Characterizing How Age-Related Changes in GABA and Glutamate Underlie Development of Working Memory Through Adolescence

by

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B.S. in Neural Science, New York University, 2016

Submitted to the Graduate Faculty of the

Dietrich School of Arts and Sciences in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2021

UNIVERSITY OF PITTSBURGH

DIETRICH SCHOOL OF ARTS AND SCIENCES

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Adolescence is a time of significant cognitive development that leads to the establishment of adult modes of operation. Working memory is a core cognitive process that continues to improve into the second decade of life and is impaired across several psychiatric disorders. However, not much is known about the neurobiological mechanisms underlying the transition to adult-like working memory ability. The prefrontal cortex (PFC) supports working memory and undergoes protracted maturation through adolescence. Postmortem human and animal studies indicate changes in PFC Gamma-Aminobutyric Acid (GABA) (inhibition) and glutamate (excitation) during adolescence. These changes may underlie shifts in excitatory/inhibitory (E/I) balance reflecting critical period plasticity initiated by relative decreased excitation and increased inhibition. However, changes in glutamate and GABA through adolescence have not been well characterized in vivo in humans. We used 7T Magnetic Resonance Spectroscopic Imaging to characterize age-related changes in glutamate, GABA, and their balance in a sample of 144 10-30 year old healthy participants. First, we found age-related decreases in glutamate in the dorsolateral prefrontal cortex (DLPFC), anterior cingulate cortex (ACC), anterior insula, and a trend-level decrease in medial prefrontal cortex (MPFC). We found decreases in GABA in ACC and anterior insula, trend-level decrease in MPFC, and no change in DLPFC. Second, we found shifts toward excitation (indexed by the ratio of glutamate to GABA) in the ACC and anterior insula. Third,

ACC and MPFC glutamate and GABA were more correlated in older age groups, suggesting greater balance. Finally, we examined whether age-related changes in these indices were associated with age-related improvements in the memory-guided saccade (MGS) task. We did not observe associations between neurotransmitter indices and MGS accuracy. However, in the youngest age group, higher correlations among GABA and glutamate within the ACC were associated with better MGS performance. These results may suggest that the *balance* of glutamate and GABA, not necessarily their individual concentrations, may be more tightly associated with cognitive ability, particularly in early adolescence. Taken together, these results provide a novel account of age-related changes in glutamate and GABA in frontal and association cortices, and their role in supporting age-related improvements in working memory.

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1.0 Specific Aims

Adolescence is a time of significant cognitive development that leads to the establishment of adult modes of operation. The adolescent period is also marked by the emergence of several mental disorders that include symptoms of cognitive impairment, such as schizophrenia and/or mood disorders. Therefore, understanding the brain mechanisms that underlie the normative transition to adult-level cognition could provide important insights into the developmental basis of psychopathology. Specifically, working memory is a core cognitive process that continues to improve into the second decade of life and is impaired across several psychiatric disorders. Thus, working memory can serve as a transdiagnostic model to probe development and integrity of the neurocognitive system. One primary brain system that supports working memory is prefrontal cortex (PFC), which undergoes protracted maturation through adolescence. Postmortem human and animal model studies indicate that there are changes in Gamma-Aminobutyric Acid (GABA) (inhibition) as well as glutamate (excitation) in PFC occurring during this time. These changes may potentially underlie shifts in excitatory/inhibitory (E/I) balance reflecting critical period plasticity initiated by relative decreased excitation and increased inhibition. Notably, GABA supports gamma oscillations necessary for working memory processing, thus providing a potential mechanism for age-related improvements. However, given the signal-to-noise (SNR) limitations of scanning at 3T that undermine the data quality of GABA and glutamate measurements, changes in glutamate and GABA through adolescence have not yet been rigorously assessed *in vivo* in humans. Thus, in Aim 1, we will characterize age-related changes in glutamate, GABA, and the ratio of glutamate/GABA in order to examine changes in E/I balance. Additionally, we will also look at the correlation between glutamate and GABA through age as another way of

conceptualizing balance between excitation and inhibition. To do this, we will use 7T Magnetic Resonance Spectroscopic Imaging (MRSI) in 144 10-30 year old healthy participants. 7T imaging provides heightened signal-to-noise measures as compared to imaging at lower field strengths, allowing for detection of low-concentration neurotransmitters, such as GABA, and better separation of overlapping signals. Additionally, as compared to single voxel MRS studies, MRSI allows us to obtain measures of GABA and glutamate from multiple frontal regions of interest. Our slice is placed to optimize coverage of dorsolateral prefrontal cortex (DLPFC), but because it is a slice, it also provides measures of other frontal regions that undergo protracted developmental trajectories and may support working memory and/or cognitive control. Thus, Aim 1 will include hypothesis-driven examination of age-related associations with glutamate and GABA in DLPFC, while also including exploratory analyses that will allow us to assess the specificity of changes in DLPFC glutamate and GABA by looking across frontal regions distinct from DLPFC. In Aim 2, we will examine whether age-related changes in neurotransmitters are associated with age-related improvements in accuracy in a working memory task, the memory-guided saccade (MGS) task. Together, these results will provide novel insight into age-related changes in glutamate and GABA in frontal and association cortices, and their role in supporting age-related improvements in working memory through adolescence.

Aim 1: To examine how glutamate, GABA, and their balance changes through adolescence in PFC.

Based on evidence from postmortem and animal models in DLPFC, we hypothesize that levels of glutamate will decrease and levels of GABA will increase, thus shifting glutamate/GABA ratios in DLPFC toward inhibition. We predict that similar patterns may emerge across all prefrontal regions due to similar developmental trajectories. We also predict that age-related

changes in the levels of glutamate and GABA may lead to changes in the correlation between glutamate and GABA; we predict glutamate and GABA will become more correlated in adulthood, akin to what is known about mechanisms underlying critical period plasticity.

Aim 2: To examine how age-related changes in glutamate, GABA, and their balance in PFC are associated with age-related improvements in working memory.

We hypothesize that changes in glutamate and GABA in the DLPFC will mediate age-related improvements in visuospatial working memory ability, as measured by accuracy in performance on the MGS task. We will also conduct exploratory analyses to examine whether changes in neurotransmitters in other frontal regions that support working memory and cognitive control are associated with age-related improvements in visuospatial working memory ability. Taken together, these results will allow us to understand whether critical period plasticity mechanisms are present in DLPFC, how specific these mechanisms are to DLPFC, and whether they are associated with cognitive development through adolescence.

2.0 Background

2.1 Adolescence

Adolescence is characterized as a transitional period between childhood and adulthood that is often said to begin with the onset of puberty and roughly encompasses 12 to 17 years of age (Spear, 2000). During this developmental phase, there is an increase in exploratory and reward-driven behaviors, as adolescents start to interact with their environments in novel ways (Spear, 2000). Concurrent with this increased exploratory drive are improvements in executive functioning. Although cognitive control capabilities, including working memory, are available in adolescence, they are unreliably engaged and continue to improve into the second decade of life (Luna et al., 2004; Luna, 2009; Luna et al., 2015; Simmonds et al., 2017).

Supporting these behavioral changes through adolescence are postmortem findings of physiological changes in the brain including synaptic pruning (Petanjek 2011) and increases in myelination (Huttenlocher 1990; Yakovlev, Lecours, 1967) in association cortices including prefrontal cortex (PFC). MRI findings concur with the physiological findings, showing protracted maturation of prefrontal cortical (Gogtay et al., 2004) and white matter structure (Simmonds et al., 2014), as well as functional connectivity (Marek et al., 2018).

2.2 Critical Periods

Despite the increased knowledge of behavioral and structural changes that occur in the brain through adolescence, the neurobiological mechanisms that drive cognitive development during this time remain poorly understood. However, recent animal and postmortem human studies are providing new evidence that suggest that critical period mechanisms may be present during adolescence in prefrontal cortex (Larsen & Luna, 2018).

Critical periods are developmental time windows during which there is heightened sensitivity in specific brain regions to experiential inputs from external stimuli; as a result, significant neural reorganization occurs that is necessary for development (Knudsen, 2004; Hensch, 2004). If brain regions undergoing critical period plasticity do not receive the necessary input during this time, certain behaviors will be permanently altered or even missing (Hubel and Weisel, 1963). Prior to and following the critical period, the brain is still responsive to the same set of inputs but they have significantly less influence on the organization of neural circuitry (Smith & Trachtenberg, 2007; Levelt & Hubener, 2012).

The visual cortex is one region in which the critical period has been highly studied and well-defined, with early landmark studies by Hubel and Weisel showing that monocular deprivation in the first few months of a kitten's life leads to irreversible visual defects (Hubel & Weisel, 1963; Hubel & Weisel, 1970). These defects are not seen when the eye of an adult cat is closed, even for prolonged periods of time. Other studies have built on these findings in the visual cortex of mice, showing that ocular deprivation during the critical period leads to altered patterns of plasticity and changes the organization of ocular dominance columns (Fagiolini et al., 1994; Gordon and Stryker, 1996).

However, this phenomenon is not specific to vision. Other sensory systems, such as auditory systems, have also been shown to undergo critical periods.Rearing rat pups under conditions of continuous noise at a moderate level leads to the delayed development of adult-like organization of auditory cortex, prolonging the typical developmental time course of the rat auditory system (Chang & Merzenich, 2003). Higher-order cognitive behaviors such as the acquisition and production of vocalizations and language are also under the control of critical period mechanisms. Studies in songbirds have demonstrated that there is a time window of 10-50 days for learning species-specific songs (Marler, 1970). Language in humans has also been shown to have a critical period. Perhaps the most well-known study of human language acquisition outside of the critical period is a case study of a girl named Genie, who was environmentally deprived until she was 13 years old, and as a result failed to learn language (Fromkin et al., 1974). Although she was able to acquire some vocabulary, she was never able to compensate for her developmental deprivation through childhood. Other studies looking at normative language development in children have shown associations between the age at which a second language was learned and the proficiency of being able to speak that language (Johnson and Newport, 1989).

Critical periods are clearly varied in where they occur, when they occur, and what stimuli are inputs for specific critical periods. However, despite this variation, critical periods share common neurobiological mechanisms that define and delineate them. One such mechanism that has been thought to initiate a critical period is the shift in the balance of excitation and inhibition in specific brain regions, a process that is thought to be driven by maturation of inhibitory neural circuitry (Dorrn et al., 2010; Hensch and Fagiolini, 2005; Toyoizumi et al., 2013). Inhibition in the brain is enacted by the primary inhibitory neurotransmitter in the brain, Gamma-aminobutyric Acid (GABA). GABA generally acts to attenuate neuronal excitability, organize neuronal activity, and

stabilize newly-formed activity-dependent connections among neurons (Hensch, 2005; Kilb, 2012). GABAergic inhibition is a core mechanism of critical periods (Hensch et al., 1998). When GABA synthesis is limited through inactivation of the GABA-synthesizing enzyme glutamic acid decarboxylase 65-kilodalton isoform (GAD65), the opening of the critical period in occipital cortex is prevented until GABA receptors are stimulated with a GABA receptor agonist (Hensch et al., 1998). Further, critical period plasticity can be prematurely induced by giving mice GABA receptor agonists prior to natural critical period onset (Fagiolini and Hensch, 2000).

Although inhibitory neural circuitry may be driving the opening of a critical period, a variety of changes also take place in aspects of excitatory neural circuitry that impact the shift in E/I balance. In fact, glutamate, the primary excitatory neurotransmitter in the brain, is involved in experience-dependent plasticity, which establishes or extinguishes neural connections through a Hebbian process of "use it or lose it" (Hebb, 1949; Malenka and Bear, 2004). Specifically, NMDA glutamatergic receptors change their subunit composition in response to neural activity supporting critical period processes (Hoffmann et al., 2000; Quinlan et al., 1999; Vallano et al., 1996). Experimental manipulations of these subunits alter experience-dependent neuronal organization and neuronal response to stimuli (Lu et al., 2001; Fagiolini et al., 2003). However, unlike the aforementioned studies looking at GABA as a means of controlling the onset and offset of the critical period, modulating excitation via glutamate and its receptors does not regulate critical period timing (Lu et al., 2001; Fagiolini et al., 2003).

Nevertheless, well-regulated excitatory activity is still a crucial component of proper circuit organization. Through the course of a critical period, as there is an increase in inhibitory influence, spontaneous excitatory activity is dampened in favor of evoked activity (Toyoizumi et al., 2013). Over time, excitation and inhibition become more tightly coupled as neural circuitry becomes

better organized (Dorrn et al., 2010). Thus, the interaction between GABAergic and glutamatergic systems in response to novel stimuli, driven by maturation of inhibitory neural circuitry, acts mechanistically to organize neuronal networks and plasticity during critical periods.

2.3 Adolescence as a Critical Period

During adolescence, cognitive systems reach a level of synchronization that results in more reliable, high-SNR input to prefrontal and association cortices, which may be a result of shifting E/I balance and critical period plasticity processes (Larsen & Luna, 2018). The ability to effectively integrate multi-sensory information would support higher-level processing that is necessary during adolescence for social interactions and adult-level cognitive reasoning. There is a growing body of evidence in support of adolescent critical period plasticity that indicates that the glutamatergic and GABAergic systems are changing in a way that is consistent with a critical period at this time in development.

The GABAergic system is adaptively readjusting throughout the lifespan, with different developmental trajectories for different aspects of the GABA metabolic pathway (for review see Kilb, 2012). For example, GAD65 is responsible for catalyzing the production of GABA that is synaptically available (Kilb, 2012). In rodents, GAD65 has been shown to increase in expression through childhood (Popp et al., 2009), but in postmortem human studies of visual cortex, it does not reach peak concentration until adolescence (Pinto et al., 2010), suggesting a possible role in human cortical development during adolescence. As mentioned previously, GAD65 in particular has been shown to be necessary for critical period plasticity to occur (Iwai et al., 2003).

However, other studies have failed to see this increase during the adolescent period for other aspects of the GABA metabolic pathway (Fung et al., 2011; Kilb, 2012). Therefore, it may be that GAD65's role in producing synaptically available GABA may be a crucial piece underlying the E/I balance shift (Pinto et al., 2010).

Another key aspect of the maturation of inhibitory circuitry involves the developmental changes in specific classes of GABAergic neurons. One such class are the parvalbumin-containing (PV+) neurons, which undergo continued maturation into adolescence, particularly in PFC (Hoftman and Lewis, 2011). Expression of parvalbumin, a marker that is tightly linked to PV+ neuron function, increases in PFC through adolescence in rats (Cabellero et al., 2014) and in primates (Erikson and Lewis, 2002). In addition, there are changes in the composition of GABA receptor subunits that contribute to changes in inhibitory neurotransmission through critical periods that are seen during adolescence (Hashimoto et al., 2009). Increases in the GABA receptor subunit $\alpha 1$ have been shown to take place through critical periods (Chen et al., 2001). In addition, there is a shift in the ratio of GABA receptor $\alpha 1$ to $\alpha 2$ from childhood to adulthood in primates, leading to a shortening in the decay rate of the inhibitory post-synaptic potential of the GABA receptors (Hashimoto et al., 2009). As will be discussed later in greater detail, fast inhibitory neurotransmission is needed in order to generate well-regulated gamma oscillations, a type of coordinated neural activity that is critical for higher-order cognitive functioning and working memory specifically (Ward, 2003; Cho et al., 2015).

Concurrent with changes in the GABAergic system is the developmental attenuation of prefrontal glutamatergic processing, contributing to the shift in overall balance away from excitation (Larsen &Luna, 2018). Much like GABA, one of the major changes that occurs in the glutamatergic system during a critical period is a change in the subunit composition of NMDA

receptors (Fagiolini et al., 2003; Bellone & Nicoll, 2007). As indicated above, glutamatergic NMDA receptors are involved in experience-dependent plasticity (Malenka & Bear, 2004). As shown in visual cortex, there is an increase in the expression of NMDA NR2A subunits and a decrease in NR2B subunits through the critical period (Sheng et al., 1994). The incorporation of NR2A subunits in NMDA receptors decreases the duration of an excitatory post-synaptic potential, which in turn, dampens plasticity (Flint et al., 1997). Deleting or reducing NR2A leads to deficits in experience-dependent weakening of synapses, as well as enhancing strengthening of synapses (Cho et al., 2009). This increase in NR2A subunits and decrease in EPSC length is temporally correlated, and they are also temporally correlated with the onset of ocular dominance plasticity, and thus the start of a critical period (Woodruff et al., 2010). In addition, an increase in NR2A subunits in PFC through adolescence into adulthood has been shown in animal models (Konstantoudaki et al., 2017). This suggests that critical period plasticity mechanisms in the glutamatergic system may also be present during adolescence.

2.4 Working Memory and Excitation/Inhibition Balance

In sum, there is a sizable volume of evidence for decreasing influence of excitation in combination with the increasing influence of inhibition in PFC during the adolescent period that coincides with known cognitive development during this developmental window. One major aspect of executive functioning that improves during adolescence is working memory. Working memory refers to the ability to hold information online to guide planned executive behavior (Baddeley 1986). Although working memory abilities are available at a rudimentary level in early childhood (Diamond & Goldman-Rakic, 1989; Diamond, Towle, Boyer, 1994), they continue to develop and

become refined through adolescence, stabilizing in the second decade of life (Demetriou et al., 2002; Luna et al., 2004). Neuroimaging studies reveal that these working memory improvements are driven by the establishment of the core circuitry through adolescence, which includes PFC and is supported by the integration of visual association regions that provide increasing precision (Geier et al., 2009, Simmonds et al., 2017). These age-related changes in brain function have been shown to enhance many aspects of working memory ability (Crone et al., 2006; Klingberg et al., 2002; Olesen et al., 2007).

One way in which change in E/I balance may lead to working memory improvements through adolescence is via enhanced coordination of neural activity. The coordination of populations of neurons occurs through an interplay between excitation and inhibition (Bartos et al., 2007). In particular, neural oscillatory activity in the frequency range of 30-90 Hz, known as gamma oscillations, is regulated by the timing and balance of excitation and inhibition in neural circuits (Brunel & Wang, 2003). Although there are a range of frequencies at which neural circuits organize into oscillatory systems, gamma oscillations in particular are thought to be involved in higher cognitive processes that continue maturing through adolescence, such as working memory (Ward, 2003). Gamma oscillations have been shown to be related to a variety of aspects of working memory functioning but in particular the ability to retain information online during a delay period (see Miller et al., 2018 for review). Therefore, synchronization of neural oscillations may be a mechanism by which the shifting balance of GABA and glutamate stabilizes executive functioning through adolescence.

GABAergic inhibition provides a mechanism by which spontaneous or evoked excitatory activity above a certain threshold is organized into a synchronized rhythm in the gamma range (Traub et al., 2003). In fact, excitation is immediately balanced by inhibition to maintain oscillatory

frequency at the proper range (Atallah and Scanziani, 2009). Further, PV+ interneurons are crucial players in modulating gamma oscillations (Sohal et al., 2009). Inhibiting PV+ neurons suppresses gamma, while driving them makes gamma oscillations emerge, leading to enhanced signal in neural circuitry and reduced noise (Sohal et al., 2009).

In addition, there is evidence suggesting that gamma oscillations actually change through adolescence. Task-induced gamma oscillations have been shown to increase in power into adolescence (Uhlhaas et al., 2009). These increases in gamma-band power may have an inverted U-shaped trajectory, peaking at around age 16 and then decreasing (Cho et al., 2015), which tracks with the development of cognitive control (Luna et al., 2004), and may track with the maturation of E/I balance.

2.5 Magnetic Resonance Spectroscopy

Although there is an extensive animal literature linking neurotransmitter changes to regional neural functional changes and behavioral outcomes, it has been difficult to examine neurotransmitters *in vivo* in humans. Thus, much of the evidence examining developmental changes in GABA and glutamate through adolescence has been done in animal models or post-mortem human tissue. Magnetic Resonance Spectroscopy (MRS) is a neuroimaging method that uses a conventional MRI in order to locate and quantify metabolites in brain tissue *in vivo* in humans. Unlike other neuroimaging modalities that can detect neurotransmitters such as Positron Emission Tomography (PET), MRS is noninvasive and therefore readily applicable to pediatric populations.

Much like conventional MRI, MRS exploits the physical principle of nuclear magnetic resonance (Gujar et al., 2005). How a given atomic nucleus responds to an applied magnetic field depends on the effective field that nucleus experiences. The effective field is determined by a given metabolite's chemical structure as well as its chemical environment, all of which can change how the nucleus experiences the magnetic field by altering things like how many electrons are shielding the nucleus. Thus, the frequency at which a given nucleus resonates with respect to the resonance of a standard reference is deemed a chemical shift. These chemical shifts are used to identify and quantify the metabolite. Traditional MRS provides a chemical spectrum per voxel that includes the chemical shifts of the metabolites detectable in that voxel. From this spectrum, chemicals are identified by the position along the spectrum that the shifts or peaks fall, and their concentration is determined by the amplitude of the spectral peaks (Alger, 2010).

However, the analysis and interpretation of MRS data remains challenging, and there is still much debate about best practices. Nevertheless, MRS has proven itself to be a promising and powerful tool to noninvasively study neurotransmitters *in vivo* in humans. While developmental studies using this method are still sparse, some have begun elucidating the changes in GABA and glutamate that may be taking place during adolescence. The first developmental study that used MRS was at 3T and found lower GABA levels in adolescents compared to adults in the anterior cingulate cortex (ACC) but not in the parieto-occipital cortex (Silveri et al., 2013). Lower GABA levels were also associated with worse response inhibition and more impulsivity. However, another 3T MRS study found increasing levels of GABA with age in a frontal voxel (Ghisleni et al., 2015). However, a study using 1.5T MRS did not find age-related changes after early childhood through adolescence (Bluml et al., 2013). Although the literature looking at developmental changes in

GABA using MRS is far from definitive, 1.5T and 3T may be too low of a magnetic field strength to accurately detect low-concentration metabolites like GABA.

Developmental MRS studies of glutamate are just as mixed as the GABA studies. MRS at 3T report age related decreases in glutamate through adolescence and into young adulthood in frontal cortex (Shimizu et al., 2017) as well as basal ganglia (Chang et al., 2009; Ghisleni et al., 2015). However, other studies at 3T have failed to find these associations between glutamate and age in frontal regions (Chang et al., 2009; Ghisleni et al., 2015; Gleich et al., 2015). Again, these discrepancies may be due to limitations in reliably measuring glutamate at relatively low MRS strengths. Despite these limitations, some studies have shown associations between glutamate and brain function. Glutamate has been shown to be associated with striatal activation in adolescence (Gleich et al., 2015). In addition, glutamate in the basal ganglia was found to mediate the association between local connectivity in the dorsal putamen and age (Ghisleni et al., 2015).

At this time, only one study has looked at normative developmental differences through adolescence in the E/I balance as measured by the ratio of glutamate to GABA, and how that might relate to cognitive development (Cohen Kadosh et al., 2015). This 3T MRS study found that a higher glutamate to GABA ratio in the inferior frontal gyrus was associated with better face processing ability in children but not adults. They did not see these associations with a separate working memory task. However, this study had a small sample size of 14 7-10 and 14 20-23 year olds and did not include any participants between the ages of 10 and 20. A more recent 3T study took a different approach at looking at E/I balance; instead of using the ratio, they looked at how correlated glutamate and GABA are, and found that they are highly correlated in a sample of 18-30 year olds (that is, people that had higher glutamate levels also had higher GABA). In this study, this was taken to mean that glutamate and GABA are already ‘in balance’ at that age range. However, this

study did not include younger adolescents, and therefore, we still do not understand what associations may be present between E/I balance and age during adolescence and how these may be related to cognitive development.

2.6 Limitations of the Current Literature

Due to the specialized nature of MRS acquisition and data analysis, few studies have been able to comprehensively examine developmental changes in GABA and glutamate. Currently, most of the studies that have been done are single voxel studies in one or two regions. This can limit the ability to look at multiple regions, in addition to not allowing for post-hoc optimization of voxel placement to maximize gray matter. Many of these studies also have small sample sizes with limited age ranges, which may restrict the ability to see complete developmental patterns and trajectories.

The proposed study will address many of these limitations with its use of 7T MRSI. First, this study will acquire a whole slice with 576 1x1x0.9 cm^3 size voxels rather than one larger voxel, which will allow for optimization of voxel placement in the gray matter and the ability to assess several brain regions. In addition, we will obtain a more comprehensive developmental sample in order to better understand the age effects of GABA and glutamate. Our 7T MRSI acquisition significantly optimizes the signal-to-noise ratio of the data, providing more reliable measures of GABA and glutamate. Most prior studies have used MRI scanners at either 3T strength or weaker, and therefore have been limited in their ability to accurately detect both GABA and glutamate. GABA is challenging to index with MRS at lower field strengths due to its low concentration in brain tissue, as well as the inability of that field strength to provide sufficient separation of

overlapping resonance signals of chemicals with similar chemical structures (Shungu et al., 2016). As such, many GABA measurements also contain a macromolecule contaminant that resonates at a frequency close to GABA and which cannot be disambiguated from the true GABA signal at 3T or less. Glutamate has also been a challenge to measure in prior studies due to overlapping signal with its metabolic precursor, glutamine (Cohen Kadosh et al., 2015; Ghisleni et al., 2015). Many studies therefore will also report their glutamate concentrations as a combination of glutamate and glutamine in order to avoid underestimating the glutamate signal, but this confounds actual glutamate measurement.

3.0 Methods

3.1 Participants

MRS data were collected on 144 participants (10 – 30 years old, 73 females, see Figure 1) recruited from the community. Participants were excluded if they had a history of head injury with loss of consciousness, a non-correctable vision problem, a history of substance abuse, a learning disability, a history of major psychiatric or neurologic conditions, a first-degree relative with a history of major psychiatric or neurologic conditions, or if they are taking certain medications. Participants were also excluded if they reported any MRI contraindications, such as non-removable metal in their body. For participants under the age of 18, parental consent and participant assent were obtained prior to data collection. For participants over the age of 18, participant consent was obtained. Participants will ultimately be brought in for 3 time points (Year 1, Year 2, and Year 3) but the scope of this project included only Year 1 data, and is thus a cross-sectional study.

3.2 Memory-Guided Saccade Task

In order to probe visuospatial working memory development, we used the memory-guided saccade task. In this task, subjects were first instructed to fixate at a central blue cross for 2 seconds. A yellow dot then appeared on the screen and subjects had 2 seconds to make a saccade to the stimulus when the cue is extinguished and the fixation cross appeared for them to return

their gaze (11.91° or 23.01°). Participants then retained fixation for a delay period of variable length (6 – 10 seconds). The crosshair then disappeared, and subjects again had 2 seconds to make a saccade to where they remember the stimulus being on the screen. For the current analyses, the outcome measure of interest will be accuracy, which is the distance from the final resting position of the last saccade to the target location of where the stimulus was. There are 4 total runs with 24 trials per run.

3.3 MRSI Data Acquisition

This study was performed at the University of Pittsburgh Medical Center Magnetic Resonance Research Center in collaboration with leaders in the field of 7T MRSI, who have largely developed the acquisition protocol and acquired the scans. Structural images were acquired using an MP2RAGE sequence (1 mm isotropic resolution, TR/TE/flip angle 1/ flip angle 2: 26000 ms/2.47 ms/4^0/5^0) for parcellation and alignment. MRSI of GABA and glutamate were acquired using a J-refocused spectroscopic imaging sequence (Pan et al., 1996) (TE/TR = 17/1500ms) in order to minimize the impact of momentary interactions between separate molecules on chemical shifts. Radiofrequency (RF) based outer volume suppression was used to minimize interference in the signal from extracerebral tissue (Hetherington et al., 2010).

One oblique axial slice (10mm thick 24x24 encodes across a FOV of 216x216 mm <1cc effective resolution) was acquired and positioned to ensure that DLPFC (roughly defined as Brodmann Area 9) was present. MRSI voxels were identified within an ROI by aligning the T1 anatomical images of each participant to an atlas space, and then aligning the MRS acquisition to the anatomical image. In order to overcome individual variability in brain morphology and slice

positioning, a program developed in our laboratory called Quantitative Partial Acquisition Slice-Alignment (Q-Pasa) was used during acquisition. Q-Pasa maps an oblique slice atlas (standard MNI space) into a participant's native space in real time so that it may guide placement of the slice acquisition. An 8x2 1H transceiver array using 8 independent RF channels was used to acquire data. To overcome potential inhomogeneity of the magnetic field at a high field strength, high order B_0 shimming was used to optimize homogeneity of the field.

3.4 Determining Metabolite Concentration

The spectral data acquired from MRSI was then fit to a model of an optimal spectrum using LCModel, a program that automates the quantification of neurotransmitters from a chemical spectrum (Provencher, 2001). LCModel output provides estimates of metabolite concentration for all metabolites present. Additionally, it provides for a way of visually inspecting spectra and model fit that is used as a first-pass exclusion criteria for data. Further, LCModel provides information about the Cramer-Rao Lower Bound (CRLB), which is an expression of the uncertainty of that estimate. The CRLB provides the lowest achievable variance for an estimator. It is used as an indicator of how well the measurement of a given metabolite fit the ideal spectrum for that metabolite, and therefore, provides a way of assessing data quality. Typically, estimates of metabolite concentrations with CRLB values above 20 are left out of analyses. Higher CRLB values indicate noisy, poor quality data due to lipid artifact, motion, or a variety of other factors.

In this study, concentration values of neurotransmitters are given as metabolite ratios of the neurotransmitter of interest to creatine (Glu:Cr or GABA:Cr) (Kwock, 1998). This is done because it allows for a shorter acquisition time (as compared to acquiring absolute metabolite

concentrations) while also providing for a way to control for inter-subject variability due to factors such as amount of cerebrospinal fluid in the voxel. Creatine is used as the denominator in metabolite ratios because it is a metabolite with a strong signal and reliable chemical shift, in addition to being relatively stable with age.

3.5 ROI-Based Analysis

Regions of interest (ROIs) were defined in MNI-space using an ROI atlas created in the lab. A nonlinear registration of the ROIs was then performed on each subjects' native space. The DLPFC (Brodmann Area 9 or 46) was the primary ROI investigated but other regions including the anterior insula, ACC, and medial PFC (MPFC) were also interrogated in exploratory analyses. Once coordinates were placed, they were inspected and their placement was optimized to ensure maximal gray matter content of the voxel.

4.0 Statistical Analysis

Aim 1: To examine how glutamate, GABA, and their balance changes through adolescence in PFC.

To examine age-related change in glutamate, GABA, and glutamate/GABA ratio, linear mixed-effects regression analyses were implemented through the R packages lme4 and lmerTest (Bates, Mächler, Bolker, & Walker, 2014; Kuznetsova, Brockhoff, Christensen, 2017). For regions that had both a left and a right hemisphere (for example, DLPFC), age-related changes in neurotransmitter measures were examined using linear mixed-effects models with participant as a random effect and ROI hemisphere, sex, and fraction of gray matter in the voxel as covariates. For regions typically characterized as one ROI (e.g., midline regions), linear regression was used, with sex and fraction of gray matter as covariates. Three forms of age were tested: age, inverse age and quadratic age; these are the functional forms that have previously characterized age-related change during this developmental period (Luna et al., 2004). The best form of age was chosen using Aikaike's Information Criterion (AIC), wherein the model with the lowest AIC (by at least 2) was chosen as the best fit model (Akaike, 1974). Correction for multiple comparisons was done using Bonferroni correction.

Age-related changes in glutamate and GABA correlations were examined in two ways; 1) First, we obtained a per-participant quantification of the degree to which glutamate and GABA were 'mismatched' (i.e., not correlated) by calculating the absolute value of residuals obtained from a linear regression model characterizing the prediction of GABA based on inverse age, glutamate, and their interaction. We did this in order to have a per-person proxy for "correlatedness", as this data is cross-sectional, that would allow us to later look at whether this

correlation or mismatch is associated with behavior. Thus, a high residual value would indicate that the values of glutamate and GABA deviated significantly from the mean association for their age cohort. That is, we are determining if having high GABA predicts high glutamate in each ROI, which may indicate that these two neurotransmitters are more 'in balance' (as per Steel et al., 2020). We then used these residuals to test whether this mismatch changed with age in a continuous manner using linear regression. Second, we binned our participants into 3 age groups of roughly equal size (Age bin 1 range = [10,16]; Age bin 2 range = [17,22]; Age bin 3 range = [22,30]), then calculated an average Pearson correlation (*r*) per age group and compared the correlations using the R package 'cocor' (Diedenhofen & Musch, 2015). This package uses the Fisher's r-to-Z transformation to compare the GABA and glutamate correlations between age groups and test whether they are significantly different from one another. We did this in order to provide another way of visualizing this change that would allow us to better understand when in development glutamate and GABA become more correlated. For these analyses, only participants with both glutamate and GABA that passed the data quality inclusion criteria defined above were included.

Aim 2: To examine how age-related changes in glutamate, GABA, and their balance in PFC are associated with age-related improvements in working memory.

The memory-guided saccade task (described previously in Methods section) was used to assess visuospatial working memory ability. The outcome measure of interest was accuracy, which refers to the distance from the endpoint of the final saccade to the actual target location. First, the association between accuracy and age was examined using linear mixed effects regression, controlling for the delay period length which was either 6, 8, or 10 seconds. Linear mixed effects regression was also used to examine the association between neurotransmitter measures and

accuracy, while controlling for the same covariates as in Aim 1 in addition to age. Correction for multiple comparisons was done using Bonferroni correction.

In order to examine whether the observed metabolite levels were specific to individual regions, or were reflective of widespread metabolite levels across multiple brain regions, we examined the correlation structure of each metabolite across ROIs, and performed two separate principal components analyses, one for GABA and one for glutamate, that included respective neurotransmitter concentrations across each ROI. Due to the missingness of data across ROIs, we conducted this PCA with the R package 'missMDA' (Audigier et al., 2014), which uses imputation to handle missing data for participants who may have bad data in some but not all ROIs, thus allowing us to retain those participants in analyses. The number of components that leads to the smallest mean square error of prediction is retained. These components were used in subsequent behavioral analyses to examine the association between global neurotransmitter content and accuracy (linear regression controlling for age as implemented above).

5.0 Results

5.1 Aim 1

We found a significant main effect of age on glutamate content in the DLPFC, with glutamate decreasing with age (β = 0.15, p = 0.004) (Figure 1A). Additionally, while these results were present in both hemispheres, there was a main effect of hemisphere (β = -0.40, p = 1.96 x 10^{-6}) showing higher concentrations in the left hemisphere than the right. We did not observe significant age-related changes in DLPFC GABA (β = -0.01, p = 0.82) (Figure 1A). We did find a main effect of hemisphere (β = -0.26, p = 0.001), with higher concentrations in the left hemisphere than the right. We also did not observe significant age-related changes in the ratio of glutamate/GABA in the DLPFC (β = 0.04, p = 0.39) (Figure 1B). In all cases, the relationships did not change after inclusion of covariates (Table 1), and we did not observe any age-related interactions. Model comparison indicated that for all models, the relationship was best explained by an inverse age fit (Table 2). Importantly, no relationship was seen between glutamate data quality (CRLB) and age (β = 0.090, p = 0.19) or GABA data quality and age (β = 0.098, p = 0.15).

In addition to our main region of interest (DLPFC), we also found age-related changes in other cortical regions. We observed significant age-related decreases in glutamate in the anterior insula (Fig. 2C; β = .29, p = 1.64×10^{-9}) and ACC (Fig. 2A; β = 0.21, p =0.0014), and a trend-level decrease in the MPFC (Fig. 2B; β = 0.12, p = 0.06). In the anterior insula, we did observe a main effect of hemisphere (β = -0.76, p = 5.51×10^{-29}), with left having overall greater concentration than right. These relationships remained significant upon inclusion of covariates (Table 3). We

observed no significant age-by-hemisphere or by-sex interactions. For most models, inverse age was the best fit; for models which did not have a difference of at least two across the three models, inverse age was chosen for consistency (Table 4). For all of these regions, there was no association between glutamate data quality and age (Table 5).

We found significant age-related decreases in GABA in the anterior insula (Fig. 2C; β = 0.20, p = 2.26x10^{-5}) and the ACC (Fig. 2A; β = 0.26, p =9.47 x 10^{-5}). Age-related change in GABA in the MPFC did not reach statistical significance (Fig. 2B; β = 0.11, p = 0.09), and the relationship was diminished following the inclusion of covariates (Table 6). In the anterior insula, we again observed a main effect of hemisphere (β = -0.49, p = 1.64 x10^{-8}), as well as a significant age-by-hemisphere interaction (β = 0.16, p = 0.048), with left having greater concentration than right, but this did not survive multiple comparisons correction. Again, for all models, inverse age was the best form of age (Table 7). For all regions except the MPFC, there was no association between GABA data quality and age (Table 5). In the MPFC, older age was associated with higher CRLB values (worse data quality) (β = 0.27, p = 0.003).

While we did not find age-related changes in the ratio of glutamate/GABA in the DLPFC, we did find associations in other prefrontal regions. We observed age-related increases in the ratio of glutamate/GABA in the anterior insula (Fig. 3C; β = 0.11, p = 0.018) and ACC (Fig. 3A; β = -0.15, p = 0.011). Additionally, we observed a significant age-by-hemisphere interaction in the anterior insula (β = -0.30, p = 1.04x10^{-4}) (Table 8). Follow-up tests revealed a significant age-related increase in the right anterior insula (β = -0.26, p = 1.2x10^{-4}) that was not significant in the left hemisphere (β = 0.03, p = 0.51). No age-related change in glutamate/GABA ratio was seen in the MPFC (Fig. 3B; β = -0.09, p = 0.23). No other age-by-covariate interactions were observed,

although there was a main effect of sex (β = 0.20, p = 0.022). Inverse age was the best form of age for these models as well (Table 9).

In order to examine whether the correlation between glutamate and GABA changes with age, we first considered age as a continuous variable by using the residuals to quantify a per-participant 'mismatch' of glutamate and GABA, as described previously in the statistical analysis section. In only the ACC, residual values decreased with age (β = 0.24, p = 0.007) (Figure 4), indicating greater glutamate and GABA coherence with increasing age. Like age-related decreases in GABA and glutamate, this association was best fit by an inverse function (Table 10). No age-related change in residuals was seen in the other regions.

In order to better understand when in development changes may be occurring and to visualize the correlation by GABA and glutamate differently, we also looked at discrete age groups, which were determined by dividing the sample into 3 groups of roughly equal number of participants. We first looked at the Pearson r value within age groups. In the ACC, MPFC, and right anterior insula, the youngest age group (10-16 years old) had the least correlated GABA and glutamate values (Table 11). Statistical tests to compare correlations between age groups revealed that the 10-16 year old group was significantly different from the older age groups in both the ACC and MPFC (Figure 5). These changes in the ACC were not driven by data quality changes (Table 5). While plausible that MPFC GABA data quality associations with age may have impacted the result in this region, the direction of the effect does not fit with the association seen here (younger participants had better data quality), and thus poor data quality is not likely driving that effect as it is only seen in the youngest age group. In the right DLPFC and right anterior insula, the youngest age group visually appeared to have reduced correlations relative to the other age groups, but this did not reach statistical significance.

5.2 Aim 2

As expected from previous studies (Luna et al., 2004; Simmonds et al., 2017), we found significant age-related improvements in MGS accuracy ($\beta = 0.30$, $p = 4.91 \times 10^{-9}$) (Figure 6). This relationship remained significant after accounting for all covariates (Table 12). We found no significant age-by-delay period interactions on accuracy.

Contrary to our hypotheses, we did not observe a main effect of DLPFC glutamate (Figure 7A; $\beta = 0.005$, $p = 0.87$), GABA (Figure 7B; $\beta = 0.014$, $p = 0.70$), or the ratio of glutamate/GABA (Figure 7C; $\beta = -0.011$, $p = 0.75$) on accuracy. For glutamate (Table 13) and GABA (Table 14), no significant interactions were seen with age and any of the covariates on accuracy. For the glutamate/GABA ratio, an interaction of ratio by delay period length was observed, although this did not survive multiple comparisons correction (Table 15).

Next, we explored the relationship between glutamate, GABA, and behavior in our other ROIs. First, to explore the interrelatedness of these neurotransmitter measures across ROIs, we ran a correlation analysis. We observed correlations among most ROIs for glutamate, and among some but not all ROIs for GABA (Figure 8). After controlling for age, these correlations largely remained significant although were overall diminished. Given the degree of correlatedness across ROIs, we conducted a PCA to reduce the dimensionality of the dataset prior to looking at associations with behavior. One component was retained for glutamate that captured 69.08% of the variance of the data, and loaded onto all ROIs. This component significantly decreased with age (Table 16; $\beta = -0.52$, $p = 4.64 \times 10^{-6}$), capturing the age-related decreases in glutamate that were observed in the ROI-specific analyses. Similarly, one component was retained for GABA that captured 58.48% of the variance, and loaded onto all ROIs. This component also significantly

decreased with age (Table 17; β = -0.34, p=2.65x10^{-5}). These two components were used in further analyses to examine associations with behavior. A component reflecting the glutamate/GABA ratio was created by making a ratio of the glutamate component over the GABA component. This ratio component, however, was not associated with age (Table 18; β = -0.07, p = 0.43).

No effect of the glutamate component was found on MGS accuracy (β = -0.002, p = 0.97) (Figure 9A). Similarly, no effect of the GABA component (Fig 9B; β = 0.11, p = 0.10) or the component reflecting the glutamate/GABA ratio (Fig 9C; β = 0.11, p = 0.13) was found. Additionally, we observed no significant component-by-age interactions on MGS accuracy.

We also assessed associations between our index of glutamate/GABA balance and behavior. For the ACC, in which the residuals showed significant age-related (age as a continuous variable) change, we observed a main effect of residuals (β = 0.23, p = 0.002), with higher residuals being associated with greater error in working memory accuracy. Upon inclusion of inverse age as a covariate, this main effect of residuals became trend-level (β = 0.13, p = 0.05). Further analyses revealed a significant interaction between residuals and age on performance (β = 0.19, p = 0.0012). Follow-up tests in the abovementioned age groups revealed that in the youngest age group (10-16), higher correlations in residuals were associated with worse working memory accuracy (β = 0.51, p = 0.017), with no association between residuals and accuracy in the older two groups (β_{middle}= -0.04, p_{middle} = 0.76; β_{oldest}= -0.03, p_{oldest} = 0.81) (Figure 10).

6.0 Discussion

This study is the first 7T MRSI study to examine changes in the primary excitatory and inhibitory neurotransmitters through adolescence in a large sample and across multiple ROIs. In line with our hypothesis in Aim 1, we found evidence for glutamate decreases in the DLPFC, although contrary to our hypothesis, GABA remained stable. Decreases in glutamate were also evident across other cortical ROIs, including the ACC, anterior insula, and a trend-level decrease in MPFC. This was additionally reflected in the component that emerged from the PCA, which decreased with age. However, although we expected to see increases in GABA through adolescence in frontal cortical regions, we saw either decreases, as in ACC and anterior insula (and trend-level decreases in MPFC), or stability, as in DLPFC. Additionally, the component emerging from the GABA PCA that decreased with age captured less of the variance in the data than did the glutamate component. Again, contrary to our hypotheses, we did not see a shift toward inhibition when examining the age-related changes in glutamate/GABA ratio in these regions; in fact, we saw either shifts toward increased excitation or no change. However, we did see changes in the glutamate and GABA correlation with age. We also saw an unexpected but consistent main effect of hemisphere, wherein the left hemisphere had higher glutamate and GABA concentrations than did the right.

The age-related decreases that we saw in glutamate are contrary to some of the findings in human spectroscopy studies at 3T that found no change from the adolescent to adult period (Bluml et al., 2013; Silveri et al., 2013; Ghisleni et al.,2015; Gleich et al., 2015). However, other studies have found age-related decreases in frontal cortex (Gallinat et al., 2007; Shimizu et al., 2017). These differences could be due to a variety of factors, including differences in scanning and

analysis protocols, as well as the inability of studies at lower field strengths to separate out the overlapping signals of glutamate and glutamine. Additionally, smaller sample sizes and various methods for grouping participants into age groups may have contributed to the inability to detect an effect. The strengths of our study are that the glutamate and glutamine signals are better resolved at 7T, our sample is larger than previous studies, and age is treated as a continuous variable which allows for the detection of the nonlinear trajectory that was observed in the current study. On a mechanistic level, our glutamate results may be a reflection of a more global mechanism rather than a regionally-specific one. We see at least trend-level decreases across all of our ROIs, in addition to finding that glutamate across most ROIs is significantly correlated. One such mechanism underlying these results may be the pruning of excitatory synapses through adolescence, a process that has been shown to take place in frontal cortical regions and continue through the adolescent period into early adulthood (Rakic et al 1986; Bourgeois et al 1993; Petanjek et al 2011). Pruning during adolescence impacts excitatory synapses that are overproduced during childhood, which may lead to decreases in overall glutamate levels.

As with glutamate, the age effects we see in our GABA data run contrary to some of the findings in the literature that used 3T MRS showing more GABA in frontal regions in adults as compared to adolescents (Silveri et al., 2013; Ghisleni et al., 2015). However, a recent study at 3T looked at the association with age after suppression of macromolecules that contaminate the GABA measure at lower field strengths and found no association between GABA and age in early adolescence (Bell et al., 2021). In our 7T study, macromolecules are better resolved from the GABA signal than studies at lower field strengths, and thus may be more reflective of true GABA. Additionally, a recent meta-analysis found sharp increases in GABA in early life that seemed to plateau at around age 11, followed by a gradual decline in adulthood (Porges et al., 2020). Thus,

if GABAergic systems increase most dramatically prior to adolescence, we may have missed it due to our relatively older age range. In our results, we see stability of GABA in DLPFC, which would be in line with these studies showing stability in adolescence, but we also see decreases in ACC and anterior insula (and trend-level decreases in MPFC), indicating continued changes in other critical prefrontal regions. A less global mechanism may be underlying this set of GABA results as compared to the glutamate results, as evidenced by the smaller amount of variance captured by the PCA as well as fewer regions that have significantly correlated GABA across them. Interestingly, those regions that are correlated may be functionally related regions; for example, anterior insula and ACC GABA are correlated in our data, and these regions are both part of the salience network (Seeley et al., 2007) which has been implicated in developmental changes occurring in affective processing through adolescence (Rosen et al., 2017). This is in line with prior studies that have shown that MRS GABA levels are correlated among functionally related brain areas (Puts et al., 2018), but not among less related brain areas (Greenhouse et al., 2016). Thus, age-related changes in GABA may be more regionally specific. Additionally, different subtypes of inhibitory neurons and different aspects of the GABAergic system show different developmental trajectories (Kilb, 2012). While expression of parvalbumin in some inhibitory neurons may increase in DLPFC through the adolescent period development, other aspects of GABAergic systems simultaneously decrease (Kilb et al., 2012; Fish et al., 2013; Caballero et al., 2014; Hoftman et al., 2017). Further, if MRS reflects more tonic GABAergic activity, the size of tonic inhibitory currents has been shown to decline through adolescence (Piekarski et al., 2017). Thus, the complex dynamics of the GABAergic system may reflect a functional remodeling of the system, wherein there is a change in the ratio of tonic to phasic GABA (Piekarski et al., 2017). Unfortunately, MRS cannot distinguish these processes. As many other

brain-behavior MRS studies have pointed out, one consideration when thinking about these results is what MRS may actually be measuring. Although 7T provides better resolution than many prior studies at lower field strengths, these voxels are still large, and therefore provide more of an "average" of all aspects of GABA in that voxel. In fact, studies have suggested that MRS may actually be a better measure of tonic GABA rather than phasic, task-evoked GABAergic activity (Dyke et al., 2017). It may be that we are unable to detect the aspects of the GABAergic system that may be increasing and changing phasic GABAergic dynamics, as they become somewhat washed out with other aspects of the GABAergic system decreasing.

To that point, we did not see associations between glutamate or GABA in any of our ROIs and accuracy in the MGS task, thus potentially reflecting the more overall phasic nature of the measured neurotransmitters. If the measured data are more phasic on average, they may not speak to task-evoked neural activity as much, and therefore may less closely relate to behavioral performance capability. If only certain aspects of the GABAergic system in the DLPFC are associated with working memory ability, we may not have the sensitivity to detect those specific aspects. However, although no prior study has examined associations between neurotransmitters and the MGS task to our knowledge, other studies have found associations between metabolites within frontal ROIs and behavior. For example, one 3T study found that lower levels of GABA in ACC, as seen in adolescents compared to adults, was found to be associated with greater impulsivity and worse performance on a Go No-Go Task (Silveri et al, 2013). This study and many others that found these associations differ from ours in a few ways that have already been mentioned, including being conducted at lower field strengths with smaller sample sizes, which could explain differences in the results. Alternatively, GABA measured with MRS may be

particularly linked to inhibitory control and not working memory, which may depend more on the coordinated action of neurotransmitters across a more distributed network.

Although we did not find associations between any specific neurotransmitter and MGS accuracy, we wanted to see whether we could probe the question of functional, dynamic change further. While E/I balance has been conceptualized in many MRS studies as a ratio of the glutamate measure to the GABA measure, one very recent study looked at correlated GABA and glutamate in a healthy adult population as a way of assessing GABA and glutamate balance (Steel et al., 2020). Although that study did not find age effects in their adult sample (18-35 years old), they saw that people in this age range tended to have more correlated glutamate and GABA in parietal cortex (i.e., people with higher glutamate tended to also have higher GABA). Thus, we applied this approach to investigate if glutamate/GABA balance changed from 10-30 years of age, which might reflect the presence of mechanisms reflecting critical period plasticity. When examining the average correlations between glutamate and GABA within age groups, we found that the youngest age group (10-16 years old) had less correlated GABA and glutamate relative to the older age groups. This effect was observed across several ROIs, including the ACC, MPFC, and right anterior insula. To our knowledge, this is the first study to find age-related differences in the correlation of glutamate and GABA. As in the Steel et al. study, older adolescents and adults showed greater glutamate and GABA correlations; we extended this finding and found that younger adolescents showed decreased correlations, providing innovative and novel evidence suggesting that there may be critical period plasticity in prefrontal regions, particularly early on in adolescence that stabilizes in later adolescence and adulthood.

The fact that we did not find changes in DLPFC may indicate that this region undergoes maturation before adolescence. This is supported by studies showing that recruitment of DLPFC

during working memory (Simmonds et al., 2017) and inhibitory control (Ordaz et al., 2013) is already at adult levels by adolescence. Instead, the ACC (Ordaz et al., 2013), which supports performance monitoring, and MPFC (Velanova et al., 2009), which provides precision, have been found using fMRI to be increasingly engaged into adulthood and to mediate developmental improvements in cognitive control. In addition, the anterior insula, has increasingly been found to play a critical role in enhancing cognitive control (Uddin et al., 2014; Uddin et al., 2017) that may support more optimal engagement. As mentioned previously, these areas have in common a protracted structural maturation with continued cortical thinning through adolescence (Gogtay et al., 2004). Thus, while DLPFC is known to support cognitive control, it is believed to support the ability to generate a goal-directed response (D'Esposito & Postle, 2015), which is available in adolescence, while these other more specialized regions may support the reliable engagement of cognitive control, which is not mature by this time (Montez et al., 2017; Montez et al., 2019). This is additionally supported by the Driven Dual Systems model (Luna & Wright, 2016), which provides evidence indicating that by adolescence, DLPFC is already available at adult-levels, but other regions that support reliable engagement of cognitive control continue to develop into adulthood.

Interestingly, the regions in which we saw changes in correlation were generally the same regions where we saw age-related decreases in GABA. Thus, it may be that age-related changes during adolescence in glutamate and GABA may be doing more than just shifting total concentrations to be "more inhibitory" when looked at as a ratio of glutamate/GABA; rather, developmental changes may act on the functional coupling of glutamate and GABA processes. While the developmental shift toward excitation reflected in the glutamate/GABA ratio in some regions such as the ACC was an unexpected result, this could be a more tightly regulated excitation

that is in greater synchrony with GABA. Indeed, although our results may not have been able to pick up on this effect in the DLPFC, excitatory activity appears to be an important piece of the puzzle in DLPFC, where excitatory inputs to inhibitory PV interneurons are pruned, but leftover synapses are strengthened, and lead to more precise excitation of inhibitory activity (Chung et al., 2017). Additionally, we did find associations with MGS accuracy and glutamate and GABA correlations in the ACC suggesting that their coordinated function, and not their relative amounts, may support working memory.

While we do find evidence for developmental changes in glutamate and GABA in prefrontal regions, this is distinct from what is see in critical period plasticity of early-life visual system circuits. Although we are not able to rule out the presence of such mechanisms in frontal cortices during adolescence, plasticity may operate in a different manner in adolescence. Critical period mechanisms to decrease noisy, spontaneous activity in favor of evoked activity may rely on changes in excitation as well as inhibition during this developmental period. The result of regaining E/I balance is similar in both mechanisms but in adolescence it may be driven by excitation supporting regulation of activity. Future studies using fMRI and EEG can assess whether developmental improvements in glutamate/GABA balance are associated with brain functional indices of age-related improvements in signal-to-noise ratio.

Some limitations of this study include determining neurotransmitter measures relative to creatine. This is a standard approach that is used often in the literature to correct for factors that may influence metabolite measurements such as the amount of cerebrospinal fluid in the voxel (Maddock & Buonocore, 2012). Without this approach, acquiring additional scans to reference metabolites to water as well as extensive post-hoc correction for cerebrospinal fluid and other factors would be needed. Creatine is often chosen as the reference due to its prominent signal that

is easy and reliable to measure, in addition to its relative stability across regions and age (Maddock & Buonocore, 2012). However, this has not been rigorously investigated through adolescence, and thus, it is possible that differences in creatine either with age or across regions could have impacted our results. Additionally, data quality decreases near the skull (DLPFC, MPFC) due to proximity to lipids, which led to the retention of fewer participants in those regions as compared to more medial regions, which may have comparatively limited power. Finally, this study is cross-sectional and thus cohort effects limit interpretation of developmental change.

7.0 Conclusion

Taken together, this study provides innovative evidence of changes and stabilization of E/I brain mechanisms that may underlie critical period plasticity in crucial regions of PFC that might have an impact on adolescent cognitive development. Understanding brain mechanisms that underlie the transition from adolescence to established adult trajectories is important to further inform our understanding of normative neurocognitive development. Additionally, a better understanding of normative trajectories can inform impaired trajectories, as in neurodevelopmental illnesses such as schizophrenia, which often first emerges in adolescence and is characterized by alterations in the balance of GABA and glutamate (Glantz & Lewis, 2000; Gonzalez-Burgos & Lewis, 2008; Hoftman & Lewis, 2011). Importantly, understanding impaired GABA and glutamate development could inform pharmaceutical therapies that alter the action of these neurotransmitters.

Appendix A Figures

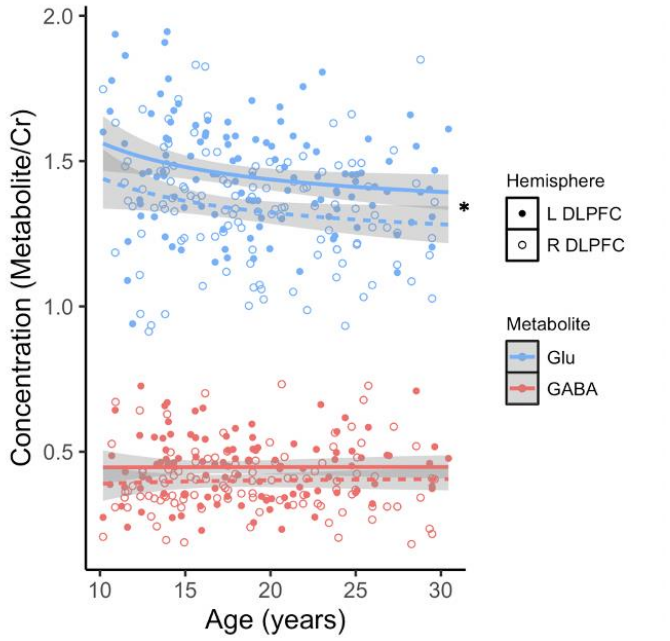

Figure 1A: Graph showing Glutamate (blue) and GABA (red) in left and right hemisphere of DLPFC by age

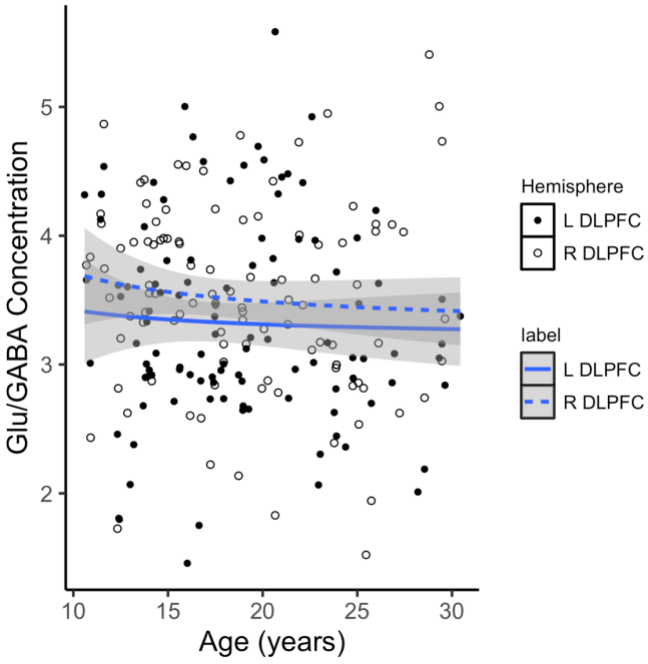

Figure 1B: Graph showing Glu/GABA ratio in left and right hemisphere of DLPFC by age

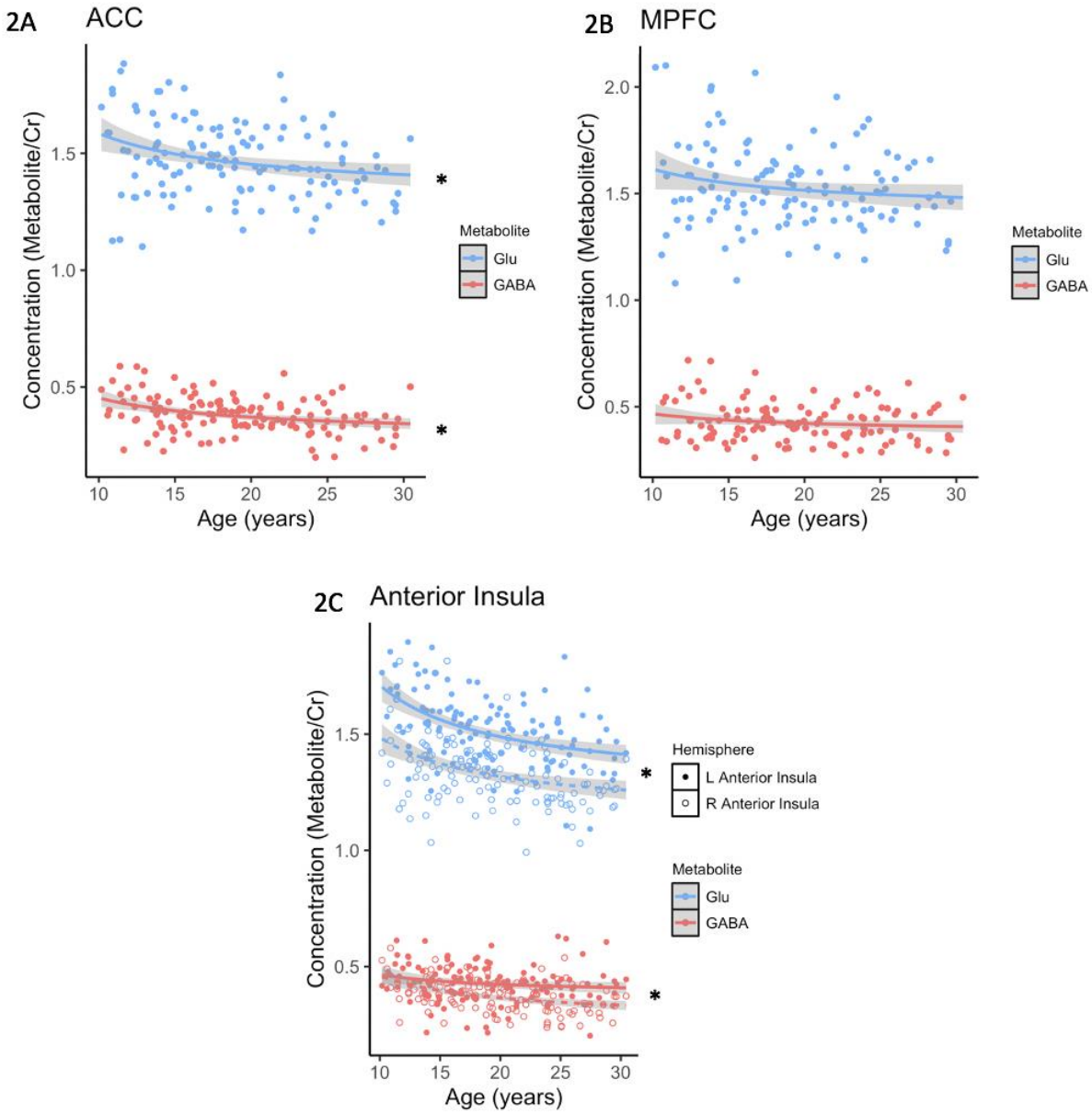

Figure 2: Graphs showing Glutamate (blue) and GABA (red) in ACC (2A), MPFC (2B), and right and left Anterior Insula (2C) by age

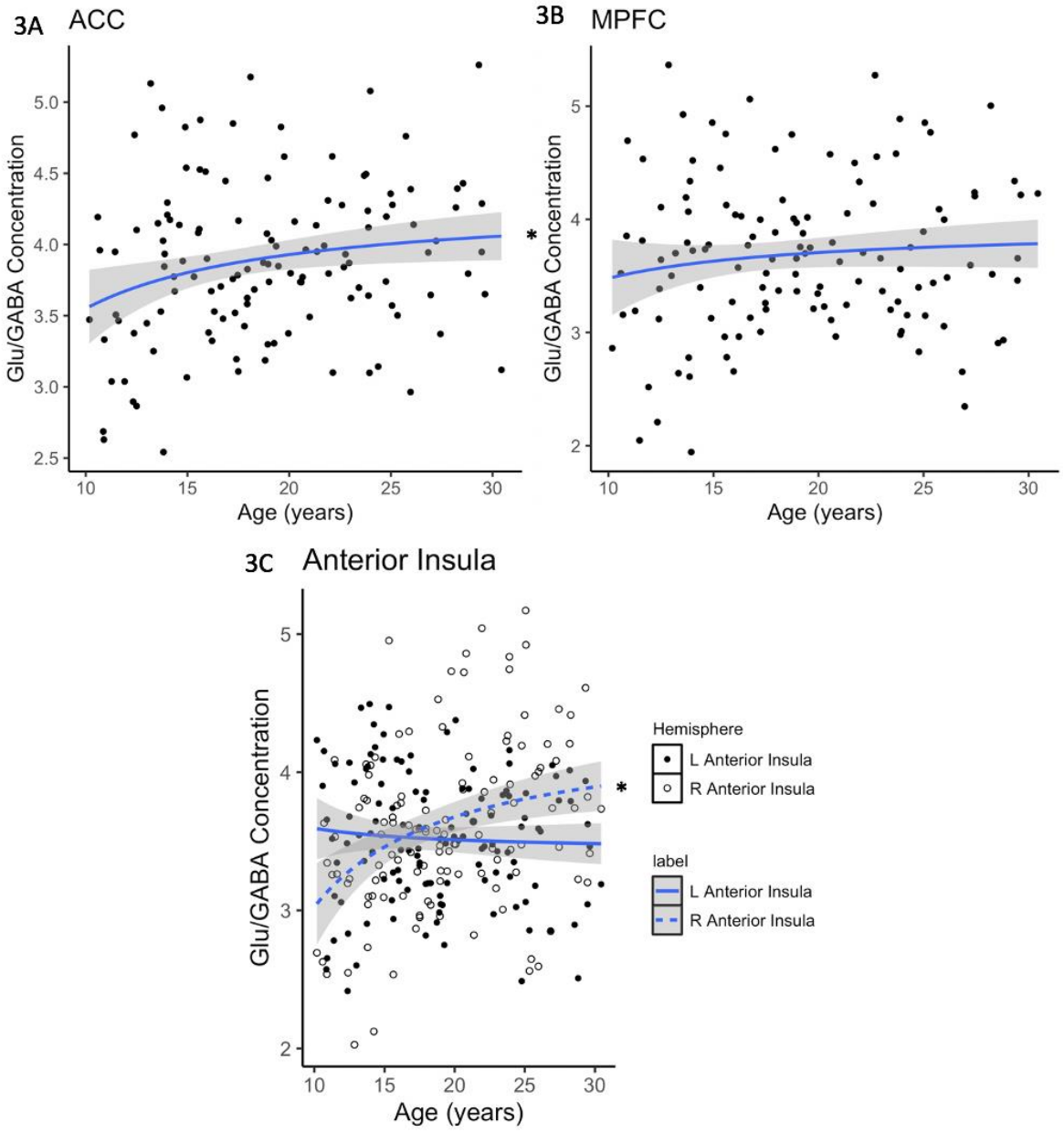

Figure 3: Graphs showing the ratio of Glu/GABA in ACC (3A), MPFC (3B), and right and left Anterior Insula (3C) by age

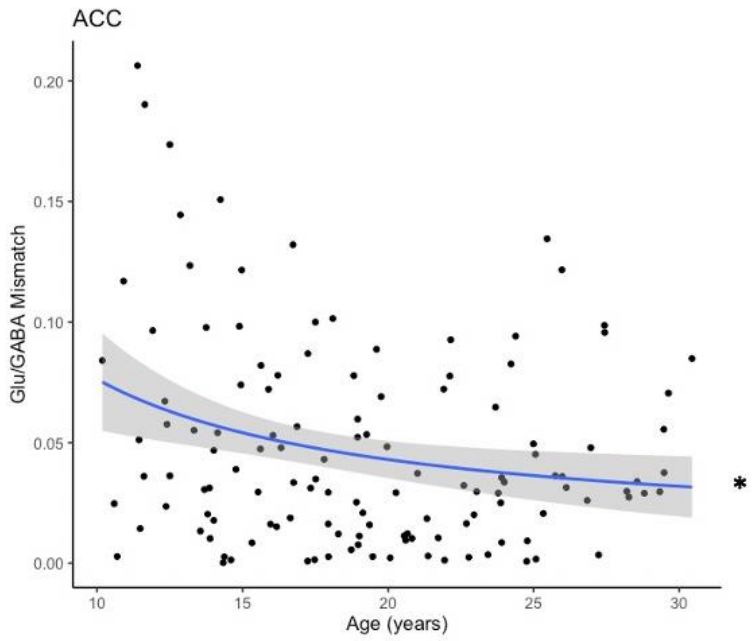

Figure 4: Graph showing age-related decrease in the amount of Glutamate and GABA mismatch

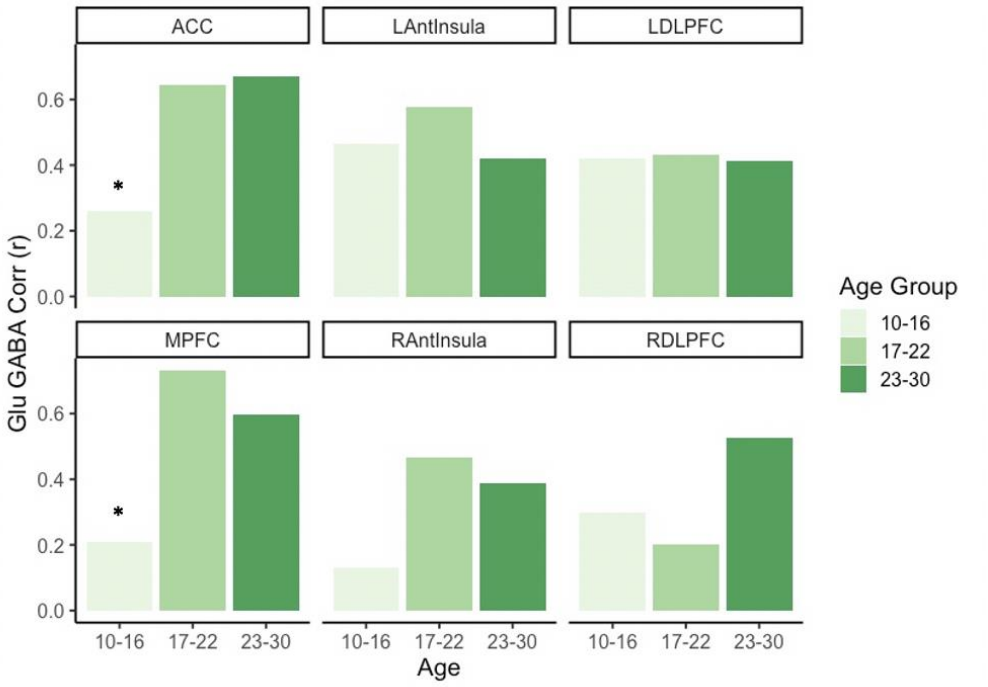

Figure 5: Bar graphs showing Pearson r value by age group in each region. Asterisk denotes group that is significantly different from other groups.

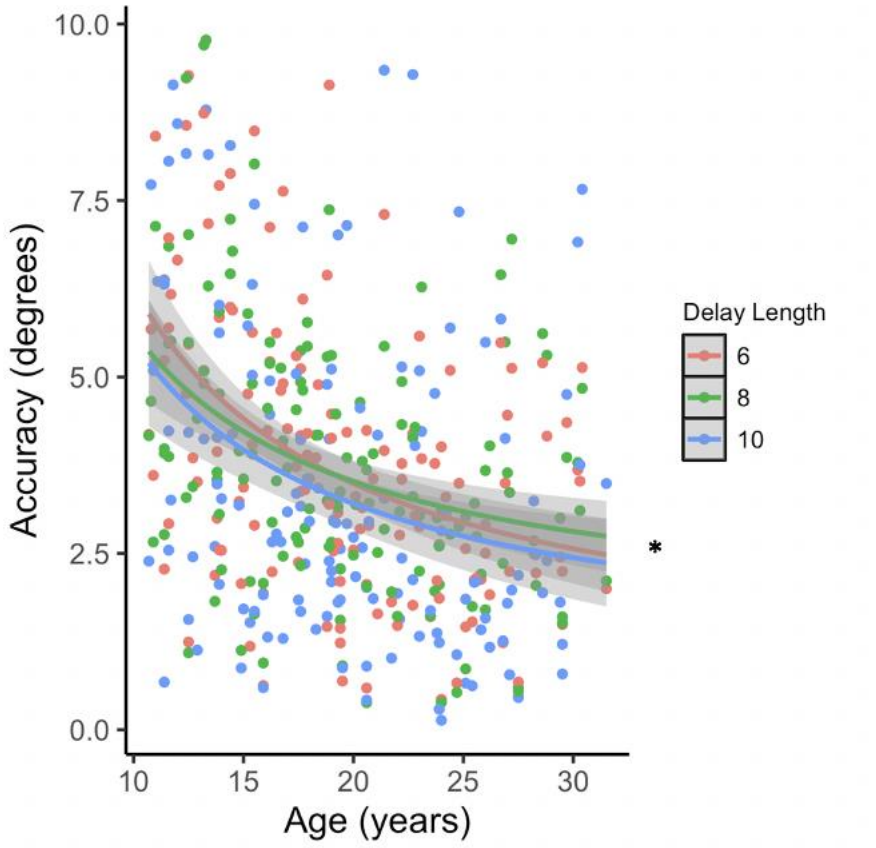

Figure 6: Graph showing accuracy improving with age for all 3 delay periods

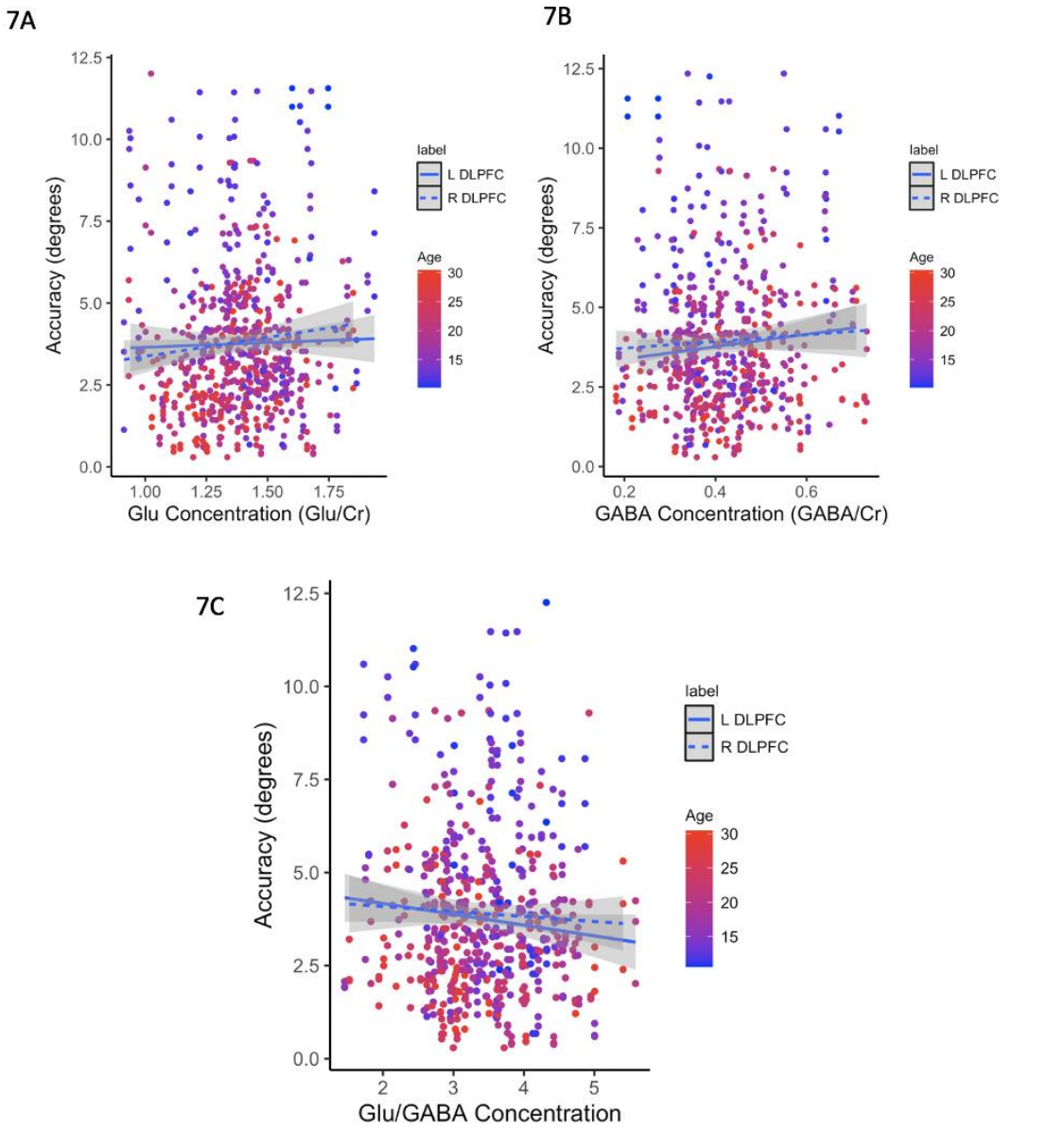

Figure 7: Graphs showing relationship between Glu (7A), GABA (7B), or Glu/GABA ratio (7C) with accuracy on the MGS task. Colored by age.

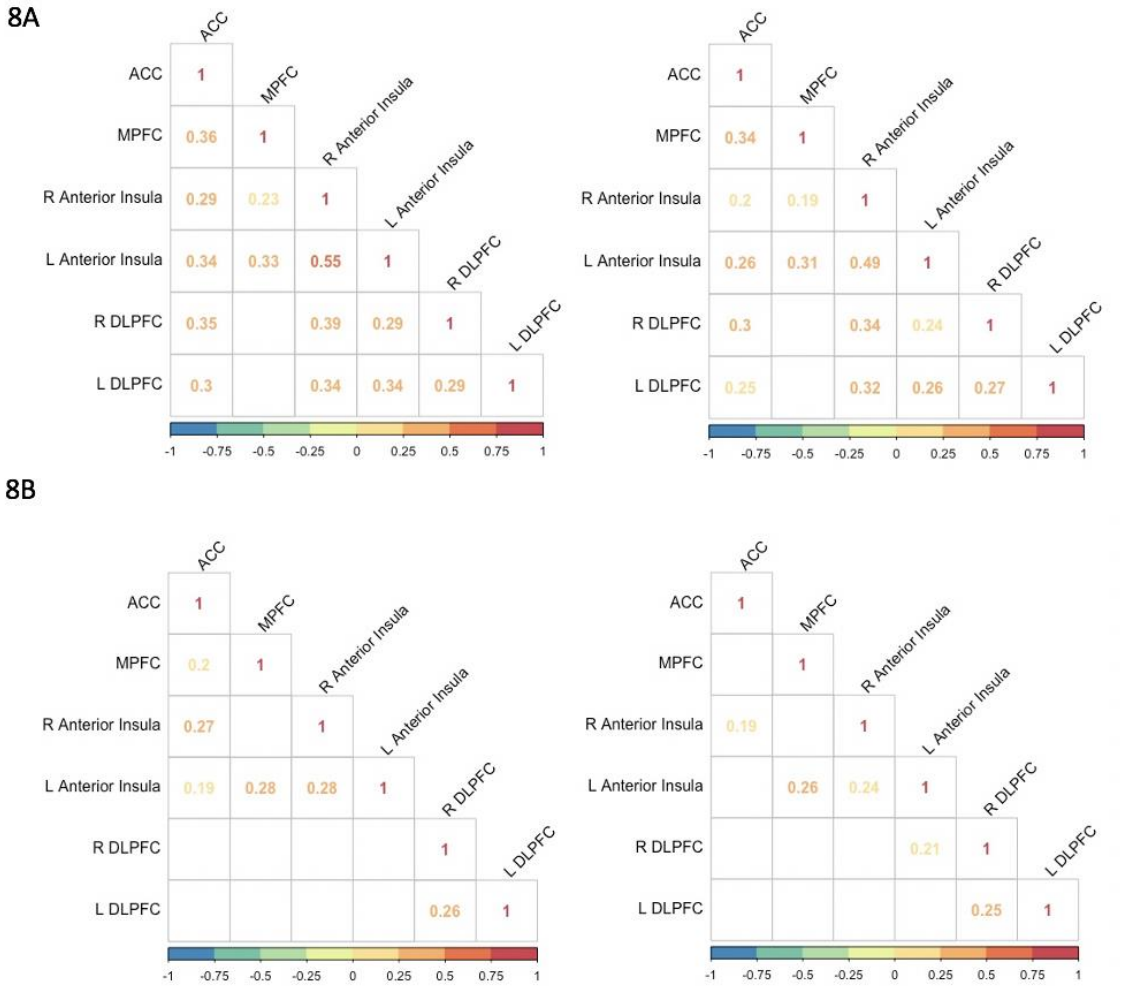

Figure 8: Correlation matrices showing correlations of Glutamate (8A) and GABA (8B) between ROIs. Matrix on the left is prior to controlling for age; matrix on the right is after controlling for age. Pearson r value is displayed in squares if significant. Blank

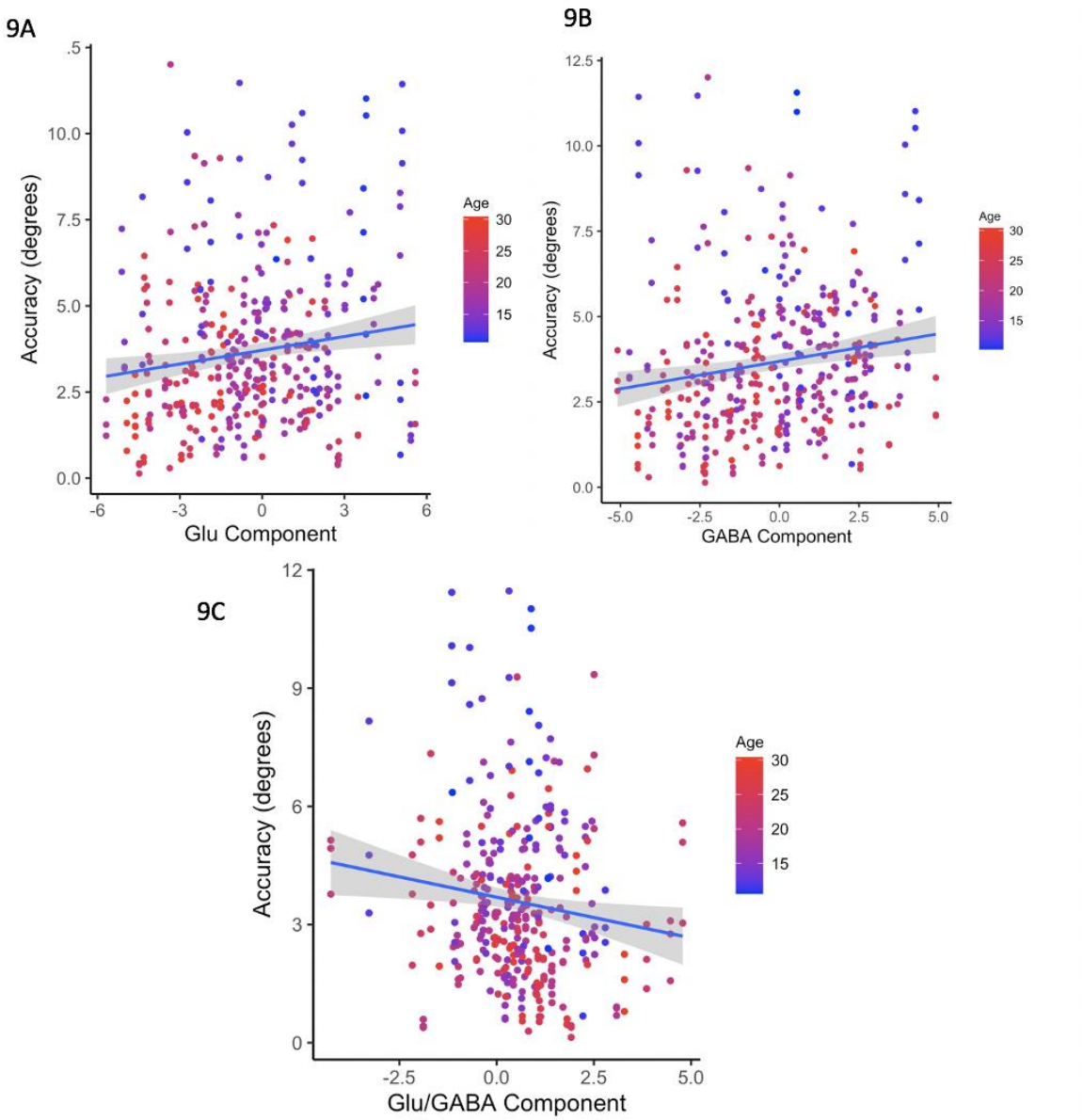

Figure 9: Graphs showing relationship between PCA components and accuracy on MGS task. Colored by age.

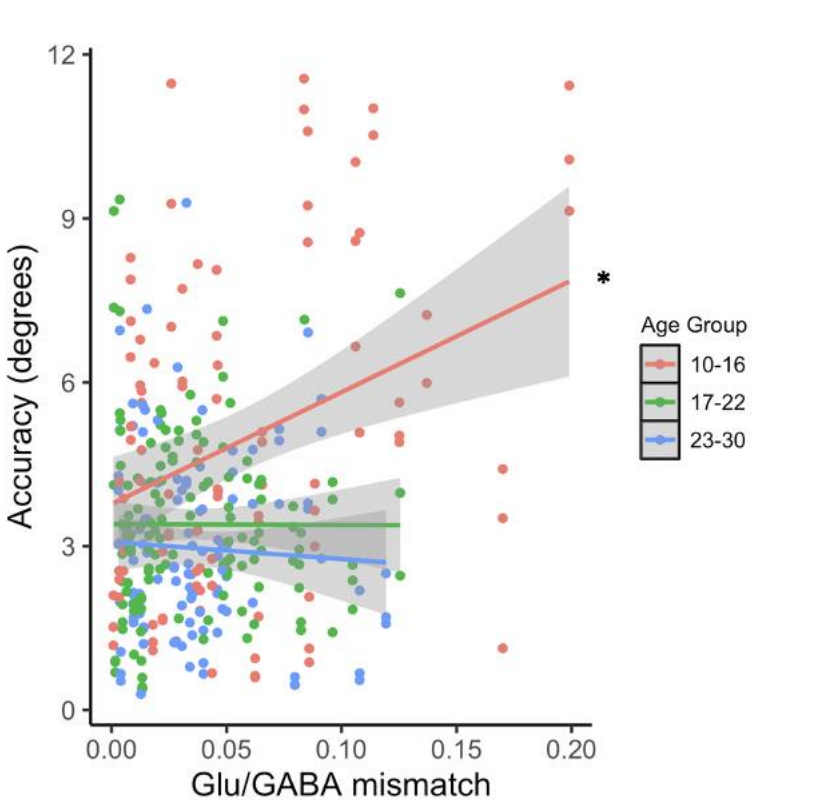

Figure 10: Graph showing age by ACC glu/GABA mismatch interaction. Colored by age group.

Appendix B Tables

Table 1 DLPFC glutamate, GABA, and glu/GABA model output

Measure	Variable	b	t	$n_{participants}$	p
Glutamate	**Age^{-1}**	0.15	2.81	132	0.006
	Sex	0.04	0.41	132	0.68
	Hemisphere	-0.40	-5.01	132	1.96×10^{-6}
	Frac GM	0.03	0.64	132	0.52
GABA	Age^{-1}	-0.03	-0.64	129	0.52
	Sex	-0.16	-1.60	129	0.11
	Hemisphere	-0.26	-3.31	129	0.001
	Frac GM	0.14	3.17	129	0.002
Glu/GABA	Age^{-1}	0.06	1.08	127	0.28
	Sex	0.04	0.39	127	0.70
	Hemisphere	0.14	1.59	127	0.12
	Frac GM	-0.13	-2.66	127	0.008

Table 2 DLPFC glutamate, GABA, and glu/GABA model comparison

Measure	Linear Age AIC	Inverse Age AIC	Quadratic Age AIC
Glutamate	-75.16	**-87.94**	-61.07
GABA	-295.31	**-307.04**	-279.43
Glu/GABA	503.13	**491.64**	515.53

Table 3 Glutamate model output for ACC, MPFC, and anterior insula

ROI	Variable	b	t	$n_{participants}$	p
ACC	**Age^{-1}**	0.21	3.248	133	0.0015
	Sex	0.02	0.15	133	0.88
	Frac GM	-0.014	-0.21	133	0.84
MPFC	Age^{-1}	0.11	1.70	125	0.093
	Sex	0.11	0.84	125	0.40
	Frac GM	0.078	1.24	125	0.22
Left & Right AI	**Age^{-1}**	0.29	6.33	137	$3.29x10^{-9}$
	Sex	0.09	1.09	137	0.28
	Hemisphere	-0.76	-14.89	137	$5.51x10^{-29}$
	Frac GM	0.06	1.68	137	0.09

Table 4 Glutamate model comparison for ACC, MPFC, and anterior insula

ROI	Linear Age AIC	Inverse Age AIC	Quadratic Age AIC
ACC	-145.48	**-145.22**	-43.56
MPFC	-97.87	**-98.78**	-97.98
Left & Right AI	-301.76	**-314.80**	-287.21

Table 5 Cramer-Rao Lower Bound and age associations

ROI	Variable	b	t	p
ACC	Glu CRLB	-0.08	-0.90	0.37
	GABA CRLB	0.10	1.24	0.22
MPFC	Glu CRLB	0.09	1.05	0.30
	GABA CRLB	0.27	3.08	0.003
Left & Right AI	Glu CRLB	-0.08	-1.23	0.22
	GABA CRLB	0.06	0.72	0.47

Table 6 GABA model output for ACC, MPFC, and anterior insula

ROI	Variable	b	t	n participants	p
ACC	**Age^{-1}**	0.27	-2.01	131	$6.38x10^{-5}$
	Sex	0.12	0.97	131	0.34
	Frac GM	-0.03	-0.47	131	0.64
MPFC	Age^{-1}	0.10	1.53	121	0.13
	Sex	0.002	0.02	121	0.98
	Frac GM	0.08	1.29	121	0.20
Left & Right AI	**Age^{-1}**	0.22	4.77	136	4.71×10^{-6}
	Sex	-0.08	-0.95	136	0.34
	Hemisphere	-0.49	-6.05	136	$1.64 x10^{-8}$
	Frac GM	-0.11	-2.46	136	0.015
	Age^{-1} x Hemisphere	0.16	1.99	136	0.048

Table 7 GABA model comparison for ACC, MPFC, and anterior insula

ROI	Linear Age AIC	Inverse Age AIC	Quadratic Age AIC
ACC	-297.87	**-298.54**	-296.44
MPFC	-225.91	**-226.34**	-224.40
Left & Right AI	-582.50	**-595.71**	-566.88

Table 8 Glu/GABA model output for ACC, MPFC, and anterior insula

ROI	Variable	b	t	n participants	p
ACC	**Age^{-1}**	-0.15	-1.28	128	0.012
	Sex	0.024	-2.54	128	0.84
	Frac GM	-0.022	-0.36	128	0.72
MPFC	Age^{-1}	-0.084	-1.15	125	0.25
	Sex	-0.04	-0.25	125	0.80
	Frac GM	-0.03	-0.44	125	0.66
Left & Right AI	**Age^{-1}**	-0.12	-3.46	138	0.010
	Sex	0.20	2.31	138	0.022
	Hemisphere	0.11	1.39	138	0.17
	Frac GM	0.11	2.51	138	0.013
	Age x Hemisphere	-0.30	-4.01	138	$1.04x10^{-4}$

Table 9 Glu/GABA model comparison for ACC, MPFC, and anterior insula

ROI	Linear Age AIC	Inverse Age AIC	Quadratic Age AIC
ACC	201.01	**200.00**	202.17
MPFC	242.71	**242.41**	244.40
Left & Right AI	402.19	**389.16**	413.82

Table 10 Residuals model comparison

Form of age	**AIC**
Linear Age	-457.32
Inverse Age	-461.35
Quadratic Age	-459.89

Table 11 Correlations (r value) by age group

Region	**Age group**	**$N_{participants}$**	**Pearson r value**
ACC	10-16	39	0.26
	17-22	46	0.64
	23-30	38	0.67
MPFC	10-16	33	0.21
	17-22	42	0.73
	23-30	38	0.60
R DLPFC	10-16	35	0.30
	17-22	33	0.20
	23-30	32	0.53
L DLPFC	10-16	36	0.42
	17-22	39	0.43
	23-30	27	0.41
R Anterior Insula	10-16	37	0.13
	17-22	45	0.46
	23-30	42	0.39
L Anterior Insula	10-16	36	0.48
	17-22	44	0.58
	23-30	36	0.42

Table 12 MGS accuracy by age model output

Measure	**Variable**	**b**	**t**	**$n_{participants}$**	**p**
Accuracy	**Age^{-1}**	0.30	6.23	149	4.91×10^{-9}
	Delay	-0.03	-2.66	149	0.0084

Table 13 DLPFC glutamate association with MGS accuracy model output

Measure	Variable	b	t	n participants	p
Accuracy	**Age^{-1}**	0.30	5.36	123	4.02 x 10 $^{-7}$
	Glu	-0.005	-0.169	123	0.87
	Delay	-0.03	-3.64	123	0.0003
	Sex	0.027	0.25	123	0.80
	Hemisphere	-0.004	-0.14	123	0.89

Table 14 DLPFC GABA association with MGS accuracy model output

Measure	Variable	b	t	n participants	p
Accuracy	**Age^{-1}**	0.30	5.35	120	4.45 x 10^{-7}
	GABA	0.022	0.62	120	0.54
	Delay	-0.034	-3.94	120	9.38 x 10^{-5}
	Sex	0.058	0.54	120	0.59
	Hemisphere	0.009	0.29	120	0.77

Table 15 DLPFC Glu/GABA association with MGS accuracy model output

Measure	Variable	b	t	n participants	p
Accuracy	**Age^{-1}**	0.30	5.24	119	7.38x10^{-7}
	Glu/GABA	-0.02	-0.59	119	0.55
	Delay	-0.033	-3.73	119	0.00021
	Sex	0.054	0.50	119	0.62
	Hemisphere	0.005	0.16	119	0.87
	Glu/GABA * Delay	0.023	2.05	119	0.041

Table 16 Glutamate component association with MGS accuracy model output

Measure	Variable	b	t	n participants	p
Accuracy	**Age^{-1}**	0.45	5.78	130	5.5x10^{-8}
	Glu component	-0.02	-0.28	130	0.78
	Delay	-0.045	-2.89	130	0.004

Table 17 GABA component association with MGS accuracy model output

Measure	Variable	b	t	n participants	p
Accuracy	**Age^{-1}**	0.43	6.11	130	6.71x10^{-8}
	GABA component	0.068	0.90	130	0.37
	Delay	-0.040	-2.74	130	0.011

Table 18 Glu/GABA component association with MGS accuracy model output

Measure	Variable	b	t	$n_{participants}$	p
Accuracy	**Age^{-1}**	0.44	5.86	114	$4.69x10^{-8}$
	Glu/GABA component	-0.11	-1.53	114	0.15
	Delay	-0.047	-2.97	114	0.014

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