Astrocyte Sensitivity to Dopamine in Culture and Ex Vivo

Ashley L. Galloway

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ASTROCYTE SENSITIVITY TO DOPAMINE IN CULTURE AND EX VIVO

by

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DEDICATION

I would like to dedicate this work to my best friend, Garrett B. Hutchison, for being by my side throughout this humbling journey. He has been irreplaceable in his efforts towards “feeding and care” of his graduate student. It is my hope that we will continue to collaborate on this journey that is life, sharing adventures and memories for the remainder of our days.

I would also like to dedicate this manuscript to my mother, Diane Galloway, who has, without fail, supported me in every possible way throughout life and most importantly, through my academic endeavors. Truly, it is to my mother that my sincerest thanks must go, for I would not have achieved such a goal without her unyielding support, understanding, and encouragement.
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I would like to thank everyone from the PPN department that I have known during my time here. Every single one has contributed in some way to this thesis.

Foremost I would like to thank my mentor, Dr. Pavel Ortinski, for his patience and encouragement throughout this project; for providing me with limitless opportunity and for opening up the world of neuro(science) to me. I have grown substantially, both as a scientist and a person, throughout our time together. May my future professional endeavors reflect the exemplary mentorship and guidance you gave me through my graduate career.

I would also like to extend a special thank you to those I regard as “graduate-school survival” mentors: Dr. Larry Reagan, Dr. Jill Turner, and Dr. David Mott. Your wisdom and guidance have helped me navigate the many twists and turns along the path to becoming a Ph.D.

To Dr. Robert (Bob) Price, thank you for teaching me the imaging techniques, without which, this dissertation would not have been possible. Your patience and enthusiasm in mentoring me on techniques of microscopy and presentation are invaluable. I was not only able to learn a challenging and rewarding scientific approach, but I was also given the opportunity to discover a creative outlet for my passion in science.
To my committee, Dr. Norma Frizzell, Dr. Robert Price, Dr. Fabienne Poulain, and Dr. Ana Pocivavsek. I am extremely grateful for your mentorship and guidance throughout my time as a Ph.D student.
ABSTRACT

Dopamine is critical for processing of reward and etiology of drug addiction. Astrocytes throughout the brain express dopamine receptors, but consequences of astrocytic dopamine receptor signaling are not well established. This thesis illustrates effects of dopamine on cultured astrocytes and astrocytes in brain slices (ex vivo). In striatal cultures, extracellular dopamine triggered changes in astrocytic Ca\(^{2+}\) signaling and rapid concentration-dependent stellation of astrocytic processes that was not a result of dopamine oxidation, but instead relied on both cAMP-dependent and cAMP-independent dopamine receptor signaling. To isolate possible mechanisms underlying these structural and functional changes, whole-genome RNA sequencing was used in identifying prominent dopamine-induced enrichment of genes containing the CCCTC-binding factor (CTCF) motif, suggesting involvement of chromatin restructuring in the nucleus. Specifically, results show that cultured astrocyte response to elevated dopamine involves PARP1-mediated CTCF genomic restructuring and concerted expression of gene networks. To examine astrocyte response to behaviorally relevant dopamine signals, astrocyte morphological and molecular profiles in ventral striatum (nucleus accumbens core; NAcC) and dorsolateral striatum were characterized following cocaine self-administration training on an extended access schedule. Findings illustrate that extended cocaine experience resulted in decreased numbers of GFAP+ cells and primary process number in the ventral striatum. This was
associated with an increase in connexin 30 mRNA, an astrocyte-astrocyte interaction gene. In the dorsolateral striatum, there was an observed increase in length of GFAP+ primary processes and downregulation of SPARC (astrocyte-neuron interaction gene) mRNA.
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<tr>
<td>α</td>
<td>alpha</td>
</tr>
<tr>
<td>β</td>
<td>beta</td>
</tr>
<tr>
<td>C°</td>
<td>degrees, Celsius</td>
</tr>
<tr>
<td>Δ</td>
<td>delta; change or difference</td>
</tr>
<tr>
<td>g</td>
<td>gram; unit of measure, the thousandth part of a kilogram</td>
</tr>
<tr>
<td>m</td>
<td>milli; unit of measure, the thousandth part of a meter</td>
</tr>
<tr>
<td>µ</td>
<td>mu, micron or micrometer; a unit of measure, the millionth part of a meter.</td>
</tr>
<tr>
<td>M</td>
<td>molarity or molar concentration</td>
</tr>
<tr>
<td>pxl</td>
<td>pixel</td>
</tr>
<tr>
<td>s</td>
<td>second(s); unit of measurement, time</td>
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</table>
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AC</td>
<td>adenylyl cyclase</td>
</tr>
<tr>
<td>Ba&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Barium</td>
</tr>
<tr>
<td>BG</td>
<td>Basal ganglia</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Calcium</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic AMP</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CX43</td>
<td>Connexin 43</td>
</tr>
<tr>
<td>CX30</td>
<td>Connexin 30</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DAR</td>
<td>Dopamine Receptor</td>
</tr>
<tr>
<td>DIV</td>
<td>Days in Vitro</td>
</tr>
<tr>
<td>DL</td>
<td>Dorsolateral</td>
</tr>
<tr>
<td>DLS</td>
<td>Dorsolateral striatum</td>
</tr>
<tr>
<td>DMS</td>
<td>Dorsomedial striatum</td>
</tr>
<tr>
<td>EA</td>
<td>Extended-access</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-signal-regulated kinase</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>FR</td>
<td>Fixed-ratio (schedule of reinforcement)</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>GIRK</td>
<td>G protein-coupled inward rectifying potassium channel</td>
</tr>
<tr>
<td>Gja1</td>
<td>Gap junction connexin 43</td>
</tr>
<tr>
<td>Gjb6</td>
<td>Gap junction connexin 30</td>
</tr>
<tr>
<td>GLT-1</td>
<td>Glutamate transporter 1/excitatory amino acid transporter 2</td>
</tr>
<tr>
<td>GLAST</td>
<td>Glutamate transporter/excitatory amino acid transporter 1</td>
</tr>
<tr>
<td>Hevin</td>
<td>SPARC-like 1 protein, SPARCL1</td>
</tr>
<tr>
<td>Hipp</td>
<td>Hippocampus</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetyl-L-Cysteine</td>
</tr>
<tr>
<td>NAc</td>
<td>Nucleus accumbens</td>
</tr>
<tr>
<td>NAcC</td>
<td>Nucleus accumbens core</td>
</tr>
<tr>
<td>NAcS</td>
<td>Nucleus accumbens shell</td>
</tr>
<tr>
<td>PAP</td>
<td>Perisynaptic astrocytic process</td>
</tr>
<tr>
<td>PFC</td>
<td>Prefrontal cortex</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SA</td>
<td>Short-access</td>
</tr>
<tr>
<td>SMBS</td>
<td>Sodium Metabisulfite</td>
</tr>
<tr>
<td>SN</td>
<td>Substantia Nigra</td>
</tr>
<tr>
<td>SNr</td>
<td>Substantia Nigra pars reticulata</td>
</tr>
<tr>
<td>SNc</td>
<td>Substantia Nigra pars compacta</td>
</tr>
<tr>
<td>STN</td>
<td>Subthalamic Nuclei</td>
</tr>
<tr>
<td>SPARC</td>
<td>Secreted protein, Acidic, Cysteine-rich</td>
</tr>
</tbody>
</table>
VM ................................................................. Ventromedial
VP ................................................................. Ventral pallidum
VTA ................................................................. Ventral Tegmental Area
CHAPTER 1
GENERAL INTRODUCTION

First noted in 1824 and first named in 1856, glia have long been acknowledged in neuroscientific literature (Virchow, 1856). Rudolf Virchow was the first to apply a name to these cells, designating them as neuroglia or “nerve-glue” (Virchow, 1856). For many years, neuroglia were regarded as inert cells charged with providing structural support in order for neurons to function. Yet, neuroglia or glia are now understood to play a multitude of diverse and crucial roles in the brain ranging from immune responses to regulation of neural networks. Neuroglia comprise an expansive population of brain cells (80 - 90%), and can be grouped based on developmental origin, morphology, and function into two main classes: microglia and macroglia (Ramon y Cajal S., 1913; Kandel et al., 2012; Oberheim et al., 2012). Cells classified as macroglia include astrocytes, oligodendrocytes, and oligodendrocyte precursor cells. Astrocytes and oligodendrocytes have discrete functional responsibilities. While oligodendrocytes ensheath CNS axons in myelin to promote rapid saltatory conduction, astrocytes are understood to play particularly diverse roles within the brain (Haydon et al., 2009; Oberheim et al., 2012; Araque et al., 2014). One of the interesting observations emerging over the

last few decades is that astrocytes can regulate neuronal activity. For example, Jones et al. (2011) found that astrocyte-secreted SPARC is stimulated by extracellular glutamate which negatively regulates synaptic AMPA receptor expression levels. This allows for a homeostatic mechanism of controlling synaptic excitability, by which increased environmental excitability (i.e., rise in glutamate) decreases synaptic excitability via astrocytes.

Astrocyte heterogeneity in form and function, both across and within brain regions, allows for refined and specialized structural and functional astrocyte-neuron interactions with implications for overall brain function and behavior. In fact, recent evidence has shown structural differences in astrocyte-neuron interactions in the mouse hippocampus and striatum. Specifically, a single astrocytic territory (which includes the cell body and associated processes) within the striatum was found to encompass up to 20 neuronal cell bodies. Yet, in the in-tact hippocampus, a single astrocyte could contain, at most, a single neuron. Moreover, astrocytic territories in the hippocampus were also found to interact with a significantly larger number of excitatory synapses with distinctions in individual astrocyte functional profiles evidenced by Ca2+ imaging analyses (Chai et al., 2017). Thus, the heterogeneous and complex nature of astrocytes in distinct brain regions and brain circuits begs for advanced techniques in investigating the morphological and functional elaborations and aberrations of these cells, as well as their role in regulating neuronal activity via astrocyte-released transmitters and molecules. The experiments and results presented in this thesis explore how astrocytes respond
to dopamine, a molecule critical for regulation of synaptic plasticity underlying drug dependence disorders.

1.1 Dopaminergic neurotransmission

Dopamine exerts its physiological effects through stimulation of D1-like (DRD1, DRD5) or D2-like (DRD2, DRD3, DRD4) metabotropic G-protein coupled receptors (Figure 1.1). Dopamine receptors span the plasma membrane 7 times and have a cytoplasmic domain that binds to and interacts with Gα and Gβγ proteins, as well as an extracellular domain that exclusively binds dopamine (Iversen et al., 2009; Kandel et al., 2012). Ligand binding to D1-like receptors stimulates Gαs/olf subunit containing G-proteins that dissociate to Gβγ and Gαolf/s-GTP. Gαolf/s-GTP then stimulates the enzyme, adenylyl cyclase (AC) which synthesizes and increases the concentration of cyclic AMP (cAMP). In turn, cAMP activates protein kinase A (PKA) which will go on to phosphorylate various targets (Kandel et al., 2012; Iversen et al., 2009). In contrast, D2-like receptors inhibit AC through activation of Gαi/o subunit containing G-proteins. Activation of D2-like receptors leads to inhibition of the AC/cAMP/PKA signaling pathway. Activation of D2-like receptors also results in Gβγ-mediated activation of PLC (Phospholipase C) resulting in upregulated IP3 (Inositol triphosphate) production. Increases in IP3 generation gives rise to the release of ER Ca2+, and the subsequent inactivation of L-type Ca2+ channels and Na+ channels (Kandel et al., 2012; Iversen et al., 2009). DRD1s and DRD2s can oligomerize to form heteroreceptors in certain populations of cells. For example, D1/D2 heteromeric receptors have been found in striatal neurons within the rat CNS (Hasbi et al.,
Ligand binding to D1/D2 heteromers causes a Gαq-mediated increase in intracellular Ca2+ in a PLC-dependent manner (Lee et al., 2004; Rashid et al., 2007).

1.2 Motivation, reward, and memory

Dopamine serves as a critical neurotransmitter of the CNS, playing important roles in regulating movement, as well as learning and motivation. Ever since its original discovery, the dopaminergic system and its intricate signaling networks have and continue to be a topic of exhaustive scientific and clinical investigation. Moreover, it is widely recognized for its role in various neurobiological disorders involving movement-related deficits (i.e., Parkinson’s) and addiction (Iversen et al., 2009).

The mesolimbic dopamine system serves as a key determinant of motivation and incentive salience, regulating an individual’s response to natural rewards, such as food and social interaction (Figure 1.2). The ventral striatum or nucleus accumbens (NAc) comprises both core (NAcC) and shell (NAcS) components and is the principal target innervated by dopamine neurons located in the ventral tegmental area (VTA). The ventral striatum partners with memory and executive function-related centers of the brain, including the hippocampus and the frontal lobe, by processing prominent glutamatergic inputs from both areas. In the hippocampus, dopamine is crucial for encoding/consolidating information into long-term declarative/explicit memory (Milner et al., 1998; Pennartz et al., 2011). In the frontal lobe, dopamine works to regulate executive control over decisions of whether or not the reward should be pursued (Bechara, 2001; Volkow et al., 2002;
These brain regions do not work independently, but rather function in a highly interactive manner to mediate an individual’s response to various environmental stimuli.

### 1.3 Roles of the hippocampus and striatum: implications in disease

Dopaminergic neurons populate two neighboring subregions of the ventral mesencephalon: the Substantia Nigra (SN) and Ventral Tegmental Area (VTA; Hokfelt et al., 1984; **Figure 1.2**). Three target areas, the striatum, the limbic system, and the frontal cortices, receive efferent projections from one of the two above mentioned midbrain regions (Hokfelt et al., 1984; Kandel et al., 2012). Each pathway formed by these projections subserves a different set of functions and is consequently implicated in distinct neuropathologies. Dopaminergic fibers stemming from the more lateral region of the substantia nigra pars reticulata (SNr) form the nigro-striatal system, a system involved in production/initiation of movement, by forming functionally distinct parallel-processing loops with the basal ganglia (Iversen et al., 2009). Deficits in this system lead to significant deficits in motor control (i.e., tremors, rigidity, postural imbalance) a pathological marker of Parkinson’s disease (Kandel et al., 2012). The mesocortical pathway originates in the VTA and terminates in the prefrontal cortex (PFC), and is involved in higher cognitive processes such as working memory and goal-directed behaviors (Zahrt et al., 1997). The mesolimbic system sends projections from the VTA to the nucleus accumbens (NAc) of the limbic system. Pathological disturbance of this pathway has been implicated in schizophrenia, obsessive-compulsive disorder, and drug addiction. A pathway directed from the PFC to the VTA and limbic system
provides a circuit encompassing both the mesocortical and mesolimbic projections. The mesolimbic pathway has been acknowledged as a regulator of sensitized incentive salience or ‘wanting’ through action in the amygdala, as well as, memory consolidation in the hippocampus (Robinson and Berridge, 2008; Li et al., 2003; Da Silva et al., 2012). Addictive substances upregulate dopaminergic activity within this complex reward system, resulting in pathologically high reward values being assigned to cues that co-occur with drug activity (Iversen et al., 2009; Kandel et al, 2012).

Strong evidence indicates a role for the hippocampal-temporal lobe system in declarative memory and the striatum and basal ganglia components in procedural learning and habit formation (Packard & McGaugh, 1992; McDonald & White, 1993; Yin & Knowlton, 2006; Pennartz et al., 2011). While the particular functions of the hippocampus (Hipp) are not fully understood, evidence shows a role of the Hipp in episodic memory, which encodes information about individually experienced events set in specific spatiotemporal context (Tulving & Markowitsch, 1998; Eichenbaum et al., 2007; Wang & Morris, 2010; Squire & Wixttted, 2011). In contrast, procedural memories are believed to be encoded by synaptic activities in the cortico-basal ganglia-thalamic loops. These circuits connect neocortical areas unidirectionally to striatal subregions, which then project to downstream structures such as the ventral pallidum (VP), ventral tegmental area (VTA), substantia nigra pars reticulata (SNr) and pars compacta (SNc). These components connect to thalamic nuclei that send projections back to the same general neocortical areas of circuit origin. Studies have identified various parallel loops associated with
different forms of motor and cognitive processes. Motor and oculomotor loops originate in the frontal eye fields and pre-motor cortices, and cognitive and motivational-affective loops are associated with the prefrontal cortex (PFC), amygdala, and the hippocampus (Alexander et al., 1990; Nambu, 2008; Pennartz et al., 2011).

The striatum can be subdivided into several subregions: the dorsolateral striatum (DLStr) mediates stimulus-response learning and habit formation, the dorsomedial striatum (DMStr) is implicated in cognitive functions and action-outcome learning, and the ventral striatum or nucleus accumbens (NAc) is involved in motivational and affective behavioral processing (Packard & McGaugh, 1992; Knowlton, 2006; Corbit & Belleine, 2003; Voorn et al., 2004; Pennartz et al., 2011; Everitt & Robbins, 2005; Yin et al., 2005; Graybiel, 2008; Balleine et al., 2009; Yager et al., 2015). The question of how striatal integration of hippocampal inputs with afferents from other areas governs motivational processes in models of reinforcement learning remains an important field of research.

Dopamine release in the ventral striatum plays an essential role in mediating affective regulation over motivated behaviors, and its dysregulation may contribute to disorders commonly associated with dysregulated dopaminergic signaling such as substance use disorders. The involvement of the ventral striatum in the reinforcing effects of drugs of abuse, such as cocaine, has led to this region being associated with addiction (Russo et al., 2010). Evidence supports the role for the ventral striatum in drug-seeking behavior in animal models of drug self-administration (Everitt & Robbins, 2005) due to its mediation of motivational effects.
of conditioned stimuli associated with the drug itself. Emerging studies reveal that this region plays a particularly important role in the early development of drug-taking and drug-seeking behaviors. Acute elevation of extracellular dopamine in the ventral striatum, but not the dorsal striatum, potentiates effects of conditioned reinforcers on lever pressing in animal operant conditioning/drug self-administration models (Di Ciano et al., 2008; Taylor & Robbins, 1984). Moreover, selective dopamine elevation by drug infusion in the NAcS of the ventral striatum has been shown to increase Hipp-dependent control over conditioned place preference (CPP). In contrast, when dopamine was directly elevated in the NAcC of the ventral striatum, Hipp-dependent control over CPP-related learning was attenuated (Ito & Hayen, 2011). These findings illustrate regional and sub-regional differences in the way dopamine regulates limbic information processing to mediate individuals’ responses to environmental stimuli (Ito & Hayen, 2011).

Taken together, evidence supports the theory of distinct cortico-striatal loops associated with processing different forms of incoming associative information. It has been established that the hippocampal-ventral striatal pathway is critical for associating contextual-positional information. Moreover, dopamine selectively regulates the strength or gain of associative control over motivated behavior in a sub-region-specific manner.

1.4 Striatum: subregion distinctions in cognition and motor function

GABAergic medium spiny neurons represent the majority of striatal neurons (MSNs; Kemp & Powell, 1971; Gerfen, 2004; Yager et al., 2015). In the dorsal striatum, two MSN populations exist and are classified based on their projection
patterns to the basal ganglia output nuclei and presence of certain neuropeptides and receptors (Figure 1.3). Direct pathway MSNs (dMSNs) send projections to the substantia nigra reticulata (SNr) and globus pallidus internal (GPI), and express dopamine D1 receptors (Gerfen & Surmeier, 2011; Wall et al., 2013; Yager et al., 2015). Indirect pathway MSNs (iMSNs) project to the globus pallidus external (GPe) and the subthalamic nucleus (STN), and express dopamine D2 receptors (Gerfen & Surmeier, 2011; Wall et al., 2013; Yager et al., 2015). Activation of direct MSNs (dMSNs) allows for initiation of intended behavior or movement. In contrast, iMSNs, when activated, work to inhibit unwanted behavior or movement (Yager et al., 2015; Lee et al., 2016). In the ventral striatum, these same MSNs and output pathways are present; however, the efferent targets of these MSNs differ from those in the dorsal striatum and the pathway distinctions are less understood. Specifically, iMSNs in the ventral striatum project to the ventral pallidum and dMSNs project to the VTA and SNr (Chang & Kitai, 1985; Lu et al., 1998; Zhou et al., 2003, Tripathi et al., 2010; Yager et al., 2015).

With these anatomical differences, there are also functional differences between the two striatal subregions (Haber & McFarland, 1999; Berke & Hyman, 2000; Haber, 2003, Pennartz et al., 2011). The ventral striatum is associated with limbic areas of the brain and regulates affective behavioral components, including motivation and emotion (Berke & Hyman, 2000; Meredith et al., 2008; Groenewegen & Trimble, 2007; Pennartz et al., 2011; Yager et al., 2015). Neurons in the NAcC and NAcS subdivisions of the ventral striatum also differ functionally. The NAcC is involved in the processing of conditioned stimuli and the NAcS is
essential in the processing of unconditioned stimuli. These distinctions are thought, in part, to be a result of heterogeneity in neuron morphology and projection patterns of these ventral striatal subdivisions (Heimer et al., 1997, Groenewegen et al., 1999, Meredith et al., 2008).

The two subdivisions of the dorsal striatum, dorsomedial and dorsolateral, both receive dense innervation from neocortical regions, including areas involved in motor processes (Berke & Hyman, 2000). The dorsomedial striatum is essential in output of goal-directed behaviors, whereas the dorsolateral striatum is thought to regulate habitual behavior (Everitt & Robbins, 2005; Pennartz et al., 2011; Yager et al., 2015). Importantly, these striatal sub-regions are not functionally independent, as limbic and cortical information can be passed from the NAcS, through the NAcC to the dorsal striatum in an ascending spiral via midbrain (VTA) dopamine neurons (Haber et al., 2000; Yager et al., 2015). This understanding provides important implications for the functional and behavioral development and persistence of addictive-like behaviors.

1.5 Dopamine and substance abuse in the striatum: evolution of addiction

During the development of drug addiction, drug behavior evolves from voluntary drug-seeking to compulsive drug-seeking behavior. This progression from voluntary to compulsive drug seeking in rodents is associated with continued drug self-administration despite aversive stimuli or consequences (e.g. mild foot shock) and can be observed following chronic, but not short periods of exposure to the drug (Yager et al., 2015; Vanderschuren & Everitt, 2004). These behavioral adaptations are associated with a transfer of control over drug-seeking behavior.
from the ventral striatum to the dorsal striatum (Vanderschuren & Everitt, 2004; Everitt & Robbins, 2013). The ventral-to-dorsal striatum shift in control over drug seeking can be seen as increase in DA efflux within the dorsolateral (DL) striatum, but not within the ventral striatum (Everitt & Robbins, 2013; Ito et al., 2002; Wilhuhn et al., 2012). In fact, lesions or dopaminergic denervation of the dorsolateral striatum disrupt both acquisition and maintenance of compulsive drug-seeking and drug-taking behaviors (Belin & Everitt, 2008; Yin et al., 2006; Faure et al., 2005). Taken together, these findings reveal that extended cocaine taking leads to compulsive drug-seeking habits which are associated with progressive involvement of dorsal striatal components.

Distinct neuronal changes in the molecular, cellular, and structural mechanisms have been established within the dorsal striatum following extended cocaine self-administration regimes (Everitt & Robbins, 2013; Everitt & Robbins 2005; Jonkman & Kenny, 2013). However, non-neuronal mechanisms and machinery which underlie the switch to habitual drug-seeking characteristic of addiction have not yet been explored.

1.6 Astrocyte morphology

Studies in brain slices and cell cultures derived from different brain regions have illustrated that astrocytes are pharmacologically, morphologically, and functionally heterogeneous, and that different subtypes of astrocytes are electrophysiologically distinct (Louise & Lovinger, 2008; Davis-Cox et al., 1994; Venance et al., 1998; Wallraff et al., 2004; Zhou et al., 2006; Chai et al., 2017). A distinction between astrocytic morphology in vitro and in vivo has been recognized
for many years. Astrocytes cultured in serum-containing media have a flat polygonal appearance, while astrocytes in vivo possess a classical star-like morphology with extending filopodia and lamellipodia (Raff et al., 1983; Miller & Raff, 1984; Lavialle et al., 2011).

In cultured conditions, two astrocytic phenotypes have been distinguished based on morphological characteristics and regional localization: type 1 cells have a flat polygonal appearance with a “mesh” of intertwined fine processes and resemble astrocytes populating grey matter; type II cells have clearly defined thicker “stellated” processes and resemble the astrocytes arranged along white matter tracts (Raff et al., 1983; Oberheim et al., 2012). Standard protocols that use serum-supplemented culture media result in predominantly polygonal, type 1 astrocytes. However, these astrocytes can be transformed into a stellated morphology by exposure to cAMP analogs (Won and Oh, 2000; Moonen et al., 1975; Paco et al., 2016). The cAMP-induced morphological transformation occurs within 2 hours and is associated with decreased cell body area and extension of distinct thick processes.

In early literature, astroglia in vivo and in vitro have been traditionally assigned into either of the two phenotypical profiles: protoplasmic of the grey matter and fibrous of the white matter. However, over the past decade emerging evidence reveals astrocytes as being a highly heterogeneous neural cell population. Moreover, this heterogeneity holds important implications for how astrocytes function within their regions and microenvironments, where astroglia
are continually interacting with diverse cells in different states in an ever-changing environment.

1.7 Astrocyte regional heterogeneity

Accumulating evidence demonstrates astrocytes to be a heterogeneous cell population in vivo and in vitro, across and within brain regions, presenting different morphologies, genomic profiles, and functions (Louise & Lovinger, 2008; Chai et al., 2017; Lidellow et al., 2017; Zamanian et al., 2012; Won et al., 200; Miller & Raff, 1984). In normal conditions, astrocytes display physiological distinctions between and within the same brain regions (Chai et al., 2017; Hoft et al., 2014; Miller 2018). In fact, researchers recently investigated differences in hippocampal and striatal astrocytes in slice and found that while the basic electrical membrane properties were the same, striatal astrocytes were more susceptible to Kir4.1 blockage in the presence of barium (Ba2+; Chai et al., 2017). In terms of morphology, unchallenged astrocytes in striatal and hippocampal slices presented similar forms having the same number of primary processes, as well as equivalent somatic and total cell volumes (Chai et al., 2017). The extent of astrocytic networks can be measured by the extent of gap-junction coupling. As with astrocyte density, studies report differing extents of gap-junction coupling within the striatum with higher numbers being observed in Louise & Lovinger et al. (2008) and Chai et al. (2017) than in Hamon et al. 2002 (Hamon et al., 2002; Louise & Lovinger, 2008; Chai et al., 2017).
1.8 Astrocyte plasticity and homeostatic control of neurotransmission

As a structurally incorporated component of the synapse, astrocytes regulate synaptic function and overall brain homeostasis via re-uptake of a variety of ions, neurotransmitters & neuromodulators, as well as metabolic, trophic, and plastic factors (Baldwin & Eroglu, 2017; Verkhratsky et al., 2016; Chung et al., 2015). Astrocytes are also responsible for synaptic pruning, a process which is crucial in organizing and remodeling synapses to ensure efficient communication (Chung et al., 2015). Astrocytes are highly connected and can regulate the neuronal environment and function by altering the concentration of various essential ions such as potassium (K+) and calcium (Ca2+). In fact, astrocytes form extensive networks of coupled cells via gap-junctions, and differences in gap-junction gene expression or protein levels often reflect a difference in astrocytic network architecture and function (Chai et al., 2017; Meunier et al., 2017; Clasadonte & Haydon, 2014). Astrocytes form this syncytium of cells interconnected through gap junction channels formed by protein connexin (Louise & Lovinger, 2008; Dermietzel et al., 1991; Dermietzel et al. 1993). Connexin 43 and connexin 30 connect the cytoplasm of adjacent cells (coupling), and are crucial for synchronization of astrocytic signaling, calcium wave propagation, and spatial buffering of potassium (Blomstrand et al., 2005; Cotrina et al., 1998; Giaume & Venance, 1998; Wallraff et al., 2006). Connexin43 (Gja1) is the major gap-junction protein connexin of astrocytes, and colocalizes with Connexin30 to couple astrocytic networks and facilitate intercellular signaling and flow of ions (Lutz et al., 2009). In fact, double knock-out of connexin 43 and connexin 30 leads to complete
ablation of astrocytic coupling, with no effects on astrocyte density (Theis et al., 2003; Wallraff et al., 2006).

Astrocytic gap-junction coupling serves a critical role in extracellular ion (i.e., Na+, K+, Ca2+) homeostasis, including regulation of potassium (K+) levels. Moreover, well-established coupling of gap-junctions has been shown to support “isopotentiality” of astrocytic networks by decreasing the membrane potential depolarization that follows increased levels of K+, thus regulating the K+ inward driving force which is necessary for effective astrocytic control of brain homeostasis (Ma et al., 2016). Importantly, one group of researchers revealed that astrocytic gap-junctions work to accelerate K+ clearance from the extracellular space and limit K+ accumulation during synchronized neuronal firing (Wallraff et al., 2006). Moreover, studies reveal that alterations in astrocytic coupling changes K+ clearance from the extracellular space which has been linked to alterations in neuronal function. Specifically, investigators have shown that deficiencies in astrocytic coupling lead to impaired potassium buffering, a major homeostatic role of astrocytes, which has been associated with dysfunction of neurons in diseases such as epilepsy (Ma et al., 2016; Wallraff et al., 2006; David et al., 2009).

Astrocytes are also able to modulate neuronal activity and synaptic transmission through release of certain chemical transmitters, such as glutamate, ATP, and d-serine in a process called gliotransmission (Haydon 2009, Araque 2014, Koizumi 2003, Fellin 2004, Fellin 2007, Panatier 2006). These transmitters provide important mechanisms by which neuronal functioning can be regulated. For example, astrocytic release of ATP facilitates inhibition of excitatory synaptic
transmission in hippocampal cell populations as evidenced by \textit{in vitro} and \textit{ex vivo} studies (Koizumi 2003, Kang 1998).

Substantial evidence reveals that astrocytes may use Ca2+ transients or waves as a signaling mechanism for release of various glia-derived molecules (Santello et al., 2012; Savtchouk & Volterra, 2018; Bazargani & Attwell, 2016). Furthermore, studies show that dopamine plays a role in modulation of Ca2+ signaling in astrocytes. Specifically, acute application of dopamine or dopamine receptor agonists, such as SKF83959, to astrocytes in culture has generally been found to induce elevations in cytosolic Ca2+ via an IP3-mediated release from intracellular stores (Vaarmann et al., 2010; Parpura & Haydon, 2000; Lee et al., 2004; Liu et al., 2009; Jennings & Rusakov, 2016; Jennings et al., 2017).

Moreover, investigation of astrocyte calcium dynamics have revealed autonomous functional domains in this cell population. That is, astrocytic calcium responses have traditionally been observed in the cell soma; yet, recent evidence shows that calcium responses can be generated in astrocytic processes that do not propagate to the soma. Importantly, while astrocytes in general are known to express neurotransmitters, studies have found that perisynaptic astrocytic processes (PAPs) also contain neurotransmitter receptors (Algulhon et al., 2008). Activation of these receptors evokes dynamic Ca2+ transients in astrocytes, which can be seen from the cell somas to the most distal astrocytic processes (Shigetomi et al., 2013). Such functionally discrete domains allow for divergent functional consequences. Depending on the morphology of discrete functional domains, a single astrocyte could exert different effects on modulation of associated local or
distant synapses (Cvelo and Araque, 2015; Volterra et al., 2014; Shigetomi et al., 2013).

1.9 Dopaminergic signaling in astrocytes

Cultured astrocytes respond with an increase in cAMP upon application of dopamine, D1-like receptor agonist SKF38393, and apomorphine, a non-selective dopamine receptor agonist (Zanassi et al., 1999). These astrocytic responses to dopamine are region-specific with the largest increase in cAMP occurring in striatal astrocytes, followed by cortical and cerebellar Bergmann astrocytes. Further, striatal astrocytes are found to express both dopamine D1 and D2 receptor mRNA and DRD1 and DRD2 proteins (Hansson et al., 1988; Bal et al., 1994; Zanassi et al., 1999; Vrana et al., 1995). Gene expression and protein level studies demonstrate evidence of dopamine receptors D1, D2, D3, D4, and D5 in striatal and basal ganglia astrocytes (Hansson et al., 1988; Bal et al., 1994; Zanassi et al., 1999; Miyazaki et al., 2004). These findings provide important physiological implications in indicating that astrocytes express functional dopamine receptors which activate intracellular downstream cascades similar to those in neurons.

Dopamine has been found to initiate large intracellular calcium increases in cultured hippocampal astrocytes (Parpura and Haydon, 2000). Another study performed in 2001 recognized a cortical population of dopamine D2 receptor expressing astrocytes in monkey using electron microscopy techniques (Khan et al., 2001). Further investigation by this group revealed dopamine D2 receptor-mediated increases in calcium transients in rat and mouse cortical astrocytes. A more recent study shows that knockout of astrocytic dopamine D2 receptors in
mice leads to disruption of αβ-crystallin production resulting in systemic inflammation and marked astrocytic reactivity (Shao et al., 2013). In another study modeling Parkinson’s Disease, interruption of dopaminergic transmission via lesioning resulted in a global increase in the astrocytic calcium dynamics (frequency, amplitude, and duration), as well as an increase in signaling synchrony and gap junction coupling within the substantia nigra reticulata (SNr) network (Bosson et al., 2015).

Interestingly, some studies indicate that astrocytes can store and release dopamine. Astrocytes have been shown to express dopamine transporters in glioma cells (Russ et al., 1996), in cortical cell cultures (Inazu et al., 1999) and in striatal tissue (Kittel-Schneider et al., 2012; Inazu et al., 2003). The norepinephrine transporter has also been identified in cultured cortical astrocytes and may be responsible for dopamine uptake in these cells (Takeda et al., 2002).

Overall, the literature is explicit in showing that astrocytic morphology may change in response to environmental stimuli, that astrocytes can express dopamine receptors, and that dopamine can induce changes in astrocytic signaling in vitro. However, the consequences of chronically elevated dopamine levels on astrocytes, as might be observed following exposure to drugs of abuse, are not clear. Such research would have major implications for understanding astrocyte function under conditions of elevated dopamine and for elucidating the physiological role of dopamine-related activity in astrocytic cell populations within the central nervous system.
1.10 Astrocyte-synapse relationships

Astrocyte structural plasticity

It is well known that neurons undergo experience-induced functional remodeling of synaptic elements. These functional alterations in synaptic transmission serve as the cellular definition of learning, and are associated with changes in dendritic spine morphology and density believed to be associated with changes in synaptic strength (Meletic-Salvativ et al., 1999; Toni et al., 1999; Wilbrect et al., 2010; Perez-Alvarez et al., 2014). The past decade has brought about increasing interest in understanding structural plasticity of astrocytes and how this structural plasticity allows for close interplay between/among astrocytes and neurons (Covelo & Araque, 2018). Astroglia cells in vivo are able to retract or extend their processes in a plastic manner to make contacts with local synapses, other astrocytic processes, or blood vessels (Oliet et al., 2001; Oberheim et al., 2012). Actin-type filaments known as lamellipodia and filopodia composing the astroglial processes exert a significant influence on synaptic architecture and physiology (Lavialle et al., 2011; Park et al., 2015; Araque et al., 2014). Neurotransmitter release by surrounding neurons may exert reciprocal regulatory action on astrocytes leading to spatial extension and plasticity of astrocytic processes. For example, oxytocin released during lactation can lead to changes in astrocyte morphology which can, in turn, blunt release of neurotransmitters (Oliet et al., 2001). In Parkinson’s Disease plastic changes in astrocyte morphology, such as upregulation of primary process numbers, increases in individual process thickness, and extension of processes have been observed to lead to an
upregulation of astroglial process-to-synapse wrapping and blanketing (Maragakis & Rothstein, 2006).

Astrocytes maintain close structural relationships with nearby synapses, physically engulfing or enwrapping synaptic terminals and dendritic spines with PAPs, an event which can be altered under various physiological conditions (Haber et al., 2006; Theodosis et al., 2008; Tanaka et al., 2013; Perez-Alvarex et al., 2014; Bernardinelli et al., 2014). In fact, investigation of astrocytic ensheathment of synapses via PAPs in various brain regions has revealed important functional implications for astrocytic control of excitatory neurotransmission (Bernardinelli et al., 2014; Lavialle et al., 2011). Specifically, activation of excitatory synapses through stimulation of metabotropic glutamate receptors briefly increases motility of PAPs which leads to stabilization of PAP-dendritic spine functional and structural interactions (Bernardinelli et al., 2014). One important actin binding protein localized to PAPs is ezrin, a protein linking the actin cytoskeleton directly to the plasma membrane (Derouiche & Frotscher, 2001; Haseleu et al., 2013). Active/open ezrin is exclusively found in PAPs and is necessary for mGluR3 and mGluR5-mediated motility of astrocytic filopodia (Lavialle et al., 2011). Specifically, the proximity of PAPs to the synaptic cleft regulates the expression and functional efficiency of astrocytic transporters, such as GLT-1 and GLAST, and thus regulates the synaptic environment to modulate synaptic transmission (Bernardinelli et al., 2014; Lavialle et al., 2011).

Another prominent actin regulator believed to play a role in astrocyte morphology and PAP formation and motility is gap junction protein connexin 30
In the rodent brain, connexin 30 is selectively expressed in protoplasmic astrocytes and certain pericytes within the brain (Nagy et al., 1999; Zhang et al., 2014; Mazare et al., 2018). *In vitro* studies show that connexin 30 (Gjb6) determines the orientation of astroglial motile protrusions through modulation of the laminin/β1 integrin/ Cdc42 polarity pathway (Ghezali et al., 2018). *In vivo*, ablation of connexin 30 (Gjb6) results in increased ramification and process length, allowing processes and PAPs to enter the synaptic cleft and cause elevated uptake of glutamate at excitatory synapses (Pannasch et al., 2014). While the molecular underpinnings of connexin 30-mediated regulation of PAPs are still largely unknown, evidence reveals involvement of connexin 30 intracellular C-terminus acting as a hub for interplay with various actin regulators (Pannasch et al., 2014).

In summary, cellular architectural remodeling, including extension or ramification of astrocyte primary processes and perisynaptic processes (PAPs), influences the expression and localization of glutamate transporters (GLT-1 and GLAST) and gap-junction contacts, connexin 43 (CX43) and connexin 30 (CX30), which serve as crucial regulators of cell signaling and metabolic homeostasis among cells in various brain regions. Thus, the nature of how astrocytes interact or contact synapses to form specialized structural relationships in different brain regions may have an influence on synaptic function (e.g., strength, plasticity, efficiency) which underlies changes in behavior.
Astrocyte functional plasticity at the tripartite synapse

Defined by its functional architecture, and termed the ‘tripartite synapse’, the relationship between astrocytes and excitatory pre- and postsynaptic neuronal synapses provides a strong argument for the regulation of synaptic transmission by astroglial interactions (Figure 1.4; Covelo & Araque 2015, Haydon 2009). Neuro-glial signaling at the tripartite synapse is reciprocal and supports the concept of astrocytes acting as integral functional components of the synapses they are locally associated with. Such association allows for input-specific regulation of synaptic transmission and plasticity by astrocytes that may serve as a feedback mechanism (Covelo and Araque, 2015) referred to as lateral astrocyte synaptic regulation (Rouach et al., 2008; Covelo & Araque 2015).

Glutamate signaling may represent a metabolic correlate of lateral astrocyte control of synaptic transmission (Covelo and Araque, 2015; Rouach et al., 2008; Fellin 2004). Specifically, astrocytes have been credited for their role in recycling glutamate, which exerts an important influence on excitatory and inhibitory synaptic transmission in regions such as the hippocampus, PFC, and NAc (Fellin et al., 2004; Fellin et al., 2007; Haydon et al., 2009; Araque 2014; Kang 1998). Glutamate release from astrocytes occurs through various mechanisms: reverse action of glutamate transporter (GLT-1), unpaired gap junction pores on cell surfaces (hemicannels), swelling-induced opening of anion channels, ionotropic purinergic receptors, Ca2+-dependent vesicular release, and glutamate exchange via the cysteine-glutamate antiporter (Scofield et al., 2016; Malarkey & Parpura, 2008). Astrocytes spontaneously release glutamate (Glut) as a gliotransmitter and
astrocytic Ca2+ has been linked to activation of extrasynaptic NMDARs within the hippocampus (Dong et al., 2013).

Emerging studies suggest these processes may involve other neurotransmitter pathways. For example, endocannabinoids released from pyramidal neurons in the hippocampus can activate astrocyte-bound CB1 receptors leading to a PLC-dependent elevation of astrocytic calcium levels (Navarette and Araque, 2010). This mobilization of calcium stores stimulates release of astroglial glutamate which activates NMDA receptors in pyramidal neurons (Navarette and Araque, 2010). In line with this, work on hippocampal neuronal and astrocytic cultures also reported that calcium uncaging in astrocytes as well as mechanical and/or electrical stimulation trigger glutamate gliotransmission that evokes slow inward excitatory currents in adjacent neurons (Fellin et al., 2004, Haydon et al., 2009, Navarette and Araque 2010, Perea and Araque, 2005). Furthermore, the threshold for inducing NMDAR-dependent long-term potentiation (LTP) within the hippocampus is lowered when astrocytic glutamate release accompanies LTP induction, suggesting that astrocytic glutamate can modulate synaptic plasticity (Park et al., 2015).

Synaptic regulation by astrocytes is not necessarily confined to local active synapses. Considering the morphological and functional characteristics of astrocytes, such as long-range propagation of the astrocyte calcium signal (Perea and Araque, 2005; Di Castro et al., 2011; Panatier et al., 2011), the intercellular coupling via gap-junctions, and the widespread branching of processes that permit a single astrocyte to make contact with a multitude of synapses (Bushong et al.,
(2002; Halassa et al., 2007; Lavialle et al., 2011), more spatially extensive regulatory processes can be conceived. A recent observation was made showing that genetic suppression of astrocytic IP3-dependent calcium signaling diminishes astrocytic coverage of hippocampal synapses (Tanaka et al., 2013). Importantly, the frequency of synaptic activity determines the capability of Ca2+ signals to exert feedback regulatory mechanisms at local active synapses or apply feedforward mechanisms at distant synapses (Haydon et al., 2009; Volterra et al., 2014; Araque et al., 2014; Perea et al., 2014). Thus, under some circumstances, gliotransmission can act in a highly localized fashion, while other conditions may allow them to act at more distant sites (Araque et al., 2014; Perea et al., 2014).

Two major astrocyte-secreted factors known to contribute to formation and specialization of synapses are Hevin (SPACL1) and SPARC. Astrocyte-secreted SPARC is stimulated by increased glutamate levels which works to reduce the expression level of synaptic AMPA receptors post-synaptically and promote specialization of silent synapses (Jones et al., 2011; Kucukdereli et al., 2011; Allen & Eroglu, 2017). In fact, astrocyte-secreted SPARC and Hevin serve opposite regulatory roles in synaptic regulation. Treatment of cultured astrocytes with Hevin triggers re-organization of excitatory synapses in vitro and in vivo. In contrast, SPARC antagonizes Hevin to prevent synaptogenesis (Singh et al., 2016; Jones et al., 2011; Kucukdereli et al., 2011; Allen & Eroglu, 2017). In adults, Hevin expression remains high, while SPARC expression is significantly downregulated (Morel et al., 2017). The opposing roles of astrocyte-secreted Hevin and SPARC allow for a homeostatic mechanism to control synaptic excitability, by which the
astrocyte-neuronal environment regulates synaptic excitability via astrocyte-mediated processes.

1.11 Alterations in astrocyte-synapse relationships: implications for substance use disorders

Chronic exposure to drugs of abuse, including cocaine, has been shown to lead to reactive astrocytosis (Bowers and Kalivas, 2003; Fattore et al., 2002). Activated astrocytes are characterized by changes in morphology including cell hypertrophy, increases in individual process thickness, and extension of processes that is often associated with an expansion of astrocytic coverage of synapses (Bowers and Kalivas, 2003; Fattore et al., 2002; Maragakis & Rothstein, 2006; Narita et al., 2006). For example, following 1 and 7 days of repeated cocaine administration, astrocytes in the mouse dentate gyrus presented marked reactivity including process thickening and cell body hypertrophy (Fattore et al., 2002). Such changes in astrocyte morphology following exposure to drugs of abuse are likely accompanied by changes in astrocytic function. For example, exposure to cocaine can lead to a reduction in astrocyte morphometric properties which is accompanied by decrease in extracellular glutamate levels due to impaired activity of astrocytic cysteine-glutamate and GLT-1 transporters (Baker et al., 2003; Uys and Reissner, 2011; Reissner et al., 2015; Moussawi et al., 2009).

Glutamate gliotransmission has received increasing attention as it has been associated with the regulation of excitatory synaptic transmission and plasticity in the striatum and other regions in animal models of substance use disorder (Scofield et al., 2016; Reeves et al., 2011; Santello et al., 2012; Savtchouk &
Glutamate transporters are responsible for reuptake of extracellular glutamate to regulate glutamate concentrations at and around the synapse. Disruption or insult to these transporters results in glutamate ‘spillover’ from the synapse leading to aberrant excitatory synaptic signaling (Reissner et al., 2015; Moussawi et al., 2009). Interestingly, astrocytic glutamate transporters, GLT-1 and cysteine-glutamate antiporter, have been suggested as potential therapeutic targets for methamphetamine and cocaine-related neuropathology and neurotoxicity (Lau et al., 2010; Reissner et al., 2015; Moussawi et al., 2009). Specifically, studies illustrate diminished levels and activity of astrocytic glutamate transporters, GLT-1 and cysteine-glutamate system, in the NAc of animals in withdrawal phase following cocaine self-administration (Reissner et al., 2015; Moussawi et al., 2009). Moreover, the drug exposure and withdrawal-induced insults to astrocytic glutamate transporters were associated with higher susceptibility to reinstatement of drug. Interestingly, via treatment with the antioxidant, N-acetyl-cysteine, investigators were able to rescue astrocytic glutamate reuptake systems to regulate excitatory synaptic transmission, as well as prevent reinstatement of drug (Reissner et al., 2015; Moussawi et al., 2009). Considering the critical role of glutamate in learning and drug-related behaviors associated with addiction (Kalivas, 2009; Kalivas and Volkow, 2011; Turner et al., 2013), neurophysiological consequences of drugs of abuse on astrocytes represent an important research venue. Identification and characterization of structural changes that astrocytes undergo in response to drugs of abuse are a critical step in understanding such consequences.
Evidence for bidirectional signal transmission from astrocytes to neurons via gliotransmission reveals a crucial role for astrocytes in their ability to actively modulate synaptic short- and long-term plasticity (Theodosis et al., 2008; Allen et al., 2012; Allen, 2014; Hahn et al., 2015; Savtchouk & Volterra, 2018); however, the responsiveness of individual synapses to alterations in their microenvironment as well as their vulnerability under pharmacological conditions such as prolonged cocaine exposure require further investigation.

In disease states, such as substance use disorders, these functional and structural relationships between astrocytes and neurons are altered to contribute to the progression of addictive-like behaviors. For example, cue-induced reinstatement of extinguished cocaine seeking behavior can be inhibited by mGluR-mediated (mGluR2 and mGluR3) release of glutamate from astrocytes (Scofield et al., 2015). Cocaine self-administration experience is also characterized by altered NMDA receptor-dependent synaptic plasticity in the NAc. In fact, it has been shown that cocaine self-administration in rats increases activation of extrasynaptic NMDA receptors (Ortinski et al., 2013) that may influence neuronal excitability in this region. Moreover, it has also been revealed that cocaine-induced deficits in NMDAR-dependent long-term potentiation (LTP) and depression result partially from reduced release of D-serine from astrocytes (Curcio et al., 2013). Administration of D-serine directly into the NAcC in vivo was able to inhibit behavioral sensitization to cocaine (Curcio et al., 2013). Thus, glutamate and D-serine appear to represent two major chemical transmitters in regulation of cocaine sensitization in the ventral striatum.
1.12 Conclusion

Astrocytes are prevalent throughout the CNS, acting as critical regulators of overall brain function. Numerous studies over the past two decades collectively reveal close structural and functional relationships between astrocytes and neurons. This revelation in recognizing astrocytes as active and dynamic participants in cell signaling within the brain challenges the idea that experience-dependent changes in neurons define the cellular mechanism of learning and elevates the idea that astrocytic processes serve crucial roles in plasticity and learning.

Serving as an integral part of the synapse, astrocytes regulate synaptic function and overall brain homeostasis via re-uptake and release of a variety of ions, neurotransmitters & neuromodulators, as well as metabolic, trophic, and plastic molecules (Baldwin & Eroglu, 2017; Verkhratsky et al., 2016; Chung et al., 2015). Specifically, astrocytes are able to modulate neuronal activity and synaptic transmission through release of certain chemical transmitters, such as glutamate and ATP (Haydon et al., 2009; Araque et al., 2014; Koizumi et al., 2003; Fellin et al., 2004; Fellin et al., 2007; Panatier et al., 2006). Moreover, astrocytes express a variety of both metabotropic and ionotropic neurotransmitter receptors, and thus are sensitive to neurotransmitter action in vitro and in vivo. Thus, these chemical transmitters provide critical mechanisms by which astrocytes and neurons are structurally and functionally regulated in both physiological and disease states.

With increasing awareness of the dynamic roles astrocytes play in regulating neuronal signaling, considerable effort is being placed on investigating
the potential roles of astrocytes in the regulation of disease states such as drug addiction. Drug-induced changes in astrocytic profiles are likely to contribute to the emergence and maintenance of addictive states, as many studies have reported morphological, functional, and molecular alterations in astrocytes as a result of drug experience. During the development of drug addiction, drug behavior evolves from voluntary drug-seeking to compulsive drug-seeking behavior, a behavioral change associated with a transfer of control over drug-seeking behavior from the ventral to the dorsal striatum. This ventral-dorsal striatal shift in control over drug seeking can be seen as increased DA efflux within the dorsolateral striatum, but not within the ventral striatum. While several studies have examined the effects of cocaine self-administration and withdrawal on astrocyte morphometric and functional properties, the impact of cocaine-induced dopamine fluctuations on astrocyte plasticity within ventral and dorsal striatum is unknown.
Figure 1.1. DAR intracellular signaling. Ligand binding to D1-like receptors stimulates Gαs/olf which dissociate to Gβγ and Gαolf/s-GTP. Gαolf/s-GTP then stimulates adenylyl cyclase (AC) which increases cyclic AMP (cAMP). In turn, cAMP activates PKA which goes on to phosphorylate various targets. D2-like receptors inhibit AC through activation of Gαi/o. Activation of D2-like receptors inhibits the AC/cAMP/PKA signaling pathway. Activation of D2-like receptors also results in Gβγ-mediated activation of PLC resulting in upregulated IP3 production. IP3 promotes release of ER Ca2+, and the subsequent inactivation of L-type Ca2+ channels and Na+ channels. DRD1s and DRD2s can oligomerize to form heteroreceptors in certain populations of cells. Ligand binding to D1/D2 heteromers causes a Gαq-mediated increase in intracellular Ca2+ in a PLC-dependent manner. Note. Figure reprinted from “Current concepts on the pathophysiological relevance of dopaminergic receptors,” by A. Ledonne, and N. B. Mercuri, 2017, Front Cell Neurosci, 11, p. 27.
**Figure 1.2. Reward pathways of the CNS.** The reward system or dopaminergic system begins in the VTA, which sends dopaminergic projections to mesocorticolimbic structures, including the NAc, PFC, and dorsal striatum. The NAc or ventral striatum receives glutamatergic inputs from PFC, Hipp, thalamus, amygdala, and VTA, and sends GABAergic projections to regions such as the VP, VTA, and lateral hypothalamus. *Note.* Figure reprinted from “Intrinsic plasticity: an emerging player in addiction,” by S. Kourrich, D. Calu, and A. Bonci, 2015, *Nature Rev Neurosci*, 16(3), p. 173 - 184. Copyright (2015), with permission to reprint from Springer Nature.
Figure 1.3. Direct & indirect pathways for movement. Direct pathway MSNs (dMSNs) express D1-like receptors. Activation of dMSNs promotes motivated behavior. Indirect pathway MSNs (iMSNs) express D2-like receptors. Activation of dMSNs inhibits motivated behavior or unwanted movement. * Nigrostriatal dopamine is particularly relevant during stereotyped (repetitive) motor behavior prominent with high doses of psychostimulants.
Figure 1.4. The tripartite synapse. Schematic showing a presynaptic (Pre) and postsynaptic (Post) terminal enwrapped by the astrocytic process (green) forming the tripartite synapse. High structural interplay of astrocytic process(es) with presynaptic and postsynaptic terminals exerts crucial roles including clearing of neuronal activity-induced accumulation of K+ ions and uptake of glutamate by glutamate transporters (i.e., \( X_c \), GLT-1, GLAST). Neurotransmitter release from presynaptic terminals can activate astrocytic mGluRs, which induce IP3-dependent release of Ca2+ from internal stores, which in turn triggers the release of several gliotransmitters. Schematic adapted from Halassa et al., 2007.
CHAPTER 2

METHODS

2.1 *In vitro* model of elevated extracellular dopamine

Primary astrocytes were dissociated from hippocampi of neonatal (postnatal day 1-2) Sprague-Dawley rat pups. The heads were removed and hippocampi dissected in Petri dishes filled with ice-cold dissection medium composed of: Hank’s Balanced Salt Solution (without Ca$^{2+}$, Mg$^{2+}$, NaHCO$_3$, or phenol red) supplemented with 10 mM HEPES (both from Sigma). The dissected hippocampi were finely chopped and dissociated by trypsin treatment (10 µl/mL; Gibco), followed by trituration with sterile glass pipettes. Cells were plated on 12 mm glass coverslips, 6-well, or 24-well plates coated with poly-l-lysine (50 µg/mL; Sigma). Cultures were maintained in DMEM/F12 (Gibco) media supplemented with 10% fetal bovine serum (FBS) and stored in an incubator at 37ºC (5% O$_2$/95% CO$_2$) for 10-14 days (DIV 10-14). This protocol produces astrocyte-enriched cultures with majority of all cells staining positive for the astrocyte marker, glial fibrillary acidic protein (GFAP). Cultured cell groups were subsequently selected and treated with dopamine (0.1, 1, 10, & 75 µM) or vehicle before being harvested for immunocytochemistry or qRT-PCR.

2.2 Intravenous catheter implantation for cocaine self-administration

Under isoflurane anesthesia (2-5\% isoflurane in O\textsubscript{2}) an indwelling silastic catheter (SAI infusion technologies, Lake Villa, IL, USA) was placed into the right jugular vein and sutured in place. The catheter was routed to a mesh backmount platform that was implanted subcutaneously dorsal to the shoulder blades. Catheters were flushed daily with 0.3 ml of antibiotic (Timentin, 0.93 mg/ml) dissolved in heparinized saline and sealed with plastic obturators when not in use. After surgery, rats were allowed 7 days to recover before behavioral testing commenced.

*Animals and housing*

Male Sprague-Dawley rats (Rattus norvegicus) weighing 250–275 g were obtained from Taconic Laboratories. Animals were individually housed and kept on a 12/12 HR light/dark cycle with the lights on at 7:00 a.m. All experimental procedures were performed during the light cycle. During the weeks of cocaine self-administration, rats were limited to 30 g of food per day (and water *ad libitum*) to minimize the effect of motivational differences due to natural variations in food intake and weight gain across rats. The experimental protocols were all consistent with the guidelines issued by the U.S. National Institutes of Health and were approved by the University of South Carolina School of Medicine’s Institutional Animal Care and Use Committee. Food and water were available ad libitum in their home cages.
2.3 Animal model of elevated extracellular dopamine signaling

The study utilizes a rat behavioral regime of cocaine self-administration under a short- or extended-access protocol (based on Wilhuhn et al., 2012) to determine the impact of cocaine duration on astrocytes in the dorsal and ventral striatum. Specifically, this project aimed to observe sub-region and experience-dependent changes in astrocytes associated with physiological shifts in dopaminergic signaling in the NAcC and dorsolateral striatum of cocaine-experienced animals.

Operant Conditioning for Cocaine Self-Administration

Each rat trained to respond for contingent cocaine infusions was paired with a yoked subject that received infusions of saline (Figure 2.1). Lever pressing for the saline-yoked rats had no scheduled consequences, but these animals received the same number and temporal pattern of infusions as self-administered by the paired cocaine-experimental rat. Cocaine-experimental rats were allowed to self-administer cocaine for 7 (short-access) or 21 days (extended access). Rats were allowed to self-administer a maximum of 40 injections per 60 minutes/daily operant session. Each operant session began with the intravenous administration of 59 μl cocaine (0.25 mg) to fill the catheter. Rats were placed in operant chambers and allowed to lever-press for intravenous infusions of cocaine (0.25 mg cocaine/59 μl saline, infused over a 5 s period) on a fixed-ratio 1 (FR1) schedule of reinforcement during which one lever press (with light cue present) results in a single infusion of cocaine. A 20 s time-out period followed each cocaine infusion, during which time active lever responses were recorded, but had no scheduled consequences.
Responses made on the inactive lever, which had no scheduled consequences, were also recorded during training sessions. Rats were decapitated following isoflurane anesthesia the day after the last drug operant session.

2.4 Immunocytochemistry and immunohistochemistry

Immunocytochemical and immunohistochemical techniques allow for the visualization of proteins, peptides and enzymes of interest using the basic properties of antibody/antigen binding interactions. Immunocytochemistry was used to assess dopamine effects on astrocyte morphology in vitro and immunohistochemistry was utilized to assess dopamine-associated alterations in astrocytes in vivo within the striatum using the following protocols:

Immunocytochemistry

Following 10-12 DIV, primary cultured cells plated on sterile glass 12mm coverslips were washed twice in PBS at room temperature (pH 7.4, RT), fixed in 4% paraformaldehyde (PFA; 10 – 20 minutes at 37°C; Affymetrix), and then washed with 1XPBS three times. The cells were next treated with 0.3% Triton-X (Sigma) for 15 minutes and washed once with 1XPBS. Nonspecific staining was blocked with PBS containing 0.1% tween 20 (Thermo-Fisher) and 10% donkey or goat serum (Sigma) for 1 – 2 hours at room temperature (RT). Coverslips were incubated with primary (mouse, polyclonal) antibodies against GFAP (1:1000; 2 HR, RT; Millipore Sigma; MAB360), and then washed with 1XPBS three times. For characterization of culture cell type content, coverslips were incubated with primary antibodies against Olig1, CD11b, or NeuN and GFAP (Refer to Table 2.2) before being washed in 1X PBS three times. Fluorescent staining was then carried out by
incubating with secondary antibodies (1:1000; Life Technologies) conjugated to AlexaFluor 488 (A11008) or AlexaFluor594 (A21203) at RT in the dark for 1 HR. Cells were then washed three times with 1XPBS and counter-stained with DAPI (5 µg/mL; Molecular Probes) for 5 minutes. Finally, coverslips were rinsed once with distilled H2O and mounted on glass slides with Prolong Antifade mounting medium (Life Technologies). Negative controls omitting primary or secondary antibodies were included in all experiments. All washes were carried out with 1XPBS (pH 7.4) at RT.

**Immunohistochemistry**

Whole brains were removed and fixed for 48hrs in 1XPBS (0.1 M) containing 4% paraformaldehyde at 4°C prior to being transferred to a 1XPBS containing 30% sucrose and stored at 4°C. When brains were sufficiently equilibrated, striatal regions were coronally sectioned at 35 µm thickness using a Vibratome (Vibratome information). Free-floating sections were collected in cold (4°C) 1XPBS for immunohistochemical processing.

Free floating tissue sections were transferred to fresh 1XPBS (RT) and rinsed with 1XPBS three times. Sections were next treated with 1XPBS (Life Technologies) containing 0.3% Triton-X (Sigma) for 20 minutes and washed once with 1XPBS. Nonspecific staining was blocked with 1XPBS containing 0.1% tween 20 (Thermo-Fisher) and 10% donkey or goat serum (Sigma) for 1 – 2 hours at room temperature (RT). Sections were incubated with a 1XPBS solution containing 2% goat serum (Sigma), .1% Triton-X (Sigma), and primary (rabbit, polyclonal) antibody against GFAP (1:500; 48 HR at 4°C; Abcam; ab7260), and then washed
with 1XPBS four times. Fluorescent staining was then carried out by incubating with secondary antibodies (1:1000; Life Technologies) conjugated to AlexaFluor647 (A21245) at RT in the dark for 2 HR. Sections were then rinsed four times with 1XPBS and counter-stained with DAPI (5 µg/mL; Molecular Probes) for 5-10 minutes. Finally, sections were rinsed once with 1X PBS and mounted on glass slides with Prolong Antifade mounting medium (Life Technologies). Negative controls omitting primary or secondary antibodies were included in all experiments. All washes were carried out with 1XPBS (pH 7.4) at RT.

2.5 Immunofluorescent imaging and analysis: density and morpho

Analyses of cultured astrocytes

Cells were examined under a widefield fluorescent microscope (Eclipse E200, Nikon). To determine the effect of exogenous dopamine on astrocyte morphology, morphometric analyses were conducted with NIH ImageJ software. (Figure 2.2a) For cell area measurements, GFAP-positive cells were manually outlined and isolated from background by applying a color threshold. For analysis of stellation, all distinct primary processes extending directly from the cell body, thinner than 40 pixels wide, and at least 30 pixels in length were counted (Figure 2.2b).

Analyses of astrocytes in slice

For slice imaging, mounted sections of rat striatum stained for GFAP (Alexa Fluor 647) were imaged using a Leica SP8 Multiphoton confocal microscope (Germany). Image stacks (Δz, .35 µm) of striatal sections were collected using a 63x oil objective with 638 nm diode and MP lasers. To evaluate the effect of
cocaine self-administration on astrocyte density and morphology, cell count and morphometric analyses were carried out using Leica SP8 3D analysis software. For density measures, individual z-stacks were rendered in 3D and GFAP+ cells depicting whole astrocytes were manually selected, counted, and reported as number of GFAP+ cells/field. For individual cell morphometric analysis, GFAP+ cells were manually selected, individually thresholded, and then isolated for measurements of cell surface area and volume and manual measurement of primary process length & count (Figure 2.3a). For analysis of stellation, all distinct primary process extending directly from the cell body were counted (Figure 2.3b,c). All objects on the edge of image field were occluded.

2.6 Western blot procedure and analysis of primary cultured astrocytes

Cultured cells were gently washed in 1XPBS before being gently scraped and collected. Cells were homogenized in 200 μL of ice-cold extraction buffer containing 50 mM Tris, 1 mM EGTA, 1 mM EDTA, 1% SDS, and 1 mM PMSF (pH 7.4). Protein concentrations were determined using a BCA assay, with bovine serum albumin as the standard. Prior to loading, 6x SDS Sample buffer (50 mM Tris, 2.5% SDS, 36% glycerol, 0.03% bromophenol blue and 1M DTT) was added to each sample, which were then boiled for 5 minutes. Equivalent amounts of protein (30 μg) for each sample were resolved in 10% SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose membranes. Membranes were incubated with LI-COR blocking buffer (LI-COR, Lincoln, NE) for 1 HR at room temperature to block non-specific binding. The blots were reacted overnight at 4°C with primary antibodies Acetyl-α-tubulin (1:1000, Lys40; Cell
Signaling Technology, Lake Placid, NY) rabbit mAb and α-tubulin (1:1000, acetyl K40; Abcam, Cambridge, MA) mouse mAB or, Acetylated-Lysine (1:1000, Cell Signaling Technology, Lake Placid, NY) rabbit pAB and alpha-tubulin (1:2000, Abcam, Cambridge, MA) mouse mAB. After washing in PBS-T, the blots were incubated in fluorescent secondary antibodies (1:20,000, LI-COR, Lincoln, NE) in LI-COR blocking buffer for 1 HR at RT. Membranes were then washed three times with PBS-T. Immunolabeling detection and densitometry measurements were performed using the LI-COR Odyssey System (LI-COR, Lincoln, NE). The blots were stripped using LI-COR Newblot stripping buffer (5x) (LI-COR, Lincoln, NE), washed, and re-probed with antibodies for acetylated alpha tubulin or acetylated lysine (1:1000, Cell Signaling Technology, Lake Placid, NY) and the reference antibody (β-tubulin). Blots were re-scanned following stripping to ensure complete removal of signal. Ratios of acetylated alpha tubulin or total acetylated lysine to β-tubulin densities were calculated for each sample and analyzed across conditions.

2.7 Genetic characterization: RNA sequencing and RT-QPCR

RNA Extraction / Sequencing

Total RNA was extracted using TRIzol (Life Technologies) and a RNeasy Mini Kit (Qiagen) according to manufacturers’ instructions. Samples were homogenized and incubated in TRIzol for 5 minutes before addition of chloroform and brief vigorous shaking for 30 seconds. Following a 3 minute incubation, samples were centrifuged at 4°C for 10 minutes at maximum speed (≥10,000rpm). The aqueous phase was aspirated and transferred to a microcentrifuge tube before addition of 70% EtOH, centrifugation at ≥10,000 rpm for 15 seconds and collection.
of the precipitate from the RNeasy mini column. This step was repeated after adding 700 µL Buffer RW1 and next 500 µL Buffer RPE to the mini column. Another 500 µL of Buffer RPE was centrifuged for 2 minutes before the sample/mini column underwent a 2 minute “dry” spin and transferred to the final collection tube. Last, 30 µL DEPC water was used to elute the sample. RNA samples were quantified using NanoDrop Spectrophotometer ND-2000 (Nanodrop Technologies) and checked for quality and degradation by Agilent 2100 Bioanalyzer. All samples were of high quality (RNA integrity number (RIN) between 9.9 and 10). Strand-specific mRNA libraries were prepared using the TruSeq Stranded mRNA Library Prep Kit (Set B, Illumina Inc.) and sequenced on the Illumina NextSeq500 in a paired-end mode with read length of 2 x 75bp.

**Sequencing data preprocessing and analyses**

To ensure there were no sequencing errors, raw sequences were checked for quality using FastQC, and then aligned to the rat genome (downloaded from iGenomes, Illumina) using the STAR aligner program (Dobin et al., 2013). Aligned SAM files from STAR were converted to BAM files using SAMtools (Li et al., 2009). BAM files were processed for read summarization using feature counts (Liao et al., 2014), and the resulting read counts were preprocessed by filtering out low read counts (read counts < 5) in R software. Processed data were then analyzed for differential expression using DESeq2 (Love et al., 2014) in R software. False discovery rate (FDR < 0.05) was used to determine the threshold of p-value for the analysis.
Functional annotation/gene ontology analyses for biological function were conducted using Panther classification system (www.pantherdb.org) accessed in September-October, 2017. CTCF binding motif analysis was performed using CTCFBSDB 2.0 (Ziebarth et al., 2013), a database for CTCF binding sites and genome organization. Top 30 differentially expressed genes (15 downregulated and 15 upregulated) from each of the treatment groups were extracted, and then matched with the downloaded CTCF binding motif database. This analysis was completed in R software.

**RT-qPCR**

Oligonucleotide primers were designed using the NCBI Primer-Designing tool, checked for specificity via the BLAST database, and supplied by Eurofins Genomics (Table 2.3). The following primers were used in our *in vitro* experiments: FosB, PDE10A, AP1S3, GPR83, Nr4a3, Klf4. For each primer tested, 1000 µL of primer mix contained: 200 µL of 100 µM forward primer, 200 µL of 100 µM reverse primer, and 600 µL of DEPC H₂O. Samples were diluted to 500 ng/µL with DEPC water before undergoing cDNA synthesis. For each sample, 10 µL of 2X SYBR Green Mix (Applied Biosystems, ThermoFisherScientific), 4 µL of DEPC H₂O, and 1 µl of cDNA sample was prepared and subsequently mixed with 5 µL of primer mix for a total volume of 20 µl before pipetting into a 385-well plate. Real-time PCR was carried out using Bio-Rad CFX384 Real-Time PCR Detection System. For all genes, the cycling parameters were as follows: initial denaturation at 95 °C for 3 minutes, 40 cycles of 95 °C for 20 s, and final cycle of 60 °C for 20 s. Transcript measurements in each sample were done in triplicates. The mRNA levels were
determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and target genes were normalized to the housekeeping genes Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH) or Hypoxanthine Phosphoribosyltransferase (HPRT). Expression values were normalized to GAPDH and reported as a fold change.

2.8 Calcium imaging of cultured astrocytes

Astrocyte cultures were incubated with 5 μL of GFAP-driven GCamp6f virus (AAV5.GfaABC1D.cytoGCamp6f.SV50, Addgene #52925, packaged by the University of Pennsylvania Vector Core) for 3 – 5 days prior to imaging at DIV 10 – 14. Coverslips were transferred to a recording chamber continuously perfused (2 – 3 ml/minute) with recording solution composed of (in mM): 145 NaCl, 5 KCl, 1 MgCl$_2$, 1 CaCl$_2$, 5 HEPES, 5 glucose, and 15 sucrose (all from Sigma) adjusted to pH 7.4 with NaOH. Two-minute videos were acquired with ORCA-Flash 4.0 (V2) digital camera mounted on an Olympus BX51WI upright microscope equipped with an LED light source (X-Cite XLED1, Excelitas Technologies). Videos were binned at 1024 x 1024 pixels and collected at 25 frames/second. Background subtraction and fluorescent signal measurements were done using ImageJ based on manual isolation of individual cells as regions of interests (ROIs). Relative fluorescence intensity within each ROI was calculated as dF/F0, where F0 is average fluorescence intensity in a one second window of baseline fluorescence for each ROI identified in a background-subtracted image. Average amplitude and duration of Ca$^{2+}$ transients exceeding three standard deviations of baseline noise were calculated following manually curated identification of individual events in Clampfit 10.4 (Molecular Devices). Ca$^{2+}$ transient duration was expressed for each cell as
half-width – the time between two frames on either side of the peak that are first to reach 50% of peak amplitude relative to baseline. Event frequency for each cell was calculated as total number of identified events per two-minute video.

2.9 Electrophysiology of cultured astrocytes

Coverslip-plated astrocytes were viewed in a perfusion chamber under an upright microscope (Olympus BX51WI) with infrared differential interference contrast optics and a 40× water-immersion objective. Recording solution was the same as described for Ca\(^{2+}\) imaging. Recording pipettes were pulled from borosilicate glass capillaries (World Precision Instruments) to a resistance of 4–7 MΩ when filled with the intracellular solution. The intracellular solution contained the following (in mM): 145 potassium gluconate, 2 MgCl\(_2\), 2.5 KCl, 2.5 NaCl, 0.1 BAPTA, 10 HEPES, 2 Mg-ATP, and 0.5 GTP-Tris, adjusted to pH 7.2–7.3 with KOH, osmolarity 280–290 mOsm. Currents were low-pass filtered at 2 kHz and digitized at 20 kHz using Digidata 1440A acquisition board (Molecular Devices) and pClamp10 software (Molecular Devices). Potassium (K\(^+\)) currents were recorded in voltage-clamp mode (\(V_{\text{holding}}=-80\) mV). Access resistance (10–30 MΩ) was monitored throughout each recording by injection of 10 mV hyperpolarizing pulses; data were discarded if access resistance changed >25% over the course of data collection. Following seal rupture, series resistance was compensated (65-75%). Inward rectifying K\(^+\) currents were evoked by a series of 10 mV, 40 ms-long steps from -180 mV to -80 mV following a 200 ms pre-step to 0 mV. Outward K\(^+\) currents were evoked by a series of 10 mV, 500 ms-long voltage steps from -80 mV to +80 mV following a 100 ms pre-step to -110 mV or -50 mV. Outward delayed
rectifier K$^+$ currents were measured following pre-steps to -50 mV. Outward A-type K$^+$ currents were isolated by subtracting traces recorded following pre-steps to -50 mV from those recorded following pre-steps to -110 mV. Inward rectifying K$^+$ currents were measured at the end of the 40 ms step. Delayed rectifier and A-type K$^+$ currents were measured at current peak. All measurements were acquired using Clampfit 10 (Molecular Devices).

2.10 Statistical analysis

Statistical analyses were performed with Excel 2016 (Microsoft) or GraphPad Prism 6 (GraphPad Software). For immunocytochemical, Ca$^{2+}$ imaging, and electrophysiological experiments, cells from a minimum of 3 different coverslips were analyzed for each reported condition. For immunohistochemistry, fields from 3 different animals per experimental group were analyzed for density measures. For individual analyses of GFAP$^+$ cell morphometric properties, 3 cells were randomly selected from 3D-rendered z-stacks formerly used to obtain GFAP$^+$ density measures. One-way analysis of variance (ANOVA) with Bonferroni post hoc tests were used for morphological analyses of GFAP$^+$ cells in immunocytochemical and immunohistochemical experiments. Due to the low size (n = 2 fields, 3 cells), ordinary two-way ANOVAs were not utilized in ex vivo analyses of GFAP$^+$ density and morphology. Immunofluorescent morphological measurements for cultured cells were made using ImageJ Fiji and immunofluorescent density and morphological measurements for GFAP$^+$ cells in slice were obtained using Leica SP8 3D Analysis software. For RNA sequencing (RNA-seq), cells from 3-4 experimental replicates were processed for analysis as
described above. For qPCR validation of RNA-seq data, one-way ANOVAs were utilized for each gene. In the hippocampal and striatal slice experiments, planned comparisons predicted effects of dopamine and/or dopamine antagonists compared to control condition, thus Student’s t-tests were performed to determine significance. For qPCR assessment of mRNA expression in cocaine-experienced animals and yoked saline controls, 5-6 biological replicates were used for each of the 6 genes and unpaired two-tailed Student’s t-test were utilized to determine significance. For electrophysiological studies, mean peak K⁺ current amplitude was analyzed with two-way repeated measures ANOVAs with current amplitude as a within-subject factor and drug treatment as a between subject factor followed by Bonferroni post hoc tests. For western blot experiment, band intensity was measured and ratios of acetylated alpha tubulin or total acetylated lysine to β-tubulin densities were calculated for each sample and analyzed across conditions. Significance was determined using two-way ANOVA. One-way analysis of variance (ANOVA) or two-way ANOVA with Bonferroni’s multiple comparison tests, or unpaired two-tailed Student’s t-test were used as indicated in results or figure legends. Data were reported as mean ± standard error of the mean and significance threshold was set at p<0.05.
**Figure 2.1. Cocaine self-administration.** (A) Depiction of a rat connected with indwelling catheter (red arrow) for i.v. delivery of cocaine in the operant chamber. (B) An active lever press (blue arrow, dashed line) elicits an infusion of cocaine (0.5 mg/kg per infusion) over 5 s followed by a 20 s time-out (house light on top is off).
Figure 2.2. Analysis of astrocyte morphology *in vitro*. (A) Example of GFAP-positive cell area measurements. We first isolated cell to eliminate noise and cell interference and applied a color threshold to the isolated cell prior to selecting the measure function. Scale bars, 100 pixels, 50 pixels. (B) Example of process length measurement. Scale bar, 50 pixels.

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Figure 2.3. Analysis of astrocyte morphology ex vivo. (A) Example of striatal slice stained for GFAP (red). Scale bar, 15 µm. (B) Isolate cell (aqua) by applying threshold. Scale bar, 5 µm. (C) Example of manual process length measurement and process count (magenta). Scale bars, 5 µm.
### Table 2.1 Summary of antibodies used in immunocytochemical and immunohistochemical assays

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### Table 2.2 Summary of oligo primer sequences used in qRT-PCR

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CHAPTER 3
EFFECTS OF ELEVATED EXTRACELLULAR DOPAMINE EXPOSURE ON ASTROCYTES IN VITRO

3.1 Introduction

To date, there have not been any published studies which report on the morphological and/or phenotypical effects of dopamine in astrocytes. Yet, studies highlight dopamine potential in inducing functional alterations through astrocyte-bound dopamine receptors which may translate to changes in astrocytic morphological and phenotypical profile. My results provide evidence that exogenous application of dopamine can trigger morphological and phenotypic alterations in primary cultured hippocampal astrocytes which may have implications for astrocytic function.

Astrocytes are a major subpopulation of the brain glial cells that mediate multiple functions, including metabolic, ionic and pH homeostasis, trophic support of neurons, antioxidant defense, the establishment and maintenance of the blood brain barrier, and regulation of neuronal activity (Kandel et al., 2012; Oberheim et al., 2012; Fattore et al., 2002; Clark et al., 2013; Zamanian et al., 2012; Clark and Adeluyi, A. A.*, O’Donovan, B., Fisher, M. L., Rao, C. N…, & Ortinski, P. I. (2018). Dopamine triggers CTCF-dependent morphological and genomic remodeling of astrocytes. Journal of Neuroscience. Reprinted here with permission from publisher. * indicates equal contributions
Barres, 2013; Freeman et al., 2010; Bushong et al., 2002; Perea et al., 2009). Such functional diversity is accompanied by morphological heterogeneity that varies with development and across brain regions (Raff et al., 1983; Miller and Raff, 1984; Khakh and Sofroniew, 2015; Chai et al., 2017). For example, astrocytic domain boundaries are observed later, but not early in development (Bushong et al., 2004) and astrocytes in the striatum have significantly more processes than hippocampal astrocytes (Chai et al., 2017; Khakh and Sofroniew, 2015).

In cultured conditions, two astrocytic phenotypes have been distinguished based on morphological characteristics: type I cells have a flat polygonal appearance with a “mesh” of intertwined fine processes and resemble astrocytes populating grey matter; type II cells have clearly defined thicker “stellated” processes and resemble the astrocytes arranged along white matter tracts (Raff et al., 1983; Oberheim et al., 2012). Standard protocols that use serum-supplemented culture media result in predominantly polygonal, type I astrocytes. However, these astrocytes can be transformed into a stellated morphology by exposure to cAMP analogs (Won and Oh, 2000; Moonen et al., 1975; Paco et al., 2016). The cAMP-induced morphological transformation occurs within 2 hours and is associated with decreased cell body area and extension of distinct thick processes.

Dopamine may trigger cAMP production via activation of D1-like dopamine receptors, expressed by astrocytes along with other dopamine receptor subtypes (Miyazaki et al., 2004; Shao et al., 2013; Mladinov et al., 2010; Khan et al., 2001; Nagatomo et al. 2017). D1-induced production of cAMP has been linked to release
of astrocytic Ca2+ from internal stores, an important hallmark of astrocyte-to-astrocyte, astrocyte-to-neuron, and astrocyte-to-vasculature signaling (Jennings et al., 2017; Chai et al., 2017). Indeed, both structural and functional astrocyte adaptations have been reported in models of neurologic disease linked to dysregulated dopamine signaling. For example, Parkinson’s disease is associated with hypertrophy of astrocytic processes, a feature of reactive astrocytosis (Booth et al., 2017), while cocaine use has been linked to a reduction of astrocyte surface area and reduced interaction with synapses (Scofield et al., 2016). Furthermore, a number of studies have demonstrated that dopamine-induced increase in astrocytic Ca2+ impacts cellular respiration mechanisms coupled to PARP1 and sirtuin activation (Requardt et al., 2010; Requardt et al., 2012; Gupte et al., 2017; Verdin, 2015). PARP1 is a critical co-factor for NAD+-dependent poly-ADP-ribosylation of CCCTC binding factor (CTCF) which promotes CTCF binding to target genes (Yu et al., 2004; Ong et al., 2013; Han et al., 2017). Recent studies have shown that CTCF regulates both chromatin remodeling (Wright et al., 2016) and chromatin insulation (Yu et al., 2004, Ong et al., 2013), thereby organizing transcriptional regulation of distinct gene suites in response to cellular signaling. However, it is unknown whether dopamine- or dopamine receptor-induced structural or genomic changes are linked to PARP1/CTCF interaction.

This study demonstrates that dopamine receptor signaling generates pronounced morphological changes in cultured astrocytes which rely on PARP1 activation and are accompanied by a dynamic regulation of the astrocyte transcriptome including, most prominently, specific enrichment of genes containing
CTCF motifs in promoter regions. Our results highlight a novel PARP1/CTCF-dependent mechanism that drives dopamine-induced morphological changes and associated transcriptional profile in astrocytes.

3.2 Characterization of primary cultures

To characterize primary culture cell-type content, this study first utilized fluorescent immunocytochemical assays to assess microglial, neuronal, and astrocyte content in untreated cells. Thus, primary cultures were evaluated for contaminating cell types via immunocytochemical assays using antibodies against CD11B (microglia), NeuN (neurons), and GFAP (astrocytes), as well as a nuclear counterstain, DAPI (Figure 3.1). Notably, the neuronal presence is negligible in these cultures and the microglial presence is similar to that seen in previous studies (Saura, 2007). Therefore, culture conditions in this study result in GFAP-positive, astroglia enriched cell population.

3.3 Elevated dopamine leads to stellation of astrocyte processes in vitro

To examine effects of dopamine on astrocyte morphology, cultured cells underwent GFAP immunostaining following treatment with dopamine (75 μM) for 1, 24, and 48 hours. This revealed a rapid and persistent dopamine-induced stellation (Figure 3.2). Following 1 HR of exposure, dopamine induced a 3.2-fold increase in the number of primary processes and also significantly reduced mean GFAP-positive (GFAP+) cell area to 81±5.6% of control values (Figure 3.2). The increase in process number was maintained following 24 and 48 hours of treatment (253±23.8% and 266±16.8%, respectively). However, reduction in GFAP+ area recovered to control levels by the 48-hour timepoint. Astrocytes exposed to control
media for 1, 24, or 48 hours had similar stellation and GFAP\(^+\) area and were pooled together for analysis (**Figure 3.2**).

This project also examined astrocyte response to dopamine concentrations in the low nanomolar to low micromolar range, similar to the reported brain dopamine levels (Smith et al., 1992; Zhang et al., 2009). Process number was significantly increased to 169±12.6% of control values following a one-hour treatment with the lowest dopamine concentration (0.1 µM) and continued to increase at a more modest rate following treatments with 1, 10, and 75 µM dopamine (196±15.9%, 219±19.9%, and 236±17.6% of control values, respectively; **Figure 3.3**). Interestingly, the decrease in GFAP\(^+\) area was strongest after a one-hour treatment with 0.1 and 1 µM dopamine (62.4±4.4% and 62.9±5.1% of control values, respectively), but failed to reach statistical significance after treatment with 10 µM or 75 µM dopamine in this experiment (73.5±10.6% and 82.9±6.2% of control values, respectively; **Figure 3.3**). These results indicated that exposure to elevated dopamine triggered a rapid, pronounced, and sustained concentration-dependent stellation of astrocyte processes. Dopamine-induced reduction in GFAP\(^+\) area was more variable between cultures and had a distinctly different concentration-dependence profile.

3.4 **Astrocyte stellation is mediated by dopamine receptors**

Dopamine can undergo oxidation in cell culture media and oxidative processes have been linked to changes in astrocyte morphology, including stellation (Clement et al., 2002; Pekny and Nilsson, 2005; Sofroniew, 2009; Vaarmann et al., 2010). Sodium metabisulfite (SMBS) and N-acetyl-L-cysteine
(NAC) can be used to prevent oxidation of dopamine (Goldstein et al., 2017; Ortinski et al., 2015). To examine whether oxidative processes play a role in dopamine-induced astrocyte stellation, we pre-treated the astrocyte cultures with NAC (5 mM) in culture media for 15 minutes prior to addition of dopamine (75 µM) for one hour. This treatment did not prevent stellation of astrocyte processes. We observed an average of 236±17.6% increase in number of processes for cells treated with dopamine alone versus an average increase of 285±29.6% in number of processes for cells treated with dopamine and NAC (Figure 3.4). GFAP+ cell area was reduced following NAC pre-treatment (42±4.5% of control levels) in excess of what was observed with dopamine alone (82.9±6.2% of control levels). This suggests that, in the absence of NAC, dopamine oxidation or background oxidative processes may oppose dopamine-induced effects on GFAP+ area. Pre-treatment with SMBS (50 µM), a different antioxidant, had similar effects: SMBS failed to prevent dopamine-induced stellation, but did facilitate reduction of GFAP+ area (Figure 3.4).

To test whether dopamine-induced stellation required activation of dopamine receptors, we examined the effects of pre-treatment with the D1-like dopamine receptor antagonist, SCH23390 (50 µM), or the D2-like dopamine receptor antagonist, sulpiride (20 µM). Cells were pre-treated with one or both antagonists for 15 minutes prior to addition of dopamine (75 µM) for one hour. The results indicated that dopamine-induced stellation could be blunted by either D1-like or D2-like dopamine receptor antagonists. Pre-treatment with SCH23390 rescued a 257±15.7% dopamine-induced increase in process number to
145±13.6% of control levels, whereas pre-treatment with sulpiride rescued dopamine-induced increase in process number to 166±14% of control levels (Figure 3.5). Pre-treatment with both SCH23390 and sulpiride, surprisingly, was less effective in blunting dopamine-induced stellation (193±21.2% of control values) than treatment with either antagonist plus dopamine. We also investigated the possibility that dopamine could drive stellation through the Gq-PLC pathway-coupled D1/D2 heterooligomers (Beaulieu and Gainetdinov, 2011). A 15-minute pre-incubation with the membrane-permeable IP3 inhibitor, 2-Aminoethoxydiphenylborane (2-APB; 50 µM), prior to the one-hour treatment with dopamine blunted dopamine-induced stellation to 158±18.2% of control values (Figure 3.5). Application of 2-APB alone had no effect on astrocyte stellation (119±21.3% of control values) or GFAP area (77±7% of control values). GFAP+ area was also not significantly affected by any of the other treatments (Figure 3.5). Altogether, these findings indicate that dopamine effects on astrocyte stellation are attributable to dopamine receptor activation with both D1- and D2-like receptors playing a role in this process. Additionally, we demonstrate a role for the IP3 receptor activation downstream of dopamine receptor signaling. Finally, while oxidative processes may play a role in dopamine-induced regulation of GFAP+ area, these effects are likely dissociable from dopamine-receptor mediated stellation.

3.5 Dopamine treatment elicits broad changes in astrocyte transcriptome

Considering that dopamine treatment can trigger activation of multiple pathways to induce rapid structural morphogenesis, we profiled genome-wide
transcriptional changes using RNA seq. We examined astrocyte transcriptomic changes following brief (20 minutes) and extended (60 minutes) exposure to exogenous dopamine (75 µM). Brief treatment with dopamine resulted in enrichment (log2fold values > 2) at 569 (116 down-regulated, 453 up-regulated) out of 17327 identified genes. Extended treatment with dopamine resulted in enrichment of 159 (37 down-regulated, 122 up-regulated) out of 17327 genes relative to control levels. The volcano plot in Figure 3.6 shows RNA-seq results from all treatment groups (control, 20 min, 60 min). Significantly enriched genes (log2 fold change >2) are color-coded in red (upregulated genes) and blue (downregulated genes) with FDR < 0.0001 (horizontal line). A number of genes with especially large magnitude changes and p-values are labeled. Gene ontology analysis indicated that biological and developmental process regulation accounted for 21.1% of differentially enriched transcripts in the 20-minute treatment condition and for 18.8% of differentially enriched transcripts in the 60-minute treatment condition. Relevant to our morphological findings, 6.2% of differentially enriched genes in the 20-minute condition were accounted for by “microtubule-based”, “cell or subcellular component movement”, and “cell projection organization” categories (Figure 3.6). None of these categories were represented in the 60-minute condition, although 3.5% of all differentially enriched genes were classified as responsible for “structure morphogenesis”. Relevant to astrocyte function, 40 genes were classified as responsible for ion transport in the 20-minute condition and 14 as responsible for calcium ion homeostasis in the 60-minute condition. Prominently represented within the ion transport category were potassium channel
genes: 8 out of 40 total. Out of those eight, six genes coded for voltage-gated potassium channels.

3.6 Functional effects of chronic dopamine

We investigated whether astrocytic voltage-gated potassium channels, involved in regulation of K⁺ ion homeostasis (Sontheimer, 1994), are affected by dopamine treatment. In whole-cell patch-clamp recordings of cells exposed to control or dopamine-containing media for 60 minutes, we found no main effect of dopamine treatment on the amplitude of: inwardly rectifying (Kᵢᵢᵣ) currents [F(1,13)=1.5, p=0.24], delayed rectifier (Kᵢᵢᵩ) currents [F(1,12)=1.44, p=0.25] or the A-type (Iₐ) currents [F(1,11)=0.01, p=0.9] (Figure 3.7Ai-i, Bi-i, Ci-i). The only significant effect was an interaction between dopamine treatment and the injected voltage step for Kᵢᵢᵣ currents [F(18,234)=1.79, p=0.03] (Figure 3.7Ai-ii). When Kᵢᵢᵣ, Kᵢᵢᵩ, and Iₐ data were normalized to the maximal current amplitude in each cell, we observed no significant main effects of dopamine treatment or treatment by voltage interactions (Figure 3.7Ai-iii, Bi-iii, Ci-iii). The resting membrane potential (RMP) was not affected by dopamine treatment (control RMP= -67.58 ± 1.72 mV; dopamine RMP= -66.8 ± 0.93 mV [t(15)=0.3, p=0.75, Student’s t-test]). A decrease in membrane capacitance after dopamine failed to reach statistical significance (control=17.1±1.8 pF; dopamine=11.72±1.9 pF [t(16)=1.99, p=0.06]). However, dopamine treatment significantly increased membrane resistance, although this was highly variable between cells (control R_m=77.6±36.35 MΩ; dopamine R_m=312.87±103.77 MΩ ([t(13)=2.43, p=0.03], Student’s t-test)). The increase in
Rm and the trend toward decreased Cm are consistent with a reduction in GFAP+ cell area seen in immunocytochemical experiments.

Given the contribution of genes that regulate Ca^{2+} ion homeostasis in the RNA seq data and functional relevance of intracellular Ca^{2+}, we also evaluated astrocytic Ca^{2+} transients. To do so, we monitored fluorescence of the GFAP-driven Ca^{2+} sensor, GCamp6f, after exposing the cells to control or dopamine-containing media for 60-90 minutes. Dopamine exposure had only a mild effect on the amplitude of spontaneous Ca^{2+} events reducing them from 4.7±1.1% of baseline fluorescence in control conditions to 2.9±0.5 % of baseline fluorescence after incubation with dopamine (p=0.08, Student’s t-test; Figure 3.8A, B). However, we observed pronounced effects on duration and frequency of astrocytic Ca^{2+} transients. In control conditions, spontaneous Ca^{2+} events were long, lasting an average of 4.1±0.25 seconds and occurred at a frequency of 0.025±0.001 Hz. Incubation with dopamine (75 µM) significantly reduced the duration of spontaneous Ca^{2+} transients to 2.05±0.11 seconds (p=1*10^{-16}, Student’s t-test), but increased their occurrence to 0.038±0.002 Hz (p=4.6*10^{-7}, Student’s t-test; Figure 3.8A, C, D). A 10-minute pre-incubation with the IP3 receptor blocker, 2-aminoethoxydiphenyl borate (2-APB, 50 µM) prior to the 60-minute treatment with dopamine+2-APB reduced the number of cells displaying spontaneous Ca^{2+} transients (from 68% of cells after dopamine alone to 19% of cells after dopamine+2-APB), had no effect on event amplitude or frequency, and led to a marked decrease in event duration (Figure 3.8B, C, D). Broad reduction in number of spontaneously active astrocytes is consistent with the IP3-mediated Ca^{2+}...
release, although additive effects of dopamine and 2-APB on event duration may suggest also an IP$_3$ receptor-independent component (Agarwal et al., 2017). In Ca$^{2+}$-free media, a very small number of cells (5%, 4 cells) continued to display spontaneous GCamp6f fluorescence. We observed a total of 11 fluorescence transients in Ca$^{2+}$-free media with amplitude, frequency and event duration similar to those measured in the presence of 2-APB (Figure 3.8B, C, D). Altogether, these results indicate that a 60-minute exposure to dopamine modulates duration and frequency of astrocyte Ca$^{2+}$ signals. However, dopamine treatment has little to no effect on activity of astrocyte voltage-gated K$^+$ channels.

3.7 Dopamine signaling in astrocytes triggers transcription of CTCF target genes

Having confirmed gene ontology results that both morphological and ionic regulatory processes were selectively induced by dopamine, we further analyzed our RNA-Seq data for mechanistic indicators of potential regulatory processes. To do this, we first plotted the top differentially enriched transcripts from the control, dopamine 20 min, and dopamine 60 min conditions in relation to each other (30 genes from each category resulting in 67 distinct genes). This revealed a clear effect of dopamine treatment and four temporally discrete patterns of transcriptomic regulation (“off/on/off”, “on/off/off”, “on/off/on”, and “off/off/on”; Figure 3.9A). To support these results, we used RT-PCR to evaluate levels of select differentially enriched transcripts in a biological replicate. In line with RNA seq data, we observed the expected regulation of FosB (off/on/off), Pde10a (on/off/off), Ap1s3 (on/off/on), and Gpr83 (off/off/on) following treatment with
dopamine (Figure 3.9Bi-iv). It is notable that in addition to FosB, the “off/on/off”
category prominently featured transient activation of other immediate early gene
family members (e.g. Nr4a3, Atf3, Egr1, Nr4a1, Egr4) among the top differentially
enriched transcripts.

We next examined whether dopamine regulates astrocytic gene expression
ex vivo in hippocampal and striatal brain slices. To do this, we examined genes
present in our in vitro RNA sequencing dataset, but selectively enriched in
astrocytes ex vivo as reported by Zhang et al. (2014). Acute hippocampal and
striatal slices were exposed to dopamine (75 µM + 50 µM SMBS) for 20 minutes
with or without pre-treatment with D1-like dopamine receptor antagonist,
SCH23390 (50 µM), or the D2-like dopamine receptor antagonist, sulpiride (20
µM). The results support our in vitro observations that dopamine induces distinct
patterns of transcriptomic regulation of astrocyte enriched-genes. Exposure to
dopamine lead to a significant increase in expression of Nr4a3 and significantly
downregulated expression of Klf4 in hippocampal slices. However, in striatal slices,
treatment with dopamine did not result in significant differential regulation of any
of the three genes screened (Nr4a3, Klf4, and FosB; Figure 3.10A, B), suggesting
there may be inherent differences among astrocyte from discrete brain regions.
Furthermore, all dopamine-induced alterations in gene expression were absent in
those slices preincubated with either D1-like or D2-like dopamine receptor
antagonists, confirming the role of both D1 and D2-like receptors in mediating
dopamine effects on the astrocyte transcriptome (Fig. 3.10A, B).
In neurons, such discrete activation patterns have been attributed to modifications in the chromatin accessibility landscape (Su et al., 2017). One protein shown to dynamically coordinate chromatin accessibility and genomic organization in the brain is CTCF, which both regulates the 3D structure of chromatin as well as defines the boundaries between active and heterochromatic DNA (Sams et al., 2016; Su et al., 2017; Phillips and Corces, 2009). To evaluate whether CTCF could mediate dopamine-elicited transcriptomic changes in astrocytes, we examined promoters of the top differentially expressed genes (Figure 3.10A) for CTCF motifs. As shown in Figure 3.9C, the results indicated that 70.15% of the top differentially enriched genes possessed a binding motif for CTCF, a substantial increase from the expected genome-wide CTCF binding site prevalence of ~33% (Holwerda and de Laat, 2013). Notably, genes with the CTCF motif included those validated by qPCR (Figures 3.9 and 3.10). Furthermore, all of the genes with the “on/off/on” pattern of dopamine-induced transcriptional regulation possessed the CTCF binding motif whereas genes with the “off/off/on” pattern had the lowest CTCF motif prevalence.

3.8 CTCF regulation recapitulates morphogenic effects of dopamine

CTCF triggers transcriptional changes by interacting with PARP1 (Yu et al., 2004; Ong et al., 2013; Han et al., 2017; Zhao et al., 2015), which has been implicated in behavioral and molecular effects of cocaine (Scobie et al., 2014). PARP1 both facilitates NAD+-dependent generation of ATP for chromatin remodeling (Wright et al., 2016) and regulates CTCF binding to target genes via NAD+-dependent poly-ADP-ribosylation of CTCF (Yu et al., 2004; Ong et al., 2013;
Han et al., 2017). While there are no direct pharmacological tools for CTCF, commonly used NAD+-precursors, nicotinamide and nicotinamide riboside, exhibit opposite effects on PARP1 activity due to their disparate effects on SIRT1, the negative regulator of PARP1 (Bitterman et al., 2002; Trammel et al., 2016; Sajish and Schimmel, 2015; Rajamohan et al., 2009). This results in nicotinamide riboside acting as an ‘antagonist’ of PARP1 and, conversely, nicotinamide acting as an ‘agonist’ of PARP1. We took advantage of this distinction to determine whether dopamine-induced morphological changes could be linked to PARP1 activation. To accomplish this, astrocytes were pre-incubated with nicotinamide (5 mM) or nicotinamide riboside (1 mM) for 15 minutes prior to a 60-minute incubation with dopamine (75 µM) or control media. Nicotinamide increased astrocyte stellation and decreased GFAP+ area in the absence of dopamine (Figure 3.11). In the presence of dopamine, nicotinamide enhanced stellation beyond that seen with dopamine alone, but maintained reduction in GFAP+ area at levels similar to that seen with dopamine alone. Nicotinamide riboside had no effect on astrocyte stellation or GFAP+ area when applied on its own, but occluded changes in both stellation and GFAP+ area when applied in combination with dopamine (Figure 3.11). Similarly, pre-incubation with PARP inhibitor, 3-aminobenzamide (3-AB; 5 mM), completely blocked dopamine-induced stellation. Notably, 3-AB increased GFAP+ cell area when applied by itself or in combination with dopamine (Figure 3.11B). Another, more potent PARP1 inhibitor, AG-14361 (100 µM), reduced dopamine-induced stellation (160±21.4% of control values), but had no effect on GFAP+ cell area (101±9.5% of control values). When applied alone, AG-14361 had
similar effects on astrocyte stellation and GFAP\(^+\) area as it did in combination with dopamine (stellation: 142±18.7\% of control; GFAP\(^+\) area: 127±38.1\% of control). Altogether, these results indicate that dopamine-induced morphological changes on astrocytes in culture rely on PARP1 activation resulting in recruitment of CTCF to target genes and remodeling of astrocyte transcriptome.

Western blot analysis revealed that treatment with dopamine or nicotinamide decreased acetylation of tubulin (Figure 3.12). These findings suggest PARP1 dependent reduction of microtubule assembly. Increased stellation, therefore may be indicative of collapse or retraction of existing membrane and processes rather than generation of new cytoskeleton. In summary, these findings have implications that dopamine treatment acts similarly to nicotinamide treatment and that this similarity may stem from regulation of NAD levels.
Figure 3.1. Characterization of primary cultures. Widefield images depicting (A) GFAP\(^+\) astrocytes, (B) NeuN\(^+\) neurons, and (C) Cd11b\(^+\) microglia present in primary culture. Imaged immunopositive fields indicate that cultures consisted of predominately (A) GFAP\(^+\) cells, some (C) Cd11b\(^+\) cell contamination, and a negligible contribution of (B) NeuN\(^+\) cells. Scale bar, 75 \(\mu\)m.
Figure 3.2. Elevated extracellular dopamine induces morphological changes in astrocytes. (A) Widefield images of astrocytes double-stained for GFAP (green) and DAPI (blue) in control conditions and after exposure to dopamine (75 µM) for 1, 24, or 48 hours (DA 1hr, DA 24hr, DA 48hr). Dopamine induces a prominent increase in the number of primary processes (arrows). Scale bars, 75 µm. (B) Quantification of the number of primary processes and average GFAP+ cell area. One-way ANOVA for number of processes (F(3,469)=56.64, p<0.0001). One-way ANOVA for GFAP+ area (F(3,469)=5.52, p=0.001); Bonferroni post-hocs: *, p<0.05, ***, p<0.001 vs. control; #, p<0.05, ##, p<0.01 vs. dopamine 1 hour. Control (n=179 cells), DA 1 hr (n=152 cells), DA 24 hr (n=58 cells), DA 48 hr (n=84 cells).
Figure 3.3. Dopamine-induced stellation of astrocyte processes is concentration-dependent. (A) Widefield images of GFAP+ astrocytes following a one-hour exposure to 0.1 µM, 1 µM, 10 µM, and 75 µM dopamine. Scale bars, 75 µm. (B) Quantification of average number of primary processes and average GFAP+ cell area. One-way ANOVA for number of processes ($F(4,325)=11.54, p<0.001$); One-way ANOVA for GFAP cell area ($F(4,325)=5.266, p<0.001$). Bonferroni post-hocs: *, p<0.05; **, p<0.01; ***, p<0.001 vs. control. Control (n=67 cells), DA 0.1 µM (n=63 cells), DA 1 µM (n=67 cells), DA 10 µM (n=54 cells), DA 75 µM (n=79 cells).
Figure 3.4. Oxidation of dopamine does not promote rapid stellation of astrocyte processes. 
(A) Representative images of GFAP+ astrocytes in the control conditions and following exposure to dopamine (75 µM) and N-acetylcysteine (5 mM; DA+NAC). Scale bars, 75 µm. 
(B) Quantification of number of primary processes and average GFAP+ cell area following a one-hour exposure to control media, dopamine, dopamine with NAC, or dopamine with sodium metabisulfite (50 µM; DA+SMBS). One-way ANOVA for number of processes (F(3,222)=17.56, p<0.001). One-way ANOVA for GFAP+ area (F(3,222)=17.99, p<0.001). Bonferroni post-hocs: ***, p<0.001 vs. control; ###, p<0.001 vs. dopamine. Control (n=67 cells), DA (n=79 cells), DA+NAC (n=41 cells), DA+SMBS (n=39 cells).
Figure 3.5. Dopamine-induced morphological changes depend on activation of D1-like and D2-like dopamine receptors. (A) Representative images of GFAP+ astrocytes after a one-hour exposure to dopamine (75 µM) or after exposure to dopamine in the presence of D1-like dopamine receptor antagonist SCH23390 (50 µM; DA+SCH), D2-like dopamine receptor antagonist, sulpiride (20 µM; DA+Sulp.), or both SCH23390 and sulpiride (DA+SCH+Sulp.). Scale bars, 75 µm. (B) Quantification of number of primary processes and average GFAP+ cell area. One-way ANOVA for number of processes (F(4,453)=17.15, p<0.001). One-way ANOVA for GFAP area (F(4,454)=2.187, p=0.07). Bonferroni post-hocs: *, p<0.05, ***, p<0.001 vs. control; #, p<0.05, ###, p<0.001 vs. dopamine. Control (n=105 cells), DA (n=116 cells), DA+SCH (n=84 cells), DA+Sulp (n=70 cells), DA+SCH+Sulp (n=84 cells).
Figure 3.6. Transcriptomic effects of dopamine. (A) Volcano plot of all differentially enriched genes in control, 20 min, and 1hr dopamine-treatment conditions. Significantly enriched genes (log2 fold change >2) are color-coded in red (upregulated genes) and blue (downregulated genes) with FDR < 0.0001 (horizontal line). Notice the abundance of immediate early gene family members among the highly upregulated genes. (B) A gene ontology analysis of over-represented “biological process” categories in the 20 min condition. The fraction of the total number of all over-represented (up- and down-regulated) genes is indicated for each category in the parentheses.
Figure 3.7. Dopamine treatment does not affect astrocytic voltage-gated potassium channels. (Ai,Bi,Ci) Representative traces from astrocytes incubated in control- or dopamine (75 µM)-containing media showing Kir (inwardly-rectifying), Kdr (delayed-rectifier), and IA (A-type) K+ currents. (Aii,Bii,Cii) Corresponding I-V plots illustrate mean amplitude ± SEM of currents for each voltage-gated conductance. (Aiii, Biii, Ciii) Same as in Aii, Bii, Cii, but with currents normalized to maximal recorded current in each cell (Kir control (n=9 cells), dopamine (n=6 cells)); Kdr control (n=8 cells), dopamine (n=6 cells)); IA control (n=7 cells), dopamine (n=6 cells)). The only significant difference is an interaction in Aii (see Results). (D) Representative wide-field image of 568-fluorescent dye filled astrocyte with GFAP conjugated to Alexa Fluor 488, and Dapi. Scale bar, 75 um.
Figure 3.8. Effects of dopamine treatment on astrocytic Ca\(^{2+}\) signals. (A) Representative “heat map” frames of GCamp6f fluorescence after a one hour exposure to control (Ai) or dopamine-containing (Aii) media. Ca\(^{2+}\) fluorescence traces for the three numbered astrocytes are presented below the image frames. Vertical dotted lines are drawn at intervals corresponding to the image frames. (B-C) Cumulative distribution histograms summarizing the effects of dopamine on amplitude and duration (half-width) of all recorded spontaneous Ca\(^{2+}\) transients (control (n=473 events); dopamine (n=1429 events)). Insets indicate mean amplitude and duration of Ca\(^{2+}\) events averaged across all identified astrocytes (control (n=172 cells); dopamine (n=325 cells). ***, p<0.001, Student’s t-test (t(495)=8.6). (D) Bar histogram, illustrating dopamine-induced increase in frequency of spontaneous Ca\(^{2+}\) transients. ***, p<0.001, Student’s t-test (t(504)=5.1).
Figure 3.9. Dopamine regulation of astrocyte transcriptome is heterogeneous and temporally dynamic. (A) Clustergram characterizing top DEGs based on expression changes between treatments. (Bl–Biv), qPCR validation of representative genes from each class of the DEGs indicated in A. Data are mean ± SEM. One-way ANOVAs: FosB (F(2,21) = 7.2, p < 0.01); Pde10A (F(2,22) = 14.6, p < 0.001); Ap1s3 (F(2,19) = 4.4, p < 0.05); Gpr83 (F(2,20) = 19.9, p < 0.001). Bonferroni post hoc: ***p < 0.001 versus control. **p < 0.01 versus control. *p < 0.05 versus control. ###p < 0.001 versus DA 20 min. #p < 0.05 versus DA 20 min).
Figure 3.10. DA exposure regulates astrocytically enriched CTCF response genes in rat hippocampal and striatal slices. (A) qPCR analysis of astrocyte-enriched genes from hippocampal slices (n = 9–12 slice experiments from 7 rats). Data are mean ± SEM. *p < 0.05. (B), qPCR screening of astrocyte-enriched genes from striatal slice (n = 9–12 slice experiments from 7 rats). Data are mean ± SEM. *p < 0.05. (C), CTCF binding motif analysis. Top Donut chart represents CTCF binding motif analysis of all top DEGs. Bottom, Four charts represent similar analysis, but for each of the DEG classes indicated in Figure 3.9A, with the validated genes in Figure 3.9B, Figure 10A,B included.
Figure 3.11. Nicotinamide recapitulates morphological effects of dopamine. (A) Representative images of GFAP+ astrocytes following exposure to dopamine and nicotinamide (DA+NAM), dopamine and nicotinamide riboside (DA+NR), nicotinamide alone (NAM), or nicotinamide riboside alone (NR). Scale bars, 75 µm. (B) Quantification of average number of primary processes and average GFAP+ cell area. One-way ANOVA for number of processes F(5,239)=18.71, p<0.001; one-way ANOVA for GFAP area F(5,239)=9.744, p<0.001; Bonferroni post-hocs: ***, p<0.001 vs. control; ##, p<0.01, ###, p<0.001 vs. dopamine.
Figure 3.12. Dopamine and nicotinamide treatment decrease acetylated tubulin in cultured astrocytes. (A) Representative blots. (B) Quantification of protein levels in primary cultured astrocytes in response to Control, Nicotinamide (NAM, 5mM), DA (75µM), or DA & NAM exposure for 1.15hours. * p<0.01 vs. control (2-way ANOVA).
CHAPTER 4

THE IMPACT OF ELEVATED EXTRACELLULAR DOPAMINERGIC SIGNALING ON ASTROCYTES EX VIVO

4.1 Introduction

Emerging evidence is challenging the traditional thought that astrocytes are morphologically and functionally homogenous throughout the brain; instead, astrocytes have been found to exhibit both circuit- and region-specific morphological, functional, and molecular distinctions in both normal and pathological conditions. Moreover, many studies illustrate how astrocytes are not only heterogeneous in form and function, but plastic in nature, responding to wide array of stimuli and conditions both in vitro and in vivo. This morphological and functional heterogeneity, as well as plasticity, allows astrocytes to serve as active participants in overall CNS function including modulation of neuronal signaling and synaptic plasticity. Such roles in overall brain function require close interplay between astrocytes and their signaling partners, where the structural and functional relationships are particularly plastic and can change depending on the individual physiological conditions.

In Chapter 3, I have shown that astrocytes in culture respond to dopamine with morphological, functional, and molecular alterations. It remains unknown whether astrocytes are sensitive to dopamine in vivo and whether this may have
physiological implications. Moreover, despite evidence of structural and functional plasticity of astrocytes in disease states associated with dysregulated dopaminergic signaling, it is not clear whether physiological differences in dopamine exposure impact astrocyte morphology. This chapter highlights a potential role for physiological increases in dopaminergic signaling over the course of cocaine self-administration in altering astrocyte morphology and expression profile of genes implicated in astrocyte-neuron interactions. While several studies have assessed the role of drug or cocaine exposure and experience on astrocytic profiles in various brain regions, including the hippocampus and striatum, the role of elevated extracellular dopamine in physiological conditions of cocaine experience has not been directly addressed.

Drug addiction develops from a voluntary-like drug-taking behavior to compulsive-like drug-taking behavior. This shift in the nature of drug-taking behavior is associated with a transfer of control over drug-taking behavior from the ventral striatum to the dorsal striatum (Vanderschuren & Everitt, 2004; Everitt & Robbins, 2013; Wilhuhn et al., 2012). The ventral-to-dorsal striatal shift in control over drug seeking is represented by an increase in phasic dopamine release within the dorsolateral (DL) striatum, but not within the NAcC, following two and three weeks of cocaine self-administration (Everitt & Robbins, 2013; Ito et al., 2002; Wilhuhn et al., 2012). Taken together, these findings reveal that extended cocaine taking leads to compulsive drug-seeking habits which are associated with a progressive increase in dorsal striatum dopamine levels.
4.2 Astrocyte morphometric and molecular properties in ventral and dorsal striatum following short duration saline experience

We characterized astrocyte density, as well as astrocyte morphometric and molecular properties in the ventral and dorsal striatum of 7 day (1 h/d) saline experienced animals. We did not find any significant differences in astrocyte GFAP\(^+\) density or average GFAP\(^+\) primary process number, process length, surface area (um\(^2\)), or volume (um\(^3\)) between the two striatal regions following 7 d experience in operant chambers. While not significant, astrocytes in the ventral striatum at baseline were depicted as trending towards a more complex structure (Scofield et al., 2016; Testen et al., 2018). Ventral striatal astrocytes possessed a slightly larger number of longer primary processes, as well as higher surface area and volume measures than those in the dorsolateral striatum (Figure 4.3). To examine baseline molecular changes, the study focused on mRNA expression of genes in three categories: intrinsic astrocytic genes (Kir4.1 & Kir.7.1), astrocyte-astrocyte interaction genes (CX43 & CX30), and astrocyte-neuron interaction genes (Hevin & Sparc; see Table 4.1 for gene descriptions). There were no significant sub-regional differences in expression of SPARC, Hevin, Gja1 (CX43), Kcnj10 (Kir4.1), or Kcnj12 (Kir2.2) mRNA (Figure 4.4). Results show significantly higher baseline expression of astrocytic Gjb6 (connexin 30) in the dorsolateral striatum compared to the ventral striatum ([t(10)= 3.333, p= 0.0076, Student’s t-test], Figure 4.4). This finding may align with the observations on trending morphometric distinctions in astrocytes at baseline, where astrocytes in the dorsolateral striatum typically present with less complex structural features,
including fewer and shorter primary processes. Moreover, these morphometric distinctions among astrocytes could be associated with baseline differences in astrocyte-astrocyte and astrocyte-neuron physical and functional interactions in the ventral and dorsal striatum. Certainly, investigation of astrocyte heterogeneity in morphology, function, and gene expression among striatal subregions and how it relates to aberrant conditions in disease warrant future study. While our data show few significant sub-regional differences in the astrocytic properties addressed, we have only begun to investigate striatal sub-regional astrocyte heterogeneity, and thus future investigations should include more thorough investigation of astrocyte morphological, molecular, and functional profiles using techniques such as electron microscopy, in vivo fast-scan cyclic voltammetry, patch-clamp recordings of astrocytes’ K⁺ currents, in vivo calcium imaging, and whole genome sequencing of astrocytes isolated from dorsal and ventral striatal brain slices. These represent only a handful of techniques which, together, could help characterize astrocyte sub-region heterogeneity in the intact striatum.

4.3 Behavioral outcomes: short and extended-access cocaine self-administration

To characterize the impact of phasic dopamine transmission on NAcC and dorsal striatal astrocytes, animals were separated into two groups with each cocaine self-administration animal being paired with a yoked saline control: short-access (7 days 1hr/daily sessions) and extended-access (21 days 1hr/daily sessions). In line with behavioral data published by Wilhuhn et. al (2012), we observed a significant increase in drug-taking behavior after 1 week (7 d) of
cocaine self-administration and this increase was largely maintained over the course of 3 weeks (21 days) cocaine self-administration (Figure 4.1A \([n = 5; F(1, 8) = 13.75, P = .006]\); Two-way ANOVA). When compared to paired yoked saline controls, both short-access and extended access groups had achieved significant increases in drug-taking behavior (active lever presses) by the final day of each experiment (Figure 4.1B; 7 (SA) or 21 (EA) d \((n = 6.5; F(3, 20) = 14.12, P < 0.0001)\) One-way ANOVA). Inactive and time-out responding (i.e., non-reinforced responding) did not change over the course of extended 21 day (1hr/daily sessions) cocaine self-administration (Figure 4.2A; \([n = 5; F(2, 18) = 0.5499, P = 0.5864]\) One-way ANOVA). However, the number of reinforced responses significantly increased over the course of the extended 21 day cocaine self-administration experiment (Figure 4.2B; \([n = 5; F(2, 18) = 23.66; P < 0.0001]\) One-way ANOVA). As a result, the ratio of reinforced to total responses (the efficiency of responding) was significantly elevated in the second and third weeks than in the first week (Figure 4.2C; \([n = 5; F(2, 18) = 28.48, P < 0.0001]\) One-way ANOVA).

4.4 Duration of cocaine self-administration differentially impacts astrocyte density and morphology in the ventral and dorsal striatum.

*Short-access cocaine SA does not alter astrocyte density in the rat striatum*

We investigated whether short access cocaine self-administration associated with a physiological increase in dopaminergic signaling in the NAcC (Wilhuhn et al., 2012) would result in changes in astrocyte density, as well as, morphological alterations to astrocytes when compared to the dorsolateral striatal astrocytes or yoked saline controls under the same behavioral regime. Our findings
reveal that short-access cocaine self-administration did not significantly impact NAcC astrocytic density or morphology (Figures 4.6 and 4.7). Surprisingly, short-access cocaine self-administration resulted in a significant increase (3.67±1.2) in the average number of primary processes per cell in the dorsolateral striatum (DLS; Figure 4.7; [n = 3; F(3, 8) = 5.526, P = .0237]; One-way ANOVA), yet no other significant morphometric alterations were found in DLS astrocytes. While not significant, this same trend was also present in GFAP+ cells within the NAcC of short duration cocaine experienced animals. Together, these findings suggest that short duration cocaine self-administration, associated with elevated dopaminergic signaling in the ventral striatum, leads to an increase in the number of GFAP+ primary processes in the ventral and dorsal striatum. Further, this increase in process number has been attributed to higher structural complexity of astrocytes (Scofield et al., 2016; Testen et al., 2018).

Extended-access cocaine SA elicits region-specific alterations in astrocyte density and morphology in the rat striatum

We next assessed whether extended access, but not short access, cocaine self-administration would lead to subregion specific alterations in density and morphology of astrocytes in the DLS when compared with NAcC or saline control groups. While there was no significant change in density of astrocytes in the DLS, there was a significant decrease from 13.5±.5 in saline conditions to 9±1 GFAP+ astrocytes per field in the NAcC following extended access cocaine self-administration (Figure 4.6; NAcC [F(3,4)=8.133, p=0.035]; One-way ANOVA)). This finding aligns with findings from Scofield et al. (2016) where 10-days
(2hr/daily) cocaine self-administration results in a decrease in GFAP expression within the ventral striatum (NAcC). Our results also indicate that extended duration of cocaine experience leads to significant decreases (NAcC: SA saline = 10.3±1.8, SA cocaine = 13.7±33, EA saline = 9±58, EA cocaine = 8±58; DLS: SA saline = 7.7±33, SA cocaine = 11.3±1.2, EA saline = 10.7±68, EA cocaine = 8.7±33) in average number of primary processes per cell in the DLS and NAcC vs yoked saline control animals and short-access animals (Figure 4.7; DLS [F(3,8 = 5.526, p = .0237]; NAcC [F(3,8 = 6.276, p = .0170] One-way ANOVAs ). Moreover, morphological analyses of astrocytes revealed an increase in the length of primary processes in the DLS following extended duration of cocaine-taking behavior (Figure 4.8; DLS EA saline = 30.1±2.4, EA cocaine = 41.6±3.2; F(3,38 = 16.82, p < .0001] One-way ANOVA). No significant changes in GFAP+ cell measures of surface area (µm²) or volume (µm³) were observed in the ventral striatum (NAcC) of animals who had undergone short or extended duration of cocaine self-administration (Figures 4.9 and 4.10). However, astrocytes within the DLS responded to extended cocaine exposure with both a significant decrease in GFAP+ surface area (Figure 4.9 and 4.10; DLS EA saline = 10887±1711um², EA cocaine = 6010±1130um²; F(3,8 = 9.4, p = .0053] One-way ANOVA) and volume (DLS EA saline = 2113±277um³, EA cocaine = 941±145um³; F(3,8 = 16.59, p = .0009] One-way ANOVA)) when compared to saline groups or short-access cocaine animals. This may indicate a shift to less complex astrocyte-synapse structural and functional interactions.
4.5 Effects of cocaine self-administration duration on gene expression in ventral and dorsal striatal astrocytes

We examined whether short- and extended-access cocaine self-administration would lead to sub-region specific regulation of astrocytic gene expression *ex vivo* in the NAcC when compared to the DLS of cocaine animals or yoked saline controls under the same regime. Results showed no significant changes in intrinsic astrocytic gene mRNA expression in the NAcC or DLS following short- or extended-access cocaine (*Figure 4.11*). Yet, we did find significant downregulation of connexin 30 (astrocyte-astrocyte) mRNA expression following short-access cocaine self-administration in the NAcC (*Figure 4.12*; [n = 6; (t(9)= 0.992) P < 0.0001]; Student’s unpaired t-test). Downregulation of connexin 30 holds important implications for astrocyte-astrocyte, as well as, astrocyte-neuron structural and functional interactions as it is often associated with regulation of PAPs into the synaptic cleft (Pannasch et al., 2014). In particular, a decrease in connexin 30 mRNA may translate to an increase in astrocytic process insertion into the synaptic cleft, and thus an increase in the efficiency of astrocytic glutamate transporters (i.e., GLT-1 & GLAST; Pannasch et al., 2014; Testen et al., 2018; Reissner et al., 2015; Scofield et al., 2016; see intro). We also observed significant downregulation of SPARC in the DLS following extended, but not short-access cocaine experience (*Figure 4.13*; [n= 6; t(9)=4.173) p < .005]; Student’s unpaired t-test). This decrease in Sparc gene expression may be linked to the morphometric increase in surface area, volume, and process length. An increase in process length and volume would suggest increased structural interactions with
nearby synapses as Sparc functions to inhibit Hevin’s role in promoting formation of synaptic specialization. There were no significant alterations in gene expression in the DLS of short-access animals. While our studies investigate short-term impact of cocaine self-administration on individual astrocytic morphometric features, future experiments will address the functional and structural properties of astrocyte-astrocyte and astrocyte-neuron interactions following elevated dopaminergic signaling.
Table 4.1  Gene descriptions for RT-qPCR of cocaine and saline-experienced animals

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cell Type</th>
<th>Finding</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Astrocyte-Astrocyte</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gja1 (CX43)</td>
<td>Astrocyte</td>
<td>The major GJ protein in astrocytes; Synchronization of astrocytic signaling, Ca²⁺ wave propagation &amp; spatial K⁺ buffering</td>
<td>1. Chai et al., 2017</td>
</tr>
<tr>
<td>Gjb6 (CX30)</td>
<td>Astrocyte</td>
<td>Regulates synaptic contact of astrocyte processes</td>
<td>2. Pannasch et al., 2014</td>
</tr>
<tr>
<td></td>
<td>Pericyte</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Astrocyte-Synapse</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sparcl1 (Hevin)</td>
<td>Astrocyte</td>
<td>Promotes the formation of both pre- and postsynaptic specializations</td>
<td>4. Risher et al., 2015</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sparc</td>
<td>Microglia &amp;</td>
<td>Inhibits the synaptogenic function of hevin</td>
<td>6. Kucukdereli et al., 2011</td>
</tr>
<tr>
<td><strong>Intrinsic</strong></td>
<td>Astrocyte</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kir4.1 (Kcnj10)</td>
<td>Astrocyte</td>
<td>Major astrocytic extracellular K⁺ uptake mechanism</td>
<td>7. Sibille, Pannasch, &amp; Rouach, 2014</td>
</tr>
<tr>
<td>Kir7.1 (Kcnj12)</td>
<td>Astrocyte</td>
<td>Extracellular K⁺ uptake mechanism</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.1. Drug-taking behavior over the course of days or weeks. (A) Active lever presses during the drug acquisition period over 7 (SA) or 21 d (EA) of self-administration (n = 6, 11). (B) Active lever presses on the final day of self-administration. Two-way ANOVA for active lever presses over course of experiments (7 days, F(1,10) = 11.67, p = 0.006; 21 days, F(1,8)=13.75, p = 0.006). One-way ANOVA for active lever final day summary (F(3,20)= 14.12, p<0.0001). One-way ANOVA for reinforced lever presses (F(2,18)= 23.66, p<0.0001). One-way ANOVA for efficiency ratio (F(2,18)=28.48, p<0.0001). Tukey post-hocs: *P < 0.05,**P < .001 ***P < 0.0001; n.s., not significant.
Figure 4.2. Drug-reinforced responding in cocaine self-administration. (A) The number of non-reinforced lever presses did not change significantly across the three-week period of drug self-administration. (B) The number of reinforced lever presses increased significantly across the three-week experiment. (C) The ratio of reinforced over total number of lever presses (efficiency) increased in the second and third weeks compared with the first week during the 21-day drug self-administration regime. One-way ANOVA for reinforced lever presses (F(2,18)=23.66, p<0.0001). One-way ANOVA for efficiency ratio (F(2,18)=28.48, p<0.0001). Tukey post-hocs: *P < 0.05, **P < .001, ***P < 0.0001; n.s., not significant.
Figure 4.3. Astrocytes in dorsal and ventral striatum: 7 days saline density and morphology. Quantification of GFAP+ (A) Density, (B) Primary process number, (C) Primary process length (um), (D) Volume (um^3), (E) Surface area (um^2). Student’s t-test. Data are mean ± SEM.
Figure 4.4. Astrocytes in dorsal and ventral striatum: 7 days saline gene expression. Quantification of (A) Hevin, (B) SPARC, (C) CX30, (D) CX43, (E) Kcnj10, & (F) Kcnj12 mRNA expression NAcC and DLS. NAcC and DLS saline 7 d (n=5, 6). Data are mean ± SEM. **, p<0.01, Student’s t-test (t(10)=3.333).
Figure 4.5. Representative confocal 3D rendered images of depth-coded GFAP\(^{+}\) astrocytes in slice of cocaine-experienced animals. (A) NAcC SA Cocaine, (B) DLS SA Cocaine, (C) NAcC EA Cocaine, (D) DLS EA Cocaine. **Red = top of stack, blue = bottom of stack.** Scale bar = 20 µm.
Figure 4.6. Duration of cocaine self-administration differentially affects ventral striatal astrocyte density. (A,B) Quantification of GFAP positive astrocytes in the (A) NAcC (ventral striatum) and (B) dorsolateral striatum (dorsal striatum) following 7 or 21 d cocaine or saline experience (n = 6, 5). Data are mean ± SEM. One-way ANOVA for GFAP+ cells/field in DLS: $F_{3,4} = 1.000$, $p = 0.4789$. One-way ANOVA for GFAP+ cells/field in NAcC: $F_{3,4} = 8.113$, *, $p = 0.0354$. The number of GFAP+ astrocytes significantly decreased in the NAc Core following extended-access cocaine self-administration.
Figure 4.7. Duration of cocaine self-administration differentially impacts dorsal and ventral striatal astrocyte primary process number. (A,B) Quantification of average GFAP$^+$ primary process per cell. (A) One-way ANOVA for process number in NAcC: $F_{3,8} = 6.27$, $p = 0.0237$. Data are mean ± SEM. (B) One-way ANOVA for process number in DLS: $F_{3,8} = 5.526$, $p = 0.0170$. Data are mean ± SEM. Bonferroni post hocs: #, $p<.05$ vs. EA cocaine; *, $p<0.05$ vs. SA cocaine. $n = 3$ cells.
Figure 4.8. Extended-access cocaine self-administration induces alterations in astrocyte process length in the dorsal striatum. (A,B) Quantification of average primary process length (um) in (A) NAcC and (B) DLS following 21 d cocaine self-administration. Data are mean ± SEM. One-way ANOVA for process number: $F_{3,38} = 16.82$, $p < 0.0001$; Bonferroni post hocs: ****, $p < 0.0001$ vs. SA cocaine or saline; **, $p < 0.001$ vs. EA saline; *, $p < 0.05$ vs. EA cocaine. SA saline, SA cocaine, EA saline, & EA cocaine (n=3 cells).
Figure 4.9. Extended-access cocaine self-administration induces alterations in astrocyte surface area in the dorsal striatum. (A,B) Quantification of GFAP+ surface area (um²) in (A) NAcC and (B) DLS following 7 or 21 d cocaine self-administration. Data are mean ± SEM. One-way ANOVA for GFAP+ surface area (um²), (DLS): F₃,₈ = 9.400, p = 0.0053; Bonferroni post hocs: **, p< 0.01 vs. EA saline; *, p< 0.05 vs. EA saline.
Figure 4.10. Extended-access cocaine self-administration induces alterations in astrocyte volume in the dorsal striatum. Quantification of GFAP+ volume (um³) in (A) NAcC and (B) DLS following 7 or 21 d cocaine self-administration. Data are mean ± SEM. One-way ANOVA for GFAP+ volume (DLS): F₃,₈ = 16.65, p = 0.0008; Bonferroni post hocs: ##, p< 0.01 vs. EA saline; **, p< 0.01 vs. EA cocaine. NAcC saline & cocaine (n=3 cells), DLS saline & cocaine (n=3 cells).
Figure 4.11. Dopamine regulation of intrinsic astrocyte gene expression. Quantification of (A, C) Kcnj10 (Kir4.1) or (B, D) Kcnj12 (Kir2.2) mRNA expression in NAcC and DLS following 7 or 21 d cocaine self-administration. Data are mean ± SEM. NAcC saline & cocaine (n=5, 6), DLS saline & cocaine (n=5, 6).
Figure 4.12. Dopamine regulation of astrocyte-astrocyte interaction-type gene expression. Quantification of (A, C) CX43 (Gja1) or (B, D) CX30 (Gjb6) mRNA expression in NAcC and DLS following 7 or 21 d cocaine self-administration. Data are mean ± SEM. ***, p<0.0001, Student’s t-test (t(9)=9.992); (n=5, 6), DLS saline & cocaine (n=5, 6).
Figure 4.13. Dopamine regulation of astrocyte-neuron interaction-type gene expression. Quantification of SPARC (A, C) and Hevin (B, D) mRNA expression in NAcC and DLS following 7 or 21 d cocaine self-administration. Data are mean ± SEM. **, p<0.005, Student’s t-test (t(9)=4.173); (n=5, 6), DLS saline & cocaine (n=5, 6).
CHAPTER 5
DISCUSSION

5.1 Summary of findings

The studies presented in this document provide novel implications for the effects of extracellular dopamine on astrocyte morphogenic, molecular, and functional profiles in vitro and in vivo. In illustrating the impact of dopamine exposure on cultured astrocytes, we found that elevated extracellular dopamine induced pronounced stellation of astrocytic processes and a reduction in astrocyte area positive for the intermediate filament marker, GFAP. This dopamine mediated effect was blunted by exposure to dopamine receptor antagonists or by inhibition of PARP1, suggesting a dopamine receptor-mediated, PARP1-dependent signaling as the source of morphological changes. Furthermore, we showed that extended exposure of astrocytes to dopamine drives a set of functional adaptations distinct from those previously reported following acute dopamine treatment in vitro. Critically, we demonstrated for the first time that at the genome-wide level CTCF target genes are a prominent marker of astrocyte transcriptional response to dopamine. In the second part of this thesis, we evaluated sensitivity of astrocytes

to region-specific physiological increases in dopamine using a behavioral model of short- and extended-access cocaine self-administration. This regime was modeled after experiments published by Wilhuhn et al., in 2012 illustrating experience-dependent and region-specific physiological increases in dopamine using a behavioral model of short- and extended-access cocaine self-administration. Using this model, results show extended cocaine experience led to a significant decrease in expression of GFAP+ astrocytes in the NAcC. Along with this observation, extended duration cocaine elicited increases in stellation of astrocytes in the NAcC. While drug experience had no effect on any other morphogenic properties of ventral striatal astrocytes, significant increases in GFAP+ process length, surface area, and volume were observed in the DLS following extended-access saline or cocaine self-administration. These findings are consistent with a significant downregulation of Gjb6 (CX30) as this would allow for extension of processes and may contribute to closer structural and functional relationships with nearby synapses. Interestingly, extended experience alone significantly affects astrocyte morphometric properties with an observed increase in GFAP+ primary process length, surface area (um^2), and volume (um^3). Furthermore, extended drug experience was found to significantly reduce GFAP+ volume and increase the average length of primary processes in the DLS when compared to yoked saline controls. While not significant, extended exposure to cocaine also led to a downward trend in GFAP+ surface area and process number when compared to paired saline control animals. Taken together, duration of cocaine self-administration exerts significant, yet subregion-specific and
temporally distinct alterations in striatal astrocyte morphogenetic and molecular properties in vivo. These experiments relied on prior findings of regional differences in dopamine levels after cocaine exposure, but did not specifically measure dopamine levels in the study’s experimental groups. Results provide evidence that duration of cocaine experience differentially impacts morphometric and molecular plasticity of astrocytes in ventral (NAcC) and dorsal (dorsolateral) striatum of rat. Future experiments will directly test the extent to which this plasticity depends on dopamine signaling in astrocytes in vivo.

5.2 Astrocyte sensitivity to duration of dopamine exposure in vitro and cocaine experience in vivo

Astrocyte sensitivity to duration of dopamine exposure in vitro

One line of evidence that supports duration of exposure as a factor relevant to dopamine-mediated response comes from our functional analysis of astrocyte Ca\(^{2+}\) signaling. Substantial evidence indicates that dopamine plays a role in modulation of Ca\(^{2+}\) signaling in astrocytes. Specifically, acute application of dopamine or dopamine receptor agonists, such as SKF83959, to astrocytes in culture has generally been found to induce elevations in cytosolic Ca\(^{2+}\) via an IP3-mediated release from intracellular stores (Vaarmann et al., 2010; Parpura & Haydon, 2000; Lee at al., 2004; Liu, 2009; Jennings & Rusakov, 2016; Jennings et al., 2017). Further, astrocytes from cortical and hippocampal cultures express a distinct phosphatidylinositol (PI)-linked D1-like receptor coupled to PLC by G\(_{\alpha}\)q, which upon activation leads to an increase in intracellular calcium levels (Liu et al., 2009, Ming et al., 2006). Our results indicate that extended treatment with
dopamine decreased the duration and amplitude of Ca\textsuperscript{2+} transients, the majority of which were dependent on IP3 receptor activation (Figure 3.8). However, a one-hour dopamine treatment increased Ca\textsuperscript{2+} event frequency (Figure 3.8). The distinction between acute and chronic effects of dopamine receptor stimulation has been reported in studies of NMDA-receptor mediated neuronal signaling (Schilström et al., 2006; Ortinski et al., 2013, Ortinski, 2014). For example, Schilström et al. (2006) indicated that a brief exposure to a D1 agonist, SKF81297 produces a delayed increase in NMDA receptor-mediated currents with an even longer delay observed after blockade of dopamine transport. Given the well-documented behavioral and molecular differences driven by temporal pattern of exposure to elevated dopamine (Ortinski et al., 2012; Knackstedt et al., 2010), understanding the extent of astrocytic contribution to these differences is an important future direction. Our morphological data from astrocytes in culture reveal a dramatic dopamine-induced shift in morphology from a fibroblast-like form to and stellated form, with over a 2-fold increase in the number of GFAP\textsuperscript{+} primary processes per cell. (Figure 3.2) These morphological changes persisted for 24- and 48-hour dopamine exposure; however, astrocytes were largely able to revert to their original polygonal form after being kept for 24-hours in dopamine-free media. Further, our sequencing data indicate that even at relatively short timescales (20 vs. 60 minutes) several distinct patterns of gene regulation can be distinguished at the genomic level (Figure 3.9). This is characterized most prominently by a sustained upregulation of genes involved in structure morphogenesis, kinase signaling, and signal transduction following a long
exposure to dopamine and a transient up-regulation of immediate early genes after a brief exposure to dopamine.

**Astrocyte sensitivity to duration of dopamine exposure in vivo**

A number of studies revealed alterations in astrocyte morphometric properties and astrocyte-synapse relationships following 10 days cocaine self-administration (Scofield et al., 2016; Testen et al., 2018; Moussawi et al., 2009). In two studies published in 2016 by Scofield et al., and 2018 by Testen et al., astrocytes within the nucleus accumbens core (NAcC) of the ventral striatum responded with decreased volume, surface area, and synaptic colocalization following cocaine self-administration and extinction, compared to NAc astrocytes from saline-administering animals. In a subsequent study, investigators assessed the effects of cocaine-self administration following the last drug self-administration session (day 10) and found no significant changes in NAc astrocytes morphometric properties after self-administration alone (Testen et al., 2018). To our knowledge, no studies exist which have examined dopamine’s effects on morphology of dorsal striatal astrocytes in a cocaine-self administration model. In line with data from Testen et al., (2018), we see no significant alterations in astrocytes morphometric properties (surface area, volume, primary process count, process length) in the NAcC following short (7 days) of cocaine self-administration experience with samples being taken 24 hrs after the final session. As it has been found that astrocytes respond to various experiences, including drug exposure, with dynamic alterations in cell morphology. Importantly, these changes depend on the conditions of drug exposure (i.e., investigator administered or self-administered),
duration, drug type, and timepoint at which samples are harvested following the last period of drug exposure. However, we did find a significant decrease in density of GFAP+ cells in the NAcC following 21 days cocaine-self administration and a significant decrease in primary process number, suggesting that long term cocaine self-administration may lead to alterations in ventral striatal astrocytes. Furthermore, our studies reveal a temporally and regionally distinct influence of cocaine self-administration on astrocytes in the dorsolateral striatum. For example, short-access (7 d) cocaine self-administration lead to a significant increase in primary process number when compared to saline controls; however, no significant alterations in number of primary processes were seen following extended-access cocaine self-administration. Furthermore, while there was no observed change in the number of primary processes following extended drug experience, there was an observed increase in primary process length in the dorsolateral striatum not seen following 7-day drug experience. Interestingly, there was an observed increase in morphometric properties following extended operant chamber experience (yoked saline controls), including an increase in process length, surface area, and volume. Since astrocytes have been found to respond to simple learning tasks and motor activity with changes in morphology, extended “training” experience in the operant chamber may explain alterations in astrocytes following extended-, but not short-access saline self-administration. These results provide insights into the influence of cocaine use on astrocytes within striatum and inform both regional heterogeneity as well as temporal dynamics of astrocyte responsiveness to cocaine self-administration.
5.3 Possible mechanisms for dopaminergic regulation of astrocyte morphological, molecular, and functional profiles in vitro: implications for role of dopamine in altering astrocytes in addictive phenotype.

*Dopamine-mediated morphological changes*

This study focused on two morphological features of cultured astrocytes, primary process stellation and GFAP\(^+\) area, as notable responses to dopamine treatment (*Figure 3.2*). Subsequent experimentation revealed these two morphological measures to be dissociable. For example, process stellation was strongest after treatment with high dopamine concentrations whereas increase in GFAP\(^+\) area was strongest after treatment with low dopamine concentrations (*Figure 3.3*). Similarly, profound suppression of GFAP\(^+\) cell area following combined treatment with dopamine and antioxidants contrasted with the lack of changes in process number following a similar treatment (*Figure 3.4*).

Treatments with cAMP analogues have been observed to trigger morphological changes in cultured astrocytes (Won and Oh, 2000; Moonen, 1975; Paco et al., 2016). However, sensitivity of astrocyte morphology to elevated dopamine has, to our knowledge, only been reported in one other study (Koppel et al., 2018). In that report, dopamine-induced stellation of cortical astrocytes was blocked by propranolol, a \(\beta\) adrenoreceptor antagonist. Our *in vitro* results support a direct role of dopamine receptors in regulation of astrocyte morphology. Thus, we found that both D1-like receptor antagonist, SCH23390, or D2-like receptor antagonist, sulpiride, were effective at blunting dopamine-induced stellation of astrocyte processes in culture (*Figure 3.5*). Interestingly, when applied together,
both D1- and D2-like receptor antagonists were less effective in blunting dopamine induced stellation than either antagonist by itself. It is possible that these dopamine-mediated receptor effects on astrocyte morphology are due to competitive binding of antagonists to D1/D2 heterooligomers (Beaulieu and Gainetdinov, 2011; Rashid et al., 2007; Hasbi et al., 2010; Lee et al., 2004; Jennings and Rusakov, 2016). The effects on GFAP^+ morphology when both D1-like and D2-like receptor antagonists were applied together prior to dopamine application were surprising given the classical notion that D2-like receptor activation decreases cytoplasmic cAMP levels. A possible explanation could involve a novel phosphatidylinositol (PI)-linked D1-like receptor found in both astrocytes and neurons, which couples to PLC by G\alpha_q and increases intracellular Ca^{2+} levels (Liu et al., 2009, Ming et al., 2006). Another explanation could involve an interaction of the antagonists with Gq-PLC pathway-coupled D1/D2 receptor heterooligomers (Beaulieu and Gainetdinov, 2011; Rashid et al., 2007; Hasbi et al., 2010; Lee et al., 2004; Jennings and Rusakov, 2016). Indeed, we show that blockade of IP_3 receptors activated downstream of PIP2 hydrolysis by PLC reduced dopamine-induced astrocyte stellation. Therefore, our results suggest that cAMP increase may not be necessary for dopamine-induced stellation.

**Dopamine and NAD^+/PARP1-dependent regulation of CTCF target genes**

We find that the dramatic alterations in astrocyte morphology triggered by dopamine treatment may be due to significant modifications in chromatin accessibility. Across the genome, the expected genome-wide binding site prevalence of CTCF is ~33% (Holwerda and deLaat, 2013). However, prevalence
of CTCF binding motifs in the top genes significantly altered by dopamine treatment was found to be between 70–100%. Complementing the evidence showing that CTCF orchestrates genomic re-organization underlying learning and memory (Sams et al., 2016), we highlight a possible NAD+ /PARP1-role linking dopamine signaling in astrocytes to epigenetic regulation of the chromatin landscape.

Our data suggest that dopamine activates PARP1, a NAD+-dependent poly-ADP-ribose polymerase that promotes CTCF binding to transcription initiation sites (Yu et al., 2004; Ong et al, 2013; Han et al., 2017; Zhao et al., 2015), leading to changes in astrocyte morphology through alterations in 3D chromatin architecture. We speculate that protein kinase C (PKC) is a likely mediator of coupling between dopamine receptors and PARP1/CTCF activation. Both D1- and D2-like dopamine receptors can stimulate PKC (Neve et al., 2004; Ortinski et al., 2015) and PKC is, in turn, known to promote PARP1 production and formation of PARP1/CTCF complexes (Henderson et al., 2017). We find that PARP1 agonist, nicotinamide, recapitulates, while PARP1 antagonists, nicotinamide riboside, 3-AB, or AG-14361, occlude dopamine-induced stellation and dopamine-induced decrease in GFAP+ area (Figure 3.11). Our observation that co-administration of dopamine and nicotinamide results in more pronounced effects than treatment with either dopamine or nicotinamide alone, suggests that dopamine may not recruit all of the available PARP1 pool. Furthermore, the nicotinamide/dopamine synergism supports PARP1-dependent generation of nicotinamide (Sajish and Schimmel, 2015) rather than regulation of PARP1 protein levels. This is indirectly supported
by the sequencing data indicating a lack of PARP1 transcript enrichment following either 20 or 60 minutes of dopamine treatment (log2 fold values of −0.130 and −0.134, respectively). Our observation that nicotinamide riboside or AG-14361 did not evoke any morphological changes by themselves and only partially suppressed morphological effects of dopamine treatment suggests that dopamine effects on astrocyte morphology involve both PARP1/NAD+ dependent as well as PARP1/NAD+ independent processes. Indeed, a recent finding demonstrated that a 3-hour dopamine treatment of cortical astrocytes induced expression of BDNF (Koppel et al., 2017), a SIRT1-dependent process (Zocchi and Sassone-Corsi, 2012). SIRT1 and PARP1 are mutually inhibitory (Sajish and Schimmel, 2015), but both SIRT1 and PARP1 rely on NAD+ as a substrate, potentially leading to a cyclical pattern of SIRT1/PARP1 activation which could serve as the basis of astrocyte sensitivity to duration of dopamine exposure. Dopamine-induced inhibition of specific sets of genes that lack CTCF binding sites in their promoter regions, highlighted by our sequencing data, may be an indicator of such a relationship.

**Dopamine regulation of astrocyte transcriptome**

Dopamine had distinct effects on the transcriptome of cultured astrocytes at 20 and 60 min post treatment. Additionally, we observed that dopamine regulates expression of astrocyte-enriched transcripts in acutely isolated brain slices and that these effects may be attenuated by antagonists at D1- and D2- dopamine receptors. The differences in dopamine effects on RNA levels between striatal and hippocampal slices reported here, extend previous work from Chai et
al. (2017), which detailed differentially expressed gene profiles in these two structures. Approximately ¾ of the top 40 genes enriched in both hippocampus and striatum in Chai et al. experiments are represented in our RNA-Seq dataset, indicating substantial overlap between ex vivo and in vitro observations at the transcriptome level. Furthermore, the genes that we validated by qPCR (FosB, Klf4, Nr4a3, PDE10a, Gpr83, Ap1s3) all possess a CTCF binding motif. Indeed, our RNA-Seq data analysis reported over-representation of CTCF binding motifs, indicating a coordinated gene network response to dopamine signaling.

**Implications for dopamine role in altering astrocytes in addictive phenotype.**

Astrocytes form extensive networks of coupled cells via gap-junctions, and changes in gap-junction gene expression or protein level often reflect a change in astrocytic network architecture and function (Chai et al., 2017; Meunier et al., 2017; Clasadonte & Haydon, 2014). For example, double knock-out of Connexin 43 and Connexin 30 has been found to completely ablate gap-junction coupling in astrocytes, and thus blunts astrocyte-astrocyte and astrocyte-neuron signaling mechanisms (Theis et al., 2003). In fact, connexin 30 (Gjb6) is distinctly found to be expressed in astrocytes and has been shown to regulate the structural plasticity (extension) of astrocytic processes into the synaptic cleft (Clasadonte & Haydon, 2014). Interestingly, our data reveal that exposure of cultured astrocytes to DA for 20 and 60 minutes leads to significant changes in gap-junction associated connexin mRNA expression, including significant upregulation of connexin 30 – a protein which may stabilize astrocytic processes during the morphological shift from a polygonal to a stellated astrocyte following DA exposure in vitro. We also
found significant upregulation of connexin 30 (astrocyte-astrocyte) mRNA expression following extended-access cocaine self-administration in the NAcC (compared to SA cocaine). Regulation of connexin 30 holds important implications for astrocyte-astrocyte, as well as, astrocyte-neuron structural and functional interactions. As the second most predominant astrocytic gap-junction connexin in the brain, connexin 30 has been shown to regulate the dynamic interactions among astrocytes and neurons and play an important role in governing CNS physiology and behavior, including synaptic plasticity and learning-associated processes (Pannasch et al., 2014; Clasadonte and Haydon, 2014). For example, investigators observed an associated increase in fine process or PAP extension into the synaptic cleft in the hippocampus of connexin 30 knockout mice (Pannasch et al., 2014). This morphological change in astrocytes was associated with an increase in astrocytic GLT-1 activity leading to an overall decrease in overall excitatory transmission, as well as impairments in contextual memory processes (Pannasch et al., 2014). Studies of effects of drug self-administration on astrocyte structural components reveal that 10 days of cocaine self-administration and/or withdrawal leads to decreases in astrocyte morphometric features, including a decrease astrocyte-neuron structural interaction (Scofield et al, 2016; Testen et al., 2018). Moreover, this decrease in morphometric properties was associated with a decrease in astrocyte-mediated control over synaptic excitability via downregulated expression and activity of astrocytic glutamate transporter, GLT-1. Thus, the observation short-access (7 d) cocaine self-administration leads to downregulated expression of connexin 30 mRNA in ventral striatal astrocytes may
be associated with the subsequent increase in primary process number in astrocytes seen in extended-access (21 d) conditions. This finding would, in theory, be associated with higher structural and functional interplay among astrocytes and neurons, and thus an increase in astrocyte-mediated glutamate uptake efficiency would be expected (Scofield et al., 2016; Pannasch et al., 2014; Clasadonte and Haydon, 2014). However, aside from an upward trend in average GFAP+ primary process number, there were no other observable alterations in morphology of astrocytes in ventral striatum of cocaine-experienced rats. Yet, GFAP represents a relatively minor fraction of overall astrocyte cell architecture and is not localized in peripheral processes where neuronal and synaptic contact is made (Scofield et al., 2016; Pannasch et al., 2014; see intro). The current imaging methods used pose limitations, as imaging of PAPs-synapse relationships and structural plasticity of PAPs requires advanced microscopic methods, such as high-resolution electron microscopy. In the future, techniques offering detailed analysis of astrocyte structural components will be adopted.

Dopamine may trigger cAMP production via activation of D1-like dopamine receptors, expressed by astrocytes along with other dopamine receptor subtypes (Miyazaki et al., 2004; Shao et al., 2013; Mladinov et al., 2010; Khan et al., 2001; Nagatomo et al. 2017). D1-induced production of cAMP has been linked to release of astrocytic Ca2+ from internal stores, an important hallmark of astrocyte-to-astrocyte, astrocyte-to-neuron, and astrocyte-to-vasculature signaling (Jennings et al., 2017; Chai et al., 2017). Indeed, both structural and functional astrocyte adaptations have been reported in models of neurologic disease linked to
dysregulated dopamine signaling. For example, Parkinson’s disease is associated with hypertrophy of astrocytic processes, a feature of reactive astrocytosis (Booth et al., 2017), while cocaine use has been linked to a reduction of astrocyte surface area and reduced interaction with synapses (Scofield et al., 2016). In contrast to these results, our in vivo experiments reveal no significant differences in GFAP+ surface volume in the NAcC following short or extended-access drug self-administration, but a dramatic increase in average GFAP+ surface volume in the dorsolateral striatum following extended-access cocaine exposure.

Furthermore, a number of studies have demonstrated that dopamine-induced increase in astrocytic Ca2+ impacts cellular respiration mechanisms coupled to PARP1 and sirtuin activation (Requardt et al., 2010; Requardt et al., 2012; Gupte et al., 2017; Verdin, 2015). CTCF triggers transcriptional changes by interacting with PARP1 (Yu et al., 2004; Ong et al., 2013; Han et al., 2017; Zhao et al., 2015), which has been implicated in behavioral and molecular effects of cocaine (Scobie et al., 2014). PARP1 both facilitates NAD+-dependent generation of ATP for chromatin remodeling (Wright et al., 2016) and regulates CTCF binding to target genes via NAD+-dependent poly-ADP-ribosylation of CTCF (Yu et al., 2004; Ong et al., 2013; Han et al., 2017). Recent studies have shown that CTCF regulates both chromatin remodeling (Wright et al., 2016) and chromatin insulation (Yu et al., 2004, Ong et al., 2013), thereby organizing transcriptional regulation of distinct gene suites in response to cellular signaling. However, it is unknown whether dopamine- or dopamine receptor-induced structural or genomic changes are linked to PARP1/CTCF interaction.
We also assessed dopamine- or dopamine receptor-induced structural or genomic changes potentially linked to PARP1/CTCF interaction. We demonstrate that dopamine receptor signaling generates pronounced morphological changes in cultured astrocytes which rely on PARP1 activation and are accompanied by a dynamic regulation of the astrocyte transcriptome including, most prominently, specific enrichment of genes containing CTCF motifs in promoter regions. Our results highlight a novel PARP1/CTCF-dependent mechanism that drives dopamine-induced morphological changes and associated transcriptional profile in astrocytes.

5.4 Caveats of investigating dopaminergic reactions in vitro

Dopamine can oxidize in culture media (Clement et al., 2002) and reactive oxygen species can trigger morphological changes in astrocytes including cell hypertrophy, swelling/thickening of processes, and increased GFAP expression, collectively referred to as reactive astrocytosis (Vaarmann et al., 2010). Of note, individual processes were found to be thicker following dopamine treatment, however, morphological changes associated with dopamine exposure persisted in the presence of antioxidants, NAC or SMBS, arguing against oxidative process-mediated reactivity. Moreover, the study observed variable, but consistent downregulation of GFAP\(^+\) area (Figures 3.2 - 3.4, Figure 3.11), arguing against swelling of existing processes as mediators of dopamine-induced morphological changes. Furthermore, RNA seq indicated only small changes in GFAP transcript (log2fold value of 1.2 at 60 minutes of treatment with dopamine), a result that was confirmed with RT-qPCR (not shown). Finally, cross-referencing with published
reactive astrocyte transcriptome revealed little to no overlap with RNA seq data. Out of the top 100 reactive astrocyte genes in Zamanian et al. (2012), three were enriched at above log2fold level in our data set (Gdf15, Srxn1, and Asns). Lcn2 and Serpina3n identified as the prominent novel markers of astrocytic reactivity (Zamanian et al. 2012) displayed minimal changes in our dataset after one hour of treatment with dopamine (log2fold values of 0.1 and –0.9, for Lcn2 and Serpina3n, respectively). Therefore, in conjunction with prior studies that support anti-inflammatory effects of dopamine (Yan et al., 2015; Beck et al., 2004; Shao et al., 2013), results illustrated that dopamine treatment did not promote a reactive state that could account for the observed morphological or transcriptome changes in our cultured astrocytes. This, however, could be different for astrocytes that experience withdrawal from elevated dopamine as reported in animals withdrawn from experimenter or self-administered cocaine (Fattore et al., 2002; Bowers and Kalivas, 2003).

5.5 Astrocytes and dopamine in vivo and ex vivo: current limitations and future directions

In this thesis, results reveal regional and exposure-dependent cocaine-induced regulation of astrocytes morphological and molecular profiles in the ventral and dorsal striatum. In trying to evoke physiological increases in dopaminergic efflux in the ventral and dorsal striatum, this study modeled methods and results published by Wilhuhn et al., 2012. Animals were trained to self-administer cocaine for 1h/d for 21 days. Using in vivo fast scan cyclic voltammetry, Wilhuhn et al., revealed that during the first week of cocaine experience dopamine levels
increased in the NAcC experience, but during the second and third weeks of cocaine self-administration dopamine levels increased in the dorsolateral striatum (2012). Behavioral results from this study recapitulate those findings from Wilhuhn et al., showing increased drug-seeking behavior with increasing duration of cocaine experience that is associated with a shift in elevated dopaminergic signaling from the ventral to the dorsal striatum (2012). Hence, observations of elevated extracellular dopamine-mediated dynamic alterations in morphological, molecular, and functional profiles of astrocytes in culture may be relevant to dopamine effects in ex vivo experiments where an impact on astrocyte morphology and gene expression is observed. Specifically, in addressing effects of elevated extracellular dopamine on cultured astrocytes results suggest that these dramatic shifts in morphological, proteomic, and genomic profile rely on PARP1/CTCF. This novel dopamine mediated PARP1/CTCF-dependent mechanism of microtubule and cytoskeletal remodeling of astrocytes was accompanied by alterations in astrocytes function. Thus, investigation of the impact of elevated dopaminergic signaling on astrocytes relating how findings of DA-induced PARP1/CTCF mediated adaptations in astrocytes in vitro relate to morphological alterations ex vivo and in vivo is needed.

In last 5 to 10 years, emerging evidence reveals that astrocytes are heterogeneous and plastic in nature, presenting discrete region-dependent functional roles in regulating synaptic transmission. Moreover, models of dysregulated dopamine signaling in neurobiological diseases reveal region-specific aberrations in astrocyte form and function which contribute to progression
of disease. Together, these findings hold important implications for dopamine’s effects on astrocytic profiles in disorders associated with alterations in physiological dopaminergic signaling. Various studies have investigated the role of drug or cocaine exposure and experience on astrocytic profiles in various brain regions, including the hippocampus and striatum; however, the role of elevated extracellular dopamine in physiological conditions of cocaine experience has not been directly addressed. Here, results show astrocytes may be differentially sensitive to duration of elevated extracellular dopamine \textit{ex vivo} using a behavioral model of short- vs. extended-access cocaine self-administration associated with temporally dynamic elevations in dopaminergic activity in striatum. Future investigations would need to address the direct and indirect impact of dopamine in exerting morphological, functional, genomic, and proteomic changes in astrocytes in models of substance abuse. Moreover, there is a crucial need of investigation of dynamic astrocyte responses in form and function, their resulting interactions with synapses, and their role in promoting disordered behaviors in the face of dysregulated dopaminergic signaling.

5.6 Conclusion

Substance use disorder(s) represent a major public health issue, as individuals diagnosed with this disease often experience severe psychological and often physical distress with serious consequences in their ability to navigate and function normally in day-to-day life. The transition from voluntary drug-seeking to compulsive drug-seeking habits in substance use disorders depends on transfer of controls over behavior from the ventral to the dorsal striatum (Yager et al., 2015;
Everitt & Robbins, 2005; Everitt & Robbins, 2013; Everitt et al., 2008). Moreover, this transition of control relies on the aberrant recruitment of dopamine-dependent plasticity processes within spiraling neuronal circuits (Haber et al., 2000). Studies now reveal how exposure to drugs of abuse, including cocaine, also trigger morphological, molecular, and functional adaptations in astrocytes within the striatum which contribute to the intrastriatal transitions observed during the development of substance use disorders. In the past decade, emerging pharmacological interventions aiming to restore the astrocytic mechanisms responsible for maintaining neuronal homeostasis have focused on the role of drug-induced alterations in glutamatergic signaling in the NAc. Importantly, novel therapeutic approaches in regulating astrocytic glutamate activity, altered by chronic exposure to substances of abuse, have been found to prevent reinstatement and relapse in both preclinical and clinical investigations. While several studies show evidence of astrocyte roles in regulating extracellular glutamate concentrations in the NAc, there is a critical need for investigation of adaptations of astrocytes in the dorsal striatum, where associative dopaminergic mechanisms govern compulsive drug-seeking behavior and reinstatement of drug use. Importantly, drug-induced alterations in glutamatergic homeostasis maintained by astrocytes in the striatum may interact with dopaminergic mechanisms associated with astrocytes and neurons to promote aberrant plasticity which may contribute to the maintenance and progression of addictive-like behaviors. Thus, the lack of research on astrocytes and their interactions with dopamine represents an essential future direction in research of substance use
disorders. Taking advantage of the growing evidence that astrocytes play a critical regulatory role in glutamate and dopamine transmission in the striatum, this study explored how dopamine signaling drives pronounced structural and functional adaptations in cultured astrocytes. These adaptations are associated with distinct short- and long-term changes in the astrocyte genome that are consistent with transcriptional effects of the PARP1/CTCF complex and PARP1-mediated NAD\(^+\) signaling. Importantly, results highlight a novel dopamine-induced PARP1/CTCF-dependent process that drives morphological changes associated with changes in transcriptional profile in astrocytes exposed to elevated concentration of dopamine. This work also reveals astrocytes may be differentially sensitive to duration of elevated extracellular dopamine using a behavioral model of short- vs. extended-access cocaine self-administration associated with temporally dynamic elevations in dopaminergic activity in striatum.

Overall, the findings from this study illustrate temporally dynamic morphological and molecular alterations in astrocytes in the presence of elevated physiological dopamine; however, the direct impact of dynamic astrocyte response to dopamine on neuronal energy homeostasis, neuronal signaling, and behavioral output in the context of dopamine-related disorders remains to be examined. Thus, future experiments will aim to elucidate the direct impact of physiological elevated dopaminergic signaling on astrocytes ex vivo and in vivo, as well as assess how these dopamine-mediated adaptations relate to findings of PARP1/CTCF mediated adaptations in astrocytes in vitro. Finally, given the putative significance and clinical relevance of elevated dopaminergic signaling aberrations in models of
substance use disorders, as well as the essential and active roles that astrocytes play in CNS physiology, approaches aiming to understand and restore astrocytic control of dopaminergic homeostasis in brain regions critical for development and maintenance of drug-taking behaviors warrant future investigation. Therefore, exploring alternative strategies targeted at astrocytes to mediate withdrawal symptoms and prevent relapse represent important future directions in basic science and clinical research.
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APPENDIX A

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