ASTROCYTES REGULATE COGNITIVE FLEXIBILITY AND NEURONAL OSCILLATIONS BY RELEASING S100β

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Abstract

Astrocyes are the most numerous cell in the human brain yet their role in behavior and brain functioning has remained incompletely explored. The goal of my dissertation is to examine the role astrocytes play in cognition. Over the course of several experiments, I show that astrocytes not only change due to experiences associated with improved cognition, but that astrocytes themselves are important contributors to cognition. My dissertation research has primarily utilized a task of cognitive flexibility that previous research in rodents, nonhuman primates, and humans suggests requires the medial prefrontal cortex. Here I show that voluntary exercise improves cognitive flexibility in rodents and that this enhancement is associated with increased astrocyte size and increased dendritic spine density in the medial prefrontal cortex. I follow up on these findings to show that impairment of astrocyte functioning in the medial prefrontal cortex, but not the orbitofrontal cortex, results in diminished cognitive flexibility. Next, I demonstrate that reducing the number of astrocytes in the medial prefrontal cortex similarly impairs cognitive flexibility, and that cognitive flexibility can be enhanced by specifically increasing Ca²⁺ signaling in astrocytes in the medial prefrontal cortex. Finally, I show that this facilitation is likely controlled by the astrocyte-specific protein S100β, and link S100β levels to changes in neuronal synchrony thought to underlie cognitive flexibility. Collectively, my work demonstrates that astrocytes are important contributors to cognitive flexibility.
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Chapter 1: Introduction
The study of neurons and their function has remained the central focus of neuroscience research since the field's inception. Countless studies have linked anatomical, chemical and electrical changes in neurons to changes in behavior. This has led to a very “neurocentric” focus that often overlooks or does not account for the function of roughly half of the other cells in the human brain (Azevedo et al., 2009). These “other” cells, glia, are a highly heterogeneous population of cells often described as being important for support functions that primarily serve the needs of neurons (Nedergaard et al., 2003; Fields 2011).

Glia, from the Greek for ‘glue’ or equally flattering, ‘cement’, is a generic term first coined by Virchow, and applied broadly to describe the connective substance in which neurons were embedded (Virchow 1858). Historically, glia were more difficult to stain and properly visualize relative to neurons, and this stalled much of the early research into these cells (García-Marín et al., 2007). New staining techniques such as the sublimated gold chloride method (Ramón y Cajal 1913a) allowed for more nuanced descriptions of arachnoid-shaped cells that made up the ‘cement,’ that Michael von Lenhossék would later term, astrocytes (von Lenhossék 1893; García-Marín et al., 2007). Ramón y Cajal went on to suggest that the word astrocyte should encompass two types of cells; one found in grey matter (protoplasmic astrocytes) and the other in white matter (fibrous astrocytes) (Ramón y Cajal 1913a). Later, Ramón y Cajal would also offer some of the first hypotheses about the function of astrocytes, suggesting that, given their proximity to blood vessels, astrocytes may be important for brain metabolism and for supplying nutrients and other factors to neurons (Ramón y Cajal 1913b). Ramón y Cajal also speculated that astrocytes may participate in the regulation neuronal signaling given that astrocytic processes often surrounded neighboring synapses (Ramón y Cajal 1913b). Despite these early predictions about the function of astrocytes, research into astrocyte function has been slow. In the last thirty years, however, evidence has confirmed that astrocytes are important for both maintaining the blood brain barrier and regulating blood flow. However, the study of astrocyte function has remained relegated to the domain of support function. As the tools to properly study astrocytes became available, initial efforts into exploring astrocyte function began using these tools to confirm previous hypotheses about the support nature of these cells rather than exploring what if any role astrocytes might play in behavior.
Despite the initial and persistent characterization as support cells (Ramón y Cajal, 1899, Barres 2008; Parpura et al., 2012), the development of better tools and the implementation of genetic screening techniques (Cahoy et al., 2008) have helped show that astrocytes are complex cells that can regulate synaptic strength (Henneberger et al., 2010). Astrocytes are highly heterogeneous and the functional complexity of these cells is only just beginning to be appreciated (Barres 2008; Ben Haim and Rowitch, 2016; Khakh and Sofroniew, 2016).

Astrocytes can release small signaling molecules known as gliotransmitters (Nedergaard et al., 2003), and in some cases these molecules have been shown to be important for the induction of LTP (Henneberger et al., 2010; Han et al., 2013), but little work has examined astrocyte signaling in conjunction with complex behaviors such as cognitive flexibility. Across evolution, the presence of astrocytes in the brain, relative to neurons, has steadily increased in species capable of more complex cognitive abilities. In C. elegans the ratio of astrocytes to neurons is 1:6, in small mammals such as rodents, the ratio is 1:3, while in humans, the ratio of astrocytes to neurons is 3:2 (Nedergaard et al., 2003). Human astrocytes are not only more numerous than their rodent counterparts, but they are approximately ten times larger and extend processes whose endfeet contact upwards of two million synapses, compared to the approximately 200,000 contacts made in rodents (Bushong et al., 2002; Oberheim et al., 2006; Oberheim et al., 2009). Individual astrocytes occupy relatively discrete territories, and have been shown to detect and respond to the inputs and outputs of upwards of hundreds of thousands of neurons (Bushong et al., 2002; Oberheim et al., 2006; Oberheim et al., 2009). While in part, changes in astrocytes across species could be attributed to scaling or increased metabolic demand, the magnitude with which the size and number of astrocytes has increased exceeds what would be expected by linear scaling and this has led some to hypothesize that astrocytes may be a crucial factor in the development of complex behaviors such as cognition (Nedergaard et al., 2003; Oberheim et al., 2009; Han et al., 2013).

Recent evidence suggests that human astrocytes when xenografted into the developing brains of immunodeficient mice maintain human-typical morphological and functional properties and intercalate with the developing mouse brain (Han et al., 2013). Human astrocytes transplanted into the mouse brain demonstrate enhanced Ca$^{2+}$ signaling, a primary means of
astrocytic signaling, compared to allograft controls, but also perform better than controls on several measures of cognitive ability (Han et al., 2013). While these findings do not provide insight into what native astrocytes may be doing in either the rodent or human brain, they are at least suggestive of the possibility that astrocytes may be contributing to cognitive function in a previously unidentified way.

Studies have demonstrated that a reduction in astrocyte number or temporary impairment of astrocyte functioning in the medial prefrontal cortex, an area of the brain important for cognition, can induce anhedonic-like behaviors in rodents (Banasr and Duman 2008; Bechtholt-Gompf et al., 2010) as well as impair some cognitive abilities (Gibbs et al., 2006a, b; Bechtholt-Gompf et al., 2010; Lima et al., 2014). Moreover, several studies have demonstrated that astrocytes contribute to synaptic plasticity, either through the induction of LTP (Henneberger et al., 2010) or through the regulation of contacts that astrocytes make with surroundings synapses (Genoud et al., 2006; Lushnikova et al., 2009; Bernardinelli et al., 2014, Perez-Alvarez et al., 2014). Astrocytes also provide over 90% of the total number of neurons in the brain with access to nutrients from the surrounding vasculature, and this neurovascular coupling has been shown to be important for the shape and the timing dynamics of the blood oxygen level-dependent (BOLD) response (Haydon and Carmignoto et al., 2006; Schummers et al., 2008; Attwell et al., 2010; Schulz et al., 2012). While speculative, these findings suggest that astrocytes may be major contributors to managing brain region-specific computation and that astrocytes may be important for shaping the coordinated output of a given brain region.

Astrocytic involvement in synchronized neuronal firing is just beginning to be investigated. One limitation to this approach is that astrocytes themselves are weakly electric and many of the electrophysiological techniques well-suited for the study of neurons must be adapted and reconfigured for the study of astrocytes. Astrocytic signaling both within the cell and between other astrocytes is often carried out through the mobilization of intracellular Ca\textsuperscript{2+} (Perea et al., 2005; Agulhon et al., 2010; Poskanzer and Yuste, 2011; Paukert et al., 2014; Poskanzer and Yuste, 2016). The importance of the spatiotemporal dynamics of this signaling is only just beginning to be explored but recent evidence from urethane-anesthetized mice has shown that astrocytes directly control state shifts between highly synchronized and desynchronized states,
where astrocytic Ca\textsuperscript{2+} signaling often predicts increased glutamate release necessary for these shifts (Poskanzer and Yuste 2011; Poskanzer and Yuste 2016). Additionally, astrocyte activity has been linked to the generation of rhythmic firing behavior in neurons associated with respiration (Gourine et al., 2010) and mastication (Morquette et al., 2015). With regards to mastication, the role of astrocyte-specific Ca\textsuperscript{2+} signaling has been expanded slightly, as rhythmogenic firing was shown to be regulated by the presence of an astrocyte specific protein S100\textbeta (Morquette et al., 2015). S100\textbeta is typically described as a Ca\textsuperscript{2+} binding protein known for the sequestering of intracellular Ca\textsuperscript{2+}. However, in this experiment S100\textbeta was shown to regulate extracellular Ca\textsuperscript{2+} and was necessary for the induction of rhythmic firing in neurons (Morquette et al., 2015). While the complex nature of astrocytic Ca\textsuperscript{2+} signaling is only just beginning to be understood, collectively these findings hint that astrocytes have the potential to play an often-overlooked role in the regulation of brain signaling. Moreover, few studies have investigated whether this role is important for the regulation of behavior, namely cognition.

For my dissertation, I sought to examine the potential role astrocytes play in cognition. I chose to focus much of my work on cognitive flexibility, as this is a highly dynamic behavior that is an essential component of cognition. Cognitive flexibility, the capacity to shift one’s attention from one response set to another, is critical for our ability to negotiate daily life and has been shown to be impaired in several neuropsychiatric disorders including schizophrenia (Zawadzki et al., 2013; Fajnerova et al., 2014). Despite the importance of this cognitive function, its underlying mechanisms remain relatively unknown. Numerous fMRI studies in humans, as well as excitotoxic lesion studies in rodents, suggest that neurons in the medial prefrontal cortex are necessary for cognitive flexibility, however, few studies have attempted to fully characterize this circuitry or have proposed a definitive mechanism by which the brain might control this behavior (Birrell and Brown 2000; Miller and Cohen 2001; McAlonan and Brown 2003).

Researchers have speculated that changes in neuronal oscillations, specifically in the theta (6-10 Hz) and gamma band (30-80 Hz), may underlie changes in attention and memory that are essential for cognitive flexibility (Canolty et al., 2006; Lee et al., 2014). Indeed, abnormalities in theta and gamma oscillations in the medial prefrontal cortex have been reported in patients with schizophrenia alongside concomitant deficits in cognitive flexibility (Basar-Eroglu
et al., 2007; Uhlhaas and Singer 2010). Little is known about the specific cellular machinery responsible for the generation of these rhythms or the functional importance changes in these rhythms has on behavior. Recent evidence has linked the activation of astrocyte Ca$^{2+}$ signaling to changes in hippocampal gamma rhythm and enhanced memory ability, however, the specific mechanism by which astrocytes influence these processes is unknown (Lee et al., 2014).

Over the course of several studies, we have shown that astrocytes in the medial prefrontal cortex play an important role in cognitive flexibility. We demonstrate that astrocytes change under conditions associated with enhanced cognitive flexibility, and that astrocytes are necessary for the maintenance of cognitive flexibility. In addition, we have identified a novel mechanism whereby astrocytes improve cognitive flexibility by releasing an astrocyte-specific Ca$^{2+}$ binding protein, S100β, which, in turn, enhances phase amplitude coupling between gamma and theta oscillations in the medial prefrontal cortex. Thus, our work suggests that astrocytes are important contributors to cognitive flexibility, and more generally, to behavior, a relatively novel and previously unknown role for these cells. Moreover, we have identified a novel mechanism by which astrocytes can directly influence neural activity, which may lead to a change in our understanding of how the brain functions.
Chapter 2: Physical Exercise Enhances Cognitive Flexibility as Well as Astrocytic and Synaptic Markers in the Medial Prefrontal Cortex
Background

Physical exercise is known to enhance cognition in humans across multiple age groups ranging from school age children to the elderly, as well as in healthy individuals and patient populations (Kramer et al., 2007; Voss et al., 2013). The types of cognitive tasks known to be improved by physical exercise in humans are varied, including tests of vocabulary (Salis 2013; Schmidt-Kassow et al., 2013), working memory (Alves et al., 2014) and executive function (Masley et al., 2009). Consistent with the wide range of improved cognitive function, physical exercise also has widespread effects on human brain structure, increasing volume in many brain regions (Kramer et al., 2007).

Understanding the cellular processes that underlie physical exercise-induced improvements in brain function will help us to understand cognition in general, as well as to identify mechanisms that might be engaged for therapeutic improvement of learning and memory. Toward this end, a considerable effort has been focused at understanding the effects of running on cognitive function in rodent models, although the vast majority of this research has focused on one brain region, the hippocampus (Kramer et al., 2007; Vivar et al., 2013). Studies in both rats and mice have shown that running enhances performance on tasks that require the hippocampus (van Praag et al., 1999), including context fear conditioning (Berchtold et al., 2010; Marlatt et al., 2012) and spatial navigation learning (Alaei et al., 2007; Greenwood et al., 2009). By contrast, relatively few studies in rodents have examined the effects of running on cognitive functions that require brain regions other than the hippocampus.

A large body of evidence suggests that running influences a variety of neuronal measures, including synaptic plasticity (Eadie et al., 2005), adult neurogenesis (van Praag et al., 1999; Stranahan et al., 2006) and dendritic spine density in the hippocampus (Stranahan et al., 2007; Marlatt et al., 2012). By contrast, relatively few studies have considered the possibility that running alters nonneuronal cells in a manner consistent with their involvement in running-induced cognitive enhancement in brain regions important for cognition beyond the hippocampus (Viola et al., 2009; Saur et al., 2014). Astrocytes, the most common nonneuronal cell type in the brain, communicate with both neurons and the brain vasculature (Haydon and Carmignoto 2006;
Barres 2008). Studies indicate that astrocytes play an important role in synaptic plasticity, including long term potentiation (LTP) (Henneberger et al., 2010), suggesting their potential involvement in learning and memory. Indeed, transplantation of human astrocytes into the mouse brain results in improved cognitive performance on a wide range of tasks potentially through enhancements in synaptic plasticity (Han et al., 2013). Despite these intriguing observations, previous studies have not established whether running-enhanced cognitive improvements are associated with changes in astrocytes. We sought to investigate this possibility as well as to examine whether brain regions that support cognition in addition to the hippocampus were affected by running.

**Methods and Materials**

**Experimental Animals**

Adult male Sprague-Dawley rats were housed in groups of 3 with or without ad lib access to a running wheel for 12 days. This time point was selected because it is sufficient to induce dramatic structural alterations in the hippocampus (van Praag et al., 1999; Stranahan et al., 2006). Running distance was recorded daily from digital counters mounted onto the running wheels. Rats were housed on a reverse 12-hour light-dark schedule (lights off at 0700), and all behavioral testing occurred between 0900 and 1400. 24 hours after the completion of behavioral testing, rats were deeply anesthetized and then perfused using 4% paraformaldehyde and their brains were processed for histology and microscopic analysis. Separate cohorts were assessed on either object memory tasks or the attentional set shifting task. Rats tested on the attentional set-shifting task were food-deprived to 85% body weight over 7 days before the start of testing.

The studies described in this chapter were approved by the Princeton University IACUC (protocol # 1852, approved June 2014) and conformed to the National Research Council Guide for the Care and Use of Laboratory Animals. Animals were deeply anesthetized with Euthasol and transcardially perfused at the end of these experiments.

**Object Memory Testing**

Runners (n=18) and sedentary controls (n=18) were tested on two object memory tasks; object in place, a task known to rely on the medial prefrontal cortex as well as the hippocampus
or perirhinal cortex, and novel object preference, a task known to rely on the perirhinal cortex (Barker and Warburton 2011). The sequence of testing was counterbalanced and rats were tested only once within a 24-hour period. Before testing, rats were habituated to an empty testing arena (17 x 17 x 17 cm) for 10 minutes/day for 3 days. On test days, rats were placed in the center of the arena and behavior was scored by an experimenter unaware of the treatment condition. Each task consisted of a 5-minute acclimation period where animals were exposed to different objects that varied in size (6 x 7 x 10 cm – 15 x 17 x 10 cm). During object in place, 4 different objects were placed in each of the 4 corners of the box 3 cm away from the arena walls. For novel object preference, 2 identical objects were placed in 2 corners of the same side of the arena 3 cm away from the walls. For both tasks, following a 5-minute break where rats were returned to their home cage, rats were again placed in the center of the testing arena for a 3-minute test. During object in place, rats were exposed to the same 4 objects, but the location of 2 of the objects was switched. During novel object preference, animals were exposed to 2 objects, 1 familiar and 1 novel. The sides of the arena in which either the orientation of the objects or the novel object were presented were counterbalanced. The time spent with the novel object or orientation was recorded. A discrimination ratio was calculated as the time spent with the novel object or orientation divided by the total time spent exploring both novel and familiar objects/orientations during the test phase (Barker and Warburton 2011).

**Attentional Set-Shifting Task**

A separate cohort of runners (n=12) and sedentary controls (n=12) were tested on the attentional set-shifting task, a test of cognitive flexibility (Birrell and Brown 2000; Leuner and Gould 2010). Attentional set-shifting consisted of 3 days of testing in which rats learned to discriminate between digging media or the texture covering the digging container to retrieve a food reward (1/4 of a Froot Loop). On testing days, rats were habituated to the testing room for 10 minutes prior to the start of testing. The attentional set-shifting task consists of 5 separate discriminations: simple discrimination, compound discrimination, intradimensional shift, reversal, and extradimensional shift. For each phase, rats must reach a criterion of 6 consecutive trials of retrieving the food reward before advancing to the next phase. Performance on the reversal is
dependent on the orbitofrontal cortex (McAlonan and Brown 2003) while performance on the extradimensional shift is dependent on the medial prefrontal cortex (Birrell and Brown 2000).

**Immunolabeling for astrocyte and synaptic markers**

Brains for immunohistochemical analysis were postfixed for 48 hours before processing. 40 µm coronal sections (1:12) were cut from half brains into a bath of 0.1M PBS using a Vibratome. For astrocyte immunolabeling, free-floating sections were rinsed in 0.1 M PBS, pH 7.5, and incubated with 3% normal donkey serum, and 0.1M PBS with 0.1% Triton X-100 and either rabbit anti-S100 (1: 10,000, Dako) or rabbit anti-aquaporin-4 (1:500; Santa Cruz Biotechnology), mouse anti-smooth muscle actin (1:200; Santa Cruz Biotechnology) and stored at 4°C. S100, an astrocyte specific Ca²⁺ binding protein, was used to visualize astrocyte cell bodies (Cahoy et al., 2008). Aquaporin-4, a water channel, was used to visualize astrocytic endfeet on blood vessels (Aoyama et al., 2012). After incubation in primary antisera for 24 hours, the sections were rinsed, incubated in donkey anti-rabbit Alexa 488 (1:250; Invitrogen) in the dark for 60 min, rinsed and mounted onto superfrost slides, and coverslipped using glycerol in TBS (3:1). All sections were counterstained with the DNA dye, Hoechst 33342 (1: 100,000, Molecular Probes). To assess the localization of S100 staining in the astrocyte population, additional sections were stained for both S100 as described above and the astrocyte cytoskeletal marker glial fibrillary acidic protein (GFAP) using guinea pig anti-GFAP (1:1000; Synaptic Systems). To assess the location of aquaporin-4 staining to that of astrocytic processes and blood vessels, additional sections were incubated in both anti-aquaporin-4 and anti-GFAP or anti-aquaporin-4 and mouse anti-smooth muscle actin (1:50 Santa Cruz Biotechnology). For analysis of synaptic markers, free-floating sections were rinsed in 0.1 M PBS, pH= 7.5, and incubated with 3% normal donkey serum, and 0.1M PBS with 0.1% Triton X-100 and either mouse anti-postsynaptic density 95 (PSD-95), a postsynaptic marker, (1:200, Millipore) or rabbit anti-synaptophysin, a presynaptic marker (1:500; Santa Cruz Biotechnology), and stored at 4°C. After incubation in primary antisera for 24 hours, sections were rinsed, incubated in donkey anti-mouse Alexa 568 (1:250; Invitrogen) to visualize PSD-95 or incubated in donkey anti-rabbit Alexa 488 (1:250; Invitrogen) to visualize synaptophysin, in the dark for 60 min. Sections were mounted and coverslipped as described above.
Slides were coded until completion of the data analysis. Images from the medial prefrontal cortex, orbitofrontal cortex, hippocampus and perirhinal cortex were taken using a Zeiss confocal microscope (LSM 700; lasers, argon 458/488; HeNe 568). Cross-sectional area measurements of 50 S100+ cells per animal per brain region (medial prefrontal cortex, hippocampus, perirhinal cortex, orbitofrontal cortex) were obtained from 20 µm image stacks using ImageJ (NIH). Regions of interest were chosen based on task relevance. Selected cells were near layer 2/3 pyramidal neurons in the medial prefrontal cortex, perirhinal cortex and orbitofrontal cortex. Cells were located in the CA1 region for the hippocampus analysis. Selected cells had to be distinct from surrounding labeled cells and be fully stained. Cross-sectional area measurements were taken from z-stacks by using ImageJ (NIH) by circling individual cells at their widest point and using the area function. Cross-sectional area covered by aquaporin-4-immunolabeled blood vessels was taken from 1 µm optical sections. All parameters for background subtraction were optimized and held constant throughout the analysis. For PSD-95 and synaptophysin analyses, mean intensity values were collected from 1 µm image stacks and background, determined from adjacent white matter bundles in olfactory bulbs or from the corpus callosum, was subtracted out.

*DiI Impregnation*

Brains of runners (n=9) and sedentary controls (n=9) were dissected and blocked down the midline. 2-3 lipophilic DiI crystals (Sigma) were implanted into the corpus callosum to backfill layer 2/3 pyramidal neurons in the medial prefrontal cortex (Kozorovitskiy et al., 2005). Tissue was incubated at 37°C for 4 weeks. 100 µm coronal sections throughout the medial prefrontal cortex were cut using a Vibratome into a bath of 0.1M PBS. Sections were mounted with PBS, coverslipped and viewed using a Zeiss confocal microscope (LSM 510; Lasers, HeNe 568) with a 40x oil objective. All settings (pinhole size, aperture gain, and offset) were initially optimized and held constant throughout the study. For each rat, 5 pyramidal neurons from layer 2/3 in the medial prefrontal cortex neurons were analyzed in 20-50 µm z-stacks by counting spines along secondary and tertiary apical and basal dendrite segments (lengths totaled 50µm/dendrite/neuron). Neurons selected for analysis were fully impregnated, had clearly identifiable secondary and tertiary branches, and were relatively isolated from other neurons.
For each brain, 50 dendritic spines were analyzed in detail (25 apical, 25 basal) for length of the spine neck.

**Statistics**

Unpaired two tailed Student’s t-tests were performed on each data set (sedentary x runner) except for the attentional set-shifting data, which were analyzed as a mixed factorial ANOVA.

**Results**

*Running enhances object memory*

Compared to sedentary controls, runners showed an enhanced discrimination ratio on the object in place task ($t_{(30)} = 5.2, p = .001$) (Figure 1), a medial prefrontal cortex-dependent task (Barker and Warburton 2011). It should be noted that sedentary rats did not exhibit the ability to discriminate on the object in place task whereas runners did. The lack of a convincing discrimination ratio among the sedentary rats is likely due to our use of Sprague Dawley rats, a strain that underperforms and shows greater variability on cognitive tasks compared to pigmented strains (Andrews et al., 1995; Turner and Burne 2014). Despite the poor performance on the object in place task by sedentary rats, running improved such performance significantly. By contrast, there was no difference in the discrimination ratios of runners and sedentary animals on the novel object preference after 12 days of running ($t_{(27)} = .839, p = .4$) (Figure 1).

*Running enhances cognitive flexibility*

Runners showed an enhancement on the simple discrimination, reversal, and extradimensional shift portions of the attentional set-shifting task in both the number of trials to reach criterion (simple discrimination: $t_{(22)} = -2.77, p = .011$; reversal: $t_{(22)} = -3.54, p = .002$; extradimensional shift: $t_{(22)} = -2.57, p = .017$) and the number of errors (simple discrimination: $t_{(22)} = -2.68, p = .014$; reversal: $t_{(22)} = -3.87, p = .0008$; extradimensional shift: $t_{(22)} = -2.56, p = .018$). There was a significant effect of test for both trials to criterion ($F_{(4,116)} = 2.9, p = .03$) and errors to criterion ($F_{(4,116)} = 2.9, p = .03$) as well as a significant interaction between condition and test for both trials to criterion ($F_{(4,116)} = 3.56, p = .01$) and errors to criterion ($F_{(4,116)} = 3.825, p = .0065$) indicating that sedentary rats performed the task as expected, however, we are unable to
determine whether runners formed an attentional set. These findings may be reflective of runners adopting a different strategy for completing the task in fewer trials and with fewer errors (Figure 1).
Figure 1. Running enhances cognitive performance on tasks known to require the medial prefrontal cortex and orbitofrontal cortex. Rats were given access to a running wheel for 12 days prior to the start of testing. 

a. Running enhances performance on the object in place (OIP) task, but not on the novel object preference (NOP) task.

b. Running results in fewer trials to criterion on the simple discrimination (SD), reversal (REV) and extradimensional shift (EDS).

c. Running results in fewer errors on the SD, REV and EDS. Error bars represent SEM. *p<0.05 compared with Sedentary rats for a-c. Complex discrimination (CD); intradimensional shift (IDS).
Running enhances astrocyte cell body area

Immunolabeling with S100 revealed many cell bodies with proximal processes stained in all brain regions examined. Double labeling of S100 with the astrocytic cytoskeletal marker GFAP revealed co-labeling of many, but not all, S100 cells (Fig. 2) with GFAP staining primarily astrocytic processes. We observed an increase in S100-labeled astrocyte cell body area in the hippocampus, medial prefrontal cortex, and orbitofrontal cortex with running compared to sedentary living (hippocampus: \( t_{(15)} = 2.651, p = .02 \), medial prefrontal cortex: \( t_{(16)} = 2.42, p = .03 \), orbitofrontal cortex: \( t_{(16)} = 3.76, p = .001 \)). No differences were observed in the perirhinal cortex between runners and sedentary controls (Figure 2). This pattern of results, with running-induced enhancements in astrocyte markers in hippocampus, medial prefrontal cortex and orbitofrontal cortex, but not perirhinal cortex, is consistent with our behavioral data demonstrating running enhanced cognitive tasks associated with the hippocampus, medial prefrontal cortex and orbitofrontal cortex (object in place, extradimensional shift and reversal attentional set-shifting), but not the perirhinal cortex (novel object preference).

Running enhances expression of the astrocytic water channel marker aquaporin-4

Aquaporin-4 immunolabeling stained blood vessels as well as numerous thin processes in all brain regions examined. Co-labeling with the astrocytic cytoskeletal marker GFAP revealed overlap with aquaporin-4 labeled blood vessels and processes and GFAP-labeled processes (Figure 2). It should be noted that GFAP does not stain astrocytes in their entirety, typically leaving the cell body and distal processes/end feet unstained. Co-labeling with aquaporin-4 and the smooth muscle marker muscle-specific actin revealed close contact between aquaporin-4 labeled astrocytic processes and blood vessels (Figure 2). Optical density measurements of aquaporin-4 staining for the 12-day running experiment revealed a brain region-specific set of changes that was identical to what we observed with S100 cell body area measurements. Compared to sedentary controls, running increased aquaporin-4 optical density in the hippocampus, medial prefrontal cortex, and orbitofrontal cortex, with no differences in the perirhinal cortex (hippocampus: \( t_{(16)} = 3.4, p = .004 \), medial prefrontal cortex: \( t_{(16)} = 10.8, p = .000 \), orbitofrontal cortex: \( t_{(16)} = 6.57, p = .000 \)) (Figure 2).
Figure 2. Running alters astrocyte morphology in regions associated with increased cognitive performance. **a.** S100+ astrocyte cell body area is increased in the hippocampus, medial prefrontal cortex, and orbitofrontal cortex. **b.** *Left:* S100+ astrocyte (red) colabeled with GFAP (green). Scale bar = 5 µm. *Right:* Representative images of astrocytes from the medial prefrontal cortex of sedentary and running animals. Scale bar = 10 µm. **c.** Optical density of aquaporin-4, a water channel found in the endfeet of astrocytes, was increased in the hippocampus, medial prefrontal cortex, and orbitofrontal cortex of runners. **d.** *Left:* Aquaporin-4 (green) colabels with GFAP (red). Scale bar = 5 µm. *Right top:* Representative images of aquaporin-4 expression in CA1 of sedentary and running animals. Scale Bar = 20 µm. *Right bottom:* aquaporin-4 labeling (green) is shown in close proximity to smooth muscle actin labeling (red). Scale bar = 20 µm. Error bars represent SEM. *p*<0.05 compared with Sedentary for A and C.
**Running enhances dendritic spine density, dendritic spine length and synaptic markers**

Running increased dendritic spine density on both apical and basal dendrites of layer 2/3 pyramidal neurons (Apical: $t_{(13)}= 6.169$, $p = .000$, Basal: $t_{(13)} = 6.037$, $p = .000$) in the medial prefrontal cortex (Figure 3). In addition to increasing spine density, the length of individual spines was also significantly increased on both apical and basal trees with running (Apical: $t_{(13)}= 4.162$, $p = .001$, Basal: $t_{(13)} = 6.614$, $p = .00004$). Immunolabeling with the presynaptic marker synaptophysin and the postsynaptic marker PSD95 revealed similar punctate staining in the medial prefrontal cortex as well as all other regions examined (Figure 3). Running resulted in a significant increase in optical intensity for both markers in the hippocampus, medial prefrontal cortex, orbitofrontal cortex and perirhinal cortex compared to sedentary controls (synaptophysin - hippocampus: $t_{(14)} = 6.27$, $p = .001$, medial prefrontal cortex: $t_{(14)} = 3.5$, $p = .003$, orbitofrontal cortex: $t_{(14)} = 5.8$, $p = .000$, perirhinal cortex: $t_{(16)} = 6.614$, $p = .001$; PSD-95 - hippocampus: $t_{(14)} = 3.13$, $p = .01$, medial prefrontal cortex: $t_{(14)} = 3.5$, $p = .003$, orbitofrontal cortex: $t_{(14)} = 4.33$, $p = .000$, perirhinal cortex: $t_{(16)} = 2.546$, $p = .02$) (Figure 3).
Figure 3. Running increases the number of dendritic spines in medial prefrontal cortex and synaptic markers in several regions supporting cognitive function. 

a. Running increases dendritic spine density on both apical and basal dendrites in the medial prefrontal cortex. 

b. Representative images of Dil labeled layer 2/3 pyramidal neuron apical dendrites in the medial prefrontal cortex and sedentary and running animals. Scale Bar = 5 µm. 

c. Running increasing the average length of spine processes. 

d. Optical intensity analysis of synaptophysin reveals increased expression in all regions studied. Inset: example of synaptophysin staining in medial prefrontal cortex. 

e. PSD-95 levels are also increased in all regions studied. Inset: example of PSD-95 staining in medial prefrontal cortex. Scale Bar = 10 µm. Error bars represent SEM. *p<0.05 compared with Sedentary for A, C-E.


Discussion

Our results indicate that a moderate duration of running (12 days) enhances performance on cognitive tasks that require the medial prefrontal cortex, such as object in place and the attentional set-shifting task. Similarly, improved performance on a reversal task suggests that orbitofrontal cortex function was augmented as well. When taken together with previous literature showing that performance on cognitive tasks requiring the hippocampus is improved by running (Viola et al., 2009; Voss et al., 2013; Vivar et al., 2013; Saur et al., 2014), our findings suggest that physical exercise has a widespread positive impact on cognition in rodents, as it does in humans (Kramer et al., 2007). We did not, however, observe an improvement in novel object preference, a task known to require the perirhinal cortex, suggesting that running-enhanced cognitive function may not generalize to all types of learning and memory at least for the time point examined.

Running is known to increase the density of dendritic spines and numbers of synapses in the hippocampus (Eadie et al., 2005; Stranahan et al., 2007). Our data extend these findings to the medial prefrontal cortex, showing similar dendritic spine and synaptic growth effects after running. We further demonstrate that running enhances the expression of both presynaptic and postsynaptic proteins in the orbitofrontal cortex and perirhinal cortex, in addition to the medial prefrontal cortex and hippocampus, strongly suggesting that this effect does not exhibit a high degree of regional specificity. It seems reasonable to surmise that increased dendritic spine density and increased levels of synaptic proteins in brain regions supporting cognition are likely mediators of running-induced improvements in cognitive function. However, it may be relevant to consider that enhanced expression of both synaptophysin and PSD-95 were observed in the perirhinal cortex and yet, no improvements in function on a task linked to this area, novel object preference, were observed. This suggests that while synaptic enhancements may be involved and in fact, necessary for improved cognition, they may not be sufficient. In this regard, it is important to consider other cellular changes that occur in brain regions linked to running-induced cognitive improvements, such as astrocytic plasticity.
Our findings suggest that astrocyte cell bodies increase in size in response to running and that this effect shows more regional specificity than running-induced changes in synaptic proteins, at least for the brain regions we considered in this study. That is, we observed significant increases in S100 labeled cell body area in the medial prefrontal cortex, orbitofrontal cortex and hippocampus, but not the perirhinal cortex. This regional pattern of results was also observed with running-induced changes in the expression of a marker of astrocytic endfeet in contact with blood vessels, aquaporin-4 (increased expression in medial prefrontal cortex, orbitofrontal cortex and hippocampus, but not the perirhinal cortex). Taken together with our behavioral results demonstrating improvements on tasks that depend on the medial prefrontal cortex and orbitofrontal cortex, as well as the literature showing improved performance on tasks that depend on the hippocampus (Voss et al., 2013; Vivar et al., 2013) and a lack of improvement on a task that depends on the perirhinal cortex, these results raise the possibility that astrocytic changes, in conjunction with synaptic changes, are necessary for the optimization of specific brain regions with physical exercise. It should be emphasized, however, that our results are correlational and do not demonstrate a causal link between astrocytic change and improved cognition. Thus, our results should be interpreted cautiously.

Astrocytes are a highly heterogeneous and relatively understudied population of cells (Barres 2008; Khakh and Sofroniew 2015). While it seems that all brain regions in the cortex have large numbers of astrocytes, regional differences in astrocyte subtypes, their function, and even size are thought to exist (Barres 2008; Gallo and Deneen 2014). This makes pinpointing a specific mechanism by which astrocytes may contribute to running-induced increases in various cognitive functions difficult. Astrocytes have been implicated in the regulation of LTP through the release of astrocyte-specific gliotransmitters, such as D-serine (Henneberger et al., 2010), as well as in the tight control of glutamate signaling which is known to modulate neuronal functioning (Bernardi et al., 2014). Additionally, astrocytes play a role in the regulation of blood flow and trophic support, both functions which have numerous implications for neuronal functioning and synaptic growth (Allen et al., 2013; Bernardi et al., 2014). It is likely that a combination of these mechanisms underlies running-induced changes in cognition but elucidating their relative roles is limited by currently available technologies (Gurden 2013). In the following chapters, I present
studies that were designed to expand on these associational results in an attempt to address the causal link between astrocytes and cognitive function.
Chapter 3: Impairment of Astrocyte Functioning Diminishes Cognitive Flexibility
Background

The results of the running experiment suggest that astrocytes change under conditions associated with enhanced cognitive flexibility (Chapter 2). Importantly, we observed that changes in both astrocytes and neurons were associated with enhancement in cognitive flexibility (Chapter 2). These results are further supported by evidence demonstrating the environmental enrichment also enhances astrocyte plasticity and that this is a potential mediator for improved cognition (Sampedro-Piquero et al., 2014). This suggests that the interplay between neurons and astrocytes may be important for cognitive flexibility in sedentary animals, however, few studies have examined whether manipulation of astrocyte functioning results in altered cognitive ability. For these experiments, we chose to take advantage of the attentional set-shifting task as this task allows us to discretely test cognitive flexibility in both the orbitofrontal cortex and the medial prefrontal cortex separately.

Across rodents, regional differences in astrocyte number have been reported in the medial prefrontal cortex and orbitofrontal cortex (Emsley and Macklis, 2006; Hewett, 2009) but these studies neither examined the size nor interaction of astrocyte processes with dendritic spines, making functional conclusions difficult to draw. It is possible that this heterogeneity in astrocyte size and number may reflect brain region specific differences in astrocyte functioning (Khakh and Sofroniew, 2015).

Our recent work has shown that experience-dependent changes in astrocyte morphology may be related to improved cognition (Chapter 2). We showed that voluntary exercise resulted in enlarged astrocytes and increased numbers of dendritic spines and synaptic markers in the medial prefrontal cortex and orbitofrontal cortex. These structural changes were associated with improvement on brain region-dependent components of the attentional set-shifting task (Chapter 2). We also noted that astrocytes in the medial prefrontal cortex were larger than those in the orbitofrontal cortex (Chapter 2). What these regional differences mean for the cognitive ability of control animals is unknown.

Central blockade of astrocyte glutamate uptake through ICV infusion of dihydrokainic acid (DHK) results in anhedonic-like behavior, as well as impaired spatial memory (Bechtolt-Gompf et al., 2010). Reducing the number of astrocytes in the medial prefrontal cortex of rodents
using an astrocyte specific toxin, L-alpha-aminoadipic acid (L-AAA) has been linked to the development of anhedonia (Banasr and Duman, 2008) and potential alterations in cognition (Lima et al., 2014). L-AAA acts as a glutamate analog which specifically kills astrocytes when taken into the cell (Khurgel et al., 1996; Banasr and Duman, 2008). L-AAA, at a concentration of 20 µg/µl, has been previously used to show a 20% depletion of astrocytes lasting approximately two weeks (Khurgel et al., 1996). Collectively, these findings may hint at a previously unidentified role for astrocytes as regulators of complex behavior.

This study first sought to characterize regional differences in astrocyte morphology as well as to investigate whether astrocytes in these brain regions are important for the regulation of cognitive behavior. First, we performed detailed analyses of astrocyte morphology and interaction with dendritic spines in the medial prefrontal cortex and orbitofrontal cortex, regions known to be involved in cognitive flexibility (Birrell and Brown, 2000; McAlonan and Brown, 2003; Brockett et al., 2015). Next, to investigate whether perturbation of astrocyte function affects cognition, we used DHK or L-AAA to either diminish their functionality or reduce the number of astrocytes respectively. Both approaches impaired medial prefrontal cortex-dependent cognitive flexibility, but not orbitofrontal cortex-dependent cognitive flexibility.

Methods

Animals

Adult male Sprague-Dawley rats, 7 weeks old, were used for these studies. Rats were housed individually to enhance recovery following surgical procedures on a reverse 12-hour light-dark schedule (lights off at 0700), and all behavioral testing occurred between 0900 and 1400. All studies were approved by the Princeton University IACUC (protocol # 1852, approved June 2014) and conformed to the National Research Council Guide for the Care and Use of Laboratory Animals (2011).

Morphological characterization of astrocytes in the medial prefrontal cortex and orbitofrontal cortex

For morphological analyses of astrocyte populations in the medial prefrontal cortex and orbitofrontal cortex, rats (n=5) were perfused with 4% paraformaldehyde and perfused at 9
weeks of age. For analysis of interaction between astrocyte processes and dendritic spines in the medial prefrontal cortex and orbitofrontal cortex, 9 week old rats (n=5), were perfused with 1.5% paraformaldehyde for Dil impregnation and analysis.

**Impairment of glutamate reuptake in the medial prefrontal cortex and orbitofrontal cortex**

DHK has been shown to temporarily inactivate astrocyte-specific glutamate uptake (Lee et al., 2007; Bechtholt-Gomp et al., 2010). DHK (Tocris Bioscience) was dissolved in sterile saline at a concentration of 25nM (Lee et al., 2007; Bechtholt-Gomp et al., 2010). Two weeks prior to testing animals underwent surgery and were fit with cannulae bilaterally targeting either the medial prefrontal cortex or orbitofrontal cortex. DHK (n=7 per brain region) or saline (n=6-7 per brain region) was infused into either the medial prefrontal cortex or orbitofrontal cortex 20 minutes prior to testing on brain region-dependent trials. Rats were briefly restrained and received a 1 µl infusion of DHK bilaterally over the course of one minute. Flowrate was controlled by a syringe pump driver (Harvard Apparatus). Following infusion, the internal cannulae were left in place for five minutes before being removed in order to minimize backflow. Dummy caps were reattached and animals were given a 5-minute break before continuing testing. One hour following the completion of behavioral testing (13 days post injection), rats were deeply anesthetized and perfused with 4% paraformaldehyde and their brains were processed for histology and microscopic analysis.

**Reduction of astrocyte number in the medial prefrontal cortex**

L-AAA is a glutamate analog that has been shown to specifically deplete the number of astrocytes (Khurgel et al., 1996; Banasr and Duman, 2008). L-AAA (Sigma) was dissolved in sterile 1M HCl at a concentration of 20 µg/µl. This concentration has been previously used to show a 20% depletion of astrocytes lasting approximately 2 weeks (Khurgel et al., 1996). The solution was sonicated at 30Hz for 10 minutes before being titrated with sterile 2M NaOH (pH = 7.5). Rats either received 1µl of L-AAA or saline infused bilaterally over the course of five minutes into the medial prefrontal cortex (n=11-12). Rats were allowed 5 days to recover before testing on the attentional set-shifting task. Next, rats were food-deprived to 85% body weight over 5 days before the start of behavioral testing. Behavioral testing occurred over the course of
3 days. Rat were perfused one hour after testing and brains were processed as described in the previous section. A separate cohort of rats (n=6) received injections of L-AAA or saline in the medial prefrontal cortex and were allowed 13 days to recover before being perfused with 1.5% paraformaldehyde for diolistic analysis of spine density.

**Surgical Procedures**

Rats were anesthetized with ketamine/ xylazine, shaved and placed in a stereotaxic instrument (David Kopf Instruments). For stereotaxic injection experiments, holes were drilled into the skull using the following coordinates: for medial prefrontal cortex, 2.7mm anterior to bregma, ±0.5mm lateral to the midline, and 4.0mm ventral from the dura, for orbitofrontal cortex, 4.0mm anterior to bregma, ±2.0mm lateral to the midline, and 4.5mm ventral from the dura (McAlonan and Brown, 2003; Banasr and Duman, 2008). Rats received a single 1µl injection bilaterally of drug over the course of five minutes. Rats were monitored for 5 days prior to any further experimental manipulation. For drug infusion experiments cannulae were implanted using the same coordinates above. For medial prefrontal cortex injections, a double cannula was implanted (26ga, 5mm pedestal, 5mm projection length; Plastics One). For the orbitofrontal cortex two separate cannulae were implanted (26ga, 5mm pedestal, 5.5mm projection length; Plastics One). Surgical screws were placed in the skull around the cannulae, and cannulae were fixed in place using dental acrylic (Bosworth). Dummy caps (Plastics One) were used to prevent clogging.

**Attentional Set-Shifting Task**

Three separate cohorts of animals (medial prefrontal cortex treated with DHK or saline, orbitofrontal cortex treated with DHK or saline, and medial prefrontal cortex treated with L-AAA or saline) were tested on the attentional set-shifting task, a well-characterized test of cognitive flexibility (Birrell and Brown, 2000; Brockett et al., 2015). All behavioral procedures were carried out in a manner consistent with those described in Chapter 2. 1-2 hours after the completion of behavioral testing, rats were injected with an overdose of Euthasol and transcardially perfused with 4% paraformaldehyde.
**Immunolabeling for astrocyte and neuronal markers.**

Brains for immunohistochemical analysis were postfixed for 48 hours before processing. 40 µm coronal sections (1:12) were cut from half brains into a bath of 0.1M PBS using a Vibratome. For astrocyte immunolabeling, free-floating sections were rinsed in 0.1 M PBS, pH 7.5, and incubated with 3% normal donkey serum, and 0.1M PBS with 0.1% Triton X-100 and either rabbit anti-aquaporin-4 (1:500, Santa Cruz Biotechnology), rabbit anti-glial fibrillary acidic protein (GFAP) (1:500, Dako), mouse anti-NeuN (1:500, Millipore), immediate early gene markers rabbit anti-egr-1 (1:500, Santa Cruz Biotechnology) or mouse anti-arc (1:500, SySy Systems) rabbit anti-S100 (1:10,000, Dako), and stored at 4°C. After incubation in primary antisera for 24 hours, the sections were rinsed, incubated in either Alexa Fluor donkey anti-rabbit 488 (1:250; Invitrogen) or Alexa Fluor donkey anti-rabbit 568 in the dark for 60 minutes, rinsed and mounted onto suprafrost slides, and coverslipped using glycerol in PBS (3:1). All sections were counterstained with the DNA dye, Hoechst 33342 (1:100,000, Molecular Probes).

Slides were coded until completion of the data analysis. Images from the medial prefrontal cortex and orbitofrontal cortex were taken using a Zeiss confocal microscope (LSM 700; lasers, argon 458/488; HeNe 568). Density measurements of GFAP+ and S100+ cells were completed on representative sections throughout the medial prefrontal cortex and orbitofrontal cortex. Density measurements were taken from cells surrounding the layer 2/3 pyramidal neurons and were obtained from 20 µm image stacks using ImageJ (NIH). Cross-sectional area measurements of 50 GFAP+ and S100+ cells per animal per brain region were obtained from 20 µm image stacks using ImageJ (NIH). Regions of interest were chosen based on task relevance. Selected cells were in close proximity to layer 2/3 pyramidal neurons in the medial prefrontal cortex and orbitofrontal cortex. Selected cells had to be distinct from surrounding labeled cells and be fully stained. Cross-sectional area measurements were taken from z-stacks by using ImageJ (NIH) by circling individual cells at their widest point and using the area function. Cross-sectional area covered by aquaporin-4-immunolabeled blood vessels was taken from 1 µm optical sections. All parameters for background subtraction were optimized and held constant throughout the analysis. For NeuN, density and area measurements were obtained in a manner
like above. Density measurements of immediate early gene expression for both Egr-1 and arc also followed the methods presented above.

**Dil impregnation and colabeling with GFAP**

To assess the differences in astrocyte populations and their interaction with surrounding neurons in the medial prefrontal cortex and orbitofrontal cortex, rats were deeply anesthetized and perfused with 1.5% paraformaldehyde. Brains were post-fixed in 1.5% paraformaldehyde for 1 hour and moved to 0.1M PBS for processing. Brains were dissected and blocked down the midline. 150 µm coronal sections throughout the medial prefrontal cortex and orbitofrontal cortex were cut using a Vibratome into a bath of 0.1M PBS. Individual sections were shot with lipophilic Dil crystals (Sigma) using the Helios Gene Gun System (BioRad) as previously described (Staffend and Meisel, 2011). Tissue was incubated for 3 hours in 0.1M PBS with .01% paraformaldehyde at 4°C. Tissue was solubilized for 10 minutes in 2% Tween-20 (Sigma), rinsed, and incubated in 0.1M PBS, 3% normal donkey serum and rabbit-anti GFAP (1:500, Dako) at 4°C. Following a 48-hour incubation in primary antisera, tissue was rinsed in 0.1M PBS and incubated in 0.1M PBS and Alexa Fluor donkey anti-rabbit 488 (1:250, Invitrogen) at 4°C. Following a 48-hour incubation in secondary antisera, tissue was mounted onto suprafrost slides and coverslipped using glycerol in PBS (3:1). Sections were visualized using a Zeiss confocal microscope (LSM 700; lasers, argon 458/488; HeNe 568) with a 63x oil objective. All settings (pinhole size, aperture gain, and offset) were initially optimized and held constant throughout the study.

In order to assess potential changes in dendritic spine density following L-AAA treatment, rats (n=6) underwent surgery to receive an injection of L-AAA as described above. Rats were perfused with 1.5% paraformaldehyde at the same time point as behaviorally tested animals. Brains were processed as described above. Individual sections were shot with lipophilic Dil crystals (Sigma) using the Helios Gene Gun System (BioRad) as previously described (Staffend and Meisel, 2011). Tissue was incubated for 1 hour in 4% paraformaldehyde at 4°C before being rinsed and mounted. Slides were coverslipped with glycerol in PBS (3:1) and sections were visualized and analyzed to assess dendritic spine density as described above.

**Statistics.**
Unpaired two-tailed Student’s t-tests were performed on each data set (for cellular analyses: saline x astrocyte manipulated, for the morphological experiments: medial prefrontal cortex x orbitofrontal cortex) except for the attentional set-shifting data, which was analyzed as a mixed factorial ANOVA with Holm’s correction for multiple comparison where appropriate.

**Results**

*Astrocytes in the medial prefrontal cortex are larger, more numerous and make more contacts with dendritic spines than astrocytes in the orbitofrontal cortex*

In order to examine whether populations of astrocytes between the medial prefrontal cortex and orbitofrontal cortex were similar, we conducted morphological analyses of astrocytes in the medial prefrontal cortex and orbitofrontal cortex of unoperated control rats. Relative to the orbitofrontal cortex, astrocytes in the medial prefrontal cortex were more numerous (GFAP+: $t(5) = 6.901, p = .0010$; S100+: $t(5) = 13.66, p = .0001$) and larger (GFAP+: $t(5) = 8.994, p = .0003$; S100+: $t(5) = 16.12, p = .0001$). Astrocytes in the medial prefrontal cortex also exhibited a greater number of astrocytic process contacts with layer 2/3 pyramidal neuron dendritic spines compared to astrocytes in the medial prefrontal cortex compared to the orbitofrontal cortex (Apical: $t(4) = 5.595, p = .0050$; Basal: $t(5) = 3.838, p = .0185$) (Figure 1), suggesting that the potential function of astrocytes may be region-dependent and inherently tied to their morphology and distribution within a particular brain region.
Figure 1. Astrocytes differ in number and size between the medial prefrontal cortex and the orbitofrontal cortex. 

a. GFAP+ ($p = .0001$) and S100+ ($p = .0001$) astrocytes respectively in the medial prefrontal cortex are more numerous than those in the orbitofrontal cortex (n=6).

b. GFAP+ ($p = .0003$) and S100+ ($p = .0001$) astrocytes in the medial prefrontal cortex are larger in cell area than astrocytes in the orbitofrontal cortex (n=6).

c. Confocal images of GFAP+ and S100+ staining in the medial prefrontal cortex. Scale bars = 10 µm.

d-e. In a separate cohort of rats (n=5) dendritic spine density of layer 2/3 pyramidal neurons was examined in the medial prefrontal cortex and orbitofrontal cortex. Despite no differences in the dendritic spine density on both apical and basal secondary/tertiary dendritic spines between the two brain regions (apical: $p = .2129$; basal: $p = .7970$), astrocytes in the medial prefrontal cortex made a greater number of contacts with the surrounding secondary and tertiary dendrites of layer 2/3 apical and basal dendrites than do astrocytes in the orbitofrontal cortex (apical: $p = .0050$; basal: $p = .0185$).

f. Sample images showing contact of GFAP+ processes (green) with basal dendrites of layer 2/3 pyramidal cells in 3D projections for both the medial prefrontal cortex and orbitofrontal cortex. Scale bar = 5 µm. *$p < .05$ compared to orbitofrontal cortex.
Inhibition of astrocytic glutamate uptake in the medial prefrontal cortex impairs cognitive flexibility, but not orbitofrontal cortex function

The attentional set-shifting task consists of five individual tasks, with the extradimensional shift and reversal tasks dependent on the medial prefrontal cortex and orbitofrontal cortex respectively (Birrell and Brown 2000, McAlonan and Brown 2003). We used the attentional set-shifting task to study the relative contributions of astrocytes in the medial prefrontal cortex and orbitofrontal cortex to these tasks. We explored whether inactivation of astrocytic glutamate reuptake with DHK altered cognitive flexibility and found impaired performance on the medial prefrontal cortex-dependent portion of the attentional set-shifting task, but not the orbitofrontal cortex-dependent portion. Infusion of DHK 20 minutes prior to the extradimensional shift significantly increased the number of trials required to reach criterion compared to saline controls ($p = .0016$) (Figure 2a). Moreover, DHK treated animals made significantly more errors compared to saline controls ($p = .0105$) (Figure 2b). By contrast, infusion of DHK into the orbitofrontal cortex 20 minutes prior to the reversal portion of the task had no effect on either trials to criterion ($p = .1104$) or errors made ($p = .6730$) on the task (Figure 3a&b). We verified that our rats correctly formed an attentional set by performing a secondary analysis of control data to show that there was a significant increase in the number of errors made on rule-dependent tasks (reversal and extradimensional shift) compared to the number of errors made during the final intradimensional shift (medial prefrontal cortex controls: $t_{(6)} = 8.629, p = .0001$; orbitofrontal cortex controls: $t_{(5)} = 3.484, p = .0176$). Analysis of brain tissue from both DHK and saline-treated controls exhibited comparable reactive gliosis consistent with chronic cannula implantation, as indicated by larger astrocytes and more intense GFAP+ staining, while no difference in the density of GFAP+ cells surrounding layer 2/3 pyramidal neurons in the medial prefrontal cortex or orbitofrontal cortex between groups was observed (medial prefrontal cortex study: $t_{(12)} = 1.117, p = .2860$; orbitofrontal cortex study: $t_{(11)} = .3552, p = .7292$) (Figure 2c; Figure 3c).
Figure 2. Temporary blockade of astrocyte specific glutamate uptake in the medial prefrontal cortex impairs cognitive flexibility. Rats were infused with either DHK (n=7) or saline (n=7) in the medial prefrontal cortex 20 minutes prior to task completion. DHK treatment altered medial prefrontal cortex-dependent performance on the attentional set-shifting task. A timeline for the DHK experiment is provided above. a-b. Rats that received DHK infusions took more trials to reach criterion on the medial prefrontal cortex-dependent extradimensional shift (EDS) when compared to saline-infused controls. DHK-infused rats also made more errors on the EDS than saline-infused controls. c. Confocal images of GFAP+ (green) staining after treatment with either DHK or saline. Scale bar = 20 μm. No differences in the number of GFAP+ astrocytes were observed in the medial prefrontal cortex of DHK-infused compared to saline-infused rats. * p<0.05 compared to saline-infused controls.
Figure 3. Temporary blockade of astrocyte specific glutamate uptake orbitofrontal cortex does not impair cognitive flexibility. DHK treatment (n=7) infused into the orbitofrontal cortex 20 minutes prior to completion of reversal (REV) portion of the attentional set-shifting task did not impair performance relative to saline-infused controls (n=6). A timeline for experiment is provided above. The attentional set-shifting task occurred over 3 days, with the reversal (REV) performed on the last day. a, b. Rats receiving infusion of DHK into the orbitofrontal cortex showed no differences in performance on the attentional set-shifting task. c. DHK infusion did not alter the number of GFAP+ astrocytes in the orbitofrontal cortex compared to saline infusion. Confocal images of GFAP+ (green) staining after treatment with either DHK or saline. Scale bar = 20 μm.
**Reduction in the number of astrocytes in the medial prefrontal cortex impairs cognitive flexibility**

Based on the positive results of the DHK experiments, we chose to focus our follow-up analyses on the medial prefrontal cortex. We next explored whether having fewer astrocytes would impair cognitive flexibility by using the astrocyte specific toxin L-AAA infused directly into the medial prefrontal cortex. We used a dose of L-AAA that significantly reduced the density of medial prefrontal cortex GFAP+ astrocytes relative to saline controls ($t_{(21)} = 2.771, p = .0115$) (Figure 4c), without altering the number of medial prefrontal cortex NeuN+ neurons ($t_{(21)} = .5333, p = .5994$); the size of medial prefrontal cortex neuronal cell body areas ($t_{(21)} = .9433, p = .3563$), the densities of dendritic spines on apical ($t_{(10)} = .4277, p = .6779$) and basal ($t_{(10)} = 1.473, p = .1715$) dendrites of layer 2/3 medial prefrontal cortex pyramidal neurons or the expression of the immediate early genes Egr-1 and Arc, proxies for neuronal activation after the attentional set-shifting task (Egr-1: $t_{(21)} = .4786, p = .6372$; Arc: $t_{(21)} = .9752, p = .3405$) in layer 2/3 medial prefrontal cortex pyramidal neurons (Figure 5a-c). Relative to saline controls, L-AAA-treated animals exhibited impaired performance on the extradimensional shift as evidenced by increases in trials to criterion ($p = .0260$) and errors made ($p = .0276$) (Figure 4a & b). Surprisingly, astrocyte reduction also enhanced performance on the compound discrimination task by reducing both trials to criterion and errors made (trials to criterion: $p = .0375$, errors made: $p = .0301$) (Figure 4a & b). Secondary analysis to verify that saline-treated rats correctly formed an attentional set revealed a significant increase in the number of errors made on the combined reversal/ extradimensional shift versus the intradimensional shift secondary analysis ($t_{(10)} = 2.994, p = .0135$). Taken together, these findings suggest that a small (~20%) reduction in the number of astrocytes in the medial prefrontal cortex hinders cognitive flexibility.
Figure 4. Reduction of astrocyte number in the medial prefrontal cortex impairs cognitive flexibility. Rats were infused with either L-AAA (n=12) or saline (n=11) in the medial prefrontal cortex 12 days before completing the attentional set-shifting task. L-AAA treatment altered medial prefrontal cortex-dependent performance on the extradimensional shift (EDS). A timeline for the L-AAA experiment is provided above. a. Rats with fewer astrocytes in the medial prefrontal cortex took fewer trials to reach criterion for the compound discrimination (CD) compared to saline-infused controls but more trials to reach criterion on the EDS portion of the task when compared to saline controls. b. Rats with fewer astrocytes also made fewer errors on the CD, but more errors on the EDS compared to saline-infused controls. c. L-AAA treatment significantly reduced the number of GFAP+ astrocytes in the medial prefrontal cortex. Confocal images of GFAP+ (green) and NeuN+ (red) staining 13 days after treatment with either L-AAA or saline. Scale bar = 20 μm. * p<0.05 compared to saline-infused controls.
Figure 5. L-AAA does not alter neuron number, size, dendritic spine density or immediate early gene expression in the medial prefrontal cortex. To rule out the possibility that L-AAA had deleterious effects on neurons, neuronal viability was characterized using several measures. 

a. Relative to saline-infused controls, both the density and cell body size of NeuN+ layer 2/3 pyramidal neurons was unchanged in L-AAA treated rats (n=11-12). Confocal images of NeuN+ staining in the medial prefrontal cortex. Scale bar = 20 μm.

b. In a separate cohort of rats, no differences in dendritic spine density for both apical and basal secondary and tertiary dendrites of layer 2/3 pyramidal neurons were found when comparing saline and L-AAA treated rats (n=6). Confocal images of Dil-labeled tertiary apical dendrites in layer 2/3 pyramidal neurons in the medial prefrontal cortex. Scale bar = 5 μm.

c. No differences in the density of Arc+ and Egr-1+ neurons in layer 2/3 of the medial prefrontal cortex were detected between saline and L-AAA -treated rats (n=11-12) after completing the attentional set-shifting task. Confocal images of Arc (green) and Egr-1 (green) and NeuN (red) immunolabeling. Scale bar = 20 μm.
Discussion

The results indicate that structural differences exist in astrocyte populations in the medial prefrontal cortex compared to those in the orbitofrontal cortex. Astrocytes in the medial prefrontal cortex are more numerous, have larger cell bodies, are more complex and make more contacts with dendritic spines than astrocytes in the orbitofrontal cortex. In addition to these differences, astrocytes in the medial prefrontal cortex seem to play a critical role in cognitive flexibility relative to those in the orbitofrontal cortex. Reducing the number of astrocytes or temporarily preventing astrocyte function impairs medial prefrontal cortex-, but not orbitofrontal cortex-dependent performance on the attentional set-shifting task. Collectively, these results suggest that the larger astrocytes of the medial prefrontal cortex may be necessary for optimal function of this brain region.

The involvement of the medial prefrontal cortex (Birrell and Brown, 2000) and orbitofrontal cortex (McAlonan and Brown, 2003) in specific components of the attentional set-shifting task has long been established. These studies have demonstrated that a loss in a relatively large number of neurons results in an impairment in functioning. However, few studies have investigated the role of astrocytes in performance on the attentional set-shifting task (Lima et al., 2014). No study has explicitly investigated differences in astrocyte morphology in brain regions and its potential functional implications. These results suggest that a relatively small decrease in the number of astrocytes in the medial prefrontal cortex, in the absence of any substantial decrease in the number of neurons, seems to induce an impairment on the extradimensional shift task similar to an impairment induced by a relatively large scale neuron loss (Birrell and Brown 2000). The gain of function manipulation mimics the results seen in previously published work showing that voluntary wheel running improves medial prefrontal cortex functioning as well as increases astrocyte size in the region (Chapter 2). In running animals, larger astrocytes correlate with enhanced cognitive performance (Chapter 2). Moreover, when human astrocytes, which are several times larger than the rodent equivalent, are placed in the rodent brain, similar cognitive benefits are also observed (Han et al., 2013). This correlational evidence may explain the brain region-dependent differences reported here. Larger astrocytes in the medial prefrontal cortex may be more directly involved in the regulation of behavior than
those in the orbitofrontal cortex. It has been shown that astrocytes in the hippocampus and perirhinal cortex are similar in size to astrocytes in the medial prefrontal cortex suggesting the orbitofrontal cortex may be an unusual brain region in this regard (Chapter 2). Future research should examine the role of astrocytes in behavior in other regions that support cognition as well as address the mechanism(s) through which astrocytes directly influence behavior.

With running, enlargement of astrocyte cell body along with increases in markers of synaptic proteins was associated with enhanced reversal learning on the attentional set-shifting task (Chapter 2). These findings suggest that astrocytes in the orbitofrontal cortex may contribute to behavior under certain circumstances, beyond those tested in our current work. Morphological differences in astrocyte number and size exist between the medial prefrontal cortex and orbitofrontal cortex. It is possible that astrocytes in the medial prefrontal cortex are simply more susceptible to changes associated with decreases in cell number or decreased glutamate uptake than those in the orbitofrontal cortex. One other possibility to explain the differences in results between the medial prefrontal cortex and orbitofrontal cortex results may be dosage. Perhaps a higher dose may have led to results more comparable to those observed in the medial prefrontal cortex. Future experimentation to determine whether increased Ca\(^{2+}\) signaling in astrocytes of the orbitofrontal cortex is sufficient to enhance reversal learning will help to determine whether astrocytes can contribute in a positive way to the functions of this brain region.

The interaction between astrocytes and neurons is an important aspect of brain functioning. While astrocytes support neurons by providing a wide array of functions from maintenance of the blood brain barrier (Iadecola and Nedergaard 2007; Attwell et al., 2010) to the regulation of glutamate (Cornell-Bell et al., 1990) and the clearance of toxins during sleep (Xie et al., 2013), few studies have linked this support to cognitive behavior specifically. Our results suggest that loss or impairment of astrocyte function in the medial prefrontal cortex, but not the orbitofrontal cortex, diminishes cognitive flexibility. Collectively, these results suggest a novel role for astrocytes in the medial prefrontal cortex in the regulation of cognitive flexibility. The extent to which abnormalities in astrocyte signaling contribute to conditions associated with deficits in prefrontal function, such as in schizophrenia and frontotemporal dementia, remains to be determined.
Chapter 4: Activation of Astrocytic Ca$^{2+}$ Signaling Leads to Enhanced Cognitive Flexibility Through S100β
Background

Previous results (Chapter 2) have shown that astrocytes change under conditions associated with enhanced cognitive flexibility and that impairment of astrocyte functioning or reduction of astrocyte number in the medial prefrontal cortex is associated with reduced cognitive flexibility (Chapter 3). However, it remains unclear how astrocytes specifically influence cognitive flexibility and more broadly medial prefrontal cortex functioning. Accumulating evidence, suggests that enhancement of medial prefrontal cortex function is associated with changes in neuronal synchrony. Changes in theta (6-10 Hz) and gamma (30-80 Hz) oscillations have been associated with enhanced working memory and cognitive control (Canolty et al., 2006; Voloh et al., 2010). At present, no study has attempted to link astrocyte functioning with changes in brain oscillations supporting medial prefrontal cortex-dependent function, however, a few studies have suggested that astrocytes may be important for the regulation of neuronal oscillations elsewhere in the brain, such as in the visual cortex (Schummers et al., 2008), brain stem regions (Gourine et al., 2010; Morquette et al., 2015), the hippocampus (Lee et al., 2014), and the cortex more broadly (Sakatani et al., 2008; Poskanzer and Yuste, 2011; Poskanzer and Yuste, 2016).

In area V1, activation of astrocytic Ca\(^{2+}\) signaling mimics neuronal firing and led to the sharpening of neuronal receptive fields (Schummers et al., 2008). While in the hippocampus, disruption of astrocyte functioning via an inducible neurotoxin selectively expressed in astrocytes, results in impaired gamma oscillations that correlated with compromised recognition memory (Lee et al., 2014). Additionally, increases in astrocytic Ca\(^{2+}\) lead to the activation of neighboring astrocytes, and the syncytial spread of astrocyte Ca\(^{2+}\) signaling has been associated with switches between highly synchronous and desynchronized cortical states thought to underlie the transition between REM and slow-wave sleep (Poskanzer and Yuste 2016). Collectively, this suggests that changes in intracellular Ca\(^{2+}\) signaling may have important effects on brain functioning and subsequently behavior. With regards to astrocytes calcium signaling, it is unclear specifically, what links changes in astrocyte Ca\(^{2+}\) signaling to changes in neuronal oscillations.

Recently, the astrocyte released Ca\(^{2+}\)-binding protein, S100\(\beta\), has been associated with enhanced neuronal oscillations in the trigeminal circuit that coordinate chewing behavior (Morquette at al., 2015). S100\(\beta\) is generally thought of as an intracellular Ca\(^{2+}\) regulator however,
its extracellular release following traumatic brain injury leads to the enhancement of neuronal oscillations (Sakatani et al., 2008). Moreover, recent evidence has implicated extracellular S100β in the regulation of rhythmogenesis in the trigeminal circuit which is important for the control of chewing behavior. These pieces of evidence, suggest that S100β can function as an extracellular regulator of Ca^{2+} as well (Sakatani et al., 2008; Morquette et al., 2015). While increased intracellular Ca^{2+} is important for astrocyte activation, somewhat paradoxically, reduced Ca^{2+} in the extracellular space has been associated with increased neuronal rhythmicity in several other systems (Angstadt and Friesen, 1991; Bouskila and Dudek, 1993; Perez-Velazquez et al., 1994; Beck et al., 2001; Hashimoto and Kita, 2006).

We hypothesized that astrocytes may coordinate their collective activity through the intracellular Ca^{2+} signaling which in turns leads to the release of S100β. S100β release then may reduce extracellular Ca^{2+} levels around the synapse leading to a change in neuronal oscillations, particularly theta and gamma that are associated with medial prefrontal cortex function and cognitive flexibility.

We first wanted to show that enhancing astrocytic Ca^{2+} signaling in the medial prefrontal cortex led to improved cognitive flexibility. Using a pharmacogenetic approach, we show that activation of astrocyte Ca^{2+} signaling improves performance on the medial prefrontal cortex-dependent portion of the attentional set-shifting task. If this improvement in cognitive flexibility is due to changes in astrocytic regulation of extracellular Ca^{2+} via S100β, then we predicted that the positive effects of pharmacogenetic activation of astrocytes could be blocked by antagonizing S100β activity through the infusion of an antibody against S100β. Blockade of S100β activity indeed prevented the boost in cognitive flexibility associated with pharmacogenetic activation of astrocytes. To further explore the role of S100β in the regulation of cognitive flexibility, we predicted that infusion of S100β would be sufficient to enhance performance on the attentional set-shifting task. Relative to infusion of genetically modified form of S100β incapable of binding Ca^{2+}(mS100β), infusion of S100β significantly enhanced cognitive flexibility. Moreover, in a separate study we show that relative to infusion of mS100β, infusion of S100β increases phase-amplitude coupling between theta and gamma oscillations, which have been associated with cognitive flexibility. Collectively, these findings suggest that astrocytes may participate in medial
prefrontal cortex function through the release of S100β which, in turn, alters theta-gamma coupling.

Methods

Animals

Adult male (7-9-week-old) Sprague-Dawley rats were used for these studies. Rats were housed individually after surgery to enhance recovery. Housing was on a reverse 12-hour light-dark schedule (lights off at 0700), and all behavioral testing occurred between 0900 and 1400. All studies were approved by the University IACUC and conformed to the National Research Council Guide for the Care and Use of Laboratory Animals (2011).

Enhancement of astrocyte Ca^{2+} signaling in the medial prefrontal cortex

rAAV-5 / GFAP-Gq-DREADD has been shown to successfully transduce astrocytes and increases astrocytic Ca^{2+} signaling in the presence of CNO (Bull et al. 2014; Scofield et al., 2015). Rats (n=15) received 1 µl bilateral infusions of rAAV5/GFAP-HA-hm3D-IRES-mCitrine (GFAP-Gq-DREADD) (University of North Carolina Vector Core, Chapel Hill, North Carolina) into the medial prefrontal cortex at a concentration of 4.0 x 10^{12} particles/ml. Virus concentration and time course was chosen based on previously published works (Bull et al., 2014; Scofield et al., 2015). Rats were allowed 3 weeks to recover before any further manipulation. Five days prior to the start of testing rats were mildly food deprived as described above. Twenty minutes prior to completing the extradimensional shift, (4 weeks post-surgery), rats received either a 3 mg/kg IP injection of clozapine-N-oxide (CNO) (n=8), a concentration known to maximally activate the GFAP-Gq-DREADD construct (Bull et al., 2014; Scofield et al., 2015) or saline (n=7).

Recent evidence has suggested that despite being an inert synthetic ligand CNO on its own can have physiological consequences on behavior after being metabolized to N-desmethylclozapine (MacLaren et al., 2016). To rule out the possibility that CNO alone may be contributing to changes in cognitive flexibility a separate cohort of animals was run on the attentional set-shifting task. Twenty minutes prior to completion of the EDS portion of the attentional set-shifting task, animals received an I.P. injection of either CNO (3 mg/kg) (n=6) or a...
comparable volume of saline (n=6). Five minutes following the injection of either CNO or saline rats completed the rest of the attentional set-shifting task before being perfused one day later.

**Antagonizing the activity of S100β signaling in the medial prefrontal cortex**

We wanted to investigate whether the beneficial effects of pharmacogenetic activation of astrocytic Ca\(^{2+}\) were mediated by the activity of S100β. Rabbit x S100β (Abcam) or non-immune rabbit IGG (Sigma) was diluted to a concentration of 40 µg/ml (Morquette et al., 2015). Three weeks prior to testing, rats underwent surgery and received 1 µl bilateral infusions of rAAV5/GFAP-HA-hm3D-IRES-mCitrine (GFAP-Gq-DREADD) (University of North Carolina Vector Core, Chapel Hill, North Carolina) into the medial prefrontal cortex at a concentration of 4.0 x 10\(^{12}\) particles/nl, and were then fit with cannulae bilaterally targeting the medial prefrontal cortex. Testing began three-four weeks after surgery in order to ensure adequate time for DREADD expression. Rats were food-deprived to 85% body weight over five days before the start of behavioral testing. Behavioral testing occurred over the course of three days (habituation, shaping, and testing). On the testing day, twenty minutes prior to the extradimensional shift (4 weeks post-surgery), rats received a 3 mg/kg IP injection of CNO, a concentration known to maximally activate the GFAP-Gq-DREADD construct (Bull et al., 2014; Scofield et al., 2015). Rats were then lightly restrained and received a 1 µl infusion of either antibody against S100β (n=9) or non-immune rabbit IGG (n=9) into the medial prefrontal cortex. Twenty minutes prior to testing on the extradimensional shift. Following infusion, the internal cannulae were left in place for five minutes before being removed in order to minimize backflow. Dummy caps were reattached and rats were given a 5-minute break before continuing testing.

**Manipulation of S100β signaling in the medial prefrontal cortex**

In order to test whether S100β was important for astrocytic regulation of cognitive flexibility we infused either S100β or a mutant form of S100β (mS100β) that has been genetically modified such that it can no longer bind Ca\(^{2+}\). Infusion of mS100β has previously been shown to have no effect on the rhythmic firing of neurons and therefore should have no effects on behavior (Morquette et al., 2015). S100β (Sigma) or mS100β (generous gift from D. Weber from the Center for Biomolecular Therapeutics, University of Maryland School of Medicine) was dissolved in sterile saline at a concentration of 1 mM (Sakatani et al., 2008; Morquette et al., 2015). Two
weeks prior to testing, rats underwent surgery and were fit with cannulae bilaterally targeting the medial prefrontal cortex. Rats were allowed five days to recover before testing on the attentional set-shifting task. Next, rats were food-deprived to 85% body weight over five days before the start of behavioral testing. Behavioral testing occurred over the course of three days (habituation, shaping, and testing). On the testing day, S100β (n=12) or mS100β (n=12) was infused into the medial prefrontal cortex twenty minutes prior to testing on the extradimensional shift. Rats were briefly restrained and received a 1 µl infusion of S100β bilaterally over the course of one minute. Flowrate was controlled by a syringe pump driver (Harvard Apparatus). Following infusion, the internal cannulae were left in place for five minutes before being removed in order to minimize backflow. Dummy caps were reattached and rats were given a 5-minute break before continuing testing.

**LFP Recordings and Analysis**

Three weeks prior to testing, rats (n=18) underwent surgery and were fit with dual cannula electrode systems (Plastics One), containing a 125 µm insulated stainless steel electrode and a guide cannula for microinfusion, targeting the medial prefrontal cortex, and a separate bare stainless steel ground wire wrapped around a skull screw above the hippocampus. This system allowed simultaneous infusion of recording of LFPs in the medial prefrontal cortex. Recordings began three weeks following surgery at a time course like the S100β behavioral experiment.

Rats received either a 1 µl infusion of S100β or mS100β directly into medial prefrontal cortex over the course of one minute, then were returned to their home cage. Testing for each drug occurred on consecutive days; treatment order was counterbalanced. Twenty minutes following the infusion, rats were placed in an open field (17 x 17 x 17 cm) for twenty minutes, LFPs were recorded, then rats were returned to their home cage.

LFP recording data were collected from the three separate recording sessions and analyzed using a within subject design. LFPs were recorded against a ground screw placed above the hippocampus. Signals were passed through a commutator, amplified 100X, and sampled at 1000 Hz using a Cambridge Electronic Design 1401 data acquisition unit and Spike2 software. Signals were notch filtered at 60 Hz, and artifacts related to movement, detected using
a voltage threshold, were cleaned from the data. Power spectral density estimates were obtained using short time Fourier transform. To obtain power estimates within theta and gamma bands, we took the average power across time for the entire session within each frequency, then took the sum of this average power across the entire frequency band.

For phase amplitude coupling analyses, theta (6-10 Hz) or alpha (12-20 Hz) and gamma (30-80 Hz) signals were filtered separately using a band-pass butterworth filter, and analytic signals were obtained using the Hilbert transformation. Peaks were clearly identifiable in the studied frequency ranges of the power spectra before filtering. Instantaneous theta or alpha phase was measured as the angle of the analytic theta signal, and instantaneous gamma amplitude was measured as the absolute value of the magnitude of the analytic gamma signal.

**Surgical Procedures**

Rats were anesthetized with ketamine/xylazine or isoflurane, shaved and placed in a stereotaxic instrument (David Kopf Instruments). For stereotaxic injection experiments, holes were drilled into the skull using the following coordinates: 2.7 mm anterior to bregma, ±0.5 mm lateral to the midline, and 4.0 mm ventral from the dura (Banasr and Duman, 2008). Rats received a single 1 µl injection bilaterally of virus in the medial prefrontal cortex over the course of five minutes. Rats were monitored for five days prior to any further experimental manipulation. For infusion experiments bilateral cannulae were implanted using the same coordinates above (26ga, 5mm pedestal, 5mm projection length; Plastics One). Surgical screws were placed in the skull around the cannulae, and cannulae were fixed in place using dental acrylic (Bosworth). Dummy caps (Plastics One) were used to prevent clogging. For recording experiments, rats were implanted a dual cannula electrode system (26 ga, 5mm pedestal, 5mm projection length; Plastics One) unilaterally targeting the medial prefrontal cortex at the coordinates described above. Prior to injection all rats received a single 1µl infusion of the GFAP-Gq-DREADD, infused slowly over the course of 5-minutes. 10-minutes post injection the dual cannula electrode system was slowly lowered again over the course of five minutes. Three self-tapping bones screws (FST) were placed surrounding the implant and one bone screw placed over the contralateral hippocampus from the implant was used as a ground for LFP recordings. A base layer of MetaBond (Parkell) was applied to the skull and implant and allowed to dry before dental acrylic
(Bosworth) was used to build up the skull cap. Placement of implants and injections were counterbalanced across the left and right hemispheres.

**Attentional Set-Shifting Task**

Four separate cohorts of animals ((1) medial prefrontal cortex GFAP-Gq-DREADD with either CNO or saline, (2) CNO or saline injected control (3) medial prefrontal cortex mS100β or S100β, (4) medial prefrontal cortex GFAP-Gq-DREADD with antibody against S100 or non-immune rabbit IGG animals treated) were tested on the attentional set-shifting task. The attentional set-shifting task was carried out following the procedures presented in Chapter 2.

**Immunolabeling for Astrocyte and Neuronal Markers.**

Rats were injected with an overdose of Euthasol and transcardially perfused with 4% paraformaldehyde 1-2 hours following the completion of behavioral testing unless otherwise noted. Brains for immunohistochemical analysis were postfixed for 48 hours before processing. 40 µm coronal sections (1:12) were cut from half brains into a bath of 0.1M PBS using a Vibratome. For astrocyte immunolabeling, free-floating sections were rinsed in 0.1 M PBS, pH 7.5, incubated with 3% normal donkey serum, 0.1M PBS with 0.1% Triton X-100 and either rabbit anti-glial fibrillary acidic protein (GFAP) (1:500, Dako, z0334), mouse anti-NeuN (1:500, Millipore, MAB377), rabbit anti-S100 (1: 10,000, Dako, zo311), and stored at 4°C. After incubation in primary antisera for 24 hours, the sections were rinsed, incubated in either Alexa Fluor donkey anti-rabbit 488 (1:250; Invitrogen) or Alexa Fluor donkey anti-rabbit 568 (1:250; Invitrogen) in the dark for 60 minutes, rinsed, mounted onto suprafrost slides, and coverslipped using glycerol in PBS (3:1). All sections were counterstained with the DNA dye, Hoechst 33342 (1: 100,000, Molecular Probes).

Slides were coded until completion of the data analysis. Images from the medial prefrontal cortex were taken using a Zeiss confocal microscope (LSM 700; lasers, argon 458/488; HeNe 568). Cross-sectional area measurements of 50 S100+ cells per animal per brain region were obtained from 20 µm image stacks using ImageJ (NIH). Selected cells were in close proximity to layer 2/3 pyramidal neurons in the medial prefrontal cortex. Selected cells had to be distinct from surrounding labeled cells and be fully stained. Cross-sectional area measurements
were taken from z-stacks by using ImageJ (NIH) by circling individual cells at their widest point and using the area function. All parameters for background subtraction were optimized and held constant throughout the analysis.

**Dil Labeling**

In order to assess potential changes in dendritic spine density following GFAP-Gq-DREADD activation, rats (n=10) underwent surgery to receive an injection of the DREADD virus (see above) following a 4-week time point consistent with the behavioral testing, rats were injected with CNO (3 mg/kg) placed back in their home cages for 3 hours and then euthanized. Rats were perfused with 1.5% paraformaldehyde at the same time point as behaviorally tested animals. Brains were processed as described above. Individual sections were shot with lipophilic Dil crystals (Sigma) using the Helios Gene Gun System (BioRad) as previously described (Staffend and Meisel, 2011). Tissue was incubated for 1 hour in 4% paraformaldehyde at 4°C before being rinsed and mounted. Following a 1 hour incubation, tissue was mounted onto suprafrost slides and coverslipped using glycerol in PBS (3:1). Sections were visualized using a Zeiss confocal microscope (LSM 700; lasers, argon 458/488; HeNe 568) with a 63x oil objective. All settings (pinhole size, aperture gain, and offset) were initially optimized and held constant throughout the study. For each rat, 5 pyramidal neurons from layer 2/3 in the medial prefrontal cortex neurons were analyzed in 20-50 µm z-stacks by counting spines along secondary and tertiary apical and basal dendrite segments (lengths totaled 50µm/dendrite/neuron). Neurons selected for analysis were fully impregnated, had clearly identifiable secondary and tertiary branches, and were relatively isolated from other neurons. Three other additional measures were used to assess changes in dendritic spines; spine length, measured as the length of the spine at its farthest point from the shaft, spine head diameter, measured as the diameter of the spine head at its largest point and spine type calculated as a percentage from a sampling of 50 spines per animal.

**Statistics**

Unpaired two tailed Student’s t-tests were performed on each data set (for cellular analyses: saline x astrocyte manipulated except for spine type data and the attentional set-shifting data. Spine type data was analyzed as Student t-tests (saline vs. CNO) and corrected for multiple comparisons using a Holm-Sidak correction. The attentional set shifting data was
analyzed as a mixed factorial ANOVA with Holm’s correction for multiple comparisons where appropriate. A secondary analysis was carried out to ensure that control animals successfully formed an attentional set. Paired t-tests were conducted comparing errors to criterion on rule-based trials, the reversal and the extradimensional shift (ED), to performance on the last learning-based trial, the intradimensional shift (ID) (O’Reilly et al., 2002). This secondary analysis was not performed on treated animals as predicted changes in performance would likely strengthen or weaken statistical testing arbitrarily (O’Reilly et al., 2002). For power spectra data in order to examine changes in theta and gamma power between treatments, paired t-tests were performed on the sum of power within the theta and gamma band, respectively, for each animal. For the phase-amplitude coupling analysis, differences in modulation index were tested using the two-sided Wilcoxon signed rank test. To calculate the modulation index, we measured average gamma amplitude within theta or alpha frequency bins (each bin 3.6 degrees), and took the difference between the maximum gamma amplitude and minimum gamma amplitude as a function of theta or alpha frequency. All data and code are available from authors upon request.

**Results**

**Enhancement of astrocytic Ca\(^{2+}\) signaling in the medial prefrontal cortex improves cognitive flexibility, which can be blocked by inactivating S100β**

We investigated whether stimulating astrocyte calcium signaling would improve cognitive flexibility. To do this, we used a pharmacogenetic approach to express the Gq-DREADD receptor in GFAP+ cells via viral injection into the medial prefrontal cortex. We first verified that DREADD expression was exclusively localized to astrocytes by demonstrating the presence of mCitrine in GFAP+ or S100+ cells, but not in NeuN+ cells. Approximately 10-15% of the astrocyte population was successfully transduced with no evidence for neuronal transduction (Figure 1e). Activation of calcium signaling in GFAP-Gq-DREADD-expressing astrocytes resulted in significantly fewer trials to criterion \((p = .0133)\) and errors made \((p = .0117)\) on the extradimensional shift portion of the attentional set-shifting task (Figure 1a&b). As before, secondary analysis of saline-treated control rats revealed a significant increase in errors made on combined reversal/ extradimensional shift trials compared to intradimensional shift trials suggesting that animals formed an attentional set \((t_{(6)} = 3.178, \ p = .0191)\). CNO-treatment did not
affect performance on any part of the attentional set-shifting task in control rats without DREADD expression (Figure 2a-c). We next investigated whether CNO treatment in DREADD-expressing astrocytes affected astrocyte morphology, given previous research showing that experiences that improve cognition are associated with larger astrocytes (Sampedro-Piquero et al., 2014; Brockett et al., 2015). Activation of astrocytic Ca\(^{2+}\) signaling by CNO increased the size of transduced and neighboring astrocytes (Figure 1f) compared to control groups with astrocyte-expressing DREADDs but no CNO treatment. There were significant main effects for treatment \((F_{(1,26)} = 23.28, p < .0001)\) and expression \((F_{(1,26)} = 7.262, p = .0104)\), as well as an interaction effect \((F_{(1,26)} = 5.717, p = .0243)\), suggesting that increases in astrocyte cell body size were driven specifically by activation of calcium signaling in astrocytes.

We also examined whether CNO activation of DREADD expressing astrocytes induced changes in dendritic architecture. In a separate cohort of behaviorally naïve rats, analysis of spine density on layer 2/3 pyramidal neurons revealed no significant differences between saline and CNO treated groups for both apical \((t_{(5)} = .8760, p = .4211)\) and basal \((t_{(5)} = .7600, p = .4815)\) dendrites (Figure 1g & h). Similarly, no differences in spine length (apical : \(t_{(5)} = .3517, p = .7395\); basal: \(t_{(5)} = .1447, p = .8906\)), spine head diameter (apical : \(t_{(5)} = .2895, p = .7838\); basal: \(t_{(5)} = 1.026, p = .3521\)) or spine type (apical : filopodia: \(t_{(5)} = 1.153, p = .3011\), thin: \(t_{(5)} = .5926, p = .5792\), mushroom: \(t_{(5)} = .9413, p = .3898\), stubby: \(t_{(5)} = .3652, p = .7299\); basal: filopodia: \(t_{(5)} = .7396, p = .4928\), thin: \(t_{(5)} = 1.066, p = .3353\), mushroom: \(t_{(5)} = 1.345, p = .2365\), stubby: \(t_{(5)} = .9825, p = .3710\) were detected in either apical or basal dendrites of layer 2/3 pyramidal neurons in the medial prefrontal cortex (Table 1a-c).

S100β is an astrocyte-specific calcium binding protein that can be released by astrocytes and has been associated with enhanced rhythmogenic firing in neurons through the regulation of extracellular calcium (Sakatani et al., 2008; Morquette et al., 2015). Because performance on the attentional set-shifting task is associated with changes in neuronal oscillations specifically in the theta and gamma frequencies (Canolty et al., 2006; Voloh et al., 2010; Lee et al., 2014), we predicted that changes in S100β levels may underlie changes in neuronal signaling that reflect
enhancement in cognitive flexibility. In order to determine whether GFAP-Gq-DREADD induced enhancement of cognitive flexibility was influenced by S100β, we examined whether temporary inactivation of S100β in the medial prefrontal cortex would block improved cognitive flexibility observed with enhanced astrocytic calcium signaling. We infused S100β antibody or a non-immune rabbit IGG into GFAP-Gq-DREADD-expressing rats treated with CNO. Compared to non-immune IGG controls, S100β antibody infusion twenty minutes prior to the extradimensional shift significantly increased the number of trials needed to reach criterion ($p = .0001$) (Figure 1c) and the number of errors made ($p = .0001$) (Figure 1d), suggesting that S100β levels may directly influence cognitive flexibility.
Figure 1. Pharmacogenetic enhancement of astrocyte calcium signaling enhances cognitive flexibility. Activation of the GFAP-Gq-DREADD with CNO (n=8) 20 minutes before completion of the EDS enhanced cognitive flexibility relative to saline-treated controls (n=7). A timeline for the GFAP-Gq-DREADD experiment is above. a, b. CNO-treated rats took fewer trials to reach criterion and made fewer errors on the EDS than saline-treated rats. c, d. In a separate cohort, CNO-treated rats that received infusions of S100β (xS100β) in the medial prefrontal cortex (n=9) took more trials to reach criterion and made more errors on the EDS than CNO-treated rats that received infusions of IGG (n=9). e. Confocal image showing Gq DREADD expression co-localized with GFAP+ astrocytes in the medial prefrontal cortex. Scale bar = 5 μm. Top: Co-localization of the astrocyte protein S100+ with Gq DREADD expression. Scale bar = 5 μm. Bottom: no co-localization between NeuN+ neurons (open arrow) and the Gq DREADD (closed arrow) was observed. Scale bar = 10 μm. f. S100+ astrocytes near mCitrine expression and in animals receiving CNO were larger compared to saline controls and when compared within-subjects to brain sections not containing mCitrine. g. Confocal images of Dil labeling in saline-treated and CNO-treated rats. Scale bar = 5 μm. h. In a separate cohort of GFAP-Gq-DREADD rats, CNO treatment (n=4) did not alter dendritic spine density on either apical or basal dendrites of layer 2/3 pyramidal neurons in the medial prefrontal cortex relative to saline (n=3). * p<0.05 compared to controls (a,b: saline-injected; c,d: IGG- infused; f: DREADD- cells).
Figure 2. CNO treatment alone does not alter cognitive flexibility. Despite being thought of as an inert synthetic ligand, recent evidence suggests that CNO can activate endogenous receptors and have behaviorally relevant effects (MacLaren et al., 2016). To rule out the possibility that CNO treatment itself affects performance on the attentional set-shifting task, unoperated rats (n=6) were injected with CNO or saline 20 min prior to the completion of the EDS. a,b. There were no differences in number of trials to reach criterion or number of errors made between CNO- and saline-treated rats. c. Secondary analysis of saline-treated rats’ performance comparing errors on the IDS and errors on the rule-based reversal (REV) and extradimensional shift (EDS) showed that controls successfully formed an attentional set. Saline-treated rats (n=6) made more errors when completing the REV/EDS portions of the task compared to the IDS. * p<0.05 compared to intradimensional shift (IDS).
Table 1. Pharmacogenetic enhancement of astrocyte calcium signaling does not alter spine morphology on secondary or tertiary dendrites of layer 2/3 pyramidal neurons in the medial prefrontal cortex. To rule out the possibility that enhancement of cognitive flexibility in GFAP-Gq-
DREADD activated rats was due to changes in dendritic spines, several parameters of spine morphology were examined. 

<table>
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<th>p-value</th>
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<td>CNO: 15 ± 2.38</td>
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a. No differences in the percentage distribution of spine types were detected between saline- (n=3) and CNO-treated (n=4) rats. 
b. No differences in spine head diameter were detected between saline- and CNO-treated rats. 
c. No differences in spine length were detected between saline- and CNO-treated animals.
Experimental increases in S100β in the medial prefrontal cortex improve cognitive flexibility and increase the modulation of theta-gamma coupling

Neuronal oscillations in the theta (6-10 Hz) and gamma (30-80 Hz) ranges, have been linked improved cognitive function and medial prefrontal cortex function, however the mechanism for by which these neuronal oscillations are regulated is unknown (Voloh et al., 2010). S100β has been associated with enhanced rhythmogenesis in brain regions other than the medial prefrontal cortex (Sakatani et al., 2008; Morquette et al., 2015). However, whether S100β can alter the relationship between theta and gamma oscillations in a behaviorally relevant context has yet to be tested. We next investigated whether infusion of the astrocyte-specific calcium binding protein S100β into the medial prefrontal cortex would enhance cognitive flexibility and change the neuronal oscillations that underlie this behavior. We found that, compared to rats infused with an inactive mutated form of the protein (mS100β) incapable of binding calcium, those infused with S100β 20 minutes prior to extradimensional shift required significantly fewer trials to reach criterion ($p = .0001$) (Figure 3a) and made significantly fewer errors ($p = .0001$) (Figure 3b), much like GFAP- Gq-DREADD animals treated with CNO. Secondary analysis comparing performance on the combined reversal/ extradimensional shift trials with performance on intradimensional shift trials revealed a significant increase in the number of errors made, indicating that these animals formed an attentional set ($t_{(22)} = 8.247, p = .0001$).

Given the evidence suggesting that S100β enhances rhythmogenic firing in neurons (Sakatani et al., 2008; Morquette et al., 2015), we also investigated whether S100β-induced enhancement of cognitive flexibility was associated with changes in medial prefrontal cortex neuronal oscillations. Using a within subject design, we treated a separate cohort of behaviorally naïve animals with mS100β and then S100β, or vice versa, and recorded local field potentials (LFPs) from the medial prefrontal cortex during 20 min exposures to an open field. To control for the effect of locomotion on neuronal oscillations, we measured the average velocity of the animal in 10 s bins throughout the session using behavioral tracking software (Biobserve). We found that the distribution of velocities measured in each 10 s bin was not significantly different between conditions ($t_{(17)} = .9880, p = .3370$) (Figure 4c). Power within the theta and gamma
bands, as well as phase-amplitude coupling between the theta and gamma bands has been associated with improved cognitive ability (Canolty et al., 2006; Tort et al., 2009; Roux and Uhlhaas 2014) so we measured theta (6-10 Hz) and gamma (30-80 Hz) power as well as coupling between theta phase and gamma amplitude (Voloh et al., 2010). Infusion of S100β into the medial prefrontal cortex did not alter theta \( \left( t_{(17)} = .4690, p = .6450 \right) \) or gamma \( \left( t_{(17)} = .8570, p = .4040 \right) \) power (Figure 4a&b). We found that the amplitude of gamma oscillations was significantly modulated by the phase of theta oscillations in rats during either treatment \( (p < .001) \) but that the modulation of gamma by theta was significantly higher in animals when S100β was infused into the medial prefrontal cortex compared to mS100β \( (z_{(17)} = 2.070, p = .0380) \) (Figure 3c&d). Additionally, we observed that the modulation of gamma amplitude by theta phase was bimodal, indicating that this effect may be driven by modulation of gamma amplitude by a higher frequency, creating a harmonic effect in the theta frequency. To test this hypothesis, we examined modulation of gamma amplitude by alpha/beta phase (12-20 Hz). We found that gamma amplitude was significantly modulated by these higher frequency amplitudes \( (p < .001) \), but there was no difference in the strength of the modulation between mS100β and S100β animals \( (z_{(17)} = -1.336, p = .1820) \) (Figure 5a&b). These data suggest that astrocytes participate in cognitive flexibility in the medial prefrontal cortex by modulating the relationship between oscillations commonly associated with cognitive ability, namely theta and gamma, via S100β.
Figure 3. Infusion of the astrocyte-specific calcium binding protein S100β in the medial prefrontal cortex enhances cognitive flexibility and theta-gamma coupling. To examine whether S100β facilitates cognitive function, rats were infused with either S100β (n = 12) or mS100β (n=12), a mutated, nonfunctional form of S100β, in the medial prefrontal cortex 20 minutes before completing the extradimensional shift (EDS). To examine whether S100β infusion alters neuronal oscillations, a separate cohort of rats was infused with S100β followed by mS100β or vice versa (n=18) in the medial prefrontal cortex and LFPs were recorded. A phase-amplitude coupling analysis was performed using within subject design to look at the role S100β plays in modulating the relationship between theta and gamma oscillations. Timelines for each experiment are provided above. For the behavioral experiment, the attentional set-shifting task occurred over 3 days, with the EDS performed on the last day. a, b. Rats infused with S100β required significantly fewer trials to reach criterion and made significantly fewer errors on the EDS compared to those infused with mS100β. c. Phase amplitude coupling analysis plotting normalized gamma power as a function of theta phase. d. Permutation tests showed significant modulation above chance for both treatment conditions (red) with S100β significantly increasing theta-gamma modulation relative to mS100β controls, indicating that increasing S100β enhances theta-gamma coupling in the medial prefrontal cortex. * p<0.05 compared to mS100β-infused.
Figure 4. S100β does not alter theta or gamma power and has no effect on locomotor behavior. Power spectra analyses were conducted to examine whether S100β alters power in the theta (6-10 Hz) and gamma (30-80 Hz) frequency ranges. a. Power spectra for low frequency ranges show no differences in the theta range. b. Power spectra for low frequency ranges show no differences in the gamma range. c. A mixed effects model revealed no differences in movement across velocity bins.
Figure 5. S100β does not affect phase-amplitude coupling between alpha and gamma oscillations in the medial prefrontal cortex. To examine whether there was harmonic influence of alpha on our original theta-gamma phase amplitude coupling analysis, a second analysis was conducted to examine the relationship between alpha and gamma oscillations. LFPs were recorded while rats (n=18) roamed an open field after infusion of S100β followed by mS100β or vice versa in the medial prefrontal cortex. A phase-amplitude coupling analysis was conducted to look at the role S100β plays in modulating the relationship between alpha and gamma oscillations. A timeline for the experiment is provided above. a. Phase amplitude coupling analysis plotting normalized gamma power as a function of alpha phase. b. Permutation results showed significant modulation about chance for both treatment conditions (red). S100β did not significantly increase alpha-gamma modulation relative to mS100β controls suggesting that S100β effects on neuronal oscillations may be specific to theta-gamma coupling in the medial prefrontal cortex.
**Discussion**

In addition to the results presented in Chapters 2 & 3, these findings further suggest that astrocytes in the medial prefrontal cortex play an important role in cognitive flexibility. Enhancing astrocyte \( \text{Ca}^{2+} \) signaling or infusing the astrocyte-specific \( \text{Ca}^{2+} \) binding protein S100\( \beta \) both improved performance on this task. Furthermore, blocking S100\( \beta \) prevented the cognitive enhancement associated with increased astrocytic \( \text{Ca}^{2+} \) signaling. Moreover, infusion of S100\( \beta \) increased the coupling between theta and gamma oscillations, a phenomenon which has been previously linked to medial prefrontal cortex function (Spellman et al., 2015). These results highlight a novel signaling mechanism whereby astrocytes can directly regulate neuronal signaling.

The behavioral results of our DREADD “gain of function” manipulation similar to those observed in the study examining the influence of physical exercise on the medial prefrontal cortex (Chapter 2). Voluntary wheel running improved performance on the extradimensional shift as well as increased astrocyte size in the medial prefrontal cortex (Chapter 2). Similar results were observed following enriched environment living in aged rodents where cognitive improvement was associated with larger astrocytes in the hippocampus (Sampedro-Piquero et al., 2014). Both physical exercise and living in an enriched environment also increase dendritic spine density in the prefrontal cortex and hippocampus (Moser et al., 1994; Eadie et al., 2005; Brockett et al., 2015). Since dendritic spines have been associated with cognitive function (Leuner et al., 2003; Brockett et al., 2015), it seems likely that an increase in their number contributes to improved cognitive flexibility associated with experience. However, our DREADD results showing that increased astrocytic \( \text{Ca}^{2+} \) signaling improves cognitive flexibility and increases astrocyte size without altering dendritic spine density, size or shape suggest that cognitive improvement is possible as a result of changes in astrocyte signaling and potentially size, without changes in dendritic spines. While it remains possible that experience-dependent improvements in cognitive function involve growth of both neurons and astrocytes, our findings suggest that alterations in astrocytes alone are sufficient to produce enhanced cognition.

Our findings suggest that one way astrocytes may improve cognitive function is through the release of the astrocyte-specific \( \text{Ca}^{2+} \) binding protein S100\( \beta \). We have found that S100\( \beta \) infusion
into the medial prefrontal cortex improves cognitive function in a manner similar to what we observed with DREADD-induced increased astrocytic Ca\(^{2+}\) signaling and conversely, that blocking S100\(\beta\) with an active antibody infused into the medial prefrontal cortex prevents improved cognitive function resulting from DREADD activation. While S100\(\beta\) has been most studied for its actions binding Ca\(^{2+}\) inside the astrocyte, some evidence suggests that it is released by astrocytes and has actions in the extracellular space as well (Zimmer et al., 1995; Donato et al, 2009). In addition, studies have shown that extracellular S100\(\beta\) participates in the generation of rhythmic firing among neurons in the brainstem (Morquette et al., 2015). Reduced Ca\(^{2+}\) in the extracellular space has been associated with increased neuronal rhythmogenesis in several other systems (Angstadt and Friesen, 1991; Bouskila and Dudek, 1993; Perez-Velazquez et al., 1994; Beck et al., 2001; Hashimoto and Kita, 2006; Kadala et al., 2015). We have shown that S100\(\beta\) infusion into the medial prefrontal cortex increases phase-amplitude coupling of neuronal oscillations in the theta and gamma ranges. Given that theta-gamma coupling in the medial prefrontal cortex has been linked to cognitive function (Canolty et al., 2006; Voloh et al., 2015), these findings suggest that astrocytes may improve cognitive function by modulating coordinated rhythmic firing through the release of S100\(\beta\). Conversely, transgenic global disruption of astrocyte function has been shown to diminish neuronal oscillations in several ranges as well as to impair hippocampal-dependent recognition memory (Lee et al., 2014).

There is no question that the interaction between astrocytes and neurons is an important aspect of brain functioning. While it is clear that astrocytes support neurons by providing a wide array of functions from maintenance of the blood brain barrier (Iadecola and Nedergaard 2007; Attwell et al., 2010) to the regulation of glutamate (Anderson and Swanson 2000; Bechtholt-Gompf et al., 2010) and the clearance of toxins during sleep (Xie et al., 2013), few studies have linked this support to cognitive function specifically. Our results suggest that augmentation of astrocyte Ca\(^{2+}\) signaling in the medial prefrontal cortex enhances cognitive flexibility presumably through the astrocytic release of S100\(\beta\), which on its own increases theta-gamma coupling. Collectively, these results suggest a novel role for astrocytes in the medial prefrontal cortex in the regulation of cognitive flexibility.
Chapter 5: Discussion
The focus of my dissertation has been to expand the role of astrocytes from cells whose chief functions are supportive, to include their essential involvement in the regulation of complex behavior. Throughout this work I have chosen to focus primarily on cognitive flexibility to explore potential mechanisms that astrocytes may employ in the regulation of behavior.

my work has shown that under conditions normally associated with enhanced cognition, astrocytes change along with neurons, and among the brain regions studied, only when we observed changes in both the neuronal and astroglial architecture did we observe changes in cognitive ability (Chapter 2). Rats given access to a running wheel performed better on the medial prefrontal cortex-hippocampal-dependent object-in-place task as well as on the medial prefrontal cortex-dependent extradimensional shift portion of the attentional set-shifting task. Rats also performed better on the orbitofrontal cortex-dependent reversal portion of the attentional set-shifting task. In the medial prefrontal cortex, hippocampus and orbitofrontal cortex, changes in dendritic spines and astrocyte cell body area as well as astrocytic endfeet coverage of the surrounding vasculature was increased. However, runners did not perform better than sedentary controls on the perirhinal cortex-dependent object in place task. While we did detect changes in proteins associated with dendritic plasticity in the perirhinal cortex, we saw no changes in astrocyte morphology, number, or staining pattern, suggesting that both changes in neurons and astrocytes may be necessary in order to support changes in cognitive ability.

We expanded on these associational findings with running, to show that in non-running animals when astrocyte functionality is diminished either by inhibition of astrocytic glutamate uptake or by reduction of astrocyte number in the medial prefrontal cortex, so too is cognitive flexibility (Chapter 3). We chose to focus these studies on cognitive flexibility as the attentional set-shifting task allows us to test medial prefrontal cortex-dependent and orbitofrontal cortex-dependent forms of cognitive flexibility separately. Surprisingly, impairment of astrocytic glutamate uptake in the orbitofrontal cortex did not result in changes in reversal learning. In considering reasons for why DHK did not impair astrocyte functioning the way it did in the medial prefrontal cortex, while running enhanced functioning in both regions, it may be relevant that running is associated with numerous robust positive benefits to brain health and functioning (Vivar et al., 2013). Thus, it is possible that in the context of running, continual exposure to...
nutrients and other trophic factors augmented the functioning of orbitofrontal cortex astrocytes beyond what is normally expected given the results of our morphological comparison study. Our morphological characterization of astrocyte populations in behaviorally naïve animals suggested that astrocyte population in the medial prefrontal cortex and orbitofrontal cortex were distinct, with astrocytes in the medial prefrontal cortex being more numerous, larger, and making a greater number of contacts with dendritic spines than their orbitofrontal cortex counterparts. It is also possible given this regional diversity that the dose of DHK was not sufficient to alter astrocyte functioning in the orbitofrontal cortex, and future studies should explore whether different doses of DHK in the orbitofrontal cortex impair reversal learning. These results potentially highlight an often-overlooked complexity in studying astrocyte function, namely regional diversity, which must be accounted for in future studies of astrocyte function and will be touched on again later in the discussion.

Next, I tested whether the positive effects of running on cognition and astrocyte physiology could be mimicked by activating astrocytes using a pharmacogenetic approach (Chapter 4). Pharmacogenetic activation of astrocyte Ca\(^{2+}\) signaling led to enhanced cognitive flexibility and increased astrocyte size in astrocytes specifically expressing or in close proximity to the DREADD construct, similar to the effects of running. No changes in dendritic spine density associated with DREADD activation were observed suggesting that this form of plasticity may not be necessary to improve cognitive flexibility.

Finally, I investigated a potential mechanism whereby astrocytes directly influence cognitive flexibility (Chapter 4). Neuronal oscillations specifically in the theta (6-10 Hz) and gamma (30-80 Hz) ranges have been shown to be important for cognitive flexibility. Some evidence linking astrocytes to neuronal oscillations suggest that S100β, an astrocyte specific protein may support rhythmic neuronal firing by sequestering extracellular Ca\(^{2+}\). We showed S100β enhances cognitive flexibility and increases phase amplitude coupling between the theta and gamma spectra. Collectively, this work has delineated a new role for astrocytes in the regulation of cognitive flexibility, and identified a novel mechanism by which astrocytes can directly influence neuronal oscillations. These findings expand on what was previously known about the role of astrocytes in brain functioning.
Technical Considerations

Throughout the course of my dissertation work, there were many technical difficulties and concerns as the tools needed to study and manipulate astrocyte function in behaving animals lag greatly behind their neuronal counterparts. Studying astrocyte morphology and function is especially difficult as the tools to label and manipulate astrocytes are relatively limited. Historically, astrocytes have been viewed as a relatively homogenous population of cells expressing the classic immunohistochemical marker glial fibrillary acidic protein, or GFAP. However, research has shown that GFAP only fills approximately 15% of the total volume of the cell and is not universally expressed by all astrocytes (Bushong et al., 2002; Cahoy et al., 2008; Khakh and Sofroniew, 2015). Similarly, other canonical markers such as S100 do not label all cell bodies, with variable overlap between GFAP+ and S100+ populations (Cahoy et al., 2008; Brockett et al., 2015). Antibodies and transgenic approaches that take advantage of better and more universal markers, such as AldH1L1 are just now commercially available (Cahoy et al., 2008; Khakh and Sofroniew, 2015). Genetic characterization methods suggest that AldH1L1 labels all astrocytes (Cahoy et al., 2008; Khakh and Sofroniew, 2015). Given the heterogeneity of astrocytes, questions can arise about whether astrocytes that label with different markers have different functions (Khakh and Sofroniew, 2015). This field of research remains practically untouched, and ultimately limits the interpretation of these results. Our results relied on two of the most highly characterized and published markers of astrocytes, GFAP and S100, as well as a less commonly used marker of astrocyte endfeet, aquaporin-4, each which labels an overlapping subpopulation of undetermined function. As our understanding of astrocyte function increases, and new tools become available, future studies should extend these findings using more targeted approaches.

Moreover, it is likely that our viral-targeting approaches suffer from similar concerns. The Gq-DREADD operates under the GFAP promoter, and it is possible that activating astrocytes in a separate subpopulation, such as S100 positive astrocytes, may have different effects on behavior. In the medial prefrontal cortex, approximately 50% of GFAP positive astrocytes co-express the protein S100 (Brockett et al., 2015). Likewise, only a subset of S100 positive astrocytes co-express GFAP (Cahoy et al., 2008). While yet to be observed, it is likely that
subsets of astrocytes have unique functional properties similar to what is seen in neurons, and this may potentially confound interpretation. This concern with regard to GFAP+ and S100+ expressing astrocyte populations is somewhat refuted by our results suggesting that the positive effects of Gq-DREADD activation can be blocked by infusion of antibody against S100β. These results seem to indicate that, at least in some way, convergent pathways are at play. Despite the limitations associated with the lack of GFAP expression by all astrocytes, DREADD expression in approximately 10-15% of the GFAP-expressing subpopulation of astrocytes in the medial prefrontal cortex was sufficient to enhance cognitive behavior associated with this brain region after CNO injection. It is worth noting that increasing Ca\(^{2+}\) signaling in a relatively small subpopulation of astrocytes may not only be sufficient, but critical, for improving performance on this behavioral task, as a manipulation that affects a greater number of astrocytes may have produced an impairment. Future studies using new tools designed to alter function of all subpopulations of astrocytes will be necessary to address this question.

With regards to studying astrocyte function, a potential limitation of pharmacological techniques for in vivo studies is the possibility of incidental damage caused by stereotaxic injection or cannula placement. Astrocytes are highly susceptible to injury, responding with hypertrophy (Takano et al., 2014; Khakh and Sofroniew, 2015). While our approach sought to minimize damage, and included vehicle controls, abnormalities in astrocyte density were evident in the controls of one of our experiments. In the DHK study, cannula-implanted animals showed a greater number of astrocytes around the cannula compared to non-operated control animals. This increase in astrocyte density is likely due to reactive gliosis, and typical of chronic cannula implantation (Takano et al., 2014; Khakh and Sofroniew, 2015). It is important to note that despite having more astrocytes than non-operated animals, saline-treated cannulated animals performed similarly to non-operated controls further suggesting that more astrocytes alone do not necessarily lead to altered behavior. Studies have shown that reactive astrocytes are heterogeneous (Anderson et al., 2014), and functional differences in reactive versus non-reactive astrocytes exist (Robel and Sontheimer, 2016), but how such differences relate to behavior remains unknown. Our findings suggest that simply having more astrocytes does not lead to altered behavioral performance, at least relative to the medial prefrontal cortex. It should be
noted that while we observed increased numbers of astrocytes following chronic cannula implantation, no such change was observed with a single injection into the medial prefrontal cortex, as in our L-AAA and DREADD experiments, suggesting surgical damage and reactive astrogliosis was relatively minimal at the time of testing.

**Potential Functional Significance of Astrocytes in the Medial Prefrontal Cortex**

Our data suggest that astrocytes in the medial prefrontal cortex may participate in cognitive flexibility by regulating extracellular Ca\(^{2+}\) which in turn is correlated with improved cognitive ability as well as enhanced phase-amplitude coupling between theta and gamma oscillations. Taken together with our results showing that both a temporary disruption of astrocytic glutamate uptake and a small (approximately 15%) decrease in the number of astrocytes in the medial prefrontal cortex produces cognitive impairment similar to large scale neuron loss in the medial prefrontal cortex (Birrell and Brown 2000), our findings suggest that astrocytes may be working to shape the overall output of a particular brain region. Astrocytic processes make direct contact with the surrounding vasculature and synapses and therefore have the potential not only to know what neurons are communicating but also bias this communication (Iadecola and Nedergaard 2007; Barres 2008; Attwell et al., 2010). S100β may represent one of several mechanisms whereby astrocytes may be able to alter the outputs of a collection of neurons in a more synchronous way, which in turn may be important for cognitive function (Canolty et al., 2006).

Astrocyte size may also be an important contributor to the regulation of cognitive ability. Whether S100β release is somehow related to astrocyte size is unknown, however, across two studies (running study and DREADD study), we have shown that astrocyte size increases and that this change in morphology is associated with enhanced cognitive ability. Moreover, we have shown that astrocyte size is highly variable across brain regions, such that astrocytes in the medial prefrontal cortex, hippocampus, and perirhinal cortex are larger than those in the orbitofrontal cortex (Chapter 2). This may reflect different evolutionary pressures on the development of these brain regions, and likely supports the notion that astrocytes are highly diverse and potentially even locally specified to a particular brain region (Ben Haim and Rowitch, 2016). These findings also offer the intriguing possibility that astrocyte size may be an important
determinant in the development of complex behavior, a possibility that is consistent with the observed differences in astrocyte size that exist between the human and rodent brain (Oberheim et al., 2009). Future research should examine the role of astrocytes in behavior in several brain regions that support cognition.

**Mechanisms Whereby Astrocytes May Influence Cognition**

Astrocytes may regulate cognitive behavior through the modulation of neuronal oscillations. Transgenic global disruption of astrocyte function leads to diminished gamma wave oscillations along with impaired hippocampal-dependent recognition memory (Lee et al., 2014). Since gamma and theta oscillations have been linked to performance on tasks that require the prefrontal cortex (Spellman et al., 2015) it seems likely that astrocytes could potentially influence oscillations in these frequency ranges associated with cognitive flexibility in the medial prefrontal cortex as well. S100β is an astrocyte specific protein known to be associated with the sequestering of Ca²⁺ intracellularly. The results of a small number of studies have suggested that extracellular S100β may be important for inducing rhythmogenic firing in neurons through the sequestering of extracellular Ca²⁺ (Sakatani et al., 2008; Morquette et al., 2015). While the mechanism by which astrocytes release S100β is unknown, we found that infusion of S100β into the medial prefrontal cortex was sufficient to enhance cognitive flexibility and increase phase-amplitude coupling between theta and gamma spectra. While the change in phase-amplitude coupling was observed in animals that were not engaging in tasks that required cognitive flexibility, these findings suggest that astrocytes may be putting the medial prefrontal cortex into a more cognitively optimal state by regulating the levels of extracellular Ca²⁺ via S100β. Future work should examine S100β-induced phase amplitude coupling changes between theta and gamma oscillations in the medial prefrontal cortex of animals undergoing a task of cognitive flexibility. Moreover, it would be interesting to show whether manipulation of astrocytes in one region ultimately changed the response and firing pattern of the subsequent downstream region. This would greatly support the notion that astrocytes regulate and optimize the output of a specific brain region in a behaviorally relevant manner. Finally, it remains unclear how decreases in extracellular Ca²⁺ lead to changes in neural synchrony, and future work should be directed towards uncovering this mechanism.
Future Directions

Historically, astrocytes have been portrayed as support cells tasked with the maintenance of brain homeostasis. Indeed, their role in the formation of the blood brain barrier, their anatomical placement that seemingly forms a scaffold around neurons, and their earliest described functions in the clearance of extracellular metabolites have suggested to many that these cells are not important contributors to behavior per se, but instead vital contributors to homeostasis. Early conceptions of the brain and how it functioned conjured ideas about a series of tubes allowing for the flow of spirits (Virchow, 1858; Ramón y Cajal, 1899; Somjen 1988; García-Marín et al., 2007; Barres 2008). Early neuroanatomists discovered tube-like cells and eventually, through a series of foundational studies, determined that instead of spirits, these tubular cells, neurons, relayed information through electrical and chemical signaling. Convenience in large part contributed to this discovery, as early histological methods such as Golgi staining preferentially stained neurons while many neuroanatomists described and complained about partially stained, non-neuronal cells that were eventually termed nervenkitt or glia (Virchow, 1858; Ramón y Cajal, 1899; Somjen 1988; García-Marín et al., 2007; Barres 2008). While unintentional, these formative ideas relegated astrocytes to the role of support cells where the perception of the functional utility of astrocytes has remained in large part until recently.

In order for us to better understand how the brain functions we need to consider the roles of all cells that make up the brain. By no means are the homeostatic functions currently assigned to astrocytes and other glia unimportant, but these frameworks often diminish the potential of these cells to contribute to more complex functions that are likely necessary for dynamic behavior. Microglial involvement in the pruning of dendritic spines has been implicated in healthy brain development (Schafer et al., 2012), while changes in oligodendrocytes and their control myelin thickness have been detected following learning and hypothesized to be correlated with enhanced signal transduction (Bengtsson et al., 2005; Fields 2015), while astrocytes have been implicated in influencing and neural signaling and now even behavior (Banasr and Duman, 2008; Henneberger et al., 2010; Han et al., 2013; Lee et al., 2014; Brockett et al., 2015). Moving forward future work needs to explore the contribution of each cell type to complex brain
functioning. With regard to astrocytes, careful genetically informed tools should be developed to effectively target specific subpopulations of astrocytes. Electrophysiology should be used both in vitro and in vivo to determine how each subpopulation contributes to brain output and specifically through which mechanisms these contributions are made possible. Tandem Ca$^{2+}$ imaging and neural recording studies will inform us about how these cell types work together. Moreover, these studies should be conducted with an eye towards complex brain functioning. While in vitro, in slice and basic in vivo experiments using anesthetized animals are informative for the basic science, these findings should be pushed toward characterizing the role of astrocytes in the context of the actual behavioral output these cells contribute to the intact brain. Finally, these findings need to inform new theories and models for how the brain functions, that move away from a predominantly “neurocentric” view of brain functioning.

**Conclusions**

My dissertation has explored the role astrocytes play in medial prefrontal cortex-dependent cognitive flexibility. Our data suggest that astrocytes change under conditions associated with enhanced cognitive flexibility, and that these cells are essential for the regulation and maintenance of cognitive flexibility. Moreover, we have identified a novel mechanism whereby astrocytes may directly influence neuronal signaling, namely the release of the calcium binding protein S100β. These findings are novel and a first step toward a better understanding of how the brain functions.
References


Fields RD. The other brain: the scientific and medical breakthroughs that will heal our brains and revolutionize our health. 2011. Simon and Schuster.: New York, New York, USA.


Moser MB, Trommald M, Anderson P. An increase in dendritic spine density on hippocampal CA1 pyramidal cells following spatial learning in adult rats suggests the formation of new synapses. Proc Natl Acad Sci USA. 1994; 91(26): 12673-12675.


Ramón y Cajal S. Textura del sistema nervioso del hombre y de los vertebrados. Tomo I: Imprenta y Libreria de Nicolas Moya. 1899.


Schafer DP, Lehrman EK, Kautzman AG, Koyama R, Mardinly AR, Yamasaki R, Ransohoff RM, Greenberg ME, Barres BA, Stevens B. Microglia sculpt postnatal


Morphological changes in hippocampal astrocytes induced by environmental enrichment in mice. Brain Res. 2009; 1274: 47-54.


