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The role of BDNF/TrkB signaling in the development of the
adolescent PFC

by

Angela Vandenberg

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Neuroscience

in the

GRADUATE DIVISION

To my grandmothers,
Connie Vandenberg and Maxine Nelson,
the embodiment of strength and resilience,
and my inspiration.

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Author Contributions

The experiments presented in Chapter 2 of this dissertation were done by Angela Vandenberg with help from Natalia Caporale, Hannah Peckler and Francisco Javier Muñoz-Cuevas. Linda Wilbrecht directed and supervised the experiments. This chapter was written by Linda Wilbrecht and Angela Vandenberg.

The experiments presented in Chapter 3 of this dissertation were done by Angela Vandenberg. Lung-Hao Tai provided technical assistance and provided help with analysis. Linda Wilbrecht directed and supervised the experiments. This chapter was written by Angela Vandenberg and edited by Linda Wilbrecht.

The experiments presented in Chapter 4 of this dissertation were done by Angela Vandenberg, Claudia Wu, Natalia Caporale, and Lung-hao Tai. Linda Wilbrecht directed and supervised the experiments. This chapter was written by Angela Vandenberg and edited by Linda Wilbrecht.

The experiments presented in Chapter 5 and the appendix were done by the Dorit Ron lab (**Fig. A1**), Angela Vandenberg (**Fig. A2**), and Steven Lieske (**Fig. A3**). Chapter 5 was written by Angela Vandenberg and Linda Wilbrecht.

Abstract

The role of BDNF/TrkB signaling in the maturation of the adolescent PFC

By Angela Vandenberg

Brain derived neurotrophic factor (BDNF) and its receptor TrkB are involved in developmental maturation of cell processes, synaptogenesis, synaptic plasticity, and neuronal differentiation and survival. BDNF has been shown to play a significant role in the regulation of sensitive period plasticity in the visual cortex and maturation of inhibitory circuits. However, little is known about how BDNF/TrkB signaling may affect the maturation of synapses and function of the prefrontal cortex (PFC), especially during adolescence, a time-period of particular relevance for the onset of mental illness. In this dissertation I seek to increase understanding of how BDNF/TrkB signaling impacts the development of the prefrontal cortex, at the level of synapses and behavior. In chapter 1, I review relevant background studies on the maturation of the frontal cortex, including the role of neurotrophins (and in particular BDNF/TrkB signaling) in the developing brain as well as in sensitive period plasticity. I also review what is currently known about the role BDNF/TrkB signaling plays in common neuropsychiatric disorders including schizophrenia, mood disorders, substance abuse disorders, and autism. In Chapter 2, I use whole-cell voltage clamp recording to show that TrkB signaling plays a specific role in the development of inhibitory but not excitatory synapses in a sub-circuit of the dorsomedial prefrontal cortex during adolescence. In Chapter 3, I show that a common

polymorphism in the BDNF gene alters flexibility in reversal learning in multiple

paradigms. In Chapter 4, I combine approaches to shed light on the role of maturation of

GABAergic inhibition on maturation of behavioral inhibition. I conclude that while

BDNF and TrkB signaling play an important role in shaping both behavior and neuronal

circuits in the periadolescent PFC, the relationship between the development of

GABAergic inhibition and behavioral inhibition is not clear-cut. Overall I hope to

provide insight into the role that BDNF/TrkB signaling has in shaping inhibitory circuits

during adolescence, and ultimately how alterations in BDNF/TrkB signaling may

contribute to frontal function disorders.

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Chapter 1: Introduction

Many of the functions of the prefrontal cortex (PFC) fall under the term 'executive function' which refers to the ability to plan and perform goal-directed actions. These include working memory, complex problem solving, planning, and behavioral inhibition. Executive functions require the integration of sensory input, internal states including emotion and cognition, and motor output, as well as the ability to anticipate the consequences of one's actions. The prefrontal cortex is highly suited for this kind of integration as it has connectivity with virtually all sensory and motor systems as well as a wide range of subcortical structures (Miller and Cohen, 2001). It also has reciprocal projections back to these systems which may allow it to exert a 'top-down' influence on a wide range of processes. Moreover, prefrontal neurons show tuning for learned associations between cues, voluntary action, and reward, making them suitable for encoding and directing goal-directed behavior (Miller and Cohen, 2001).

Development of the PFC

In the human brain the developmental trajectory of the PFC is relatively protracted compared to other cortical areas, marking adolescence as a period of profound neuroanatomical changes. Cortical grey matter in the frontal lobes increases during childhood and peaks at puberty onset, after which they decline into early adulthood (**Fig. 1.1**) (Gogtay et al., 2004; Paus et al., 2008). White matter also shows a linear increase throughout childhood and adolescence, peaking in adulthood (Paus et al., 2008). In parallel to anatomical changes in the PFC during adolescence there are changes in

functional measures. For instance, there is an age-related increase in the blood-oxygenation-level-dependent (BOLD) signal in the prefrontal and parietal cortex during the performance of working memory tasks, as well as tasks that involve response inhibition such as the Stroop task and go/no-go task (Rubia et al., 2000; Luna et al., 2001; Adleman et al., 2002; Bunge et al., 2002; Tamm et al., 2002). There are also improvements in some aspects of executive function including delayed gratification, working memory, problem solving, as well as tasks that require response inhibition (Woo et al., 1997; Chambers et al., 2003).

These improvements in executive functions are thought to reflect the maturation of the functional architecture of the frontal cortex. A series of classic studies on 'working memory' illustrates how the changes in PFC anatomy may augment task performance. In the delayed response task the subject must retain knowledge of information provided by a sensory cue in order to produce the appropriate behavioral response (i.e. maintain the correct response in working memory) (Lewis, 1997). The ability to carry out the task appears between 2 and 4 months of age in monkeys and one year in humans, but continues to improve until after puberty in monkeys and humans (Goldman, 1971; Alexander and Goldman, 1978; Levin et al., 1991). Ablation of the dorsolateral PFC (DLPFC) in infant monkeys does not produce the same degree of impairment as in adults (Alexander and Goldman, 1978). Moreover, reversible cooling of the DLPFC disrupts performance in monkeys in an age dependent manner, with cooling producing the most pronounced deficits in adult monkeys when compared with juvenile or adolescent animals (Lewis, 1997). This change in function has been linked to a change in PFC

circuitry. Populations of neurons in the PFC have been shown to have activity that is temporally linked to the delayed response task (Fuster et al., 1982). Some PFC neurons show elevated firing rates during the delayed period of the task and the loss of this activity is associated with errors (Bauer and Fuster, 1976; Funahashi et al., 1989).
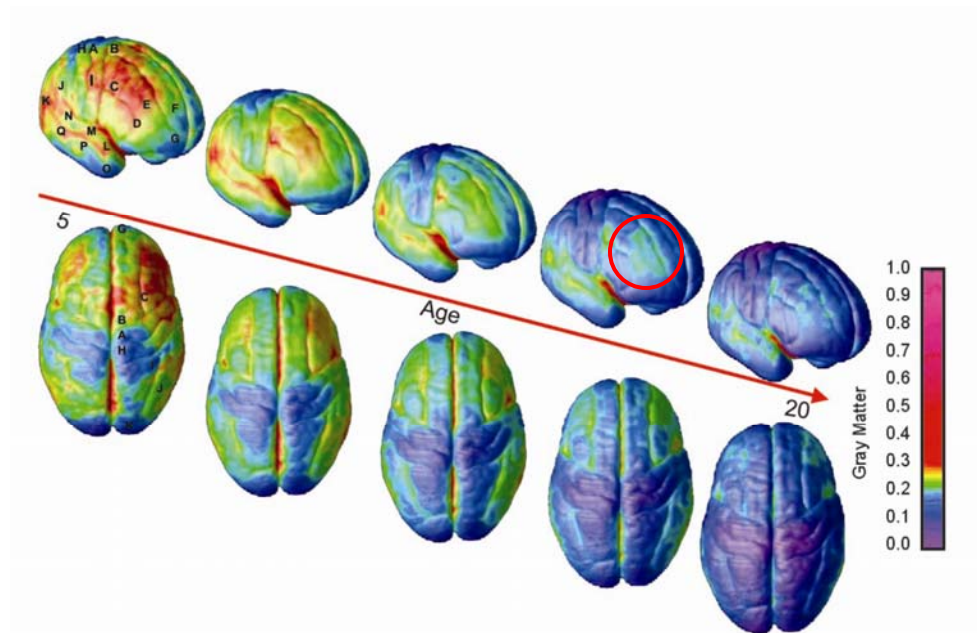
Figure 1.1 Gray matter maturation over the cortical surface. Gray matter thins in a back-to-front fashion with the frontal cortex (circled) thinning last during adolescence. The side bar shows a color representation in units of gray matter volume. Figure adapted from Gogtay *et al.* 2004

During postnatal development in macaques the percentage of DLPFC neurons that exhibit delay period doubles between 12 and 36 months of age, suggesting that developmental changes in DLPFC circuitry facilitated the recruitment of these neurons to a functional role (Lewis, 1997). This developmental change was not seen in other areas that subserve the delayed response task such as the medio-dorsal thalamic nucleus,

caudate nucleas, or posterior parietal cortex, suggesting that the adolescent refinement of this circuit is limited to the PFC (Lewis, 1997).

Adolescence is also associated with changes in the degree to which the frontal cortex is connected with other structures. Studies comparing functional connectivity (the correlation values of BOLD activity in various structures) in children and young adults have found that functional brain networks (networks that support a specific cognitive function) move from a more local to distributed organization with age (Jolles et al.; Fair et al., 2009). For instance, one study has shown decreased correlated activity in short-range frontal connections, and an increase in correlated activity in long-range connections with age (Fair et al., 2007). Adolescence is also associated with an increase in the degree to which two cerebral hemispheres can process information independently, as well as the amount of hemispheric asymmetry evident in EEG (Merola and Liederman, 1985; Anokhin et al., 2000).

Many insights into the development of the PFC have come through rodent studies. Though the rodent PFC is not as anatomically complex as the primate PFC, many of the functional and neuroanatomical characteristics are preserved. The medial portion of the rodent PFC (mPFC) which includes the anterior cingulate, prelimbic and infralimbic cortices, is likely homologous to the primate cingulate region and supplementary motor region and potentially DLPFC (including Broadman's area 46) (Bizon et al., 2012). Like the primate PFC, the rat and mouse mPFC receives afferents from limbic structures and medial thalamus, and sends efferents to the striatum (Uylings et al., 2003; Bizon et al.,

2012). The rodent mPFC also receives monoaminergic innervation from the locus

coereleus, ventral tegmental area (VTA), and raphe nuclei (Uylings et al., 2003). The

rodent mPFC also underlies many of the same functions as the primate PFC, including

working memory, and behavioral flexibility (Birrell and Brown, 2000; Ragozzino and

Rozman, 2007; Seamans et al., 2008; Johnson and Wilbrecht, 2011). Since rodents lend

themselves to genetic, cellular and biochemical approaches, rodent models have become

important tools in understanding the neurobiological mechanisms that underlie executive

function. Work from our own lab has shown that juvenile mice (P21-25) show more

flexible learning strategies than adult mice (**Fig. 1.2**), in an odor based 4-choice reversal

task known to depend on mPFC function (Johnson and Wilbrecht, 2011).
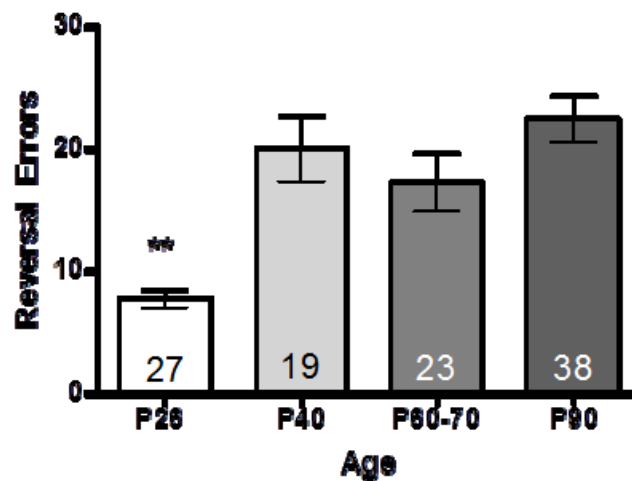
Figure 1.2 Developmental changes in reversal learning performance in a 4 choice task. Age affects performance on a reversal task (1 way ANOVA $F_{(3,106)}=11.2$, $p<0.0001$). **Juvenile mice (P25-26) make fewer reversal errors (errors in which the animal digs in the previously rewarded pot) than mice P40 and older (Bonferroni post-hoc test). N is shown in bar for each group.

Behavior becomes adult like after P40 (**Fig. 1.2**), the age of puberty onset in mice. We hypothesize that P25-P40 may be a sensitive period for development of the mouse frontal cortex function. Recent studies of the impact of social isolation on oligodendrocyte development of the mouse PFC have also isolated P21-35 as a critical period for social experience, PFC myelination, and working memory development in mice (Makinodan et al., 2012). One of the goals of my thesis is to shed light on how the maturation of the mouse PFC during this potential sensitive period (P25-P40) is regulated by neurotrophin signaling and how this impacts the maturation of executive function.

Research in the frontal cortex of rodents and primates have suggested that adolescence is a period of dynamic structural changes in both innervating long-range excitatory and neuromodulatory fibers as well as local inhibitory synapses. Synaptic density increases during early postnatal development and declines in adolescence in both humans and monkeys (Huttenlocher, 1979; Lewis, 1997). Spine density in rodents has also been shown to decrease during the periadolescent period (Holtmaat et al., 2005; Zuo et al., 2005). In rats, glutamatergic axons from the basolateral amygdala (BLA) continue to innervate the mPFC during the post-weanling period (Cunningham et al., 2008). Parvalbumin-IR terminal density also increases through adolescence in the primate PFC (Erickson and Lewis, 2002) and in macaques, there is dynamic regulation of $GABA_A$ receptor subunits, as well as parvalbumin (PV) and GABA membrane transporter (GAT1) in "cartridge" axon terminals (**Fig. 1.3**) (Gonzalez-Burgos et al., 2008). Modulatory neurotransmitter circuits also undergo changes during adolescence.

Maturation of Layer III in Macaque Frontal Cortex
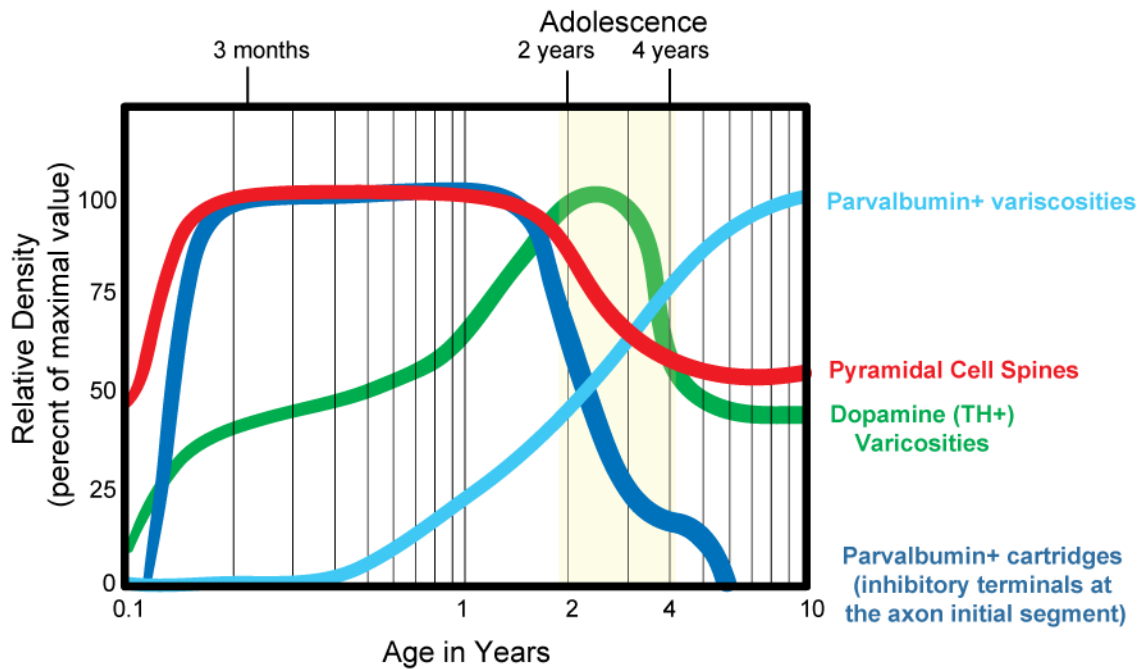
Figure 1.3 Maturation of Layer III in Macaque Frontal Cortex. During adolescence the relative density of pyramidal cell spines and the number of parvalbumin+ cartridges decreases in layer 3 of the macaque frontal cortex, as the number of parvalbumin+ varicosities continues to increase until adulthood. Dopamine varicosities show a curvilinear increase and then decline in relative density during adolescence. Figure adapted from Hoftman and Lewis, 2011

Dopamine (DA) terminal densities, a crucial component of the reward pathway, increase into adulthood in both rodents and primates (Rosenberg and Lewis, 1995). Moreover, there is a curvilinear increase in DA varicosities found in contact with inhibitory interneurons in the PFC of rats, but not excitatory neurons, suggesting that changes in the DA system may have direct effect on the balance of excitation and inhibition in frontal circuits (Benes et al., 1996).

Adolescence is a period of dramatic reorganization of neural circuits. The number of "moving parts" during this process of reorganization may make this a period of

vulnerability for psychological disorders. In fact, the results of the National Comorbidity Survey Replication study, conducted from February 2001 to April 2003, show that the peak age of onset for any mental health disorder is 14 years of age (Kessler et al., 2005; Kessler and Wang, 2008). Anxiety disorders, mood disorders, schizophrenia, and substance abuse all emerge during adolescence (**Fig. 1.4**) (Paus et al., 2008).
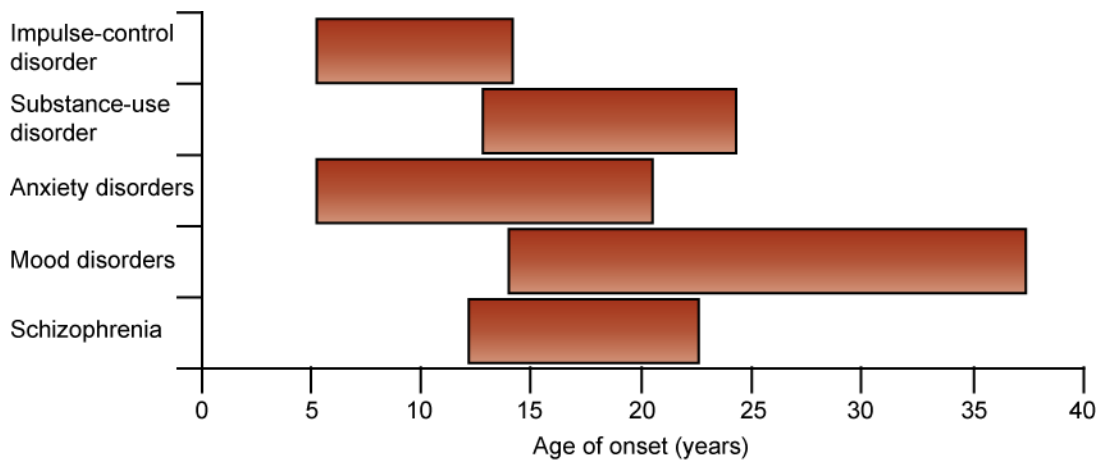
Figure 1.4 Ranges of onset for common neuropsychiatric disorders.
Figure adapted from Paus *et al.* 2008.

Many studies have investigated the myriad of potential biological mechanisms that could alter brain development during this vulnerable period. Disruption of neurotrophic factor signaling is one candidate mechanism that could significantly impact the trajectory of development of the frontal cortex leading to vulnerability to psychiatric disease. Neurotrophic signaling is likely to play a significant role in prefrontal cortex development (and brain development in general) because neurotrophic factors (NTFs) regulate cell proliferation, migration, differentiation and growth. Any disruption or abnormality in the regulation of NTFs and their receptors could potentially bring about maladaptive changes in neural circuitry which may then manifest as neuropsychiatric

disorders. In the next section I will first review the role of NTFs in neural development and plasticity with a focus on brain derived neurotrophic factor (BDNF) and the TrkB receptor receptors. I will then discuss links that have been made between BNDF/TrkB signaling and psychiatric disease.

Neurotrophins in brain development

Neurotrophins are homodimeric proteins that include brain derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin (NT)-3, and NT-4 (Lessmann and Brigadski, 2009). Neurotrophins function primarily through tropomyosin receptor kinase (Trk) family of tyrosine kinase receptors with NGF binding TrkA, BDNF and NT-4 binding TrkB, and NT-3 binding with TrkB and TrkC (Reichardt, 2006). BDNF and its receptor TrkB have long been recognized as playing a crucial role in the survival, differentiation, and growth of neurons. They also participate in activity dependent plasticity mechanisms such as long-term potentiation, synaptogenesis, learning and memory (Martinez et al., 1998; Hanover et al., 1999; McAllister et al., 1999; Tanaka et al., 2008). BDNF is initially synthesized as proBDNF, which is transported to the Golgi body for sorting into either constitutive or regulated secretory vesicles. ProBDNF may then be converted into mature BDNF, which is preferentially sorted into regulated secretory vesicles, while proBDNF is preferentially sorted into vesicles that are constitutively released (McAllister et al., 1999; Reichardt, 2006). It had long been thought that only mature BDNF is biologically active, but there is accumulating evidence that proBDNF, the immature form of BDNF, induces apoptosis via activation of the p75 receptor (Nickl-Jockschat and Michel; Reichardt, 2006).

In humans, BDNF and its receptors are developmentally regulated, peaking during adolescence: a period of enhanced neuronal growth, differentiation, and synaptogenesis in the prefrontal cortex (Knott et al., 2006; Casey, 2009). This makes them of particular interest in neuropsychiatric disorders with a late developmental onset. Postnatally, BDNF and its receptor TrkB play a key role in assembling neural circuits, both in way of promoting excitatory and inhibitory synaptogenesis, and in modulating activity-dependent morphological changes and long-term potentiation.

BDNF/TrkB signaling in neuron growth and morphology

BDNF plays an important role in morphological plasticity and neural growth in the post-natal brain. BDNF has been shown to regulate the dendritic and axonal growth of pyramidal neurons in the developing neocortex (Mcallister et al., 1995; Cao et al., 2007). BDNF is also necessary for the proper development of Purkinje cell dendritic complexity (Segal et al., 1995; Schwartz et al., 1997). In the retina, local addition of BDNF reduces ganglion cell dendritic arborizations, while increasing axon arborizations in the tectum, suggesting that BDNF can differentially modulate dendritic and axonal arborizations in a single population of cells (Lom et al., 2002). Additionally, overexpression of BDNF in the visual cortex leads to a destabilization of dendrites and spines of pyramidal cells in slice preparations (Horch et al., 1999).

BDNF has also been recognized as modulator of synaptic plasticity (Minichiello et al., 1999; Itami et al., 2003; Abidin et al., 2006; Minichiello, 2009). Locally applied BDNF

up-regulates postsynaptic Ca^{2+} transients and synaptic potentiation at spines through NMDA receptor activation (Kovalchuk et al., 2002). Moreover, BDNF has also been also been implicated in the conversion of NMDAR-only containing synapses (silent synapses) into AMPAR-containing synapses during the critical period in the mouse barrel cortex (Itami et al., 2003).

Activity-regulated BDNF secretion also plays a key role in assembling neural circuits in post-natal development. BDNF treatment increases the numbers of excitatory synapses in hippocampal cultures (Vicario-Abejon et al., 1998), and BDNF heterozygous KO mice have decreased excitatory synaptic strength as well as deficits in neuronal plasticity in the visual cortex (Abidin et al., 2006). TrkB KO mice also have decreased numbers of excitatory synapses (Martinez et al., 1998), and a conditional TrkB KO suggests that BDNF works pre and postsynaptically to regulate glutamatergic synapse formation (Luikart et al., 2005).

BDNF also plays a crucial role in inhibitory synaptogenesis. In hippocampal cultures BDNF has been shown to increase $GABA_A$ receptor expression after 48 hours of treatment (Yamada et al., 2002). Moreover, a mutation that disrupts the ability of CREB to bind to BDNF promoter IV results in deficits in miniature IPSCs, reduced expression of GABAergic markers, and fewer inhibitory synapses in cultured cortical neurons (Hong et al., 2008). Mice that lack promoter IV BDNF transcription show decreased PV staining in the PFC as well as deficits in GABAergic, but not glutamatergic, synaptic transmission (Sakata et al., 2009). TrkB hypomorphic mice also show a gene dependent

decrease in both GAD67 and PV mRNA (Hashimoto et al., 2005). Moreover, in BDNF

heterozygous KO mice the overall balance in the strength of cortical excitation to

inhibition is shifted towards decreased inhibition (Abidin et al., 2008). These studies

suggest that BDNF plays an important role in regulating the balance of inhibition and

excitation (I/E) through activity-dependent positive or negative feedback loops.

The role of BDNF/TrkB signaling in sensitive period plasticity

BDNF has been shown to play an important role in regulating sensitive period plasticity

in the post-natal brain due to its role in promoting GABAergic development (Cabelli et

al., 1997; Vicario-Abejon et al., 1998; Hanover et al., 1999; Huang et al., 1999; Baldelli

et al., 2005; Henneberger et al., 2005). Its role in critical period remodeling has been best

studied in ocular dominance plasticity in the visual cortex. The absence of visual input to

children, but not adults, has long been known to result in a permanent loss of visual

acuity (amblyopia)(Hensch, 2005). A classic experiment in cats showed that monocular

deprivation of one eye during the critical period leads to an expansion of the inputs from

the spared eye at the expense of areas targeted by the deprived eye (Wiesel and Hubel,

1963). Subsequent experiments have shown that GABAergic inputs play a critical role in

controlling the onset of this kind of critical period plasticity. For instance, mice lacking

GAD65 show a lack of ocular dominance shift during the height of the critical period

(P25-P27). This effect could be compensated by increasing GABA activity with the use

of benzodiazepines (Reiter and Stryker, 1988; Hensch et al., 1998; Fagiolini and Hensch,

2000). Moreover, in GAD65 KO mice critical period plasticity can be initiated at any age with diazepam infusion (Iwai et al., 2003). It has also been shown that the onset of ocular dominance plasticity requires a specific type of interneuron, namely PV+ basket cells, and that $GABA_A$ $\alpha 1$ subunits on the pyramidal cells are also required to trigger normal ocular dominance plasticity (Fagiolini et al., 2004). These experiments indicated that sufficient levels of inhibition are required for the critical period to occur.

BDNF is integral in establishing normal IE balance during development, as well as triggering the normal opening and closing of the critical period for ocular dominance plasticity. This idea has been borne out in experiments in which BDNF levels are modulated during early post-natal development. BDNF overexpression, which leads to the rapid maturation of GABAergic inhibition, has been shown to produce a precocious development of ocular dominance plasticity, while BDNF knockout has been shown to delay the onset of ocular dominance plasticity indefinitely (Cabelli et al., 1997; Hensch et al., 1998; Hanover et al., 1999; Huang et al., 1999) (**Fig. 1.5**). The role of BDNF on GABA neurons can also explain the classic effect of dark rearing. Animals that have been reared from birth without visual experience have a delayed critical period that can be abolished by diazepam infusion or BDNF overexpression (Castren et al., 1992; Chen et al., 2001; Morales et al., 2002; Gianfranceschi et al., 2003). These animals have reduced BDNF release and therefore reduced GABA function in the visual cortex (Chen et al., 2001; Morales et al., 2002). Interestingly, enriched environments, which have been shown to increase cortical BDNF levels, can also abolish the expected delay of the critical period in dark reared mice (Bartoletti et al., 2004).
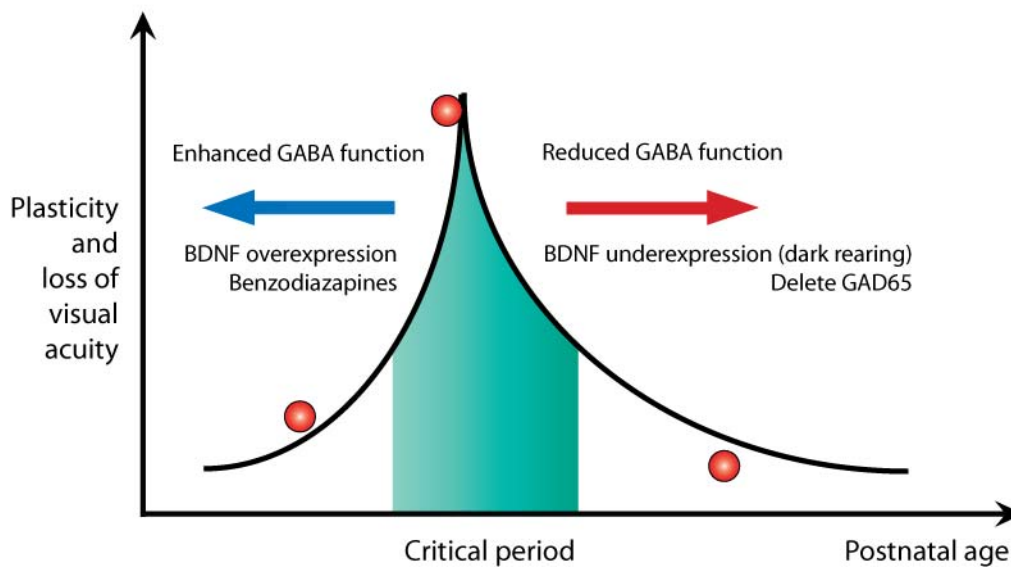
Figure 1.5 Modulating BDNF and GABA activity can alter the onset of the critical period for ocular dominance plasticity. The red circles represent the onset, peak, and end of amblyopia due to monocular deprivation. The onset of plasticity can be delayed by a reduction of GABA function in the visual cortex. This can be done by dark rearing and thereby reducing BDNF secretion, or deleting GAD65 which encodes an enzyme that synthesizes GABA (Chen et al., 2001; Morales et al., 2002; Gianfranceschi et al., 2003). Conversely, the onset of the critical period can be brought forward by enhancing GABA function either through BDNF overexpression (Hanover et al., 1999; Huang et al., 1999) or the use of benzodiazepines (Fagiolini and Hensch, 2000; Iwai et al., 2003; Fagiolini et al., 2004). Figure adapted from Hensch, 2005

Though adolescence marks a particularly dynamic time in frontal cortex development, it is unclear whether there is a critical period for development in the PFC. The role that BDNF/TrkB signaling and GABAergic inhibition plays in the development of prefrontal cognitive function is an idea that will be explored in this dissertation.

BDNF/TrkB signaling in psychiatric disorders

Because of its key role in synaptic plasticity, neuronal survival, and growth, BDNF/TrkB signaling has been discussed in many studies as playing a role in the pathophysiology of

psychiatric disorders, as well as a possible target of therapeutic drugs used to treat these disorders.

Schizophrenia

Schizophrenia is a neurodevelopmental disease with an onset during adolescence and early adulthood (**Fig. 1.4**). Though psychosis is usually the most striking clinical aspect of schizophrenia, the core features of the illness includes disturbances in cognitive processes, such as impairments in attention, memory, and executive function (Lewis et al., 2005). Schizophrenia has several hallmarks that suggest the involvement of the DLPFC, including working memory deficits as well as reduced DLPFC activation when attempting to carry out tasks that require working memory. Moreover, the severity in the deficits in the activation of the DLPFC predicts the severity of other schizophrenic symptoms such as disorganized thinking (Perlstein et al., 2001; Lewis et al., 2005).

Many aspects of schizophrenia are suggestive of neurodevelopmental processes gone awry during adolescence. For instance, PFC gray matter volume has been found to be decreased in schizophrenic subjects (Andreasen et al., 1994). Though the number of pyramidal neurons does not seem to be different in schizophrenics, there is a decrease in cortical thickness and increase in cell packing density, suggesting a decrease in dendritic spine density and complexity as well as in axon terminals (Pakkenberg, 1987, 1993; Akbarian et al., 1995; Daviss and Lewis, 1995; Selemon et al., 1995). Moreover, mean neuronal size and the density of dendritic spines on layer 3 pyramidal cells is decreased in the DLPFC of individuals with schizophrenia (Rajkowska et al., 1994; Garey et al.,

1995; Glantz and Lewis, 1995). Despite the many differences found in the brains of schizophrenic individuals, the exact pathophysiology is unknown. There are however many models that attempt to explain both the positive and negative symptoms of schizophrenia. Some of these include disinhibition in the frontal cortex, gamma oscillation disruptions, dopamine hyperfunction, NMDAR hypofunction, and abnormal connectivity between brain regions (Lisman, 2012). These models are not mutually exclusive. Here I will review three models: abnormal connectivity, disruptions in neural oscillations, and disinhibition in the PFC.

Abnormal connectivity

The aberrant maturation of white matter during adolescence has been a proposed factor in the pathophysiology of schizophrenia (Woo and Crowell, 2005; Uhlhaas and Singer, 2011). Multiple lines of evidence suggest the myelin in the PFC may be disturbed in schizophrenia. For example, many genes that regulate oligodendrocyte function appear to be disregulated in schizophrenic patients (Hakak et al., 2001; Pongrac et al., 2002; Davis et al., 2003; Kubicki et al., 2003; Lehrmann et al., 2003; Tkachev et al., 2003) and both the density and distribution of oligodendrocytes have been shown to be altered (Hof et al., 2003). Electron microscopic studies have also noted myelin impairments in the brains of schizophrenic patients (Uranova et al., 2001), and diffusion tensor imaging studies (DTI) have shown that the anisotropy of fiber tracts of the frontal lobes is altered. In particular the collosal fibers connecting language centers of the PFC and temperoparietal cortices have shown decreased anisotropy compared to controls (Kubicki et al., 2003; Shergill et al., 2007; Whitford et al., 2010; Whitford et al., 2011), as well as

the cingulum bundle that links the cingulate to other limbic structures (Kubicki et al., 2003).

Myelin has been shown to increase until early adulthood, particularly for corticocortical tracts (Benes, 1989; Uhlhaas and Singer, 2011). This has led to the theory that myelination of the corticolimbic system may trigger the onset of schizophrenia during adolescence in susceptible individuals (Benes, 2003). This theory is strengthened by the fact that pathology characteristic of demylination has not been observed in schizophrenics, suggesting that schizophrenia may be neurodevelopmental in origin (Uhlhaas and Singer, 2011). Brain imaging studies also suggest that the developmental increase in white matter volume seen during normal brain maturation is absent in patients with schizophrenia (Bartzokis, 2002, 2003; Flynn et al., 2003).

Increased myelination during adolescence decreases conduction times, reducing the latency of responses (Salami et al., 2003). Thus the development of white matter during adolescence is thought to be important coordinating synchronous activity in the brain, which also increases in adolescence (Uhlhaas and Singer, 2011) and is thought to underlie important functions that are impaired in schizophrenia such as working memory, perception, and attention (Uhlhaas and Singer, 2010).

Abnormal neural oscillations

Neural oscillations are an important mechanism in enabling coordinated activity during normal brain activity (Uhlhaas and Singer, 2010). Oscillations in the high frequency

ranges (beta and gamma) provide synchronization in local cortical networks (Gray et al., 1989; von Stein et al., 2000; Womelsdorf et al., 2007) while oscillations in the low frequency ranges (theta and alpha) establish synchronization over longer distances (von Stein et al., 2000). Neural oscillations are important for establishing precision in spike timing which is crucial for synaptic plasticity (Pavlides et al., 1988; Huerta and Lisman, 1993; Singer and Gray, 1995; Wespatat et al., 2004). Synchronized oscillations also allow only the most excitable cells to fire, thereby selecting the cells that form an ensemble (de Almeida et al., 2009), as well as allowing the ensemble to be recognized by downsteam networks via coincidence detection (Singer, 2009). Oscillations are closely linked to behavioral responses such as working memory, stimulus selection, and perceptual grouping, all of which are disrupted in schizophrenia (Uhlhaas et al., 2009).

Schizophrenic patients have been shown to have changes in neural oscillations in the resting state as well as during cognitive and perceptual stimulation across a wide rage of frequencies (Uhlhaas and Singer, 2010). During resting state, individuals with schizophrenia show an increase in slow oscillations, particularly over frontal electrodes (Boutros et al., 2008). Sensory evoked oscillations are also impaired, including deficits in the amplitude and phase locking of alpha, beta, and gamma oscillations during auditory and visual stimulation (Spencer et al., 2003; Hirano et al., 2008; Haenschel et al., 2010). Patients with schizophrenia also show a reduction of self-generated synchronized network activity during particular tasks. For instance, patients with schizophrenia have been shown to have a deficit in gamma-band activity during a perceptual organization task which was accompanied by impaired performance on the

task (Muller et al., 2009). Other studies have found reduced gamma and theta

oscillations in frontal regions during executive and working memory tasks (Lewis and

Moghaddam, 2006; Boutros et al., 2008; Uhlhaas and Singer, 2010). Phase synchrony of

induced oscillation have also been shown to be impaired in schizophrenic patients,

suggesting that there may be disturbances in the integration of neural responses in

distributed cortical networks in schizophrenic individuals (Spencer et al., 2003; Uhlhaas

et al., 2006).

Changes in the amplitude and synchronization of neural oscillations continue until early

adulthood, suggesting that abnormal neural oscillations in the schizophrenic brain may be

reflective of irregular neurodevelopmental changes that occur during adolescence

(Uhlhaas and Singer, 2011). The disturbances in the maturation of neural synchrony are

likely due to the aberrant development of anatomical and physiological parameters that

are necessary for driving oscillatory activity. One of these may be abnormal maturation

of white matter as mentioned above. It may also be due to disturbances in the normal

maturation of GABAergic neurons, which have also been implicated in the

pathophysiology of schizophrenia.

Imbalances in excitation and inhibition in the PFC

Besides changes in grey and white matter, GABAergic markers are also changed in the

brain of schizophrenics (**Fig. 1.6**). Many studies have confirmed that there is a decrease

in GAD67 mRNA (an enzyme that synthesizes GABA) in schizophrenic patients

(Akbarian et al., 1995; Guidotti et al., 2000; Mirnics et al., 2000; Volk et al., 2000;

Knable et al., 2002; Vawter et al., 2002; Hashimoto et al., 2005; Lewis et al., 2005) as well as a decrease of the GABA membrane transporter 1 (GAT1) (Volk et al., 2001). Moreover, this reduction seems to occur in PV-expressing neurons in which PV mRNA expression was reduced, but still detectable (Lewis et al., 2005). These studies suggest that while the number of PV-expressing GABA neurons is not reduced in individuals with schizophrenia, both synthesis and reuptake of GABA are reduced, and PV-expressing neurons might therefore be functionally impaired.

Evidence for impaired GABAergic function in schizophrenic individuals also comes from looking at abnormalities in gamma band oscillations in schizophrenic patients. Gamma band oscillations are induced and sustained during the delay period of working memory tasks (Tallon-Baudry et al., 1998; Le Magueresse and Monyer, 2013). The amplitude or power of the gamma band oscillations in the DLPFC seem to increase in proportion to working memory load (Howard et al., 2003). In schizophrenia, impairments in working memory are accompanied by disruptions in phase-locking of gamma activity, as well as a reduction in gamma band power during the delay period of working memory task (Park and Holzman, 1992; Spencer et al., 2003). Moreover, injection of GABA antagonists into the DLPFC disrupt working memory (Sawaguchi et al., 1989). Notably, gamma band oscillations are formed by PV neurons (calbindin neurons give rise to theta band oscillations), the same inhibitory neuronal subtype that shows reduced mRNA expression for GABAergic markers in schizophrenic individuals (Lewis et al., 2005; Sohal et al., 2009).

It is possible that many of the neurodevelopmental impairments seen in schizophrenia may be at least partially explained by reductions in BDNF or TrkB levels. Both mRNA and protein levels for BDNF and TrkB are reduced in the DLPFC of individuals with schizophrenia (Weickert et al., 2003; Hashimoto et al., 2005), whereas TrkC, the receptor tyrosine kinase for NT-3, is unchanged (Lewis et al., 2005). Moreover, signaling by BDNF through TrkB (which is predominantly expressed by parvalbumin GABA neurons, but not calretinin) promotes the development of GABA neurons and induces the expression of GABA-related proteins including GAD67, GAT1, and parvalbumin (Cellerino et al., 1996; Huang et al., 1999; Yamada et al., 2002). In postmortem brains of schizophrenic individuals, changes in TrkB and GAD67 mRNA were strongly correlated, suggesting that TrkB expression might be a pathogenic mechanism that results in a reduction of GABA-related gene expression (Hashimoto et al., 2005).

This hypothesis has been borne out in part in studies using animal models. TrkB hypomorphic mice in which the insertion of floxed TrkB cDNA resulted in decreased TrkB expression (Xu et al., 2000) showed lower TrkB mRNA in the PFC as well as decreased GAD67 and parvalbumin mRNA in the PFC. Calretinin mRNA, however, remained unaffected (Xu et al., 2000). Heterozygous BDNF KO mice, on the other hand, showed no change in GAD67 or parvalbumin mRNAs (Hashimoto et al., 2005), suggesting that changes in TrkB expression, and not in BDNF expression, plays a primary role in the regulation of GABA-related gene expression in the adult PFC. These studies support the idea that deficiency in TrkB signaling is one mechanism that can

result in reduced GABA synthesis in the PV+ subpopulation of neurons and that potentially may underlie neural dysfunction leading to schizophrenia.

Mood Disorders and Anxiety Disorders

Mood disorders include major depressive disorder (MDD), which affects about 17% of the population at some point in life, and bipolar disorder (BPD), which affects about 1 % of the population aged 18 and older at any given year (Kessler et al., 1994; Belmaker and Agam, 2008). The typical age of onset of mood or anxiety disorder is adolescence or early adulthood (**Fig. 1.4**). Though the neurobiology underlying these disorders is unknown, BDNF is thought to play a role in their pathophysiology, and has even been proposed as a potential biomarker (Hashimoto et al., 2004; Hashimoto, 2010).

Evidence for the role of BDNF in the etiology of mood disorders comes from studies looking at the levels of BDNF and its receptor TrkB in the brains and blood serum of patients with MDD or BPD. Studies have shown reduced mRNA levels of both BDNF and TrkB in the PFC and hippocampus of suicide subjects (Dwivedi et al., 2003; Karege et al., 2005; Pandey et al., 2008; Ernst et al., 2009; Keller et al., 2010). Moreover, studies of blood serum has shown reduced BDNF levels in MDD in antidepressant-naïve patients (Karege et al., 2002; Shimizu et al., 2003), which one study found was negatively correlated with the severity of the depression (Karege et al., 2002). A meta-analysis study demonstrated that patients with BPD also have lower levels of BDNF when compared to healthy controls (Lin, 2009) although the differences were only significant when patients were in a manic or depressed state. These studies have made blood levels

of BDNF of interest as a potential biomarker for MDD and a state-dependent bio-marker BPD.

Other evidence for the role of BDNF in mood disorders comes from looking at the outcome of current treatments, which have been reported to increase BDNF levels in the brain (Deltheil et al., 2008). Mood stabilizers like lithium and valproate have been shown to increase expression of BDNF in the rat brain (Hashimoto et al., 2002) while treatment with K252a, a protein kinase inhibitor, suppresses the neuroprotective effects of lithium (Hashimoto, 2010). BDNF may be a downstream target of a variety of antidepressant treatments, since studies have reported increases in BDNF protein levels after chronic, but not acute, treatments with antidepressents (Deltheil et al., 2008). This is consistent with the time course required for the therapeutic action of antidepressants to take effect (Nibuya et al., 1995; Nibuya et al., 1996; Russo-Neustadt et al., 2001). Other modes of therapy for MDD have also been found to increase BDNF mRNA levels including electroconvulsive therapy (ECT) (Altar et al., 2003) and repetitive transcranial magnetic stimulation (rTMS) (Muller et al., 2000; Hashimoto et al., 2002).

BDNF has also been linked to animal models of depression, including learned helplessness (Siuciak et al., 1997). Forced swimming causes a decrease of BDNF mRNA in the hippocampus of rats, which either antidepressant treatment or physical activity can mitigate (Russo-Neustadt et al., 2001). Moreover, a single bilateral infusion of BDNF into the dentate gyrus of the hippocampus (Shirayama et al., 2002) has been shown to produce antidepressant effects in both the learned helplessness and the forced-swim test

paradigms 3-10 days after infusion. These antidepressant effects seem to be mitigated by the TrkB receptor since infusion of a TrkB inhibitor K252a blocked the antidepressant effects of BDNF (Shirayama et al., 2002). Moreover, the TrkB.T1-overexpressing transgenic mice, which show reduced TrkB activation in the brain, are resistant to the effects of antidepressants when undergoing the forced swim test, suggesting that normal TrkB signaling is required for the behavioral effects produced by antidepressants (Saarelainen et al., 2003). Phosphorylation of CREB was found to be increased in the PFC by the administration of antidepressants, concomitant with increased phosphorylation of TrkB, and this response was reduced in TrkB.T1 over expressing mice (Saarelainen et al., 2003). These findings have been interpreted as suggesting that antidepressants may work to acutely increase TrkB signaling in the cerebral cortex (via BDNF), and that this mode of signaling is required to achieve therapeutic effects (Saarelainen et al., 2003).

The fairly recent discovery of a single nucleotide polymorphism in the BDNF gene (Val66Met), found only in humans, has also motivated study of the contributions of BDNF to affective disorders (Casey, 2009). The polymorphism leads to a Met substitution for Val in the BDNF prodomain at codon 66. This substitution is found in about 20%-30% of individuals in Caucasian populations, and it has been found to correlate with alterations in human carriers, including smaller hippocampal volumes (Bueller et al., 2006) and poorer performance on hippocampal-dependent memory tasks (Egan et al., 2003; Hariri et al., 2003). The mechanism that contributes to altered BDNF Met function is trafficking abnormalities which reduces the activity dependent secretion of BDNF (Egan et al., 2003; Chen et al., 2004; Chen et al., 2005b). The link between the

BDNF Val66Met polymorphism and mood disorders is unclear, although studies have suggested that BDNF signaling in the PFC is required for the behavioral effects of chronic antidepressant administration to take place (Saarelainen et al., 2003). Recently two mouse models with a BDNF knock-in Met allele have been created (Chen et al., 2006; Warnault et al., 2013). One line shows some of the phenotypic hallmarks of human carriers including reduced hippocampal volume and memory deficits (Chen et al., 2006). Although these animals do not show signs of increased behavioral measures of depression, they do show enhanced anxiety related behaviors that, unlike in controls, were not attenuated by the antidepressant fluoxetine (Chen et al., 2006).

Substance Use Disorders

Drug addiction is characterized by compulsive drug use despite adverse consequences. Drug seeking behavior is thought to be mediated by neuronal activity in the in the ventral tegmental area (VTA) and many of its projection targets including the PFC, nucleus accumbens (NAc), amygdala, bed nucleus of stria terminalis (BNST), and hippocampus, among other structures (Ghitza et al., 2010). A current hypothesis is that drug addiction is caused by drug-induced neuroadaptations in the dopamine and corticolimbic circuitry (Nestler, 2001; Kalivas and Volkow, 2005). This hypothesis has inspired studies into the role of BDNF in the survival and function of adult dopamine neurons, as well as the maladaptive synaptic plasticity involved in addiction (Ghitza et al., 2010).

Animal studies suggest that within the limbic system, BDNF is a largely positive modulator of psychostimulant and opiate reward. For instance, BDNF/TrkB signaling in

the VTA or nucleus accumbens contributes to cocaine and morphine conditioned place preference (CPP), while signaling in the accumbens has also been shown to contribute to increased self-administration (Graham et al., 2007; Bahi et al., 2008; Graham et al., 2009; Vargas-Perez et al., 2009).   In contrast to this, BDNF/TrkB signaling in the PFC decreases cocaine seeking, potentially by inhibiting glutamate transmission in the accumbens (Berglind et al., 2007; Berglind et al., 2009).

Drug related changes in BDNF and other growth factors during abstinence have also been associated with reinstatement and drug seeking in animal models of relapse (Sinha, 2011). For instance, elevated BDNF in the PFC after cocaine withdrawal has been shown to facilitate LTP by suppressing GABA inhibition (Lu et al., 2010). Activation of BDNF/TrkB signaling in the VTA or accumbens has also been found to potentiate reinstatement induced by cocaine priming and footshock stress (Lu et al., 2004; Graham et al., 2007) as well as prime synapses in the VTA for increased potentiation (Pu et al., 2006). Moreover, a human study showed serum BDNF levels in abstinent cocaine abusers to be significantly higher than that of controls. These levels were highly predictive of shorter time to cocaine relapse and higher amounts of cocaine used (D'Sa et al., 2011; Sinha, 2011). The BDNF Val66Met polymorphism has also been associated with opiate dependence with Met/Met carriers having a higher incidence of heroin-dependence as well as an earlier onset of abuse than Val/Val carriers (Cheng et al., 2005; Bath and Lee, 2006; Meng et al., 2012). These studies support the notion that BDNF-mediated synaptic plasticity in limbic structures causes enhanced responsiveness of these neurons to drug cues or stress.

It is interesting to note that, like many psychiatric disorders, addictive disorders identified in adults commonly have an onset in adolescence or young adulthood (Chambers et al., 2003). Growing evidence suggests that adolescence is a period of heightened vulnerability to the addictive properties of drugs. Animal studies suggest that part of the reason for this may be that drugs of abuse are more rewarding in adolescence than in adults. For instance, upon amphetamine treatment periadolescent rats will exhibit greater preference for environments previously paired with amphetamine delivery than adult rats (Adriani et al., 1998). Periadolescent rats also show greater behavioral sensitization and striatal dopamine release after repeated psychostimulant injections than adult rats (Laviola et al., 1995; Bucholz, 1999; Laviola et al., 2001).

Evidence that adolescence is a period of vulnerability for drug addiction also comes from looking at the comorbity of substance use disorders with other neuropsychiatric diseases that commonly onset in adolescence. The prevalence of substance use disorders is elevated in individuals with schizophrenia, major affective disorders, and pathological gambling (Regier et al., 1990; Bucholz, 1999; Blanco et al., 2009). This suggests that a common brain mechanism may underlie vulnerability to substance use disorders and psychiatric disorders identified with disturbances in reward motivation. Both psychiatric disorders and adolescence are commonly associated with poor impulse control (Damasio et al., 1994; Dervaux et al., 2001; Moeller et al., 2001), which is thought to be mediated in large part by the PFC. Damage to the PFC causes pervasive impulsivity associated with poor decision making and executive planning, emotional instability, and inability to

read social cues (Damasio et al., 1994). Moreover, individuals with poor impulse control

show a tendency to engage in risky behaviors characterized by long-term negative

outcomes, including drug abuse. This has lead to the hypothesis that an underdeveloped

or abnormally developed PFC may increase the probability of performing impulsive

activities, like drug taking, which may in turn lead to neuroplastic changes resulting in

further drug use (Chambers et al., 2003).

Autism

Autism appears during infancy with diagnosis often being established before the age of

three (Nickl-Jockschat and Michel, 2011). Autistic individuals typically present with

deficiencies in language and social interaction as well as sensory disturbances. They are

also prone to exhibit repetitive and stereotyped movements as well as having restricted

interests (Volkmar and Pauls, 2003; Minshew and Williams, 2007). Autism spectrum

disorder (ASD) is a neurodevelopmental disorder with a heterogeneous clinical

presentation that is often co-morbid with other disorders such as Angelman syndrome,

Rett syndrome, and Fragile X syndrome (Kana et al., 2011; Le Magueresse and Monyer,

2013). Like in schizophrenia, BDNF serum levels are often found to be disrupted in

individuals with autism (Horwitz et al., 1988; Lam et al., 2008; Ben-Sasson et al., 2009)

suggesting a possible role for BDNF/TrkB signaling in the etiology of the disorder.

Although the exact mechanism of autism is unknown, disruptions in BDNF/TrkB

signaling make up the core of several hypotheses, two of which will be reviewed here.

Disrupted cortical connectivity theory

Neurotrophins are known to modulate axonal and dendritic growth as well as synapse

formation and plasticity (Weng et al., 2010). Their role in neuronal growth and plasticity

suggests that any changes in brain structure and function might reflect altered

neurotrophin levels (Kana et al., 2011). One of the most consistent findings in autism is

an abnormally accelerated brain growth during development (Mundy et al., 1990;

Sheinkopf and Siegel, 1998; Weiss and Harris, 2001). It has been suggested that this is

due to improper formation or elimination of inappropriate connections. Magnetic

resonance imaging studies (MRI) have also shown reduced axodendritic pruning in

autistic individuals (Kjelgaard and Tager-Flusberg, 2001; Tager-Flusberg and Joseph,

2003; Lam et al., 2008; Ben-Sasson et al., 2009). Reduced anatomical connectivity have

been found in several regions including the collosum (Barnea-Goraly et al., 2004; Waiter

et al., 2005), the frontal lobe (Hendry et al., 2006) and the superior temporal gyrus

(Alexander and Goldman, 1978). Along with anatomical changes, functional

connectivity in ASD patients describe a pattern of reduced fronto-parietal connectivity

during response inhibition (Gallese and Goldman, 1998), sentence comprehension

(Gallese and Goldman, 1998) and working memory tasks (Gazzola et al., 2006). This is

paralleled by a decreased performance on executive function tasks throughout

development (Rizzolatti et al., 1996; Keller et al., 2007).

A particularly striking aspect of ASD behavior is a strong preference for structure and

routine, as well as propensity to engage in repetitive behaviors. It has been proposed that

preferences for structure may be due to reduced cognitive flexibility or set shifting (Kana

et al., 2011). Difficulty in behavioral flexibility can lead to perseveration and the inability to switch from one task to another under changing conditions. One commonly used behavioral measure of cognitive flexibility in humans is the Wisconsin Card Sorting Task (WCST) (Ozonoff, 1995). In the WCST participants are instructed to match response cards to stimulus cards based on either shape, color, or number of shapes. During the test the category changes without warning and the participant must recognize the change in contingencies and adapt to it (Bennetto et al., 1996). Participants with ASD struggle during the WCST and tend to perseverate on one category or rule (Ozonoff, 1995; Bennetto et al., 1996; Goldstein et al., 2001; Tsuchiya et al., 2005; Pellicano et al., 2006). One study found significant positive correlation with repetitive behaviors and preservative responding on the WCST in adolescents with ASD (South et al., 2007). Interestingly, patients with other frontal disorders, including schizophrenia, have also been shown to perseverate on the WCST (Anderson et al., 1990; Mattes et al., 1991).

Functional MRI data have suggested that the deficits in cognitive flexibility in ASD are related to decreased activation in structures involved in cognitive set shifting (Kana et al., 2011). For example, Shafritz et al. found decreased activity in the DLPFC, premotor cortex, anterior cingulate cortex, and basal ganglia in ASD patients, as well as poorer behavioral responses when compared to the control group. Moreover, the severity of repetitive behavior was correlated with decreased activation in the anterior cingulate and posterior parietal in the ASD group (Shafritz et al., 2008).

According to the functional underconnectivity theory (Just et al., 2004; Kana et al., 2006), individuals with ASD have weaker connections between the prefrontal and posterior brain regions, and this lack of connectivity causes cognitive inflexibility and reduced executive function. How these changes in frontal cortex connectivity come about is open to some debate. In one view, early frontal lobe disorganization leads to its inability to connect with other brain areas which reduces its ability to complete executive function tasks (Courchesne and Pierce, 2005). Another theory posits that early medial temporal lobe dysfunction causes a breakdown in connections that are normally recruited during complex cognitive tasks and that this breakdown may trigger abnormal development of the PFC, which develops much later in life, and in turn, deficits in executive functions (Belmonte et al., 2004; Courchesne and Pierce, 2005). Regardless of directionality, deficits in connectivity may be due to deficits in BDNF/TrkB signaling.

IE ratio hypothesis

Another proposed mechanism for ASD is the inhibition-excitation (IE) ratio hypothesis. This hypothesis suggests that a common mechanism behind autism and other related disorders is an imbalance in excitation and inhibition leading to cortical hyperexcitability (Rubenstein and Merzenich, 2003). This in turn may lead to the broad-ranging abnormalities in perception, memory, and cognition seen in ASD. Because of the role BDNF plays in establishing post-natal IE ratios, disruptions BDNF/TrkB signaling may underlie abnormalities in excitation and inhibition. This hypothesis is borne out in looking at the clinical presentation of autistic patients. One third of autistic individuals will have epileptic seizures, again suggesting that autism may be related to a disruption in cortical inhibition (Gillberg and Billstedt, 2000). Moreover, common comorbid disorders

such as Angelman syndrome, Rett syndrome, and Fragile X syndrome are also associated

with an increased susceptibility to seizures. 50-70% of autistic children have ongoing

'sharp spike' EEG activity during sleep which is also suggestive of noisy and unstable

cortical networks (Lewine et al., 1999).

There is also evidence for disturbances in IE ratio from postmortem studies of autistic

patients. The expression of the 65 kDa isoform of glutamate decarboxylase (GAD65)

and GAD67 (both of which convert glutamate to GABA) is decreased in autistic

individuals, as well as the expression of $GABA_A$ and $GABA_B$ receptors (Fatemi et al.,

2002; Yip et al., 2007; Fatemi et al., 2010; Oblak et al., 2011). There is also evidence of

smaller minicolumns in autistic brains due to the loss of inhibitory projections (Casanova

et al., 2003; Geschwind and Levitt, 2007; Pardo and Eberhart, 2007; Gogolla et al.,

2009). This may result in altered long-range connectivity, necessary for communication

between different neocortical areas and hemispheres (Brock et al., 2002; Rippon et al.,

2007).

Several polymorphisms of genes commonly associated with inhibitory neuron function

have also been associated with increased risk of autism. This includes polymorphisms in

or near the DLX1/2 genes which govern the migration and differentiation of forebrain

GABAergic neurons (Anderson et al., 1997a; Anderson et al., 1997b; Liu et al., 2009).

Polymorphisms of the gene that codes for the $GABA_B3$ receptor subunit are also

associated with higher risk of autism (Menold et al., 2001), a finding consistent with

decreased expression of the subunit in the brain of autistic patients (Samaco et al., 2005).

Mouse models have also implicated alterations in inhibition as a mechanism for autism. For instance, there are reduced numbers of PV+ interneurons in mice treated with valpoic acid (Gogolla et al., 2009; Le Magueresse and Monyer, 2013). Moreover, mice engineered to carry a mutation in the gene encoding urokinase plasminogen activator receptor, which is also associated with autism, have decreased numbers of interneurons including a complete lack of PV+ neurons (Powell et al., 2003). A study using optogenetic stimulation has also shown that elevation, but not reduction, of IE balance within the mPFC elicits social impairments and increased gamma oscillations, both of which are present in autism (Yizhar et al., 2011).

There is also evidence from animal models of Angelman, Rett, and Fragile X syndromes that GABA-dependent plasticity mechanisms are disturbed during development. In mice lacking the maternal copy of Ube3a, a model of Angelman syndrome, ocular dominance plasticity is strongly impaired during the critical period (Yashiro et al., 2009; Sato and Stryker, 2010). There's a similar finding of reduced ocular dominance plasticity in a Fmr1 knockout mice, a mouse model of Fragile X syndrome (Dolen et al., 2007). These results suggest that critical period plasticity may be disrupted in autism-related diseases.

embryo   pathological maturation of the GABAergic system   adult

| Migration | ↔ | Synaptic integration | ↔ | Network activity/plasticity | ↔ | Behavioral or clinical phenotype |

increased density of interneurons in superficial white matter

altered tangential migration (DISC-1 knock-down, loss of ErbB4/NRG1 signaling in mice)

increased α2 at ChC-PC synapse

decreased α1 at PV+BC-PC synapse

NMDAR hypofunction

decreased BDNF/TrkB signaling

decreased axo-axonic synapses decreased glu synapses onto PV+BC (mouse model)

decreased PV expression in PV+ cells

decreased GAD67 expression in PV+ cells

increased γ oscillations in sensory cortices

decreased excitability of PV+ cells (mouse model)

schizophrenia

altered expression and polymorphisms of DLX genes

decreased expression/polymorphisms of GABAAR subunits

increased GABA transmission in PCs (mouse model)

network hyperexcitability

Altered OD plasticity in visual cortex (mouse model)

decreased γ oscillations in auditory cortex

decreased number of PV+ cells (mouse model)

Autism/related disorders

Figure 1.6 The pathological development of the GABAergic system in schizophrenia and autism. Alterations in GABA function at different developmental epochs have been implicated in underlying frontal disorders such as schizophrenia and autism. Abbreviations: PV+BC, parvalbumin-expressing basket cell. ChC, Chandelier cell. Adapted from Magueresse and Monyer, 2013

Chapter 2: Adolescent maturation of inhibition in the cingulate cortex is pathway specific and TrkB dependent

Introduction

Interneurons in the prefrontal cortex are known to synchronize the firing of excitatory projection neurons, as well as influence synaptic plasticity, both of which are thought to modulate executive functions, such as planning and working memory (Hensch, 2005; Sakata et al., 2009; Gonzalez-Burgos et al., 2011; Le Magueresse and Monyer, 2013). Inhibitory neurotransmission matures during the periadolescent period in both mice and humans and may regulate sensitive periods of brain plasticity (Hensch, 2005). Among important regulators of the maturation of inhibition in post-natal life are brain derived neurotrophic factor (BDNF) and its receptor TrkB (Hashimoto et al., 2004; Le Magueresse and Monyer, 2013). Disruptions of BDNF and TrkB have been linked to multiple late-onset psychiatric disorders, including schizophrenia (Angelucci et al., 2005; Gratacos et al., 2007; Wong et al., 2009; Guo et al., 2010) and mood disorders. For instance, a study of postmortem human brains found that BDNF and TrkB expression in the prefrontal cortex (PFC) was reduced in two cohorts of schizophrenics along with parvalbumin (PV) and GAD67 (Hashimoto et al., 2005). It is currently unclear, however, how local GABAergic circuits regulate specific excitatory subnetworks in the frontal cortex (Krook-Magnuson et al., 2012). It is also unknown whether TrkB signaling acts to regulate to the maturation of inhibition onto different neuron subtypes in a homogenous or independent fashion.

Recent studies investigating the specificity of GABAergic connectivity onto different subtypes of pyramidal neurons within the cortex have found evidence for both homogeneity and specificity. One set of sister-studies have suggested that interneuron connectivity in the cortex is locally dense, "promiscuous," and stable across development (Fino and Yuste, 2011; Packer and Yuste, 2011), while others have found evidence for specificity within these dense local connections that vary by connectivity or projection neuron subtype (Yoshimura and Callaway, 2005; Yoshimura et al., 2005; Varga et al., 2010; Krook-Magnuson et al., 2012).

To investigate the differences in inhibitory input onto different projection neurons of layer 5 of the cingulate cortex, we recorded from subtypes of pyramidal neurons with distinct projection targets. Across the neocortex, pyramidal neurons can be subdivided into roughly two classes: those with mainly intratelencephalic connections (IT-type), and neurons that project subcortically to the pons, pyramidal tract (PT) and other subcortical targets (PT-type) (Reiner et al., 2010; Krook-Magnuson et al., 2012). The IT-type, which projects within cortex or to the contralateral hemisphere, shows spike adaptation and tends to have a more simple apical tuft morphology (Hattox and Nelson, 2007; Miller et al., 2008; Yu et al., 2008; Sohal et al., 2009; Gee et al., 2012). The PT-type, which projects subcortically and subcerebrally, typically has no spike adaptation and a complex apical tuft morphology. Both PT and IT-type neurons project to the striatum, but may also differentially innervate the two hemispheres and the direct and indirect pathway (Reiner et al., 2010) (**Fig. 2.1**).

To record from these different populations we used two strategies from which we obtained similar outcomes. In the first we made use of the YFPH mouse line in which only a subset of layer 5 pyramidal neurons are labeled (Feng et al., 2000; Sugino et al., 2006) (**Fig. 2.2B**). YFP+ and YFP- cells in this line have previously been shown to have PT- and IT-type firing properties, respectively, (Sugino et al., 2006; Yu et al., 2008), to project to different targets consistent with PT and IT-type neurons (Porrero et al., 2010), and to receive differential inputs from excitatory neuron in layers II/III of the motor cortex (Yu et al., 2008). In a second strategy we used fluorescent retrobeads injected in the ipsilateral pons and the contralateral anterior cingulate to label PT- and IT-type neurons, respectively (**Fig. 2.2C**). To investigate the differences in input onto different projection neurons of layer 5, we made whole-cell recordings of miniature excitatory (mEPSCs) and inhibitory (mIPSCs) postsynaptic currents in different projection neurons in the mouse anterior cingulate cortex (**Fig. 2.2A**). We found that while mEPSC measures did not differ between PT- and IT-type neurons, PT-type neurons had a significantly higher mIPSC frequency compared to adjacent IT-type neurons. We also found that the IT-type population showed a TrkB dependent increase in mIPSC amplitude over periadolescent development.

Our data show that inhibitory synapses on PT and IT-type pyramidal neuron subtypes are differentially regulated over the periadolescent period, and that some aspects of this differential regulation depends on signaling through TrkB. Knowledge of this kind can help to isolate candidate subcircuits to better understand the etiology of diseases that

show onset during late development. Changes in IT-type neurons observed in our study may be particularly relevant to changes observed in long-range cortical connectivity, synchrony, and commissural white matter observed in patients with schizophrenia.

Results

Adjacent PT- and IT-type neurons show differences in mIPSCs

The first thing we wanted to determine was whether synaptic inputs differ between different types of projection neurons. Comparing mPSC amplitude and frequency in serially recorded pairs of YFP+ and YFP- neurons in juvenile mice (P21-25), we found that mEPSCs did not differ between cell types (**Fig. 2.3A-C**). However, the frequency, but not amplitude, of mIPSCs was significantly lower in YFP- neurons compared to adjacent YFP+ neurons (Wilcoxon matched-pairs signed rank test p=0.016)(**Fig. 2.3D-F**). In older animals (P40-50) the frequency of YFP+ neurons was also larger than YFP- neurons (Mann Whitney, p=0.016) (**Fig. 2.4F**).

In a follow up experiment we used green and red retrobeads (**Fig. 2.2C**) to identify PT-type neurons that project to the pontine nucleus (PN) or IT-type neurons that project to the cingulate of the opposite hemisphere (commissural cells, COM). Recording serial, adjacent pairs in P21-25 WT mice, we found that the frequency of mIPSCs was significantly lower in COM neurons compared to adjacent PN neurons (Wilcoxon matched-pairs signed rank test p=0.007) (**Fig. 2.3H,I**), while mIPSC amplitude was comparable between projection neuron types (**Fig. 2.3G**). Our result suggests that there are cell-type and pathway-specific differences in mIPSC frequency.

mIPSCs increase in IT- but not PT-type neurons during adolescence

We next wanted to determine whether inputs onto different subtypes of pyramidal

neurons changed across periadolescent development. To study the maturation of

inhibition onto layer 5 pyramidal neurons we made mPSC recordings in young adult mice

at P40-50 and compared these recordings to those made in juveniles (P21-25) in the same

anterior cingulate region. Comparisons revealed no significant change in mEPSC

amplitude or frequency measures from P21-25 to P40-50 in YFP+, YFP-, or in COM

neurons (**Fig. 2.4A,B**). However, developmental comparisons revealed a significant

increase in mIPSC amplitude (**Fig. 2.4C-E**) and a trend toward an increase in mIPSC

frequency (**Fig. 2.4F-H**) in IT-type neurons: YFP- (amplitude p<0.001; frequency

p=0.08) and COM neurons (amplitude p=0.03, frequency p=0.09). This periadolescent

increase in mIPSC measures was similar between male and female mice (**Fig. 2.5**). In

contrast, mIPSC amplitude and frequency in PT-type YFP+ neurons remained constant

(**Fig. 2.4C,F**). These data suggest that while mEPSCs do not change in cingulate PT and

IT-type neurons across peri-adolescence (P25-40), mIPSCs show a developmental

increase in IT- type neurons alone.

To further elucidate cell-type specific differences in mIPSC development, we also

recorded from neurons identified by Cre expression in a dopamine D1 receptor BAC

transgenic line crossed to a Cre reporter line (Ai-14 td tomato). Studies from other labs

suggest that cortical D1-Cre+ neurons are IT-type neurons, while cortical D2-Cre+

neurons overlap with PT-type populations (**Fig. 2.1**). By crossing Thy-1-YFPH mice

with the D1-Cre x Ai14 tdTomato line, we found that D1+ neurons had no overlap with

YFP+ neurons in adult animals (**Fig. 2.6A**) (were YFP-). However, when recording from these neurons we did not see a periadolescent increase in mIPSC amplitude as expected from other IT-type neurons (**Fig. 2.6B**). These data suggest that the PT and IT-type neuron classes likely contain additional sub-types (Fame et al., 2011; Otsuka and Kawaguchi, 2011) that may follow different patterns of maturation.

Periadolescent disruption of TrkB signaling stalls maturation of mIPSCs in IT-type neurons

We next wanted to study the role that TrkB signaling played in the developmental increase of mIPSCs in layer 5 projection neurons. To study this we used a chemical-genetic approach to inhibit TrkB signaling. We bred Thy1 YFPH mice to the $TrkB_{F616A}$ line. In the $TrkB_{F616A}$ line, a phenylalanine to alanine substitution in the ATP binding pocket allows for the temporally specific inhibition of TrkB when it is in the presence of a small molecule inhibitor 1NM-PP1 (Chen et al., 2005a). Importantly, the mutation is functionally silent without the inhibitor (Chen et al., 2005a; Kaneko et al., 2008). We used osmotic minipumps to systemically deliver either 1NM-PP1 or vehicle (DMSO in saline) in these mice from P23 to the day of recording between P40-50 (**Fig. 2.7A**).

Comparisons of mEPSC measures in periadolescent vehicle treated $TrkB_{F616A}$ mice (white) and 1NM-PP1 treated $TrkB_{F616A}$ mice (blue) showed no significant treatment effect for YFP- neurons (**Fig. 2.7B,C**) or YFP+ neurons (data not shown).

Vehicle treated control mice achieved expected developmental increase in mIPSC amplitude in YFP- neurons by P40-50, but 1NM-PP1 treated animals did not (**Fig. 2.7D,E**) (one-way ANOVA, $F(4,43)=3.565$, $p=0.01$; Bonferroni post-hoc test P40-50 1NM-PP1 treated animals vs. age matched vehicle treated controls ($p<0.05$), untreated juvenile mice vs. P40-50 day old 1NM-PP1 treated animals ($p>0.05$)). mIPSC amplitude in YFP+ neurons recorded from the same TrkB$_{F616A}$ mice were not affected by the presence of 1NM-PP1 (One-way ANOVA, $F(4,43)=3.565$, $p=n.s.$)(**Fig. 2.7D**). Comparisons between treatment groups also revealed differences in mIPSC frequency between periadolescent vehicle treated mice and 1NM-PP1 treated mice in YFP- neurons (One-way ANOVA, $F(4, 43)=5.867$, $p<0.001$). A Bonferroni post-hoc test confirmed that mIPSC frequency in YFP- neurons in 1NM-PP1 treated mice was lower than in the vehicle treated control group ($p<0.05$)(**Fig. 2.7F,G**). In YFP+ neurons, mIPSC frequency was unaffected by treatment with 1NM-PP1 (**Fig. 2.7F**).

To ensure that 1NM-PP1 did not have unanticipated off-target effects, we also recorded mIPSCs in YFP- neurons in P40-50 WT mice treated with either 1NM-PP1 or vehicle over the same time period as TrkB$_{F616A}$ mice discussed above. We found no significant difference in mIPSC amplitude between vehicle and 1NM-PP1 treated WT mice ($p=0.30$, vehicle n=12; 1NM-PP1 n=8, data not shown).

Our data show that the IT-type sub-population of Layer 5 neurons (YFP-) show a TrkB dependent late developmental (P25-P40) increase in mIPSC amplitude and frequency,

while a neighboring subtype (PT-type, identified using YFP+), that send axons subcortically and subcerebrally, do not.

Discussion

By recording mPSCs on identified cell types in the mouse anterior cingulate cortex, we found that inhibitory synapses onto layer 5 pyramidal neurons that project to different targets show clear differences (**Fig. 2.3**). Specifically, we found that PT-type neurons (defined by YFP+ expression in the Thy1 YFP-H transgenic line or retrobead fluorescence after pons injection) have higher mIPSC frequency rates than adjacent IT-type neurons (defined by YFP- expression or retrobead fluorescence after contralateral cingulate injection) in juvenile mice. Although we cannot yet say whether this due to difference in number of inhibitory synapses or probability of release, our data suggest that interneurons in the cingulate cortex selectively regulate different information-processing streams.

Our second finding is that in the adolescent transition to young adulthood, inhibitory synapses on IT-type neurons increase in amplitude (**Fig. 2.4**) while adjacent PT-type neurons do not. This finding suggests that cortical interneuron synapses onto pyramidal neurons differ in developmental trajectory depending on the principal neuron projection target. Furthermore, these data suggest there is cell-type specific remodeling of information processing between the frontal hemispheres across adolescence. Interestingly, in healthy human subjects there is a decrease in functional connectivity

between frontal hemispheres from childhood to adulthood (Fair et al., 2007) that could potentially be mediated by an increase in inhibition onto commissural projecting cells.
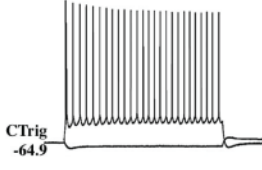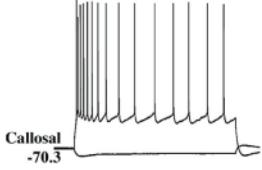
Finally we find that the late maturation of inhibitory synapses on IT-type neurons can be blocked by disrupting TrkB signaling without affecting mEPSCs on the same IT-type neurons or inhibitory synapses on neighboring PT-type neurons (**Fig. 2.7**). Moreover, we find that inhibiting TrkB signaling during periadolescence has different effects on neuronal subtypes with different projection targets. Namely, TrkB inhibition reduced the frequency of mIPSCs in IT-type neurons, suggesting either a decrease in probability of release and/or number of synapses, while having no significant effect on PT-type neurons. Our results support the conclusion that inhibition can specifically target and regulate specific subcircuits, and that different subtypes of cortical neurons are differently affected by TrkB activity during periadolescent development.

Relevance to mental illness

These results have implications for disorders thought to be neurodevelopmental in origin, particularly schizophrenia. Developmental studies in healthy human subjects show changes in cortical-cortical oscillatory activity and increased cortical synchrony over the adolescent period (Fair et al 2007; Uhlhaas and Singer, 2011). Moreover, schizophrenic patients have been found to display decreased synchrony between long-range cortical areas (Uhlhaas and Singer, 2010) and abnormalities of white matter in the cingulum bundle (Kubicki et al 2003; Whitford et al. 2010). Here we show that mIPSC amplitudes increase across the periadolescent period (P21-25 to P40-50) in IT-type neurons. Our data

suggest there is inhibition-mediated remodeling of information processing between the

frontal hemispheres across adolescence. We predict these changes might enhance

synchronization between the two hemispheres, and that reducing inhibition in IT-type

neurons, by TrkB inhibition or other means, may produce behavioral abnormalities such

as seen in schizophrenia. These findings suggest deficits in IT-type neuron maturation

might be particularly relevant to schizophrenia and other late onset developmental

disorders.

Figures

	PT-type	IT-type
Target Hattox and Nelson 2007 Porrero *et al.* 2010	Pyramidal tract Superior Colliculus Pons	Telencephalic Ipsi and Contra Cortex
Morphology Angulo *et al.* 2003 Hattox and Nelson 2007 Dembrow *et al.* 2010	Tufted	Simple or non-tufted
Firing Pattern Hattox and Nelson 2007 Dembrow *et al.* 2010 Sheets and Shepherd 2011	CTrig -64.9 Non-Adapting	Callosal -70.3 Adapting
I_h Current Dembrow *et al.* 2010 Gee and Sohal 2012	Present	Absent
D1 or D2 containing Seong and Carter 2012 Gee and Sohal 2012	D2	D1
YFP or Non-YFP **enriched in Thy1-YFPH** Miller *et al.* 2008 Sheets and Shepherd 2011	YFP	Non-YFP

Figure 2.1 Subtypes of Layer V pyramidal neurons. Layer 5 pyramidal neurons fall into two major categories that can be distinguished on the basis of their projection site, morphology, physiological properties and major receptors. IT-type: neurons with only intratelencephalic connections, PT-type: neurons sending their main axon to the brainstem via the pyramidal tract (Reiner et al., 2010). Figure adapted from Molnar and Cheung 2006
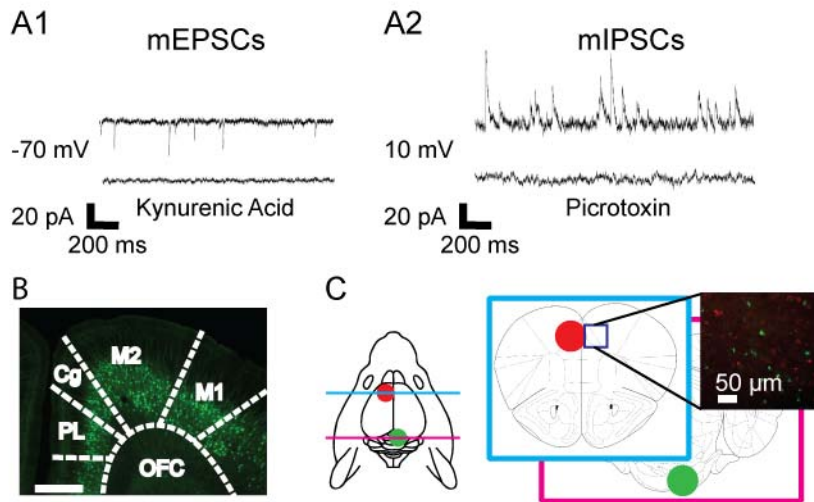
Figure 2.2 Methods. *A1.* Miniature excitatory post synaptic currents (mEPSCs) were recorded at -70 mV in the presence of TTX (top). mEPSCs were abolished with the nonspecific glutamate antagonist, kynurenic acid (bottom). *A2.* Miniature inhibitory post synaptic currents (mIPSCs) were recorded in the same neurons at -70 mV (top), and were abolished by the GABA$_A$ receptor antagonist, picrotoxin (bottom). *B.* Miniature post synaptic currents were recorded from YFP+ and YFP- pyramidal cells in the anterior cingulate (Cg) of Thy-1 YFPH line mice. PL, Prelimbic; OFC, Orbitofrontal cortex; M2, secondary motor cortex; M1, primary motor cortex (Franklin and Paxinos, 2008). *C.* Retrograde beads were injected into WT mice, allowing us to record from pontine-projecting and cingulate-projecting neurons within the same slice. Recordings were targeted to layer 5 of the Cg region.
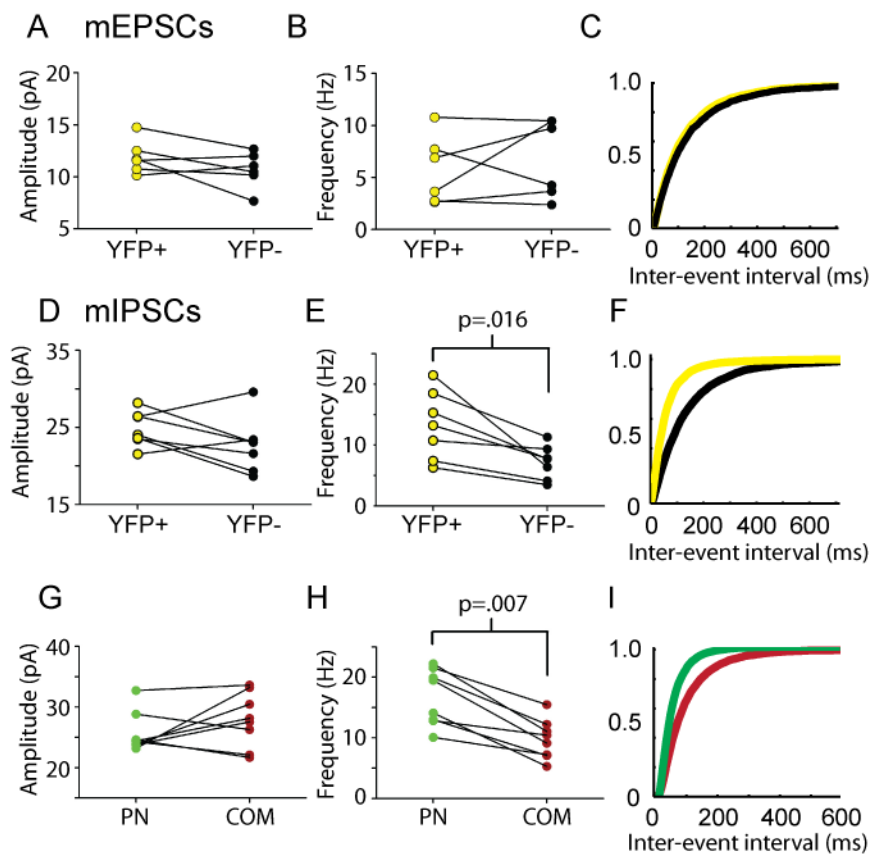
Figure 2.3 mIPSC frequency varies by pyramidal cell subtype. *A.* Comparison of mEPSC between serially recorded pairs of YFP+ and YFP- neurons revealed that mEPSC amplitude and frequency (*B, C*) were similar across pyramidal cell subtypes. *D.* Comparison of mIPSC between serially recorded pairs of YFP+ and YFP- neurons revealed that while mIPSC amplitudes were not different between cell types, mIPSC frequency was higher in YFP+ neurons than in YFP- neurons (*E, F*). *G.* In pairs of adjacent cortico-pontine (PN, green) projecting neurons and commissural (COM, red) neurons, PN neurons have similar amplitudes to adjacent COM neurons, but increased mIPSC frequency compared to adjacent COM neurons (*H, I*).
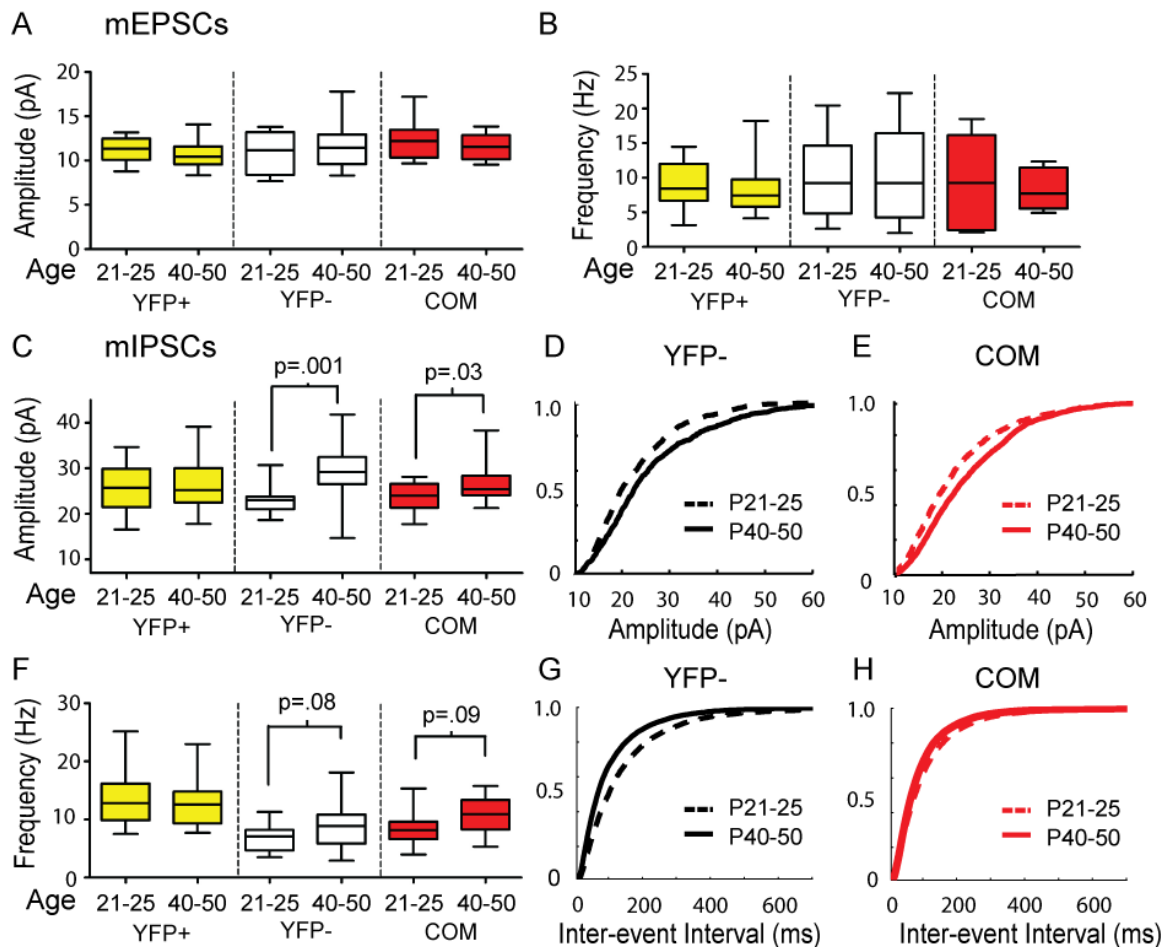
Figure 2.4 mIPSC amplitude increases over the periadolescent period in a subtype of neuron in the mouse cingulate. *A*. Over the periadolescent period (P21-25 to P40-50), there is no change in mEPSC amplitude or frequency (*B*) in YFP+, YFP-, or COM neurons. *C.* Layer 5 YFP- and COM neurons, but not YFP+ neurons, showed an increase in mIPSC amplitude over the periadolescent period. *D*. Cumulative probability graphs of mIPSC amplitude in YFP- populations further illustrate the developmental increase from P21-25 to P40-50 (KS test, p<0.0001). *E*. Cumulative probability graphs of mIPSC amplitude in COM neurons illustrate an increase in amplitude in COM neurons with age (KS test, p<0.0001). *F*. Average values of the inter-event interval in YFP- and COM neurons showed a trend toward increasing mIPSC frequency during the periadolescent period, while YFP+ neurons did not. *G*. More sensitive cumulative probability graphs of inter-event interval showed a significant increase in mIPSC frequency over peri-adolescence in YFP- (KS test, p<0.0001) and COM neurons (*H*) (KS test, p<0.0001).
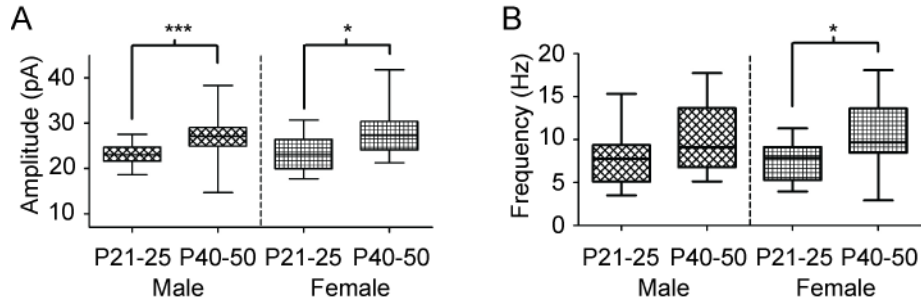
Figure 2.5 mIPSC amplitude increases over the periadolescent period in a subtype of neuron in both males and females. *A.* Layer 5 YFP- neurons show a similar increase in mIPSC amplitude over the periadolescent period in both males left (p21-25 n=15, p40-50 n=25, p=.0006) and females (p21-25 n=9, p40-50 n=23, p=0.03). *B*. Average values of the inter-event interval in YFP- neurons showed a trend toward increasing mIPSC frequency during the periadolescent period in males (p=0.07) and a significant increase in females (p=0.05).
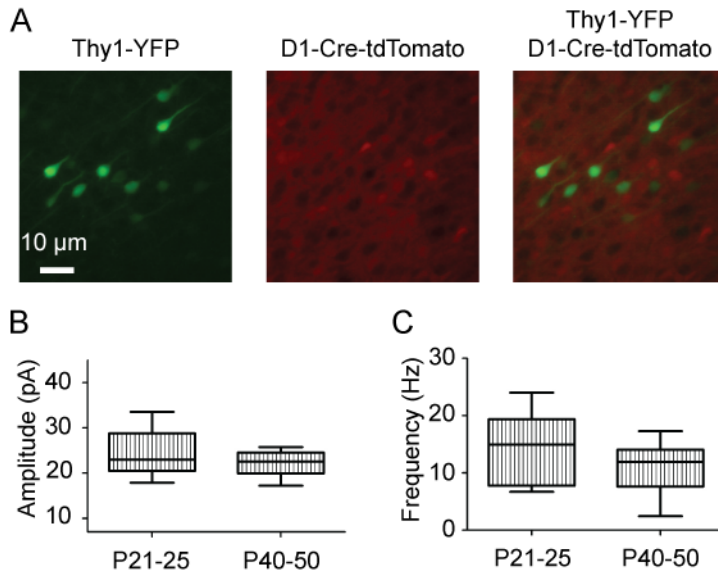
Figure 2.6 mIPSC amplitude does not increase over the periadolescent period in D1+ neurons. *A.* Anterior cingulate of Thy-1-YFPxD1-Cre-tdTomato adult mouse showing YFP+ neurons (left panel), tdTomato+ neurons (middle) and overlap (right). There is little to no overlap between YFP+ neurons and D1+ neurons. *B.* Despite having considerable overlap with YFP- neurons, there is no increase in mIPSC amplitude or (*C*) frequency in D1+ neurons over peri-adolescence (P21-25 n=9, P40-50 n=19).
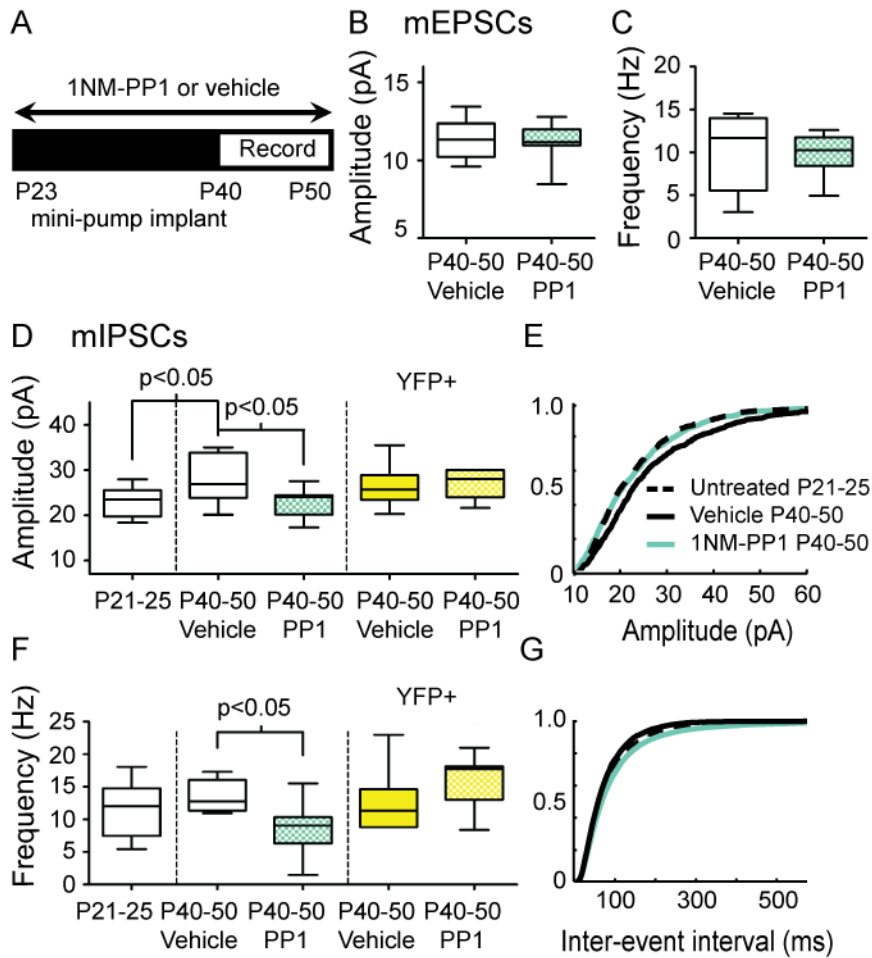
Figure 2.7 Inhibiting TrkB blocks the periadolescent maturation of inhibition in YFP- neurons. *A.* Experimental timeline. *B.* Comparisons between juvenile and periadolescent vehicle-treated TrkB$_{F616A}$ mice (white) and 1NM-PP1 treated TrkB$_{F616A}$ mice (blue) showed no significant differences in mEPSC amplitude or frequency (*C*) between groups. *D.* In non-YFP neurons TrkB$_{F616A}$ mice (white) and 1NM-PP1 treated TrkB$_{F616A}$ mice (blue) showed significant differences in mIPSC amplitude at P40-50 (one-way ANOVA, $F_{(4,43)}=3.565$, $p=0.01$), while YFP+ neurons did not. *E.* Cumulative probability graphs: untreated juvenile TrkB$_{F616A}$ mice (P21-25, dotted line) are significantly different from P40-P50 vehicle controls (solid line) (KS test, $p<0.0001$). 1NM-PP1 treated TrkBF$_{F616A}$ mice (blue) overlapped with juveniles (n.s.) and showed a significant difference from age matched vehicle treated TrkB$_{F616A}$ controls (KS test, $p<0.0001$). *F.* Comparisons between juvenile TrkB$_{F616A}$ mice and periadolescent drug and vehicle treated TrkB$_{F616A}$ mice showed significant differences in mIPSC frequency between groups (one-way ANOVA, $F_{(4,43)}=5.867$, $p<0.001$) in non-YFP neurons but not YFP+ neurons. *G.* Cumulative probability graphs of inter-event intervals in TrkB$_{F616A}$ mice: untreated juvenile TrkB$_{F616A}$ mice (P21-25, dotted line) are significantly different from P40-P50 vehicle controls (solid line) (KS test, $p<0.0001$). 1NM-PP1 treated TrkB$_{F616A}$ mice (blue) also showed a significant difference from age-matched vehicle treated TrkB$_{F616A}$ controls (KS test, $p<0.0001$).

Methods

Animals. All experimental procedures were approved by the Ernest Gallo Clinic and Research Center Animal Care and Use Committee. WT (n=12) and Thy1-eYFP line H mice (YFPH) (n=20) (Jackson Labs, line 003782)(Feng et al., 2000) were used to identify subtypes of neurons in the frontal cortex. We made use of the YFPH line of mice because only a subset of Layer 5 pyramidal neurons are labeled in these mice (Feng et al., 2000; Sugino et al., 2006) (Fig. 1A). YFP+ and YFP- cells in this line have previously been shown to receive differential inputs from excitatory neuron in layers II/III of the motor cortex (Yu et al., 2008), to have different firing properties (Sugino et al., 2006; Yu et al., 2008), and to project to different targets (Porrero et al., 2010). A major proportion of YFP+ neurons have been shown to send descending axons to the pons, spinal cord, and pyramidal tract (Miller et al., 2008; Porrero et al., 2010), whereas YFP- layer 5 neurons are thought to mainly correspond to populations that communicate within the telencephalon and across the corpus callosum (Porrero et al., 2010). Both male and female mice were used for experiments and no significant sex differences were found (mIPSCs YFP- and WT COM pooled: P21-P25 p=0.87, n=15 male and n=9 female; P40-50 p=0.73, n=25 male and n=23 female).

C57BL/6J BAC transgenic mice expressing Cre recombinase (Gong et al., 2007) under the regulatory elements for the D1 receptor (D1-Cre) were obtained from Mutant Mouse Regional Resource and crossed to an Ai14-Cre reporter strain, which express the red fluorescent protein variant, tdTomato, following Cre-mediated recombination (n=11).

Ai14 mice were hemizygous for Rosa-CAG-LSL-tdTomato-WPRE::deltaNeo conditional allele and were obtained from the Allen Institute.

TrkB$_{F616A}$ mice obtained from the David Ginty lab via the Michael Stryker lab (Chen et al., 2005a) were crossed to Thy1 YFPH mice. Resultant mice were used to study the impact of blocking TrkB signaling during a specific window of development using 1NM-PP1 (n=11).

Injection of retrogradely transported microspheres. Surgical procedures were conducted under deep isoflurane anesthesia (1.5-3% in oxygen). Anesthetized mice were placed in a stereotaxic apparatus for craniotomy. Fluorescent retrobeads (undiluted, LumaFluor, Inc.) were injected into the left cingulate cortex and/or right pontine nucleus. Coordinates for cingulate (in millimeters relative to bregma) were: 2.1 anterior-posterior (AP), 0.4 mediolateral (ML), and 0.5, 0.7 and 1.0 dorsoventral (DV). Coordinates for injections into the pontine nucleus (PN) were: -4.26 AP, 0.4 ML, and 4.6 DV (Franklin and Paxinos, 2008). Animals were sacrificed 72 hours after microsphere injection.

1NM-PP1 and minipump implantation. 1NM-PP1 (Cayman Chemical) or vehicle solution (4% (vol/vol) DMSO and 2% (vol/vol) Tween-20 in saline) was administered via Alzet minipumps. Under isoflurane anesthesia, minipumps were implanted subcutaneously under the scruff of the neck at P23 and remained in place until animals were sacrificed for electrophysiology (before P51). 1NM-PP1 was delivered at the rate of 6.25 nmol/h, a concentration used in previous reports (Kaneko et al., 2008).

Slice preparation. Mice were deeply anesthetized with a lethal dose of ketamine and

xylazine and transcardially perfused with ice-cold cutting solution containing (in mM):

110 choline-Cl, 2.5 KCl, 7 $MgCl_2$, 0.5 $CaCl_2$, 25 $NaHCO_3$, 11.6 Na-ascorbate, 3 Na-

pyruvate, 1.25 NaH_2PO_4, and 25 D-glucose, and bubbled in 95% O_2 5% CO_2. Coronal

sections (300 μm thick) were cut in ice-cold cutting solution and then transferred to

ACSF containing (in mM): 120 NaCl, 2.5 KCl, 1.3 $MgCl_2$, 2.5 $CaCl_2$, 26.2 $NaHCO_3$, 1

NaH_2PO_4 and 11 Glucose. Slices were bubbled with 95% O_2/5% CO_2 in a 35° C bath for

30 minutes, and allowed to recover for at least 30 minutes at room temperature before

recording.

Electrophysiology. Recordings were obtained from layer 5 pyramidal neurons in the

cingulate cortex (Cg) (Franklin and Paxinos, 2008). Whole cell voltage clamp miniature

excitatory post synaptic currents (mEPSCs) were recorded at -70 mV and inhibitory post

synaptic currents (mIPSCs) were recorded in the same neurons at 10 mV (**Fig. 2.2A**).

Kynurenic acid and picrotoxin confirmed the currents recorded at this voltage were

consistent with EPSCs and IPSCs, respectively (**Fig. 2.2A**). Recording pipettes having a

series resistance of 3-4 MΩ were filled with intracellular solution (in mM): 135 Cs-

methanesulfonate, 10 HEPES, 10 BAPTA, 10 Na2-phosphocreatine, 4 MgCl2, 4 Na2-

ATP, 0.4 Na-GTP, 3 Na-L-ascorbate. In some experiments 30 μM of Alexa-594 was

added to the internal solution in order to visualize recorded neurons. Recordings were

made using Multiclamp 700B amplifier and were not corrected for liquid junction

potential. Miniature postsynaptic currents (mPSCs) were pharmacologically isolated by

bath application of 1 µM tetrodotoxin. Only recordings with series resistance of <25MΩ were included in the analysis. Series resistance was recorded every 30 seconds, and cells were discarded if parameters changed more than 20%. All experiments were conducted at 32°C. All chemicals were from Sigma, Tocris Biosciences, or Fluka.

Statistics. Mann-Whitney test and Wilcoxon tests were used to compare groups and a D'Agostino and Pearson omnibus test was used to test normality (Graphpad Prism) unless otherwise noted. Two-sample Kolmogorov-Smirnov (KS) test (Matlab, Mathworks) was used to compare cumulative distributions. Results are presented as mean±SEM, and differences were considered significant if $p < 0.05$.

Chapter 3: BDNF Val68Met KI mice show greater behavioral flexibility in reversal learning tasks than WT littermates

Introduction

Many of the functions of the prefrontal cortex (PFC) fall under the term 'executive function,' which refers to the ability to plan and perform goal-directed actions. These include working memory, complex problem solving, planning, and behavioral inhibition (Alfimova et al., 2012). Executive function abnormalities are a key clinical pathology in many psychiatric disorders including schizophrenia, autism, and bipolar disorder (Pellicano et al., 2006; Wobrock et al., 2009).

Studies of executive functions in humans have identified common genetic polymorphisms that impact behavioral performance presumably by altering PFC function (Alfimova et al., 2012). A recently discovered variant in the gene that encodes brain derived neurotrophic factor (BDNF) (Egan et al., 2003) has been of considerable interest due to the known role of BDNF in neurodevelopment and plasticity. This common single nucleotide polymorphism (SNP) encodes a valine (Val) to methionine (Met) substitution at codon 66 at the prodomain of the gene (Val66Met). The Met substitution confers a trafficking deficit which results in decreased activity dependent release of BDNF (Chen et al., 2006). This polymorphism occurs in 20-30% of the population and has been linked to deficits in select forms of memory (Egan et al., 2003), as well as susceptibility to certain psychiatric disorders (Angelucci et al., 2005; Cheng et al., 2005; Chen et al.,

2006; Gratacos et al., 2007; Joffe et al., 2009). Previous studies of human subjects have indicated that BDNF Met carriers have compromised cognitive function (Miyajima et al., 2008; Alfimova et al., 2012). However, these results can be reinterpreted in a potentially positive light. There is a growing body of literature suggesting that the Met allele may confer benefits in certain executive functions (Erickson and Lewis, 2002; Ventriglia et al., 2002; Matsushita et al., 2005; Beste et al., 2010a; Beste et al., 2010b; Gajewski et al., 2011, 2012; Getzmann et al., 2013). A study by Beste *et al.* showed that carriers of the Met allele had fewer false alarms on a Go/No-Go task, which correlated with larger Nogo-N2 event related potentials (ERPs) in Met carriers (Beste et al., 2010b). Another 10 year longitudinal study looked at executive function in elderly individuals. Only Val homozygotes demonstrated a significant reduction in performance over time, suggesting that the Met allele may have a neuroprotective effect against cognitive decay in old age (Erickson and Lewis, 2002). These results have inspired the hypothesis that the BDNF Val66Met polymorphism may be a risk factor during periods of development when BDNF levels are high (early adolescence), and a protective factor when levels are low, such as in early childhood or old age (Casey et al., 2009). Given the critical role of BDNF signaling in regulating the onset of sensitive period plasticity in the sensory cortex we were particularly interested in the impact of the BDNF Val66Met polymorphism on the timing of the maturation of PFC function. Previous studies from our lab have found that mice show changes in reversal learning over adolescent development (Johnson and Wilbrecht, 2011)(**Fig. 1.2**). We predicted that BDNF Val66Met polymorphism might delay the maturation of reversal behavior to adult levels.

In this study we used a mouse model with a Met knock-in mutation at codon 68 (in mice codon 68 is homologous to the human codon 66) (**Fig. A1**). This animal has been shown to have anxiety phenotypes similar to the published mouse and humans with the Met polymorphism (Warnault et al, submitted; Chen et al 2006). We tested BDNF Val68Met mice on a battery of tasks that require behavioral inhibition and cognitive flexibility to ascertain the effect of the BDNF mutation on executive functions. As the Val66Met polymorphism is thought to confer distinct behavioral and functional consequences at different developmental periods, we looked at performance on these tasks at two different ages: At P25, when BDNF levels peak (Katoh-Semba et al., 1997; Patz and Wahle, 2006; Casey, 2009; Guo et al., 2010); and in adulthood (P60-90). We find that animals with the Met allele show more rapid reversal learning on two different paradigms but learn at rates comparable to Val homozygotes on a discrimination task, a go/no-go task, and have similar rates of appetitive extinction. Moreover, we find that differences in reversal performance between Val/Val and Met/Met mice are more pronounced in adults than at P25 which suggests that the behavioral consequences of the BDNF trafficking deficit become more pronounced when BDNF levels are decreasing. Our results suggest that BDNF Met carriers are likely to respond differently to changes in the environment and to use different cognitive strategies for decision making.

Results

Previous studies have suggested that human carriers of the Met allele have fewer errors on a go/no-go task than homozygous Val individuals (Beste et al., 2010a). To determine if the same was true in Val68Met knock-in mice we tested adult mice (P60-90) on an

automated odor discrimination go/no-go task (**Fig. 3.1A**). In this task animals would

nose-poke in a center poke for odorant cue (either a "go" or "no-go" cue) and then move

to an adjacent port to receive water. Water reward was given for a correct "go" response

and a 4 second time-out was given for incorrect "no-go" responses. There was a 30%

probability of receiving a "no-go" cue. This task had three phases: a shaping phase

where the animals learned the task with odorants A ("go" cue vanilla) and B ("no-go" cue

cinnamon); a phase where novel odorants C ("go" cue bay) and D ("no-go" cue basil)

were introduced; and a reversal phase where D became the "no-go cue" and C the "go"

cue. We found that homozygous Val and Met littermates (P60-90) performed similarly

on the first two phases of the task, making a similar number of "no-go" errors (**Fig. 3.2

A,B**) (Val/Val n=10, Met/Met n=10). However, on the reversal phase of the task Met

homozygous animals reversed faster and made fewer numbers of reversal errors than Val

homozygous mice (**Fig. 3.2C**). A two-way repeated measures (rm) analysis of variance

(ANOVA) showed a significant main effect of genotype and session number (genotype

$F(1,99)=6.93$, $p=0.0098$, session $F(5,99)=23.16$, $p<0.0001$) but no significant interaction

between the two ($F(5,99)=0.55$, $p>0.05$) **Fig. 3.2C**).

In order to determine whether the faster reversal rates in Met animals were due to faster

rates of extinction learning or reversal learning, we tested another cohort of Val68Met

littermates on an extinction task using the go/no-go paradigm. The first two phases of the

task were identical to the go/no-go task, above. On the final phase of the task animals

were given 30 minutes of "maintenance" trials (where water reward was made available

for correct "go" trials and a time-out for incorrect "no-go" responses) followed by one

hour of within session extinction trials (wherein nose-poke responses had no consequence and water delivery was unavailable). We found similar extinction rates for both homozygous Val and Met animals when we looked at either nose-pokes into the cue port (**Fig. 3.2D**) or number of completed trials (nose-poke followed by water port or "no-go" response.) (Val/Val=8, Met/Met=7) (**Fig. 3.2E**). These experiments suggest that differences in extinction learning do not explain differences in reversal learning found in BDNF Val68Met mice.

To explore the maturation of behavioral flexibility between BDNF Val68Met mice, we tested mice on a 4-choice odor discrimination task (**Fig. 3.1B**). This task has been used previously to test behavioral flexibility in rats (Ragozzino and Rozman, 2007) and mice (Johnson and Wilbrecht, 2011). During the discrimination phase of this task animals were taught to dig for buried food reward in pots with differently scented shavings. Only one scent was rewarded and pots were shifted after each trial, ending when the animal reached 8 out of 10 correct trials. We found that adult Val/Val and Met/Met mice learned the discrimination task at similar rates (**Fig. 3.3A**). However, during the reversal phase, in which a previously irrelevant odor predicted the location of the reward, Met/Met mice accumulated fewer total errors before reaching criterion (**Fig. 3.3B**) (Val/Val n=14, Met/Met n=14, $p<0.0001$). Isolation of errors to reversal errors (trials in which they dug in the previously rewarded pot) also showed fewer cumulative reversal errors before criterion in Met/Met vs. Val/Val mice (**Fig. 3.3C**). ($p<0.0001$). Upon further breakdown of errors, we found Val/Val and Val/Met animals made more errors before the first correct trial than Met/Met mice (**Fig. 3.3D)**. A one way ANOVA did not reveal any effect

of genotype on latency to dig, (F(2,35)=1.289, p=0.1074), or the number of entries into different quadrants (F(3,47)=1.582, p=0.097) between the three genotypes. However, a post-hoc test for linear trend found a gene "dose dependent" decrease in trial latency (p=0.03) and number of entries into different quadrants (p=0.03) between Val/Val, Val/Met, and Met/Met mice (**Fig. 3.3E,F**). These results suggested that in adults the number of copies of the Met mutation in the BDNF gene can impact some behaviors in a dose-dependent fashion.

We next tested a cohort of juvenile (P25) BDNF Val68Met mice on the 4-choice discrimination and reversal task. We found that, as in adults, young homozygous Val and Met animals performed similarly on the discrimination phase of the task (**Fig. 3.4A**)(Val n=10, Met=11). However, during the reversal phase there was no significant effect of genotype on accumulation of errors before reaching criterion (both for total errors or reversal type errors)(**Fig. 3.4B,C**). When we isolated our analysis to errors before 1 correct (**Fig 3.4D**), a two way rm ANOVA found that there was no effect of genotype (F(1,133)=1.12, p>0.05) or interaction between age and genotype (F(7,133)=1.49, p>0.05). However, a Bonferroni post-hoc comparison did find that juvenile Val/Val mice made more errors than Met/Met mice prior to receiving one reward (similar to adults) (p<0.01). To further quantify the difference between young and adult animals, we also plotted the difference in cumulative errors between Val/Val and Met/Met mice in young and adult cohorts (**Fig. 3.4E,F**). The area under the curve was significantly larger for the adult cohorts than the young both in terms of total errors (p=0.0006)(**Fig. 3.4E**, inset) and reversal errors (p=0.0028) (**Fig. 3.4F**, inset), suggesting that there is a greater difference

in behavioral flexibility between adult Val/Val and Met/Met mice than in young mice. An analysis of the number of perseverative errors (trials in which the animal dug in the previously rewarded pot before getting one correct trial) also revealed differences between genotypes at different ages. A two way ANOVA revealed a significant main effect of genotype ($F_{(1,38)}=8.07$ $p<0.01$), but not age ($F_{(1,38)}=2.06$), or a significant interaction between age and genotype ($F_{(1,38)}=1.81$). A Bonferroni post-hoc test revealed that there was a significant difference in perseverative errors between adult homozygous Val and Met animals ($p<0.01$), but not young animals. These data support the idea that behavioral differences between homozygous Val and Met mice may change during different developmental epochs. Finally, two way ANOVAs of latency and number of entries found no significant main effect of genotype (entries $F_{(1,44)}=1.03$, latency $F_{(1,44)}=0.83$) or age (entries $F_{(1,44)}=0.29$, latency $F_{(1,44)}=0.30$), or an interaction between the two (entries $F_{(1,44)}=1.92$, latency $F_{(1,44)}=2.99$).

Discussion

This study demonstrates that although BDNF Val/Val and Met/Met mice perform similarly on odor discrimination tasks, homozygous Met mice show faster reversal learning than Val/Val mice on two separate tasks. We also show that the difference between BDNF Val/Val and Met/Met mice is not due to differences in behavioral inhibition alone, since both Val/Val and Met/Met mice made a similar number of "no-go" errors on a go/no-go task. Similarly, the difference between BDNF Val/Val and Met/Met mice cannot be attributed to faster appetitive extinction learning in Met/Met mice, since homozygous Val and Met mice showed similar extinction rates. Our study suggests that

adult BDNF Met/Met mice have greater cognitive flexibility than Val/Val mice in reversal tasks known to depend on the integrity of the PFC (Ragozzino and Rozman, 2007; Bissonette et al., 2008; Johnson and Wilbrecht, 2011). BDNF Val68Met mice, which model a common human mutation, respond more rapidly to changing environmental contingencies in a manner that is consistent across distinct tasks. Our data suggest humans that carry this polymorphism may have different decision making styles and strategies.

Testing of BDNF Val68Met mice at juvenile and adult ages suggest that these cognitive differences emerge with development. While adult mice showed clear genotype differences in reversal learning performance, juvenile homozygous Val and Met mice (P25) were similar for most measures. It may be that differences in behavioral flexibility in BDNF Val68Met mice may be less pronounced during early adolescence when BDNF levels are peaking (about P25) (Katoh-Semba et al., 1997; Ivanova and Beyer, 2001; Patz and Wahle, 2006; Guo et al., 2010) than later periods when BDNF levels are relatively low. It could also be that maturation of the frontal cortex is delayed or stalled in these mice due to later initiation of BNDF dependent sensitive period mechanisms.

In either case, our data are in line with gene-environment interaction models, which suggest that specific polymorphisms, instead of predicting risk or resilience, confer a differential responsiveness to the environment that changes with age (Belsky et al., 2009; Casey, 2009). For example, a recent longitudinal study that looked at children that were raised with either quality foster care or reared in an institution, showed that children with the BDNF Met allele demonstrated the highest level of indiscriminant behavior

(unrestrained social boundaries) in the institutional setting and the lowest level of indiscriminant behavior in the foster care environment. Val/Val children however, demonstrated little difference in levels of indiscriminant behavior in either environment (Drury et al., 2012). This study is in line with many recent studies that suggest that 'vulnerability' genes such as the BDNF Met polymorphism may predict greater responsiveness to both enriched and deprived environments (Belsky et al., 2009; Casey, 2009; Drury et al., 2012). In this context it is interesting to note that the animals in this study were raised in a semi-enriched environment (co-housed with bedding and toys). In future studies we plan to use maternal separation to model early life adversity and test if BDNF Met mice show different impacts of stress on cognitive flexibility when compared to Val/Val littermates. Future studies will also be important to determine how brain development is altered in BDNF Val68Met mice, particularly in prefrontal cortical circuits and other brain regions known to support flexibility in reversal learning.

Figures
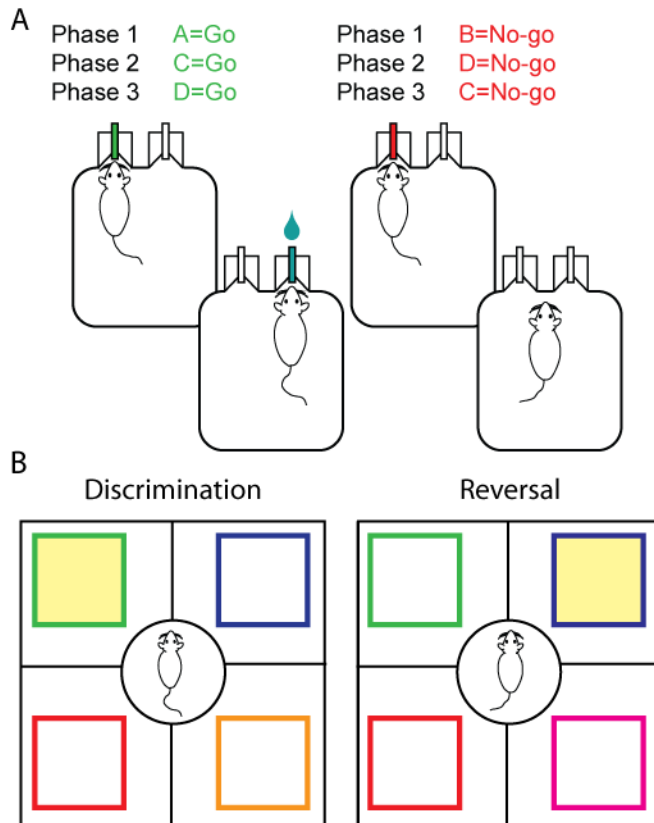
Figure 3.1 Methods. *A. Automated go/no-go task.* Mice learned to nose-poke at the cue port for odorant cue. Cues indicated either "go" to the water port to receive water, or "no-go." Incorrect trials initiated a 4 second time-out. The task had three phases: In phase 1 (shaping) animals learned the task by responding to odorants A (go cue) and B (no-go cue); In phase 2 new odorants C (go cue) and D (no-go cue) were introduced; In phase 3, odorants C and D were reversed so that C became the "no-go" cue and D became the "go" cue. *B. 4-choice discrimination and reversal learning task.* Scented shavings were introduced in 4 pots. During the initial discrimination training animals learned to discriminate odors in order to find a buried food reward. Pots were shifted after each trial and the task ended when the animal reached 8 out of 10 correct trials. During the reversal task a previously irrelevant odor predicted the location of the reward and a novel odor was introduced.
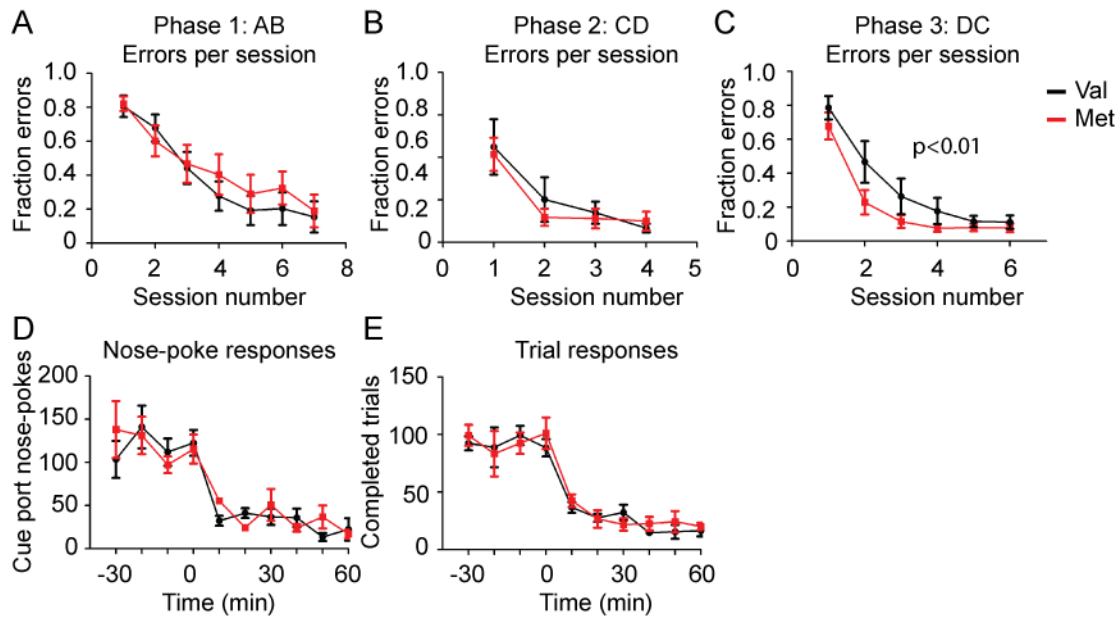
Figure 3.2 Met/Met animals have a comparable number of errors on the no-go trials of a go/no-go tasks to Val/Val animals, but reverse faster. *A.* Val/Val (n=10) and Met/Met (n=10) animals have a similar number of errors on the no-go trials of a go/no-go task during the initial shaping session (A=go cue, B=no-go cue). *B.* They also perform similarly during session 2 where novel odorants C (go) and D (no-go) are introduced. *C.* However, Met/Met animals reach criterion faster than Val/Val animals (80% correct trials) when the contingencies are reversed (D=go and C=no-go). A repeated measures two-way analysis of variance (ANOVA) showed a significant main effect of genotype and session number (genotype $F_{(1,99)}=6.93$, $p=0.0098$, session $F_{(5,99)}=23.16$, $p<0.0001$) but no significant interaction between the two ($F_{(5,99)}=0.55$, $p<0.05$). *D.* Animals trained on the first 2 sessions of the go/no-go show similar rates of extinction when looked at either by number of nose-pokes at the cue port or *E.* number of completed trials (nose-poke at the cue port, followed by the water port or a "no-go" response). During extinction sessions animals were given 30 minutes of post-training "maintenance" trials before one hour of within session extinction trials (Met/Met n=7, Val/Val n=8).

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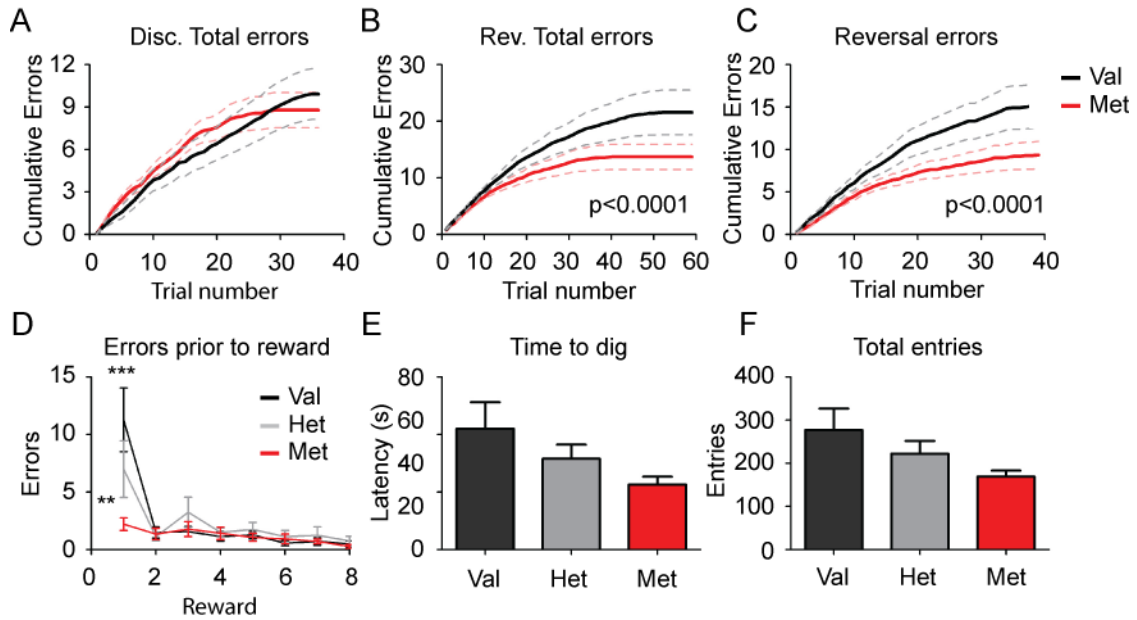
Figure 3.3 Adult BDNF Val68Met KI mice reverse faster on a 4-choice odor based task. *A.* BDNF Val/Val and Met/Met mice performed similarly on the discrimination phase of a 4-choice task. *B.* Val/Val animals (n=14) made more total errors (errors of any kind) on the reversal phase of a 4-choice task than Met/Met animals (n=14) (two way rm ANOVA showed a significant interaction between genotype and trial number for accumulation of errors $F(1,1508)= 2.626$ $p<0.0001$). *C.* Val/Val animals also made more reversal errors (trials in which they dug in the previously rewarded pot, O1) than Met/Met animals (Two way rm ANOVA $F(1, 988)=3.154$, $p<0.0001$). *D.* Both Val/Val and Val/Met mice (n=8) made more errors prior to the first reward than Met/Met animals. A two way rm ANOVA showed significant interaction between trial number and genotype ($F(1,988)=3.15$, $p<0.0001$), and a Bonferroni post-hoc test revealed a significant difference between genotypes in the number of errors prior to the first reward (Val/Met n=8, Val/Val vs. Met/Met $p<0.0001$, Val/Val vs. Val/Met $p<0.001$). *E.* Average time per trial before digging. There was no effect of genotype in latency to dig, (1 way ANOVA $F(2,35)=1.289$, $p=0.1074$). A post-hoc test for linear trend found a gene "dose dependent" decrease in trial latency ($p=0.03$) *F.* Average number of entries into different quadrants before digging showed no effect of genotype (1 way ANOVA $F(3,47)=1.582$, $p=0.097$). A post-hoc test for linear trend found a gene "dose dependent" decrease number of entries into different quadrants ($p=0.03$).
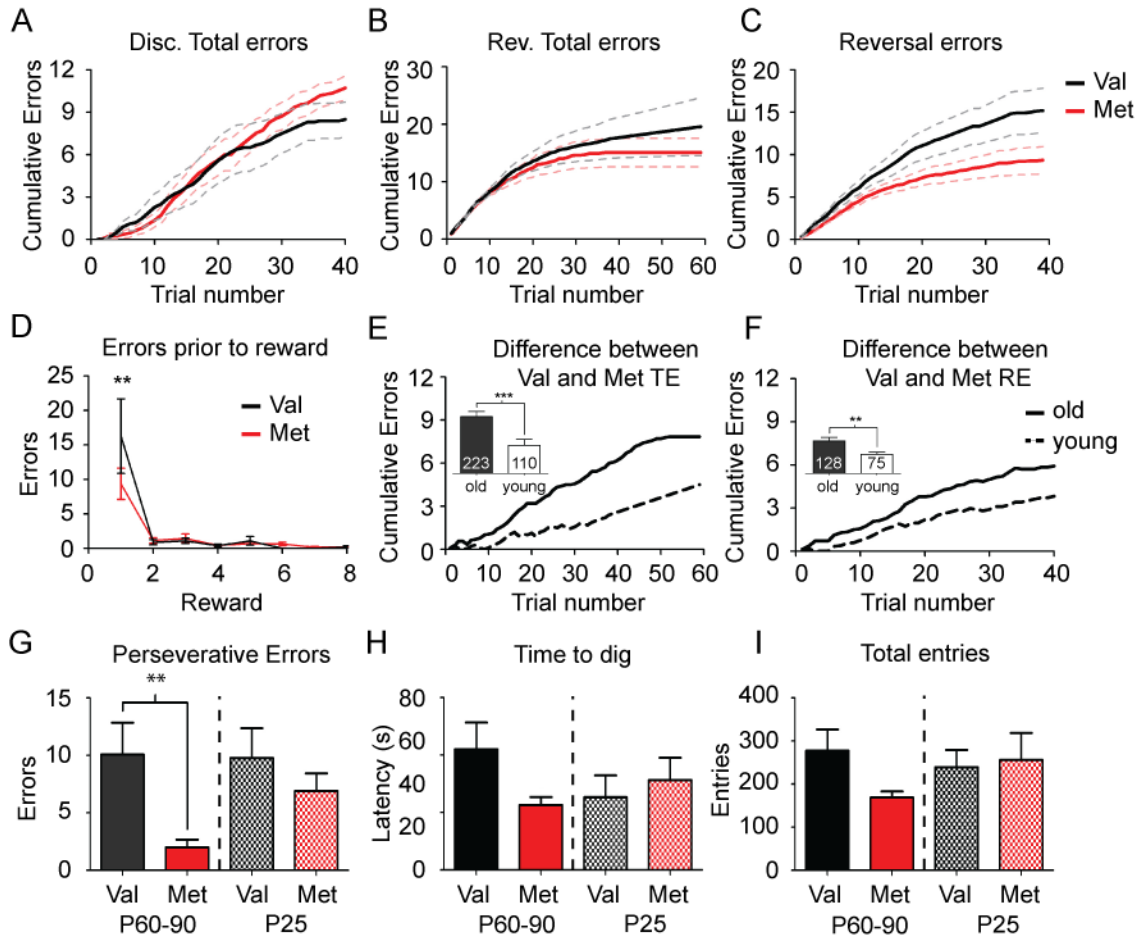
Figure 3.4 Comparing performance on a 4-choice discrimination and reversal task between young and old Val/Val and Met/Met mice. *A.* Young (P25) Val/Val (n=10) and Met/Met (n=10) mice performed comparably on the discrimination phase of a 4-choice task. *B.* Young homozygous Val and Met animals made a similar number of total errors (errors of any kind) as well as reversal errors (RE, errors back to previously rewarded pot)(*C*) during the reversal phase of a 4-choice task. *D.* As in adults, young Val/Val mice made more errors prior to the first reward than Met/Met animals (p<0.01). *E.* Difference in cumulative errors and (*F*) reversal errors between Val/Val and Met/Met mice in young and old cohorts. Inset shows area under the graph measures which were significantly larger in the old cohort than the young cohort. *G.* An analysis of the number of perseverative errors (trials in which the animal dug in the previously rewarded pot before getting one correct trial) revealed differences between genotypes at different ages. A two way ANOVA revealed a significant main effect of genotype ($F_{(1,38)}=8.07$ p<0.01) but not age ($F_{(1,38)}=2.06$), nor a significant interaction between age and genotype ($F_{(1,38)}=1.81$). A Bonferroni post-hoc test revealed that there was a significant difference in perseverative errors between adult homozygous Val and Met animals (p<0.01), but not young animals. *H.* Comparison of average latency to dig between young and old animals. *I.* Total number of entries into different quadrants.

Methods

Animals. All animal procedures were approved by the Ernest Gallo Clinic and Research Center Institutional Animal Care and Use Committee. BDNF Val68Met mice were generated by the Dorit Ron lab (Warnault et al., unpublished) see Appendix for targeting strategy (**Fig. A1**). For the four choice discrimination and reversal task, male and female BDNF Val68Met mice (Ron lab) (n=56, female n=26, male n=26) were bred in our animal facility and were co-housed on a 12h/12h reverse light-dark cycle (lights on at 10PM) in a semi-enriched environment with bedding and toys. Food restriction began two days before behavioral pre-training. During food restriction, juvenile mice continued to gain weight throughout the experiment and all mice were maintained at 90% of their ad lib weight. Water was freely available both in the homecage and in the maze during all phases of behavioral testing.

For the Go/No-go task, male and female BDNF Val68Met mice (n=35, female n=19, male n=16) (Ron lab line) were water restricted for two days before and throughout behavioral training, receiving 1-2ml per day through behavioral training, as well as supplementary water in their home cage after training as needed. Mice were maintained at 90% of their ad lib weight. *Apparatus.* The 4-choice maze was a square box 12"×12"×9" with 4 internal walls measuring 3" wide which partially divided the four quadrants. Odor stimuli were presented in ceramic pots. Pots were sham baited with a Honey Nut Cheerio (General Mills, Minneapolis, MN) secured underneath a mesh screen at the bottom. A 6" diameter removable cylinder fit in the center of the maze and was

lowered between trials (after a digging response) to isolate the mouse from the rest of the maze.

The apparatus for the automated Go/No-go odor discrimination task and extinction task was 5"x7"x5." The initiation port was located in the middle of one wall, and two choice ports were located 2.5" to the left and right of the initiation port (center to center), but only the center port and the right port were made available during the task. An infrared photodiode/phototransistor pair was placed on either side of the port to report the times of port entry and exit (Island Motion). The water valves (Neptune Research) were calibrated to deliver a volume of water (2 µl) for rewarded choices.

Four choice discrimination and reversal task. The 4-choice odor discrimination and reversal task was adapted from Kim and Ragozzino (2005). Training took place over three days after an initial two days of food restriction.

On day one the animals were habituated. Small pieces of Honey Nut Cheerio (approximately 10 mg each) were placed inside of four empty digging pots, one in each of the four quadrants. The mice were allowed to explore the maze and consume the cereal pieces for 30 minutes. Pots were rebaited every 10 minutes.

On the second day of pre-training mice were taught to find cereal pieces buried in pine wood shavings (Hartz Mountain Corporation, Secaucus, NJ). One pot with increasing amounts of wood shavings covering the cereal reward was used in this shaping phase. The quadrant containing the pot was alternated in each trial and all quadrants were

rewarded equally. Trials were untimed and most animals retrieved the reward in the 12 total shaping trials within one hour.

On the third day the animals were tested. During the initial discrimination phase, the animal had to discriminate among four initial odors (anise, clove, litsea and thyme) and learn which one was associated with a buried Cheerio reward (anise). The stimulus presentation was pseudo-randomized such that an odor was never in the same quadrant two trials in a row. Each trial began with the mouse confined to the central start cylinder, which was equidistant to all the odor pots. At the beginning of the discrimination phase the animals were given a 'hint' trial in which a bowl with the rewarded scent was placed in the center and the animal was allowed to retrieve the Cheerio reward. Timing began when the cylinder was lifted. *Entry* to each quadrant was recorded if all four paws crossed beyond the perimeter of the start area. The mouse could freely explore the arena until it chose to dig in a pot. *Latency* was defined as the time elapsed before digging. Digging was defined as purposefully moving the shavings with both front paws, but not as superficial sniffing or chewing of the shavings. A trial was terminated if no choice was made within three minutes and was recorded as an omission. *Criterion* was met when the animal completed 8 out of 10 consecutive trials correctly.

Once criterion was met on the discrimination phase, the animal moved on to the reversal phase immediately within the same session. All shavings were replaced with new shavings to prevent discrimination via unintentional cues. Odor 4 was swapped out for a novel odor (eucalyptus) and a new odorant (clove) became the rewarded odorant.

Perseverative errors were defined as trials in which the mouse dug in the pot of the previously rewarded odor before getting one correct trial. ***Reversal errors*** were trials in which the mouse dug in the pot of previously rewarded odor, both before and after correct trials. Irrelevant errors were trials in which the mouse dug in the pot with the odor that was never rewarded (litsea). Novel errors were trials in which the mouse dug in the newly introduced odor (eucalyptus), which was also never rewarded. To complete the reversal, the mouse had to reach criterion by completing 8 out of 10 consecutive trials correctly. Animals typically completed both discrimination and reversal phases within three hours.

Go/No-go task. Mice learned to nose poke in the center port for odorant cue. Cues indicated either "go" to the right port to receive water, or "no-go" with a 30% probability of receiving a "no-go" cue. Incorrect trials initiated a 4 second time-out. The Go/No-go task included three phases: in the first phase (shaping) the animals learned the task by responding to odorants A (go cue) and B (no-go cue); in the second phase new odorants C (go cue) and D (no-go cue) were introduced; in the final phase (reversal), odorants C and D reversed contingency so that C became the "no-go" cue and D the "go" cue. Odorants used were as follows: cinnamon (A), vanilla (B), bay (C), and basil (D). Animals were trained in each phase until they reach criterion of 80% correct trials.

Extinction task. The extinction was similar to the Go/No-go task above. The first two phases of the task were identical to that of the Go/No-go task. In the third phase, odorants C and D were still delivered after a center nose-poke, but after 400 priming

trials all subsequent trials were unrewarded and no time-out was initiated. The numbers of nose-pokes in the cue port as well as the number of trials completed (nose-poke in cue port followed by water port or "no-go" response) were measured. Odorants used and training criterion were the same as in phase 2 above.

Statistics. Statistical analyses were conducted using two-way ANOVAs (repeated measures or regular), one-way ANOVAs, or Student's t-tests (or Mann-Whitney test) where appropriate. A D'Agostino and Pearson omnibus test was used to test normality. Where significant main effects or interactions were indicated, post hoc comparisons were made using the Bonferroni test or a test for linear trend (Graphpad Prism). Statistical significance was set at $p < 0.05$ for all analyses.

Chapter 4: The role of GABAergic inhibition in behavioral inhibition

Introduction

The term "inhibition" can be used to refer to both to the function of inhibitory neurotransmission to block depolarization of cells and to the function of the brain to control or regulate behavior. Both of these functions are known to increase through postnatal development, with the maturation of inhibition in the prefrontal cortex (**Fig. 2.4**) potentially following the same time course as the maturation of inhibition of impulsive behaviors (**Fig. 1.2**). Given the important role of inhibitory neurons in underlying complex circuit functions across cortex, it has been assumed that interneurons in the prefrontal cortex play an important role in the function of the prefrontal cortex through the synchronization of neuronal oscillations, gating of inputs, and influence on synaptic plasticity (Sakata et al., 2009; Le Magueresse and Monyer, 2013)

In humans response inhibition and the ability to switch between response sets emerges over childhood and early adolescence. By age 5 most children can switch between response sets for simple stimuli (Espy, 1997). By age 11-12 the ability to shift between attention sets reaches adult-like levels (Chelune and Baer, 1986; Luciana and Nelson, 1998; Crone et al., 2004; Romine et al., 2004; Somsen, 2007). Functional changes in behavioral inhibition parallel changes in cortical inhibitory circuits. Parvalbumin-IR terminal density increases throughout adolescence in the primate PFC (Erickson and Lewis, 2002) and in macaques, there is dynamic regulation of $GABA_A$ receptor subunits,

as well as parvalbumin (PV) and GABA membrane transporter (GAT1) in "cartridge" axon terminals that show dramatic changes at puberty onset (**Fig. 1.3**) (Gonzalez-Burgos et al., 2008). Because of this developmental overlap and the role of inhibition in the maturation of the sensory cortices (Hensch et al., 2005) it is tempting to hypothesize that the maturation of cortical inhibitory circuits may at least in part drive the developmental change in behavioral inhibition.

Other parallels between behavioral inhibition and GABAergic inhibition can be made by looking at similarities between neurodevelopmental psychiatric disorders such as schizophrenia and autism. In both of these disorders deficits in PV+ immunoreactivity and GAD67 are accompanied by impaired executive functions such as complex problem solving, behavioral inhibition, and working memory (Gonzalez-Burgos et al.; Daviss and Lewis, 1995; Benes et al., 1996; Benes, 1997; Guidotti et al., 2000; Benes and Berretta, 2001; Lewis et al., 2005; Gonzalez-Burgos et al., 2010; Gonzalez-Burgos et al., 2011; Lisman, 2012). Both are also characterized by behavioral inflexibility, or the inability to update behaviors in the face of changing environmental contingencies. A classic measure of cognitive flexibility in humans is the Wisconsin Card Sorting Task (WCST) (Ozonoff, 1995). Participants with schizophrenia (Anderson et al., 1990) and ASD (Ozonoff, 1995; Bennetto et al., 1996; Goldstein et al., 2001; Tsuchiya et al., 2005; Pellicano et al., 2006) tend to perseverate on one category or rule when tested in the WCST. This can be seen as a deficit in inhibition of a prepotent response, sorting using the initially established rule. Perseverative behavior patterns may also be related to repetitive behavior observed in autism spectrum disorders (Kana et al., 2011). Individuals with schizophrenia and

ASD also have impairments on tasks that measure behavioral inhibition, such as the Go/No-go or Stroop task (Han et al., 2011; Gigaux et al., 2013). Despite these parallels, it is unknown what role deficits in cortical inhibition play in perseveration in cognitive inflexibility.

In this study we used mouse genetics to investigate the role cellular inhibition plays in behavioral inhibition in reversal, go/no-go and extinction tasks. We found that disruption of GABAergic inhibition in the prefrontal cortex by two genetic methods does not necessarily impact behavioral inhibition thought to depend on the prefrontal cortex. In a mouse line showing disruption of behavioral inhibition (deficits in reversal learning), we also found normal measures of GABAergic inhibition. We discuss the relevance of these results to understanding the maturation of GABAergic and behavioral inhibition and suggest potential future directions for investigation.

Results

I. Deficits in GABAergic inhibition do not necessarily impact behavioral inhibition
We used two established genetic methods to disrupt cellular inhibitory function in the mouse cortex: systemic inhibition of TrkB receptor signaling using a chemical-genetic approach and GAD65 KO mice (Hensch et al., 1998). We then tested these mice on measures of behavioral inhibition adapted for mice including an odor based 4-choice discrimination and reversal task, and an odor based automated go/no-go task with a reversal phase.

Inhibiting TrkB during peri-adolescence does not produce differences in reversal learning performance. In the TrkB$_{F616A}$ line, a phenylalanine to alanine substitution in the ATP binding pocket allows for the temporally specific inhibition of TrkB when it is in the presence of a small molecule inhibitor 1NM-PP1 (Chen et al., 2005a; Kaneko et al., 2008). In Chapter 2, we showed that inhibiting TrkB in these mice with systemic mini-pump delivery of 1NM-PP1 from P23 to P40-50 resulted in impaired development of mIPSCs into pyramidal neurons (**Fig. 2.7**). In this study we used the same approach to determine whether disrupting the normal development of cortical mIPSCs affected reversal learning performance. To do this we used the 4-choice odor discrimination and reversal task described in Chapter 3 (**Fig. 3.1B**). We found that vehicle or 1NM-PP1 treated TrkB$_{F616A}$ animals performed similarly on the task, during the discrimination and reversal phase (**Fig 4.1A-D**). Moreover, vehicle and 1NM-PP1 treated mice had a similar latency to dig per trial (**Fig. 4.1E**) as well as the total number of entries (**Fig. 4.1F**) suggesting that exploratory strategies were also similar. These data suggest that preventing the maturation of inhibitory synapses onto cortical pyramidal neurons via TrkB inhibition during post-natal development is not sufficient to produce deficits on this kind of reversal learning task.

GAD65 KO mice perform similarly to WT animals on reversal learning tasks.
GABA is synthesized by glutamic acid decarboxylase, which is produced by two genes, GAD65 and GAD67. GAD65 knock-out mice have reduced cortical GABA content but are viable (Tian et al., 1999), and have previously been used to show the dependence of ocular dominance plasticity on GABAergic inhibition (Hensch et al., 1998). We tested

GAD65 KO animals and WT control littermates at P40 on the 4-choice discrimination and reversal task. We found that KO and WT controls performed comparably, having a similar number of errors both on the discrimination phase (**Fig 4.2A**) and reversal phase (**Fig 4.2B-D**) of the task. GAD65 KO mice also had similar digging latencies (**Fig. 4.2E**) as well as total number of entries (**Fig. 4.2F**) as WT mice.

To further explore the role of inhibition on reversal learning and behavioral inhibition, we also tested GAD65 KO mice on a go/no-go reversal task (test age P90-P200)(**Fig. 3.1A**). In this task animals were taught to nose-poke in a center poke for odorant cue (either a "go" or "no-go" cue) and then move to an adjacent port to receive water. Water reward was given for a correct "go" response and a 4 second time-out was given for incorrect "no-go" responses. This task had three phases: a shaping phase where the animals learned the task with odorants A ("go" cue vanilla) and B ("no-go" cue cinnamon); a phase where novel odorants C ("go" cue bay) and D ("no-go" cue basil) were introduced; and a reversal phase where D became the "no-go" cue and C the "go" cue. We found that GAD65 KO mice performed similarly to WT mice on all phases of the task (**Fig. 4.3**). These data provide further evidence that reducing GABAergic inhibition is not sufficient to produce deficits on reversal learning tasks.

II. Mutant mice with developmental differences in behavioral inhibition show comparable development of GABAergic inhibition

mIPSC measures between BDNF Val68Met mice.

As discuss in Chapter 3, BDNF Val68Met knock-in mice model a human polymorphism

in the BDNF gene which confers a trafficking deficit in BDNF and a reduction in

activity-regulated release. We also show in Chapter 3 that BDNF Val68Met Met/Met

mice reverse faster on a variety of reversal tasks (**Fig. 3.2 and 3.3**). Given this difference

in reversal learning performance, as well as the known role of activity dependent BDNF

release on the maturation of GABAergic neurons (Abidin et al., 2008), we hypothesized

that a reduction in GABAergic inhibition in Met/Met mice might underlie reversal

learning performance differences.

mIPSCs were recorded in P40-50 BDNF Val68Met mice crossed to the Thy-1 YFPH line

(**Fig 2.2B**). Because performance on the 4-choice reversal task has previously been

shown to be dependent on the anterior cingulate in rats (Ragozzino and Rozman, 2007)

and mice (Johnson and Wilbrecht, 2011), we recorded mIPSCs in the anterior cingulate.

We found no difference in mIPSC amplitude (**Fig 4.4C**) or frequency (**Fig. 4.4D**) in

either YFP+ or YFP- neurons in BDNF Val/Val and Met/Met mice. We also found no

difference in mEPSC measures between Val/Val and Met/Met mice in either cell type

(**Fig. 4.4A,B**). These data suggest that the BDNF Val68Met polymorphism does not

impact the development of differences in inhibitory input onto layer 5 pyramidal neurons

as described in Chapter 2. It also suggests that age dependent changes in mIPSC

amplitude in the anterior cingulate does not account for the behavioral differences

between genotypes discussed in Chapter 3. However, these data do not rule out the

changes in other excitatory or inhibitory synapses in regions we did not measure.

Discussion

In Chapter 2 we show that there is a TrkB dependent developmental increase in mIPSC amplitude in a subset of layer 5 pyramidal neurons during peri-adolescence (P21 to P40-50). Over this same period, reversal learning behavior reaches adult-like levels (**Fig. 1.2**)(Johnson and Wilbrecht, 2011). We predicted that these cellular and behavioral changes would be correlated based on previous studies of ocular dominance plasticity in the visual cortex. However, despite the fact that changes in inhibitory currents in the anterior cingulate occur over the same time frame as the behavioral maturation in reversal learning performance, blocking the maturation of mIPSC amplitude with 1NM-PP1 in TrkB$_{F616A}$ mice did not significantly impact reversal learning performance at P40-50 (**Fig. 4.1**). These data suggest that the maturation of inhibition in layer 5 projection neurons in the anterior cingulate during peri-adolescence is not necessary for the simultaneous maturation of a behavior dependent on this region. In other studies covered in Chapter 3, we did find that a common polymorphism in the BDNF gene (BNDF Val68Met) does impact reversal leaning performance. It may be that interrupting neurotrophin signaling from an early post-natal time-point may be required to bring about behavioral differences in reversal learning performance seen in BDNF Val68Met mice (**Fig. 3.3**). This is discussed more in the following chapter.

GAD65 KO mice perform similarly to WT animals on reversal learning tasks.
GAD65 KO mice have been shown to have delayed maturation of inhibition and delayed critical period plasticity in the visual cortex (Hensch et al., 1998; Hensch, 2005). We therefore selected these mice as an additional way to investigate the maturation of

inhibition and function of the prefrontal cortex. We did not measure the maturation of

cellular inhibition in prefrontal cortex directly in these mice, but we did test them on a 4-

choice reversal task at P40 to test if GAD65 KO mice are behaviorally delayed compared

to littermates. We predicted that if reversal behavior was developmentally regulated by

GABAergic activity, GAD65 KO mice might reverse faster than WT animals of the same

age, a phenotype that would look more like juvenile mice. However, we found that,

similar to TrkB manipulations, there was no impact of GAD65 KO genotype on 4-choice

reversal learning at P40. Testing of a smaller number of mice on a go/no-go reversal task

at 3-6 months of age also revealed no significant genotype differences in acquisition or

reversal. These findings, although negative, are interesting as they conflict with what

might be expected from studies of the visual cortex sensitive period regulation. They

also suggest that the maturation of frontal cortex function may be regulated by different

mechanisms than other sensory cortices. It could also be that GAD65 KO is not

sufficient to alter GABAergic transmission in a significant manner in the PFC. Pehrson

et al. (2013) found inhibiting GABA neurotransmission in the anterior cingulate cortex

(ACC) using a $GABA_A$ agonist or an inverse agonist of the benzodiazepine binding site

of $GABA_A$ receptors reduced performance on attention tasks, but inhibiting GAD locally

by microinfusions of the GAD inhibitor 3-MPA into the ACC, even at high

concentrations, did not. The authors suggest that this may be due to compensatory

mechanisms that conserve the available supply of GABA when GABA metabolism is

reduced (Pehrson et al., 2013). However, given negative results in reversal behavior in

1NM-PP1 treated $TrkB_{F616}$ mice where GABAergic synapses were shown to be altered,

we might reject this possibility.

Our study was intended to investigate the relationship of cellular inhibition and behavioral inhibition to better understand both the development of the healthy brain and the etiology of mental illness. Postmortem evidence from schizophrenic patients has suggested that GAD and GABA abnormalities are involved in its pathophysiology. This includes increased expression of $GABA_A$ receptors and reduced GAD67 expression in the PFC (Akbarian et al., 1995; Volk et al., 2000; Hashimoto et al., 2005). These findings have been interpreted as evidence of reduced prefrontal GABA neurotransmission in schizophrenia, and researchers have sought to connect this to behavioral dysfunction. However, it has recently been shown that reduced GAD67 expression can also be caused by altering glutamate neurotransmission during development (Belforte et al., 2010) or by ventral hippocampal lesions (Francois et al., 2009; Nakazawa et al., 2012). It may be that reduced GAD67 is not a primary pathology, but instead reflects a compensatory response to other changes in the brain. If true, then GAD 65 KO may be modeling a symptom rather than a cause of prefrontal dysfunction.

Altered maturation of prefrontal cortex dependent behavior is not accompanied by altered maturation of GABAergic inhibition.

In a second line of investigation we also looked at the maturation of GABAergic inhibition in a mouse line that shows alterations in the maturation of behavioral inhibition on reversal tasks, the BDNF Val68Met line (Warnault et al, unpublished; Chen et al. 2006) (**Fig 3.2C and 3.3B-D**). Note these mice do not show global differences in behavioral inhibition as Met/Met and Val/Val mice are comparable for no-go learning

(**Fig. 3.2A,B**) and appetitive extinction (**Fig. 3.2D,E**). However, because reversal

learning matures over the same time scale as GABAergic inhibiton in the cingulate

cortex, these mice provided an opportunity to investigate connections between cellular

and behavioral measures. When we measured mEPSCs and mIPSCs in two subtypes of

layer 5 neurons in the anterior cingulate of homozygous BDNF Val68Met mice we found

no significant differences (**Fig. 4.4**). Other studies have found significant deficits in

excitatory and inhibitory transmission in BDNF heterozygous knock-out mice (Abidin et

al., 2006; Abidin et al., 2008), and mice that lack promoter IV BDNF transcription show

decreased parvalbumin staining in the PFC as well as deficits in GABAergic, but not

glutamatergic, synaptic transmission (Sakata et al., 2009). However, it should be noted

that unlike the aforementioned manipulations, the BDNF Val68Met mutation affects only

activity-regulated secretion (Chen et al., 2004) and not constitutive release. This may

reduce the impact of this mutation compared to BDNF KOs and other more severe

manipulations. It should also be noted that the Met mutation is present throughout

development, and there may be corresponding compensatory mechanisms (such as

increased constitutive release) that reduce effects on synaptic transmission. Finally, it is

possible that the striatum, which does not produce its own BDNF and requires activity

dependent release of BDNF from the cortex (Kokaia et al., 1998), may show more

dramatic impact than the cortex after BDNF Val68Met knockin (see Rauskolb et al.,

2010).

Conclusion

I conclude the relationship between the development of GABAergic inhibition and behavioral inhibition is not clear-cut. Further studies will be needed to confirm that subtler aspects of GABAergic inhibition do not play a larger role in behavioral inhibition. However, these studies suggest mechanisms that have been found to regulate maturation of the visual cortex in mice will not directly translate to the prefrontal cortex despite overlap in timing and the gross anatomical similarity of the two cortices. This difference may be important for translational models.

In terms of understanding the maturation of behavioral inhibition, we find the phenotype of BDNF Val68Met mice provides an interesting new lead. In the next chapter we will explore additional experiments that may help delineate the role that BDNF and TrkB signaling may have in shaping the maturation of behavioral flexibility.

Figures
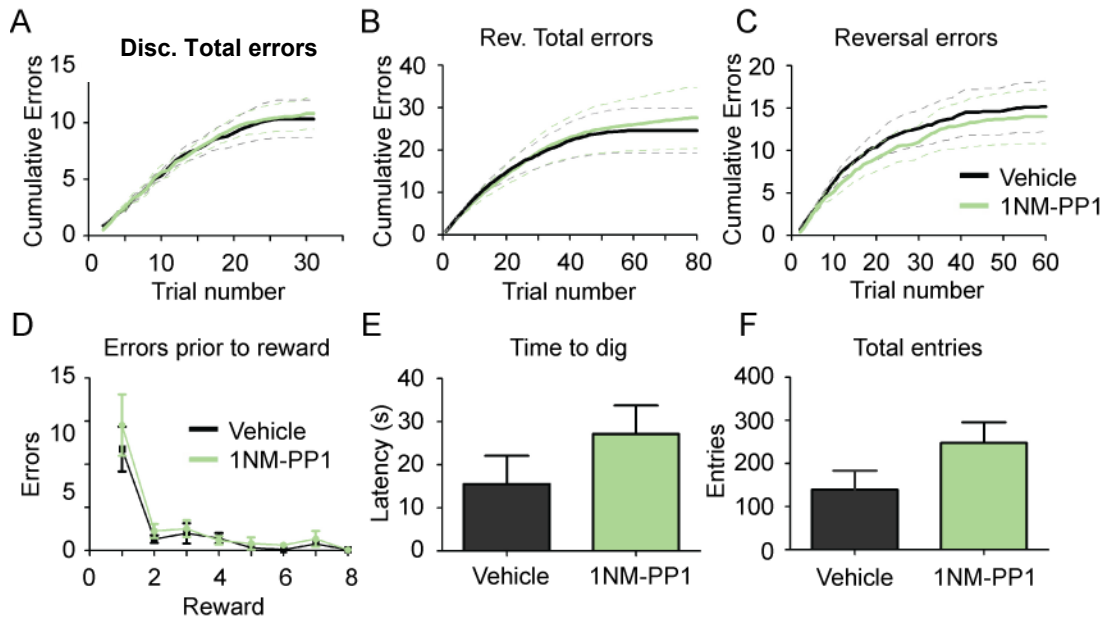
Figure 4.1 TrkB inhibition during the periadolescent period does not affect performance on a 4-choice discrimination and reversal task. *A.* TrkB$_{F616A}$ mice given either vehicle (n=9) or 1NM-PP1 (n=11) via osmotic minipump from P23 to P40-50 (**Fig. 2.6A**) had similar performance on the discrimination phase (SD) of the 4-choice reversal task. A two way repeated measures (rm) ANOVA showed that there was no significant main effect of treatment or interaction between treatment and trial number (treatment $F_{(1,522)}=0.01$, $p>0.05$, interaction $F_{(29,522)}=0.06$, $p>0.05$) *B.* Mice given either 1NM-PP1 or vehicle also performed similarly on the reversal phase of a 4-choice reversal task, having similar numbers of total errors (treatment $F_{(1,1422)}=0.02$, $p>0.05$, interaction $F_{(79,1422)}=0.13$, $p>0.05$) and reversal errors (treatment $F_{(1,1044)}=0.06$, $p>0.05$, interaction $F_{(58,1044)}=0.16$, $p>0.05$) (*C*). *D.* 1NM-PP1 and vehicle-treated mice have a similar number of errors prior to reward (treatment $F_{(1,126)}=0.27$, $p>0.05$, interaction $F_{(7,126)}=0.72$, $p>0.05$). *E.* Average time per trial before digging (p=0.31). *F.* Average number of entries into different quadrants before digging (p=0.13).
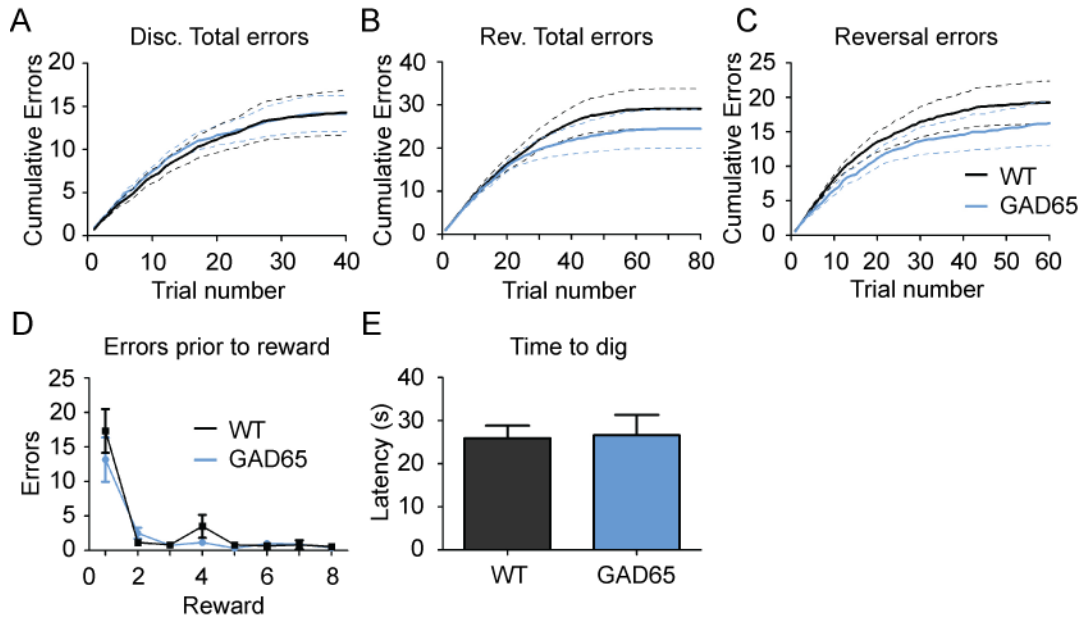
Figure 4.2 GAD65 KO mice perform similarly to WT mice on a 4 choice discrimination and reversal task. *A.* GAD65 (n=12) and WT (n=12) mice performed similarly on the discrimination phase of a 4-choice task. A two way rm ANOVA analysis of cumulative errors showed that there was no significant main effect of genotype or interaction between genotype and trial number (genotype $F_{(1,858)}=0.02$, $p>0.05$, interaction $F_{(39,858)}=0.07$, $p>0.05$). *B.* KO and WT mice also performed similarly on the reversal phase of a 4-choice task with no difference in accumulation of total errors (two way rm ANOVA genotype $F_{(1,1738)}=0.55$, $p>0.05$, interaction $F_{(79,1738)}=0.62$, $p>0.05$) and (*C*) reversal errors (genotype $F_{(1,1298)}=0.98$, $p>0.05$, interaction $F_{(59,1298)}=0.43$, $p>0.05$). *D.* Number of errors prior to reward in reversal was also comparable (genotype $F_{(1,154)}=1.033$, $p>0.05$, interaction $F_{(7,154)}=0.98$, $p>0.05$) *E.* Average time per trial before digging ($p=0.54$).
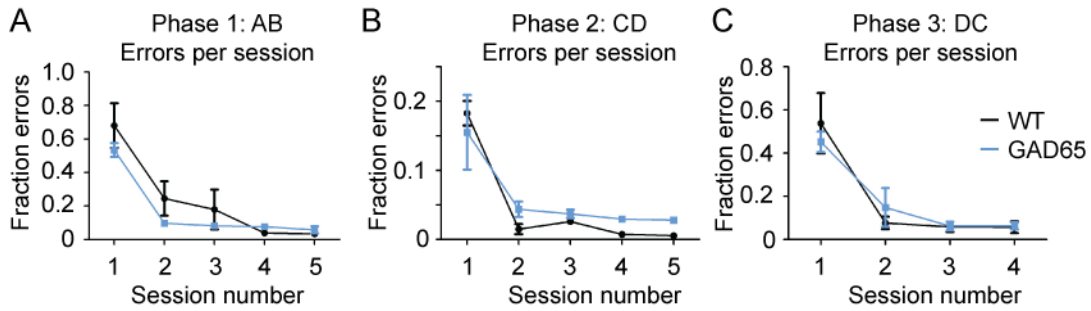
Figure 4.3 GAD65 KO mice perform similarly to WT mice on a go/no-go discrimination and reversal task. *A,* GAD65 KO (n=5) and WT (n=3) littermates commit a similar number of errors on the no-go trials of a go/no-go task during the initial shaping session (A=go cue, B=no-go cue). A two way repeated measures ANOVA showed that there was no significant main effect of genotype or interaction between genotype and session (genotype $F_{(1,30)}=3.89$, $p>0.05$, interaction $F_{(4,30)}=1.48$, $p>0.05$). *B,* KO and WT mice also perform similarly during phase 2 where novel odorants C (go) and D (no-go) are introduced (genotype $F_{(1,40)}=0.93$, $p>0.05$, interaction $F_{(4,40)}=0.74$, $p>0.05$) as well as during the reversal phase (*C*) when the contingencies are reversed (D=go and C=no-go) (genotype $F_{(1,24)}=0.0005$, $p>0.05$, interaction $F_{(3,24)}=0.54$, $p>0.05$).
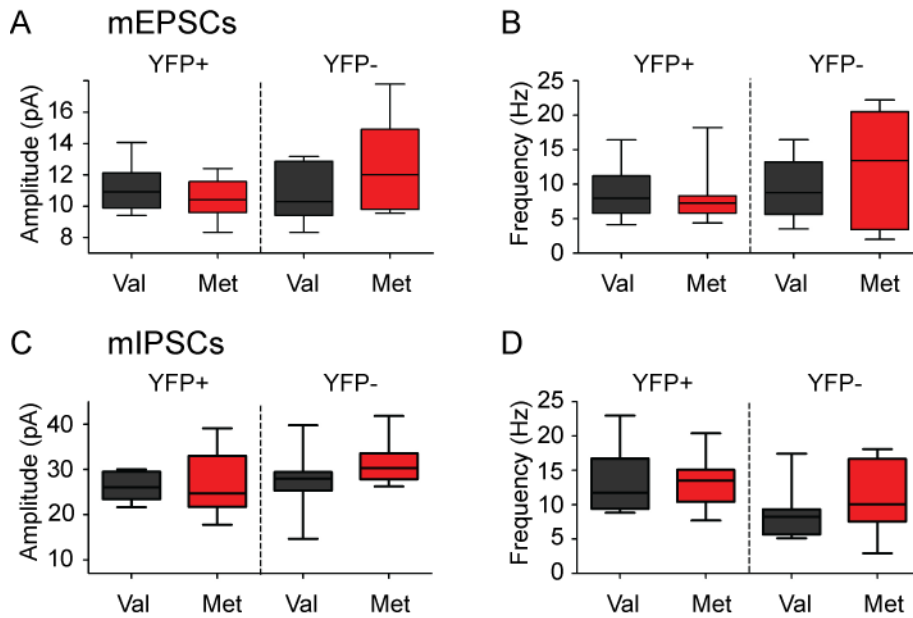
Figure 4.4 mPSC amplitude and frequency are similar between BDNF Val/Val and Met/Met mice in L5 of the anterior cingulate. *A*, In YFP+ and YFP- neurons there is no significant difference in mEPSC amplitude (Mann Whitney, YFP+ p=0.30, YFP- p=0.37) or frequency (YFP+ p=0.55, YFP- p=0.60) (B) between P40-50 BDNF Val/Val (n=9) and Met/Met mice (n=18). *C,* In YFP+ and YFP- neurons there is also no difference in mIPSC amplitude (YFP+ p=0.86, YFP- p=0.08) or (*D*) frequency (YFP+ p=0.75, YFP- p=0.18) between Val/Val (n=9) and Met/Met (n=10) mice.

Methods

Animals. In electrophysiology experiments, BDNF Val68Met mice (Ron lab) crossed to the Thy1-eYFP line H mice (YFPH) (n=21) (Jackson Labs, line 003782)(Gonzalez-Burgos et al.; Feng et al., 2000) were used to identify subtypes of neurons in the frontal cortex. We made use of the YFPH line of mice because only a subset of Layer 5 pyramidal neurons are labeled in these mice (Feng et al., 2000; Sugino et al., 2006) (Fig. 1A).

For the 4-choice discrimination and reversal task Thy1 YFPH mice crossed to $TrkB_{F616A}$ mice (n=20) as well as GAD65 mice (n=24) were bred in our animal facility and were co-housed on a 12h/12h reverse light-dark cycle (lights on at 10PM) in a semi-enriched environment with bedding and toys. Food restriction and water restriction were as stated previously in Chapter 3, Methods. All animal procedures were approved by the Ernest Gallo Clinic and Research Center Institutional Animal Care and Use Committee.

The 4-choice discrimination and reversal task and Go/No-go task was as described in Chapter 3, Methods.

1NM-PP1 and minipump implantation. 1NM-PP1 or vehicle solution (4% (vol/vol) DMSO and 2% (vol/vol) Tween-20 in saline) was administered via Alzet minipumps. Under isoflurane anesthesia, minipumps were implanted subcutaneously under the scruff of the neck at P23 and remained in place throughout behavioral testing. 1NM-PP1 was

delivered at the rate of at the rate of 6.25 nmol/h, a concentration used in previous reports (Kaneko et al., 2008).

Slice preparation and Elecrophysiology. All electrophysiology methods were as described in Chapter 2, Methods.

Statistics. Statistical analyses were conducted using two-way repeated measures ANOVAs, or Student's t-tests, or Mann-Whitney test where appropriate. D'Agostino and Pearson omnibus test was used to test normality (Graphpad Prism) and statistical significance was set at $p < 0.05$ for all analyses.

Chapter 5: Conclusion and Future Directions

In this dissertation we make several findings. First, in Chapter 2, we find that in the mouse anterior cingulate neuronal inhibition varies for pyramidal neurons with different projection targets, both in terms of developmental trajectory as well as in its dependence on TrkB signaling. Specifically we find that neurons that project within the cortex, or IT-type neurons, have a lower frequency of mIPSCs than adjacent subcortical projecting, or PT-type neurons (**Fig. 2.3**). We also find that while IT-type neurons show a developmental increase in mIPSC amplitude, adjacent PT-type neurons do not (**Fig. 2.4**). Finally, we show that while IT-type neurons are sensitive to TrkB inhibition over periadolescence, showing both a decrease in mIPSC amplitude and frequency when it is inhibited, PT-type neurons are not affected (**Fig. 2.7**). Overall our data suggest that inhibition onto pyramidal neurons in the mouse anterior cingulate is cell-type specific. Our data also suggest that cortical-projecting neurons within the anterior cingulate may be particularly vulnerable to disruptions in TrkB signaling over the periadolescent period.

In Chapter 3 we find a novel behavioral phenotype in the BDNF Val68Met line of mice. We find that Met knock-in mice have enhanced reversal learning when compared to Val/Val littermates (**Fig. 3.2A-C and 3.3**). We also show that this difference in reversal learning is not due to differences in appetitive extinction learning since both Val/Val and Met/Met mice extinguish at similar rates (**Fig. 3.2D,E**). Finally, we show that the difference in reversal learning between Val/Val and Met/Met mice changes with age, showing a developmental decrease during adolescence when BDNF levels peak, and

increasing during adulthood when BDNF levels decrease (**Fig. 3.4**). Our results suggest

that the BDNF Val66Met mutation may be beneficial for some forms of reversal learning.

Our results are also in line with the "plasticity allele" hypothesis, which suggests that the

BDNF Met polymorphism, rather than conferring "risk" for greater vulnerability for

psychiatric disorders per-se, may be better thought of as increasing the probability of both

positive and negative effects of enriched and impoverished environments respectively.

Finally in Chapter 4 we explore the effect of GABAergic inhibition on tests of behavioral

inhibition and flexibility. We find that reducing GABA signaling, either through

inhibiting TrkB during the periadolescent period (**Fig. 4.1**), or by using constiuitive

GAD65 KO mice (**Fig. 4.2 and 4.3**), does not significantly affect behavioral inhibition or

flexibility in a 4-choice odor discrimination and reversal task and a Go/No-go task.

Moreover, we find that mIPSCs recorded from layer 5 in BDNF Val68Met mice do not

differ in amplitude or frequency (**Fig. 4.4**) suggesting that differences in synaptic

transmission in the anterior cingulate are unlikely to be the mechanism for differences in

behavioral flexibility discussed in Chapter 3. However, since BDNF is important for

many aspects of neuronal growth, development, and plasticity, there are many different

candidate mechanisms downstream of the BDNF Val68Met mutation. Here we explore

three future experiments that may later help to delineate the role that BDNF and TrkB

signaling plays in shaping behavioral flexibility in the behavioral phenotypes we

described in Chapter 3.

Inhibit TrkB signaling during early vs. late post-natal developmental windows

We presume periadolescent activity-dependent BDNF signaling is largely mediated

through TrkB signaling in the brain. However, we were not able to phenocopy the BDNF

Val68Met mice by inhibiting TrkB at P23-40. In Chapter 2 of this dissertation we show

that there is a TrkB dependent developmental increase in mIPSC amplitude in a subset of

layer 5 pyramidal neurons during periadolescence (P21-40) which is blocked by

inhibiting TrkB (**Fig. 2.7**). We also show that blocking the maturation of mIPSC

amplitude in Trk$_{F616A}$ mice did not impact reversal learning performance (**Fig. 4.1**).

However, we did not determine whether inhibiting TrkB during earlier or longer

developmental windows affects reversal learning performance. Preliminary results using

water delivery of 1NM-PP1 (P1 to P40-50, compared to vehicle) suggests that inhibiting

TrkB during either longer or earlier developmental periods in TrkB$_{F616A}$ mice may

significantly affect reversal learning performance (**Fig. A2**) in a manner similar to BDNF

Val68Met mutation. In ongoing experiments we plan to inhibit TrkB though water

delivery of 1NM-PP1 to TrkB$_{F616A}$ mice during either early (P1-21) or late (P21-50)

development to determine the critical time-period over which TrkB inhibition produces

changes in reversal behavior. By determining the critical window for the development of

behavioral flexibility we can hone in on possible neurodevelopmental changes that might

underlie behavioral differences between animals.

Striatal D1/D2 excitability

We show in this dissertation that BDNF Val68Met mutation leads to behavioral differences in reversal learning performance (**Fig. 3.2 and 3.3**), yet in **Fig. 4.4** we show that there is no difference in mPSCs in the anterior cingulate between BDNF Val68Met Val/Val and Met/Met mice. In ongoing investigations we are looking at maturation of the striatum in BNDF Val68Met mice. The striatum relies on activity dependent release of BDNF from the cortex (Kokaia et al., 1998), and may be particularly sensitive to deficits in BDNF signaling (Rauskolb et al., 2010). Using a conditional BDNF KO, Rauskolb *et al.* have shown that BDNF deficits can impact striatal cell survival, spine density, and dendritic complexity without detectable effects on hippocampal neurons. Based on these data I propose an alternate hypothesis: that trophic support and synaptic changes between the dorsomedial cortex (including the anterior cingulate region) and the dorsomedial-striatum (DMS), a brain region also known to support behavioral flexibility (Balleine and O'Doherty, 2010) and reversal learning (Ragozzino and Rozman, 2007), may underlie performance differences seen in reversal learning tasks in BDNF Val68Met mice. Lobo et al. has shown that deletion of TrkB in the nucleus accumbens affects cocaine reward by increases neuronal excitability in striatal medium spiny neurons. This change alters cocaine conditioned place preference in a direction dependent on whether the deletion is targeted to dopamine D1 or D2 receptor-bearing medium spiny neurons. When targeting is non-specific behavioral profiles look as if D2 type cells are targeted, suggesting they are dominant or more sensitive to TrkB receptor deletion (Lobo et al., 2010).

It is possible that comparable changes may occur in the dorsal-medium striatum in BDNF Val68Met mice. A reduction of activity dependent BDNF release may produce an effect

similar to TrkB deletion, that is, an overall increase in MSN activity with D2+ neurons being most affected. An increase in D2+ excitability, as part of the indirect pathway, might be expected to increase inhibitory control enhancing behavioral flexibility and task switching performance (Stelzel et al., 2010; Smith et al., 2011; Yawata et al., 2013). We have begun preliminary experiments looking at the excitability of MSNs in BDNF Val68Met mice as well as the D2/D1 ratio in the striatum using radioligand binding. Though the results are preliminary, we do see a subtle difference in excitability between BDNF Val68Met homozygous mice (**Fig. A3**). These and future experiments may shed light on the mechanism that underlies differences in behavioral flexibility.

Neuronal Synchrony

In this dissertation we use two manipulations to disrupt cortical inhibition: a chemical genetic approach to block TrkB systemically during the periadolescent period and a constitutive GAD65 KO. Neither of these manipulations had a significant effect on reversal learning performance and GAD65 KO did not impact go/no-go learning (Chapter 4). I concluded that there is not a straightforward relationship between cellular inhibition in the prefrontal cortex and behavioral inhibition associated with prefrontal function. However, it may be that there are other ways to disrupt inhibition that perhaps we did not accomplish with our manipulations. Network oscillations reflect the temporal coordination of primary cell populations, and have been recognized as being important in the coding of neural information. It has recently been shown that different GABAergic cell types that innervate specific domains on principle cells are active during discrete phases of the local field potential cycle (Varga et al., 2012). It is possible that disruption

of the timing of inhibition might have a more significant impact on behavior than the amount of inhibition. For example, using a mouse model of schizophrenia, Nakazawa et al. showed that disruptions of inhibitory neuron synapses that effect the correlation of neural firing may have more impact on behavior than those that do not.   It is possible that the manipulations used in the study, while reducing inhibition, did little to alter neuronal synchrony. It would therefore be interesting in future experiments to record cell attached activity from units and local field potentials from BDNF Val68Met, 1NM-PP1 treated TrkB$_{F616A}$, and GAD65 KO mice to determine if neural synchrony is impacted in any of these mice. These measures could be used to compare relative timing of local pyramidal cells and specific interneuron types to the LFP phase. They could also be used to measure phase synchrony/locking across brain regions, such as across the commissures (potentially relevant to results in Chapter 2). If synchrony was critical for reversal task performance, we would predict that synchrony was disrupted in BDNF Val68Met KI mice, but not after 1NM-PP1 treatment in TrkB$_{F616A}$ mice or GAD65 KO mice. Future experiments could also use optogenetic techniques in PV-Cre and somatostatin-Cre mice to determine whether disruption of their activity alters reversal learning or other tasks which require behavioral inhibition.

Appendix
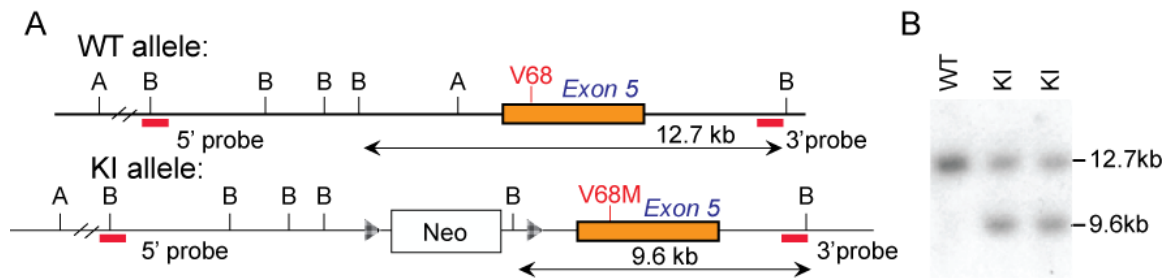
Figure A1. Generation of the Dorit Ron lab BDNF (Val68Met) mice. *A.* WT and k/in alleles of the BDNF gene. The introduction of the point mutation (Val68Met) into the coding region of the BDNF gene (exon 2) is indicated. A neomycin resistance cassette (neo) flanked by loxP sites (depicted as triangles) was also introduced into the k/in allele. Restriction sites are as follows: A, Afl II and B, Bgl II. B. Southern blot analysis of *Bgl*II-digested genomic ES cell DNA to identify homologously recombined ES cell clones. Hybridizations depict the WT allele (12.7 kb) and the k/in allele (9.6 kb) by using the 3' flanking probe. (Warnault et al., 2013)
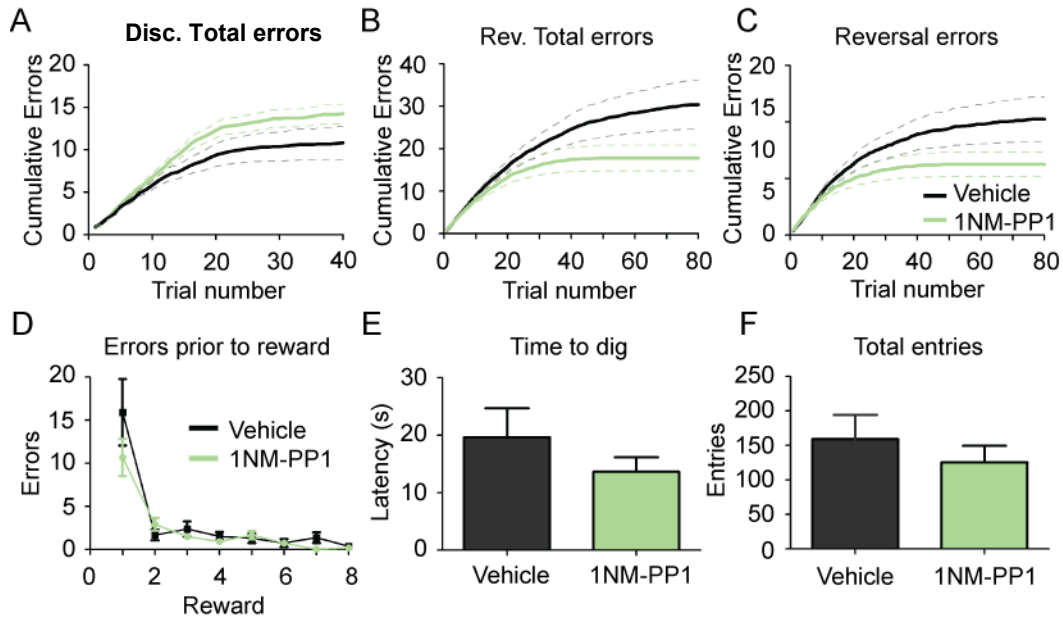
Figure A2. 1NM-PP1 treatment in drinking water from P1 to P40-50 impacts both discrimination and reversal learning in TrkBF616A mice. *A.* TrkB$_{F616A}$ mice given 1NM-PP1 (n=16) via drinking water from P1 to P40-50 accumulated more errors than vehicle (n=13) treated mice during the discrimination phase (SD) of the 4-choice reversal task. A two way repeated measures ANOVA showed that there was no significant main effect of treatment but a significant interaction between treatment and trial number for accumulation of errors (treatment $F(1,1053)=3.2$, $p>0.05$, interaction $F(39,1053)=2.27$ $p<0.0001$) *B.* Mice given 1NM-PP1 accumulated fewer total errors than vehicle treated control mice during the reversal phase of the 4-choice reversal task (treatment $F(1,2212)=3.21$, $p>0.05$, interaction $F(79,2212)=3.78$, $p<0.0001$) as well as reversal errors (*C*) (treatment $F(1,2212)=2.82$, $p>0.05$, interaction $F(79,2212)=3.4$ $p<0.0001$). *D.* 1NM-PP2 and vehicle treated mice have a similar number of errors prior to reward (treatment $F(1,189)=2.06$, $p<0.05$, interaction $F(7,189)=1.24$, $p<0.05$). *E.* Average time per trial before digging (Mann Whitney test, $p=0.08$). *F.* Average number of entries into different quadrants before digging ($p=0.17$).
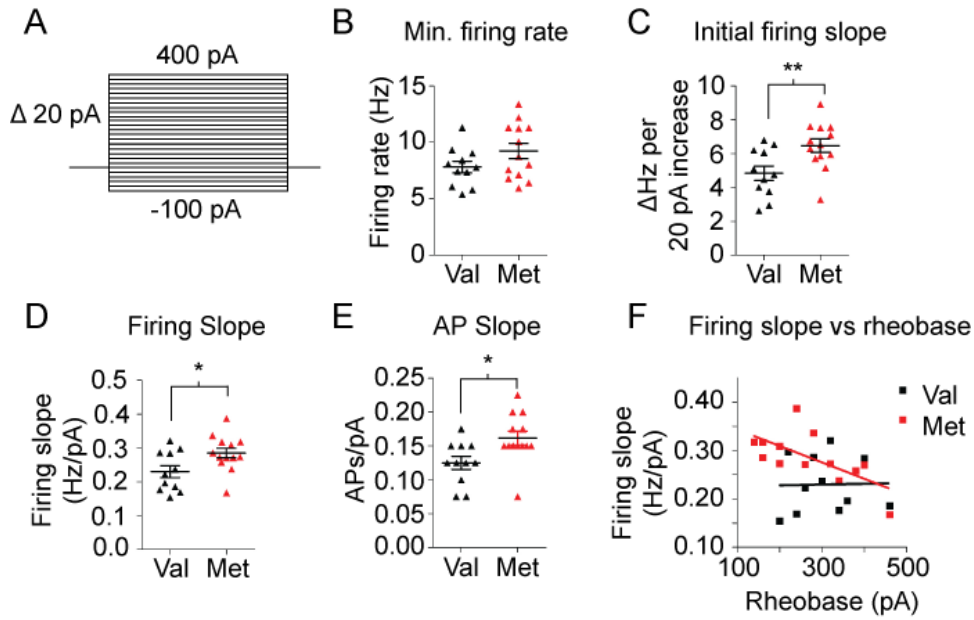
Figure A3. Whole cell current clamp recordings from MSN neurons in BDNF Val68Met mice shows evidence for differences in MSN excitability.
A. Stimulation protocol. *B.* Firing rate in first sweep with a measurable firing rate (at least 2 APs). *C.* Firing slope defined as slope of the firing rate between the first sweep with a measurable firing rate and the second sweep. *D.* Slope of a fit line computed over the first three sweeps that have a measurable firing rate. *E.* A similar measure to that in D, but using the number of action potentials rather than inter-spike interval to compute firing rate. *F,* Scatterplot of the firing slope vs. rheobase.

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