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Differential expression of cytoskeletal regulatory factors in the adolescent prefrontal cortex: Implications for cortical development

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Abstract

The prevalence of depression, anxiety, schizophrenia, and drug and alcohol use disorders peaks during adolescence. Further, up to 50% of “adult” mental health disorders emerge in adolescence. During adolescence, the prefrontal cortex undergoes dramatic structural reorganization, in which dendritic spines and synapses are refined, pruned, and stabilized. Understanding the molecular mechanisms that underlie these processes should help to identify factors that influence the development of psychiatric illness. Here we briefly discuss the anatomical connections of the medial and orbital prefrontal cortex (mPFC and OFC, respectively). We then present original findings suggesting that dendritic spines on deep-layer excitatory neurons in the mouse mPFC and OFC prune at different adolescent ages, with later pruning in the OFC. In parallel, we used western blotting to define levels of several cytoskeletal regulatory proteins during early, mid-, and late adolescence, focusing on tropomyosin-related kinase receptor B (TrkB) and β 1-integrin-containing receptors and select signaling partners. We identified regional differences in the levels of several proteins in early and mid-adolescence that then converged in early adulthood. We also observed age-related differences in TrkB levels, both full-length and truncated isoforms, Rho-kinase 2 (ROCK2), and synaptophysin in both PFC subregions. Finally, we identified changes in protein levels in the dorsal and ventral hippocampus that were distinct from those in the PFC. We

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conclude with a general review of the manner in which TrkB- and β 1-integrin-mediated signaling influences neuronal structure in the postnatal brain. Elucidating the role of cytoskeletal regulatory factors throughout adolescence may identify critical mechanisms of PFC development.

Keywords

BDNF; Arg; Abl2; p120RasGAP; p190RhoGAP; cortactin; LIM kinase; PSD95; Rho-kinase II; review

Part 1. General Introduction

Adolescence represents a critical period of neurodevelopment, defined by significant structural and synaptic maturation within the prefrontal cortex (PFC). Synapses and dendritic spines, the primary contact sites of excitatory synapses in the brain, are refined, pruned, and stabilized (*e.g.*, (Bourgeois et al 1994, Rakic et al 1994, Huttenlocher & Dabholkar 1997, Gourley et al 2012b, Koss et al 2014)). Additionally, changes in gross PFC volume can be detected across rodent-human species (Van Eden & Uylings 1985, Giedd et al 1999). This structural remodeling is believed to establish a foundation for the transition to adulthood. It may also, however, open a window of vulnerability to the development of psychiatric illness.

The prevalence of depression, anxiety, schizophrenia, and drug and alcohol use disorders peaks during adolescence worldwide (Whiteford et al 2013, Davidson et al 2015). Furthermore, approximately 50% of “adult” mental health disorders emerge during adolescence (Kessler et al 2005, Belfer 2008). Abnormalities in PFC maturation are associated with neuropsychiatric illness. For example, an MRI study of adolescents determined that depression symptoms are associated with thicker ventromedial PFC volume (Ducharme et al 2014), potentially suggestive of a failure in neuropil pruning. Another recent study determined that complement component 4A, a protein known to facilitate synaptic pruning during adolescence in mice, is 1.4-fold higher in individuals suffering from schizophrenia than healthy controls (Sekar et al 2016). Elevated complement component 4A could contribute to excessive synapse elimination in schizophrenia, in line with evidence that schizophrenia is characterized by decreased dendritic spine density in the PFC and progressive cortical thinning that is also associated with symptom severity (Garey et al 1998, Glantz & Lewis 2000, Cannon et al 2002, Cannon et al 2015). Understanding the molecular mechanisms regulating structural remodeling of neurons in the PFC – under both typical and pathological circumstances – during adolescence could provide valuable insight into the mechanisms underlying adolescent-onset neuropsychiatric illness.

The PFC can be grossly divided into the medial and orbital PFC (mPFC and OFC, respectively). These structures appear to have distinct developmental trajectories; for example, the maximum volume of the mPFC occurs at postnatal day (P) 24 in rats, whereas the maximal OFC volume is detectable later, at P30 (Van Eden & Uylings 1985).

In this review, we will briefly outline key differences in some of the afferent and efferent projections of the mPFC and OFC. Next, we will introduce a constellation of proteins that

regulates neuronal structural plasticity and stability in the postnatal brain. Then, we will present original data illustrating differential expression of these synaptic, neurotrophic, and cytoskeletal regulatory signaling factors during early, mid-, and late adolescence in the PFC subregions of the mouse. The distinct developmental trajectories of protein levels may be critical for the typical maturation of mPFC and OFC neurons. For some targets, we also compare protein levels in the dorsal and ventral hippocampus (DHC and VHC, respectively). Finally, we conclude with a general review of the manner in which TrkB and β 1-integrin and associated signaling partners are currently believed to regulate neuronal structure.

Part 1.1. Connections of the PFC

The mPFC contains the anterior cingulate, prelimbic, and infralimbic cortices, as well as the medial OFC, situated at the base of the medial wall (Heidbreder & Groenewegen 2003) (Fig. 1a). The OFC extends across the ventral surface of the rostral PFC and generally refers to the ventrolateral and dorsolateral OFC regions, as well the agranular insular cortex (Ongur & Price 2000) (Fig. 1a). Reciprocal connections between the mPFC and OFC allow for communication within the PFC (Heidbreder & Groenewegen 2003, Hoover & Vertes 2011). Additionally, the mPFC and OFC both have rich connections (both direct and via relays) with structures such as the amygdala, thalamus, hippocampus, and striatum.

In addition to these similarities, there are key distinctions between the mPFC and OFC. For example, sensory modalities converge in the OFC but spare the mPFC (Carmichael & Price 1995, Reep et al 1996, Hoover & Vertes 2011). The mPFC and OFC each also have discrete projections to the striatum. Within the mPFC, the ventral prelimbic and infralimbic cortices project to the nucleus accumbens shell, whereas the dorsal prelimbic and cingulate cortices preferentially project to the nucleus accumbens core (Berendse et al 1992, Heidbreder & Groenewegen 2003, Rodriguez-Romaguera et al 2015) (Fig. 1a), and these patterns are associated with the differential roles of the dorsal and ventral mPFC in drug-seeking behaviors in animal models of addiction (Moorman et al 2015, Gourley & Taylor 2016). The mPFC also innervates the medial and central aspects of the dorsal striatum (Berendse et al 1992, Mailly et al 2013) (Fig. 1a), connections associated with PFC control over goal-directed action (Hart et al 2014).

In contrast, the agranular insular cortex projects to the ventral compartment of the nucleus accumbens, and the medial OFC targets the dorsal compartment, with relatively sparse ventral striatal innervation by the ventral, lateral, and dorsolateral regions of the OFC (Berendse et al 1992, Schilman et al 2008, Hoover & Vertes 2011, Rodriguez-Romaguera et al 2015) (Fig. 1a). The OFC projects to the dorsal striatum in a topographically-organized fashion, with the medial OFC innervating the medial-most region adjacent to the lateral ventricles, and the ventrolateral and dorsolateral OFC innervating the central and lateral aspects, respectively. Finally, the agranular insula targets the ventral and lateral compartments of the dorsal striatum (Berendse et al 1992, Schilman et al 2008, Hoover & Vertes 2011, Zimmermann et al 2015). OFC-striatal interactions are associated with selecting behaviors based on the likelihood that they will be reinforced (Gourley et al 2013a) or the value of the reinforcer (Gremel & Costa 2013), as well as the expression of

conditioned fear (Rodriguez-Romaguera et al 2015). Both the mPFC and OFC can receive indirect input from the striatum relayed through thalamic nuclei (Hoover & Vertes 2007).

The mPFC and the OFC also share reciprocal connections with the basolateral amygdala (BLA), however there are differences in their innervation patterns (Kita & Kitai 1990, Heidbreder & Groenewegen 2003, Cho et al 2013, Zimmermann et al 2015). The OFC shares reciprocal projections with the basolateral amygdala (BLA). This connectivity is critical for goal-directed decision making, as disconnecting the BLA from the ventrolateral OFC results in habit-based behavior (Zimmermann et al 2015). Although indirect projections from the OFC to the BLA via the thalamus have been reported in primates and rats, this circuitry is disputed in mice (van Vulpén & Verwer 1989, Matyas et al 2014, Timbie & Barbas 2014).

In the rodent, the mPFC neurons targeting the amygdala do not appear to relay through the mediodorsal thalamus, and have a broader projection pattern innervating multiple nuclei of the amygdala (Heidbreder & Groenewegen 2003, Matyas et al 2014). Within the mPFC, neurons in the prelimbic subregion project to the basal nucleus of the amygdala, whereas the medial orbital and the infralimbic cortices send projections to the BLA (Sesack et al 1989, McDonald et al 1996, Groenewegen et al 1997) and the central nucleus (McDonald et al 1996, Hoover & Vertes 2011). Neurons from the caudal region of the BLA innervate the infralimbic cortex and the ventral section of the prelimbic cortex, while neurons in the rostral region of the BLA project to the lateral OFC and agranular insular cortex (Sarter & Markowitsch 1983, Kita & Kitai 1990).

In contrast to the striatum and the amygdala, there are no direct projections from the PFC to the hippocampus (Laroche et al 2000, Heidbreder & Groenewegen 2003, Hoover & Vertes 2007, Hoover & Vertes 2011). Rather, reciprocal connections from the thalamus to the hippocampus and PFC could be responsible for shuttling information from the PFC subregions to the hippocampus (Heidbreder & Groenewegen 2003, Vertes 2006, Vertes et al 2007, Hoover & Vertes 2011, Ito et al 2015). Further, neurons in both the mPFC and OFC innervate the entorhinal cortex (Heidbreder & Groenewegen 2003, Vertes 2004, Hoover & Vertes 2011, Kondo & Witter 2014), a primary interface between the hippocampus and cortex (van Groen et al 2003, Agster & Burwell 2013). The entorhinal cortex has widespread projections throughout the mPFC and the OFC, whereas the DHC and the VHC have more limited innervation patterns in comparison (Insausti et al 1997, Delatour & Witter 2002, Hoover & Vertes 2007). The mPFC nevertheless receives direct input from the DHC and VHC, with markedly more substantial input from the VHC (Hoover & Vertes 2007). VHC projections to the OFC are present, but they are sparser by comparison (Jay et al 1989, Jay & Witter 1991, Cenquizca & Swanson 2007, Hoover & Vertes 2007, Vertes et al 2007).

Part 1.2. Brain-derived neurotrophic factor (BDNF)-TrkB and β 1-integrin-mediated signaling influence neuronal morphology

BDNF is a member of the Nerve Growth Factor family and is involved in neuronal development, morphology, and synaptic plasticity. BDNF is synthesized as a 32 kD pro-peptide, known as proBDNF, which contains a prodomain that can be cleaved to yield the 14 kD mature neurotrophin, mBDNF. ProBDNF activates the p75 receptor, whereas mBDNF

binds with highest affinity to TrkB, and can also activate truncated forms of the receptor (TrkB.T1) (Lu et al 2005, Fenner 2012).

What are the functions of TrkB receptors? Full-length TrkB receptors are comprised of an extracellular region containing a ligand-binding domain, a transmembrane region, and an intracellular section with a tyrosine kinase domain. Upon BDNF binding, the TrkB receptor dimerizes, and the tyrosine kinase domains autophosphorylate. Activated tyrosine kinase domains provide docking sites for the recruitment of intracellular signaling molecules that contribute to the widespread effects of BDNF/TrkB activity (Deinhardt & Chao 2014). Truncated isoforms of TrkB receptors lack the intracellular kinase domain and can dimerize with full-length receptors but are unable to activate the canonical signaling cascades (Deinhardt & Chao 2014). Although less is known about the truncated isoforms, there is evidence that they can initiate distinct signaling cascades (Ohira et al 2005).

Aberrant BDNF expression and TrkB signaling is associated with several neuropsychiatric disorders including anxiety and depression, bipolar disorder, and schizophrenia (Duman & Monteggia 2006, Autry & Monteggia 2012). Further, typical and atypical antidepressants increase BDNF and *Bdnf* expression in the human and rodent hippocampus and increase TrkB activation throughout cortico-hippocampal regions (Nibuya et al 1995, Chen et al 2001, Saarelainen et al 2003, Rantamaki et al 2007). Antidepressant treatment also restores cortico-hippocampal BDNF levels in rodents with a history of stress hormone exposure (modeling risk factors in depression) (*e.g.*, (Gourley et al 2012a, Gourley et al 2012c)). Given that dysregulated BDNF-TrkB signaling is implicated in neuropsychiatric illness, and that restoration of signaling is associated with recovery from these illnesses, we characterized the developmental expression profile of proBDNF and mBDNF, as well as full-length and truncated forms of the TrkB receptor in the mPFC, OFC, DHC, and VHC.

We also focused on β 1-integrin and downstream signaling factors. Integrin receptors are cell adhesion factors activated by extracellular matrix proteins. β 1-integrin is one subunit of a heterodimeric integrin receptor and is localized to synapses (Mortillo et al 2012). β 1-integrin-containing receptors are critical for synaptic transmission, long-term potentiation (LTP), synapse maturation, and dendrite and dendritic spine stability in the postnatal brain (Chan et al 2006, Huang et al 2006, Chan et al 2007, Warren et al 2012, Kerrisk et al 2013). Knockout of *Itgb1*, encoding β 1-integrin, in excitatory neurons of the forebrain results in significant dendrite and synapse destabilization in hippocampal CA1 between P21 and P42, the equivalent of adolescence in rodents (Warren et al 2012). These findings suggest that β 1-integrin is necessary for structural stabilization during adolescence. Studies using *in vitro* approaches further indicate that β 1-integrin signaling is essential for the formation and maturation of dendritic spines and synapses, as shRNA or antibodies against β 1-integrin interfere with these processes (Orlando et al 2012, Ning et al 2013). In neurons, activation of β 1-integrin-containing receptors stimulates Abl2/Arg kinase, also essential for dendrite and dendritic spine stabilization in hippocampal and cortical neurons (Sfakianos et al 2007, Gourley et al 2012b, Warren et al 2012). For example, integrin-Arg signaling activates p190RhoGAP to inhibit RhoA GTPase (Rho) signaling, ultimately stabilizing dendrite arbors.

Despite the numerous studies elucidating the functions of these various cytoskeletal regulatory proteins, few have established the developmental trajectory of these proteins across adolescence, particularly in the PFC. Here we first enumerated dendritic spines in deep-layer mPFC and OFC and then characterized levels of several synaptic, neurotrophin, and cytoskeletal regulatory factors during adolescent development. We find that dendritic spine elimination in deep-layer mPFC occurs earlier than in the OFC, in parallel with gross volumetric changes reported in these regions (Van Eden & Uylings 1985). Additionally, proteins associated with $\beta 1$ -integrin-mediated signaling are expressed at higher levels in the OFC than mPFC early in adolescence, whereas the levels of full-length and truncated isoforms of the TrkB receptor and synaptic markers are differentially expressed later, in mid-adolescence. These spatiotemporal differences in protein levels may be associated with the differential timing of PFC neuron structural maturation.

Part 2. Methods and results associated with original research findings

Methods

Subjects

Subjects for biochemical analyses were wild type C57BL/6 female mice (Jackson Labs). We chose to use females mice since females are largely underrepresented in preclinical neuropsychiatric research (Clayton & Collins 2014), despite the fact that women report significantly higher rates of mood and anxiety disorders than men (Eaton et al 2012). For dendritic spine analyses, mice of both sexes expressed Yellow Fluorescent Protein (YFP) under the control of neuron-specific elements in the *Thy1* gene (Feng et al 2000) and were back-crossed for at least 10 generations onto a C57BL/6 background. When possible, a single litter contributed to multiple time points. Throughout, mice were randomly assigned to early, mid-, or late adolescent treatment groups. Ages are indicated, with adolescence defined as P28–56 (Spear 2000). Mice were housed 2–8 per cage, maintained on a 12-hour light cycle (0700–0800 on), and provided food and water *ad libitum*. Procedures were Emory University IACUC-approved.

Dendritic spine analysis

Mice were briefly anaesthetized with isoflurane and euthanized by rapid decapitation. Brains were extracted and submerged in chilled 4% paraformaldehyde for 48 hr, then transferred to 30% w/v sucrose and sectioned into 40 or 50 μm coronal sections on a freezing microtome held at -15°C .

We compiled dendritic spine counts from 2 independent studies. Apical dendrites of mPFC neurons with cell bodies located in layer V were imaged using a Leica SP8 laser scanning confocal microscope with a 100X 1.4 numerical aperture objective and a 0.1 μm step size. We then confirmed at 10X magnification that the images were collected from the ventral prelimbic subregion of the mPFC, corresponding to plates 14–16 in *The Mouse Brain in Stereotaxic Coordinates* (Paxinos & Franklin 2002). Dendrites were located 25–150 μm from the cell body. 2–8 segments, each 20–25 μm in length, were imaged per mouse and 5–11 mice were used/time point. Each mouse considered an independent sample.

Deep-layer OFC neurons were imaged in separate experiments contributing to ref. (Gourley et al 2012b). Briefly, dendritic segments were imaged on a laser scanning confocal microscope (Olympus Fluoview FV1000) using a 100X 1.4 numerical aperture objective and Z-steps of 0.5 μm . After imaging, we confirmed that the image was collected from the ventrolateral OFC, with 2–8 dendrites per mouse and each mouse considered an independent sample. Dendrites were collected from secondary branches within 50–150 μm of the soma and were 11–85 μm in length. Due to the relatively stellate appearance of OFC neurons, apical vs. basal branches were not distinguished in this analysis (for direct comparison to mPFC neurons, see (Liston et al 2006, Kolb et al 2008))

Throughout, collapsed z-stacks were analyzed using NIH ImageJ: Each protrusion $\geq 4 \mu\text{m}$ was considered a spine (Peters & Kaiserman-Abramof 1970) and counted. If a spine bifurcated, only the longest arm was measured and counted. Individual planes were also evaluated to detect protrusions extending perpendicular to the collapsed z-stack. Total spine number for each segment was normalized to the length of the dendritic segment to generate density values. All scoring was conducted by blinded raters. Because mPFC vs. OFC images were collected as part of 2 independent studies using different methods, we caution against directly comparing between structures. Rather, we aim to highlight differences in dendritic spine densities between ages *within* each structure.

Immunoblotting

Mice were briefly anaesthetized with isoflurane and euthanized by rapid decapitation, and brains were extracted and frozen at -80°C . Frozen brains were sectioned using a chilled brain matrix into 1 mm sections, and tissue was extracted using a 1 mm diameter tissue core. OFC tissue punches would be expected to include the dorsolateral and ventrolateral OFC, as well as agranular insular cortex, whereas mPFC tissue punches included anterior cingulate, prelimbic, and infralimbic cortices, as well as the medial OFC. The DHC and VHC punches would be expected to contain CA1, CA3, and the dentate gyrus sub-regions of the hippocampus. Tissues were homogenized by sonication in lysis buffer (200 μl : 137 mM NaCl, 20 mM tris-HCL [pH=8], 1% igeal, 10% glycerol, 1:100 Phosphatase Inhibitor Cocktails 2 and 3 [Sigma], 1:1000 Protease Inhibitor Cocktail [Sigma]), and stored at -80°C . Protein concentrations were determined using a Bradford colorimetric assay (Pierce).

Samples from mice of every age were loaded onto each gel. This organization allowed for normalization within and between gels. Equal amounts of protein (15 μg) were separated by SDS-PAGE on either 4–20% or 7.5% gradient tris-glycine gels (Bio-rad). Following transfer to PVDF membranes, blots were blocked with 5% nonfat milk for 1 hr. Membranes were incubated with primary antibodies at 4°C overnight and then incubated in horseradish peroxidase secondary antibodies for 1 hr. Immunoreactivity was assessed using a chemiluminescence substrate (Pierce) and measured using a ChemiDoc MP Imaging System (Bio-rad). Densitometry values were normalized to the corresponding loading control (HSP-70 or GAPDH). All densitometry values were then normalized to the control sample mean from the same membrane in order to control for variance in fluorescence between gels.

Antibodies

See table 1.

Statistical analyses

Dendritic spine densities and densitometry values were compared by 2-tailed *t*-tests or one- or two-factor ANOVA, as appropriate, using SigmaStat and Graphpad Prism with $\alpha = 0.05$. In the case of significant interactions or main effects, Tukey's post-hoc comparisons were made and are indicated graphically. PFC TrkB data were subjected to transformation to improve normality. Throughout, values lying two standard deviations outside of the mean were considered outliers and excluded.

Results

We aimed to enumerate dendritic spines on deep-layer pyramidal neurons in the mPFC and OFC during adolescence. In parallel, we sought to characterize levels of several neurotrophic and cytoskeletal regulatory elements in the mPFC and OFC during adolescent development. For several factors, we compared levels across time in the DHC and VHC as well.

Dendritic spines in the mPFC and OFC are eliminated during adolescence

We measured dendritic spine densities on excitatory layer V PFC neurons at 3 developmental time points, corresponding to early, mid-, and late adolescence in rodents (Spear 2000). Densities in the mPFC decreased between P31 and P42, approaching adult-like levels (compare to P56) [$F_{(2,19)}=16.173$, $p=.0001$] ($n=5-9/\text{group}$) (Fig. 1b). Dendritic spine densities in the OFC, on the other hand, were unchanged between P31–P39, before decreasing by P56 [$F_{(2,22)}=8.184$, $p=.003$] ($n=6-11/\text{group}$) (Fig. 1b). Thus, adolescent-onset dendritic spine pruning appears to occur earlier in the mPFC.

Regional and age-dependent patterns in synaptic, neurotrophic, and cytoskeletal regulatory factors during adolescence

Next we compared the protein levels of several synaptic, neurotrophic, and cytoskeletal regulatory factors at P35, P42, and P56 in mPFC and OFC tissue. First, we assessed levels of the presynaptic marker, synaptophysin, and the post-synaptic marker, post-synaptic density protein 95 (PSD95). We found no regional or age-related changes in PSD95 (all $p>.05$, $n=7-10/\text{group}$) (Fig. 2a), however synaptophysin levels decreased with age [main effect $F_{(2,45)}=5.770$, $p=.006$] ($n=7-10/\text{group}$) (Fig. 2b), consistent with synaptic pruning. By contrast, TrkB.T1 and TrkB levels increased with age [main effect $F_{(2,43)}=3.258$, $p=.048$] ($n=7-10/\text{group}$) [main effect $F_{(2,45)}=3.820$, $p=.029$] ($n=6-10/\text{group}$) (Fig. 2c–d). ProBDNF, mBDNF, $\beta 1$ -integrin, Abl2/Arg, cortactin, p120RasGAP, Rho and LIMK2 levels were fairly consistent across ages (all main effect and interaction $p>.05$, $n=5-13/\text{group}$) (Fig. 2e–i,k,l,n). Finally, both p190RhoGAP and ROCK2 levels increased in both PFC subregions from P35–P42 before decreasing by P56 [main effect $F_{(2,26)}=5.270$, $p=.012$] ($n=10-13/\text{group}$) [main effect $F_{(2,61)}=5.365$, $p=.007$] ($n=3-7/\text{group}$) (Fig. 2j,m).

Casting a spotlight on early and mid-adolescence

Throughout these analyses, we noted several instances in which proteins associated with $\beta 1$ -integrin-mediated signaling (Fig. 3a) appeared slightly elevated in the OFC at P35. We thus compared protein levels in a separate series of western blots specifically using tissues collected at P35. This approach revealed a main effect of region [$F_{(1,117)}=3.067$, $p=.05$] ($n=7-13/\text{group}$) (Fig. 3b), indicating overall higher protein levels in the OFC. We did not detect any interaction effects, which precluded post-hoc comparisons, but this general pattern, albeit modest, could contribute to Abl2/Arg-mediated stabilization of dendritic spines in the OFC beginning early in adolescence (Gourley et al 2012b). We did not observe any regional differences in levels of these proteins at P42 or P56 (not shown).

A targeted approach similarly revealed that at P42, mid-adolescence, PSD95, synaptophysin, and mBDNF were more abundant in the mPFC than OFC, whereas TrkB levels were greater in the OFC than mPFC [interaction $F_{(5,85)}=7.489$, $p<.0001$] ($n=4-9/\text{group}$) (Fig. 4b,e). There were no regional differences in protein levels detected at earlier, at P35, or later, at P56 (all $ps>.05$, $n=6-17/\text{group}$) (Fig. 4a,c,d,f).

Finally, we characterized expression patterns of several of these proteins in the hippocampus, a region with a distinct developmental trajectory. PSD95 levels were higher in the DHC than VHC, especially during early adolescence [main effect of region $F_{(1,36)}=4.867$, $p=.034$] ($n=7/\text{group}$) (Fig. 5a). Synaptophysin levels were stable and did not differ (all $ps>.05$, $n=7/\text{group}$) (Fig. 5b). TrkB.T1 levels increased progressively in both regions [$F_{(2,35)}=5.665$, $p=.007$] ($n=6-7/\text{group}$) while TrkB remained unchanged (all $ps>.05$, $n=6-7/\text{group}$) (Fig. 5c,d). proBDNF levels were higher in the DHC than VHC [$F_{(1,36)}=14.794$, $p=.0005$] ($n=7/\text{group}$) (Fig. 5e), whereas mBDNF levels were comparable between regions ($p>.05$, $n=6-7/\text{group}$) (Fig. 5f).

Together, these results point to early and mid-adolescence as key periods during which integrin- and neurotrophin-related signaling may differ between the mPFC and OFC, while protein levels largely converge by early adulthood. These patterns contrast those detected in the hippocampus, where regional differences, but fewer age-related patterns, were identified.

Part 3. General Review

During adolescence, the PFC undergoes dramatic structural remodeling and synaptic reorganization. The mPFC and OFC compartments are apposed to one another and interconnected, but they also have distinct projection patterns, and can be differentially impacted by various environmental stimuli, such as stressors and drugs of abuse. Our findings concur with those of others, revealing that these structures also have different developmental trajectories during adolescence. Specifically, we report that the dendritic spine elimination on deep-layer excitatory pyramidal neurons occurs earlier in the mPFC than the OFC. Furthermore, several neurotrophic and cytoskeletal regulatory factors exhibit different protein levels between structures during adolescence, potentially contributing to differential structural maturation trajectories.

A number of studies report that exposure to environmental stimuli such as social interactions or stressors during adolescence can have opposing effects on dendrite structure and dendritic spine density in the mPFC and OFC. For example, social play during adolescence modestly increases dendrite arborization in the OFC, while decreasing arborization in the mPFC, an effect that could be interpreted as enhanced pruning (Bell et al 2010). Exposure to the primary stress hormone corticosterone during adolescence eliminates dendritic spines in the mPFC and OFC, however only mPFC spine counts readily recover following the corticosteroid exposure period (Gourley et al 2013b). It is possible that regional differences in the levels of key stability-regulating proteins could contribute to these instances of differential responsivity to play and stress hormone exposure.

Part 3.1. PFC dendritic spine density and synaptic marker levels change during adolescence

We directly compared dendritic spine densities on excitatory pyramidal neurons in deep-layer mPFC and OFC in early, mid-, and late adolescence/young adulthood in rodents (Spear 2000). Dendritic spine densities dropped appreciably in the mPFC and approached adult-like densities between P31 and P42. Juraska and colleagues have reported considerable elimination of dendrites and dendritic spines in the mPFC between P35 and P90 (Koss et al 2014). Our data suggest that key pruning events occur within a relatively sharp window between P35 and P42 in the mPFC. In contrast, OFC dendritic spine densities decreased significantly only between P39 and P56, suggesting that pruning occurs later in the OFC than the mPFC. We note however that elimination of PFC dendritic spines is not necessarily “complete” by late adolescence/early adulthood, as Milstein et al. report elimination of spines on distal dendritic segments in both mPFC and OFC between ages P49 and 8 months (Milstein et al 2013). Overall, our findings suggest that at least some aspects of the mPFC mature before the OFC, which is consistent with neuroimaging studies in humans revealing that white matter volume in the mPFC reaches an adult-like state earlier than in the OFC (Tamnes et al 2010).

Our dendritic spine counts include mature dendritic spines that likely contain synapses, as well as immature spines that likely do not host synapses. In an attempt to evaluate synapse maturation during adolescence we also compared levels of the synaptic markers PSD95 and synaptophysin. PSD95 is a scaffolding protein critical for organizing the post-synaptic density of excitatory synapses (El-Husseini et al 2000, Garner et al 2000, Aoki et al 2001, Kim & Sheng 2004, Chen et al 2011, Chen et al 2015). More specifically, PSD95 interacts with several receptors and ion channels to regulate their insertion into the post-synaptic density, as well as their internalization (El-Husseini et al 2000, El-Husseini et al 2002, Ehrlich & Malinow 2004, Kim & Sheng 2004). Furthermore, PSD95 binds receptor subunits and intracellular signaling molecules to couple activation of receptors to intracellular signaling cascades (Amano et al 1996). PSD95 is also critical for both synaptic function and maturation, as knockdown of PSD95 in hippocampal cell culture decreases both AMPA- and NMDA receptor-mediated excitatory postsynaptic currents and prevents the increase in dendritic spine density and mushroom-shaped spine formation that is associated with dendritic spine maturation (Ehrlich et al 2007).

Synaptophysin is a membrane-bound protein expressed on the surface of synaptic vesicles (Navone et al 1986). It is located presynaptically at both excitatory and inhibitory synapses. Synaptophysin is among the most abundant synaptic vesicle proteins, yet surprisingly, synaptic formation and transmission appear to be relatively normal in synaptophysin knockout mice (McMahon et al 1996, Tarsa & Goda 2002, Takamori et al 2006). The synaptophysin knockout mice do exhibit deficits in learning and memory tasks, however (Schmitt et al 2009), indicating that synaptophysin deficiency likely impacts neuronal function.

In the human PFC, PSD95 levels are highest between ages 11–15, and they decrease only slightly over the next few years before plateauing. Synaptophysin levels, on the other hand, peak between ages 6–10 before decreasing substantially to adult levels around age 16 (Glantz et al 2007). We observed a similar developmental trajectory in the mouse PFC, as PSD95 levels remained relatively stable, whereas synaptophysin levels in both the mPFC and OFC decreased significantly between P35 and P56. A similar pattern of synaptophysin loss was reported in the rat mPFC between P35 and P45 (Drzewiecki et al 2016). Interestingly, this effect was specific to females, and not males. We did not include males here, so we cannot comment on whether this sex difference occurs in mice as well.

Although the expression pattern of PSD95 did not parallel the elimination of dendritic spines as synaptophysin did, it is important to note that PSD95 is also considered an indicator of synaptic strength (Beique & Andrade 2003, Stein et al 2003). No net change in PSD95 levels could thus conceivably reflect a combination of both dendritic spine pruning and increases in stronger, more mature synapses during this window of time.

In several experiments, we included samples collected from the hippocampus, which has distinct developmental timing relative to the PFC. PSD95 levels were higher in the DHC than VHC but fairly stable over time in both regions. In the rat hippocampus, by contrast, PSD95 levels decrease between P10 and P60. This effect is strongest in CA3 compared to CA1, however regional differences between the dorsal and ventral CA zones were not considered in this prior report (Elibol-Can et al 2014). Synaptophysin levels were also consistent between early adolescence and young adulthood (Fig. 5); however, unlike PSD95, regional differences were not detected (see also (Elibol-Can et al 2014)). This pattern is in agreement with mRNA expression in adolescent *vs.* adult humans and rats (Eastwood et al 2006).

Part 3.2. BDNF-TrkB signaling regulates cell structure in the postnatal brain

Neurotrophin signaling contributes greatly to dendritic and dendritic spine morphology and synaptic plasticity (Zagrebelsky & Korte 2014). For example, acute application of BDNF to hippocampal primary neurons results in transient phosphorylation of its high-affinity receptor TrkB and neurite elongation and dendritic spine head enlargement. Longer-term application prolongs TrkB phosphorylation and increases dendrite branching and dendritic spine neck elongation (Ji et al 2005). Conversely, decreasing TrkB levels reduces dendritic spine density (Orefice et al 2013). Thus, BDNF mediates dendrite and dendritic spine growth through TrkB, and the duration of signaling determines its effects on neural structure. Further, mice with *Bdnf* knocked out specifically in the central nervous system

have smaller brains and a greater proportion of immature dendritic spines in hippocampal CA1 compared to wild type mice (Rauskolb et al 2010).

Experiments using cortical neuron cultures reveal similar findings, in that BDNF overexpression (Horch et al 1999) or bath application of BDNF (McAllister et al 1997) increases the number of basal dendrites on BDNF-releasing neurons. The morphological effects of BDNF are eliminated with the addition of a TrkB-blocking antibody, again indicating that BDNF is acting through TrkB (McAllister et al 1997). *In vivo* studies further indicate that both TrkB and BDNF are critical for the stability of dendrite and dendritic spine structure throughout the lifespan. For example, transgenic mice with a 47% reduction in cortical TrkB have 50% thinner apical dendrites, simplified dendritic arbors, and smaller cell bodies in layer II/III neurons relative to control mice (Xu et al 2000). Further, layer II/III cortical neurons from forebrain-specific *Bdnf* knockout mice appear normal at P15, but have decreased cell body size by P21, and a 29% reduction in branch points by P35 (Gorski et al 2003). Mice with late-onset, forebrain-specific *Bdnf* knockdown, causing a 97% reduction in BDNF by postnatal week 4, have normal dendritic spine density at P35, but a 30% reduction in density by P84 (Vigers et al 2012). Interestingly, dopamine transporter knockout mice have reduced dendritic spine density in the mPFC and CA1 region of the hippocampus (Kasahara et al 2015), as well as on medium spiny neurons of the striatum (Berlanga et al 2011). This is pertinent because these same mice also have a 50% reduction in BDNF mRNA and protein levels in the PFC (however BDNF protein levels are unaffected in the striatum and hippocampus) (Fumagalli et al 2003, Li et al 2010). This loss of neurotrophic support in the PFC may contribute to the loss of PFC dendritic spines in these mice.

In contrast to mature BDNF, proBDNF appears to enhance dendritic spine *pruning*, as overexpression decreases dendritic spine density, and this effect is dependent on the p75 receptor (Orefice et al 2013). Interestingly, these influences are detectable only in mature cultures, suggesting that proBDNF-p75 signaling could be involved in dendritic spine pruning relatively late in development (Orefice et al 2013).

In humans, ~30% of individuals have a small nucleotide polymorphism known as Val66Met, a coding variant in the proBDNF region of the gene that impairs BDNF transport and subsequent release (Egan et al 2003, International HapMap 2003). These individuals have relatively impaired executive functioning, increased risk of depression, and reduced hippocampal volume (Frodl et al 2007, Gatt et al 2009, Duman & Duman 2015). Similarly, mice that are genetically manipulated to express this coding variant have elevated plasma corticosterone levels and increased depression-like and anxiety-like behavior following restraint stress (Yu et al 2012). In the mPFC, dendritic spine counts and lengths are reduced (Liu et al 2012), consistent with a reduction of BDNF levels, as discussed above. Furthermore, the polymorphism is associated with decreased dendrite length and branch points in hippocampal CA3 neurons (Chen et al 2006). Similarly, *Bdnf*^{+/-} mice have decreased hippocampal volume and reduced dendritic branching in hippocampal CA3 compared to wild type counterparts (Magarinos et al 2011).

Bdnf mRNA increases to adult levels in late adolescence in the human dorsolateral PFC (Webster et al 2002). Late adolescence/early adulthood corresponds to approximately P56 in

the mouse (Spear 2000), however we did not observe variability in PFC BDNF protein levels at this time. It is important to note, though, that BDNF is subject to both anterograde and retrograde transport, so *Bdnf* mRNA is not necessarily a reliable indicator of local BDNF protein levels (DiStefano et al 1992, Conner et al 1997).

In the hippocampus, proBDNF levels were consistently higher in the DHC than VHC, and we did not identify any age-related changes in either proBDNF or mBDNF levels. These findings were not wholly unexpected, given that in humans, *Bdnf* mRNA within both hippocampal CA1 and CA3 appears fairly stable across the lifespan (Webster et al 2006). Further, Silhol et al., 2005 reported that *Bdnf* expression in the rat hippocampus is unchanged throughout postnatal development (from P1–22 months), and BDNF protein (both pro and mature forms) reaches adult levels by P7. In the mouse hippocampus, *Bdnf* mRNA similarly increases during early development (Ivanova & Beyer 2001). Interestingly, one recent investigation found that mBDNF levels increase further later, between P21 and P42 (Lauterborn et al 2016). Given that we did not observe any changes in mBDNF between P35 and P56, we suggest that levels could be particularly dynamic up to P35, and then they stabilize.

Part 3.3. Does TrkB.T1 impact neuron structure?

Initially, it was believed that TrkB.T1 was a dominant-negative receptor, preventing BDNF from binding to TrkB (Fenner 2012). Further studies revealed that this may not fully be the case; for example, TrkB.T1 promotes neurite outgrowth on dendrites distal from the soma in cortical slices, an effect that is independent of BDNF (Yacoubian & Lo 2000). Further, BDNF-mediated activation of TrkB.T1 stimulates a GTPase inhibitor that prevents the activation of RhoA, promoting spindle-shaped cell morphology in a glioma cell line (Ohira et al 2006). TrkB.T1 knockout mice have decreased dendrite arborization and decreased dendrite length in the basolateral amygdala, with no apparent effects on neural morphology in the hippocampus (Carim-Todd et al 2009). Together, these findings suggest that TrkB.T1 indeed regulates dendrite and dendritic spine morphology in both BDNF-dependent and -independent manners (Fenner 2012).

We detected a significant increase in both full-length TrkB and TrkB.T1 between P35–P56, with a qualitatively greater increase in TrkB.T1. Additionally, we found higher levels of both receptors in the OFC than mPFC at P42, corresponding to a mid-adolescent period (Spear 2000). The regional difference could potentially be associated with our finding that mPFC dendritic spine density is approaching a mature adult-like state by P42, whereas the OFC appears to be under-going a significant degree of pruning at this point (see again, Fig. 1). TrkB receptors may be elevated to support dynamic periods of pruning and spine stabilization in the OFC (Deinhardt & Chao 2014, Zagrebelsky & Korte 2014), however additional studies are needed to clarify the role of TrkB receptors, including TrkB.T1 receptors, in PFC dendritic spine dynamics.

As in the PFC, we observed a progressive increase in TrkB.T1 during adolescence in the VHC and DHC. By contrast, full-length TrkB appeared constant in both hippocampal regions. A similar pattern was reported in the rat hippocampus — TrkB protein decreases slightly throughout the first three weeks of life – before our tissue collection here – and then

reaches stable adult-like levels (Silhol et al 2005). In contrast, Lauterborn et al. report that both TrkB and TrkB.T1 levels decrease slightly between P21 and P42 in the mouse hippocampus (Lauterborn et al 2016); we suggest that this drop occurs prior to P35, since we observed stable (TrkB) or even increased (TrkB.T1) protein levels between P35–42.

Part 3.4. β 1-integrin-mediated cell adhesion systems regulate postnatal neural development

Integrins are heterodimeric cell adhesion receptors that mediate cell binding to the extracellular matrix. Integrin receptors are comprised of α and β subunits that determine ligand binding specificity (*e.g.*, laminin vs. fibronectin). There are 18 α subunits and 8 β subunits that are believed to form 24 distinct integrin receptors, 14 of which are expressed in the developing or mature central nervous system (Pinkstaff et al 1999, Hynes 2002, Reichardt & Prokop 2011). Ligand binding can induce a conformational change in the α and β subunit that exposes surfaces on the β cytoplasmic tail to engage downstream signaling proteins, including non-receptor tyrosine kinases (Woodside et al 2001, Woodside et al 2002, Arias-Salgado et al 2003, de Virgilio et al 2004, Harburger & Calderwood 2009, Simpson et al 2015). In addition, proteins of the talin and kindlin families can bind to the β tail and induce a conformational change to the activated high affinity binding state, so-called inside out signaling (Calderwood et al 2013).

The β 1-integrin subunit is highly expressed in both the cortex and hippocampus (Pinkstaff et al 1998, Pinkstaff et al 1999), and numerous studies indicate that β 1-integrin is critical for normal synaptic transmission, synapse maturation and neuroplasticity (Chan et al 2006, Kramar et al 2006, Shi & Ethell 2006, Warren et al 2012). LTP, synaptic transmission, and spine stabilization are facilitated by β 1-integrin-containing receptors, in particular α 3 β 1 (Kramar et al 2002, Chan et al 2007, Kerrisk et al 2013). Also, β 1-integrin is necessary for hippocampal-dependent learning and memory, PFC-dependent behavioral flexibility, and working memory (Chan et al 2006, Babayan et al 2012, Warren et al 2012).

In addition to LTP, integrin α 3 β 1 regulates postnatal dendritic spine development (Kerrisk et al 2013). Presumably upon binding its target ligand, the integrin β 1 subunit engages and stimulates Abl2/Arg kinase (Warren et al 2012, Simpson et al 2015) (see Fig. 3a). Abl2/Arg interacts with cortactin to stabilize actin filaments and promote new actin branch nucleation by the Arp2/3 complex (Lin et al 2013, Courtemanche et al 2015). In addition, Abl2/Arg phosphorylates p190RhoGAP, driving it into a complex with p120RasGAP that inhibits Rho (Sfakianos et al 2007). Active Rho stimulates ROCK2 to destabilize dendrite structure. The integrin-Arg-p190RhoGAP axis thus stabilizes dendrite branches by inhibiting Rho (Sfakianos et al 2007). ROCK2 can also influence the activity-dependent remodeling of dendritic spines (Murakoshi et al 2011), although this pool of ROCK2 may not be regulated by integrin signaling through Abl2/Arg (Lin et al 2013).

Elimination of β 1-integrin in excitatory neurons has revealed that β 1-integrin is essential for cortical, though not hippocampal, lamination (Huang et al 2006). Nonetheless, when β 1-integrin is eliminated starting in embryonic development, mutant mice exhibit a destabilization of postnatal hippocampal synapses and dendrites beginning after P21 (Warren et al 2012). This dendrite and synapse destabilization was not reported when β 1-

integrin was eliminated starting in postnatal week 3 (Chan et al 2006), suggesting that early-life β 1-integrin tone influences later neuronal structure, as well as behavior (Warren et al 2012). When we evaluated β 1-integrin levels across time here, we did not observe any significant changes. This was somewhat surprising, given the clear effects of β 1-integrin on dendrite morphology and synaptic density during adolescence (Warren et al 2012). It is possible that adult-like protein levels are reached prior to P35. Or, integrin ligand levels may be dynamically regulated, rather than β 1-integrin itself. Further, little is known about the postnatal development of intracellular signaling cascades that shift integrins into their activated conformation. All of these factors could contribute to the effects of β 1-integrin-mediated signaling in adolescence.

Part 3.5. Abl2/Arg kinase and cortactin determine cell structure

We also assessed the levels of Abl2/Arg, an Abl-family nonreceptor tyrosine kinase highly expressed in the nervous system and concentrated at synapses (Koleske et al 1998). Cortical neurons in adult mice lacking Abl2/Arg have shorter basal dendrites and fewer branch points than neurons from wild type controls (Moresco et al 2005). Importantly, these dendrites develop normally in *arg*^{-/-} mice and reach their full size by P21, but then simplify by P42, indicating that Abl2/Arg is critical for postnatal dendrite stability, but not the initial formation of these cortical dendritic arbors. Similarly, Abl2/Arg-deficient dendrites on hippocampal CA1 neurons exhibit normal morphology at P21, but later destabilize and have smaller arbors with fewer dendrite branch points by P42 (Sfakianos et al 2007). In hippocampal CA1 and the OFC, spine densities in *arg*^{-/-} mice do not differ from wild type at P21 and P24, but then diverge at P31. Densities become reduced in *arg*^{-/-} mice, and dendritic spines on deep-layer OFC pyramidal neurons are longer, a phenotype that may reflect less mature or destabilizing synapses (Gourley et al 2012b). Correspondingly, adult but not pre-adolescent mice lacking Abl2/Arg kinase have reduced levels of PSD95 and dopamine D1- and D2-family receptors in the PFC (Gourley et al 2009).

As expected, loss of Abl2/Arg function influences behaviors that are OFC- and hippocampus-dependent. Abl2/Arg-deficient mice are impaired in novel object and spatial reversal tasks and also hypersensitive to the locomotor-activating effects of cocaine (Sfakianos et al 2007, Gourley et al 2009, Gourley et al 2012b). These behavioral phenotypes are detectable only when testing occurs after P21, corresponding to time points when Abl2/Arg serves to stabilize dendrites and dendritic spines. Interestingly, we did not observe any significant changes in Abl2/Arg levels across adolescence. It is likely that levels peak prior to P35, as Abl2/Arg is actively regulating neuronal morphology in the OFC by P31 (Gourley et al 2012b).

Abl2/Arg likely stabilizes dendritic spines through interactions with its binding partner cortactin. Cortactin is highly enriched in dendritic spines where it co-localizes with actin filaments and is required for dendritic spine stability (Hering & Sheng 2003). For instance, knockdown of cortactin in hippocampal cell cultures decreases dendritic spines and synapses, whereas overexpression results in an increase in dendritic spine length, reflecting an immature spine phenotype (Hering & Sheng 2003). Cortactin promotes Arp2/3 complex-mediated actin branch nucleation on existing actin filaments and also stabilizes these

branches (Uruno et al 2001, Weaver et al 2001, Ammer & Weed 2008). Also, Abl2/Arg binding to actin filaments promotes increased recruitment of cortactin, and the proteins synergize to stabilize actin filaments and promote branch formation (MacGrath & Koleske 2012). In addition to stabilizing and promoting branch point formation, cortactin also interacts with several synaptic scaffolding proteins (Naisbitt et al 1999, Hajdu et al 2015, MacGillavry et al 2015). As a result, it is believed that cortactin plays a role in organizing the postsynaptic density.

Within a dendritic spine, cortactin is concentrated in two discrete pools: one at the post-synaptic density and one within the dendritic shaft (Racz & Weinberg 2004). Cortactin trafficking to the post-synaptic density is facilitated by BDNF, whereas trafficking from the post-synaptic density to the core is mediated by NMDA receptor activity (Hering & Sheng 2003, Iki et al 2005, Lin et al 2013). Although we did not examine cortactin localization, qualitatively, levels appeared to be elevated at P56, particularly in the OFC, relative to earlier time points. This is particularly interesting given that both TrkB and TrkB.T1 are also most highly expressed at P56. These parallels in timing raise the possibility that synergies between cortactin and neurotrophin signaling facilitate cortical development during adolescence. Further studies could address this possibility.

Part 3.6. p190RhoGAP-p120RasGAP complex – Rho interactions

Rho is a member of the Rho-family GTPases, comprised of several GTPases, including, Rho, Rac, and Cdc42. GTPases are regulated by guanine exchange factors that catalyze GDP to GTP to promote activity, whereas GTPase-activating proteins stimulate hydrolysis of GTP to GDP to inhibit activity (Schmidt & Hall 2002, Bernards & Settleman 2004). Together, Rac, Cdc42, and Rho regulate actin polymerization, bundling, contractility, and severing, and via these processes they coordinate changes in neuronal shape (Govek et al 2005). Within the family of Rho GTPases, Rho is associated with cell contraction, while Rac and Cdc42 promote actin-based protrusion (Govek et al 2005). For example, constitutively active Rho reduces dendritic spine density and spine length in both hippocampal and cortical slices (Nakayama et al 2000, Tashiro et al 2000), whereas inhibition of Rho *increases* spine density and promotes spine elongation (Tashiro et al 2000). One study reported that in whole-brain homogenate, Rho expression does not change from P14 to adulthood (P84–98) (Komagome et al 2000). Our findings are consistent with these stable expression patterns, as we did not observe any significant age-related changes in Rho levels.

p120RasGAP is a Ras GTPase activating protein that inhibits activity of the Ras GTPases by promoting hydrolysis of GTP to GDP. Tyrosine phosphorylation of p190RhoGAP, a Rho GTPase-activating protein, promotes assembly of a p190RhoGAP-p120RasGAP complex (Hu & Settleman 1997). Integrin-mediated activation of Abl2/Arg and Src family kinases increases p190RhoGAP phosphorylation to drive its association with p120RasGAP at the cell membrane (Bradley et al 2006). This complex inhibits Rho activity, which is a likely mechanism for reduced appearance of actin stress fibers following an increase in p190RhoGAP phosphorylation (Chang et al 1995, Sharma 1998, Bradley et al 2006). As with Rho, we did not observe any robust age-related changes in p120RasGAP. We did however observe that p190RhoGAP levels peaked at P42 in both PFC subregions.

Adolescent *p190rhogap*^{+/-} mice are more susceptible to stress hormone-induced anhedonic-like behavior and dendritic spine elimination in the OFC (Gourley et al 2013b). With the caveat that other ages were not tested in this prior report, robust p190RhoGAP levels during adolescence could thus contribute to the stabilization of dendrites and dendritic spines that are *not* pruned, combatting the influence of pathological insults such as stressors (Gourley et al 2013b) or cocaine (Gourley et al 2012b).

Throughout these analyses, we noted several instances when protein levels appeared slightly elevated in the OFC at P35. We thus generated a separate series of western blots specifically using tissues collected at P35. This approach revealed higher overall protein levels in the OFC as compared to mPFC. We did not detect any interaction effects, which precluded post-hoc comparisons, but qualitatively, expression patterns differed most notably for p120RasGAP, Abl2/Arg kinase, and downstream factors. There is evidence that OFC maturation is slower relative to the mPFC (Fig. 1) (Van Eden & Uylings 1985); this might account for differential protein levels at P35.

Part 3.7. ROCK2 and LIMK2: Key cytoskeletal regulatory elements in the postnatal brain

The ROCK serine/threonine kinases are major effectors of active Rho. There are two isoforms of ROCK, ROCK1 and ROCK2. The isoforms share 64% homology, and have different patterns of expression within the central nervous system, as ROCK2 is primarily expressed in neurons, whereas ROCK1 is confined to glia (Iizuka et al 2012). ROCKs are activated when GTP-bound Rho binds to its Rho-binding domain, inducing a conformational change that releases ROCK from its auto-inhibited conformation (Mueller et al 2005). Once activated, ROCK phosphorylates LIMK, which phosphorylates cofilin and inhibits its ability to sever actin filaments (Arber et al 1998, Yang et al 1998, Bamburg & Wiggan 2002). Actin severing contributes to actin filament turnover, but can also stimulate actin polymerization because it provides a new free actin filament plus end that can elongate, and both processes are likely critical for dendritic spine remodeling (Okamoto et al 2004, Pontrello & Ethell 2009, Shi et al 2009). Actin polymerization and filament turnover are believed to occur in distinct subcellular compartments within the dendritic spine, and are modulated by various cytoskeletal regulatory proteins localized to these regions (Oser & Condeelis 2009, van Rheenen et al 2009).

ROCK2 activity prevents neurite outgrowth, and accordingly, ROCK2 inhibition induces neurite formation *in vitro* (Hirose et al 1998, Da Silva et al 2003). *In vivo*, ROCK2 inhibition causes dendrite elongation in hippocampal CA1 pyramidal neurons (Couch et al 2010). Furthermore, ROCK2 inhibition blocks spine loss associated with increased Rho protein levels (Xing et al 2012) and promotes formation of labile, filopodia-like protrusions (Tashiro & Yuste 2004). Nonetheless, elimination of *Rock2* impairs synaptic transmission and LTP in hippocampal neurons, and *Rock2* knockout mice have increased dendritic spine area but decreased dendritic spine density (Zhou et al 2009). This somewhat surprising finding – that *Rock2* elimination decreased dendritic spine density – suggests that some degree of ROCK2 signaling is nonetheless critical for normal dendritic spine formation.

ROCK2 levels dramatically increase from P7 to P14 in whole brain homogenate (Komagome et al 2000), however no later ages were examined in this prior report. We

observed that ROCK2 levels peaked at P42 in both PFC subregions examined. This timing is logical, as ROCK2 prevents neurite outgrowth, and P42 represents a period of considerable dendritic spine pruning, in which dendritic and dendritic spine retraction, rather than cell elaboration, is favorable. On the other hand, ROCK2 *inhibition* can, under certain circumstances, *facilitate* dendritic spine pruning (Murakoshi et al 2011). The precise function of elevated ROCK2 in the adolescent PFC has not, to our knowledge, yet been resolved.

As noted in the prior sections, LIMK is another critical regulator of the actin cytoskeleton. There are two isoforms, LIMK1 and LIMK2, which share 50% homology (Acevedo et al 2006). We focused on LIMK2 because it is activated by ROCK2, while LIMK1 is not (Amano et al 2001, Sumi et al 2001). Furthermore, LIMK2 is confined to neurons, whereas LIMK1 is expressed in both neuronal and non-neuronal cells (Foletta et al 2004, Acevedo et al 2006). Active LIMK2 phosphorylates cofilin, inhibiting actin severing. Interestingly, *Limk2* knockout mice have normal synaptic transmission and LTP (Meng et al 2004). This could be a result of redundancy in the LIMK family (Meng et al 2004, Cuberos et al 2015). We observed that LIMK2 levels are stable during adolescence in both the mPFC and OFC.

Part 3.8. Limitations of our current studies

This study provides novel insight into the developmental trajectory of cytoskeletal and regulatory factors in the mPFC and OFC during adolescence. One limitation of our study, however, is the lack of comparison between males and females, since only females were used for protein quantification, and the sexes were combined in our dendritic spine studies in the interest of statistical power. Importantly, sex differences in the postnatal maturation of dendritic spines and synapses within the mPFC *are* reported (Koss et al 2014, Drzewiecki et al 2016). Furthermore, gonadal hormones may impact the effect of neurotrophic factors on cytoskeletal regulatory systems (Carrer & Cambiasso 2002, Hill et al 2012, Kramar et al 2013). Directly comparing dendritic spine density trajectories, as well as protein levels during adolescence in male and female mice, could provide further insight into differences between mPFC and OFC postnatal development.

Another caveat is that we do not normalize protein levels to the total volume of the mPFC or OFC. The volume of the prefrontal cortex changes during adolescence in both humans and rodents (*e.g.*, (Van Eden & Uylings 1985, Giedd et al 1999)). During this time, there is a decrease in gray matter and an increase in white matter, as well as a loss of neurons and a gain of glia (Lenroot & Giedd 2006, Markham et al 2007). As a result, it is important to note that our dissections collected at P35 could be comprised of a different ratio of cell bodies to axons, and neurons to glia, than our P42 or P56 dissections, which could also differ from each other. Lastly, our OFC, mPFC, DHC, and VHC punches contain multiple subregions of the PFC and hippocampus respectively. These subregions have distinct properties and circuitry, as discussed in our anatomy section (for further comparison of hippocampal subregions, see (Vinogradova 2001, Agster & Burwell 2013)).

Conclusions

Our objective was to evaluate the developmental trajectory of key proteins involved in postnatal neural development and structural plasticity. Throughout, we compared protein levels between the mPFC and OFC, two brain regions implicated in mood regulation and psychiatric disorders, and that have differential involvement in behavioral flexibility, executive control, and complex decision making, but that are nonetheless often treated as a single unit. We compared protein expression between early, mid-, and late adolescent periods, revealing several differences in protein abundance at the earlier time points, followed by convergence in late adolescence/young adulthood. In several cases, we noted that expression patterns differed from those identified in the hippocampus. Further characterization of these developmental patterns and their functional consequences may provide insights into the mechanisms of psychiatric illnesses with neurodevelopment etiologies.

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Significance Statement

Up to 50% of “adult” mental health disorders emerge in adolescence. During this time, the prefrontal cortex (PFC) undergoes dramatic structural reorganization, *e.g.*, dendritic spines are refined, pruned, and stabilized. Here we identify and discuss differences between distinct subregions of the PFC in the levels of several cytoskeletal regulatory proteins throughout adolescence that then converge in early adulthood. These variabilities could provide insight into critical periods of structural change during adolescence and provide a better understanding of vulnerability to psychiatric illness. We conclude with a review regarding how TrkB- and β 1-integrin-mediated signaling events influence neuronal structure in the postnatal brain.

Figure 1. Subregions of the rodent PFC and dendritic spine elimination during adolescence

A. Subregions of the mouse PFC are highlighted on images from the Mouse Brain Library (Rosen et al 2000). These subregions are considered functionally and anatomically distinct. For example, the OFC and mPFC project to the striatum in a topographically-organized fashion. The LO and VO project to the LS and central/medial striatum respectively, whereas the MO, PL and IL all project to regions of the DMS. The mPFC also innervates the nucleus accumbens core and shell, with the more dorsal section of the PL innervating the core while the ventral PL and IL target the shell. Meanwhile, projections to the nucleus accumbens from the OFC originate primarily in the AI and MO subregions. **B.** Dendritic spines on excitatory deep-layer pyramidal neurons in the mPFC (PL region) and OFC (VLO region) were enumerated during adolescence, with time points indicated, revealing dynamic changes throughout development. In the mPFC, dendritic spine density decreased between P31 and P42, while in the OFC, dendritic spine density decreased later, between P39 and P56. Means + SEMs, * $p < 0.05$, ** $p < 0.01$. Each mouse is considered an independent sample.

Abbreviations: AI: agranular insular cortex, AID: dorsal agranular insular cortex, AIV: ventral agranular insular cortex, CG: cingulate cortex, core: nucleus accumbens core, DMS: dorsomedial striatum, IL: infralimbic cortex, LO: lateral orbital cortex, LS: lateral striatum, MO: medial orbital cortex, PL: prelimbic cortex, shell: nucleus accumbens shell, VO: ventral orbital cortex.

Figure 2. Levels of several synaptic and cytoskeletal regulatory factors change during adolescence

A. PSD95 levels were consistent across development and between PFC subregion. **B.** Synaptophysin levels decreased in both the mPFC and OFC between P35 and P56. **C.** Both TrkB.T1 and full-length TrkB (TrkB.FL) (**D**) levels increased in the mPFC and OFC during adolescence. **E.** ProBDNF and mBDNF (**F**) did not significantly change between regions or across time. Similarly, no regional or age-related changes in β 1-integrin (**G**) or Abl2/Arg kinase (**H**) were observed. **I.** Cortactin levels increased slightly in the OFC while staying constant in the mPFC, but this effect was statistically non-significant. **J.** In both PFC subregions, p190RhoGAP levels increased between P35 and P42 and then decreased by P56. **K.** p120RasGAP levels trended higher in the OFC than mPFC. **L.** Rho levels were variable, but with no statistically significant changes. **M.** ROCK2 levels increased at P42 and then decreased by P56 in both subregions of the PFC. **N.** LIMK2 levels were consistent across ages and between regions. Representative blots below were loaded in the following order:

mPFC P35, OFC P35, mPFC P42, OFC P42, mPFC P56, OFC P56, with 15 µg of total protein/lane throughout. Loading controls (GAPDH, 37 kD or HSP70, 70 kD) are the bottom band. Means + SEMs, * $p < 0.05$, ** $p < 0.01$.

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Figure 3. Regional differences in proteins associated with β_1 -integrin signaling during early adolescence

A. Extracellular matrix proteins bind to α/β_1 -containing integrin receptors. Once activated, β_1 -integrin stimulates Abl2/Arg kinase, which can then stimulate cortactin or p190RhoGAP (forming a complex with p120RasGAP). Activation of cortactin contributes to lamellipodia formation, whereas activation of p190RhoGAP inhibits Rho. When Rho is active, it stimulates ROCK2, which subsequently activates LIMK2. Rho signaling has been associated with dendrite retraction in multiple biological systems, thus inhibition stabilizes dendrite structure. **B.** In the course of conducting the experiments described in Fig. 2, subtle differences in protein levels were noted between regions early in adolescence. Here we show the results of independent analyses indicating that at P35, early adolescence, protein levels in this signaling cascade were indeed generally higher in the OFC than the mPFC. Western blotting was performed using 15 μ g of protein/sample/lane. Means + SEMs, * $p=0.05$, main effect of region.

Figure 4. Regional differences in synaptic marker and neurotrophic factor levels at P42

A. We also conducted single time point analyses for synaptic marker and neurotrophic factors. At P35, we found no regional differences in the levels of PSD95, synaptophysin (SYN), TrkB.T1 and full-length TrkB receptor isoforms (TrkB.FL), or proBDNF and mBDNF. **B.** At P42, however, PSD95, synaptophysin, and mBDNF were elevated in the mPFC compared to OFC, while levels of both TrkB receptor isoforms were higher in the OFC than mPFC. **C.** At P56, no regional differences were observed. **D.** The same data are represented as scatter plots of protein levels at P35, **(E)** P42 and **(F)** P56. Western blotting was performed using 15 μ g of protein/sample/lane. Means + SEMs, * p <0.05, ** p <0.01 following interaction effects.

Figure 5. Regional differences in synaptic markers and neurotrophic factors in the hippocampus during adolescence

A. As a point of contrast, we assessed the same markers as in Fig. 4 in the hippocampus. PSD95 levels were higher in the DHC than VHC. **B.** Synaptophysin levels were, however, equivalent between both subregions, and also consistent during adolescence. **C.** TrkB.T1 levels progressively increased across adolescence. **D.** No regional or age-related changes in full-length TrkB (TrkB.FL) levels were noted. **E.** ProBDNF levels were consistently higher in the DHC than VHC. **F.** mBDNF levels, on the other hand, were equivalent in the VHC vs. DHC and not variable during adolescence. Representative blots below were loaded in the following order: DHC P35, VHC P35, DHC P42, VHC P42, DHC P56, VHC P56, with 15 μ g of total protein/lane throughout. Loading controls (GAPDH, 37 kD or HSP70, 70 kD) are the bottom band. Means + SEMs, * p <0.05, ** p <0.01.

Table 1**Antibody list**

The antibodies used in immunoblotting are indicated with their respective immunogen, host, manufacturer, product number, lot number and effective concentration.

Antibody	Immunogen	Host	Manufacturer, product number, lot number	Conc.
anti-GAPDH	Proprietary sequence	Mouse-monoclonal	Sigma #G8795, lot 044m4808v	1:5000
anti-HSP70	aa 580-601	Mouse-monoclonal	Santa Cruz Biotechnology #7298, lot F0413	1:5000
anti-β1-integrin (CD29)	aa 76-256	Mouse-monoclonal	BD Biosciences #610468, lot 4101803	1:100
anti-p120RasGAP (GTPase Activating Protein)	Full length sequence	Mouse-monoclonal	Thermo-Scientific #Ma4-001, lot Of185059	1:500
anti-Abl2/Arg kinase	aa 766-1182	Mouse-monoclonal	generously provided by Dr. Peter Davies	1:250
anti-RhoA GTPase	aa 120-150	Mouse-monoclonal	Santa Cruz #418, lot K1213	1:500
anti-ROCK2	aa 1-100	Rabbit-polyclonal	Abcam #71598, lot Gr51275-1	1:1000
anti-LIM kinase 2	aa 561-638	Rabbit-polyclonal	Santa Cruz #5577, lot E0707	1:500
anti-TrkB	Proprietary sequence	Rabbit-monoclonal	Cell Signaling #4603s, lot 3	1:250
anti-PSD95	Proprietary sequence	Rabbit-monoclonal	Cell Signaling #3450s, lot 2	1:1000
anti-synaptophysin	C terminus domain	Rabbit-monoclonal	Abcam #32127, lot Gr196393-2	1:20,000
anti-cortactin	aa 309-499	Rabbit-polyclonal	Santa Cruz #11408, lot F3010	1:1000
anti-p190RhoGAP	aa 1-1513	Mouse-monoclonal	BD Biosciences #610149, lot 5273884	1:500
anti-BDNF	Proprietary sequence	Mouse-monoclonal	Sigma #B9436, lot 098k0575	1:100
anti-BDNF	Proprietary sequence	Rabbit-monoclonal	Abcam #108319, lot GR115071	1:250