ASTROCYTES REGULATE CORTICAL ACh RELEASE VIA KYNURENIC ACID: IMPLICATIONS FOR COGNITIVE IMPAIRMENTS IN SCHIZOPHRENIA

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ABSTRACT

The basal forebrain cortical cholinergic system (BFCS) innervating the prefrontal cortex (PFC) is essential for normal attentional processing and higher order cognition. Aberrations in this neural system contribute to cognitive deficits seen in various neuropsychiatric disorders, including schizophrenia. Dopamine (DA) and glutamate (Glu) are two neurotransmitters that have been shown to modulate cortical acetylcholine (ACh) release locally within the PFC and across distributed, yet functionally interconnected areas like the nucleus accumbens (NAC). The NAC aids in the linking of discriminative cues to salient stimuli and can bias the attentional resources of the BFCS under conditions of heightened motivation; modulation of cortical ACh by NAC DA and Glu represent a chemoanatomical representation of the NAC-BF-PFC circuit. Dysfunctional interactions between these neurotransmitter systems and brain regions are all implicated in schizophrenia.

Astrocytes, and their associated neuroactive ‘gliotransmitters,’ are non-neuronal sources that also influence chemotransmission. For instance, the neuroinhibitory metabolite kynurenic acid (KYNA) is synthesized and released by astrocytes and antagonizes α7 nicotinic ACh receptor function at physiological concentrations. Modest elevations or reductions in KYNA reduce or facilitate extracellular dopamine (DA) and
glutamate (Glu) release respectively. Dysregulation of endogenous KYNA may contribute to the pathophysiology of several neuropsychiatric disorders, including schizophrenia. Elevated KYNA is found in the brain tissue and cerebrospinal fluid of schizophrenic patients, and chronic neuroleptic treatment reduces brain KYNA concentrations, further implicating KYNA in the disorder. The present thesis determined whether KYNA regulates cortical ACh by local or transynaptic interactions and assessed a potential role for KYNA in PFC-mediated tests of cognitive flexibility that require switching response strategies between various stimulus dimensions (i.e. extra-dimensional shift, EDS) depending on dynamic task rules. Schizophrenic patients are characteristically impaired in performance of these tasks. The first set of experiments found administration of KYNA or its precursor kynurenine had minimal impact on cortical ACh. In contrast, reducing endogenous KYNA by inhibiting its synthesis augmented ACh release, indicating ACh is tonically inhibited by KYNA. Moreover, the second experiment revealed cortical ACh is tonically regulated by KYNA acting within the NAC, though the precise mechanisms underlying this effect remain to be elucidated. The final set of experiments assessed whether increased KYNA levels would impair ED shifting. We used an attentional set-shifting task that requires the animals to discriminate between two distinct stimulus dimensions for a food reward. On day 1 (Set 1), rats were trained to discriminate between stimuli (e.g. the black arm is always reinforced) until reaching a criterion performance level (8 consecutive correct responses). On day 2 (Set
2), animals were trained on the alternative (ED) discrimination strategy. Administration of kynurenine (50 mg/kg, i.p.), which transiently increases cortical KYNA levels by ~800% did not impair initial rule acquisition when administered prior to Set 1 training. On day 2 (Set 2) the kynurenine-treated animals were impaired in learning the EDS (28% reached criterion) relative to saline-treated controls (83% reached criterion). Direct intra-PFC infusion of kynurenine produced similar results as systemic kynurenine; treatment prior to Set 1 produced no deficit in rule acquisition while intra-PFC infusion of kynurenine prior to Set 2 caused a marked impairment in EDS (16% reached criterion). The present results, together with previous studies, suggest that elevated KYNA levels may play a role in the neurotransmitter dysfunctions and cognitive deficits seen in schizophrenia. As such, selective pharmacotherapies aimed at normalizing brain KYNA may therefore represent novel and potentially more efficacious treatments for cognitive dysfunction in schizophrenia.

**Keywords:** KYNA, cognitive flexibility, schizophrenia, ACh, glutamate, PFC
Dedicated to:

The Mother, because the title says it all

To Andy, I can’t imagine life without you

And to Kelly Weiler- you have *always* been an inspiration
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CHAPTER 1

INTRODUCTION

“Consciousness gradually loses its coherence. One's center gives way...No core holds things together, providing the lens through which to see the world, to make judgments and comprehend risk...”

Elyn Saks, The Center Cannot Hold: My Journey Through Madness

Schizophrenia is a debilitating disorder affecting approximately 1% of the general population worldwide (Lewis and Lieberman, 2000). Diagnosis usually occurs from the late teens to early twenties, and includes three main classes of symptoms that include positive, negative and cognitive symptoms. Positive symptoms include hallucinations, delusions, and disorganized thinking, representing an excess or distortion of normal function. In contrast, symptoms reflecting a loss of normal function constitute the negative symptoms of schizophrenia, and include anhedonia, flattened affect, social withdrawal, and deficits in motivation. Cognitive deficits represent the most stable and enduring of the disorder’s core features, and are characterized by impairments in attention, learning and memory, and executive functioning (Lewis and Lieberman, 2000).
Moreover, the development of efficacious treatments targeting cognitive dysfunction has proven exceptionally challenging. These core symptoms, united by a foundation of cognitive dysfunction, culminate to form a disorder in which patients cannot appropriately monitor and respond to the world around them.

1.1 Attentional processing and cognition

Attention represents a critical early mediator of cognitive function. Attentional processing is critical for acute stimulus detection and provides a framework for higher-order cognitive functions like learning and memory (Coull, 1998; Sarter et al., 2003; Pepeu and Giovannini, 2004). It involves the distribution of processing resources to salient or relevant stimuli (Coull, 1998), necessitating the detection, selection, and discrimination among stimuli and the active filtering of irrelevant stimuli (Sarter et al., 2003). Consequently, aberrations in the neural systems mediating effective attentional processing may underlie cognitive dysfunctions inherent to a variety of neuropsychiatric disorders.

1.2 Schizophrenia and attention

Deficits in aspects of attentional processing, including the selection and filtering of stimuli, contribute to cognitive dysfunction in schizophrenia. Specifically, the inability to focus attention and inhibit inappropriate responses may underlie problems in executive functioning (Waters et al., 2006). Consequently, schizophrenics cannot easily modify their response strategies to changing environmental cues, and are characteristically impaired in tasks of cognitive flexibility like the Wisconsin Card
Sorting Task (WCST; Weinberger and Gallhofer, 1997; Robbins, 2007). A recent study measuring automatic shifts of attention found that reduced attentional engagement in schizophrenics mediates deficits in voluntary responding (Reilly et al., 2008).

Attentional impairments compound preexisting cognitive dysfunction, culminating in an inability to focus attention and filter distracters (Reilly et al., 2008). Affected adults often show impairments in sustained attention (Michie et al., 2000; Addington and Addington, 1997; Thaden, et al., 2006) as do their unaffected siblings (Michie et al., 2000; Delawalla et al., 2008). Moreover, attentional deficits in childhood may be a marker for later schizophrenic development (Cornblatt and Erlenmeyer, 1985), and are an enduring feature of the disorder (Addington and Addington, 1997; Thaden et al., 2006). As such, chronic impairments in attentional processing provide an unstable foundation with which to interact with the world, thus spawning and nurturing chronic cognitive impairment.

1.3 The basal forebrain cholinergic system and regulation of attentional processing

The basal forebrain (BF) is a key structure in the distributed systems responsible for regulation of attentional resources (Figure 1). Anatomically, it sends cholinergic, glutamatergic, and GABA-ergic projections to cortical areas (Mesulam and Geula, 1988; Eckenstien, et al., 1988; Zaborsky and Duque, 2000; Manns et al., 2001, 2003). Efferents project in a topographic manner with target areas receiving input from distinct BF nuclei (Zaborsky et al., 1999; Eckenstien, et al., 1988). The prefrontal cortex (PFC) receives the majority of its cholinergic input from the nucleus basalis of Meynert/nucleus basalis
magnocellularis (Ch4) region of the BF (Mesulam and Geula, 1988; Eckenstien, et al., 1988).

A vast body of evidence highlights the central role of the basal forebrain cholinergic system (BFCS) in attention. Cortical cholinergic transmission is critical for performance in sustained and visuo-spatial attentional tasks (Dalley et al., 2004b; McGaughy et al., 1996; McGaughy and Sarter, 1998; Muir et al., 1994), and performance in these tasks stimulates release of acetylcholine (ACh) in cortical regions known to play key roles in the mediation of attentional processing (Arnold et al., 2002; Dalley et al., 2001; Himmelheber et al., 2000; Passetti et al., 2000). Excitotoxic lesions of the BF, or lesions using the selective cholinergic toxin 192-IgG saporin, increase response latencies in various attentional tasks (Turchi and Sarter, 1997; McGaughy et al., 1996; McGaughy and Sarter, 1998; Muir et al., 1994), and the degree of performance deficits correlate with reductions in cortical AChE-positive fiber density (Turchi and Sarter, 1997; McGaughy et al., 1996). Importantly, intra-basalis infusions of drugs that bi-directionally modulate cholinergic efferent activity have correlated impacts on attentional performance (Holley et al., 1995). Furthermore, administration of muscarinic antagonists impairs performance in tasks that require attentional processing in humans (Koller et al., 2003; Mauri et al., 1994) and laboratory animals (Chudasama et al., 2004; Chen et al., 2004) and increases in BFCS activation correlate with challenges to attentional performance (Himmelheber et al., 2000; Gill et al., 2000; Kozak et al., 2006). Finally, increased cortical cholinergic activity after administration of nicotinic receptor agonists (e.g. epibatidine and ABT-418) may improve attentional processing in rats (Hahn et al., 2003) and humans (Kumari et al.,
Collectively, the evidence clearly demonstrates a central role for the BFCS in attention; since attentional processing is a critical early mediator of higher-order cognitive processes, aberrations in the functional integrity of the BFCS may contribute to cognitive impairments in schizophrenia.

Given that the integrity of the cortical cholinergic system holds such significance for normal attentional processing, and therefore higher order executive functions, understanding the distributed systems governing its regulation is critical. The nucleus basalis of Meynert/substantia innominata (nbm/SI) region of the BF receives excitatory inputs from multiple regions including the cortex, amygdala and brainstem (Kirouac and Ganguly, 1995; Zaborsky et al., 1997). The ventral tegmental area (VTA), thalamic nuclei, and lateral hypothalamus provide additional excitatory and neuromodulatory input to the BF (Grove, 1988; Givens and Sarter, 1997). Of these various converging inputs, the role of inhibitory projections from the nucleus accumbens (NAC) in regulating BFCS activity is a central focus of this thesis (Zaborsky et al., 1997; Mogenson et al., 1983). With its roles in motivation and reward (Wyvell and Berridge, 2001; Yun et al., 2004a,b), and translation of limbic information to motor output (Kelley, 1999), the NAC is a critical component to the attentional functions of the BFCS.

1.4 Nicotinic cholinergic receptors

The PFC contains an extensive population of cholinergic receptors, including high levels of both muscarinic and nicotinic receptors. Muscarinic subtypes are single subunit metabotropic G-protein coupled receptors, designated M1-M5 (Gu, 2002; van der Zee
and Luiten, 1999). They are widely expressed in cortical and in various sub-cortical regions, evoking slow-acting neuronal responses through second messenger systems (Levey et al., 1991; Wamsley et al., 1981; Hyde and Crook, 2001). In contrast, neuronal nicotinic receptors are fast-acting ligand-gated ion channels. In humans and rodents, nicotinic receptor binding is primarily present on pyramidal cells (Bravo et al., 1992; Lobron et al., 1995) and GABAergic interneurons (Krenz et al., 2001; Albuquerque et al., 2000; Bandyopadhyay et al., 2006) in layers I, II, and V of the cortex, with additional layers and cell types showing activity (Sihver et al., 1998; Marutle et al., 1998; Tribollet et al., 2004; Lavine et al., 1997). The nAChR consists of five trans-membrane combinations of various α and β subunits circling a central water-filled pore (Dani and Bertrand, 2007). They can be found both pre- and post-synaptically, allowing fine tuning of transmitter release and neuronal excitability (Lubin et al., 1999; Sher et al., 2004).

Nicotinic ACh receptors containing α4β2 subunits are the most common of these subtypes; they are distributed throughout the cortex, hippocampus, thalamus, and in various subcortical and brainstem regions (Wada et al., 1989; Gotti et al., 2006; Milhailescu and Drucker-Colin, 2000; see also Sher et al., 2004). They are found pre-synaptically as both auto- and heteroreceptors (Wilkie et al., 1996) and post-synaptically as well (see Sher et al., 2004). Of the nicotinic receptors, those containing α4β2 have the highest affinity for nicotine and have roles ranging from working memory to mediating the rewarding effects of nicotine (Broide and Leslie, 1999).
1.5 The α7 nicotinic cholinergic receptor: localization and function

A few nicotinic subunits are capable of forming homomeric receptors: the α7, α8, and α9 units. Of these, the α7 subunit is the only one widely expressed in the brain (Sher et al., 2004; Dani and Bertrand, 2007). These receptors have a low affinity for their endogenous ligands ACh and choline, are blocked by αbungarotoxin (αBgtx), and are rapidly activated and desensitized (Papke et al., 1996; Albuquerque et al., 2000; Dani and Bertrand, 2007). High concentrations of α7 nACh receptors are found in the cortex and hippocampus, and are also distributed throughout various subcortical regions including the amygdala, VTA, and others (Gotti et al., 2006; Sher et al., 2004). In contrast, low levels are found in the basal ganglia and thalamic regions (Gotti et al., 2006).

Homomeric α7 nACh receptors are extremely permeable to Ca$^{+2}$ having a fractional current ranging from 6-12% (Fucile, 2004; Fucile et al., 2003). In contrast, heteromeric nicotinic receptors have a much lower fractional current with Ca$^{+2}$ mediating only 2-5% of the whole-cell current (Fucile, 2004). Stimulation of nACh receptors activates voltage-gated Ca$^{+2}$ channels (Rathouz and Berg, 1994), and can also generate Ca$^{+2}$ currents in the absence of cell depolarization by permitting ion flux directly through the receptor pore (Dajas-Bailador and Wonnacott, 2004; Rathouz and Berg, 1994; Dajas-Bailador et al., 2002). The unique ability to modulate many sources of Ca$^{+2}$ underscores the multi-faceted and powerful role of the α7 receptor in modulating neuronal receptivity, transmitter release, and synaptic communication.
Presynaptic α7 nACh receptors located on glutamatergic terminals have especially important roles in facilitating neurotransmitter release (Gotti et al., 2006; Sher et al., 2004), though evidence does not support a role for these as autoreceptors on cholinergic terminals (Wilkie, et al., 1996). Activation of α7 nACh receptors facilitates the release of multiple neurotransmitters, thereby providing many opportunities for modulation of synaptic communication. Stimulation of pre-synaptic α7 receptors directly facilitates glutamate (Glu) and γ-aminobutyric acid release (GABA; Wonnacott et al., 2006; Dani and Bertrand, 2007). Dopamine (DA), norepinephrine and serotonin are indirectly modulated by α7 receptor-induced facilitation of Glu and GABA release in various brain regions (Kaiser and Wonnacott, 2000; Gotti et al., 2006; Wonnacott et al., 2006; Dani and Bertrand, 2007; Sher et al., 2004; Gotti et al., 2006). The α7 nACh receptor is located post-synaptically on pyramidal cells (Bravo and Karten, 1992; Lobron et al., 1995; Lubin, et al., 1999), and other cell types (Lubin, et al., 1999; Lavine et al, 1997). They are distributed on somato-dendritic areas of GABAergic and glutamatergic neurons in the cortex, and on cholinergic and GABAergic interneurons of the cortex, hippocampus and in multiple subcortical regions (Lubin et al., 1999; Alberquerque et al, 2000; Krenz et al., 2001; Sher et al., 2004; Gotti et al., 2006). Ultrastructural analyses reveal α7nAChR are expressed almost ubiquitously in the CA1 stratum radiatum region of the hippocampus (Fabian-Fine et al., 2001). Functionally, nAChR activity can relay inhibitory or disinhibitory information to pyramidal neurons, providing ample substrate for modulation of cortical neuronal circuits (Alkondon et al., 2000). The role of the α7 receptor extends well beyond regulation of neurotransmitter release, having critical involvement in neuronal differentiation and synapse formation in the developing nervous
system, and in higher order cognitive processes like learning and memory (Adler et al., 1998; Chan et al., 2007; Ochoa and Lasalde-Dominicci, 2007).

1.6. Schizophrenia and the cholinergic system

Along with attentional impairments, several other lines of evidence implicate an abnormal cholinergic system in the pathophysiology of schizophrenia. It has been shown that muscarinic receptors are altered in a regionally-specific manner, with increased binding found in the orbital frontal cortex and putamen, and decreased receptor binding in parietal, temporal and frontal cortices (see Hyde and Crook, 2001). Moreover, the severity of positive symptoms correlates with reduced densities of muscarinic M1 and M4 receptors in the PFC (Raedler et al., 2003; Crook et al., 2001). High-affinity nACh receptors are reduced in various brain regions including the cortex, hippocampus, striatum and thalamus (Breese et al., 2000). Reductions in α7 nACh receptors have been reported in the hippocampus and frontal cortex, but not in parietal cortex (Freedman et al., 1995; Hyde and Crook, 2001).

A vast majority (80-90%) of schizophrenics smoke (Ochoa and Lasalde-Dominicci, 2007). Moreover, elevated urinary concentrations of cotinine, a nicotine metabolite, were found in schizophrenic patients compared to controls matched for smoking frequency (Olincy et al., 1997). Patients utilized deeper inhalation patterns while smoking, enabling greater extraction of nicotine per cigarette than controls (Olincy et al., 1997). Heavy smoking in these patients has been hypothesized as a form of self-medication to reduce anxiety, improve mood and alleviate certain deficits in information
processing (see Janunen and Ahtee, 2007). Moreover, chronic smoking up-regulates nAChRs in controls, but fails to do so in schizophrenics (Breese et al, 2000; Ochoa and Lasalde-Dominicci, 2007; Levin and Rezvani, 2007), indicating cholinergic receptor activity is functionally abnormal as well.

Schizophrenics characteristically show impairments in sensorimotor gating like prepulse inhibition (PPI), further implicating aberrant cholinergic system in the pathophysiology of schizophrenia (Leonard and Freedman, 2006). In normal individuals, presentation of two auditory stimuli 500 msec apart yields a characteristic auditory evoked potential (P50) to the first stimulus that diminishes with presentation of the second stimulus. This P50 response in not voluntarily controlled, and as such, represents an unconscious inhibitory mechanism for the filtering of extraneous stimuli (Leonard and Freedman, 2006). In contrast to control subjects, schizophrenics are unable to suppress responding to the second tone, reflecting a deficit in sensory gating (Adler et al., 1998; Ochoa and Lasalde-Dominicci, 2007). The P50 gating deficit is widespread, found in upwards of 90% of patients and in 50% of their unaffected relatives (George et al., 2006). Functionally, this translates to an inability to filter competing stimuli, and further drains already compromised processing resources. Patients who smoke have comparable PPI to unaffected controls, an effect that is worsened with smoking abstinence and improved by acute nicotine exposure (Hyde and Crook, 2001; George et al., 2006; Martin et al., 2004). Administration of mecamylamine, a nACh receptor antagonist, attenuates nicotine-facilitated improvements in sensory gating indicating central nicotinic receptor activity mediates this filtering (George et al., 2006). Impairments in P50 response suppression
are also associated with problems in sustained attention (Martin et al., 2004).

Administration of nicotine, or nicotinic agonists, improves learning and memory (Levin and Rezvani, 2007) and galantamine, an allosteric potentiator of the α7 receptor currently used in the treatment of Alzheimer’s disease, may be a useful adjunct to currently utilized therapies, as has been shown with the atypical neuroleptic risperidone (see Ochoa and Lasalde-Dominicci, 2007). Moreover, the exaggerated frequency and intensity of smoking in schizophrenic patients may enable nicotine concentrations to be sufficiently elevated to activate low-affinity α7 receptors, thereby improving sensory gating defects by an α7 nACh receptor-dependent mechanism (Adler et al., 1998).

The widespread sensory gating deficits in patients and their unaffected relatives and the heritability factor in schizophrenia implicate cholinergic system genetic abnormalities in disease vulnerability (Martin et al., 2004). The P50 response is linked to the 15q14 chromosome of the α7 receptor gene, CHRNA-7, implicating reductions in α7 receptor function in deficient P50 inhibitory responses (Freedman et al., 1997). A recent study suggests certain polymorphisms of the choline acetyltransferase (ChAt) gene are associated with increased susceptibility to schizophrenia and in responsiveness to olanzapine treatment (Manama et al., 2007). Collectively, the above examples clearly implicate a substantial role for disturbances in the cholinergic system in the pathophysiology of schizophrenia.
1.7. Astrocytes and neuronal communication

While studies on neuronal communication have focused on the influence of other neurotransmitter systems, the importance glial activity plays in regulating neuronal transmission is becoming increasingly evident. Traditionally, astrocytes were conceptualized to interact via passive associations with neurons. Their roles were thought to be restricted to providing structural support and maintainence of the neuronal territories they innervate (Halassa et al., 2007; Fields and Stevens-Graham, 2002). However, mounting evidence suggests astrocytes are morphologically and behaviorally suited for dynamic interactions with neurons.

Anatomically, astrocytes are located in close apposition to both pre- and post-synaptic cells. Astrocytes have extensive end processes that can reach 100 µM from the cell body and make contact with upwards of 300-600 dendrites (Halassa et al., 2007). This intimate association provides ample opportunity for astrocyte-neuron communication. In addition to contacting neuronal cells, astrocytes also innervate nearby capillaries (Nemeth et al., 2005; Halassa et al., 2007), enabling efficient sequestering of nutrients from the blood supply. This regulates both the blood flow and metabolic requirements of the synaptic microenvironment according to energy needs (Halassa et al., 2007).

Astrocytes utilize various strategies to monitor and refine neuronal activity. They can respond to presynaptic neurotransmitter release; they contain receptors and transporters for a variety of neurotransmitters, including Glu, GABA, ACh and serotonin
(Hatton, 2002; Fellin and Carmignoto, 2004), indicating astrocytes have rich interactions with various neurotransmitter systems. As such, astrocytes monitor and can modulate Glu release from neighboring neurons. They can dynamically alter the length and reach of their processes, fine-tuning information transfer by adjusting their proximity to neurons and regulating the rate of Glu clearance from the synapse (Hatton et al., 2002; Piet et al., 2004; Ni et al, 2007; Oliet et al, 2007), perhaps increasing the specificity of information transferred.

The strength of incoming stimuli may modify the nature of astrocytic communication. For instance, low frequency stimulation elevates Ca\(^{+2}\) levels only within a single astrocyte. In contrast, high frequency stimulation yields spreading waves of Ca\(^{+2}\) that may extend to neighboring astrocytes (Fellin and Carmignoto, 2004; Ni et al., 2007). Binding of astrocytic glutamate receptors can elevate intracellular Ca\(^{+2}\) levels, stimulating glutamate release from astrocytes themselves, and subsequently impacting neuronal excitability (Araque et al., 2001; Carmignoto and Fellin, 2006). Propagating Ca\(^{+2}\) waves can extend hundreds of micrometers, providing a mechanism for neuronal synchronization by glia (Halassa, 2007; Araque et al., 2001). Collectively, this evidence suggests a sophisticated role for astrocytes in the fine tuning of information processing and the efficacy of synaptic transmission.

Astrocytes may also communicate directly with neurons by releasing a multitude of ‘gliotransmitters.’ These are glial-derived neuroactive compounds capable of dramatically influencing neuronal behavior. Glycine, D-serine, and Glu are examples of
the neuroactive chemical messengers released by astrocytes capable of impacting synaptic strength and communication (Guthrie 1999; Halassa et al., 2007).

1.8. The Kynurenine pathway

While the gliotransmitters mentioned above may all have roles in maintaining astrocyte-neuron relationships, much attention has recently focused on the role of neuroactive compounds generated by the kynurenine pathway (KP; Figure 2) in neuronal support and communication. The KP begins with the breakdown of tryptophan into L-kynurenine and ultimately generates the essential co-factors nicotinic acid and nicotinamide adenine dinucleotide (NAD+; Stone, 2001).

L-Kynurenine is generated in the periphery and the CNS by the enzymes tryptophan dioxygenase (TDO) and indolamine dioxygenase (IDO), respectively (Schwarcz and Pellicciari, 2002) and readily transported across the blood brain barrier by the neutral amino acid transporter (Nemeth et al., 2005; Speciale and Schwarcz, 1990). Its breakdown in various glial cells generates metabolites that potently modulate neuronal excitability (Nemeth et al., 2005). Two microglia-derived products, 3-hydroxykynurenine (3HK) and quinolinic acid (QUIN) are neurotoxic; 3HK generates free radicals while QUIN is excitotoxic. In contrast, kynurenic acid (KYNA) is an inhibitory KP derivative that functionally opposes 3HK and QUIN (Figure 2; Wu et al., 2000; Nemeth et al., 2005; Schwarcz and Pellicciari, 2002). Under normal conditions, the KP is primarily neuroprotective. However, disruptions between these KP intermediaries are implicated in various conditions including Huntington’s disease,
Alzheimer’s disease, AIDS dementia complex (especially in patients with psychotic symptoms), ischemia, schizophrenia, and many more (see Nemeth et al., 2005; Baran et al., 1999; Guillemin et al., 2001; Stone and Darlington, 2002; Atlas et al., 2007).

1.9. Kynurenic acid and neuronal communication

KYNA is an inhibitory metabolite produced in a dead-end arm of the KP with neuroprotective and anticonvulsant properties. Several studies have demonstrated that reducing endogenous KYNA concentrations increase the vulnerability to excitotoxic insults (Carpendo et al., 1994; Poeggeler et al., 1998; Erhardt et al., 2000). At physiologically-relevant concentrations, KYNA acts as a non-competitive antagonist at α7 nAChRs and can also competitively block the glycine site of the NMDA receptor complex (Hilmas et al., 2001; Erhardt et al., 2000; Schwarcz and Pellicciari, 2002). It is generated from the breakdown of kynurenine by the kynurenine aminotransferase (KAT) enzymes (Figure 2; Schwarcz and Pellicciari, 2002; Swartz et al., 1990; Turski et al., 1989) and is generated preferentially in astrocytes, as microglia and neuronal cells lack the enzymatic machinery necessary for KYNA production (Swartz et al., 1990; Schwarcz and Pellicciari, 2002; Guillemin et al., 2001). KAT–reactive astrocytes are distributed throughout several regions including the neocortex, striatum, thalamus, substantia nigra, hippocampus and cerebellum (Guidetti et al., 2007). Furthermore, many of these astrocytes are found contacting capillary walls, thereby providing strategic positioning for the efficient acquisition and transportation of blood-borne kynurenine into astrocytes (Guidetti et al., 2007).
The majority of KYNA in the brain is produced locally, as very little crosses the blood brain barrier (Swartz et al., 1990). Following generation, it is rapidly released into the extracellular space to modulate neuronal signaling. KYNA influences neuronal communication by modulating the release of neurotransmitters like DA and Glu through antagonism of presynaptic α7 nAChRs (Rassoulpour et al., 2005; Kaiser and Wonnacott, 2000; Wu et al., 2007; Carpendo et al., 2001; Alkondon et al., 2004).

Humans and rodents exhibit two distinct kynurenine aminotransferase (KAT) enzymes, KAT I and KAT II, responsible for the irreversible catabolism of kynurenine to KYNA (Guidetti et al., 1997). Two additional KYNA-generating enzymes have been recently characterized, though neither appear to contribute significantly to KYNA generation in either the rat or human (Guidetti et al., 2007; Yu et al., 2006). Despite a lower expression than KAT I, KAT II generates approximately 75% of available KYNA in rodents, and has a predominant role in human KYNA production as well (Gramsbergen et al., 1997; Guidetti et al., 1997; Kiss et al., 2003). Additionally, KAT II operates most efficiently at a physiological pH, whereas KAT I has a much higher optimum pH of 9.5-10 (Schwarcz and Pellicciari, 2002).

Localization studies reveal KAT II is expressed exclusively in astrocytes, with no discernable labeling present in neurons or microglia (Guidetti et al., 2007). Reactive astrocytes are widely distributed, found throughout the neocortex, striatum, thalamus, hippocampus, substantia nigra, and cerebellum (Guidetti et al., 2007). Moreover, these astrocytes are found in close contact to nearby capillaries, enabling efficient harvesting of
kynurenine from blood supplies (Guidetti et al., 2007; Speciale et al., 1989; Turski et al., 1989).

1.10. KYNA and Schizophrenia

Though not typically known as an astrocytic disorder, an intriguing role for KYNA in schizophrenia is emerging. Elevated KYNA levels have been found in both the cerebral spinal fluid (CSF; Erhardt et al., 2001) and post mortem brain tissue of schizophrenic patients (Schwarcz et al., 2001; Nilsson-Todd et al., 2007). Moreover, KYNA levels are normalized following treatment with first or second generation antipsychotics (Ceresoli-Borroni et al., 2006). In parallel with the therapeutic latency for clinical efficacy, KYNA is only attenuated by chronic neuroleptic exposure. Interestingly, elevating endogenous KYNA in rats significantly disrupted PPI, though PPI was restored following administration of haldol or clozapine (Erhardt et al., 2004). Attenuation of pathological elevation in KYNA may be a mechanism by which neuroleptics achieve their therapeutic efficacy.

The neural systems mediating attentional processing work in concert to optimize the detection of stimuli under changing conditions. As a critical early mediator of higher order cognitive processes, compromises in the functional integrity of the anatomical areas or neurotransmitter systems regulating attention may lead to distortions in information processing, and could ultimately contribute to pathological dysfunctions in cognition. Given the potent inverse relationship between KYNA and the various neurotransmitters that positively modulate cortical ACh release, and based on evidence implicating elevated
KYNA in schizophrenia, we propose KYNA may have a regulatory capacity over the BFCS as well. The experiments in this thesis will link KYNA, and hence astrocytic activity, to the regulation of basal ACh release in the PFC using the overall working hypothesis: **If the astrocyte-derived metabolite KYNA has a critical role in the regulation of basal ACh transmission, then changes in extracellular levels of KYNA will modulate ACh release both locally in the PFC and in functionally interconnected areas such as the nucleus accumbens (NAC). Furthermore, these KYNA-based changes should also manifest in changes in PFC-based cognitive behaviors, such as cognitive flexibility measured by an attentional set shifting task.**

Three main experiments were conducted to examine the role of KYNA on cortical ACh release: Experiment 1 first tested the capacity of KYNA to modulate basal ACh transmission in the PFC and then extended this modulation to the role of *endogenous* tonic concentrations of KYNA. Since the enzymatic machinery necessary for the generation of KYNA is primarily located in astrocytes, this experiment tests the capacity of *astrocytic* function to regulate ACh. Experiment 2 determined whether this astrocytic regulation of PFC ACh release can also be evidenced following KYNA manipulations in the NAC. The NAC was chosen based on its essential role in modulating the detection and selection of stimuli for processing, which involves the recruitment of attentional resources and modulation of ACh transmission in the PFC. Integrating the first two experiments, Experiment 3 used an attentional set shifting task to extend KYNA’s role into the realm of cognitive flexibility. The collective evidence clearly implicates KYNA and ACh dysfunction in schizophrenia, though the functional relationship between them
remains unknown. The results from these experiments are expected to aid substantially in characterizing KYNA and ACh interactions, and may ultimately lead to the development of novel pharmacotherapies for the treatment of schizophrenia.
CHAPTER 2

GENERAL METHODS

2.1. Subjects

Male Wistar rats (Charles River Labs, Wilmington, MA, USA) weighing between 300-400 grams were utilized for all studies. Animals were maintained on a 12:12 hour light:dark cycle (lights on: 0600) in a temperature- and humidity-controlled room. Animals were individually housed in plastic cages lined with corn cob bedding (Harlan Teklad, Madison, WI, USA) and had access to food and water *ad libitum*. All procedures involving animals were approved by The Ohio State University Institutional Animal Care and Use Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Three days prior to surgery, animals were acclimated to the microdialysis testing environment in clear plastic bowls (35 cm height x 38 cm diameter; CMA, Stockholm, Sweden) lined with corn cob bedding. Animals remained in the bowls for a minimum of four hours/day, and returned to their home cages at the end of each acclimation period.
2.2. Surgery

Following their three days of acclimation, animals were anesthetized using isoflurane gas (2%, 0.6 L/min, O₂ delivery) and unilaterally implanted with microdialysis guide cannulae (0.38 mm o.d.; Sci Pro Inc., Sanborn, NY, USA) into the mPFC (A = 4.2, L = 0.6, V = 0.6 mm, 20º rostral angle relative to Bregma and dura). Additional groups of animals were implanted with a guide cannula in the mPFC as described above, plus an additional cannula implanted in the ipsilateral shell region of the NAC (A = 1.3, L = 1.0, V = 5.8, vertical placement, relative to Bregma and dura). Animals used in set-shifting tasks were implanted with bilateral microdialysis cannulae in the mPFC (infusion cannulae: A = 4.2, L = 0.6, V = 1.5 mm, 20º rostral [one hemisphere] and P = 1.5, L = 0.6, V = 1.5 mm, 20º caudal [the other hemisphere] angle relative to Bregma and dura). All guide cannula were fixed to the skull using stainless steel screws and dental acrylic. Stylets, ending flush with the guide cannula, were inserted to prevent cannula occlusion. The surgical site was swabbed with a topical antibiotic ointment (Neosporin), and animals also received a prophylactic dose of the antibiotic chloromphenicol (100 mg/ml; 0.125 ml administered subcutaneously). Following surgery, animals were returned to their home cages and allowed to recover for 3 days prior to microdialysis testing or resuming behavioral training.

2.3. General microdialysis procedures

Microdialysis sessions were conducted using repeated perfusions, with each animal receiving three different pharmacological manipulations, one every other day. This repeated testing paradigm has the advantage of decreasing variability among
treatment conditions because each subject serves as its own control. Furthermore, it allows for paradigms such as dose-response analyses, as well as agonist-antagonist interactions to be studied in the same animal. The procedure has been repeatedly validated by demonstrating that basal cortical ACh efflux does not significantly change over repeated dialysis sessions, and that the effects of behavioral, pharmacological, or sensory manipulations on ACh levels do not interact with dialysis sessions (Bruno et al., 1999; Nelson et al., 2002).

On the fourth day following surgery, animals were brought to the testing environment, stylets were removed and probes (Sci Pro, Inc., 0.2 mm o.d., 3 mm membrane tip for PFC, 0.2 mm o.d., 2.0 mm membrane tip for NAC) were inserted into each guide. Inlet and outlet lines for the dialysis probes were attached to a two-channel liquid swivel (Instech, Plymouth Metting, PA).

Probes were continuously perfused with artificial cerebral spinal fluid (aCSF) (containing in mM: NaCl 166.5, NaHCO3 27.5, KCl 2.4 CaCl2 1.2, Na2SO4 0.5, KH2PO4 0.5, glucose 1.0, pH 7.1) at a flow rate of 1.25 μl/min. No acetylcholinesterase inhibitor was utilized in any experiment. A three-hour wash out period was observed after probe insertion to allow ACh efflux to reach a stable baseline that was maximally sensitive to TTX, and therefore impulse-dependent, before beginning collections (Moore et al., 1992). All microdialysis experiments conducted followed this general paradigm.
2.4. Quantification of acetylcholine

Dialysis samples were stored at -80º C until analyzed using high performance liquid chromatography (HPLC) with electrochemical techniques. A volume of 15 µl of each sample was injected by an autosampler (ESA Inc., Chelmsford, MA). ACh and choline were separated by an UniJet microbore analytical column (BAS Inc.; 1 x 50 mm) using a sodium phosphate mobile phase (35 mM Na₂HPO₄, 484 μM EDTA, 0.005% microbicde reagent Proclin, pH = 8.5), flowing at 0.15 ml/min. A post-column IMER containing acetylcholinesterase and choline oxidase was used to break down ACh into H₂O₂ (Potter et al., 1983), and quantified using a peroxidase-coated glassy carbon electrode.

2.5. Histology

Following the final microdialysis session, animals were given an overdose of sodium pentobarbital and trans-cardially perfused with 0.9% heparinized saline followed by 10% formalin. Brains were removed and stored in 10% formalin for at least 24 hours, and were then transferred to a 30% sucrose solution for three days. Brains were sectioned using a cryostat and sections (50 µm) were mounted on gelatin-coated slides, stained using Cresyl Violet, and examined under a light microscope. Subjects whose probe and injection cannula placements were located outside any of the targeted regions were excluded from further analysis.
2.6. Data analysis

Changes in basal ACh efflux (fmol/15 µl) across sessions and treatment groups were analyzed using one-way repeated measures analysis of variance (ANOVAs). Basal efflux was then defined as the mean of the four baseline collections, and subsequent data were expressed as percent change from that mean baseline. Statistical analysis of drug effects was conducted using a two-way, within-subjects ANOVA with drug GROUP and TIME as within-subjects measures. Significance was defined as $P < 0.05$, and the Huynh-Feldt correction was utilized to reduce Type I errors associated with repeated measures ANOVAs (Vasey and Thayer, 1987). All statistical tests were preformed using SPSS for windows (version 16.0).

2.7. Attentional Set-shifting

Apparatus

The set-shifting maze is constructed out of pressed wood (0.7 cm thick) and is sealed with a polyurethane primer. The maze is composed of a central platform (14 cm each side) and four arms (40 x 14 x 20 cm each) radiating from the central platform. A hexagonal plastic food dish was placed at the end of each arm and was sufficiently deep where a food reward could not be seen when entering the arm. Maze arms varied along two stimulus dimensions: brightness and texture. Two arms were covered with a faux leather material in black and the other two were covered in the identical material in white. The smooth texture (on the floor) consisted of the same faux leather material covering the walls, and the rough texture consisted of rough grade sandpaper spray painted either black or white to correspond with the appropriate colored arms. Arm combinations were:
white/smooth, white/rough, black/smooth and black/rough. The maze was attached to a rotary platform. The holding cage used for inter-trial intervals was a standard rat housing cage lined with corn-cob bedding.

**Set-Shifting Training**

All training and testing paradigms were modeled after procedures used by Stefani and Moghaddam (2005). Animals were first food restricted (given 15-20 g/day) and maintained at a minimum of 85% of their free-fed weight. During the pre-acclimation phase, animals were given a food reward consisting of a sugary cereal (Froot Loops) in their home cages to familiarize them with the taste and odor of the reward, and to reduce possible neophobic effects of reward introduction in the maze.

Following a week of handling and food restriction, animals began one of two phases of maze acclimation. Animals were acclimated in a maze that differed in stimulus dimensions from the testing maze, and conducted all acclimation procedures in the training maze before moving to the testing maze to begin Set 1 training. The training maze was constructed from identical dimensions as the testing maze, though the brightness stimuli included dull (plain wood walls) and shiny (aluminum foil covered walls), and the texture stimuli consisted of wood (plain wood flooring) and cotton (plastic-backed cotton material). During the first day of acclimation, multiple animals were placed in the maze and were allowed 10 minutes to explore and consume any food rewards there. Following this first day, animals were individually placed in the maze in which all four food wells were baited with pieces (1/3 of an individual cereal piece) of
Froot Loops. Rats were given 10 minutes to roam freely and explore the apparatus, to condition the rats to receiving food rewards while in the maze. On subsequent days of acclimation, each food well was baited with one piece of Froot Loop per well, and rats were allowed to explore until all food had been consumed, up to 10 minutes. Upon removal from the maze, rats were briefly placed in a holding cage before returning to their home cages. Surgical implantation (see above) of injection cannulae were done following this first phase of acclimation.

During the second phase of acclimation, the maze was set up in a T configuration (with a divider placed to block one arm). Animals received 8 trials per day, consisting of two starts from each arm. Animals were placed in the central arm, allowed to turn left or right (choosing one arm) and consume any food reward there. Arms were reinforced randomly. Between trials, rats were removed from the maze and placed in a holding cage (inter-trial interval approx. 15 s). Once animals actively and reliably turned left or right (made a choice) and readily consumed any available food reward, they were advanced to set shifting testing the following day. In most cases, this second phase of acclimation took 1-4 days.

**Set-Shifting Testing**

The attentional set-shifting task consists of two sessions on consecutive days, with the maze set up in a T configuration, like during the second phase of acclimation. On day 1 (Set 1) animals were randomly trained to discriminate between brightness (black verses white arms) or texture (smooth verses rough arms) dimensions, and were trained to reach
a criterion performance level consisting of 8 consecutive correct trials (Stefani and Moghaddam, 2005). Animals were allowed 2 minutes to make a ‘choice’ by which they needed to leave the start arm and enter one choice arm far enough so all four feet passed into that arm. Animals were allowed up to 120 trials to reach criterion, and any individuals who failed to learn the initial discrimination after 120 trials were removed from study. On day 2 (Set 2), animals were trained on the alternative (extra-dimensional) discrimination strategy for 80 trials, regardless of performance level to allow for a normalized basis of comparison (Stefani and Moghaddam, 2005).

**Set-Shifting Data Analysis**

All statistical analyses were conducted according to tests described in Stefani and Moghaddam (2005). Dependent measures including trials and time to criterion (Set 1) and time to criterion (Set 2) were analyzed using one-way analysis of variance (ANOVA). Because the trials to criterion for Set 2 were restricted under a cap of 80 trials (Stefani and Moghaddam, 2005), these data were analyzed using the non-parametric Kruskal-Wallis test. The percentage of correct scores was analyzed for each 8-trial block (over 10 consecutive blocks) to examine performance across blocks of trials for Set 2. Performance across trial blocks was analyzed using mixed-design 2-way, repeated measures ANOVAs, with drug treatment as the between subjects variable and trial block as the within subjects measure (Stefani and Moghaddam, 2005). Analysis of perseverative responding during Set 2 was conducted by comparing the percent error scores from each start-arm designation. Animals made perseverative errors when they chose arms that were previously reinforced on day 1 but were irrelevant according to the
new rule of day 2. Reinforced responses were choices that were previously reinforced on day 1 and still relevant for the new rule of Set 2. For example, animals that learned to choose the black arm during Set 1 but need to learn the smooth arm for Set 2 would be making perseverative errors if they repeatedly chose the black/rough (incorrect) arm over the white/smooth (correct) arm. Animals may also make errors in which they fail to learn the new strategy and repeatedly choose into never-reinforced arms which includes the white/rough arm in this example.
EXPERIMENT 1: CHANGES IN LOCAL KYNURENIC ACID LEVELS
NEGATIVELY MODULATE ACETYLCHOLINE RELEASE IN THE
 PREFRONTAL CORTEX

3.1. Introduction

Modulation of neurotransmitter release by kynurenic acid

In addition to its neuroprotective and anti-convulsant properties (Turski et al., 1989; Chiarugi et al., 1995; Wu et al., 2000), KYNA directly influences the release of a variety of neurotransmitters. Following production, KYNA is rapidly liberated from astrocytes where it readily interacts with neurons as a competitive antagonist at the glycine\textsubscript{B} site of the NMDA receptor, and a non-competitive antagonist at the \(\alpha7\) nACh receptor (Turski et al., 1989; Kessler et al., 1989; Hilmas et al., 2001). In the absence of glycine, KYNA has similar affinities to both receptors (IC\textsubscript{50} at the glycine\textsubscript{B} site without glycine \(\sim15\) \(\mu\text{M}\); IC\textsubscript{50} at the \(\alpha7\) receptor \(\sim7\) \(\mu\text{M}\); Kessler et al., 1989; Hilmas et al., 2001). However, KYNA’s actions at the NMDA receptor are inversely proportional to the
amount of glycine present; because glycine is an obligatory co-agonist for the NMDA receptor and not saturated under normal conditions, KYNA’s efficacy at this site is probably limited (IC\textsubscript{50} at the glycine\textsubscript{B} site in the presence of glycine \(\sim 250\ \mu\text{M} \); Hilmas et al., 2001; Scharfman et al., 1999). Endogenous KYNA levels range from 10-150 nM in rodents and 0.1-1.5 \(\mu\text{M} \) in humans; at these concentrations KYNA likely targets \(\alpha7\) nACh receptors preferentially (Moroni et al., 1988; Turski et al., 1988; Swartz et al., 1990).

KYNA inversely regulates Glu and GABA transmission in multiple brain regions (Carpenedo et al., 2001; Wu et al., 2007; Rassoulpour et al., 2005), most likely through antagonism of pre-synaptic \(\alpha7\) nACh receptors (Carpendo et al., 2001; Alkondon et al., 2004). Mice null for the KAT II enzyme display attenuated KYNA levels corresponding with both dramatically increased \(\alpha7\) nACh receptor activity and GABA release in the hippocampus (Alkondon et al., 2004). KYNA’s modulatory influence also extends to the DA system; elevations in KYNA significantly attenuate DA release by first targeting \(\alpha7\) nACh receptors on Glu nerve terminals (Wu et al., 2007; Rassoulpour et al., 2005; Grilli et al., 2006; Nemeth et al., 2005). The subsequent reduction in \(\alpha7\)-facilitated Glu release fails to excite ionotropic Glu receptors on DA nerve terminals, thereby attenuating DA release (Kaiser and Wonnacott, 2000; Wonnacott et al., 2006). In the striatum, KAT II knock-outs show reductions in KYNA (by 67\%), accompanied by a dramatic elevation in DA release (by 170\%). Moreover, co-infusion of the \(\alpha7\) allosteric modulator galantamine, but not D-serine, blocked KYNA-induced decreases in DA. Collectively, these data suggest KYNA potently modulates DA and Glu release primarily through an
α7 nACh receptor-dependent mechanism (Wu et al., 2007), and highlights a role for KYNA as a viable and potent mediator of chemotransmission.

**Regulation of basal cortical ACh efflux by local KYNA levels**

Modest increases in physiological KYNA concentrations *in vivo* antagonize α7 receptors and reduce DA and Glu release (Carpendo et al., 2001; Rassoulpour et al., 2005). Because cortical ACh release is positively modulated by DA and Glu (Laplante et al., 2004; Hernandez et al., 2007; Del Arco et al., 2007), we thus propose that this inverse modulation also extends to ACh release. As such, KYNA-mediated attenuation of DA and Glu should have a correlated impact on ACh release. Due to the predominant role of KAT II in KYNA production, we also utilized a KAT II inhibitor to reduce synthesis of endogenous KYNA (see General Introduction). The present experiments test the contributions of KYNA and α7 nACh receptors in maintaining basal levels of ACh. We propose KYNA tonically inhibits cortical ACh release, and predict that bi-directional changes in KYNA will have correlated, yet inverse, impacts on ACh locally in the PFC.

### 3.2. Methods

**Subjects**

See Chapter 2 for details regarding all methods for this and subsequent experiments. Male Wistar rats (Charles River Labs, Wilmington, MA, USA) weighing between 300-400 grams were utilized for all studies. Animals were maintained on a 12:12 hour light:dark cycle in a temperature and humidity controlled room with access to food and water *ad libitum*. 
**Acclimation and Surgery**

Three days prior to surgery, animals were acclimated to the testing environment in clear plastic bowls for a minimum of 4 hours per day, and were returned to their home cages at the conclusion of each acclimation period.

Following their three days of acclimation, animals were anesthetized using isoflurane gas (2%, 0.6 L/min, O2 delivery) and unilaterally implanted with microdialysis guide cannulae (0.38 mm o.d.; Sci Pro Inc., Sanborn, NY, USA) into the medial prefrontal cortex (PFC; in mm from bregma: AP + 4.2, ML ± 0.6, DV – 0.6 at a 20° rostral angle). Cannulae were inserted with dummy stylets to prevent occlusion. Guide cannulae were fixed using dental cement and three stainless steel skull screws. The surgical site was swabbed with a topical antibiotic ointment (lidocane, 5%), and animals also received a prophylactic dose of the antibiotic chloromphenicol (100 mg/ml; 0.125 ml) administered subcutaneously. Animals were allowed to recover for three days following surgery while being further acclimated daily to the microdialysis testing environment.

**Microdialysis Procedures**

Microdialysis sessions were conducted using a repeated measures paradigm, with each animal receiving four different pharmacological manipulations, one every other day. An important advantage to this repeated perfusion paradigm is that each animal is able to serve as his own control, thereby reducing variability among treatment conditions. This
procedure has been previously validated by demonstrating that basal cortical ACh efflux does not significantly change over repeated dialysis sessions, and the effects of behavioral, pharmacological, or sensory manipulations on ACh levels do not interact with dialysis sessions (Moore et al., 1995; Nelson et al., 2002).

On the fourth day following surgery, animals were brought to the testing environment; dummy stylets were removed and microdialysis probes (Sci Pro, Inc., 0.2 mm o.d., 3 mm membrane tip) were inserted into the guides. Probes were continuously perfused throughout the duration of the experiment with artificial cerebral spinal fluid (aCSF) at the rate of 1.25 µl/min. Following each dialysis session, probes were removed, stylets re-inserted, and animals were returned to their home cages.

Experiment 1: Intra-PFC perfusion: KYNA dose response

Refer to the General Methods (Chapter 2) for detailed experimental procedures regarding the present and all subsequent experiments. Animals were tested four times, with a different pharmacological manipulation (vehicle-aCSF, 100 nM, 300 nM, and 1 µM KYNA) administered, in counterbalanced order, every other day. Exposure to 100 nM KYNA, a physiologically-relevant concentration, has previously been shown to substantially reduce extracellular Glu (to 70% of controls) and DA (50% of controls; Rassoulpour et al., 1995; Wu et al., 2007). Following a 3 hour washout period, four baseline samples were collected from the PFC probe; fifteen-minute collections intervals were observed with all time points. After the baseline period, the syringe containing CSF was then switched to one containing aCSF + drug. Following a 15 min wash out, four
collections of aCSF + drug were taken before switching the syringe back to aCSF alone. Once lines were switched back to aCSF, three additional post-drug recovery samples were collected.

**Experiment 2: Intra-PFC perfusion: kynurenine dose response**

Animals were tested three times with varying concentrations of kynurenine (Kyn; vehicle-aCSF, 2 µM and 5 µM Kyn) administered every other day. Following the four baseline samples, the syringe containing CSF was then switched to one containing aCSF + drug. Following a 15 min wash out, four collections of aCSF + drug were taken before switching the syringe back to aCSF alone. Once lines were switched back to aCSF, three additional post-drug recovery samples were collected before study completion.

**Experiment 3: S-ESBA dose response studies**

Four different pharmacological manipulations (vehicle-aCSF, 500 µM, 2 mM, and 5 mM S-ESBA) were administered. This range of concentrations was selected in part because 5 mM S-ESBA reduces KYNA by 30% and stimulates Glu release by 150% (R Schwarcz, personal communication). Following the washout period, four baseline samples were taken before switching the syringe to one containing aCSF + drug (vehicle-aCSF, 500 µM, 2 mM, or 5 mM S-ESBA). Four drug samples were collected, the syringe was then changed back to aCSF alone, and three post-drug samples were also harvested.
Experiment 4: Endogenous KYNA tonically inhibits cortical ACh

Animals received four different pharmacological manipulations (vehicle-aCSF, 100 nM KYNA, 5 mM S-ESBA, and 100 nM KYNA + 5 mM S-ESBA). After baselines were collected, the syringe was changed to one containing aCSF + drug; four drug samples were collected, the line was switched back to one containing the vehicle alone, and three recovery samples were taken.

HPLC analysis

Dialysis samples were stored at -80°C until analyzed using high performance liquid chromatography (HPLC) with electrochemical techniques. A volume of 15 µl of each sampler was injected by an autosampler (ESA Inc., Chelmsford, MA). ACh and choline were separated by a microbore column Bio Analytical Systems, USA) using a sodium phosphate mobile phase (35 mM Na₂HPO₄, 484 µM EDTA, 0.005% microbicidal reagent Proclin, pH = 8.5). A post-column IMER containing acetylcholinesterase and choline oxidase was used to break down ACh into H₂O₂ (Potter et al., 1983), and the resulting H₂O₂ was quantified using a peroxidase-wired ceramic glassy carbon electrode (Model #5041 microdialysis analytical cell, ESA Inc.).

Histology

Following the final microdialysis session, animals were given an overdose of sodium pentobarbital and transcardially perfused with 0.9% heparinized saline followed by 10% formalin. Brains were removed and stored in 10% formalin for at least 24 hours, and were then transferred to a 30% sucrose solution for three days. Brains were
sectioned using a cryostat and sections (50 µm) were mounted on gelatin-coated slides, stained using Cresyl Violet, and examined under a light microscope. Subjects whose probe placements were located outside the mPFC were excluded from further analysis.

Data analysis

Changes in basal ACh efflux (fmol/15 µl) across sessions and treatment groups were analyzed using one-way repeated measures analysis of variance (ANOVAs). Basal efflux was then defined as the mean of the four baseline collections, and subsequent data were expressed as percent change from that mean baseline. Statistical analysis of drug effects was conducted using a two-way, within-subjects ANOVA with drug GROUP and TIME as within-subjects measures. Significance was defined as P < 0.05, and the Huynh-Feldt correction was utilized to reduce Type I errors associated with repeated measures ANOVAs (Vasey and Thayer, 1987). All statistical tests were preformed using SPSS for windows (version 16.0).

3.3. Results

Guide cannulae placements

Figure 3 shows a representative placement of the mPFC. Any animals whose probe placement fell outside the mPFC were excluded from further analysis.

Experiment 1: Intra-PFC perfusion: KYNA dose response

This experiment examined the effects of increasing concentrations of exogenous KYNA on basal ACh release. Figure 4 illustrates the effects of aCSF-vehicle, 100 nM,
300 nM, and 1 μM KYNA on cortical ACh efflux (n = 6). Basal ACh efflux remained stable over the four dialysis sessions (SESSION, F_{3,15} = 0.670, P = 0.539), and across all drug treatments (DRUG, F_{3,15} = 1.506, P = 0.269), as revealed by one-way ANOVAs. Basal levels of ACh (mean ± S.E.M., fmol/15 μl) were 10.7 ± 1.2, 8.1 ± 1.9, 12.0 ± 1.4, and 10.3 ± 1.5 for aCSF, 100 nM, 300 nM and 1 μM KYNA sessions, respectively. Given that basal levels of ACh efflux did not differ over session or group, all subsequent values were expressed and analyzed as a percent change from session baseline. Basal cortical ACh was attenuated by perfusion of KYNA (DRUG, F_{3,15} = 3.654, P = 0.037), without impacting any other measure (TIME F_{10,50} = 1.288, P = 0.275; DRUG x TIME, F_{30,150} = 0.696, P = 0.865). Given the overall main effect of drug, a series of smaller 2-way ANOVAs were conducted to look for differences between drug pairs. Interestingly, only the concentration closest to physiological levels of KYNA (100 nM) reduced ACh below baseline levels (DRUG, F_{1,5} = 7.530, P = 0.041; TIME F_{10,50} = 0.768, P = 0.658; DRUG x TIME, F_{10,50} = 0.883, P = 0.494). The 300 nM concentration was without significant impact on cortical ACh (DRUG, F_{1,5} = 3.462, P = 0.122; TIME F_{10,50} = 0.762, P = 0.646; DRUG x TIME, F_{10,50} = 0.846, P = 0.546), though the highest concentration (1 μM) did show a modest decrease of cortical ACh (DRUG, F_{1,5} = 5.230, P = 0.071) without any additional impact (TIME F_{10,50} = 0.431, P = 0.924; DRUG x TIME, F_{10,50} = 0.657, P = 0.730).

**Experiment 2: Intra-PFC perfusion: kynurenine dose response**

This experiment examined the effects of increasing concentrations of *endogenous* KYNA by administering its precursor, kynurenine (Kyn). Figure 5 illustrates the effects
of aCSF-vehicle, 2 μM Kyn, and 5 μM Kyn on cortical ACh efflux (n = 7). Basal ACh efflux remained stable over the four dialysis sessions (SESSION, F_{2,12} = 0.409, P = 0.599), and across all drug treatments (DRUG, F_{2,12} = 0.202, P = 0.746), as revealed by one-way ANOVAs. Basal levels of ACh (mean ± S.E.M., fmol/15 µl) were 7.3 ± 2.7, 6.3 ± 0.5, 7.5 ± 1.1, for aCSF, 2 μM Kyn, and 5 μM Kyn sessions, respectively. Given that basal levels of ACh efflux did not differ over session or group, all subsequent values were expressed and analyzed as a percent change from session baseline. Cortical ACh remained completely unaffected by escalating Kyn concentrations (DRUG, F_{2,12} = 0.150, P = 0.817; TIME F_{10,60} = 1.022, P = 0.427; DRUG x TIME, F_{20,120} = 0.922, P = 0.530).

**Experiment 3: KYNA tonically inhibits ACh efflux in the mPFC**

This experiment utilized the KAT II inhibitor S-ESBA to assess the potential tonic regulation of ACh by reducing synthesis of endogenous KYNA. Figure 6 illustrates the effects of aCSF-vehicle, 500 μM S-ESBA, 2 mM S-ESBA, and 5 mM S-ESBA on cortical ACh efflux (n = 5). Basal levels of ACh efflux remained stable over the four dialysis sessions (SESSION, F_{3,12} = 2.021, P = 0.165), and across all drug treatments (DRUG, F_{3,12} = 1.103, P = 0.385), as revealed by one-way ANOVAs. Basal levels of ACh (mean ± S.E.M., fmol/15 µl) were 8.2 ± 1.8, 7.2 ± 1.4, 5.3 ± 0.9, and 8.6 ± 0.9 for aCSF, 500 μM, 2 mM, and 5 mM S-ESBA, sessions, respectively. Given that basal levels of ACh efflux did not differ over session or group, all subsequent values were expressed and analyzed as a percent change from session baseline. Intra-cortical perfusion of drugs led to differential effects on cortical ACh efflux (DRUG, F_{3,12} = 13.929, P = 0.001). These effects were found to vary across collection interval (TIME F_{10,40} = 4.593, P = 0.020;
DRUG x TIME, F_{30,120} = 2.076, P = 0.128). Considering the overall main effects comparing all four treatment groups, a series of smaller 2-way ANOVAs were conducted to look for differences between pairs of groups.

All three concentrations of S-ESBA significantly increased ACh from controls (CSF/500 μM: DRUG F_{1,4} = 29.533, P = 0.006; TIME, F_{10,40} = 3.217, P = 0.004; DRUG x TIME, F_{10,40} = 2.409, P = 0.035) (CSF/2 mM: DRUG F_{1,4} = 14.776, P = 0.018; TIME, F_{10,40} = 3.385, P = 0.184; DRUG x TIME, F_{10,40} = 3.385, P = 0.070) (CSF/5 mM: DRUG F_{1,4} = 39.672, P = 0.003; TIME, F_{10,40} = 1.964, P = 0.182; DRUG x TIME, F_{10,40} = 4.497, P = 0.009). The magnitude of ACh evoked by 500 μM S-ESBA did not differ from 2 mM S-ESBA (DRUG F_{1,4} = 3.462, P = 0.136); both concentrations increased ACh similarly over time (TIME F_{10,40} = 4.035, P = 0.040; DRUG x TIME F_{10,40} = 0.876, P = 0.515). The highest concentration used, 5 mM, facilitated cortical ACh release to a far greater extent than 500 μM S-ESBA (DRUG F_{1,4} = 10.381, P = 0.032; TIME F_{10,40} = 4.560, P = 0.002; DRUG x TIME F_{10,40} = 1.838, P = 0.202). Additionally, S-ESBA’s effects at 5 mM were not significantly different than those elicited by the 2 mM concentration (DRUG F_{1,4} = 4.751, P = 0.095; TIME F_{10,40} = 4.718, P = 0.030; DRUG x TIME F_{10,40} = 1.011, P = 0.421. Collectively, these data show S-ESBA dose-dependently augments ACh release. Administration of S-ESBA increased cortical ACh to 150% above baseline. This effect is especially surprising given the relative paucity of effects generated from either the application of increasing exogenous KYNA concentrations or by augmenting endogenous production of KYNA.
Experiment 4: Inhibiting endogenous KYNA potentiates cortical ACh release

This experiment utilized a combination of the maximally effective concentrations of KYNA and the KAT II inhibitor, S-ESBA established in the previous experiments as a framework for interpreting the increase in ACh release facilitated by administration of S-ESBA. If the increases in cortical ACh are driven by reductions in extracellular KYNA concentrations, then addition of exogenous KYNA was hypothesized to attenuate S-ESBA-induced facilitation of ACh. Figure 8 illustrates the effects of aCSF-vehicle, 100 nM KYNA, 5 mM S-ESBA and 100 nM KYNA + 5 mM S-ESBA on cortical ACh efflux (n = 7). Basal levels of ACh efflux remained stable over the four dialysis sessions (SESSION, $F_{3,18} = 0.808, P = 0.474$), and across all drug treatments (DRUG, $F_{3,18} = 3.181, P = 0.077$), as revealed by one-way ANOVAs. Basal levels of ACh (mean ± S.E.M., fmol/15 µl) were 3.1 ± 0.8, 5.2 ± 0.7, 2.7 ± 0.3, and 5.4 ± 1.1 for aCSF, 100 nM KYNA, 5 mM S-ESBA, and 100 nM KYNA + 5 mM S-ESBA sessions, respectively. Given that basal levels of ACh efflux did not differ over session or group, all subsequent values were expressed and analyzed as a percent change from session baseline. Intracortical perfusion of drugs led to differential effects on cortical ACh efflux (DRUG, $F_{3,18} = 29.080, P < 0.001$). These effects were found to vary across collection interval (TIME $F_{10.60} = 3.638, P = 0.001$; DRUG x TIME, $F_{30,180} = 2.787, P = 0.002$). Considering the overall main effects comparing all four treatment groups, a series of smaller 2-way ANOVAs were conducted to look for differences between treatment groups.
Administration of KYNA did not significantly decrease basal levels of ACh from CSF controls (DRUG $F_{1,6} = 2.156, P = 0.192$; TIME $F_{10,60} = 0.609, P = 0.615$; DRUG x TIME, $F_{10,60} = 0.816, P = 0.615$), which stands in contrast to Experiment 1. However, reducing KYNA by $S$-ESBA dramatically elevated cortical ACh to a greater extent than both aCSF (DRUG $F_{1,6} = 44.826, p = 0.001$; TIME $F_{10,60} = 4.899, p < 0.001$; DRUG x TIME $F_{10,60} = 2.930, p = 0.032$) and KYNA levels (DRUG $F_{1,6} = 50.520, p < 0.001$; TIME $F_{10,60} = 3.186, p = 0.004$; DRUG x TIME $F_{10,60} = 1.020, p = 0.436$). Moreover, $S$-ESBA administration significantly increased ACh within the first 15 minutes of perfusion above CSF (collection 5, $t_5 = -3.773, P = 0.009$) and KYNA (collection 5, $t_5 = -3.887, P = 0.008$) conditions.

$S$-ESBA-stimulated elevations in PFC ACh were attenuated with co-perfusion of KYNA (DRUG $F_{1,6} = 67.171, p < 0.001$; TIME $F_{10,60} = 4.595, p < 0.001$; DRUG x TIME $F_{10,60} = 3.729, p = 0.003$). ACh efflux did not differ between aCSF and KYNA+$S$-ESBA conditions (DRUG $F_{1,6} = 0.058, p = 0.818$), indicating the attenuation of stimulated ACh release was complete. KYNA inhibited ACh efflux slightly more when administered alone then when given in combination with $S$-ESBA (DRUG $F_{1,6} = 6.369, p = 0.045$) without any effect on time (TIME $F_{10,60} = 0.925, P = 0.517$ DRUG x TIME $F_{10,60} = 1.020, P = 0.436$), perhaps indicating a slight elevation by the co-administered $S$-ESBA.

3.4. Discussion

This set of experiments was designed to elucidate potential modulatory effects of KYNA on cortical ACh efflux. The results suggest basal ACh is tonically inhibited by
endogenous KYNA, as perfusion of a KAT II inhibitor dramatically potentiated ACh release. The facilitory effects of the KAT II inhibitor were dependent upon reductions in cortical KYNA concentrations, as concurrent application of KYNA and the KAT II inhibitor S-ESBA, blocked the facilitation of ACh release. The following discussion examines relevant questions raised from these findings.

Unexpectedly, exogenous KYNA exposure produced only modest effects on basal ACh. In fact, only perfusion of the KYNA concentration closest to physiological levels (100 nM) significantly decreased cortical ACh, and only in 1 of the 2 experiments in which it was utilized. These results are particularly intriguing given that inhibition of KYNA synthesis augmented ACh efflux by 150%. While the results clearly show that KYNA tonically inhibits cortical ACh release, no substantial bi-directional influence was evident. This is also surprising considering KYNA inversely regulates many other neurotransmitter systems. In the striatum, perfusion of 100 nM KYNA reduced extracellular Glu and DA by 70% and 50% respectively (Carpendo et al., 2001; Rassoulpour et al., 1995; Wu et al., 2007). In contrast, the maximal reduction in cortical ACh observed in these studies barely reached 40%, despite using the identical concentration of KYNA.

The mechanisms underlying this modulation remain unstudied, though the results highlighted here suggest KYNA likely regulates ACh and Glu in differential capacities. Anatomical evidence does not support a role for α7s acting as autoreceptors on cholinergic terminals (Wilkie et al., 1996; Sher et al., 2004). Therefore, direct facilitation
by presynaptic $\alpha 7$ receptors is unlikely and instead suggests, unlike Glu, KYNA mediates ACh release indirectly.

Antagonism of $\alpha 7$ nACh receptors on glutamatergic terminals may provide an initial step in regulating cortical ACh (Kaiser and Wonnacott, 2000; Dani and Bertrand, 2007). In the frontal cortex, activation of nACh receptors facilitates $[^{3}H]$D-aspartate release from terminals, a process thought to rely predominantly on activation of non-$\alpha 7$ nACh receptors (Gioanni et al., 1999; Lambe et al., 2003). However, Wonnacott and colleagues established a putative role for presynaptic $\alpha 7$ nACh receptors in direct facilitation of Glu release in the frontal cortex (Rosseau et al., 2005). These receptors are also found extrasynaptically on glutamatergic neurons in areas like the striatum, suggesting a role for volume transmission in these effects (Agnati and Fuxe, 2000; Pakkanen et al., 2005). ACh can be modulated within local PFC circuits by Glu (Nelson et al., 2005; Parikh and Sarter, 2008) and GABA (Giovannini et al., 2001). Alternatively, KYNA could tonically inhibit ACh by longer-loop circuits that include PFC-NAC-BF projections (Figure 8), or through recurrent projections from the VTA (Grace 2000; Seamans and Yang, 2004).

KYNA can modify synaptic transmission in several capacities. For example, DA release is mediated indirectly by responding to KYNA-driven suppression of Glu release. Consequently, AMPA receptors on DA neurons are insufficiently stimulated, secondarily attenuating DA as well (Rassoulpour et al., 2005; Wu et al., 2007). KYNA-facilitated reductions in extracellular DA are mimicked by the $\alpha 7$ selective antagonist, MLA, and
blocked by co-application of choline or galantamine (Rassoulpour et al., 2005; Wu et al., 2007), indicating these effects are mediated by $\alpha_7$ nACh receptors and not NMDAR activity. Cholinergic neurons contain AMPA and NMDA receptors and are responsive to Glu innervation (Martin et al., 1993; Fouriner et al., 2003); as such it is tempting to speculate KYNA modulates cortical ACh through indirect mechanisms, possibly through secondary regulation by Glu and DA.

KYNA could also indirectly modulate ACh release via post-synaptic effects. Immunocytochemical analyses revealed $\alpha$-Bgtx binding is co-localized with markers for both glutamatergic terminals and cell bodies, suggesting $\alpha_7$ post-synaptic receptor activity also mediates Glu release (Rosseau et al., 2005). These nicotinic receptors are found on somatodendritic regions of GABAergic interneurons in the cortex and hippocampus (Albuquerque et al., 2000), with extensive functional connections with innervating cholinergic neurons (Van Der Zee and Luiten, 1999). GABAergic interneurons can differentially modulate pyramidal cells via nicotinic receptor activity. The desensitization of these receptors by continuous exposure to endogenous agonists excites pyramidal cells by dampening GABAergic transmission. Alternatively, nAChR activity can also facilitate GABA release directly and thereby inhibit target neuronal activity (Albuquerque et al., 2000). BF cholinergic neurons have been recently demonstrated to be tonically excited by PFC afferents (Rasmusson et al., 2007). In context of the present data, by acting on pre- and post-synaptic $\alpha_7$ receptors within the PFC, KYNA could inhibit activity of glutamatergic afferents projecting to the BF, and tonically suppress the excitatory drive of cortically projecting cholinergic neurons.
These studies do not exclude the possibility that KYNA is impacting cholinergic transmission through NMDA receptors directly (and not by first altering extracellular Glu levels). However, while KYNA’s affinity for the glycine site of the NMDA receptor is comparable to that of the α7 receptor in the absence of glycine, and while the glycine site is not saturated under physiological concentrations, the affinity of KYNA at this site in the presence of glycine is significantly higher (IC\textsubscript{50} = 250 μM; Hilmas et al., 2001). Additionally, DA release was not attenuated by 7-Cl-KYNA administration, which has preferential selectivity for the glycine\textsubscript{B} site (Rassoulpour et al., 2005; Hilmas et al., 2001), suggesting this modulatory relationship is likely mediated by α7 receptor activity. However, administration of either D-serine or glycine in conjunction with neuroleptic treatment improves negative and cognitive symptoms in schizophrenic patients (Tsai et al., 1998, Heresco-Levy et al., 1999, 2005; Coyle and Tsai, 2004), which may indicate KYNA can inhibit NMDA receptor function at physiological levels. In contrast, other reports suggest high doses of glycine are ineffectual in aiding performance in neurocognitive tasks in healthy controls (Palmer et al., 2000). Clearly the mechanisms underlying KYNA-based modulation of transmitter release require additional characterization, though they do reflect a potential role for KYNA to act at both α7 nACh and NMDA receptors. Most evidence suggests the α7 receptor is the preferential target of KYNA as its influence on NMDA receptors may co-vary with the amount of glycine or D-serine present and hence be viable only under certain conditions.
The differential regulation of ACh and Glu suggests factors like floor effects could confound the interpretation of these results. The possibility that our detection limits are not sufficient to capture true decreases in cholinergic transmission must be considered and warrants further investigation. However, slight reductions in ACh were seen, which complicates the interpretation of these results, and may suggest the absence of a bi-directional KYNA effect is not necessarily due to an insensitive HPLC method incapable of detecting decreases in basal cholinergic transmission. It is possible cortical ACh is not as tightly regulated by KYNA as are transmitters like DA and Glu, or that other excitatory influences preserve basal α7 nACh receptor function with increasing KYNA concentrations.

**Astrocytic modulation of neuronal communication**

*S-ESBA*-facilitated increases in ACh were hypothesized to be driven by corresponding reductions in endogenous KYNA. The present results support this hypothesis, as the addition of a physiologically-relevant concentration of exogenous KYNA attenuated the *S-ESBA*-induced potentiation of ACh release. Because KAT II is not expressed in neurons or microglia, these data highlight a role for astrocytes in modulating basal cortical cholinergic transmission. Astrocytes interact with neuronal systems on many levels (see General Introduction) and have important roles in the fine-tuning of synaptic communication. They can initiate cross-talk with neurons by altering their intrinsic activity—alterations in astrocyte-derived Ca^{2+} or Glu release influence a variety of neuronal processes (Ni et al., 2007). In this regard, KYNA could potentially modulate neuronal communication by inhibiting astrocytic α7AChRs, thereby dampening
Ca\(^{2+}\) release from intracellular stores or by reducing astrocyte-derived Glu release (Sharma and Vijayaraghavan, 2001; Parpura and Haydon, 2000). Collectively, these data establish a substantial role for KYNA in the tonic inhibition of basal ACh release, extending the role of astrocytes from mere support cells to active participants in cortical cholinergic communication.
CHAPTER 4

EXPERIMENT 2: KYNURENIC ACID MANIPULATIONS WITHIN THE NUCLEUS ACCUMBENS TRANSYNAPTICALLY MODULATE ACETYLCHOLINE RELEASE IN THE PREFRONTAL CORTEX

4.1. Introduction

The nucleus accumbens (NAC) has long been recognized in its role as an interface between limbic input and motor output (Kelley, 1999; Mogenson et al., 1980; Mogenson and Yang, 1991; Pennartz et al., 1994), with fundamental roles in the selection of appropriate behaviors in response to novel stimuli (Legault and Wise, 1999; Rebec et al., 1997) and matching of discriminative cues with incentive salience (Berridge and Robinson, 1998; Neigh et al., 2004; Nicola et al., 2004a,b; Yun et al., 2004a,b). The NAC can direct the activity of its projection neurons to amplify reward-predictive cues and bias motor output (Deadwyler et al., 2004; Ghitza et al., 2004; Schultz et al., 2003), and the linking of behavioral responses to cues that predict reward is a central feature of motivated behavior. A central role for the NAC in modulating the neuronal circuitry that
contributes to the processing of discriminative cues is becoming increasingly evident. Through experience, animals become biased to direct their limited attentional resources toward the identification, selection and processing of stimuli that are novel or have acquired incentive salience (Franken, 2003; Sarter et al., 2003; Dalley et al., 2004a).

**The nucleus accumbens and cortical cholinergic activity**

The NAC is one of several structures that play a key role in mediating BFCS excitability. Anatomically, GABAergic projections from the NAC terminate on cholinergic neurons within the basal forebrain (Zaborszky and Cullinan, 1992) to modulate cortical ACh release via BF GABA$_A$ receptor activity (Moore et al., 1992). Additionally, administration of DA agonists into the NAC facilitates firing of basal forebrain neurons (Yang and Mogenson, 1984), suggesting DA may have an inhibitory influence on NAC GABAergic MSNs. Previous studies have established a functional linkage between DA and Glu receptor activity within the NAC and ACh release in the PFC. Increases in cortical ACh efflux induced by the benzodiazepine receptor partial inverse agonist, FG 7142, were significantly attenuated by intra NAC administration of D2 antagonists (Moore et al., 1999). Futhermore, neuronal activity in the NAC is necessary for task-induced increases in cortical ACh. Neigh et al., (2004) showed intra-NAC administration of TTX blocked the incentive salience task-induced increase in cortical ACh efflux, thus demonstrating the NAC’s prominent role in regulating BF excitability.
Dopamine and glutamate interactions in the NAC, a role for KYNA too?

The activity of these MSN projections is largely determined by Glu input from areas including the amygdala, hippocampus, and PFC (Yim and Mogenson, 1982; Kirouac and Ganguly, 1995; Wright and Groenwegen, 1996; Zahm, 2000) that converge with modulatory DA input from the VTA (Zahm, 2000; Forster and Blaha, 2000; Yim and Mogenson, 1982). MSN projection neurons from the NAC contain ionotropic NMDA, AMPA, and kainate receptors, metabotropic Glu subunits, and DA receptor subtypes D1, D2, D3, and D4 (Lu et al., 1999; Tarazi et al., 1998; Jongen-Relo et al., 1995). These inputs converge in close proximity to one another on the dendrites of NAC MSNs (Sesack and Pickel, 1990), providing multiple opportunities for complex interactions between DA, Glu, and potentially KYNA, to influence NAC excitability.

The convergence of glutamatergic and DA inputs on NAC MSNs provides a chemoanatomical substrate for understanding the linking of discriminative cues with behavioral output. Excitatory inputs from the PFC, amygdala and hippocampus are involved in distinguishing between different discriminative cues (Schoenbaum et al., 1998, 1999) while DA inputs from the VTA are activated following novel or salient stimuli (Legault and Wise, 1999; Rebec et al., 1997), particularly for cues that predict reward (Nicola et al., 2004a,b; Schultz et al., 2003). The interaction of these inputs has been best conveyed in theories speculating that DA selectively gates the corticolimbic inputs to NAC MSNs to bias processing resources toward certain behavioral responses at the expense of others (Pennartz et al., 1994; Nicola et al., 2004a,b; Yun et al., 2004a,b).
These neurotransmitter interactions within the NAC filter salient and incentive-laden stimuli from irrelevant stimuli, functionally acting to match predictive cues with appropriate behavioral responses (Yun et al., 2004b). Interactions between DA-Glu within the NAC modulate neuronal communication locally within the NAC itself, and also in more widely distributed systems. Previously, we reported that NMDA perfused into the NAC shell significantly increased ACh in the mPFC, an effect positively and negatively modulated by D1 and D2 receptor activity, respectively (Zmarowski et al., 2005; Brooks et al., 2007). Thus, local changes in Glu and DA signaling within the NAC can bias BFCS activity and transynaptically modulate cortical ACh release.

Guided by recent demonstrations of NAC Glu-DA gating of cue-evoked goal-directed behavior (Yun et al., 2004a, 2004b), the clear anatomical and functional links between NAC efferents and the BF, and the bi-directional influence of KYNA over DA and Glu, we hypothesized that PFC ACh is under tonic inhibition of NAC KYNA. As such, reducing endogenous KYNA within the NAC will transynaptically increase ACh release in the PFC. Such modulation would establish astrocytes as active participants in the direction and recruitment of BFCS-driven attentional resources, thus assisting in the coordinated interplay between discriminative cues in the environment and the appropriate behavioral response.
4.2. Materials and Methods

Subjects

Male Wistar rats (Charles River Labs, Wilmington, MA, USA) weighing between 300-400 grams were utilized for all studies. Animals were maintained on a 12:12 hour light:dark cycle in a temperature and humidity controlled room with access to food and water *ad libitum*. All procedures involving animals were approved by The Ohio State University Institutional Animal Care and Use Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Acclimation and Surgery

As described in the General Methods (Chapter 2), three days prior to surgery, animals were acclimated to the testing environment in clear plastic bowls (35 cm height x 38 cm diameter; CMA, Stockholm, Sweden) that were lined with corn cob bedding. Animals remained in the bowls for a minimum of 4 hours per day, and were returned to their home cages at the conclusion of each acclimation period.

Following their three days of acclimation, animals were anesthetized using isoflurane gas (2%, 0.6 L/min, O2 delivery) and unilaterally implanted with microdialysis guide cannulae (0.38 mm o.d.; Sci Pro Inc., Sanborn, NY, USA) into the nucleus accumbens shell (NAC; in mm from bregma: AP + 1.3, ML ± 1.0, DV – 5.8), and the ipsilateral medial prefrontal cortex (PFC; in mm from bregma: AP + 4.2, ML ± 0.6, DV – 0.6 at a 20° rostral angle). Cannulae were inserted with dummy stylets to prevent occlusion. Guide cannulae were fixed using dental cement and three stainless steel skull
screws. The surgical site was swabbed with a topical antibiotic ointment (lidocaine, 5%), and animals also received a prophylactic dose of the antibiotic chloromphenicol (100 mg/ml; 0.125 ml) administered subcutaneously. Animals were allowed to recover for three days following surgery while being further acclimated daily to the microdialysis testing environment.

**Microdialysis Procedures**

On the fourth day following surgery, animals were brought to the testing environment, dummy stylets were removed and microdialysis probes (Sci Pro, Inc., 0.2 mm o.d., 2 mm membrane tip for NAC, 0.2 mm o.d., 3 mm membrane tip for PPC) were inserted into the guides. Probes were continuously perfused with aCSF (Chapter 2) at a rate of 1.25 µl/min throughout the duration of the experiment. Following each dialysis session, probes and injection needles were removed, stylets were re-inserted and animals were returned to their home cages.

**Experiment 1: Astrocytes regulate cortical ACh across distributed brain regions**

This experiment utilized the maximally-effective concentrations of KYNA and S-ESBA established in Chapter 3 to establish a possible role for KYNA in the NAC to modulate cortical ACh release. Animals were tested four times, with vehicle-aCSF, 100 nM KYNA, 5 mM S-ESBA, and 100 nM KYNA + 5 mM S-ESBA) perfused into the NAC shell in counterbalanced order, every other day while ACh was harvested from the PFC probe. After the 3 hour washout period, four baseline samples were collected after which the syringe leading to the NAC was switched from aCSF to one containing aCSF + drug
(100 nM KYNA, 5 mM S-ESBA, or 100 nM KYNA + 5 mM S-ESBA; aCSF was perfused for an additional four collections in the vehicle session). Following a fifteen-minute wash out, four additional collections of aCSF + drug were taken before switching the syringe back to one only containing aCSF. Once lines were switched back to aCSF, three additional post-drug recovery collections were taken before probes were removed, stylets were replaced, and animals were returned to their home cages.

**HPLC analysis**

As described previously, dialysis samples were stored at -80° C until analyzed using HPLC with electrochemical techniques. A volume of 15 µl of each sample was injected by an autosampler (ESA Inc., Chelmsford, MA). ACh and choline were separated by a microbore column Bio Analytical Systems, USA) using a sodium phosphate mobile phase (see Chapter 2). A post-column IMER containing acetylcholinesterase and choline oxidase was used to break down ACh into H₂O₂ (Potter et al., 1983), that was degraded further and quantified using a peroxidase-coated carbon electrode (Model #5041, ESA Inc.).

**Histology**

Following the final microdialysis session, animals were given an overdose of sodium pentobarbital and transcardially perfused with 0.9% heparinized saline followed by 10% formalin. Brains were removed and stored in 10% formalin for at least 24 hours, and were then transferred to a 30% sucrose solution for three days. Brains were sectioned using a cryostat and sections (50 µm) were mounted on gelatin-coated slides,
stained using Cresyl Violet, and examined under a light microscope. Subjects whose probe placements were located outside any of the targeted regions were excluded from further analysis.

Data analysis

Changes in basal ACh efflux (fmol/15 µl) across sessions and treatment groups were analyzed using one-way repeated measures analysis of variance (ANOVAs). Basal efflux was then defined as the mean of the four baseline collections, and subsequent data were expressed as percent change from that mean baseline. Statistical analysis of drug effects was conducted using a two-way, within-subjects ANOVA with drug GROUP and TIME as within-subjects measures. Significance was defined as P < 0.05, and the Huynh-Feldt correction was utilized to reduce Type I errors associated with repeated measures ANOVAs (Vasey and Thayer, 1987). All statistical tests were performed using SPSS for windows (version 16.0).

4.3. Results

Guide cannulae placements

Figure 9 shows representative placements of cannulae in the mPFC and the NAC. Any animals whose probe placement fell outside either of these regions were excluded from further analysis.
Experiment 1: Astrocytes regulate cortical ACh across distributed brain regions

Previous results support a functional linkage between DA and Glu activity in the NAC and cortical cholinergic transmission. As such, this experiment examined a possible contribution of KYNA in the NAC to transsynaptically regulate cortical ACh release. Figure 10 illustrates the effects of intra-NAC perfusion of aCSF-vehicle, 100 nM KYNA, 5 mM S-ESBA, and 100 nM KYNA + 5 mM S-ESBA on cortical ACh efflux (n = 8). Basal levels of ACh efflux remained stable over the four dialysis sessions (SESSION, F_{3,21} = 0.771, P = 0.435), and across all drug treatments (DRUG, F_{3,21} = 2.015, P = 0.193), as revealed by one-way ANOVAs. Basal levels of ACh (mean ± S.E.M., fmol/15 µl) were 4.0 ± 0.4, 5.2 ± 0.8, 3.3 ± 0.4, and 8.6 ± 3.2 for aCSF, 100 nM KYNA, 5 mM S-ESBA, and 100 nM KYNA + 5 mM S-ESBA sessions, respectively.

Given that basal levels of ACh efflux did not differ over session or group, all subsequent values were expressed and analyzed as a percent change from session baseline. Intra-NAC perfusion of KYNA ligands had differential effects on cortical ACh efflux (DRUG, F_{3,21} = 13.777, P < 0.001), that varied across collection interval (TIME F_{10,70} = 2.747, P = 0.007; DRUG x TIME, F_{30,210} = 3.494, P < 0.001). Considering the overall main effects comparing all four treatment groups, a series of smaller 2-way ANOVAs were conducted to look for differences between group pairs.

Intra-NAC administration of KYNA did not significantly reduce cortical ACh release (DRUG, F_{1,7} = 1.422, P = 0.272; TIME F_{10,70} = 0.734, P = 0.667; DRUG x TIME, F_{10,70} = 0.844, P = 0.589). In contrast, inhibiting endogenous KYNA synthesis locally in...
the NAC transynaptically increased ACh release in the PFC by 150% (DRUG, F_{1,7} = 17.278, P = 0.004; TIME F_{10,70} = 5.486, P < 0.001; DRUG x TIME, F_{10,70} = 4.199, P = 0.001). ACh was significantly higher than controls within the first 15 minutes of S-ESBA perfusion (Collection 5; t_7 = -2.384, P = 0.0245, 1-tailed), and returned to control levels by the first post-drug collection (Collection 9; t_7 = -0.169, P = 0.871), suggesting endogenous KYNA may be tightly regulated and capable of rapid normalization.

Importantly, concurrent application of KYNA with the KAT II inhibitor significantly attenuated ACh release as compared to S-ESBA administered alone (DRUG, F_{1,7} = 35.072, P = 0.001; TIME F_{10,70} = 4.033, P < 0.001; DRUG x TIME, F_{10,70} = 6.359, P < 0.001). Indicating S-ESBA elevated PFC ACh by reducing NAC KYNA concentrations. Moreover, this efflux was indistinguishable from controls within the first 15 minutes of exposure (DRUG, F_{1,7} = 1.512, P = 0.259; TIME F_{10,70} = 0.661, P = 0.746; DRUG x TIME, F_{10,70} = 1.381, P = 0.207; Collection 5 t_7 = 0.281, P = 0.500).

Interestingly, the results here effectively mirror the phenomenon established locally in the PFC (Chapter 3), and show cortical ACh is under tonic inhibition of KYNA, and hence astrocytes, within the NAC.

4.4. Discussion

The present study elucidated a possible role for astrocytes within the NAC in regulating cortical ACh release, and shows ACh in the PFC is tonically inhibited across multiple brain regions by NAC KYNA. Thus, the present results establish a novel role for astrocytes in one brain area in regulating neurotransmitter release in a distal, yet
functionally interconnected region. Perfusion of KYNA alone did not reduce ACh efflux in the PFC, though inhibiting KYNA synthesis within the NAC stimulated cortical ACh release to the same extent as reported locally in the PFC (Chapter 3). KYNA may therefore function in the same capacity as other modulatory transmitters to directly influence NAC neuronal excitability. Possible mechanisms underlying transynaptic regulation of cortical ACh are discussed in the context of regulating intra-NAC DA and Glu release.

**Astrocytes transynaptically regulate cortical ACh release**

Unexpectedly, these data are strikingly similar to those reported in Chapter 3. Intra-NAC perfusion of KYNA did not reduce cortical ACh efflux, though inhibiting KYNA synthesis within the NAC significantly potentiated ACh release in the PFC. Thus, cortical ACh is tonically inhibited by endogenous KYNA within the NAC. The mechanisms enabling an astrocyte product released in one brain region to regulate neurotransmitter release in a distal area are unknown. Functionally, gliotransmitters enable astrocytes to signal independently of gap junctions, thereby modulating neuronal excitability directly (Guthrie et al., 1999). With its inverse regulation of DA and Glu, KYNA likely drives NAC output by fine-tuning transmission of these two neurotransmitters.

**KYNA modulates NAC output via dopamine and glutamate**

The present results effectively mirror the interactions observed locally within the PFC, and therefore provides additional support for these effects being mediated by a
long-loop pathway proposed in Chapter 3 (Figure 8). Functional interactions between DA and Glu at the level of the NAC have important implications for the direction of goal-oriented behaviors. At the level of transmitter release, activation of DA receptors facilitates Glu transmission (Dalia et al., 1998), and local NMDA receptor activity within the NAC can reciprocally stimulate DA release (Grace, 1991; Floresco et al., 2001; Howland et al., 2002). Functionally, Glu afferents are involved in distinguishing between different discriminative cues (Schoenbaum et al., 1998, 1999) while DA inputs from VTA are activated following novel or salient stimuli (Legault and Wise, 1999; Rebec et al., 1997), particularly those cues that predict reward (Nicola et al., 2004a,b; Schultz et al., 2003). Corticolimbic input to the NAC is selectively gated by DA afferents that biases the expression of certain behavioral responses at the expense of others (Pennartz et al., 1994; Nicola et al., 2004a,b; Yun et al., 2004a,b).

Excitatory input from the mPFC modulates firing rates of VTA DA neurons via NMDA and AMPA receptors (Karreman et al., 1996). Interestingly, endogenous ACh also modulates firing rates of VTA DA neurons via nicotinic ACh receptors. Activation of $\alpha_4\beta_2$ receptors switched DA neurons from resting to excited states while $\alpha 7$ nACh receptors were subsequently responsible for fine tuning information processing (Mameli-Engvall et al., 2006), indicating KYNA can modulate NAC DA release via Glu and cholinergic mechanisms in the VTA.
KYNA signaling in the NAC modulates attentional resources of the BFCS

Medium spiny neurons of the NAC have bi-stable membrane potentials consisting of relatively depolarized ‘up states’ and hyperpolarized ‘down states’ where cells are more or less responsive to incoming stimuli, respectively (Grace 2000). Despite constituting a major excitatory input, the PFC does not have unregulated influence over NAC output activity and only evokes EPSPs when MSNs are in up states. Furthermore, these up states are dependent on excitatory drive from the hippocampus; consequently, PFC afferents require hippocampal input to modulate NAC activity (O’Donnell and Grace, 1995; Grace 2000). Input from the PFC is tonically suppressed by DA acting on D2 receptors. However, behaviorally-relevant stimuli induce burst firing and phasic DA transmission (see Grace 2000). Interestingly, phasic DA transiently reduces KYNA levels (Rassoulpour et al., 1998; Poeggeler et al., 2007) which would disinhibit Glu transmission, and thus provide one mechanism by which KYNA could regulate transmission along the NAC-BF-PFC circuit.

Burst firing of DA neurons encodes salience and reward (Berridge and Robinson, 1998), allowing top-down drive from the PFC to bias the goal-directed responding of NAC output neurons. As such, increasing DA with burst firing will reduce KYNA occupation of α7 receptors within the NAC, thereby facilitating Glu transmission and PFC throughput. In this regard, α7 activity could have a role in phasic responses to unanticipated stimuli (Mamell-Engvall et al., 2006). Under phasic DA stimulation, the combination of increased D1 receptor activity driving hippocampal reactivity, the consequent up states that are permissive for incoming prefrontal input, and decreased
PFC inhibition by a switch from tonic D2 receptor stimulation, would allow a strong PFC drive on NAC activity. In normal individuals goal-directed NAC output is balanced between hippocampally-mediated contextual constraints and the emotional valence of stimuli provided by the amygdala (Grace, 2000). Conversely, in the schizophrenic brain, pathological dysfunction of excitatory innervation to the NAC could disrupt tonic inhibitory DA levels and induce inappropriate phasic DA firing (as a failure of corticoaccumbens projections to down regulate phasic DA). Abnormal information flow within the NAC of the schizophrenic brain is hypothesized to involve faulty hippocampal gating of PFC input that produces abnormal behavioral output from an inability to use contextual cues or filter competing stimuli (see Grace 2000). Interestingly, reduced DA and NMDA receptor activity in the frontal cortex is hypothesized to drive hyper-reactivity the mesolimbic DA system (see Grace 2000; Del Arco and Mora 2008). Elevated KYNA in the PFC of schizophrenic patients contributes to the reduced excitatory drive of the VTA and NAC, thereby failing to stimulate tonic D2 receptors that would normally dampen abnormal phasic activity (Erhardt and Engberg, 2002; Erhardt et al., 2000). Moreover, increased KYNA in the VTA induces phasic activation and burst firing of DA neurons by action on NMDA receptors (Erhardt and Engberg, 2002). Collectively, interactions between these two dysfunctional systems contribute to imbalances in NAC/PFC excitability and throughput that culminate to form inappropriate information processing (Grace, 2000).

Communication across astrocytes provides an alternative, though less probable, mechanism by which KYNA may regulate cortical ACh release. Astrocytes have α7
nACh receptors and thus may provide an additional source of KYNA-modulated glutamate to interact with neuronal signaling. KYNA may be targeting α7 nACh receptors on other astrocytes and thereby reduce astrocyte-derived Glu release. While astrocytes can communicate with one another over vast networks through induction of propagating Ca$^{+2}$ waves (Guthrie et al., 1999; Araque et al., 2001) the transynaptic effects reported here are likely mediated by other mechanisms as this example requires propagating astrocyte-astrocyte interactions from the NAC to the cortex, and then necessitates additional astrocyte-neuron communication locally in the PFC for these effects. As such, modulation of DA and Glu interactions within the NAC remains the most parsimonious mechanism by which KYNA can transynaptically modulate cortical ACh.

The results described herein indicate a novel role for astrocytes in the NAC to actively regulate cortical ACh release. Moreover, this is the first demonstration of astrocytes in one brain region actively modulating neurotransmitter release across multiple regions. Thus, dysfunctions in the balance between KYNA, Glu and DA may exacerbate dysfunctional cortical and subcortical communication and thus contribute to inappropriate information processing in neuropsychiatric disorders like schizophrenia.
CHAPTER 5

EXPERIMENT 3: ELEVATING ENDOGENOUS KYNURENIC ACID IMPAIRS COGNITIVE FLEXIBILITY

5.1. Introduction

Cognitive flexibility

Cognitive flexibility is an executive function requiring the shifting of one’s response strategy in accordance with changing environmental contingencies or task rules (Owen et al., 1991). In rodents, flexible responding is measured by set-shifting tasks that require the shifting of attention between features of stimuli that vary on at least two perceptual dimensions. An intradimensional shift (ID) requires subjects to shift between stimuli of the same exemplars (i.e. color 1 to color 2). In contrast, the shifting of strategies between different exemplars (i.e. color to texture) constitutes an extradimensional shift (ED). Shifting between dimensions is more cognitively demanding as it requires abandoning a previously reinforced strategy and also
necessitates attending to a previously irrelevant stimulus (Owen et al., 1991; Birrell and Brown, 2000; Brown and Bowman, 2002). An attentional set is formed when certain stimulus response patterns stay relevant over time, serving to bias processing resources or behavioral responses to optimize learning of set-related information (Robbins and Robers, 2007). The Wisconsin Card Sort Task (WCST) is the human corollary to the attentional set shift task described for rodents (Robbins, 2007; Birrell and Brown, 2000). Subjects are required to choose a target stimulus based on knowledge of previous rules and experimenter feedback from current responses. Subjects must update working memory caches for information on previous rules and utilize current feedback to choose between multiple types of stimuli to adapt response strategies with changing task rules. If their response is correct, subjects are likely to stay with the reinforced strategy, but will modify an existing strategy after negative feedback (see Weinberger and Gallhofer, 1997). In contrast, schizophrenics and individuals with PFC damage are severely impaired in tests of cognitive flexibility, including the WCST (Robbins, 2007; Jazbec et al., 2007; Thoma et al. 2007, Everett et al. 2001). Specifically, schizophrenics have difficulties in ED shifting (Robbins, 2007; Jacbec et al., 2007) subserved by additional behavioral impairments that include poor response inhibition, perseveration, and attentional deficits (Thoma et al., 2007).

Successful performance in these tasks depends heavily on effective PFC function in humans (Pantelis et al., 1999; Jacbec et al., 2007), primates (Dalley et al., 2004; Robbins, 2007), and rodents (Ragozzino et al., 1999; Birrell and Brown 2000). The PFC aids in executive functioning, and distinct subdivisions of this region have dissociable
roles in aspects of cognitive flexibility. The dorsolateral PFC (dIPFC; primates) and mPFC (rodents) are required for reconfiguration of cognitive sets (i.e. cognitive flexibility), while the orbitofrontal cortex (OFC) is essential for reversal learning (Birrell and Brown, 2000; Crone et al., 2006; Dalley et al., 2007). Inactivation of the OFC impairs reversal learning in the primate (Robbins et al., 2002) and rat (McAlonan and Brown, 2003; Kim and Ragozzino, 2005), without any disruption to set formation.

Effective formation of attentional sets also depends on extensive communication between cortical and subcortical regions. Interactions between distributed structures like the PFC, NAC, thalamus, and dorsal striatum (DS) greatly contribute to the overall efficacy of flexible responding (Ragozzino et al., 2002; Block et al., 2007). The OFC depends on cross-talk with the amygdala to alter behavior in response to changing reinforcer value (Baxter et al., 2000) and communication between the mPFC and the cingulate cortex couples error detection with cognitive control to optimize performance, especially under challenging conditions (Duncan and Owen, 2000; Sarter et al., 2006). The NAC also aids in cognitive flexibility: inactivation of the core region selectively impairs ED shifting but not rule acquisition. In contrast, NAC shell inactivation does not impair rule learning or EDS, though inactivation of the shell prior to Set 1 training improves EDS performance during Set 2, indicating the shell may mediate learning about irrelevant stimuli (Floresco et al., 2006).
Cognitive flexibility requires DA and Glu transmission

Effective Glu and DA activity are each critical for performance in tests of cognitive flexibility. Blockade of cortical NMDA receptors selectively impairs ED shifts while AMPA receptor antagonism produces more general cognitive impairments (Stefani and Moghaddam, 2005). Dopamine has a substantial influence over many aspects of cognitive flexibility. Dissociable changes in DA release vary with aspects of task performance including rule learning, uncertainty, and reward and across distributed regions including the PFC, NAC and DS (Stefani and Moghaddam, 2006). Multiple DA receptor subtypes mediate cognitive flexibility at the level of the cortex (Floresco et al., 2006; Ragozzino, 2006) and the NAC (Janhunen and Ahtee, 2007). Moreover, DA activity within the mPFC mediates the generation of new strategies and the inhibition of previously relevant rules via D1 and D2 receptor activation (Ragozzino, 2006; Floresco et al., 2006).

The necessity of cortical cholinergic function for successful performance in attentional tasks has been extensively documented (Sarter and Bruno, 1997; Salmond et al., 2005) though the role of the BFCS in flexible responding remains poorly understood. The literature on cholinergic involvement in cognitive flexibility is mixed; some evidence suggests selective damage to BF cholinergic neurons only impacts attentional shifting when isolated to certain sensory modalities (McGaughy et al., 2002), though more contemporary studies indicate ACh has a negligible role in cognitive flexibility. Administration of the muscarinic receptor antagonist scopolamine impaired reversal learning and ED shifts in attention (Chen et al., 2004), suggesting cholinergic activity
plays some role in maintaining set-shifting performance. However, the locus of this action has not yet been specified, and likely involves cholinergic mechanisms outside the PFC (Chen et al., 2004). Furthermore, recent studies by Tait and Brown (2008) and McGaughey and colleagues (2008) show that selective cholinergic lesions or deafferentation of the BF spare ED shifting. As such, a role for cortical ACh in set shifting is likely minor and perhaps limited to modulating the activity of neurotransmitters like Glu and DA.

**Role of kynurenic acid in behavioral flexibility**

As determined by Chapters 3 and 4, KYNA has a regulatory influence over DA and Glu in the both PFC and NAC. As interactions between these neurotransmitters and anatomical regions are critical for effective cognitive flexibility, elevating endogenous KYNA could impair behavioral responding mediated by these systems. Moreover, KYNA may also have direct influences on behavior. For example, increases in KYNA can alter the processing of conditioned stimuli in part by impairing response inhibition to irrelevant stimuli (Chess and Bucci, 2006). Additionally, mice null for the KAT II enzyme with dramatically reduced KYNA levels perform better in hippocampal-dependent learning tasks. Conversely, the beneficial effects on performance are abolished in these mutants when KYNA levels naturally return to wild-type control levels following puberty (Potter et al., 2005, 2006; Alkondon and Alburquerque, 2005).

With its modulatory role in both anatomical areas and transmitter systems that play critical roles in cognitive processing, augmenting KYNA may have dramatic
impacts on behavioral responding. Given the selective ED impairments schizophrenics have in tests of cognitive flexibility, and guided by recent demonstrations that KYNA is elevated in the frontal cortex and CSF of schizophrenic patients (Schwarcz et al. 2001; Erhardt et al., 2001), we hypothesize that augmenting endogenous KYNA will selectively impair ED shifting in an attentional set shifting task.

5.2. Methods

Subjects

Male Wistar rats (Harlan Sprague-Dawley, Indianapolis, IN, USA) weighing between 300-400 grams were utilized for all studies. Animals were maintained on a 12:12 hour light:dark cycle in a temperature and humidity controlled room. Animals were individually housed in plastic cages lined with corn cob bedding (Harlan Teklad, Madison, WI, USA) and had access to food and water ad libitum. All procedures involving animals were approved by The Ohio State University Institutional Animal Care and Use Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Surgery

Animals were implanted with bilateral microdialysis cannulae in the mPFC (infusion cannulae: A = 4.2, L = 0.6, V = 1.5 mm, 20° rostral [one hemisphere] and P = 1.5, L = 0.6, V = 1.5 mm, 20° caudal [the other hemisphere] angle relative to Bregma and dura) after completing the initial stage of maze acclimation. All guide cannula were fixed to the skull using stainless steel screws and dental acrylic. Stylets, ending 2 mM beyond
the guide cannula, were inserted to prevent occlusion. The surgical site was swabbed with a topical antibiotic ointment (Neosporin), and animals also received a prophylactic dose of the antibiotic chloromphenicol (100 mg/ml; 0.125 ml administered subcutaneously). Following surgery, animals were returned to their home cages and allowed to recover for 3 days prior to microdialysis testing or resuming behavioral training.

**Attentional Set-shifting**

**Apparatus**

The set-shifting maze is constructed out of pressed wood (0.7 cm thick) and sealed with a polyurethane primer. The maze is composed of a central platform (14 cm each side) and four arms (40 x 14 x 20 cm each) radiating from the central platform (Figure 11). A hexagonal plastic food dish was placed at the end of each arm and was sufficiently deep where a food reward could not be seen when entering the arm. Maze arms varied along two stimulus dimensions: brightness and texture. Two arms were covered with a faux leather material in black and the other two were covered in the same material in white. The smooth texture (on the floor) consisted of the faux leather material covering the walls, and the rough texture consisted of rough grade sandpaper spray painted either black or white to correspond with the appropriate colored arms. Arm combinations were: white/smooth, white/rough, black/smooth and black/rough. The maze was attached to a rotary platform. The holding cage used for inter-trial intervals was a standard rat housing cage lined with corn-cob bedding.
Set-Shifting Training

All training and testing paradigms were modeled after procedures used by Stefani and Moghaddam (2005). Animals were first food restricted (given 15-20 g/day) and maintained at a minimum of 85% of their free-fed weight. During the pre-acclimation phase, animals were given a food reward consisting of a sugary cereal (Froot Loops) in their home cages to familiarize them with the taste and odor of the reward, and to reduce possible neophobic effects of reward introduction in the maze. Following a week of handling and food restriction, animals began one of two phases of maze acclimation. During the first day of acclimation, multiple animals were placed in the maze and were allowed 10 minutes to explore and consume any food rewards there. Following this first day, animals were individually placed in the maze in which all four food wells were baited with pieces (1/3 of an individual cereal piece) of Froot Loops. Rats were given 10 minutes to roam freely and explore the apparatus, to condition the rats to receiving food rewards while in the maze. On subsequent days of acclimation, each food well was baited with one piece of Froot Loop per well, and rats were allowed to explore until all food had been consumed, up to 10 minutes. Upon removal from the maze, rats were briefly placed in a holding cage before returning to their home cages. Surgical implantation (see above) of injection cannulae were done following this first phase of acclimation.

During the second phase of acclimation, the maze was set up in a T configuration (with a divider placed to block one arm). Animals received 8 trials per day, consisting of two starts from each arm. Animals were placed in the central arm, allowed to turn left or
right (choosing one arm) and consume any food reward there. Arms were reinforced randomly. Between trials, rats were removed from the maze and placed in a holding cage (inter-trial interval approx. 15 s).

**Set-Shifting Testing**

The attentional set-shifting task consists of two sessions on consecutive days, with the maze in a T configuration as it was during the second phase of acclimation. On day 1 (Set 1) animals were trained to discriminate between brightness (black verses white arms) or texture (smooth verses rough arms) dimensions, and were trained to reach a criterion performance level consisting of 8 consecutive correct trials. Animals were allowed 2 minutes to make a ‘choice’ by which an animal had to leave the start arm and enter one choice arm far enough so all four feet passed into the choice arm. Animals were allowed up to 120 trials to reach criterion, and animals who failed to learn the initial discrimination following 120 trials were removed from study. On day 2 (Set 2), animals were trained on the alternative (extra-dimensional) discrimination strategy for 80 trials, regardless of performance level (Stefani and Moghaddam, 2005).

Animals with bi-lateral infusion cannulae targeting the mPFC received drug infusions prior to each day of testing. Animals were brought into the testing room, sylets removed and infusion needles were inserted. Drugs (vehicle-aCSF or Kyn 10 μM/ 0.5 μl) were infused at a rate of 0.25 μl/min with a total volume of 0.8 μl was infused. Infusion needles were left in place for an additional 10 minutes before initiating testing.
**Data Analysis**

All statistical analyses were conducted according to tests described in Stefani and Moghaddam (2005). Dependent measures including trials and time to criterion (Set 1) and time to criterion (Set 2) were analyzed using one-way analysis of variance (ANOVA). Because the trials to criterion for Set 2 were restricted under a cap of 80 trials (Stefani and Moghaddam, 2005), these data were analyzed using the non-parametric Kruskal-Wallis test. The percentage of correct scores was analyzed for each 8-trial block (over 10 consecutive blocks) to examine performance across blocks of trials for Set 2. Performance across trial blocks was analyzed using mixed-design 2-way, repeated measures ANOVAs, with drug treatment as the between subjects variable and trial block as the within subjects measure (Stefani and Moghaddam, 2005). Analysis of perseverative responding during Set 2 was conducted by comparing the percent correct scores from each of two start-arm designations (perseveration and reinforcement arms) within each block of trials. Perseveration arms consisted of the start arms that yielded an incorrect response during Set 2, but were previously reinforced during Set 1. Reinforcement arms were designated as those yielding a correct response during Set 2 when responding according to the strategy that was successful during Set 1. Perseverative arm and reinforcement arm performance was analyzed by comparing percent correct scores using mixed design 2-way, repeated measures ANOVAs with treatment group as the between-subjects and trial block as the within-subjects variables. Using this paradigm, animals can make 2 kinds of errors in learning the new strategy for Set 2 (see Chapter 2 for a detailed explanation). These include perseverative errors.
where animals choose the previously reinforced dimension. For example, animals that learned to choose the black arm during Set 1 but need to learn the smooth arm for Set 2 would be making perseverative errors if they repeatedly chose the black/rough (incorrect) arm over the white/smooth (correct) arm. Animals may also repeatedly enter never reinforced arms (here, the white/rough arm), indicating errors are due to a failure to learn the new strategy.

5.3. Results

Experiment 1a: Elevated KYNA does not impair rule acquisition

The role of KYNA in cognitive flexibility was assessed using three experiments. We first determined whether elevating endogenous KYNA impairs general cognitive function by impairing initial rule acquisition. Figure 12 represents the rule acquisition for the first day of testing for animals given saline (n = 6) or kynurenine (50 mg/kg i.p.; n = 6) prior to Set 1; both groups received saline before Set 2. Kynurenine-treated and control animals required 43.2 ± 9.7 and 36.5 ± 9.2 trials (mean ± SEM) to reach criterion, respectively, indicating they learned the stimulus rule at a similar rate (F{sub 1,11} = 1.491, P = 0.250). Moreover, the percentage of correct responses for Set 2 acquisition varied across blocks of trials (BLOCK F{sub 9,90} = 8.466, P < 0.001), but not between drug pre-treatment (GROUP F{sub 1,10} = 0.514, P = 0.490; BLOCK X GROUP F{sub 9,90} = 0.464, P = 0.831), which represents gradual learning of the discriminative role over repeated trials. Thus, elevating endogenous KYNA does not impair initial rule acquisition. Analysis of ED shifting determined there were no differences between Kyn and Saline treatment groups in making the ED shift with both groups making more correct choices over blocks (Figure
Due to the imposed limit of 80 trials for Set 2, trials to criterion were examined using the Kruskal-Wallis test for nonparametric data. Kyn/Sal animals (treated with Kyn before Set 1 and saline before Set 2) made the ED shift in 47.4 ± 9.2 trials, while controls ( saline before Set 1 and 2) learned in 57.4 ± 7.7 trials, respectively. Figure 14 is a composite graph that shows the trials to criterion for each Set in these animals (mean ± SEM; top panel). The number above Set 2 indicates the percentage of animals in each group to learn the ED shift (83.3% of animals in each group). The bottom panel of Figure 14 depicts the percent of subjects to reach criterion for each day of testing and the corresponding trials to criterion for each group are noted above each bar graph. Both groups made the EDS at a similar rate ($X^2 = 0.521$, P = 0.470). Moreover, no differences were found between the 2 groups when considering perseverative (Figure 15; $PA \ F_{1,10} = 0.144$, P = 0.712) or reinforcement error rate (data not shown; $F_{1,11} = 0.403$; P = 0.540), indicating Kyn administered prior to Set 1 had no unexpected effects on set shifting performance.

**Experiment 1b: Elevated KYNA impairs cognitive flexibility**

Given evidence supporting elevated KYNA in schizophrenia (Schwarcz et al., 2001; Erhardt et al., 2001) and deficits in cognitive flexibility common to the disorder, this experiment tested the hypothesis that increasing *endogenous* KYNA, by administering its precursor Kyn, will specifically impair ED shifting. Animals were divided into 2 groups: animals treated with saline over both testing days (Sal/Sal), and animals who were treated with saline prior to Set 1 and Kyn (50 mg/kg) prior to Set 2 testing (Sal/Kyn; $n = 7$ for each group). Figures 16 and 17 represents the gradual
acquisition of Sets 1 and 2 respectively, and shows the % scores for control animals that were treated with saline before Set 1 and Set 2; and animals that received saline prior to Set 1 and Kyn before Set 2 were separated into subgroups that were able to learn the ED shift (n = 2), and those that failed to make the behavioral switch (n = 5). On day 1, the groups required a similar number of trials to reach criterion (Sal/Sal = 39.4 ± 18.4 trials; Sal/Kyn = 38.3 ± 10.6 trials) indicating there were no differences in the groups’ abilities to learn the initial discrimination rule (F_{1,13} = 0.020, P = 0.889).

On day 2, only 2 of the 7 animals pre-treated with Kyn acquired the new discrimination rule. Collectively, animals in this group required significantly more trials to make the ED shift than controls given saline before each day of testing (X^2 = 4.545, P = 0.033). Figure 18 is a composite graph depicting trials to criterion for Set 1 and 2 in these animals (mean ± SEM; top panel). The number above Set 2 indicates the percentage of animals in each group to learn the ED shift (100% of animals in the control group and 28.5% of animals given KYNA prior to Set 2). The bottom panel of Figure 18 depicts the percent of subjects to reach criterion for each day of testing and the corresponding trials to criterion for each group are noted above each bar graph. Both the control group and the subgroup of Kyn-treated animals that learned the Set 2 rule made the EDS at a similar rate (X^2 = 0.521, P = 0.470). Moreover, no differences were found between the Kyn subgroup and controls when considering perseverative (Figure 19; PA F_{1,10} = 0.144, P = 0.712) or reinforcement error rate (data not shown; F_{1,11} = 0.403; P = 0.540), indicating Kyn administered prior to Set 1 had no unexpected effects on set shifting performance. Analysis between the subgroup of animals that did not learn and
controls revealed animals that did not learn \((n = 5)\) did have higher rates of perseverative errors \((F_{1,10} = 7.858, P = 0.019)\). Interestingly, when the subset of Sal/Kyn animals that learned Set 2 were analyzed separately \((n = 2)\), they were found to acquire the EDS over a comparable number of trials as controls \((\text{GROUP}; F_{1,12} = 2.758, P = 0.123)\). Sal/Sal animals reached Set 2 criterion in \(50.4 \pm 5.0\) \((n = 7)\) trials, while Sal/Kyn animals that learned on day 2 reached criterion in \(56.3 \pm 7.2\) \((n = 2)\). Trials to criterion in Set 2 were analyzed using the Kruskal-Wallis test for nonparametric data because of the imposed 80-trial cap in this test. While the \% correct choices of Set 2 varied over blocks of trials \((\text{BLOCK} F_{9,108} = 5.508, P < 0.001)\), they did not differ based on drug treatments nor did they differ over the task duration \((\text{GROUP} F_{1,12} = 2.758, P = 0.123; \text{BLOCK} \times \text{GROUP}; F_{9,108} = 1.385, P = 0.227)\).

**Experiment 2: Local elevations of cortical KYNA impairs cognitive flexibility**

While the results from the systemic studies are intriguing, they do not reveal the locus of action mediating these effects. Given the central role of the PFC in cognitive flexibility and ED shifts of attention, we propose KYNA impairs ED shifting by acting within the mPFC. As such, we hypothesized intra-PFC infusions of Kyn would parallel the cognitive impairments characterized in Experiment One by elevating KYNA locally within the cortex. Figure 20 represents bilateral infusion cannulae placements in the mPFC. Figure 21 represents the \% correct scores during Set 1 acquisition for 3 groups: animals infused with aCSF prior to both testing days \((\text{aCSF/aCSF}; n = 6)\), animals infused with Kyn prior to Set 1 and aCSF prior to Set 2 \((\text{Kyn/aCSF}; n = 5)\), and animals given aCSF before Set 1 and Kyn before Set 2 \((\text{aCSF/Kyn}; n = 6)\). All groups acquired
Set 1 over a similar number of trials (aCSF/aCSF = 40.6 ± 5.8, Kyn/aCSF = 42.8 ± 4.6, aCSF/Kyn = 39.2 ± 4.1) indicating there were no differences in the groups’ abilities to learn the initial discrimination rule (F_{2,14} = 0.131, P = 0.878).

Figure 22 represents the % correct scores during Set 2 performance for the 3 groups, there were no differences between groups in the % correct achieved per block while learning the ED shift (BLOCK F_{9,126} = 6.638, P < 0.001), there was no differential effect of drug (DRUG F_{2,14} = 1.358, P = 0.289; BLOCK X DRUG F_{9,108} = 1.385, P = 0.227). Figure 23 is a composite graph depicting trials to criterion for Set 1 and 2 in these animals (mean ± SEM; top panel). The number above Set 2 indicates the percentage of animals in each group to learn the ED shift. Of the treatment groups, 66% (4 of 6) of vehicle-treated animals and 60% (3/5) of animals infused with Kyn pre Set 1 acquired the ED shift (Figure 23). The percentage of control animals to learn is lower than indicated in Experiment One, and may be a consequence of the limited sample size. However, a mere 16% (1 of 6) of animals in the CSF/Kyn group effectively switched response strategies, indicating acute elevations in cortical KYNA impair flexible responding. Moreover, the single aCSF/Kyn subject that learned the new rule reached criterion at the 80^{th}, and very last, trial. The bottom panel of Figure 23 depicts the percent of subjects to reach criterion for each day of testing and the corresponding trials to criterion for each group are noted above each bar graph. Due to the imposed 80-trial cap for Set 2, trials to criterion were analyzed using the Kruskal-Wallis test for nonparametric data (X^2 = 3.889, P = 0.143) and found no significant difference between
aCSF/aCSF and Kyn/aCSF group. The single aCSF/Kyn animal was not included in this analysis.

Analysis of perseverative errors made while learning Set 2 (Figure 24) revealed a significant effect of block (BLOCK F_{9,126} = 3.964, P < 0.001) as animals gradually learned the task over time. There was no effect of drug (DRUG F_{2,14} = 1.358, P = 0.289) or drug by block interaction (BLOCK X DRUG F_{9,126} = 1.005, P = 0.456), indicating the cognitive deficits observed here are not due to an inability to abandon a previously reinforced strategy.

5.4. Discussion

The present results demonstrated that acutely elevating endogenous KYNA impairs flexible responding in an attentional set shifting task. Several major findings can be reported. Experiment One determined that systemic Kyn impaired shifting of response strategies to previously irrelevant stimuli (i.e. ED shifting), presumably by elevating endogenous KYNA. In contrast, elevating KYNA prior to Set 1 did not impair initial rule acquisition, which suggests ED shifting deficits are not simply due to impairments in general cognitive function. Experiment Two revealed shifting deficits were mediated by actions within the PFC because local and systemic Kyn produced comparable behavioral deficits. These results parallel clinical literature that show schizophrenics are selectively impaired in shifting attentional sets. As such, the following discussion addresses questions raised from these findings in the context of
anatomical areas and potential neurotransmitter interactions that could mediate these results.

The present finding that elevating KYNA within the PFC impairs ED shifts of attention is supported by evidence that cognitive flexibility is largely dependent on the integrity of PFC function in humans and rodents (see Introduction). It is likely these effects are mediated by inhibiting DA and Glu transmission since flexible responding is critically dependent on effective Glu and DA transmission within the PFC. Extradimensional shifting is impaired with antagonism of PFC NMDA receptors by MK-801 (Stefani and Moghaddam, 2005) and multiple DA receptor subtypes mediate cognitive flexibility at the level of both the cortex (Floresco et al., 2006; Ragozzino, 2006) and NAC (Janhunen and Ahtee, 2007). As such, by modulating Glu and DA transmission, KYNA also mediates flexible responding in set shifting tasks.

While this thesis centers on the relationship between KYNA and cortical cholinergic transmission, the precise role for the cholinergic system in flexible responding remains poorly understood. KYNA likely impairs cognitive flexibility by inhibiting Glu and DA transmission, and is not heavily dependent on cholinergic activity. Two recent investigations found selective cholinergic lesions or deafferentation of the BF spare ED shifting (Tait and Brown, 2008; McGaughy et al., 2008), indicating ACh has little influence over flexible responding. However, the lesions and deafferentation were not complete in either study, indicating some cholinergic function may be spared. It is possible residual cholinergic activity has a subtle yet behaviorally relevant contribution to

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performance in these tasks. A recent study found ED shifting and reversals were spared with reduced PFC ACh, though additional measures like serial reversals were impaired (Robbins and Roberts, 2007). KYNA preferentially targets α7 nACh receptors and increased production of endogenous KYNA significantly impairs task performance. Because ACh and choline are the two endogenous ligands for the α7 receptor, a role for cholinergic transmission in cognitive flexibility cannot be completely dismissed. Since Glu and DA transmission are each paramount for successful ED shifts (Floresco et al., 2006; Stefani and Moghaddam, 2005), cholinergic innervation could aid in the processing of reward-predictive cues, in part by facilitating Glu and DA transmission in the PFC. Alternatively, cholinergic transmission may influence cognitive flexibility by modulating the representation of relevant stimuli. Cortical ACh increases signal to noise ratios by amplifying thalamic inputs and suppressing associational information from other cortical areas (Detari et al., 1999; Hasselmo and Cekic, 1996) and enhances synchronized activity of GABAergic interneurons in the PFC (Bandyopadyay et al., 2006). Functionally, this aids in the allocation of processing resources to behaviorally-relevant stimuli (Stefani and Moghaddam, 2006; Ragozzino et al., 2002; Floresco et al., 2006) and therefore may indirectly assist in the DA and Glu-mediated formation of attentional sets. As such, these examples suggest ACh may have subtle but relevant roles in modulating cognitive flexibility, potentially through multiple indirect mechanisms.

Though the studies here implicate the PFC in ED impairment, they do not exclude the possibility that additional pathways contribute to these effects. Various subcortical regions aid the PFC in forming attentional sets, and interactions between multiple
interconnected brain regions contribute to the overall efficacy of flexible responding. For instance, connections between mediodorsal thalamus-PFC and between the PFC-NAC are also critical for ED shifts (Block et al., 2007; Shafritz et al., 2005). As suggested by the results of Chapter 4 and the systemic results presented here, KYNA could impair performance through the NAC as well. Elevated KYNA could impair burst-firing of DA neurons induced by reward-predictive cues (Ishikawa et al., 2008). Alpha-7 receptor activation preferentially facilitates Glu release from terminal regions in the PFC, NAC and striatum (Jänhunen and Ahtee, 2007; also see General Introduction), which subsequently facilitates DA release by stimulating NMDA receptors (Schilstrom et al., 1998; Svensson et al., 1998). As such, KYNA likely impairs cognitive flexibility by targeting the anatomical areas and neurotransmitter systems that critically mediate set shifting performance.

**Schizophrenics show impairments in cognitive flexibility: a KYNA-based mechanism?**

The present studies revealed deficits in ED shifting that parallel the cognitive dysfunctions observed in schizophrenia and suggest a hyper-KYNA tone may mediate some of the neurocognitive impairments inherent to the disorder. Schizophrenic patients may also show problems with additional aspects of cognitive flexibility including difficulty in forming stable representations of stimuli (Jazbec et al., 2007) which supports the present observations that acute KYNA elevations impair flexible responding without increasing perseveration. The version of the set shifting task utilized in this thesis was based on work done by Stefani and Moghaddam (2005). Many variants of the task exist
(Floresco et al., 2006; Ragozzino et al., 2002), and other versions like those used by Birrell and Brown (2000) utilize more complex analyses including various ID and ED shifts along with simple and compound discriminations. The Stefani and Moghaddam version of the task was more than sufficient to uncover the deficits in ED shifts reported in this chapter, though versions like Birrell and Brown’s may be required for more in-depth analyses of specific learning deficits.

The degree of cognitive flexibility, measured with WCST performance, correlates with general social functionality in schizophrenia and is predictive of long-term therapeutic outcome (Addington and Addington, 1999; Breier et al., 1991; Green et al., 2000). While executive impairments are almost ubiquitous in schizophrenic populations, the degree of cognitive dysfunction can be highly variable between individuals since some schizophrenic patients make ED shifts at rates comparable to controls (Weinberger and Lipska, 1995; Green et al., 2000). A priori, the examples of normal variance from the human literature may aid in interpreting the results of the present study; as such, the subset of animals with elevated KYNA that acquired the ED shift may reflect normal variance in executive ability.

Attentional dysfunction represents a core deficit by which interacting abnormal transmitter systems collectively produce inefficient information processing. Impairments in information processing depend on the degree of functional demands on the cortex and requires communication between PFC and temporolimbic cortices (Weinberger and Lipska, 1995). The convergence of information between multiple dysfunctional brain
regions like the PFC and NAC culminate to exacerbate existing cognitive deficits.
Along with KYNA’s potent regulation of Glu and DA transmission, evidence KYNA is augmented in schizophrenic patients (Schwarcz et al., 2001; Erhardt et al., 2001), and normalized with chronic neuroleptic treatment (Ceresoli-Borrini et al., 2006), these data further support a role for elevated KYNA imbalance in cognitive dysfunction inherent to schizophrenia.
6.1. Role of the KP and KYNA in neuronal communication

In human neurons, normal activity of the KP is primarily neuroprotective, capable of inhibiting tumor development and regulating immune function, and KYNA greatly contributes to this role (Guillemin et al., 2007). Moreover, QUIN generated from normal KP activity ultimately contributes to the protective role of the KP as it helps replenish sources of NAD+ depleted by NMDA receptor activation. Because exhaustion of NAD+ leads to cell death, physiological QUIN concentrations also contribute to the KP’s overall protective influence (Stone et al., 2007). The KP maintains a delicate balance between these products for optimum neuronal function. For instance, dysfunctional KP activity is hypothesized to facilitate excitotoxic cell death and tumor growth in human neuroblastoma cell lines by generating excessive QUIN (Guillemin et al., 2007) and suggests maintaining proper QUIN:KYNA ratios may be a critical factor in the function of many biological processes.
Under normal conditions, KYNA may fine-tune neuronal excitability, in essence weighing the potential for excitotoxic insult and modifying neurotransmitter release accordingly. However, sustained increases in KYNA may pathologically impair neuronal communication. Furthermore, dysfunctions in neurotransmitters like Glu, DA, GABA, and ACh have each been implicated in the pathophysiology of schizophrenia, and are all negatively modulated by KYNA (Carpena et al., 2001; Alkondon et al., 2004; Rassoulpour et al., 2005; Wu et al., 2007). Evidence supporting hyper reactivity of the mesolimbic DA system in schizophrenics may appear contradictory given KYNA’s inverse relationship with DA transmission. However, elevations in KYNA can induce burst firing of VTA and nigral DA neurons by inhibiting NMDA receptors on GABAergic interneurons (Erhardt and Engberg, 2002; Erhardt et al., 2000; Linderholm et al., 2007). Clozapine attenuates burst firing of these neurons by acting at the NMDA receptor, but only in animals with KYNA-induced hyper-DA states (Schwieler et al., 2003; Linderholm et al., 2007; Schwieler et al., 2004). While the precise mechanisms of this dynamic relationship remain to be elucidated, these examples do reflect aberrations between neuron and astrocyte communication in schizophrenia mediated by NMDA and α7 nACh receptors. Therefore, understanding KYNA’s influence over chemotransmission creates potential for the development of novel and more efficacious therapeutics that might selectively target impaired cognitive function inherent to the disorder.
6.2. Distributed systems mediating cognitive deficits in schizophrenia

Enduring executive dysfunctions in schizophrenia are products of inappropriate interactions between cortical-cortical and cortical-subcortical structures, of which PFC-NAC dysfunctions are of particular interest (Weinberger and Lipska, 1995). The NAC provides incentive drive to initiate top-down controls of attention (Sarter et al., 2006). Gating of dopaminergic tone biases subjects towards certain behavioral responses at the expense of others (Pennartz et al., 1994; Nicola et al., 2004a,b; Yun et al., 2004a,b). Interestingly, pharmacological manipulations that augment DA efflux show corresponding reductions in extracellular KYNA, indicating neuronal activity also modulates KYNA (Poeggeler et al., 2007). Intra-NAC DA receptor activation increases Glu transmission (Dalia et al., 1998), possibly driven by attenuation of extracellular KYNA. Increases in DA release in response to behaviorally-relevant cues could transiently reduce extracellular KYNA and thus disinhibit excitatory transmission in the NAC for amplification of behaviorally relevant cues, thus allowing NAC drive to bias PFC activity. Functionally, this modulatory relationship enables the sufficient linkage of salient cues with attentional processing resources.

Activity of the NAC is determined by the convergence of excitatory information from cortex, hippocampus, and amygdala with modulatory DA innervation from the VTA (Groenewegen et al., 1999; O'Donnell, 1999; Grace, 2000). Dopamine receptor activity selectively gates the corticolimbic inputs to NAC medium spiny neurons, and is heavily dependent on glutamatergic stimulation of the VTA. NMDA-mediated Glu activity in
the VTA tonically facilitates NAC DA release, while AMPA receptor stimulation contributes to phasic DA activation in the NAC (Karreman et al., 1996).

Excitatory input to the NAC is selectively gated by tonic and phasic DA receptor activity. For instance, information flow from the PFC is tonically inhibited by NAC D2 receptor activation, while D1 receptors modulate hippocampal inputs under phasic stimulation (Goto and Grace 2005). Under conditions requiring little attentional effort, the PFC influence over NAC output is relatively minimal. However, under conditions of heightened attentional demand, phasic DA transmission reduces the tonic suppression of PFC inputs that are subsequently activated and bias subpopulations of NAC efferents. PFC afferents require hippocampal input to modulate NAC activity (O’Donnell and Grace, 1995). Under phasic DA stimulation, increased D1 receptor activity stimulating hippocampal afferents drives MSNs into up states that are permissive for incoming PFC input, and decreased tonic D2 receptor stimulation, collectively allow a strong PFC drive on NAC activity. Furthermore, burst firing of DA neurons encode salience and reward (Berridge and Robinson, 1998), allowing top-down drive from the PFC to bias goal-directed responding of NAC output neurons.

As discussed in the Introduction, NMDA and DA receptors modulate the firing rates of GABAergic projections, which then regulate BFCS excitability directly via cholinergic projections from SI to the entire neocortical mantle (Mogenson et al., 1983; Zaborszky et al., 1999; Semba, 2000). By targeting α7 nACh receptors, and therefore Glu transmission, KYNA may have profound control of this interconnected system.
Alpha-7 receptors are critical in stimulating DA release from the VTA, indicating a potential role for KYNA in modulating NAC DA tone (Jones et al., 1999). Activation of nACh receptors enhances Glu signaling with phasic, but not tonic, stimulation, which indicates KYNA may modulate multiple aspects of neuronal communication (Alkondon et al., 2003). Conversely, neuronal activity is also capable of impacting astrocytic communication. Neurons can induce plasticity in astrocytes via increased Ca$^{2+}$ (Araque et al., 2001). Collectively, bi-directional neuron/astrocyte communication provides myriad possibilities for fine tuning information processing. Neurotransmitters like Glu, DA, and norepinephrine all increase astrocytic Ca$^{2+}$ levels, and may be one mechanism for neurons to drive astrocytic activity (Parpura and Haydon, 2000). Interestingly, pharmacological manipulations that augment DA efflux show corresponding reductions in extracellular KYNA (Poeggeler et al., 2007), indicating neuronal activity can also modulate KYNA. In the striatum, metabotropic Glu agonists like quisqualate reduce KYNA by inhibiting KAT II activity (Gramsbergen et al., 1997; Battaglia et al., 2000). Reciprocal interactions between neurons and astrocytes provide a local feedback circuit to fine-tune information transfer at the synapse (see for details Carmignoto and Fellin, 2006).

### 6.3. Functional implications

The schizophrenic brain has distributed and functionally interconnected abnormalities that can be exacerbated by cognitively-challenging conditions (McGuire et al., 2008). Interactions between multiple dysfunctional brain regions yield inappropriate or inefficient information processing (Andreasen et al., 1997). The degree of cortical
activation is task- and context-dependent (Callicott et al., 2000; Schlosser et al., 2008), collectively suggesting dysfunctions in multiple aspects of information processing (Schlosser et al., 2008).

Abnormalities in NAC-PFC communication may lead to enduring problems in a variety of executive functioning and mediate many symptomotologies inherent to schizophrenia (Weinberger and Lipska, 1995). The NAC conveys information of the incentive salience of stimuli by selectively amplifying signals that predict reward. Prefrontal innervation of the NAC is selectively gated by hippocampal input, modulating NAC activity under heightened attentional demand. Enhanced PFC drive of mesolimbic information augments ACh release in the PFC, to enhance executive control over information processing with increased functional demand on attentional resources (Kozak et al., 2005; Sarter et al., 2006). Exaggerated KYNA in the NAC or locally within the cortex dysregulates Glu, DA and ACh signaling and contribute to abnormal information processing. Dysfunctions in these interactions may be especially detrimental under attentionally-taxing conditions, interfering normal information processing, thus impairing a wide array of cognitive functions. Collectively, the data presented in this thesis determined KYNA regulates cortical ACh release and cognitive behavior and implicate chronically elevated KYNA in the pathophysiology of schizophrenia. Development of pharamacotherapies aimed to normalize aberrant KYNA concentrations, like KAT II inhibitors, or treatments designed to augment α7 nACh receptor activity like selective α7 nACh receptor agonists may provide novel and efficacious treatments for the cognitive deficits in schizophrenia. Recent studies found α7 agonists improve a variety of
symptoms including sensory gating, negative symptoms and cognitive deficits in schizophrenia (Martin et al., 2004).Administration of galantamine competitively antagonizes KYNA’s actions on the α7 nACh receptor (Lopes et al., 2007), may be a useful adjunct to neuroleptic treatment. Administration of galantamine to patients already treated with risperidone significantly improved measures of attention (Schubert et al., 2006). Moreover, selective α7 agonists work across the same neuroanatomical systems as antipsychotic drugs, and induce c-fos expression in the PFC and NAC shell, but not the DS (Hansen et al., 2007).

6.4. Conclusions

These studies collectively demonstrate a remarkable regulatory capacity for KYNA mediating neurotransmitter release and behavior. Whether the transynaptic regulation of ACh by KYNA is attributable to neuronal networks or the reciprocal communication between these systems remains unclear. The research presented here is both innovative and significant because it is the first demonstration to link astrocytic activity and cortical cholinergic transmission locally in the PFC and across anatomically distinct brain regions. These results reveal an added layer of complexity previously unknown regarding the mechanisms regulating neuronal communication and is also the first to uncover the role of KYNA in regulation of prefrontally-mediated cognitive flexibility. Collectively, these studies implicate KYNA-ACh dysfunction in neuropsychiatric disorders and may ultimately pave the way for the development of more efficacious pharmacotherapies for the treatment of cognitive deficits seen in schizophrenia.
APPENDIX: FIGURES
Figure 1. The distributed systems mediating basal forebrain cholinergic system excitability and attentional processing. Cortical ACh release is determined by activity of the substantia innominata (SI) region of the basal forebrain (BF). BF activity is modulated by GABAergic projections from the nucleus accumbens (NAC) that is regulated by converging Glu input from the cortex, amygdala (AMYG), and hippocampus (HIP). The NAC also receives modulatory DA input from the ventral tegmental area (VTA). Under normal conditions, cortical drive of the NAC is fairly minimal. However, under conditions of heightened attentional demand, PFC input can modulate NAC activity and bias attentional resources of the BFCS via a PFC-NAC-BF circuit.
Figure 2. The Kynurenine Pathway of Tryptophan Degradation
**Figure 3.** Panel A depicts a schematic representation of a mPFC probe placement. Guides were implanted so that when probes were inserted, the membrane tip (3.0 mm) was located at: $A = 4.2$, $L = 0.6$, $V = 3.6$ from dura at 20º rostral. Panel B is a representative photomicrograph of the mPFC placement illustrated in Panel A. All coordinates were calculated according to Paxinos and Watson (1998).
Figure 4. Comparison between ACh efflux measured from the mPFC after intra-cortical administration of 100 nM, 300 nM or 1 μM KYNA during separate dialysis sessions. Mean (± S.E.M.) ACh efflux in the mPFC (n = 6). Following baseline collections (collections 1-4), vehicle or KYNA was administered for 60 minutes (collections 5-8). Upon conclusion of the 60 minute perfusion, aCSF alone was perfused for 45 minutes until the end of the dialysis period (collections 9-11). Cortical ACh release was significantly reduced below baseline levels by perfusion of 100 nM KYNA only.
Figure 5. Comparison between ACh efflux measured from the mPFC after intra-cortical perfusion of kynurenine (Kyn). Mean (± S.E.M.) ACh efflux in the (n = 7) receiving, in counterbalanced order, vehicle (aCSF), 2 or 5 µM Kyn during separate dialysis sessions. Following baseline collections (collections 1-4), vehicle or Kyn was administered for 60 minutes (collections 5-8). Upon conclusion of the 60 minute perfusion, aCSF alone was perfused for 45 minutes until the end of the dialysis period (collections 9-11). No concentration of Kyn significantly reduced cortical ACh release.
Figure 6. Comparison between ACh efflux measured from the mPFC with intra-cortical administration of the KAT II inhibitor S-ESBA designed to reduce endogenous KYNA production. Mean (± S.E.M.) ACh efflux in the and mPFC (n = 5) received, in counterbalanced order, vehicle (aCSF), 500 μM, 2 mM, or 5 mM S-ESBA into the mPFC during separate dialysis sessions. Following baseline collections (collections 1-4), vehicle or S-ESBA was administered for 60 minutes (collections 5-8). Upon conclusion of the 60 minute perfusion, aCSF alone was perfused for 45 minutes until the end of the dialysis period (collections 9-11). Cortical ACh was dose-dependently increased by S-ESBA administration.
Figure 7. Comparison between ACh efflux measured from the mPFC following administration of vehicle, KYNA, the KAT II inhibitor S-ESBA and a combination of KYNA+S-ESBA. Mean (± S.E.M.) ACh efflux in the and mPFC (n = 7) received, in counterbalanced order, vehicle (aCSF), 100 nM KYNA, 5 mM S-ESBA, or KYNA+ S-ESBA into the mPFC during separate dialysis sessions. Following baseline collections (collections 1-4), vehicle or drug was administered for 60 minutes (collections 5-8). Upon conclusion of the 60 minute perfusion, aCSF alone was perfused for 45 minutes until the end of the dialysis period (collections 9-11). The ACh facilitated by S-ESBA administration was blocked with concurrent perfusion of exogenous KYNA, indicating ACh released by S-ESBA is causally driven by reductions in endogenous KYNA.
Figure 8. Distributed systems underlying astrocytic modulation of PFC ACh release. KYNA targets α7 nACh receptors on pyramidal cells and interneurons in the cortex, and can modulate ACh release within local PFC circuits or through a long-loop pathway that includes possible PFC-NAC-BF connections.
Figure 9. Panel A depicts a schematic of a mPFC placement. Guides were implanted so that when probes were inserted, the membrane tip (3.0 mm active tip) was located at: AP + 4.2, M = 0.6, V = 3.6 from dura with the tip of the guide oriented at 20º rostrally. Panel B is a photomicrograph depicting a representative mPFC placement. Panel C is a schematic representing probe placement into the NAC shell. Guides were implanted so that when probes were inserted (2 mm active tip), the membrane tip was located at A = 1.3, M =1.0, V = 7.8 from dura matter. Panel D is a photomicrograph of a representative NAC placement illustrated in panel C. All coordinates were calculated according to Paxinos and Watson (1998).
Figure 10. Comparison between ACh efflux measured from the mPFC following intranAC administration of vehicle, KYNA, the KAT II inhibitor, S-ESBA and a combination of KYNA+S-ESBA. Mean (± S.E.M.) ACh efflux in the and mPFC (n = 8) received, in counterbalanced order, vehicle (aCSF), 100 nM KYNA, 5 mM S-ESBA, or KYNA+S-ESBA into the mPFC during separate dialysis sessions. Following baseline collections (collections 1-4), vehicle or drug was administered for 60 minutes (collections 5-8). Upon conclusion of the 60 minute perfusion, aCSF alone was perfused for 30 minutes until the end of the dialysis period (collections 9-11). PFC ACh release was significantly elevated by S-ESBA, indicating cortical ACh release is tonically inhibited by KYNA within the NAC.
Figure 11. The attentional set shifting maze. Arms vary on 2 stimulus dimensions: brightness (black and white arms) and texture (smooth and rough arms). Animals are tested with one arm of the maze blocked so they must choose between 1 of 2 possible choice arms after leaving the start arm.
Figure 12. Mean (± S.E.M.) % correct scores for animals acquiring Set 1 after pre-treatment with saline (n = 6) or Kyn (n = 6; 50 mg/kg, i.p.). No differences in the rate of rule acquisition were seen between drug treated and control animals. Therefore, Kyn administration prior to Set 1 did not impair initial rule acquisition.
Figure 13. Mean (± S.E.M.) % correct scores for animals during acquisition of day 2 (Set 2) of testing. Animals were administered either saline or kynurenine (50 mg/kg) before Set 1 testing, and both groups were given saline prior to Set 2. There were no differences between groups in ability to make the ED shift.
Figure 14. Composite graph showing trials to criterion (mean, ± SEM; top panel) and the % of subjects to reach criterion and (bottom panel). The percentages listed above the Set 2 bar graphs (top panel) represent the percent of subjects to reach Set 2 criterion, and therefore make an ED shift. The numbers in parentheses above the bar graphs (bottom panel) indicate the average trials to criterion reached for each group per day.
Figure 15. Perseverative error rate of animals acquiring Set 2 that received saline or Kyn prior to learning Set 1. Administration of Kyn pre Set 1 did not impact Set 2 performance.
Figure 16. Mean (± S.E.M.) % correct scores for animals during acquisition on day 1 (Set 1) of testing. Animals were administered saline or kynurenine (50 mg/kg) prior to Set 2 testing; animals were not treated prior to Set 1 testing. Drug-treated animals were sub-divided into groups of animals that learned (n=2) or that failed to learn (n=5) the new response strategy when tested on day 2.
Figure 17. Mean (± S.E.M.) % correct scores for animals during acquisition of Set 2 (day 2 of testing). Animals were administered saline or kynurenine (50 mg/kg) prior to testing. Drug-treated animals were sub-divided into groups of animals that learned (n=2) or that failed to learn (n=5) the new response strategy.
Figure 18. Composite graph showing trials to criterion (mean, ± SEM; top panel) and the % of subjects to reach criterion and (bottom panel). The percentages listed above the Set 2 bar graphs (top panel) represent the percent of subjects to reach Set 2 criterion, and therefore make an ED shift. The numbers in parentheses above the bar graphs (bottom panel) indicate the average trials to criterion reached for each group per day. Administration of Kyn prior to Set 2 severely impaired ED shifting with only 28.5% of those subjects learning the new rule.
Figure 19. Mean (± S.E.M.) perseverative error rate for animals on day 2 (Set 2) of testing. Animals were administered saline or kynurenine (50 mg/kg) prior to testing. Drug-treated animals were sub-divided into groups of animals that learned (n=2) or that failed to learn (n=5) the new response strategy. Perserverative errors were similar between saline-treated controls and the subset of Kyn-treated animals that successfully made the ED shift while Kyn-treated animals that did not learn maintained higher rates of perseverative errors.
Figure 20. This depicts a photomicrograph of a representative placement of dual mPFC infusion cannulae. Cannulae were implanted so that infusion needles were inserted the tip was located at: AP + 4.2, L = 0.6, DV – 1.5 from dura at 20º rostral angle (right arrow). The second mPFC infusion cannula was placed at P – 1.5, L = 0.6, DV – 1.5 at 20º caudal angle from dura matter. All coordinates were calculated according to Paxinos and Watson (1998).
Figure 21. Mean (± S.E.M.) % correct scores for animals during acquisition of day 1 (Set 1) of testing. Animals were administered either saline (0.5 μl/at 0.25 μl/min) or kynurenine (10 μM/0.5 μl) before Set 1 testing (n = 6 for each group). Drug treatment had no effect on initial rule acquisition.
Figure 22. Mean (± S.E.M.) % correct scores for animals during acquisition of day1 (Set 1) of testing. Animals were administered either aCSF or kynurenine (10 μM/0.5 μl) before Set 1 testing and either aCSF or Kyn prior to Set 2. Drug treatment had no effect on initial rule acquisition, but it did impair ED shifting in 5 of 6 animals infused with Kyn prior to Set 2.
Figure 23. Composite graph showing the % of subjects to reach criterion (mean, ± SEM; top panel) and trials to criterion (bottom panel). The percentages listed above the Set 2 bar graphs (top panel) represent the percent of subjects to reach Set 2 criterion, and therefore make an ED shift. The numbers in parentheses above the bar graphs (bottom panel) indicate the average trials to criterion reached for each group. Administration of Kyn prior to Set 2 severely impaired ED shifting with only 16.7% of those subjects learning the new rule (1 of 6).
Figure 24. Mean (± S.E.M.) perseverative error rate for animals on day 2 (Set 2) of testing. Animals were infused with aCSF or Kyn (10 μM/0.5 μl) prior to testing. Perserverative errors were similar between all groups.
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