A Role for Astrocytes in Dopamine-Glutamate Interactions of the Prefrontal Cortex

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To my wife, Denay, for her love and support,

To my daughter, Kinley, for her unrelenting happiness,

and

To my amazing parents, Anne and John Vollbrecht, both endlessly encouraging
and supportive throughout the years

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CHAPTER 1

THE PREFRONTAL CORTEX

The expanded cognitive abilities of man have been attributed to the fact that the frontal cortex of humans is enlarged relative to lower primates and mammals. For example, Brodmann (1912) argued that the human frontal cortex is 6% larger than the chimpanzee's, based on "total frontal lobe surface". However, subsequent studies emphasized that the frontal cortex in human brains is not larger, relative to the rest of the brain, when compared to a number of closely related great apes, including the chimapanzee (Semendeferi et al., 2001; Bush and Allman, 2004). Understanding the frontal cortex and its functions has been of significant interest for over a hundred years. However, due to moral (and legal) complications, significant manipulations in man are not often pursued, making the definition of a homologous region in a model organism critical to the study of the frontal cortex.

The frontal cortex can be separated into a number of different areas with distinct cytoarchitectonics, including what has been termed the prefrontal cortex (PFC). The prefrontal cortex can be subdivided into even smaller regions. Significant differences have been reported in the relative volume of these subdivisions, specifically Brodmann's areas 10. Brodmann's area 10 is the most anterior portion of the frontal cortex, and is significantly enlarged in humans (1.2% of total brain volume) relative to great apes and other primates (0.46-0.74%; (Fuster, 2001; Semendeferi et al., 2001). Lesions of this region have been associated with an impairment of future planning (Fuster, 2001), thus increased volume of this region suggests that humans may have an enhanced ability to perform higher-order cognitive tasks.

Absolute size of the brain, including the frontal cortex, is much enlarged in primates, relative to rodents such as the rat and mouse. Differences in relative size also exist in the prefrontal cortex of humans and non-human primates when compared to lower organisms. Because of the functional attributes of the prefrontal cortex as seen in primates, and the limited ability to study these attributes in humans and great apes in detail, considerable attention has been devoted to establishing that non-primate species, particularly rats and mice, have a prefrontal cortex (see Uylings et al., 2003).

Defining the Prefrontal Cortex

Cytoarchitectonics. Homologous regions of the brain across different species are arguably best defined based on three criteria: cytoarchitecture, connectivity, and function. In 1870, Gustav Fritsch and Eduard Hitzig published “On the Electrical Excitability of the Cerebrum,” which established that “a part of the convexity of the hemisphere of the brain of the dog is motor…another part is not motor. The motor part, in general, is more in front, the non-motor part more behind. By electrical stimulation of the motor part, one obtains combined muscular contractions of the opposite side of the body” (Fritsch and Hitzig, republished 2009). Furthermore, these mapping studies demonstrated that there was another area rostral to the motor cortex that was “electrically inexcitable” (Fritsch and Hitzig, republished 2009), meaning that stimulation of this area, did not result in peripheral muscle contractions. These studies were followed by cytoarchitectonic analyses, which noted that the previously described inexcitable area could also be distinguished from the premotor cortex by the existence of a granular layer IV, located rostral to the agranular premotor regions (Kolb, 1984; Uylings et al., 2003). However, the granular layer IV rostral to the premotor area of the frontal cortex, as described by Brodmann and others, was found to be extremely thin or even non-existent in lower mammals, suggesting that, by this definition, the prefrontal cortex is limited to primates, and is not present in lower order mammals (Preuss, 1995). Thus, in rodents there are 5 layers, including a cell-sparse layer I, layers II and III (which collectively provide most of the efferent projections to other cortices), a wide layer V with deep and superficial sublamina (the major source of subcortical projections), and a layer V of multiple cell types that gives rise to thalamic projections.

Connectivity. Cytoarchitectonic findings led to further efforts to define a brain region in lower animal species that resembled the primate PFC through connectivity studies. Rose and Woolsey demonstrated that the thalamic nuclei project to distinct regions of the cortex (Rose and Woolsey, 1949). Building from the work of Rose and Woolsey, Akert suggested that homologs of the prefrontal cortex could be defined by the medial dorsal thalamic (MD) input, which sends a massive projection to the primate and rodent frontal cortices (Akert and Monakow, 1980; Semendeferi et al., 2002; Bush and Allman, 2004). In addition to the MD projection to the frontal cortex, there is a dopamine innervation of the frontal cortex derived from the midbrain ventral tegmental area (VTA; Divac et al., 1978a; Slopsema et al., 1982; Fuster, 2001; Semendeferi et al., 2001) that overlaps with the MD input. Similarly, there is a projection of the basolateral amygdala

(BLA) onto the frontal cortex that overlaps in part with that of the MD input (Gerfen and Clavier, 1979; Fuster, 2001). These anatomical studies, together with functional data, led Divac to suggest that it is not the input from any one area, but a zone of overlap of distinct afferents which defines the PFC. Specifically, Divac argued for the overlap of the MD, VTA, and BLA inputs in defining the frontal region designated the PFC in rodents, cats, opossum, tree shrews, and pigeons (Björklund et al., 1978; Lindvall et al., 1978; Markowitsch et al., 1978; Divac et al., 1978a; 1978b; 1985; Uylings et al., 2003). Specifically, in the rat this overlap occurs in the prelimbic cortex (area 32), as well as in a region dorsal to the rhinal sulcus.

Functional studies. Disruption of the prefrontal cortex in both humans and non-human primates leads to significant changes in working memory, response inhibition, and other cognitive functions (Brutkowski, 1965; Jacobs et al., 2007; Fritsch and Hitzig, 2009). A famous example of the functional effects of frontal lobe damage is the case of Phineas Gage. Gage, working on construction crew laying railroad tracks in the mid-19th century, suffered an accident in which a large metal rod was driven through his left frontal lobe. He survived for 12 years. After his accident, friends and family members described significant personality changes, including speaking “grossest profanity”, displaying “little deference for his fellows” and an inability to stick with plans. These changes have since been attributed to the loss of frontal lobe function. Over a hundred years after his death, Gage’s case continues to garner significant interest. (Ratiu et al., 2004). Two recent studies used *in vivo* imaging techniques, including x-ray, CAT scan, and 3-D modeling, to reconstruct the exact trajectory of the rod that passed through Gage’s brain and to determine what frontal cortical regions were affected (Ratiu et al., 2004; Damasio et al., 1994). These studies both concluded that there was dorsolateral prefrontal cortex damage, although one study suggested that the damage was confined to one hemisphere (Ratiu et al., 2004), while the other concluded that there was some degree of bilateral damage (Damasio et al., 1994). The case of Phineas Gage and subsequent experimental studies (Jacobsen and Nissen, 1937; Leonard, 1969; Van Haaren et al., 1985; Preuss, 1995; Joel et al., 1997) have concluded that the frontal cortex, and more specifically the medial prefrontal cortex (not the orbitofrontal cortex), is critically involved in executive function, attention, response inhibition, and working memory.

In 1936, Jacobsen and Nissen described deficits in a delayed-response task following lesions of the frontal association areas in monkeys, over delay periods of 10 to 100 seconds between the stimulus presentation and the monkey’s responses. The authors suggested that these changes could be attributed to working memory deficits. Studies in rats and other mammals subsequently described similar working memory deficits after lesions of prelimibic cortex, suggesting that subdivisions of the prefrontal cortex in lower animals, including rats, are functionally homologous to the regions of the primate prefrontal cortex (Rose and Woolsey, 1949; Brutkowski, 1965; Van Haaren et al., 1985; Joel et al., 1997).

Performance of the Wisconsin Card Sorting Task (WCST) is a working memory test that is commonly used in neuropsychological assessments of humans. Deficits in performance on the WCST, particularly perseverative errors, are seen in humans with PFC damage (Nelson, 1976; Nyhus and Barceló, 2009). Similarly, non-human primates sustaining lesions of the PFC, mediodorsal thalamus, or the dopaminergic input to the frontal cortex arising from the VTA, exhibit performance deficits in tasks analogous to the WCST (Brozoski et al., 1979; Sawaguchi and Goldman-Rakic, 1994; Joel et al., 1997; Birrell and Brown, 2000; Mitchell and Dalrymple-Alford, 2005). This last sentence sounds like the monkeys had triple lesions.

It can be argued that the prelimbic and infralimbic cortices of the medial wall of the rodent frontal (pregenual) cortex are homologous to, but not isomorphic with the primate dorsolateral PFC. Despite lacking a clearly-identifiable Layer IV, the rat medial PFC shares with its primate counterparts key connections with the MD, BLA, and VTA, and shares with the primate PFC roles in working memory and response inhibition. I do not include area 24b, or the region dorsal and lateral to area 24b, as the PFC; these regions appear to correspond to the primate anterior cingulate cortex and regions that, in the primate, spatially segregate as the frontal eye field, premotor, and supplementary motor cortex. I will use the term PFC to refer to the medial aspects of the rodent prelimbic and dorsal infralimbic cortices, as substantial and compelling evidence now exists that rats have prefrontal cortical areas (Kolb, 1984; Brown and Bowman, 2002; Uylings et al., 2003; Roth, 2011).

Cells of the Prefrontal Cortex

Neurons have dominated neuroscience research as a whole, and PFC research has been no different. However, the brain is composed of a number of different cell types, including both neurons and glia.

Neurons. Broadly, neurons in the PFC can be divided into pyramidal cells and interneurons. As indicated by their name, pyramidal cells have a distinct pyramidal-shaped soma. Typical pyramidal cells consist of a large apical dendrite, which undergoes extensive branching in Layer I to form the apical tuft. Basal dendrites extend from the soma laterally and into deeper cortical layers (DeFelipe and Fariñas, 1992; Elston, 2003). Dendrites of pyramidal cells are studded with small protrusions known as dendritic spines, which are sites of glutamatergic input. Pyramidal cell axons typically enter the white matter of the forceps or corpus callosum, and project to and terminate in more distant subcortical or cortical targets, including but not limited to the MD, ventral tegmental area, amygdala, and contralateral PFC (Sesack et al., 1989; Carr and Sesack, 2000). In contrast, the axons of interneurons remain local; interneurons exhibit a wide variety of morphologies.

Pyramidal cells use glutamate as their neurotransmitter and make up roughly 70-80% of neurons within the prefrontal cortex (deFelipe and Farinas, 1992). We are only beginning to appreciate the heterogeneity of pyramidal cells. These neurons differ substantially in soma size, dendritic arbor, and projection target, as well as exhibiting clear molecular differences (DeFelipe and Fariñas, 1992; Molyneaux et al., 2007).

Interneurons have a paucity of dendritic spines and use γ-aminobutyric acid (GABA) as their transmitter. These local circuit neurons can be further subdivided on the basis of morphology, calcium-binding protein expression, and different neuropeptide co-transmitters. Early researchers defined numerous subtypes based on morphology and laminar distribution (for review see DeFelipe, 2002). Outside of morphological definitions, these cells are often classified by their differential, non-overlapping expression of three EF-hand calcium-binding proteins: calbindin, parvalbumin, and calretinin. However, these proteins are not expressed exclusively by any one morphologically-distinct type of interneuron. Similarly, the peptide transmitters, somatostatin (SOM), vasoactive intestinal peptide (VIP), cholecystokinin (CCK), and neuropeptide Y (NPY) are often used as markers of interneurons but are not exclusive to a distinct morphologically defined subtype. Interneurons have also been classified by their physiological properties, including fast-spiking, low-threshold-spiking, burst-spiking, irregular-spiking, and late-spiking subtypes. Once again, these physiological characteristics do not appear to correspond to any singular morphology, calcium-binding protein expression, or peptide transmitter. All of these different characteristics can be used to define a large number of distinct types of interneurons.

Glia. While neurons have consistently received the most interest from researchers and the general population, there is a growing interest in the role that glial cells play in the brain. The name glia literally means glue, suggesting that these cell types are the “glue” of the brain. Like neurons, glial cells have heterogeneous morphologies and functions. There are three major types of glia: microglia, oligodendrocytes, and astrocytes.

Microglia can be divided into a number of subtypes , including amoeboid, activated, ramified, phagocytotic, and others (Harry and Kraft, 2012). Microglia are primarily involved in the inflammatory response of the central nervous system (CNS), and have received significant attention in a variety of neurological and psychiatric conditions (for reviews see Frick et al., 2013; Tsuda et al., 2013).

Oligodendrocytes form the myelin sheath that surrounds certain axons (Simons and Lyonns, 2013). Although subtypes of oligodendrocytes have been suggested (Butt et al., 1998), this idea has not received wide acceptance (Richardson et al., 2006). Loss or damage to oligodendrocytes has been implicated in diseases such as multiple sclerosis (see Compston and Coles, 2008).

Astrocytes have been classically considered to be critically involved in ion homeostasis and blood-brain barrier function, and to play a support role for neurons. The name astrocyte suggests the star-like morphology that these cells exhibit, particularly in the human brain. Astrocytes have long been separated into fibrous and protoplasmic astrocytes, but are very diverse morphologically. The structural heterogeneity of these cells is only recently starting to be explored (Matyash and Kettenmann, 2010; Oberheim et al., 2012).

Because the prefrontal cortex and astrocytes continue to garner significant attention, understanding the role that astrocytes play in the prefrontal cortex is of particular importance. Understanding astrocytic involvement in neuronal and intracellular signaling may shed light on dysfunction of the prefrontal cortex that occurs in a number of neurological disorders.

CHAPTER 2

ASTROCYTES

Astrocytes were first identified by Camillo Golgi in 1873, and were later given their name by Michael von Lenhossek in 1891 based on their star-shaped morphology (Kettenmann and Verkhratsky, 2008). Shortly thereafter, based on their location and morphology, astrocytes were categorized as protoplasmic or fibrous astrocytes, residing in the grey and white matter respectively (Andriezen, 1893). During the initial period of interest following the discovery of astrocytes, several prominent neuroscientists, including Golgi and Santiago Ramon y Cajal, suggested potential roles for astrocytes. Hypotheses ranged from metabolic support for neurons to direct regulation of synaptic connectivity (Verkhratsky et al., 2011). Unfortunately, due to technical limitations, these hypotheses remained uninvestigated for nearly 100 years, and astrocytes continued to be known only as the "glue" holding neurons together. In the late 20th century, investigations began to evaluate the specific functional attributes of astrocytes, including metabolic and neurotrophic support, neurotransmitter uptake, neurotransmitter release, and even synapse formation.

Astrocytes and Glutamate

Astrocytes are critical in maintaining the levels of a number of extracellular molecules, such as K^+, within homeostatic levels (Walz et al., 1984). Similarly, aquaporin proteins localized to astrocytic membranes play an important role in regulating osmolality (Nagelhus et al., 1998; Manley et al., 2000). These studies began to shift the perception of astrocytes from “glue” holding neurons together, to playing an active role in intercellular communication.

Astrocytes are also critically involved in the metabolism of glutamate, from release to inactivation of signaling. Glutamine synthetase, the enzyme responsible for the synthesis of the glutamate precursor glutamine, is expressed only in astrocytes (Erecińska and Silver, 1990). Glutamate, once released from axon terminals, is not enzymatically inactivated in the extracellular space. Instead, the termination of glutamate signaling occurs only through diffusion or uptake of the transmitter (Niciu et al., 2012). Astrocytes are the major cellular elements that accumulate glutamate from the extracellular space, and are responsible for >90% glutamate uptake in the adult cortex (Rothstein et al., 1996; Tanaka et al., 1997).

Glutamate Uptake. High concentrations of glutamate are excitotoxic (Mehta et al., 2013). Accordingly, considerable attention has been devoted to understanding the mechanisms involved in maintenance of extracellular glutamate levels. Excitatory amino acid transporters (EAATs) are the primary means of glutamate uptake and the resultant termination of glutamate signaling in the brain. This group of transporters has five members, designated EAAT 1-5 in humans (Grewer et al., 2014). In rodents, EAAT 1-3 correspond to GLAST, GLT-1, and EAAC1, respectively, while EAAT4 and EAAT5 share their nomenclature with their human homologues (Bridges and Esslinger, 2005). The rodent nomenclature will be used throughout the extent of this dissertation.

Human Nomenclature	**Rodent Nomenclature**	**Localization**
EAAT1	**GLAST**	Primarily astrocytes, some neurons
EAAT2	**GLT-1**	Astrocytes
EAAT3	**EAAC1**	Neurons
EAAT4	EAAT4	Cerebellum
EAAT5	EAAT5	Retina

Table 1. Nomenclature and cellular localization of the excitatory amino acid transporters (EAATs). GLAST, GLT-1 and EAAC1 (**Bold**) are those transporters localized to the rodent PFC and this nomenclature is used throughout the dissertation.

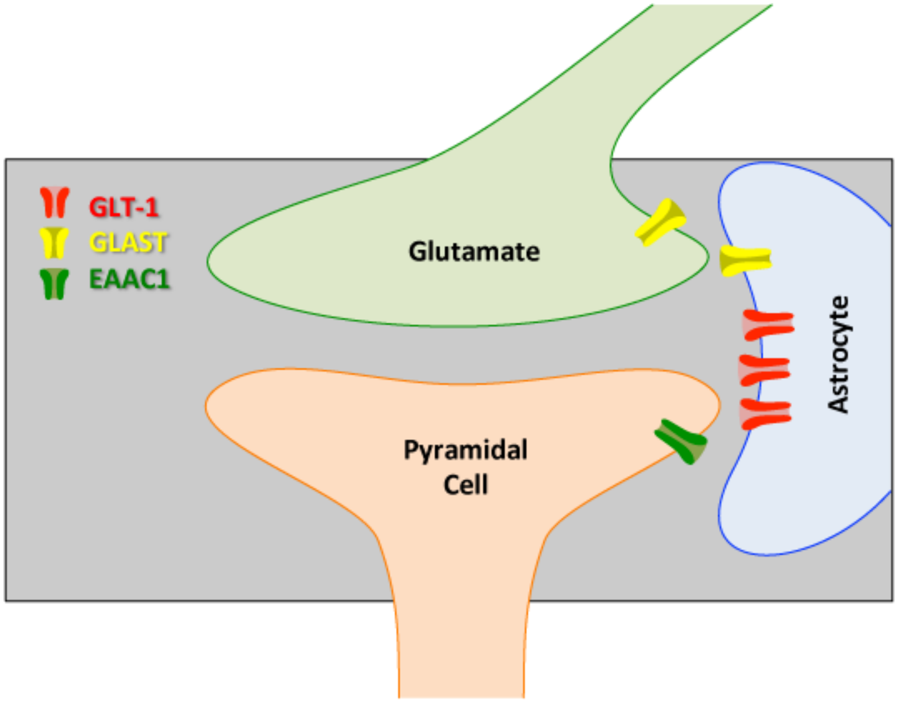

Figure 1. Illustration of a synapse with presynaptic (glutamate), postsynaptic (pyramidal cell) and astrocytic elements, displaying the current understanding of the cellular localizations of the three glutamate transporters expressed in the prefrontal cortex. GLT-1 expression in the PFC is considered to be exclusive to astrocytes in the normal adult rat. Expression of EAAC1 as determined by ultrastructural studies appears to be present on neuronal cell bodies as well as dendrites, with an absence of axonal expression (Rothstein et al., 1994). GLAST is primarily expressed by astrocytes, although some evidence suggests minor neuronal expression.

Of the five EAAT proteins, GLAST, GLT-1, and EAAC1 are expressed in the cerebral cortex, but have different cellular distributions (Table 1, Figure 1). GLAST and GLT-1 are localized primarily to astrocytes, while EAAC1 is localized to neurons. EAAT4 is localized to neurons of the cerebellum while EAAT5 is expressed exclusively in the retina (for reviews see Danbolt, 2001; Bridges and Esslinger, 2005; Kanai et al., 2013; Grewer et al., 2014).

Examining the functional consequences of manipulating levels of these transporters has demonstrated their importance in preventing excitotoxity (Meldrum et al., 1999). For example, Rothstein and colleagues chronically treated spinal cord cultures with antisense oligonucleotides, effectively knocking out GLAST or GLT-1. This resulted in increased excitotoxicity with antisense GLT-1 leading to 32-fold increases in extracellular glutamate (Rothstein et al., 1996). Null mutant GLT-1 mice exhibit seizures early in life, with 50% of animals dying by 6 weeks, further demonstrating the physiological importance of glutamate transporters, particularly GLT-1 (Tanaka et al., 1997). Tanaka et al. also reported that GLT-1 is responsible for >90% of glutamate uptake in the cortex (1997). These studies demonstrate a critical role for this family of transporters, particularly those localized to astrocytes, in the uptake of glutamate and termination of glutamate signaling.

Glutamate Release. Although it has been known for the past 35 years that astrocytes are critically involved in glutamate uptake (Hertz et al., 1980; Walz et al., 1984), more recent studies indicate that astrocytes also release transmitters, including glutamate (for review see Hamilton and Attwell, 2010). In 1990, Cornell-Bell et al. were the first to report that glutamate induces a rise in intracellular calcium levels in astrocytes, and that these increases can be propagated across astrocytes (Science). Furthermore, increasing calcium levels in astrocytes, as induced by glutamate, appears to lead to calcium influx by neighboring neurons and astrocytes (Cornell-Bell et al., 1990; Parri et al., 2001; Perea and Araque, 2005). Finally, a glutamate-induced increase in intracellular astrocyte calcium levels evokes the release of glutamate and other molecules (such as ATP and D-serine) from astrocytes into the extracellular space (Guthrie et al., 1999; Mothet et al., 2005; Hamilton et al., 2008). These data indicate that astrocytes release glutamate and other neurotransmitters, a process that has been termed gliotransmission (Bezzi and Volterra, 2001).

Gliotransmission, in particular a process resembling neuronal vesicular release of neurotransmitters, remains controversial. A number of studies have shown that synaptic machinery necessary for vesicle fusion is present in the astrocyte, including synaptobrevin 2, syntaxins 1, 2 and 4, and also SAP23 (for review see Montana et al., 2006). However this machinery is also required for endosomal fusion (Proux-Gillardeaux et al., 2005; Oishi et al., 2006). Similarly, cultured astrocytes express a number of voltage gated-calcium channels including N-, L-, R-, and T-type channels (Latour et al., 2003), some of which are critical for neurotransmission. While much of the machinery exists for vesicular release of glutamate, one basic point has left some investigators skeptical: vesicles comparable to those found in neurons have not been described. Electron microscopy studies have reported the existence of vesicle-like structures in astrocytes that contain vesicular proteins such as the vesicular glutamate transporters 1 and 2 (Bezzi et al., 2004). However, these astrocytic “vesicles” are not organized in the densely-packed and organized manner that is present in axon terminals, and they vary in size from 30 to 300nm compared to approximately 40 nm in neurons (Bezzi et al., 2004). While these factors suggest that the vesiscular-like organelles in axons may differ from those seen in astrocytes, single fusion events of these vesicle-like compartments to the plasma membrane of astrocytes can be visualized using total internal reflection fluorescence (TIRF) microscopy (Bowser and Khakh, 2007). For a review of gliotransmission refer to Parpura and Zorec (2010), which discusses exocytotic (vesicular-like) release of glutamate, ATP, peptides, and other signaling molecules from astrocytes.

While vesicular release of gliotransmitters from astrocytes has been somewhat controversial other, less contested, mechanisms exist for astrocytic release of glutamate into the extracellular space, including channels, reversal of amino acid transporters, and antiporters. The antiporter system xC$^{-,}$ expressed by astrocytes, is a heterodimer comprised of both a light chain (xCT) and a heavy chain (4F2hc), and exchanges glutamate from the astrocyte to the extracellular space in exchange for a cystine molecule with a 1:1 stoichiometry (Niciu et al., 2012). Substrate specificity of system xC^{-} is determined by xCT while 4F2hc is necessary for trafficking of xCT to the membrane (Sato et al., 2000; Wang et al., 2003; Sato et al., 2005). The cystine brought into the cell is used in the synthesis of glutathione. Astrocytes, by virtue of their role in glutathione production, have neuroprotective effects under certain conditions. For example, the cytotoxic effects of nitric oxide or glutamate exposure of primary cortical cultures are attenuated in the presence of a dialysate probe coated in astrocytes (Chen et al., 2001), while NMDA-induced excitotoxicity remains unaffected. Because of the importance of cystine for glutathione production, many studies of xC- have focused on the influx of cystine and the production of glutathione rather than the consequences of glutamate efflux into the extracellular space (Shih et al., 2006).

It has been difficult to untangle the relative contributions of astrocytes and neurons to extracellular glutamate levels *in vivo*. As new methods emerge, it will be important to determine to what degree, and under which physiological or pathophysiological conditions, astrocytic release of glutamate is functionally significant *in vivo*. However, the body of work that was sparked by the suggestion of glutamate release from astrocytes has turned attention from the homeostatic role of astrocytes to their active role in signaling in the brain.

Astrocyte Expression of Neurotransmitter Receptors

Neurotransmitter receptors have mainly been studied from the vantage point of neurons. The hypothesis that glia participate in synaptic signaling has been strengthened by the fact that many transmitter receptors are expressed by astrocytes.

Dopamine Receptors. Autoradiographic studies using tritiated dopamine binding suggested the presence of dopamine receptors on astrocytes more than 25 years ago (Hösli and Hösli, 1986). Both anatomical and molecular studies subsequently confirmed the presence of D_2 receptor protein and mRNA in astrocytes, including prefrontal cortical astrocytes (Bal et al., 1994; Khan et al., 2001). Consistent with these findings, stimulation of D_2 receptors in astrocytic cultures with relatively high (50 μM) concentrations of dopamine has been reported to increase astrocytic calcium levels (Parpura and Haydon, 2000). Despite the identification of functional dopamine receptors on astrocytes, the downstream effects of dopamine binding to these receptors remains largely unexplored.

Glutamate Receptors. Glutamate receptors are broadly classified into two groups, ionotropic and metabotropic glutamate receptors. Ionotropic glutamate receptors are ligand-gated ion channel complexes, and include three different subtypes of receptors: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate receptors. Glutamate binding to these heteromeric receptors results in the influx of Na^+ and Ca^{2+}. Immunohistochemical and functional studies have led to the conclusion that both NMDA and AMPA receptors are localized to astrocytes (Bowman and Kimelberg, 1984; Kettenmann et al., 1984; Conti et al., 1994; Schipke et al., 2001; Matthias et al., 2003; Lalo et al., 2006).

Metabotropic glutamate receptors (mGluRs) are G protein-coupled receptors. These proteins are divided into Group I (mGluR1 and 5), Group II (mGluR2 and 3), and Group III (mGluR4, 6, 7, and 8) receptors, based on sequence similarity, pharmacology, and G protein-coupling. The group I receptor mGluR5 is expressed cultured astrocyte processes and, *in vivo*, in cortical astrocytes, albeit at lower levels than in observed cultures. (Petralia et al., 1996). However, neither mGluR1 mRNA or protein has been detected in astrocytes Ohishi et al. (1993) described expression of the Group II receptor mGluR3 mRNA in astrocytes. Subsequent studies using an antibody that does not discriminate between the two different types of Group II mGluRs detected protein in astrocytes (Petralia et al., 1996; Mineff and Valtschanoff, 1999). Because mGluR2 is not expressed by astrocytes, it appears that the Group II mGluR found in astrocytes is mGluR3. Interestingly, activation of Group I and Group II mGluRs can elicit astrocyte motility in cultures (Lavialle et al., 2011). Functional studies have included determination of neuroprotective effects of Group II mGluR activation on astrocytes (Yao et al., 2005). Specifically, Caraci et al demonstrated, using glial conditioned media, that astrocytic mGluR3 activation is neuroprotective in the face of an amyloid-beta challenge (Caraci et al., 2011).

Other Neurotransmitter Receptors. A number of other neurotransmitter receptors have been localized to astrocytes using immunohistochemical and functional approaches. These include purine receptors ($P2X_7R$;Fellin et al., 2006), $GABA_A$ receptors, serotonin receptors ($5HT_{5A}$, $5HT_{2B}$;Carson et al., 1996; Sandén et al., 2000), α_1- and β-adrenergic receptors (Pearce et al., 1985; Aoki, 1992), muscarinic acetylcholine receptors using immunohistochemistry (Van Der Zee et al., 1993), and histamine H1, H2, and H3 receptors (Arbonés et al., 1988; Jurič et al., 2011). Interestingly, some of these receptors have different, even opposite effects on astrocytes than they do on neurons. For example, $GABA_A$ receptor activation leads to an efflux of Cl^- from astrocytes (Fraser et al., 1995). While the functional significance of the astrocytic forms of neurotransmitter receptors continues to be investigated, their existence suggests that a variety of neurotransmitters act on astrocytes.

Astrocytes and Synapse Formation

Astrocyte contributions to neuron development, and specifically to synapse formation, are becoming increasingly popular areas of study (Clarke and Ben A Barres, 2013). Studies in cultured retinal ganglion cells (RGCs) show that synapse formation increases dramatically when these cells are cultured with astrocytes or astrocyte–conditioned media (Ullian et al., 2001; Diniz et al., 2012). These synapses contain functional AMPA receptors when RGCs are cultured with neurons from the superior colliculus, a projection target of RGCs (Ullian et al., 2001). Subsequent studies have suggested that extracellular matrix proteins elaborated by astrocytes are responsible for the formation of certain synapses (DeFreitas et al., 1995; Eroglu et al., 2009; Crawford et al., 2012). In particular, a class of astrocyte-secreted proteins known as thrombospondins (TSPs) are involved. The TSPs are five large extracellular matrix proteins involved in both cell-cell and cell-matrix interaction, acting at specific membrane receptors (Christopherson et al., 2005). TSP1 and TSP2 are highly expressed early in rodent brain development, corresponding to a period of high levels of synapse formation, and are down-regulated in adults (Christopherson et al., 2005). While the addition of TSPs alone to cultures of RGCs can induce synapse formation, including the formation of postsynaptic densities, these synapses contain few AMPA receptors and are therefore silent, suggesting that a different factor(s) may be involved in inducing AMPA receptor insertion into the postsynaptic density (Christopherson et al., 2005). Further work has since determined that two more astrocytic proteins are involved in synaptogenesis, hevin and SPARC: hevin is sufficient for synapse formation in RGC cultures (Kucukdereli et al., 2011).

As interest in astrocytes has grown, there has been an expansion of the concept of synaptic signaling to include not only the presynaptic and postsynaptic neuronal elements, but also the astrocyte. Astrocytes are closely associated with excitatory synapses, enveloping excitatory pre- and post-synaptic neuronal elements (Eroglu and Barres, 2010). The release of neurotransmitters from astrocytes, the involvement of astrocytes in terminating the action of certain neurotransmitters, and the role of astrocytes in elaborating extracellular matrix proteins and in determining synapse formation, all point to gliotransmission as being an active process in neuronal signaling, leading to the coining of the term tripartite synapse.

CHAPTER 3

DOPAMINE AND GLUTAMATE HYPOTHESES OF SCHIZOPHRENIA

A number of hypotheses on the pathophysiological basis of schizophrenia have been advanced, ranging from environmental to genetic components and everything in between (Eyles et al., 2012; Stachowiak et al., 2013; Maziade and Paccalet, 2013; Schmitt et al., 2014). These hypotheses often converge on the dopamine and/or glutamate signaling pathways. Both the dopamine and glutamate hypotheses arose largely through observations of pharmacological manipulations, and have undergone significant refinement over the years.

The Dopamine Hypothesis. The dopamine hypothesis of schizophrenia was first put forward in the middle of the 20th century. Following the introduction of typical antipsychotic drugs (APDs), a remarkable positive correlation between D2 affinity and the average daily dose of the APD (or plasma level of the drug) was observed, and was the major development responsible for the wide-spread acceptance of the dopamine hypothesis of schizophrenia (Creese et al., 1976; Seeman et al., 1976). As the dopamine hypothesis continued to develop, it appeared that the effectiveness of antipsychotic agents in controlling positive symptoms could be linked to their affinity for the D_2 dopamine receptor. Further evidence for the importance of the dopamine receptor in the treatment of psychosis came from studies involving amphetamine, which increases monoamine levels within the synapse, and can lead to psychotic symptoms (Peselov et al., 1985; Davidson et al., 1987). This first iteration of the dopamine hypothesis posited that the cause of schizophrenia was hyperdopaminergia, and that treatment with a dopamine receptor antagonist could relieve these symptoms. Davis et al. (1991) and Deutch (1992) put forth the second version of the dopamine hypothesis, which suggested that positive symptoms of schizophrenia arise from an increase in dopamine tone in subcortical structures, including the striatum, while negative symptoms arise from decreased dopamine in the prefrontal cortex. Studies from several investigators, including both Pycock (1980) and Deutch (1990), suggested that cortical hypodopaminergia might lead to excessive dopaminergic activity in subcortical regions.

Pharmacological manipulation of PFC dopamine receptors in animal models causes deficits in executive functions, including processes such as working memory and attention Currently, an “inverted U” model suggests that PFC dopamine must be tightly regulated, as too much or too little dopamine leads to deficits in working memory (Sawaguchi and Goldman-Rakic, 1991; Arnsten 2011). The involvement of dopamine in PFC function suggests a central role for dopaminergic signaling in the cognitive symptoms of schizophrenia.

The Glutamate Hypothesis. Uncompetitive NMDA receptor antagonists, such as PCP and ketamine, produce psychotomimetic effects in healthy control subjects, and, at doses that do not exert such effects in healthy control subjects. Administration of NDMA antagonists to schizophrenic subjects leads to an acute exacerbation of the psychotic process and the emergence of cognitive deficits. For example, studies as early as the

1960s suggested that the NMDA antagonists, PCP and ketamine, administered to healthy control subjects produced effects consistent with positive, negative, and cognitive symptoms of schizophrenia (Davies and Beech, 1960; Bakker and Amini, 1961). More recent studies have reported that ketamine, PCP, and MK-801 result in cognitive deficits in a number of widespread domains, including working memory and executive processes, (Ghoneim et al., 1985; Oye et al., 1992; Krystal et al., 1994). These findings have slowly given rise to the glutamate hypothesis of schizophrenia, which in its current form posits that NMDA receptor hypofunction in PFC interneurons may contribute to the pathophysiology of schizophrenia (Homayoun and Moghaddam, 2007; Belforte et al., 2010; Gonzales-Burgos and Lewis, 2012).

HYPOTHESIS

While convincing evidence exists to support both the dopamine and glutamate hypotheses of schizophrenia, changes to one of these transmitter systems results in changes in the other (Gonzalez-Islas and Hablitz, 2003; Tseng and O’Donnell, 2004; Sokoloff et al., 2013; Usun et al., 2013; Yuen et al., 2013). Therefore, understanding interactions between these two transmitter systems will be critical to an increased appreciation of the pathophysiology schizophrenia and ultimately the development of better treatment approaches for the illness. Astrocytes may be a critical locus for dopamine-glutamate interactions, I hypothesize that dopamine depletion of the PFC will lead to significant changes in glutamate-related proteins. In particular, I posit that dopamine depletion will lead to alterations in the astrocytic glutamate transporter GLT-1, which plays a critical role in cortical glutamate uptake. In the following chapters, I will explore, and present data to support, this hypothesis, through the use of immunoblotting, immunohistochemistry, RT-PCR, stereology, and functional assays.

CHAPTER 4

GLUTAMATE TRANSPORTER EXPRESSION FOLLOWING DOPAMINE DEPLETION OF THE PREFRONTAL CORTEX

Excitatory amino acid transporters (EAATs) are the primary means of termination of glutamate signaling in the brain. Alterations in glutamate transporter levels or activity have been reported in a number of neurological diseases, including schizophrenia (Bauer et al., 2010; Rao et al., 2012). Excessive synaptic glutamate concentrations, as would be seen with a decrease in glutamate reuptake, can cause excitotoxicity. In the striatum, a loss of dopamine increases GLT-1 protein levels (Massie et al., 2010). Similar studies in the PFC have not been performed. In the following experiment, I investigated the effect of dopamine depletion of the PFC on glutamate transporter protein levels.

Methods

Animals. Adult male Sprague–Dawley rats (Harlan; Indianapolis, IN) were group-housed with food and water *ad libitum,* and kept on a 12 hour light-dark cycle. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals as promulgated by the National Institutes of Health.

Surgical procedures. Animals underwent bilateral 6-hydroxydopamine (6-OHDA) lesions of the VTA, the source of the dopamine innervation of the PFC. We used this approach, rather than direct PFC manipulations, in order to avoid mechanical damage to the PFC and a resultant astrocytosis. In order to prevent uptake of 6-OHDA into noradrenergic axons coursing through and above the VTA, rats were injected with desipramine (12.5 mg/kg, i.p.) 30 and 15 minutes before infusion of 1.0 μL of 6-OHDA HBr (4.0 μg/μL free base in 0.02% ascorbic acid at 4^0 C). The 6-OHDA solution was infused through a pump-controlled microsyringe fitted with a 33g needle at a rate of 250 nL/min at the following coordinates: AP: -5.4, ML: +/- 0.6, DV: -8.4 (Paxinos and Watson, 1998). Sham-operated animals served as controls. Animals were sacrificed 3 weeks following surgery (N =6 control and 13 lesions).

Catecholamine determinations. The medial PFC dorsal to the prelimbic cortex (including the pregenual cingulate [area 24b] and shoulder cortices; see Figure 2) was dissected for subsequent determination by HPLC-EC of catecholamine concentrations (Deutch and Cameron, 1992).

Immunoblotting. The medial PFC, including both the prelimbic (area 32) and infralimbic (area 25) cortices (see Figure 2), was dissected from 1.0 mm thick coronal slices and stored at – 80° C for up to 2 months before fractionation and subsequent immunoblot. Samples were subjected to a subcellular fractionation method using a series of buffers that contained no detergent, 1% Triton X-100, or 1%Triton/deoxycholate, yielding cytosolic-, membrane-, and postsynaptic density (PSD)-enriched fractions, respectively

(Gustin et al., 2010). Samples were then mixed with sample buffer containing beta-mercaptoethanol and heated at 65 °C for 10 minutes and separated on a 10% SDS-polyacrylamide gel by electrophoresis. Proteins were transferred to nitrocellulose membranes, which were then stained with Ponceau-S and digitally scanned in order to compare total protein levels in each lane.

Membranes were blocked in 4% nonfat dry milk in PBS, after which they were incubated in primary antibodies against GLT-1, GLAST, and EAAC1 (Table 2) in 4% milk in PBS overnight at 4^{o} C. Two different GLT-1 antibodies (see Table 2) were used in order to confirm our results. The membranes were washed in PBS and incubated in appropriate peroxidase-conjugated donkey antibodies (Jackson ImmunoResearch Laboratories Inc; West Grove, PA). Membranes were developed using a chemiluminescent protocol (Plus-ECL;Perkin-Elmer; Waltham, MA). The bands representing the individual proteins of interest were densitometrically scanned using ImageJ, and normalized to total protein levels from the Ponceau-stained membranes (Romero-Calvo et al., 2010).

Antibody	Company	Antigen	Catalog #	Dilution
GLT-1	Tocris	amino acids 554-573 of GLT-1 protein	#2063	1:100k
GLT-1	Cell Signaling Technologies	C-terminus peptide	#3838	1:5000
GLAST	Novus Biologicals	20aa residue C-terminus peptide	NB100-1869	1:3000
EAAC1	Alpha Diagnostics	14aa residue C-terminus peptide	#EAAC11-A	1:500

Table 2. Source information and dilutions for antibodies used to examine changes in glutamate transporters following dopamine depletion.

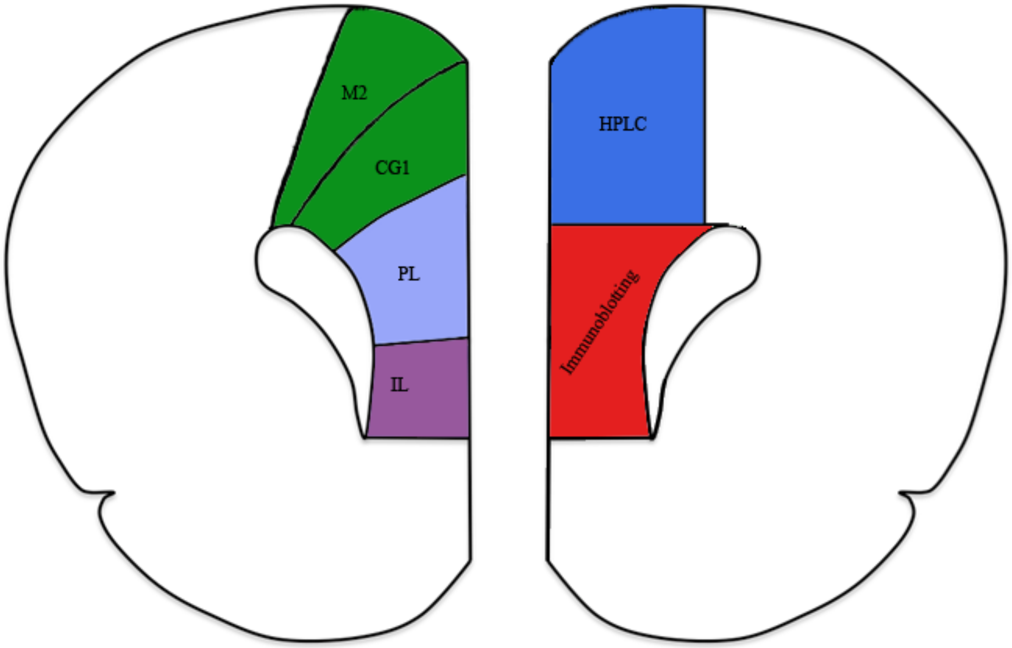

Figure 2. Coronal sections illustrating the regions dissected for HPLC analysis of catecholamine concentrations, and for immunoblot analysis. **Left)** Coronal section indicating the prelimbic (PL) and infralimbic (IL) cortices that make up the rodent PFC, as well as cingulate cortex (CG1) and secondary motor cortex (M2) **Right)** A coronal section illustrating the regions used for various aspects of our experiments including HPLC analysis of dopamine levels, and immunoblotting.

Results

Extent of dopamine depletion. In our initial 6-OHDA lesion experiment, dopamine depletion levels were decreased by 53.3 ± 6.6% (t_{14}= 5.83, $p<0.001$; Table 3). The dopamine concentration in the PFC of control animals averaged 1.06 ± 0.074 ng/mg protein. Norepinephrine concentrations (control value of 6.191 ± 0.343 ng/mg protein) were not significantly affected by dopamine denervation of the PFC (Table 3). Serotonin levels (control value of 8.743 ± 0.483 ng/mg protein) were reduced in animals receiving a 6-OHDA lesion by 25.2 ± 5.8% ($p<0.05$, Table 3). In subsequent cohorts prefrontal cortical dopamine concentrations in 6-hydroxydopamine-lesioned rats were reduced by 70.4 ± 3.6% (t_{17}= 9.02, $p<0.001$; Table 3) and 59.1 ± 3.4% (t_{15}= 4.40, $p<0.001$; Table 3). No significant change was seen in norepinephrine in either of the subsequent runs while serotonin was reduced in cohorts II and III by 28.9 ± 4.0% (t_{17}= 4.85, $p<0.001$; Table 3) and 15% (t_{15}=2.56, $p<0.05$; Table 3).

	Dopamine	Norepinephrine	Serotonin
	Concentration (ng/mg protein)		
Cohort I			
Control	1.006 ± 0.087	6.191 ± 0.343	8.743 ± 0.483
Lesion	0.485 ± 0.022 **	5.839 ± 0.187	6.541 ± 0.509 *
Cohort II			
Control	1.050 ± 0.086	5.137 ± 0.192	9.851 ± 0.346
Lesion	0.311 ± 0.039 **	5.083 ± 0.228	6.999 ± 0.398 **
Cohort III			
Control	0.459 ± 0.063	3.651 ± 0.143	7.298 ± 0.386
Lesion	0.188 ± 0.016 **	3.415 ± 0.112	6.102 ± 0.285 *

Table 3. Concentrations of dopamine, norepinephrine, and serotonin in the prefrontal cortex following 6-OHDA lesion of the ventral tegmental area (VTA).

** $p < 0.001$; * $p < 0.05$

PFC GLT-1 protein levels and dopamine depletion. Both GLT-1 antibodies revealed a clear band at ~62 kDa, with a much lighter “smear” visible between 150 and 250 kDa. The majority of GLT-1 was seen in the membrane-enriched fraction (Figure 3). Following dopamine depletion, PFC levels of GLT-1 across all lesioned animals were increased by 240 ± 28.2% in the membrane-enriched fraction compared to control animals (t_{16}=2.88, p=0.011). No changes in either GLAST or EAAC1 were observed (Figure 4). No differences were seen in glutamate transporter levels of any of the three transporters in the cytosolic- or PSD-enriched fractions. In subsequent cohorts we were able to achieve similar increases in GLT-1 levels of 150.0 ± 17.7% and 173.6 ± 19.4% using two different antibodies to GLT-1. As was demonstrated in the initial cohort, no changes were observed in GLT-1 in cytosol- or PSD-enriched fractions. Likewise, no changes were observed in any fraction for GLAST or EAAC1.

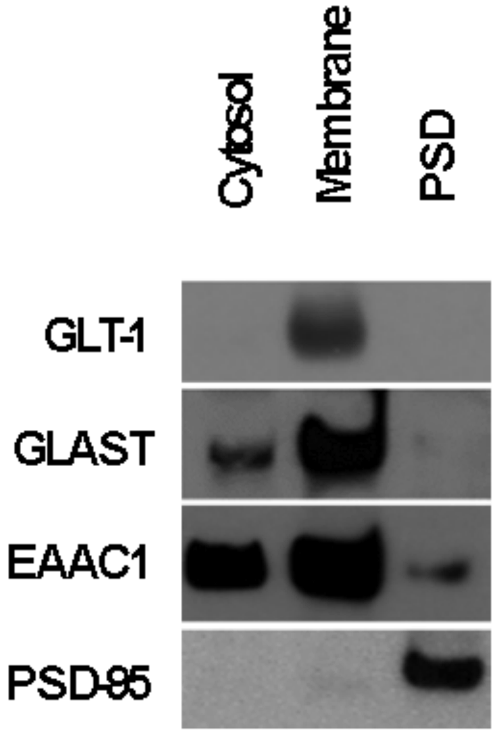

Figure 3. Fractionation of samples. Use of increasingly harsh detergents yields cytosolic-, membrane-, and post-synaptic density (PSD) –enriched fractions. GLT-1 GLAST, and EAAC1 are primarily localized to the membrane-enriched fraction. PSD-95, localized to the post-synaptic density, was used as a control to verify the effectiveness of our method.

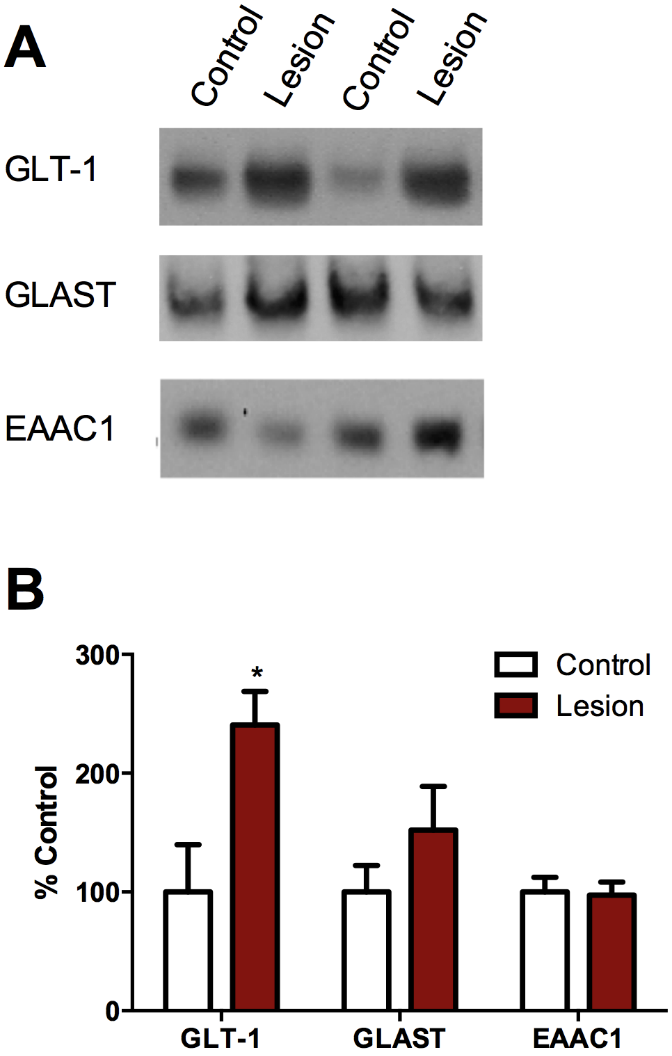

Figure 4. The effects of PFC dopamine loss on glutamate transporter protein levels in the PFC. Levels of GLT-1 are significantly increased by ~240% following dopamine depletion, without a significant change in levels of GLAST or EAAC1.

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* = $p < 0.01$

Discussion

Dopamine in the PFC. Animals injected with 6-OHDA into the PFC averaged ~70% loss of dopamine relative to controls, spanning a range from 40 to 91% depletion. This rather variable degree of dopamine denervation is consistent with previous studies, including those that injected 6-OHDA directly into the PFC (Bubser, 1994; King and Finlay, 1995). Even when 6-OHDA is directly injected into the PFC, dopamine depletion averages only ~80% (King and Finlay, 1995), and needle insertion causes gliosis in the region of interest. Gliosis, as mentioned in Chapter 2, is an activation of astrocytes and other glia following injury to the brain. In this study, an astrocytic response to mechanical damage caused by a needle being lowered into the PFC, in the form of protein increases or decreases, would confound results aimed at determining astrocytic protein changes that are a result of dopamine depletion. Therefore, VTA lesions appear to be the most appropriate method of dopamine denervation of the PFC for these experiments. The partial dopamine denervation achieved with this method presumably reflects an inability to take up 6-OHDA by some dopamine neurons innervating the PFC, because of a very low or absent expression of the dopamine transporter (Freed et al., 1995; Sesack et al., 1998).

Dopamine depletion of the PFC through injection of 6-OHDA into the VTA does lead to dopamine depletion of other areas, such as the nucleus accumbens (>90% depletion) and striatum (~70% depletion). Ockham’s razor would have us believe that the simplest answer is the right one until proven differently. Therefore, our current hypothesis is that dopamine depletion of the PFC itself, rather than depletion of areas projecting to the PFC, leads to increases in PFC GLT-1 levels. However, it is important to keep in mind that observations in the PFC may be a downstream effect of dopamine depletion of one of these other areas, and this idea may be worth investigating in the future.

Similarly, use of 6-OHDA injections (into the prefrontal cortex or the VTA) may have effects that are not specific to dopaminergic cells, destroying noradrenergic or serotonergic axons. To prevent a loss of noradrenergic axons, animals were treated with desipramine to attenuate 6-OHDA uptake through the norepinephrine transporter (NET). We measured levels of norepinephrine and serotonin in all of our runs, and determined that norepinephrine levels did not change. Levels of serotonin were reduced in the PFC of lesioned animals, albeit to a much lower extent than dopamine (~25% depletion compared to ~70% for dopamine). Alternative approaches could be used to verify that the effect we observe is specific to dopamine depletion rather than changes in other transmitters. These include the use of pharmacological methods (dopamine receptor antagonists), or knockout or transgenic models (again targeting dopamine receptors, although these animals would likely need to be flox recombinant via inducible Cre in order to prevent developmental complications).

Increases in GLT-1 following dopamine depletion. In these experiments we determined the levels of three glutamate transporters by immunoblot analyses of cytosolic-, membrane-, and postsynaptic density-enriched fractions of the PFC. All three glutamate transporters were most abundant in the membrane-enriched fraction. The fractionation approach that we used yields fractions that are enriched in the named target, although there may be trace amounts of off target proteins. Nonetheless, this approach yields data that agrees well with anatomical analyses of protein localization. Immunohistochemical studies have found that GLT-1 and GLAST are primarily localized to the plasma membrane (Chaudhry et al., 1995). Furthermore, our observations are consistent with ultrastructural data indicating that EAAC1 is localized to both the plasma membrane and, to a lesser degree, the cytosol(Conti et al., 1998).

We observed an increase in GLT-1 in the membrane-enriched fraction of dopamine-denervated PFC. Increases in GLT-1 levels were also observed in replicate experiments using two different GLT-1 antibodies. These antibodies were both directed towards the C-terminus, with one antigen raised against a synthetic peptide derived from the amino acids 554-573 of the rat protein. The second antibody was derived from a different region of the C-terminus as verified by the vendor, although an exact peptide sequence was not disclosed. Results using these two antibodies were similar, with increases in GLT-1 levels of approximately 150% and 175%. The use of two antibodies yielding comparable results, as well as replication of the finding across multiple cohorts, makes us particularly confident in our findings. The increase in membrane-associated GLT-1 was not accompanied by any detectable change in levels of the transporter in the cytosolic or membrane-enriched fractions, suggesting that the increase is due to GLT-1 protein accumulation in membranes.

Accumulation of GLT-1 in membranes may be a response to increased extracellular glutamate following a loss of dopamine. Our hypothesis suggests that dopamine depletion leads to increases in extracellular glutamate levels and that GLT-1 levels increase as a compensatory mechanism in an effort to prevent excitotoxic effects that can result from prolonged increases in extracellular glutamate. However, we did not measure extracellular glutamate levels in our studies. Glutamate levels increase following dopamine depletion of the striatum (Meshul et al., 1999). Consistent with these findings, previous data from our lab also suggest that dopamine depletion increases glutamate levels in the striatum (Garcia et al., 2010). To the best of our knowledge, extracellular glutamate concentrations following dopamine depletion of the PFC have not been examined, and could be a subject for further studies.

Studies using pharmacological disruption of dopamine signaling have reported that neither acute, nor chronic treatment with the D_2 antagonist haloperidol changes extracellular glutamate levels in the PFC (Daly and Moghaddam, 1993; Yamamoto and Cooperman, 1994). These studies did not use no-net flux dialysis, and thus were unable to compare basal levels, but rather measured relative changes in response to a challenge to baseline levels. This led Yamamoto and colleagues (Yamamoto and Cooperman, 1994) to assess evoked release of glutamate. They found that K^+-elicited depolarization led to a non-significant trend toward an increase in PFC glutamate levels in rats that were chronically treated with haloperidol. Unfortunately p-values for non-significant results were not reported in their paper; similarly, the N was not reported for their figure. However, all other figures included in the manuscript had an N of 5-7 rats, leading us to infer that the same is probably true for these results as well. Thus, it may be that with a larger number of animals, a significant result might have been observed.

Potential for post-translational modification. Several possibilities exist to explain increased PFC levels of GLT-1 observed after dopamine depletion, including an increase in GLT-1 gene expression (Chapter 6), an increase in astrocyte number or activation (Chapter 7), or changes in the turnover rate of the protein.

Post-translational modifications can lead to changes in the function and localization of proteins. Our immunoblot analyses showed a clear band for GLT-1 corresponding to ~65-70 kDa, consistent with previous reports (Schmitt et al., 2003; Zou et al., 2011). However, there was a faint smear of high molecular weight (150-250 kDa) protein species, suggesting the existence of post-translationally modified forms of GLT-1. Analyzing these high molecular weight species, we uncovered a significant decrease following moderate dopamine depletion of the PFC. Previous studies have demonstrated that ubiquitination of GLT-1 is required for its internalization and subsequent degradation (González et al., 2005; Boehmer et al., 2006; Donovan and Poronnik, 2013). Our own observation of decreased high molecular weight species, coupled with these studies, suggest that accumulation of GLT-1 may be a result of decreased ubiquitination following moderate dopamine depletion.

Unfortunately, our attempts to detect polyubiquitinated GLT-1 in the PFC using immunoprecipitation of GLT-1 and ubiquitin, as well as pull-down assays using ubiquitin-binding small molecules, failed. Several technical challenges make *in vivo* detection of ubiquitin difficult. Some of these include the transient nature of polyubiquitinated proteins, the high expression of deubiquitilyases in tissue, and the lack of ubiquitin antibodies with a robust immunoprecipitation profile. Thus, while difficult to demonstrate in vivo, we speculate that decreases in ubiquitination, and the subsequent degradation, of GLT-1 may explain accumulation of GLT-1 protein in membrane-fractions following dopamine depletion.

CHAPTER 5

DOPAMINE DEPLETION EXTENT REVEALS DIFFERENTIAL EFFECTS ON GLT-1

In Chapter 4, we observed that dopamine denervation of the PFC increases levels of the glutamate transporter GLT-1, but not the two other cortical transporters, GLAST and EAAC1. Although there is a significant overall increase in membrane-associated GLT-1, we noticed that the distribution of GLT-1 levels in the individual animals followed what appeared to be a bimodal distribution. We therefore determined whether there was a correlation between the extent of dopamine depletion and the magnitude of the increase in GLT-1. A significant negative correlation was uncovered ($r=0.77$, $p < 0.01$; Figure 5). When we examined the scatterplot illustrating the correlation, we noticed that the lesioned animals appeared to fall into two groups with a separation at approximately 65% dopamine depletion. We therefore did a post-hoc analysis examining GLT-1 levels in cohort II animals described in Chapter 3, with "moderate" depletions of 40-65% of control values, and "severe" depletions of >65% dopamine depletion.

Dopamine levels were reduced by 58.0 ± 3.2% in "moderate" depletion animals, while animals with the more extensive "severe" depletions averaged 78.9 ± 2.5% dopamine depletion compared to controls (Table 4). Norepinephrine levels were unchanged compared to controls when separated by dopamine depletion group (Table 4).

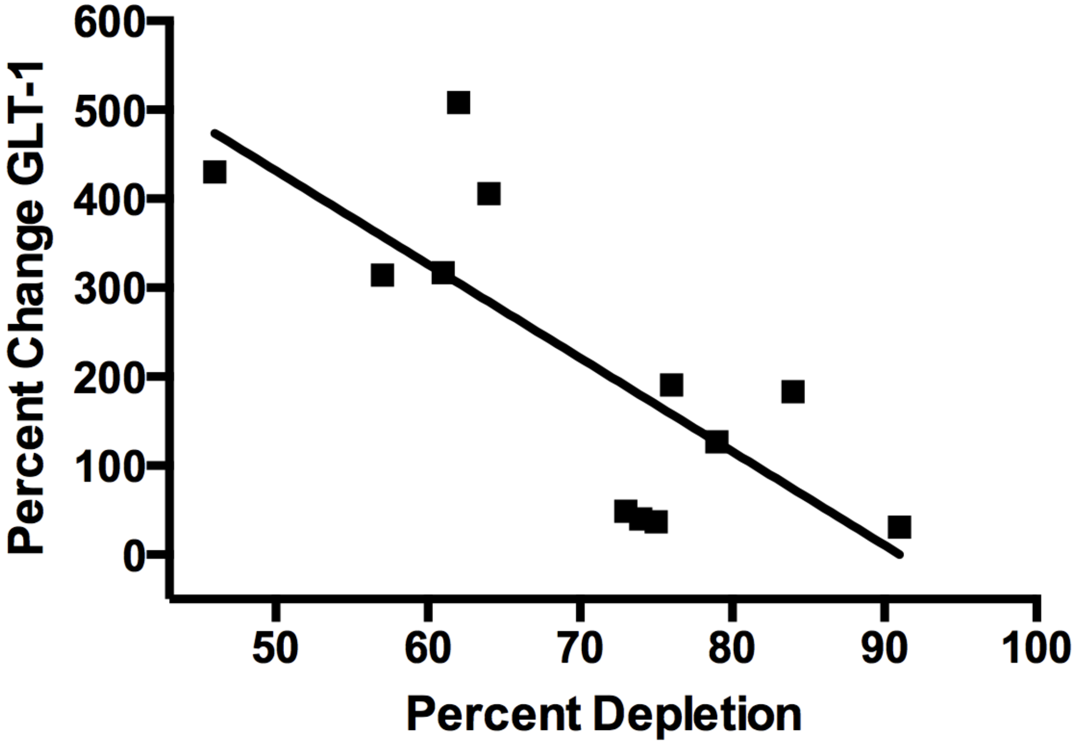

Figure 5. Negative correlation between extent of dopamine depletion and changes observed in GLT-1 levels. As dopamine depletion becomes more "severe" GLT-1 levels appear comparable to control levels.

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r = -0.77 p <0.01

	Dopamine	Norepinephrine	Serotonin
	Concentration (ng/mg protein)		
Control	1.050 +/- 0.086	5.137 +/- 0.192	9.851 +/- 0.346
Moderate Depletion (40-65%)	0.440 +/- 0.035 *	5.612 +/- 0.447	8.106 +/- 0.378 *
Severe Depletion (>65%)	0.219 +/- 0.026 *	4.704 +/- 0.103	6.209 +/- 0.425 *

Table 4. Neurotransmitter concentrations following separation of animals in cohort II by the extent of dopamine depletion. ANOVA analysis revealed significant decreases in both dopamine depletion groups compared to controls and a decrease in serotonin levels in animals with a severe dopamine depletion.

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* $p < 0.05$

A one-way between subjects ANOVA ($F_{2,16} = 24.03$, $p < 0.001$) revealed that serotonin levels were significantly decreased in both the “moderate” and “severe” depletion groups relative to controls (Table 4).

The extent of PFC dopamine depletion had a significant effect on GLT-1 ($F_{2,16} = 27.74$, $p < 0.001$; Figure 6). Post-hoc comparisons using the Tukey test indicated that in animals sustaining moderate PFC dopamine depletion, there was a marked increase in GLT-1 levels (see Figure 6). In contrast, there was no significant difference in GLT-1 levels, relative to controls, in animals with the more extensive (“severe”) dopamine depletion (see Figure 6). Furthermore, no changes were observed in GLAST or EAAC1 in either the “moderate” or “severe” dopamine depletion groups compared to controls (Figure 6). Post-hoc analyses using these “moderate” and “severe” dopamine depletion groups in prior experiments, as well as subsequent studies, were consistent with findings from cohort II.

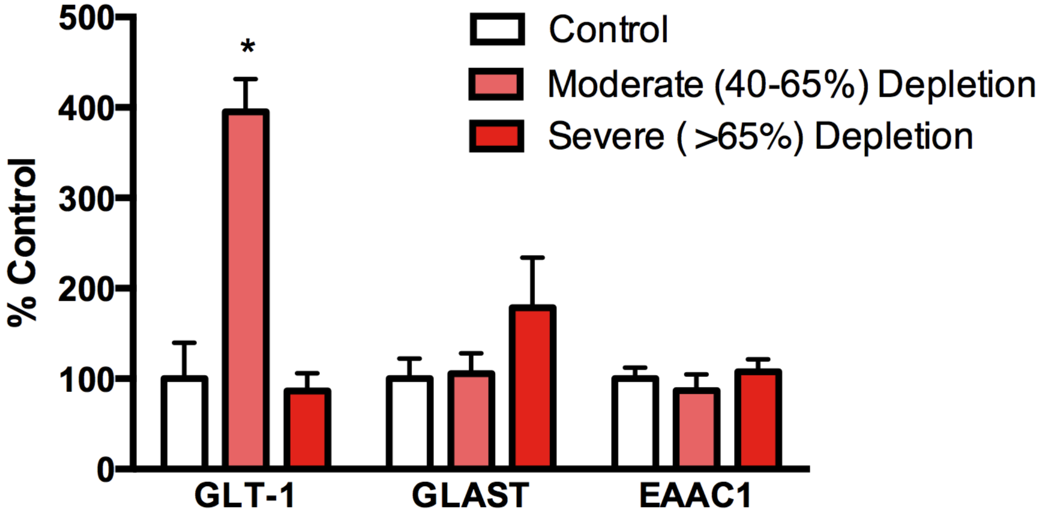

Figure 6. Glutamate transporter changes as a function of severity of dopamine depletion. Lesioned animals were separated based on the extent of dopamine depletion, with “moderate” depletion being classified as 40-65% dopamine loss, and “severe” dopamine depletion being characterized by >65% dopamine loss in the PFC. Animals with a “moderate” depletion showed an almost 400% increase in GLT-1 levels while “severe” depletion resulted in normal levels of GLT-1. No significant changes were observed in GLAST or EAAC1 in either group.

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* $p < 0.001$

Discussion

We demonstrated previously that dopamine depletion of the PFC leads to significant increases in protein expression of GLT-1 across all lesioned animals (see Figure 4). However, when animals are separated based on the extent of PFC dopamine depletion into “moderate” and “severe” depletion groups, only animals with moderate depletion display significantly increased levels of GLT-1. This finding is particularly striking as it suggests that different mechanisms are activated, depending on the extent of dopamine depletion.

In subsequent replications of our finding that only moderate PFC depletion elicits an increase in GLT-1 protein levels, we used two different methods of injecting 6-OHDA in an attempt to obtain the full range of cortical dopamine depletion. One was the original approach, in which a microsyringe with a 33 gauge needle was mounted in a pump apparatus to continuously eject the 6-OHDA solution. We also used a procedure in which the plunger of a microsyringe equipped with a 23 gauge needle was advanced manually; this would presumably result in a somewhat larger extent of diffusion of 6-OHDA. We found that the pump method usually produced less severe depletions than the manual method, although there was some overlap in the magnitude of depletion (Figure 7).

VTA lesions by 6-OHDA injection can have effects on other neurotransmitters, such as norepinephrine and serotonin. In this experiment norepinephrine levels were unchanged in lesioned animals compared to controls (See Table 4). However,

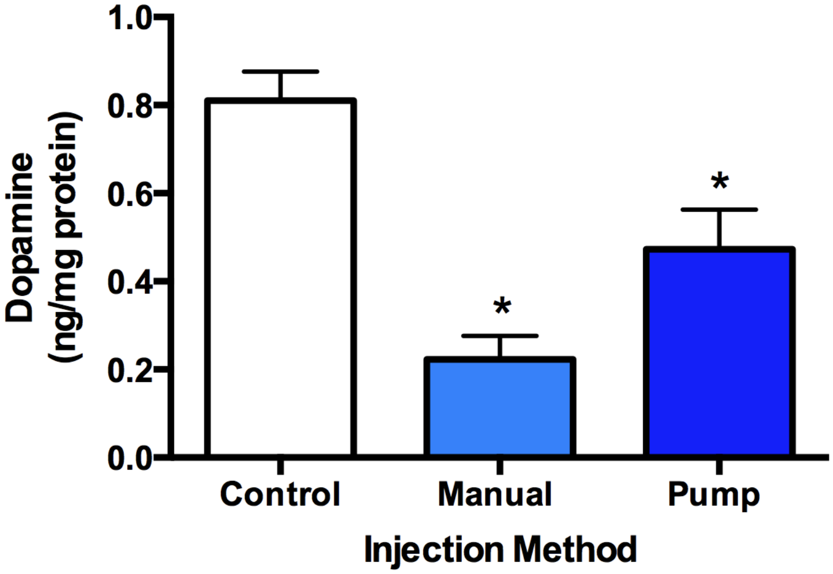

Figure 7. Different methods of 6-OHDA delivery result in different extents of PFC dopamine loss. Manual injections consistently resulted in more severe dopamine depletion when compared to injections using the microsyringe/pump. Both methods were used in order to observe a varied extent of dopamine depletion.

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* $p < 0.05$

serotonin levels were significantly reduced in lesioned animals compared to controls. Therefore, we cannot rule out the possibility that serotonin may play a role in the differential effects observed in these two groups of lesions. Future studies that could address this possibility will be discussed in greater detail in Chapter 11.

We have posited that dopamine depletion leads to increases in PFC glutamate, based on studies performed in the striatum. No studies have shown the effects of dopamine depletion on glutamate levels in the PFC. Future studies addressing this may be valuable not only to our own studies but to the field of researchers studying the interactions between dopamine and glutamate within the PFC.

Differential effects following dopamine depletion. There are several examples of differential changes in proteins, behavior, and mRNA following manipulation of the nigrostriatal pathway (Arbuthnott and Ungerstedt, 1975; Nisenbaum et al., 1996; Paillé et al., 2010). For example, “partial” striatal dopamine depletion (~75%) increases levels of the NMDA receptor subunit GluN2A (NR2A), while no change in GluN2A occurs following “full” (>95%) dopamine depletion (Paille et al., 2010). Interestingly, decreases in GluN2B (NR2B) were observed only in animals with a “full” dopamine depletion (Paille et al., 2010).

Arbuthnott and Ungerstedt (1975) demonstrated that with lower levels of electrical stimulation (reported as uColoumbs) of the nigrostriatal pathway (electrodes placed at the following coordinates AP= -4.0, ML= 1.7, DV= 7.6) in rats, sniffing behavior was induced, but at higher stimulation levels rotational behavior was observed (Arbuthnott and Ungerstedt, 1975).

In another study, direct injection of 6-OHDA into the striatum led to significant changes in mRNA levels encoding for preprotachykinin (PPT) and preproenkephalin (PPE) (Nisenbaum et al., 1996). Striatal PPT gene expression was significantly reduced regardless of depletion extent. However, when enkephalin mRNA levels were evaluated relative to the extent of dopamine depletion, significant increases in PPE gene expression enkephalin mRNA were only observed in animals with greater than 90% dopamine depletion, suggesting that different processes may be induced depending on the extent of dopamine depletion. These examples suggest that differential responses that are dependent on the extent of dopamine loss occur at the level of protein, behavior, and genes.

The mechanism through which somewhat less extensive PFC dopamine depletions increase GLT-1 levels, but greater degrees of dopamine depletion do not elicit any detectable change in GLT-1 levels, is not known. The decreases in GluN2B described by Paille and colleagues following extensive dopamine loss in the striatum raises the interesting possibility that alterations in this or other proteins involved in glutamate signaling may be responsible for a neuronal compensatory response following extensive dopamine depletion of the PFC in our animals. Thus, another possible explanation for the differential response to varying extents of dopamine depletion in the PFC is that following moderate dopamine depletion, GLT-1 increases reflect an astrocytic compensatory response to increased extracellular glutamate, but that more severe dopamine depletion elicits a neuronal response. This idea will be further explored in Chapters 9, 10 and 11.

CHAPTER 6

GLUTAMATE TRANSPORTER GENE EXPRESSION FOLLOWING DOPAMINE DEPLETION OF THE PREFRONTAL CORTEX

Dopamine depletion of the prefrontal cortex leads to an increase in levels of the glutamate transporter GLT-1 but not the two other cortical glutamate transporters, GLAST and EAAC1. The increase in membrane-associated GLT-1 in the PFC may be due to a selective increase in GLT-1 but not GLAST or EAAC1 mRNAs. We therefore examined the relative abundance of the mRNAs encoding the three cortical glutamate transporters in control and PFC dopamine-denervated rats.

Methods

The methods used in this experiment are the same as described in Chapter 4, with the exception that we used RT-PCR to assess glutamate transporter gene expression instead of monitoring protein levels.

RT-PCR. The prelimbic and infralimbic PFC was dissected (control N=12; 6-OHDA N=12) and stored at -80°C. RNA was extracted from the tissue using an RNeasy Mini kit including DNaseI treatment (Qiagen; Germantown, MD). After extraction,

Gene	Forward (5’-3’)	Reverse (5’-3’)
GLT-1	ATGCCGCACACAACTCTGTCGT	TCAGCTGACTTTCCATTGGCCGC
GLAST	CCTCAGGCCGGTCTAGTCACCA	GGTGGTGGTTCGGAGGCGGT
EAAC1	CTTCCTGCGGAATCACTGGCTG	GAGCTCACTGTGTCCTCGAACC
GAPDH	GGGCTCTCTGCTCCTCCCTGT	CCAGGCGTCCGATACGGCCA

Table 5. Primers designed using NCBI-Primer Blast

cDNA synthesis was performed using Superscript Vilo reverse transcriptase (Life Technologies; Grand Island, NY). RT-PCR was performed with SYBR Green master mix (Bio-Rad; Hercules, CA) as the reporter using the primers described in Table 5. Primers were designed using NCBI-Primer Blast. All primers were optimized for 60 degree annealing and two-step cycling was performed from 95 degrees (10s) to 60 degrees (30s) for 40 cycles. Melting curves were inspected after every run performed on BioRad CFX96 real time cyclers. Cycle threshold values were subjected to statistical analyses after normalization to *gapdh*.

Results

Extent of dopamine depletion. The dopamine concentration in the PFC of control animals averaged 0.49 ± 0.05 ng/mg protein. Prefrontal cortical dopamine concentrations in 6-hydroxydopamine-lesioned rats were reduced across all lesioned subjects by 74.29 ± 5.1% (t_{27}= 7.36, $p<0.001$). Norepinephrine concentrations (control value of 4.34 ± 0.25 ng/mg protein) were significantly reduced by 29.9% after dopamine denervation of the PFC (t_{27}=2.92, $p<0.01$). Serotonin levels were unchanged (See Table 6). We also analyzed mRNA levels in animals separated into moderate and severe dopamine depletion groups, as described in Chapter 4. The average decrease in PFC dopamine concentrations was 50.27% in the moderate group, and 89.6 % depletion in the severe group (Table 6). Norepinephrine levels were unchanged in the “moderate” dopamine depletion group, while levels of norepinephrine were reduced by 28.85 ±6.8% in the “severe” dopamine depletion group (Table 6). Similar to the combined depletion groups, serotonin levels remained unchanged when animals were separated by dopamine depletion extent (Table 6).

Glutamate transporter mRNA levels. Real-time PCR revealed no significant change in mRNA levels of any of the glutamate transporters examined (GLT-1, GLAST, and EAAC1) in response to DA depletion across all animals (Figure 8). Similarly, when evaluated based on the extent of dopamine depletion, no changes were observed in relative abundance of the mRNAs for GLT-1, GLAST, and EAAC1 in animals with moderate or severe dopamine depletion compared to controls (Figure 8).

	Dopamine	Norepinephrine	Serotonin
	Concentration (ng/mg protein)		
Control	0.497 +/- 0.050	4.338 +/- 0.252	6.958 +/- 0.422
All Lesioned Animals	0.128 +/-0.025 *	3.162 +/- 0.344 *	5.386 +/- 0.493
Moderate Depletion (40-65%)	0.247 +/- 0.013 *	4.318 +/- 0.269	5.644+/- 0.733
Severe Depletion (>65%)	0.052 +/- 0.014 *	2.266 +/- 0.276 *	5.222 +/- 0.682

Table 6. Concentrations of dopamine, norepinephrine, and serotonin in the prefrontal cortex of animals for RT-PCR analysis following 6-OHDA lesion of the ventral tegmental area. HPLC analysis uncovered significant decreases between control and lesion animals in both dopamine and norepinephrine in the PFC, but not in serotonin. When animals were separated by the extent of dopamine depletion, animals with severe depletion had significantly reduced norepinephrine levels, while animals with moderate depletion showed no change. Separation of animals into moderate and severe depletion did not reveal any differences in serotonin levels

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* $p < 0.05$

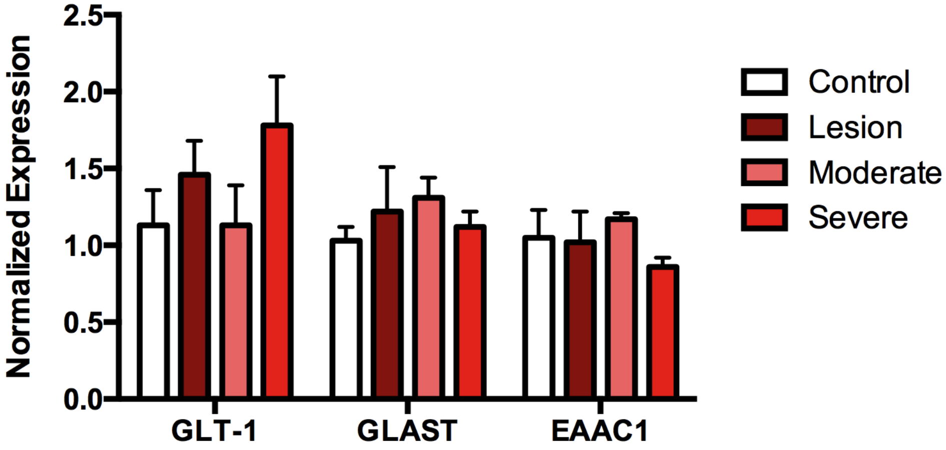

Figure 8. No change was observed in mRNA expression levels of GLT-1, GLAST or EAAC1.

Discussion

No changes in the relative abundance of GLT-1, GLAST, or EAAC1 mRNA levels were seen in the PFC of dopamine-depleted compared to control animals. Similarly, separating animals into moderate and severe cortical dopamine denervation groups did not reveal a difference in mRNA levels across groups. The large increase in GLT-1 protein levels that we observed in response to cortical dopamine denervation without a concurrent increase in steady-state mRNA levels suggests that the increase in GLT-1 protein is not due to *de novo* itranscription of the transporter.

Schmitt *et al.* (2003) reported that chronic treatment with the D_2 receptor antagonist haloperidol decreases GLT-1 mRNA in the PFC. In contrast, we did not observe a change in GLT-1 gene expression after cortical dopamine depletion. The difference between our results and those of Schmitt and colleagues may be attributable to the fact that the dose of haloperidol used by Schmitt *et al.* results in complete occupancy of D_2 receptors *in vivo* (see Perez-Costas et al., 2008), whereas our lesions resulted in an incomplete dopamine denervation, even in what we designate the "severe" depletion group. Furthermore, rather than preventing dopaminergic signaling at all dopamine receptors, haloperidol has a high affinity only for D2 receptors (including D_2, D_3, and D_4), whereas dopamine depletion disrupts signaling through D2 but also D1 receptors, which are expressed at higher density in the PFC than D2 family receptors.

Another possibility is that decreases in norepinephrine levels observed in our samples, which were not observed when assessing protein levels of the transporters (see Chapters 4 and 5), may lead to a differential response. However, when lesioned animals are separated into moderate and severe dopamine depletion groups, the moderate depletion group closely resembles animal cohorts used in the immunoblot studies. Thus, differences in norepinephrine levels do not explain the differential regulation of GLT-1 protein and mRNA expression.

Another explanation for the lack of an observed increase in GLT-1 mRNA levels is that levels of mRNA actually do increase following dopamine depletion, but we fail to capture this increase at our three-week post-surgery timepoint. The half-life of GLT-1 protein is suggested to be approximately 24 hours (Zelenaia and Robinson, 2000). Therefore, it seems unlikely that an elevation in protein levels would be observed without detection of a concurrent increase in mRNA levels, if increases in transcript levels are responsible for increases in protein levels. These experiments, therefore, suggest that increases in GLT-1 protein levels are not due to increases in protein production as predicted by mRNA levels. Two more possible explanations for the increase in GLT-1 observed following dopamine depletion are examined in subsequent chapters of this dissertation, including astrocytosis and transporter degradation.

CHAPTER 7

ASSESMENT OF ASTROCYTE NUMBER AND ACTIVATION

In Chapter 4, I demonstrated that a significant increase in GLT-1 occurs as a result of dopamine depletion. Three possibilities were put forward that may explain the increase in GLT-1 levels, including gene expression, an increase in astrocyte number or activation and decreased protein degradation. In Chapter 6, we tested the hypothesis that increases in GLT-1 are a result of increased gene expression, and observed that mRNA levels of GLT-1 did not change following dopamine depletion. These experiments showed that increased GLT-1 protein levels seen after PFC dopamine depletion were not attributable to increased GLT-1 gene expression. In the current chapter, I discuss experiments designed to explore the possibility that dopamine depletion of the PFC leads to an increase in astrocyte number or activation, increasing GLT-1 protein levels.

Gliosis refers to the response of glial cells to brain injury. Astrogliosis refers to a number of changes in astrocytes, including alterations in morphology, gene and protein expression, astrocyte function, and astrocyte number (Pekny et al., 2014). Astrogliosis can occur in response to neurodegenerative conditions, as well as more acute conditions such as stroke (Tilleux and Hermans, 2007; Li et al., 2008). Classically, reactive astrocytes that invade an area of acute damage or inflammation show an increase in the extent of their processes and increased levels of certain proteins, such as the astrocytic marker glial fibrillary acidic protein (GFAP; Pekny and Pekna, 2004;

Bramanti et al., 2010). In an effort to determine whether changes in GLT-1 occur because of an increased number, or activation, of astrocytes, we measured GFAP levels in the PFC by immunoblot, and determined the number GFAP-immunoreactive (-ir) astrocytes in the PFC following PFC dopamine depletion.

Methods

Immunoblotting. Tissue preparation, immunblotting, and analysis was performed as described in Chapter 4. An antibody generated against GFAP was used at a 1:1000 dilution (Chemicon, #1540), using a donkey anti-rabbit antibody and ECL detection.

Stereology. The number of GFAP-immunoreactive (-ir) positive astrocytes in the PFC of sham-lesioned animals (n=4) and animals receiving 6-OHDA lesions of the VTA (n=4) was determined using the optical dissector stereological method (2500 μm^2 sampling area; MBF Bioscience; Williston, VT) on serial sections collected through the extent of the PFC and immunostained with the previously described GFAP antibody. The number of sampling regions varied by section but ranged between 10-20 regions of interest (ROIs)/hemisphere/section. The activation of astrocytes, as reflected by branching complexity, was determined by measuring the number of GFAP-ir processes crossing two of the four the stereological counting square boundaries (50 μm/side).

Results

GFAP expression. Western blot analysis failed to reveal a change in PFC levels of the astrocytic marker GFAP across all lesioned animals or in animals separated into moderate and severe PFC dopamine depletion groups (see Figure 9).

Astrocyte number and morphology. Consistent with the lack of change in GFAP levels, stereological studies found no change in the number of PFC astrocytes after dopamine denervation of the PFC (Figure 10). Because the dopamine innervation of the PFC is more dense in layer V than layers II/III (Kalsbeek et al., 1988), we also looked for a lamina-specific change in astrocyte number after dopamine denervation; there was no change (Figure 10). Similarly, we did not observe an increase in the number of GFAP-ir astrocytic processes (Figure 10) across the PFC.

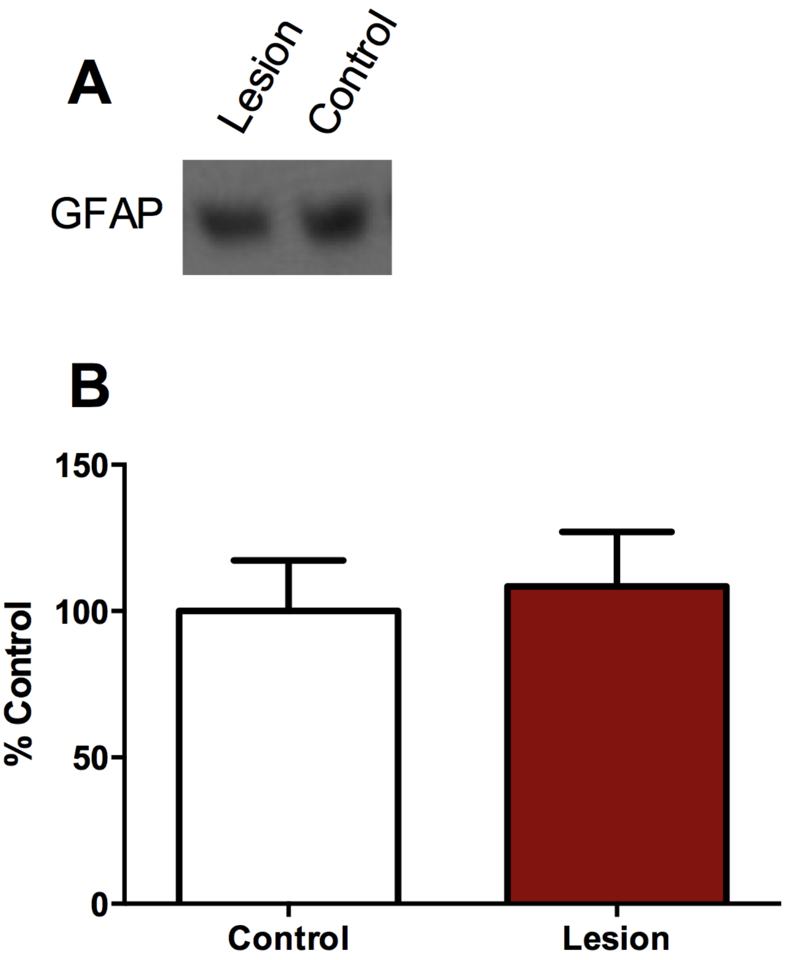

Figure 9. A) Representative blots of GFAP. **B)** Immunoblot assessment revealed that GFAP levels were unchanged following dopamine depletion of the PFC.

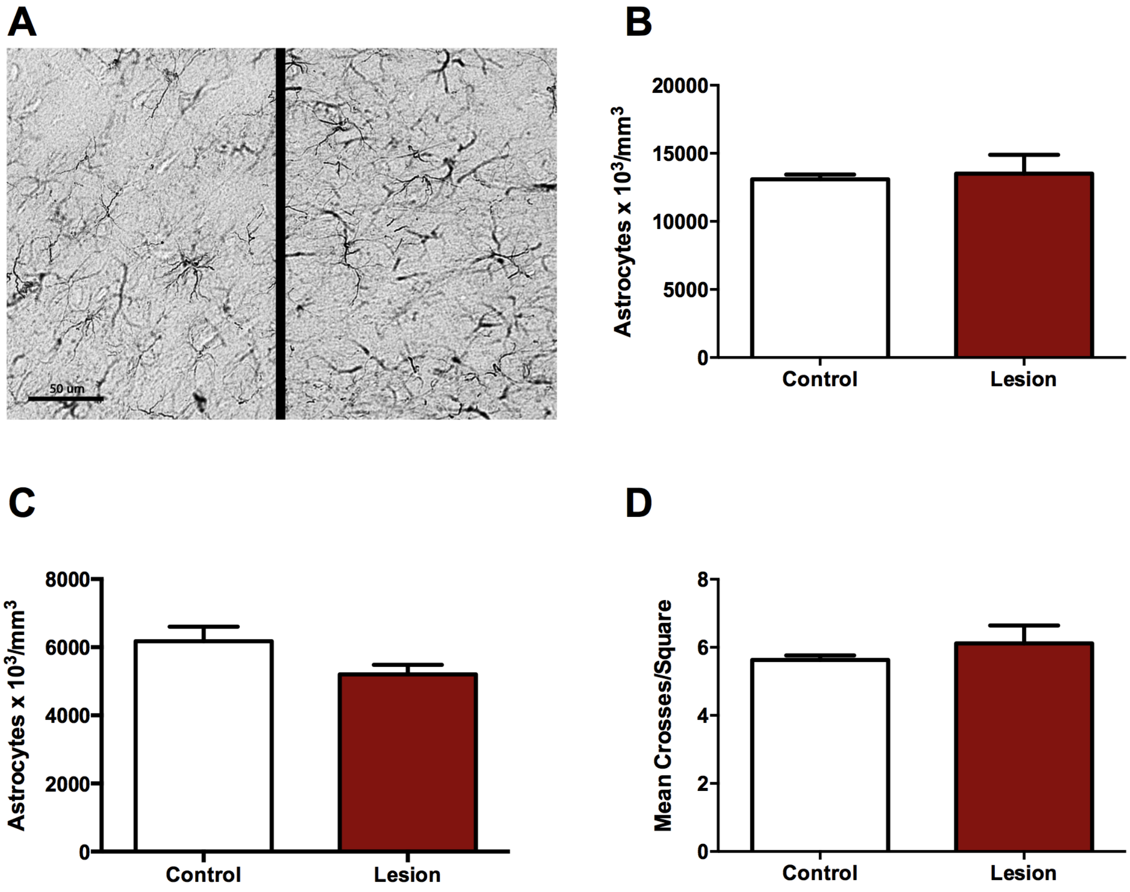

Figure 10. Assessment of astrocyte number and activation. **A)** Representative images of GFAP staining of the PFC from a control (left) and a lesioned animal (right); scale bar =50 µm **B)** Stereological methods failed to reveal a difference in total astrocyte number in the PFC. **C)** Similarly, analysis of only deep layers (V/VI) of the PFC failed to reveal a significant change in astrocyte number. **D)** Assessment of astrocyte activation did not uncover differences between control and lesioned animals

Discussion

It is common for gliosis to occur in areas surrounding a site of injection into the brain. In an effort to prevent an astrocytic response to mechanical damage we injected 6-OHDA into the A10 dopamine cell group in the VTA, which is the sole source of the dopamine innervation of the PFC. The results are not consistent with an increased number of astrocytes or astrocytic processes contributing to the dopamine denervation-induced increase in GLT-1 levels in the PFC.

Immunoblot analysis failed to reveal a significant change in GFAP protein levels following dopamine depletion. Even when animals were separated into moderate (40-65%) and severe (>65%) dopamine depletion groups, no differences were observed when compared to control animals. GFAP is a commonly utilized marker of astrocytes, and its expression is increased in reactive astrocytes (Pekny and Pekna, 2004). Therefore, these data suggest that there is not an increase in PFC astrocyte number or reactivity following dopamine depletion.

To further verify our imunnoblot data, we performed stereological assessment of GFAP positive cells in the prefrontal cortex, observing no difference between control and dopamine-denervated animals. Furthermore, existing astrocytes do not display increased reactivity as measured by GFAP levels and astrocyte branching complexity. Thus, despite an astrocytic response to dopamine depletion of the PFC as reflected by increased levels of GLT-1, increases in GLT-1 cannot be attributed to morphological changes in astrocytes. Three possible explanations for increased levels of GLT-1 protein expression following dopamine depletion were identified in the discussion of Chapter 4: increased gene expression, increased astrocyte number or reactivity, and decreased GLT-1 degradation. Thus far, I have shown that gene expression and astrocyte number or activation, are not responsible for increased GLT-1 protein levels. In the following chapter, I will explore the effects of dopamine depletion on glutamate uptake in an effort to determine whether glutamate transporter activity increases in parallel with GLT-1 protein expression.

CHAPTER 8

GLUTAMATE UPTAKE IN THE PREFRONTAL CORTEX FOLLOWING DOPAMINE DEPLETION

We have seen that dopamine denervation of the prefrontal cortex increases GLT-1 protein levels, specifically in those animals with moderate (40-65%) PFC dopamine depletion. This change cannot be attributed to an increase in GLT-1 gene expression, nor to an increase in astrocyte number or astrocyte activation. Just as increases in gene expression do not always lead to increased protein expression, increases in protein levels do not always lead to increases in protein function. GLT-1 is the primary mechanism for glutamate uptake in the cortex. Although we observed an increase in levels of GLT-1, the exact stoichiometry of GLT-1 protein:glutamate transport is not known. In the current chapter I explore the effects of dopamine depletion on glutamate uptake in the PFC.

Methods

The methods used in this experiment are the same as described in Chapter 4, with the exception that we assessed high affinity uptake of radiolabeled glutamate in PFC tissue minces.

Glutamate Uptake Assay. Animals were deeply anesthetized, sacrificed, and their brains removed. The prelimbic and dorsal infralimbic cortices were dissected on ice. Tissue was minced using a razor blade and suspended in Krebs Ringer Hepes buffer (120 mM NaCl, 4.7 mM KCl, 1.2 mM$KHPO_4$, 10.0 mM HEPES). 250 µL of the tissue suspension was added to 250 µL of KRH buffer in glass tubes. Samples were then heated to a 37 °C water bath shaking at 100 rpm. 50 µL of [3H] glutamate (10 nM radiolabelled and 90 nM unlabelled glutamate) was added to samples, which were incubated for 10 minutes at 37 °C under constant agitation. On a Brandell filter apparatus, uptake was terminated by filtration through a GF/B Whatman filter pre-soaked in 0.3 % PEI (poly-ethyleneimine) in water, washing 3 times with ice-cold PBS. Filters were incubated with 7 mL scintillation liquid Ecoscint H overnight on rotary shaker at temp (37°C or room temp). Each sample was run in triplicate. In order to determine non-specific uptake, the same procedures were followed, except that samples were kept at 4°C rather 37°C. Non-specific uptake was subtracted from values observed at 37°C. Averages of the three replicates were determined and adjusted for non-specific up-take. Protein concentrations of individual samples were determined using a BCA-assay, and were used to normalize glutamate uptake values.

Results

Neurotransmitter levels. The dopamine concentration in the PFC of control animals averaged 0.67 ± 0.05 ng/mg protein. Prefrontal cortical dopamine concentrations across all 6-hydroxydopamine-lesioned rats were reduced by 63.2 ± 6.3% (t_{16}= 7.62, $p<0.001$).

ANOVAs were conducted to compare the effect of dopamine depletion on dopamine, norepinephrine, and serotonin concentrations in control, moderate and severe dopamine depletion conditions. Significant main effects were observed for all three monoamines: dopamine: $F_{2,15} = 181.0$, $p < 0.001$, norepinephrine: $F_{2,15} = 5.546$, $p = 0.016$, and serotonin: $F_{2,15} = 15.21$, $p < 0.001$. Tukey multiple comparison tests were used to compare concentration differences across dopamine depletion groups. The severe depletion group had significantly reduced dopamine concentrations compared to the moderate depletion group (Table 7). Norepinephrine levels were significantly reduced in the severe dopamine depletion group, while the moderate dopamine depletion group showed no significant difference from controls. Similarly, serotonin levels were significantly reduced in the severe but not moderate dopamine depletion group, compared to controls, (Table 7).

Glutamate Uptake. No significant change in glutamate uptake was observed when all lesioned animals were compared to controls (Figure 11A). However, when data from animals with moderate and severe dopamine depletions were compared to control subjects by ANOVA, a significant effect of dopamine depletion on glutamate uptake was uncovered ($F_{2,15} = 7.33$, $p = 0.006$; Figure 11A). Post-hoc analysis with the Tukey test found that glutamate uptake in animals with severe dopamine depletions was significantly greater than uptake in control rats (Figure 11B); No such difference was observed comparing glutamate uptake in animals with moderate PFC dopamine loss to control subjects.

	Dopamine	Norepinephrine	Serotonin
	Concentration (ng/mg protein)		
Control	0.716 +/- 0.059	6.776 +/- 0.539	10.13 +/- 0.322
All Lesioned Animals	0.298 +/-0.051 *	3.345 +/- 0.843 *	8.758 +/- 0.459
Moderate Depletion (40-65%)	0.464 +/- 0.015 *	4.368 +/- 1.211	10.070+/- 0.408
Severe Depletion (>65%)	0.160 +/- 0.032 *	2.493 +/- 1.145 *	7.665 +/- 0.367 *

Table 7. Concentrations of dopamine, norepinephrine, and serotonin. Significant decreases were observed in dopamine and norepinephrine when all lesions were examined together, however, no change was observed in serotonin. When separated into moderate and severe dopamine depletion groups both groups showed significant reductions in dopamine levels, while only the severe dopamine depletion group exhibited decreased norepinephrine and serotonin levels.

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* = $p < 0.05$

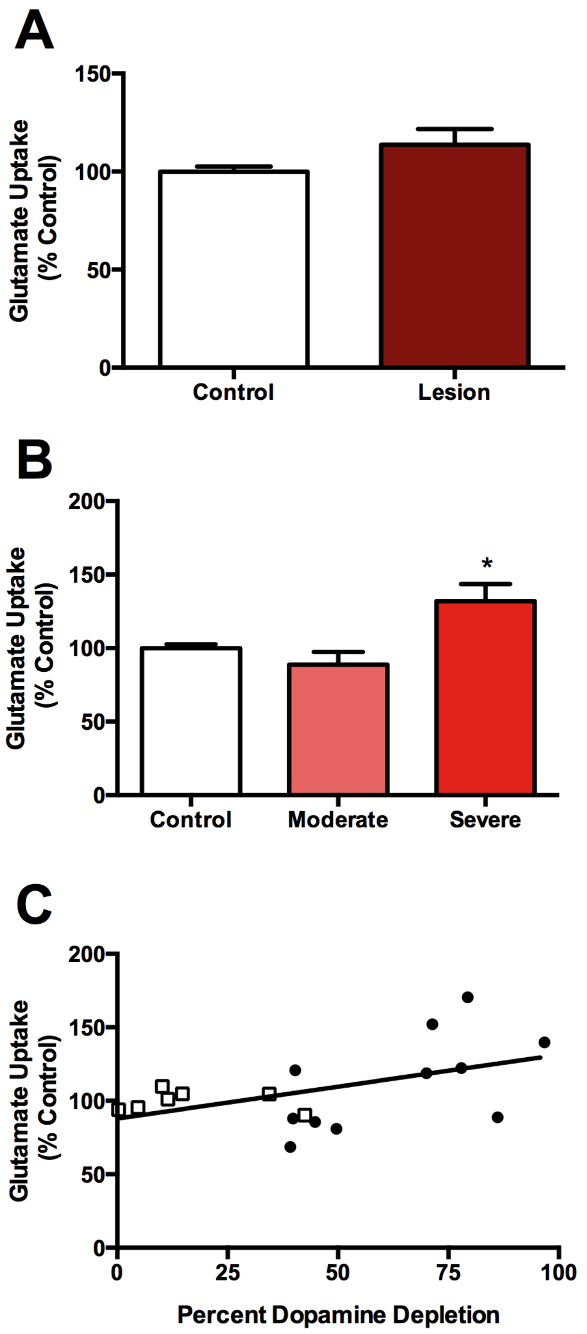

Figure 11. Glutamate uptake was assessed in tissue from the PFC using tridiated glutamate. **A)** No difference was seen in lesions as a whole. **B)** Separation of animals into moderate and severe depletion groups revealed a significant increase in glutamate uptake in animals with a severe dopamine depletion (* = p <0.05). **C)** When glutamate uptake was compared to dopamine depletion a significant correlation was observed (r = 0.494 p=0.04).

These results can be better appreciated when comparing the relationship between extent of dopamine depletion and high affinity glutamate uptake: a significant correlation is present (r=0.49, p= 0.037; Figure 11C).

Discussion

High affinity glutamate uptake was increased in the PFC of animals with cortical dopamine loss, but only if the extent of dopamine depletion exceeded about 70%. Interestingly, the relationship between glutamate uptake and extent of dopamine depletion is different than the relationship between GLT-1 protein levels and extent of cortical dopamine loss. In the latter case, only animals with moderate degrees of dopamine depletion showed an increase in membrane-associated GLT-1 levels, but the activity of the transporter (glutamate uptake) was increased only in animals with extensive PFC dopamine loss. Thus, it appears that increases in membrane-bound GLT-1 protein levels are not accompanied by an increase in functional protein, but rather an accumulation of non-functional protein. This further suggests the importance of understanding whether post-translational modifications to GLT-1 occur following dopamine depletion, as discussed in Chapter 5. In addition, it will be important to determine whether these changes are dependent upon the extent of depletion, which may explain differences in GLT-1 levels and glutamate uptake between “moderate” and “severe” dopamine depletion groups.

Neurotransmitter levels. The effects of VTA lesions on dopamine concentration are similar to results of previous experiments discussed in this thesis, i.e., a significant decrease in dopamine levels as a whole. The magnitude of this decrease was somewhat lower (~63% depletion) as compared to previous experiments (~70% depletion). Interestingly, when separated into moderate and severe depletion groups the average dopamine depletion of the moderate depletion group was ~43% compared to the ~50% depletion seen in previous experiments. The average depletion in the severe dopamine depletion group (~80%) was similar to that reported in previous studies (~78%). Finally, decreases were observed in norepinephrine and serotonin levels in the severe depletion group but not in the moderate depletion group. As was mentioned in a previous chapter, the simplest explanation for changes resulting from a VTA lesion is likely to be due to decreases in dopamine concentrations. Dopamine depletion has been shown to have an effect on GLT-1 protein expression in the striatum (Massie et al. 2010), while to the best of our knowledge the effect of norepinephrine and serotonin manipulation on GLT-1 expression has not been investigated. Studies examining these effects may prove useful in the future if differential responses in GLT-1 function and expression between moderate and severe depletion groups cannot be explained solely through the effects of dopamine depletion.

Glutamate transporter contributions to glutamate uptake. Keeping samples on ice is an effective method to stop transport by all three glutamate transporters. This method was used with the understanding that >90% of glutamate uptake in the cortex occurs through GLT-1. However, our glutamate uptake data appears to contradict our protein expression findings, with increased uptake in the severe depletion group, which did not show elevated changes in GLT-1 protein levels. One possibility is that another of the transporters has significantly increased function, while protein expression remains unchanged. This data suggests that further investigations of glutamate uptake are warranted. Specifically, separating the relative contributions to glutamate uptake made by the individual transporters GLT-1, GLAST, and EAAC1, may shed light on the effects of dopamine depletion on glutamate uptake. This could be done using transporter-specific inhibitors. Previous studies have shown that EAAC1 is upregulated following knockdown of GLT-1 expression (Salvatore et al., 2012).

Across all lesioned animals there was no significant change in glutamate uptake. However, when separated by extent of dopamine depletion, animals with more severe dopamine depletion exhibited higher rates of glutamate uptake. This suggests that a mechanism separate from the increased GLT-1 expression observed in animals with moderate dopamine depletion, may lead to increased glutamate uptake following more extensive dopamine depletion. Possible neuronal mechanisms are examined in Chapters 9 and 10.

CHAPTER 9

ALTERATIONS IN EXPRESSION OF GLUTAMATE-RELATED PROTEINS FOLLOWING DOPAMINE DEPLETION OF THE PREFRONTAL CORTEX

In Chapter 4, I discussed the effect of dopamine depletion on glutamate transporters within the prefrontal cortex, and suggested that these changes are in response to increased extracellular glutamate levels. The glutamate transporters are but one of a variety of mechanisms that contribute to glutamate homeostasis. In this chapter, I will discuss a number of glutamate-related proteins, including ionotropic NMDA and AMPA receptor subunits, the cystine/glutamate antiporter subunit xCT, and group II metabotropic glutamate receptors.

<u>Ionotropic glutamate receptors</u>. Ionotropic glutamate receptors, including NMDA and AMPA receptors, are primarily localized to postsynaptic terminals. However, expression of NMDA and AMPA receptors on astrocytes has also been reported (Bowman and Kimelberg, 1984; Kettenmann et al., 1984; Conti et al., 1994; Schipke et al., 2001; Matthias et al., 2003; Lalo et al., 2006). Both AMPA and NMDA receptors are critical for the establishment of long-term potentiation, which is a long lasting increase in the strength (or transmission) of a synapse. Binding of glutamate to these receptors causes a depolarization of the postsynaptic membrane through the transport of Na^+ and Ca^{2+} into the cell.

AMPA receptors are made up of several subunits, including GluA1, GluA2, GluA3, and GluA4. These subunits form heterotetramers that typically consist of two GluA2 subunits along with a dimer of one of the other three subunits (Shepherd and Huganir, 2007). The presence of GluA2 subunits generally renders AMPA receptors impermeable to calcium (Burnashev et al., 1992).

NMDA receptors also consist of a heterotetramer of subunits, including two GluN1 and two GluN2 subunits. Eight splice variants of GluN1 exist, however GluN1-1a, is the predominant GluN1 isoform found in the prefrontal cortex (Paoletti et al., 2013). The GluN2 subunit has four variants labeled A through D, with GluN2A and GluN2B being the most common variants in the adult rodent PFC (Paoletti et al., 2013). The GluN2 subunit present in the heterotetramer confers biophysical function to the receptor (Wyllie et al., 2013).

In order to gain a better understanding of changes in glutamate systems after cortical dopamine denervation, I analyzed protein levels of ionotropic glutamate receptor subunits from both the NMDA receptor (GluN1, GluN2B) and the AMPA receptor (GluA1, GluA2), following dopamine depletion.

Cystine/Glutamate antiporter system. I previously discussed System xC^- in Chapter 2. This antiporter system exchanges intracellular glutamate for extracellular cystine in a 1:1 stoichiometry. The subunit xCT confers substrate specificity to the antiporter.

Group II metabotropic glutamate receptors. Group II metabotropic glutamate receptors are primarily considered to be presynaptic proteins. However, one of the Group II receptors, mGluR3, is also localized to astrocytes (Ohishi et al., 1993; Petralia et al., 1996). On the presynaptic terminal, group II mGluRs couple to inhibitory G-proteins to inhibit glutamate release. The functional effect of astrocytic mGluR3 remains poorly defined. Emerging evidence suggests that activation of mGluR3 in astrocytic cultures may lead to increases in GLT-1 protein expression (Aronica et al., 2000).

In this chapter, I explore the effects of dopamine depletion on a number of glutamate-related proteins with differing localizations, ranging from presynaptic to postsynaptic to astrocytic. By using HPLC-EC determination of dopamine depletion extent followed by immunoblot analysis, I was able to determine whether changes in glutamate-related proteins occurred broadly across all lesioned animals, or as a result of varying levels of dopamine depletion.

Methods

The methods of this chapter are the same as those used in Chapter 4, with the exception of the antibodies used (see Table 8). I attempted to use antibodies against mGluR2 and mGluR3 specifically but was not satisfied with the specificity of these antibodies, thus an antibody against Group II mGluRs was utilized.

Antibody	Company	Antigen	Catalog #	Dilution
GluN1	Millipore	C-terminus peptide aa 900-938	AB9864	1:2000
GluN2B	BD Biosciences	C-terminus peptide aa 891-1051	#610417	1:500
GluA1	Millipore	Cytoplasmic domain peptide	AB1504	1:1000
GluA2	Millipore	C-terminus peptide	AB1768	1:500
mGluR2/3	Millipore	C-terminus peptide: NGREVVDSTTSSL	AB1553	1:1000
xCT	Novus Biologicals	N-terminus peptide	NB300-318	1:1000
xCT	Abcam	50 aa N-terminus peptide	Ab37185	1:1000
xCT	David Baker		--	1:500
xCT	Santa Cruz	N-terminus	sc-79359	1:1000

Table 8. Source information and dilutions for antibodies used in immunoblot studies.

Results

Extent of dopamine depletion. The dopamine concentration in the PFC of control animals averaged 1.05 ± 0.085 ng/mg protein. Prefrontal cortical dopamine concentrations in 6-hydroxydopamine-lesioned rats were reduced by 70.4 ± 3.6% (t_{17}= 9.02, $p<0.001$). Norepinephrine concentrations (control value of 5.137 ± 0.192 ng/mg protein) were not significantly affected by dopamine denervation of the PFC (t_{17}=0.16, NS). Animals were further separated into moderate and severe depletion groups, with moderate depletion being those animals with dopamine depletions of 40-65% and severe depletion being those animals with depletions greater than 65%. For more detailed information, please refer to Table 6 in Chapter 5.

No effect on NMDA or AMPA receptor subunit expression. Antibodies selective for GluN1 and GluN2B detected distinct bands at 116 and 108 kDa, respectively. The majority of protein expression for these proteins was observed in the PSD-enriched and membrane-enriched fractions, respectively (Figure 12A). Levels of GluN1 and GluN2B in their respective fractions were not affected by dopamine depletion when all lesioned animals were analyzed together (Figure 12B), nor were differences seen when animals were divided into moderate and severe depletion groups (Figure 12B).

Antibodies against AMPA receptor subunits GluA1 and GluA2 detected bands at 106 and 99 kDa, consistent with their predicted masses, and were localized to the membrane-enriched fraction (Figure 12A). I detected no significant change in levels of

GluA1 or GluA2 in all dopamine-denervated animals or in the moderate and severe depletion groups, relative to control subjects. (Figure 12B).

Altered mGluR 2/3 expression. The antibody to mGluR2/3 detected a clear band at 96 kDa, as well as a higher molecular weight band of ~225 kDa. The majority of protein expression was seen in the membrane-enriched fraction (Fig 12A). Following dopamine depletion, PFC levels of the Group II metabotropic glutamate receptors were markedly increased in the membrane-enriched fraction compared to control animals (Fig 12B; $p < 0.05$). Interestingly, when animals were separated into moderate and severe depletion groups, I observed that animals with moderate dopamine depletion had significantly greater expression of mGluR2/3 than controls ($p<0.01$), while animals with severe depletion showed no difference from control animals. No differences were seen in the remaining fractions.

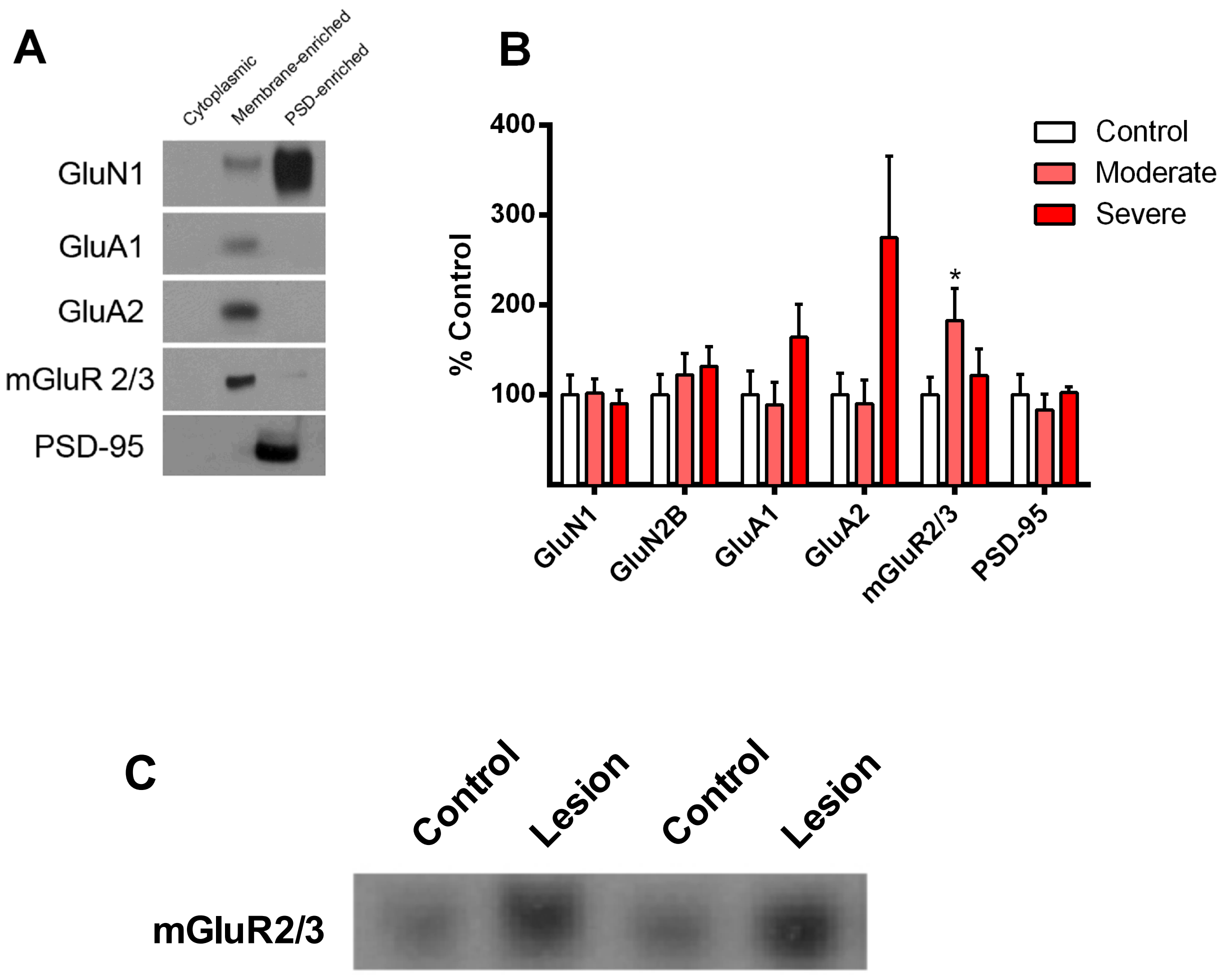

Figure 12. Effect of dopamine depletion on glutamate-related proteins. **A)** Fraction localization of proteins examined by western blot. **B)** Moderate dopamine depletion leads to a significant increase in mGluR2/3 levels, while levels of other glutamate related-proteins remain consistent with controls following severe dopamine depletion. All other proteins were unaffected following both moderate and severe dopamine depletion. **C)** Representative blot showing increased mGluR2/3 following moderate dopamine depletion.

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* $p < 0.01$

xCT antibody characterization. I tried a number of different antibodies generated against xCT in an attempt to find one that was specific for xCT (see Table 8). Tests with all of these antibodies resulted in multiple bands when probing the membrane fraction, including, in some cases, bands at the predicted mass for xCT (see Figure 13). In order to help determine if any of these antibodies recognized xCT in addition to other proteins, I obtained tissue from xCT knockout mice, courtesy of Dr. Hideyo Sato (Yamagata University, Japan) (Sato et al., 2005). Unfortunately, studies with the knockout cortex failed to reveal that any of the 4 antibodies recognized xCT (Figure 13). I therefore did not pursue further studies aimed at evaluating changes in xCT in response to PFC dopamine depletion.

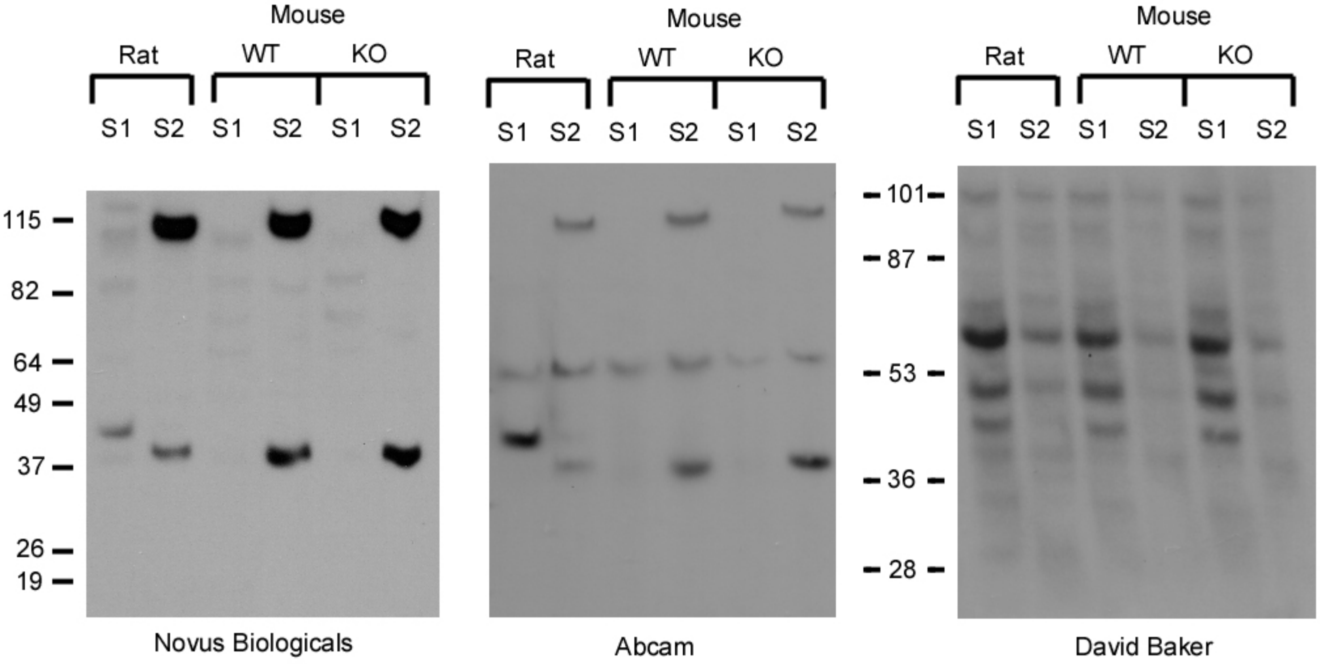

Figure 13. Failure of xCT antibodies to detect differences between wildtype and xCT-knockout animals. Antibodies from three sources are shown, in which no difference is observed between wildtype and knockout tissue, illustrating the ineffectiveness of the currently available antibodies.

Discussion

In Chapter 4, I demonstrated that dopamine depletion leads to increases in protein levels of the astrocytic glutamate transporter GLT-1. Interestingly, this increase is not accompanied by a parallel increase in the neuronal glutamate transporter EAAC1, suggesting that dopamine depletion may lead to an astrocyte-specific upregulation of glutamate uptake. The current chapter focuses on proteins involved in glutamate homeostasis that are localized to neurons, and have also, in a few cases, been described on astrocytes (AMPA and NMDA receptors and mGluR3). The functional significance of the astocytically expressed proteins remains unclear.

Proteins known to be critical to synaptic glutamate signaling, including the AMPA receptor and NMDA receptor subunits, showed no significant changes in response to dopamine depletion. We have not explored the possibility of functional changes in these proteins following dopamine depletion. Subunit composition of NMDA receptors can lead to significant variations in function and localization (Wyllie et al 2013). Similarly, phosphorylation states, which contribute to the functional effects of these proteins, were not examined. Thus, studies aimed at determining NMDA receptor function and composition may help us to better understand the effects of dopamine depletion on glutamatergic signaling through ionotropic glutamate receptors.

Unfortunately, none of the four putative xCT antibodies that I tested specifically recognized xCT, despite the fact that some have been used in previous studies (Knackstedt et al., 2009; Pampliega et al., 2011). However, in these earlier papers the full gel is not shown, only a narrow area of the gel surrounding the predicted mass of xCT, and thus it is not possible to know if they used an antibody from a different animal or a different lot (bleed) that did recognize xCT. In the absence of specific xCT antibodies, future studies could evaluate radiolabeled cystine uptake to evaluate system xC^-.

I observed a significant increase in mGluR2/3 protein levels in the dopamine-denervated PFC. Interestingly, while mGluR2 and 3 are both found on the presynaptic terminal, mGluR3 is also expressed on astrocytic membranes (Ohishi et al., 1993; Petralia et al., 1996). Presynaptic Group II mGluRs inhibit presynaptic release of glutamate, if the coupling mechanisms to astrocytic vesicule-like bodies that contain Glu are the same in astrocytes as in neurons there might be a corresponding decrease in Glu release. However, to the best of my knowledge this possibility has not been examined. Future studies, aimed at determining the relative contributions of mGluR2 and mGluR3 to the group II mGluR immunoreactivity increases in the present study, may shed light on the different roles of astrocytic and neuronal mGluRs. The functional effects of mGluR3 activation in astrocytes have only recently started to be explored.

Interestingly, several studies have suggested that mGluR3 and GLT-1 may interact, although the exact mechanism remains unclear (Aronica et al., 2003; Huang et al., 2004; Bellesi and Conti, 2010). Thus, one must consider the possibility that increases in GLT-1 (also observed only following moderate dopamine depletion) may lead to increases in mGluR2/3, or vice versa. Intriguingly, Aronica and colleagues showed that application of an mGluR2/3 agonist to cultured astrocytes leads to increased levels of GLT-1 and GLAST (2003). It will therefore be interesting to determine whether the parallel increases in GLT-1 and mGluR2/3 seen in our own study may be a result of interactions between the two proteins.

CHAPTER 10

TRANSCRIPT EXPRESSION OF GLUTAMATE-RELATED PROTEINS FOLLOWING DOPAMINE DEPLETION OF THE PREFRONTAL CORTEX

I found that dopamine depletion of the prefrontal cortex increases mGluR2/3 protein levels, as discussed in the previous chapter. However, I unable to determine if this increase reflected a change in mGluR2, which is restricted to neurons (Ohishi et al., 1998), or mGluR3, which is expressed by astrocytes and neurons (Mudo et al., 2007; Harrison et al., 2008). Similarly, the lack of a specific antibody prevented me from assessing levels of xCT. In order to gain some insight into potential changes in xCT and group II mGluRs after PFC dopamine depletion, I examined the relative abundance of the mRNAs encoding xCT, mGlur2, and mGluR3, as well as several other glutamate-related proteins.

Methods

Methods used in this chapter are the same as those used in Chapter 6, with the exception of the primers used for these studies (see Table 9).

Gene	Forward (5’ to 3’)	Reverse (5’ to 3’)
GluN1	ATGGAGGCCCGGGAACTGGAG	CATTGCGGCTGCGCGGTACA
GluA1	GGTGCGGTTGTGGGTGCCAAT	GGGCTCCGTGAGTTGTGACAAAG
mGluR 2	CAGCAAGCGGGAACCGGAGC	GAGGAGGAAACAGGGGCCAGGA
mGluR 3	CTCACCGCTGCCGCTACCAC	GGCTGAAAGAGCCCGTCACCG
xCT	CGCCGAGGAGCTGTTGCAGTC	ATGGAGCCGAAGCAGGAGAGGG

Table 9. Primers designed using NCBI-Primer Blast for study of glutamate-related protein transcripts.

Results

Extent of dopamine depletion. The dopamine concentration in the PFC of control animals averaged 0.49 ± 0.05 ng/mg protein. Prefrontal cortical dopamine concentrations in 6-hydroxydopamine-lesioned rats were reduced across all lesioned subjects by 74.3 % (t_{27}= 7.36, $p<0.001$). Norepinephrine concentrations (control value of 4.34 ± 0.25 ng/mg protein) were significantly reduced by 29.9% after dopamine denervation of the PFC (t_{27}=2.92, $p<0.01$). Serotonin levels were unchanged. We also analyzed mRNA levels in animals separated into moderate and severe dopamine depletion groups, as described in Chapter 5. The average decrease in PFC DA concentrations was 50.3% in the moderate group, and 89.6% depletion in the severe group (see Table 6 in Chapter 7). Norepinephrine levels were unchanged in the "moderate" dopamine depletion group, while levels of norepinephrine were reduced by 28.9% in the "severe" dopamine depletion group (Table 6). Similar to the combined depletion groups, serotonin levels remained unchanged when animals were separated by dopamine depletion extent (Table 6).

Effects of PFC dopamine depletion on mGluR2, mGluR3, and xCT mRNA levels. When mRNA levels of mGluR3, mGluR2, GLUN1, GluR1, and xCT were evaluated by RT-PCR, no change was observed in dopamine-depleted animals when compared to controls in any of the transcripts measured (Figure 14). Similarly, no change was observed in mGluR3, GLUN1, GluR1 or xCT transcripts when animals were separated by extent of dopamine depletion (Figure 14). However, in samples taken from animals with “severe” dopamine depletion, a significant decrease in mGluR2 mRNA levels was observed (~50%; Figure 14).

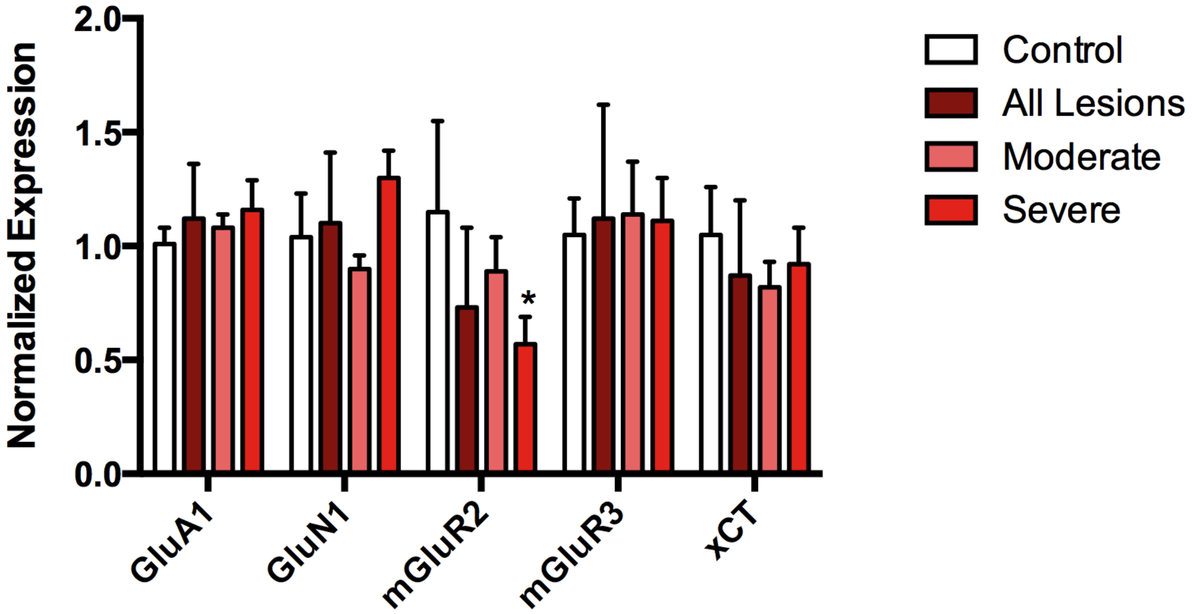

Figure 14. RT-PCR following dopamine depletion of the PFC uncovers a significant decrease in mGluR2 mRNA levels. No change was observed in mRNA expression of the AMPA and NMDA receptor subunits GluA1 and GluN1 respectively. Similarly no change was observed in mGluR3 or xCT. A significant decrease was observed in mGluR2 mRNA levels in animals with a severe dopamine depletion.

$p<0.05$

Discussion

mGluR2 and mGluR3 gene expression. We found that the mRNA encoding mGluR2 was selectively increased in animals with severe dopamine depletion of the PFC. In contrast, we observed that mGluR2/3 protein levels were increased, but only in those animals with moderate, not severe, dopamine loss in the PFC.

Unfortunately, this leaves us no closer to understanding how the increase we observed in mGluR2/3 protein levels in the group of animals sustaining moderate depletion of dopamine in the PFC occurs. Indeed, the increase in mGluR2 mRNA that we uncovered in the severely dopamine depleted animals is difficult to put into any consistent framework with the other data we obtained. We replicated the increase in mGluR2/3 protein levels twice, and thus have a total of three different experiments giving the same result. However, we have not replicated in separate cohorts the mRNA data, which should be done. Because the group II mGluRs function as release-modulating autoreceptors, physiological studies in mGluR2 and mGluR3 knockout mice may offer the best chance of untangling how the contrasting changes we obtained in protein levels and mRNA relate.

While the precise relationship between group II protein and mRNA levels remains obscure, it is of considerable interest that group II mGluR agonists applied to astrocytic cultures significantly increase GLT-1 levels (Aronica et al., 2003). This suggests that increases in mGluR2/3 immunoreactivity may drive the observed increase in GLT-1 in our *in vivo* experiments. Furthermore, these receptors appear to play an important role in the astrocytic response to neuronal glutamate release, as activation of these receptors leads to increases in intracellular Ca^{2+} in astrocytes (Winder and Conn, 1996).

xCT gene expression. Immunoblot studies of xCT protein levels produced inconclusive results, as discussed in Chapter 9. The data reported in this chapter suggest that xCT gene expression was unchanged following dopamine depletion of the PFC. Interestingly, like mGluR3, xCT has been linked to GLT-1. Knackstedt et al. showed that following cocaine administration, both xCT and GLT-1 were downregulated, and that administration of the beta-lactem antibiotic ceftriaxone, as well as N-acetylcysteine, could restore levels of both xCT and GLT-1 (2010). GLT-1 plays an important role in bringing glutamate into astrocytes; xCT exchanges intracellular glutamate for extracellular cystine, which is then used for production of the reducing agent glutathione (Sato et al., 2005; Shih et al., 2006). Therefore, the connection between these two proteins may be difficult to untangle if a challenge leads to the production of glutathione, as both are critical to glutathione production and disruption of one or the other of these proteins will effect the function of the other. Our data suggest that, rather than production of glutathione, restoration of glutamate homeostasis is the primary goal of the astrocyte following dopamine depletion, and therefore, xCT levels (and glutamate transport into the extracellular space) remain at normal levels, while increases in GLT-1 likely result in increased intracellular glutamate levels and reduced extracellular glutamate.

CHAPTER 11

FUNCTIONAL IMPLICATIONS, DISCUSSION, AND FUTURE DIRECTIONS

Through the course of this dissertation I demonstrated that dopamine depletion increases protein levels of the astrocytic glutamate transporter GLT-1, without increasing GLT-1 mRNA levels. The increase in protein was not attributable to increased astrocyte number or activation. Furthermore, glutamate transport strongly correlated with the extent of dopamine depletion. No changes were observed in other glutamate transporters, nor a variety of proteins related to glutamate homeostasis, with the exception of mGluR2/3 protein, which showed the same relationship to dopamine denervation as GLT-1. In this chapter, I will discuss the functional implications, limitations and future directions of this dissertation.

Functional Implications

Schizophrenia is a debilitating disease classically characterized by positive and negative symptoms. Positive symptoms include hallucinations, delusions, and thought disorder. Negative symptoms include flattened affect, a lack of motivation, and alogia. Cognitive symptoms of schizophrenia have gained significant attention in the last 30 years, and include deficits in executive function, working memory, and attention. Improvements in cognitive symptoms correlate positively with patients’ overall outcome (Green, 2006; Tabarés-Seisdedos et al., 2008), which has led researchers to investigate new therapeutics that aim to improve not only the positive and negative symptoms, but the cognitive deficits as well.

The prefrontal cortex is commonly associated with cognition. Morphological changes in the PFC, including decreases in volume as well as cellular changes, have been reported in patients with schizophrenia (Glantz and Lewis, 2000; Shenton et al., 2010). The decreased volume is not accompanied by an overall loss of neurons, which has given rise to the reduced neuropil hypothesis of schizophrenia (Selemon and Goldman-Rakic, 1999). This hypothesis suggests that the loss of dendritic or axonal processes contributes to PFC volume reduction. Consistent with this idea, loss of dendritic spines in the PFC is a consistently replicated pathological finding in schizophrenia (Garey et al., 1998; Glantz and Lewis, 2000; Black et al., 2004). However, the mechanism behind the loss of neuropil remains unclear.

Two primary hypotheses dominate current discussions of the pathophysiology of schizophrenia: the dopamine hypothesis and the glutamate hypothesis. Both PFC dopamine and glutamate have been shown to affect cognitive function and dendritic spine number in the prefrontal cortex (Sawaguchi and Goldman-Rakic, 1991; Krystal et al., 1994; Arnsten and Li, 2005; Carli et al., 2011). Current treatment options predominantly target subcortical hyperdopaminergia, which is thought to give rise to positive symptoms. Unfortunately, cognitive deficits remain one of the most difficult aspects of schizophrenia to treat. Thus, novel therapeutic strategies continue to be explored, including those that target aspects of glutamate signaling (de Bartolomeis et al., 2012; Kantrowitz and Javitt, 2012; Takahashi and Sakurai, 2013). Among the novel therapeutics aimed at reducing cognitive dysfunction, are various players in the glutamate pathway, including mGluRs and allosteric modulatory sites on the NMDA receptor, including D-cycloserine and the the glycine transporter (which provides an essential NMDA co-agonist).

Yet the basic science question remains: do changes in dopamine lead to changes in glutamate, or vice versa? Understanding the interactions between dopamine and glutamate appears to be critical for understanding the role that the PFC plays in cognition. Therefore, schizophrenia is one field (of many) in which a detailed understanding of the interactions between these transmitters may lead to significant advances in therapeutics, particularly in the treatment of cognitive deficits seen in the disorder.

Dopamine-Glutamate Interactions. As studies continue to explore the roles of both dopaminergic and glutamatergic signaling dysfunction in schizophrenia, it is becoming clear that the disease is not likely to be explained fully by one or the other, but rather by an understanding of the interactions between dopamine and glutamate. Thus, studies such as those described in this thesis, as well as others, which describe interactions and effects of dopamine on glutamate, and vice versa, are of particular importance. Moghaddam and colleagues used subanesthetic doses of the NMDA receptor antagonist ketamine, and measured dopamine and glutamate levels in the PFC and striatum using microdialysis (1997). These studies found that ketamine increases extracellular glutamate and dopamine levels in the PFC. Researchers have also investigated the effects of dopamine and pharmacological manipulation of dopamine receptors on glutamate receptor activation (Tseng and O'Donnell, 2004; Sokoloff et al., 2013; Yuen et al., 2013). In a study using slice electrophysiology, bath application of dopamine to the PFC resulted in a dose dependent increase in EPSC amplitude recorded in Layer II/III pyramidal cells, suggesting alterations in the postsynaptic response (Gonzalez-Islas and Hablitz, 2003). In similar experiments with adult animals, agonists specific to D_1 (SKF38393) or D_2 (quinpirole) have differential effects, as SKF38393 elicits an increase in pyramidal cell excitability, while quinpirole induces a dose-dependent decrease in excitability (Tseng and O'Donnell, 2004). These data suggest that targeting dopamine receptors as a whole may be too broad a tool, and that subtype specific drugs may allow more specific treatments, if basic research is able to tease apart the contributions of these receptors to various disease states. Furthermore, Paille and colleagues demonstrated that dopamine depletion of the striatum produces differential effects on NMDA receptor subunit levels. Partial dopamine depletion (~75%) leads to increases in GluN2A, while a full depletion (>95%) has no effect on GluN2A, but leads to significantly lower levels of GluN2B (Paille et al., 2010). These data suggest that dopamine-glutamate interactions are quite complex, being dependent on concentrations of the transmitters involved, as well as the receptors expressed. Dopamine can impact presynaptic release of glutamate, as well as the postsynaptic response to released glutamate (Wang, 2001, Gonzalez-Islas et al., 2003). Similarly, glutamate can have a significant impact on dopamine release (Usun et al., 2013). While the interactions between dopamine and glutamate continue to be explored and defined, it is becoming increasingly clear that manipulation of the dopamine system is likely to have implications for the glutamate system, and vice versa. Thus far, investigation of dopamine-glutamate interactions has focused primarily on neurons. Over the past decade it has become clear that glia, and particularly astrocytes, can no longer be regarded as passive support cells in the brain.

Previous work examining the effects of dopamine on glutamate release has used pharmacological manipulation (Daly et al., 1997 Yamamoto and Cooperman, 1994), but there has been no work using chronic dopamine depletion as appears to occur in schizophrenia. Likewise, little work has been done investigating the effects of dopamine depletion on astrocytic glutamate-related proteins in any brain region, and no investigations have been done in the PFC. I therefore investigated the effect of dopamine depletion on both neuronal and astrocytic glutamate-related proteins in the PFC in an effort to fill this knowledge gap.

In the previous chapters, I have demonstrated that dopamine depletion of the prefrontal cortex leads to increases in GLT-1 and mGluR2/3, two proteins that are expressed (although not exclusively) by astrocytes. When samples were separated by the extent of dopamine depletion, the observed effect was limited to those animals suffering a “moderate” depletion as opposed to a “severe” depletion. The observed changes were specific to the membrane-bound fraction, and were not paralleled by changes in gene expression. Furthermore, increases were not attributable to astrocytosis, leading us to suggest that disrupted post-translational modification may be the cause of GLT-1 increases, specifically. Interestingly, as seen in Chapter 8, functional assays revealed that the extent of dopamine depletion correlates positively with the rate of glutamate uptake. All of these data point to an astrocytic response to dopamine depletion of the prefrontal cortex.

Limitations and Future Studies

As with all scientific studies, those studies described in this thesis have limitations, some of which have been mentioned within the previous chapters. Several of these limitations, and experiments that could address them, will be discussed in more detail in the following section.

What happens to extracellular glutamate levels? I have hypothesized that dopamine depletion of the PFC leads to increases in extracellular glutamate, and thus leads to changes in proteins such as GLT-1 and mGluR2/3. At this point studies demonstrating this increase in glutamate levels have not been performed. However, preliminary studies aimed at addressing this hypothesis have been done using electrophysiological assessment of spontaneous EPSC number. Contrary to our expectations, no difference was seen in the frequency of spontaneous EPSCs in lesioned animals compared to controls. However, when these experiments were performed, we had not yet established differences between “moderate” and “severe” dopamine depletion. Therefore, the extent of dopamine depletion was not addressed in these experiments. Future studies aimed at determining the effect of dopamine depletion of the PFC on extracellular levels of glutamate would likely prove to be very useful, not only for improving the interpretation of our own data, but also for numerous other labs studying the interactions of these transmitters.

While electrophysiology can produce valuable insight into the release of glutamate, it is difficult to determine if basal extracellular levels of glutamate are increased using these techniques. A more direct measure of glutamate levels following dopamine depletion could be achieved using microdialysis. However, traditional microdialysis requires establishing a baseline concentration, before administration of a challenge, after which the response to this challenge can be determined. This technique has been utilized in the presence of dopamine antagonists. Traditional microdialysis experiments are not capable of measuring true concentrations of glutamate for comparison between control animals and animals receiving a VTA lesion. Rather traditional microdialysis requires the addition of a challenge, often K^+ stimulation. In order to determine the extracellular glutamate levels following dopamine depletion, a technique known as no-net-flux or stable-isotope-labelling should be used (Olson and Justice, 1993; Hershey and Kennedy, 2013). These methods allow the experimenter to determine a true concentration of glutamate or other molecules without the use of a challenge or stimulation. Use of these techniques, while difficult, could prove to be very helpful in determining the effect of chronic dopamine depletion on extracellular glutamate concentrations. Furthermore, these experiments could help to elucidate the source of the differential response to varied dopamine depletions. It could be that moderate and severe dopamine depletions have differential effects on extracellular glutamate levels, leading to increases or activation of different sets of proteins. However, while these experiments could help to explain why there are different responses to varied extents of dopamine depletion, it would do little to determine what those responses might be.

Why is there an increase in GLT-1 and mGluR2/3 following moderate dopamine depletion and no increase following severe dopamine depletion? One question that has come out of the work presented in this thesis is: why is there an increase in GLT-1 and mGluR2/3 levels following moderate dopamine depletion, but not after severe dopamine depletion? This question certainly deserves significant attention. I have hypothesized that differential mechanisms likely exist following moderate depletion when compared to severe dopamine depletion. Thus an astrocytic response, as seen through increases in GLT-1, occurs following moderate depletion, while another, as yet undefined, mechanism may occur after severe dopamine depletion. A number of proteins that may be potential players in a secondary neuronal mechanism, including NMDA receptor subunits and AMPA receptor subunits, have been examined without an observed change (see Chapter 9). However, the phosphorylation states of these proteins were not examined. Differences in phosphorylation can lead to significant functional changes in a protein (Moon et al., 1994; Rostas et al., 1996; Giese et al., 1998; Strack, 1998). Therefore, examining some of these proteins more thoroughly, with regards to post-translational modifications might provide insight into a potential response to severe dopamine depletion.

Another possible explanation for increased GLT-1 and mGluR2/3 following moderate dopamine depletion with no change following severe dopamine depletion, which was raised in Chapter 5, is that following severe dopamine depletion there is a loss of dendritic spines that may not be observed after moderate dopamine depletion. Previous work by myself and others in our lab have laid the groundwork for studies exploring the effects of varied extents of dopamine depletion on dendritic spine density of PFC pyramidal cells. Previously published work has demonstrated that dopamine depletion of the PFC results in a loss of dendritic spines on Layer V pyramidal cells (Wang and Deutch, 2008). Since the publication of this data, our lab has switched from Golgi staining to intracellular fills using the dye Lucifer Yellow, which allows for the selection of non-overlapping neurons for processing, and also allows us to take intermittent sections for immunohistochemistry. The sections can then be stained with a TH antibody in an effort to determine the extent of dopamine depletion in individual animals. In order to verify that this method produces a good representation of dopamine depletion, we correlated dopamine concentration, as determined by HPLC analysis, to the total length of TH positive axons in the PFC (Figure 15). Using intracellular fills along with TH axon length, experiments can now be performed to evaluate the effects of dopamine depletion on the number of dendritic spines. This could help to determine whether dendritic spine loss might be a mechanism that is restricted to those animals with severe dopamine depletion, while GLT-1 and mGluR2/3 increases only occur in animals with moderate dopamine depletion.

It may be that both of these experiments will fail to yield significant differences between moderate and severe dopamine depletion, and that the observed difference in protein expression will need to be explained through another, currently undefined, mechanism. However, understanding why there is a differential response in moderate, compared to severe dopamine depletions remains a critical question that should be addressed by future studies.

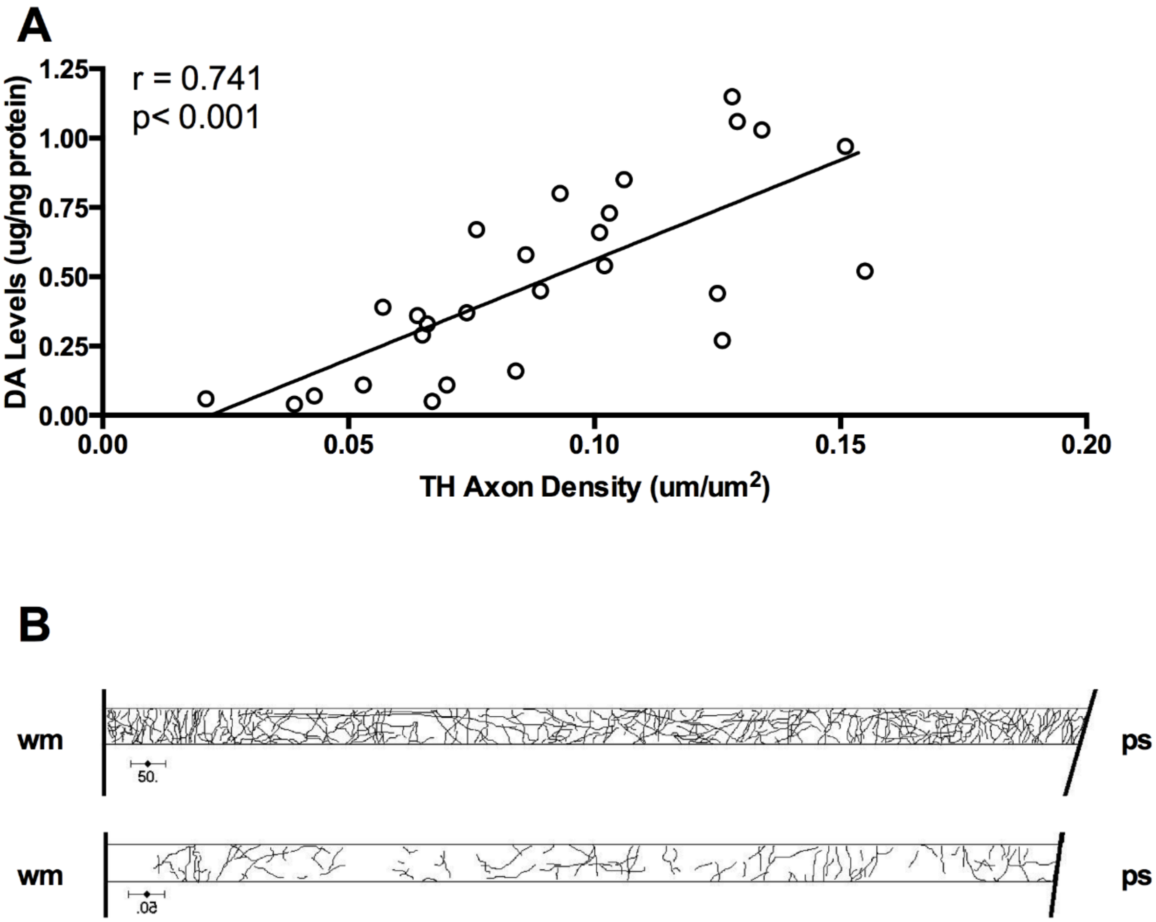

Figure 15. Comparison of TH axon density and HPLC dopamine levels in the PFC following dopamine depletion. Two methods were used to determine the extent of dopamine depletion of the same animals, HPLC analysis, and TH axon density. **A)** TH axon density strongly correlated with dopamine levels determined by HPLC analysis. **B)** Representative images of TH tracings for a control (top) and a lesioned animal (bottom). wm=white matter; ps= pial surface

Can we be certain that GLT-1 increases are astrocytic? Some controversy exists over the localization of GLT-1. The majority of researchers appear to be in agreement that GLT-1 protein is exclusively, or nearly exclusively, expressed by astrocytes in the adult rodent brain under normal conditions (Danbolt, 2001; Kanai et al., 2013). However, research has also demonstrated that there are circumstances in which GLT-1 can be expressed by neurons. When hippocampal neurons are grown in culture, for example, these cells have been shown to express the glutamate transporter GLT-1 (Mennerick et al., 1998). Furthermore, ischemic conditions *in vivo* have been shown to lead to neuronal expression of GLT-1 protein (Martin et al., 1997). Interestingly, GLT-1 mRNA has been observed in neurons *in vivo*, despite a lack of protein expression (Schmitt et al., 1997; Torp et al., 1997). These data suggest that under certain conditions, neuronal GLT-1 expression may be induced. Our results demonstrate that there is no increase in levels of GLT-1 mRNA following dopamine depletion, yet there is a significant increase in protein expression. We have suggested that this is due to an accumulation of GLT-1 in astrocytic membranes, since the majority of researchers believe that GLT-1 is overwhelmingly expressed in astrocytes. Future experiments using electron microscopy should be performed to determine the localization of GLT-1 protein expression following dopamine depletion, resolving this issue more concretely. However, the increases in GLT-1 that we have observed, regardless of neuronal or astrocytic localization, represent a substantial response in a protein involved in glutamate signaling following dopamine depletion. This further solidifies the need for extensive research into the interactions of these two neurotransmitter systems.

Are glutamate uptake results solely attributable to GLT-1? In Chapter 8, the effect of dopamine depletion on glutamate uptake was determined. In this experiment, specific glutamate uptake was determined by subtracting non-specific uptake as determined by running samples through the same uptake protocol while keeping samples on ice, effectively preventing all transport. We have assumed that glutamate uptake in the cortex occurs almost entirely through the GLT-1 transporter, as has been reported by multiple investigators (Rothstein et al., 1996, Tanaka et al., 1997). However, following inhibition of GLT-1, upregulation of EAAC1 transport has been reported (Salvatore et al., 2012), suggesting that under certain conditions the burden of transport can shift away from GLT-1.

Chapter 8 also demonstrated that glutamate uptake positively correlated with the extent of dopamine depletion, contrary to the negative correlation observed between dopamine depletion and GLT-1 protein expression. This suggests that GLT-1 function differs between varied extents of dopamine depletion, or that glutamate uptake shifts from being mediated primarily by GLT-1 to include a greater contribution by other transporters (i.e. GLAST or EAAC1).

Experiments aimed at determining glutamate uptake specific to the different transporters following dopamine depletion may prove useful in determining whether there is a shift from transport primarily through GLT-1 to one of the other transporters. Through the use of inhibitors specific to GLT-1 (dihydrokainate; Bunch et al., 2009), GLAST (UCPH-1;Abrahamsen et al., 2013) and EAAC1 (HIP-B;Callender et al., 2012), it could be determined whether major changes in transporter function occur as a result of varied dopamine depletions. However, despite being used as specific inhibitors, one would need to be careful in interpreting results from these studies, closely examining the specificity of the inhibition, not just between glutamate transporters, but other targets as well.

Does dopamine depletion directly affect astrocytes, or do effects of dopamine depletion on neurons lead to the astrocytic response? The experiments contained in this document do not address the question of which cell type is directly affected by dopamine depletion. For example, loss of dopamine signaling at astrocytic dopamine receptors may lead to intracellular signaling which ultimately leads to increased expression of GLT-1 and mGluR2/3. In contrast, a decrease in dopamine signaling at neuronal dopamine receptors may lead to increased glutamate release and a subsequent increase in levels of glutamate-related proteins including GLT-1 and mGluR2/3.

Astrocytes and neurons each express dopamine receptors, making this question difficult to address through *in vivo* experiments. *In vitro* cultures using astrocytes and neurons may provide an experimental model that allows for the separation of neuronal and astrocytic effects, although complications also exist with this technique. Initially, a simple astrocyte culture system using dopamine receptor antagonists (towards both D1 and D2 receptors) followed by western blot analysis of GLT-1 and mGluR2/3 levels could give some insight into astrocytic responses to dopamine depletion. Unfortunately, astrocytes and neurons have been shown to have very different protein expression patterns *in vitro* when compared to *in vivo* (Lehmann and Harris, 1995; Mennerick et al., 1998). Thus, changes observed in cell-type specific cultures could be significantly different from *in vivo* responses that are presumably more closely resemble the human in vivo condition.

Co-culture of astrocytes and neurons is frequently used to better represent the environment present *in vivo*, and a recent co-culture system allows for the culturing of astrocytes and neurons from different genetic backgrounds (Jones et al., 2012). Using genetically modified animals lacking the dopamine receptors, cultures could include unmodified neurons and astrocytes without dopamine receptors. Using these cultures one could determine whether a lack of dopamine signaling at astrocytic dopamine receptors leads to GLT-1 and mGluR2/3 protein increases. Similarly, neurons lacking dopamine receptors could be cultured with unmodified astrocytes to determine whether a lack of dopamine signaling on neurons leads to increased GLT-1 or mGluR2/3 protein. However, modeling chronic dopamine depletion, as we have utilized in our *in vivo* models, would remain difficult.

Relevant techniques available to tease apart the effects of dopamine depletion on astrocytes and neurons remain limited. To effectively mimic our experimental paradigm *in vivo*, a co-culture including astrocytes and neurons, in which one cell–type could be treated, but not the other, would be ideal. To the best of my knowledge this technique does not yet exist. However, through the use of optogenetics, one might be able to transfect astrocytes or neurons with light-inhibited dopamine receptors. By culturing these, along with wild-type neurons or astrocytes, respectively, one might be able to tease apart the different contributions of astrocyte and neuron dopamine depletion to GLT-1 and mGluR2/3 changes. Unfortunately, the methods available to us in pursuing the effects of dopamine depletion on a complete astrocyte-neuron system, appear to be inadequate. Advances in culture (*or in vivo*) systems may make these experiments possible in the future.

Conclusions

This work has demonstrated that dopamine depletion of the prefrontal cortex, similar to that observed in schizophrenia, can lead to alterations in the expression of glutamate-related proteins. In particular, we show that dopamine depletion has a significant effect on the astrocytic glutamate transporter, GLT-1. The study of astrocytes continues to expand, and our work suggests that astrocytes may be an integral part of dopamine-glutamate interactions. Future studies, building off the work in this thesis, may demonstrate that these once overlooked cells play a critical role in linking the dopamine and glutamate hypotheses of schizophrenia.

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