity during early development. To investigate whether the observed increase in

neurogenesis and synaptogenesis translate to an actual reinstatement in cortical plasticity, Maya Vetencourt and colleagues (2008) examined the effects of fluoxetine (Prozac ®) on visual cortex plasticity of adult rats. Fluoxetine is a known selective serotonin reuptake inhibitor (SSRI) and therefore increases the levels of serotonin available for transmission in the synaptic cleft (van Harten et al., 1993). Using visually evoked potentials and behavioural tests assessing visual acuity after MD and reverse occlusion, it was found that fluoxetine reinstated OD plasticity and promoted the recovery of visual functions in adult amblyopes (Maya Vetencourt et al., 2008). Utilizing in vivo brain microdyalisis, they further found reduced extracellular GABA levels in visual cortex of fluoxetine treated rats. Intracortical infusion of diazepam prevented the effects of fluoxetine serving as a further confirmation of the role of inhibition in visual cortical plasticity.

The effects of serotonin on the excitatory cortical network were investigated by Reynolds et al (1988). Using voltage clamp techniques and an application of NMDA, an excitatory amino acid agonist, it was found that serotonin produced a long lasting enhancement of the amplitude of an EPSP (Reynolds et al., 1988). Also, monocular deprivation elicits changes to physical properties of dendritic spines (Hofer et al., 2009), which are the main post-synaptic structure for excitatory synapses (Eilers & Konnerth, 1997). AMPA receptors are colocalized with NMDA receptors at excitatory synapses. Thus, the involvement of AMPAR-mediated excitatory transmission during an adult reinstatement of plasticity cannot be ruled out.

Because AMPA receptors mediate the majority of excitatory neurotransmission, and are key in the establishment of the E/I balance (Beston et al., 2010), we set out to investigate if there are any changes in the expression of proteins involved in AMPA receptor trafficking after fluoxetine administration.

Methods

Subjects and Rearing Conditions

Twenty-eight adult Long-Evans rats were used in this study. Animals were group housed under standard conditions with food and water available *ad libitum.* At P70, half (n = 14) the animals started to receive daily administrations of the antidepressant fluoxetine hydrochloride in their drinking water (0.2 mg/ml water). The rest of the animals continued with normal drinking water and served as a control group.

To assess OD plasticity, MD for 7 days was performed by suturing the eyelid of the right eye in half the animals in each group (n = 7 from fluoxetine group; n = 7 from control group). The adult animals were anaesthesized with isoflurane (3.0-5% in oxygen) at P90, mounted on a stereotaxic apparatus and eyelid closure was performed with 5-0 vicryl suture using aseptic surgical techniques. The eyelid was monitored and topical antibiotics were applied daily to prevent infection up until proper healing at the site of occlusion was observed.

At P98, all animals were euthanized and transcardially perfused.
All experimental procedures were approved by the McMaster University Animal Research Ethics Board.

Tissue Collection, Tissue Preparation, and Immunoblotting

These steps of the procedure were carried out as previously discussed in Chapter 2. For a detailed list of steps involved in tissue collection, tissue

preparation and immunoblotting, please refer to pg. **14** Tissue samples were taken from both hemispheres and used in analysis.

Analysis

Protein expression was quantified and analyzed in tissue from both hemispheres. Expression levels for each animal were normalized to the average of the protein expression for the 7 animals in the control group (i.e., binocular vision (BV) and no fluoxetine).

We plotted bar graphs for each antibody within each cortical area. The plots included the average expression for each experimental group as well as the standard error of the means of each group.

Index calculations for trafficking proteins GRIP and PICK were carried out as previously described (See Methods in Chapter 2, pg. 14) and plotted for visual cortex.

Results

We assessed the expression of four proteins involved in AMPAR trafficking in the visual and frontal cortex of adult rats treated with fluoxetine for 1 month and monocularly deprived during the last week of treatment.

Plasticity in the adult visual cortex

For visual cortex, expression levels of each of the proteins of interest in V1 are depicted relative to the average expression of the control group (BV). Graphed are the averages for each experimental group. Looking at GluR2 expression in adult rodents, fluoxetine administration alone had no effect; however monocular deprivation caused a large and significant upregulation ($p < 0.05$) in protein levels in visual cortex contralateral to the deprived eye (**Figure 3.1A**). The group of rodents that received fluoxetine as well as monocular deprivation also had significantly increased levels of GluR2 expression ($p<0.05$). Such was the case with pGluR2 expression levels, where there was also a significant ($p<0.05$) increase in protein expression in the group of rodents that were monocularly deprived as well as those administered fluoxetine (**Figure 3.1B**).

There was a modest but significant ($p<0.05$) increase in GRIP expression in visual cortex of monocularly deprived animals relative to the control animals reared with binocular vision (**Figure 3.1C**). The differences in PICK expression (**Figure 3.1D**) between the groups paralleled those of GRIP, where there was a

significant ($p<0.05$) upregulation in protein expression following monocular deprivation.

Knowing that there were significant changes in all four proteins studied, we quantified the index for GRIP and PICK, proteins involved in AMPAR trafficking to see if there was any difference in balance that was induced by the experimental conditions. **Figure 3.2** shows the indices of GRIP:PICK expression for the four groups, quantified for both the contralateral and ipsilateral hemispheres. There was a striking and significant ($p<0.05$) contralateral increase in the GRIP:PICK balance in favour of GRIP in animals that were monocularly deprived. In animals who were administered fluoxetine, the balance was also shifted towards more GRIP; however, the difference did not reach significance.

Assessment of plasticity in frontal cortex

Because of the results we got with visual cortex, we expected not to see any differences between experimental groups in protein expression in frontal cortex. However, since we saw significant differences in the developmental trajectories of GluR2 and pGluR2 (Chapter 2), we investigated whether the plasticity differences extend beyond V1.

Figure 3.3 shows the expression profiles of GluR2 (**A**), pGluR2 (**B**), GRIP (**C**), PICK (**D**) for both contralateral and ipsilateral cortices. No significant differences were observed for any of the comparison groups. The were also no significant differences across the experimental groups in the balance of

GRIP:PICK in frontal cortex (**Figure 3.4**).

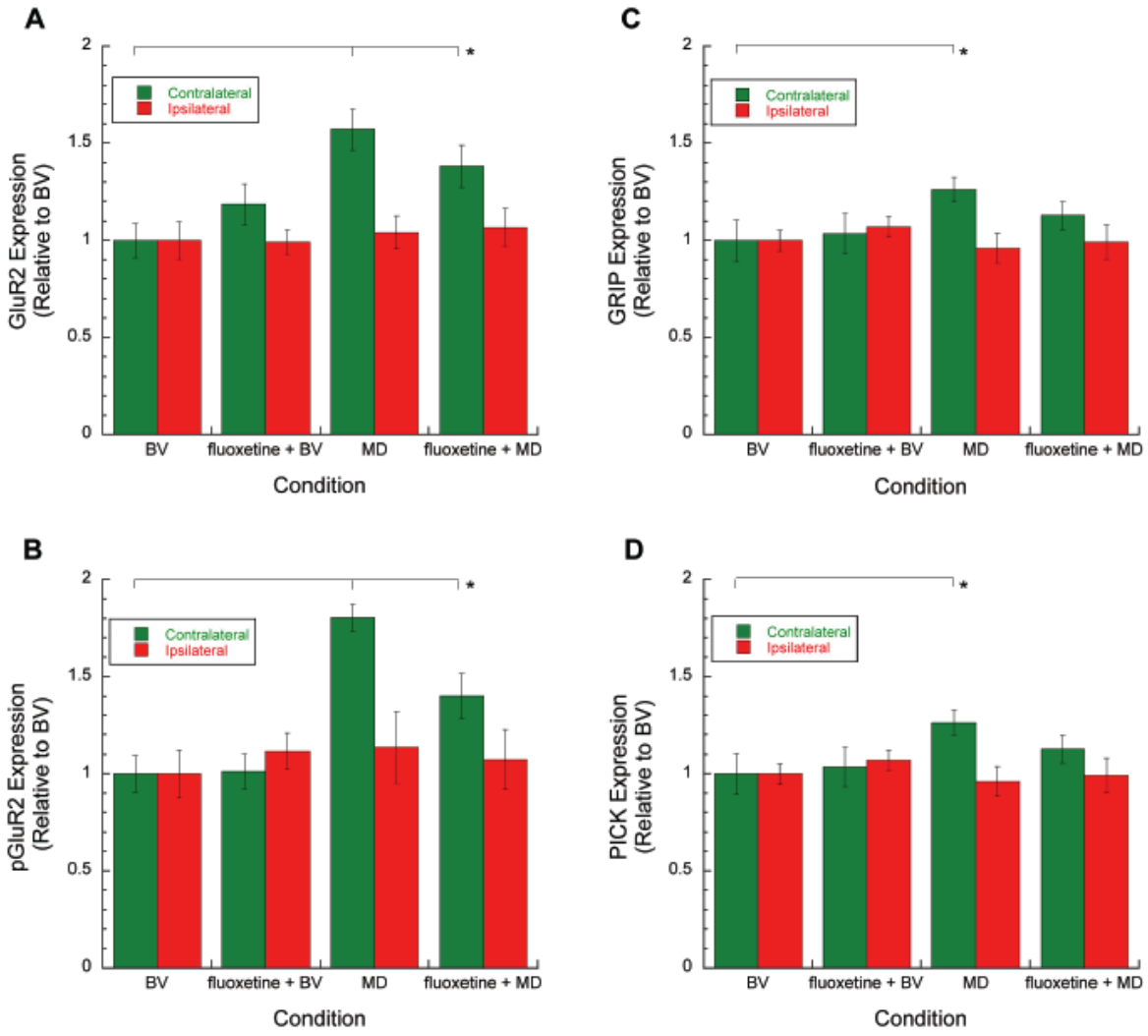

Figure 3.1. AMPA receptor expression in adult visual cortex. For each experimental group, expression levels are depicted for cortical tissue contralateral and ipsilateral to the deprived eye. Each bar represents the average levels of protein expression in each group relative to the expression of the control group that received binocular vision.
Monocular deprivation induces a significant increase in expression levels of GluR2 (**A**), pGluR2 (**B**), GRIP (**C**), and PICK (**D**).
Lines above the graphs identify the groups that were significantly different (Tukey's post hoc HSD, *p<0.05)

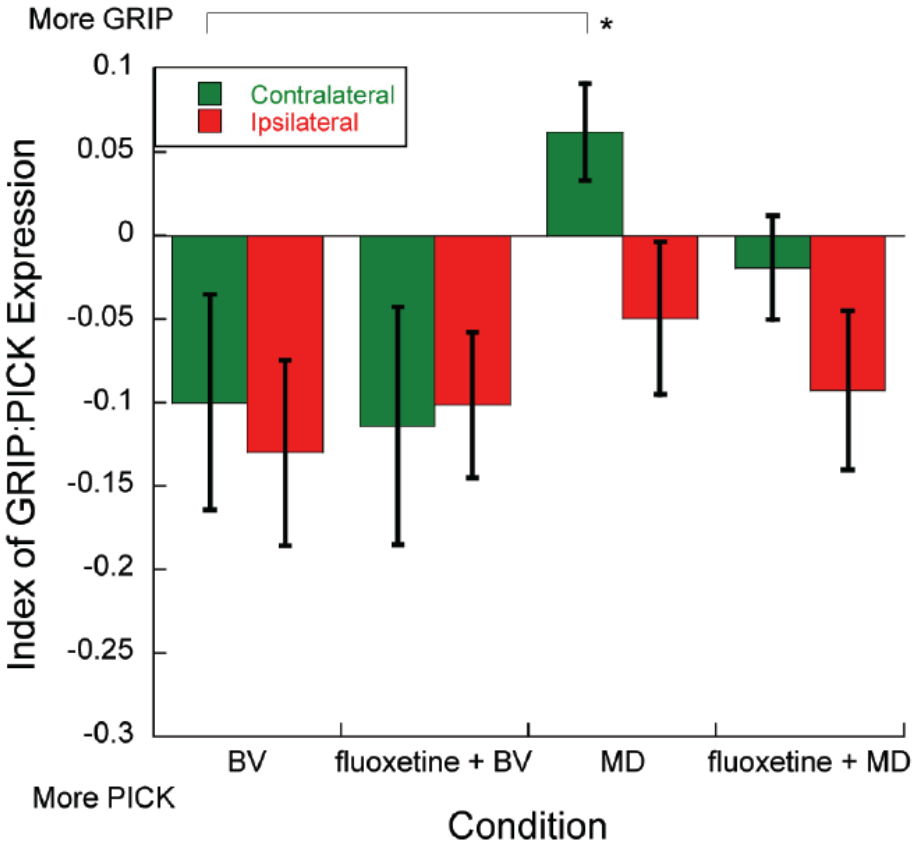

Figure 3.2. Relative changes in AMPA trafficking proteins in adult visual cortex. Graph follows the same convention as described in Chapter 2. There was a significant ($p<0.05$) increase in the relative expression of GRIP in visual cortex contralateral to the deprived eye.

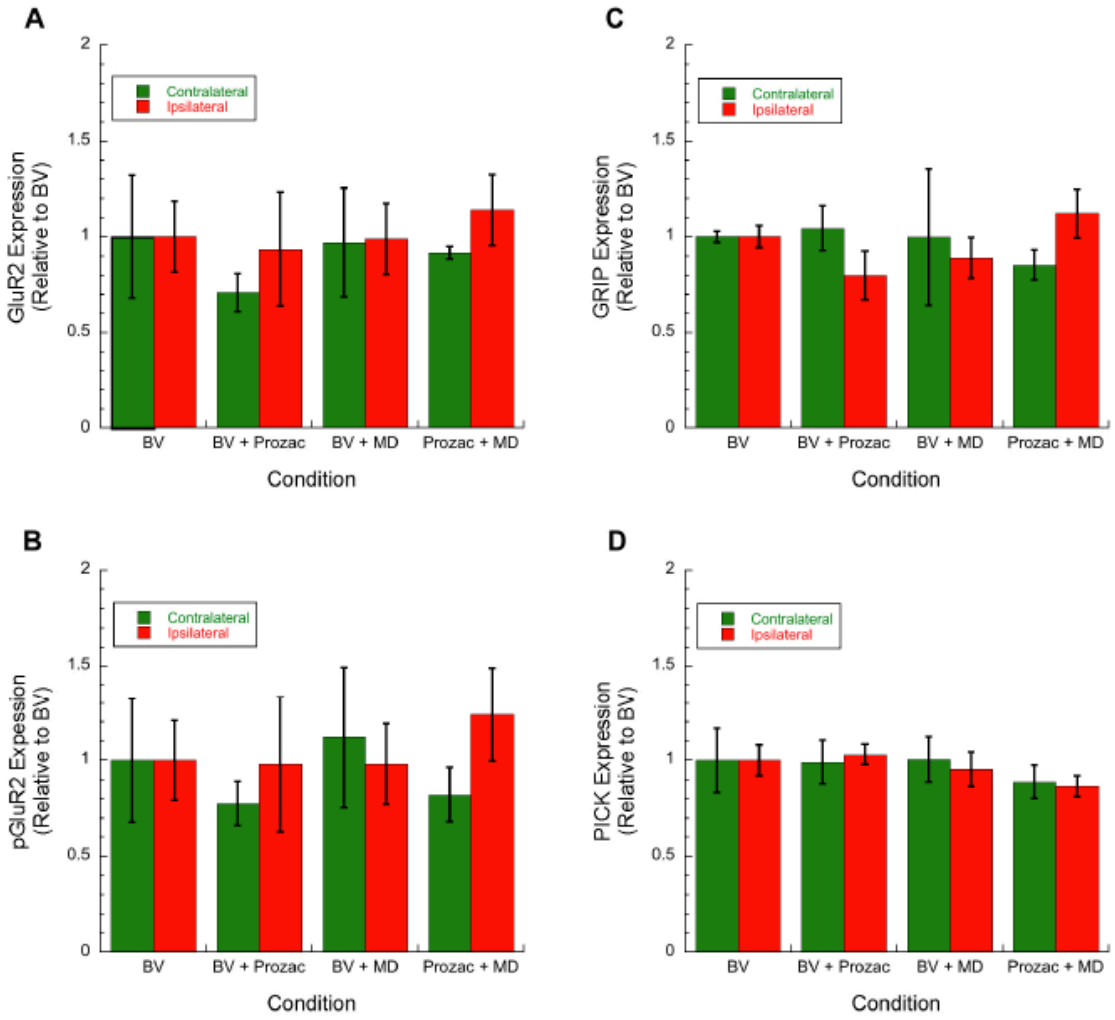

Figure 3.3. AMPA receptor expression in adult frontal cortex. For each experimental group, expression levels are depicted for cortical tissue contralateral and ipsilateral to the deprived eye. No significant differences were observed in protein expression for GluR2 (**A**), pGluR2 (**B**), GRIP (**C**), and PICK (**D**).

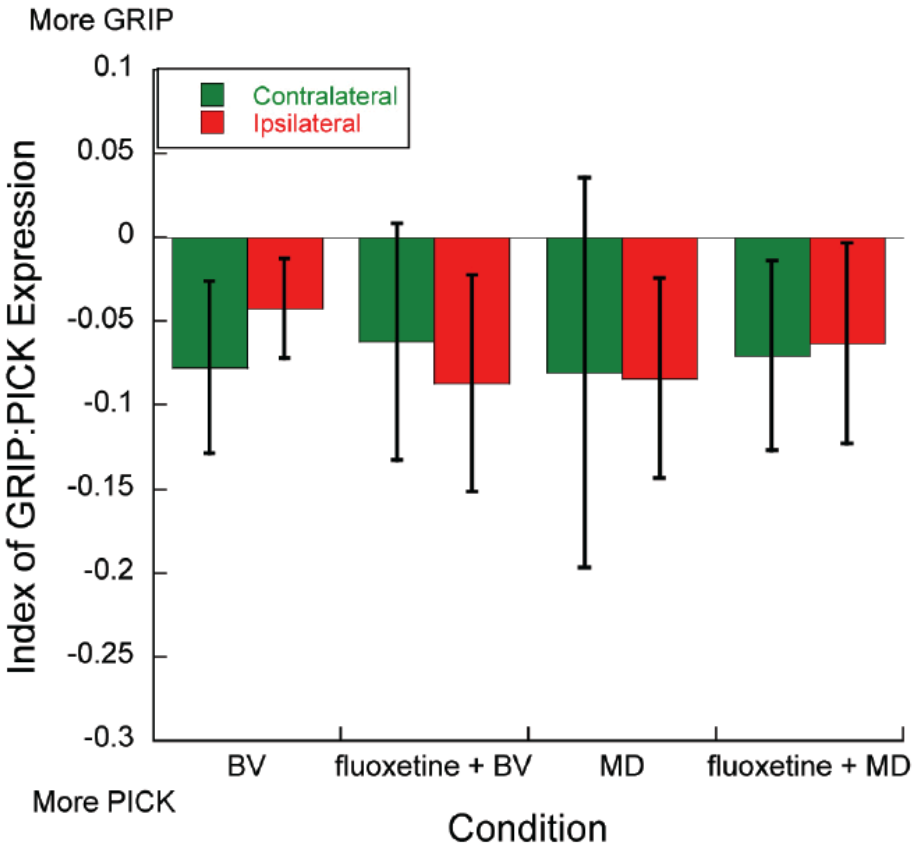

Figure 3.4. Relative changes in AMPA trafficking proteins in adult frontal cortex. No significant differences were observed in the relative levels of expression.

Discussion

Expression of proteins involved in AMPAR trafficking did not change following fluoxetine administration alone in visual cortex of adult animals. However, there was a significant upregulation in expression levels of all four proteins following monocular deprivation, which may reflect the existence of AMPAR mediated plasticity mechanisms.

AMPARs are located at dendritic spines and it has been shown that an increase in spine density is associated with increases in GluR2 expression (Passafaro et al., 2003). The fact that the expression levels of all four proteins involved in AMPAR trafficking were increased with MD suggests that the cortex may be trying to compensate for the lack of input to the deprived eye, and that synaptic restructuring is taking place. The increases in expression levels of GRIP and PICK were modest when compared to GluR2 and pGluR2 following MD, but there was a profound change in the balance of GRIP:PICK in favour of more GRIP. As mentioned before, GRIP is involved in the stabilization of the AMPA receptor at the synaptic surface (Dong, 1997). Taken together, our results suggest a possible mechanism for dealing with reduced synaptic activity by scaling up AMPAR trafficking as well as keeping more AMPARs at the synaptic surface, thereby making them available for signal transmission. This idea is consistent with synaptic scaling (Turrigiano & Nelson, 2004), where MD in the presence of TTX results in an increase in GluR2 expression (Gainey et al., 2009).

The notion of adult visual cortex plasticity has been previously investigated in mice. Hofer and colleagues (2006) demonstrated a shift in OD plasticity in adult mice following MD. This shift was significantly more profound if there was a previous case of MD earlier in life (Hofer et al., 2006). Furthermore, the timing of the first period of MD (i.e. during the critical period or in adulthood) did not matter as the second case of MD still produced faster and more persistent shifts in OD. Importantly, brief MD (3 days) was not sufficient to cause a shift in OD in adult mice, when prolonged MD was clearly associated with plastic changes (Hofer et al., 2006).

Previous studies have looked at AMPAR involvement in altering synaptic transmission after MD. Heynen and colleagues (2003) showed that brief periods of MD during early development lead to changes in phosphorylation of GluR subunits and a decrease in surface expressed AMPARS, resulting in LTD in the deprived cortex (Heynen et al., 2003). We saw a significant increase in pGluR2 expression following MD, which is consistent with these results. However, our animals were significantly past the age of the established critical period of OD plasticity (Fagiolini et al., 1994). Nevertheless, in our experiment the period of MD was prolonged, and this could account for the increased levels of expression that we found in phosphorylated GluR2. In fact, it was previously reported that, in adult mice, 5 days of MD, but not 3 produce significant shifts in OD plasticity (Sawtell et al., 2003).

Over the past decade, research delving into the mechanisms of depression and antidepressant function has shown accumulating evidence supporting a role for glutamate and ionotropic glutamate receptors (Skolnick et al., 2001; Skolnick et al., 2002; Stoll et al., 2006). It is possible that the increase in serotonin, which is made available by fluoxetine has no effect on the AMPA receptor. Consistent with this idea, it was shown that treatment with certain tricyclic antidepressants (TCAs) induces changes in AMPA receptor subunit expression, which in turn influence calcium permeability and receptor localization (Martinez-Turrillas et al., 2002; Stoll et al., 2006). Exploring this further, Stoll et al. (2006) found that TCAs but not SSRIs directly bind to the GluR2 subunit of the AMPA receptor. This leads us to believe that other pharmacological agents may be associated with changes in adult cortical plasticity, specific to the AMPA receptor.

A recent study looking at effects of chronic fluoxetine administration on glutamate receptor subunits in subcortical structures found treatment induced increases in GluR2 as well as the NMDA receptor subunit NR2A (Ampuero et al., 2010). These findings are in contrast with our results, as we did not see any significant changes in protein expression following fluoxetine administration in visual and frontal cortex. The treatment induced increases in glutamate receptor subunit expression were specific to the retrosplenial granular region of the cortex (Ampuero et al., 2010), which was not part of our frontal cortex sample and it is likely that, at the interface of limbic and subcortical areas, differences in AMPAR

expression exist. It is also possible that the effects of fluoxetine on AMPAR expression in our results was overshadowed by the effects of MD and the clear but not significant change in the GRIP:PICK index in visual cortex following fluoxetine administration in MD suggests that fluoxetine may function to bring the balance in the trafficking mechanism back to a normal state.

Chapter 4

General Discussion

In this thesis, I looked at expression levels of a selected set of proteins involved in AMPA receptor trafficking. In the first study (Chapter 2), I focused on expression during normal development and compared these levels across the cortical areas. Although I found significant differences in expression of the GluR2 subunit as well as its phosphorylated form, pGluR2, between the visual and frontal cortex, expression levels of GRIP and PICK, the trafficking mechanism for GluR2 were consistent across the brain. Furthermore, looking at the correlation between the surface and internalized components as well as the differences in the development of the components between the cortical areas, it became evident that the AMPAR trafficking system development is tightly regulated across the cortex.

The goal of the second part of my thesis (Chapter 3) was to look at adult plasticity, with respect to the AMPA receptor using the same set of proteins in visual and frontal cortex. For this experiment, I looked at protein expression in cortical tissue of adult rats treated with fluoxetine, an agent that was previously found to reinstate a juvenile state of plasticity to the adult visual cortex (Maya Vetencourt et al., 2008). Results demonstrated that plasticity was present in the adult visual cortex as monocular deprivation in the absence of fluoxetine induced an upregulation in expression levels of all four proteins.

Taken together, results presented in Chapter 2 and Chapter 3 provide insight into the cortical plasticity in development and in the adult in a primary sensory area and one involved in higher order cognition.

Methodological Considerations

The immunoblotting procedures used in the experiments allowed us toquantify the expression of AMPA receptors (GluR2, pGluR2), as well GRIP and PICK, the proteins involved in AMPAR trafficking in tissue samples from rodent visual and frontal cortex. Furthermore, using the synaptoneurosome preparation we were able to quantify the synaptic expression of excitatory receptors (Hollingsworth et al, 1985). The subcellular fractionation technique enriches for excitatory synaptic proteins so that the level of expression of our proteins of interest was quantified at the site that is most important for understanding synaptic plasticity. To be able to draw links between physiological changes in synaptic efficacy and the neural plasticity mechanisms that underlie those changes, it is essential to know the level of expression of functional receptors at the synapse.

The Western Blot technique quantifies the amount of protein but does not provide information about laminar development, the arrangement of neurons expressing the proteins, or the types of neurons expressing AMPA receptors. These types of questions can be answered utilizing other techniques such as in situ hybridization. Also, combining our results with electrophysiological methods would provide insight into the receptive fields of the cells studied, which would be especially useful for Chapter 3, as it would allow us to gain insight into the quality of the action potential after monocular deprivation. Because our methods allowed us to look at absolute protein expression, we couldn't say with certainty whether

the increase in AMPAR expression was due to the weakening of the deprived eye or the strengthening of the nondeprived eye.

In our lab, we have been using index calculations to get a more comprehensive view of changes in protein expression that occur at the synapse during development and aging (Murphy et al., 2005; Beston et al., 2010; Williams et al., 2010; Pinto et al., 2010). In Chapter 2, indices were especially useful in seeing the developmental balances that persisted between AMPAR proteins despite significant differences in the individual trajectories.

The processes that govern neuronal function and organization within the brain are incredibly complex and many biological pathways are uniquely adapted to perform the intricate information processing achieved by the brain. It is estimated that there are ten thousand proteins present at mammalian synapses (Pocklington et al., 2006). Receptors are dynamic structures and their expression at the synapse at any given time will surely affect cortical network functioning. The trafficking of AMPA receptors is crucial for the modulation of synaptic transmission (Shepherd & Huganir, 2007). In my thesis, I have quantified the expression of a set of four proteins involved in AMPAR trafficking. My results serve as an important step towards the elucidation of mechanisms involved in synaptic development.

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