CELL AND MOLECULAR SIGNALING, AND TRANSPORT PATHWAYS INVOLVED IN GROWTH FACTOR CONTROL OF SYNAPTIC DEVELOPMENT AND FUNCTION

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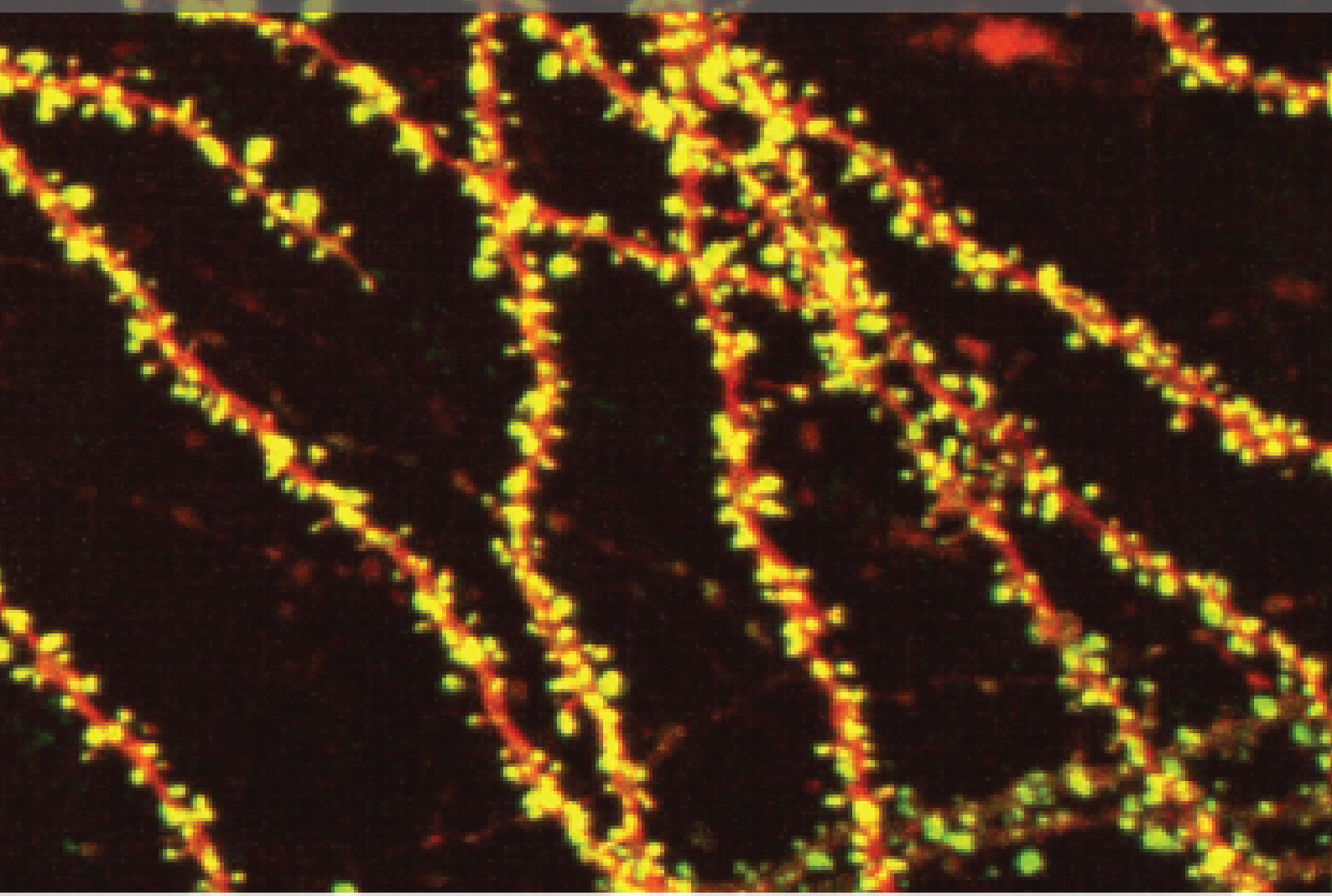

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CELL AND MOLECULAR SIGNALING, AND TRANSPORT PATHWAYS INVOLVED IN GROWTH FACTOR CONTROL OF SYNAPTIC DEVELOPMENT AND FUNCTION

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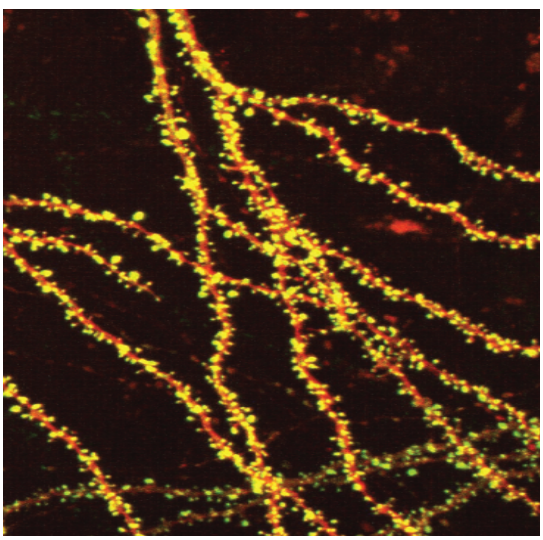

The image shows stretches of the apical dendrite of a CA3 hippocampal pyramidal neurons co-transfected by single cell electroporation with one expression plasmid for mCherry, labeling the membrane in red and one for eGFP-tagged actin. Note the obvious accumulation of actin-eGFP within dendritic spine heads.
Image by Martin Korte

Brain derived neurotophic factor (BDNF) and its receptor tropomyosin receptor kinase B (TrkB) signaling has been extensively studied for its roles in the central nervous system (CNS) ranging from cell survival, axonal and dendritic growth and synapse formation. Intracellular signaling pathways triggered by BDNF activate gene transcription, translation, post-translational functions, trafficking of key synaptic proteins, and synaptic release mechanism. BDNF-TrkB signaling mediates long-lasting activity-modulated synaptic changes on excitatory and inhibitory neurons and plays significant roles in circuit development and modulation. Furthermore, this pathway is critical for learning, memory, sensory processing and other cognitive functions, and is implicated in neurological and psychiatric diseases.

In addition to BDNF, more recent studies have identified new "growth" factors that play important roles in the development, maturation and maintenance and modulation of synaptic function. However, details of the cytoplamic signaling systems downstream of these synaptogenic factors are often less understood than conventional neurotophin signaling. This e-Book has collected original studies and review articles that present cellular and molecular mechanisms concerning activity-dependent synapse formation and their implications for behavior and brain disorders.

It is our hope that readers will perceive this volume as a showcase for diversity and complexity of synaptogenic growth factors, and will stimulate further studies in this field.

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EDITORIAL
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Editorial: Cell and molecular signaling, and transport pathways involved in growth factor control of synaptic development and function

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Keywords: BDNF, neurotrophic factors, synapse formation, FGF7, FGF22, UNC-5, UNC-6 Netrin

Since the discovery of nerve growth factor (NGF) more than a half century ago (Levi-Montalcini and Cohen, 1960), the prototypic neurotrophin family has included brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4). Neurotrophins bind to the Trk family of receptors, as well as the p75 receptor, to activate multiple intracellular signaling cascades (reviewed by Reichardt, 2006). BDNF receptor tropomyosin receptor kinase B (TrkB) signaling has been extensively studied for its roles in the central nervous system (CNS) ranging from cell survival, axonal and dendritic growth and synapse formation. The pathway mediates long-lasting activity-modulated synaptic changes on excitatory and inhibitory neurons and plays critical roles in circuit development and maintenance. In addition to BDNF, many studies have identified other "growth" or signaling factors in the CNS that play important roles in the development, maintenance, and control of synaptic and circuit function. However, details of the intracellular signaling systems downstream of these events are frequently unexplored. In this Research Topic, we have collected original studies and review articles that present cellular and molecular mechanisms concerning activity-dependent synapse formation and their implications for behavior and brain disorders.

Vadodaria and Jessberger discuss synapse maturation in adult-born dentate granule cells and the role of BDNF-TrkB and several other signaling pathways that activate Cdc42, Rac1, and RhoA (Vadodaria and Jessberger, 2013). These small Rho GTPases regulate polymerization of actin and microtubules, and are consequently involved in aspects of neuronal maturation ranging from cell migration, to dendritic arborization, spine maturation, and synaptic integration of these newborn hippocampal neurons.

Kellner et al. show that BDNF is critical for activity-dependent maintenance of mature spines through F-actin polymerization (Kellner et al., 2014). Integrity of this spine cytoskeleton is also critical for the vesicular transport, carried out by molecular motor proteins. For example, Myosin Va is a plus end actin, vesicular motor protein that carries postsynaptic density protein 95 (PSD-95), Synapse-associated protein 90/postsynaptic density-95-associated protein (SAPAP) and Shank, an essential glutamate receptor scaffold complex along actin to the postsynaptic membrane at the tip of dendritic spines (Hammer and Wagner, 2013; Yoshii et al., 2013). Furthermore, BDNF-TrkB signaling triggers transport of the vesicles containing PSD-95 and its associated complex

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to postsynaptic membranes via activation of PI3-kinase-Akt pathway (Yoshii and Constantine-Paton, 2007).

BDNF-TrkB signaling pathway also regulate the molecular assembly of synaptic membrane. Zonta and Miniciello discuss the emerging role of lipid raft, the detergent resistant lipid microdomain enriched with cholesterol and sphingolipid, in synaptic plasticity as a result of neurotrophin signaling (Zonta and Minichiello, 2013). When BDNF binds to the TrkB receptor, the ligand-receptor complex shifts to lipid rafts via activation of tyrosine kinase Fyn (Pereira and Chao, 2007; Suzuki et al., 2007). Lipid raft is enriched in postsynaptic membrane, and facilitates localization of palmitoylated proteins such as PSD-95, which is a critical regulator of synaptic plasticity at excitatory synases. Palmitoylation of PSD-95 in the cell body is also mediated by BDNF-TrkB signaling via activation of phospholipase Cγ (PLCγ) and protein kinase C (PKC). The PKC inhibitors chelerythrine as well as a synthetic *z*eta *i*nhibitory *p*eptide (ZIP) designed to block the brain-specific PKC isoform protein kinase Mζ (PKMζ) were used to demonstrate this effect (Yoshii et al., 2011). However, additional studies in the hippocampus began to raise concerns about the specificity of ZIP (Lee et al., 2013; Volk et al., 2013). A follow-up study by Yoshii and Constantine-Paton in this Topic confirmed that, while both chelerythrine and ZIP could suppress the postsynaptic localization of PSD-95, PKMζ knock-down with RNA interference did not exhibit this effect. The result indicates that the ZIP peptide, widely used as a "specific" PKMζ antagonist, may block another PKC variant that is the kinase actually involved in PSD-95 palmitoylation in cell body.

BDNF is critical for mechanisms underlying various modalities of sensory processing, cognition and behaviors. These roles have been studied in hippocampal learning and memory (reviewed by Minichiello, 2009), in maturation and plasticity of the CNS and also in neurological disease (reviewed by Yoshii and Constantine-Paton, 2010). It is not surprising that BDNF is associated with brain disorders such as epilepsy, autism, depression, and schizophrenia since all of these have chronic effects on synaptic function. However, Andersen and Sonntag have studied the effect of juvenile exposure to psychostimulants on the risk of cocaine addiction in adulthood. They found that treatment with methylphenidate, frequently used to improve attention in children, has a long-lasting suppressive effect on cocaine-induced increases in BNDF expression (Andersen and Sonntag, 2014). Further studies will hopefully identify the mechanisms underlying the critical period effect on addiction as well as long term effect of stimulants.

Neurotrophic factors also affect feeding. Maekawa et al. have shown that low BDNF expression in the ventromedial hypothalamus correlates with blood glucose level, increased leptin secretion and eating, and visceral fat accumulation in a type 2 diabetic rat line (Maekawa et al., 2013). The results indicate BDNF and leptin play major roles in central regulation of energy metabolism and dysregulation of the neurotrophin signaling result in obesity.

Harvey discusses that leptin regulates synaptic functions (Harvey, 2013). This dietary hormone activates extracellular-signal-regulated kinase (ERK) and facilitates GluN2B-mediated synaptic depression during early postnatal development while it regulates LTP through PI3-kinase pathway in adult hippocampus (Moult and Harvey, 2011).

It is now clear that variety of synaptogenic growth factors are wider than previously thought. Two articles review diversity of these factors. Williams and Umemori discuss members of the fibroblast growth factor (FGF) family in the context of synaptic development (Williams and Umemori, 2014). FGFs have been shown to organize presynaptic vesicle clustering. Remarkably, FGF7-null mice exhibit a specific deficit in hippocampal inhibitory synapse formation while FGF22-null mice are deficient in excitatory synapses (Terauchi et al., 2010). Furthermore, FGF7-null animals are prone to develop epilepsy after kindling, while FGF22-null are resistant to this seizure induction.

Poon et al. provide a comprehensive review of Netrin, Wnt, transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), all of which were first identified for their roles other than synaptogenesis (Poon et al., 2013). For example, UNC-6/netrin and its receptor UNC-5 were originally described as axonal guidance molecules. However, UNC-6 and UNC-5 facilitate localization of presynaptic proteins to axons by excluding them from dendrites (Poon et al., 2008). Consequently, these pathway are all involved in regulating axonodendritic polarity.

The field of neuronal growth factors is continuing to grow, and new discoveries, some which are highlighted in this volume, will prompt new questions. For example, do these growth factors work together, competitively or separately? Is there cell-type specificity for each factor? Do they play a deterministic or a modulatory role in synaptic specificity during development ? Advances in genomics and proteomics will help us understand not only single cascade but also multiple signaling pathways as network. Various genetic tools allow spacial and temporal controls of gene expression and neuronal activities (Boyden et al., 2005; Arenkiel and Ehlers, 2009; Konermann et al., 2013). Super-resolution microscopy enables observations of signaling molecules at synapse and organelles in unprecedented manner. Multi-photon microscopy has been invaluable to study a wide range of structures from individual spines to neuronal circuitry. Applications of these new technologies will create exciting opportunities to tackle the above and other questions.

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Maturation and integration of adult born hippocampal neurons: signal convergence onto small Rho GTPases

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Adult neurogenesis, restricted to specific regions in the mammalian brain, represents one of the most interesting forms of plasticity in the mature nervous system. Adult-born hippocampal neurons play important roles in certain forms of learning and memory, and altered hippocampal neurogenesis has been associated with a number of neuropsychiatric diseases such as major depression and epilepsy. Newborn neurons go through distinct developmental steps, from a dividing neurogenic precursor to a synaptically integrated mature neuron. Previous studies have uncovered several molecular signaling pathways involved in distinct steps of this maturational process. In this context, the small Rho GTPases, Cdc42, Rac1, and RhoA have recently been shown to regulate the morphological and synaptic maturation of adult-born dentate granule cells *in vivo*. Distinct upstream regulators, including growth factors that modulate maturation and integration of newborn neurons have been shown to also recruit the small Rho GTPases. Here we review recent findings and highlight the possibility that small Rho GTPases may act as central assimilators, downstream of critical input onto adult-born hippocampal neurons contributing to their maturation and integration into the existing dentate gyrus (DG) circuitry.

Keywords: neurogenesis, Rac1, Cdc42, RhoA, synaptic integration, spine growth, dendrite, *in vivo*

INTRODUCTION

Throughout lifespan new neurons are continuously born in the mammalian hippocampus. It is now widely accepted that the process of adult neurogenesis is not merely a remnant of embryonic development, but a highly responsive and regulated process that appears to be critically involved in hippocampus-dependent behavior in health and disease (Sahay and Hen, 2007; Zhao et al., 2008; Danzer, 2012). The number of newborn neurons in the adult hippocampus is not static but strongly influenced by many positive and negative stimuli that influence the neurogenic process at distinct developmental stages. Positive extrinsic regulators include physical exercise, environmental enrichment, antidepressants, and learning, whereas stress and aging negatively regulate the number of newly generated neurons (Ma et al., 2009). These stimuli are thought to impact adult neurogenesis via a number of regulatory pathways including growth factors, neurotransmitters, developmental signaling molecules, and hormones. Inspired by previous studies in the context of embryonic neurogenesis, a number of cellular and molecular mechanisms, involved in the control of neural stem/progenitor cells (NSPC) activity and subsequent integration of newborn granule cells within the adult hippocampus, have been identified (Ming and Song, 2011). Even though it has been demonstrated that the neurogenic process in the adult hippocampus shares many properties with embryonic neurogenesis, it principally differs from embryonic neurogenesis in that, NSPCs and maturing neurons are present in an entirely different (adult) environment and must integrate into a preexisting circuit, presumably in the absence of large amounts of developmental guidance cues (Conover and Notti, 2008). Thus, understanding the molecular underpinnings of how adult-born neurons integrate into the dentate gyrus (DG) circuitry requires experiments that analyze the cellular mechanisms and signaling pathways in their endogenous adult niche.

Recently, a few reports demonstrate stage-specific roles for small Rho GTPases, Cdc42, Rac1, and RhoA in adult hippocampal neurogenesis *in vivo*. These studies suggest an important and potentially central role for the small Rho GTPases in the maturation and integration of newborn neurons in the adult hippocampus. In this mini-review, we discuss the main findings of recent *in vivo* studies and then focus on discussing how known upstream intrinsic regulators (neurotrophins, neurotransmitters, developmental signaling molecules, and intermediate signaling molecules) maybe recruiting Rho GTPases for mediating their effects on neuronal maturation in adulthood. We begin by describing the maturation process of adult born hippocampal neurons and give a broad overview of neuronal Rho GTPase signaling, followed by a discussion of evidence showing how important regulators of neuronal maturation may be modulating small Rho GTPases as downstream effectors. We conclude with hypotheses on mechanisms for signal convergence and a brief discussion of how Rho GTPases may act to assimilate multiple upstream signals to decisively influence cell cytoskeleton and neuronal cytoarchitecture. For a more detailed discussion on Rho GTPase signaling in neurons, please refer to other reviews (Auer et al., 2011; Govek et al., 2011; Chen et al., 2012).

MATURATION OF ADULT-BORN HIPPOCAMPAL NEURONS

Adult hippocampal NSPCs go through distinct stages of maturation on their way to becoming fully mature newborn granule cells (**Figure 1**). It is currently assumed that radial glia-like NSPCs (type 1 cells) give rise to non-radial glia-like transit amplifying precursors (type 2 cells) that divide and generate immature neurons. These immature neurons grow an apical dendrite towards the molecular layer and send axonal processes to their target area, the CA3, several days after they are born (Zhao et al., 2006) (**Figure 1A**). During this period of maturation, newborn cells display distinct electrical properties, including gamma-aminobutyric acid (GABA)-induced depolarization, contributing to their survival and functional integration into the adult hippocampal circuitry (Ge et al., 2006) (**Figures 1B**,**C**). As these neurons further mature, they start receiving excitatory synaptic input, develop dendritic spines, and display extensive dendritic arborization. Adult-generated young granule cells display hyperexcitability as compared to granule cells generated during development (Wang et al., 2000; Schmidt-Hieber et al., 2004; Marin-Burgin et al., 2012). This property conceivably enables cohorts of newborn neurons to encode time (temporal context) within memory and allows the separation of patterns that are closely related, spatially or temporally (Aimone et al., 2010; Deng et al., 2013). In the rodent DG, it takes ∼8 weeks for adult-born granule cells to become nearly indistinguishable from developmentally-generated granule cells (Laplagne et al., 2006). Notably, each successive developmental stage is sensitive to a number of extrinsic and intrinsic regulators (Zhao et al., 2008; Ming and Song, 2011).

Molecular regulators of maturation and integration of adult-born neurons include neurotrophins such as brain derived neurotrophic factor (BDNF), neurotransmitters such as GABA and glutamate, and signaling molecules like Disrupted-in-Schizophrenia 1 (DISC1) (**Figure 1C**). These regulators recruit diverse downstream pathways to finally influence distinct aspects of neuronal maturation such as migration, dendritic arborization, spine maturation and synaptic integration of newborn hippocampal neurons (Jagasia et al., 2006; Hagg, 2007). Thus, one may speculate that these molecules and their downstream effectors may partially impinge on some common signaling pathways to influence neuronal maturation. Several lines of evidence indicate that the small Rho GTPases are important and central regulators of cell cytoarchitecture in different cell types and play important roles in modulating cell migration, neurite outgrowth, survival, as well as synapse formation in neurons (Govek et al., 2005; Newey et al., 2005; Watabe-Uchida et al., 2006; Svitkina et al., 2010). Hereon, we focus on how the aforementioned regulators interact with and influence small Rho GTPase signaling to possibly modulate neuronal integration in the adult hippocampus.

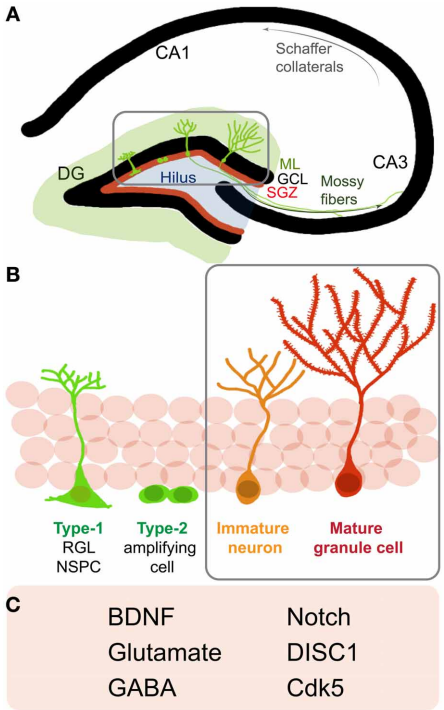

FIGURE 1 | Neurogenesis in the adult rodent hippocampus (A) Schematic of a coronal section of the hippocampus: cornu ammonis (CA) regions and the dentate gyrus (DG). Depicted are neural stem/progenitor cells (NSPCs) (green cells) residing in the subgranular zone (SGZ, red line) at the border of the granule cell layer (GCL) and molecular layer (ML). (Gray box) NSPCs divide, mature and go through the different stages of development and send out dendrites into the molecular layer and axons to the CA3 region via the mossy fiber pathway. **(B)** A closer look at neurogenesis reveals that adult NSPCs go through distinct stages of maturation where Type-1, radial glia-like stem cells give rise to Type2 transit amplifying cells, which divide to generate immature neurons that start developing characteristic DG granule cell morphology and finally mature and integrate into the DG circuitry as mature granule cells. **(C)** Listed are some notable regulators of later stages **(B**, gray box**)** of newborn neuron maturation and integration: brain derived neurotrophic factor (BDNF), gamma-Aminobutyric acid (GABA), Disrupted-in-Schizophrenia 1 (DISC1), cyclin-dependent kinase 5 (Cdk5).

SMALL RHO GTPase SIGNALING

Rho GTPases are part of the larger Ras superfamily of monomeric GTPases. These small GTPases are thought to act as binary molecular switches, transducing upstream signals to downstream effectors by alternating between the "active" GTP-bound and the "inactive" GDP-bound state (Schmitz et al., 2000) (**Figure 2**). Conversion from one state to the other is tightly regulated by guanine nucleotide exchange factors (GEFs), the GTPases activating proteins (GAPs) and the guanine nucleotide dissociation inhibitors (GDIs). Small Rho GTPase activity states are regulated by the activating GEFs, which promote exchange of GDP for GTP; the inactivating GAPs (GTPases activating proteins), which enhance the intrinsic capacity of the small GTPases for hydrolyzing GTP to GDP; and the inactivating GDIs that prevent dissociation of GDP from the GTPases (as there are usually higher levels of intracellular GTP than GDP) (Jaffe and Hall, 2005). GDIs further inhibit GTPase activity by sequestering them in the cytoplasm, as opposed to the cell membrane where they functionally localize due to posttranslational lipid modifications such as prenylation and palmitoylation (Kang et al., 2008; Samuel and Hynds, 2010; Navarro-Lerida et al., 2012). GEFs, GAPs, and GDIs are specific to different Rho GTPases, and

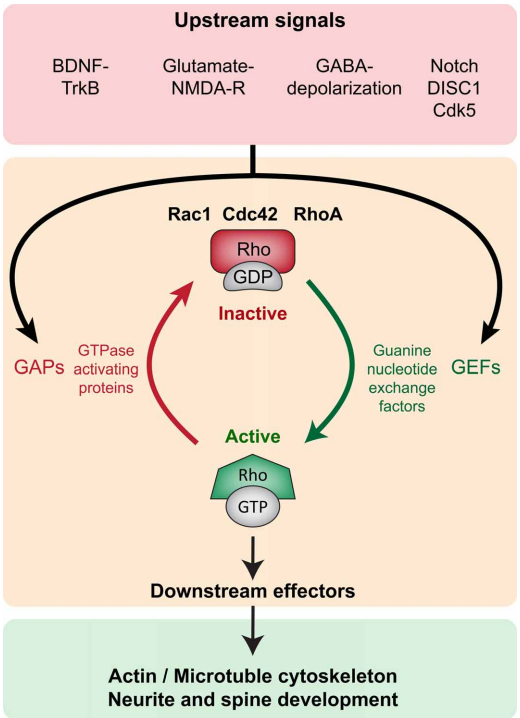

FIGURE 2 | Small Rho GTPase signaling in neurite and spine maturation (Top panel, red). Upstream signaling such as BDNF-TrkB, Glutamate-NMDAR, GABA-induced depolarization, Notch, DISC1 and Cdk5 modulate small Rho GTPase activity. (Middle panel, orange) Upstream signals modulate GEFs and GAPs thereby regulating the activity of small Rho GTPases Rac1, Cdc42, and RhoA. GEFs promote activation (GTP-bound conformation) and GAPs promote inactivation (GDP-bound conformation). **(Bottom panel, green)** In the active state, small Rho GTPases bind to several downstream effectors exerting influence on local actin and microtubule networks, thereby influencing neurite and spine growth.

their expression and subcellular localization is crucial to the spatial and temporal regulation of Rho GTPase activity. Activated GTPases (GTP-bound) bind to several downstream effectors that directly modify the actin and microtubule cytoskeletal network, thereby influencing a variety of processes such as cell growth, survival, proliferation, membrane trafficking, transcriptional activation, adhesion, mechanosensation, and migration (Van Aelst and D'Souza-Schorey, 1997; Schmitz et al., 2000; Govek et al., 2005; Watabe-Uchida et al., 2006; Govek et al., 2011; Keung et al., 2011) (**Figure 2**).

Cdc42 (cell division cycle 42), Rac1 (ras-related C3 botulinum toxin substrate 1) and RhoA (ras homolog family member A) are the best-studied members of the small Rho GTPase family, especially in the neuronal context. Despite early indications of important roles of the Rho GTPases in neuronal maturation *in vitro*, there are far fewer reports examining their role in the mammalian CNS *in vivo* (Heasman and Ridley, 2008). This is due to the fact that straight knockouts of Cdc42 and Rac1 are lethal during embryogenesis, with death occurring by E9.5 (Sugihara et al., 1998; Chen et al., 2000). Newer studies, using region-specific conditional deletion of the small GTPases during development, demonstrate diverse roles for Cdc42, Rac1 and RhoA in embryonic neurogenesis and neuronal maturation (Luo et al., 1996; Cappello et al., 2006, 2012; Chen et al., 2007; De Curtis, 2008; Fuchs et al., 2009; Haditsch et al., 2009; Leone et al., 2010; Vaghi et al., 2012). Only very recently have studies begun investigating the role of the small GTPases in the adult CNS *in vivo*. These studies show roles for Rho GTPase signaling in SVZ/OB (Ding et al., 2010; Leong et al., 2011) and hippocampal neurogenesis (Keung et al., 2011; Christie et al., 2013; Vadodaria et al., 2013). Here, we focus on recent findings demonstrating specific roles for Rho GTPases in adult hippocampal neurogenesis.

In their study, using retrovirus mediated overexpression of constitutively active (CA, GTP-bound) and dominant negative (DN, GDP-bound) forms of RhoA in adult hippocampal neurons *in vivo*, Keung et al. found an inverse relationship between RhoA activity and the percentage of cells differentiating into neurons (Keung et al., 2011). However, it is possible that this effect on neuronal differentiation was due to an effect on survival of newborns and not differentiation per se, as shown recently by Christie et al. Here, the authors blocked RhoA signaling pharmacologically *in vivo* and observed enhanced spatial memory in the Y-maze along with an increase in newborn neuron survival, and no differences in NSPC proliferation or differentiation (Christie et al., 2013). These findings suggest that RhoA may have a "negative" role in neuronal survival and maturation. However, due to off-target effects of DN/CA forms and pharmacological inhibitors, additional studies genetically knocking down RhoA *in vivo*, would help clarify its precise role in adult hippocampal neurogenesis. In our study, using both genetic deletion *in vivo* and DN-form overexpression, we found stage-specific roles for Cdc42 and Rac1 in adult hippocampal neurogenesis. Cdc42 activity was found to be important for normal levels of proliferation, as well as dendritic and spine maturation. In contrast, Rac1 was found to be specifically important for later stages of dendritic and spine maturation (Vadodaria et al., 2013). Collectively, results from the above mentioned studies suggest that Cdc42 and Rac1 are involved in proliferation, and dendritic and spine maturation, whereas RhoA may inhibit survival and growth of newborn neurons. In addition to playing a role in adult hippocampal neurogenesis, Rac1 and its epigenetic regulation was found to regulate synaptic plasticity and spine remodeling in the adult nucleus accumbens, where it is functionally involved in addiction (Dietz et al., 2012).

Taken together, these findings hint at stage-specific roles of the Rho GTPases in adult hippocampal neurogenesis. This may be due to differential recruitment of the Rho GTPases by upstream factors during different stages of neuronal maturation. We next discuss how upstream signaling molecules may recruit Cdc42, Rac1, and RhoA, for mediating their effects on maturation and integration of newborn neurons in the adult hippocampus.

REGULATORS OF NEURONAL MATURATION: TIES WITH SMALL RHO GTPase SIGNALING

NEUROTROPHIN SIGNALING: BDNF

BDNF is among the most well studied neurotrophins and has been shown to have a specific role in maturation and integration of newborn neurons in the adult hippocampus (Schmidt and Duman, 2007). Conditional mutants lacking BDNF exhibit a

specific defect in newborn neuron maturation, but not in proliferation, cell fate specification, or survival. In particular, loss of BDNF leads to an accumulation of immature neurons with significantly shorter dendrites (Chan et al., 2008). Although BDNF binds multiple Trk receptors as well as the non-specific p75 neurotrophin receptor (NTR), it appears that the effects of BDNF deletion on neuronal morphology are likely mediated by its main receptor, TrkB (tropomyosin receptor kinase B). Conditional deletion of TrkB in adult NSPCs leads to a specific reduction in dendritic arborization and spine density (Bergami et al., 2008). Further, stimuli such as stress, exercise, and antidepressants that are known to regulate adult neurogenesis (and hippocampus-dependent behavior) act via regulation of BDNF signaling (Duman and Monteggia, 2006). These studies suggest an indispensible role for BDNF-TrkB signaling in the maturation and integration of newborn neurons in the adult hippocampus.

The above-mentioned *in vivo* studies shed light on BDNF's role in hippocampal neuron maturation, and the molecular mechanisms downstream of BDNF-TrkB signaling have been explored mainly using primary cultures *in vitro*. Several studies have established Rho GTPases to be downstream of TrkB activation. BDNF-TrkB signaling has been shown to specifically modulate cell morphology of hippocampal neurons *in vitro*, via activation of GEF Tiam1 and Rac1, and through Cdk5 dependent activation of Cdc42 (Miyamoto et al., 2006; Cheung et al., 2007). In addition to directly targeting Cdc42 and Rac1, TrkB also regulates dendritic morphogenesis by activating geranylgeranyltransferase-I, which performs lipid modifications (prenylation) of the Rho GTPases, enabling their localization at the cell membrane (Zhou et al., 2008). This may be particularly relevant as recruitment of Rac1 to lipid rafts in hippocampal neurons is crucial to the biological activity of neurotrophins like, nerve growth factor (Fujitani et al., 2005). These results suggest that BDNF-TrkB signaling recruits and activates the positive regulators of neurite outgrowth Cdc42 and Rac1. BDNF and its protein precursor proBDNF bind to the non-specific neurotrophin receptor p75 (p75NTR), which has been shown to negatively impact cell survival and neurite outgrowth (Nykjaer et al., 2005; Blochl and Blochl, 2007). Interestingly, proBDNF-p75NTR signaling promotes growth cone collapse via downstream activation of RhoA (Sun et al., 2012). This fits in well with the notion that RhoA generally promotes growth cone collapse, whereas Cdc42 and Rac1 promote neurite growth and stabilization (Huang and Reichardt, 2003). Taken together, these studies indicate a strong link between BDNF-TrkB signaling and the regulation of small Rho GTPases for modulation of neurite growth dynamics. Given this, it is possible to hypothesize that Cdc42, Rac1 and RhoA may be recruited in a similar fashion downstream of BDNF for maturation of adult-born hippocampal neurons *in vivo*.

NEUROTRANSMITTER SIGNALING: GLUTAMATE AND GABA

Neuronal activity is central to the development of neurons, and neurotransmitters have been shown to regulate distinct stages of maturation of adult-born neurons (Vaidya et al., 2007). Glutamate and GABA primarily regulate excitation and inhibition in the adult nervous system, respectively, and have been shown to play important roles in the maturation of adult-born hippocampal neurons. Previous studies using pharmacological tools have demonstrated the importance of activity in neuronal maturation, and a key role for N-Methyl-D-aspartic acid receptor (NMDAR)-driven excitation in neuronal maturation has since long been proposed (Brewer and Cotman, 1989; McKinney et al., 1999; Richards et al., 2005; Nacher and McEwen, 2006). More recent studies have capitalized on the development of finer molecular biology tools to examine the stage-specific roles of glutamate and GABA in distinct stages of neuron maturation. Tashiro et al. showed that NMDAR-driven excitation is critical for survival and integration of newborn neurons using retrovirus-mediated single-cell knockout of NMDAR in adult-born hippocampal neurons, (Tashiro et al., 2006). Not only is glutamatergic excitation important but also GABA-induced depolarization is crucial during early stages of newborn neuron maturation. Early excitatory GABAergic input critically regulates the survival and maturation of adult-born hippocampal neurons (Ge et al., 2006, 2008). The effects of early GABAergic input on newborn neurons are likely mediated via cAMP response element-binding protein (CREB) signaling (Jagasia et al., 2009). Furthermore, recent studies have revealed which GABA receptors and subunits are involved in mediating the effects of GABA on the newborn progenitors. Adult-born hippocampal neurons of mutant mice lacking the α4-subunit-containing $GABA_A$Rs and α2-subunit-containing $GABA_A$Rs show defects in dendritic arborization, and cell migration in a temporally distinct manner (Duveau et al., 2011). This differs from the role of γ2-subunit-containing $GABA_A$Rs, which regulate early stages of stem cell division, as NSPCs lacking the γ2-subunit exit quiescence and exhibit increased symmetrical divisions (Song et al., 2012). Collectively, these results indicate that glutamatergic and GABAergic input play important roles in the maturation of newborn hippocampal neurons *in vivo*.

The small Rho GTPases appear to be regulated by both glutamatergic and GABAergic neurotransmission via specific GEFs. Although glutamate initially and transiently activates RhoA (Jeon et al., 2002), longer activation of NMDAR leads to robust activation of the Rac1 GEF, Tiam1, specifically in dendrites and spines of hippocampal neurons *in vitro* (Tolias et al., 2005). This suggests that glutamate may transiently upregulate RhoA activity via AMPA receptors initially, whereas, longer or coincident excitation via NMDARs likely recruits Rac1 for mediating its positive influence on neurite growth and spine motility (Tashiro and Yuste, 2008). This is significant given that localized activation of the Rho GTPases has been directly linked to plasticity at single dendritic spines (Murakoshi et al., 2011; Yasuda and Murakoshi, 2011). GABAergic signaling has also been linked to the Rho GTPases Cdc42 and Rac1. Meyer et al. show that Rac1 activity is required for optimal $GABA_A$ receptor activity, as a loss of Rac1 activity reduced $GABA_A$ receptor activity, possibly through loss of receptor clustering and recycling (Meyer et al., 2000). The effects of GABA on neural progenitors appear to be highly specific to the subunit composition of GABA receptors, and it remains to be elucidated how distinct subunit-containing receptors recruit different Rho GTPases. Interestingly, not only does GABAergic signaling recruit the Rho GTPases, it appears that Rho GTPases can also regulate components of the GABAergic postsynapse. The Cdc42-specific GEF Collybistin has been shown

to regulate GABAergic neurotransmission as well as clustering of the main inhibitory postsynaptic scaffolding protein Gephyrin (Tyagarajan et al., 2011; Korber et al., 2012). This raises the interesting possibility that the small GTPases may also be playing a role in feedback or homeostatic mechanisms, influencing plasticity at the inhibitory postsynapse (Papadopoulos and Soykan, 2011). Collectively these results suggest that glutamatergic and GABAergic neurotransmission may be influencing newborn neuron maturation and spine development, at least in part by modulating small Rho GTPase activity.

DEVELOPMENTAL SIGNALING MOLECULES: NOTCH

Notch signaling involves the activation of transmembrane heterodimer receptors, activated by Delta-like or Jagged, which are membrane-bound ligands present on neighboring cells. Ligand binding results in cleavage of the transmembrane domain of the notch receptor, releasing the notch intracellular domain (NICD), which translocates to the nucleus where it interacts with RBP-J to activate transcription of several genes. Notch signaling has been extensively explored during development and shown to be critical for neuronal cell-fate choice and neurite development during embryonic neurogenesis (Sestan et al., 1999; Redmond et al., 2000). Recently, studies have found Notch to also play important roles at different stages during adult neurogenesis (Ehm et al., 2010; Ables et al., 2011). In their study, using a conditional knockout of Notch1 in adult neural stem/progenitor cells, Breunig et al. show that in addition to playing a role in proliferation and differentiation, Notch1 is also important for dendritic arborization of newborn neurons (Breunig et al., 2007). Recent evidence indicates that non-canonical Notch signaling recruits the Trio-Rac1 (GEF-GTPase) complex for axon guidance in drosophila motor neurons, indicating that non-canonical Notch signaling may be specifically involved in neurite development via modulation of small Rho GTPase activity as compared to canonical Notch signaling (Song and Giniger, 2011). Although it has been hypothesized that Rho GTPase signaling likely mediates the effects of canonical Notch signaling on neurite growth, this remains to be confirmed (Redmond and Ghosh, 2001).

INTERMEDIATE SIGNAL REGULATORS

Recently, other intermediate signaling molecules such as Disrupted in schizophrenia 1 (DISC1) and Cyclin-dependent kinase 5 (Cdk5) were found to regulate the maturation of adult born neurons (Wu et al., 2013). In a hallmark study, Duan et al. found that cell-autonomous downregulation of DISC1 accelerated morphological maturation and aberrant integration of newborn neurons in the adult hippocampus. Their study revealed DISC1 to be a key regulator in maintaining the pace of maturation and integration of adult-born neurons (Duan et al., 2007). Following this study, several other groups found DISC1 to also play important roles in other aspects of neuronal maturation, such as axonal targeting, cell-cell/cell-matrix adhesion, and neurite outgrowth (Hattori et al., 2010; Kvajo et al., 2011). Interestingly, DISC1 has also recently been shown to be downstream of regulators previously shown to regulate neuronal maturation, for example, NMDARs (Namba et al., 2011; Wu et al., 2013). Despite the relatively recent discovery of DISC1's role in newborn neuron maturation, some studies have reported Rac1 to be specifically downstream of DISC1 in regulating neuronal maturation. A study in primary neurons *in vitro* found that DISC1 anchors the Rac1-GEF Kalirin-7 to the postsynaptic density, and regulates local Rac1 activity and spine morphology downstream of NMDAR activation (Hayashi-Takagi et al., 2010). Another study, using a heterologous DISC1 transgenic system in *C. elegans* motor neurons, found DISC1-mutant neurons to have abnormal axonal morphology, phenocopying Rac1-mutant defects. Further, it was found that DISC1 directly interacts with the Rac1 GEF, Trio, promoting Rac1 recruitment, suggesting that the observed axonal defects are likely due to impaired downstream Rac1 signaling (Chen et al., 2011). Additional studies are required to precisely characterize the role of small GTPases downstream of DISC1 in regulating neuronal maturation of adult-born hippocampal neurons.

Cdk5 has been shown to phosphorylate a number of neuronal proteins specifically involved in neuronal migration and synaptic plasticity (Jessberger et al., 2009; Lopes and Agostinho, 2011). A number of GEFs and small Rho GTPase effectors such as Ephexin1 (Fu et al., 2007), Ras Guanine nucleotide release factors 1 and 2 (Kesavapany et al., 2004, 2006), and Wave1 (Kim et al., 2006; Cheung and Ip, 2007) have been shown to be phosphorylated by Cdk5. In the context of adult hippocampal neurogenesis, Cdk5 appears to regulate adult neurogenesis, both in a cell-autonomous and non-cell-autonomous way. Retrovirus mediated overexpression of dominant negative Cdk5 leads to aberrant dendritic targeting and impaired spine maturation in a significant fraction of targeted adult-born hippocampal neurons, suggesting an intrinsic role for Cdk5 in neuronal maturation (Jessberger et al., 2008; Tobias et al., 2009). On the other hand, conditional deletion of Cdk5 in the NSPC population affected survival of immature neurons, an effect phenocopied in mice lacking the activating cofactor p35 (Lagace et al., 2008). This is particularly interesting because the p35/Cdk5 complex has been shown to be present at the growth cone, where it directly associates with Rac1, hyperphosphorylating its effector Pak1 kinase, resulting in an attenuation of Rac1 signaling (Nikolic et al., 1998). Interestingly, a recent study has placed Cdk5 downstream of BDNF in dendritic spines, *in vitro*, by showing that TrkB phosphorylation at specific serine residues (S478) is Cdk5 dependent. This phosphorylation of TrkB at S478 regulates its interaction with Rac1-GEF TIAM1, and downstream Rac1 activation (Lai et al., 2012). These results suggest that small Rho GTPases are downstream of Cdk5 signaling, and that Cdk5 may have distinct roles in adult neurogenesis via the differential recruitment of specific GEFs and GAPs. Taken together, data from these studies indicate strong links between DISC1, Cdk5, and Rac1 signaling in modulating neurite outgrowth dynamics *in vitro*, allowing us to speculate about such interactions *in vivo*.

MECHANISMS FOR SIGNAL CONVERGENCE IN ADULT-BORN HIPPOCAMPAL NEURONS

Research over the last two decades has given us a large amount of information regarding the molecular mediators downstream of diverse cellular inputs. We now understand how an upstream

activator may have diverse effects on cellular behavior by recruiting multiple signaling cascades. In the context of maturation and integration of adult-born neurons, cellular behaviors such as neurite and spine growth are readouts, used as indicators of the state in which the cell is, as well as key endpoints with functional relevance. Neurite outgrowth and spine morphogenesis, at their core, are regulated by subcellular events leading to the stabilization, extension, or collapse of local cytoskeletal elements. Therefore, despite simultaneous activation of multiple signaling pathways, decisions of actual neurite growth or retraction are consequences of single subcellular events via modulation of local microtubules or actin filaments. *In vivo*, newborn neurons receive diverse input, each of which may simultaneously activate different pathways that can "instruct" neurite growth or neurite retraction. However, given the limited number of potential outcomes in neurite dynamics (i.e., either growth, stability, or collapse), we hypothesize that there must be mechanisms that allow for the convergence of multiple pathways leading to a single decision resulting in either neurite extension, stabilization, or collapse at a given time.

Studies exploring different signaling pathways have revealed how the bifurcation or divergence of signals via diverse signaling cascades enables different cellular responses to the same input, but it remains unclear how convergence of pathways might be taking place. One possible mechanism for signal convergence could be that following the peak of signal divergence some signaling cascades "funnel-in" by having fewer and fewer downstream targets until they directly influence cell neurite growth. For example, pathways such as mitogen-activated protein (MAP) kinase and CREB are common targets of many upstream regulators and have been hypothesized to act as signal integrators for certain cellular processes (Wagner and Nebreda, 2009; Merz et al., 2011). Given that an upstream input can recruit different signaling entities and the fact that there are limited context-specific cellular responses that can occur, there exists a dichotomy between high signal divergence initially followed by signal convergence, resulting in a single cellular event. Spatial and temporal segregation of signaling cascades obviously enables such divergence to take place. On the same note, spatial and temporal segregation can also facilitate convergence by promoting interaction of pathways within designated spatial and temporal contexts, for example at the post-synaptic density (Sheng and Kim, 2011). Similar concepts of signal convergence for pathways involving insulin, DAG/PKC, TGF-beta/BMP, G-proteins Gz/Gi have been previously discussed (Ho and Wong, 2001; Yang and Kazanietz, 2003; Taniguchi et al., 2006).

Central decision-making moieties are another mechanism through which signal convergence may occur. Among others, small Rho GTPases, due to their switch-like nature may serve to function as decision-making entities, for modulating neurite growth or collapse. As they are known to be downstream of a variety of activators, we further speculate that multiple upstream signals impinge upon the small Rho GTPases leading to decisive events shaping neurite and spine architecture. A way by which Rho GTPases may be assimilating upstream signal is by setting an activation (GTP-bound GTPase) threshold for upstream signaling. Further, regulating the concentration and regional availability of Rho GTPases may additionally enable localized assimilation of upstream signal. Sufficient input regulating the Rho GTPases' activity may come from multiple upstream pathways, where the Rho GTPases may be facilitating detection of coincident upstream input. Whether or not this is actually the case in maturation of newborn neurons remains to be resolved.

Clearly, the small Rho GTPases Cdc42, Rac1, and RhoA have functional ties with intrinsic regulators of neuronal maturation. Given their switch-like nature and a large repertoire of GEFs and GAPs regulating their state, it is likely that they are among the "decision-making" moieties via which diverse upstream regulators maybe decisively influencing neuronal architecture in the adult brain. So far, a majority of studies exploring the role of signaling molecules on neuronal function have utilized knockdown or overexpression models, *in vitro* and *in vivo*. While these approaches have generated valuable information about the basic roles of the Rho GTPases, their dispensability (knockout or dominant-negative constructs), and/or maximum functional capacity (overexpression or constitutively active constructs), we may have missed the nuanced roles of these signaling pathways of these signaling pathways under physiological conditions in neurons. Understanding whether such convergence indeed plays a role in signal transduction leading to decisive cellular events would minimally require experiments investigating activation of endogenous small GTPases, in real time, *in vivo*. This would provide us with significant insights into the physiological roles of these molecules and the decisive influence of regulatory pathways in mediating specific cellular behavior in the context of adult hippocampal neurogenesis.

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The BDNF effects on dendritic spines of mature hippocampal neurons depend on neuronal activity

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The fine tuning of neural networks during development and learning relies upon both functional and structural plastic processes. Changes in the number as well as in the size and shape of dendritic spines are associated to long-term activity-dependent synaptic plasticity. However, the molecular mechanisms translating functional into structural changes are still largely unknown. In this context, neurotrophins, like Brain-Derived Neurotrophic Factor (BDNF), are among promising candidates. Specifically BDNF-TrkB receptor signaling is crucial for activity-dependent strengthening of synapses in different brain regions. BDNF application has been shown to positively modulate dendritic and spine architecture in cortical and hippocampal neurons as well as structural plasticity *in vitro*. However, a global BDNF deprivation throughout the central nervous system (CNS) resulted in very mild structural alterations of dendritic spines, questioning the relevance of the endogenous BDNF signaling in modulating the development and the mature structure of neurons *in vivo*. Here we show that a loss-of-function approach, blocking BDNF results in a significant reduction in dendritic spine density, associated with an increase in spine length and a decrease in head width. These changes are associated with a decrease in F-actin levels within spine heads. On the other hand, a gain-of-function approach, applying exogenous BDNF, could not reproduce the increase in spine density or the changes in spine morphology previously described. Taken together, we show here that the effects exerted by BDNF on the dendritic architecture of hippocampal neurons are dependent on the neuron's maturation stage. Indeed, in mature hippocampal neurons *in vitro* as shown *in vivo* BDNF is specifically required for the activity-dependent maintenance of the mature spine phenotype.

Keywords: dendrites, spines, structural plasticity, hippocampus, neurotrophins

INTRODUCTION

Neurotrophins are essential for multiple aspects of neuronal development and function. Especially Brain-derived Neurotrophic Factor (BDNF) has been shown to play an important role in neuronal survival and in the maintenance of several neuronal systems. In addition, BDNF has been implicated in numerous processes of functional and structural synaptic plasticity (Gottmann et al., 2009; Park and Poo, 2012; Zagrebelsky and Korte, 2013). The role of BDNF has been extensively analyzed *in vitro* upon the application of exogenous BDNF. The data obtained support the notion that during development BDNF is involved in regulating the fine-tuning of the cortical network by selectively enhancing dendritic growth in an activity-dependent manner (Mcallister et al., 1995, 1996; Horch et al., 1999). Application of exogenous BDNF to developing primary hippocampal neurons has been shown to result in a significant increase in the number of primary neurites as well as an increase in neurite complexity and length (Ji et al., 2005; Kwon and Sabatini, 2011). Furthermore, dendritic spine density and morphology of mature primary hippocampal neurons are significantly influences by a BDNF application (Ji et al., 2005, 2010). Similarly, mature organotypic hippocampal neurons treated with BDNF show a significant increase in dendritic spine density and in the number of synapses (Tyler and Pozzo-Miller, 2001, 2003). Taken together, the *in vitro* studies described above strongly support the notion that, in the hippocampus exogenous BDNF promotes dendritic formation and growth during development and regulates dendritic spine density and morphology in mature neurons. But as strong as the evidence for a role of BDNF in modulating dendritic architecture might appear, the preparation techniques (Danzer et al., 2004) and the culture conditions (Chapleau et al., 2008) have been shown to influence the expression levels as well as the cellular response to BDNF, possibly confounding the analysis under these conditions. Suggestive for a role of BDNF *in vivo* is the correlation between the physiological variability in BDNF expression levels in the mouse dentate gyrus and the dendritic spine density in granule cells (Stranahan, 2011). Moreover, a reduction in BDNF serum levels is associated to a reduction in hippocampus volume in aging humans (Erickson et al., 2011) as well as in dendritic complexity and spine density in senescent rats (Von Bohlen Und Halbach, 2010). The *in vivo* role of BDNF has been very difficult to evaluate in the post-natal brain as $bdnf^{-/-}$ mouse mutants die too early for the role of BDNF to be assessed after its increased post-natal expression caused by neuronal activity (Zafra et al., 1990; Hong et al., 2008). The analysis of $bdnf^{+/-}$ mouse mutants, showing

reduced BDNF levels provided evidence that, *in vivo* BDNF plays an important role in the induction of LTP in the hippocampus (Korte et al., 1995), the acquisition of extinction learning (Psotta et al., 2013) and in the structural rearrangement of adult cortical circuitry upon increased sensory input (Genoud et al., 2004). On the other hand, in *bdnf*$^{+/-}$ mice synapse density and spine morphology are indistinguishable from those in WT mice (Korte et al., 1995; Genoud et al., 2004) and a compensatory increase in TrkB receptor expression occurs (Carreton et al., 2012), leaving open the question of whether BDNF modulates dendritic architecture *in vivo*. Thus, a number of studies have been performed using different conditional gene targeted mouse lines and Cre-loxP-mediated excision of *bdnf* (Rios et al., 2001; Gorski et al., 2003; Baquet et al., 2004; He et al., 2004; Chan et al., 2006, 2008; Monteggia et al., 2007; Unger et al., 2007; Rauskolb et al., 2010). Surprisingly, the effect on excitatory neurons in the hippocampus and cortex of a global BDNF deprivation throughout the central nervous system (CNS) is extremely mild when compared to the effects observed upon a BDNF application *in vitro*. Rauskolb et al. (2010) showed that in a Tau-BDNF^{KO} mouse the volume of the cortex is only slightly reduced and the one of the hippocampus unchanged. Accordingly, dendritic complexity of CA1 pyramidal cells is only mildly reduced and while no changes could be observed in dendritic spine density the spine type distribution in Tau-BDNF^{KO} neurons is significantly shifted toward a more immature phenotype, pointing to a specific role of BDNF in the maintenance of mature dendritic spines in the mature hippocampus. These results are consistent with the observation that a conditional deletion of TrkB in the hippocampus does not affect the gross dendritic morphology of CA1 pyramidal neurons (Minichiello et al., 1999; Luikart et al., 2005).

So overall it is noteworthy that while most of the published data supporting a role for BDNF in modulating dendrite and dendritic spine morphology derive from *in vitro* experiments applying exogenous BDNF, a role for endogenous BDNF in this context is still unclear. Moreover, *in vivo* the effects of endogenous BDNF in modulating the structure of neurons seem to be extremely specific, depending on the developmental stage, the brain area as well as the cell-type. Therefore, in this study we set out to address the discrepancies in the role of BDNF in modulating the architecture of mature hippocampal neurons. In order to contribute to a better understanding of the BDNF activity, we analyzed the effects of several manipulations of its signaling on dendrites and dendritic spines at different developmental stages and under different levels of neuronal activity in primary neuronal cultures. The results we obtained in our culture system confirm the observations previously obtained *in vivo* and show that BDNF exerts a highly precise role on different aspects of neuronal architecture in an age-dependent manner. In mature hippocampal neurons *in vitro,* as shown *in vivo* endogenous BDNF is specifically required for the activity-dependent maintenance of the mature spine phenotype.

MATERIALS AND METHODS

PREPARATION OF PRIMARY HIPPOCAMPAL CULTURES

Primary hippocampal cultures were prepared from C57BL/6 mice at embryonic day 18. All procedures were approved by guidelines from the Animal Committee on Ethics in the Care and Use of Laboratory Animals of TU Braunschweig. Embryos were decapitated and the brains were kept in ice-cold Gey's balanced salt solution (GBSS) containing of 1% kynurenic acid, 1% glucose and adjusted to pH 7.2. The dissected hippocampi were incubated for 30 min in trypsin/EDTA at 37°C and then mechanically dissociated. Cells were plated at high density (10^5/well) on poly-L-lysine-coated coverslips (13 mm) and kept in Neurobasal medium (Invitrogen) supplemented with 2% B27 (Invitrogen) and 0.5 mM Glutamax at 37°C, 5% CO_2, and 99% humidity. For analysis of neurite growth in 3–6 days *in vitro* to the cells were plated at a density of 10^4 cells per well. For the experiments performed in high Magnesium (Mg^{2+}) containing medium, the cells were plated at a density of 70,000 cells per well and kept in Neurobasal medium (Gibco) supplemented with 2% BSA (Gibco), 200 µM L-Glutamin and 1% 100 × N2 (Invitrogen) at 37°C, 5% CO_2, and 99% humidity.

TRANSFECTION OF PRIMARY HIPPOCAMPAL NEURONS

Hippocampal neurons were transfected at various time points with a farnesylated form of the enhanced green fluorescent protein (fEGFP) using Lipofectamine 2000 according to the manufacturer's protocol. To specifically label F-actin without adverse effects on actin dynamics the cells were co-transfected with fEGFP and an expression plasmid for the Lifeact peptide fused to a red fluorescent protein (RFP-LA) (Riedl et al., 2008, 2010). Briefly, 0.8 µg DNA or, for a co-transfection 0.4 µg pfEGFP and 0.6 µg pRFP-LA and 2 µl Lipofectamine 2000 per well were mixed in 100 µl Neurobasal medium, and added to the cultures dropwise. After 50 min of incubation the transfection medium was exchanged for the original Neurobasal medium. The cultures were kept for 24 h at 37°C, 5% CO_2, and 99% humidity before the treatment.

TREATMENT OF PRIMARY HIPPOCAMPAL NEURONS

Twenty-four hours after transfection, 1/3 of the culture medium was exchanged for a medium containing 0.1% Bovine Serum Albumine (BSA, Sigma; 0.1% BSA/PBS) as a control treatment or 0.1% BSA combined with BDNF (Recombinant Human BDNF, 40 ng/ml, R&D Systems), BDNF blocking antibodies (BDNF-Abs, 1:500, M. Sendtner, Wuerzburg Germany) or TrkB receptor bodies (TrkB-Fc, Recombinant Human TrkB-Fc, 500 ng/ml, R&D Systems). The BSA was added to the medium to prevent BDNF or the antibodies from sticking to the plastic (Chen et al., 1999). In a second set of control experiments the cultures were kept in Neurobasal medium without addition of BSA. As no significant difference between the two control conditions could be observed only the BSA control was used in the result part. The cultures were fixed 24 h after the treatment in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PBS), washed and mounted with an antifading aqueous mounting medium (Fluoro-Gel Emsdiasum).

In a second set of experiments neuronal activity was reduced in primary hippocampal cultures by increasing the Mg^{2+}concentration in the medium. Indeed, Mg^{2+} decreases the activation of voltage-gated channels thereby reducing neuronal excitability (Mayer and Westbrook, 1987; Dribben et al., 2010). To avoid effects on the early developmental stages Mg^{2+} concentration in the medium was increased starting at 7 DIV from 1.5

to 3.5 mM by adding $MgCl_2$, for 2 weeks and fresh medium was added once a week. The treatment with BDNF or TrkB receptor bodies was performed for 24 h either in low (1.5 mM) or high (3.5 mM) Mg^{2+} containing medium.

IMMUNOCYTOCHEMISTRY

Primary hippocampal cultures (6 or 23 DIV) were fixed with 4% PFA, permeabilized and blocked with a solution containing 0.2% Triton X-100, 10% goat serum, and 0.1% BSA in PBS. A rabbit polyclonal antibody anti-c-fos (Santa Cruz Biotechnology, Inc., sc-52; dilution 1:500), a mouse monoclonal antibody anti-CaMKII (Invitrogen, dilution 1:500), a rabbit polyclonal anti-MAP2 (Abcam, dilution 1:500) or a primary rabbit monoclonal anti-phospho-TrkA (Tyr674/675)/TrkB (Tyr706/707) antibody (Cell Signaling Technology; dilution 1:200) were diluted in blocking solution (10% goat serum and 0.1% BSA in PBS) and incubated at 4°C overnight. Secondary anti-rabbit or anti-mouse antibodies conjugated with Cy2, Cy3, or Cy5 (Jackson Immuno Research) were diluted 1:500 in PBS and incubated for 2 h at room temperature. Alexa Fluor 350 phalloidin (Lifetechnologies) was diluted 1:50 in PBS and incubated for 3 h at room temperature to selectively stain F-actin. Finally, cultures were counterstained with DAPI (4′,6-diamidino-2-phenylindole) (Biotium) diluted 1:1000 in PBS for 5 min, washed and mounted using an anti-fading aquous mounting medium (Fluoro-Gel Emsdiasum).

NEURON SELECTION

The transfection of the fEGFP expression plasmid under the control of the CMV promoter resulted in the intense labeling of the entire dendritic arbor as well as of all dendritic spines of individual pyramidal neurons. A small number of individual pyramidal neurons were transfected in each culture allowing to following the complete dendritic processes of each labeled neuron. Only non-overlapping labeled neurons were selected for our analysis allowing for an unambiguous reconstruction of the entire dendritic tree as well as the analysis for the spine density. Degenerating neurons were identified according to the presence of retraction bulbs and fragmentation of the dendritic processes and excluded from the analysis. Due to a recent report showing that Mg^{2+} (5 to 10 mM) increases neuronal apoptosis in primary hippocampal neurons (Dribben et al., 2010), special care was used in selecting neurons kept in high magnesium medium (3.5 mM). Indeed, all neurons selected for the analysis showed a healthy cell body and dendritic tree with no signs of apoptosis.

NEURONAL IMAGING AND ANALYSIS

The neurons selected for analysis were imaged using an Axioplan 2 microscope equipped with an ApoTome module (Zeiss). Each neuron was first imaged using a 20× objective [1.25 numerical aperture (NA)] and the images were used to analyze dendritic complexity and length using a Sholl analysis (Sholl, 1953). The neurons were traced using the Neurolucida software (MicroBrightField), and the Sholl analysis was obtained with Neuroexplorer software (MicroBrightField), calculating the cumulative number of dendritic intersections at 10 μm interval distance points starting from the cell body. The total number of crossings for each cell was used as an index for total dendritic complexity. The total neurite length, number of primary neurites and number of branching points of 6 DIV neurons were analyzed by tracing MAP2 labeled neurites using the NeuronJ (Meijering et al., 2004) plugin for ImageJ (NIH) Software. Spine density was measured separately for portions of selected second or third order dendrite branches and imaged using a 63× objective (1.32 NA) acquiring three-dimensional stacks z-sectioned at 0.5 μm. The number of spines and the dendritic length were measured using the ImageJ software. The number of spines was normalized per micrometer of dendritic length and a minimal length of 250 μm of dendrite per cell was analyzed. The maximal spine head diameter and maximal spine length (from the dendritic shaft to the spine head tip) were measured on three-dimensional stacks using the ImageJ (NIH) software. To examine the actin content in dendritic spine heads the mean fluorescent intensity of RFP-lifeact was measured with the area selection tool of ImageJ and normalized to the mean fluorescent intensity of the dendritic shaft (for normalization of the RFP overexpression, **Figure 3B**, indicated with the arrow heads). RFP-Lifeact and the immunocytochemistry for c-fos and phosphorylated TrkB were imaged keeping a constant exposure time. The mean fluorescent intensity values for the immunocytochemistry of c-fos and phosphorylated TrkB were measured with ImageJ (NIH) and normalized to the control condition. The analysis was performed blindly.

CALCIUM IMAGING

Primary hippocampal cultures 16 and 23 DIV were loaded with calcium indicator by incubating them for 15 min with the indicator solution (50 μg Oregon Green 488 BAPTA-1 AM (Invitrogen), dissolved in 50 μl DMSO and 2.5% Pluronic acid) at 37°C, 5% CO_2, and 99% humidity. The cells were then transferred to HBSS (Hanks balanced salt solution) and kept 45 min at 37°C, 5% CO_2, and 99% humidity. The coverslips were thentransferred to the recording chamber, continuously perfused with HBSS and kept at 32°C. The live imaging was performed using an Olympus BX61WI microscope system (Cell^M software) and a CCD camera (VisiCam QE, Visitron Systems). Images were recorded at 6 Hz for 75 sec with an Olympus 20× water immersion objective (0.95 NA XLUMPLAN FL, Olympus), whereby a pixel resolution of 0.77 μm/pixel was achieved. A frame rate of 150 ms and an exposure time of 61 ms were used. Primary hippocampal cultures were treated with BSA (serial 0.1% BSA/1× PBS) or BDNF (Recombinant Human BDNF, 40 ng/ml, R&D Systems) by a 5 min bath-application.

The analysis of calcium transients was performed using ImageJ (NIH) and calculating the fluorescence changes as average of all pixels within regions of interests (ROIs). The ROIs were set around the cell bodies of the neurons. For the background subtraction the ROI was drawn on a neighboring area containing neuropil. The resulting changes in the intracellular calcium concentration are represented as $\Delta F/F_0$ in percent, where F_0 is the fluorescence at resting conditions [$\Delta F/F_0 = (F - F_0)/F_0$] taken before the application of BDNF (Lang et al., 2006). In addition to it we generated using the plugin "reslice" of the ImageJ Software a pseudo-line scan to visualize changes in the fluorescence intensity for the whole ΔF-stack. In the pseudo-line scan the y-axis

corresponds to the respective line drawn on the stack whereas the x-axis corresponds to time.

STATISTICAL ANALYSIS

The statistical analysis was performed using Microsoft Excel or GraphPad Prism. The data obtained were compared between two different experimental conditions using an unpaired two-tailed Student's *t*-test. Data including more than 2 different groups were analyzed using a One-Way ANOVA followed by a *post-hoc* Tukey's Multiple Comparison Test. Values of $p = 0.05$ were considered significant and plotted as follows $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. All data are indicated as mean ± s.e.m.

RESULTS

ENDOGENOUS BDNF REGULATES DENDRITIC SPINE DENSITY AND MORPHOLOGY IN MATURE PRIMARY HIPPOCAMPAL NEURONS

In view of the discrepancy existing between a described action of exogenous BDNF in modulating the dendritic architecture of hippocampal neurons *in vitro* (Tyler and Pozzo-Miller, 2001; Ji et al., 2005, 2010) and previous data showing that a global deprivation of BDNF throughout the CNS *in vivo* results in only minimal morphological alterations of mature hippocampal neurons (Minichiello et al., 1999; Zakharenko et al., 2003; Rauskolb et al., 2010, for a review see Zagrebelsky and Korte, 2013), we analyzed the role of BDNF in modulating dendritic spine number and spine morphology as well as dendritic architecture of primary hippocampal neurons. The dendrite and dendritic spine architecture of mature 3-week-old (22 DIV) primary hippocampal neurons (Papa et al., 1995), expressing a membrane targeted farnesylated EGFP (fEGFP; **Figure 1B**) and identified as excitatory by their pyramidal like morphology (**Figure 1B**) and by the expression of the Ca^{2+}/calmodulin-dependent protein kinase (CaMKII; **Figure 1B** inserts) were analyzed upon the application of either a gain- or loss-of-function approach for BDNF (**Figure 1A**). While 22 DIV fEGFP-expressing neurons treated for 24 h with exogenous BDNF showed no obvious alterations in dendritic spine density when compared to control neurons, (**Figure 1B′**), treating the neurons with BDNF function blocking antibodies (BDNF-Abs) led to a strong reduction in dendritic spine density (**Figure 1B′**). This observation was confirmed by the quantification of dendritic spine density showing no significant difference between BDNF and control treated cells (**Figure 1C**, Table S1). On the other hand, a significant reduction in dendritic spine density was observed for neurons treated with BDNF blocking antibodies (**Figure 1C**, Table S1; control vs. BDNF-Abs, $p < 0.001$; BDNF vs. BDNF-Abs $p < 0.001$). These observations suggest that while the endogenous BDNF may be crucial for the maintenance of dendritic spines in mature primary hippocampal neurons, exogenous BDNF does not play a role in this context. We next assessed whether BDNF regulates spine morphology in mature hippocampal neurons by analyzing the effects of adding or blocking BDNF on dendritic spine head width and length. When compared spine head width as well as spine length (**Figure 1B′**) did not show any major difference after a 24 h treatment with BDNF in comparison to the control condition. On the other hand neurons treated with BDNF blocking antibodies showed clearly elongated and thinner spines than control neurons (**Figure 1B′**). Indeed, quantification of spine morphology upon BDNF antibody treatment showed a significant decrease in spine head width (**Figure 1D**, Table S1; control vs. BDNF-Abs and BDNF vs. BDNF-Abs, $p < 0.05$) associated to a significant increase in dendritic spine length (**Figure 1E**, Table S1; control *vs.* BDNF-Abs $p < 0.01$, BDNF vs. BDNF-Abs $p < 0.001$). Treatment with exogenous BDNF did not result in any significant change in dendritic spine head width (**Figure 1D**, Table S1). Under these conditions dendritic spine length was only slightly reduced when compared to controls but significantly lower than in neurons treated with BDNF blocking antibodies (**Figure 1E**, Table S1; BDNF vs. BDNF-Abs, $p < 0.001$). Next dendritic spines were separated according on their stage of maturation into four categories based on their head width. Comparing the effect of the different treatments within each spine head category showed a significant increase in spines with a small head ($<0.3\,\mu m$; control vs. BDNF-Abs $p < 0.001$, BDNF vs. BDNF-Abs $p < 0.01$) accompanied by a significant decrease in larger spine heads ($\geq 0.5\,\mu m$ $<1\,\mu m$; control vs. BDNF-Abs $p < 0.01$, BDNF vs. BDNF-Abs $p < 0.001$) when a treatment with BDNF blocking antibodies was compared to a control treatment or to a treatment with exogenous BDNF (**Figure 1F**). Application of BDNF (gain-of-function experiment) did not result in any significant alteration in spine head size when compared to the control conditions (**Figure 1F**). Binning the spines according to their length did not show any difference for BDNF treated neurons but resulted in a significant alteration in spine length distribution upon BDNF antibody treatment (loss-of-function) (**Figure 1G**). While under these conditions spines shorter than $1.5\,\mu m$ were significantly decreased (**Figure 1G**; $\geq 0.5\,\mu m$ $<1\,\mu m$ $p < 0.01$ to the BDNF treatment; $\geq 1\,\mu m$ $<1.5\,\mu m$ $p < 0.05$ to the control), those longer that $1.5\,\mu m$ were significantly increased when compared to the control conditions or the BDNF treatment (**Figure 1G**; $p < 0.01$ to the control, $p < 0.001$ to BDNF treatment). Finally, the effects of either a gain- or a loss-of-function for BDNF on dendritic architecture were analyzed using a Sholl analysis to compare dendritic complexity. No significant differences in dendritic complexity could be observed between the three different treatments (**Figure 1H**; control, BDNF, BDNF-Abs). Accordingly, also total dendritic complexity was not significantly different in BDNF or BDNF- antibody-treated cells vs. control cells (**Figure 1H′**, Table S1).

Taken together, these results demonstrate that the exogenous application of BDNF, in mature primary hippocampal neurons, does not influence dendritic spine density, morphology and dendrite complexity. On the other hand, the loss-of-function experiments show that the endogenous BDNF is essential for maintaining spine density and the mature morphology of dendritic spines in primary hippocampal neurons.

ENDOGENOUS BDNF REGULATES DENDRITIC SPINE DENSITY AND MORPHOLOGY IN DEVELOPING PRIMARY HIPPOCAMPAL NEURONS

In the next series of experiments we tested whether the different effects of exogenous vs. endogenous BDNF on neuronal structure might depend on the age of the treated primary hippocampal neurons. To this aim we analyzed whether a gain- or a loss-of-function approach for BDNF regulates the dendritic

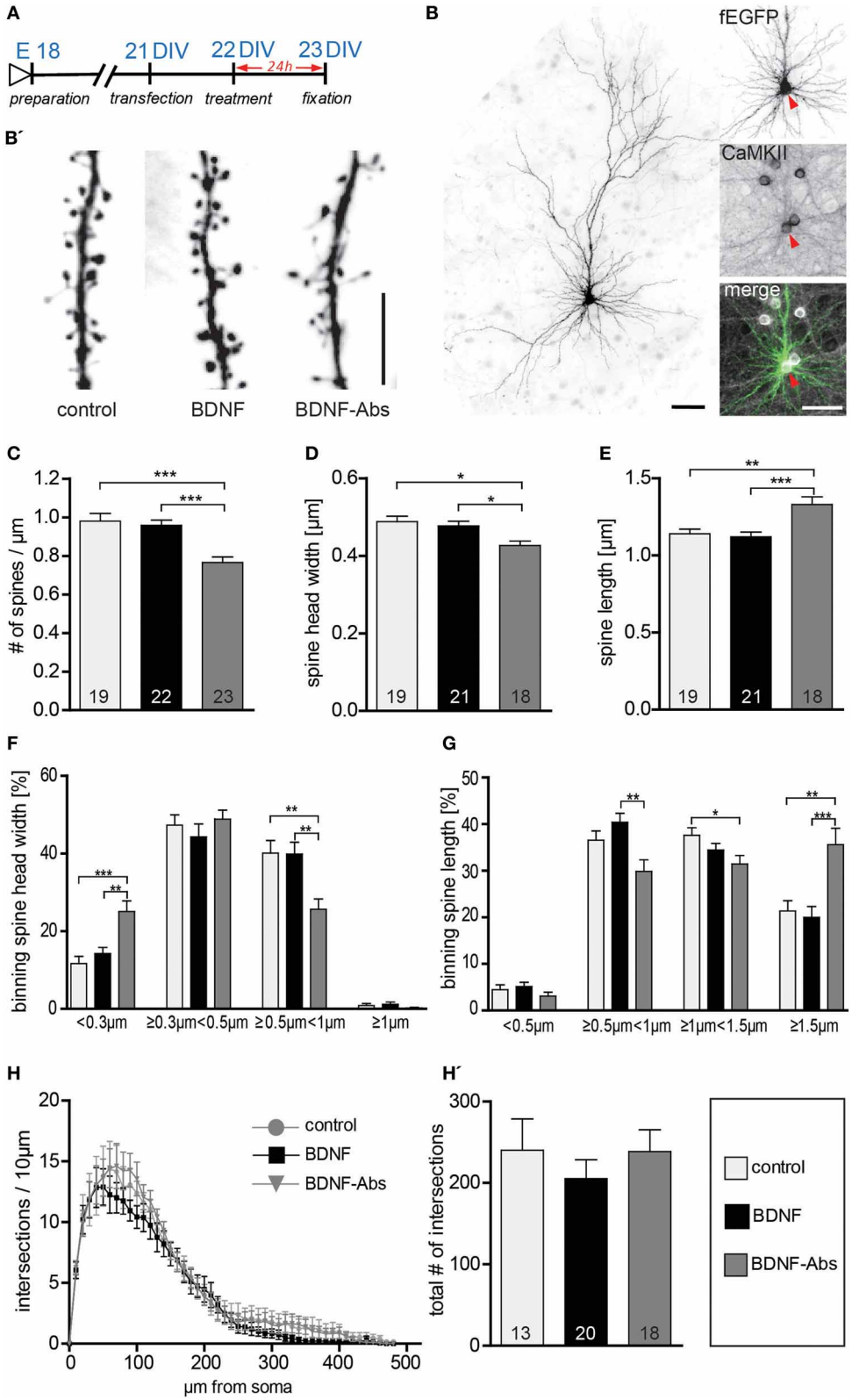

FIGURE 1 | (A) Experimental timeline. DIV23 hippocampal neurons were treated for 24 h before fixation. **(B)** Representative image of a typical DIV23 fEGFP expressing hippocampal neuron used in the experiments; the inserts on the right show a close up of the cell body labeled with fEGFP (above) or with an immunohistochemistry against CaMKII (middle) merged to show colocalization (below): scale bars are 50 µm. **(B′)** High-magnification image of typical dendritic stretches from control (left), BDNF (middle) and BDNF

(Continued)

FIGURE 1 | Continued
antibodies (right) treated neurons. Scale bar, 5 μm. **(C)** Graphs comparing dendritic spine density between BSA (control), BDNF and BDNF antibodies (BDNF-Abs) treated DIV22 hippocampal neurons. **(D)** Histogram comparing the spine head width between hippocampal neurons upon BSA (control), BDNF or BDNF antibodies (BDNF-Abs) treatment. **(E)** Histogram of the dendritic spine length in control, BDNF or BDNF antibodies (BDNF-Abs) treated neurons. **(F)** Graph showing the binning of spines according to their spine head width and comparing the proportion of spines within each category in response to a BSA (control), BDNF or BDNF antibodies (BDNF-Abs) treatment. **(G)** Graph showing the binning of spines according to their spine length and comparing the proportion of spines within each category in response to a BSA (control), BDNF or BDNF antibodies (BDNF-Abs) treatment. **(H)** Graph plotting dendritic complexity in relation to the distance to the cell body of hippocampal neurons under control conditions or treated with BDNF or BDNF antibodies (BDNF-Abs). **(H′)** Histogram of the total number of intersections for BSA (control), BDNF and BDNF antibodies (BDNF-Abs) treated neurons. The number in the columns represents the number of cells analyzed. Significance is indicated as follows: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. Error bars indicate s.e.m. ANOVA, *post-hoc* Tukey's Multiple Comparison Test.

architecture or spine number and morphology in 2 weeks-old primary hippocampal neurons (15 DIV; **Figure 2A**). When 15 DIV fEGFP-expressing neurons treated for 24 hours with BDNF were compared to control neurons, no obvious difference in dendritic spine density or morphology could be observed. Accordingly, the quantification of spine density did not result in any significant difference between BDNF treated and control neurons (**Figure 2B**, Table S1). However, as for mature hippocampal neurons, also here the application of BDNF blocking antibodies led to a highly significant reduction in dendritic spine density (**Figure 2B**, Table S1; control vs. BDNF-Abs and BDNF vs. BDNF-Abs $p < 0.001$). We then assessed whether BDNF regulates spine morphology comparing the maximal spine head width and spines length between different treatments. Application of exogenous BDNF for 24 h did not result in any significant alteration of the spine head width (**Figure 2C**, Table S1) and length (**Figure 2D**, Table S1) in comparison to the control condition. Interestingly, while the loss-of-function experiments did not result in any significant change in spine head width (**Figure 2C**, Table S1) a significant increase in dendritic spine length could be observed after application of BDNF blocking antibodies in comparison to both the control and the BDNF treatment (**Figure 2D**, Table S1; control vs. BDNF-Abs and BDNF vs. BDNF-Abs $p < 0.001$). Binning the spines according to their head width and comparing the different treatments within each spine head category resulted in a significant increase in the proportion of spines with a small head for the BDNF blocking antibody treatment only when compared to the BDNF treatment (**Figure 2E**; BDNF vs. BDNF-Abs $p < 0.05$). No significant changes could be observed for the spine head width distribution upon BDNF application (**Figure 2E**) when compared to the control condition. Binning the spines according to their length showed for neurons treated with BDNF blocking antibodies a significant decrease in the shorter spine category (**Figure 2F**; <0.5 μm, control vs. BDNF-Abs $p < 0.01$, BDNF vs. BDNF-Abs $p < 0.001$; and ≥ 0.5 μm <1 μm, control vs. BDNF-Abs $p < 0.01$, BDNF vs. BNF-Abs $p < 0.001$) accompanied by a significant increase in longer spines (**Figure 2F**; >1.5 μm, control vs. BDNF-Abs $p < 0.01$, BDNF vs. BDNF-Abs $p < 0.001$). Finally, we also analyzed whether the effect of BDNF on dendrite architecture might be age dependent. However, also for 15 DIV hippocampal neurons when either a BDNF or a BDNF antibody treatment was compared to the controls, no significant alteration in dendritic complexity could be observed for the Sholl analysis (**Figure 2G**). Accordingly, we did not observe any significant differences in total complexity between the different treatments (**Figure 2G′**, Table S1).

All in all the results described above indicate that endogenous BDNF is necessary for dendritic spine growth and to regulate spine length in developing primary hippocampal neurons. Interestingly, the spine head width in younger hippocampal neurons is not dependent on endogenous BDNF signaling.

ENDOGENOUS BDNF REGULATES ACTIN CONCENTRATION WITHIN DENDRITIC SPINE

Because the actin cytoskeleton is involved in regulating cell mobility and morphology and actin is highly enriched within the dendritic spine head—for a review see (Cingolani and Goda, 2008)—we hypothesized that BDNF may regulate actin in dendritic spines in order to shape and change spine morphology. To test this hypothesis we used RFP-Lifeact (Riedl et al., 2008 and 2010) to detect possible changes in the F-actin content in dendritic spines upon changes in BDNF signaling. The RFP-Lifeact expression plasmid was co-transfected with fEGFP and resulted in 99.7% colocalization of RFP-Lifeact within spines (**Figures 3A/B**) as well as with F-actin labeled using Alexa Fluor 350 Phalloidin (**Figure 3B**). In line with our observations the exogenous application of BDNF for 24 hours to 22 or 15 DIV primary hippocampal neurons did not result in any change in the normalized mean fluorescent intensity for RFP-Lifeact in comparison to control (**Figures 3C,D**, Table S1). However, in the loss-of-function experiments the treatment with BDNF blocking antibodies resulted in a highly significant decrease in the F-actin content within the head of dendritic spines both in 16 DIV (**Figure 3D**, Table S1; control vs. BDNF-Abs $p < 0.001$, BDNF vs. BDNF-Abs $p < 0.001$) and 23 DIV primary hippocampal neurons (**Figure 3D**, Table S1; control vs. BDNF-Abs $p < 0.01$, BDNF vs. BDNF-Abs $p < 0.001$).

Taken together our results show that endogenous BDNF modulates the actin cytoskeleton within dendritic spines as well as their morphology.

MATURE PRIMARY HIPPOCAMPAL NEURONS ARE RESPONSIVE TO AN ACUTE TREATMENT WITH EXOGENOUS BDNF

Our results so far clearly support a role for endogenous BDNF in the hippocampus by completely reproducing *in vitro* the mild alterations observed in the morphology of hippocampal neurons *in vivo* upon a global BDNF deprivation (Rauskolb et al., 2010). On the other hand our data fail to replicate previous work showing an effect of BDNF application in regulating dendrite and spine architecture (Tyler and Pozzo-Miller, 2001; Ji et al., 2005). To address this discrepancy we first confirmed that the BDNF used in this study is indeed active and that it is able

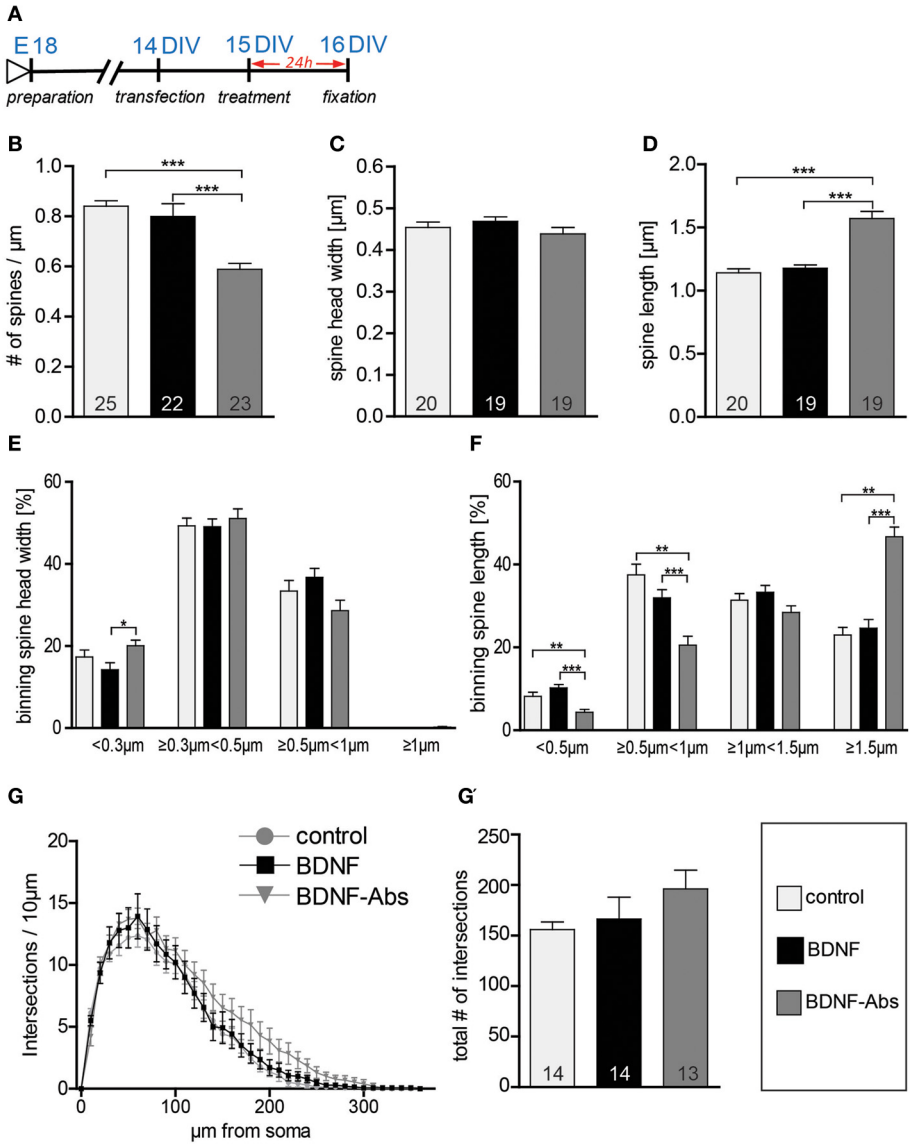

FIGURE 2 | (A) Experimental timeline. DIV16 hippocampal neurons were treated for 24 h before fixation **(B)** Graphs comparing dendritic spine density between control, BDNF and BDNF-antibodies (BDNF-Abs) treated DIV16 hippocampal neurons. **(C)** Histogram comparing the spine head width between hippocampal neurons upon BSA (control), BDNF or BDNF antibodies treatment. **(D)** Histogram of the dendritic spine length in BSA (control), BDNF or BDNF antibodies (BDNF-Abs) treated neurons. **(E)** Graph showing the binning of spines according to their spine head width and comparing the proportion of spines within each category in response to a BSA (control), BDNF or BDNF antibodies (BDNF-Abs) treatment. **(F)** Graph showing the binning of spines according to their spine length and comparing the proportion of spines within each category in response to a BSA (control), BDNF or BDNF antibodies (BDNF-Abs) treatment. **(G)** Graph plotting dendritic complexity in relation to the distance to the cell body of hippocampal neurons under control conditions or treated with BDNF or BDNF antibodies (BDNF-Abs). **(G′)** Histogram of the total number of intersections for BSA (control), BDNF and BDNF antibodies (BDNF-Abs) treated neurons. The number in the columns represents the number of cells analyzed. Significance is indicated as follows: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Error bars indicate s.e.m. ANOVA, *post-hoc* Tukey's Multiple Comparison Test.

to elicit clear neuronal responses in primary hippocampal neuronal cultures. To this aim we tested three critical events known to occur upon a BDNF application: (a) activation of the TrkB receptor was tested by analyzing its phosphorylation levels (Ji et al., 2010); (b) calcium imaging was used to test the ability of BDNF to induce local calcium transients in hippocampal neurons (Berninger et al., 1993) and (c) immunohistochemistry was used to visualize the activation of the immediate early gene (IEG) *c-fos* (Marty et al., 1996). An immunohistochemistry against phosphorylated TrkB receptor performed 15 min after BDNF application (**Figure 4A**) showed an obvious increase in the levels of TrkB receptor phosphorylation when compared to control treated neurons (**Figure 4B**). This observation was confirmed by a significant increase in the normalized intensity for the phospho-TrkB

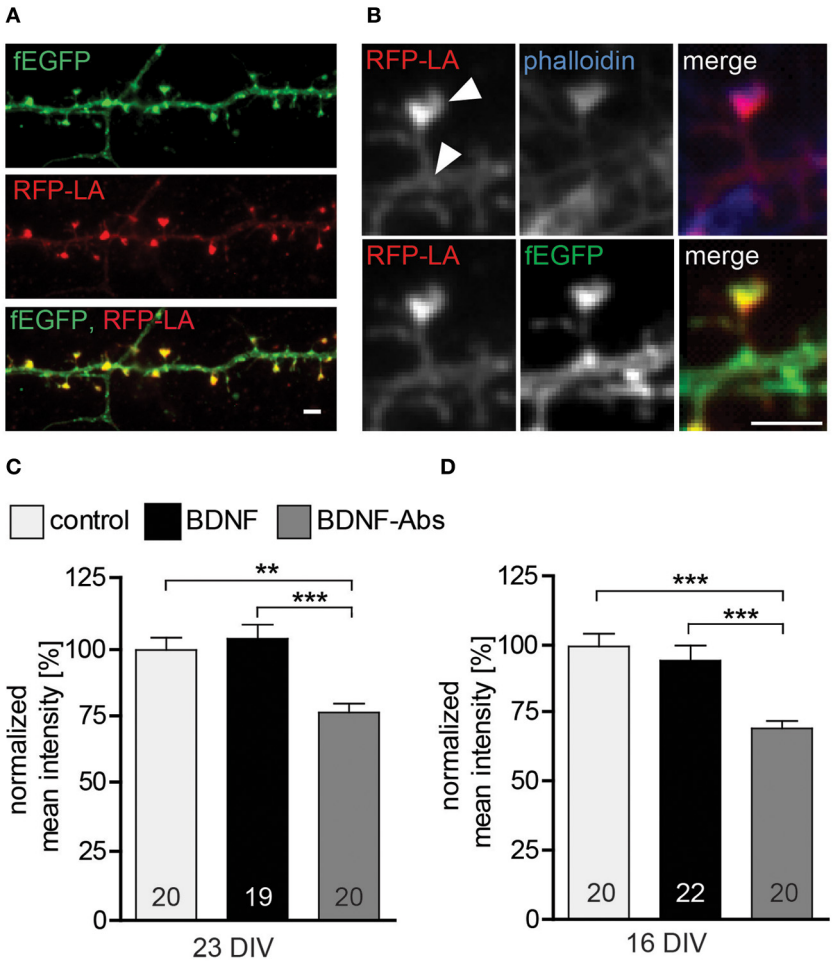

FIGURE 3 | (A) Maximal intensity projection of a representative dendrite of hippocampal neurons expressing fEGFP (above) and RFP-Lifeact (middle) merged to show colocalization (below). Scale bar, 2 μm. **(B)** High magnification images showing a single dendritic spine expressing fEGFP (above) and RFP-Lifeact (middle) and merged to show their colocalization in the spine head (below) Scale bar, 2 μm. **(C)** Histogram plotting the normalized mean fluorescent intensity for RFP-Lifeact within the spine head of DIV23 BSA (control), BDNF and BDNF antibodies (BDNF-Abs) treated neurons. **(D)** Histogram showing the normalized mean intensity for RFP-Lifeact within the spine head of DIV16 hippocampal neurons. The number in the columns represents the number of cells analyzed. Significance is indicated as follows: $^{**}p < 0.01$; $^{***}p < 0.001$. Error bars indicate s.e.m. ANOVA, *post-hoc* Tukey's Multiple Comparison Test.

immunohistochemistry (**Figure 4C**, Table S2; $p < 0.001$) as well as by a clear shift in the proportion of positive vs. negative cells for phospho-TrkB. Indeed, upon BDNF treatment significantly more neurons resulted to be positive for phospho-TrkB immunohistochemistry and significantly less to be negative for it (**Figure 4D**, Table S2; $p < 0.01$). The activation of the PLCγ signaling pathway upon BDNF binding to the TrkB receptor leads to an increase in the intracellular calcium levels (Segal and Greenberg, 1996; Blum and Konnerth, 2005; Minichiello, 2009) and was specifically shown for hippocampal cultures (Berninger et al., 1993; Canossa et al., 1997; Finkbeiner et al., 1997). Here we analyzed the frequency of global calcium transients as readout for a neuronal response to an acute BDNF application (**Figure 4E**; Lang et al., 2007; Lohmann, 2009). A clear increase in the frequency of calcium transients in DIV22 primary hippocampal neurons could be observed upon the acute application of BDNF (**Figure 4F**). Indeed, at a quantitative analysis calcium transient frequency resulted to be significantly increased after BDNF treatment in comparison to a control treatment (**Figure 4G**, Table S2; BDNF vs. control $p < 0.05$). The increase in the frequency in calcium transients upon BDNF application was transient as 10 min after BDNF washout the frequency was back to the pre-treatment levels (**Figure 4G**, Table S2). Finally, to verify that the application of BDNF leads to the expression of immediate early genes (IEGs) we used an immunohistochemistry for c-fos known to be activated upon an acute treatment with BDNF (**Figure 4H**; Marty et al., 1996; Gascon et al., 2005). An immunohistochemistry for c-fos showed a clear colocalization between c-fos and the nucleus of neurons (**Figure 4E**). Quantitative analysis performed 3 h after the stimulation with BDNF (**Figure 4H**) showed significantly higher intensity values for the c-fos immunohistochemistry (**Figure 4J**, Table S2; $p < 0.01$) and a significant increase in the number of c-fos positive cells in comparison to control (**Figure 4K**, Table S2; $p < 0.001$).

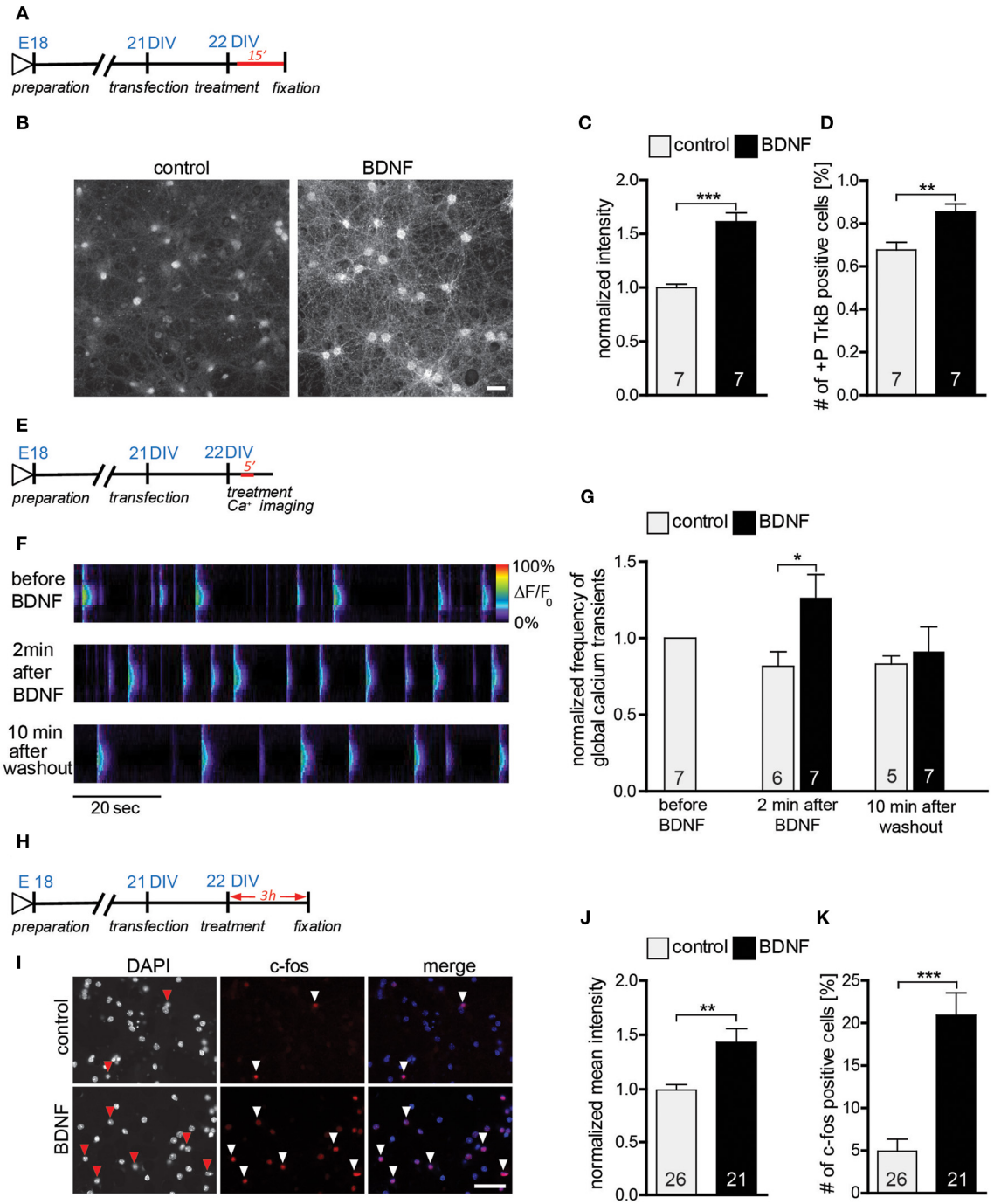

FIGURE 4 | (A) Experimental timeline. DIV22 primary hippocampal neurons were treated 15 min with BDNF before fixation. **(B)** Images of primary hippocampal cultures (22 DIV) treated with either BSA (control) (left) or BDNF (right) and stained with an anti-phospho-TrkB receptor antibodies. Scale bar, 40 μm. **(C)** Histogram showing the normalized fluorescence intensity for the phospho-TrkB receptor under control conditions or treated with BDNF. **(D)** Graph showing the number of phospho-TrkB positive hippocampal neurons in control and BDNF treated hippocampal primary cultures. **(E)** Experimental timeline. DIV22 primary hippocampal neurons were treated 5 min with BDNF during calcium imaging. **(F)** Pseudo line-scan showing the global calcium transients occurring in hippocampal primary cultures before, during and after BDNF applications. **(G)** Graph showing the frequency of calcium transients in hippocampal primary neurons before, during and after BDNF application. **(H)** Experimental timeline. DIV22 primary hippocampal neurons were treated for 3 h with BDNF before fixation. **(I)** Images of primary hippocampal cultures (22 DIV) treated with either BSA (control) (above) or BDNF (below) and stained with an anti-c-fos antibody. Scale bar, 20 μm. **(J)** Graph comparing the normalized mean intensity for the immunohistochemistry against c-fos in control or BDNF treated hippocampal primary neurons. **(K)** Histogram of the number of c-fos positive neurons in control and BDNF treated hippocampal cultures. The number in the columns represents the number of analyzed experiments. Significance is indicated as follows: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. Error bars indicate s.e.m. Unpaired two-tailed Student Test.

In summary we could show that the BDNF used in these experiments is active and clearly leads to an activation of the TrkB-receptor, an increase in the frequency of calcium transients and an activation of transcription (up regulation of the immediate early gene *c-fos*) in mature primary hippocampal neurons.

EXOGENOUS BDNF REGULATES NEURITE OUTGROWTH AND COMPLEXITY IN YOUNG PRIMARY HIPPOCAMPAL NEURONS

Previous studies have shown BDNF to promote neurite outgrouth and dendritic complexity (Mcallister et al., 1995; Shimada et al., 1998). In view of the lack of effects on dendritic architecture observed in this study upon BDNF application to either DIV15 or DIV22 hippocampal primary neurons, we tested whether the effect of BDNF on dendrites might be age-dependent. Indeed, BDNF has been shown to regulate neurite outgrowth in development hippocampal neurons (Ji et al., 2005, 2010). Therefore, we treated young hippocampal neurons 3 DIV with either a gain- or a loss-of-function approach for BDNF. After 3 days of treatment (**Figure 5A**) an immunohistochemistry for Microtubule-associated protein 2 (MAP2) was used to visualize the developing neurites (**Figure 5B**; up control, middle BDNF, lower TrkB-Fc). Morphometric analysis for neurite growth was performed by quantifying three parameters: total neurite length, number of primary neurites and number of branching points (**Figures 5C–E**, Table S3). The total neurite length was higher after BDNF application than in control treated neurons (**Figure 5C**, Table S3; BDNF vs. control, $p < 0.001$). Interestingly the loss-of-function experiments with either a BDNF-Abs or TrkB-Fc did not show any significant difference in neurite length when compared to the control conditions (**Figure 5C**, Table S3). The specificity of the TrkB-Fc in neutralizing BDNF was determined by a co-application of BDNF and TrkB-Fc. Quantitative analysis revealed that application of TrkB-Fc completely prevented the positive effect of a BDNF treatment on the total neurite length (**Figure 5C**, Table S3). The number of primary neurites after BDNF application showed a slight increase when compared to the control (**Figure 5D**, Table S3; BDNF vs. control, $p < 0.01$), whereas the treatment with BDNF-Abs or TrkB-Fc did not alter neurite number (**Figure 5D**, Table S3). The significant increase in primary neurites observed after BDNF treatment could be prevented by a co-treatment with TrkB-Fc (**Figure 5D**, Table S3). Next we determined whether a BDNF application might affect neurite complexity by analyzing the number of branching points of MAP2 labeled processes. Indeed, a BDNF treatment resulted in a 2-fold increase in complexity in comparison to control (**Figure 5E**, Table S3; BDNF vs. control, $p < 0.001$). On the contrary, treatment with BDNF-Abs or TrkB-Fc did not influence the number of branching points when compared to the control (**Figure 5E**, Table S3). Additionally, the positive effect of BDNF on neurite complexity was prevented by a co-treatment with BDNF and TrkB-Fc (**Figure 5E**, Table S3).

Our results suggest that the application of BDNF promotes neurite outgrowth by increasing both neurite elongation and branching in developing DIV3 hippocampal primary neurons. Interestingly, two loss-of-function approaches for BDNF did not affect the neurite architecture of primary hippocampal neurons (3–6 DIV) during their early development.

ROLE OF NEURONAL ACTIVITY IN REGULATING THE SENSITIVITY OF MATURE HIPPOCAMPAL NEURONS UPON EXOGENOUS BDNF APPLICATION

BDNF synthesis and release have been shown to occur in an activity-dependent way (Thoenen, 1991; Gärtner and Staiger, 2002). Therefore, one possible explanation for the discrepancies in the effect of BDNF application on neuronal morphology *in vitro* (Ji et al., 2005, 2010; Rauskolb et al., 2010) may lie in the different levels of neuronal activity in the primary cultures. Indeed, in cultures with a higher neuronal activity the levels of endogenous BDNF may be already high and might mask the effects of BDNF application. To test this hypothesis primary hippocampal neurons were kept for 2 weeks in a medium containing a higher concentration of Magnesium (3.5 mM Mg^{2+}; **Figure 6A**) and the effects of a 24 h treatment with BDNF (in NB medium with low, 1.5 mM Mg^{2+}) on the number and structure of dendritic spines were analyzed. Calcium imaging was used to compare the levels of neuronal activity in hippocampal neurons grown under high Mg^{2+} to those of neurons grown under control (low Mg^{2+}) conditions. While no significant difference could be detected regarding the amplitude of global calcium transients between the two groups (**Figure 6C**, Table S4), their frequency was significantly decreased in neurons kept in high Mg^{2+} when compared to control neurons (**Figure 6B**, Table S4; $p < 0.001$) suggesting a long-term reduction in neuronal activity. Accordingly, while dendritic complexity was unchanged (**Figure 6D**, Table S4), a significantly lower dendritic spine density could be observed when comparing control neurons kept in high Mg^{2+} with control neurons kept in low Mg^{2+} medium (**Figure 6E**, Table S4; $p < 0.001$). On the other hand, no significant differences in spine density could be detected upon a BDNF or a TrkB-Fc treatment for neurons grown under high Mg^{2+} conditions when compared to the control high Mg^{2+} conditions (**Figure 6E**, Table S4). Next, a possible effect of BDNF on dendritic spine morphology was analyzed. Interestingly, compared to control treated neurons BDNF treatment in cultures kept in high Mg^{2+} resulted in a slight increase in dendritic spine width (**Figure 6F**, Table S4) and a slight decrease in spine length (**Figure 6G**, Table S4), but the difference did not reach significance. No significant differences were observed both for the head width and for the length when TrkB-Fc treated cells in high Mg^{2+} were compared to high Mg^{2+} controls (**Figures 6F,G**, Table S4). To better clarify the mild spine head increase observed in neurons kept in high Mg^{2+} medium upon BDNF treatment spines were binned into four categories according to their head width. Comparing spine head width within each category showed a significant increase upon BDNF treatment in the proportion of spines with larger heads (**Figure 6H**; $\geq 0.5\,\mu m$ $< 1\,\mu m$; $p < 0.05$) with a comparable significant decrease in the proportion of spine with small heads (**Figure 6H**; $< 0.3\,\mu m$; $p < 0.05$) when compared to control neurons cultivated in high Mg^{2+}. No significant difference was observed between the control and TrkB-Fc treatments in any of the spine head width categories under the same conditions (**Figure 6H**). Comparing the effect of a control, a BDNF or a TrkB-Fc treatment within different categories of dendritic spine binned according to their length did not show any significant

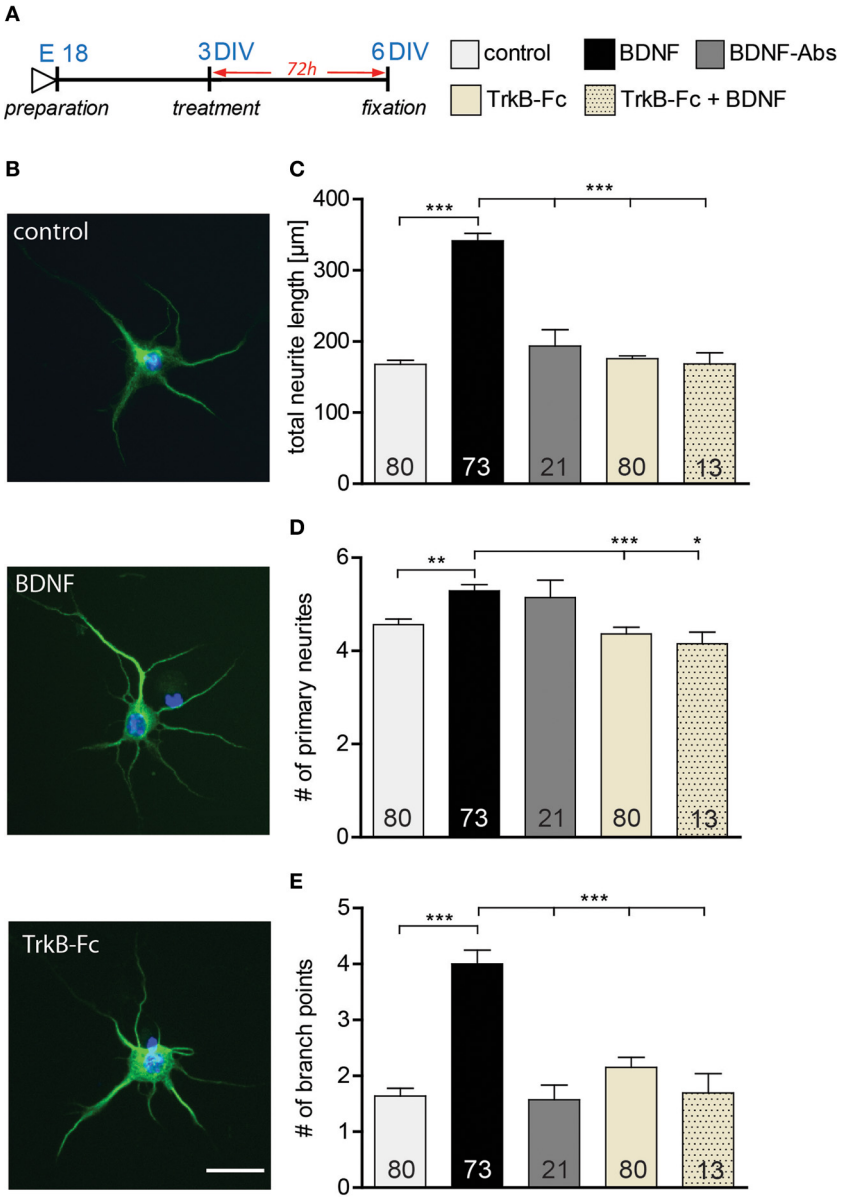

FIGURE 5 | (A) Experimental timeline. BSA, BDNF, BDNF antibodies (BDNF-Abs) or TrkB receptor bodies (TrkB-Fc) were applied on DIV3 hippocampal neurons for 72 h. **(B)** Micrographs showing MAP2 positive BSA (control) (above), BDNF (middle) or BDNF antibodies (BDNF-Abs; below) treated primary hippocampal neurons. Scale bar, 25 µm. **(C)** Histogram comparing neurite length of DIV6 hippocampal neurons after application of BSA (control), BDNF, BDNF antibodies (BDNF-Abs), TrkB receptor bodies (TrkB-Fc) or combined BDNF and TrkB receptor bodies (TrkB-Fc). **(D)** Histogram comparing the number of primary neurites of DIV6 hippocampal neurons after application of BSA (control), BDNF, BDNF antibodies (BDNF-Abs), TrkB receptor bodies (TrkB-Fc) or combined BDNF and TrkB receptor bodies (TrkB-Fc). **(E)** Histogram comparing the number of branching points for the dendrites of DIV6 hippocampal neurons after application of BSA (control), BDNF, BDNF antibodies (BDNF-Abs), TrkB receptor bodies (TrkB-Fc) or combined BDNF and TrkB receptor bodies (TrkB-Fc). The number in the columns represents the number of cells analyzed. Significance is indicated as follows: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. Error bars indicate s.e.m. ANOVA, *post-hoc* Tukey's Multiple Comparison Test.

difference (**Figure 6I**). The data described above suggest that indeed a chronic reduction in neuronal activity during the cultivation time might reduce the endogenous BDNF levels in hippocampal primary neurons and thereby increase their sensitivity to an exogenous BDNF application to modulate dendritic spine morphology.

THE ACTION OF EXOGENOUS BDNF IN MODULATING DENDRITIC SPINE MORPHOLOGY DEPEND ON NEURONAL ACTIVITY

BDNF has been shown to modulate dendritic spine morphology upon activity-dependent synaptic plasticity Tanaka (Tanaka et al., 2008). To assess whether under our culture condition neuronal activity governs the BDNF effect on dendritic spine

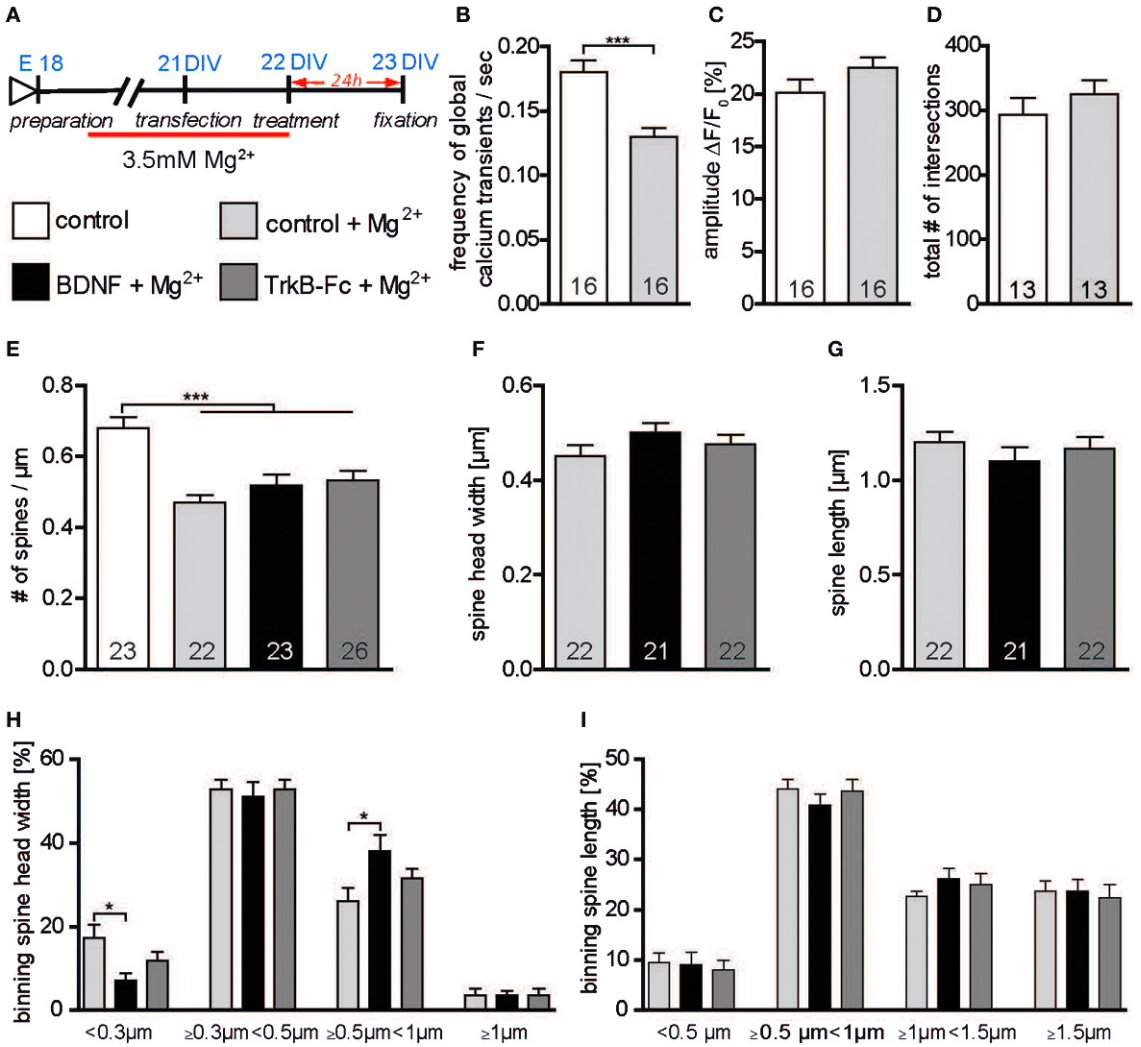

FIGURE 6 | (A) Experimental timeline. Primary neurons were cultivated for 2 weeks in high (3.5 mM) Mg^{2+} medium and treated with BSA (control), BDNF or TrkB-Fc in low (1.5 mM) Mg^{2+}. **(B)** Graph comparing the frequency of calcium transients in hippocampal primary neurons cultivated in high or low Mg^{2+} containing medium. **(C)** Graph comparing the amplitude of calcium transients in hippocampal primary neurons cultivated in high or low Mg^{2+} containing medium. **(D)** Histogram of the total number of intersections in hippocampal primary neurons cultivated in high or low Mg^{2+} containing medium. **(E)** Graphs comparing dendritic spine density between neurons cultivated in low or high Mg^{2+} and treated with BSA (control), BDNF and TrkB receptor bodies (TrkB-Fc). **(F)** Histogram comparing the spine head width between primary hippocampal neurons cultivated in high Mg^{2+} and treated with BSA (control), BDNF and TrkB receptor bodies (TrkB-Fc). **(G)** Histogram of the dendritic spine length between primary hippocampal neurons cultivated in high Mg^{2+} and treated with BSA (control), BDNF and TrkB receptor bodies (TrkB-Fc). **(H)** Graph showing the binning of spines according to their spine head width and comparing the proportion of spines within each category in response to a BSA (control), BDNF or BDNF antibodies (BDNF-Abs) treatment. **(I)** Graph showing the binning of spines according to their spine length and comparing the proportion of spines within each category in response to a control, BDNF or TrkB receptor bodies (TrkB-Fc) treatment. The number in the columns represents the number of cells analyzed. Significance is indicated as follows: $*p < 0.05$; $***p < 0.001$. Error bars indicate s.e.m. Unpaired two-tailed Student Test and ANOVA, *post-hoc* Tukey's Multiple Comparison Test.

morphology, hippocampal primary neurons were kept for 2 weeks and treated with BDNF in a high Mg^{2+} (3.5 mM) medium (**Figure 7A**) in order to reduce neuronal activity. Under these conditions a significantly lower dendritic spine density could be observed when comparing control neurons kept in high Mg^{2+} to control neurons in low Mg^{2+} medium (**Figure 7B**, Table S4; $p < 0.05$). Moreover, dendritic spine density of neurons treated either with BDNF or with TrkB-Fc in high Mg^{2+} was significantly lower than in controls (low Mg^{2+}) neurons (**Figure 7B**, Table S4; BDNF vs. low Mg^{2+} control $p < 0.05$; TrkB-Fc *vs.* low Mg^{2+} control $p < 0.001$). Interestingly, both a BDNF treatment as well as a treatment with TrkB-Fc performed in high Mg^{2+} did not affect either spine head width (**Figure 7C**, Table S4) or spine length (**Figure 7D**, Table S4) when compared to a control treatment in high Mg^{2+}. Also binning the spines according to their head width (**Figure 7E**) or to their length (**Figure 7F**) did not show any alteration in the spine size distributions both for a BDNF and a TrkB-Fc

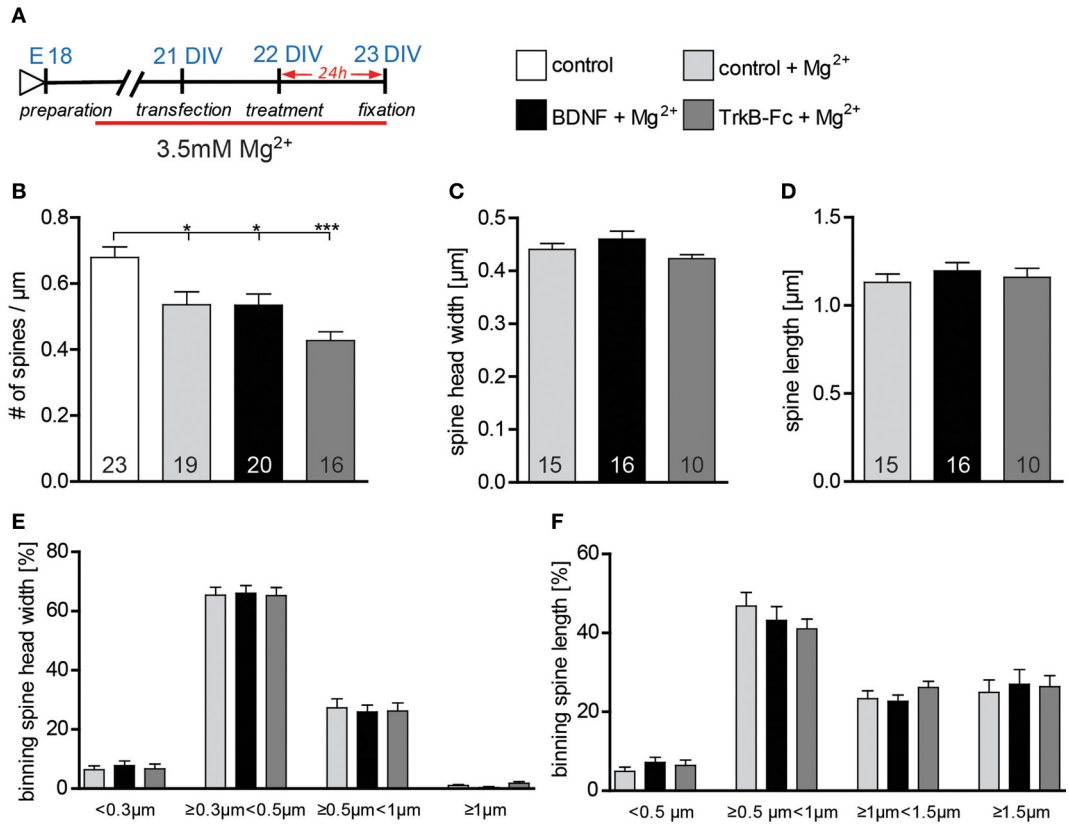

FIGURE 7 | (A) Experimental timeline. Primary neurons were cultivated for 2 weeks in high Mg^{2+} medium and treated with BSA (control), BDNF or TrkB-Fc in high Mg^{2+}. **(B)** Graphs comparing dendritic spine density between neurons cultivated in low or high Mg^{2+} and treated with BSA (control), BDNF and TrkB receptor bodies (TrkB-Fc) in high Mg^{2+}. **(C)** Histogram comparing the spine head width between primary hippocampal neurons cultivated and treated in high Mg^{2+} with BSA (control), BDNF and TrkB receptor bodies (TrkB-Fc). **(D)** Histogram of the dendritic spine length between primary hippocampal neurons cultivated and treated in high Mg^{2+} with BSA (control), BDNF and TrkB receptor bodies (TrkB-Fc). **(E)** Graph showing the binning of spines according to their spine head width and comparing the proportion of spines within each category in response to a BSA (control), BDNF or BDNF antibodies (BDNF-Abs) treatment in high Mg^{2+}. **(F)** Graph showing the binning of spines according to their spine length and comparing the proportion of spines within each category in response to a BSA (control), BDNF or TrkB receptor bodies (TrkB-Fc) treatment in high Mg^{2+}. The number in the columns represents the number of cells analyzed. Significance is indicated as follows: $*p < 0.05$; $***p < 0.001$. Error bars indicate s.e.m. Unpaired two-tailed Student Test and ANOVA, *post-hoc* Tukey's Multiple Comparison Test.

applications compared to a control treatment performed in high Mg^{2+}.

The results above indicate that BDNF application indeed modulates dendritic spine architecture in an activity-dependent manner.

DISCUSSION

The main findings in this study are that the effects exerted by BDNF on the dendritic architecture of hippocampal neurons are dependent on the neuron's maturation stage and that BDNF is specifically required for the activity-dependent maintenance of the mature spine phenotype in mature excitatory hippocampal neurons *in vitro*. Indeed, our current results reproduce previous results obtained *in vivo* (Rauskolb et al., 2010). These observations further support the notion, that BDNF is a precise mediator of specific effects on neurons rather than a pleiotropic molecule unspecifically promoting cell health and survival. Indeed, besides its role in promoting neurite outgrowth (Ji et al., 2005, 2010), which we can confirm in the data presented here, BDNF is required for the activity-dependent maturation and stabilization of dendritic spines.

Our results are in line with previous work showing a surprising selectivity in the brain areas requiring BDNF-signaling for normal post-natal development (Minichiello et al., 1999; Zakharenko et al., 2003; Rauskolb et al., 2010; Zagrebelsky and Korte, 2013). Indeed, while a global BDNF deprivation throughout the CNS impairs the proper post-natal growth of striatal medium spiny neurons, BDNF deprivation fails to cause major alterations in the fine structure of mature CA1 pyramidal neurons (Rauskolb et al., 2010). These observations suggest a crucial but rather limited and specific effect of BDNF in maintaining the mature phenotype of dendritic spines without affecting the general architecture of hippocampal neurons. Our results *in vitro* show a very similar effect upon the application of two loss-of-function approaches for BDNF (function blocking BDNF antibodies, TrkB receptor bodies) confirming a physiological role of the endogenous BDNF in this context. On the other hand, our experiments fail to reproduce previously published data showing a highly significant increase

in dendritic spine density and alterations in spine morphology upon the application of exogenous BDNF to mature hippocampal primary neurons (Ji et al., 2005, 2010) or to hippocampal slice cultures (Tyler and Pozzo-Miller, 2001). The discrepancy between the data presented here and other previously published data regarding the role of exogenous BDNF in regulating the architecture of mature hippocampal neurons may have different reasons. One possible explanation is the different animal species used for the experiments. While studies showing an effect of a BDNF loss-of-function either *in vivo* (Rauskolb et al., 2010) or *in vitro* (our current study) were mostly performed in mouse derived cultures, most of the previous *in vitro* work describing an effect for a BDNF gain-of-function was done in rat hippocampal neurons (Tyler and Pozzo-Miller, 2001; Ji et al., 2005, 2010) suggesting a possible difference in the sensitivity to BDNF signaling in the two different species—or simply a different level of BDNF release from neurons into the medium. Moreover, culture conditions have been shown to possibly influence the expression levels as well as the cellular response to BDNF (Chapleau et al., 2008). Indeed, here we show that the levels of neuronal activity in the cultures determine the responsiveness to the exogenous or the endogenous BDNF (**Figures 6**, **7**). Low levels of neuronal activity during the cultivation period result in lower spine density values and an increased responsiveness of the hippocampal neurons to the applications of exogenous BDNF. Indeed, the synthesis and secretion of BDNF have been shown to be directly controlled by neuronal activity (reviewed in Thoenen, 1991; see also Gärtner and Staiger, 2002). Accordingly, control neurons in previous publications showing a strong effect upon a BDNF application (Ji et al., 2005, 2010) had a spine density which is about 10 times smaller than the one measured in the current study or described *in vivo* (De Simoni et al., 2003) and 5 times smaller than the one described for a more complex *in vitro* system (De Simoni et al., 2003; Rauskolb et al., 2010). It should also be noted that in our culture system the levels of neuronal activity as well as the cellular responsiveness to BDNF were tested in different ways (**Figures 4A–K**). Application of exogenous BDNF has been shown to trigger a direct response of the neuronal network via a transient elevation of the intracellular calcium concentration (Berninger et al., 1993; Lang et al., 2007). In contrast, blocking intrinsic BDNF signaling was shown to reduce the frequency of spontaneously occurring calcium rises in developing rat hippocampal neurons (Lang et al., 2007). In our study calcium imaging was used first to test the responsiveness of the hippocampal neurons to BDNF and also to test the effects on neuronal activity of manipulating the Mg^{2+} concentration in the culture medium. Thereby we could confirm that increasing Mg^{2+} concentration in the medium results in a long lasting reduction in neuronal excitability and network activity of primary hippocampal cultures (Dribben et al., 2010). While this reduction in neuronal activity does not influence dendritic morphology and complexity, it's accompanied by a significant decrease in dendritic spine density as well as by an increased cellular sensitivity to the application of exogenous BDNF. The cellular responsiveness to BDNF was tested also quantifying the changes in TrkB phosphorylation as well as the activation of the IEG c-fos, two critical events known to occur upon the acute activation of BDNF signaling (Marty et al., 1996; Ji et al., 2005, 2010). Our results indicate that in spite of the lack of morphological effects of an application of BDNF, mature excitatory, hippocampal neurons under the culture conditions used in this work are responsive to BDNF. In addition, their responsiveness is increased under low neuronal activity conditions. Whether the increase neuronal responsiveness to BDNF is due to an activity-dependent decrease in BDNF release from neurons or, as recently described from microglia (Parkhurst et al., 2013) will be very interesting to address. Indeed, changes in magnesium concentration have been shown to possibly influence ATP release (Li et al., 1997) and could thereby indirectly influence BDNF release from microglia (Trang et al., 2009; Parkhurst et al., 2013). Moreover, it will be crucial to explore the possibility that different amounts of microglia may influence the BDNF levels in the primary hippocampal cultures and thereby their responsiveness to a BDNF exogenous application.

Our results show a requirement for neuronal-activity during the application for BDNF to exert its effect on dendritic spines. This observation is in line with previous work showing that during the early development of cortical pyramidal neurons in ferrets BDNF regulates dendritic architecture in an activity-dependent manner (Mcallister et al., 1996). Moreover, in cultures of cerebellar neurons, BDNF increases the spine density of Purkinje cells only in the presence of granule cells suggesting a crucial role of neuronal activity in mediating this BDNF effect (Shimada et al., 1998). In line with this observation is the role of BDNF in regulating structural changes at dendritic spines upon activity-dependent synaptic plasticity in mature hippocampal neurons (Tanaka et al., 2008).

One important open question concerns the functional consequences of the observed structural changes at spines regulated by BDNF signaling. Our results upon a BDNF loss-of-function confirm the structural alterations at spine observed already *in vivo* (Rauskolb et al., 2010). Specifically, a BDNF deprivation results in a decrease in dendritic spine density accompanied by a decrease in spine head width and an increase in spine length suggesting a loss of the mature phenotype of spines possibly resulting in a decrease and a weakening of synaptic connections under these conditions. Indeed, spine head width is correlated with the size of the post-synaptic density and the number of AMPA receptors (Holtmaat and Svoboda, 2009) and a shrinking of the spine head width has been correlated to the synaptic weakening occurring upon long term depression induction (Zhou et al., 2004). Moreover, the spine neck has been suggested to act as a diffusion barrier regulating the biochemical compartmentalization in the spine head (Sjostrom et al., 2008; Holthoff et al., 2010). Indeed, spines with long necks have been shown to be electrically silent at the soma, although their heads are activated by an uncaging event (Araya et al., 2006) and a BDNF-dependent shortening of the spine neck was observed upon inducing long term potentiation at single spines (Tanaka et al., 2008). It has been show that upon maturation the proportion of mushroom spines increases while the one of thin long spines decreases (De Simoni et al., 2003). Taken together these data suggest that BDNF signaling plays a crucial role in maintaining the mature architecture of dendritic spines.

Finally, we show in our experiments that endogenous BDNF modulates the actin cytoskeleton within dendritic spines as well as their morphology. This is of interest with respect to the fact that activity-dependent as well as spontaneous structural changes of dendritic spines are dependent on the modulation of actin dynamics within spines (Krucker et al., 2000; Fukazawa et al., 2003; Kramar et al., 2004). Noteworthy in this context is the observation that BDNF modulates actin regulatory binding proteins which control the turnover of the actin cytoskeleton as well as cytoskeleton-associated proteins like Arc/Arg3.1 (Ying et al., 2002; Messaoudi et al., 2007; Rex et al., 2007; Bramham, 2008). Interestingly, while a gain-of-function approach performed in this study did not affect the F-actin content within dendritic spines, a loss-of-function experiment resulted in a significant decrease in polymerized actin (see **Figure 3**). This is in line with the results of Rex et al. (2007) which show that exogenous application of BDNF to rat hippocampal slices increased the number of F-actin labeled spines only when theta burst stimulation was applied (Rex et al., 2007). Moreover, the effect of theta burst stimulation on F-actin was completely abolished by a BDNF scavenger (TrkB-Fc; Rex et al., 2007) suggesting a role for the TrkB receptor in this context. On the other hand, BDNF might also modulate actin dynamics via the p75 Neurotrophin Receptor ($p75^{NTR}$). In a series of gain- and loss-of-function experiments we previously showed that the $p75^{NTR}$ negatively modulates dendritic spine density and morphology (Zagrebelsky et al., 2005), in addition to its effect on long-term depression (Rösch et al., 2005; Woo et al., 2005). It has been shown that signaling via the $p75^{NTR}$ controls the activity of the small GTPase RhoA, thereby regulating the actin cytoskeleton (Yamashita et al., 1999; Yamashita and Tohyama, 2003; Gehler et al., 2004) and possibly preparing it for processes of negative synaptic plasticity.

In summary our study reports highly specific effects of BDNF on the maintenance of mature spines in excitatory hippocampal neurons and it also shed some light on why published *in vivo* and *in vitro* results concerning morphological effects of BDNF are often contradictory.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fnsyn.2014.00005/abstract

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Synaptic membrane rafts: traffic lights for local neurotrophin signaling?

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Lipid rafts, cholesterol and lipid rich microdomains, are believed to play important roles as platforms for the partitioning of transmembrane and synaptic proteins involved in synaptic signaling, plasticity, and maintenance. There is increasing evidence of a physical interaction between post-synaptic densities and post-synaptic lipid rafts. Localization of proteins within lipid rafts is highly regulated, and therefore lipid rafts may function as traffic lights modulating and fine-tuning neuronal signaling. The tyrosine kinase neurotrophin receptors (Trk) and the low-affinity p75 neurotrophin receptor ($p75^{NTR}$) are enriched in neuronal lipid rafts together with the intermediates of downstream signaling pathways, suggesting a possible role of rafts in neurotrophin signaling. Moreover, neurotrophins and their receptors are involved in the regulation of cholesterol metabolism. Cholesterol is an important component of lipid rafts and its depletion leads to gradual loss of synapses, underscoring the importance of lipid rafts for proper neuronal function. Here, we review and discuss the idea that translocation of neurotrophin receptors in synaptic rafts may account for the selectivity of their transduced signals.

Keywords: neurotrophin signaling, lipid rafts, synaptic rafts, BDNF, TrkB receptor translocation

INTRODUCTION

Neurons are highly polarized cells. They are organized in distinct morphological regions, namely cell soma, dendrites and axon. Each of these regions performs specific functions. In particular, dendrites receive information from other neurons, whereas the axon conveys information from one neuron to another through connections known as synapses. The axon-dendrite polarity ensures unidirectional transmission of the signal from pre-synaptic cells to post-synaptic targets (Barnes and Polleux, 2009). The synapses are highly specialized subdomains. It is clear by now that heterogeneous multiprotein signaling complexes assemble at pre- and post-synaptic parent neurons thus providing diversity to the molecular composition and functional properties of chemical synapses in the mammalian central nervous system (CNS) (O'Rourke et al., 2012).

One key question is how proteins are specifically recruited to these compartments.

It is widely accepted that proteins and lipids do not freely diffuse along cell membranes as initially proposed (Singer and Nicolson, 1972); instead their lateral mobility is constrained. After observing heterogeneity in lipid phases of cell membranes, it has been suggested that lipids are organized into domains, also known as lipid rafts, which are enriched in sphingolipids and cholesterol (Karnovsky et al., 1982). The presence of cholesterol makes lipid domains more insoluble than the rest of the membrane and, after differential centrifugation in density gradients, they can be isolated from soluble fractions (Simons and Toomre, 2000). Lipid rafts have been proposed to function as platforms orchestrating the specific lipid-protein and protein-protein interactions that activate cellular processes, including signal transduction, cell-adhesion, membrane trafficking and molecular sorting. In particular, they are believed to localize signaling molecules, allowing these to interact with each other but not with those that are excluded from the rafts (Simons and Toomre, 2000; Allen et al., 2007; Lingwood and Simons, 2010; Simons and Sampaio, 2011).

However, the raft concept has undergone severe scrutiny. Since its formal introduction in 1982 (Karnovsky et al., 1982), the concept of membrane rafts has been quite controversial due to the difficulty in characterizing the cell membrane structure. Initially, the controversy stemmed from the employment of indirect methods to prove the existence of lipid rafts. These included detergent extraction protocols to isolate insoluble membrane fractions, which turned out to be highly variable in composition depending on the type of detergent, the time of extraction and the temperature. Additionally, cholesterol depletion experiments have implicated lipid rafts in processes where the results reflected more global perturbations of the cell membrane than specific effects (Munro, 2003; Simons and Gerl, 2010).

The advancement in imaging technology, lipidomic analysis and biophysical assays has allowed to directly visualize lipid rafts in living cells and to define their characteristics using primarily synthetic membranes (Simons and Gerl, 2010). More specifically, the concept of lipid rafts has evolved to include short-lived and highly dynamic structures that exist as nanoscale assemblies, in which components are either permanently or just transiently associated. Activation of distinct signaling pathways would depend on their protein/lipid composition and cell type (Pike, 2003; Lingwood and Simons, 2010).

Despite persisting skepticism among scientists the appealing nature of the lipid raft concept has prompted researchers

to hypothesize possible biological functions of lipid rafts at synapses. This is based on the observation that detergent resistant synaptic membranes (i.e., synaptic rafts) are enriched in cholesterol and proteins, including neurotransmitter receptors, signaling molecules and cytoskeletal-adaptor proteins (Suzuki, 2002). In addition, there is increasing evidence of a physical interaction between post-synaptic densities and post-synaptic lipid rafts (Suzuki et al., 2011). The latter have also been proposed to play a role in membrane trafficking and receptor sorting near synaptic active sites (Torres et al., 1998; Bruckner et al., 1999; Suzuki et al., 2011). Moreover, the importance of synaptic membrane rafts is underscored by the fact that their disruption leads to depletion of synapses, loss of dendritic spines and instability of neurotransmitter receptors (Hering et al., 2003).

The neurotrophin Trk receptors and the $p75^{NTR}$ low affinity receptor were found enriched in neuronal lipid rafts together with the intermediates of downstream signaling pathways, suggesting a possible role of lipid rafts in neurotrophin signaling (Higuchi et al., 2003; Suzuki et al., 2004). Various models have been proposed for the involvement of lipid rafts in neurotrophin signaling in the context of cell survival and neurite outgrowth (Paratcha and Ibanez, 2002; Suzuki et al., 2011). Here, we aim to review recent evidence supporting the idea that compartmentalization of neurotrophin receptors in lipid rafts at synapses may account for the selectivity of their transduced signals.

NEUROTROPHIN SIGNALING AT SYNAPTIC RAFTS

The neurotrophin family of growth factors, including brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF), are diffusible molecules synthesized as precursors (pro-neurotrophins) and subsequently cleaved by proteases and convertases to produce mature neurotrophins. Pro-neurotrophins preferentially binds to the $p75^{NTR}$ receptor, a single transmembrane protein of the superfamily of tumor necrosis factor (TNF) receptors, whose activation is usually associated with apoptosis (Lee et al., 2001; Lu et al., 2005).

In their mature form, BDNF and NGF bind with high affinity to TrkB and TrkA receptors, respectively, members of the tyrosine kinase family of receptors. The signaling pathways activated by these two receptors promote survival, differentiation, axon growth, dendrite pruning and other aspects of nervous system maturation and function (Chao, 2003).

BDNF has been the most extensively studied among the neurotrophins and found to play, among other biological functions, a pivotal role in synaptic transmission and activity-dependent synaptic plasticity (Minichiello, 2009; Edelmann et al., 2013). A very active research area is focused on the role of BDNF in modulating synaptic efficacy at specific synaptic sites (Poo, 2001). One fascinating idea is that partitioning of neurotrophin receptors in synaptic rafts may explain the selectivity of their transduced signals. Importantly, the receptors mediating BDNF signaling have been found to localize in lipid rafts together with the effectors of their downstream signaling pathways (Higuchi et al., 2003; Suzuki et al., 2004; Pereira and Chao, 2007).

LIPID RAFT REGULATION OF BDNF SIGNALING AT SYNAPSES

BDNF/TrkB signaling is known to activate multiple transduction pathways, which include the phosphatidylinositol 3–kinase (PI3K)–AKT, ERK/MAPK, and the phospholipase C-γ (PLC-γ) pathways. In principle the three pathways can work in parallel and have all been implicated in controlling various aspect of synaptic plasticity, including translation, transport and translocation of synaptic proteins (Yoshii and Constantine-Paton, 2010).

Several experiments suggest that BDNF-induced synaptic modulation is selective for both active synapses and specific synapses. For instance, in hippocampal slices, exogenous application of BDNF enhanced synaptic responses (Figurov et al., 1996). Moreover, addition of BDNF to cultured hippocampal neurons potentiated glutamatergic but not GABAergic synapses from the same pre-synaptic neuron, suggesting that different terminals could be independently modified (Schinder et al., 2000). Although we should always be cautious in correlating *in-vitro* observations with physiological conditions found in neuronal circuits of an intact organism, these observations point to a potential mechanism allowing BDNF to promote different synaptic responses.

It is still unclear how a freely diffusible protein, such as BDNF, is able to attain local effects at synapses and to modulate specific synapses. However, it is known that glutamatergic and GABAergic synapses are different in terms of molecular composition, morphology and ultimately function (O'Rourke et al., 2012), and therefore may differentially regulate compartmentalization of signaling complexes. Various mechanisms have been proposed to explain BDNF-induced specific synaptic effects, including local synthesis and secretion of BDNF at active synapses, activity-dependent synthesis and transport of TrkB mRNA, and TrkB receptor insertion into the plasma membrane (Lu, 2003). On the other hand, BDNF secreted at active synapses may induce TrkB receptors relocation from extra-synaptic sites to particular membrane microdomains enriched in synaptic zones (Nagappan and Lu, 2005), which could represent a complementary mechanism to the ones mentioned above.

Indeed, Suzuki et al. (2004) were the first to demonstrate that BDNF stimulation of cultured neurons induced translocation of TrkB receptors in lipid rafts of detergent insoluble neuronal membranes. Most importantly, this translocation was dependent upon TrkB autophosphorylation and it was accompanied by increased phosphorylation of ERK in lipid rafts, thus suggesting specific activation of the MAPK pathway (Suzuki et al., 2004). In addition, they found that BDNF-induced translocation and activation of TrkB in lipid rafts was critically relevant for neurotransmitter release and synaptic plasticity, since raft disruption by cholesterol depletion abolished the acute potentiating effect of BDNF on evoked synaptic transmission in culture, and the enhancement of the synaptic response to tetanus in hippocampal slices (Suzuki et al., 2004). However, TrkB translocation to lipid rafts was not important for neuronal survival. Treatment of neuronal cortical cultures with cholesterol synthesis inhibitors, known to effectively deplete lipid rafts, induced cell death, whereas addition of BDNF significantly enhanced cell viability in these conditions. This suggests that lipid rafts are not required for BDNF/TrkB

signaling effect on neuronal survival. Thus, TrkB receptors in lipid rafts initiate local signaling, whereas, outside lipid rafts, they may activate alternative pathways, possibly involved in retrograde signaling (Suzuki et al., 2004). Regarding these, there are numerous studies (extensively reviewed elsewhere Harrington and Ginty, 2013), highlighting the importance of retrograde signaling of neurotrophin/Trk receptors to control the survival of different neuronal populations such us sympathetic and sensory neurons. In addition, Trk effectors, for example PI3K–AKT and Erk5, have been suggested to promote survival of neurons supported by target-derived neurotrophins (Harrington and Ginty, 2013). Therefore, we can speculate that depending on TrkB localization and local availability of effector molecules similar signaling pathways could alternatively be activated outside of lipid rafts.

Several components of pre- and post-synaptic regions have been described in lipid rafts, including ionotropic receptors as well as G-protein coupled receptors and their effectors (reviewed in Allen et al., 2007). Lipid rafts are also believed to contribute to trafficking of these receptors to and from the cell membrane (Pediconi et al., 2004), to their stability at the cell surface, and to ligand binding efficacy (Allen et al., 2007). In addition, similarly to BDBF/TrkB, also neurotransmitter receptors have been suggested to perform different functions depending on their localization in lipid rafts or outside these lipid microdomains. For example, NMDA receptors have been shown to mediate neurotoxicity when recruited within lipid rafts (Frank et al., 2004), whereas outside these microdomains they stimulate growth cone guidance (Guirland et al., 2004).

Although lipid rafts are enriched at synapses (Nagappan and Lu, 2005), whether BDNF/TrkB signaling role in lipid rafts occurs at synapses and whether it takes place pre-synaptically and/or post-synaptically remains to be elucidated. Not only BDNF is secreted from pre-synaptic terminals and post-synaptic boutons, but also TrkB receptors are present on the membrane on either side of the synaptic cleft (Edelmann et al., 2013).

In terms of signal transduction mechanisms, it was suggested that BDNF facilitates neurotransmitter release via the TrkB/MAPK/synapsin I signaling pathway (Jovanovic et al., 2000). Moreover, BDNF was found to stimulate cholesterol biosynthesis and increase the level of pre-synaptic proteins in lipid rafts (Suzuki et al., 2007). Taken together, these observations would support a role of BDNF/TrkB signaling at pre-synaptic terminals.

To our knowledge, so far no studies have specifically addressed BDNF-induced activation of TrkB receptors in the context of their partitioning at post-synaptic rafts, although a plethora of studies attributes a role of post-synaptic BDNF-TrkB signaling in synapse formation and plasticity (Yoshii and Constantine-Paton, 2010).

It has been shown that BDNF-TrkB signaling is required to deliver PSD95 to the post-synaptic membrane via vesicular transport (Yoshii and Constantine-Paton, 2007) and for the palmitoylation of PSD95 via activation of PLCγ and the brain-specific PKC variant protein kinase ζ (Yoshii et al., 2011). Interestingly, palmitoylation of proteins is a post-translational modification which is known to facilitate sequestration of proteins to lipid rafts (Linder and Deschenes, 2007). For instance, palmitoylation of the A-kinase anchoring protein 79/150 (AKAP79/150 is important to target AMPA receptors to post-synaptic rafts together with PSD-95, inducing synaptic potentiation (Keith et al., 2012).

Therefore, one can hypothesize that BDNF-stimulated TrkB signaling could potentially coordinate the delivery of key molecules at post-synaptic lipid rafts to promote synaptic plasticity (**Figure 1**). This idea is supported by combined *in vitro* and

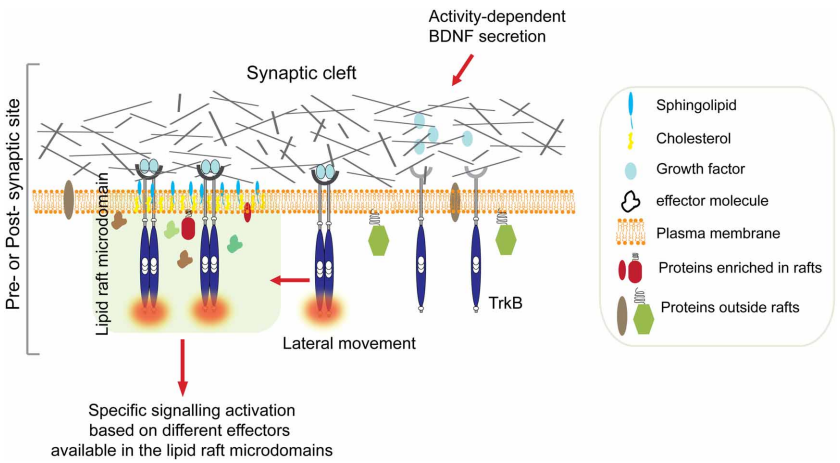

FIGURE 1 | Ligand-induced TrkB translocation into lipid raft at active synapses. Synaptic activity stimulates BDNF secretion from both pre-synaptic terminals and post-synaptic boutons. High concentration of BDNF in the synaptic cleft is followed by TrkB receptors autophosphorylation and translocation into lipid rafts on membranes of either sides of the synaptic cleft. This is followed by activation of specific signaling depending on the kind of effectors available in the raft microdomain. For example, translocation of activated TrkB in lipid rafts at the pre-synaptic site stimulates the MAPK/synapsin I signaling pathway promoting neurotransmitter release (Jovanovic et al., 2000). In contrast, translocation of TrkB receptors to the post-synaptic site promotes palmitoylation and delivery of PSD95, and possibly other key molecules, to post-synaptic membrane rafts via activation of PLC-γ and PKC pathways thus promoting synaptic plasticity (Yoshii et al., 2011).

in vivo observations that provide the first genetic evidence for a role of the non-receptor tyrosine kinase Fyn in the selective BNDF-induced translocation of TrkB receptors to intracellular lipid rafts (Pereira and Chao, 2007).

Internalization of the BDNF/TrkB receptor complex is an important step in the regulation of TrkB signaling, and of neurotrophin retrograde signaling in general (Harrington and Ginty, 2013). Fyn is a member of the Src family of tyrosine kinases protein, which has been previously shown to co-immunoprecipitate with TrkB receptors and to localize in lipid rafts (Iwasaki et al., 1998).

In Fyn knockout neurons, after BDNF stimulation, TrkB receptors translocation in lipid rafts was reduced and this was accompanied by diminished activation of the PLC-γ pathway (Pereira and Chao, 2007). Moreover, Fyn knockout mice have severe neurological defects, including deficits in hippocampal LTP (Grant et al., 1992), which are similar to those found in mouse mutants where the BDNF/TrkB/PLC-γ pathway is genetically impaired (Patterson et al., 1996; Minichiello et al., 2002).

Taken together, these observations illustrate an important mechanism mediated by Fyn in facilitating BDNF/TrkB induced PLC-γ signaling cascade, possibly at synapses, through receptor localization in intracellular lipid rafts. However, these findings are in contrast to Suzuki and colleague's observations, in which BDNF-induced TrkB receptor activation in lipid rafts stimulated the ERK/MAPK pathway (Suzuki et al., 2004). The discrepancy is likely to be due to differences in the sensitivity of their detection methods, in the employment of pure versus mixed neuronal populations, and/or in the amount of exogenous BDNF stimulation applied to their respective neuronal cultures. Possibly the relative abundance of BDNF may account for the selective activation of signaling pathways depending on TrkB receptor enrichment in lipid rafts either at the cell surface or in internal compartments. Indeed, using antibody cross-linking, TrkB receptor colocalizing with lipid rafts was also visualized on the surface of living cells (Pereira and Chao, 2007).

NGF-INDUCED SIGNALING AT SYNAPTIC RAFTS

There is no direct evidence for a role of NGF signaling at synaptic rafts. Because of its low abundance in the CNS, NGF action at synapses has been studied prevalently in sensory neurons. However, evidence for activity dependent-release of NGF in the hippocampal-septal system has been recently provided (Guo et al., 2012). Both TrkA and $p75^{NTR}$ receptors were found enriched in lipid raft membranes (Peiro et al., 2000; Higuchi et al., 2003; Pryor et al., 2012). In neural crest-derived PC12 cells, NGF treatment stimulates translocation of TrkA receptors to lipid rafts and this localization is required for activation of ERK. Translocation appears to be mediated by association with flotilin, a component of lipid rafts, and the adapter protein c-CbI associated protein (CAP) (Limpert et al., 2007). Although the biological activity of TrkA receptors in PC12 cells are likely to differ from those found in neurons, these observations underscore a potential common mechanism among neurotrophins in organizing and compartmentalizing signal transduction.

Recent evidence points to a possible role of NGF signaling in organizing the association of lipid rafts with the cytoskeleton (Pryor et al., 2012). Indeed, the latter plays an essential role in stabilizing specific sets of proteins localized at the plasma membrane, in molecular trafficking, cell adhesion, migration and remodeling of growth cones and synapses (Tolias et al., 2011). Cytoskeletal proteins also interact with specific lipids (Janmey and Lindberg, 2004) and are enriched in lipid rafts. For example, RhoGTPases have been isolated from insoluble membrane fractions (Dupree and Pomicter, 2010) and are considered key regulators of the actin cytoskeleton, playing an important role in synapse remodeling (Tolias et al., 2011).

Pryor et al. (2012) have recently shown *in vitro* that NGF specifically enhances the binding of TrkA, but not $p75^{NTR}$, to microtubules in detergent resistance membranes, and that this binding does not require phosphorylation of TrkA (Pryor et al., 2012). Since activation of neurotrophin receptors in endosomes is required for their trafficking (Harrington and Ginty, 2013), this finding suggests a possible mechanism for local signaling that is distinct from the one associated with retrograde transport. Indeed, in PC12 cells Rac1, a member of small GTPases, was targeted to lipid rafts when activated by NGF. In addition, Rac1 activation was impaired by treatment with a cholesterol-sequestering agent (Fujitani et al., 2005). These observations indicate that reorganization of the cytoskeleton at specific sites in the membrane might be controlled by NGF-dependent targeting of key molecules to lipid rafts. However, further studies are needed to elucidate this mechanism and the specific neurotrophin receptors involved (Harrington and Ginty, 2013).

CONCLUDING REMARKS

In summary, the literature reviewed here is alluding to the idea that neurotrophin signaling may occur by recruitment of neurotrophin receptors at specific sites on the cell surface and/or in internal compartments that might be distinct from signaling endosomes. This recruitment, if occurring at synaptic rafts, might elicit activation of distinct signaling pathways involved in synaptic plasticity.

In the context of neurotrophin signaling, the lipid raft hypothesis is quite attractive. One can speculate that the temporally controlled availability of neurotrophins at synapses can stimulate lipid rafts to function as traffic lights, directing the spatial recruitment and time of activation of specific receptors and effector molecules with the exclusion of others. In doing so, they could potentially fulfil a critical role in modulating and fine-tuning synaptic signaling, by partitioning signal transduction mechanisms along the synaptic and extra-synaptic membranes, and within the synapse itself. In addition, since lipid rafts are highly dynamic they can bring together various combinations of adaptor molecules and enzymes whose availability could depend on the strength of synaptic activity and the cell type, adding an additional level of specificity in time and space (Simons and Toomre, 2000).

However, the studies presented here did not use synaptosomal preparations and were based on the assumption that the current methods used to isolate raft–associated proteins and to study their biological functions are valid methods. Nevertheless, these

methods are prone to produce conflicting or inconsistent results because they cannot capture the level of complexity predicted by the idea that lipid rafts are fluctuating nanoscale entities and therefore may vary in composition in a timescale that cannot be resolved by available tools. Therefore, progress in this active area of research will depend on the development of new technologies to study lipid rafts biology especially *in vivo*.

Whether lipid rafts coordinate neurotrophin signaling at synapses *in vivo* is assumed but not demonstrated. Only technological advancement will test this idea, and as Karl Popper's says: "Every genuine test of a theory is an attempt to falsify it, or to refute it. Testability is falsifiability; but there are degrees of testability: some theories are more testable, more exposed to refutation, than others; they take, as it were, greater risks" (Popper, 1963).

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Postsynaptic localization of PSD-95 is regulated by all three pathways downstream of TrkB signaling

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Brain-derived neurotrophic factor (BDNF) and its receptor TrkB regulate synaptic plasticity. TrkB triggers three downstream signaling pathways; Phosphatidylinositol 3-kinase (PI3K), Phospholipase Cγ (PLCγ) and Mitogen activated protein kinases/Extracellular signal-regulated kinases (MAPK/ERK). We previously showed two distinct mechanisms whereby BDNF-TrkB pathway controls trafficking of PSD-95, which is the major scaffold at excitatory synapses and is critical for synapse maturation. BDNF activates the PI3K-Akt pathway and regulates synaptic delivery of PSD-95 via vesicular transport (Yoshii and Constantine-Paton, 2007). BDNF-TrkB signaling also triggers PSD-95 palmitoylation and its transport to synapses through the phosphorylation of the palmitoylation enzyme ZDHHC8 by a protein kinase C (PKC; Yoshii et al., 2011). The second study used PKC inhibitors chelerythrine as well as a synthetic zeta inhibitory peptide (ZIP) which was originally designed to block the brain-specific PKC isoform protein kinase Mζ (PKMζ). However, recent studies raise concerns about specificity of ZIP. Here, we assessed the contribution of TrkB and its three downstream pathways to the synaptic distribution of endogenous PSD-95 in cultured neurons using chemical and genetic interventions. We confirmed that TrkB, PLC, and PI3K were critical for the postsynaptic distribution of PSD-95. Furthermore, suppression of MAPK/ERK also disrupted PSD-95 expression. Next, we examined the contribution of PKC. While both chelerythrine and ZIP suppressed the postsynaptic localization of PSD-95, RNA interference for PKMζ did not have a significant effect. This result suggests that the ZIP peptide, widely used as the "specific" PKMζ antagonist by many investigators may block a PKC variant other than PKMζ such as PKCλ/ι. Our results indicate that TrkB regulates postsynaptic localization of PSD-95 through all three downstream pathways, but also recommend further work to identify other PKC variants that regulate palmitoylation and synaptic localization of PSD-95.

Keywords: synapse formation, BDNF, TrkB, PSD-95, PKMζ, protein kinase C, MAP kinase, PI-3 kinase

INTRODUCTION

The Brain-derived neurotrophic factor (BDNF) and its receptor TrkB are critical for maturation of both excitatory (Gorski et al., 2003; Wirth et al., 2003; Chakravarthy et al., 2006; Tanaka et al., 2008; Kaneko et al., 2012) and inhibitory neurons (Hanover et al., 1999; Huang et al., 1999). In the visual cortex, BDNF increases 2 weeks after birth largely due to the visual activity after eye opening (Castren et al., 1992). BDNF-TrkB signaling regulates excitation–inhibition balance (Schinder et al., 2000) and facilitates activity-dependent formation of neuronal circuits (Cabelli et al., 1995, 1997; Heimel et al., 2010) as well as critical period closure of ocular dominance (Hanover et al., 1999; Huang et al., 1999).

In excitatory neurons, BDNF-TrkB signaling regulates dendritic growth (Xu et al., 2000), spine maturation, stabilization (Gorski et al., 2003; Wirth et al., 2003; Chakravarthy et al., 2006; Tanaka et al., 2008; Kaneko et al., 2012), and long-term potentiation (LTP; Kang and Schuman, 1995; Figurov et al., 1996; Patterson et al., 1996; Tanaka et al., 1997; Frerking et al., 1998; Gottschalk et al., 1998; Huber et al., 1998). BDNF-TrkB signaling also plays a critical role in the development of synapses by regulating the transport of the membrane associated guanylate kinase the post-synaptic density protein PSD-95. In the visual pathway of rodents upon eye-opening, PSD-95, which is the major scaffolding protein at mature glutamate synapses (Yoshii and Constantine-Paton, 2007; Yoshii et al., 2011), is transported to young synaptic contacts by BDNF/TrkB. SAP102, the dominant MAGUK in neonatal period, does not require an activation of BDNF-TrkB signaling for its postsynaptic localization (Yoshii and Constantine-Paton, 2007) and moves to early synapses in association with the NR2B subunit GluN2B (Washbourne et al., 2004). In our previous work, we reported two distinct mechanisms whereby the BDNF-TrkB pathway controls trafficking of PSD-95. BDNF activates the Phosphatidylinositol 3-kinase (PI3K)-Akt pathway and triggers synaptic delivery of PSD-95 via vesicular transport (Yoshii and Constantine-Paton, 2007). BDNF-TrkB signaling is also necessary for PSD-95's initial association with membranes.

It initiates PSD-95 palmitoylation through the phosphorylation of the palmitoylation enzyme ZDHHC8 by Phospholipase Cγ (PLC γ) and a protein kinase C (PKC; Yoshii et al., 2011). Here we verify our previous observations on the roles of the PI3K-Akt and PLC γ- PKC pathways in the transport of PSD-95 to synapses. We also extend these analyses of BDNF-TrkB signaling to the role of the third pathway downstream of TrkB activation: namely, the Mitogen activated protein kinase/Extracellular signal-regulated kinase (MAPK/ERK) pathway. Finally, in light of recent publications indicating that ZIP, the reagent we used to block the C kinase PKMζ, is not specific for PKMζ in LTP, (Lee et al., 2013; Volk et al., 2013) we test whether another PKC may also be involved in the palmitoylation of PSD-95.

MATERIALS AND METHODS

ANIMALS

$TrkB^{F616A}$ mice carrying a modification in the ATP binding site of this kinase (Chen et al., 2005) were kindly provided by Dr. David Ginty. Wild type (WT) C57BL6 mice were obtained from Charles River Laboratories. All manipulations were performed in accord with the guidelines of the MIT-IACUC.

PRIMARY NEURON CULTURE, LIPOFECTION, IMMUNOCYTOCHEMISTRY

Occipital cortices of E15.5 mouse brains were dissected, digested with a solution containing papain and DNase for 25 min. Cells were dissociated using fire polished glass pipets and plated at the density of 0.5×10^6 cells per cm^2 after counting with a hemocytometer. Coverslips were coated with laminin and poly-D-lysine. Transfections of DNA constructs encoding either small interfering RNA (siRNA) against PKMζ or its scrambled sequence (kindly provided by Dr. Richard Huganir, Johns Hopkins University) was performed at day *in vivo* (DIV) 8 using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. On DIV15, cultured neurons were treated with the following reagents. 1NM-PP1 to block activation of TrkB or its control construct Bph-PP1 (kindly provided by Dr. Kevan Shokat, UCSF). U73122 (1 μM), Chelerythrine (2.5 μM), PD98059 (50 μM), and Wortmannin (100 nM) were used to block PLC, PKC MAPK, and PI3K, respectively, and were purchased from Sigma-Aldrich. The PKMζ inhibitory pseudosubstrate ZIP (myr-SIYRRGARRWRKL-OH) and scrambled peptide (myr-RLYRKRIWRSAGROH; Pastalkova et al., 2006) were synthesized in the MIT Biopolymers Laboratory.

Twenty-four hours after each exposure, cultures were fixed with 4% paraformaldehyde (15 min). Neurons were permeabilzed with 0.3% TritonX-100 for 5 min and washed. Cultures were blocked with 9% BSA (1 h), and incubated with an antibody for PSD-95 (Neuromab #K28/43, 1:1000) at room temperature overnight. After washing with PBS (3x), cultures were incubated with secondary antibody Alexa Fluor 488 (Molecular Probes) for 2 h.

Images were captured using a 60x objective on a Nikon PCM 2000 confocal microscope and a 6–8 μm, Z-series, of optical sections were taken at intervals of 0.5 μm. 12/4 μm areas containing isolated typical secondary dendritic branches from pyramidal neurons were selected. For each area, a threshold was set to optimally represent PSD-95 puncta and exclude diffuse label in dendritic shafts (See **Figure 2A** middle and bottom rows). The same threshold was applied to all neurons in the set of cultures in each experiment. Immunocytochemical experiments were repeated twice for each treatment condition. Eight cells from eight culture dishes were imaged. Using ImageJ, total pixel intensity was calculated as the sum of each pixel number multiplied by its intensity as measured above threshold for each cell. This value was then averaged across all dendritic segments sampled in the experiment and presented as total pixel intensity of PSD-95 puncta. Averaged cell body intensity was also measured in ImageJ by manually selecting soma (yellow circles in **Figure 2A**) and applying the Measure function in ImageJ. Subsequently, total pixel intensity of PSD-95 puncta was divided by the averaged cell body intensity for normalization.

TUNEL ASSAY

FragEL™ DNA Fragmentaion Detection Kit (Calbiochem) was used for the TUNEL assay. The procedures were performed according to the manufacturer's instructions. Cell numbers in 250 μm × 250 μm were counted and the apoptosis incidence was calculated by dividing the TUNEL (+) cell number with total cell number.

STATISTICS

A Student's *t*-test was used for comparison of two groups. One-way ANOVA with *post hoc* Tukey tests were used for comparisons of more than two groups. $p < 0.05$ were considered significant and indicated as * in graphs. $p < 0.01$ and $p < 0.001$ are indicated as ** and ***. Numeric data are presented as average ±SD in the "Results" section. Error bars in graphs represent SEM.

RESULTS

We previously studied trafficking of PSD-95 in dendrites of cultured occipital cortical neurons using Fluorescent recovery after photobleaching (FRAP) and showed that bath application of BDNF as well as a BDNF-coated beads facilitated transport of GFP-tagged PSD-95 in dendrites (Yoshii and Constantine-Paton, 2007). Furthermore, BDNF application resulted in an increase of the intensity of PSD-95 immunolabeled puncta.

In the current experiments we first examined the postsynaptic distribution of PSD-95 in dissociated cultured neurons prepared from E15.5 cortices of $TrkB^{F616A}$ mice. This mouse strain has a single amino acid mutation in the intracellular domain of the TrkB receptor allowing the signaling activities of the receptor to be selectively blocked by the synthetic compound 1NM-PP (Chen et al., 2005). At DIV 15, we treated neurons with either 1NM-PP1 or the "control" non-blocking molecule Bph-PP1for 24 h. Using the TUNEL assay, we examined the incidence of apoptotic cells at various concentrations of 1NM-PP1 (**Figure 1**). We found that the cell death incidence was not significantly different at 1 (0.036 ± 0.020; total of 123 apoptotic cells out of 3430 cells in 33 images) and 3 μM (0.036 ± 0.021; total of 244 apoptotic cells out of 6652 cells in 33 images) as compared with no treatment (0.033 ± 0.025; total of 132 apoptotic cells out of 3961cells in 33 images) (ANOVA relative the no treatment group or the control Bph-PP1 group $p = 0.96$ and 0.79, respectively). We determined 9 μM of 1NM-PP1 is toxic because 40% of cells are $TUNEL^+$. Consequently, PSD-95 puncta intensity was accessed using 1NM-PP1at 3 μM or less. First, we

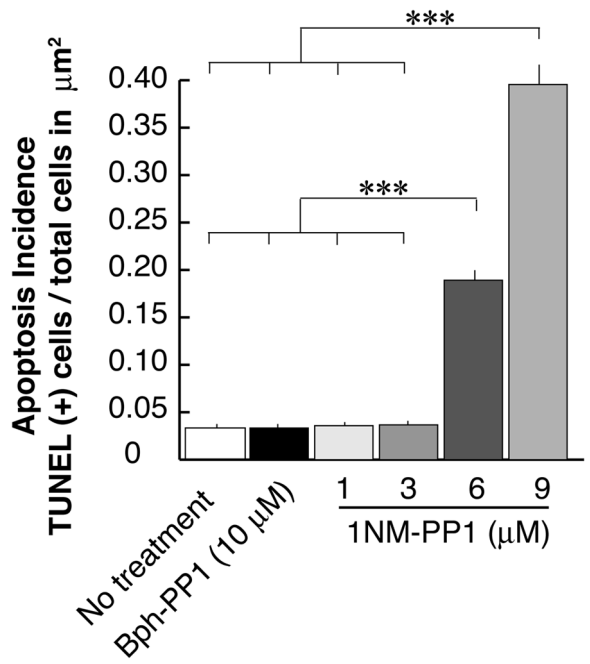

FIGURE 1 | Apoptosis rate of neurons that undergo TrkB signaling inhibition using the chemical genetic approach. Ratio's of Tunel signal positive cells to total cell number (~3,500–6,600 cells examined in 250 μm × 250 μm from eight cultures for each condition) are presented. Error bars represent SEM.

measured the averaged pixel intensity of the cell bodies (encircled areas with yellow dashed lines in **Figure 2A**) and found that PSD-95 expression level was slightly but significantly decreased in cultures treated with 2 (41.5 ± 7.6; $N = 8$) or 3 μM (41.5 ± 8.7; $N = 8$) of 1NM-PP1 as compared with 1 μM (50.4 ± 10.1; $N = 8$) or Bph-PP1 control (49.8 ± 10.5; $N = 8$). Therefore, we normalized the total pixel intensity of PSD-95 puncta by the intensity of soma. First we selected the defined area (4 μm × 12 μm squares in **Figure 2A**) of secondary dendritic branches and acquired total pixel intensities of PSD-95 puncta (See Materials and Methods; Yoshii and Constantine-Paton, 2007; Yoshii et al., 2011). These crude PSD-95 total pixel intensities were significantly reduced in neurons treated with 1NM-PP1 (1 μM; 57,800 ± 23,200; $N = 16$) as compared to neurons without treatment (23,100 ± 11,600; $N = 16$; **Figure 2C**; ANOVA between 1 μM and the no treatment groups; $p = 0.0043$). Next, each intensity value was divided by averaged somal PSD-95 intensity of the same neuron. Normalized PSD-95 total pixel intensities remained significantly reduced in neurons treated with 1NM-PP1 as compared to control neurons treated with Bph-PP1 (**Figure 2D**; ANOVA between 1 μM and the control groups; $p = 0.0089$). Higher concentration of 1NM-PP1 caused more suppression of PSD-95 puncta (ANOVA between 1 and 3 μM groups; $p = 0.043$; **Figure 2D**).

To examine each of the three TrkB downstream signaling pathways, we used antagonists against PLC, PI3K, and MAPK/ERK (**Figure 3**). First, we examined the PSD-95 immunolabel intensities of the somata and found that the blocker of the MAPK/ERK (38.3 ± 6.2; $N = 8$) but not two other signaling pathways (PI3K; 49.7 ± 7.4; $N = 8$ and PLC; 45.5 ± 4.7; $N = 8$) resulted in a decrease of somal PSD-95 expression (Control; 49.9 ± 10.5; $N = 8$; **Figure 3A**). Next we examined PSD-95 total puncta intensities in dendrites (**Figure 3B**). As expected from our previous works, blockade of PLC with U73122 (31,700 ± 10,200; $N = 16$) or PI3K with Wortmannin (23,100 ± 11,600; $N = 16$) caused suppression of PSD-95 puncta intensities as compared to the no treatment controls (50,900 ± 35,100; $N = 16$; ANOVA; $p = 0.042$ and 0.025, respectively). Furthermore inhibition of the MAPK/ERK pathway with PD98059 (21,300 ± 19,500; $N = 16$) resulted in a decrease of PSD-95 puncta intensities (ANOVA to the no treatment group; $p = 0.0017$). Normalization to averaged cell body intensity also confirmed that all three downstream pathways were involved in the normal postsynaptic delivery and/or expression of PSD-95 (**Figure 3C**).

We previously showed that activation of PLC by BDNF-TrkB signaling is necessary for PSD-95 palmitoylation and its transport to synapses. This mechanism depends on the phosphorylation of the palmitoylation enzyme ZDHHC8 by a PKC (Yoshii et al., 2011), which is activated by PLC. In that study, we used the PKC inhibitor Chelerythrine as well as a synthetic PKMζ inhibitory peptide ZIP which was originally thought to specifically block this brain-specific PKC isoform (Ling et al., 2002). We confirmed that ZIP treatments resulted in a reduction of PSD-95 puncta intensity (25,100 ± 13,900; $N = 16$; **Figure 4A**; ANOVA to control; $p = 0.02$) as was also reported in the hippocampus (Shao et al., 2012), subsequent to our original finding in the visual cortex. Chelerythrine treatment also showed a similar result as ZIP treatment (28,000 ± 13,000; $N = 16$; ANOVA to control; $p = 0.035$).

However, in contrast to earlier experiments suggesting that ZIP was a specific PKMζ antagonist (Ling et al., 2002), recent studies using PKMζ knockout mice have not shown the expected LTP defect even though they did confirm that ZIP suppressed the LTP response (Lee et al., 2013; Volk et al., 2013). These reports raised the concern about the specificity of ZIP for PKMζ. Consequently, we tested PKMζ directly by suppressing its expression using RNAi. We transfected DNA constructs encoding siRNA against PKMζ or the scrambled sequence at DIV8 and examined it at DIV16. RNAi for PKMζ did not have a significant effect on postsynaptic localization of PSD-95 (30,600 ± 21,200; $N = 16$) as compared to neurons transfected with the scrambled sequence construct (33,600 ± 20,300; $N = 16$; Student's t-test; $p = 0.73$; **Figure 4B**).

DISCUSSION

SYNAPTIC EXPRESSION OF PSD-95 DEPENDS ON PKC BUT NOT PKMζ

In our previous *in vitro* study, we applied BDNF to neurons expressing PSD-95 tagged with GFP and saw an increase in puncta intensity in 60 min (Yoshii and Constantine-Paton, 2007). We also used FRAP and pharmacological treatments and showed that the BDNF effect on vesicular transport of PSD-95 requires the PI3K/Akt pathway (Yoshii and Constantine-Paton, 2007). In the following *in vivo* study, we used $TrkB^{F616A}$ mice and showed that our synchronized eye-opening regime, which triggers PSD-95 redistribution to synapses in rats (Yoshii et al., 2003), also activates TrkB in visual cortex and regulates the synaptic

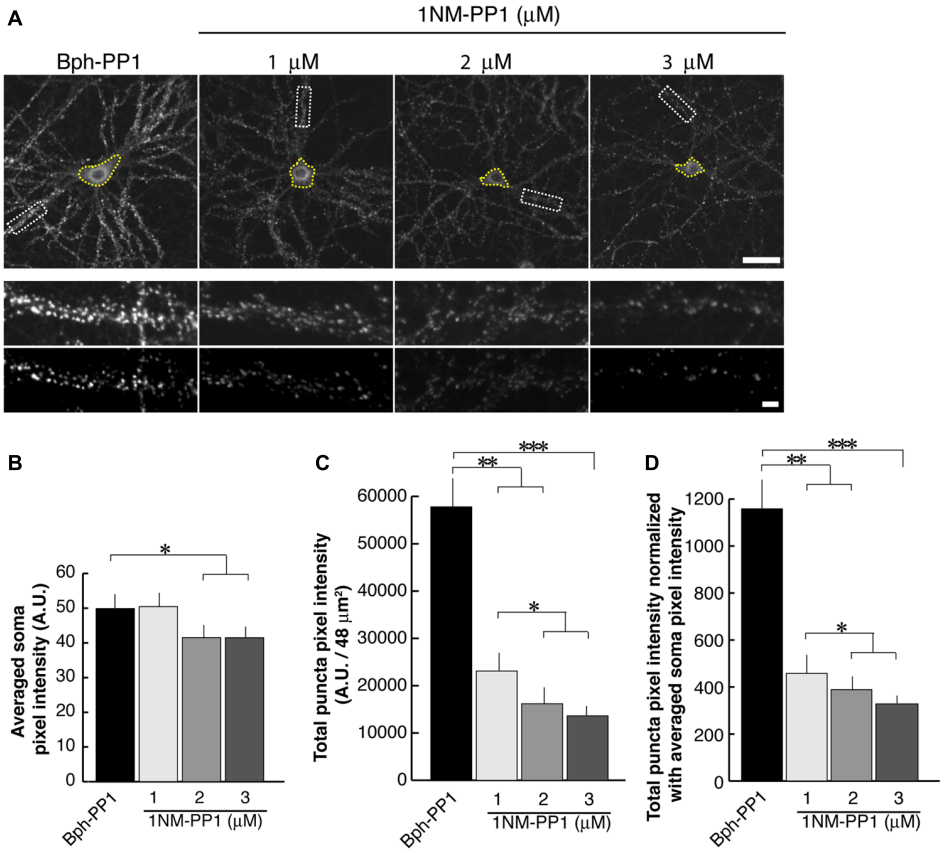

FIGURE 2 | 1NM-PP1 suppresses punctate distribution of PSD-95 dose-dependently in TrkB^{F616A} neurons. (A) The top row shows representative neurons immunostained with anti-PSD-95. Typical secondary dendritic branches analyzed are shown in 48 μm^2 rectangles in the middle row. These branches are processed after the thresholding shown in the bottom row. The scales in the top and bottom rows show 10 and 1 μm, respectively. **(B)** Graph showing averaged PSD-95 intensities in somata. **(C)** Graph showing quantification of PSD-95 puncta total pixel intensities. Note that all three concentrations of 1NM-PP1 result in reduced total PSD-95 puncta intensity. **(D)** In this graph, PSD-95 puncta intensities are normalized to somal PSD-95 intensities. In each condition, 16 branches from eight cells (chosen from the two different dissociations) were analyzed. Error bars represent SEM.

localization of PSD-95. Furthermore, the TrkB activation is necessary for palmitoylation of PSD-95 in somata through PLC, PKC, and the palmitoylation enzyme ZDHHC8 (Yoshii et al., 2011). In the current study on cultured visual cortical neurons using the same pharmacological intervention, we confirmed that postsynaptic distribution of PSD-95 depends on BDNF-TrkB signaling, and that PI3K and PLC are necessary for increased PSD-95 at synaptic puncta. However, even though ZIP suppressed PSD-95 puncta intensity, knockdown of PKMζ using RNAi has no effect on postsynaptic expression of PSD-95. This result is consistent with recent reports showing PKMζ knockout mice do not show an abnormal LTP response (Lee et al., 2013; Volk et al., 2013). One possibility for these LTP results, suggested by Lisman (2012), is that the concentration of ZIP is too high and causes non-specific inhibition (Lisman, 2012). Alternatively, ZIP may not be a specific PKMζ inhibitor rather it may also interact with other PKC variants such as another atypical PKC (PKCλ/ι; Lee et al., 2013; Volk et al., 2013). These results necessitated revising our previous interpretation (Yoshii et al., 2011). The current results suggest that a PKC variant other than PKMζ should be present in the developing visual cortex and is likely to phosphorylate the palmitoylation enzyme ZDHHC8.

MAPK/ERK IS INVOLVED IN SYNAPTIC EXPRESSION OF PSD-95

The current work also indicates that the MAPK/ERK pathway plays a role in the synaptic expression of PSD-95 as its inhibitor PD98059 significantly suppresses PSD-95 intensities of the soma as well as dendrite. This is consistent with the previous report showing that BDNF-induced increase in dendritic spine density is mediated by MAPK/ERK1/2 (Alonso et al., 2004). MAPK/ERK regulates protein-synthesis dependent plasticity by increasing phosphorylation of eukaryotic initiation factor 4E (eIF4E), the 4E-binding protein 1 (4E-BP1) and ribosomal protein S6 (Kelleher et al., 2004; Klann and Dever, 2004), therefore this pathway may directly initiate translation of PSD-95 gene either in the cell bodies of young neurons or in response to local activity in dendritic spines.

Brain-derived neurotrophic factor also activates the elongation of translation, which is mediated by PI3K and MAPK/ERK (Inamura et al., 2005). These two kinases turn off the eukaryotic

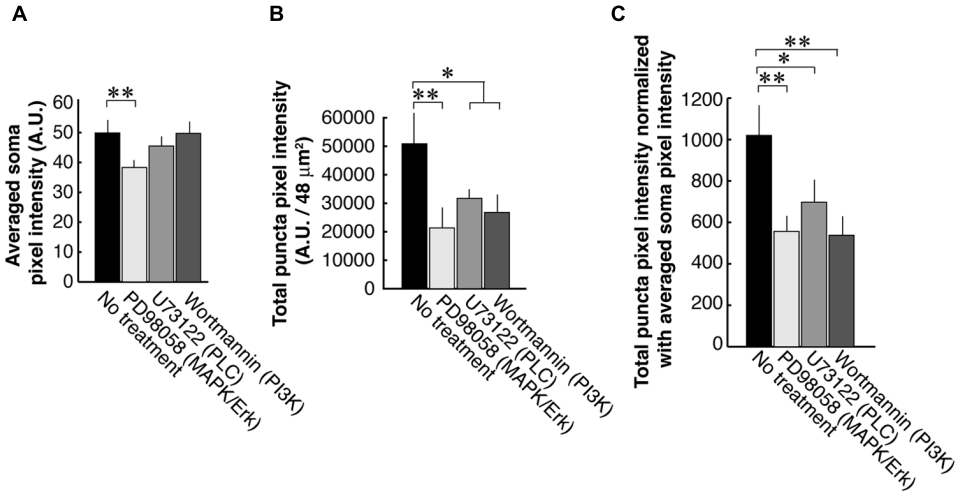

FIGURE 3 | PSD-95 puncta intensities in neurons that are treated with inhibitors of signaling molecules downstream of TrkB. Neurons are treated with blockers of the PLC (U73122, 1 μM), MAPK (PD98059, 50 μM), or PI3K (Wortmannin, 100 nM). Quantification was performed in the same manner as data presented in **Figure 2**. The graphs show averaged PSD-95 intensities in somata **(A)**, total pixel intensities of PSD-95 puncta **(B)**, and puncta intensities normalized to somal intensities **(C)**. In each condition, 16 branches from eight cells were analyzed. Error bars represent SEM.

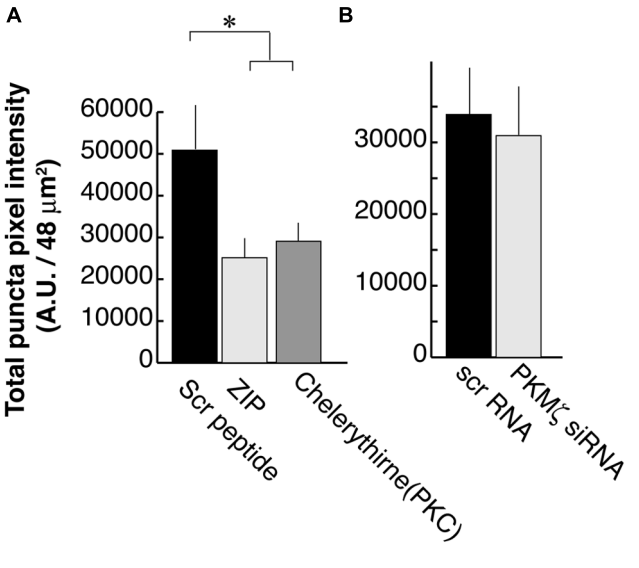

FIGURE 4 | Zeta inhibitory peptide (ZIP) but not PKMζ knockdown causes suppression in PSD-95 puncta intensity. (A) Neurons are treated with ZIP (1 μM), PKC (Chelerythrine, 2.5 μM), or the scrambled ZIP peptide (1 μM). **(B)** Neurons are transfected with DNA constructs encoding siRNA for PKMζ or the scrambled sequence. In both experiments, 16 branches from eight cells were analyzed. Error bars represent SEM.

elongation factor 2 kinase (eEF2K), in two parallel mechanisms. In turn, the suppression of eEF2K results in an increase of dephosphorylated and active eukaryotic elongation factor 2 (eEF2; Wang et al., 2001). This laboratory previously showed that NMDAR activation and visual activity rapidly induces phosphorylation of eEF2 at the synapse (Scheetz et al., 1997). This suppresses synthesis of most proteins but facilitates CaMKII synthesis. In rat dentate gyrus, BDNF application *in vivo* has been shown to induce LTP via ERK pathway, increase phosphorylation of eEF2 at non-synaptic sites and enhance expression of CaMKII-α and Arc (Kanhema et al., 2006). Furthermore, Worley and his colleagues obtained similar results for eEF2 phosphorylation via activation of mGluR5 in the hippocampus where the general shutdown of protein synthesis at the synapse significantly increased Arc/Arg3 translation (Park et al., 2008).

Another potential mechanism is that MAPK/ERK may be involved is mRNA transport. PSD-95 transcripts have been shown to exist in dendrites and interact with the fragile X mental retardation protein (FMRP). This enhances stability of the PSD-95 transcript and represses its translation during mRNA transport (Zalfa et al., 2007). A recent study shows that FMRP forms a complex with CYFIP1, a newly identified 4E-binding protein, and represses translation during mRNA transport (Napoli et al., 2008). BDNF can release this translational repression (Napoli et al., 2008). Whether MAPK/ERK meditates BDNF-dependent protein synthesis via translation and/or mRNA transport awaits future studies.

Brain-derived neurotrophic factor application also regulates transcription via MAPK/ERK which phosphorylates the cAMP-response element binding transcription factor (CREB) at serine133 residue (Bonni et al., 1995, 1999; Finkbeiner et al., 1997; Shaywitz and Greenberg, 1999; Pizzorusso et al., 2000; Ying et al., 2002). Interestingly, CREB can activate the *Bdnf* gene through promoter IV (Hong et al., 2008) and amplify BDNF-dependent synapse maturation. Therefore, we predict that MAPK/ERK regulates either or both transcription and translation of PSD-95. However, it remains to be studied whether CREB directly activates transcription of PSD-95 itself or upregulates BDNF, which further facilitates posttranscriptional regulation of PSD-95, i.e., translation, palmitoylation, or vesicular transport.

CONCLUSION

It is now clear that the increases in PSD-95 at synapses are mediated by all three signaling pathways downstream of TrkB. They are involved in various processes regulating protein expression. MAPK/ERK could regulate transcription through CREB and other transcription factors. MAPK/ERK and PI3K-Akt pathway play major roles in translation (Kelleher et al., 2004; Klann and Dever, 2004). The PI3K pathway also facilitates vesicular transport of PSD-95 from ER to Golgi apparatus, then along microtubule. PSD-95 is synthesized in the cytoplasm and requires palmitoylation to become attached to membranes and to get delivered to postsynaptic membranes. This post-translational modification is regulated by BDNF-TrkB signaling via PLC–PKC. These same mechanisms are likely to regulate BDNF-dependent long-term plasticity. Furthermore, PSD-95 itself and its interaction with TrkB signaling have been implicated in various brain diseases, especially neurodevelopmental disorders such as autism spectrum disorders (Tsai et al., 2012), Angelman syndrome (Cao et al., 2013), and schizophrenia (Mukai et al., 2008).

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Juvenile methylphenidate reduces prefrontal cortex plasticity via D3 receptor and BDNF in adulthood

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Background: Early drug intervention in childhood disorders aims to maximize individual potential in the short- and long-term. Consistently, juvenile exposure to psychostimulants, such as methylphenidate (MPH), reduces risk for substance use in animals and sub-populations of individuals with attention deficit hyperactivity disorder (ADHD). We investigated the effects of MPH on brain plasticity via dopamine receptor D3 (D3R) and brain-derived neurotrophic factor (BDNF) expression in developing rats.

Methods: Between postnatal days 20–35, rat pups were administered saline vehicle (Veh) or MPH (2 mg/kg), the D3R-preferring agonist ±7-OHDPAT, or the antagonist nafadotride (0.05 mg/kg) alone, or in combination with MPH twice a day. In adulthood, subjects were challenged to Veh or cocaine (10 mg/kg for two days). The prefrontal cortex was analyzed for protein and mRNA levels of total BDNF, its splice variants I, IIc, III/IV, and IV/VI, and D3 receptors. A separate group of subjects was assessed for splice variants at 20, 35, 40, and 60 days of age.

Results: Across age strong correlations were evident between *Drd3* and *Bdnf* mRNA levels ($r = 0.65$) and a negative relationship between *Drd3* and exon IIc after MPH treatment ($r = -0.73$). BDNF protein levels did not differ between Veh- and MPH subjects at baseline, but were significantly lower in MPH-treated and cocaine challenged subjects ($30.3 \pm 9.7\%$). *Bdnf* mRNA was significantly higher in MPH-treated subjects, and reversed upon exposure to cocaine. This effect was blocked by nafadotride. Furthermore, $Bdnf_{\text{total}}$ and *Bdnf* splice variants I, IIc, III/IV, and IV/VI changed across the transitions between juvenility and late adolescence.

Conclusions: These data suggest a sensitive window of vulnerability to modulation of BDNF expression around adolescence, and that compared to normal animals, juvenile exposure to MPH permanently reduces prefrontal BDNF transcription and translation upon cocaine exposure in adulthood by a D3R-mediated mechanism.

Keywords: ADHD, adolescence, child, methylphenidate, ritalin, sensitive period

INTRODUCTION

Children with Attention Deficit Hyperactivity Disorder (ADHD) often respond favorably to psychostimulant therapy, contributing to its use by 61% of children with ADHD between the ages of 6–13 years (Dalsgaard et al., 2013). The most commonly prescribed stimulant is methylphenidate (MPH). MPH predominantly affects the dopamine and noradrenergic systems, with recent evidence showing a role of the dopamine D3 receptor (D3R) in ADHD-associated behaviors in animals (Andersen et al., 2008; Barth et al., 2013). For example, D3R antagonists reduce hyperactivity and facilitate object recognition in DAT knockout mice, which have been used to model an ADHD phenotype. Other studies further localize D3R actions in object recognition to the prefrontal cortex (Watson et al., 2012).

Childhood/juvenile exposure to psychostimulants during this time of elevated plasticity produces effects that are opposite of those observed in drug-exposed adult animals (Brandon et al., 2001; Dow-Edwards and Busidan, 2001; Andersen et al., 2002; Bolanos et al., 2003; Andersen, 2005). For example, juvenile, but not adult, exposure to cocaine reduces drug seeking later in life (Dow-Edwards and Busidan, 2001; Andersen et al., 2002). Similarly, juvenile MPH decreases *Drd3* mRNA in the prefrontal cortex (PFC) (Andersen et al., 2008), in contrast to an increase in the same region in adult, stimulant-exposed animals (Le Foll et al., 2005). While most adult studies have focused on D3R changes in the nucleus accumbens (Everitt and Robbins, 2000; Le Foll et al., 2005), the reduction in D3R following juvenile MPH exposure is not evident in that region (Andersen et al., 2008). Rather, the juvenile MPH effect on D3R and an MPH-induced place aversion was recapitulated by juvenile treatment with the D3R agonist ±7-OHDPAT (Andersen et al., 2008). Finally, microinjections of ±7-OHDPAT into the PFC reversed aversion, resulting in a preference for cocaine-associated environments.

Cue responsiveness to drug-associated contexts depends on neuroplasticity associated with brain-derived neurotrophic factor (BDNF) levels in adult animals within the prelimbic (pl) PFC

(Berglind et al., 2007). Adult BDNF levels are transiently elevated following an acute injection of cocaine in both the PFC and the nucleus accumbens (NAc), with sustained elevations of *Bdnf* and *Drd3* mRNA found 60 days later in the NAc (Le Foll et al., 2005). Juvenile exposure to MPH reduces *Bdnf* mRNA in the striatum and the hypothalamus with no change in the cingulate cortex during peri-adolescence (Chase et al., 2007). Similar findings of no change in *Bdnf* mRNA were evident in the ventral tegmental area both immediately after treatment and long-term (Warren et al., 2011). However, the enduring effects of MPH exposure interact with development to manifest fully later in life (Andersen, 2005; Brenhouse and Andersen, 2011). Therefore, the lack of changes in BDNF expression as observed by Chase et al. (2007) in the PFC and Warren et al. (2011) in the ventral tegmental area could have been undetectable, because the window of observation was too early. In order to investigate the time course of D3 receptors and BDNF expression in the developing PFC, we examined the postnatal expression of these two indices and their inter-relationship (Experiment 1). We also examined how previous exposure to MPH may or may not modify BDNF levels to cocaine later in life (Experiment 2). As we suspect that D3 receptors modify BDNF expression in the medial PFC, we manipulated D3 activity *in vivo* using juvenile exposure to MPH, ±7-OHDPAT, and the D3R antagonist nafadotride (Experiment 3). Ultimately, we were interested in how juvenile exposure to MPH or a D3R-preferring agonist modulates BDNF expression later in life at baseline and following a 2-day unbiased place conditioning paradigm to 10 mg/kg cocaine.

Environmental influences, such as drug exposure during sensitive periods, can further modulate synaptic structure by altering the expression of *Bdnf* splice variants (Boulle et al., 2012). Such specific modulation of splice variants permits both spatial and temporal regulation of BDNF expression that in turn, can lead to the precise building of synaptic structure and respond to environmental demands. Understanding how specific splice variants of the *Bdnf* gene change across age may provide insight into the nature of maturation of different parts of the neuron. Exon I and II are specific to neurons, whereas exons III and IV are found in non-neural tissue as well (Nakayama et al., 1994). The exon I splice variant is expressed primarily in the soma and dendrites, whereas exon II is predominantly in the dendrites, and exon IV is restricted to the soma (Boulle et al., 2012).

Empirical data suggest that different exons are altered following experiences in a semi-unique manner. For example, exon II seems to be preferentially regulated in reward circuits (McCarthy et al., 2012). Sadri-Vakili et al. (2010) demonstrated that chronic cocaine increased *Bdnf* exon IV in the PFC of adult rats. Exon IV contains the binding sites for CREB and MeCP2 and plays a role in cognitive processes and learning and memory—such as those required for encoding drug-associated cues (Boulle et al., 2012; McCarthy et al., 2012). While BDNF changes have already been associated with addiction-related alterations in structure and function, the majority of these studies have been performed following adult drug exposure paradigms. We are particularly interested in whether exon IV changes following juvenile exposure to MPH, which we have shown increases CREB expression (Andersen et al., 2002). To investigate whether stimulants have unique effects on *Bdnf* splice variants, we determined how $Bdnf_{\text{total}}$, and different 5′ exons splice variants (exons I, IIc, III/IV, and IV/VI), change across age within the late maturing PFC (Experiment 4). Here, we used quantitative Real-Time PCR (qRT-PCR) to examine changes in *Bdnf* exons, as protein-labeling antibodies for the specific exons are not yet available.

MATERIALS AND METHODS

SUBJECTS

Lactating female Sprague-Dawley rats obtained from Charles River were housed with their litters on a 12 h/12 h light/dark cycle with lights on at 07:00 h with food and water provided *ad libidum*. Litters were culled to 8 pups of equal numbers of males and females on postnatal day 1 (P1), and were weaned at P21. Care was taken to distribute each condition to one male from each litter. Only males were used for these studies, with the remaining littermates used in other studies. The overall experimental design is found in **Figure 1**.

DRUGS

MPH HCl (d,l-MPH), ±7-OHDPAT, nafadotride, and cocaine HCl were obtained from Sigma (St. Louis, MO). Drugs were dissolved in 0.9% saline (vehicle) and administered in a volume of 1 ml/kg. Our earlier study demonstrated no behavioral difference with i.p. vs. oral administration in place conditioning (Brenhouse et al., 2009).

qRT-PCR

qRT-PCR was performed as described (Andersen et al., 2008). Briefly, brains were rapidly dissected into the mPFC. Sections were snap-frozen in TriReagent solution (Sigma) and stored at −80°C until further analysis. Samples were homogenized, total RNA extracted, DNAse digested (Ambion, Austin, TX), and reverse transcribed into cDNA using the SuperScript II

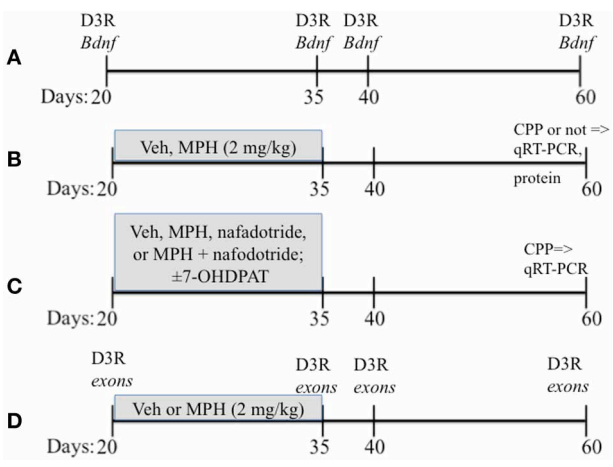

FIGURE 1 | A schematic showing the overall experimental design that includes the timing of drug treatments. Vehicle (Veh), methylphenidate (MPH), conditioned place preferences (CPP). Ages of rats are in days. **(A–D)** presents a schematic of the paradigms used for Experiments 1–4.

First-Strand Synthesis System (Life Technologies, Foster City, CA). cDNA samples were analyzed by qPCR using the iQ SYBR Green Supermix (BioRad). Amplifications were performed in a total volume of 20 μl with 40 nMol primers for each reaction on an Opticon MJ Thermocycler; MJ Research (Watertown, MA). The following published primer sequences were used: *Bdnf* exons I, II, II, and (Tsankova et al., 2006) $Bdnf_{\text{total}}$ (Asai et al., 2007), and *Drd3* and *Gapdh* (Andersen et al., 2008).

$Bdnf_{\text{total}}$: F: 5′-ACTCTGGAGAGCGTGAATGG-3′ R: 5′-TAC TGTCACACACGCTCAGC-3′
Exon I: F: 5′-CCTGCATCTGTTGGGGAGAC-3′ R: 5′-GCC TTGTCCGTGGACGTTTA-3′
Exon II: F: 5′-CTAGCCACCGGGGTGGTGTAA-3′ R: 5′-AGGA TGGTCATCACTCTTCTC-3′
Exon III F: 5′-CTTCCTTGAGCCCAGTTCC-3′ R: 5′-CCGTGG ACGTTTACTTCTTTC-3′
Exon IV F: 5′-CAGAGCAGCTGCCTTGATGTT-3′ R: 5′-GCC TTGTCCGTGGACGTTTA-3′
Drd3: F: 5′-AAGCGCTACTACAGCATCTGC-3′ R: 5′-GGATAA CCTGCCGTTGCTGAG-3′
Gapdh: F: 5′-AACTCCCATTCTTCCACCTTTG-3′ R: 5′-CCC TGTTGCTGTAGCCATATTC-3′

The *Bdnf* exon primers were verified by sequence alignment using deposited sequences in GenBank: Exon I, transcript variant I (Accession EF125675); Exon II: transcript variant IIc (Accession EF125678); Exon III: transcript variant III (Accession EF125686); Exon IV: transcript variant IV (Accession EF125679). A new nomenclature for *Bdnf* transcript variants has recently been suggested by Aid et al. (2007). Accordingly, our primer sequences for exon II, III, and IV amplify new exons IIc, IV, and VI, respectively. Thus, for each exon both the old and the new nomenclature are indicated.

DESIGN

Experiment 1: The relationship between Drd3 and Bdnf mRNA IN VIVO

Subjects and Design. mRNA from the PFC was assessed to determine the nature of the relationship between $Bdnf_{\text{total}}$ and *Drd3* across age. Rats were sacrificed at 4 ages: P25, 35, 40, and 60 representing juvenile, early adolescent, mid adolescent and late adolescent stages respectively (**Figure 1A**). The PFC was rapidly (<1 min) dissected out onto chilled glass plates. The sample was then processed for qRT-PCR as described in detail above (3.3). An $n = 6$ for P25 and $n = 5$ for P35, 40, and 60 were used.

Experiment 2: Measurement of $Bdnf_{total}$ mRNA and BDNF protein levels following MPH treatment with and without cocaine conditioning

Drug treatment paradigm. Subjects were weighed daily at 09:00 h and injected with either MPH (2 mg/kg) or saline vehicle (Veh) between P20-35 (see **Figure 1B**; Andersen et al., 2002, 2008. The second injection was administered 4 h later at 13:00 h. The dose of MPH was selected on the basis of previous studies (Andersen et al., 2002), and approximates a clinically relevant dose in humans based on plasma levels (Wargin et al., 1983; Kuczenski and Segal, 2002). After P35, the rats received no further drug treatment until behavioral testing or sacrifice for the pharmacological studies 25 days later at P60.

Design. To determine whether PFC BDNF levels and MPH exposure interacted with place conditioning to cocaine, we examined mRNA and protein levels. Drug-exposed subjects were housed with littermates and grown to P60, when they either (1) remained naïve to cocaine exposure (cocaine-naïve) or 2 underwent place conditioning to cocaine (10 mg/kg, i.p.). The 10 mg/kg dose of cocaine was chosen based on prior studies (Andersen et al., 2002; Carlezon et al., 2003) that demonstrated a clear differentiation of place conditioning effects between Veh animals. Our earlier study with these animals showed that the Veh subjects demonstrated a minimal preference for cocaine-associated environment, whereas the MPH animals demonstrated a significant place aversion (Andersen et al., 2008). Two sets of subjects were run: one set for *Bdnf* mRNA ($n = 6$) and the second set for BDNF protein ($n = 5$).

Cocaine place conditionin. Unbiased place conditioning, according to standard laboratory methods (Andersen et al., 2002; Carlezon et al., 2003), occurred in a 3-chamber apparatus. This chamber consisted of two large ($24 \times 18 \times 33$) side compartments separated by a small ($12 \times 18 \times 33$ cm) middle compartment. Screening was conducted for 30 min on day 1. Rats were placed in the middle compartment and allowed to freely explore the apparatus. Rats that demonstrated a clear preference for either side (>18 of 30 min) were eliminated from further testing. Two days of conditioning (with 2 sessions per day) occurred on day 2 and 3 for 60 min, and robust conditioning under these conditions was observed (Andersen et al., 2002; Carlezon et al., 2003). During the conditioning sessions, rats received a 1 ml/kg, i.p. injection of Veh in the morning (09:00 h) and confined to one side. The animals were then returned to the home cage. Four hours later, rats received 10 mg/kg cocaine and confined to the other side. The dose of cocaine for the place conditioning experiments was based on previous results that demonstrated consistent place aversion in MPH-exposed male rats at P60 (Andersen et al., 2002; Carlezon et al., 2003). Sides differed in floor texture, wall colors, and lighting, and assignments were counterbalanced. On day 4, rats were permitted to freely explore the entire apparatus for 30 min in a drug-free state.

BDNF protein immunoassays. BDNF protein was extracted from the PFC. Dissected tissue was weighed and homogenized in 10 vol/wt of cold extraction buffer consisting of: 100 mM Tris/HCl, ph = 7.0, 2% bovine serum albumin, 4 mM EDTA, 2% Triton-X, and the protease inhibitors aprotinin (5 μg/ml), benzamidine HCl (157 μg/ml), pepstatin A (0.1 μg/ml), and phenylmethylsulfonyl fluoride (PMSF; 17 μg/ml). Homogenates were centrifuged at 14,000 rpm for 30 min at 4°C and the supernatant was used in the assay. BDNF protein was quantified using the ChemiKine BDNF Sandwich ELISA kit (Chemicon, Temecula, CA) according to the manufacturer's directions. Samples were run in duplicate and quantified

using standards run simultaneously with the experimental samples.

Experiment 3: Relationship between $Bdnf_{total}$ mRNA and D3R drug treatment

Subjects. Subjects were exposed to ±7-OHDPAT (0.3 mg/kg; $n = 6$) between P20-35 or Veh ($n = 6$; **Figure 1**). To further confirm that the effects of MPH were mediated by the D3 receptor, a separate group of subjects was treated with MPH (2 mg/kg, i.p.; $n = 6$), nafadotride (0.05 mg/kg; $n = 6$), a combination of MPH and nafadotride (MPH/Nafad; $n = 6$) twice daily between the ages of P20-35 (Andersen et al., 2002).

Design. Subjects were tested with place conditioning to environments associated with 10 mg/kg cocaine at P60 (Andersen et al., 2008) and sacrificed 24 h after the last cocaine injection, the PFC rapidly dissected out, and *Bdnf* mRNA changes determined by qRT-PCR (**Figure 1C**).

Experiment 4: Age and drug effects on the Bdnf splice variants exons I, IIc, III/IV, IV/VI and Drd3

Subjects. Rats the ages of 20, 35, 40, and 60 days were used in these studies ($n = 6$/age). The 20 and 35 days ages were selected to characterize the expression of exons during the drug exposure period, 40 and 60 days represent short- and long-term ages for the paradigm.

Design. The PFC was dissected and processed for mRNA for exons I, IIc, III/IV, IV/VI, and Drd3 across age. *Bdnf* exons were assessed from subjects exposed to MPH or Veh as juveniles (**Figure 1D**).

DATA ANALYSIS

The expression of the *Bdnf* exons was normalized to the housekeeping gene product *Gapdh*. Linearity ($r = 0.9$ or better) and detection limit of the assay were determined in successive 10-fold serial dilutions, performed in triplicate, and an optimal amount of template was chosen for the quantitative analysis. Quantification was performed at a threshold detection line ("threshold cycles," *Ct* value). The Ct of each gene was normalized against *Gapdh*, which was run simultaneously for each marker. Expression levels and differences between treatment and Veh groups were determined according to the $2^{-\Delta Ct}$ or $2^{-\Delta\Delta Ct}$ method, respectively (Livak and Schmittgen, 2001). Comparisons were made between drug groups with a two-tailed Student's *t*-test or an ANOVA (SPSS); statistical significance was set at $p < 0.05$. Data were expressed as mean ± s.e.m.

RESULTS

EXPERIMENT 1: THE RELATIONSHIP BETWEEN Drd3 AND *Bdnf* mRNA *IN VIVO*

mRNA from the PFC across age of animals was measured to determine a relationship between $Bdnf_{\text{total}}$ and *Drd3*. Age itself had a significant effect on *Drd3* [$F_{(3,17)} = 7.87$, $P < 0.001$], but did not significantly influence *Bdnf* mRNA ($p = 0.1$). Regression analysis with ANCOVA showed that *Drd3*, when co-varied for age, significantly predicted *Bdnf* mRNA [$F_{(1,19)} = 18.20$, $P < 0.001$] and accounts for 49% of *Bdnf* changes (**Figure 2**).

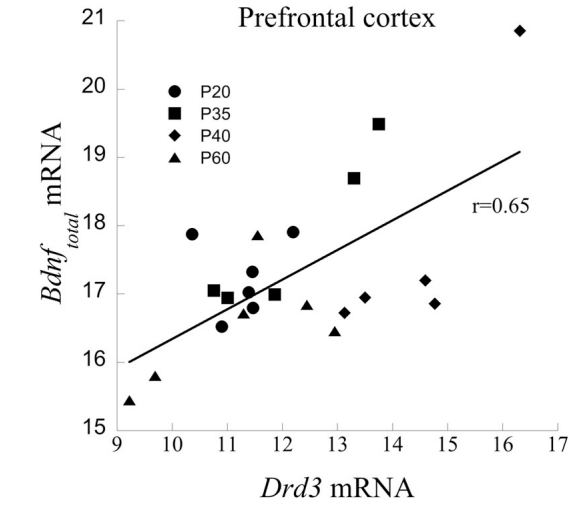

FIGURE 2 | Correlation between Drd3 and *Bdnf* mRNA from the PFC of developing rats at the ages of 20, 35, 40, and 60 days.

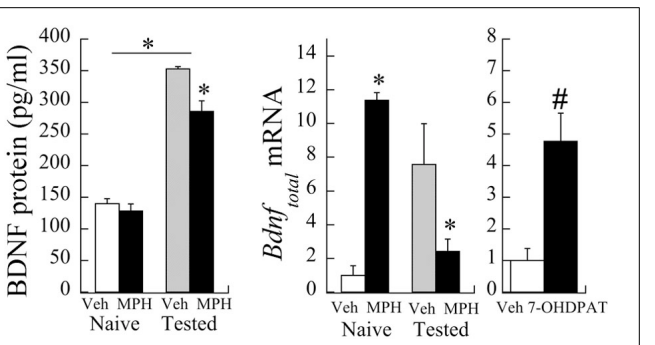

FIGURE 3 | *Bdnf* mRNA changes following juvenile MPH treatment between 20 and 35 days of age relative to controls. (left) Veh or MPH rats were assessed at P60 without prior cocaine place conditioning (naïve) or after place conditioning to 10 mg/kg cocaine for 2 days (tested). $^*p < 0.05$. **(right)** A separate group of subjects was exposed to the D3R-preferring agonist ±7-OHDPAT between P20-35 and assessed for *Bdnf* at P60. #$p < 0.06$ Mean ± *SE* for $n = 6$ subjects shown.

EXPERIMENT 2: $Bdnf_{\text{total}}$ mRNA AND PROTEIN LEVELS FOLLOWING MPH TREATMENT

The relationship between PFC *Bdnf* mRNA levels and MPH exposure interacted significantly with whether or not the animals were behaviorally tested with cocaine [Exposure × Testing interaction: $F_{(1,12)} = 6.01$, $P < 0.05$]. *Bdnf* mRNA levels were elevated in MPH subjects relative to Veh controls, and this relationship reversed following behavioral testing to cocaine at P60 [**Figure 3** (left)]. Similar to the effects of MPH exposure, juvenile treatment with the D3R-preferring agonist ±7-OHDPAT also increased *Bdnf* mRNA [**Figure 3** (right)] in behaviorally-naïve subjects.

As observed in the mRNA levels, a significant interaction was observed in BDNF protein levels as a result of cocaine testing [Exposure × Testing interaction: $F_{(1,29)} = 5.35$, $p < 0.05$]. Baseline levels of BDNF protein (pg/ml) did not significantly

differ between MPH and Veh subjects [$t_{(8)} = 0.83$, $p = 0.4$; see **Figure 3**). After cocaine administration at P60, however, BDNF protein levels in Veh subjects increased 253.1 ± 28.9% relative to cocaine naïve controls. In contrast, MPH-exposed subjects had a slightly smaller percent increase (222.2 ± 24.4%) relative to cocaine naïve levels, consistent with lower *Bdnf* mRNA levels in this group.

EXPERIMENT 3: RELATIONSHIP BETWEEN *Bdnf*$_{total}$ mRNA, D3R, AND MPH TREATMENT

Consistent with the data in Experiment 2, following juvenile MPH exposure and place conditioning to cocaine, *Bdnf* mRNA levels were significantly reduced 30.3 ± 9.7% when compared to the Veh exposed subjects [$t_{(11)} = 2.69$, $p < 0.03$; **Figure 4**]. In contrast, *Bdnf* mRNA significantly increased 35% following juvenile exposure to the D3R antagonist, nafadotride, when compared with Veh controls [$t_{(12)} = 2.65$, $p < 0.03$]. No significant difference was observed between the Veh and the MPH/Nafad subjects [$t_{(13)} = 0.74$, $p > 0.4$].

EXPERIMENT 4: AGE AND DRUG EFFECTS ON *Bdnf* SPLICE VARIANTS I, IIc, III/IV, IV/VI

Bdnf mRNA exons change across age

Individual ANOVAs of exons I, IIc, III/IV, IV/VI were run to determine significant changes across Age; only exon IIc and IV/VI were significantly different [$F_{(3,20)} = 3.35$ and 4.45, respectively, $p < 0.05$; **Figure 5**]. Exons I and III/IV did not significantly change across maturation ($p > 0.6$), nor did *Bdnf* $_{total}$ ($p < 0.14$).

Bdnf exon IIc and IV/VI mRNA, Drd3, and MPH exposure

No significant main effect of MPH exposure was evident at P60 ($p > 0.7$) for exon IIc. However, a strong correlation existed between *Drd3* and exon IIc [Pearson's $r = -0.73$, $p < 0.01$; **Figure 6** (left)]. Exon IV/VI was significantly reduced following MPH exposure [$F_{(1,10)} = 42.43$, $P < 0.001$], as shown in **Figure 6** (right), however, this relationship did not correlate with changes in D3 receptors.

DISCUSSION

Our data show that juvenile exposure to MPH or manipulation of the D3 dopamine receptor has enduring effects on the expression of BDNF that are related to D3 receptor changes. D3R activation during development did not significantly change BDNF protein levels, but increased *Bdnf* $_{total}$ mRNA while decreasing its splice variants IIc and IV/VI in adulthood. A negative correlation

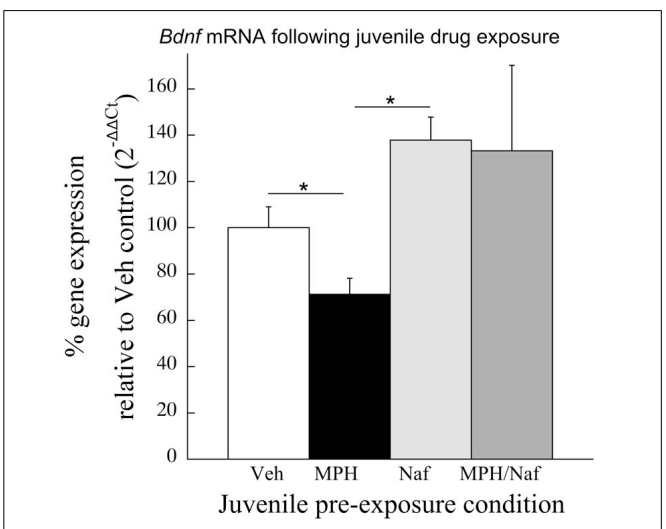

FIGURE 4 | The effects of juvenile treatment with Veh, MPH, the D3R antagonist nafadotride or MPH in combination with nafadotride on *Bdnf* mRNA levels in the PFC following place conditioning. Means ± *SE* are presented for $n = 7$–8 subjects/condition. *indicates significant difference at $p < 0.05$.

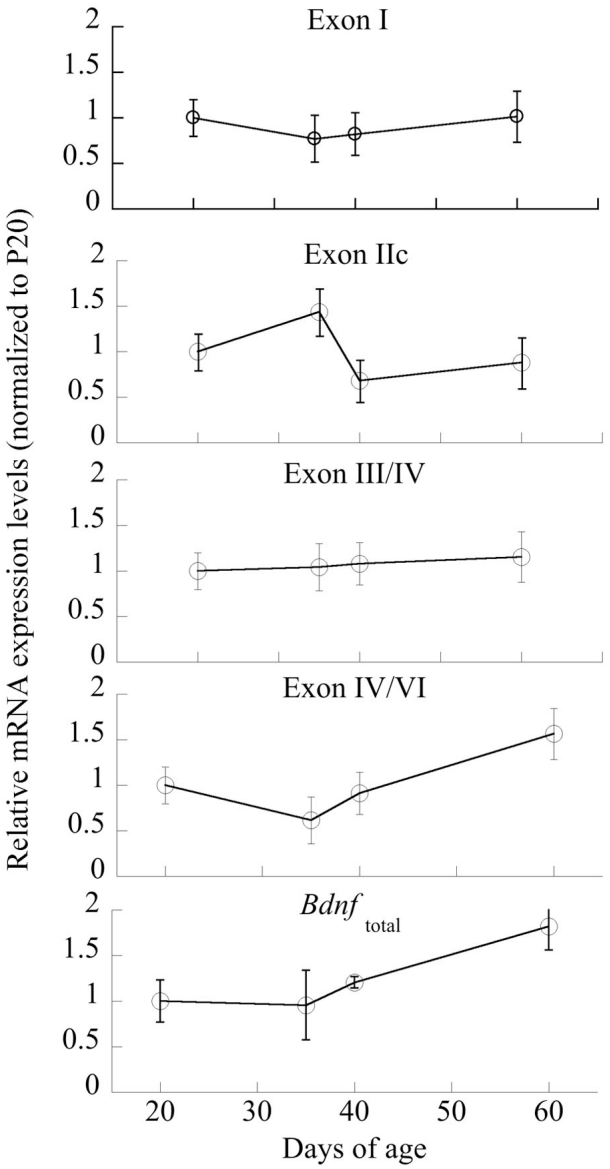

FIGURE 5 | Changes in mRNA expression of *Bdnf* and its exon-specific splice variants I, IIc, III/IV, and IV/VI as a function of age. Data are expressed following correction for GAPDH levels run simultaneously. $n = 5$–6/age, with Means ± *SE* presented.

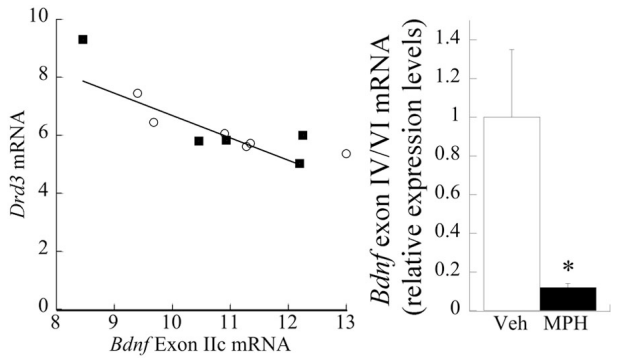

FIGURE 6 | Relationship between D3R, MPH, and BDNF exon splice variants. (left) *Drd3* mRNA negatively correlates (Pearson's $r = -0.73$) with exon IIc expression. The filled squares represent the MPH subjects at P60, whereas the open circles are Veh. **(right)** Juvenile treatment with MPH significantly reduces exon IV/VI expression at 60 days of age relative to the Veh. $n = 6$/group, with Means ± *SE* presented. $^*p < 0.05$.

between *Drd3* and exon IIc mRNA expression was observed. In contrast, a reduction in $Bdnf_{\text{total}}$ mRNA and protein were evident in CPP tested animals following D3 receptor activation during development.

Bdnf gene regulation occurs in response to environmental stimuli. As D3R antagonism attenuates cue-induced drug-seeking behavior in adult animals (Gal and Gyertyan, 2005), the observed decrease in *Drd3* mRNA is consistent with a reduction in place preferences for cocaine-associated cues in animals exposed to MPH during the juvenile period (Andersen et al., 2002). More importantly, changes in *Bdnf* and its association with *Drd3* suggest that the long-term plasticity observed in addiction (Sokoloff et al., 2001) is reduced in MPH animals. Clinically, a subtype of children that is treated with MPH shows reduced substance use disorder (Mannuzza et al., 2008), suggesting that BDNF may play a role.

A sensitive period exists for manipulations of PFC development, with pre-pubertal exposure to psychostimulants offering a window of opportunity for reduced substance abuse disorders (SUD; Andersen, 2005). D3 receptors are localized on dopamine terminals and non-terminal fields (Stanwood et al., 2000), which continue to develop into adolescence (Andersen et al., 2008). During adolescence, regulatory processes in the PFC change markedly (Andersen et al., 1997; Dumont et al., 2004; Tseng and O'Donnell, 2007) that lead to an increase in dopamine function. One of these effects is the loss of a dopamine auto-regulatory-type process mediated by D3 receptors (Booth et al., 1994; Andersen et al., 1997). The current data show that D3R changes across age account for 49% of the changes in *Bdnf* mRNA, suggesting that D3R may differentially sculpt the pre- vs. post-adolescent PFC by altering neurotrophic expression. Relative to controls, MPH-exposed animals show a significant increase in $Bdnf_{\text{total}}$ mRNA, but no changes in protein at baseline without challenge. However, MPH animals had a reduction of $Bdnf_{\text{total}}$ mRNA and protein following cocaine conditioning. BDNF is released with dopamine in the PFC in response to drug cues (Altar et al., 1998; McCarthy et al., 2012). The adult literature shows that cocaine-induced increases of BDNF suppress inhibitory GABAergic interneuron activity in the mPFC [resulting in increased long-term potentiation (LTP) (Lu et al., 2010)]. By extrapolation, a decline in BDNF following juvenile MPH and drug challenge in adulthood would reduce cortical output in response to drug cues relative to controls. A recent study shows that juvenile MPH reduces the number of spikes in PFC neurons in adulthood—consistent with this hypothesis (Urban et al., 2012).

Modulation of BDNF occurs by histone acetylation or post-transcriptional regulation (Mellios et al., 2008; Caputo et al., 2011; Boulle et al., 2012; Leal et al., 2014), and our data provide evidence for epigenetic modifications in BDNF splice variants by MPH or D3R. In development, the splice variants II and IV are typically decreased during the transition from juvenile to adolescence. These exons were also reduced in adult animals after juvenile MPH exposure. A negative correlation between exon IIc and *Drd3* mRNA suggests less neuronal plasticity as D3 receptors decrease either following typical aging or that facilitated by juvenile MPH treatment (Andersen et al., 2008). Exon II has been localized to neurons and its expression is activity-dependent in visual cortex (Pattabiraman et al., 2005), supporting a potential role in modulating neuronal plasticity. Exon IV/VI was also significantly lower in MPH subjects consistent with previous observations in juvenile MPH rats studied by Fumagalli et al. (2010). It should be noted that the observed up-regulation of $Bdnf_{\text{total}}$ mRNA in adult animals after juvenile treatment with MPH did not correlate with BDNF protein levels. There is emerging evidence that protein expression is regulated on the post-transcriptional level by small non-coding RNAs, including micro (mi) RNAs. Several miRNAs have been identified that target the *Bdnf* transcript (Mellios et al., 2008; Caputo et al., 2011), indicating that other epigenetic mechanisms may be involved in regulating BDNF expression.

The enduring effects in juveniles are predictably opposite to the increase in *Bdnf* exon IV/VI expression in the PFC of adult, cocaine self-administering rats that may underlie craving and relapse (Sadri-Vakili et al., 2010; Schmidt et al., 2013). Exon IV/VI contains the binding sites for CREB and MeCP2 and plays a role in synaptic plasticity, cognitive processes and learning and memory—such as those required for encoding drug-associated cues (McCarthy et al., 2012). While earlier studies have demonstrated reduced cue responsiveness in rats exposed to MPH during the juvenile period (Andersen et al., 2002; Carlezon et al., 2003; Argento et al., 2012), others have shown that peri-adolescent treatment with MPH reduces object recognition later in life (Leblanc-Duchin and Taukulis, 2007). Interestingly, object recognition increases following D3 receptor blockade in the adult PFC (Watson et al., 2012). Together, these data suggest that MPH may also permanently modulate object recognition later in life. Object recognition measures whether a novel object is recognized, as new or not, which is impaired in ADHD.

Changes in BDNF are important during sensitive period when the programming of drug-seeking behavior occurs (Andersen et al., 2002; Andersen, 2005). Further understanding of the molecular mechanisms regulating these changes can potentially lead to novel ways of harnessing developmental relationships to redirect an off-course trajectory. For example, Shaw et al. (2007)

have shown that gray matter development of the PFC of ADHD children and teens is delayed. Pharmacological targeting of BDNF itself is difficult, but treatment with a D3R agonist pre-pubertally may reduce behaviors that are associated with ADHD, including risk for SUD, changes in object recognition, and activity levels.

AUTHOR CONTRIBUTIONS

Both Drs. Andersen and Sonntag contributed equally to the design, execution, and preparation of the manuscript.

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Brain-derived neurotrophic factor in VMH as the causal factor for and therapeutic tool to treat visceral adiposity and hyperleptinemia in type 2 diabetic Goto–Kakizaki rats

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We previously reported that the type 2 diabetic Goto–Kakizaki (GK) rats at young adult ages (6–12 weeks) exhibited increased visceral fat mass and hyperleptinemia, due to hyperphagia caused primarily by neuropeptide Y (NPY) overexpression in the hypothalamic arcuate nucleus. Later, we found that GK rats continued to exhibit mesenteric fat accumulation and hyperleptinemia at least until 26 weeks of age, while hyperphagia and NPY overexpression ceased at 15 weeks of age. Therefore, we hypothesized that the long-lasting fat accumulation and hyperleptinemia are due to unidentified brain dysfunction other than NPY overexpression. In GK rats aged 26 weeks, glucose transporter-2 (GLUT2) mRNA expression in ventromedial hypothalamus (VMH) was markedly reduced in parallel with significant decreases in brain-derived neurotrophic factor (BDNF) mRNA level and BDNF-expressing cell numbers in the VMH. Pharmacologic inhibition of glucose utilization reduced BDNF mRNA expression in VMH *in vivo* and *in vitro*. The results suggested that impaired glucose utilization caused the reduction of BDNF. On the other hand, intracerebroventricular injection of BDNF for 6 days ameliorated hyperleptinemia in a long-lasting manner concurrently with feeding suppression in GK rats. Restricted feeding paired to BDNF-treated rats reduced plasma leptin level only transiently. BDNF treatment also reduced mesenteric fat mass in GK rats. These results reveal a novel action mode of BDNF to long-lastingly counteract visceral adiposity and hyperleptinemia in addition to and independently of its anorexigenic action. These results suggest that visceral fat accumulation and hyperleptinemia are at least partly due to the reduction of BDNF in VMH primarily caused by impaired glucose utilization in GK rats. The BDNF supplementation could provide an effective treatment of visceral obesity, hyperleptinemia and leptin resistance in type 2 diabetes.

Keywords: BDNF, type 2 diabetes, adiposity, visceral fat, hyperleptinemia, glucose, VMH

INTRODUCTION

The Goto–Kakizaki (GK) rat was established as an animal model for type 2 diabetes by selecting Wistar rats which exhibited glucose intolerance (Goto et al., 1976) and has been widely used to investigate the mechanisms of glucose intolerance and complications of hyperglycemia (Yagihashi et al., 1982; Picarel-Blanchot et al., 1996; Moreira et al., 2007). It has been reported that glucose intolerance in GK rats is mainly caused by reduced β-cell mass and impaired glucose-induced insulin secretion in β-cell (Portha et al., 1991). Glucose intolerance of GK rats is also thought to be partly due to impaired insulin sensitivity (Farese et al., 1994).

Our particular concern in this study is how impaired insulin sensitivity develops and is sustained in GK rats. We previously reported that impaired insulin sensitivity and increased visceral fat mass occurred in parallel in young adult (6–12 weeks) GK rats (Maekawa et al., 2006a). The result indicated a possibility that the increased visceral fat mass induced impairment of insulin sensitivity. In young adult (6–12 weeks), the increased fat mass was initiated by hyperphagia which was caused by enhanced neuropeptide Y (NPY) mRNA expression and impaired intracellular signaling of leptin in the hypothalamic arcuate nucleus (ARC) (Maekawa et al., 2006a). In GK rats, it has been also reported that insulin sensitivity is impaired not only at young adults but at middle-aged adults (Ndisang and Jadhav, 2009). Middle-aged GK rat in our colony (22–35 weeks) exhibited hyperleptinemia and higher mesenteric fat weight, while hyperphagia and overexpression of NPY mRNA in ARC were no longer observed (Maekawa et al., 2006a). Therefore, it is speculated that the long-lasting fat accumulation and

hyperleptinemia at middle-age are due to decreased energy expenditure.

In this study, we focused on the relationship of brain-derived neurotrophic factor (BDNF) to fat accumulation and plasma leptin level in GK rat at middle-aged adult. In addition to well-defined role of BDNF in the regulation of development, survival, differentiation and synaptic plasticity in the nervous system (Tapia-Arancibia et al., 2004), it is also implicated in feeding behavior and energy balance (Noble et al., 2011; Rios, 2013). Heterozygous BDNF mutant mice and brain-specific BDNF knockout mice, in which the BDNF gene is selectively deleted in the brain after birth, showed increased food intake and body weight (Lyons et al., 1999; Kernie et al., 2000; Rios et al., 2001). Intracerebroventricular (icv) BDNF injection markedly reduced body weight in several obesity models by reducing appetite (Pelleymounter et al., 1995) and increasing energy expenditure (Nakagawa et al., 2000). In the hypothalamus, BDNF-expressing neurons are mainly localized in ventromedial hypothalamic nucleus (VMH) and paraventricular nucleus (PVN) (Noble et al., 2011). Since BDNF knockout in medial basal hypothalamus containing VMH induced hyperphagia and obesity, the BDNF neurons in this region is thought to be critical for regulating energy metabolism (Unger et al., 2007). BDNF neurons of VMH possibly project to the neurons expressing corticotrophin-releasing hormone (CRH) in PVN, since the CRH neurons express TrkB, a receptor for BDNF, and icv injection of BDNF increases CRH mRNA (Toriya et al., 2010). BDNF injection decreased respiratory quotient and increased rectal temperature, and these effects were antagonized by simultaneous treatment with α-helical $CRH_{9–41}$, a CRH receptor antagonist (Toriya et al., 2010). These results suggested that the projection of BDNF neurons to CRH neurons in PVN plays a critical role in energy expenditure.

It has been reported that the BDNF expression in hypothalamus is regulated by several factors, which include neurotransmitters such as melanocortins (Nicholson et al., 2007; Vanevski and Xu, 2013), metabolic factors such as leptin (Komori et al., 2006), insulin and glucose (Unger et al., 2007), and environmental cues such as stress and environmental enrichment (Cao et al., 2010). In addition, BDNF level in hypothalamus is altered in obesity and/or diabetes models such as db/db, agouti yellow (Xu et al., 2003) and SF-1 knockout mice (Tran et al., 2003). These reports suggest that metabolic changes in obesity and diabetes result in and/or result from the reduction of BDNF expression.

Here, we found that BDNF expression is reduced specifically in VMH in GK rats at middle-age. We investigated the mechanism underlying the reduction of BDNF in VMH and examined whether BDNF supplementation induces lipolysis and ameliorates visceral obesity.

MATERIALS AND METHODS

ANIMALS

The GK rats purchased from Japan SLC (Shizuoka, Japan) were maintained by breeding in the Center for Experimental Medicine, Jichi Medical University. Adult male or pregnant Wistar rats were purchased from Japan SLC. The rats were housed under a controlled temperature (26°C) and photoperiod (12L:12D). The rats received pellet-type food (CE-2, Japan CLEA, Tokyo, Japan) and tap water *ad-libitum*. The animal protocols were approved by the Jichi Medical School Institute of Animal Care and Use Committee and were in accord with the Japanese Physiological Society's guidelines for animal care.

mRNA EXTRACTION FROM BRAIN TISSUES AND cDNA SYNTHESIS

A portion of hypothalamus in each rat was dissected using a brain slicer. The coronal slice of 2-mm thickness covering the anterior part of VMH to the posterior part of ARC was obtained. The tissues of the VMH and ARC were dissected with incisions, and homogenized with TRIzol (Invitrogen, Carlsbad, CA). Total RNAs were extracted following the protocol indicated by the manufacturer. DNase (1 U/10 μl RNA solution, Promega, Madison, WI) was added and the mixtures were incubated for 1 h at 37°C. Following the inactivation of DNase by heat, cDNA was synthesized from 2 μg total RNA with SuperscriptII Reverse Transcription Kit (Invitrogen) utilizing $oligo(dT)_{20}$ primer.

MIXED CULTURE OF CELLS FROM THE MEDIOBASAL HYPOTHALAMUS AND mRNA EXTRACTION

The mediobasal hypothalamic tissue was isolated from the brain of 6-day-old pups of Wistar rats, followed by dissociation of neurons according to the procedures reported previously (Kohno et al., 2007) with slight modification. Briefly, brain slices in 1 mm-thickness were prepared, from which the tissues containing the VMH and ARC were obtained. The 5–6 dissected tissues were mixed and washed with 10 mM HEPES-buffered Krebs-Ringer bicarbonate buffer (HKRB) [(in mM): NaCl 129, $NaHCO_3$ 5.0, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 2.0, $MgSO_4$ 1.2 and HEPES 10 at pH 7.4] containing 10 mM glucose. Then they were incubated in HKRB supplemented with 20 units/ml papain (Sigma-Aldrich, St. Louis, MO), 0.015 mg/ml DNase, and 0.75 mg/ml BSA for 12 min at 36°C in a shaking water bath, followed by graded trituration. The cell suspension was incubated on ice for 5 min and a supernatant was centrifuged at 500 rpm for 5 min. The pellet was resuspended in the culture medium containing 50% minimal essential medium, 25% Hank's balanced salt solution, 25% horse serum (#12360-038, #24020-117, #16050-122, Gibco BRL) and 10 mM glucose. The cells were distributed onto 96-well plates and short-term cultured. Each well-contained ∼20% of cells removed from the brain of a pup. Three h after the treatment with 2-deoxy-d-glucose (2-DG) at various dosages, total RNA was extracted and cDNA was synthesized.

mRNA MEASUREMENTS BY FLUORESCENCE REAL-TIME RT-PCR

Quantification of mRNAs was carried out using ABI PRISM ®7900-HT system (Applied Biosystems, Foster City, CA). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) level was measured as an internal control. PCR primer sets for amplification and TaqMan®probes with carboxyfluorescein in 5′end and carboxytetramethyl-rhodamine in 3′end were purchased from Sigma Genosys (Hokkaido, Japan) (**Table A1**). To detect GLUT8, SybrGreen PCR was performed. Dividing each mRNA level by the GAPDH level resulted in a normalized mRNA value. The

fluorescence real-time PCR was performed by the method as described previously (Maekawa et al., 2006a). Dividing each mRNA level by the GAPDH level resulted in a normalized mRNA value.

Icv CANNULAE IMPLANTATION

Rats were anesthetized with Avertin (a mixture of 2,2,2-tribromoethanol (T4840-2, Sigma-Aldrich) and Tert-amyl alcohol (24,048-6, Sigma-Aldrich), 200 mg/kg, intraperitoneal). In a stereotaxic frame, a 23-gauged stainless steel guide cannula was inserted into the brain with the tip in the third cerebral ventricle, and secured to the skull with screws and cement. The cannula tip was located at 0.9 mm caudal to the bregma and 7.0 mm below the skull. After surgery, rats were allowed to recuperate for 7 days. Handling of the operated animals was performed for 10 min everyday. For injecting substances, the internal cannula was inserted into the guide cannula and injection was executed for 1 min under free-moving conditions.

IMMUNOHISTOCHEMISTRY

A chicken anti-human BDNF polyclonal antibody (1:100, G1641, Promega), a sheep anti-α-MSH antibody (1:30,000, AB5087, Chemicon, Temecula, CA), a biotinylated rabbit anti-chicken IgY (1:300, G2891, Promega), a biotinylated rabbit anti-sheep IgG (1:300, BA-6000, Vector Laboratories, Burlingame, CA) were used. Rats were intracerebroventricularly treated with 10 μl colchicine (100 μg/10 μl in distilled water). Forty-eight h after injection, rats were perfused transcardially with 100 ml of 2% paraformaldehyde in 50 mM phosphate buffer (PB), pH 7.5, followed by 50 ml of 50 mM phosphate-buffered saline under deep urethane anesthesia. Brains were removed, postfixed by the same fixative for 2 h, cryoprotected with 30% sucrose in 50 mM PB for 2–3 days at 4°C, sectioned coronally at 40-μm thickness with a freezing microtome. The sections collected at every 160-μm interval were used. The localization of the target proteins was determined as described previously (Maekawa et al., 2006b).

Icv BDNF INJECTION, PLASMA HORMONE AND METABOLITES LEVELS

Wistar and GK rats were intracerebroventricularly injected with BDNF (15 μg/5 μl saline/head) or vehicle once a day for 6 days. Plasma samples were obtained at the day before injection, the 6th, 10th, 21st day after injection. Blood was withdrawn from tail vein under unanesthetized condition. Glucose level was determined by a conventional blood glucose-measuring device (Glucocard, Arkray, Kyoto, Japan). Intraperitoneal glucose tolerance test (IpGTT) was performed under overnight fasting conditions. Glucose (1 g/kg body weight) was injected and then blood samplings from the tail vein were performed up to 3 h. For hormonal assay, plasma was immediately separated. Plasma leptin and insulin concentrations were measured using ELISA kits for leptin and insulin, respectively (#200728 and #200718, Morinaga Institute of Biological Science, Yokohama, Japan). Plasma non-esterified free fatty acid (NEFA) concentration was measured using an assay kit for NEFA (#279-75401 NEFA *C*-test Wako, Osaka, Japan).

MEASUREMENTS OF FOOD INTAKE AND BODY WEIGHT COMBINED WITH PAIR-FEEDING

Food intake over 24 h was calculated by weighing the remaining food pellets and body weight was measured between 11:00 and 12:00 h every day. For pair-feeding experiment, the amount of food consumed by the BDNF-treated group over the course of 24 h was measured at 11:00 h, and a corresponding amount of pellets was given to the pair-fed group over a 24-h period.

MEASUREMENT OF FAT MASSES

At the completion of each experiment, interscapular, epididymal, mesenteric, and perirenal fat were dissected and weighed. Fat masses were calculated as percentage of body weight.

DATA ANALYSES

All data are expressed as mean ± SEM. The number of animals used is indicated in the parenthesis. One-Way ANOVA with Holm's *post-hoc* test was used for **Figures 3D**, **4**, **5A,B, 6**, **7A,B, Table 2**. Two-Way ANOVA with Tukey's *post-hoc* test was used for **Figures 1B**, **8**, and **Table 1**. Other data was analyzed by Student's *t*-test with Microsoft Excel 2008 for Mac (Microsoft, Redmond, WA). All statistical analyses except Student's *t*-test were performed using R (The R Foundation for Statistical Computing, Vienna, Austria). $p < 0.05$ was considered significant.

RESULTS

METABOLIC INDICES OF GK RATS AT 26 WEEKS OF AGE

Casual blood glucose level in GK rat was significantly higher than that in Wistar rat at 11 weeks and it increased further at 26 weeks (**Figure A1A**). Intraperitoneal glucose tolerance test in GK rats revealed that glucose intolerance progressed at 24 weeks, compared to 14 weeks of age (**Figure A1B**). Mesenteric and perirenal fat weights were larger in GK rats than in Wistar rats at 11 and 26 weeks of age (**Table 1**).

REDUCED BDNF EXPRESSION IN VMH OF GK RATS AT 26 WEEKS OF AGE

By comparison between Wistar and GK rats at 11 and 26 weeks of age, BDNF mRNA level examined by real-time RT-PCR was found to be reduced in VMH of GK rat specifically at 26 weeks of age (**Figure 1A**, $p < 0.05$). Furthermore, immunohistochemistry revealed marked reduction in the number of BDNF expressing neurons selectively in VMH but not in PVN and nucleus tractus solitarius (NTS) of GK rat at 26 weeks of age (**Figures 1B–J**, $p < 0.05$ in **B**).

MECHANISM UNDERLYING THE REDUCED BDNF EXPRESSION IN VMH OF GK RATS

α-MSH, melanocortin-4 receptor (MC4R), insulin, leptin and glucose have been reported to affect BDNF expression. Hence, we examined possible involvement of these factors in reduction of BDNF expression in GK rat. Based on the previous report showing a link between BDNF and α-MSH (Xu et al., 2003; Nicholson et al., 2007), we examined a possible alteration of melanocortin system in GK rats. By immunohistochemistry, localization of α-MSH-immunoreactive neurons in the ARC of GK rats was identical to that in control Wistar rats (**Figures 2A,B**). The cell numbers of α-MSH-immunoreactive neurons in the ARC were

Table 1 | Interscapular, epididymal, mesenteric and perirenal fat weights (% body weight) in control Wistar and diabetic GK rats at 11 and 26 weeks of age.

Age	Strain	No.	Weight of fat pad (% BW)			
			Interscapular	Epididymal	Mesenteric	Perirenal
11 weeks	Wistar	6	0.09 ± 0.01	1.47 ± 0.08	0.63 ± 0.05	0.93 ± 0.04
	GK	5	0.21 ± 0.02*	1.04 ± 0.05*	1.08 ± 0.04*	1.78 ± 0.11*
26 weeks	Wistar	5	0.08 ± 0.02	1.52 ± 0.11	0.89 ± 0.05	1.55 ± 0.14
	GK	6	0.12 ± 0.01	1.16 ± 0.07*	1.27 ± 0.12*	1.97 ± 0.11*

*$p < 0.05$ vs Control Wistar rats (Two-Way ANOVA with Tukey's post-hoc test).

not different between GK and Wistar rats (**Figure 2C**). Similarly, no difference in MC4R mRNA expression was found in the ARC and VMH in GK rats (**Figure 2D**).

Next, the possible association of insulin and/or leptin with BDNF reduction was examined. We tested whether the supplementation of insulin would rescue BDNF reduction in GK rats. GK rats were treated with intraperitoneal administration of insulin (1 U/kg body weight) twice a day for 3 days, which failed to restore the BDNF mRNA levels in the VMH (**Figure 2E**). To examine the acute effect of insulin to increase BDNF mRNA level in the VMH, single icv injection of insulin (10 mU/10 μl saline) to Wistar rats was performed. However, it did not increase the BDNF mRNA expression (**Figure 2F**). Icv administration of leptin (5 μg, twice a day for 3 days) in Wistar rats under fasting condition failed to alter BDNF mRNA level (**Figure 2G**).

Since transcription of BDNF in the VMH has been reported to be regulated by glucose (Unger et al., 2007), we examined the mRNA expression of genes related to the glucose utilization in the VMH of GK rats using real-time RT-PCR. There was no difference in GLUT4, GLUT8 and glucokinase mRNA expressions between Wistar and GK rats (**Figure 3A**). In contrast, the GLUT2 mRNA level was significantly reduced at 26 weeks compared to 11 weeks of age (**Figure 3B**). To test the possibility that reduction of glucose utilization affects BDNF mRNA level in the VMH, 2-DG (5 mg/10 μl saline), an inhibitor of glycolysis, was administered icv to Wistar rats. 2-DG markedly reduced BDNF mRNA level in the VMH as well as in hippocampus at 2 h after treatment (**Figure 3C**). In a parallel *in vitro* experiment, bath application of 2-DG to primary cultured neurons of medial basal hypothalamus including the VMH dose-dependently reduced BDNF mRNA expression (**Figure 3D**). These results indicate that lowered glucose utilization in the VMH directly reduces BDNF mRNA expression.

EFFECT OF BDNF TREATMENT ON PLASMA LEPTIN LEVEL AND FAT MASS IN GK RATS

The effect of daily treatment with BDNF (15 μg/5 μl saline/head) for 6 days on plasma leptin level and fat mass was examined in Wistar and GK rats at 26 weeks. Plasma leptin level in GK rats (GK-Vehicle group and GK-BDNF group) was significantly higher than that in Wistar rat before treatment (Day 0) (**Figure 4**), confirming our previous report (Maekawa et al., 2006a). GK rats subjected to treatment with saline revealed higher plasma leptin level during experiment (GK-Vehicle group, **Figure 4**). By contrast, the treatments with BDNF significantly decreased plasma leptin level in both Wistar and GK rats (Wistar-BDNF and GK-BDNF groups, respectively) at the timing of termination of treatment (Day 6) and at 4 days after termination of treatment (Day 10) ($p < 0.01$, **Figure 4**). Plasma leptin level at 15 days after termination of treatment (Day 21) returned to the level before treatment in GK rats of GK-BDNF group, while it was still low in Wistar-BDNF group. At Day 21, weights of fat mass in all groups were measured (**Table 2**). The weight of mesenteric fat in BDNF-treated GK rats (GK-BDNF group) was significantly lower than that in saline-treated GK rats (GK-Vehicle group, $p < 0.05$), although it was higher than that in BDNF-treated Wistar rats.

EFFECT OF BDNF TREATMENT ON FOOD CONSUMPTION, BODY WEIGHT, GLUCOSE TOLERANCE, AND NEFA LEVEL IN GK RATS

Daily food intake was significantly lowered both in Wistar-BDNF and GK-BDNF groups during BDNF treatment (**Figure 5A**). After termination of treatment, the food intake in Wistar-BDNF and GK-BDNF groups rebounded to the same level as that in GK-Vehicle group (**Figure 5A**). BDNF treatments also reduced body weights in the rats of Wistar-BDNF and GK-BDNF groups (**Figure 5B**). After termination of treatment, the body weight of the BDNF-treated groups rebounded to the level close to that before BDNF treatment (**Figure 5B**). Although the blood glucose level in GK-BDNF level did not change during BDNF treatment, after termination of treatment it became lower than that in GK-Vehicle group, demonstrating the late-onset effect of BDNF on glucose control (**Figure 6**). To investigate the mechanism how BDNF lowered the casual glucose level in GK-BDNF group, we performed IpGTT and measured glucose and insulin levels during IpGTT (**Figure 7**). Blood glucose levels during IpGTT in GK-Vehicle and GK-BDNF groups were markedly higher than that in Wistar-BDNF groups at Day 0 (before treatment), Day 7 (immediately after termination of treatment), and Day 11 (after termination of treatment) (**Figure 7A**). Blood glucose level during IpGTT at Day 7 tended to increase, rather than decrease, in GK-BDNF group. The higher glucose level at Day 7 in GK-BDNF group is probably due to the lowered insulin level at Day 7 (**Figure 7B**). These results taken together suggest that BDNF treatments lowered casual blood glucose level by changing insulin sensitivity but not by improving insulin secretion in GK

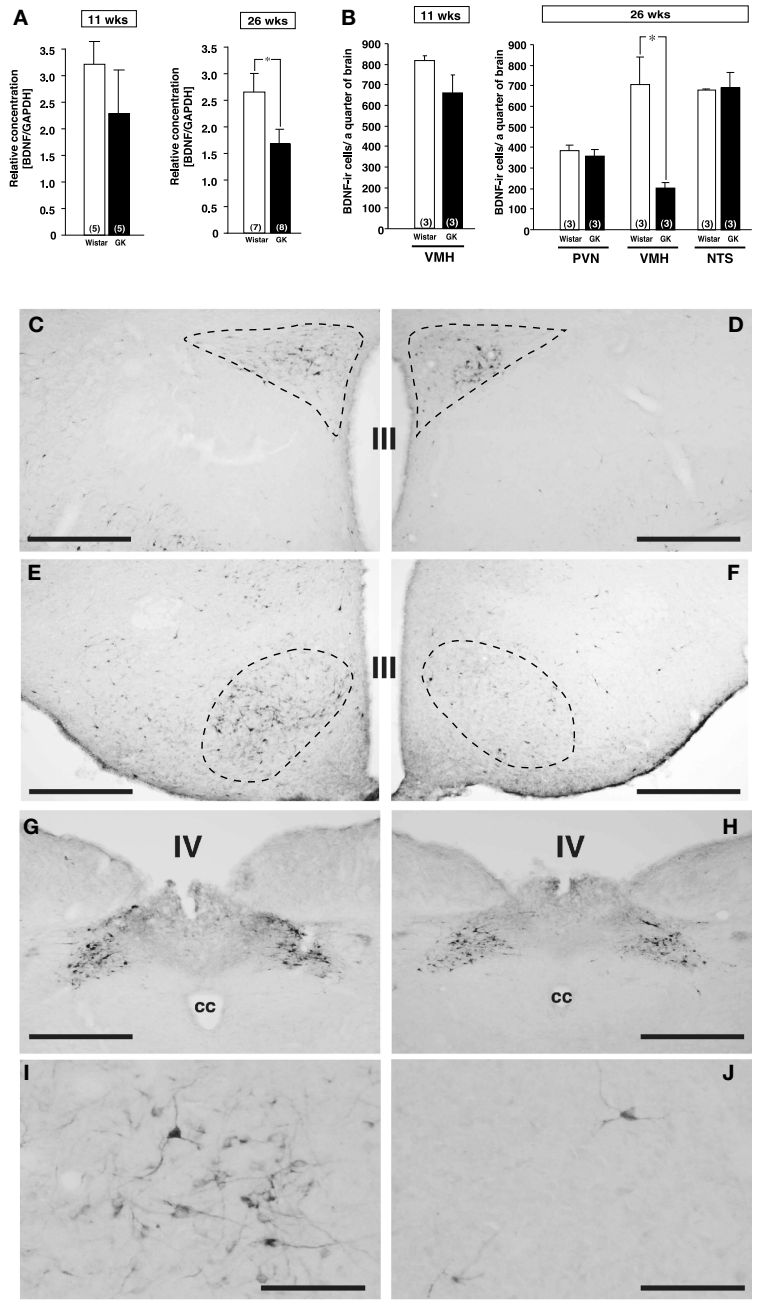

FIGURE 1 | Expressions of BDNF mRNA and BDNF-immunoreactive cells in Wistar and GK rats at 11 and 26 weeks of age. (A) BDNF mRNA levels in VMH of Wistar and GK rats. Significant reduction of BDNF mRNA level was found in GK rats at 26 weeks of age ($*p < 0.05$, Student's *t*-test). **(B)** At 26 weeks, significant reduction of the number of BDNF-immunoreactive neurons was found in VMH, but not PVN and NTS, of GK rats ($*p < 0.05$, Two-Way ANOVA with Tukey's *post-hoc* test). This reduction in VMH was not different at 11 weeks. **(C–J)** Representative images of BDNF-immunoreactive neurons in Wistar rats **(C,E,G,I)** and GK rats **(D,F,H,J)** at 26 weeks of age. Broken lines in each image indicate the outline of PVN **(C,D)** and VMH **(E,F)**. No apparent difference was found in the PVN **(C,D)** and NTS **(G,H)**. By contrast, the number of BDNF-immunoreactive neurons in the VMH of GK rats **(F)** was fewer than that of Wistar rats **(E)**. At higher magnification, the difference between Wistar **(I)** and GK **(J)** rats was obvious. III; the third ventricle, IV; the fourth ventricle, cc; central canal. Scale bar = 500 μm **(C–H)** or 100 μm **(I** and **J)**.

rats. To examine whether exogenous BDNF treatment changes plasma NEFA level in GK rats, we measured plasma NEFA level in fed and 12h-fasted conditions. In GK-Vehicle group, plasma NEFA level was higher in fasted condition at all time points measured (**Figure 8**). In GK-BDNF and Wistar-BNDF groups, plasma NEFA level was significantly increased both in fed and fasted conditions at Day 6(fed)/7(fasted) and returned to pre-treatment level at Day 10 (fed)/11(fasted) (**Figure 8**), indicating

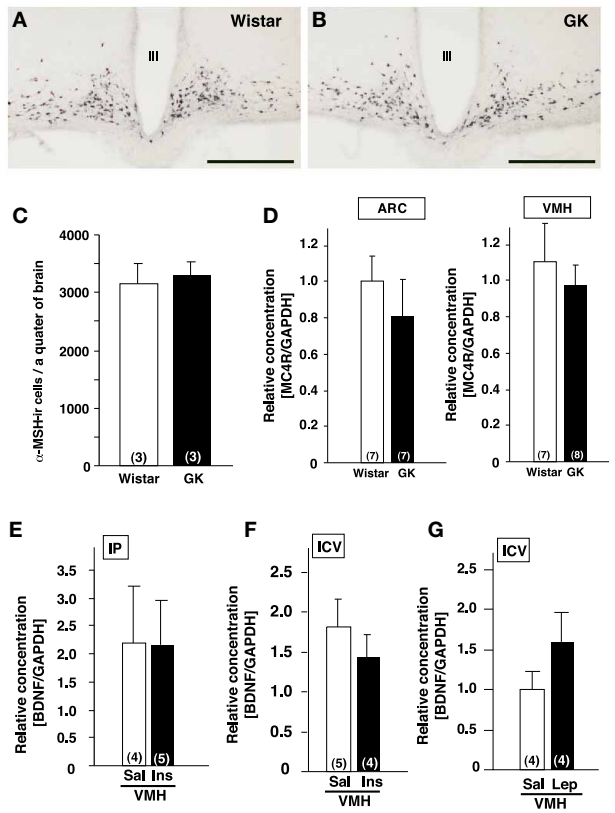

FIGURE 2 | Lack of alterations of melanocortines and effects of insulin and leptin on BDNF mRNA expression in GK rats. (A,B) Representative images of α-MSH-immunoreactive neurons in ARC of Wistar **(A)** and GK **(B)** rats. III; the third ventricle, Scale bar = 500 μm. **(C)** Numbers of α-MSH-immunoreactive neurons in Wistar and GK rats. **(D)** MC4R mRNA expressions in ARC and VMH were not different between Wistar and GK rats. **(E)** Repeated intraperitoneal injection of insulin (1 U/kg body weight, twice a day for 3 days) in GK rats did not alter BDNF mRNA level in VMH. **(F)** Single icv injection of insulin (10 mU) in Wistar rats did not alter BDNF mRNA levels in VMH. **(G)** Repeated icv injection of leptin (5 μg, twice a day for 3 days) to the fasting Wistar rats had no significant effect on BDNF mRNA levels in VMH of Wistar rats.

the possibility that BDNF not only works as a feeding suppressant but also increases lipolysis in GK rats.

COMPARISON OF PLASMA LEPTIN LEVELS BETWEEN GK-BDNF AND GK-VEHICLE PAIR-FED GROUPS

We examined plasma leptin levels in GK-BDNF and GK-Vehicle pair-fed groups (**Figure 9**). In GK-Vehicle pair-fed group, GK rats were treated with saline and the amount of food supplied was controlled to the same level as consumed by GK-BDNF group. In GK-BDNF group, at Day 6 and 10 plasma leptin levels were markedly reduced (**Figure 9**). In GK-Vehicle pair-fed group, plasma leptin level was lowered to the same level as in GK-BDNF group at Day 6. However, at Day 10 it increased to a level significantly higher than that in GK-BDNF group, indicating that exogenous BDNF counteracted visceral adiposity and hyperleptinemia via two modes of action: in acute phase via anorexigenic action and in late long-lasting phase via food intake-independent mechanisms.

DISCUSSION

In this study, we found that middle-aged GK rats show abdominal fat accumulation and hyperleptinemia, reduction in BDNF mRNA expression and protein levels specifically in VMH, and reduction in GLUT2 mRNA in VMH. Pharmacological blockade of glucose utilization *in vivo* and *in vitro* reduced BDNF expression in VMH, suggesting that glucose availability positively regulates BDNF expression in VMH. In rescue experiment, BDNF supplementation for 6 days ameliorated hyperleptinemia and higher adiposity in a long-lasting manner. These results reveal that reduction of BDNF expression due to decreased GLUT2 expression and glucose utilization in VMH is linked to visceral fat accumulation and hyperleptimemia in GK rats.

FAT ACCUMULATION AND GLUCOSE INTOLERANCE IN MIDDLE-AGED GK RATS

It has been reported that glucose intolerance in human type 2 diabetes progresses with age (Gong and Muzumdar, 2012). A major cause for this progression is insulin resistance due to genetic and/or environmental factors. Especially, visceral fat accumulation is of particular concern as a causal factor to induce insulin resistance (Pouliot et al., 1992). Our breeding colony of GK rats displayed these characteristic features common for the type 2 diabetes. First, hyperglycemia and glucose intolerance progressed markedly at 24 weeks of age compared to 14 weeks (**Figure A1**). Second, the visceral adiposity in mesenteric and perirenal fat pads in GK rats was greater at 26 weeks of age than at 11 weeks. Third, progression of hyperglycemia paralleled with progression of adiposity. These findings suggested that higher visceral adiposity is related to the progression of glucose intolerance and that GK rat is a suitable animal model to study interaction of glucose intolerance and visceral adiposity in type 2 diabetes.

REDUCTION OF BDNF EXPRESSION IN VMH OF GK RATS AT MIDDLE AGE

BDNF in the brain plays a critical role in regulating feeding and metabolism. The BDNF neurons expressed in VMH and PVN have been reported to contribute to the regulation of feeding and metabolism (Noble et al., 2011). We found that the GK rats aged 26 weeks display reduction of the BDNF mRNA and protein in the VMH in parallel with progression of higher adiposity. Regarding regulation of BDNF, it has been reported that BDNF mRNA expression is modulated by the melanocortine receptor 4 (MC4R) and downstream pathway (Xu et al., 2003; Nicholson et al., 2007). Therefore, we examined a possible alteration of the α-MSH-MC4R system as a cause of BDNF reduction in GK rats. However, neither the number of α-MSH-immunoreactive neurons nor the MC4R mRNA levels in the ARC and VMH were altered in GK rats at 26 weeks of age. We also examined whether insulin and/or leptin influence BDNF expression in VMH. We could not find convincing evidence that insulin and leptin regulate BDNF mRNA level in GK and Wistar rats.

We found that the GLUT2 mRNA level in VMH was significantly reduced in GK rats. Previous studies in GK rats

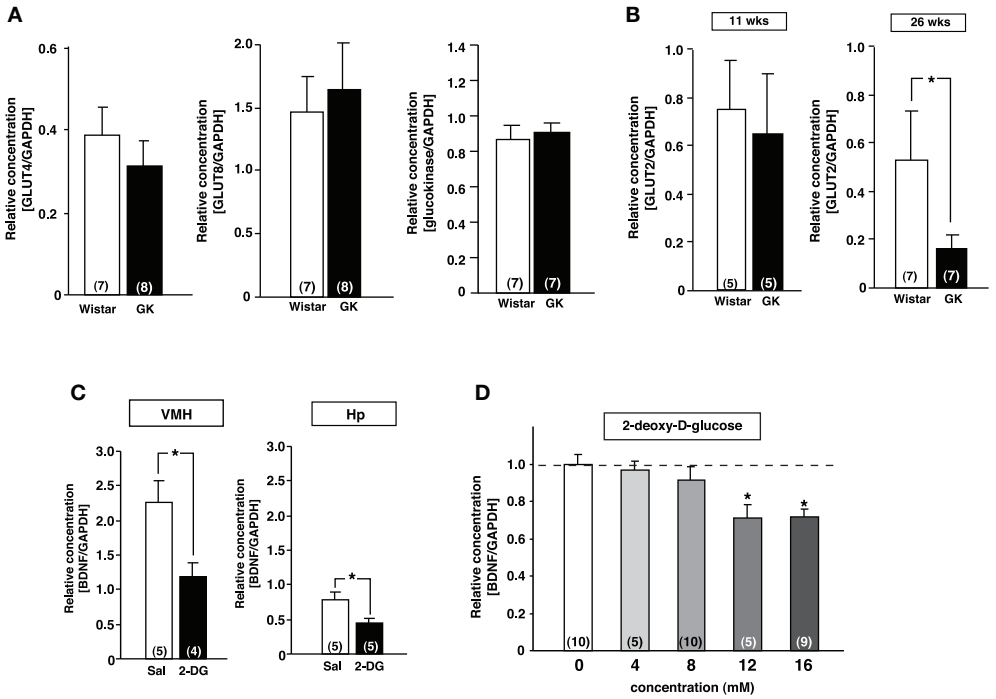

FIGURE 3 | Reduction of GLUT2 paralleled with reduction of BDNF expression in GK rats. (A) GLUT4, GLUT8 and gluokinase mRNA expressions in VMH were unchanged in GK rats at 26 weeks. **(B)** GLUT2 mRNA expression in VMH was significantly reduced in GK rats at 26 weeks, but not at 11 weeks (*$p < 0.05$, Student's *t*-test). **(C)** Effects of icv 2-DG injection on BDNF mRNA levels in VMH were determined at 2 h after icv injection of 2-DG (5 mg/10 μl) or saline (Sal). BDNF mRNA expression in VMH and hippocampus (Hp) was significantly reduced by 2-DG (*$p < 0.05$, Student's *t*-test). **(D)** Treatment with 2-DG for 3 h dose-dependently suppressed BDNF mRNA level in cultured mediobasal hypothalamic cells. The effects of 12 and 16 mM 2-DG were significant (*$p < 0.05$, One-Way ANOVA with Holm's *post-hoc* test).

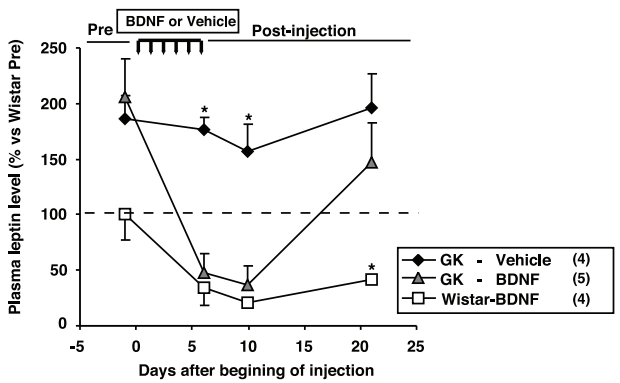

FIGURE 4 | Effect of icv BDNF treatment daily for 6 days on plasma leptin levels in GK rats. Plasma leptin levels at 0, 6th, 10th, 21st days after beginning of BDNF or vehicle injections. BDNF treatment significantly reduced plasma leptin level in GK rats. *$p < 0.05$ vs. other groups (One-Way ANOVA with Holm's *post-hoc* test).

reported reductions of GLUT1 in the retina (Fernandes et al., 2004), GLUT2 in the pancreatic islets (Ohneda et al., 1993) and GLUT4 in the heart (Desrois et al., 2004). Glucose uptake in the skeletal muscle was also reported to be impaired in GK rats (Krook et al., 1997). As a causal factor of GLUT reduction, it has been implicated that over-activation of hexosamine pathway by hyperglycemia decreases the expression level of GLUT2 in the pancreatic β-cells, eventually leading to the inhibition of glucose-stimulated insulin secretion and induction of apoptosis in β-cells (Yoshikawa et al., 2002). The down-regulation of GLUT2 is expected to suppress the glucose uptake and utilization in a specific subset of neurons presumably including BDNF-expressing neurons, which could suppress BDNF mRNA in the VMH of GK rats. In support of this speculation, icv injection of 2-DG in Wistar rats, a procedure used to impair the glucose utilization and mimics the impairment in GK rats, significantly decreased BDNF mRNA level in the VMH. Furthermore, the bath application of 2-DG to the cells isolated from the medial basal hypothalamus including VMH, significantly decreased BDNF mRNA level. Our *in vitro* results demonstrate that BDNF expression in VMH is directly regulated by glucose availability within BDNF neurons. Oomura et al. originally found subpopulations of neurons in the VMH that respond to hyperglycemic or hypoglycemic condition (Oomura et al., 1969; Mizuno and Oomura, 1984; Nakano et al., 1986). Experimental cytosolic Ca^{2+} imaging combined with single-cell RT-PCR showed that up to 60% of glucose-sensing neurons in the VMH express glucokinase mRNA (Kang et al., 2004). It could be speculated that BDNF neurons in VMH have machinery to sense extracellular glucose level and change BDNF mRNA expression. GLUT2 and glucokinase are the candidate machineries. It has been reported that GLUT2 is expressed in a specific subset of VMH neurons in the brain

Table 2 | Effect of BDNF on interscapular, epidiymal, mesenteric and perirenal fat weights (% body weight) in GK rats.

Strain	Treatment	No.	Weight of Fat pad (% BW)			
			Interscapular	Epididymal	Mesenteric	Perirenal
GK	Vehicle	6	0.15 ± 0.01	1.02 ± 0.11	1.14 ± 0.08	1.18 ± 0.14
GK	BDNF	5	0.17 ± 0.02	0.80 ± 0.07	0.80 ± 0.09*	0.97 ± 0.17
Wistar	BDNF	4	0.05 ± 0.01*	0.99 ± 0.08	0.55 ± 0.06*	0.56 ± 0.07*

*Fat weights of saline-treated GK rats (GK-Vehicle group, 5 μl saline/head, n = 6), BDNF-treated GK rats (GK-BDNF group, 15 μg/5 μl saline/head, n = 5) and BDNF-treated Wistar rats (Wistar-BDNF group, 15 μg/5 μl saline/head, n = 4) at Day 21 after the beginning of injections were examined. Interscapular: *p < 0.05 vs. other groups, Mesenteric: *p < 0.05 vs. GK-Vehicle, Perirenal: *p < 0.05 vs. GK-Vehicle, One-Way ANOVA with Holm's post-hoc test.*

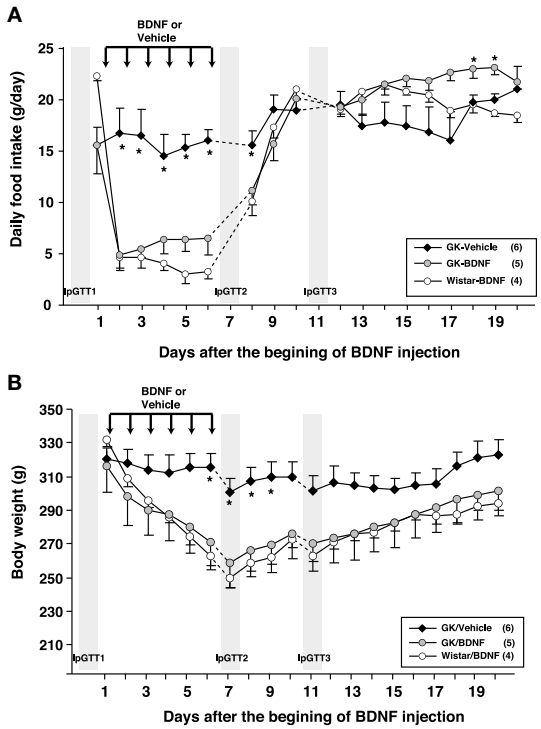

FIGURE 5 | Effect of BDNF treatment on food intake and body weight in GK rats. (A) Daily food intake was examined before and after BDNF treatment (15 μg/5 μl saline/head) for 6 days. BDNF treatment significantly reduced daily food intake during period of treatment in both GK-BDNF and Wistar-BDNF groups. *$p < 0.05$ vs. other groups (One-Way ANOVA with Holm's *post-hoc* test). **(B)** Body weight was examined before and after treatment of BDNF for 6 days. BDNF treatment significantly reduced body weight in both GK-BDNF and Wistar-BDNF groups. *$p < 0.05$ vs. other groups (One-Way ANOVA with Holm's *post-hoc* test).

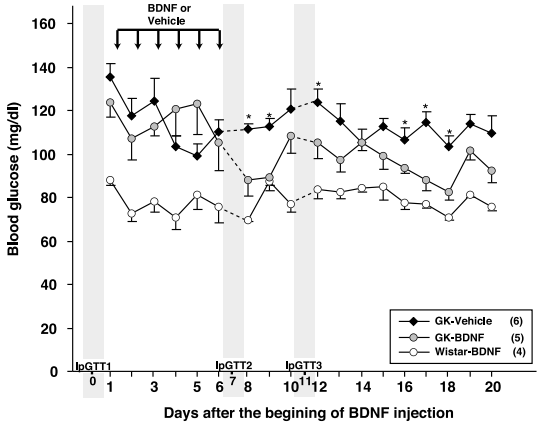

FIGURE 6 | Effect of BDNF treatment on casual blood glucose level in GK rats. Although BDNF did not alter blood glucose level during treatment, significant reduction of blood glucose level was observed after termination of BDNF treatment in GK-BDNF group (*$p < 0.05$ vs. other groups, One-Way ANOVA with Holm's *post-hoc* test).

(Arluison et al., 2004) and certain population of GLUT2-expressing neurons in VMH have a property to respond to change of extracellular glucose concentration (Kang et al., 2004). Considering that both GLUT2-expressing and BDNF-expressing neurons are able to respond to glucose, the two populations could be overlapped. This hypothesis should be examined by either double immunostaining or *in situ* hybridization of BDNF and GLUT2 in future study. Taken together, our finding demonstrates the possibility that the BDNF mRNA expression in the VMH is controlled by glucose metabolism in which glucokinase and GLUT2 might play a role.

In middle-aged GK rats with reduced level of BDNF in VMH, hyperphagia was not found as shown in previous study (Maekawa et al., 2006a). On the other hand, it has been reported that the reduced level of BDNF in medial basal hypothalamus by conditional BDNF gene knockout causes hyperphagia (Unger et al., 2007). What causes the difference between these phenotypes? It has been reported that the BDNF neurons in VMH regulate various physiological functions such as feeding, energy expenditure, and blood glucose control (Noble et al., 2011). It could be speculated that each function is assigned to a specific subgroup of BDNF-expressing neurons in VMH. Since the BDNF-expressing neurons at the level up to 30% of that in Wistar rats persisted in middle-aged GK rats, such subgroup of BDNF neurons might play a role in preventing hyperphagia whereas the subgroup might take no part in the regulation of lipolysis. This possibility should be validated in future study.

CENTRAL BDNF SUPPLEMENTATION AMELIORATED HIGHER ADIPOSITY IN GK RATS

It has been reported that lesioning the VMH induces not only hyperphagia but also a marked visceral fat accumulation in Wistar (Bray and Nishizawa, 1978) and GK rats (Yoshida et al., 1996), and that the loss of neurons in the VMH induces dysfunction of lipolysis. The neurons in VMH are known to project to various

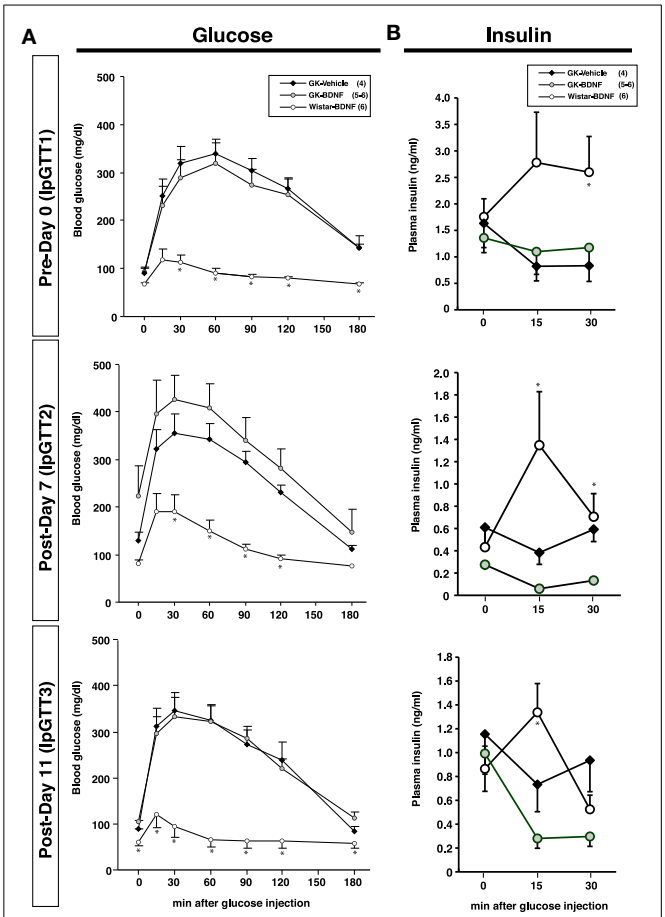

FIGURE 7 | Intraperitoneal glucose tolerance test before and after BDNF treatment in GK rats. BDNF treatment did not attenuate glucose intolerance **(A)** and impaired insulin release **(B)** during glucose tolerance test in GK rats throughout experiment. **(A)** $*p < 0.05$ vs. GK-Vehicle and GK-BDNF (One-Way ANOVA with Holm's *post-hoc* test). **(B)** $*p < 0.05$ vs. GK-Vehicle in Day 0 (30 min), Day 7 (15 min), $*p < 0.05$ vs. GK-BDNF in Day 7 (15 min, 30 min) and 11 (15 min), One-Way ANOVA with Holm's *post-hoc* test.

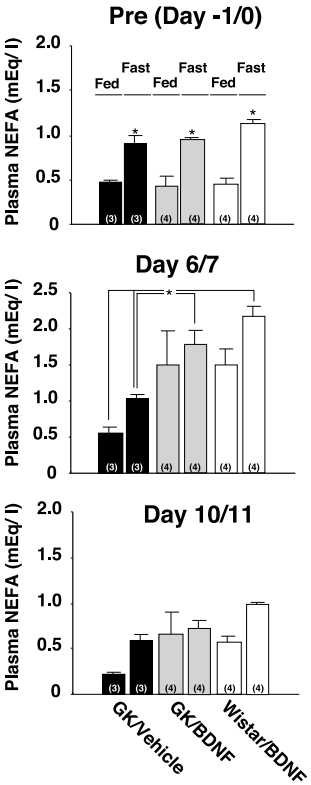

FIGURE 8 | Effect of BDNF on NEFA levels before and after treatment to GK rats in fed and fasted conditions. Plasma NEFA levels in fed and 12 h-fasted conditions at time points before treatment (Day –1/0), immediately after termination of treatment (Day 6/7), and 4/5 days after termination of treatment (Day 10/11) were measured. Plasma NEFA level was higher in fasted condition at all time points measured in all groups [Pre(Day –1/0), $p < 0.05$ vs. fed conditions, Two-Way ANOVA with Tukey's *post-hoc* test]. At time point immediately after termination of treatment (Day 6/7), plasma NEFA levels in Wistar-BDNF and GK-BDNF groups were increased both at fed and fasting conditions. Especially, plasma NEFA levels in Wistar-BDNF and GK-BDNF groups at fasting condition were significantly higher than those in GK-Vehicle group at fasting condition ($*p < 0.05$, Two-Way ANOVA with Tukey's *post-hoc* test). Such tendency continued to Day 10/11 but there was no significant difference at this time point.

brain areas. In our previous report using Wistar rats (Maekawa et al., 2006b), only 19% of BDNF neurons of VMH project to midbrain central gray, a region known to be innervated by VMH neurons. PVN is one of possible candidates for primary synaptic transmission of VMH BDNF neurons. Our previous report suggested that TrkB receptor, a neurotrophin receptor specific for BDNF, is expressed in CRH neurons of PVN and icv BDNF injection increases the CRH mRNA in PVN (Toriya et al., 2010). Although there is no direct evidence that BDNF neurons of VMH are connected to CRH neurons in PVN, these results obtained by physiological experiments indirectly support the possibility that the VMH BDNF neurons project to CRH neurons in PVN. It has been demonstrated that CRH neuron in PVN regulates both sympathetic tone and hypothalamic-pituitary-adrenal axis and that CRH neurons positively control lypolysis (Yada et al., 2012). Our previous report demonstrated that lypolytic effect of BDNF was countracted by simultaneous treatment with CRH receptor antagonist, suggesting that the lypolytic effect of BDNF is mediated by CRH release from CRH neurons in PVN (Toriya et al., 2010). Thus, our present and previous results taken together suggest a possibility that the reduction of BDNF in VMH impairs lipolysis via reducing CRH release in GK rats. Other than CRH release, TrkB and CRH receptor in hypothalamus including PVN might be changed concomitantly with the change in CRH release in GK rats. Such possibilities should also be taken into account when elucidating the etiology of visceral fat accumulation in GK rats.

BDNF has been reported to regulate the binding of cAMP response-element binding protein (CREB) and coactivator proteins to CRH promoter and thereby positively control transcription of CRH (Jeanneteau et al., 2012). Activation of phospholipase Cγ might mediate the BDNF-TrkB signaling by triggering Ca^{2+} increase, activating adenylate cyclase and subsequently increasing cAMP concentration (Ji et al., 2005). On the other hand, BNDF works not only as a neurotransmitter but as a regulator of synaptic structural plasticity (Yoshii and Constantine-Paton, 2010).

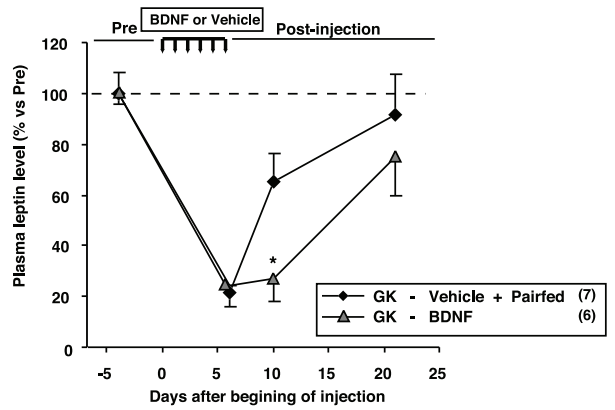

FIGURE 9 | Comparison of plasma leptin levels between GK-BDNF and GK-Vehicle pair-fed groups. In both groups, plasma leptin levels at Day 6 were decreased after BDNF and pair-fed treatments. At Day 10, plasma leptin level in GK-BDNF group remained low while that in GK-Vehicle pair-fed group was significantly elevated (*$p < 0.05$, Student's *t*-test).

The regulation includes spine formation by altered localization of postsynaptic proteins such as PSD-95 (Yoshii et al., 2003). Regulation of synaptic plasticity by BDNF, a process thought to be requisite for learning both at juvenile and adult ages (Poo, 2001; Lu et al., 2008; Suzuki et al., 2012), might also be related to the control of feeding and energy metabolism.

FUTURE PERSPECTIVE

The molecular mechanisms connecting lower glucose metabolism to BDNF reduction remained unclear in this study. One of key proteins that link glucose metabolism to BDNF expression is SIRT1, a metabolic sensor by working as a NAD^+-dependent deacetylase. It has been reported that SIRT1 controls BDNF expression by deacetylating MeCP2 (Zocchi and Sassone-Corsi, 2012), by enhancing CREB-TORC1 transcriptional activity (Jeong et al., 2011), and by suppressing expression of specific microRNA which binds to BDNF mRNA (Gao et al., 2010). Another possible protein to link glucose metabolism to BDNF expression is neuron-restrictive silencer factor (NRSF). The NRSF, a transcriptional repressor, has been reported to recruit the NADH-binding co-repressor CtBP to BDNF promoter. Notably, it has been also reported that glycolysis inhibition by 2-DG injection reduces BDNF transcription by NRSF-CtBP-dependent change of histone modification (Garriga-Canut et al., 2006). Involvements of such proteins to BDNF reduction should be examined in future study.

In this study, we found that BDNF treatments reduce fat mass and lower casual blood glucose level by changing insulin sensitivity. However, BDNF could not restore the insulin secretion in GK rats. Therefore, to improve the glycemic control in type-2 diabetes more effectively, the simultaneous treatment with BDNF and the drug having a function to alleviate impaired insulin secretion might be required. Therapeutic potential of the combination of BDNF with the drug enhancing insulin secretion against diabetes should be investigated in future study.

CONCLUSION

In this study, we present the following scenario to explain how the higher visceral adiposity continues in GK rats until middle-aged adult. (1) The hyperglycemia and/or other diabetes-associated factors suppress the GLUT2 expression to lower the glucose availability in VMH. (2) The reduction of glucose availability leads to impair BDNF expression in VMH. (3) The reduced BDNF level attenuates lypolysis and accelerates glucose intolerance. Further studies are required to validate whether this scenario in GK rats is more generally applicable to other diabetes and/or obesity models.

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APPENDIX

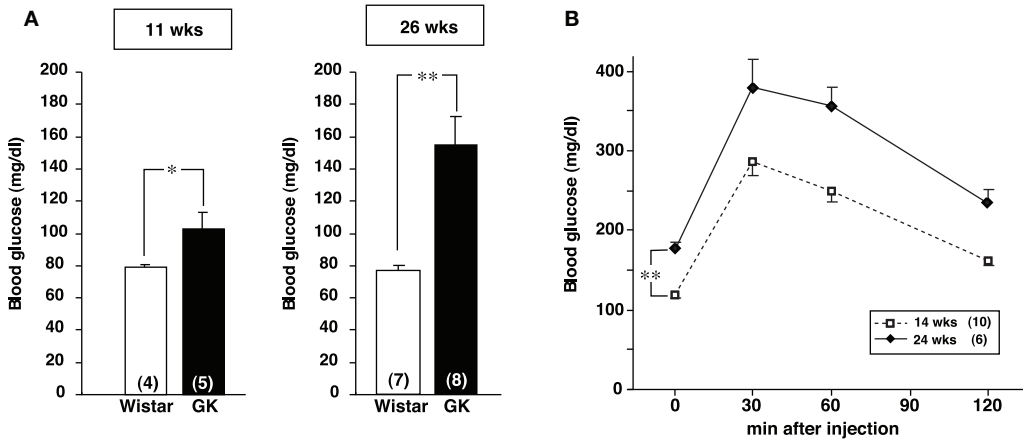

FIGURE A1 | Glycemic profiles of GK rats. (A) The blood glucose level under fed conditions in Wistar and GK rats at the age of 11 and 26 weeks. The blood glucose level in GK rats was significantly higher than that in Wistar rats at both 11 and 26 weeks (*$p < 0.05$ and **$p < 0.01$, respectively, Student's *t*-test). The difference was greater at 26 weeks. **(B)** Profiles of IpGTT in GK rats at 14 and 24 weeks. One g/kg body weight glucose (0.1 g/ml in distilled water) was injected at 0 min and blood glucose was measured at 0, 30, 60, and 120 min after injection. The glucose intolerance was progressed in GK rats at 24 weeks of age (**$p < 0.01$, Student's *t*-test).

Table A1 | Primer sequences used for real-time RT-PCR.

mRNA (Abbreviation)	Genbank accession number (amplicon)	Primers	Probe (5′-FAM, 3′-TAMRA)
Brain-derived neurotrophic factor (BDNF)	NM012513 (504-571)	Fwd: CCATAAGGACGCGGACTTGTAC Rvs: GAGGAGGCTCCAAAGGCACTT	CTTCCCGGGTGATGCTCAGCAGT
Melanocortin-4 receptor (MC4R)	NM013099 (731-819)	Fwd: TGGCGAGGCTTCACATTAAGA Rvs: CAAGGTAATTGCGCCCTTCA	CACGGGTACCATCCGACAGGGTG
Glucose transporter-2 (GLUT2)	NM012879 (818-898)	Fwd: GTCCAGAAAGCCCCAGATACC Rvs: TGCCCCTTAGTCTTTTCAAGCT	TTGCCCTGACTTCCTCTTCCAAATTTAGGT
Glucose transporter-4 (GLUT4)	NM012751 (818-907)	Fwd: CCCCCGATACCTCTACATCATC Rvs: GCATCAGACACATCAGCCCAG	CTGCCCGAAAGAGTCTAAAGCGCCT
Glucose transporter-8 (GLUT8)	NM053494 (1049-1235)	Fwd: TCATGGACAGAGCAGGGCG Rvs: GCCAGCCAGGCCAGCCCCA	Not used
Glucokinase	NM012565 (1330-1414)	Fwd: CAAGCTGCACCCGAGCTT Rvs: TGATTCGATGAAGGTGATTTCG	TCAGCCTGCGCACACTGGCG

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Leptin regulation of neuronal morphology and hippocampal synaptic function

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The central actions of the hormone leptin in regulating energy homeostasis via the hypothalamus are well documented. However, evidence is growing that this hormone can also modify the structure and function of synapses throughout the CNS. The hippocampus is a region of the forebrain that plays a crucial role in associative learning and memory and is an area also highly vulnerable to neurodegenerative processes. Recent studies indicate that leptin is a potential cognitive enhancer as it modulates the cellular processes underlying hippocampal-dependent learning and memory including dendritic morphology, glutamate receptor trafficking and activity-dependent synaptic plasticity. Here, we review the recent evidence implicating the hormone leptin as a key regulator of hippocampal synaptic function and discuss the role of leptin receptor-driven lipid signaling pathways involved in this process.

Keywords: leptin, synaptic plasticity, morphology, receptor trafficking, hippocampus, AMPA receptor, PTEN

INTRODUCTION

LEPTIN RECEPTOR SIGNALING

The endocrine hormone, leptin is a 167 amino acid protein that is mainly produced by adipocytes and circulates in the plasma at levels closely correlated with body fat content (Maffei et al., 1995). Although leptin acts on a number of peripheral tissues, leptin also readily targets the CNS, following its transport across the blood brain barrier. Several lines of evidence indicate the expression of leptin mRNA and protein in a number of brain regions, thereby raising the possibility that leptin is also synthesized and released locally in the CNS. The most well documented roles for leptin focus on its actions in the hypothalamus where it participates in the regulation of energy homeostasis, bone formation as well as reproductive function (Spiegelman and Flier, 2001; Karsenty, 2006). However the central actions of leptin are not confined to the hypothalamus. Indeed, numerous studies have detected high levels of leptin receptor expression in extra-hypothalamic brain regions and evidence is growing that leptin has widespread actions throughout the CNS.

The biological actions of leptin are mediated by leptin receptors; members of the class I cytokine receptor superfamily (Tartaglia et al., 1995). The leptin receptor (ObR) is encoded by the diabetes gene (*db*) and alternative splicing of this gene generates six leptin receptor isoforms (ObRa-f) with identical N-terminal domains but vary in the length of their C-terminal region. All the isoforms, except ObRe, have a membrane spanning region consisting of 34 amino acids. ObRe is thought to buffer free circulating leptin levels in the plasma. The membrane spanning ObRs all contain an intracellular proline-rich box which enables association with janus tyrosine kinases, in particular JAK2. However, only the long leptin receptor isoform (ObRb) contains additional intracellular signaling motifs necessary for full JAK-STAT (signal transducer and activation of transcription) signaling. Following leptin binding to ObRb and JAK2 activation, JAK2 associates with and promotes phosphorylation of tyrosine residues within the C-terminal domain. The phosphorylated tyrosine residues enable recruitment and activation of various downstream signaling pathways including the STAT (signal transducers and activators of transcription) family of transcription factors, phosphoinositide 3-kinase as well as adaptor proteins associated with the Ras-Raf-MAPK (mitogen activated protein kinase) pathway. Leptin is capable of activating all of these ObR-driven signaling in central neurons (Harvey, 2003).

LEPTIN RECEPTOR EXPRESSION IN THE CNS

In accordance with its role in regulating energy balance, high levels of leptin receptor expression have been detected on specific hypothalamic nuclei involved in energy homeostasis (Schwartz et al., 1996). Leptin receptors are also expressed throughout the CNS with high levels of expression reported in the amygdala, brainstem and cerebellum. High levels of ObR mRNA and immunoreactivity have also been detected in hippocampal CA1, CA3, and dentate gyrus regions (Mercer et al., 1996; Håkansson et al., 1998). In hippocampal cultures, ObR expression is localized to principal neurons and glial cells. Moreover, dual labeling studies indicate that ObR is expressed at somato-dendritic regions and also in close proximity to NMDA receptors (Shanley et al., 2002b; O'Malley et al., 2007). Although the main source of leptin is from peripheral tissues, leptin readily gains access to the brain via a saturable transport process at the blood brain barrier (Banks et al., 1996). Short ObR isoforms expressed on brain microvessels are implicated in this process. High levels of ObRa are also expressed at the choroid plexus thereby supporting a possible role for the cerebrospinal fluid in transporting leptin to the CNS. Direct diffusion of leptin to the hypothalamus may also occur as the main leptin-sensitive hypothalamic neurons are located close to the median eminence.

LEPTIN REGULATION OF HIPPOCAMPAL SYNAPTIC FUNCTION

The hippocampus is a key brain area involved in learning and memory. Activity-dependent forms of hippocampal synaptic plasticity, such as long-term potentiation (LTP) and long-term depression (LTD) are key cellular events underlying learning, memory and habituation (Bliss and Collingridge, 1993). Recent studies indicate that leptin can modify excitatory synaptic transmission at hippocampal CA1 synapses (Shanley et al., 2001; Xu et al., 2008). Various forms of activity-dependent hippocampal synaptic plasticity are also regulated by this hormone. Indeed, electrophysiological studies have detected impairments in both LTP and LTD at hippocampal CA1 synapses in slices from leptin-insensitive rodents (*fa/fa* rats; *db/db* mice; Li et al., 2002). Defects in hippocampal memory tasks have also been observed in leptin-insensitive rodents (Li et al., 2002; Farr et al., 2006). Administration of leptin directly into the hippocampus not only enhances LTP but also boosts hippocampal-dependent learning and memory (Wayner et al., 2004). Cellular studies performed by Shanley et al. (2001) established that exposure to leptin promotes the conversion of short-term potentiation (STP) into LTP in acute hippocampal slices.

Although NMDA receptors contribute little to basal synaptic transmission, activation of NMDA receptors is essential for the induction of hippocampal LTP (Collingridge et al., 1983). Like other hormones and growth factors, leptin potently enhances NMDA receptor function which contributes to its ability to facilitate LTP. Indeed, leptin is capable of enhancing Ca^{2+} influx via native NMDA receptor channels in hippocampal cultures and NMDA evoked currents in *Xenopus* oocytes expressing recombinant NMDA receptors (Shanley et al., 2001; Harvey et al., 2005). Conversely, other studies have reported either no effect or attenuation of NMDA responses by leptin in hippocampal neurons (Oomura et al., 2006) which may be due to differences in the experimental approach or age of tissue used. Nevertheless, more recent studies support a crucial role for NMDA receptors, as distinct GluN2 subunits play a key role in leptin's effects on excitatory synaptic transmission at different stages of postnatal development and aging (Moult and Harvey, 2011). In addition, divergent signaling pathways are implicated in the age-dependent effects of leptin such that the ERK pathway mediates the synaptic depressions evoked by leptin early in postnatal development whereas leptin-driven increases in excitatory synaptic strength in adult are PI 3-kinase dependent (Moult and Harvey, 2011). Previous studies revealed that distinct signaling pathways link leptin receptors to facilitation of molecularly distinct NMDA receptors in cerebellar granule cells (Irving et al., 2006). Thus, early in postnatal development it is possible that leptin driven ERK activation facilitates GluN2B-mediated responses thereby resulting in either persistent or transient depressions in excitatory synaptic transmission. Conversely in adult hippocampus, leptin increases GluN2A-mediated responses via PI 3-kinase which in turn promotes a long lasting increase in synaptic efficacy (Moult and Harvey, 2011; **Figure 1**).

LEPTIN-INDUCED LTD

Two main forms of LTD occur at mammalian central synapses that are generated by the synaptic activation of NMDA and metabotropic glutamate receptors, respectively (Massey and Bashir, 2007). Several studies have shown that hormones and growth factors are also capable of inducing novel forms of LTD. Similarly, under conditions of enhanced excitability, exposure to leptin results in the induction of a novel form of NMDA receptor-dependent hippocampal LTD (Durakoglugil et al., 2005). Leptin-induced LTD shares similar expression mechanisms to activity-dependent LTD as LTD induced by low frequency stimulation (LFS) occludes leptin-induced LTD (Durakoglugil et al.,

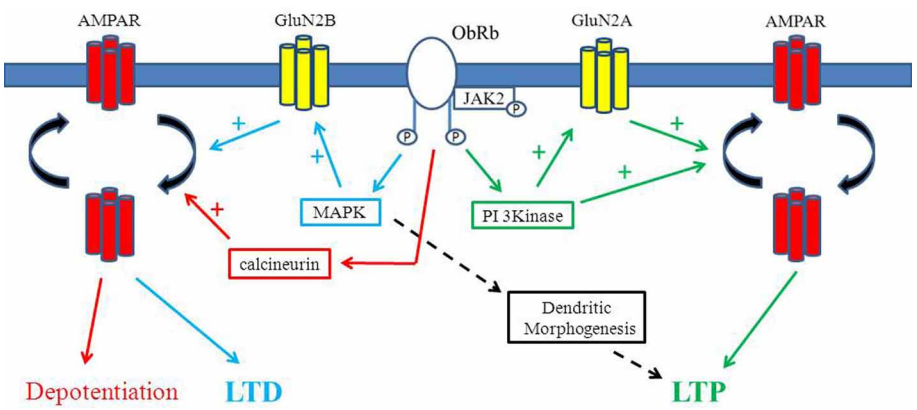

FIGURE 1 | Divergent effects of leptin on hippocampal synaptic function. Schematic representation of the key signaling pathways that contributes to the diverse effects of leptin in the hippocampus. Activation of leptin receptors triggers PI 3-kinase stimulation which results in AMPA receptor exocytosis and a sustained increased in synaptic efficacy (leptin-induced LTP). Leptin driven stimulation of PI 3-kinase also enhances GluN2A activity which in turn promotes AMPA receptor delivery to synapses and subsequent LTP at adult hippocampal CA1 synapses. In contrast, at early stages of postnatal development, leptin dependent activation of the ERK (MAPK) signaling cascade facilitates GluN2B-mediated responses resulting in either persistent (LTD) or transient depressions in excitatory synaptic transmission. Leptin is also capable of depotentiating hippocampal synapses via a process involving the activation of calcineurin and subsequent endocytosis of GluA2-lacking AMPA receptors. Rapid alterations in dendritic morphology are also evoked by leptin that are mediated by the MAPK (ERK) signaling cascade.

2005). Moreover, like *de novo* LTD, leptin-induced LTD has a postsynaptic locus of expression as no alteration in the paired pulse ratio was observed during leptin-induced LTD. Moreover, inhibition of PI 3-kinase increased the magnitude of leptin-induced LTD suggesting that PI 3-kinase negatively regulates this process. Similarly, inhibitors of serine/threonine protein phosphatase 1/2A, but not 2B, also enhanced the synaptic depression induced by leptin, indicating that leptin-induced LTD is negatively regulated by phosphatase 1/2A (Durakoglugil et al., 2005).

LEPTIN REVERSES ESTABLISHED LTP

It is known that LTP can be readily reversed shortly after induction via a process known as depotentiation. This phenomenon is thought to boost the capacity of neuronal networks to store information by preventing saturation of potentiated synapses. Several studies indicate that application of theta burst stimuli or LFS can reverse LTP at hippocampal CA1 synapses (Bashir and Collingridge, 1994). Recent evidence indicates that leptin can also reverse established LTP, in a time and concentration-dependent manner (Moult et al., 2009). The ability of leptin to depotentiate hippocampal CA1 synapses is not associated with any change in paired-pulse ratio or coefficient of variance (CV), suggesting a postsynaptic expression mechanism. Leptin-induced depotentiation also requires the activation of NMDA receptors as blockade of NMDA receptors with D-AP5 prevented the effects of leptin. A decrease in the rectification properties of synaptic AMPA receptors accompanied leptin- induced depotentiation. Moreover the effects of leptin were mirrored by application of philanthotoxin, a selective inhibitor of GluA2-lacking AMPA receptors, indicating that a reduction in the density of GluA2-lacking AMPA receptors at hippocampal synapses underlies leptin-induced depotentiation (Moult et al., 2009). The involvement of AMPA receptor internalization in leptin-induced depotentiation displays parallels to other studies as removal of AMPA receptors from synapses also contributes to the reversal of hippocampal LTP by LFS, mGluRs, and neuregulin (Zho et al., 2002; Kwon et al., 2005).

Previous studies have identified a role for the stress-activated protein kinase JNK (c-Jun N-terminal kinase) in LFS-induced depotentiation (Zhu et al., 2005). In contrast, however, treatment of slices with selective inhibitors of JNK failed to alter the magnitude of leptin-induced depotentiation suggesting involvement of a JNK-independent process (Moult et al., 2009). However, calcineurin (protein phosphatase 2B) is implicated in NMDA receptor-driven removal of GluA2-lacking AMPA receptors from synapses (Beattie et al., 2000). In accordance with this, leptin-induced depotentiation is blocked by selective inhibitors of calcineurin, indicating a role for PP2B in this process (**Figure 1**). As dephosphorylation of GluA1 on Ser845 is pivotal for NMDA receptor-driven AMPA receptor endocytosis (Man et al., 2007), it is likely that leptin promotes the activation of calcineurin and subsequent dephosphorylation of GluR1 which in turn results in removal of GluA2-lacking AMPA receptors from hippocampal synapses.

LEPTIN EVOKES A NOVEL FORM OF LTP IN ADULT HIPPOCAMPUS

Application of leptin to acute hippocampal slices from juvenile (2–3 weeks old) rats results in a transient and readily reversible depression of excitatory synaptic transmission (Shanley et al., 2001; Xu et al., 2008). Conversely, in adult leptin evokes a long lasting increase in hippocampal excitatory synaptic strength (leptin-induced LTP) which is sustained following washout of leptin (Moult et al., 2010). Activation of leptin receptors is crucial for leptin-induced LTP as leptin has no effect in slices from Zucker *fa/fa* rats, whereas robust LTP is observed in Zucker lean animals. NMDA receptor activation is also essential as NMDA receptor blockade with D-AP5 prevents leptin-induced LTP (Moult et al., 2010). The ability of leptin to induce LTP in adult is likely to be mediated by GluN2A containing NMDA receptors as the leptin-driven increase in synaptic efficacy is completely blocked by NVP-AAM077, an NMDA receptor antagonist with preferential selectivity for GluN2A subunits (Auberson et al., 2002), but is unaffected by inhibition of GluN2B subunits with ifenprodil (Moult and Harvey, 2011; **Figure 1**).

Activation of NMDA receptors is pivotal for the induction of hippocampal LTP (Collingridge et al., 1983) as well as promoting AMPA receptor trafficking to synapses during LTP (Collingridge et al., 2004). Recent evidence indicates that hippocampal synaptic plasticity is also associated with alterations in the subunit content of AMPA receptors as synaptic activity increases the density of synaptic GluA2-lacking AMPA receptors (Isaac et al., 2007). Alterations in the molecular composition of AMPA receptors also accompany leptin-induced LTP as AMPA receptor rectification increases after exposure to leptin whereas application of philanthotoxin to block GluA2-lacking AMPA receptors reverses the leptin-induced increase in synaptic efficacy (Moult et al., 2010). The surface expression of GluA1, but not GluA2, subunits is also elevated in hippocampal slices and cultured neurons treated with low nanomolar concentrations of leptin.

PI 3-kinase, which catalyses phosphorylation of PtdIns(45)P_2 into PtdIns(3,4,5)P_3, is not only an integral part of neuronal leptin receptor-driven signaling but it is also critical for trafficking AMPA receptors to hippocampal synapses during LTP (Man et al., 2003). Similarly, the ability of leptin to increase GluA1 surface expression involves activation of PI 3-kinase as an increase in PtdIns(3,4,5)P_3 levels accompanied the effects of leptin and the leptin-driven increase in both GluA1 expression and PtdIns(3,4,5)P_3 levels were blocked by selective PI 3-kinase inhibitors. However, PtdIns(3,4,5)P_3 levels are also controlled by PTEN, a phosphatase that promotes conversion of PtdIns(3,4,5)P_3 to PtdIns(4,5)P_2, and thus antagonizes PI 3-kinase activity. In support of a role for PTEN, the leptin-driven increase in GluA1 surface expression is combined with an elevation in P366-PTEN staining in hippocampal cultures. Moreover, application of leptin to hippocampal slices resulted in a robust increase in the phosphorylation of PTEN (Moult et al., 2010). In accordance with studies revealing that casein kinase 2 (CK2) phosphorylates PTEN, inhibition of CK2 prevented the effects of leptin on GluA1 surface expression, PTEN phosphorylation and excitatory synaptic transmission. Thus, it is feasible that leptin activates CK2 which phosphorylates and inhibits PTEN thereby increasing PtdIns(3,4,5)P_3 levels and in turn driving alterations in AMPA receptor trafficking and excitatory synaptic strength.

In further support of a role for PTEN, expression of dominant-negative PTEN mutants (C124S or G129E) in neurons

or pharmacological inhibition of PTEN with the phosphatase inhibitor bisperoxovanadium (Bpv) mirror and occlude the effects of leptin on GluA1 trafficking and hippocampal synaptic function (Moult et al., 2010). As inhibition of PTEN leads to an elevation in PtdIns(3,4,5)P_3 levels, it is likely that alterations in the levels of this inositol lipid are crucial for modifying AMPA receptor trafficking processes. Recent evidence supports this possibility as the availability of PtdIns(3,4,5)P_3 is reported to maintain AMPA receptor clustering and synaptic function at hippocampal synapses (Arendt et al., 2010). Although the exact role of PtdIns(3,4,5)P_3 remains to be determined, it is likely that PtdIns(3,4,5)P_3 influences AMPA receptor trafficking by either altering actin cytoskeletal dynamics (Zhou et al., 2001) or promoting Akt-driven inhibition of glycogen synthase kinase 3 (GSK-3; Peineau et al., 2007).

LEPTIN PROMOTES MORPHOLOGICAL CHANGES IN HIPPOCAMPAL DENDRITES

Alterations in the morphology of dendrites and spines are reported to occur after hippocampal activity-dependent synaptic plasticity and these structural changes are thought to contribute to modifications in excitatory synaptic strength (Maletic-Savatic et al., 1999). In addition, several hormones are reported to induce rapid morphological changes in neurons which can enable further regulation of neuronal connectivity and synaptic strength. Likewise, exposure of hippocampal neurons to leptin results in marked changes in the density and motility of dendritic filopodia within a few minutes (O'Malley et al., 2007). Time lapse confocal microscopy studies also reveal that leptin rapidly increases the density and motility of dendritic filopodia in hippocampal neurons transfected with a cytosolic EGFP construct (O'Malley et al., 2007). The actin cytoskeleton is reported to play a key role in the morphological changes that occur during synaptic plasticity (Matus, 2000; Smart and Halpain, 2000). Thus, as leptin can rapidly alter the structure of actin filaments in hippocampal neurons and hypothalamic cell lines (O'Malley et al., 2005; Ning et al., 2006) it is feasible that leptin-driven formation of new filopodia involves alterations in actin cytoskeletal dynamics. Indeed, the leptin-dependent increase in dendritic filopodia is accompanied by a significant reduction in polymerized actin staining in proximal dendrites, which is consistent with leptin driven redistribution of actin filaments from the dendritic shaft to dendritic filopodia.

It has been shown that activation of synaptic NMDA receptors stimulates the appearance of dendritic protrusions (Maletic-Savatic et al., 1999) and neuronal exposure to glutamate regulates the formation and motility of dendritic filopodia (McKinney et al., 1999; Fischer et al., 2000). In accordance with these studies, the synaptic activation of NMDA receptors is pivotal for the effects of leptin as the morphology of dendrites is unaffected by leptin in neurons treated with either tetrodotoxin or the competitive NMDA receptor antagonist, D-AP5. However, blockade of GluN2B containing NMDA receptors with ifenprodil failed to alter the leptin-driven structural changes indicating the likely involvement of GluN2A containing NMDA receptors (O'Malley et al., 2007). As GluN2A subunits are predominantly expressed at synaptic loci, it is likely that the activation of synaptic, as oppose to extrasynaptic NMDA receptors is pivotal for the morphological changes induced by leptin. It is known that PI 3-kinase and MAPK are two of the major signaling pathways activated by neuronal leptin receptors (Niswender et al., 2001; Harvey, 2003; Irving et al., 2006). Moreover, the ability of leptin to enhance NMDA receptor function in the hippocampus is mediated by both PI 3-kinase and MAPK (Shanley et al., 2001). However, only the MAPK signaling cascade is required for the rapid structural changes induced by leptin as inhibitors of MEK but not PI 3-kinase attenuated the effects of leptin on dendritic morphology (**Figure 1**). Our previous studies indicate that actin filament reorganization underlies the activation of large-conductance Ca^{2+}-activated K^+ (BK) channels by leptin (Shanley et al., 2002a; O'Malley et al., 2005). However, a PI 3-kinase, rather than MAPK- dependent process mediates actin filament disruption and subsequent activation of BK channels by leptin (Shanley et al., 2002a). Activation of PI 3-kinase by leptin has been shown to trigger localized elevations in phosphatidylinositol-3,4,5-triphosphate [PtdIns(3,4,5)P_3; (O'Malley et al., 2005)] levels in close proximity to the plasma membrane. Thus, it is likely that leptin-driven reorganization of actin filaments via this pathway occurs in a highly compartmentalized manner. In contrast, however, activation of MAPK by leptin may promote more extensive alterations in the actin cytoskeleton as numerous downstream targets for MAPK are widely expressed in dendritic and somatic regions in hippocampal neurons (Thomas and Huganir, 2004).

During the earliest stages of synaptogenesis there is increased movement and extension of dendritic filopodia (Fiala et al., 1998; Munno and Syed, 2003). Dendritic filopodia are also thought to play an active role in initiating synaptic contacts during synaptogenesis (Ziv and Smith, 1996). Thus, it is feasible that leptin, by increasing the density and motility of dendritic filopodia, alters the number of synaptic connections. Indeed, O'Malley et al. (2007) evaluated if leptin altered synaptic connectivity using immunocytochemical approaches to assay the relative number of presynaptic terminals. Exposure of hippocampal cultures to leptin for 30 min resulted in elevations in the density of actin-rich spines and also synapsin-1-positive puncta, which is consistent with leptin increasing the number of hippocampal synapses (O'Malley et al., 2007).

The structural changes induced by leptin display many parallels to the morphological changes observed following activity-dependent synaptic plasticity. Indeed, increases in the density of actin-rich spines and dendritic filopodia occur following hippocampal LTP, which parallels the morphological changes induced by leptin in hippocampal neurons (Maletic-Savatic et al., 1999; Fukazawa et al., 2003). However, the appearance of dendritic filopodia has also been linked to the induction of hippocampal LTD (Bourne and Harris, 2007). Thus, the possibility that leptin-driven alterations in dendritic filopodia play a role in the persistent synaptic depressions induced by leptin cannot be excluded. The time course of the morphological changes induced by leptin is similar to those associated with LTP (Maletic-Savatic et al., 1999), and like LTP, the rapid growth of dendritic filopodia induced by leptin requires the synaptic activation of NMDA receptors (Maletic-Savatic et al., 1999).

Activation of MAPK is implicated in the structural changes associated with activity-dependent synaptic plasticity as NMDA receptor activation promotes increases in MAPK activity and dendritic spine density in hippocampal neurons (Goldin and Segal, 2003), whereas new hippocampal dendritic spines and filopodia are formed after sustained activation of MAPK (Wu et al., 2001). Similarly, a role for MAPK is also thought to underlie the effects of leptin as leptin failed to induce structural changes following blockade of MAPK activation (O'Malley et al., 2007).

In addition to regulating hippocampal neuron morphology, recent evidence indicates that leptin has the capacity to alter the morphology of neuronal populations. Thus, leptin promotes neurite outgrowth from hypothalamic (Bouret et al., 2004) and cerebellar purkinje neurons (Oldreive et al., 2008). Exposure to leptin also alters the size of axonal growth cones in cortical neurons (Valerio et al., 2006). Studies in leptin deficient (*ob/ob*) mice have identified significant alterations in the density of both excitatory and inhibitory synapses compared to wild type mice (Pinto et al., 2004). Furthermore, these alterations are normalized within 6 h of systemic administration of leptin to *ob/ob* mice. However, the morphological changes induced by leptin in hypothalamic, cerebellar, and cortical neurons occur on a much slower time scale than in the hippocampus, as structural changes were only evident after treatment with leptin for several hours (Bouret et al., 2004; Valerio et al., 2006; Oldreive et al., 2008).

POTENTIAL CROSS-TALK BETWEEN LEPTIN AND OTHER HORMONAL SYSTEMS IN THE HIPPOCAMPUS

Numerous studies indicate that leptin is an important modulator of activity-dependent synaptic plasticity at different stages of postnatal development and aging. However, although numerous growth factors and hormones are capable of regulating hippocampal synaptic function, it remains to be determined if the effects of leptin are influenced by other hormonal systems. It is feasible that there is not only convergence of function but also potential cross-talk between hormonal systems at the level of the signal transduction pathways. Indeed, the endocrine hormone insulin that is secreted by pancreatic beta cells in response to elevated glucose levels displays many parallels to leptin in terms of its central actions and also the signaling pathways that it activates. Thus, in a manner similar to leptin, insulin reduces food intake when administered centrally (Schwartz et al., 1992). Insulin derived from peripheral sources is also readily transported into the brain where it can regulate hippocampal synaptic function. Indeed, at hippocampal CA1 synapses, insulin promotes endocytosis of GluA2 and subsequent induction of a novel form of NMDA receptor-dependent LTD (Man et al., 2000; Ahmadian et al., 2004; Huang et al., 2004). Like leptin, insulin is capable of facilitating NMDA responses and increasing NMDA receptor exocytosis (Liu et al., 1995; Skeberdis et al., 2001). Furthermore, exposure of hippocampal neurons to insulin stimulates an increase in the cell surface expression of GluA1 (Man et al., 2000; Passafaro et al., 2001), which parallels the actions of leptin on GluA1 trafficking processes (Moult et al., 2010). Thus, given the overlapping functional effects of leptin and insulin in the hippocampus, it is likely that there is also cross-talk between the hormonal driven signaling pathways in this brain region. This possibility is supported by studies in hypothalamic neurons as insulin not only mimics the ability of leptin to activate ATP-sensitive K^+ (K_{ATP}) channels but channel activation by both hormones involves a PI 3-kinase-dependent process (Harvey et al., 2000; Spanswick et al., 2000). PI3-Kinase is also implicated in the activation of hypothalamic large-conductance Ca^{2+}-activated K^+ (BK) channels by leptin and insulin (Yang et al., 2010). Thus, modulation of PI3K signaling may act as a point of convergence for the regulation of hippocampal synaptic function by the leptin and insulin hormonal systems.

Both the PI 3-Kinase and the Ras/Raf/MEK signaling pathways are activated by other class I cytokines, including interleukin 6 (IL-6). Moreover, as evidence is growing that IL-6 is a potent regulator of hippocampal synaptic function (Tancredi et al., 2000; Nelson et al., 2012), the possibility that there is also convergence between leptin and IL-6 driven signaling cascades in hippocampal neurons cannot be excluded. It is also feasible that other hormonal systems indirectly influence leptin-driven regulation of hippocampal synaptic function by modulating leptin levels. In support of this possibility, insulin-like growth factor-1 (IGF-1) is reported to increase the expression of leptin in organotypic hippocampal slices (Marwarha et al., 2011). Although there is some evidence supporting potential interactions between leptin and other hormonal systems in the hippocampus, it is clear that a greater understanding of the interplay between different hormones is required. In particular it is key that the complex cellular events underlying synaptic plasticity and how such processes are modulated by hormonal systems are more fully understood.

CONCLUSION

It is well established that the endocrine hormone leptin regulates many central processes including energy homeostasis. However, evidence is growing that the structure and function of hippocampal CA1 synapses is also markedly influenced by leptin. Recent studies indicate that leptin has cognitive enhancing properties as it rapidly alters glutamate receptor trafficking, dendritic morphology and different forms of activity-dependent hippocampal synaptic plasticity. Regulation of NMDA receptor activity by leptin appears to be key for its ability to influence multiple aspects of hippocampal synaptic function, although it is not entirely clear how leptin-driven activation of NMDARs leads to such opposing effects on hippocampal synaptic function. However, emerging studies indicate not only that distinct NMDA receptor subunits are pivotal for leptin's effects on excitatory synaptic transmission, but also that distinct signaling pathways couple leptin receptors to molecularly distinct NMDA receptors at different developmental stages. Although it is established that leptin plays a pivotal role in normal brain function, disruption of the leptin system is also linked to neurodegenerative disorders, like Alzheimer's disease. Thus, the ability of leptin to regulate neuronal morphology and synaptic efficacy is likely to have important implications not only in health but also in diseases associated with leptin dysfunction.

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The best-laid plans go oft awry: synaptogenic growth factor signaling in neuropsychiatric disease

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Growth factors play important roles in synapse formation. Mouse models of neuropsychiatric diseases suggest that defects in synaptogenic growth factors, their receptors, and signaling pathways can lead to disordered neural development and various behavioral phenotypes, including anxiety, memory problems, and social deficits. Genetic association studies in humans have found evidence for similar relationships between growth factor signaling pathways and neuropsychiatric phenotypes. Accumulating data suggest that dysfunction in neuronal circuitry, caused by defects in growth factor-mediated synapse formation, contributes to the susceptibility to multiple neuropsychiatric diseases, including epilepsy, autism, and disorders of thought and mood (e.g., schizophrenia and bipolar disorder, respectively). In this review, we will focus on how specific synaptogenic growth factors and their downstream signaling pathways might be involved in the development of neuropsychiatric diseases.

Keywords: synapse, synaptogenesis, growth factor, psychiatry, mental illness

INTRODUCTION

Neuropsychiatric diseases are increasingly recognized to have developmental origins. Some of these illnesses, such as autism and ADHD, must be diagnosed based on symptoms identified during early childhood (Association, 2013). Others, such as bipolar disorder and schizophrenia, are usually diagnosed in adulthood, but are recognized to have some manifestations in childhood as well (Martin and Smith, 2013; Schulz et al., 2014). Although these illnesses were initially studied in isolation from each other, there is increasing evidence that these clinically disparate diseases may have common genetic origins (Smoller and Finn, 2003; Lichtenstein et al., 2010; Sullivan et al., 2012; Cross-Disorder Group of the Psychiatric Genomics et al., 2013). To take this idea further, if these diseases begin early in development and have identifiable common genetic origins, it is possible, and perhaps even likely, that perturbations in some common developmental pathways may be involved in their pathogenesis.

One major set of signaling molecules that are important in neural development are synaptogenic growth factors. These growth factors, including brain-derived neurotrophic factor (BDNF), the fibroblast growth factor (FGF) family, Wnts, and insulin-like growth factors (IGFs), are important not only in cell fate specification and neurogenesis, but specifically in the formation and maintenance of synapses (Vicario-Abejon et al., 1998; Barros et al., 2009; Terauchi et al., 2010; Guillemot and Zimmer, 2011; Corvin et al., 2012; Rosso and Inestrosa, 2013). Appropriate partnering of pre- and postsynaptic neurons is critical for the establishment of individual neuronal circuits, which in turn is the fundamental basis of overall wiring of the functional brain. Problems in these synaptogenic signaling pathways, which could occur either due to mutations in individual growth factors or their receptors, or inappropriate conduction of those signals through intracellular signaling pathways, could lead to abnormal connections between neurons or aberrant neuronal circuitry (**Figure 1**).

Several lines of evidence suggest that synaptogenic growth factors are involved in the pathogenesis of neuropsychiatric diseases. First, it is known that many mouse models with mutations in synaptogenic growth factors or their receptors have behavioral abnormalities, which may be analogous to neuropsychiatric disease in humans. For example, mice lacking FGF7 are predisposed to epilepsy in a kindling protocol (Terauchi et al., 2010). Second, some humans with mutations in growth factors have observable behavioral and cognitive problems. For example, people with a valine to methionine substitution at position 66 (V66M) in the proBDNF polypeptide have impaired episodic memory and increased risk of mood disorders (Egan et al., 2003; Schumacher et al., 2005). Finally, there is growing evidence that maintenance of proper networks and synaptogenesis and plasticity are impaired in neuropsychiatric illnesses (Brennand et al., 2011; Uddin et al., 2013), and growth factors are known to have a major role in all of these processes.

We propose that the critical stage of interest for studying these illnesses is during synaptogenesis, as this is when neurons are wired together to form functional circuits. For our purposes, "synaptogenesis" includes synapse development, maturation, and maintenance, as these steps are all essential for a mature, functional synapse. It is important to note that synapse maturation and modulation occur throughout life, and are likely to contribute to variations in disease presentation as development progresses. For example, FGF2 has been hypothesized as an "online" modulator of mood and anxiety in adults (Turner et al., 2006). Synaptogenic growth factors are released from both the pre- and postsynaptic neurons to assist synaptogenesis (**Figure 2**).

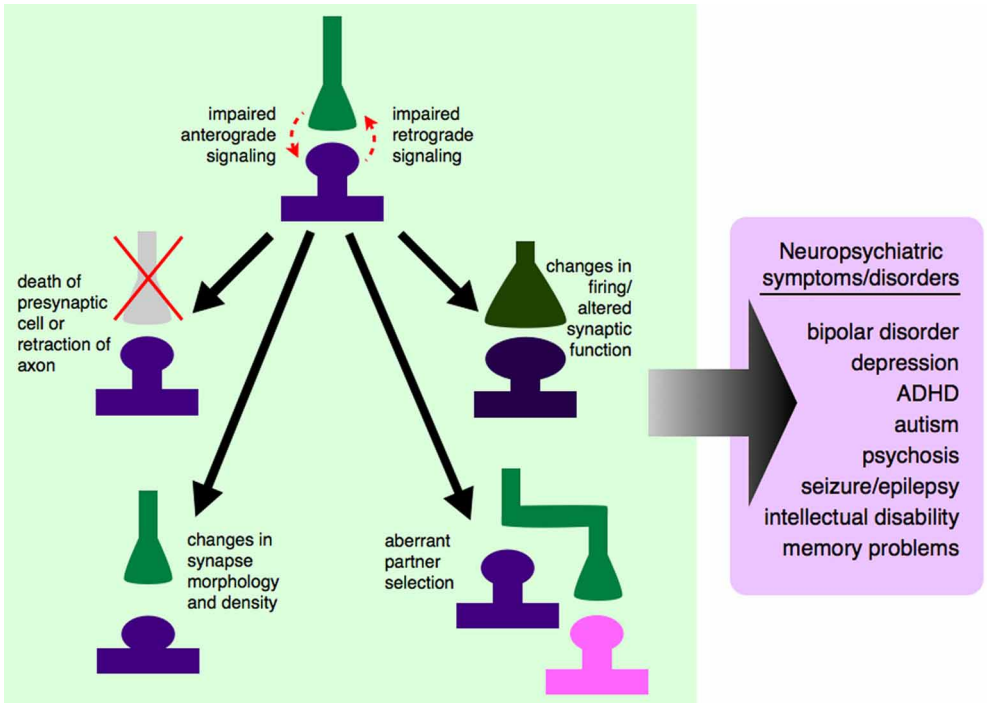

FIGURE 1 | Dysregulated growth factor signaling can lead to synaptic-level defects and neuropsychiatric disease. Synaptogenic growth factors signal in both anterograde and retrograde directions, depending on the specific growth factor involved. If this signaling is disrupted, due to genetic mutations, changes in expression level, or changes in secretion pattern, synapses will not be established properly. Some growth factors exert trophic functions at the level of the synapse, and dysregulated signaling could lead to the death of the presynaptic cell or retraction of that axon from its appropriate postsynaptic partner. Even if the presynaptic axon is not retracted, impaired growth factor signaling between synaptic partners could cause changes in synaptic morphology and density, and ultimately to a non-functional synapse. Impaired signaling could also lead an axon to bypass its correct partner entirely and establish a synapse with a non-preferred partner, leading to aberrant neuronal circuitry. If appropriate signals are not passed between pre- and postsynaptic cells, this could lead to changes in action potential firing rates and altered communication between cells. It is still unclear which of these processes contribute to which neuropsychiatric diseases, although there are data to support neuron and synapse loss in certain cortical and hippocampal areas in mood disorders like depression and bipolar disorder (Manji et al., 2001; Stockmeier et al., 2004; Stockmeier and Rajkowska, 2004), aberrant brain connectivity in autism (Chung et al., 2013; Lynch et al., 2013; Uddin et al., 2013), and aberrant feed-forward loops (Yilmazer-Hanke et al., 2007) and neuronal circuitry (Aliashkevich et al., 2003) in epilepsy.

Other developmental processes, such as neurogenesis and programmed cell death are also important in brain development, and occur throughout life; their potential contributions to the pathogenesis of neuropsychiatric diseases have been reviewed elsewhere (Margolis et al., 1994; Mennerick and Zorumski, 2000; Gigante et al., 2011; Petrik et al., 2012).

There are many molecules that act as synaptogenic growth factors in the brain. The most well-studied of these is BDNF, which has been linked to multiple neuropsychiatric diseases including bipolar disorder, depression, and schizophrenia (Neves-Pereira et al., 2002, 2005; Schumacher et al., 2005). The FGFs are a large family of growth factors, which are important in many processes throughout development. FGFs have recently been shown to be important in the development of glutamatergic and GABAergic synapses (Flajolet et al., 2008; Stevens et al., 2010; Terauchi et al., 2010) and have been implicated in a wide number of neuropsychiatric diseases (Evans et al., 2004; Perez et al., 2009; Terwisscha Van Scheltinga et al., 2010; Yamanaka et al., 2011; Turner et al., 2012). Wnts and their receptors have been implicated in learning and memory (Tabatadze et al., 2012; Fortress et al., 2013), autism (Wassink et al., 2001), and some forms of epilepsy (Lako et al., 1998). Although the insulin-like growth factor (IGF) family of factors and receptors was previously recognized primarily for its importance in neurogenesis, development, and aging, this family is also now known to have roles in cortical plasticity (Tropea et al., 2006) and memory (Chen et al., 2011). Other families of growth factors have also been shown to be active in synaptogenesis and brain development, such as the TGFβ, GDNF, and EGF/neuregulin families (Mei and Xiong, 2008; Paratcha and Ledda, 2008; Williamson and Hiesinger, 2008; Van Kesteren et al., 2008; Krieglstein et al., 2011). The contributions of growth factors are summarized in **Table 1**.

Growth factor signaling between pre- and postsynaptic neurons ensures that proper connections between both individual neurons and brain regions are made. Here we describe how dysregulation of these systems may lead to neuropsychiatric disease. Since many of these synaptogenic growth factors promote intracellular signaling through common signal transduction pathways, it is possible that modulation of one or a few of these pathways could lead to significant improvement of clinical symptoms.

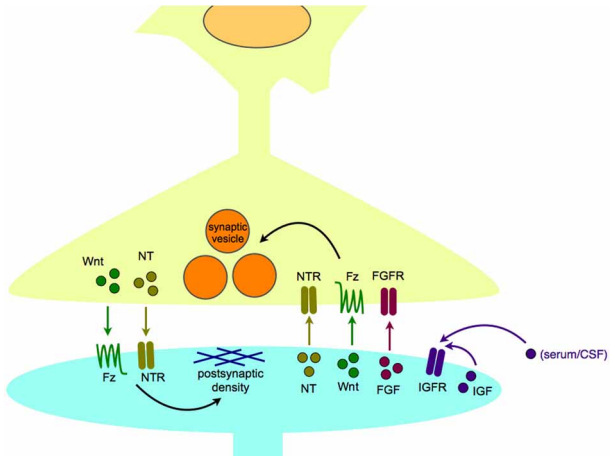

FIGURE 2 | Synaptogenic growth factors in normal synapse formation and maintenance. Synaptogenic growth factors, including neurotrophins, Wnts, and FGFs, are secreted from the postsynaptic cell to induce appropriate differentiation of the presynaptic terminal, including clustering of synaptic vesicles. In turn, growth factors, including neurotrophins, and Wnts, can be released from the presynaptic cell to organize the differentiation of the postsynaptic density. IGFs are thought to act in a paracrine or autocrine fashion, and may be able to bind receptors within the synapse as well as at extrasynaptic sites. Abbreviations used: FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; Fz, Frizzled receptor; IGF, insulin-like growth factor; IGFR, insulin-like growth factor receptor; NT, neurotrophin; NTR, neurotrophin receptor.

BDNF AND THE NEUROTROPHIN FAMILY OF GROWTH FACTORS

The neurotrophin family of growth factors includes BDNF, pro-BDNF, NGF, NT-3, and NT-4. Neurotrophins bind to the Trk family of receptors, as well as the p75 receptor, to activate multiple intracellular signaling cascades. BDNF binds primarily to TrkB, NGF to TrkA, NT-3 to TrkC and TrkB, and NT-4 primarily to TrkB. All neurotrophins bind with relatively low affinity to the p75 receptor, and proBDNF binds only the p75 receptor. When neurotrophins bind to Trks, they support the survival and growth of neurons. Neurotrophins secreted from the postsynaptic cell promote the survival and health of the presynaptic neuron, and maintain a synapse between the two cells. One exception to this rule is the binding of proBDNF to p75, which is usually a pro-apoptotic signal to neurons (Teng et al., 2005). BDNF may also participate in postsynaptic organization (Johnson-Venkatesh and Umemori, 2010; Yoshii et al., 2011), but it is unclear whether this is independent of its presynaptic organizational activities.

BDNF signaling plays a major role in CNS synaptogenesis. It is involved in development of both excitatory and inhibitory synapses (Itami et al., 2000; Fiorentino et al., 2009), and is important for strengthening excitatory synapses through long-term potentiation, a form of cellular and network learning and memory (Minichiello, 2009). BDNF is released from neurons both constitutively and in an activity-dependent fashion (Farhadi et al., 2000; Zha et al., 2001; Egan et al., 2003), and its activity-dependent expression is important for the development and maintenance of cortical inhibitory synapses (Hong et al., 2008). Mice with a hypomorphic or null TrkB allele in hindbrain neurons demonstrate impaired climbing fiber pruning at the climbing fiber-Purkinje cell synapse (Johnson et al., 2007), suggesting that TrkB signaling is important for developmental synaptic pruning, possibly in an activity-dependent fashion. BDNF also plays a role in shaping dendritic morphology, which is an important aspect of synaptogenesis. Mice homozygous (knock-in) for the V66M BDNF mutation show decreased cortical spine density and diameter (Liu et al., 2012), as well as decreased hippocampal and cortical dendritic complexity (Chen et al., 2006b; Yu et al., 2009). BDNF is upregulated in mouse hippocampus under conditions of environmental enrichment (Hu et al., 2013), which is known to enhance dendritic arborization (Turner et al., 2003) and number of hippocampal synapses (Gogolla et al., 2009; Babic and Zinsmaier, 2011). BDNF may have differential effects depending on where its mRNA is translated in the cell; recent evidence shows that somatic BDNF is important for dendritic spine formation, whereas dendritic BDNF expression is important for spine head growth and spine pruning (Orefice et al., 2013). Other neurotrophins, including NT-3 and NT-4, have not been shown conclusively to be involved in synaptogenesis; these do have importance in neurogenesis and other aspects of CNS development (**Table 1**).

BDNF has been implicated in the pathogenesis of multiple neuropsychiatric diseases, including depression (Schumacher et al., 2005), schizophrenia (Zintzaras, 2007), and Rett syndrome, a severe developmental disorder with autistic features (Larimore et al., 2009; Zeev et al., 2009). The V66M mutation in BDNF impairs activity-dependent release of BDNF in hippocampal cultures and is associated with impaired episodic memory, both in patients with schizophrenia and people without neurologic or psychiatric illness (Egan et al., 2003). Interestingly, this mutation may be both a risk factor for depression (Schumacher et al., 2005) and a protective factor against bipolar disorder (Geller et al., 2004), although not all genetic studies in humans support these associations (Neves-Pereira et al., 2002). Data from post-mortem patient tissue supports changes in mRNA and protein levels of BDNF and TrkB in patients with mood and psychotic disorders (Issa et al., 2010; Thompson Ray et al., 2011; Tripp et al., 2012; Qi et al., 2013). Data from animal models with deficits in neurotrophin signaling support the links between BDNF signaling and behavior. Mice lacking TrkB in forebrain neurons show impaired spatial learning, delay in fear conditioning, and impaired hippocampal LTP (Minichiello et al., 1999), as well as behavioral rigidity when faced with changing environmental conditions (Vyssotski et al., 2002). Another group, using the same forebrain-specific TrkB knockout mice, observed increased behavioral hyperactivity and impulsivity (Zorner et al., 2003). Mice with reduced BDNF expression levels display increased alcohol consumption (Hensler et al., 2003; McGough et al., 2004). The same is observed in mice when trkB expression levels are reduced (Jeanblanc et al., 2006). Although it is unknown whether NT-4 functions specifically in synaptogenesis, NT-4 null mice have deficits in fear conditioning and hippocampal LTP (Xie et al., 2000), which may have implications for human anxiety and cognitive disorders.

Table 1 | Growth factors and their receptors in synaptogenesis and neuropsychiatric disease.

Family	Factor/receptor	Role in synaptogenesis and/or brain development	Mutation/signaling defect	Pathology/disorder
Neurotrophins	BDNF	Excitatory and inhibitory synapse development, LTP	Haploinsufficiency	Increased aggressiveness and hyperphagia (Lyons et al., 1999)
			V66M (heterozygous knockin)	Increased immobility in forced swim test and decreased sucrose intake after stress (depressive endophenotype) (Yu et al., 2012)
			V66M (homozygous knockin)	Decreased hippocampal volume, decreased hippocampal dendritic complexity, increased anxiety-related behaviors (Chen et al., 2006b), decreased volume and dendritic complexity in vmPFC with impaired extinction learning (Yu et al., 2009), decreased spine density and diameter in PFC (Liu et al., 2012)
			Val66 (most common allele in general population)	Bipolar disorder (Geller et al., 2004)
			V66M	Depression (Schumacher et al., 2005), bipolar disorder (Neves-Pereira et al., 2002), episodic memory deficit in both homozygous and heterozygous people (Egan et al., 2003), childhood onset OCD (Hall et al., 2003), eating disorders (Ribases et al., 2003, 2004), schizophrenia (Neves-Pereira et al., 2005)
			None identified	Decreased serum levels in depression (Sen et al., 2008)
			Haploinsufficiency	WAGRO (complex medical syndrome that includes intellectual disability) (Han et al., 2008)
	NT3	Inhibition of myelination (Cosgaya et al., 2002); axonal arborization in CNS sensory neurons (Lilley et al., 2013)	Cortical neuron-selective knockout	Defects in thalamocortical pathways with secondary visual system impairment (Ma et al., 2002)
	NT4		Knockout	Loss of specific sensory neurons (Liu et al., 1995); deficit in fear conditioning and attenuated hippocampal LTP (Xie et al., 2000)
	NGF, beta subunit	Role in synaptogenesis unknown; primarily functions in growth and differentiation of sympathetic and subset of sensory neurons (Levi-Montalcini, 1987)	Homozygous loss of function ("functional null")	Hereditary sensory and autonomic neuropathy, mild intellectual disability (Carvalho et al., 2011)
	TrkA	Apoptotic factor for developing neurons (Nikoletopoulou et al., 2010); promotes survival of cholinergic neurons in forebrain nucleus basalis (Fagan et al., 1997)	Reduction in expression	Loss of cortical TrkA correlates with cognitive impairment by MMSE (Counts et al., 2004)
			Receptor blockade via autoantibodies	Blockade of TrkA correlated with sensory axonal neuropathy and axonal dysfunction (Mutoh et al., 2005)
			Multiple loss of function mutations	Congential insensitivity to pain with anhidrosis (Smeyne et al., 1994; Mardy et al., 1999)
			Knockout	Loss of forebrain cholinergic neurons around time of synaptogenesis (Fagan et al., 1997)

(Continued)

Table 1 | Continued

Family	Factor/receptor	Role in synaptogenesis and/or brain development	Mutation/signaling defect	Pathology/disorder
	TrkB	Excitatory and inhibitory synapse development, LTP	Partial (75%) loss of function	Hyperphagia and weight gain (Xu et al., 2003)
			Chemical inhibition of TrkB	Spontaneous seizures, anxiety-like behavior, and loss of hippocampal neurons (Liu et al., 2013)
			S478A homozygous knock-in (phosphorylation-deficient TrkB)	Impaired hippocampal LTP and impaired spatial memory (Lai et al., 2012)
	TrkC	Apoptotic factor for developing neurons (Nikoletopoulou et al., 2010)	Mutations in 3′-UTR of TrkC	Anxiety disorders (Muinos-Gimeno et al., 2009)
	NGFR/p75 (NTR)	Apoptosis of neurons	Knockout	Impaired hippocampal LTD, and alterations in expression of AMPA receptor subunits GluR2 and GluR3 (Rosch et al., 2005)
FGF	FGF1		None identified	Decreased in dorsolateral prefrontal cortex in MDD (Evans et al., 2004)
	FGF2	Posteriorizing cortical pattern (Doniach, 1995), proper migration and differentiation of neurons (Dono et al., 1998; Ortega et al., 1998)	Knockout/targeted disruption	Decreased neuronal density in multiple layers of cortex and spinal cord, ectopic neurons in hippocampal commissure (Dono et al., 1998; Ortega et al., 1998)
			None identified	Decreased in anterior cingulate in MDD (Evans et al., 2004)
	FGF3	Inner ear development (Frenz et al., 2010), works cooperatively with FGF8 in zebrafish retinal development (Martinez-Morales et al., 2005)	Multiple mutations, likely loss of function	Congenital deafness (Tekin et al., 2007, 2008; Alsmadi et al., 2009; Sensi et al., 2011)
	FGF7	Presynaptic organizing molecule for inhibitory synapses onto CA3 neurons (Umemori et al., 2004; Terauchi et al., 2010)	Knockout	FGF7-null mice are sensitive to PTZ kindling of seizures (Terauchi et al., 2010); enhanced mossy fiber sprouting and increased dentate gyrus neurogenesis (Lee et al., 2012)
	FGF10	Presynaptic organizing molecule (Umemori et al., 2004)		
	FGF22	Presynaptic organizing molecule for excitatory synapses onto CA3 neurons (Umemori et al., 2004; Terauchi et al., 2010)	Knockout	FGF22-null mice are resistant to PTZ-induced kindling (Terauchi et al., 2010), do not have induction of DG neurogenesis or ectopic hilar cells with PTZ treatment despite having seizures (Lee and Umemori, 2013)
	FGF8	Specifying anterior cortical positional identity (Fukuchi-Shimogori and Grove, 2001), dorsal identity (Gunhaga et al., 2003)	Multiple (H14N, P26L, F40L, K100E, R127G)	Idiopathic hypogonadotropic hypogonadism with or without anosmia; R127G mutation associated with color blindness and bilateral hearing loss (Falardeau et al., 2008)

(Continued)

Table 1 | Continued

Family	Factor/receptor	Role in synaptogenesis and/or brain development	Mutation/signaling defect	Pathology/disorder
			T229M	Brain defects, seizures, severe neurologic impairment in one family member, above-average intelligence with subtle midline abnormalities in two other family members with mutation (Arauz et al., 2010)
			Hypomorphic alleles in compound heterozygotes	Brain malformations including loss of midbrain and anterior hindbrain and reduced size of telecephalic vesicles (Meyers et al., 1998)
	FGF17	Neuronal fate specification, patterning of cortex	Knockout	Reduced size of dorsal frontal cortex with rostral shift of sensory cortical areas (Cholfin and Rubenstein, 2007); impaired social behaviors (Scearce-Levie et al., 2008)
			Multiple missense mutations, probable loss of function	Hypogonadotropic hypogonadism with or without anosmia (Miraoui et al., 2013)
	FGF9	Mitogen for astrocytes	None identified	Decreased in anterior cingulate in MDD (Evans et al., 2004)
	FGF20	Highly expressed in cerebellum (Jeffers et al., 2001)	951C/T polymorphism leading to increased FGF20 expression	Parkinson disease (Wang et al., 2008; but see Wider et al., 2009); other SNPs also implicated in PD but not always found in replication studies (Van Der Walt et al., 2004; Clarimon et al., 2005)
	FGFR1		Multiple loss-of-function alleles	Hypogonadotropic hypogonadism with or without anosmia (Dode et al., 2003)
			Transgenic expression of forebrain-specific FGFR1 antagonist	Locomotor hyperactivity, abnormal forebrain cortical organization, reduced number of pyramidal neurons and reduced dendritic arborization (Shin et al., 2004)
	FGFR2		Multiple (S351C, delD273, W290C)	Pfeiffer syndrome type III, includes seizures and severe developmental delay (Tartaglia et al., 1997; Gripp et al., 1998)
			None identified	Decreased expression in dorsolateral prefrontal cortex and anterior cingulate in MDD (Evans et al., 2004)
	FGFR3		P250R	Muenke coronal synostosis, early bone fusion syndrome with some patients exhibiting sensorineural hearing loss and/or intellectual disability (Muenke et al., 1997; Reardon et al., 1997), deafness (Hollway et al., 1998)
			Heterozygous missense	CATSHL syndrome (includes hearing loss) (Toydemir et al., 2006)
			R248C	Intellectual disability, seizures, cortical and subcortical atrophy (Garcia-Vargas et al., 2008)
			K650M	SADDAN dysplasia, includes seizures, neurologic impairments, profound developmental delay (Francomano et al., 1996; Tavormina et al., 1999)
			None identified	Decreased expression in dorsolateral prefrontal cortex and anterior cingulate in MDD (Evans et al., 2004)
			Knockout	Profound deafness (Colvin et al., 1996)
			CNS-specific K664E (equivalent to human K650M)	Asymmetric changes in cortical thickness and cerebellar abnormalities, premature oligodendrocyte progenitor differentiation in spinal cord (Lin et al., 2003)

(Continued)

Table 1 | Continued

Family	Factor/receptor	Role in synaptogenesis and/or brain development	Mutation/signaling defect	Pathology/disorder
Wnt	Wnt1		S295X	Osteogenesis imperfecta with learning and developmental delays (Pyott et al., 2013)
	Wnt2		Nonconservative coding sequence variants; linkage disequilibrium with 3′UTR SNP	Autism particularly with severe language deficits (Wassink et al., 2001; McCoy et al., 2002)
	Wnt3	Branching of motoneuron axons (Krylova et al., 2002), hippocampal neurogenesis (Lie et al., 2005), axonal guidance (Schmitt et al., 2006)	None identified	Injection of amphetamine into nucleus accumbens causes increase in Wnt3 expression (Macleod et al., 2012)
	Wnt3a	Patterning of brain, especially rostrocaudal and dorsoventral	Application of anti-Wnt3a antibody	Inhibits LTP in acute hippocampal slices (Chen et al., 2006a)
	Wnt5a	Axonal differentiation (Zhang et al., 2007), promotion of excitatory synapse formation (Varela-Nallar et al., 2010), reduction of excitatory synapse formation (Davis et al., 2008)	Knockout of Wnt5a receptor (Ryk)	Reduction in ventral midbrain progenitor cells and loss of dopaminergic precursor cells with decrease in dopaminergic neurons (Blakely et al., 2013)
	Wnt7a	Presynaptic organizer in cerebellum, synapse clustering, growth cone remodeling (Hall et al., 2000; Ahmad-Annuar et al., 2006); promotes excitatory synapse formation (Davis et al., 2008)	Knockout	Deficit in cerebellar synapse formation, defects in neurotransmitter release in cerebellar synapses (Hall et al., 2000; Ahmad-Annuar et al., 2006)
	Wnt7b	Dendritic branching (Rosso et al., 2005), promotes excitatory synapse formation (Davis et al., 2008)		
	Wnt8b	Posteriorizing signal in posterior forebrain and midbrain (Houart et al., 2002)	Linkage disequilibrium in chromosome 10q24	Potential linkage to partial epilepsy with auditory features (Lako et al., 1998)
IGF	IGF1R	Implicated in ES cell survival and clonogenicity (Bendall et al., 2007)	R59X (early termination)	Microcephaly and mild intellectual disability (Raile et al., 2006); IGFR1 mutations in general are linked to generalized growth retardation
GH	GH	Enhances excitatory glutamatergic neurotransmission (Ramsey et al., 2005; Molina et al., 2013)	Point mutation in intron 3 resulting in very low GH levels	Deficit in spatial learning and memory in rats with GH mutation (Li et al., 2011)
			Viral-mediated GH overexpression in amygdala	Enhanced freezing to auditory cue after fear conditioning (Meyer et al., 2013)
			Untreated GH deficiency	Increased incidence of anxiety, depression, psychosis, and cognitive deficits in women deficient in GH (Bulow et al., 2002)

(Continued)

Table 1 | Continued

Family	Factor/receptor	Role in synaptogenesis and/or brain development	Mutation/signaling defect	Pathology/disorder
EGF	EGFR		Hypomorphic mutation in EGFR	Excessive daytime locomoter activity that is nonresponsive to light (Kramer et al., 2001)
	NRG1	maturation of dendritic spines	CNS-specific knockout	Dendritic spines form but do not grow properly, impaired glutamatergic signaling (Barros et al., 2009)
TGFβ	TGF1	Stimulates proliferation, differentiation and other cell functions in many cell types, negative autocrine growth factor	Knockout	Apoptotic neurons, loss of neocortical presynaptic differentiation, reduced laminin expression, microgliosis, death at postnatal day 21 (Brionne et al., 2003)
			Astrocyte-specific TGFβ1 overexpression	Increased Aβ deposition in aged AD mice and postmortem AD human brains (Wyss-Coray et al., 1997), but expression in microglia might be protective against amyloid plaque development (Wyss-Coray et al., 2001)
	TGFβR1		Heterozygous mutations	Loeys-Deitz syndrome (Loeys et al., 2005; Ades et al., 2006)
	TGFβR2		Transgenic expression of kinase-deficient TGFβR2	Accelerates age-dependent neurodegeneration and dendritic loss in AD mouse model (Tesseur et al., 2006)
			None identified	Significantly decreased in AD brains but not other forms of dementia (Tesseur et al., 2006)
			Neural stem cell-specific knockout	Embryonic defects similar to DiGeorge syndrome, which in humans includes learning disabilities, seizures, psychiatric illness (Wurdak et al., 2005)
			Heterozygous mutations	Loeys-Deitz syndrome, with widespread problems with development including mental retardation; Increased collagen and connective tissue growth factor suggestive of enhanced TGFβ signaling (Loeys et al., 2005)
GDNF	GDNF	Dopaminergic neuron trophic factor	Direct infusion of GDNF to ventral tegmental area	Decreased drug sensitivity (cocaine and morphine) and reversal of drug-induced plasticity (Messer et al., 2000); prevented and reversed neuropathic pain (Boucher et al., 2000); decreased ethanol self-administration (Carnicella et al., 2008)
VEGF	VEGF-A	Angiogenic mitogen	Viral transduction	Enhanced hippocampal-dependent learning in rats (Cao et al., 2004)
	VEGF-D	Angiogenic mitogen	RNAi knockdown	Reduced cortical dendrite length and complexity, and memory impairments in hippocampal-dependent memory tasks (Mauceri et al., 2011)

Growth factors proposed to be involved in brain development are listed, along with available evidence for their involvement in neuropsychiatric disease. Evidence from animal models is listed in blue, evidence from studies in humans or tissue derived from humans is listed in orange. Abbreviations used: 3′-UTR, 3′-untranslated region; AD, Alzheimer disease; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BDNF, brain-derived neurotrophic factor; CA3, cornu ammonis area 3; CATSHL, camptodactyly, tall stature, scoliosis, and hearing loss; CNS, central nervous system; DG, dentate gyrus; EGF, epidermal growth factor; ES cell, embryonic stem cell; Fz, Frizzled receptor; GDNF, glial-derived neurotrophic factor; GH, growth hormone; GluR, AMPA glutamate receptor subunit; JNK, c-Jun N-terminal kinase; LTP, long-term potentiation; MDD, major depressive disorder; miR, microRNA; MMSE, mini-mental status exam; NGF, nerve growth factor; NRG, neuregulin; NT-3, neurotrophin-3; NT-4, neurotrophin-4; OCD, obsessive compulsive disorder; PD, Parkinson disease; PFC, prefrontal cortex; PTZ, pentylenetetrazol; RNAi, RNA interference; Ryk, atypical receptor tyrosine kinase; SADDAN, severe achondroplasia with developmental delay and acanthosis nigricans; SNP, single nucleotide polymorphism; TGF, transforming growth factor; vmPFC, ventromedial prefrontal cortex; WAGRO, wilms tumor, aniridia, genitourinary anomalies, mental retardation, and obesity syndrome.

FIBROBLAST GROWTH FACTORS AND THEIR RECEPTORS

The FGF family includes 22 FGF genes, which are clustered into groups based on phylogenetic similarity and receptor specificity (Umemori, 2009). Among them, there are 4 FGF homologous factors (originally called FGFs 11–14, now known as FHFs), which are solely intracellular signaling molecules and do not bind to FGF receptors (FGFRs). Other FGFs bind to FGFRs, of which there are 4 genes that can be alternatively spliced into multiple receptor subtypes (Umemori, 2009). FGF signaling is important in organogenesis and growth throughout development (Beenken and Mohammadi, 2009). In the CNS, FGFs have many functions, including neurogenesis, fate specification, and neuronal survival (Dono, 2003; Mason, 2007). FGFs also play roles in axon guidance and target recognition. For example, FGF8 has been shown to be an axon guidance molecule for trochlear nerve axons in a cultured rat midbrain explant model of neuronal pathfinding (Irving et al., 2002), and FGF2 gradients help retinal ganglion cell axons find their targets in the optic tectum in developing Xenopus (McFarlane et al., 1995). Although some FGFs act by an endocrine mechanism in the periphery, (such as FGF19, FGF21, and FGF23), FGFs that are active in the CNS are released by postsynaptic cells to stimulate presynaptic organization (Terauchi et al., 2010), and therefore act primarily by local mechanisms within the CNS.

Evidence is accumulating for the importance of FGFs in synaptogenesis. In cultured rat hippocampal neurons, addition of FGF2 to the culture medium generates an increase in excitatory synapses via a MAPK-dependent mechanism (Li et al., 2002). In cultured neurons, FGF7 and FGF22 function as presynaptic organizers (Umemori et al., 2004; Terauchi et al., 2010). FGF7-null mice have a deficit in hippocampal inhibitory synapse formation while FGF22-null mice are deficient in hippocampal excitatory synapses (Terauchi et al., 2010), consistent with the roles of FGF7 and FGF22 in presynaptic organization (Umemori et al., 2004). Other FGFs, including FGFs 4, 6, and 9 also promote synaptic vesicle clustering in cultured neurons (Umemori et al., 2004), but their roles in synaptogenesis *in vivo* are unknown.

The evidence linking FGFs and behavioral abnormalities is growing. FGF7-null are prone to develop epilepsy after kindling, while FGF22-null are resistant to seizure induction (Terauchi et al., 2010), providing a link between synaptogenic defects and a neurobehavioral phenotype. Mice overexpressing FGF21 primarily in the liver, which is known to function in metabolism and insulin sensitivity, also show dysregulation in circadian rhythms, which is a common feature of mood disorders (Bookout et al., 2013); it is unknown whether these mice have other behavioral abnormalities consistent with mood alterations. It is also unknown whether FGF21 plays a role in synaptogenesis, although it is known to cross the blood-brain barrier (Bookout et al., 2013). Mice globally lacking FGF17 have impaired social interactions, a key diagnostic feature of autism (Scearce-Levie et al., 2008). Interestingly, FGF17 was found to induce neurite branching in cultured neurons (Umemori et al., 2004), suggestive that abnormal connectivity between neurons may underlie these behavioral changes in FGF17-null mice. Peripheral administration of FGF2 to rats with endogenously high levels of anxiety was found to reduce anxiety-like behaviors (Perez et al., 2009), while lentiviral shRNA-mediated knockdown of FGF2 in rat hippocampus increased anxiety-like behaviors (Eren-Kocak et al., 2011). Although no studies of FGF expression in anxiety disorder patients have been published, the body of literature supports the idea that FGF2, if not other FGFs, is an important regulator of many emotional states. Alterations in FGF expression in humans have also been associated with depression (Evans et al., 2004), substance abuse (Turner et al., 2012), and schizophrenia (Terwisscha Van Scheltinga et al., 2010). Mutations in FGFR2 are causative for Pfeiffer Syndrome, some severe forms of which manifest intellectual disability (Priolo et al., 2000; Shotelersuk et al., 2002). There are multiple other examples of FGFs and FGFRs linked to neuropsychiatric disease, which are detailed in **Table 1**. Overall, the data underscore the importance of normal FGF signaling both for normal synapse formation and normal neuropsychiatric functioning.

Wnt SIGNALING MOLECULES AND THEIR RECEPTORS

Wnts are a family of 19 highly-conserved secreted signaling glycoproteins that play important roles in embryogenesis and fate specification in early development. When they bind to their receptors, the Frizzled proteins and LRP coreceptors, they can trigger several different types of intracellular signaling pathways. The best characterized intracellular signaling pathway is the Wnt/Frizzled/β-catenin/GSK3-β pathway, also known as the canonical pathway. Wnts 1, 2, 3a, 7a, and 7b generally signal through the canonical pathway. There are also several non-canonical pathways that have been identified which do not signal via β-catenin, the most well-studied of which are the planar cell polarity (PCP) and the Wnt/calcium pathways. Wnts 4a and 5a signal through the PCP pathway, which is involved in neuronal migration as well as cell polarity (Okerlund and Cheyette, 2011). The Wnt/calcium pathway is important for control of calcium release from the endoplasmic reticulum (ER) for calcium-dependent intracellular signals (De, 2011). There are also a number of other Wnt signaling pathways, but these are generally less well-understood (Niehrs, 2012). Although Frizzled and LRP are the most well-studied receptors for Wnts, Wnts are also known to bind to many other cell surface receptors, including Ryk, ROR2, and others (Niehrs, 2012).

Wnt signaling pathways have many roles in CNS synaptogenesis, and can both increase or decrease synapse formation depending on the Wnt pathways and cell types involved. The role of Wnts in non-mammalian and peripheral nervous system synaptogenesis has been reviewed extensively elsewhere (Park and Shen, 2012; Poon et al., 2013). Wnt7a is a retrograde signal derived from cerebellar granule cells to presynaptic mossy fiber terminals in the cerebellum (Hall et al., 2000). Wnt7a binds to Dvl1, a mouse homolog of Disheveled, and induces clustering of synapsin I and axon growth cone remodeling (Hall et al., 2000; Ahmad-Annuar et al., 2006). In mice globally lacking either Wnt7a or Dvl1, there are deficits in cerebellar synapse formation, while mice null for both Wnt7a and Dvl1 have an additional defect in neurotransmitter release at mossy fiber-granule cell synapses (Hall et al., 2000; Ahmad-Annuar et al., 2006). Wnt7a also has a role in synaptic differentiation in the hippocampus, particularly enhancing the number and strength of excitatory synapses (Davis et al., 2008;

Ciani et al., 2011); this is also true for Wnt7b (Davis et al., 2008). Wnt5a has been shown to increase the formation of glutamatergic synapses and maturation of dendritic spines in cultured neurons via a calcium-dependent mechanism (Varela-Nallar et al., 2010). However, in a separate study, application of Wnt5a to neuronal cultures resulted in a decrease in glutamatergic synapses (Davis et al., 2008), suggesting that Wnt5a effects may be dependent on culture conditions or downstream signaling pathways (canonical vs. non-canonical). Taken together, the data demonstrate the importance of Wnt signaling in synaptogenesis in both pre- and postsynaptic compartments.

Although many knockout mouse models for Wnts have been developed, most do not survive embryogenesis (Uusitalo et al., 1999; Van Amerongen and Berns, 2006), and therefore cannot be assessed for behavioral phenotypes. However, there are mouse models where other mediators of Wnt signaling have been genetically manipulated, which implicate Wnt signaling in behavior. Mice null for Dvl1 have diminished social interactions, a core feature of autism, as well as abnormal prepulse inhibition, which is observed in both autism and schizophrenia (Lijam et al., 1997). These mice also have deficits in hippocampal dendritic branching and cerebellar synaptogenesis (Lijam et al., 1997; Rosso et al., 2005). Forebrain-specific reduction of expression of β-catenin, the putative downstream signaling molecule for Dvl1, generates subtle behavioral changes in the tail suspension test, a depression-like endophenotype (Gould et al., 2008). The lack of similar behavioral deficits between the forebrain-specific β-catenin knockout and Dvl1-null mice could be due to the fact that the β-catenin knockout was limited to the forebrain, whereas the synaptic changes noted in Dvl1-null mice are primarily noted in hippocampus and cerebellum, or may be attributable to the multiplicity of downstream effectors of Wnt signaling. Another way to modulate Wnt signaling is by overexpressing Axin, a scaffolding protein that negatively regulates Wnt signaling. When mice overexpressing Axin are trained in a fear-conditioning paradigm, they exhibit an increase in freezing to contextual conditioning as well as changes in cued fear conditioning, suggestive that alterations in Wnt signaling could increase anxiety-related behaviors (Kim et al., 2011).

Wnts have been implicated in multiple genetic studies of human neuropsychiatric disease. Some data suggest that mutations in Wnt2 are linked with forms of autism with severe language deficits (Wassink et al., 2001), although not all studies have confirmed this association (McCoy et al., 2002). Interestingly, the *CHD8* gene has been identified in multiple genetic studies of autism and related neurodevelopmental disorders (Neale et al., 2012; O'Roak et al., 2012a,b; Talkowski et al., 2012). The CHD8 protein binds β-catenin and negatively regulates Wnt/β-catenin signaling (Nishiyama et al., 2012). Wnts also can activate the retinoid-related orphan receptor alpha, RORA, which has been implicated by GWAS in several neuropsychiatric diseases, including autism (Nguyen et al., 2010; Sarachana and Hu, 2013), bipolar disorder (Le-Niculescu et al., 2009; but see McGrath et al., 2009), depression (Terracciano et al., 2010; Utge et al., 2010), and PTSD (Logue et al., 2013). Additionally, both lithium and valproic acid, medications commonly used to treat neuropsychiatric diseases, are known to inhibit GSK3β, a downstream effector of the canonical Wnt signaling pathway (Lucas and Salinas, 1997; Hall et al., 2002), and lithium treatment in mice activates Wnt signaling in various regions of the brain including amygdala and hippocampus (O'Brien et al., 2004).

INSULIN-LIKE GROWTH FACTORS AND THEIR RECEPTORS

IGFs are peptide growth factors identified based on their similarity to the peptide hormone, insulin. The family consists of two growth factor ligands (IGF1 and IGF2), two receptors (IGF1R and IGF2R), and multiple IGF binding proteins (IGFBPs) and IGFBP-related proteins (Fernandez and Torres-Aleman, 2012). IGF1 is a neurotrophic factor that enhances the survival of neurons in culture (Meyer-Franke et al., 1995; Arnaldez and Helman, 2012; O'Kusky and Ye, 2012). IGF2 has also been implicated in neurogenesis, synaptogenesis, myelination, and dendritic branching (Agis-Balboa et al., 2011; Fernandez and Torres-Aleman, 2012; Schmeisser et al., 2012). The mechanism of IGF1 action on neurons may be both endocrine and autocrine, as it circulates in the bloodstream and can cross the blood-brain barrier, in addition to being secreted locally by neurons (Nunez et al., 2003) (**Figure 2**). IGF2 may also serve a neurotrophic function, at least for young hippocampal neurons, since increasing IGF2/IGFBP7 signaling via a fear-conditioning paradigm in mice leads to enhanced survival of newborn hippocampal neurons (Agis-Balboa et al., 2011).

The primary physiologic receptor for the IGFs is IGF1R, although IGF1 can also bind the insulin receptor. Like many growth factor receptors, IGF1R is a receptor tyrosine kinase, and when bound by IGF1, can activate several different intracellular cascades (Arnaldez and Helman, 2012). IGF2R can bind IGF2, but not IGF1. IGF2R is thought primarily to sequester IGF2 at the cell surface, and in most cases this binding does not generate transmembrane signals. IGFBPs regulate IGF activity by binding to IGFs and IGF1R, and this binding can inhibit or facilitate the binding of IGFs to IGF1R, or prolong the half-life of IGFs, depending upon the IGF/IGFBP pair and the specific microenvironment (O'Kusky and Ye, 2012).

IGF1 is widely expressed throughout the brain throughout development (Garcia-Segura et al., 1991), and IGF1 is upregulated in neurons during the developmental periods associated with dendritic maturation and synapse formation (Bondy, 1991). Application of IGF1 to cultured cortical neurons causes an increase in puncta containing PSD-95 and synapsin, but not puncta containing gephyrin, suggestive that IGF1 treatment increases the number of excitatory rather than inhibitory synapses in the cortex (Corvin et al., 2012). Interestingly, in mice modeling a severe form of autism, Rett syndrome, treatment with an active IGF1 peptide fragment partially restores spine density, synaptic function, PSD-95 localization and levels, and synaptic plasticity (Tropea et al., 2009). IGF2 is expressed in neurons and may localize to synaptic sites, and application of IGF2 to cultured hippocampal neurons causes an increase in spine formation via an IGF2R-dependent mechanism (Schmeisser et al., 2012), in contrast to previous data suggesting that IGF2R functions only as a reservoir to bind IGF2 at the cell surface. IGF1R is found in both pre- and

postsynaptic areas in certain hypothalamic nuclei and the cerebellum (Garcia-Segura et al., 1997), suggestive that IGF signaling may play roles in both pre- and postsynaptic organization. IGF2R also localizes to postsynaptic densities (Schmeisser et al., 2012).

Animal models have demonstrated the importance of IGFs in normal synaptogenesis as well as neuropsychiatric disease. In rat pups, environmental enrichment during youth is also known to reduce anxiety-like behaviors during adulthood, but this effect of environmental enrichment is lost when IGF1 activity is blocked by systemic injection of blocking peptide during environmental enrichment. Interestingly, IGF1 injection during youth mimics the anxiolytic effects of environmental enrichment when the rats reach adulthood (Baldini et al., 2013). Blockade of IGF1 during youth, and the concomitant increase in anxiety-like behaviors in adulthood, is correlated with increased hippocampal IGF1R expression at postnatal day 12 in rats and increased glucocorticoid receptor expression at postnatal day 60 (Baldini et al., 2013). Interestingly, IGF1 infusion into the CSF of adult rats improved their performance on both cognitive and affective reactivity tasks (Markowska et al., 1998).

There are limited data from humans on the potential role of IGF signaling in neuropsychiatric disease, but there are some lines of evidence that implicate IGF signaling may be important. Lithium is one of the most effective treatments available for bipolar disorder, and it is known to inhibit GSK3β (Hedgepeth et al., 1997; Chalecka-Franaszek and Chuang, 1999). In patient-derived lymphoblastoid cell lines, bipolar disorder patients who respond to lithium have higher levels of IGF1 than bipolar disorder patients who do not respond to lithium (Squassina et al., 2013). This suggests that IGF1 may act upstream of GSK3β in modulating lithium response (Cui et al., 1998; Chalecka-Franaszek and Chuang, 1999). There is also significant evidence that insulin and IGF signaling promote the aging process in many animals (Bartke, 2008; Kenyon, 2010), raising the intriguing possibility that age-related cognitive decline may be mediated by the effects of insulin and IGFs on transcription factors and synapse function.

SYNAPTOGENIC GROWTH FACTOR SIGNALING PATHWAYS

There is significant crossover in the intracellular downstream signaling pathways activated by synaptogenic growth factors. These pathways include (a) the MAPK/ERK pathway, (b) the PI3K/Akt pathway, and (c) the PLC/IP3/CAMK pathway. Significantly, all of these pathways have been implicated in several different neuropsychiatric diseases. We will address the evidence linking each individual pathway to synaptogenesis and disease, and then present a model that may help explain how these systems are linked in disease pathogenesis.

THE MAPK/ERK PATHWAY

The MAPK/ERK pathway is a common signal transduction pathway for many synaptogenic growth factors, including BDNF, FGFs, some Wnts, and IGF1 (Easton et al., 1999; Perron and Bixby, 1999; Quevedo et al., 2000; Bikkavilli et al., 2008). This signaling cascade begins when a synaptogenic growth factor binds its receptor, often itself a receptor tyrosine kinase except in the case of some Wnt receptors, and activates it. This results in binding of intracellular signaling proteins, which ultimately activate MAPK, which activates ERK. ERK can activate multiple transcription factors, including CREB, RSK, and myc. There are many ways in which alterations in this signaling pathway can contribute to neuropsychiatric disease. A mutation in RSK2, one of the downstream effector molecules of this pathway, can cause Coffin-Lowry syndrome, an X-linked form of severe intellectual disability (Morice et al., 2013). Mutations of the RSK2 gene in humans are associated with smaller volumes of hippocampus, cerebellum and temporal lobe, while a mouse model of Coffin-Lowry syndrome lacking Rsk2 demonstrates defects in hippocampal spine morphology and hippocampus-dependent learning (Morice et al., 2013). There is also evidence that environmental stressors can alter the MAPK/ERK pathway. In rhesus monkeys who were abused or neglected by their mothers during childhood, decreased CSF serotonin metabolites were correlated with both activated p38 MAPK in serum monocytes as well as increased risk of anxiety behaviors, delayed social development and reduced exploration as adolescents (McCormack et al., 2006; Sanchez et al., 2007).

THE PI3K/Akt PATHWAY

Another critical intracellular signaling pathway, the PI3K/Akt pathway, is activated when synaptogenic growth factor receptors phosphorylate PI3K. PI3K activation then leads to phosphorylation of Akt. Akt can translocate into the nucleus to regulate other transcription factors, leading to changes in levels of synaptic proteins, and can also activate mTOR (mammalian target of rapamycin) and thereby indirectly influence the growth and survival of cells. In neurons, the PI3K/Akt pathway is activated by growth factors including BDNF and IGF1 (Stroppolo et al., 2001; Bondy and Cheng, 2004; Li and Thiele, 2007). One study suggests that exogenous FGF1 may activate this pathway in astrocytes (Ito et al., 2013), although whether this is also the case for neurons is unknown. There is some evidence that this pathway can be activated by FGFR2 in oligodendrocytes independent of any FGF ligand at all (Bryant et al., 2009). This pathway is also activated by FGF7 in lung tissue (Ray et al., 2003), but it is not known whether FGF7 can also activate this pathway in neurons. Environmental enrichment, which is known to have multiple beneficial effects on anxiety-like behaviors in rodents, upregulates the Akt pathway and leads to downregulation of GSK3β (Hu et al., 2013). Akt itself has been implicated as a risk factor for schizophrenia susceptibility, as a specific *AKT1* haplotype causes decreased Akt levels and is associated with illness (Emamian et al., 2004). Another Akt family member, AKT3, has been shown to be important in some cases of brain malformation and epilepsy (Poduri et al., 2012b), linking this pathway to multiple aspects of brain development, including circuit formation, circuit activity, and neuronal survival. Finally, the specific serotonin reuptake inhibitor, fluoxetine, increases phosphorylation of Akt as well as ERK in rat neural stem cells (Kitagishi et al., 2012; Huang et al., 2013). The fact that fluoxetine and other medications in its class are useful for a wide variety of neuropsychiatric illnesses outside of depression lends further support to the idea that these illnesses may have common origins.

THE PLC/IP3/CAMK PATHWAY

A third common signal transduction pathway activated by many synaptogenic growth factors and implicated in neuropsychiatric disease is the PLC/IP3/CAMK pathway. It is activated by BDNF, many FGFs, and some Wnts (Klint and Claesson-Welsh, 1999; Reichardt, 2006). It may be induced by IGF1, although it is unknown whether this is via direct IGFR1 activation of PLC or if this occurs indirectly (Chattopadhyay and Carpenter, 2002). In this pathway, activation of receptor tyrosine kinases by extracellular binding of synaptogenic growth factors leads to activation of phospholipase C (PLC, most commonly PLCγ1) and generation of the second messenger IP3. IP3 diffuses to the ER where it binds to its receptor, IP3R. IP3R is a calcium channel that releases calcium from the ER. When released into the cytosol, calcium can bind to a number of calcium-dependent proteins, such as calmodulin, which activates a number of important intracellular enzymes, including the calmodulin-dependent kinases (CAMKs). CAMKs are important effector molecules for a number of neuronal functions, including long-term potentiation (Sanhueza et al., 2007) and calcium-response element (CRE)-dependent transcription (Kang et al., 2001). One particular CAMK protein, Camk2B, is expressed widely in the CNS, and levels of *CAMK2B* mRNA were found to be upregulated 2-fold in the frontal cortex of post-mortem schizophrenia patient brains compared to control brains (Novak et al., 2000). In a single patient, a point mutation in *CAMK2G* (R29P) was associated with a number of phenotypic abnormalities, including severe intellectual disability (De Ligt et al., 2012). Mice lacking Camk4 have deficits in fear learning, with corresponding reductions in phosphorylated CREB in brain areas associated with fear memory after training (Wei et al., 2002). One of the upstream signaling factors in this pathway, PLCβ1, has been implicated in severe forms of epilepsy (Kurian et al., 2010; Poduri et al., 2012a). All of these lines of evidence point to the importance of this pathway in normal neural development and function, and there is clear evidence of synaptic dysfunction and behavioral phenotypes when these pathways are altered.

CROSSTALK BETWEEN SIGNALING PATHWAYS

Most synaptogenic growth factors can activate multiple downstream signaling pathways depending on which receptor they bind, and in which cell type the receptor is expressed. Due to the fact that activated growth factor receptors can bind promiscuously to various intracellular second messengers, it is unlikely that any single growth factor signaling cascade will account for all of the phenotypes observed in a given neuropsychiatric disease. It is far more likely that the complex interplay of a number of signaling pathways will generate an observable phenotype, such as autism or depression. However, all three pathways described above have intermediary signaling molecules (ERK, Akt, and CaMKII) that can activate cAMP/calcium-response element binding protein (CREB) and lead to CRE-dependent transcription of genes (**Figure 3**). CREB-mediated transcription is critical for expression of a number of genes, including some synaptogenic growth factors as well as c-fos and other activity-dependent genes (Benito and Barco, 2010). CREB may serve as a key integrator of signals of neuronal activity, such as NMDA receptor activation-mediated calcium influx, with synaptogenic growth factor signaling (such as the cascades described above). Activation of CREB then leads to transcription of activity-dependent genes that play roles in synaptogenesis. One such activity-dependent gene is the L-type voltage-gated calcium channel (VGCC), which has recently been the focus of much interest as SNPs within the alpha subunit of one L-type VGCC has been implicated as a risk factor in multiple neuropsychiatric diseases (Andreassen et al., 2013; Cross-Disorder Group of the Psychiatric Genomics et al., 2013).

Another possible cellular focus for synaptogenic growth factor signal integration is the WAVE regulatory complex (WRC), a large five-subunit complex that controls actin cytoskeleton dynamics (Pollitt and Insall, 2009). Recently, two papers were published which describe how cell surface receptors containing a WRC interacting receptor sequence (WIRS) domain interact with the WRC and the actin cytoskeleton to direct synapse formation and changes in neuronal morphology including axonal branching (Chen et al., 2014; Chia et al., 2014). Many synaptic proteins have potential WIRS domains, including some synaptogenic growth factor receptors (Chen et al., 2014). The WAVE complex might be another major intergrator of synaptogenic growth factor signaling in neurons.

Crosstalk between multiple growth factor pathways occurs as well, further underlining how interconnected these systems are in the brain. For example, Wnt signaling triggers transcription of FGF4 in tooth development (Kratochwil et al., 2002), sequential signaling by Wnt3a and FGF8 are required to induce dorsalization during brain development (Gunhaga et al., 2003), and both FGF19 and Wnt8C signaling are required for successful inner ear development (Ladher et al., 2000). Cooperative signaling of the Wnt and FGF systems is also critical in spinal cord specification (Nordstrom et al., 2006). Recently, crosstalk between FGF and Wnt signaling in *C. elegans* sensory organs was described on a transcriptional level, where FGF activates the MAPK/ERK pathway and regulates a downstream Wnt effector molecule (Squarzoni et al., 2011). Therefore, it will be important to consider that modulation of a single synaptogenic growth factor or intracellular signaling pathway will likely affect other systems as well.

CONCLUSIONS AND FUTURE DIRECTIONS

Growth factor signaling between pre- and postsynaptic neurons is critical for proper connections between individual neurons, and for the development of appropriate brain circuitry. Synaptogenic growth factors play a key role in ensuring that synapses develop properly and are modulated appropriately over time, so that suitable emotional and behavioral responses to the environment are generated when necessary. As described, dysregulation of these systems may lead to inappropriate emotional and behavioral responses to either internal or external stimuli, which is associated with functional decline. Modulation of synapses over time is also critical for learning and memory when the environment changes, and dysfunction in these processes likely contributes to cognitive impairment. Ongoing synaptogenic dysregulation caused by defects in growth factor signaling may cause these illnesses not to improve (as in autism) or worsen and become increasingly difficult to treat (such as schizophrenia) over a patient's lifetime.

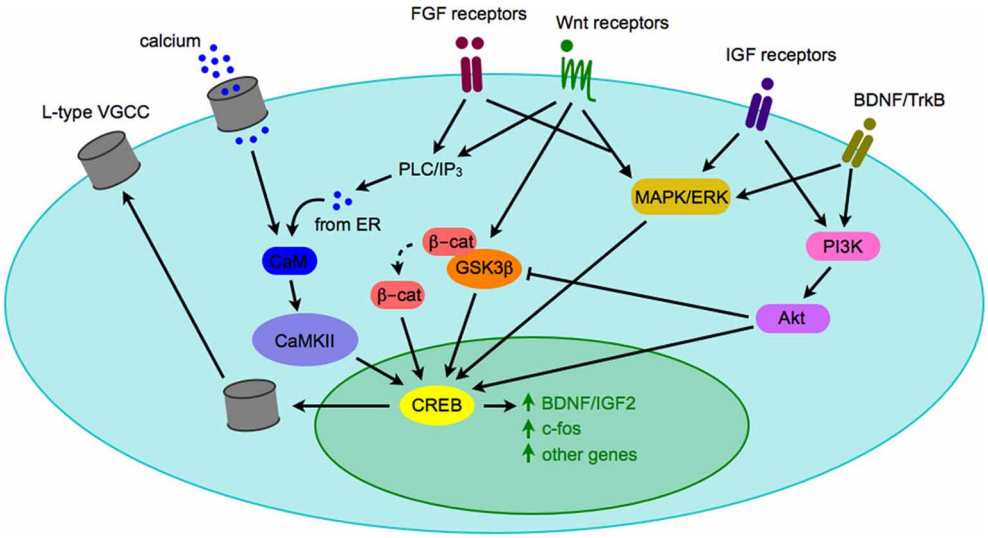

FIGURE 3 | Crosstalk between signaling pathways implicated in pathogenesis of neuropsychiatric diseases. When synaptogenic growth factors bind to their respective receptors, they can trigger a number of intracellular signaling cascades. Depicted here are the PLC/IP3/CAMK pathway, the MAPK/ERK pathway, and the PI3K/Akt pathway. Although there are a number of downstream effectors of each pathway, they may converge on the CREB transcription factor and gene expression, including expression of genes that modify neuronal activity, such as BDNF and the L-type VGCC. Although only one cell is depicted here, the model could apply to a presynaptic or postsynaptic cell. Abbreviations used: β-cat, β-catenin; BDNF, brain-derived neurotrophic factor; CaM, calmodulin; CaMKII, calmodulin-dependent kinase II; CREB, calcium response element binding protein; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; GSK3β, glycogen synthase kinase 3β; IGF, insulin-like growth factor; IP3, inositol 1,4,5-trisphosphate; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositide 3-kinase; PLC, phospholipase C; TrkB, tyrosine receptor kinase B; VGCC, voltage-gated calcium channel.

Complicating this picture is the possibility that the specific functions of growth factors may change throughout development. Conversely, at different times throughout the life cycle, different growth factors may be required for similar functions. For example, at the neuromuscular junction (NMJ), laminin-β2 is a critical presynaptic organizer in the neonate, whereas collagen IV performs this function in the adult (Nishimune et al., 2004; Fox et al., 2007). Therefore, it is possible that the mutations or abnormalities in growth factors may only be relevant at specific developmental times, or in different locations, for specific neuropsychiatric diseases. Additionally, many other genetic risk factors for neuropsychiatric diseases are associated with synapse-specific proteins, including the synaptic scaffolding Shank proteins (Guilmatre et al., 2014), the synaptic adhesion molecules contactin/caspr and neurexin/neuroligin (Sudhof, 2008; Vernes et al., 2008; Kenny et al., 2013; Zuko et al., 2013), and proteins in the mTOR pathway, which is critical for synapse-specific protein synthesis (Hoeffer and Klann, 2010; Russo et al., 2012; Wong, 2013). These molecules and pathways may interact with the growth factor pathways (Patzke and Ernsberger, 2000; Iki et al., 2005; Hoeffer and Klann, 2010; Williams and Casanova, 2011; Russo et al., 2012; Wong, 2013; Bennett and Lagopoulos, 2014). The myriad ways in which these pathways may be linked requires further exploration.

Nevertheless, since many receptors for synaptogenic growth factors act through common intracellular signal transduction pathways, it may be that modulation of one or a few of these pathways could lead to significant resolution of clinical symptoms. In addition, growth factor binding proteins often act as regulators of growth factor binding and localization, which have the added benefit of functioning in the extracellular space rather than intracellular compartments. This could significantly reduce the difficulty of getting treatments to their target sites. Additionally, in the case of FGFs, heparan sulfate proteoglycans (HSPGs) are required for binding of FGFs to their receptors at high affinity (Klint and Claesson-Welsh, 1999). Modulation of certain HSPGs could alter FGF binding to particular FGFRs. Such an approach may also be possible with other synaptogenic growth factors.

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Growth factors in synaptic function

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Synapses are increasingly recognized as key structures that malfunction in disorders like schizophrenia, mental retardation, and neurodegenerative diseases. The importance and complexity of the synapse has fuelled research into the molecular mechanisms underlying synaptogenesis, synaptic transmission, and plasticity. In this regard, neurotrophic factors such as netrin, Wnt, transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), and others have gained prominence for their ability to regulate synaptic function. Several of these factors were first implicated in neuroprotection, neuronal growth, and axon guidance. However, their roles in synaptic development and function have become increasingly clear, and the downstream signaling pathways employed by these factors have begun to be elucidated. In this review, we will address the role of these factors and their downstream effectors in synaptic function *in vivo* and in cultured neurons.

Keywords: netrin, Wnt, TGF-β, TNF-α, synaptogenesis, synaptic transmission and plasticity

INTRODUCTION

Human perception, learning, and memory are only possible when the nervous system is functioning normally. The primary building blocks of the nervous system are neurons–specialized cells that form connections, or synapses, with specific targets. Loss or malfunction of synapses leads to mental retardation, schizophrenia, and neurodegenerative diseases like Alzheimer's or Parkinson's disease.

As a functional synapse is a fundamental requirement for the brain to process any task, synaptic function is tightly regulated. This regulation occurs at multiple steps, such as recruitment and assembly of molecular machinery, synapse formation and stabilization, coordinated release of neurotransmitters, downstream signaling of receptors, maintenance, plasticity, and eventual loss of the synapse.

To study synaptic function, neurobiologists have utilized multiple model systems, including *C. elegans*, *Drosophila*, the vertebrate neuromuscular junction (NMJ), primary mammalian neurons, brain slice cultures, and rodent models. Pioneering work in invertebrates led to the identification of novel roles for growth factors in synaptic function (Zhang et al., 1997; Aberle et al., 2002; Chin et al., 2002; Marques et al., 2002; Packard et al., 2002; McCabe et al., 2003; Ziel and Sherwood, 2010), and subsequent studies have demonstrated similar synaptic functions for these growth factors in mammals (Krieglstein et al., 2011; Salinas, 2012; Horn et al., 2013).

Abbreviations: AA, arachidonic acid; ABI-1, Abl-interacting protein-1; Abl, Abelson tyrosine-protein kinase 1; AC, adenyl cyclase; AChR, acetylcholine receptor; ACR-, acetylcholine receptor; AD, Alzheimer's disease; ADAM17, a disintegrin and metallopeptidase domain 17; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; APC, adenomatous polyposis coli; Aβ, amyloid-β; Babo, baboon; BMP, bone morphogenetic protein; BMPR, BMP receptor; CAM-, CAN cell migration defective; CaMKII, calcium/calmodulin-dependent protein kinase II; Cdc42, cell division cycle 42; CDK-5, cyclin-dependent kinase 5; CED-, cell death abnormality; CNS, central nervous system; CREB, cAMP response element-binding protein; CWN-, *C. elegans* WNT family; CYY-, cyclin Y; dactivin, *Drosophila* activin; Dad, daughters against decapentaplegic; Daw, dawdle; DBL-, decapentaplegic/BMP-like; DCC, deleted in colorectal cancer; DD, death domain; DFz2, *Drosophila* Frizzled-2; DGRIP, *Drosophila* glutamate receptor interacting protein; DOCK180, dedicator of cytokinesis 1; Drl, derailed; DSCAM, down syndrome cell adhesion molecule; DSH-, dishevelled related; Dvl, dishevelled; EJC, end-plate junctional current; Eps15, epidermal growth factor receptor pathway substrate 15; ERK, extracellular signal-regulated kinase; Evi/Wls/Srt, Evenness Interrupted/Wntless/Sprinter; FADD, fas-associated DD; FNI, frizzled nuclear import; $GABA_A$, γ-aminobutyric acid A; GAP, guanosine triphosphatase-activating protein; Gbb, glass bottom boat; GEF, guanine nucleotide exchange factor; GluN2B, an NMDAR subunit; GSK3β, glycogen synthase kinase 3β; HIV, human immunodeficiency virus; HFS, high frequency stimulation; HSPG, heparan sulfate proteoglycan; IP_3, inositol-1,4,5-trisphosphate; JNK, c-Jun-amino-terminal kinase; LIMK1, LIM domain kinase 1; LIN-, abnormal cell lineage; LPS, lipopolysaccharide; LTD, long-term depression; LTP, long-term potentiation; Ly6, lymphocyte antigen 6; Mad, mothers against decapentaplegic; MAP, microtubule associated protein; MAPK, mitogen-activated protein kinase; Mav, maverick; MCPG, α-methyl-4-carboxyphenylglycine; mEJC, miniature end-pate junctional current; mEPSC, miniature excitatory postsynaptic current; mGluR, metabotropic glutamate receptor; MIG-, abnormal cell migration; mIPSC, miniature inhibitory postsynaptic current; MPEP, 2-methyl-6-(phenylethynyl)pyridine; MuSK, muscle-specific kinase; NF-κB, nuclear factor kappa B; NFAT, nuclear factor of activated T-cells; NGL, netrin G ligand; NMDAR, N-methyl-D-aspartate-type glutamate receptor; NMJ, neuromuscular junction; PCP, planar cell polarity; PCT-1, Pctaire kinase 1; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PPF, paired pulse facilitation; PSD-95, postsynaptic density protein 95; Rac1, ras-related C3 botulinum toxin substrate 1; RIPK1, receptor-interacting protein kinase 1; ROR, receptor tyrosine kinase-like orphan receptor; Ryk, receptor-like tyrosine kinase; Sax, Saxophone; Smad, Mad homolog; Src, Rous sarcoma oncogene; TGF-β, transforming growth factor-β; Tkv, Thickveins; TNF-α, tumor necrosis factor-α; TNFR, TNF receptor; TRADD, TNFR-associated DD protein; TRAF2, TNFR-associated factor-2; TRPV1, transient receptor potential subtype V1; UNC-, Uncoordinated; VDCC, voltage-dependent calcium channel; VTA, ventral tegmental area; Wg, Wingless; Wit, wishful thinking.

Through these studies, the role of growth factors such as netrin, Wnt, transforming growth factor-β (TGF-β), and tumor necrosis factor-α (TNF-α) in synaptogenesis, synaptic transmission, and plasticity is gradually being elucidated. Netrin, Wnt, and TGF-β both enhance and suppress synaptogenesis, and their effects are mediated through a variety of pathways (Shen and Cowan, 2010; Krieglstein et al., 2011; Koles and Budnik, 2012; Salinas, 2012). In addition, the netrin receptor Deleted in Colorectal Cancer (DCC) is implicated in synaptic plasticity (Horn et al., 2013) while members of the Wnt superfamily enhance synaptic function *in vivo* and *in vitro* primarily through the planar cell polarity (PCP) and calcium Wnt signaling pathways (Koles and Budnik, 2012; Salinas, 2012). In contrast, the TGF-β superfamily and TNF-α enhance excitatory synaptic transmission, while suppressing inhibitory synaptic transmission (Krieglstein et al., 2011; Santello and Volterra, 2012). In this review, we will focus on the function of netrin, Wnt, TGF-β, and TNF-α in the various aspects of synaptic function and the downstream signaling pathways employed. Roles of other growth factors like brain-derived neurotrophic factor (BDNF), fibroblast growth factor (FGF), and glial cell line-derived neurotrophic factor (GDNF) are discussed elsewhere (Shen and Scheiffele, 2010; Wu et al., 2010; Duarte et al., 2012; Park and Poo, 2013).

NETRIN

The netrin family of laminin-related proteins is known for its critical role in axon guidance during neuronal development. Over the past two decades, netrins have been implicated in diverse processes in multiple tissues, including cell adhesion (Baker et al., 2006), cell survival (Ko et al., 2012), and tumorigenesis (Arakawa, 2004). Within the nervous system, there is emerging evidence for netrins as novel regulators of synaptogenesis and synaptic function (Shen and Cowan, 2010; Flores, 2011). As it is challenging to isolate a synaptogenic function of netrin that is independent of its function in guidance, the role for netrin at synapses has mostly been addressed in simple and genetically tractable systems like *C. elegans*, *Drosophila*, and *Xenopus* (Winberg et al., 1998; Colon-Ramos et al., 2007; Poon et al., 2008; Manitt et al., 2009). Nonetheless, as tools that allow temporal-specific perturbation of netrins or their signaling components become available (Lai Wing Sun et al., 2011; Horn et al., 2013), more studies addressing the synaptogenic role of netrin should follow.

The founding member of the netrin family, uncoordinated-6 (UNC-6), was first identified as a component of the extracellular matrix that guides dorsoventral migration in *C. elegans* (Ishii et al., 1992). In mammals, the netrin family is composed of five members: netrin 1, 3 and 4, which are secreted and highly conserved, and netrin G1 and G2, which are glycophosphatidylinositol (GPI)-linked and vertebrate-specific. Netrin signaling is transduced through receptors such as DCC/Frazzled/UNC-40, neogenin, the UNC-5 family, and Down syndrome cell adhesion molecule (DSCAM) (Lai Wing Sun et al., 2011). The effectors that lie downstream of DCC, neogenin, and UNC-5 receptors comprise regulators of the cytoskeleton like the Rho family of GTPases, Src-family kinases, focal adhesion kinase and microtubule-associated proteins (Li et al., 2004b; Rajasekharan and Kennedy, 2009). In contrast, netrin Gs bind to netrin G ligands (NGLs) NGL-1/LRRC4C and NGL-2/LRRC4 (Nakashiba et al., 2000, 2002; Lin et al., 2003; Kim et al., 2006). The NGL family also includes NGL-3, a member that does not bind netrin Gs. As these membrane-anchored netrins and their ligands are less characterized, their signaling pathways remain unclear.

Though netrins and their receptors are widely studied for their role in nervous system development, they are continually expressed throughout adulthood (Livesey and Hunt, 1997; Manitt and Kennedy, 2002; Horn et al., 2013), suggesting that they play additional roles that are distinct from early developmental events. In addition, both netrin 1 and its receptor DCC are present in synaptosomes (Horn et al., 2013) and may thus act locally at synapses. Netrin Gs are similarly highly expressed in the adult brain and exhibit complex non-overlapping expression patterns (Nakashiba et al., 2002; Yin et al., 2002).

Netrin signaling in the nervous system is further altered when neuronal activity is perturbed. Levels of netrin receptors and netrin G2 are regulated by psychostimulant drugs (Yetnikoff et al., 2007; Argento et al., 2012), endocannabinoid receptor antagonists (Argaw et al., 2011), and epilepsy-induced activity (Pan et al., 2010). Amphetamine treatment elevates the expression of DCC and UNC-5 receptors in the mesocorticolimbic dopamine system in adult rats (Yetnikoff et al., 2007), while methylphenidate lowers the expression of DCC in the ventral tegmental area (VTA) of adult mice (Argento et al., 2012). It is intriguing to note that this down-regulation of DCC levels is associated with diminished sensitivity to cocaine (Argento et al., 2012). Taken together, these studies suggest that drugs that induce plasticity in the dopamine system regulate netrin receptor levels. Treatment of cultured primary cortical neurons with endocannabinoid receptor antagonists elevates surface expression of DCC (Argaw et al., 2011), suggesting that synaptic transmission of endocannabinoids regulates DCC activity. Netrin G2 levels are also elevated in the cortex of epileptic patients and mice (Pan et al., 2010), indicating that netrin G2 expression may be regulated by alterations in neuronal activity induced by epilepsy. While the significance, consequence, and underlying mechanisms of the regulation of netrin and its receptors are still being addressed, these studies provide preliminary evidence for a putative role for netrins in synaptic function. We will next explore the known functions of netrins in synaptogenesis, synaptic transmission, and plasticity.

ROLE OF NETRINS IN SYNAPTOGENESIS

Work in *Drosophila* motor neurons was the first to suggest a synaptogenic role for netrins. Overexpressing netrin in ventral muscles leads to DCC/frazzled-dependent formation of ectopic synapses in the transverse nerve in flies (Winberg et al., 1998). Similarly, addition of netrin into the *Xenopus* optic tectum augments the number of pre-synaptic sites in retinal ganglion cell axons in a DCC-dependent manner (Manitt et al., 2009). However, the downstream signaling components remain unknown.

Subsequent studies in *C. elegans* provided further evidence for the synaptogenic function of netrin (**Figure 1A**). Secretion of netrin/UNC-6 by glia coordinates innervation between AIY and RIA, two interneurons that mediate thermotaxis (Colon-Ramos

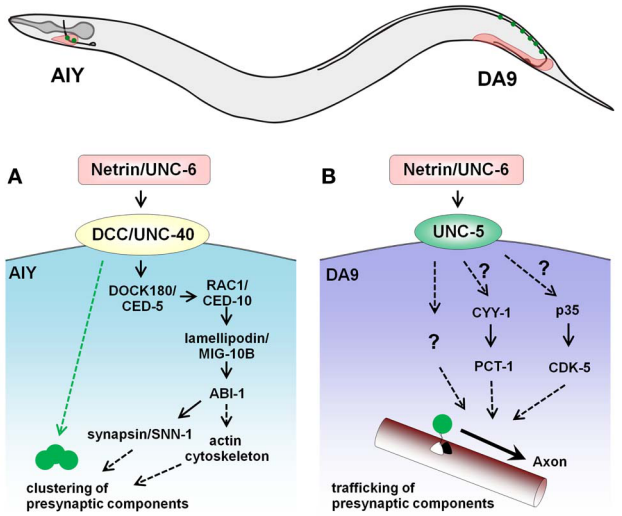

FIGURE 1 | Opposing roles of netrin/UNC -6 on synaptogenesis in the *C. elegans* AIY interneuron and DA9 motor neuron. (A) In the head region, glial sheath cells secrete UNC-6 and promote pre-synaptic assembly in AIY. UNC-6 signals through the DCC/UNC-40 receptor in AIY and recruits DOCK180/CED5 to pre-synaptic sites, leading to actin cytoskeleton remodeling and the pre-synaptic vesicle clustering. In addition, activation of UNC-40 clusters active zone proteins in a DOCK180/CED5-independent fashion (green dotted arrow). **(B)** In the tail, UNC-6 secreted by the ventral muscles acts through the UNC-5 receptor to prevent mislocalization of pre-synaptic components to the dendrite of the DA9 motor neuron. Pctaire kinase PCT-1, cyclin CYY-1, cyclin-dependent kinase CDK-5 and its activator p35 also regulate the kinesin/dynein-mediated localization of pre-synaptic components and may act downstream of UNC-6. The black arrow indicates the trafficking of components to the axon.

et al., 2007). Loss of netrin/UNC-6 or its receptor DCC/UNC-40 leads to defects in pre-synaptic assembly in AIY without affecting axon guidance. The DCC/UNC-40 receptor interacts with and localizes DOCK180/CED-5, which signals through Rac1/CED-10, lamellipodin/MIG-10B, and a component of Wiskott-Aldrich syndrome protein family, Abelson-interacting protein-1 (ABI-1) to regulate the actin cytoskeleton at pre-synaptic sites (Stavoe and Colon-Ramos, 2012; Stavoe et al., 2012). In addition, synaptic vesicle clustering is regulated through synapsin/SNN-1, which lies downstream of ABI-1 and lamellipodin/MIG-10B. These studies in the *C. elegans* AIY interneuron have thus elucidated the signaling effectors responsible for netrin-mediated synaptogenesis.

In addition to promoting synaptogenesis, netrin/UNC-6 expressed by ventral tissue also inhibits ectopic synapse formation in *C. elegans* (**Figure 1B**). Removal of netrin/UNC-6 or its receptor UNC-5 results in mislocalization of pre-synaptic components to the ventral dendrite of the DA9 motor neuron, and this effect is independent of guidance defects (Poon et al., 2008). Considering how netrin/UNC-6 drives synaptogenesis in AIY through the DCC/UNC-40 receptor, it is not surprising that netrin/UNC-6 functions in an opposite manner in DA9 when signaling through UNC-5. Since several intracellular regulators like the novel cyclin CYY-1, a cyclin-dependent kinase CDK-5, and the Pctaire kinase PCT-1 govern proper localization of pre-synaptic components in DA9 (Ou et al., 2010), it is possible that these factors lie downstream of netrin/UNC-6-UNC-5 signaling.

Apart from regulating pre-synapse formation, the netrin receptor DCC/UNC-40 also directs differentiation of muscles that are post-synaptic to motor axons and egg-laying motor neurons in *C. elegans*. Absence of this receptor leads to a reduction in post-synaptic muscle arm extensions (Alexander et al., 2009) and abolishes vulval muscle arms (Li et al., 2013a). In both instances, however, netrin/UNC-6 is not required. Taken together, the above studies demonstrate that netrin and its receptors modulate synaptogenesis in invertebrates and *Xenopus*. However, this begs the question: does netrin function similarly in mammals?

The role of netrin 1 and its receptor DCC in the mesocorticolimbic dopamine system has been explored (Flores, 2011). Adult mice lacking DCC have reduced dendritic spine densities in layer V pyramidal neurons in the medial prefrontal cortex (Grant et al., 2007; Manitt et al., 2011), suggesting that DCC is required for post-synaptic differentiation. These mice also exhibit defects in pubertal maturation of synaptic connectivity of dopaminergic neurons in this brain area, where numbers of tyrosine hydroxylase-positive varicosities are elevated (Manitt et al., 2011). Further work is needed to confirm if these varicosities are functional pre-synaptic terminals and if this is a secondary effect of axon misguidance. In addition, knocking down DCC in dopaminergic neurons *in vitro* suppresses the formation of autaptic axon terminals (Xu et al., 2010), consistent with a pro-synaptogenic role for DCC. While studies in *C. elegans* have provided some insights into netrin-mediated signaling pathways involved in regulating synaptogenesis, further studies in the mammalian central nervous system (CNS) are pertinent to elucidating the synaptogenic function of netrins.

Unlike secreted netrins, which act as both positive and negative regulators of synaptogenesis, the netrin G2 receptor NGL-2 primarily promotes synaptogenesis in cultured hippocampal neurons (Kim et al., 2006). NGL-2 was first identified as a novel binding partner of the post-synaptic scaffolding protein PSD-95. Overexpressing NGL-2 elevates the number of dendritic spines while knocking it down causes a loss in excitatory, but not inhibitory synapses. In hippocampal slices, removing NGL-2 leads to selective loss of spines in CA1 dendrites in the stratum radiatum, and spine formation requires NGL-2-netrin G2 binding (Denardo et al., 2012). Intriguingly, netrin G2 knockout mice have no detectable anomalies in the density of PSD-95 clusters in the hippocampus (Nishimura-Akiyoshi et al., 2007). Work in cultured hippocampal neurons further indicates that NGL-2-induced post-synaptic differentiation occurs via multiple mechanisms that are PSD-95-dependent or -independent (Kim et al., 2006). In addition to driving post-synaptic differentiation, NGL-2, like the post-synaptic cell adhesion molecule neuroligin, is sufficient to induce pre-synaptic differentiation (Kim et al., 2006). NGL-2 likely binds to netrin G2 and other factors to mediate this process since netrin G2 alone is insufficient to induce post-synaptic differentiation. Understanding the signaling pathways downstream of NGL-2 will be critical for comprehending the mechanisms of NGL-2 function.

ROLE OF NETRINS IN SYNAPTIC TRANSMISSION AND PLASTICITY

Given that netrins and their signaling components are expressed in adulthood (Livesey and Hunt, 1997; Manitt and Kennedy, 2002; Horn et al., 2013) and regulate synaptogenesis (Winberg et al., 1998; Kim et al., 2006; Colon-Ramos et al., 2007; Poon et al., 2008; Manitt et al., 2009; Flores, 2011), one may expect netrins to regulate synaptic transmission and plasticity. Several groups employing DCC-deficient mice and mammalian hippocampal cultures have attempted to explore this possibility.

Mice lacking DCC have altered dopamine transmission and are insensitive to the stimulant drug of abuse amphetamine (Grant et al., 2007; Yetnikoff et al., 2007, 2010). These mice exhibit enhanced amphetamine-induced dopamine release in the medial prefrontal cortex, but display the opposite response in the nucleus accumbens (Grant et al., 2007). A reduction in DCC also suppresses the rewarding effects of amphetamine on behavior and neuronal activity (Grant et al., 2007), and this effect is likely due to loss of DCC activation in the VTA (Yetnikoff et al., 2010). A deficiency in DCC also abolishes the amphetamine-induced increase in the expression of dendritic spine-associated protein spinophilin in the VTA (Yetnikoff et al., 2010).

A recent study further implicates DCC in synaptic plasticity in forebrain pyramidal neurons in the adult (Horn et al., 2013). Forebrain neurons in which DCC is deleted late in development had shorter dendritic spines, impaired long-term potentiation (LTP) but not long-term depression (LTD), and diminished expression of N-methyl-D-aspartate-type glutamate receptor (NMDAR) subunit GluN2B, Src, phosphorylated phospholipase C γ1, and phosphorylated Src family kinase Fyn. As deficits in LTP displayed by the DCC knockout mouse are rescued by Src activation or NMDAR function enhancement, it is likely that DCC regulates NMDAR-dependent plasticity through Src (Horn et al., 2013).

Using heterozygous mutants or conditional knockout mice, the previous studies showed that DCC is required for plasticity in the limbic system and the hippocampus. What about the ligand? To examine if netrin 1 affects synaptic function and plasticity, Bayat and colleagues infused netrin 1 into the hippocampus of mice after cerebral ischemia (Bayat et al., 2012). This treatment improved spatial memory impairment, basal evoked potential, and LTP, suggesting that netrin 1 is sufficient to enhance synaptic transmission. However, the underlying mechanism was not determined and the effects observed may be secondary to a pro-survival function of netrin 1. Nonetheless, this is the first study investigating an *in vivo* role for netrin in mammalian synaptic function and plasticity.

Apart from secreted netrins and their receptors, NGL-2 is also required for proper synaptic transmission. As previously described, NGL-2 drives synaptogenesis in cultured hippocampal neurons. Knocking down NGL-2 diminishes the frequency, but not the amplitude of miniature excitatory post-synaptic currents (mEPSCs) and has no effect on inhibitory currents (Kim et al., 2006). In hippocampal slices, removal of NGL-2 reduces synaptic transmission at Schaffer collateral synapses in the stratum radiatum of the CA1 region (Denardo et al., 2012). Hence, in addition to promoting synaptogenesis, NGL-2 drives synaptic transmission in distinct regions in the hippocampus, and regulates excitatory but not inhibitory synaptic function.

Wnts

First identified as key regulators of embryonic development, Wnt proteins have gained prominence over the past decade for their role in synapse formation and function in both the central and peripheral nervous system (Budnik and Salinas, 2011; Koles and Budnik, 2012; Salinas, 2012). These secreted lipo-glycoproteins are evolutionarily conserved and the mammalian genome comprises 19 Wnt genes (Willert and Nusse, 2012).

To achieve a wide spectrum of functions, Wnt proteins act through a diverse number of pathways—the canonical, divergent canonical, PCP, calcium Wnt signaling, and Frizzled nuclear import (FNI) pathways (Kuhl et al., 2000; Mlodzik, 2002; Ciani et al., 2004; Logan and Nusse, 2004; Speese and Budnik, 2007). These pathways lie downstream of the seven-pass transmembrane Frizzled receptors, and with the exception of the FNI pathway, activate the scaffolding protein Dishevelled (Dvl). In the canonical pathway, Dvl inhibits the Axin/Adenomatous Polyposis Coli (APC)/Glycogen synthase kinase 3β (Gsk3β) complex, and β-catenin is imported into the nucleus where it activates gene transcription. In the divergent pathway, inhibition of Gsk3β leads to decreased phosphorylation and augmented activity of microtubule-associated proteins. In the PCP pathway, Dvl regulates the cytoskeleton by activating the small Rho GTPases RhoA and Rac1, and c-Jun-amino-terminal kinase (JNK). In the calcium Wnt signaling pathway, Dvl increases intracellular calcium levels, thus activating multiple targets, including calcium/calmodulin-dependent protein kinase II (CaMKII), protein kinase C (PKC), and calcineurin, which results in the nuclear import of nuclear factor of activated T-cells (NFAT). In the FNI pathway, Frizzled-2 is internalized, processed, and imported into the nucleus. Though less characterized, Wnts also signal through members of the receptor tyrosine kinase-like orphan receptor (ROR) and the tyrosine kinase-like receptor Derailed (Drl)/Ryk families. Two recent reviews describe the Wnt signaling pathways in further detail (Koles and Budnik, 2012; Mulligan and Cheyette, 2012).

Given their importance in neuronal development and function, it is not surprising to note that Wnt ligands and their signaling components are present in neurons and regulated by activity. Neuronal activity-mediated regulation of Wnt signaling is prevalent in systems ranging from the *C. elegans* and *Drosophila* NMJ to the vertebrate CNS. Neuronal stimulation leads to secretion of the *C. elegans* Wnt ligand CWN-2 (Jensen et al., 2012), as well as release of the *Drosophila* Wnt1 ligand Wingless (Wg) from synaptic boutons in the larval NMJ (Ataman et al., 2008) and the fly olfactory sensory neuron (Chiang et al., 2009). In a central serotonergic neuron in *Drosophila*, activity triggers Wnt signaling and leads to dendritic refinement (Singh et al., 2010). Similarly, during activity-dependent dendrite development in hippocampal neurons, activity elevates Wnt release (Yu and Malenka, 2003) and Wnt2 transcription (Wayman et al., 2006). Wnt3a is also released at synapses in the hippocampus during tetanic stimulation (Chen et al., 2006). Altering activity with exposure to different environments or learning paradigms also changes Wnt

levels in the hippocampus. Wnt7a/b levels in post-synaptic CA3 neurons rise when mice are kept in an enriched environment (Gogolla et al., 2009); mice undergoing spatial learning in the Morris water maze have augmented levels of Wnt7, Wnt5, but not Wnt3. Lastly, levels of surface Frizzled-5, a receptor of Wnt7a in the hippocampus, increase with high frequency stimulation (HFS) in a Wnt-dependent fashion (Sahores et al., 2010). Taken together, the tight interplay between neuronal activity and Wnt signaling suggests a critical role for Wnts and their downstream effectors to modulate synaptogenesis, synaptic transmission, and plasticity.

ROLE OF Wnts IN SYNAPTOGENESIS

In the larval NMJ of *Drosophila*, members of the Wnt family promote formation of both pre- and post-synapses (**Figure 2**). Loss of Wg leads to defective pre- and post-synaptic specializations (Packard et al., 2002). During development, pre-synaptic vesicular release of the Wg-binding protein Evenness Interrupted/Wntless/Sprinter (Evi/Wls/Srt) leads to proper Wg secretion and recruitment of a *Drosophila* glutamate receptor interacting protein (dGRIP) to post-synaptic sites (Korkut et al., 2009). Wg binds the *Drosophila* Frizzled-2 (DFz2) receptor that is located both in the motor neuron and muscle. Several studies indicate that divergent signaling pathways are employed both in the pre-synaptic motor neuron and in the post-synaptic muscle. In the case of the latter, DFz2 is endocytosed from the post-synaptic membrane and transported to the nucleus by binding dGRIP, and this process is required for assembly of the post-synapse (Mathew et al., 2005; Ataman et al., 2006, 2008; Speese et al., 2012). On the pre-synaptic side, Wg signaling involves components of the canonical pathway like Arrow/Low-density lipoprotein receptor-related protein Dvl and Shaggy/Gsk3β to regulate bouton number (Ataman et al., 2008; Miech et al., 2008). Anterograde Wg signaling also modulates NMJ growth through the retrograde signal laminin A and the pre-synaptic integrin pathway (Tsai et al., 2012). Thus, Wg signals bi-directionally and utilizes distinct pathways in pre- and post-synaptic compartments. Recently, Kamimura and colleagues found that bi-directional signaling by Wg is regulated by a secreted heparan sulfate proteoglycan (HSPG) perlecan/trol (Kamimura et al., 2013). Coincidentally, Wg levels are also altered by HSPG sulfation (Dani et al., 2012). In addition to Wg, loss of Wnt5 leads to a reduction in the number of pre-synaptic boutons and suppresses active zone formation (Liebl et al., 2008). Wnt5 signals through its post-synaptic receptor Drl but some of its functions are Drl-independent. Taken together, these studies suggest that Wg and Wnt5 drive synaptogenesis in the fly NMJ.

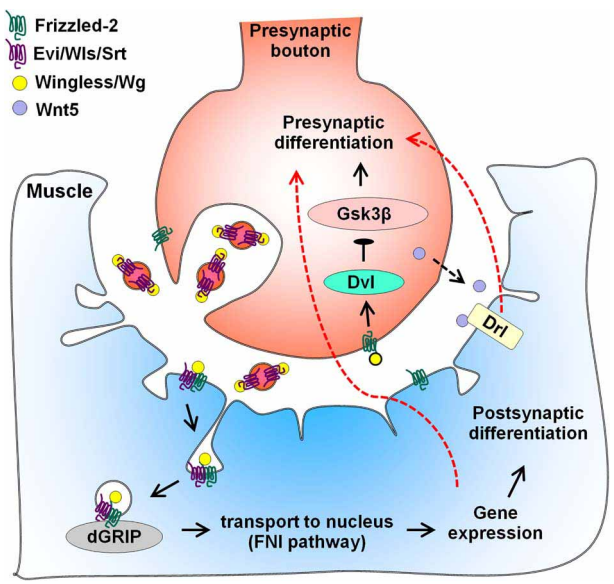

FIGURE 2 | Wnt regulation of larval NMJ differentiation. Vesicular release of the Wnt-binding protein Evenness Interrupted/Wntless/Sprinter (Evi/Wls/Srt) facilitates pre-synaptic secretion of Wingless/Wg. In the pre-synaptic bouton, binding of Wg to the Frizzled-2 receptor (DFz2) activates components of the canonical pathway and leads to pre-synaptic differentiation. In the post-synaptic muscle, Wg binds to DFz2, inducing endocytosis of the receptor. As part of the Frizzled nuclear import (FNI) pathway, Evi/Wls/Srt recruits the Wg receptor-interacting protein dGRIP, leading to transport of DFz2 to the nucleus. Entry of DFz2 into the nucleus alters gene expression, promoting post-synaptic, and possibly, pre-synaptic differentiation (red dotted arrow). Additional regulators of Wg signaling excluded from this figure include laminin A, integrin, the HSPG perlecan/trol and HSPG sulfation. In addition, Wnt5 is also secreted by the pre-synaptic bouton (black dotted arrow) and acts through the tyrosine kinase-like receptor Derailed (Drl) to promote pre-synaptic differentiation retrogradely (red dotted arrow).

In the vertebrate NMJ, Wnt3 and Wnt11r enhance synaptogenesis. Wnt3 augments acetylcholine receptor (AChR) clustering in the chick wing NMJ and in cultured myotubes via the non-canonical PCP pathway involving Rac1 activation and Rho signaling (Rattner et al., 1997; Weston et al., 2003; Niehrs, 2006; Henriquez et al., 2008). Similarly, the non-conventional Wnt11r is required for AChR clustering in zebrafish, but acts through the muscle-specific kinase (MuSK)/unplugged receptor and Dvl1 (Jing et al., 2009). In addition to Wnt3 and Wnt11r, Wnt signaling components like Dvl1, Dvl1-interacting protein p21-activated kinase1 (Luo et al., 2002), APC (Wang et al., 2003), and β-catenin (Zhang et al., 2007; Li et al., 2008) are implicated as positive regulators of NMJ development.

Studies in the glutamatergic cerebellar glomerular rosette, a multi-synaptic structure formed between mossy fibers and granule cells, provided the first glimpse into the synaptogenic role of Wnts in the vertebrate CNS. Loss of Wnt7a or Dvl1 delays the maturation of glomerular rosettes and leads to defects in the localization of pre-synaptic markers while expression of Wnt7a in granule cells induces clustering of the pre-synaptic protein synapsin I in mossy fiber axons (Hall et al., 2000; Ahmad-Annuar et al., 2006). Wnt7a similarly stimulates clustering of pre-synaptic markers in hippocampal neurons and acts through the Frizzled-5 receptor (Cerpa et al., 2008; Sahores et al., 2010). In addition to regulating the pre-synapse, Wnt7a signaling also promotes dendritic spine growth and PSD-95 clustering through Dvl1 and CaMKII (Ciani et al., 2011). Lastly, mice exposed to an enriched environment have an increased number of synapses in their hippocampus and this effect is dependent on Wnt7a/b (Gogolla et al., 2009).

Apart from Wnt7a/b, Wnt5a also regulates synaptogenesis in the hippocampus. There are conflicting reports on the effect of Wnt5a on the pre-synapse while the role of Wnt5a at the post-synapse is less controversial. Several studies report that Wnt5a increases clustering of pre-synaptic proteins and synaptic contacts (Varela-Nallar et al., 2012), and acts through ROR1/2 receptors to promote pre-synaptic assembly in cultured hippocampal neurons (Paganoni et al., 2010). However, other studies indicate that Wnt5a decreases the number of pre-synaptic terminals or has no effect on hippocampal neurons (Davis et al., 2008; Farias et al., 2009). In dendrites, Wnt5a increases calcium levels, spine size, and spine number during development (Varela-Nallar et al., 2010), and clusters PSD-95 through a JNK-dependent signaling pathway (Farias et al., 2009). On top of regulating synapse formation in the pre- and post-synaptic compartments of excitatory synapses, Wnt5a also augments the insertion and clustering of γ-aminobutyric acid A ($GABA_A$) receptors in hippocampal neurons by activating CaMKII (Cuitino et al., 2010).

Like Wnt7 and Wnt5a, Wnt3 also induces pre-synaptic protein clustering in hippocampal neurons and drives synapse formation between sensory and motor neurons in the spinal cord. Through the pre-synaptic Frizzled-1 receptor, Wnt3a elevates the number of Bassoon clusters in axons (Varela-Nallar et al., 2009). In motor neurons, Wnt3 secretion induces synapsin clustering and regulates terminal arborization of sensory neurons in a Gsk3β-dependent manner (Krylova et al., 2002). Hence, Wnts, particularly Wnt7a/b, Wnt5a, and Wnt3, regulate synaptogenesis in vertebrate cerebellar, hippocampal, and spinal neurons through diverse signaling mechanisms. Whereas Wnt7a signals through Frizzled-5, Dvl1, and/or CaMKII, Wnt5a signaling occurs via ROR receptors, JNK, or CaMKII, and Wnt3 acts through Gsk3β.

While the previously described Wnt ligands are generally positive regulators of synaptogenesis, other Wnt ligands in *Drosophila*, *C. elegans*, and mice also negatively regulate synaptogenesis. Wnt4 is preferentially expressed in the *Drosophila* muscle cell M13. Absence of Wnt4, its receptor DFz2, Drl2, or Dvl results in the formation of ectopic synapses by motor neuron 12 onto M13 (Inaki et al., 2007). Similarly, in the *C. elegans* cholinergic motor neuron DA9, LIN-44, a Wnt ligand secreted by the tail hypodermal cells, inhibits ectopic synapse formation in the posterior segment of the neuron through the LIN-17/Frizzled receptor and DSH-1/Dvl (Klassen and Shen, 2007). Since other known canonical, PCP, calcium Wnt signaling pathway effectors have no effect, a pathway comprising novel mediators may be employed. In vertebrates, Wnt3a inhibits post-synapse formation by reducing AChR clustering in cultured myotubes through the canonical pathway involving β-catenin (Wang et al., 2008). Taken together, Wnts both enhance and suppress synaptogenesis through the engagement of both canonical and non-canonical pathways (**Table 1**).

ROLE OF Wnts IN SYNAPTIC TRANSMISSION AND PLASTICITY

Just as the fly NMJ provided important insights into how Wnts regulate synapse formation, further studies utilizing this model system have yielded additional roles for Wnt5 and Wnt1/Wg in synaptic transmission and plasticity. Absence of Wnt5, but not Drl, lowers the amplitude of evoked end-plate junctional currents (EJCs) and lowers the frequency of miniature EJCs (mEJCs), indicating defects in pre-synaptic transmission (Liebl et al., 2008). The *wg* mutant also has suppressed activity-dependent synaptic growth (Ataman et al., 2008). In addition, Wg is a potential negative regulator of homeostatic compensation, where it is inhibited by the paired box protein Pax3/7 homolog gooseberry (Marie et al., 2010). However, the downstream mechanisms remain elusive.

A recent study on the *C. elegans* Wnt CWN-2 at the NMJ provides some mechanistic insight (Jensen et al., 2012). In contrast to another Wnt ligand LIN-44 that inhibits pre-synapse formation, CWN-2 promotes synaptic strength by regulating the translocation of an AChR ACR-16/α7 to the synapse (Jensen et al., 2012). Reduction in AChR enrichment and synaptic current is observed both in the absence of CWN-2 in the motor neuron and during the loss of LIN-17/Frizzled, ROR receptor tyrosine kinase CAM-1 or DSH-1/Dvl in muscles. Other Frizzled receptors and Ryk/Drl are not required for the elevation in post-synaptic strength induced by CWN-2. The identities of the effectors downstream of DSH-1/Dvl responsible for AChR translocation remain to be elucidated.

In addition to the synaptogenic functions of Wnt7a, Wnt5a, and Wnt3a, these ligands also increase synaptic transmission in cerebellar and hippocampal slices. In the mossy fiber-granule cell synapses of Wnt7a/Dvl1 double mutant mice, neurotransmitter release is diminished (Ahmad-Annuar et al., 2006). Wnt7a and post-synaptic Dvl1 also increase the frequency of mEPSCs, indicating larger neurotransmitter release in CA3-CA1 synapses in the hippocampus (Cerpa et al., 2008; Ciani et al., 2011). Ciani and colleagues further observed a CaMKII-dependent increase in the amplitude of mEPSCs in hippocampal neurons induced by Wnt7a, suggesting that this ligand acts through the calcium Wnt pathway in dendrites to augment synaptic strength (Ciani et al., 2011). On the other hand, Wnt5a increases both excitatory and inhibitory synaptic transmission and signals through the PCP and calcium Wnt signaling pathways in hippocampal neurons. Wnt5a and JNK, a component of the PCP pathway, regulate glutamatergic synaptic transmission (Farias et al., 2009). In addition, Wnt5a facilitates LTP by augmenting the proportion of GluN2B-containing NMDARs at the synapse, as well as the amplitude of NMDAR currents through the elevation of calcium and the activation of CaMKII (Varela-Nallar et al., 2010; Cerpa et al., 2011). In contrast, through the same calcium Wnt signaling pathway, Wnt5a also increases $GABA_A$ receptor recycling and miniature inhibitory post-synaptic currents (mIPSCs) (Cuitino et al., 2010). Wnt3a is likely to have a similar effect as Wnt5a since blocking its activity decreases LTP in hippocampal slices (Chen et al., 2006). Consistent with the previous finding, Wnt3a enlarges neurotransmitter release through pre-synaptic Frizzled-1 in hippocampal neurons (Varela-Nallar et al., 2009) and enhances excitatory transmission in hippocampal slices (Beaumont et al., 2007).

The studies mentioned above suggest that Wnts increase synaptic function *in vivo* and *in vitro* through the PCP pathway, calcium Wnt signaling, and possibly other pathways (**Table 1**). However, do Wnts affect neural circuit function? In the

Table 1 | Known functions of Wnts in synaptogenesis and synaptic function.

Wnt	System	Function	Pathway	References
Wg/Wnt1	*Drosophila* NMJ	Pre-synaptic differentiation	Canonical (Arrow/LRP, Dvl, Gsk3β)	Packard et al., 2002; Ataman et al., 2008; Miech et al., 2008
		Post-synaptic differentiation	FNI	Mathew et al., 2005; Ataman et al., 2006; Speese et al., 2012
		Activity-dependent synaptic growth	Unclear	Ataman et al., 2008
Wnt4	*Drosophila* NMJ	Inhibit ectopic synapses	Dfz2, Drl2, Dvl	Inaki et al., 2007
Wnt5	*Drosophila* NMJ	Pre-synaptic differentiation	Drl	Liebl et al., 2008
		Pre-synaptic transmission	Not through Drl	Liebl et al., 2008
LIN-44	*C. elegans* DA9 neuron	Inhibit ectopic synapses	LIN-17/Fz, Dvl	Klassen and Shen, 2007
CWN-2	*C. elegans* NMJ	AChR clustering, synaptic transmission	LIN-17/Fz, Dvl, ROR/CAM-1	Jensen et al., 2012
Wnt3	Chick wing NMJ, cultured myotubes	AChR clustering	PCP (Rac1, Rho)	Rattner et al., 1997; Weston et al., 2003; Niehrs, 2006; Henriquez et al., 2008
Wnt11r	Zebrafish	AChR clustering	MuSK, Dvl1	Jing et al., 2009
Wnt3a	Hippocampal neurons	Bassoon clustering	Fz1	Varela-Nallar et al., 2012
	Hippocampal neurons, slice	Excitatory transmission	Fz1	Beaumont et al., 2007; Varela-Nallar et al., 2012
	Sensory neurons	Synapsin clustering	Gsk3β	Krylova et al., 2002
	Cultured myotubes	Reduce AChR clustering	Canonical (β-catenin)	Wang et al., 2008
Wnt5a	Hippocampal neurons	Pre-synaptic differentiation	ROR1/2	Paganoni et al., 2010; Varela-Nallar et al., 2012
		Decrease pre-synapse number or no effect	Unclear	Davis et al., 2008; Farias et al., 2009
		Spine growth	Calcium Wnt	Varela-Nallar et al., 2012
		PSD-95 clustering	PCP (JNK)	Farias et al., 2009
	Hippocampal slice	Synaptic transmission	PCP (JNK)	Farias et al., 2009
	Hippocampal neurons	GABAR insertion and clustering	Calcium Wnt (CaMKII)	Cuitino et al., 2010
	Hippocampal slice	Inhibitory transmission	Calcium Wnt (CaMKII)	Varela-Nallar et al., 2010; Cerpa et al., 2011
		Excitatory transmission	Calcium Wnt (CaMKII)	Varela-Nallar et al., 2010; Cerpa et al., 2011
Wnt7a	Cerebellar granule cells	Synapsin clustering	Dvl1, Gsk3β?	Hall et al., 2000; Ahmad-Annuar et al., 2006
	Cerebellar slice	Pre-synaptic transmission	Dvl1	Ahmad-Annuar et al., 2006
	Hippocampal neurons	Pre-synaptic differentiation	Fz5	Cerpa et al., 2008; Sahores et al., 2010
		Spine growth, PSD-95 clustering	Calcium Wnt (Dvl1, CaMKII)	Ciani et al., 2011
	Hippocampal slice	Pre-synaptic transmission	Calcium Wnt (Dvl1, CaMKII)	Cerpa et al., 2008; Ciani et al., 2011

developing *Xenopus* optic tectum, Lim and colleagues reported that Wnt secreted from tectal cells enhances visual experience-dependent plasticity of receptive fields of cells in the dorsal tectum (Lim et al., 2010). This suggests that regulation of synapse formation and function by Wnt signaling likely leads to downstream effects on circuit function.

TRANSFORMING GROWTH FACTOR-β

TGF-β signaling is critical for multiple biological processes, including proliferation, development, patterning, and regeneration (Kubiczkova et al., 2012). The TGF-β superfamily consists of more than 30 secreted members in humans that are broadly classified into two ligand subfamilies: the TGF-β-activin-Nodal

group and the bone morphogenetic proteins (BMPs) group (Shi and Massague, 2003). Different members signal through distinct subtypes of heterotetrameric receptor complexes composed of specific type I and II receptors, leading to phosphorylation of R-Smads and inducing their binding to Smad4. Upon entering the nucleus, the Smad complex interacts with transcription factors to enhance gene expression. Massague provides a detailed description of TGF-β signaling in two recent reviews (Massague, 2012a,b).

Multiple members of the TGF-β superfamily play a role in the developing nervous system and several are regulated by neuronal activity. For instance, developmental expression of TGF-β in the mammalian neocortex is required for axon initiation *in vivo* and *in vitro* (Yi et al., 2010). Depolarization of primary hippocampal neurons with high levels of potassium or glutamate leads to the release of TGF-β (Specht et al., 2003) and the elevated expression of TGF-β2 and TGF-β3 (Lacmann et al., 2007). In addition, both kainate-induced seizures and HFS augment levels of activin βA mRNA in the hippocampus (Andreasson and Worley, 1995; Inokuchi et al., 1996) while sensory deafferentation of the visual cortex reduces activin βA mRNA levels in cortical neurons in specific layers (Andreasson and Worley, 1995). It was also recently reported that lowering activity in the *C. elegans* AVA command interneuron by exposure to pathogenic bacteria enhances release of TGF-β/DBL-1 (Zhang and Zhang, 2012). This precise regulation of TGF-β and activin levels by synaptic input suggests an activity-dependent function for these TGF-β family members in synaptogenesis, synaptic transmission, and plasticity.

ROLE OF TGF-β IN SYNAPTOGENESIS

Studies in the *Drosophila* NMJ have provided key mechanistic insights into how TGF-β family members act as positive regulators of synaptogenesis (**Figure 3**). Multiple reports from the early 2000s have demonstrated that the BMP homolog Glass Bottom Boat (Gbb) secreted by muscle cells signals through pre-synaptic receptors wishful thinking (Wit), thickveins (Tkv) and saxophone (Sax) to promote NMJ synapse formation (Aberle et al., 2002; Marques et al., 2002; McCabe et al., 2003; Rawson et al., 2003). Gbb binding both activates the LIM-domain kinase LIMK1 to stabilize the synapse (Eaton and Davis, 2005) and phosphorylates the R-Smad transcription factor Mothers against decapentaplegic (Mad) to increase the number of synapses (Rawson et al., 2003). Several downstream targets have been identified, including the Rac guanine nucleotide exchange factor (GEF) Trio (Ball et al., 2010; Kim and Marques, 2010). This signaling requires dynein-mediated retrograde axonal transport of BMP receptors (Smith et al., 2012b). The Tkv receptor and Mad transcription factor are also present in the muscle and may affect post-synaptic development and function (Dudu et al., 2006). To prevent synaptic overgrowth, this pathway is negatively regulated by several factors in the motor neuron, including the cysteine-rich transmembrane BMP regulator 1 homolog that antagonizes BMP signaling (James and Broihier, 2011), the inhibitory Smad Daughters against decapentaplegic (Dad) and the E3 ubiquitin ligase Highwire (McCabe et al., 2004). In addition, the Cdc42 pathway inhibits post-synaptic Gbb secretion (Nahm et al., 2010a,b) while endocytic and endosomal machinery lower surface levels of BMP

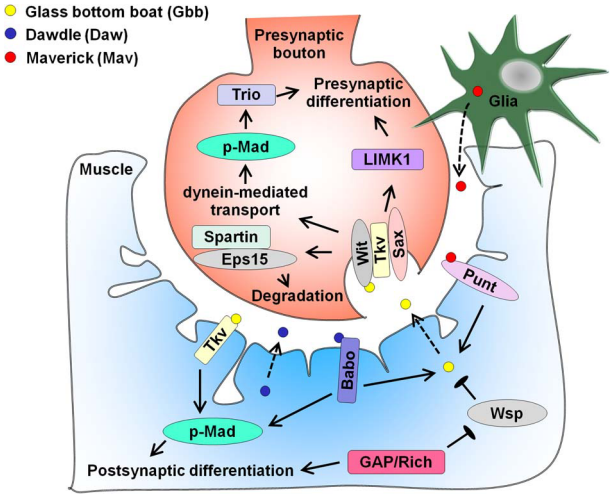

FIGURE 3 | Bone Morphogenetic Protein (BMP) homolog Glass bottom boat (Gbb), activin ligand Dawdle (Daw), and TGF-β ligand Maverick (Mav) regulate larval NMJ differentiation. Gbb secreted from the muscle (dotted arrow) signals through pre-synaptic BMP receptors wishful thinking (Wit), thickveins (Tkv), and saxophone (Sax) to enhance synaptogenesis. Gbb binding activates LIMK1 and dynein-mediated retrograde axonal transport of the BMP receptors leads to phosphorylation of the Mad transcription factor, thus driving pre-synaptic differentiation. One of the downstream targets of phosphorylated Mad (p-Mad) is the Rac GEF Trio. Gbb signaling is inhibited by spartin, which binds endocytic adaptor Eps15 and enhances endocytic degradation of Wit. Cysteine-rich transmembrane BMP regulator 1 homolog, the inhibitory Smad Dad, and Highwire are additional negative regulators of Gbb signaling absent from this figure. Gbb also binds Tkv in muscle and regulates gene expression through p-Mad. Apart from Gbb, muscle-derived Daw (dotted arrow) binds Baboon (Babo) to enhance post-synaptic differentiation, while promoting Gbb expression to drive pre-synaptic differentiation. Lastly, glia-derived Mav (dotted arrow) binds Punt and augments Gbb transcription and release. In addition, the Cdc42-selective GAP Rich inhibits the Cdc42 effector Wiskott-Aldrich syndrome protein Wsp, thus stimulating Gbb secretion from the muscle. Rich also promotes post-synaptic development independently of Cdc42.

receptors in neurons (Sweeney and Davis, 2002; Wang et al., 2007; O'Connor-Giles et al., 2008). Gbb secretion is further modulated by HSPG sulfation (Dani et al., 2012). Spartin, which binds to endocytic adaptor Eps15 was recently found to inhibit synaptic growth at the NMJ by promoting endocytic degradation of BMP receptor Wit (Nahm et al., 2013). This leads to elevated levels of fragile X mental retardation protein, a translational repressor of Futsch/microtubule associated protein MAP1B mRNA. Apart from Gbb, the activin ligand Dawdle (Daw) and the TGF-β ligand Maverick (Mav) are also present at the NMJ. Daw acts through the post-synaptic activin type I receptor Baboon (Babo) and Smad2 transcription factor to promote synaptogenesis at the NMJ (Ellis et al., 2010). Daw and Babo further regulate pre-synaptic differentiation by regulating Gbb expression (Ellis et al., 2010). Secreted by glia, Mav regulates synaptic growth by binding muscle activin-type receptor Punt and by increasing Gbb signaling (Fuentes-Medel et al., 2012). Taken together, the BMP homolog Gbb and the activin ligand Daw are potent activators of synapse growth at the NMJ and achieve this by promoting gene expression through the Smad transcription factors. The TGF-β ligand

Mav, the activin ligand Daw, and a host of other intracellular components regulate Gbb signaling to ensure strict control of synaptogenesis at the NMJ.

TGF-β family members also enhance synapse formation in mammalian neurons *in vitro*. Treating primary neurons with TGF-β1, activin, or BMP7 augments synapse formation through different effectors. TGF-β1 secreted from astrocytes increases synaptogenesis in cortical neurons by inducing secretion of D-serine, the co-agonist of the NMDAR (Diniz et al., 2012). Activin promotes synaptic development and alters spine morphology in hippocampal neurons by modulating actin dynamics. This process is independent of protein and RNA synthesis (Shoji-Kasai et al., 2007). BMP7 accelerates hippocampal dendrite development and elevates the rate of synaptogenesis, but the underlying mechanism remains unclear (Withers et al., 2000). Though these findings indicate a synaptogenic role for TGF-β1, activin, and BMP7, it is uncertain if these TGF-β family members function likewise *in vivo* in the vertebrate CNS. Recently, Xiao and colleagues examined the auditory system of conditional BMPR1a and BMPR1b double knockout mice and observed smaller synapses with fewer docked synaptic vesicles, as well as multiple inputs, at the calyx of Held (Xiao et al., 2013). Hence, BMP signaling regulates synapse size and elimination *in vivo* at the calyx of Held.

While other TGF-β family members have primary roles in driving synaptogenesis, *Drosophila* activin (dactivin) and myoglianin, a *Drosophila* TGF-β2 ligand, are involved in synaptic patterning in the visual system and NMJ, respectively (Ting et al., 2007; Awasaki et al., 2011; Yu et al., 2013). In the *Drosophila* visual system, mutations in Babo and the Smad2-interacting nuclear import protein importin-α3 lead to overlap of R7 photoreceptor axon terminals with those in neighboring columns (Ting et al., 2007). Similar defects in tiling occur in the absence of dactivin or Smad2. Hence, activin regulates activity of Smad2 to ensure formation of appropriate pre-synaptic contacts. In the larval NMJ, TGF-β2/myoglianin secreted from muscle acts through Babo to prevent formation of ectopic synapses and this process is regulated by the immunoglobulin superfamily transmembrane protein Plum, as well as the ecdysone receptor-B1 (Yu et al., 2013). However, the downstream signaling mechanism has not been characterized.

ROLE OF TGF-β IN SYNAPTIC TRANSMISSION AND PLASTICITY

In addition to driving synaptogenesis, members of the TGF-β family are implicated in promoting excitatory synaptic transmission. Work on long-term synaptic facilitation in the marine mollusk *Aplysia californica* provided the earliest evidence of the ability of TGF-β1 to sculpt synaptic transmission (Zhang et al., 1997). This was followed by the finding that TGF-β1 induces long-term increases in neuronal excitability by activating mitogen-activated protein kinase (MAPK), a well-established regulator of LTP in *Aplysia* (Chin et al., 2006). TGF-β1 also acutely activates MAPK, altering distribution of the pre-synaptic protein synapsin and reducing synaptic depression in the *Aplysia* sensorimotor synapse (Chin et al., 2002). Treatment of cultured hippocampal neurons with TGF-β2 also led to an analogous effect—decreased short-term synaptic depression of evoked post-synaptic currents (Fukushima et al., 2007). This observation is associated with heightened phosphorylation of cAMP response element-binding protein (CREB). Consistent with a role for TGF-β2 in promoting synaptic transmission, TGF-β2 knockout mice have impaired transmission in GABAergic/glycinergic and glutamatergic synapses in the brainstem where both frequency of mEPSCs and total charge transfer are suppressed (Heupel et al., 2008). This effect on pre-synaptic transmission by TGF-β1 and TGF-β2 is reminiscent of diminished neurotransmitter release in the fly NMJ when BMP signaling is disrupted, and this process is likely partially mediated through the lymphocyte antigen 6 (Ly6) neurotoxin-like molecule target of Wit (Aberle et al., 2002; Marques et al., 2002; Baines, 2004; McCabe et al., 2004; Nahm et al., 2010b; Kim and Marques, 2012). Conversely, chordin null mice that have elevated BMP signaling exhibit augmented pre-synaptic neurotransmitter release, as reflected from enhanced paired pulse facilitation (PPF) and LTP (Sun et al., 2007). This observation is unlikely due to transduction through Smad4 since Smad4 knockout mice have stronger, instead of weaker, PPF in excitatory synaptic transmission in the hippocampus (Sun et al., 2010). In addition, at the calyx of Held synapse, knocking out both BMPR1a and BMPR1b reduced the amplitude of EPSCs and lengthened decay times, indicating that a loss in BMP signaling reduces synaptic transmission (Xiao et al., 2013).

Besides TGF-β1, TGF-β2, and BMP, activin also enhances excitatory synaptic transmission. In cultured hippocampal neurons, activin phosphorylates NMDARs, possibly inducing LTP (Kurisaki et al., 2008). This signaling occurs through Src family tyrosine kinases, PDZ proteins, and activin receptor interacting protein 1. Coherent with this finding, transgenic mice with impaired activin function have reduced NMDA currents and LTP in glutamatergic synapses in the hippocampus (Muller et al., 2006). Similarly, inhibiting activin by overexpressing follistatin in mouse forebrain neurons also impairs hippocampal late-LTP and long-term memory formation during contextual fear conditioning (Ageta et al., 2010). What are the downstream mediators that induce LTP in the presence of BMP and activin? As Smad4-deficient mice do not exhibit defects in LTP or spatial memory, it is possible that BMP and activin regulate hippocampal LTP through non-canonical signaling pathways that might include MAPK (Zhou et al., 2003; Sun et al., 2010).

Activin also suppresses inhibitory synaptic transmission, but this may occur through the canonical Smad-dependent pathway (Krieglstein et al., 2011). Impairing activin function by expressing a dominant-negative mutant of activin receptor in forebrain neurons enhanced spontaneous GABA release and $GABA_B$ receptor function in hippocampal neurons and suppressed anxiety-like behavior in mice (Zheng et al., 2009). Since Smad4 knockout mice have larger paired-pulse depression of $GABA_A$ currents in the hippocampus (Sun et al., 2010), it is possible that activin regulates GABAergic synapses through Smad4. Lastly, activin indirectly affects the excitatory-inhibitory balance by decreasing the number of GABAergic interneurons while increasing that of dentate gyrus granule cells (Sekiguchi et al., 2009). Taken together, the TGF-β superfamily enhances excitatory synaptic transmission, while suppressing inhibitory synaptic transmission. The

downstream effectors differ for the different members and likely involve both Smad-dependent and Smad-independent pathways. Several other reviews cover further details on the effect of TGF-β on synapses and behavior (Krieglstein et al., 2011; Salinas, 2012).

TUMOR NECROSIS FACTOR-α

TNF-α is a type II transmembrane 26 kDa precursor molecule which is proteolytically cleaved by the metalloprotease TNF-α converting enzyme, a disintegrin and metallopeptidase domain 17 (ADAM17) to generate a soluble 17 kDa homotrimeric pro-inflammatory cytokine (Horiuchi et al., 2010). Both membrane-bound and soluble forms of TNF-α contribute to a broad range of physiological and pathological activities, including cell proliferation, differentiation, apoptosis, and inflammatory responses in various cells (Wang et al., 2005; Chapard et al., 2012).

TNF-α is secreted by a variety of cells such as macrophages, monocytes, neutrophils, T cells, natural killer cells, adipocytes, and fibroblasts (Fahey et al., 1995; Jovinge et al., 1996; Cawthorn and Sethi, 2008; Ambler et al., 2012; Brotas et al., 2012; Zakka et al., 2012), and its signaling is transduced through TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2). Soluble TNF-α binds preferentially to TNFR1, which is expressed in neurons (Brambilla et al., 2011) whereas transmembrane TNF-α binds to TNFR2, which is mainly expressed in immune cells such as those of the myeloid lineage, lymphocytes, and macrophages (McCoy and Tansey, 2008).

TNFRs regulate both cell death and survival depending on the cellular environment and context. Activation of TNFR1 recruits the intracellular death domain (DD)-containing adaptor TNFR-associated DD protein (TRADD), which can also recruit the receptor-interacting protein kinase 1 (RIPK1) and TNFR-associated factor-2 (TRAF2). This complex leads to the activation of the transcription factor AP-1 through MAPK and JNK pathways that prevent the triggering of cell death processes. In contrast, TRADD can also promote the recruitment of the Fas-associated DD protein (FADD), which is associated with a caspase-dependent or caspase-independent cell death signaling process known as apoptosis or necrosis, respectively (Chu, 2013). These cell death processes require the internalization of the TNFR (Schneider-Brachert et al., 2004).

Numerous studies have recently shown that TNF-α is involved in inflammatory events in the CNS and have opposing effects depending on their levels in the brain (Hoffmann et al., 2009; Mc Guire et al., 2011; Smith et al., 2012a). TNF-α is secreted by non-neural cells in the brain, including activated astrocytes and microglial cells (Santello and Volterra, 2012) in response to pathological brain conditions and diseases, which can play a protective role in neurons. Under physiological conditions, TNF-α controls the inflammatory response, hence defending against infection. However, excessive amounts of TNF-α are indicative of acute and chronic neuroinflammation. Not surprisingly, TNF-α is involved in several neurodegenerative disorders associated with neuroinflammation and neuronal cell death such as Alzheimer's disease (AD), Parkinson's disease, and HIV-associated dementia (Brabers and Nottet, 2006; Frankola et al., 2011). Consistent with these reports, chronic expression of neuronal TNF-α enhances neuronal cell death in an AD mouse model (Janelsins et al., 2008).

Since the effect of TNF-α signaling is largely dependent on its concentration, multiple factors including neuronal activity, excitotoxicity, and neuroinflammation are involved in TNF-α regulation. Elevating neuronal activity by whisker stimulation elevates TNF-α expression in the somatosensory cortex, as measured by immunostaining (Churchill et al., 2008). Excitotoxicity induced by chronic treatment of NMDA also enhances the expression of TNF-α and other neuroinflammatory markers (Chang et al., 2008). Lastly, treatment with lipopolysaccharide (LPS) augments the expression of TNF-α (Ikeda et al., 2007; Dholakiya and Benzeroual, 2011; Welser-Alves and Milner, 2013). TNF-α released from microglia and astrocytes up-regulates gene transcription for arachidonic acid (AA) cascade enzymes via the nuclear factor kappa B (NF-κB) pathway, which has been shown to damage neurons by activating pro-apoptotic factors and caspase-3 (Rao et al., 2012).

During neuroinflammation, an elevation in TNF-α levels and AA signaling alters synaptic protein expression and leads to the loss of synapses (**Figure 4A**). LPS-induced neuroinflammation lowers the protein levels of several key molecules including the pre-synaptic vesicle protein synaptophysin, the neuron-specific post-synaptic F-actin-binding protein drebin, and PSD-95 (Kellom et al., 2012; Rao et al., 2012). Reductions in these pre- and post-synaptic proteins suggest that high TNF-α levels induced by neuroinflammation may enhance synaptic loss. Furthermore, synaptic loss induced by LPS is abolished in neurons cultured with microglia that produce less TNF-α, indicating that TNF-α mediates LPS-induced synapse loss (Xing et al., 2011; Kellom et al., 2012).

ROLE OF TNF-α IN SYNAPTIC TRANSMISSION AND PLASTICITY

TNF-α has been reported to play important roles in neuronal functions such as microglia activation, synaptic transmission, and synaptic plasticity (Stellwagen et al., 2005; Watters and O'Connor, 2011). Activated astrocytes and microglia increase the expression and secretion of TNF-α (Santello and Volterra, 2012), and also promote glutamatergic excitatory synaptic transmission and plasticity (Stellwagen and Malenka, 2006; Kawasaki et al., 2008; Wheeler et al., 2009; Steinmetz and Turrigiano, 2010; Park et al., 2011a; Zhang and Dougherty, 2011; Zhang et al., 2011; O'Connor, 2013).

TNF-α was shown to regulate calcium currents at the post-synapse through the NF-κB pathway (Furukawa and Mattson, 1998), as well as block LTP in the hippocampus (Butler et al., 2004; Pickering et al., 2005) (**Figure 4A**). In cultured hippocampal neurons, long-term but not short-term treatment with TNF-α augments calcium currents through post-synaptic L-type voltage-dependent calcium channels (VDCCs), and decreases glutamate receptor agonist-induced currents. In addition, TNF-α blocks the early phase of LTP but not the late phase through the activation of TNFR1 and metabotropic glutamate receptors (mGluRs) in a p38 MAPK–dependent manner (Butler et al., 2004; Pickering et al., 2005). TNF-α activation of mGluRs leads to inositol-1,4,5-trisphosphate (IP_3) receptor-mediated calcium release via phospholipase C (PLC), which elevates intracellular calcium concentration to impair LTP (Pickering et al., 2005). Group I/II mGluR antagonist MCPG and the selective mGluR5 antagonist

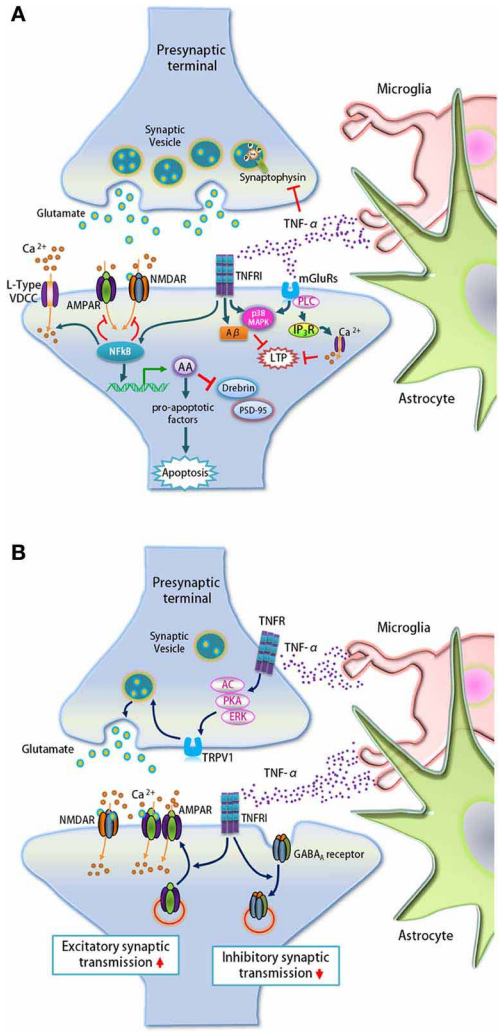

FIGURE 4 | TNF-α regulates synaptic loss, synaptic transmission, and plasticity. (A) TNF-α secreted by microglia and astrocytes up-regulates arachidonic acid (AA) via the NF-κB pathway that leads to synaptic loss by activating pro-apoptotic factors. The elevated AA signaling also down-regulates the expression of post-synaptic proteins drebrin and PSD-95, and pre-synaptic protein synaptophysin. In addition, TNF-α regulates intracellular calcium levels through the NF-κB pathway by regulating calcium entry through VDCCs, while suppressing glutamate receptor agonist-induced currents in hippocampal neurons. Lastly, TNF-α blocks the early phase of LTP through the activation of TNFR1 and mGluRs and this process is dependent on p38 MAPK, IP_3 receptor-mediated calcium release via PLC and Aβ. **(B)** Glia-derived TNF-α signals through pre- and post-synaptic TNFR1 to regulate pre-synaptic neurotransmitter release and the surface expression of AMPARs and $GABA_A$ receptors, respectively. In spinal cord outer lamina II neurons, TNF-α increases spontaneous EPSC frequency via TRPV1-mediated glutamate release that is mediated by AC, PKA, and ERK pathways from pre-synaptic terminals. At the post-synapse, TNF-α promotes GluA2-lacking AMPAR trafficking to the surface and induces $GABA_A$ receptor endocytosis.

MPEP significantly attenuate the inhibition of LTP by TNF-α (Cumiskey et al., 2007). The inhibition of LTP by TNF-α was significantly reversed by ryanodine, which blocks the release of intracellular calcium from ryanodine-sensitive stores. This implicates the involvement of ryanodine-sensitive intracellular calcium stores in TNF-α-mediated inhibition of LTP (Cumiskey et al., 2007).

Apart from impairing LTP by regulating calcium stores, TNF-α is also involved in LTP inhibition mediated by amyloid-β (Aβ), a major component of plaques in AD brains (**Figure 4A**). Many studies have shown that hippocampal LTP is blocked by Aβ (Cullen et al., 1997; Lambert et al., 1998; Itoh et al., 1999; Chen et al., 2000; Stephan et al., 2001; Vitolo et al., 2002; Walsh et al., 2002; Raymond et al., 2003; Wang et al., 2005; Kotilinek et al., 2008; Jo et al., 2011; Li et al., 2011; Kimura et al., 2012; Olsen and Sheng, 2012; Li et al., 2013b). In addition, expression of TNF-α and its receptor TNFR1 is up-regulated in the brain and plasma of AD patients (Tarkowski, 2002; Li et al., 2004a). Using mutant mice null for TNFR1 and TNF-α, as well as inhibitors, Wang and colleagues reported that TNF-α and TNFR1 are required for Aβ-mediated LTP inhibition. This TNF-α-mediated inhibition of LTP is dependent on the activation of p38 MAPK and mGluR5 (Wang et al., 2005).

While the earlier studies focused on how pathological levels of TNF-α impair LTP in the hippocampus, the role of TNF-α in the spinal cord has also been explored. In several models of neuropathic pain, expression of TNF-α and TNFR1 is up-regulated in the spinal dorsal horn (Ikeda et al., 2007; Wei et al., 2007), and elevated levels of TNF-α induce spinal LTP in a JNK-, p38 MAPK-, and NF-κB-dependent fashion (Liu et al., 2007). Inhibition of TNF-α signaling abolishes LTP (Zhong et al., 2010), and intriguingly, inhibition of Src-family kinases leads to HFS-induced LTD, instead of LTP, and this inhibitory effect on spinal LTP is reversed by TNF-α addition (Zhong et al., 2010).

In addition to its effect on synaptic proteins, calcium levels, and LTP, TNF-α also enhances excitatory synaptic transmission and suppresses inhibitory synaptic transmission by regulating the surface expression of post-synaptic receptors (**Figure 4B**). Immunocytochemistry and electrophysiology revealed that treatment with TNF-α or astrocyte-derived conditioned media containing TNF-α elevates the surface levels of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) as well as the frequency of mEPSCs in cultured hippocampal neurons (Beattie et al., 2002). Glial TNF-α signaling through TNFR1 was shown to be involved in this AMPAR-mediated control of synaptic strength (Beattie et al., 2002). Furthermore, genetic approaches have shown that deletion of TNFR1 but not TNFR2 lowers the surface expression and synaptic localization of AMPARs, suggesting a critical role of TNFR1 signaling in AMPAR-mediated synaptic functions (He et al., 2012). Intriguingly, the effect of TNF-α on surface AMPARs preferentially affects GluA2-lacking AMPARs (Stellwagen et al., 2005). In contrast to its effect on AMPARs, TNF-α induces $GABA_A$ receptor endocytosis, diminishing surface expression of $GABA_A$ receptors and inhibitory synaptic strength (Stellwagen et al., 2005). Taken together, TNF-α affects both excitatory and inhibitory synaptic transmission, suggesting an important role of TNF-α in the homeostasis of neural circuits.

In addition, TNF-α also enhances synaptic transmission in the spinal cord. In spinal cord outer lamina II neurons, TNF-α increases spontaneous EPSC frequency but not amplitude via

pre-synaptic transient receptor potential subtype V1 (TRPV1)-mediated glutamate release that is dependent on adenylyl cyclase (AC), PKA, and the extracellular signal-regulated kinase (ERK) in pre-synaptic terminals (Park et al., 2011a). However, this spinal cord LTP induction is abolished in Tnfr1$^{-/-}$ mice, Tnfr2$^{-/-}$ mice, and Trpv1$^{-/-}$ mice. This observation indicates the importance of TNFR and TRPV1 in spinal cord LTP (Park et al., 2011a).

PERSPECTIVES

Growth factors like netrin, Wnt, TGF-β, and TNF-α were first identified for their roles in axon guidance, embryonic development, cell proliferation, and inflammation, respectively. Over the past decade, they have gained prominence as regulators of the synapse. Similar to other patterning molecules such as sonic hedgehog (Salie et al., 2005), these growth factors play multiple roles during development. By utilizing effectors that multitask, the nervous system can carry out multiple functions more efficiently. For a single factor that has to fulfill various roles, diverse regulatory and signaling pathways that are spatiotemporally restricted must be put in place for it to achieve distinct functional outcomes.

Synapse formation largely involves transport, recruitment, and assembly of molecular machinery, cytoskeletal remodeling, and eventual stabilization of the synapse. As described earlier, netrin, Wnt, TGF-β, and TNF-α largely promote synaptogenesis and/or synaptic transmission but several including netrin/UNC-6, Wnt5a, Wnt4, Wnt3a, Wnt/LIN-44, TGF-β2, and activin also act as negative regulators. It is intriguing to note how some of these factors have opposing effects on synaptogenesis. In the case of netrin, the use of distinct receptors—DCC/UNC-40 or UNC-5 determine its effect on the synapse (Colon-Ramos et al., 2007; Poon et al., 2008). For the Wnt family, only a few members have inhibitory effects on synaptogenesis and distinct pathways are utilized for this purpose (**Table 1**). Lastly, only two members of the TGF-β family negatively regulate synaptogenesis: dactivin and myoglianin in *Drosophila* (Ting et al., 2007; Yu et al., 2013). As a pro-synaptogenic role for both these ligands has yet to be identified, they may activate pathways to specifically inhibit ectopic synapse formation. In addition, a putative mechanism coordinating synapse formation and elimination within a single neuron is discussed in a recent paper by Park and colleagues (Park et al., 2011b).

Apart from the opposing effects of several growth factors on synaptogenesis, TNF-α also appears to have conflicting effects on LTP and surface levels of AMPARs. Many studies have reported that an increased level of TNF-α impairs LTP in the hippocampus (Butler et al., 2004; Cumiskey et al., 2007; Liu et al., 2007, 2012) and also elevates the surface level of GluA2-lacking Ca^{2+}-permeable AMPARs in cultured hippocampal neurons (Beattie et al., 2002; Stellwagen et al., 2005). It is important, however, to note that TNF-α increases the insertion of Ca^{2+}-permeable AMPARs to both synaptic and extrasynaptic sites (Ferguson et al., 2008; Leonoudakis et al., 2008). In addition, Ca^{2+}-permeable AMPARs are incorporated into the surface during LTP (Plant et al., 2006). Thus, one plausible mechanism is that TNF-α elevates the surface level of AMPARs at both synaptic and extrasynaptic sites, leading to excessive calcium influx through synaptic and extrasynaptic AMPARs, hence impairing LTP.

Considering how the synapse is key to proper communication between neurons, one would expect a complex interplay of multiple molecular mechanisms to ensure tight regulation of synaptic function. Studies in the *C. elegans* AIY interneuron and the *Drosophila* NMJ have provided strong mechanistic insights into how netrin, Wnt, and TGF-β regulate synaptic function. However, it remains unclear if these growth factors utilize similar pathways in mammals. Identification of the target genes that lie downstream of the different signaling pathways will elucidate how the diverse growth factors differentially regulate synaptic function.

Given that synaptic function is compromised in a majority of neurological diseases, further understanding of the signaling pathways of netrin, Wnt, TGF-β, and TNF-α may contribute to novel therapeutic approaches for these debilitating disorders.

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