Endocannabinoids (eCBs) are important mediators of synaptic plasticity, acting as retrograde messengers because they are released on demand from postsynaptic sites and activate presynaptic type I cannabinoid (CB1) receptors to suppress neurotransmitter release at both excitatory and inhibitory synapses. Similarly, brain derived neurotrophic factor (BDNF) is also a potent neuromodulator of synaptic transmission. The effect of BDNF on synaptic transmission is mediated by tropomyosin receptor kinase B (trkB) receptors. Both CB1 and trkB receptors are highly expressed at synapses throughout the neocortex and hippocampus. There is a growing evidence of cross talk between eCB and BDNF signaling. In particular, studies in our lab have shown that BDNF can trigger the release of endogenous endocannabinoids via phospholipase C signaling at inhibitory synapses in somatosensory cortex. It is not known whether BDNF-induced eCB release also occurs at excitatory synapses and in regions other than the neocortex. In the present studies, we examined a potential crosstalk between BDNF and eCB release at excitatory synapses in neocortex and inhibitory synapses in the hippocampus.

Using whole cell patch clamp recordings and pharmacological manipulations in mouse brain slices, we found at layer 5 excitatory synapses of somatosensory cortex, BDNF by
itself had little effect on spontaneous excitatory activity. However, blocking CB1 receptors or disrupting eCB release unmasked a significant BDNF-induced increase in the frequency of spontaneous excitatory synaptic events. These results suggest that BDNF induces the release of endogenous eCBs at these synapses that has a mitigating effect on the direct presynaptic effects of BDNF. **We also found** evidence for BDNF-induced eCB release at inhibitory synapses in hippocampus. Acute application of BDNF reduced spontaneous inhibitory synaptic activity in CA1 pyramidal neurons and this effect of BDNF was triggered by postsynaptic trkB activation. The suppressive effect of BDNF was mediated by eCB signaling because it was completely prevented by either blocking CB1 receptors or by inhibiting eCB release. Further, we identified 2-AG as the specific eCB released by BDNF because blocking the synthesis of 2-AG prevented the effect of BDNF, whereas blocking 2-AG degradation enhanced the effect of BDNF. Collectively, these results suggest that in the hippocampus, BDNF-trkB signaling induces the release of the endogenous cannabinoid 2-AG, which acts as a retrograde messenger at presynaptic CB1 receptors to suppress GABA release. Taken together, these studies indicate that BDNF induces the release of eCBs at both inhibitory and excitatory synapses in neocortex, and the effects of BDNF at inhibitory synapses extend to the hippocampus. These studies contribute to the understanding of the physiological roles of BDNF and eCB signaling in the context of synaptic plasticity.
BDNF-Induced Release of Endogenous Cannabinoids at Inhibitory and Excitatory Synapses

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Doctor of Philosophy Dissertation

BDNF-Induced Release of Endogenous Cannabinoids at Inhibitory and Excitatory Synapses

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DEDICATIONS

To my beloved grandparents Sadasivam Ramasamy (1937-2018), Rajamani Sadasivam, Sethukkarasi Annamalai (1928-2001), Annamalai (1932-2011) and parents, Selvam Annamalai and Janaki Selvam for everything they have given me.
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Chapter 1
Introduction, Background and Rationale

1.1 Overview

The most fascinating property of the brain is its plasticity – the ability to change throughout life as a result of experiences. Experience, whether it is a stressful event, recovery after injury, or learning in class, causes the brain to adapt, refine and modify neural activity and the organization of neural circuits. The most important and well-studied mechanism by which neural activity modifies brain function is synaptic plasticity. Synaptic plasticity is integral for learning and memory in both the developing and adult mammalian brain. Synaptic plasticity is embodied in Hebb’s postulate: “cells that fire together wire together”. It specifically refers to activity-dependent modulation that can potentiate or depress synaptic strength in a time frame lasting hours to days and sometimes even longer (Citri and Malenka, 2008). The cerebral cortex and hippocampus are two brain regions where extensive studies have been conducted to study and understand the mechanisms of synaptic plasticity. The cerebral cortex is composed of six distinct layers of synaptically-connected cells. The cortex controls sensory and motor perceptions along with higher cognitive tasks. Local circuitry exists in the cortex that integrates and transmits information received across all layers thereby shaping synaptic plasticity (Bannister, 2005). On the other hand, the hippocampus is important for spatial and non-spatial forms of declarative memory. Different cortico-hippocampal circuits exist that transmit information to other brain regions. Hippocampal regions encode information pertaining to long term memory which is again heavily dependent on synaptic plasticity (Basu and Siegelbaum, 2015; Basu et al., 2013).
Synaptic plasticity plays a key role in the formation and maturation of synaptic connectivity in the developing nervous system. Synaptic plasticity is a complex process that involves multiple synaptic structures and numerous molecular mechanisms that are required for the formation and maintenance of a functional neuronal circuit. Synaptic plasticity occurs at both developmental and adult stages (Chaudhury et al., 2016). Both excitatory synapses and inhibitory synapses exhibit this phenomenon. In a typical CA1 neuron, ~30,000 glutamatergic synaptic inputs and ~1700 GABAergic inputs are spread throughout the dendritic tree. The excitatory neurotransmitter glutamate and the inhibitory neurotransmitter GABA are the primary mediators of “fast” synaptic transmission, however their effects are regulated by neuromodulators such as neurotrophins, endocannabinoids and neuropeptides (Basu and Siegelbaum, 2015; Basu et al., 2013). In particular, endocannabinoids (eCBs) and brain-derived neurotrophic factor (BDNF) have been identified as important modulators of synaptic plasticity. Though these two systems have been studied extensively in the context of synaptic plasticity, the interdependence between these systems is relatively unexplored. The proposed studies will (1) explore potential interactions between BDNF and eCBs in neocortex and its functional relevance in the context of long-term synaptic plasticity and (2) examine the role of BDNF and eCBs in the hippocampus.

1.2 The endogenous cannabinoid system

The endocannabinoid system is comprised of cannabinoid receptors, endogenous ligands, transporters, and enzymes required for ligand biosynthesis and degradation.
Each component is described in detail below. The physiological role of the endocannabinoid system is described in section 1.3.

1.2.1 Cannabinoid receptors

Two distinct cannabinoid receptors have been identified, type 1 and type 2 cannabinoid receptors (CB1R and CB2R), both of which are $G_{i/o}$ linked G-protein coupled receptors (GPCRs). While CB1Rs are highly expressed in the brain (Howlett et al., 2002), CB2Rs are predominantly expressed in the immune system. More recent studies have shown that CB2 is also present in both glia and neuronal processes in different brain regions, including cerebral cortex, hippocampus, amygdala, and striatum (Gong et al., 2006; Van Sickle et al., 2005). Recent studies have also suggested that chronic activation of CB2R enhances excitatory synaptic transmission but has no acute effect on synaptic transmission (Kim and Li, 2015). CB2R knockouts shows impairments in excitatory synaptic transmission, long-term potentiation, and dendritic spine density in hippocampus (Li and Kim, 2016; Stempel et al., 2016). In the present studies, I will focus on CB1Rs and their function in synaptic plasticity as CB1R is the predominant cannabinoid receptor expressed in neurons.

The CB1R is one of the most highly expressed GPCR receptors in brain (Howlett, 1998; Howlett et al., 2002), found in hippocampus, cerebral cortex, cerebellum, basal ganglia and nucleus accumbens (Egertova et al., 2003; Herkenham et al., 1990; Matsuda et al., 1993). CB1Rs are localized predominantly at presynaptic axonal terminals of both
GABAergic and glutamatergic cells (Katona et al., 1999; Katona et al., 2006). The main physiological effect of CB1R activation is to suppress presynaptic neurotransmitter release (Kreitzer and Regehr, 2001; Wilson et al., 2001; Wilson and Nicoll, 2001). The presynaptic localization of CB1Rs allows endocannabinoids to act as retrograde synaptic signals (Alger, 2002). Activation of CB1R negatively regulates N-type and P/Q-type voltage gated calcium channels (VGCCs) (Mackie et al., 1995; Twitchell et al., 1997). Further, activation of CB1R also can lead to suppression of adenylyl cyclase and cAMP production as well as modulation of D- and M-type potassium channels (Mu et al., 1999; Schweitzer, 2000).

CB1R expression in the cortex displays a cell type-specific and laminar-specific pattern. High levels of CB1R expression have been reported in terminal and preterminal axons of cholecystokinin (CCK)-containing and calbindin-positive interneurons that surround pyramidal neurons (Bodor et al., 2005; Eggan and Lewis, 2007; Harkany et al., 2005; Marsicano and Lutz, 1999). CCK cells are basket cells that innervate the perisomatic pyramidal neuron area (Neu et al., 2007) while calbindin-positive interneurons can be found in distal dendrites of pyramidal neurons in the cortex (DeFelipe et al., 1989; Wedzony and Chocyk, 2009). Higher amounts of CB1Rs are found at excitatory terminals compared to inhibitory terminals. CB1Rs are not detected in parvalbumin-, somatostatin-, or vasoactive intestinal peptide-containing interneurons in the cortex (Bodor et al., 2005; Marsicano and Lutz, 1999). The lamina-specific pattern of CB1R in neocortex in rodents is characterized by high levels of expression in layers 2/3, 5a, and 6 (Deshmukh et al., 2007; Egertova et al., 2003; Egertova and Elphick, 2000;
Marsicano and Lutz, 1999; Tsou et al., 1998). In contrary, in the prefrontal cortex of monkeys, CB1R is highly expressed in layer 4 (Eggan and Lewis, 2007). This differential distribution of CB1R across cortical layers and across species is an important factor in understanding the role of eCBs in synaptic plasticity and cortical function.

In addition to the classical cannabinoid receptors, two other related receptors, transient receptor potential vanilloid type-1 ion channel (TRPV1) and GPR55 receptor also engage in eCB signaling. TRPV1 is a non-selective cation channel expressed in peripheral sensory neurons and its activation regulates synaptic transmission related to pain sensation (De Petrocellis et al., 2000). Anandamide (AEA) is an endogenous cannabinoid ligand that acts as a full agonist at TRPV1 channels indicating a crosstalk between the endovanilloid and eCB systems. However, it is not known if TRPV1 can modulate excitatory or inhibitory synaptic activity (Maccarrone et al., 2008; Ryberg et al., 2007). On the other hand, GPR55 is an orphan GPCR that has been recognized as a novel cannabinoid receptor. GPR55 can be activated by several ligands including THC, CP55, 940 but not by WIN55,212-2, a synthetic CB1 agonist. The function of GPR55 is currently unknown (Ryberg et al., 2007).

1.2.2 Endogenous ligands in the brain and their synthesis

Endogenous ligands are the lipid molecules that are synthesized in brain and bind to CB1 receptors. There are several endogenous ligands, 2-arachidonoylglycerol (2-AG), anandamide (AEA), noladin ether (Hanus et al., 2001), virodhamine (Porter et al., 2002).
and oleamide (Leggett et al., 2004). The two best-characterized ligands are 2-AG and AEA. Unlike typical neurotransmitters, eCBs are not packaged and stored in vesicles before release. Instead, they are synthesized and released ‘on-demand’ from membrane lipid precursors.

N-arachidonylethanolamide, derived from arachidonic acid in the plasma membrane, was the first identified eCB and was isolated from pig brain (Devane et al., 1992). Based on the euphoria associated with marijuana use and CB1 activation, N-arachidonylethanolamide was named ‘anandamide’ meaning bliss in Sanskrit. AEA acts as a partial agonist for both CB1 and CB2Rs and full agonist for TPRV1 receptors (Ross et al., 2001; Starowicz et al., 2007). Multiple pathways exist for the production of anandamide. Three well known pathways will be described here: (1) the precursor of N-arachidonyl phosphatidylethanolamine (NArPE) is synthesized in a Ca\(^{2+}\)-dependent manner by the enzyme N-acyl transferase (NAT). NArPE is cleaved to AEA and phosphatidic acid through the action of NArPE specific phospholipase D (Di Marzo et al., 1994). (2) phospholipase C-mediated hydrolysis of NAPE yields phosphoanandamide (p-AEA) (Liu et al., 2008). This p-AEA is dephosphorylated to yield AEA. (3) Deacylation of NAPE by α,β-hydrolase 4 (Abhd4) and the subsequent cleavage of glycerophosphate yields anandamide (Liu et al., 2008).

2-AG is a full agonist of CB1R and CB2R and the concentration of 2-AG is higher than that of AEA in brain tissue (Stella et al., 1997; Sugiura et al., 1995). The major synthesis
pathway of 2-AG involves hydrolysis of phosphatidylinositol by phospholipase C (PLC) and subsequent hydrolysis of 1,2–diacylglycerol (DAG) by a DAG lipase (DAGL) (Stella et al., 1997). Two isoforms of DAGL have been identified: DGLα and DGLβ (Bisogno et al., 2003). DGLα is related to 2-AG synthesis and release (Kano et al., 2009). Alternatively, DAG can be synthesized from phospholipase A2 or D from phosphatidic acid (Nakane et al., 2002). 2-AG and AEA are synthesized via different lipid metabolism pathways and mediate different functions upon activation of CB1Rs and CB2Rs (Piomelli, 2003; Piomelli et al., 1998).

1.2.3 Endocannabinoid mobilization and metabolism

The synthesized ligands are released in a non-vesicular manner and travel retrogradely to activate presynaptic CB1Rs. Several mechanisms have been known to induce eCB release. (1) Calcium-induced eCB release is driven by postsynaptic depolarization of the cell resulting in increase of intracellular Ca$^{2+}$ via VGCCs (Pitler and Alger, 1992) and NMDA receptors (Ohno-Shosaku et al., 2007). Ca$^{2+}$-ER is independent of PLC signaling, because PLC inhibitors failed to disrupt depolarization-induced eCB release in hippocampus and cerebellum (Chevaleyre and Castillo, 2003; Edwards et al., 2012; Engler et al., 2006). Genetic ablations of various isoforms of PLC also did not alter eCB-mediated short term depression (Hashimotodani et al., 2008; Hashimotodani et al., 2005; Maejima et al., 2005). (2) Receptor-driven eCB release (RER): Activation of group 1 metabotropic receptor glutamate receptors (mGluR1) (Maejima et al., 2001), M1/M3 muscarinic acetylcholine receptors (Kim et al., 2002; Straiker and Mackie, 2007), or orexin receptors (Haj-Dahmane and Shen, 2005) can activate PLCβ. This activation can lead to
eCB release that is independent of postsynaptic Ca\(^{2+}\) elevation (Hashimotodani et al., 2007b; Maejima et al., 2001). (3) Ca\(^{2+}\)-assisted RER: In certain cases, combination of increased intracellular Ca\(^{2+}\) along with RER can induce eCB release. Calcium entry through postsynaptic depolarization in combination with mGluR or muscarinic agonists such as carbamylcholine or dihydroxyphenylglycine (DHPG) can enhance eCB release (Martin and Alger, 1999; Ohno-Shosaku et al., 2002; Straiker and Mackie, 2007). (4) BDNF-induced release of eCBs, which requires BDNF, TrkB signaling, PLC \(\gamma\) signaling and elevated intracellular Ca\(^{2+}\) (Lemtiri-Chlieh and Levine, 2010; Zhao and Levine, 2014). Each of these forms of eCB release share similarities yet have distinct mechanisms.

After release from the postsynaptic cell and activation of CB1 receptors, both AEA and 2-AG are rapidly degraded by specific intracellular hydrolyzing enzymes. AEA is degraded into arachidonic acid and ethanolamine by fatty acid amide hydrolase (FAAH). FAAH is mostly expressed in postsynaptic membranes (Cravatt et al., 1996; Hillard et al., 1995; Schmid et al., 1985). On the other hand, 2-AG is degraded by MAGL into glycerol and fatty acid. MAGL is located in the presynaptic terminals (Dinh et al., 2002). FAAH (Goparaju et al., 1998) and ABHD6 (Blankman et al., 2007) can also degrade 2-AG, however, the majority of 2-AG is degraded by MAGL. It is still unclear how eCBs are transported in and out of the membrane for release and reuptake processes. It has been proposed that the reuptake process involves carrier-mediated transport into cells and subsequent intracellular hydrolysis, however the identification and cloning of the transporter protein is still lacking (reviewed in (Piomelli, 2003)). Another thought is that, rather than a typical transporter, fatty acid binding proteins (FABPs) are possible
intracellular AEA carriers that can transport AEA across the membrane. FABPs are expressed in the central nervous system and can help the movement of fatty acids freely inside the cells providing a rapid substrate delivery system of AEA to FAAH (Elmes et al., 2015; Kaczocha et al., 2009).

1.3 Endocannabinoid-mediated synaptic plasticity

E CBs acutely suppress inhibitory and excitatory neurotransmission in several brain regions as well as mediate several forms of short- and long-term depression at both excitatory and inhibitory synapses.

1.3.1 Endocannabinoid-mediated short-term plasticity

Endocannabinoid-mediated short-term depression (eCB-STD) includes depolarization-induced suppression of inhibition (DSI) and excitation (DSE). eCB-mediated DSI was first demonstrated at GABAergic synapses in CA1 pyramidal cells of hippocampus and cerebellum (Kreitzer and Regehr, 2001; Pitler and Alger, 1992; Wilson and Nicoll, 2001). Thereafter, eCB-mediated DSE at glutamatergic synapses was reported in the cerebellum and hippocampus (Kreitzer and Regehr, 2001). In neocortex, DSI was first reported by our group in layer 2/3 auditory and visual cortex (Trettel and Levine, 2003). Later studies reported DSI/DSE in amygdala (Zhu and Lovinger, 2005), basal ganglia (Hashimotodani et al., 2013), ventral tegmental area (Melis et al., 2004) and hypothalamus (Jo et al., 2005).
In DSE/DSI, in response to postsynaptic depolarization-induced calcium influx, eCBs are released from postsynaptic sites and act retrogradely via CB1Rs to suppress neurotransmitter release. The suppression is very transient, lasting 2 to 3 minutes (Wilson and Nicoll, 2001). The critical step for releasing eCBs is a rise in intracellular calcium levels in the postsynaptic sites which can happen in few different ways: (a) via voltage gated calcium channels (VGCC) (Kreitzer and Regehr, 2001; Pitler and Alger, 1992; Wilson and Nicoll, 2001), (b) group I metabotropic receptors (mGluRs) and (c) muscarinic acetylcholine receptors, which mobilize stored calcium via phospholipase C (PLC) (reviewed in (Xu and Chen, 2015)). Elevation of intracellular Ca\(^{2+}\) induces STD while decrease of Ca\(^{2+}\) blocks STD. The transient suppression is believed to be mediated by direct βγ subunit of G\(_{i/o}\) protein interacting with N-type and P/Q-type voltage gated calcium channels leading to a rapid suppression of presynaptic Ca\(^{2+}\) influx (Wilson and Nicoll, 2001). Specifically, our group has found that pyramidal neurons (PNs) can express DSI, but not interneurons (INs), even though all subsets of neurons tested received cannabinoid-sensitive inhibitory inputs (Lemtiri-Chlieh and Levine, 2007).

DSI also exhibits laminar-specific expression pattern in the somatosensory cortex – most layer 2/3 and a small population of layer 5a pyramidal neurons display eCB-mediated DSI (Bodor et al., 2005). Further, our lab have reported that the eCBs have differential effects on excitation and inhibition across cortical layers. We have shown that glutamatergic and GABAergic inputs to layer 5 PNs of mouse somatosensory cortex are differentially regulated following depolarization-induced endocannabinoid release, with
DSE much more prominent than DSI as a majority of inhibitory inputs were cannabinoid insensitive (Fortin and Levine, 2007; Lemtiri-Chlieh and Levine, 2007).

The attempts to identify the specific endogenous ligand involved have yielded contradicting results. Inhibition of MAGL extends DSI, thus 2-AG is considered as the eCB that mediates DSI (Hashimotodani et al., 2007c; Makara et al., 2005). In DAGLα mice, DSI and DSE are abolished suggesting the involvement of DGL. However, many other studies using various DAGL inhibitors have reported no inhibition of DSI/DSE (Min et al., 2010) indicating that 2-AG is not involved in the process. In light of negative results, we cannot exclude the role of 2-AG in modulating synaptic plasticity as there are other alternative pathways to synthesize 2-AG. 2-AG can be synthesized by hydrolysis of PI via phospholipase A1 (PLA 1) and lysophosphatidylinositol specific PLC (Tsutsumi et al., 1994) or by conversion of 2-arachidonoyl lysophosphatidic acid (LPA) to 2-AG (Nakane et al., 2002)

1.3.2 Endocannabinoid-mediated long-term plasticity

eCBs mediate several forms of long-term depression (LTD) at both excitatory (eCB-LTD) and inhibitory synapses (eCB-iLTD). The first eCB-LTD at excitatory synapses was reported in dorsal striatum (Gerdeman et al., 2002) and nucleus accumbens (Robbe et al., 2002). At the same time, eCB-iLTD was observed in the amygdala (Marsicano et al., 2002). Later LTD/iLTD was observed in sensory cortex (Bender et al., 2006; Crozier et al., 2007; Huang et al., 2008; Jiang et al., 2010; Lefort et al., 2013; Min and Nevian, 2012; Nevian and Sakmann, 2006; Sjostrom et al., 2003; Sjostrom et al., 2004), prefrontal
cortex (Chiu et al., 2010; Lafourcade, 2009), hippocampus (Chevaleyre and Castillo, 2003; Chevaleyre and Castillo, 2004; Chevaleyre et al., 2007; Yasuda et al., 2008), cerebellum (Safo and Regehr, 2005) and basal lateral amygdala (Azad et al., 2004; Marsicano et al., 2002). Induction of eCB-LTD also requires an increase in intracellular Ca\(^{2+}\) and activation of CB1 receptors. However, CB1 receptor activation is not necessary for the maintenance phase of eCB-LTD (Chevaleyre and Castillo, 2003). Different signaling pathways mediate the long-lasting suppression of transmitter release compared to eCB-STD. The cyclic AMP/protein kinase A (cAMP/PKA) pathway might be a pathway for the downstream signaling cascade mediating eCB-iLTD (Heifets and Castillo, 2009). Indeed, theta burst stimulation (TBS)-induced eCB-iLTD in hippocampus is prevented by blocking presynaptic cAMP/PKA signaling. Further, the active zone protein RIM1α, which can be phosphorylated by PKA, is required for eCB-iLTD, indicating that activation of CB1R may lead to regulation of the release machinery for GABA (Chevaleyre et al., 2007).

eCB-mediated timing dependent plasticity (tLTD) has been reported at different excitatory synapses in the cortex and hippocampus. For example, at layer 5 pyramidal neurons in visual cortex tLTD at excitatory synapses requires eCB release. The tLTD induction requires both CB1R and NMDA receptor activation (Sjostrom et al., 2003). A similar tLTD protocol has been reported at layer 4 to layer 2/3 excitatory synapses of the somatosensory cortex (Bender et al., 2006). In prefrontal cortex, 10 minutes of 10 Hz stimulation at layer 2/3 afferents has been reported to induce eCB-LTD in layer 5/6 pyramidal neurons which requires activation of postsynaptic mGluR5 and PLC signaling.
The authors also suggest that 2-AG is the specific ligand involved in this form of LTD (Lafourcade, 2009). In layer 5 of prefrontal cortex, coactivation of CB1R and dopamine type 2 receptors triggers eCB-iLTD. This iLTD also requires activation of mGluR1 for induction (Chiu et al., 2010). eCB-iLTD can be induced by a combination of electrical and chemical stimulation. For instance, pairing subthreshold stimulation with a low dose of DHPG induced iLTD comparable to strong TBS alone in hippocampal CA1 neurons (Younts et al., 2013). Yet another study in hippocampus indicates that high frequency stimulation (HFS, 100Hz/1s, 2 trains with 20s inter-train interval) can elicit mGluR dependent eCB-iLTD (Chevaleyre and Castillo, 2003). The LTD can be developmentally regulated, suggesting a role in shaping cortical circuits during postnatal development. In layer 2/3 pyramidal neurons of visual cortex, TBS of layer 4 axons induces eCB-LTD in mGluR 5 dependent, but NMDA receptor independent. However, no LTD is observed in these animals past their critical period (Huang et al., 2008).

It has been hypothesized that eCBs may modulate LTP of glutamatergic transmission via disinhibition of postsynaptic neurons through the suppression of GABA release. This form of LTP was first observed in the hippocampus. Carlson and others recorded whole cell EPSCs and field EPSPs simultaneously in the CA1 region of hippocampus. A combination of postsynaptic depolarization and weak presynaptic stimulation together induced LTP of EPSCs but not fEPSPs. EPSC-LTP was completely abolished in the presence of CB1 receptor antagonists, indicating that eCB-STD at inhibitory synapses could facilitate LTP induction at glutamatergic synapses (Carlson et al., 2002). Conversely, eCB-LTD at inhibitory synapses can also facilitate LTP induction at nearby
glutamatergic synapses in hippocampal slices. This form of LTP requires both GABAergic synaptic function and eCBs (Chevaleyre and Castillo, 2004). However, further studies are needed to understand the mechanisms underlying eCB-mediated LTP of excitatory synaptic transmission.

Two recent studies reported a novel form of LTP mediated by endocannabinoids. In rat hippocampal slices, LTP was induced in the lateral perforant path (LPP) pathway by high frequency stimulation. This induction protocol activates NMDA and mGluR5 type glutamate receptors and increases postsynaptic calcium content and engages in production of 2-AG. 2-AG acts on presynaptic CB1 receptors thereby initiating signaling via small GTPases. This leads to the assembly and reorganization of latrunculin-sensitive actin filaments causing an enhancement of evoked transmitter release (Wang et al., 2016). Further, the authors show that the CB1Rs activate two signaling cascades – pregnenolone sensitive and pregnenolone insensitive. The pregnenolone-insensitive cascade stimulates integrin-associated focal adhesion kinases (FAK), which targets small GTPases. Integrin, CB1R and activation of small GTPases act together to aid in reorganization of actin filaments resulting in enhanced glutamate release probability by increased vesicle docking. The involvement of FAK in synaptic plasticity indicates that these kinases can modulate changes in gene expression and support the neural changes along with eCBs in LTP (Wang et al., 2018). A more recent study in 2018, in acute hippocampal slices, has shown that low frequency stimulation coupled with action potential and slow depolarization induces eCB-LTP at these synapses. The authors confirm that eCB-mediated DSI during stimulation facilitates LTP at these synapses. In
addition, the stimulation causes release of endogenous BDNF which facilitates LTP at these synapses (Maglio et al., 2018). This is a novel form of LTP where eCBs facilitate LTP via interaction with BDNF signaling. In the next section, we will discuss BDNF and its role in synaptic plasticity.

To summarize this section,

(i) eCBs are retrograde messengers that act on presynaptic CB1 receptors to suppress neurotransmitter release (including GABA and glutamate).

(ii) eCBs can be released via calcium induced release, receptor driven release or calcium assisted receptor driven release (as explained in section 1.2.3).

(iii) The best characterized ligands are 2-AG and AEA – however, most studies have identified 2-AG as the endogenous ligand mediating short-term and long-term synaptic plasticity.

(iv) eCBs can modulate LTP of glutamatergic transmission via disinhibition of postsynaptic neurons through the suppression of GABA release.

eCB-LTD is well established at both excitatory and inhibitory synapses, yet it is unclear how long is the maintenance phase of LTD? What are the accompanied changes due to long lasting depression – is there a structural change of the synapses, dendritic spines and how it does relate to synaptic function of the synapses? Can these associated changes in synapses be reversed in the maintenance phase? Instead of using existing
stimulation protocols to elucidate LTD, can we use DSI stimulation paradigms repeatedly to induce LTD?

To further add to the complexity of the eCBs is its interaction with other neuromodulatory systems (explained in detail in section 1.6). How do these interactions occur? What are the associated changes at synaptic and functional levels? What are the mechanisms and signaling pathways that aid in these interactions? These questions needs to be addressed in the future. These interactions might represent a novel class of eCB-mediated plasticity, for instance, CB1Rs may stimulate the release of a key factor or activate a signaling pathway that helps in inducing synaptic plasticity at these synapses as discussed in these (Maglio et al., 2018; Wang et al., 2018; Wang et al., 2016) studies.

1.4 Brain-derived neurotrophic factor (BDNF)

1.4.1 BDNF, TrkB receptors

Brain derived neurotrophic factor (BDNF) belongs to the neurotrophin gene family and promotes neuronal development and differentiation. It is also a potent modulator and/or mediator of synaptic plasticity. Acutely, BDNF enhances glutamatergic transmission and suppresses GABAergic transmission via both pre- and postsynaptic mechanisms. BDNF can be released in an activity-dependent manner and has been found to be essential for long-term potentiation and some forms of long-term depression (Gottmann et al., 2009).
BDNF produces its effects through different classes of receptors: the high affinity full length tropomyosin receptor kinase B (TrkB, \( K_d \sim 10^{-11} \text{M} \)) that mediates most of BDNF signaling including its role in synaptic plasticity; the truncated form of TrkB (TrkB-T1) which lacks protein tyrosine kinase activity, and the lower affinity, pan-neurotrophin receptor, p75NTR (Berg et al., 1991; Kaplan et al., 1991; Rodriguez-Tebar and Barde, 1988). TrkB-T1 is widely distributed in the brain and its level of expression increases with age (Fryer et al., 1996; Ohira et al., 1999). It has been implicated as a dominant negative inhibitor of full length TrkB function, however, the underlying signaling pathways are largely unknown (Eide et al., 1996; Saarelainen et al., 2000). On the other hand, p75NTR primarily acts in opposition to BDNF/TrkB effects, for example, its activation promotes apoptosis (Barrett, 2000; Bunone et al., 1997; Majdan et al., 1997) and inhibits neurite outgrowth (Kohn et al., 1999; Walsh et al., 1999; Yamashita et al., 1999).

The high affinity TrkB receptor is a single transmembrane domain receptor tyrosine kinase. Upon binding, TrkB receptors dimerize and phosphorylate tyrosine residues which enhances catalytic activity of the kinase. Sometimes, TrkB receptors can autophosphorylate to activate downstream signaling pathways. Upon BDNF binding and receptor activation, three downstream signaling pathways are activated via tyrosine phosphorylation: phospholipase C\( \gamma \) pathway (PLC\( \gamma \)), Ras/mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinases (PI3K)/Akt pathways. Activation of the PI3K/Akt and MAPK pathways are critical for neuronal survival and differentiation. However, MAPK pathways are also involved in certain forms of chemically-induced LTP that requires endogenous BDNF/TrkB signaling. Activation of PLC\( \gamma \) leads to cleavage of
phosphatidylinositol 4,5-bisphosphate (PIP₂) into second messengers inositol triphosphate (IP₃) and diacylglycerol (DAG). IP₃ induces Ca²⁺ release from calcium stores upon binding to its receptor and thus increases intracellular calcium concentration. DAG is involved in several intracellular signaling pathways, one of which is stimulation of protein kinase C, which is required for neurite outgrowth. DAG also serves as a precursor to the endocannabinoid 2-arachidonoylglycerol via DAG lipase. Both these pathways have been found to be involved in modulating the effect of BDNF on inhibitory synaptic transmission (Henneberger et al., 2002; Jovanovic et al., 2004).

1.4.2 Distribution of BDNF and TrkB receptors

TrkB mRNA and protein are widely distributed across the brain, with highest densities in the neocortex, hippocampus, amygdala, striatum, cerebellum and dorsal root ganglia (Ernfors et al., 1990; Gorba and Wahle, 1999; Hofer et al., 1990). The mRNA levels reach their peak at birth and remain stable throughout adulthood in most regions of rat forebrain (Fryer et al., 1996), while TrkB protein reaches peak density as well as stable layer distribution around postnatal day 10, with highest density in neocortical layers 2/3 and 5 (Cabelli et al., 1996). At the synaptic level, TrkB is expressed in both postsynaptic dendrites and presynaptic terminals in the hippocampus and cortex (Aoki et al., 2000; Drake et al., 1999). Immunoreactive TrkB receptors are expressed at both glutamatergic and GABAergic synapses (Drake et al., 1999). The TrkB presence in both pre- and postsynaptic components suggests that BDNF can modulate synaptic plasticity at both locations.
BDNF mRNA is widely distributed throughout the brain including cerebral cortex (Hofer et al., 1990), hippocampal formation, thalamus, hypothalamus, amygdala, olfactory lobes and spinal cord (Ernfors et al., 1990; Hofer et al., 1990; Phillips et al., 1990; Wetmore et al., 1990). BDNF protein is widely distributed in cerebral cortex, hippocampus, amygdala and thalamus (Conner et al., 1997; Wetmore et al., 1991). In rodents BDNF mRNA and protein levels peak shortly after birth and remain stable (Friedman et al., 1991; Maisonpierre et al., 1990; Patz and Wahle, 2006). BDNF can be found both in neurons and glia and can be released from both neurons and glia in response to activity (Conner et al., 1997; Wetmore et al., 1990; Wu et al., 2004). At the subcellular level, BDNF mRNA is distributed in somatic structures and in dendritic compartments but absent in axons (An et al., 2008; Capsoni et al., 1999). At the subcellular level, BDNF protein is observed in axons within the superior colliculus, amygdala, dentate gyrus granule cells and CA3 pyramidal cells. BDNF protein is also found in the postsynaptic density and dendritic shafts of cerebral cortical pyramidal neurons. Further, ultramicroscopic studies have identified BDNF in endoplasmic reticulum and in vesicles at extrasynaptic, presynaptic, and postsynaptic sites indicating that the BDNF can be released synaptically as well as extrasynaptically (reviewed in (Edelmann et al., 2014)).

1.4.3 Release of endogenous BDNF

After transport of BDNF-containing vesicles into axons and dendrites, BDNF is secreted constitutively and in an activity-dependent manner in response to electrical activity. BDNF release can be triggered by (i) calcium influx through ionotrophic glutamate
receptors or voltage gated channels (VGCCs) (Hartmann et al., 2001); (ii) activation of Group I mGluR receptors, which subsequently triggers IP$_3$-mediated Ca$^{2+}$ release from intracellular stores (Canossa et al., 2001). BDNF can be released from both pre- and postsynaptic sites. The most common stimulation protocols, high frequency stimulation or theta burst stimulation (TBS), can induce BDNF release in hippocampal or cortical neurons (Dean et al., 2012). In addition, depolarization of hippocampal neurons, rhythmic neuronal discharges and spontaneous electrical network activity can induce BDNF release (Matsuda et al., 2009) also reviewed in (Edelmann et al., 2014)). For instance, Aicardi and colleagues have shown that BDNF can be released with low frequency stimulation in cortical slices. BDNF release is also regulated by intravesicular pH (Aicardi et al., 2004).

Besides the distribution of receptors, induction and release of BDNF, yet another factor that needs to be addressed is the time window for BDNF function – since BDNF can act instructively and permissively at synapses. For instance, using specific antibodies to BDNF, neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5), Chen and colleagues have identified that BDNF but not NT-3 or NT-4/5 impairs LTP induced by TBS. However, they show that LTP is impaired only when BDNF is blocked before and/or during TBS stimulation but not after stimulation, suggesting the release of BDNF occurred in a specific time period (Chen et al., 1999). In contrast, Kossel and colleagues show that blocking BDNF shortly after LTP induction is sufficient to impair LTP indicating that the released BDNF acts in an instructive capacity to maintain LTP (Kossel et al., 2001). Taken together, these studies suggest that the induction paradigms, release sites of BDNF and
time window of BDNF presence have an impact on the effect of BDNF on synaptic transmission.

1.5 Functional role of BDNF in synaptic plasticity

It has been widely established that BDNF is an important modulator of synaptic plasticity. However, very little is known about the release and time of action of BDNF when synaptic plasticity is induced in a given paradigm. The most pressing open ended questions in the field about BDNF are: (i) Does BDNF act as a modulator or mediator of synaptic plasticity. (ii) Is BDNF secreted from presynaptic, postsynaptic, and/or glial sites to induce the synaptic changes? What are the relevant patterns of activity that trigger BDNF release? (iii) What factors decide whether a BDNF dependent synaptic change is mediated by pre- or post-synaptic alterations? The below section will attempt to answer some of these questions and enhance our knowledge of the role of BDNF in synaptic plasticity.

1.5.1 Acute effect of BDNF on synaptic transmission

Acute application of BDNF modulates synaptic transmission at both excitatory and inhibitory synapses in cerebral cortex and hippocampus. For instance, BDNF enhances glutamate transmission in hippocampal and cortical cultures (Lessmann and Heumann, 1998; Levine et al., 1995; Li et al., 1998) in hippocampal acute slices (Madara and Levine, 2008) and in layer 2/3, 5 (Madara and Levine, 2008) of cortical slices from rodents. These results match up with anatomical locations in which TrkB receptors are expressed.
BDNF enhances excitatory synaptic transmission both presynaptically via enhancement of glutamate release as well as postsynaptically via NMDA receptor enhancement. Specifically, BDNF rapidly enhances action-potential independent current or miniature excitatory postsynaptic current (mEPSC) decay time and frequency suggesting BDNF has both presynaptic and postsynaptic mechanisms in the same preparation (Madara and Levine, 2008). By selectively blocking postsynaptic TrkB receptors by intracellular application of trk inhibitor inhibits the mEPSC decay time but not the mEPSC frequency. The presynaptic effect is mediated by presynaptic TrkB and NMDA receptors as blocking with the receptor antagonist inhibited the effect (Madara and Levine, 2008).

However, BDNF does not affect evoked glutamate release in response to single spikes in hippocampus (Frerking et al., 1998) or in layer 5 somatosensory cortex (Madara and Levine, 2008). This suggests that the effects of BDNF on evoked release may depend on specific action-potential activity patterns. It is also important to note that the spontaneous and evoked glutamate release may utilize different vesicle pools that are regulated differently. The visualization of glutamate release events at individual synapses shows that there is a preference for evoked versus spontaneous glutamate transmission that is determined by release machinery at active zones (reviewed in (Andreae and Burrone, 2018; Atasoy et al., 2008; Crawford and Kavalali, 2015; Kaeser and Regehr, 2014; Walter et al., 2014)). For instance, Melom and colleagues have reported that spontaneous release occurs from all active zones but suggest that some zones are incapable of supporting evoked release and hence remain as spontaneous synapses, early in
development. However, as synapses mature, additional regulatory proteins are acquired that allow for better control of synaptic release and thus enable evoked release rather than spontaneous release (Melom et al., 2013). Spontaneous release at glutamatergic synapses has been associated with formation of dendritic spines, synaptic stabilization and long-term forms of synaptic plasticity (reviewed in (Andreae and Burrone, 2018; Melom et al., 2013; Peled et al., 2014; Walter et al., 2014)). For instance, application of BDNF on layer 4 visual cortex increases the growth of basal and apical dendritic arbors. The authors show that BDNF-induced growth is completely abolished in the presence of glutamate receptor antagonists in the basal dendrites, suggesting that spontaneous glutamate release is needed for this BDNF-induced growth of dendritic arbors (McAllister et al., 1996). In hippocampus, disruption of spontaneous glutamate release for several hours leads to homeostatic regulation of inhibitory synapses through a mechanism that relies on activation of postsynaptic metabotropic glutamate and cannabinoid receptors and release of endocannabinoids (Zhang et al., 2009b). During the developmental period where neurotransmitter release is exclusively spontaneous, blocking NMDA receptors with an antagonist causes a reduction in dendritic arbor complexity while blocking NMDA at later stages did not have any effect. This study suggests that the glutamate might act as a growth-promoting cue. In cultured hippocampal cells, spontaneous glutamate release activates NMDA receptors to suppress local protein synthesis in dendrites (Sutton et al., 2006; Sutton et al., 2004). The majority of the published work has focused on investigating the role of spontaneous glutamate release. The mechanisms by which spontaneous GABA release occurs is relatively unknown compared to glutamate release.
While most studies show that the acute application of BDNF suppresses GABAergic transmission, the mechanisms by which BDNF modulates inhibitory neurotransmission are unclear. In CA1 region of hippocampus, BDNF application causes a reduction in the amplitude of evoked inhibitory postsynaptic currents. Also, BDNF causes a reduction in the paired-pulse ratio and decreases in the coefficient of variation suggesting a presynaptic site of action (Frerking et al., 1998). In rat hippocampal cultures, BDNF causes a rapid reduction in postsynaptic GABA receptor number that is responsible for a decrease in mIPSC amplitude (Brunig et al., 2001). A study by Mizoguchi et al., 2006 suggests that the effect of BDNF on GABA release is dependent on age. BDNF suppresses mIPSC frequency and amplitude in hippocampal preparations of age P14 and P21 rats but not in P7 rats (Mizoguchi et al., 2006). A decrease in mIPSC frequency, mIPSC amplitude and decrease in release probability is observed in visual cortex of BDNF knockout mice suggesting that the BDNF impairment is presynaptic (Abidin et al., 2008; Abidin et al., 2006). In cerebellar granule cells, BDNF decreases both the amplitude and frequency of spontaneous and miniature postsynaptic currents suggesting both pre- and postsynaptic effects (Cheng and Yeh, 2003). This effect of BDNF is blocked by inclusion of TrkB antagonist to the postsynaptic cell in cerebellar granule cells (Cheng and Yeh, 2003).

Other studies have reported acute postsynaptic effects of BDNF triggered by TrkB activation in hypothalamus (Hewitt and Bains, 2006), cerebellum (Brunig et al., 2001), cultured neurons from hippocampus (Cheng and Yeh, 2003; Jovanovic et al., 2004), mouse superior colliculus (Henneberger et al., 2005), visual cortex (Mizoguchi et al.,
Collectively, these diverse effects explain the varied findings regarding BDNF on synaptic transmission as the effects of BDNF depend on several factors including age, area of interest, developmental stage of brain, and cell type. Further, these early studies did not account for the role of retrograde messengers in their preparations. For instance, at inhibitory synapses of somatosensory cortex, BDNF causes reduction in the amplitude of spontaneous inhibitory activity via postsynaptic TrkB receptors and the expression is presynaptic. We have shown that the eCBs are the retrograde messengers that mediate the effect of BDNF in this brain region (Lemtiri-Chlieh and Levine, 2010). Similarly, we have also shown that BDNF triggers the release of eCBs at layer 5 excitatory synapses of somatosensory cortex (Yeh et al., 2017). Taken together, these results suggest BDNF utilizes several mechanisms to modulate inhibitory neurotransmission resulting in different outcomes, which adds to the complexity of understanding the effect of BDNF on synaptic transmission across different brain regions.

1.5.2 BDNF and long-term synaptic plasticity

Along with its acute effects on synaptic transmission, endogenously-released BDNF plays an important role in several forms of activity-dependent plasticity, including long-term potentiation (LTP). The endogenous role of BDNF/TrkB signaling is well established in CA1, CA3 of hippocampus and cerebral cortex. The released BDNF can modulate
synaptic efficacy either by changes in presynaptic transmitter release or by postsynaptic transmitter sensitivity (Itami et al., 2003; Levine et al., 1995) to induce a long lasting potentiation. The role of endogenous BDNF was reported first in heterozygous BDNF knockout mice, in which LTP was dramatically reduced at Schaffer collateral-CA1 synapses (Korte et al., 1995). Similar results have been reported in animals that lack TrkB receptors in CA1 (Xu et al., 2000) or forebrain (Minichiello et al., 1999). In the knockout, LTP can be rescued by exogenous application of BDNF for several hours (Patterson et al., 1996) or by re-expression of BDNF through viral-mediated gene transfer (Korte et al., 1996). Several other studies have indicated a role for endogenous BDNF in hippocampus, cortex, amgydala and ventral tegmental area. Application of a TrkB receptor antagonist or the BDNF scavenger TrkB-IgG fusion protein prevented LTP in hippocampus and cortical slices (Akaneya et al., 1997; Chen et al., 1999; Kang et al., 1997). The requirement for BDNF in plasticity is experience-dependent. In hippocampal slices, it has been shown that the high frequency stimulation-induced LTP does not require BDNF signaling while endogenous BDNF is required for mild stimulation. Similarly, some forms of LTD require BDNF while short term depression or DSI does not require BDNF (Aarse et al., 2016).

The role of BDNF in LTP is highly diverse depending on the type of preparation and stimulation protocol. For example, LTP induced by tetanic stimulation in the hippocampus is prevented by global knockout of TrkB receptors, but is not impaired by selective knockout of postsynaptic TrkB, suggesting that presynaptic BDNF/TrkB signaling mediates this effect (Xu et al., 2000). Similarly, in layer 5 of the visual cortex, BDNF is
secreted from postsynaptic sites and acts on presynaptic TrkB receptors to induce LTP (Inagaki et al., 2008). In cocaine-withdrawn animals, BDNF facilitates LTP induction at layer 2/3 to layer 5 synapses in medial prefrontal cortex by suppressing GABAergic inhibition, thereby enhancing pyramidal neuron excitability (Lu et al., 2010). This effect of BDNF was mediated by postsynaptic TrkB receptors as downregulation of postsynaptic TrkB receptors with a specific siRNA blocked LTP expression (Lu et al., 2010). In transgenic TrkB\textsuperscript{F616A} mice (in these mice, TrkB can be rapidly and reversibly inhibited using nanomolar concentrations of 1NMPP1, a derivative of the kinase inhibitor PP1), the authors found that postsynaptic BDNF/TrkB signaling was not required for pairing-induced LTP, but it was critical for presynaptic facilitation of immature CA3-CA1 synapses. This study indicates that BDNF binds to presynaptic TrkB receptors, initiating signaling cascades resulting in an increase in transmitter release at these synapses (Sallert et al., 2009). In addition, the F616A mutation in TrkB receptor has little or no effect on BDNF-mediated TrkB signaling under basal conditions, i.e. in the absence of 1NMPP1 (Chen et al., 2005). Similar to acute slices, LTP observed in hippocampal cultures also shows mixed pre- and postsynaptic roles (reviewed in (Edelmann et al., 2014)).

Spike timing-dependent plasticity (STDP) is considered a more physiological form of plasticity compared to high frequency stimulation-induced plasticity. STDP has been observed in different brain regions including visual cortex (Seol et al., 2007), amgydala (Jung et al., 2010), hippocampus (Buchanan and Mellor, 2010; Edelmann et al., 2015; Edelmann and Lessmann, 2013; Zhang et al., 2009a) and striatum (Pawlak and Kerr, 2008). A few studies have indicated a role of endogenous BDNF in this form of STDP.
For immature hippocampal mossy fiber (MF) synapses at early postnatal stages, a BDNF and cAMP/PKA dependent STDP was demonstrated (1:1 pairing 10x at 0.1 Hz) (Sivakumaran et al., 2009). BDNF was assumed to be released postsynaptically under these conditions, acting retrogradely and inducing presynaptic expression of STDP (Sivakumaran et al., 2009). It has also been shown that the STDP is impaired in pyramidal neurons in the infralimbic medial prefrontal cortex from BDNF<sup>met/met</sup> mice. (Pattwell et al., 2012). These mice recapitulate the specific phenotypic properties of the human BDNF Val66Met polymorphism, impairing regulated release of BDNF, but not constitutive release of BDNF (Chen et al., 2006). TrkB-IgG are scavengers of extracellular BDNF, consisting the BDNF binding region of BDNF specific TrkB receptor fused to the human Fc region. Upon application, TrkB-IgG binds and inactivates the released BDNF. In acute hippocampal slices, using TrkB-IgG to block BDNF signaling, Lu and colleagues found that BDNF is required for STDP as blocking BDNF disrupted the STDP. The authors found that the effects of BDNF application can be mimicked by the low frequency pairing of glutamate release and postsynaptic spiking as this protocol is sufficient to trigger the release of BDNF in a timing-dependent manner (Lu et al., 2014). In acute hippocampal slices, three different pairing protocols were used to induce STDP. Interestingly, the expression mechanism (pre versus post) and the dependency of BDNF was strikingly different between the protocols. The pairing protocol is pre-post pairings with either a 1EPSP/1 AP (70-100 repeats at 0.5Hz; 1:1) or 1EPSP/4AP (20-35 repeats at 0.5Hz; 1:4). The authors found that 1:4 protocol is postsynaptic and requires BDNF for the induction and maintenance of STDP while the 1:1 protocol is presynaptic and is independent of BDNF/TrkB signaling (Edelmann et al., 2015). Taken together, these
studies show both pre- and/or postsynaptic BDNF effects in LTP indicating that BDNF can be released both pre- and postsynaptically and can alter pre- and postsynaptic functions at a given synapse.

BDNF can also contribute to LTD by interacting with endocannabinoids. At layer 2/3 inhibitory synapses of somatosensory cortex, theta burst stimulation (TBS) induces an eCB-mediated iLTD that is independent of mGluR signaling. This form of iLTD requires endogenous BDNF signaling and PLC \( \gamma \) signaling to trigger the release of eCBs (Zhao et al., 2015). Similar to the study described above, weaker stimuli did not cause LTD, however, in combination with exogenous BDNF, weaker stimuli yielded a long-lasting LTD (Zhao et al., 2015). In another study, BDNF has been shown to induce two different forms of depression – short term depression and long-term depression in ventral tegmental area dopamine neurons. Both forms of plasticity require eCB signaling as blocking CB1 receptors blocked the depression at these synapses (Zhong et al., 2015). These studies helped in understanding of synaptic plasticity to highlight the interactions between BDNF and other neuromodulatory systems.

Several lines of evidence described in this section suggest that BDNF regulates short- and long-term synaptic plasticity. To summarize these studies,

(i) BDNF can be secreted from axons, dendrites and astrocytes.

(ii) BDNF can affect synaptic transmission at both inhibitory and excitatory synapses in different brain regions.
(iii) The effect of BDNF on synaptic transmission is also dependent on age, sex, developmental stage, and cell type.

Depending on the context – BDNF

(i) can be both, a mediator (that is critical for triggering a set of events in synaptic plasticity) or modulator (favoring either induction or maintenance of synaptic plasticity) of synaptic plasticity,

(ii) can be released either pre- or postsynaptically in neurons, and

(iii) can alter pre- and postsynaptic functions at the same synapse. Whether BDNF has a permissive or instructive function in a given LTP paradigm is relatively unexplored.

Future research in the field should be focused on (i) investigation of BDNF release sites and mode of release (constitutive vs regulated). (ii) Investigation of role of BDNF in response to different patterns of electrical stimulation. (iii) Investigation of BDNF and TrkB signaling at GABAergic and/or glutamatergic synapses in astrocytes and microglia. (iv) Investigation of role of BDNF as permissive (facilitating the synaptic responses that favor the induction phase of LTP) or instructive (BDNF signaling is required only for expression of LTP) in a given plasticity paradigm and (iv) investigation of BDNF/TrkB system interactions with other neuromodulatory systems. BDNF interactions with eCBs are described in detail in the following section.
1.6 Crosstalk between BDNF and endocannabinoid signaling

Traditionally, BDNF is known for its trophic role in neurodevelopment, such as neuronal migration, neuronal survival and neurogenesis and neuronal maturation (Barde, 1994; Lykissas et al., 2007; Newton and Duman, 2004). Over the past two decades, BDNF signaling has been highlighted as an important modulator of synaptic transmission and synaptic plasticity (Edelmann et al., 2014; Gottmann et al., 2009). On the other hand, endocannabinoid signaling was discovered and initially studied in the context of synaptic plasticity (reviewed in (Castillo et al., 2012)) and recently has also been shown to be involved in embryonic neuronal development, axonal navigation, neural progenitor proliferation and pyramidal specification in developing brain ((Oudin et al., 2011), reviewed in detail (Diaz-Alonso et al., 2012; Galve-Roperh et al., 2013)). Further, the expression profile of TrkB receptors and CB1 receptors have an overlapping pattern – high expression of both receptors are present in cerebral cortex, hippocampus, amygdala, striatum and cerebellum of brain regions (Egertova et al., 2003; Fryer et al., 1996; Gomes et al., 2006; Hofer et al., 1990; Masana et al., 1993; Matsuda et al., 1993; Moldrich and Wenger, 2000; Tsou et al., 1998). Within the neocortex, TrkB is highly localized to layers 2/3 and 5 of cortex (Cabelli et al., 1996; Fryer et al., 1996; Miller and Pitts, 2000) and CA1 of hippocampus and CB1 receptors are highly expressed in the same regions (Egertova et al., 2003; Marsicano and Lutz, 1999; Matsuda et al., 1993; Tsou et al., 1998). These anatomical studies corroborate the possibility of functional interactions between BDNF and eCB signaling in the neocortex and hippocampus.
During development, eCBs provide a mechanism for the molecular control of interneuron migration and differentiation by regulating BDNF actions. During cortical interneuron development, AEA acts as a chemo-attractant and regulates migration of CCK-expressing interneurons by TrkB receptor transactivation. It also blocks BDNF-induced neural differentiation of cortical interneurons which suggests that eCBs govern interneuron placement, migration and specification during corticogenesis (Berghuis et al., 2005). Relevant studies about BDNF-eCB interactions came from animal models of depression, in which activation of one system affects the other system. For instance, BDNF and eCBs are both well known for their neuroprotective effects against depression, disruption of either of the signaling pathways has been found to be related to depression. It has been suggested that the anti-depressant effects of eCBs are mediated by BDNF. CB1R activation leads to the increased expression of BDNF (Aso et al., 2008; Vinod et al., 2012). Chronic exposure to THC, the psychoactive component of marijuana, causes an upregulation of BDNF in vivo in areas implicated in reward processes (Butovsky et al., 2005). Further, BDNF administration to hippocampus reversed the enhanced stress response in CB1R knockouts (Aso et al., 2008) while BDNF levels are decreased in CB1R-null mice (Aso et al., 2008). Also, eCBs modulate the level of BDNF released by activating the downstream signaling pathways of BDNF in regulating the fear extinction model in mice (Bennett et al., 2017). In another study of primary cerebellar neurons, corticotrophin releasing hormone (CRH) increases the expression of BDNF while the effect is blocked by inhibiting the eCB system. Similarly, depolarization-induced BDNF synthesis is blocked by the prolonged application of a CB1 agonist (Bayatti et al., 2005).
BDNF also regulates endocannabinoid signaling (Luongo et al., 2014). For instance, in cultured cerebellar granule neuronal cultures, BDNF enhances neuronal sensitivity to eCBs by increasing the expression of CB1Rs and decreasing MAGL expression (Maison et al., 2009). These interactions between BDNF and endocannabinoids are also observed at synaptic level. Theta burst stimulation (TBS)-induced LTP and LTD simultaneously at layer 4 to layer 2/3 excitatory synapses in visual cortex, however, the net effect was a potentiation of synaptic transmission. The LTP is mediated by the endogenous release of BDNF by the TBS paradigm while the LTD is mediated by TBS-induced eCBs as the effect is blocked by TrkB receptor and CB1 receptor antagonists respectively. Thus, the net effect observed is due to competing components of BDNF-mediated LTP against eCB-mediated LTD (Huang et al., 2008). Our lab has shown that at layer 2/3 inhibitory synapses of somatosensory cortex, BDNF triggers the release of endogenous cannabinoids via phospholipase C \( \gamma \) (PLC \( \gamma \)) signaling pathway that act retrogradely on presynaptic CB1 terminals to suppress GABA release (Lemtiri-Chlieh and Levine, 2010; Zhao and Levine, 2014). We also found that TBS induces a form of endocannabinoid-mediated LTD that requires endogenous BDNF and PLC \( \gamma \) signaling (Zhao et al., 2015). Similar to the visual cortex study described above, at layer 5 excitatory synapses of somatosensory cortex, we have shown that BDNF induces the release of endogenous cannabinoids and this effect opposes the direct presynaptic effects of BDNF (Yeh et al., 2017).

In the striatum, BDNF inhibits CB1 receptor function selectively at inhibitory synapses by altering cholesterol metabolism and membrane lipid raft function (De Chiara et al.,
2010; De Chiara et al., 2013). Yet another study in striatum shows that the CB1 receptor protects striatal cells from excitotoxicity via phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin complex 1 pathway. This pathway triggers the production of BDNF via activation of BDNF gene promoter IV (Blazquez et al., 2015). These studies in striatum indicate different mechanisms by which BDNF and eCBs interact.

In hippocampus, prolonged BDNF treatment in vitro suppresses mGluR-induced eCB signaling while enhancing depolarization-induced eCB signaling at excitatory synapses, suggesting that BDNF employs different mechanisms to regulate eCB-mediated effects (Roloff et al., 2010). In dopamine neurons of ventral tegmental area of midbrain, BDNF facilitates two forms of eCB-mediated plasticity – DSI and LTD at inhibitory synapses. BDNF is also required for cocaine-induced reduction of GABAergic inhibition. However, in BDNF knockout mice, there is an impairment of cocaine-induced reduction of GABAergic inhibition, eCB-mediated iLTD at inhibitory synapses and potentiation of glutamatergic excitation indicating that BDNF interacts with eCBs for cocaine-induced synaptic plasticity (Zhong et al., 2015). In rat barrel cortex, at layer 5 distal basal excitatory synapses, action potential barrages facilitate the release of eCBs. The released eCBs suppress inhibitory transmission but facilitate Ca²⁺ spike facilitation and BDNF dependent LTP of excitatory synaptic transmission. In addition, both BDNF and eCBs are endogenously released and facilitate this novel form of LTP indicating another mode of interaction between the neuromodulators (Maglio et al., 2018).
Collectively, these studies suggest that the physiological and functional relevance of BDNF-eCB interactions might have a diverse impact on synaptic plasticity at both excitatory and inhibitory synapses in different brain regions, although much is still unexplored at this stage. Understanding these interactions will help us in elucidating the differences between normal and diseased state of brain on synaptic transmission in different brain regions.

In this section, I have discussed interactions between BDNF and eCBs in different brain regions during neuronal development and in relation to synaptic plasticity. The set of studies in this thesis contributes further to our understanding of BDNF and its role in synaptic transmission and plasticity. BDNF has diverse effects depending on the particular context – excitatory vs inhibitory synapses, pre- versus postsynaptic receptor expression, and site of release. In addition, BDNF interacts with other neuromodulators and activates signaling pathways that can alter transcription factors and protein synthesis. Developing new approaches and tools to interfere with the expression and secretion of endogenous BDNF will be crucial to further advance our understanding of BDNF regulation of synaptic function.

1.7 Rationale and hypothesis

The studies carried out in this dissertation examine the physiological interactions of BDNF and eCBs at hippocampal inhibitory synapses and cortical excitatory synapses. Our lab has previously shown that, in layer 2/3 of somatosensory cortex, the effect of
BDNF on inhibitory postsynaptic currents (IPSCs) is mediated by a retrograde effect on presynaptic terminals. We found that BDNF caused a suppression of IPSCs, which is blocked by the trk inhibitor K252a or the calcium chelator BAPTA applied to the postsynaptic cell, indicating the involvement of postsynaptic TrkB receptor activation and subsequent increase in intracellular calcium. Unexpectedly, the suppression of IPSCs was due to a decrease in presynaptic GABA release and not due to altered postsynaptic GABA receptor responsiveness. BDNF causes an increase in the paired pulse ratio, increase in the coefficient of variation of evoked responses and a decrease in the frequency of miniature postsynaptic currents (mIPSCs), all of which indicate decreased presynaptic release probability. A presynaptic expression and a postsynaptic induction suggest an involvement of a retrograde signal. Indeed, we showed that the BDNF effect is blocked by CB1R antagonists or an eCB transport inhibitor. The DAG lipase inhibitors, THL and RHC-80627 also blocked the effect of BDNF, indicating that the BDNF induces the synthesis of 2-AG. These results, taken together, indicate the BDNF causes the release of endogenous cannabinoids at layer 2/3 inhibitory synapses of somatosensory cortex (Lemtiri-Chlieh and Levine, 2010). Next, we determined that the phospholipase Cγ (PLC-γ) signaling pathway is required by BDNF to release endogenous endocannabinoids at neocortical inhibitory synapses. We showed that the BDNF effect is completely abolished by broad-spectrum PLC inhibitors, U73122 and edelfosine. We also confirmed that the effect of BDNF on inhibitory transmission does not require protein kinase C (PKC), mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) signaling nor mGluR signaling. These results indicate that BDNF triggers the release of eCBs via phospholipase Cγ (PLCγ pathway) (Zhao and Levine, 2014). There
is growing evidence of cross talk between BDNF and endocannabinoid signaling; our lab is interested in understanding the functional relevance of BDNF-eCB interactions in regulating activity-dependent long term depression at inhibitory synapses (iLTD). We found that the theta burst stimulation (TBS) protocol can induce a form of eCB-mediated iLTD at inhibitory synapses that does not require mGluR signaling. However, this eCB-mediated iLTD required endogenous BDNF and TrkB signaling as it is blocked by a TrkB receptor antagonist and also requires activation of DAG lipase (DAGL). Taken together, these results suggest that TBS can cause the release of endogenous BDNF, which triggers DAGL-dependent eCB release and cannabinoid receptor-dependent iLTD at layer 2/3 inhibitory synapses of somatosensory cortex (Zhao et al., 2015). However, it is unclear if similar interactions occur between BDNF and eCBs at cortical excitatory synapses or in hippocampus.

The highest levels of expression of TrkB and CB1 receptors are present in layers 2/3 and 5 of somatosensory cortex. In layer 5 excitatory synapses of somatosensory cortex, we have shown that BDNF can modulate glutamatergic transmission while the predominant effect of eCBs is to suppress glutamate release. This suggests that the net effect on glutamate release depends on the balance between BDNF and eCB influences. In the first part of the present study, we examine whether BDNF can cause the release of eCBs at layer 5 excitatory synapses. We hypothesize that BDNF induces eCB release at excitatory synapses which could have a mitigating or opposing effect on the direct presynaptic effects of BDNF.
We use long-term potentiation paradigms to examine the functional relevance of BDNF-eCB interactions at layer 5 excitatory synapses. TBS, high frequency stimulation (HFS) and chemical induction protocols are known to induce various forms of long term potentiation at excitatory synapses. Interestingly, some forms of TBS and HFS can also trigger the release of endogenous BDNF. We hypothesize that these LTP paradigms can cause release of endogenous BDNF. Released BDNF can activate postsynaptic TrkB receptors which then induces the release of eCBs and further leads to eCB-dependent forms of LTP. Our goal in this study is to identify paradigms that can cause LTP/LTD at layer 5 excitatory synapses and the involvement of BDNF and eCB signaling in the LTP/LTD paradigms.

Finally, the last part of the study focuses on understanding the interactions between BDNF and eCBs in CA1 inhibitory synapses of hippocampus. In hippocampal cells, acute application of BDNF causes a reduction in IPSCs and the effect is mediated by postsynaptic TrkB receptor activation (Cheng and Yeh, 2003; Frerking et al., 1998; Lemtiri-Chlieh and Levine, 2010; Levine et al., 1995). The suppressive effect of BDNF on IPSCs is expressed presynaptically in the hippocampal CA1 region (Frerking et al., 1998) whereas several other studies indicate a postsynaptic involvement of TrkB receptors (Hewitt and Bains, 2006; Tanaka et al., 1997). The mechanisms that are involved in mediating the effects of BDNF are unknown. We hypothesize that BDNF mediates its effect via endocannabinoids which causes a suppression in inhibitory neurotransmission at CA1 inhibitory synapses of hippocampus.
The studies presented in this dissertation examined: 1) the interactions between BDNF and eCBs at cortical excitatory synapses and their functional relevance in the context of long term synaptic plasticity; and 2) the interactions between BDNF and eCBs at hippocampal inhibitory synapses. Understanding the physiological role of BDNF and eCBs interactions at different brain regions will help us in elucidating the modulation of cortical and hippocampal circuitry. Further, both neuromodulators are widely implicated in an overlapping set of neurological and psychiatric disorders, a better and refined understanding of these interactions may help in uncovering the therapeutic strategies for disease treatments. The results of the present studies will be presented in chapters 2 and 3 and will be discussed in Chapter 4.
Chapter 2

BDNF-endocannabinoid interactions at cortical excitatory synapses

Part of the work in this chapter (including Figures 2.1 and 2.2) is taken from Yeh ML, Selvam R and Levine ES, “BDNF-induced endocannabinoid release modulates neocortical glutamatergic neurotransmission” Synapse 2017: 71 (5).

For that manuscript, M.L.Y. and E.S.L. were responsible for conception and design of research; M.L.Y. and R.S. performed experiments and analyzed data; M.L.Y. drafted the manuscript and all authors edited and revised the manuscript.

The remainder of the work presented in this chapter is in progress and unpublished data carried out by R.S.

2.1 Introduction

In the central nervous system, synapses are specialized sites of cell-to cell contact that form the basic substrate of information transfer within neuronal networks. Chemical neurotransmission, be it excitatory or inhibitory, is regulated by a range of neuromodulators. It is now widely accepted that the proper and precise modulation of synapses is critical for brain development as well as many cognitive functions in the adult. In the mammalian neocortex, the endocannabinoid (eCB) system and neurotrophin signaling pathways have both been well characterized and identified as important regulators of synaptic activity and neurotransmitter release. Extensive research within the
past decade has firmly established the role of eCBs as retrograde messengers suppressing neurotransmitter release in either a transient or long-lasting manner at both inhibitory and excitatory terminals (Alger, 2012; Chevaleyre et al., 2006; Freund and Hajos, 2003; Kano et al., 2009). Additionally, the eCB signaling system is now recognized as a regulator of various neural functions, including cognition, motor control, feeding behaviors and pain (Hillard et al., 2012; Mechoulam and Parker, 2013). Further, the dysregulation of the eCB signaling system has been implicated in neuropsychiatric disorders such as depression and anxiety, providing a clear and understudied experimental model for potential therapeutic interventions. Likewise, neurotrophins, specifically brain-derived neurotrophic factor (BDNF), have been shown to modulate the efficacy of synaptic transmission and their expression at the synapse is activity dependent (Berninger and Poo, 1996; Katz and Shatz, 1996; Minichiello, 2009; Park and Poo, 2013). Interestingly, multiple reports have described evidence for cross-talk between neurotrophin and eCB signaling. Specifically, (Zhong et al., 2015) showed that BDNF in midbrain dopamine neurons regulates eCB responses, cocaine-induced synaptic plasticity, and associative learning by selectively knocking down BDNF expression in dopaminergic neurons. In the visual cortex, BDNF has been shown to oppose eCB-mediated forms of heterosynaptic long-term depression (LTD) at activated synapses by inducing homosynaptic long term potentiation (Huang et al., 2008). Furthermore, there is evidence of BDNF and its involvement as a modulator of eCB-mediated synaptic plasticity in the hippocampus (Roloff et al., 2010). Lastly, BDNF and eCB interactions have been demonstrated in mediating neuronal survival and protection against excitotoxicity (Khaspekov et al., 2004; Maison et al., 2009).
BDNF may also be an important trigger for eCB synthesis and release. It is well established that eCB mobilization can be triggered by increases in intracellular calcium or activation of Gq coupled receptors and subsequent phospholipase C (PLC)-dependent increase in diacylglycerol (DAG), the precursor to 2-arachidonoylglycerol (2-AG) (Castillo et al., 2012; Hashimotodani et al., 2007a). In addition, we have recently shown that BDNF/TrkB signaling also triggers PLC-dependent eCB release. BDNF, acting through postsynaptic TrkB receptors, induces 2-AG release from pyramidal neurons at neocortical inhibitory synapses, which in turn suppresses GABA release from presynaptic terminals (Lemtiri-Chlieh and Levine, 2010; Zhao and Levine, 2014). In fact, the suppressive effect of BDNF seems to be completely mediated by eCBs, because the effect of BDNF at these inhibitory synapses is completely prevented by blocking cannabinoid type 1 (CB1) receptors or interfering with eCB synthesis or release. Furthermore, we also showed that theta burst stimulation induced release of endogenous BDNF can also trigger eCB synthesis and release resulting in inhibitory long-term depression (iLTD) (Zhao et al., 2015).

In the present studies, we addressed two questions: (1) Whether BDNF can trigger the release of eCBs at excitatory synapses. It is known that BDNF can directly potentiate glutamatergic neurotransmission by enhancing presynaptic release probability (Carmignoto et al., 1997; Lessmann and Heumann, 1998; Li et al., 1998; Schinder and Poo, 2000; Tyler and Pozzo-Miller, 2003), as well as enhancing postsynaptic NMDA receptor responsiveness (Crozier et al., 1999; Levine et al., 1998; Lin et al., 1998; Madara and Levine, 2008). Conversely, eCB signaling decreases release probability at synapses
throughout the neocortex. However, it is not known if BDNF induces eCB release at excitatory synapses. We hypothesized that, if it occurs, BDNF-induced eCB release at excitatory synapses would act to mitigate the direct effects of BDNF. (2) Examine the functional interactions between BDNF and eCBs in long-term potentiation/long-term depression (LTP/LTD) at layer 5 excitatory synapses. Endogenous BDNF has been shown to play an important role in LTP while eCBs play a major role in mediating LTD. The patterns of activity used to induce these opposite forms of plasticity, however, likely cause the release of both eCBs and BDNF. We hypothesize that the direction of plasticity (potentiation or depression) induced by a given pattern of activity/stimulation depends on the relative levels of BDNF and eCBs that are released. In particular, we hypothesized that endogenous BDNF and eCBs can act opposing to each other to mediate LTP and/or LTD.

2.2 Materials and methods

1. Animal handling and slice preparation

All animal procedures were conducted using protocols approved by the University of Connecticut Institutional Animal Care and Use Committee. Postnatal Day 15–27 Swiss CD1 (Charles River, Wilmington, MA) and C57BL/6J (The Jackson Laboratory, Bar Harbor, ME) mice were anesthetized by 3.5% isoflurane inhalation, followed by decapitation. Experiments were conducted on Swiss CD-1 mice except where noted. Whole brains were removed and immersed in ice-cold slicing solution containing (in mM) 110 choline chloride, 2.5 KCl, 1.25 NaH₂PO₄·H₂O, 25 NaHCO₃, 0.5 CaCl₂, 7 MgCl₂·6H₂O, 25 D-glucose, 11.6 sodium ascorbate, and 3.1 sodium pyruvate, equilibrated with 95%
O₂-5% CO₂ (pH 7.3, 310±5 mosmol/kg). Transverse slices (300 µm) containing somatosensory cortex were cut with a Dosaka EM DTK-1000 vibratome (Kyoto, Japan) and transferred to an incubating chamber. Slices were then incubated for 15 min at 33–35°C in carboxygenated incubating solution containing (in mM) 125 NaCl, 2.5 KCl, 1.25 NaH₂PO₄·H₂O, 25 NaHCO₃, 0.5 CaCl₂, 3.5 MgCl₂·6H₂O, 25 D-glucose, 4 sodium lactate, 2 sodium pyruvate, and 0.4 ascorbic acid (pH 7.3, 310±5 mosmol/kg) before being transferred to room temperature. Slices were then individually transferred to a recording chamber (room temperature) fixed to the stage of an Olympus BX51WI upright microscope fitted with an x40 water-immersion objective lens (0.8 NA). The recording chamber was continuously perfused at 1.5–2.0 ml/min with carboxygenated artificial cerebrospinal fluid (aCSF) consisting of (in mM) 125 NaCl, 2.5 KCl, 1.25 NaH₂PO₄·H₂O, 25 NaHCO₃, 2 CaCl₂, 2 MgCl₂·6H₂O, and 25 D-glucose (pH 7.3, 305±5 mosmol/kg).

2. Electrophysiology

Whole cell recordings were obtained from layer 5 somatosensory cortex pyramidal neurons. Neurons were visually identified by their morphology and position under infrared differential interference contrast video microscopy. Patch electrodes (2–4 MX) were pulled from borosilicate glass capillaries using a Flaming/Brown P-97 micropipette puller (Sutter Instrument, Novato, CA). Pipette internal solution contained (in mM) 4 KCl, 125 K-Gluconate, 10 HEPES, 10 Phosphocreatine, 1 EGTA, 0.20 CaCl₂, 4 Na₂-ATP, and 0.30 Na-GTP. The sodium equilibrium potential (ENa) with the use of the above internal and external solutions was close to +90 mV; thus EPSCs were recorded as inward currents. Cells were voltage clamped at -70 mV during recording. Miniature excitatory postsynaptic
currents (mEPSCs) were isolated by bath perfusion of tetrodotoxin (TTX, 500 nM). Spontaneous excitatory postsynaptic currents (sEPSCs) were isolated without TTX.

A bipolar tungsten electrode (1 MΩ; WPI; Sarasota, FL) was positioned 70-150 µm intralaminaly to the patched pyramidal neuron to elicit electrically evoked excitatory postsynaptic currents (eEPSCs). Extracellular stimuli consisted of individual square wave current pulses (170 µs; 4-30 µA) and were delivered every 20 secs through an ISO-flex stimulus isolator (Jerusalem, Israel). Every third evoked response will be delivered using a paired pulse protocol with a 75 ms interpulse interval. Stimulation strength was set to a level that evoked 30-70% of maximal response of each individual cell. For recording sEPSCs and eEPSCs, we used a pipette internal solution containing (in mM): 130 CsCl, 10 HEPES, 1 EGTA, 0.1 CaCl₂, 1.5 MgCl₂, 4 Na₂-ATP, 0.3 Na-GTP, 10 phosphocreatinine and 5 QX-314 (pH 7.3, 290 ± 5 mOsm/kg).

All electrical currents were filtered at 2.9 kHz and digitized at >6 kHz using a HEKA EPC9 amplifier and ITC-16 digitizer (HEKA Elektronik, Darmstadt, Germany). Series resistance (Rs) was compensated up to 50% at 10–100 ls lag. Input resistance (Ri) was monitored with 10 mV (50 ms) hyperpolarizing voltage steps at the end of each sweep. Cells were rejected from analyses if Ri changed by>15% or fell below 50 MΩ during the course of an experiment.
3. Chemicals

Unless otherwise stated, all drugs were obtained from Tocris Biosciences (Bristol, UK) and were delivered by bath perfusion. Drugs were first prepared as concentrated stock solution in solvents and stored at -20°C. Stock solutions of WIN55-212,2, ANA-12, SR141716A, AM404, K252a, forskolin and DHPG were dissolved in 100% dimethyl sulfoxide (DMSO). The stock solution of BDNF, rolipram and cyclotraxin-B (CTX-B) was dissolved in 18 MΩ water. The stock solution of TTX was dissolved in aCSF. Drug stock solutions were diluted in aCSF on the day of recording to the final concentrations. The final concentration of DMSO did not exceed 0.1%, which by itself had no effect on synaptic transmission.

4. Data analysis

Off-line analysis of whole-cell patch clamp electrophysiological recordings was carried out using Clampfit 10 (Molecular Devices, Sunnyvale, CA) and Prism 6 (GraphPad Software, La Jolla, CA). Group data are reported as means±SE. Statistical comparisons were made using one-way ANOVA and Dunnett’s multiple comparison test or paired Student’s t test for post hoc comparison. p <.05 was taken as a statistically significant effect.

2.3. Results

2.3.1 Effect of BDNF on spontaneous release probability at excitatory synapses

We first confirmed that CB1 and TrkB receptors are colocalized in layer 5 excitatory synapses of somatosensory cortex. Then, we examined the effect of exogenous BDNF
(20 ng/ml, 0.8 nM) on isolated AMPA-mediated mEPSCs in layer 5 of mouse somatosensory cortex. As illustrated by sample traces and group data in Figure 2.1a, bath application of 20 ng/ml BDNF (0.8 nM) had no significant effect on mEPSC frequency. The amplitude of mEPSCs was also not affected by bath-applied BDNF in any cells sampled (Figure 2.1a bottom). A higher concentration of BDNF (50 ng/ml, 2.0 nM) also failed to alter mEPSC frequency or amplitude in layer 5 of somatosensory cortex (Figure 2.1b). In light of the negative results obtained with BDNF in layer 5 of somatosensory cortex, we also examined the effect of BDNF in areas where it had been previously shown to increase mESPC frequency, including layer 5 of visual cortex (Madara and Levine, 2008), and hippocampus (Alder et al., 2005; Gibon et al., 2016; Leal et al., 2015). In contrast to the lack of effect in layer 5 of somatosensory cortex, BDNF (0.8 nM) increased mEPSC frequency in hippocampal CA1 PNs (ANOVA (F)\(_{14,74}=2.75, p<.05\); Baseline, 6.63±1.83 Hz; BDNF, 7.87±2.01, n=7 cells, 2 animals) (Figure 2.1c) and in layer 5 PNs of visual cortex (ANOVA (F)\(_{14,73}=1.86, p<.05\); Baseline, 2.05±0.88 Hz; BDNF, 2.54±0.83, n=6 cells, 2 animals) (Figure 2.1d). As expected, BDNF did not enhance the amplitude of AMPA-mediated mEPSCs in these areas (Figure 2.1c,d). Lastly, we confirmed the role of BDNF/TrkB signaling in mediating the increase in mEPSC frequency in both CA1 of hippocampus and layer 5 of visual cortex by bath-applying BDNF in the presence of the TrkB receptor specific antagonist, ANA-12 (10 µM) (Figure 2.1c,d). We did not observe any effect of ANA-12 alone on mEPSC frequency or amplitude. Furthermore, ANA-12 blocked the increase in mEPSC frequency elicited by exogenous BDNF (2.0 nM) application in the CA1 of hippocampus and layer 5 of visual cortex.
cortex (Figure 2.1c,d). The effect of BDNF and its block by ANA-12 were seen in both CD1 and C57 mice, thus data from the two strains were pooled for the above analysis.
Figure 2.1: **BDNF has region-specific effects at cortical excitatory synapses.** (a) Group time courses showing lack of effect of 0.8 nM BDNF on mEPSC frequency (top) and amplitude (bottom) in layer 5 pyramidal neurons of somatosensory cortex (n=14 cells; 5 animals). Insets, in this and following panels, show sample traces during baseline and in the presence of BDNF from a representative experiment. Scale bars in all sample traces are 10 pA, 100 ms. (b) Similar lack of effect of 2.0 nM BDNF on mEPSC frequency (top) and amplitude (bottom) in layer 5 of somatosensory cortex (n=12 cells; 3 animals). (c) Effect of BDNF (0.8 nM) on mEPSC frequency and amplitude in CA1 hippocampal pyramidal cells (n=7 cells; 2 animals—1 CD1, 1 C57). Data from individual experiments compare mEPSC frequency during minutes 3–5 of BDNF exposure to baseline. Bottom graph, effect of BDNF on mEPSC frequency is blocked in the presence of the TrkB receptor antagonist, ANA12 (10 µM; n=4 cells; 1 CD1, 1 C57). (d) Effect of BDNF (0.8 nM) on mEPSC frequency and amplitude in layer 5 of visual cortex (n=6 cells; 1 CD1, 1 C57). Data from individual experiments compare mEPSC frequency during minutes 3–5 of BDNF exposure to baseline. Bottom graph, effect of BDNF on mEPSC frequency is blocked in the presence of the TrkB receptor antagonist, ANA12 (10 µM; n=4 cells; 1 CD1, 1 C57). *p<.05
2.3.2. Endocannabinoid signaling opposes BDNF-mediated potentiation of excitatory neurotransmission

We hypothesized that the inability of BDNF to produce a net increase in mEPSC frequency in layer 5 somatosensory cortical neurons may be the result of BDNF-TrkB induced release of eCBs that have an opposing effect on presynaptic release probability. We first confirmed the effects of CB1 receptor activation at these excitatory synapses with the CB1 receptor agonist WIN 55,212–2 (WIN; 5 mM). Bath application of WIN decreased mEPSC frequency (Baseline 1.82±0.45 Hz; WIN 1.03±0.25 Hz; Student’s t test; n=8, 4 animals) (Figure 2.2d) with no change in mEPSC amplitude. Because CB1 receptor signaling typically attenuates presynaptic release via inhibition of voltage-gated calcium channels (VGCCs) (Kreitzer and Regehr, 2001; Lozovaya et al., 2009; Szabo et al., 2014), we examined the effect of WIN in the presence of cadmium (Cd2+; 100 µM) to block VGCCs. As shown in Figure 2.2d, bath application of Cd2+ alone reduced mEPSC frequency (Baseline 2.75±0.97 Hz; Cd2+ 1.18±0.48 Hz; Student’s t test, n=4, 1 animal). Subsequent addition of WIN in the presence of Cd2+ had no further effect on mEPSC frequency (Figure 2.2d). Taken together, these data indicate that VGCCs contribute to spontaneous glutamatergic release at these synapses, and that cannabinoid-mediated suppression of release requires VGCC activity, similar to what was reported for layer 2/3 inhibitory synapses (Madara and Levine, 2008; Trettel and Levine, 2002)(Madara & Levine, 2008; Trettel & Levine, 2002). To test whether BDNF-induced eCB release was mitigating the direct effect of BDNF, we examined the effect of BDNF (0.8 nM) in the presence of the CB1 receptor-specific inverse agonist, SR141716A (SR; 10 mM) (Figure 2). In this experiment, SR was bath-applied for 10 minutes, followed by a 10 min perfusion
of BDNF (0.8 nM) in the continued presence of SR. Representative traces from a single experiment are shown in Figure 2.2a. Application of SR resulted in a small but nonsignificant and transient increase in the frequency of mEPSCs in the majority of cells tested, as shown in the group data presented in Figure 2.2b.

The amplitude of mEPSCs was not affected by SR alone (Figure 2.2c). As shown in the group data presented in Figure 3b, exogenous BDNF (0.8 nM) application in the presence of SR resulted in a statistically significant increase in mEPSC frequency. mEPSC frequency was increased to 159.4%±13.5% at 5 min of BDNF exposure in the presence of SR (ANOVA (F)14,131=1.76, p<.05; SR 1.36 Hz±0.27; SR+BDNF 1.97 Hz±0.36; n=15, 5 animals) (Figure 2.2b). We have shown that the direct effects of BDNF can be unmasked...
either by blocking CB1 receptors or by inhibiting postsynaptic eCB release from pyramidal neurons. These results support our hypothesis that BDNF/TrkB signaling causes the release of eCBs at excitatory synapses, which directly opposes direct BDNF-mediated potentiation of glutamatergic release probability.

2.3.3 Pharmacologically-induced long term potentiation (LTP)

A combination of forskolin, an adenylyl cyclase activator (50 µM) and rolipram, a phosphodiesterase inhibitor (0.1µM) in the presence of 0 Mg²⁺ (Otmakhov et al., 2004) was used to induce LTP at excitatory synapses onto layer 5 pyramidal neurons of somatosensory cortex. Forskolin/rolipram causes a stable increase in intracellular cAMP levels thereby activating protein kinase A and the removal of Mg²⁺ enhances NMDA receptor activation. We induced LTP by exposure to this induction cocktail for 10 or 15 minutes. We examined frequency and amplitude of spontaneous excitatory postsynaptic currents (sEPSC) recorded from layer 5 pyramidal neurons. As shown in figure 2.3a, this chemical induction protocol induced potentiation of excitatory transmission lasting ~40 minutes. As shown in figure 2.3a, 10 minutes of forskolin/rolipram/0 Mg²⁺ exposure increased the frequency of spontaneous excitatory postsynaptic currents (ANOVA, $F_{(6,84)}= 29.57; p< 0.05; n=7; 3$ animals). The induction paradigm did not have any significant effect on amplitude (Fig 2.3b) nor on the amplitude distribution (Fig 2.3c). Next, we determined if the chemically-induced form of LTP can be potentiated with 15 minutes
of treatment. As shown in figure 2.3d, evoked EPSC amplitude (eEPSC) was potentiated in all 5 cells tested. In contrast to 10 minutes treatment, 15 minutes of treatment induced potentiation of excitatory transmission lasting for ~90 minutes (figure 2.3E). Similar to 10 minutes treatment, the 15-minute treatment increased the frequency of spontaneous
excitatory postsynaptic currents (figure 2.3E) but not amplitude of sEPSCs (Figure 2.3F; n=8, 2 animals).

2.3.4 Excitatory LTP is independent of BDNF signaling

Because many forms of LTP require endogenous BDNF signaling, we examined the effects of the chemical induction protocol during bath application of TrkB receptor antagonists. We hypothesized that the chemical induction protocol causes the release of endogenous BDNF. We used three TrkB receptors antagonists to test the effects on both 10 minutes and 15 minutes of chemical induction – (1) K252a (200 nM), tyrosine kinase inhibitor that inhibits TrkB at lower concentrations (Lemtiri-Chlieh and Levine, 2010; Zhao and Levine, 2014); (2) ANA12 (5 µM), a TrkB selective non-competitive antagonist (Zhao et al., 2015) and (3) Cyclotraxin B (10 µM), a TrkB selective non-competitive antagonist (Maglio et al., 2018; Zhao et al., 2015). As shown in figures 2.4 and 2.5, blocking BDNF signaling did not prevent the induction of LTP at layer 5 excitatory synapses. If endogenous BDNF is involved in the paradigm, we expected to see a decrease in the frequency of sEPSCs in the presence of TrkB receptor antagonists. As shown in figure 2.4a, c, e, 10 minutes of forskolin/rolipram/ 0 Mg²⁺ treatment in the presence of TrkB receptor antagonists had no change in the sEPSC frequency compared to forskolin/rolipram/ 0 Mg²⁺ treatment alone. The amplitude of sEPSCs was not affected by the forskolin/rolipram/ 0 Mg²⁺ treatment in the presence of TrkB receptor antagonists (Fig 2.4b, d, f). Similarly, 15 minutes of forskolin/rolipram/ 0 Mg²⁺ treatment in the presence of TrkB receptor antagonists had no change in the sEPSC frequency (fig 2.5). With both 10 and 15 minutes of forskolin/rolipram/ 0 Mg²⁺ treatment, the amplitude of
sEPSCs had no effect. This result led to three conclusions: (1) BDNF is not released in this induction paradigm or (2) The effect of endogenous BDNF is masked by effect of eCBs in sEPSC frequency similar to the lack of exogenous BDNF effect in mEPSC frequency observed and reported in layer V excitatory synapses or (3) The combination of forskolin and rolipram causes a very strong stimulation that it activates several downstream signaling molecules and pathways causing a ceiling effect. Hence, even if

Figure 2.4: Excitatory LTP is independent of BDNF signaling with 10 minutes of forskolin/rolipram treatment (A) Group time course of sEPSC frequency and (B) sEPSC amplitude in the presence of K252a (n=12; 4 animals). (C) Group time course of sEPSC frequency and (D) sEPSC amplitude in the presence of ANA-12 (n=7; 2 animals). (E) Group time course of sEPSC frequency and (F) sEPSC amplitude in the presence of cyclotraxin-B (CTX-B; n=8; 2 animals). Arrow indicates 10 minutes of forskolin treatment. Black data points in all graphs are from controls (from figure 2.3a, 2.3b).
BDNF is released here, BDNF does not affect the effectiveness of the LTP stimulation as the stimulation from forskolin/rolipram is much stronger than BDNF alone.

2.3.5. **Excitatory LTP is independent of endocannabinoid signaling**

Assuming that there is an involvement of endogenous eCBs in excitatory synapses, we hypothesized that by blocking CB1 receptors we expect to see an increase in sEPSC frequency and amplitude in the induction protocol. However, as shown in figure 2.6, we did not see any change in sEPSC frequency and amplitude in the presence of CB1 receptor antagonist (SR141716A; 10µM). This led to conclusion that neither BDNF nor endocannabinoids are involved in this pharmacologically-induced form of LTP.
2.3.6 Electrically induced forms of LTP

Similar to other brain areas including hippocampus, striatum, amgydala and visual cortex, we used electrically induced protocols to elicit LTP at layer 5 synapses as opposed to chemical stimulation. The available induction paradigms in literature are: high frequency stimulation (HFS), theta burst stimulation (TBS), pairing protocols (PP). Nevertheless, I was not able to elicit a LTP with any of the paradigms which could be partly due to the inability to recapitulate the required circuitry, stimulation patterns of where and how to stimulate in this brain region. Below is the table summarizing the induction protocols and the stimulation pattern that have been used:

<table>
<thead>
<tr>
<th>Induction Protocol</th>
<th>Stimulation paradigm</th>
<th>Position of electrode in layer</th>
<th>Brain region reported in literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBS</td>
<td>7X TBS delivered with a 5s intertrain interval. Each TBS train contained 10 bursts</td>
<td>5, 2/3</td>
<td>Somatosensory cortex (Zhao and Levine, 2014)</td>
</tr>
<tr>
<td></td>
<td>(200ms interburst interval), each burst consisted of 5 stimuli at 100Hz.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HFS</td>
<td>25 pulses given at 100Hz</td>
<td>5</td>
<td>Hippocampus (Wang et al., 1997)</td>
</tr>
<tr>
<td>TBS</td>
<td>3X TBS delivered with a 5s intertrain interval. Each TBS train contained 10 bursts</td>
<td>5, 2/3</td>
<td>Hippocampus (Aarse et al., 2016)</td>
</tr>
<tr>
<td></td>
<td>(200ms interburst interval), each burst consisted of 5 stimuli at 100Hz.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 2.6: **Excitatory LTP is independent of eCB signaling** (A) Group time course of sEPSC frequency and (B) sEPSC amplitude in the presence of SR (n=5; 2 animals). Arrow indicates 10 minutes of forskolin treatment. Black data points in all graphs are from controls (from figure 2.3a, b).
<table>
<thead>
<tr>
<th><strong>TBS</strong></th>
<th>10X TBS delivered with a 10s intertrain interval. Each episode of TBS contained 20 bursts at 5 Hz, each burst containing five pulses at 100 Hz. <em>One cell out of 12 cells in this experiment showed LTP</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HFS</strong></td>
<td>Two trains of 50 pulses at 100Hz (HFS) given at 5 min intervals</td>
</tr>
<tr>
<td><strong>TBS</strong></td>
<td>10 bursts of four pulses given at 100Hz, in the form of one train</td>
</tr>
<tr>
<td><strong>HFS</strong></td>
<td>50 Hz, 1 s was applied 10 times at intervals of 10 s</td>
</tr>
<tr>
<td><strong>HFS</strong></td>
<td>100 Hz, 1 s was applied 4 times at 10 s intervals</td>
</tr>
<tr>
<td><strong>Pairing protocol</strong></td>
<td>4 brief high frequency tetani (50 pulses at 50 Hz; 4 s intervals) paired with a short depolarization of 15s</td>
</tr>
<tr>
<td><strong>Pairing protocol</strong></td>
<td>4 brief high frequency tetani (50 pulses of 50 Hz; 4s intervals) paired with a long depolarization of 3 minutes</td>
</tr>
<tr>
<td><strong>Pairing protocol</strong></td>
<td>Low frequency protocol (200 pulses, 1.4Hz) paired with long depolarization of 3 minutes</td>
</tr>
<tr>
<td><strong>Pairing protocol</strong></td>
<td>3X TBS delivered at 30 s intervals. Each TBS consists of 5 bursts each consisting of 4 stimuli at 100 Hz with a 250 ms interburst interval. TBS was paired with postsynaptic depolarization (5 depolarizing steps to -20 mV, 60 ms long). Repeated this paradigm with 7 trains and 10 trains of TBS yet did not observe LTP.</td>
</tr>
<tr>
<td><strong>Pairing protocol</strong></td>
<td>Stimulation protocol consisted of presynaptic activation of 10 bursts, each bursts with 5 pulses at 100Hz, spaced at 200ms, repeated 3 times at 10s intervals and postsynaptic injection of a depolarizing current pulse (1.5 nA, 40 ms) during each burst, with a 5ms interval between the onset of pre- and postsynaptic stimulation. <em>This protocol works</em></td>
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</table>

- **Layers II/III/V of sensorimotor cortex** (Chen et al., 2009)
- **Hippocampus** (Aarse et al., 2016)
- **Layer V of visual cortex** (Inagaki et al., 2008)
- **Medium spiny neurons in striatum, hippocampus** (Jia et al., 2010)
- **Hippocampus** (Wang et al., 1997)
- **Hippocampus** (Lapointe et al., 2004)
- **Medial Prefrontal cortex** (Lu et al., 2010)
2.3.7. Chemical induction by DHPG induces LTD

Using a chemical induction paradigm (Izumi and Zorumski, 2012; Peterfi et al., 2012), we examined LTD at excitatory synapses of layer 5 pyramidal neurons of somatosensory cortex. Induction paradigm consisted of DHPG application to slices for 10 minutes to induce plasticity. Cells were then perfused with ACSF and held for 90 mins post-induction. sEPSC amplitude and frequency were measured. Example traces of baseline, DHPG application and 30 minutes post induction were shown in Figure 2.7a. As shown in figure 2.7b and 2.7c, bath application of DHPG (50 µM) decreased the amplitude but not the frequency of spontaneous excitatory postsynaptic currents (n=3 cells, 3 animals). As shown in figure 2.8 DHPG also decreased the amplitude of evoked responses (eEPSC).

Figure 2.7: Long-term depression of spontaneous excitatory activity in pyramidal neurons (A) Sample traces of sEPSCs. Scale bar: 50 pA, 5 s. (B) Time course of sEPSC amplitude. (C) Time course of sEPSC frequency for the same neurons. (n=3 cells from 3 animals).
and increased the paired pulse ratio (PPR), a measure of release probability. Example traces and individual cells during baseline, DHPG application and post-induction period were shown in figure 2.8a.

Group data showed that the eEPSC amplitude decreased during and 90 minutes after DHPG treatments to 75% and 74% of eEPSC amplitude before exposure respectively (Fig 2.8b). Similarly, group data of all cells showed an increase in PPR during and 90 minutes after DHPG treatment (Fig 2.8c) indicating that the expression of LTD includes a presynaptic component in this paradigm (n=3 cells, 3 animals). This form of chemically induced LTD can be used to test the involvement of BDNF and eCBs at these synapses.

Figure 2.8: **Long-term depression of evoked responses in pyramidal neurons** (A) Top: Sample sweeps of eEPSCs application during baseline (1), DHPG (2), 30(3), 60 (4) and 90 minutes (5) post-DHPG. Scale bar: 200 pA, 20 ms. Bottom: An example time course of eEPSC amplitude showing LTD at 30, 60 and 90 minutes (circles). Increase in paired pulse ratio (stars) of a single pyramidal cell is also shown during the same time course. (B) Group data showing a decrease in eEPSC amplitude during and at 90 minutes post-DHPG application. (C) Paired pulse ratio showing an increase with DHPG and 90 minutes indicating that the LTD observed here is presynaptic in expression. (n=3 /3 animals).
Using low frequency stimulation (LFS), we examined LTD at layer 5 pyramidal neurons of somatosensory cortex. Stimulation consisted of 900 pulses at 1 Hz (Izumi and Zorumski, 2012). This protocol induced a stable long-lasting suppression of excitatory transmission. Figure 2.9a shows the sample traces and an example time course of a cell showing a decrease in eEPSC amplitude after LFS and no change in PPR. Group data showed a significant decrease in eEPSC amplitude at 30 and 60 minutes post induction (Fig 2.9b; ANOVA, F(2,9) = 17.14; p<0.05. BL: 1053 ± 72.11 pA; 30 minutes: 494.3 ± 35.56 pA; 60 minutes: 496 ± 107.7
pA). No change observed in PPR suggests that LTD has a postsynaptic component in the LFS paradigm (fig 2.9c). These results indicate that LTD can be obtained consistently in acute slices with both paradigms and the magnitude of duration of LTD is 90 minutes. We can now use these LTD paradigms to examine the role of endogenous BDNF and/or endogenous cannabinoids in mediating these forms of plasticity.

2.4 Discussion

We recently showed that BDNF induces release of eCBs from postsynaptic pyramidal cells in layers 2/3 of somatosensory cortex (Lemtiri-Chlieh and Levine, 2010). This effect of BDNF is initiated by postsynaptic TrkB signaling, requires downstream phospholipase-Cγ (PLCγ) signaling and is independent of metabotropic glutamate receptor (mGluR) activation (Zhao and Levine, 2014). Furthermore, we established a role for endogenous BDNF in eCB-mediated plasticity at cortical inhibitory synapses, where strong theta burst stimulation (TBS) in layers 2/3 of somatosensory cortex can induce iLTD that requires BDNF-TrkB and diacylglycerol (DAG) signaling (Zhao et al., 2015). To complement these studies of BDNF and eCB interactions at cortical inhibitory synapses, we asked whether BDNF also triggers the release of eCBs at cortical excitatory synapses. We have shown that BDNF and eCBs exert opposing effects at cortical excitatory synapses (Fortin and Levine, 2007; Madara and Levine, 2008). However, whether these two distinct signaling systems interact and the net functional outcome of these influences at excitatory synapses remains unexplored. We then examined the synaptic effects of eCB-CB1 receptor signaling and BDNF-TrkB receptor signaling on spontaneous transmission at excitatory terminals within layer 5 of the somatosensory cortex. Surprisingly, we found that BDNF by itself did not increase mEPSC frequency (or amplitude) at these synapses.
To confirm previous reports of BDNF effects on mEPSC frequency in other brain regions (Carmignoto et al., 1997; Li et al., 1998; Schinder and Poo, 2000; Tyler and Pozzo-Miller, 2003), we examined the effect of BDNF in pyramidal neurons in the hippocampal CA1 region and layer 5 of visual cortex. We found that BDNF significantly increased mEPSC frequency in these regions, these effects were blocked by a TrkB receptor antagonist, and these effects were consistent across two different mouse strains.

We next determined whether a BDNF-induced release of eCBs at excitatory synapses in layer 5 of somatosensory cortex might offset the direct presynaptic effects of BDNF. Blocking eCB signaling with a CB1 receptor antagonist unmasked a BDNF-induced increase in mEPSC frequency. Blocking postsynaptic release of eCBs had a similar effect. These results suggest that BDNF-TrkB signaling induces the release of eCBs at excitatory synapses. While our group and others have previously described an increase in presynaptic release probability as a result of acute BDNF exposure in other brain regions, this is the first study examining synaptic effects of BDNF in layer 5 of mouse somatosensory cortex. In this region, we find that BDNF activates presynaptic TrkB receptors to enhance spontaneous release probability, while simultaneously triggering the postsynaptic release of eCBs that act to decrease release via presynaptic CB1 receptors. The presynaptic effects of BDNF on spontaneous glutamate transmission may have functional consequences of their own (Carmignoto et al., 1997) (Lessmann and Heumann, 1998) (Schinder and Poo, 2000; Tyler and Pozzo-Miller, 2003) and may also translate to changes in evoked AP-dependent release. However, there is a lack of clear evidence for effects of BDNF on evoked glutamate release. This may in fact reflect the
offsetting effect of BDNF-induced eCB release, and/or the effects of BDNF on evoked release may depend on specific AP activity patterns. It is also important to recognize that evoked (action potential-dependent) and spontaneous (action potential-independent) neurotransmitter release may utilize distinct vesicle pools that can be differentially regulated ((Huntwork and Littleton, 2007; Littleton et al., 1993; Maximov et al., 2007; Pang et al., 2011; Yoshihara et al., 1999). Furthermore, nonoverlapping populations of postsynaptic NMDA receptors have been implicated in differentiating between spontaneous and evoked release (Atasoy et al., 2008; Melom et al., 2013).

In summary, our published study contributes to uncovering region-specific roles of the eCB-CB1 receptor and BDNF-TrkB receptor signaling pathways at cortical excitatory synapses. Moreover, we provide evidence that BDNF-TrkB signaling may play an important role in triggering eCB release at cortical excitatory synapses. These two signaling systems have been implicated in various neurological disease states, including autism spectrum disorders, schizophrenia and epilepsy. There is, at present, a continuously growing body of evidence that the eCB system and BDNF system interact at various levels across multiple fields, including synaptic plasticity (De Chiara et al., 2010; Maison et al., 2009), neuropathic pain (Luongo et al., 2014), cortical development (Berghuis et al., 2005; Galve-Roperh et al., 2013) and neuroprotection in depression and epileptic seizures (Aguado et al., 2007; Aso et al., 2008; Khaspekov et al., 2004; Marsicano et al., 2003; Vinod et al., 2012). Careful examination and elucidation of these interactions at excitatory and inhibitory synapses will be essential to the development and implementation of novel and effective therapeutic strategies.
In the context of understanding the interactions between BDNF and eCBs, the second part of the chapter focused on long term plasticity and how these two neuromodulators interact. We first determined the chemical induction paradigms to induce LTP at excitatory synapses of somatosensory cortex. Compared to 10 minutes induction paradigm, 15 minutes induction paradigm caused a longer lasting potentiation of excitatory transmission (~90 minutes). To test for the involvement of BDNF and eCBs in this paradigm, we used antagonists to block the relevant receptors. We concluded that neither endogenous BDNF nor eCBs were required to elucidate LTP in this paradigm. This result led to several conclusions – (i) the chemical induction paradigm of forskolin and rolipram may be too strong by itself, hence causing large increases in cAMP levels which makes the contribution of BDNF nor eCBs insignificant, if any. To this effect, we will perform a dose-response with different concentrations and/or duration of forskolin/rolipram/0 Mg^{2+} to determine the role of BDNF and/or eCBs in this system. (ii) BDNF contributes more to some forms of LTP than others and that pattern determines the dependence of resulting potentiation of BDNF. Maybe, in this paradigm, BDNF release is minimal compared to eCB release, hence, we do not see an effect of BDNF. To ascertain if there is an effect of eCBs at these synapses, we can perform DSE to determine eCB-mediated suppression (Fortin et al., 2004). Further, blocking CB1 receptors did increase the magnitude of forskolin in 5 cells, obtaining additional cells might help us resolve this question. (iii) This chemical induction protocol is truly independent of BDNF signaling in this brain area. We will examine other induction paradigms that are dependent in BDNF (Abidin et al., 2006; Edelmann et al., 2015; Lu et al., 2010). We will also explore the same protocol in CA1 hippocampus to determine if
there is a region specificity. (iv) Increase in cAMP levels can cause the release of BDNF which in turn activates MAPK pathway (directly or through translocation of MAPK) downstream to potentiate at the synapses (Patterson et al., 2001). It will be interesting to find out whether BDNF can activate both MAPK pathway (to exert its effects on LTP) and PLC γ signaling to trigger eCB release.

We also tested several electrical stimulation protocols to elicit LTP at layer 5 excitatory synapses. Despite my efforts, we could not obtain potentiation at these synapses. The lack of plasticity in this region could be for the following reasons: (i) inability to recapitulate the circuitry in our preparation. (ii) the level of eCBs is high in these slices and masks the potentiation. To address this concern, we performed several DSE protocols to determine if there is high eCB tone. Unfortunately, we did not observe DSE in any of the protocols.

Finally, we identified both chemical and electrical induction paradigms to induce LTD. DHPG-induced LTD is known to be dependent on endocannabinoids (Izumi and Zorumski, 2012). However, LFS-LTD may not require endocannabinoids as we did not observe any change in PPR. Future experiments will address the role of eCBs and BDNF in these paradigms to compare and contrast these forms of LTD. There are multiple forms of LTP and LTD, so experiments using different protocols and different experimental preparations may not share molecular mechanisms. Identifying which mechanisms are recruited under physiological conditions and disease conditions is critical. To this extent, the current work provides a repertoire of LTP and LTD paradigms to understand the role of BDNF and eCBs and their mechanism of action.
Taken together, this chapter elucidates the interactions at cortical excitatory synapses. The first part addresses the effect of exogenous BDNF and BDNF-induced eCB release on excitatory neurotransmission. In the second part of the study, we explored different plasticity paradigms to study the functional relevance of these neuromodulator interactions.
Chapter 3

Endogenous cannabinoids mediate effect of BDNF at CA1 inhibitory synapses in the hippocampus

This chapter is a duplicate version of a manuscript currently under review: Selvam R, and Levine ES. Endogenous cannabinoids mediate effect of BDNF at CA1 inhibitory synapses in the hippocampus.

R.S. and E.S.L. were responsible for conception and design of research; R.S. performed experiments and analyzed data. R.S. and E.S.L. interpreted the results of the experiments; R.S. prepared the figures and drafted the manuscript; R.S. and E.S.L. edited and revised the manuscript.

3.1 Introduction

Brain derived neurotrophic factor (BDNF), a member of the neurotrophin gene family, plays a pivotal role in neuronal survival and differentiation, as well as the formation and maturation of synapses during development (Gottmann et al., 2009). BDNF binds to the tropomyosin receptor kinase B (TrkB) receptor as well as the pan-neurotrophin receptor p75. TrkB receptors are expressed throughout the central nervous system with the highest levels of expression in the neocortex and hippocampus (Masana et al., 1993). At the subcellular level, TrkB receptors are expressed in postsynaptic dendrites and axon terminals of GABAergic and glutamatergic neurons (Aoki et al., 2000; Cabelli et al., 1996;
Drake et al., 1999; Fryer et al., 1996; Gomes et al., 2006). In addition to its effects on survival and differentiation, BDNF modulates synaptic transmission at both inhibitory and excitatory synapses (Gottmann et al., 2009). Several studies have also shown the involvement of BDNF in long term potentiation (LTP), a cellular mechanism thought to be required for learning and memory (Lu et al., 2008; Minichiello, 2009).

BDNF modulates GABAergic neurotransmission, with acute effects that tend to be suppressive, but the mechanisms involved are unclear. In CA1 region of hippocampus, BDNF application reduced the amplitude of evoked inhibitory postsynaptic currents. A reduction in the paired-pulse ratio and a decrease in the coefficient of variation suggested a presynaptic site of action for this effect (Frerking et al., 1998). In rat hippocampal cultures, BDNF caused a rapid reduction in postsynaptic GABA receptor number that was responsible for a decrease in mIPSC amplitude (Brunig et al., 2001). A study by Mizoguchi et al. (Mizoguchi et al., 2006) suggested that the effect of BDNF on GABA release was dependent on age. BDNF suppressed mIPSC frequency and amplitude in hippocampal preparations of age P14 and P21 rats but not in P7 rats. A decrease in mIPSC frequency, mIPSC amplitude and decrease in release probability was also observed in visual cortex of BDNF heterozygous knockout mice suggesting that the BDNF may have both presynaptic and postsynaptic effects (Abidin et al., 2008; Abidin et al., 2006). In cerebellar granule cells, BDNF decreased both the amplitude and frequency of spontaneous and miniature postsynaptic currents also suggesting both pre- and postsynaptic effects (Cheng and Yeh, 2003). The effect of BDNF was blocked by inclusion of a TrkB antagonist in the postsynaptic cell in cerebellar granule cells (Cheng and Yeh, 2003). Taken
together, these results suggest that postsynaptic TrkB receptors are often required to initiate the effects of BDNF at GABAergic synapses, however the diversity of these effects indicates that the effects of BDNF at inhibitory synapses depend on several factors including age, brain area, and cell type.

In somatosensory cortex, we previously found that BDNF decreases the amplitude of spontaneous inhibitory postsynaptic currents, and this effect is initiated by postsynaptic TrkB activation but is expressed presynaptically. This effect was shown to be mediated by the postsynaptic release of endocannabinoids (eCBs) that act in a retrograde manner to suppress GABA release (Lemtiri-Chlieh and Levine, 2010). BDNF triggers the release of eCBs via phospholipase C pathway (Zhao and Levine, 2014; Zhao et al., 2015). In the hippocampus, BDNF has also been shown to acutely suppress presynaptic GABA release, however underlying mechanisms involved were not identified (Frerking et al., 1998). In the present study, we focused on understanding the mechanism by which BDNF suppresses GABAergic neurotransmission in CA1 hippocampal pyramidal neurons.

### 3.2 Materials and methods

1. **Slice preparation**

Briefly, Swiss CD1 mice (postnatal day 14–24) were anesthetized by 3.5% isoflurane inhalation, followed by decapitation. Whole brains were harvested quickly and immersed in ice-cold slicing solution containing (in mM) 110 choline chloride, 2.5 KCl, 1.25 NaH₂PO₄·H₂O, 25 NaHCO₃, 0.5 CaCl₂, 7 MgCl₂·6H₂O, 25 D-glucose, 11.6 sodium ascorbate, and 3.1 sodium pyruvate, equilibrated with 95% O₂-5% CO₂ (pH 7.4, 310±5
mosmol/kg). Sagittal slices of 300 µm containing hippocampus were cut with a Dosaka EM DTK-1000 vibratome (Kyoto, Japan) and transferred to an incubating chamber. Slices were then incubated for 15 min at 34°C in incubating solution containing (in mM) 125 NaCl, 2.5 KCl, 1.25 NaH₂PO₄·H₂O, 25 NaHCO₃, 0.5 CaCl₂, 3.5 MgCl₂-6H₂O, 25 D-glucose, 4 sodium lactate, 2 sodium pyruvate, and 0.4 ascorbic acid (pH 7.3, 310±5 mosmol/kg) before being transferred to room temperature. Slices were then individually transferred to a recording chamber (room temperature) fixed to the stage of an Olympus BX51WI upright microscope fitted with a x40 water-immersion objective lens (0.8 NA). The recording chamber was continuously perfused at 2.0 ml/min with carboxygenated artificial cerebrospinal fluid (aCSF) consisting of (in mM) 125 NaCl, 2.5 KCl, 1.25 NaH₂PO₄·H₂O, 25 NaHCO₃, 2 CaCl₂·2H₂O, 2 MgCl₂-6H₂O, and 25 D-glucose (pH 7.3, 305±5 mosmol/kg). All animal procedures were conducted using protocols approved by the University of Connecticut Institutional Animal Care and Use Committee.

2. Electrophysiology

Whole cell recordings were obtained from hippocampal CA1 pyramidal neurons. Neurons were visually identified by their morphology and position under infrared differential interference contrast video microscopy. Patch electrodes (6–7 MΩ) were pulled from borosilicate glass capillaries using a Flaming/Brown P-97 micropipette puller (Sutter Instrument, Novato, CA). Pipette internal solution contained (in mM) 120 CsCl, 10.0 HEPES, 10.0 phosphocreatine, 1.0 EGTA, 0.1 CaCl₂, 4.0 Na₂-ATP, 1.5 MgCl₂, 0.4 Na-GTP and 5.0 QX-314 (pH 7.4, 290±5 mosmol/kg). In all experiments, the chloride equilibrium potential (ECl) was close to 0 mV; thus spontaneous inhibitory postsynaptic
currents were recorded as inward currents at a holding potential of -70 mV. The ionotropic glutamate receptor antagonists 6,7-dinitroquinoxaline-2,3-dione (DNQX; 10 µM) and 3-[(R)-2-carboxypiperazin-4-yl]-propyl-1-phosphonic acid (CPP; 3 µM) were added to the bath solution to isolate inhibitory activity. To enhance spontaneous inhibitory activity, slices were treated with the muscarinic agonist carbachol (CCh; 10 µM). CCh, DNQX and CPP were present throughout all experiments and all drugs were delivered by bath perfusion, unless otherwise stated. Electrical currents were filtered at 2.9 kHz and digitized at >6 kHz using a HEKA EPC9 amplifier and ITC-16 digitizer (HEKA Elektronik, Darmstadt, Germany). Series resistance ($R_s$) was compensated up to 50% at 100 µs lag. Cells were rejected from analyses if input resistance ($R_i$) changed by >15% or fell below 100 MΩ during the experiment.

3. Analysis

Off-line analysis was carried out using Clampfit 10.2 (Molecular Devices, Sunnyvale, CA) and Prism 7 (GraphPad Software, La Jolla, CA). Group data are reported as mean ± SE. Statistical comparisons were made using one-way ANOVA and Dunnett’s multiple comparison test or Student’s paired t-test for post hoc comparison.

4. Chemicals

Unless otherwise stated, drugs were obtained from Tocris Biosciences (Bristol, UK) and were delivered by bath perfusion or by intracellular application through patch pipette, as indicated. Stock solutions of BDNF (PeproTech, Rocky Hill, NJ), CPP (Sigma-Aldrich, St.Louis, MO) and carbachol were dissolved in 18 MΩ water. Stock solutions of WIN55-
212,2 (Cayman Chemicals, Ann Harbor, MI), DNQX (Sigma-Aldrich, St. Louis, MO), K252a, ANA-12, SR141716A, AM404, JZL184, URB597 and THL were dissolved in 100% dimethyl sulfoxide (DMSO). The final concentrations of the drugs were obtained by dilution in aCSF from stock solutions on the day of recording. The final concentration of DMSO did not exceed 0.1%, which by itself had no effect on synaptic transmission.

3.3 Results

3.3.1. **BDNF depresses inhibitory transmission via postsynaptic TrkB receptors**

The first set of experiments examined the effect of BDNF on pharmacologically-isolated spontaneous inhibitory postsynaptic currents (sIPSCs) in CA1 pyramidal neurons in hippocampal slices. Under basal conditions, bath application of BDNF (0.8 nM) caused a significant decrease in the mean frequency (BL: 9.6 ± 0.9 Hz; BDNF: 8.1 ± 1.2 Hz; n=6, 2 animals, p=0.0093) but no effect on mean amplitude (BL: 45.6 ± 7.7 pA; BDNF: 46.8 ± 8.1 pA). Because spontaneous inhibitory activity was very low in the presence of glutamate receptor antagonists, the muscarinic agonist carbachol (CCh; 10 µM) was used to enhance action potential firing in interneurons (Kawaguchi, 1997; Lemtiri-Chlieh and Levine, 2007; Lemtiri-Chlieh and Levine, 2010; Trettel et al., 2004). In the presence of CCh, mean sIPSC amplitude increased to ~two-fold from 67.4 ± 9.1 pA to 132.2 ± 15.0 pA (n=10, 2 animals, p=0.0035) and the frequency increased from 7.4 ± 0.6 Hz to 11.8 ± 0.9 Hz (p=0.0001). As shown in the example sweeps in Fig. 3.1A, BDNF (0.8 nM) applied in the presence of carbachol caused a decrease in both mean sIPSC frequency (BL: 12.1 ± 1.2 Hz; BDNF: 10.6 ± 1.1 Hz; n=21, 8 animals) and amplitude (BL: 119.5 ± 8.2 pA; BDNF: 99.8 ± 7.9 pA). In order to capture changes in both the frequency and the
amplitude of action potential-dependent synaptic events, we integrated the area of individual IPSCs within specified bins to determine the total synaptic charge (Q; pA ms). As shown in Figure 1B, BDNF decreased mean sIPSC charge within 3-4 minutes of BDNF exposure. On average, the mean sIPSC charge decreased significantly to 79.4 ± 4.6% of CCh baseline (BL) after 8-10 mins of BDNF application (n=21, 8 animals, ANOVA F(20,240)=84.56, p<0.0001). Shown at the right are the data from individual cells comparing charge during a 3 min BL to min 8-10 of BDNF application (paired t-test, p=0.0002).

Generally, sIPSC charge returned to baseline values ~ 10 minutes following BDNF wash out (105.5± 4.2 % of CCh BL; n=5, 1 animal). A similar effect was observed with BDNF at 2.0 nM concentration (Figure 3.1C; 3.1H). The mean sIPSC charge decreased significantly to 82.5 ± 3.9% of CCh BL (n=13, 3 animals, ANOVA F(12,144)=269.8, p=0.0006). Shown in the right panel are data from individual cells (paired t-test, p=0.0074).
Figure 3.1: BDNF depresses carbachol (CCh)-induced inhibitory synaptic currents in CA1 pyramidal neurons via activation of postsynaptic TrkB receptors. (A) Representative traces of spontaneous inhibitory postsynaptic currents (sIPSCs) during CCh baseline (BL) and in the presence of BDNF (0.8 nM). Scale bar: 200 pA, 5 s. (B) Left, Group data time course showing the effect of 0.8 nM BDNF on sIPSC charge (n=21; 8 animals). Right, Data from individual cells during BL and minutes 8-10 of BDNF exposure. (C) Similar layout to (B), showing effect of 2.0 nM BDNF on sIPSC charge (n=13; 3 animals). (D) Similar layout to (B), showing lack of effect of 0.8 nM BDNF in the presence of the TrkB receptor antagonist ANA-12 (10 µM; n=7; 2 animals). (E) Similar layout to (B), showing lack of effect of 0.8 nM BDNF with intracellular loading of the TrkB inhibitor K252a (200 nM; n=10; 2 animals). (F) Example traces for two separate exposures to BDNF in the same cell. Scale bar: 200 pA, 5 s. (G) Representative time course showing the effect of repeated exposure to BDNF (0.8 nM) on a single cell. (H) Group data showing the effect of BDNF on CCh-induced inhibitory activity under various conditions. *p<0.05.
Next, we determined if the effect of BDNF on sIPSCs required TrkB receptor activation. As shown in the group time course and individual cells in Figure 3.1D and 3.1H, application of BDNF had no significant effect on sIPSC charge in the presence of the competitive TrkB receptor antagonist ANA-12 (10 µM; 110.3 ± 15.6% of CCh BL; n=7, 2 animals) suggesting that TrkB receptors are required to initiate the effect of BDNF. To address whether pre- or postsynaptic TrkB receptors are involved, we used the trk tyrosine kinase inhibitor K252a (200 nM) in the patch pipette to selectively block postsynaptic trk receptors (Madara and Levine, 2008). Intracellular application of K252a blocked the effects of BDNF on sIPSC charge as shown in Figure 3.1E and 3.1H (113.9 ± 15.5% of CCh BL; n=10, 2 animals). These results suggest that postsynaptic TrkB receptor activation is required for the effect of BDNF on CCh-induced inhibitory synaptic activity.

As evident from the data from individual experiments, the magnitude of the BDNF effect is highly variable across cells. We therefore examined whether the effect of repeated application of BDNF is consistent within a particular cell, which is important when employing manipulations predicted to alter the magnitude of the BDNF effect. For these experiments, BDNF was applied for two 5-minute exposures separated by 20 min. In each instance, the magnitude of the BDNF effect was normalized to the CCh BL prior to each application. Example traces and an individual time course for repeated BDNF exposure on the same cell is shown in Figure 3.1F and G. As can be seen in this example, and in the group data in Figure 3.1H, the effect of five-minute exposure to BDNF is
reversible and the magnitude of the effect is similar across trials (BDNF 1: 79.3 ± 3.3% of CCh BL; BDNF 2: 81.2 ± 3.7 % of CCh BL; n=9, 2 animals).

3.3.2. Effect of BDNF requires activation of CB1 cannabinoid receptors:
Figure 3.2: **BDNF suppression of inhibitory transmission requires activation of type I cannabinoid (CB1) receptors.** (A) Representative traces during BL and in the presence of CB1 receptor agonist WIN (5 µM). Scale bar: 200 pA, 2 s. (B) Group data time course showing the suppressive effect of WIN on sIPSC charge (n=9; 2 animals). (C) Representative traces of sIPSCs in the presence of the CB1 receptor antagonist SR141716A (SR; 10 µM) alone and in the presence of SR and BDNF. Scale bar: 200 pA, 2 s. (D) Group data time course showing lack of effect of 0.8 nM BDNF in the presence of SR (n=18; 5 animals). (E) Group data showing the effect of WIN and BDNF on CCh-induced inhibitory activity under various conditions. Also shown for comparison are the BDNF group data from Figure 1G (red). (F) Data from an individual cell showing the effect of 0.8 nM BDNF on sIPSC charge in the first trial and the lack of effect of BDNF in the presence of SR in the second trial. (n=8; 2 animals). Each data point is individual cells by color. *p<0.05.
Based on our previous results in somatosensory cortex (Lemtiri-Chlieh and Levine, 2010), we hypothesized that the effect of BDNF could be mediated by eCB release from postsynaptic CA1 neurons in hippocampus. We first examined whether the effect of BDNF was mimicked by application of a CB1 receptor agonist. As shown in example traces and group time course in Figure 3.2A and 3.2B, acute application of the cannabinoid agonist WIN55,212-2 (5 µM) caused a reduction of sIPSC charge to 70.4 ± 8.0% of CCh BL. This effect lasted for the duration of WIN exposure and did not recover to baseline values during the experiment (n=9; 2 animals, paired t-test, p=0.0108). This suppressive effect of WIN is similar to the effect of BDNF (Figure 3.2E). Next, we tested the effects of BDNF in the presence of the CB1 receptor antagonist SR141716A (SR; 10 µM). A representative trace of effect of BDNF in the presence of SR is shown in Figure 3.2C. The group time course shown in Figure 3.2D and 3.2E illustrates that BDNF failed to suppress inhibitory activity in the presence of SR (101.2 ± 5.7% of BL; n=18; 8 animals).

Next, we tested the effects of SR in the presence of BDNF using the within-cell experimental design. For the second application, BDNF was applied in the presence of SR. Consistent with the results obtained above, BDNF decreases sIPSC charge to 68.2 ± 4.8% of BL in 1st trial and SR prevented the suppressive effects of BDNF to 92.1 ± 3.3% of BL in 2nd trial as shown in Figure 3.2F (n=8; 2 animals, paired t-test, p=0.02). All cells showed a decreased effect of BDNF in the presence of SR. Together, these results suggest that CB1 receptors are required for the suppressive effects of BDNF on carbachol-induced inhibitory activity.
3.3.3 Effect of BDNF is mediated by postsynaptic endocannabinoid release:

Because the effect of BDNF requires activation of postsynaptic TrkB receptors, we hypothesized that endocannabinoids are released from postsynaptic CA1 neurons. It has previously been established that intracellular loading of the membrane-impermeable eCB transport inhibitor AM404 can be used to block eCB release (Lemtiri-Chlieh and Levine, 2010; Maglio et al., 2018; Ronesi et al., 2004; Yeh et al., 2017; Zhao et al., 2015). As shown in the representative traces in Fig. 3.3A, we found that including AM404 (5 µM) in the patch pipette disrupted the effect of BDNF. The group time course is shown in Figure 3.3B (109.2 ± 9.2% of CCh BL; n=8; 1 animal). These results suggest that endocannabinoids are released from the postsynaptic cell.

Figure 3.3: Effect of BDNF is mediated by endocannabinoids. (A) Example traces during BL and BDNF in the presence of intracellular transport inhibitor AM404 (5 µM). Scale bar: 200 pA, 2 s. (B) Group time course showing lack of effect of 0.8 nM BDNF with intracellular loading of AM404 (5 µM; n=8; 1 animal). (C) Example traces of BDNF on first exposure and BDNF in the presence of reuptake inhibitor AM404 (25 µM) on second exposure on the same cell. Scale bar: 200 pA, 5 s. (D) Group data showing the effect of 0.8 nM BDNF alone and in the presence of AM404 on sIPSC charge (25 µM; n=19; 5 animals). *p<0.05.
AM404 can also be used extracellularly to block reuptake of eCBs, and bath application of AM404 in vitro has been shown to enhance the magnitude of eCB-mediated plasticity, including depolarization-induced suppression of inhibition (DSI) and long term depression (LTD) (Beltramo et al., 1997; Du et al., 2013; Fortin et al., 2004; Sheinin et al., 2008; Trettel et al., 2004; Wilson and Nicoll, 2001). Because of the variability in the magnitude of the BDNF effect across cells, we examined the effect of bath-applied AM404 using the within-cell experimental design. Representative traces from a single experiment are shown in Figure 3.3C, showing a greatly enhanced effect of BDNF in the presence of AM404, while AM404 itself did not affect baseline activity. Data for each experiment is shown in Figure 3.3D. BDNF in the presence of AM404 significantly depressed sIPSC charge to 73.1 ± 4.9% of BL in 2nd trial compare to BDNF alone (98.5 ± 5.8% of BL; n=19; 5 animals, paired t-test, p=0.0003).) In this cohort of cells, there were several cells that did not show a decrease to the first BDNF application, however most of those cells did show a decrease to BDNF in the presence of AM404. These results suggest that BDNF triggers the release of eCBs that in turn mediate the effect on CCh-induced inhibitory activity.

3.3.4. Effect of BDNF is mediated by 2-AG:

We have previously shown that, at inhibitory synapses of somatosensory cortex, BDNF causes release of 2-AG that acts retrogradely on presynaptic terminals to suppress GABA release (Lemtiri-Chlieh and Levine, 2010). We therefore investigated whether the eCB released by BDNF at hippocampal inhibitory synapses is also 2-AG. To address this, we blocked the synthesis of 2–AG using the diacylglycerol (DAG) lipase inhibitor
tetrahydrolipstatin (THL; 25 µM). As shown in the sample traces (Figure 3.4A) and group time course, bath application of THL completely prevented the effects of BDNF (Figure 3.4B; 103 ± 5% of BL; n=13; 2 animals). We further explored the involvement of 2-AG using the monoacylglycerol lipase (MAGL) inhibitor JZL184 to increase 2-AG levels by preventing breakdown (Kiritoshi et al., 2016; Pan et al., 2009; Schlosburg et al., 2010; Wang et al., 2016). As shown in Fig. 3.4C and 3.4D, BDNF in the presence of JZL184 depressed sIPSC charge to 69.3 ± 4.4% of BL, a significantly enhanced effect compared to BDNF alone (91.8 ± 3.6% of BL) (n=18; 5 animals, paired t-test, p=0.0125). These results suggest the BDNF causes release of 2-AG to suppress spontaneous inhibitory activity.
The potential role of BDNF-induced anandamide release was explored by blocking anandamide degradation with the fatty acid amide hydrolase (FAAH) inhibitor URB597 (2 µM). Example traces of BDNF and BDNF in the presence of URB597 are shown in Figure 3.4E. BDNF caused reduction in charge to 88.2 ± 2.7% of BL in 1st trial and in the presence of URB597, BDNF similarly decreased the charge to 91.0 ± 2.9% of BL in 2nd trial as shown in Figure 3.4F (n=17; 3 animals). These results suggest that blocking anandamide degradation does not alter the effects of BDNF.
3.4. Discussion

BDNF and eCBs are potent neuromodulators that are highly expressed throughout the forebrain, including the hippocampus, and play critical roles in many behavioral and physiological processes. Interestingly, disruption of either BDNF or eCB signaling is associated with an overlapping set of neurologic and psychiatric diseases, including anxiety, depression, seizure disorders, and schizophrenia (Hill and Patel, 2013; Lupica et al., 2017; McNamara and Scharfman, 2012). As a result, these systems are of high interest for the development of novel therapeutics (Autry and Monteggia, 2012; Patel et al., 2017).

The present results indicate that the BDNF-induced suppression of inhibitory transmission at CA1 pyramidal cell synapses results from activation of postsynaptic TrkB receptors, which causes mobilization of 2-AG that acts retrogradely on presynaptic CB1 receptors. On average, BDNF (0.8 nM) caused a ~25% decrease in CCh-induced inhibitory activity. This is consistent with the results obtained in other studies on the effect of BDNF on GABAergic transmission (Frerking et al., 1998; Tanaka et al., 1997). The effect of BDNF was triggered by postsynaptic activation of TrkB receptors as loading the postsynaptic neuron with the trk tyrosine kinase inhibitor K252a was sufficient to prevent the BDNF effect. We should also note that K252a is membrane permeable drug and therefore could diffuse outside the cell to block presynaptic TrkB receptors. Previous work in our lab, however, found that intracellular K252a prevented the postsynaptic effect of BDNF at glutamatergic synapses but did not block the presynaptic effect on mEPSC frequency (Madara and Levine, 2008). The effect of BDNF in the present studies was
also blocked by a CB1 receptor antagonist and mimicked by a CB1 receptor agonist, suggesting that activation of CB1 receptors is required for BDNF suppression of GABAergic transmission. Further, we found that the effect of BDNF was disrupted by blocking 2-AG synthesis and enhanced by blocking 2-AG degradation.

Presynaptic effects of BDNF at inhibitory synapses have been identified in multiple brain regions. In an earlier study of CA1 pyramidal neurons in hippocampus, BDNF caused a decrease in the evoked inhibitory response and an increase in the paired-pulse ratio suggesting a presynaptic mechanism (Frerking et al., 1998). Chronic application of BDNF on solitary neurons cultured from rat visual cortex caused a reduction in mIPSC frequency but not in mIPSC amplitude also suggesting a presynaptic effect (Palizvan et al., 2004). We have previously shown that the effect of BDNF at layer 2/3 inhibitory synapses of somatosensory cortex is expressed presynaptically using three different approaches: an increase in the paired pulse ratio (PPR), an increase in mIPSC frequency with no change in mIPSC amplitude, and a decrease in the coefficient of variation (Lemtiri-Chlieh and Levine, 2010). Further, several studies have presented evidence of synaptic localization of CB1 and TrkB receptors – both are highly expressed in cortex and hippocampus (Cabelli et al., 1996; Egertova et al., 2003; Fryer et al., 1996; Marsicano and Lutz, 1999; Matsuda et al., 1993; Miller and Pitts, 2000; Tsou et al., 1998). The present study provides evidence that the effects of BDNF at CA1 synapses are mediated by eCBs, which may generalize to inhibitory synapses throughout the nervous system.
BDNF-induced mobilization of eCBs most likely involves 2-AG, as blocking DAG lipase to prevent 2-AG synthesis disrupted the effect of BDNF, and blocking MAG lipase to increase 2-AG levels enhanced the effect of BDNF. Although the specific signaling mechanisms for BDNF-induced 2-AG release have not been identified, it is likely that postsynaptic PLC\(\gamma\) signaling is involved. TrkB receptor activation and subsequent PLC\(\gamma\) signaling can generate diacylglycerol (DAG) which can then be converted to 2-AG by DAG lipase. This signaling pathway is similar to 2-AG mobilization in response to metabotropic glutamate receptor (mGluR) or muscarinic receptor activation. Our previous work at layer 2/3 inhibitory synapses in somatosensory cortex supports the conclusion that BDNF-induced eCB release requires PLC\(\gamma\) signaling and is independent of mGluR activation (Zhao and Levine, 2014). Similarly, in cerebellar Purkinje cells, BDNF modulates GABA receptor function via TrkB receptor-PLC\(\gamma\) signaling cascade (Cheng and Yeh, 2005). Further, elevated calcium is not necessary for mGluR-induced (Hashimotodani et al., 2005; Maejima et al., 2005; Ohno-Shosaku et al., 2005) or CCh-induced eCB release (Kano et al., 2009). However, BDNF requires elevated calcium through calcium intracellular stores or calcium influx to mediate its effects (Amaral and Pozzo-Miller, 2012). It has been shown at inhibitory synapses of somatosensory cortex, intracellular calcium is required for BDNF to trigger the release of eCBs (Lemtiri-Chlieh and Levine, 2010). It is thus possible that different signaling mechanisms could be involved in mGluR-induced, CCh-induced and BDNF-induced 2-AG release.
There is a growing body of evidence supporting interactions between BDNF and eCB signaling. In the current study, we have shown that BDNF induces eCB release to suppress GABAergic neurotransmission in the hippocampus, similar to previous results in somatosensory cortex. We have also shown that endogenous BDNF, released in response to theta-burst stimulation, triggers the release of eCBs that mediate inhibitory long-term depression (iLTD) at layer 2/3 inhibitory synapses of somatosensory cortex (Zhao et al., 2015). In addition, BDNF induces eCB release at layer 5 excitatory synapses of somatosensory cortex, where eCB signaling acts to limit the direct potentiation of glutamate release triggered by BDNF (Yeh et al., 2017). BDNF also appears to increase eCB production in ventral tegmental area dopamine neurons to facilitate DSI and inhibitory LTD (Zhong et al., 2015). BDNF can inhibit CB1 receptor function in striatum and this effect is mediated by cholesterol metabolism (De Chiara et al., 2010). On the other hand, BDNF has been shown to mediate CB1 receptor-dependent protection against excitotoxicity (Khaspekov et al., 2004; Marsicano et al., 2003). CB1 receptor signaling results in striatal neuroprotection from excitotoxicity via the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin complex 1 pathway, which, in turn, induces BDNF expression through BDNF gene promoter IV (Blazquez et al., 2015). In visual cortex, BDNF opposes eCB-mediated heterosynaptic LTD by inducing homosynaptic long term potentiation (Huang et al., 2008). In addition, eCBs released by action potential bursts of layer 5 pyramidal neurons of barrel cortex are crucial for the induction of BDNF-mediated LTP at excitatory synapses (Maglio et al., 2018). Understanding the mechanistic basis of these varied interactions will provide insights into the physiological roles of BDNF and eCBs in regulating synaptic transmission.
Chapter 4
Discussion and Conclusions

4.1 Summary and interpretation of findings

The studies conducted in this dissertation explored interactions between BDNF and endocannabinoids in the context of cortical excitatory synaptic transmission and hippocampal inhibitory synaptic transmission. In Chapter 2, the first set of experiments examined the interactions between BDNF and eCBs at cortical excitatory synapses. We chose layer 5 excitatory synapses as we have shown that these synapses receive cannabinoid-sensitive inputs, whereas inhibitory synapses in this layer are relatively cannabinoid-insensitive (Fortin and Levine, 2007). We found that BDNF by itself did not alter excitatory neurotransmission. However, in the presence of a CB1R

Figure 4.1 Simplified scheme summarizing the interactions between BDNF and eCBs at cortical excitatory synapses. BDNF modulates presynaptic release probability at cortical glutamatergic synapses via competing effects. BDNF activates presynaptic TrkB receptors to directly enhance release probability, while simultaneously triggering the postsynaptic release of endocannabinoids that act to decrease release via presynaptic CB1 receptors.
antagonist, BDNF produced an increase in excitatory neurotransmission. Similarly, blocking the release of eCBs also unmasked the effect of BDNF. Thus, we were able to detect the effect of BDNF by blocking eCB release or CB1Rs. Consistent with our previous results in layer 2/3 somatosensory cortex, BDNF triggers the release of eCBs from postsynaptic cells to act on presynaptic CB1 receptors. This effect opposes the direct presynaptic facilitatory effect of BDNF on glutamate release. Thus, the net effect on glutamate release we observe at these synapses depends on the influence of BDNF and eCBs. These findings are summarized in figure 4.1.

In the latter part of Chapter 2, we focused on examining the functional relevance of BDNF and eCBs in the context of long term synaptic plasticity – potentiation and depression (LTP/LTD). Using a chemical induction protocol of forskolin and rolipram, we identified a form of LTP in layer 5 excitatory synapses. This form of LTP does not require BDNF signaling. In addition, blocking CB1Rs did not alter the LTP indicating that eCBs are also not involved in this form of chemical LTP. We also used several electrical stimulation protocols to induce LTP, nevertheless, we do not have a paradigm to study LTP at layer 5 excitatory synapses. We have identified several forms of LTD- mGluR dependent LTD and low frequency stimulation (LFS) to study the role of BDNF and eCBs in these paradigms at layer 5 excitatory synapses.
The second data chapter (chapter 3) of the thesis focused on examining whether BDNF induces the release of eCBs at CA1 inhibitory synapses of hippocampus. Consistent with our previous results in the neocortex, we found that the acute application of BDNF caused a reduction in GABAergic transmission onto pyramidal neurons in CA1 hippocampus. These results indicate that BDNF indeed triggers the release of eCBs from the postsynaptic cell and subsequent activation of presynaptic CB1Rs. We also implicated the specific ligand released by BDNF as 2-AG since blocking the synthesis of 2-AG disrupted the effect of BDNF while blocking the degradative enzyme of 2-AG enhanced the effect of BDNF. Similar to somatosensory cortex, we

Figure 4.2 Schematic summarizing the findings of hippocampal excitatory synapses. BDNF suppress inhibitory activity at GABAergic synapses at CA1 hippocampus. BDNF activates postsynaptic TrkB receptors and triggers the release of 2-arachidonoylglycerol (2-AG) that acts on presynaptic CB1 receptors to suppress GABA release.

receptors. By blocking eCB release, we disrupted the effect of BDNF on spontaneous inhibitory activity. By blocking the reuptake of eCBs, we enhanced the effect of BDNF on spontaneous inhibitory activity. These results indicate that BDNF indeed triggers the release of eCBs from the postsynaptic cell and subsequent activation of presynaptic CB1Rs. We also implicated the specific ligand released by BDNF as 2-AG since blocking the synthesis of 2-AG disrupted the effect of BDNF while blocking the degradative enzyme of 2-AG enhanced the effect of BDNF. Similar to somatosensory cortex, we
identified a BDNF/TrkB signaling that can induce eCB release to act retrogradely to inhibit GABA release at CA1 inhibitory synapses of hippocampus. These results are summarized in figure 4.2.

4.2. Functional impact of BDNF-eCB interactions on cortical and hippocampal synaptic plasticity

The major cortices identified in mammals include sensory cortex, motor cortex and association cortices (like prefrontal cortex). Within sensory cortices, we have visual, auditory and somatosensory cortex. These cortices are processed by assemblies of neurons arranged into 6-layer cortical columns. Layer 5 is the main output layer of cortical processing. Layer 5 also receives inputs from thalamus in addition to cortex. Layer 2/3 pyramidal neurons have principally intracortical connections. Inhibitory interneurons are also crucial for information processing and signal integration. Understanding the local inhibitory and excitatory microcircuits is essential to understand how information is processed in a given cortical circuit (Méndez and Bacci, 2011).

Somatosensory cortex is a good model system because of its accessibility in the rodent brain and also because of the somatotopic representation of whiskers. Receptive field mapping studies have enabled us to identify the somatosensory topographic maps in this region. The somatosensory cortex is well defined and studied in terms of circuitry and function, laminar specificity, neural network and plasticity, sensorimotor integration, coding and tactile perception. The “critical period” (defined as the time period during which the presence of certain factors are crucial for the development) concept was first identified
in somatosensory cortex using whisker-barrel development from the ocular dominance plasticity studies. Cortical circuits are refined by sensory experience during critical periods of development as well as in adulthood. eCBs and BDNF have been found to be involved in experience-dependent refinement of cortical circuits (Huang et al., 2008; Jiang et al., 2010). For instance, GABAergic circuits are regulated by sensory experience via release of endogenous BDNF. Knocking out BDNF reduced GABA release from fast spiking interneurons and decreased perisomatic inhibition. Thus, activity dependent secretion of eCB and BDNF makes them suitable models to study the shaping of cortical microcircuits (Jiao et al., 2011).

Endocannabinoid-dependent forms of plasticity are good models to study local modulation of somatosensory cortex. CB1R is highly expressed in layer 2/3 and 5 of cortex. In layer 2/3, both inhibitory and excitatory inputs are sensitive to endocannabinoids. However, in layer 5 only excitatory inputs are sensitive to endocannabinoids and hence we used layer 5 as our model system to study synaptic plasticity. CB1Rs are predominantly expressed on presynaptic basket cells and calbindin-positive cells. By selectively suppressing perisomatic inhibitory transmission and increasing pyramidal neuron excitability, eCB signaling may enhance the output from layer 2/3 to layer 5. eCB signaling in layer 2/3 of the cortex mediates STDP by enhancing dendritic backpropagation. For instance, layer 5 PNs, pre and post-pairing produced LTP at proximal synapses while producing LTD at distal synapses. With boosting of dendritic backpropagation, LTD at distal inputs switched to LTP in response to the same induction protocols. The eCBs are involved in modulation of dendritic backpropagation which may
have consequences for regulating the magnitude and direction of STDP (Hsieh and Levine, 2013). Collectively, eCB signaling in different cortical layers exhibits diverse effects and each could have an impact on different aspects of cortical information processing.

Other sensory cortex like motor cortex or visual cortex also share same laminar patterns of CB1R distribution and we can hypothesize that layer 2/3 of the cortical layers may all behave the same in sensory cortices – BDNF modulating the release of eCBs to suppress GABA release. There might be differences across cortical layers (comparison of layer 2/3 to layer 5) in each of these sensory cortices. In general, in all these cortices, CB1R is more prominently observed in GABAergic terminals. Hence, we can extend our findings in somatosensory cortex to other layers/areas of cortex and how they are functionally relevant in the context of synaptic plasticity.

On the other hand, prefrontal cortex, an association cortex is involved in higher cognitive and executive functions like mood, working memory, planning and attention. TrkB receptors are highly expressed in cortical layers II-VI in prefrontal cortex. CB1Rs are also highly expressed in all layers of prefrontal cortex but predominantly localized in GABAergic interneurons. There is a striking similarity in the distribution pattern of CB1 and TrkB receptors in prefrontal cortex and somatosensory cortex. This makes it possible to address the questions that have be addressed in somatosensory cortex to this region. What layer of prefrontal cortex acts similar to layer 2/3 inhibitory synapses and layer 5
excitatory synapses of somatosensory cortex? Further, the discovery of a single nucleotide polymorphism (SNP) resulting in a Val to Met switch at codon 66 of the gene encoding for BDNF has facilitated the study of BDNF role in synaptic plasticity and cognitive function in motor cortex and hippocampus, but has not been addressed in prefrontal cortex. Cortical circuits are refined by sensory experience in somatosensory cortex. Similarly, in prefrontal cortex, the circuity are refined by sensory experience, insult/injury, paternal affection – yet another question that we can explore is how does cortical circuits are shaped in prefrontal cortex. CB1R interacts with the dopaminergic system in medial prefrontal cortex to regulate GABA mediated inhibition of pyramidal output neurons as described in the (Chiu et al., 2010) study. These evidences suggest that eCBs might interact with dopaminergic system/reward pathways in prefrontal cortex as they are involved in higher order cognitive functions. The few questions that can be addressed in the context of synaptic plasticity are: Is there any interaction between BDNF and eCBs in this region? What is their functional relevance?

The hippocampus is important for both spatial and non-spatial forms of declarative or explicit memory. Information flows through the cortico-hippocampal circuit. In the classical pathway, CA1 is the major output pathway of hippocampus. Most of the excitatory input comes from entorhinal cortex. The information is transmitted through the trisynaptic path. CA1 has local GABAergic interneurons; these target CA1 pyramidal neuron somas or axons to modulate pyramidal neuron activity in a domain specific manner. CB1R is highly expressed in hippocampal layers including CA1, CA3 and dentate gyrus. Our results in CA1 inhibitory synapses of hippocampus are very similar to
the results we obtained in layer 2/3 inhibitory synapses of somatosensory cortex. In both cases, BDNF causes the release of eCBs to modulate GABAergic neurotransmission. We can extend these studies to excitatory synapses of CA1 or CA3 to identify if the effects of BDNF are similar to layer 5 excitatory synapses of somatosensory cortex (Basu and Siegelbaum, 2015; Basu et al., 2013). Understanding how each hippocampal layer functions and its relevance in synaptic plasticity is crucial to understanding how information is processed in different hippocampal circuits. Similar to somatosensory cortex, endocannabinoid-mediated plasticity is very well studied in hippocampus. DSI was first observed in hippocampus. Tetanus stimulation causes a long lasting suppression of inhibition termed as LTD and eCBs are potent mediators of LTD in this region (reviewed in (Castillo et al., 2012; Heifets and Castillo, 2009)). BDNF also mediates several forms of LTP in hippocampus (reviewed in (Edelmann et al., 2014)). Endogenous BDNF is required for the presynaptic component of LTP but not for the postsynaptic component at CA1-CA3 synapses. Further, BDNF release in hippocampus is also experience dependent. Short-term depression does not require BDNF but long-term depression requires BDNF (Aarse et al., 2016).

To sum up, activity-dependent secretion of BDNF and eCBs makes them a viable model to study the activity-dependent cortical information flow or to study the corticohippocampal circuit. In summary, studying the input-specific and region-specific effects of eCB and BDNF signaling as well as their interactions will provide insights in understanding the regulation of corticohippocampal or cortical circuits and the same questions can be addressed in other cortical areas as well.
4.3 BDNF and eCB signaling at cortical excitatory synapses

We focused on somatosensory cortex, where CB1 and TrkB receptors are highly expressed in layers 2/3 and 5. Further, in layer 2/3, depolarization-induced eCB release targets both inhibitory and excitatory neurotransmission. The net effect is an increase in pyramidal neuron excitability due to disinhibition. However, in layer 5, depolarization-induced eCB release results in DSE at excitatory synapses and the majority of inhibitory inputs are cannabinoid-insensitive. Thus, the net effect of a brief depolarization in layer 5 is a decrease in pyramidal neuronal excitability (Fortin et al., 2004). In contrast, we and others have shown that BDNF can modulate excitatory neurotransmission presynaptically (Crozier et al., 1999; Levine et al., 1998; Levine et al., 1995). Thus, layer 5 is an ideal place to address interactions at excitatory synapses. We used this as our model system to address the question whether BDNF induces eCB release.

One of the future questions that needs to be addressed is how BDNF and eCBs interact to modulate action potential-dependent glutamate release. We have shown that at inhibitory synapses in layer 2/3 of somatosensory cortex, BDNF causes a reduction in evoked IPSCs by triggering eCB release (Lemtiri-Chlieh and Levine, 2010). However, there is clearly lack of evidence for the effects of BDNF on evoked glutamate release. In addition, evoked and spontaneous glutamate release derive from distinct vesicle populations and use distinct mechanisms (Atasoy et al., 2008; Melom et al., 2013; Peled et al., 2014; Walter et al., 2014). Hence, it is critical to identify if BDNF and eCBs interact differently with regard to evoked glutamate release.
Similar to the effect of exogenous BDNF on glutamate release, we determined the role of endogenous BDNF in mediating synaptic plasticity, LTP. The chemical induction protocols did not require BDNF or eCB signaling. It is possible that the net effect of BDNF on LTP is so negligible that we do not see it. The chemical induction protocol regulates cAMP levels to activate downstream signaling pathways to potentiate at these synapses (Otmakhov et al., 2004). However, we know that eCBs can inhibit adenylyl cyclase thereby reducing cAMP levels (Castillo et al., 2012; Chevaleyre et al., 2007). On the other hand, BDNF can increase cAMP levels to facilitate LTP (Lu et al., 2008). There are three key players in regulating cAMP levels in this form of plasticity (cyclic AMP synthesis and breakdown, BDNF and eCBs) and this complexity meant that it was not possible to determine the effect. Another factor to add to this equation is the direct presynaptic effects of BDNF against BDNF triggered eCB release at postsynaptic sites. Depending on the net effect of glutamate release, the direction of plasticity will change. The outcome of this interaction might vary and be complicated, yet, it will be interesting to examine the roles of each component in this plasticity. One possibility is that subthreshold concentrations of BDNF in combination with shorter duration of forskolin induction will reveal a role for BDNF.

Assuming the eCBs are potently inhibiting glutamate release, blocking CB1 receptors will unmask the effects of BDNF. Adding TrkB receptor antagonists to the existing equation will help us in determining the role of BDNF in LTP. Another possibility that we will consider is BDNF might activate TrkB receptors which in turn will activate MAPK
signaling pathway to induce LTP (Patterson et al., 2001). This type of BDNF-induced LTP will be an eCB-independent form of LTP.

Electrical stimulation protocols at layer 5 excitatory synapses, including HFS, TBS, and pairing protocols, did not elicit LTP in our studies. This could be due to several possibilities: (a) inability to recapitulate the microcircuit and (b) the stimulation patterns are not sufficient to induce LTP. To rule this out, we will repeat the stimulation patterns in CA1 or CA3 hippocampus to elucidate LTP. If we obtain LTP in hippocampus, it suggests that there is region-specificity and layer-specificity to LTP in cortex. Alternatively, if the inhibition of excitatory inputs is high then we will not be able to induce LTP as the endogenous eCBs levels are high and the net effect on glutamate release favors the eCB influence. If this is true, we should be able to obtain DSE at these synapses. However, with stimulation protocols we used at these synapses, we failed to induce DSE indicating that the eCBs levels are not sufficient to block LTP.

Similarly, we used different LTD-inducing paradigms to test the involvement of BDNF and eCBs. Bath application of DHPG induces mGluR-dependent LTD which also requires eCB signaling. We will test the involvement of BDNF in this form of LTD. Low frequency stimulation induces eCB-independent LTD. Having eCB-dependent/independent and BDNF-dependent/independent forms of plasticity will help uncover the mechanisms involved in each form of LTD/LTP. We can compare and contrast these forms of plasticity as these interactions might have significant roles in shaping synaptic plasticity thereby contributing to the brain circuits’ activity.
4.4 Future directions for studying BDNF-eCB interactions at cortical excitatory synapses

Over the past decade, there has been growing evidence for BDNF-eCB interactions, yet the mechanisms involved are largely unknown. The studies presented in this dissertation characterized the effects of BDNF and eCBs at layer 5 cortical excitatory synapses as well as CA1 hippocampal inhibitory synapses. Many questions still remain to be answered. For instance, an immediate question is to determine whether BDNF and eCBs interact to modulate glutamate release in response to stimulation (high frequency, low frequency or theta burst stimulation paradigms). Next, we would like to determine the role of endogenous BDNF in excitatory neurotransmission. Although we have some preliminary evidence suggesting that BDNF and eCBs are not involved in a chemically-induced form of LTP, we would like to refine the answers to these questions. An immediate question we will address is whether there is an enhancement of forskolin-induced potentiation at three time points (5 minutes, 10 minutes and 15 minutes of treatment) at layer 5 excitatory synapses. We will use subthreshold concentrations of BDNF in combination with forskolin induction to induce LTP. If the LTP is dependent on BDNF, we will identify the downstream signaling pathway from BDNF that causes LTP induction.

Similar to the effect of exogenous BDNF we observed at inhibitory synapses of cortex and hippocampus, we will extend the current findings to CA1 excitatory synapses of hippocampus as well. An immediate question we will address at CA1 excitatory synapses is whether BDNF triggers the release of eCBs as we have already shown that
BDNF can induce eCB release at layer 5 excitatory synapses. Next, we will perform a set of experiments with chemical induction paradigms to elucidate LTP and to determine the role of BDNF in this form of plasticity. We also know that we can elicit LTP using electrical stimulation protocols in hippocampus. Hence, we will identify LTP paradigms that are BDNF dependent and independent to probe for the role of eCBs. Taken together, studying the layer- and area-specific effects of BDNF and eCB signaling may provide insights in understanding activity-dependent synaptic plasticity in physiological conditions and in disease states.

4.5 BDNF and eCB signaling at hippocampal inhibitory synapses

4.5.1 Experimental controls

Since muscarinic receptor activation has been shown to selectively depolarize interneurons, we applied carbachol (CCh) to increase the firing of interneurons. In particular, CCh increases action potential firing in a subset of interneurons that generate large amplitude synaptic currents with fast rise time (Lemtiri-Chlieh and Levine, 2010). BDNF (0.8 nM) application in the presence of CCh causes a significant decrease in mean sIPSC frequency and in mean sIPSC amplitude. The cumulative distribution of sIPSC amplitude suggests that the BDNF caused a decrease in both miniature events and in action-potential dependent events. The shift in amplitude causes a corresponding decrease in sIPSC frequency. As sIPSC amplitude and frequency are dependent on each other, we use charge as a parameter to determine the effects of drugs tested in the study. Charge is defined as the quantal area per unit time and accounts for changes in both amplitude and frequency. Further, note that one subset of interneurons that can be
activated by CCh is CCK-positive basket cells (and presumably expressing CB1) and it has been shown that CCh can also stimulate the release of eCBs. However, we have shown that CCh is not required for BDNF-evoked eCB release since we have obtained similar results for BDNF effects on evoked responses in the absence of CCh (Lemtiri-Chlieh and Levine, 2010).

Prior to these studies, our lab established the selective involvement of CB1Rs in the effect of BDNF with a series of control experiments. We examined the potential role of TRPV1 receptors as SR141716A can antagonize TRPV1 receptors as well. However, the TRPV1 receptor antagonist capsazepine had no effect on BDNF-induced suppression of eIPSC amplitude confirming the specific involvement of CB1 receptors. We have also confirmed that intracellular calcium is required by BDNF to exert its effect on GABA release (Lemtiri-Chlieh and Levine, 2010). We used K252a, a Trk tyrosine kinase antagonist as well as ANA-12, a selective TrkB receptor antagonist, to confirm that the effects of BDNF on spontaneous inhibitory activity are TrkB-mediated (Zhao et al., 2015).

4.5.2. BDNF-induced eCB release at CA1 inhibitory synapses

BDNF can act both via pre- and postsynaptic mechanisms. Frerking and colleagues established in 1998 that BDNF reduces inhibitory synaptic transmission that was associated with presynaptic expression. However, they speculated the mechanism of BDNF in inhibiting neurotransmission requires a retrograde messenger (Frerking et al., 1998). A decade later, we have identified a mechanism by which BDNF suppresses
GABA release by adding eCB signaling into the equation. The current study establishes that BDNF inhibits synaptic transmission at GABAergic synapses by inducing eCB release. With this study, we have extended our results from somatosensory cortex to hippocampal inhibitory synapses. We have, now, shown at two different brain regions, BDNF induces eCB release to suppress GABA release. Further studies can help us determine the signaling pathway required for BDNF-induced eCB release.

GABAergic interneurons have a very different developmental pattern in terms of brain region, transcriptional regulators and migration patterns from excitatory neurons, and mature GABAergic synapses show high levels of spontaneous release. However, we do not know if such spontaneous release is seen during developmental stages and the role it might play (Andreae and Burrone, 2018). In addition, BDNF is shown to alter interneuron phenotype during development (Jones et al., 1994). We also do not have a clear understanding of how BDNF modulates GABA release in response to stimulation. These questions can be addressed in the future.

### 4.6 Future directions of BDNF-eCB interactions at hippocampal inhibitory synapses

The current study confirms and extends our findings in layer 2/3 somatosensory cortex to hippocampus. Future experiments will focus on understanding the role of endogenous BDNF and eCBs in synaptic plasticity paradigms. One of the fundamental questions we will address is whether BDNF regulates eCB-mediated DSI. In the first set
of experiments, we will explore the role of endogenous BDNF in regulating eCB-mediated DSI by using the BDNF scavenger TrkB-IgG and TrkB antagonists. In the second set of experiments, we will examine the effect of exogenous BDNF on eCB-mediated DSI. We will compare the effects of different depolarization protocols with or without added (exogenous) BDNF. DSI will be induced with stimulation protocols (1) in conjunction with 0.8 nM BDNF or (2) using a theta burst stimulation paradigm (TBS) that has been shown to induce endogenous release of BDNF (Zhao et al., 2015). This set of studies will examine the effect of exogenous BDNF on eCB-mediated DSI.

Next, we want to address the role of endogenous BDNF in long term depression as we have identified an eCB-mediated iLTD at layer 2/3 inhibitory synapses. Finally, there is wealth of information available about the regulation of BDNF in LTP. We will use these BDNF-dependent LTP paradigms to identify the role of endocannabinoids. As mentioned in the introduction, BDNF can be released from pre- and postsynaptic sites and act both pre- and postsynaptically. Understanding the role of eCBs in these forms of LTP will help us in uncovering insights regarding the sites of BDNF secretion and regulation of BDNF in LTP. Taken together, these studies will help us in understanding region-specific differences in mediating synaptic plasticity at inhibitory synapses.

4.7 Conclusions

In summary, our understanding of eCB and BDNF function has grown tremendously in the last two decades, although BDNF-eCB interactions is still a growing field. The current work adds to the accumulating evidence of BDNF-eCB interactions in the brain.
We have examined both inhibitory and excitatory synapses to understand the physiological relevance of these interactions. We used cortical layer 5 pyramidal neurons as a model to study excitatory synapses and CA1 pyramidal neurons of hippocampus as a model to study inhibitory synapses. In future, we will extend these findings to CA3 and DG of hippocampus. Understanding short- and long- term synaptic plasticity in these region- and layer- specific patterns will play a significant role in shaping synaptic plasticity and neural circuits. With the studies presented in this dissertation, I hope to shed some light towards future studies of synaptic plasticity mediated by either BDNF or eCBs.
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