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BRAIN-DERIVED NEUROTROPHIC FACTOR AND
ENDOCANNABINOID FUNCTIONS IN GABAERGIC
INTERNEURON DEVELOPMENT

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voor Annemieke

papa, mama en Monique
ABSTRACT

Formation of the cerebral cortex is controlled by tightly regulated processes: the proliferation and survival decisions of neural progenitors, radial and tangential migration of neuronal precursors, axon guidance and dendritic growth underpinning the formation of synapses. Patterning of the neocortex has been extensively studied and significant progress has been made in understanding the principles of cortical neuronal circuit formation. However, the cellular and molecular factors that coordinate the establishment of specialized neuronal subnetworks remain unclear.

In this thesis the roles of brain-derived neurotrophic factor (BDNF) and endocannabinoids, critical regulators of synaptic plasticity, were studied for their functions in corticogenesis. We showed that BDNF, along with membrane depolarization, is critical for fast-spiking interneurons (FS cells) to establish functional inhibitory microcircuits. In enriched FS cell cultures, BDNF promoted interneuron differentiation by increasing the somatic diameter, neurite outgrowth and branching, and the frequency of action potential firing. BDNF treatment led to a significant up-regulation of synaptophysin and vesicular GABA transporter expressions reflecting the accelerated maturation of functional synapses. Next, we addressed the role of BDNF and its receptor TrkB in the differentiation of GABAergic interneurons in the main olfactory bulb in vivo. We used mice lacking BDNF, mice carrying neurotrophin-3 (NT3) in the place of BDNF, and TrkB signaling mutant mice with a receptor that exclusively activates phospholipase Cγ (PLCγ). The absence of BDNF resulted in a compressed olfactory bulb. A significant loss of parvalbumin (PV) immunoreactive GABAergic interneurons in the external plexiform layer was dependent on the recruitment of the adaptor proteins Shc/Frs2 to the TrkB receptor. In contrast, PLCγ signaling was sufficient for dendrite growth.

Since in utero exposure to Δ⁹-tetrahydrocannabinol, the active component from marijuana, induces cognitive deficits, we asked whether endocannabinoids can alter the BDNF-dependent maturation of cortical GABAergic interneurons. We identified endocannabinoids as chemoattractants for migrating GABAergic interneurons with their effect mediated by Src-dependent TrkB receptor transactivation. Simultaneously, endocannabinoids suppressed BDNF-stimulated neurite outgrowth. Based on the findings that interneurons arbors were affected by endocannabinoids, we studied the mechanisms involved in endocannabinoid-regulated axonal growth and guidance. We identified a selective enrichment of CB₁Rs in isolated growth cone particles. Agonist stimulation induced CB₁R trafficking in motile growth cones and activated the Erk1/2 pathway. Endocannabinoids were identified as repellant cues for cultured rodent GABAergic interneurons through the activation of the small GTPase RhoA. Similarly, endocannabinoids diminished galvanotropism of Xenopus laevis spinal neurons. These results are consistent with the increased density of inhibitory afferents in the neocortex in mice lacking CB₁Rs in forebrain GABAergic interneurons.

Overall, this thesis provides compelling data showing that the antagonism of BDNF and endocannabinoid signaling during the late embryonic and perinatal periods of cortical development is essential for the establishment of the cortical microarchitecture.


* denotes equal contribution

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<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>2-AG</td>
<td>2-Arachidonoylglycerol</td>
</tr>
<tr>
<td>AEA</td>
<td>Anandamide</td>
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<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
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<tr>
<td>cAMP</td>
<td>cyclic Adenosine Monophosphate</td>
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<tr>
<td>CB</td>
<td>Calbindin D28K</td>
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<tr>
<td>CB1R</td>
<td>Type 1 Cannabinoid receptor</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin-8</td>
</tr>
<tr>
<td>CGE</td>
<td>Caudal ganglionic eminence</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CR</td>
<td>Calretinin</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DIV</td>
<td>Days <em>in vitro</em></td>
</tr>
<tr>
<td>DSI</td>
<td>Depolarization-induced suppression of inhibition</td>
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<tr>
<td>EF</td>
<td>Electrical field</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EPL</td>
<td>External plexiform layer</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FAAH</td>
<td>Fatty acid amide hydrolase</td>
</tr>
<tr>
<td>Fgf8</td>
<td>Fibroblast growth Factor 8</td>
</tr>
<tr>
<td>FS Cell</td>
<td>Fast-spiking interneuron</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GAD65/67</td>
<td>Glutamic acid decarboxylase 65/67</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating Protein</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial cell-derived neurotrophic factor</td>
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<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GNEF</td>
<td>Guanine nucleotide exchange factor</td>
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<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
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<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
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<tr>
<td>IMCS</td>
<td>Immuno-magnetic cell sorting</td>
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<tr>
<td>IP3</td>
<td>Inositol 1,4,5-trisphosphate</td>
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<tr>
<td>LGE</td>
<td>Lateral ganglionic eminence</td>
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<tr>
<td>LPA</td>
<td>Lysophosphatic acid</td>
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<tr>
<td>LTP</td>
<td>Long term potentiation</td>
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<tr>
<td>MGE</td>
<td>Medial ganglionic eminence</td>
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<tr>
<td>MGL</td>
<td>Monoglyceride lipase</td>
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<tr>
<td>NAPE-PLD</td>
<td><em>N</em>-acyl-phosphatidyl-ethanolamine-selective Phospholipase D</td>
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<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
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<tr>
<td>NT-3/4</td>
<td>Neurotrophin-3/4</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-OH Kinase</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>----------------------------------------------------</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>POa</td>
<td>Preoptic area</td>
</tr>
<tr>
<td>PV</td>
<td>Parvalbumin</td>
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<tr>
<td>RGC</td>
<td>Radial glia</td>
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<tr>
<td>RMS</td>
<td>Rostral migratory stream</td>
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<tr>
<td>Shh</td>
<td>Sonic hedgehog</td>
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<tr>
<td>SST</td>
<td>Somatostatin</td>
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<tr>
<td>SVZ</td>
<td>Subventricular zone</td>
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<tr>
<td>TCA</td>
<td>Thalamocortical axon</td>
</tr>
<tr>
<td>THC</td>
<td>$\Delta^9$-Tetrahydrocannabinol</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>Trk</td>
<td>Tropomyosin-related kinase</td>
</tr>
<tr>
<td>VGAT</td>
<td>Vesicular GABA transporter</td>
</tr>
<tr>
<td>VGLUT</td>
<td>Vesicular glutamate transporter</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal polypeptide</td>
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1 CORTICAL DEVELOPMENT

It is generally believed that the cortex is the neuronal structure that allows us to speak, makes us think and stores our memories, processes that raise humans above any living organism on earth. The cortex comprises the neocortex, the hippocampus and the outer layers of the olfactory bulb. Generally, the neocortex can be described as a layered structure, subdivided into different subregions, each subserving particular information processing tasks. The neurons populating the neocortex are excitatory (glutamatergic) projection neurons and local-circuit inhibitory (GABAergic) interneurons. The vast majority of cortical neurons are the projecting pyramidal cells located in layers 2 to 6, under the control of local interneurons. The interconnectivity of the cortical neurons is organized into multiple, small repeating circuits forming the cortical microcircuitry (Silberberg et al., 2002). These neuronal subnetworks process inputs ariving from their connected neighbors and from various cortical and sub-cortical afferent. The defined cortical organization raises the intriguing question how its structural specification occurs during embryonic development? Which factors participate in the formation of the precise synaptic connections that define cortical circuitries? These are the basic questions addressed in this thesis, through studying the functions and signaling mechanisms of brain-derived neurotrophic factor (BDNF) and the endocannabinoids, two major factors in cortical synaptic communication in the adult, and their role in neuronal network formation.

1.1 CORTICAL LAYER SPECIFICATION

During early CNS development, the cortex develops from the ventricular zone located in the dorsocaudal part of the telencephalic vesicles. Specification of the various cortical areas is directed by signaling centers, located along the edges and midline of the neural plate, that secrete molecules important for positional information (Crossley et al., 2001). In the ventral telencephalon, expression of sonic hedgehog (Shh) is important for initial patterning and morphogenesis events (Ohkubo et al., 2002). Structural development of the hippocampus and neocortex is further initiated by the release of the bone morphogenic protein (Bmp) and Wnt families of ligands from the dorsal midline (Furuta et al., 1997; Grove et al., 1998). At these early stages, at around E10.5 in the mouse embryo, the cerebral cortex is merely a thin layer, densely packed with neuronal cells and is termed the cortical plate. Subsequently, the inward migration of new cortical neurons separates the cortical plate into two layers; a superficial layer called the marginal zone (which later develops into cortical layer I) and a transient underlying layer called the cortical subplate (see fig. 1).

New-born neurons continue to migrate into the cortex from two important proliferative areas termed the ventricular zone (VZ), containing proliferating cells at the ventricular surface, and the subventricular zone (SVZ) that contains neuronal progenitor cells (Letinic et al., 2002). Cortical progenitors migrate according to an inside-out sequence: meaning that the most recently generated neurons migrate towards the pial surface through previously established layers of cells (Uylings, 2000). Accordingly, neurons in layer II are generated later than layer VI and layer V neurons. Most studies on
corticogenesis have focused on the neocortex, however similar ontogenic processes contribute to the development of the hippocampus (Soriano et al., 1994). Early hippocampal progenitors are located outside the cortical plate in the marginal zone and the subplate. Similar to the neocortex, hippocampal neurons follow an inside-out pattern to form a cortical plate (Super et al., 1998). An exception to this rule is the formation of the dentate gyrus that creates the granular cell layer according to an outside-in pattern (Bayer, 1980). During development, the cerebral wall is dominated by GFAP positive radial glial cells (RGCs) (Zecevic, 2004). RGCs are anchored in the ventricular wall, with their basal feet ending on the pial surface. The shafts of RGCs provide a physical scaffold that allows newborn neurons to migrate the long distance from the ventricular zones towards the pial surface (fig. 1). This is most remarkable in the enlarged primate cortex, where RGCs have evolved to provide a more permanent scaffolding, likely supporting neuronal migration along larger distances (Rakic, 2006). Besides providing a scaffold, recent data identified RGCs as precursors of neuronal progenitors as well as directly postmitotic neurons (Fishell and Kriegstein, 2003; Gaiano and Fishell, 2002; Noctor et al., 2001). RGCs undergo asymmetric division resulting in self-renewal of the radial cell and the generation of new neurons. RGCs are now being recognized as the major source of neuronal progenitors in the developing cortex (Fishell and Kriegstein, 2003). Previously it was thought that all neocortical neurons were derived from the neocortical (sub)ventricular zone progenitors, however this view drastically changed at the discovery that GABAergic interneurons originated in the subpallium (Anderson et al., 1997). It is now generally believed that pyramidal neurons migrate radially from the cortical subventricular zone of the cerebral wall, while GABAergic interneurons are generated in the ganglionic eminences and undergo long-distance tangential migration to populate the developing neocortex.

1.1.1 Cortical interneuron development

Immunolabeling for GABA on developing embryo’s in combination with fluorescence dye labeling of cultured telencephali identified streams of migrating GABAergic cells undergoing long distance tangential migration to reach final positioning in specific cortical layers (de Carlos et al., 1996; DeDiego et al., 1994). Putative GABAergic interneurons originate in the subpallial telencephalon from around E12 onwards in the mouse and follow precise migration pathways towards the neocortex (Anderson et al., 2002). At the onset of migration all cells avoid the developing striatum and their routes diverge into a population entering the cortex superficially, with another subset migrating from below the striatal mantle (Ang et al., 2003; Marin et al., 2001). Finally, the cells that penetrate the cortex deep from the striatum traverse the marginal zone and the subplate and inhabit the superficial cortical layers, whereas superficially migrating neurons travel close to the SVZ. Tangentially-migrating cortical cells have multiple origins within the subpallium, however comparisons of the migratory behaviour of the lateral and caudal ganglionic eminence (L/CGE) vs. the medial ganglionic eminence (MGE) indicates that cells originating from the MGE have the greatest propensity to migrate into the cortex (Anderson et al., 2001; Wichterle et al., 1999). This is underscored by the fact that mice lacking the homeobox transcription factor Nkx2.1 fail to develop an MGE and exhibit a reduced migration of cortical neurons. Nkx2.1 mutants have a ∼50% reduction in cortical GABA-positive cell numbers, as compared to wild-type littermates (Sussel et al., 1999). All progenitor regions in the ganglionic
Fig. 1. The generation of neuronal diversity during cortical development

(a) Schematic drawing of telencephalic development delineating transient embryonic neural progenitor zones and the acquiring of layer patterning and subcortical innervation in the neocortex. Note the progressive reduction of progenitor zones and the establishment of distinct brain regions with specific connectivity patterns. Abbreviations: Cn, neocortical neuroepithelium; CP, cortical plate; Ctx, neocortex; f, fornix; HC, hippocampus; hn, hippocampal neuroepithelium; Iv, lateral ventricle; Pn, pallidal neuroepithelium; Sn, striatal neuroepithelium; svz, subventricular zone; tca, thalamocortical axons; Th, thalamus; vz, ventricular zone.

(b) The concept of layer specification during corticogenesis (E11 - P0). The diagram illustrates the relationship between radial glial cells and radially migrating neurons from the ventricular zone (VZ), and tangentially migrating interneurons from the ganglionic eminences. The subplate (SP) confers to a transient zone that is populated by migrating neurons and the initial contingent of cortical afferents. Abbreviations: CP, cortical plate; IZ, intermediate zone; L, neocortical layer; MZ, marginal zone; SVZ, subventricular zone.

eminences specifically express Dlx1, Dlx2 and Mash1 transcription factors that contribute to the timing of differentiation into a pro-GABAergic phenotype (Anderson et al., 1997; Fode et al., 2000). Mice with a mutation in both Dlx1 and Dlx2 (Dlx1/Dlx2) cease tangentially interneuron migration and thus have reduced numbers of cortical interneurons (Anderson et al., 1997). Alternatively, Mash1 appears to be critical for the ventralization of neuronal progenitors and the acquisition of a GABAergic phenotype (Fode et al., 2000). Tangential migration of putative GABAergic interneurons is evolutionarily preserved and has been observed in mice, rats, ferrets and humans (Anderson et al., 1997; Anderson et al., 2002; de Carlos et al., 1996; Wichterle et al., 1999) However, in the developing human brain terminal mitosis appears to occurs in the SVZ (Letinic et al., 2002).
Over the past few years several diffusible factors, involved in the coordinated migration of GABAergic interneurons have been identified. The ventral to dorsal direction of interneuron migration is regulated by repulsive guidance cues derived from the preoptic area (POa), motogenic factors present in the ganglionic eminences and permissive and attractive cues released by the cortex. Repellent factors expressed in the POa have not been identified yet, however the release of Slit1 and Slit2 from the POa has been proposed to repress dorsal to ventral cell migration patterns (Zhu et al., 1999). However, the chemorepulsive activity is still present in mice with targeted mutations in both Slit1 and Slit2, suggesting that Slit does not play a crucial role in the repulsive properties of the POa (Marin et al., 2003). Motogenic substances promote migration rates and could increase the total number of contingents of migrating interneurons. A few well characterized motogenic factors include hepatocyte growth factor (HGF) and the neurotrophins BDNF and NT-4 (Polleux et al., 2002; Powell et al., 2001). In slice cultures, HGF increases the number of cells migrating away from the subpallium, whereas antibodies against HGF inhibit cell movement and reduced the number of calbindin D28-expressing interneurons in the neocortex (Powell et al., 2001). Similar effects have been shown for PI3K-dependent BDNF and NT-4 signaling (Polleux et al., 2002). Furthermore, point mutations in the PLCγ and Shc/Frs2 docking sites of the TrkB receptor have further highlighted a crucial role for TrkB in controlling cortical stratification through the timing of neuronal migration (Medina et al., 2004). Most recently, the expression of glial cell line-derived neurotrophic factor (GDNF) and its receptor GFRα1 was identified in the MGE and the neocortex along the pathway of interneuron migration. GDNF acts as a potent attractant through the activation of GFRα1 and accordingly a loss of GABAergic intracortical cells was observed in both GDNF and GFRα1 knockout mice (Pozas and Ibanez, 2005).

Even though cortical interneurons represent only a small fraction (~20%) of the total neuronal population in the neocortex, they are critical modulatory components of cortical neuronal microcircuits. Unlike the rather homogeneous population of excitatory pyramidal cells, cortical interneurons represent a highly heterogeneous group based on their morphological, physiological, molecular and synaptic characteristics (for further details see section 2.5.1) (Kawaguchi and Kondo, 2002; Markram et al., 2004). Recent evidence suggests that the diversity of cortical interneurons is generated during early stages of development. Cells migrating from the proliferative zones in the GE constitute a heterogeneous population regarding their neurochemical properties and are different in their route toward the cortex, indicating that early migrating neurons react differently to guidance cues (Ang et al., 2003; Tanaka et al., 2003). One other important factor for the diversification of interneurons subtypes is the spatial segregation of GABAergic precursors in the GE. The distinguished three domains of the subpallium (LGE, MGE and CGE) that contribute cortical interneurons could provide anatomical explanation for interneuron diversification. Indeed, in vitro experiments indicate that parvalbumin (PV)-containing FS cells and somatostatin/calbindin D28k (SST) burst-spiking interneurons are predominantly derived from the MGE, whereas regular-spiking calretinin/vasoactive intestinal polypeptide (CR/VIP)-positive neurons are generated in the CGE (Xu et al., 2004). More recent experiments using GAD65-GFP transgenic mice to study subpopulations of cortical interneurons, confirmed that calretinin-expressing interneurons derive from the CGE, by as a tool (Lopez-Bendito et al., 2004).
Besides their anatomical localization, temporal differences also shape interneuron diversity. The earliest wave of migrating cells leaving the GEs migrates towards the hippocampus, whereas later-departing interneurons inhabit the cortical mantle. The temporal dynamics of cell migration could therefore be important for subtype specification and for defining distinct sets of interneurons destined for different cortical areas. Furthermore, the birth-date of progenitors also correlates with the progressive inside-out lamination of the cortical plate (Anderson et al., 2002; Valcanis and Tan, 2003), suggesting that the timing of neuronal birth and migration determines their layer specificity. For example, explants derived from the CGE at E13.5 give rise to distinct classes of regular-spiking interneurons, while later neurons generated in the CGE at E15.5 develop into different subclasses and migrate to the more superficial layers of the cortex (Butt et al., 2005). Overall, the above findings show that the complexity of GABAergic interneuron phenotypes appears to be shaped by tightly-controlled and temporal patterning rules.

1.1.2 Heterogeneity of cortical interneurons

Cortical interneurons can be divided into multiple subclasses according to their neurochemical heterogeneity, intrinsic discharge behaviors, location of their synapses on principal cells, and connectivity patterns with other interneurons. Neurochemically, the major subset of perisomatic inhibitory cells contains the Ca$^{2+}$-binding protein PV (Kawaguchi and Kondo, 2002). Although PV is generally used as a classification marker for these interneurons, they display additional cytochemical hallmarks that define their discharge properties (Wang et al., 2002). FS cells selectively express voltage-dependent potassium channel (Kv) subunits, e.g. Kv3.1b, which facilitates the fast re-polarization of their membranes (Weiser et al., 1995). Many PV-containing cells are surrounded by poly-anionic chondroitin sulphate-rich perineuronal nets that serve as local buffers of excess cation changes (Hartig et al., 1999). Synapses of PV-positive cells contain M$_2$ muscarinic acetylcholine receptors (Hajos et al., 1998) that regulate GABA release by P/Q-type Ca$^{2+}$ channels (Wilson et al., 2001), while postsynaptic GABA$_A$ receptors contain α1 subunits in their efferent synapses on pyramidal cells. This subtype exhibits high discharge frequencies (≥100 Hz) with limited or no accommodation, labeling them as fast-spiking cells. The discharge waveform of PV-containing interneurons displays striking differences that permit the identification of e.g., stuttering, late-spiking, accommodating, and non-accommodating subtypes (Gupta et al., 2000). PV-containing interneurons predominantly belong to the class of basket cells as they innervate the somatic region and proximal dendrites of pyramids (Gupta et al., 2000; Kawaguchi and Kondo, 2002; Kawaguchi and Kubota, 1997), while a fraction, termed axo-axonic cells, selectively forms synapses on the axon initial segments of principal neurons (Pawelzik et al., 2002; Szabadics et al., 2006). Interestingly, excitatory afferents outnumber the inhibitory input on PV-containing basket cells by ~15-fold (Gulyas et al., 1999), suggesting a principal regulatory role of local excitation on this cell type (Holmgren et al., 2003). Both PV-containing basket and axo-axonic cells fire counter-phase with pyramidal cells during theta activity (Klausberger et al., 2003). In the neocortex and hippocampus, PV-containing basket cells can be connected by electric (gap) and chemical junctions that presumably suit the entrainment of large pyramidal cell populations at γ frequency oscillations (Tamas et
al., 2000). However, their electrical coupling is differentially regulated in the hippocampus and neocortex. While the incidence of coupling declines as a function of postnatal age in the hippocampus, it remains unaltered in layer 2/3 of the neocortex (Meyer et al., 2002).

Another subset of perisomatic inhibitory neurons contains CCK and co-expresses VIP (Kawaguchi and Kondo, 2002) and vesicular glutamate transporter 3 (Harkany et al., 2004; Somogyi et al., 2004). CCK-containing interneurons express a unique composition of receptors, including pre-synaptic type 1 cannabinoid (Katona et al., 1999), postsynaptic 5-HT<sub>3</sub> serotonin (Ferezou et al., 2002), and nicotinic acetylcholine receptors (Porter et al., 1999). In contrast to PV-containing interneurons, GABA release from axon terminals of CCK-containing cells is mediated by N-type Ca<sup>2+</sup> channels (Wilson et al., 2001) under the control of CB<sub>1</sub> (Katona et al., 1999) and GABA<sub>B</sub> receptors (Davies and Collingridge, 1993). In addition, their efferent synapses predominantly contain GABA<sub>A</sub> receptors with α2 subunits. CCK cells are primarily regular spiking, with a maximum firing rate of ~50 Hz (Pawelzik et al., 2002). In addition to perisomatic regions, CCK cells also innervate all dendritic layers of the hippocampus that correlates with their morphological variability (Pawelzik et al., 2002). CCK-containing interneurons receive much less synaptic contacts than PV cells with a high proportion of inhibitory synapses (Matyas et al., 2004). Similar to PV cells, though with lower incidence, CCK-containing interneurons also innervate each other. Importantly, CCK interneurons, targeted by cholinergic and serotonergic afferents originating in basal forebrain and median raphe nucleus (Freund et al., 1990), respectively, are critical for the tonic modulation of excitatory input in the hippocampus.

1.1.3 Long-range axonal connections afferents between subcortical and cortical regions

Sensory information from the periphery is relayed by thalamic nuclei towards the specialized somatosensory and association areas of the neocortex (Lopez-Bendito and Molnar, 2003). As thalamocortical axons (TCAs) reach a given cortical area, they project topographically within the target area and their synaptic connections are refined. Developmental mechanisms underlying TCA pathfinding towards the neocortex as well as their intracortical navigation are still poorly understood as yet. In the neocortex the main thalamic afferents enter via the transient subplate by E14 in the mouse (Lopez-Bendito et al., 2006). The location and timing of cortical entrance of TCAs is coinciding with outgoing projections derived from ‘pioneer’ neurons, possibly providing the substrate and directional guidance cues for growing TCAs (Allendoerfer and Shatz, 1994). More recent experiments have identified a variety of factors involved in TCA guidance. Mutations in the transcription factors Ebf1 and Dlx1/2, mainly expressed in the ventral telencephalon, display a defective topography of thalamocortical projections (Garel et al., 2002). Furthermore, the repulsive axon guidance cue ephrin-A5 appears to control their intracortical mapping (Dufour et al., 2003). Recently, development of TCAs was shown to depend on the early tangential migration of a population of neurons derived from the ventral telencephalon. Tangentially migration neurons derived from the LGE contribute to the establishment
of a permissive corridor which channels axons from the dorsal thalamus towards the neocortex. Additionally, this study showed the involvement of neuregulins in TCA pathfinding. These mechanisms define the coincidence of neuronal tangential migration and the timely arrangement of axon guidance cues as a novel mechanism to control TCA formation (Lopez-Bendito et al., 2006).

1.2 ANATOMICAL COMPLEXITY OF THE MAIN OLFACTOR Y BULB

Olfactory perception begins with the binding of odor molecules, dissolved in the nasal mucus, to specific receptors on the cilia of olfactory sensory neurons in the nasal cavity (Firestein, 2001). Each olfactory sensory neuron belongs to a population specifically expressing one olfactory receptor and sends unmyelinated axons through the olfactory nerve. Sensory neuron axons terminate onto the dendrites of mitral/bitufted principal neurons that assemble into glomeruli, analogues of the cortical barrels and columns (Lledo et al., 2004; Mombaerts et al., 1996). Subsequently, the mitral/tufted cells project to a narrow region of the ventro-lateral part of the telencephalon, close to the pial surface, where they form the lateral olfactory tract. The olfactory bulb is not just a relay system, as information processing takes place by tuning the activity of the output cells, the mitral/bitufted neurons. This is controlled by various interneurons, mainly consisting of two major types; periglomerular interneurons surrounding the glomeruli and the granule cells in the external plexiform layer (EPL). These unusual interneurons lack an axon and instead release their GABAergic contents from dendritic spines at specialized reciprocal contacts with dendrites of the principal cells of the olfactory bulb (Schoppa and Urban, 2003). Similar to the neocortex, olfactory bulb interneurons are heterogeneous in their morphology, neurochemistry and connectivity. They can be distinguished according to their expression of Ca\(^{2+}\)-binding proteins. Calbindin D28k and calretinin-expressing cells are most frequently found in the periglomerular regions, whereas PV-immunoreactive neurons predominate in the EPL.

Most cells in the nervous system are born during the embryonic stages and perinatal period. However, the interneurons of the olfactory bulb are continuously generated throughout postnatal life to replace older neurons within mature circuits (Gage, 2002). During embryonic development local circuit interneurons are in part derived from the dorsal region of the ganglionic eminences (Marin and Rubenstein, 2003), but after birth the production of new neurons becomes restricted to the SVZ, surrounding the lateral ventricles (Luskin, 1993). Stem cell-derived neuroblasts migrate tangentially along the rostral migratory stream (RMS) towards the olfactory bulb where they migrate radially and differentiate into phenotypically-defined mature functioning neurons (Lois and Alvarez-Buylla, 1994). Little is known about the processes that drive the integration of novel interneurons in an already function circuit, however.

1.3 AXON GUIDANCE MECHANISMS

During embryonic development neuronal circuitries are formed by differentiating neurons that, while becoming polarized, sprout an axon with a highly motile structure, called the growth cone, at its leading edge. Growth cones sense their surroundings for a multitude of attractive and repellant guidance cues, and steer axons to their appropriate postsynaptic targets. Over a century ago, Ramón y Cajal already proposed that axon
guidance might be mediated by long-range chemoattraction, reminiscent of the chemotaxis of motile cells and bacteria (Tessier-Lavigne and Goodman, 1996). Numerous diffusible factors have been identified that influence directional axonal turning over long distances, of which some of the best characterized are the neurotrophin family of ligands (see further details in Chapter 2). Besides diffusible factors, elongating axons are also controlled by short-ranged contact mediated substances. Growth cones require specific substrates that create a permissive environment for their growth, like extra-cellular matrix proteins and non-diffusible membrane bound molecules.

The cytoskeletal machinery of a growth cone consists of a tubulin-dense central domain and motile actin-rich lamellipodia and filopodia (Dent et al., 2003). Axonal elongation progresses through 3 distinct stages; rapid actin polymerization leads to a protrusion at the plasma membrane, followed by the filling of the central domain by microtubules allowing the transport of vesicles and cell organelles (engorgement). Finally, the proximal part of the growth cone reshares to a cylindrical form associated with actin depolymerisation, allowing microtubules to enter and stabilize the newly-formed axon segment (Goldberg and Burmeister, 1986).

Growth cones are densely loaded with cell surface receptors, that interpret the presence of extracellular guidance cues through stabilizing or destabilizing the actin cytoskeleton, and hence induce an attractive or repellent response (Bentley and O'Connor, 1994). These receptors include single- and seven-transmembrane receptors that converge onto the activation of members of the Rho family of small GTPases. The Rho family of GTPases plays important roles in the regulation of actin cytoskeletal dynamics and has been associated with axon growth and turning (Yuan et al., 2003). In growth cones, Rac and Cdc42 promote actin polymerization at the leading edge, allowing filopodial extension and axonal elongation. In contrast, activation of Rho family members is associated with growth cone retraction and collapse through the activation and contraction of actomyosin. Rho family GTPases exist in a GTP-bound active and a GDP-bound inactive form and their activity state is highly regulated by two classes of factors; the guanine nucleotide exchange factors (GNEFs) activates GTPases by promoting the conversion of GTP to GDP, and the GTPase-activating proteins (GAPs), that hydrolyse GTP (Hall, 1994; Lamarche and Hall, 1994). Many well-established guidance factors, including slit, semaphorin, ephrins, netrins, and BDNF have been shown to regulate intracellular Rho GTPase activity (Li et al., 2002; Wahl et al., 2000; Whitford and Ghosh, 2001; Wong et al., 2001; Yuan et al., 2003). It has further been proposed that a local increase in intracellular Ca$^{2+}$ activates members of the Rho family, manipulating growth cone turning (Rajnicek et al., 2006b). It must be noted however that Rho GTPases, independently of the effects on the cytoskeletal dynamics, also convey signals from membrane receptors to the cell nucleus (Ridley, 1996). It is now recognized that an asymmetry of Rho GTPase activity across a single growth cone is the major determinant of the axonal turning response induced by extracellular guidance factors (Etienne-Manneville and Hall, 2002).
2 BDNF

Neurotrophins are important regulators of target-directed axonal pathfinding, the survival of various neuronal subtypes, and their differentiation into mature neurons and the establishment of synaptic contacts. The neurotrophin hypothesis postulates that the secretion of limiting survival factors is required to stimulate organ innervation and the survival of the appropriate number of input neurons, in order to maintain a balance between organ size and the number of neuronal connections a particular organ receives. The initial discovery of neuronal growth factors was based on the notion that removal of target tissue in developing embryos drastically reduced the survival rate of the sympathetic neurons in the peripheral nervous system (PNS) (Korsching, 1993). The first neurotrophin to be isolated was nerve growth factor (NGF) by Levi-Montalcini and Cohen in the early 50’s of the last century (Cohen et al., 1954), which they were later awarded the Nobel prize for. The identification of NGF provided the first molecular factor confirming the neurotrophin hypothesis (Cohen and Levi-Montalcini, 1956; Levi-Montalcini and Booker, 1960; Levi-Montalcini and Cohen, 1956). The discovery of an entire family of neurotrophins made a significant impact on the scientific community and stimulated the research aimed at understanding the mechanisms of neuronal survival, differentiation and damage-related neurorepair.

To date four mammalian neurotrophins have been characterized: NGF, BDNF, neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). These factors are derived from a common ancestral gene and are largely similar in their structure and functions, hence the collective name neurotrophins (Hallbook, 1999). Neurotrophin actions are mediated through at least two distinct receptor classes: neurotrophin-specific receptors of the tropomyosin-related kinase receptors (Trk) family and the low-affinity p75 neurotrophin receptor, a member of the tumor necrosis factor (TNF) superfamily. Each Trk receptor is activated by one or more of the four neurotrophins; nerve growth factor (NGF) binds and activates TrkA, BDNF and NT-4 both act through TrkB, while NT-3 predominantly activates TrkC, but is also know to interact with TrkA and TrkB (Kaplan and Miller, 2000). Recent genetic evidence however contrasts the above rules, as in the cochleovestibular system, TrkB and TrkC receptors are not redundant for neuronal survival and target innervation mediated by NT-3 (Stenqvist et al., 2005). Although originally discovered as an oncogene, by fusing the intracellular kinase domain of TrkA was fused to tropomyosin (Martin-Zanca et al., 1986), the discovery that neurotrophins activate Trk receptors is a key step in promoting neuronal survival stimulated the research in neurotrophin signaling and its relation to the various effects on cellular development (Hallbook, 1999; Huang and Reichardt, 2001).

2.1 TRKB RECEPTOR SIGNALING

The binding of neurotrophins to the immunoglobulin (Ig-like) extracellular domain of Trk receptors induces their homo-dimerization and the subsequent activation of the intracellular effector kinases. Trk receptors contain 10 evolutionarily conserved tyrosines within the cytoplasmic domain of which 3 are present in the autoregulatory loop that further activates the kinase upon ligand binding (Hallbook, 1999). Phosphorylation of the remaining tyrosine residues initiates signaling by creating docking sites for adaptor proteins that couple the receptor to intracellular signaling...
cascades (see fig. 2), such as the Ras/extracellular signal regulated kinase (ERK) protein kinase pathway, the phosphatidylinositol-3-OH kinase (PI3K)/Akt kinase pathway and phospholipase C-γ (PLC-γ) (Huang and Reichardt, 2001).

![BDNF](BDNF.png)

**Fig. 2 Schematic diagram of TrkB receptor signaling.**

Binding of BDNF to TrkB induced receptor dimerization and recruitment of the adaptor molecules, that subsequently activate signaling cascades. The three best characterized pathways are Ras/extracellular signal regulated kinase (ERK), the phosphatidylinositol-3-OH kinase (PI3K) and phospholipase C-γ (PLC-γ).

Binding of BDNF to the TrkB receptor phosphorylates tyrosine residue Y515 leading to the recruitment and phosphorylation of shc. Shc forms a complex with the adaptor proteins Grb-2 and the Ras exchange factor son of sevenless (SOS), initiating in the transient activation of Ras. In turn, Ras activates both the PI3K and p38 MAPK/MAPK-activating protein kinase 2 pathways and the c-Raf/ERK pathway (Xing et al., 1996). As one of the major signaling pathways relaying the effects of neurotrophins, the Ras/ERK protein kinase pathway, promotes a variety of cellular mechanisms, including neuronal survival, proliferation and differentiation. Interestingly, these effects depend on the timing and duration of ERK activity (Grewal et al., 1999). Transcriptional activity initiated by the ERK pathway is through phosphorylation of the cAMP response element binding protein (CREB) and several other transcriptional factors (Xing et al., 1996; Xing et al., 1998). Activated CREB is commonly associated with increased neuronal survival and has a role in long-term potentiation (Bonni et al., 1999; Riccio et al., 1999; Walert and Weller, 2003). The second effector pathway stimulated by Ras is the PI3K pathway, an important regulator of neuronal survival. PI3K is predominantly activated through Ras, however PI3K can also be activated through the adaptor proteins Shc, Grb-2 and Gab-1 in certain cells (Holgado-Madruga et al., 1997). PI3K activates the protein kinase Akt (also known as PKB) at the inner leaflet of the membrane through intermediate lipid products and the Akt activating kinases 3-phosphoinositide-dependent kinases (PDKs) (Datta et al., 1999). Protein substrates of Akt inhibit the actions of several apoptotic proteins, for instance through the Bcl-2 family member Bad by binding to its phosphorylated form (Datta et al., 1997). The third major pathway activated by neurotrophins is the PLCγ pathway: within the C-terminal extremity of the TrkB receptor, PLCγ is recruited and activated at phosphorylated Y816 and generates diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP3) by hydrolyzing phosphatidylinositides. The main action of IP3 is the release of Ca^{2+} from intracellular stores to increase and to activate various pathways, such as protein kinase C (PKC) and Ca^{2+}-calmodulin-regulated protein kinases. Besides the 3 major pathways described so far, neurotrophins also regulate the cytoskeleton in different cellular domains, e.g. the growth cones and the cell soma. Rapid cytoskeletal dynamics induced by Trk activation are controlled by Rho, Rac and Cdc42 of the Rho family of small GTPases, leading to the assembly of contractile...
actin/myosin filaments, protrusive actin-rich lamellipodia, and protrusive actin-rich philopodia (Yuan et al., 2003).

Exciting recent data demonstrated that tyrosine kinase receptors can be trans-activated by G protein–coupled receptors (GPCRs). Pioneer experiments showed that the GPCR ligands endothelin-1, lysosphatic acid (LPA) and thrombin rapidly phosphorylated the epidermal growth factor receptor (EGFR) (Daub et al., 1996). This process was found to be dependent on the cleavage of membrane-linked proHB-EGF by metalloproteinases that were activated by GPCR ligands (Prenzel et al., 1999). Trk receptors share the ability of transactivation, although the temporal dynamics of the receptor-specific mechanisms are strikingly different. TrkA activation through adenosine treatment is observed after a 60-90 minutes stimulation period compared to EGFR activation, which occurs rapidly, within only minutes (Daub et al., 1996; Lee and Chao, 2001; Prenzel et al., 1999). The mechanisms underlying Trk activation by GPCRs are just being elucidated. The simplest hypothesis is a direct interaction of the GPCR ligand with the Trk receptor, inducing dimerization and subsequent activation of the receptor in a manner resembling that of the neurotrophins. This is unlikely as the binding capacity of NGF to TrkA is not affected in the presence of adenosine (Arevalo et al., 2000) and the long lag time for receptor activation is inconsistent with the short half-life of adenosine. De novo synthesis or release of neurotrophins by adenosine treatment and an indirect activation of Trk receptors (similar to the conversion of proHB-EGF to active EGF) could explain the slow time course. In PC12 cells however, adenosine does not release NGF and no NGF-like activity was observed in situ, suggesting that TrkA receptor transactivation mechanisms are independent of neurotrophins (Lee et al., 2002a).

The major candidates mediating GPCR-induced transactivation are the Src kinase family of non-receptor tyrosine kinases. Members of this family include Src, Fyn, Lyn, Lck and Yes and play fundamental roles in the control of cell proliferation, survival, adhesion, cytoskeletal dynamics and vesicular trafficking of surface receptors (Luttrell and Luttrell, 2004). As Src kinases are activated downstream from both Trk receptors and GPCRs, they provide a possible link for receptor activation. In support of this concept, incubation of COS-7 cells with the Src inhibitor PP1 led to a reduced EGFR transactivation by LPA. The responsiveness of the EGFR to EGF was not reduced showing that the action of PP1 was not directly targeted to the receptor itself (Daub et al., 1997). In parallel to the involvement of Src in EGFR phosphorylation, the activation of Trk receptors is partly mediated by the Fyn-kinase via a direct protein-protein interaction at the SH-3 domain of this kinase (Rajagopal and Chao, 2006). Furthermore, the colocalisation of Trk receptors with Src family kinases on intracellular membranes (e.g. Golgi system) has been proposed as the cellular site of transactivation (Rajagopal et al., 2004). The significance of GPCR and Trk receptor interactions is evidenced by studies focusing on survival and proliferation in neuronal and cancer cell types. The survival of various tumor cell lines is dependent on EGFR transactivation, with the intrinsic involvement of the anti-apoptotic kinase Akt (Schafer et al., 2004). Moreover, GPCR ligands stimulate tumour cell migration and invasion through metalloproteinase activation and EGFR phosphorylation (Schafer et al., 2004). Trk receptor phosphorylation by GPCR ligands is commonly associated with increased neuronal survival, also through activation of the Akt pathway (Lee et al., 2002a).
Surprisingly, Trk receptor transactivation does not stimulate the differentiation of neuronal cell lines by affecting, e.g., the outgrowth of both dendrites and axons (Lee and Chao, 2001; Lee et al., 2002b). Instead, Trk receptor transactivation by GPCRs is now recognized as an important mechanism allowing cells to integrate a multitude of extracellular stimuli and provides a link between two large families of cell surface receptors to produce an enhanced spectrum of multi-modal cellular responses.

2.2 ROLES OF BDNF IN CNS DEVELOPMENT

TrkB receptors are expressed at early embryonic stages in the CNS. In situ hybridization studies have detected TrkB transcripts as early as E8.5 in a variety of structures in the developing nervous system, including the forebrain, caudal midbrain, hindbrain, spinal cord, the trigeminal ganglion and neural crest cells (Klein et al., 1990a; Klein et al., 1990b). TrkB receptor expression reflects similar patterns as in the adult CNS and PNS in the midgestation embryo (E13.5) and is maintained during the late fetal periods (Klein et al., 1990b). In the adult CNS, the highest amounts of TrkB receptors are found in the cerebral cortex, the granular layer of the dentate gyrus and the pyramidal layer of the hippocampus, whereas it is absent in the corpus callosum and the fimbria-fornix hippocampus. In the cerebellum high mRNA levels are confined to the Purkinje cell layer and the caudal peduncle (Klein et al., 1990b). Adult TrkB expression is highly regulated in response to neuronal injury. Fimbria-fornix lesions and lesions of the perforant path in the hippocampus increased mRNA levels in non-neuronal cells 6-14 days after injury, indicating a role for BDNF in neuronal repair mechanisms, including neuronal sprouting and the regeneration of synaptic contacts (Beck et al., 1993).

Targeted deletion in the TrkB gene (TrkBtk-/-) disrupts TrkB signaling throughout development and postnatal life and TrkBtk-/- mice develop until birth, but die during the first postnatal week. Although no gross malformations were observed in the head, lip or palate, the animals die due to malnourishment already observed as early as 12 hours after birth (Klein et al., 1993). TrkBtk-/- mice exhibit behavioural abnormalities accompanied by reduced neuronal densities (30-80%) in certain PNS structures. Among these, highly affected regions are the trigeminal ganglion, the nodose/petrosal ganglia, the dorsal root ganglia and spinal cord (lumbar region). Irrespective of the high levels of TrkB receptor transcripts in the cerebral cortex, hippocampus and dentate gyrus, these structures reveal no morphological deficits in TrkBtk-/- mice. This could be partially explained by compensatory mechanisms that replace the function of TrkB during development through receptor promiscuity (see above), possibly by signaling through the TrkC receptors in these structures. However, recent reports using novel transgenic strains showed more detailed analyses of the neuronal deficits caused by the disruption of TrkB signaling. Mice with conditionally TrkB receptor deletion restricted to the forebrain during postnatal development survive into adulthood but display impaired learning and LTP. Changes in synaptic plasticity correlate with specific reductions in spine densities and a significant increase in spine length of apical and basal dendrites in the CA1 area of the hippocampus (von Bohlen und Halbach et al., 2006). Conditional and knock in mutagenesis of the TrkB receptor, selectively targeting the Shc/FRS2 and PLCγ adaptors revealed that TrkB controls cortical stratification
As described previously, both BDNF and TrkB receptors are expressed in the developing and adult hippocampus and neocortex (Cellerino et al., 1996; Klein et al., 1990b) suggesting a role in the formation of cortical microcircuits. In the neocortex, TrkB receptors are expressed in both pyramidal neurons and cortical GABAergic interneurons, although highest expression is observed in PV-expressing FS cells (Cellerino et al., 1996). However, BDNF production and release is restricted to pyramidal cells (Cellerino et al., 1996). BDNF expression is regulated by sensory input and is dependent on electrical activity, as shown by upregulation of BDNF during hippocampal seizure induction (Kornblum et al., 1997). In addition, neuronal activity elevates the responsiveness to BDNF by increasing both mRNA expression and the availability of functional Trk receptors in the cytoplasmic membrane via docking of TrkB-loaded intracellular vesicles (Castren et al., 1992; Meyer-Franke et al., 1998). The dynamic regulation of TrkB receptor and BDNF availability could explain the differences between active and inactive synapses in their responsiveness to BDNF and thereby regulate philopodial number and size, the activity of e.g. glutamate receptors, and various other functions associated with synaptic strength. The hypothesis that BDNF underscores the formation of neuronal microcircuits is supported by the fact that BDNF application increases the complexity of axonal and dendritic arbours in a variety of cell types (Horch, 2004). In cortical slices, BDNF regulates the dendritic morphology of pyramidal cells in a layer and cell specific manner (Horch et al., 1999). BDNF promotes the dendritic arborization of neurons in layers IV and V, while conversely, it decreases dendritic complexity of neurons in layer VI (McAllister et al., 1997). Moreover, using Cre-mediated TrkB removal from neurons expressing CaMKII, primarily pyramidal neurons, resulted in a striking reduction of the dendritic tree of pyramids in conjunction with a significant loss of neuronal cell numbers (Xu et al., 2000b). Recent data also support that BDNF acts as a diffusible chemo-attractant for axonal growth: elongating axons of Xenopus spinal neurons change direction towards a microscopic gradient of BDNF aimed at the axonal growth cone (Ming et al., 2002; Song et al., 1997), resembling bacterial migration in response to a chemoattractive substance. The attractive response to BDNF is dependent on several factors of with intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{i}\)) signaling being one of the most studied. Depending on the type of neurons and the experimental conditions, [Ca\(^{2+}\)]\(_{i}\) has been reported to induce growth cone motility and axonal steering as well as inhibition of neurite growth. The [Ca\(^{2+}\)]\(_{i}\) can be altered by the release of calcium from intracellular stores or by the influx of calcium from the extracellular space. In turn, Ca\(^{2+}\) itself plays a crucial role in the activation of intracellular signaling cascades. BDNF can trigger an asymmetric [Ca\(^{2+}\)]\(_{i}\) distribution with a clear elevation at the proximal side of the growth cone facing the gradient of BDNF (Kao et al., 2002; Ming et al., 2002). The bimodal directionality of growth cone guidance and the involvement of Ca\(^{2+}\) dependent kinases and phosphatases show a remarkable resemblance to the synaptic modifications induced by Ca\(^{2+}\) changes associated with neuronal activity. Adaptations of growth cone turning responses to BDNF could also result in the activity levels of cAMP or PKA. Under standard conditions BDNF induces an attractive response in Xenopus spinal neurons in culture. However, in the presence of a competitive analogue for cAMP or specific PKA inhibitors diverts axonal growth (Song et al., 1997). Therefore, the effect of diffusible
guidance cues (e.g., BDNF) on axonal directional turning is dependent on the intracellular context enabling a cell to simultaneously translate and interpret multiple extracellular stimuli.

Changes in synaptic strength are important for memory formation and mood stability (Malenka and Bear, 2004). Activity-dependent changes in synaptic strength, e.g., LTP, are generally believed to underlie synapse consolidation. BDNF triggers synaptic strengthening by the activation of CREB, a transcriptional regulator, controlling the expression of target genes and de novo synthesis of proteins involved in LTP and long-term memory (Kang and Schuman, 1996; Messaoudi et al., 2002). The role of BDNF is underscored by a reduction of LTP formation in bdnf-null mutant mice and trkB-conditional-forebrain-null mutant mice (Minichiello et al., 1999; Xu et al., 2000a). More recent experiments, using mice carrying single amino acid mutations in the TrkB gene that disrupt the binding site for PLCγ (Tyr816), showed a crucial involvement for PLCγ in the establishment of LTP (Minichiello et al., 2002). In contrast to previous data (Ying et al., 2002), PLCγ mediated LTP appeared not dependent on the ERK-CREB pathway, but acted through elevated Ca2+ levels and CaMKIV to phosphorylate CREB (Minichiello et al., 2002).

2.3 BDNF SIGNALING IN DISEASE

The wide variety of neurotrophin actions on neuronal development and synaptic functions prompted the idea of using neurotrophic factors as therapeutic agents. Most emphasis has been on the symptomatic interventions in impaired neuron circuitries. Since the 1980s, Alzheimer’s disease is associated with a loss of cortical NGF availability that is critical to sustain subcortical afferents originating from basal forebrain cholinergic neurons. Accordingly, cholinergic neurons show improved survival upon NGF application in association with partial rescue of mental functions (Williams et al., 1986). BDNF, in turn, promotes the survival of motor neurons in wobbler mice, a model of progressive motor neuron disease (Tsuzaka et al., 2001). These striking effects of neurotrophins in animal disease models created great optimism and clinical trials were initiated as soon as neurotrophins became available by recombinant technology. Unfortunately, clinical trials indicated that subcutaneous or intrathecal delivery of BDNF in ALS patients had minimal effects and produced severe side-effects including pain and intestinal malfunctions (Thoenen and Sendtner, 2002). The discrepancy between the failed human and successful rodent trials may derive from the high BDNF doses used in humans thus causing robust reductions to TrkB expression. Another major obstacle is the delivery of neurotrophins to their targets in the CNS, as they do not penetrate the blood-brain barrier. However, since the discovery that Trk receptors can be transactivated by GPCRs, small GPCR ligands that do cross this barrier, emerged and could provide alternatives for neurotrophin receptor modulation.

Additional evidence suggests that human nervous system disorders are related to a malfunction in neurotrophin signaling. A single nucleotide polymorphism in the BDNF gene, leading to a Val→Met substitution (BDNFmet) at position 66 in the pro-domain has been linked to memory impairments and increased susceptibility to Alzheimer’s and Parkinson’s diseases, depression, eating disorders and bipolar symptoms (Momose
et al., 2002; Ribases et al., 2003; Sen et al., 2003; Sklar et al., 2002; Ventriglia et al., 2002). As the mutated pro-domain plays a crucial role in the loading of BDNF into secretory vesicles, activity-dependent BDNF_{met} release is reduced compared to wild-type BDNF (Egan et al., 2003). A de novo missense mutation in the kinase domain of TrkB (Tyr722→Cys) markedly impairs receptor dimerization and autophosphorylation with a subsequent reduction in MAPK signaling. These patients develop an unique human syndrome of hyperphagic obesity and display impaired memory, learning and nociception (Yeo et al., 2004). Although CNS disorders caused by a hampered BDNF/TrkB signaling are rare, they pinpoint the requirement of BDNF signaling networks during physiological neurodevelopment.
THE ENDOCANNABINOID SYSTEM

The identification of the endocannabinoid ligands and their receptors vastly broadened our understanding of the role of endocannabinoids in neuronal signaling (Mackie and Stella, 2006). Endocannabinoids and the psychoactive substance Δ⁹-tetrahydrocannabinol (THC), derived from the Cannabis sativa plant, activate cannabinoid receptors on both neurons and glia. Originally identified as retrograde messengers, endocannabinoids are released at postsynaptic terminals by various inhibitory and excitatory neurons and subsequently activate presynaptic type 1 cannabinoid receptors (CB₁Rs), which inhibit presynaptic neurotransmitter release (Freund et al., 2003; Wilson and Nicoll, 2001). The pathogenic effects of maternal marijuana smoking during pregnancy on children, e.g. cognitive, motor, and social deficits, that endure into adulthood (Bernard et al., 2005), stimulated the interest in the function of cannabinoids and their receptors during prenatal and perinatal development. It is now clear that endocannabinoids are involved numerous developmental processes, including the proliferation, differentiation, migration, and survival of neural progenitors (Guzman et al., 2001) and dictate the phenotypic differentiation of neurons (Galve-Roperh et al., 2006).

3.1 ENDOCANNABINOID EXPRESSION DURING DEVELOPMENT

The most accurately resolved expression patterns are available for the CB₁R in the developing CNS. CB₁Rs have been detected as early as the pre-implantation period in the embryonic mouse. From day 11 of gestation in the rodent, comparable to 5-6 weeks in the human, CB₁R mRNA and protein expression gradually increases throughout the prenatal period (Berrendero et al., 1999). Similar developmental patterns of CB₁Rs were found during human pre- and postnatal development. CB₁Rs were detected at week 14 of gestation with high expression levels in the CA2-CA3 subfields of the hippocampus and the basal nuclear group of the amygdala at week 20 of gestation (Mato et al., 2003). Gradually increasing CB₁R mRNA levels were furthermore shown in the frontal cortex, hippocampus, basal ganglia and cerebellum between the fetal period and adulthood. Elongating axonal trajectories in the white matter of the mouse and human fetus also transiently express CB₁R. Strikingly, CB₁R disappear from axonal tracts at the time when the formation of synapses is completed (Fernandez-Ruiz et al., 2000). Furthermore, the functional activity of CB₁Rs at all stages of development, was shown by ligand induced [35S]GTPγS binding (Mato et al., 2003). The morphological evidence supports the idea that the endocannabinoid system is expressed and ideally positioned during CNS development, to be involved in a variety of developmental programs in both neuronal progenitors and developing neuronal circuitries.

The main endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG), are present throughout embryonic development already from the earliest stages (Berrendero et al., 1999; Fernandez-Ruiz et al., 2000). AEA concentrations in the uterus define receptivity for embryo implantation between days 4 - 6 of pregnancy (Paria et al., 2001). Synchronization is achieved by simultaneous down-regulation of
intra-uterine AEA levels and expression of CB$_1$ and CB$_2$ receptor in the pre-implantation embryo prior to implantation (Paria et al., 2001). Low AEA concentrations are present in the CNS at mid-gestation with gradually increasing levels throughout the perinatal period (Berrendero et al., 1999). 2-AG concentrations (2 - 8 nmol/g tissue) exceed those of AEA (3 - 6 pmol/g tissue) during brain development (see fig. 3). In rats, fetal 2-AG levels are similar to those in young and adult brains with a remarkably distinct peak on the first day after birth (Berrendero et al., 1999; Fernandez-Ruiz et al., 2000).

Several biosynthetic routes for the generation of 2-AG and AEA have been described. The $\alpha$ and $\beta$ isoforms of sn-1 diacylglycerol lipase (DAGL$\alpha$/\$\beta$) are characterized as 2-AG-synthesizing enzymes (Bisogno et al., 2003). However, the identities of enzymes contributing to AEA synthesis are still controversial. N-acyl-phosphatidyl-ethanolamine-selective phospholipase D (NAPE-PLD) has been considered as a prime candidate for AEA synthesis. Recent evidence further suggest that $\alpha/\beta$-hydrolase 4 (Simon and Cravatt, 2006) and various phosphatases (Liu et al., 2006) contribute in the biosynthesis of AEA. The uncertainty of the enzymes that participate in AEA and 2-AG synthesis in the developing CNS has hampered their histochemical mapping. In contrast, monoglyceride lipase (MGL) (Dinh et al., 2002) and fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996) have been established as the catabolic enzymes of 2-AG and AEA, respectively. Whereas the cellular distribution of MGL during ontogenesis is as yet unknown, FAAH has been detected in radial glia during late gestation as well as postnatally (Aguado et al., 2006). The presence of FAAH and the promoting actions of endocannabinoid on astrogliogenesis could suggest the involvement of endocannabinoid signaling in neural progenitor differentiation.
3.2 PRENATAL MARIJUANA IMPAIRS CNS DEVELOPMENT

One-third of THC in the plasma crosses the placenta after marijuana intake during pregnancy (Hutchings et al., 1989). Cognitive defects due to intra-uterine THC are consistent with the high expression of CB1Rs in the developing hippocampus (Wang et al., 2003) and in developing axonal tracts during pathfinding and target selection. Retrospective studies have associated behavioral abnormalities with prenatally marijuana-exposed children. The pathological representations include an exaggerated startle response, poor habituation, hyperactivity, attention disorders, and cognitive retardation (Fried et al., 2003; Huizink and Mulder, 2006; Richardson et al., 1995). Experimental studies substantiate these findings (Antonelli et al., 2005; Mereu et al., 2003) and directly link behavioral and cognitive deficits and emotional responsiveness to prenatal exposure to cannabis. More importantly, THC has been suggested to influence the functions of endocannabinoids by increasing their (particularly AEA) synthesis and release in a concentration-dependent manner that is reliant on phospholipase D (PLD) activity (Hunter and Burstein, 1997). The decreased AEA concentrations in the hippocampus of CB1R-/- mice (Di Marzo et al., 2000) further indicates that CB1Rs regulate the availability of endocannabinoids. In addition, THC lowers CB1R expression desensitization (Fernandez-Ruiz et al., 2000) in a region-specific manner that could trigger the onset of epileptiform activity (Bernard et al., 2005). The possible involvement of CB1Rs in the maturation of neuronal networks provides a base for understanding the developmental processes underlying THC-induced cognitive abnormalities.

3.3 ENDOCANNABINOID SIGNALING IN NEURAL PROGENITORS

A fine-tuned balance between progenitor cell proliferation and programmed death guarantees the generation of adequate quantities of neural cells during brain development. It is now becoming increasingly evident that endocannabinoids and related lipid mediators regulate neural progenitor commitment and survival (Aguado et al., 2006; Guzman et al., 2001; Guzman et al., 2002). Neural progenitors express both CB1Rs and the FAAH (Aguado et al., 2006). CB1R activation promotes progenitor cell proliferation and the differentiation into an astroglial lineage (Aguado et al., 2006). CB1R-/- mouse brain, progenitor proliferation and astrogliogenesis are decreased. Accordingly, elevated levels of AEA in FAAH-/- increases progenitor proliferation (Aguado et al., 2005). In contrast, the impact of CB1R activation on neurogenesis seems variable. The non-hydrolysable AEA analogue methanandamide significantly decreases neurogenesis in the adult dentate gyrus, as measured by incorporation of the S-phase marker 5-bromo-2’-deoxyuridine (Galve-Roperh et al., 2006). In addition, endocannabinoids decrease the expression of selective markers of early and terminally-differentiated neurons, β-III-tubulin and neuron-specific nuclear protein, respectively, and inhibit neurite outgrowth. Conversely, HU-210, a synthetic CB1R agonist, has been shown to expand hippocampal neurogenesis and exert anxiolytic and antidepressant effects (Jiang et al., 2005). The selective CB1R antagonist SR141716 increases neuronal differentiation of neural progenitors (Jin et al., 2004; Rueda et al., 2002). The contradicting findings on the effects of different cannabinoid ligands and the divergent actions on distinct cellular populations indicate that the cellular context in which cannabinoid signaling occurs determines the balance between neuron versus glia production during brain development.
Box 1. Cannabinoids and BDNF as synaptic retrograde messengers

The strength of synaptic transmission is controlled by the release of retrograde messengers from the post-synaptic neurons, and modulate neurotransmitter release through activation of presynaptic receptors (Alger, 2002). Retrograde cannabinoid signaling at inhibitory hippocampal and cerebellar synapses induces transient depression of spontaneous synaptic activity, also termed depolarization-induced suppression of inhibition (DSI) (Llano et al., 1991; Pitler and Alger, 1992). In various brain regions it has been demonstrated that postsynaptic depolarization-induced [Ca^{2+}]_{i} increases causes the release of endocannabinoids through active transport or passive diffusion over the membrane (Maejima et al., 2001; Wilson and Nicoll, 2001). Alternatively, increased [Ca^{2+}]_{i} stimulates cannabinoid production via the activation of NAPE-PLD and DAGLs, which are critical for the production of AEA and 2-AG, respectively. Postsynaptic cannabinoid release activates G_{i/o}-linked CB_{1}Rs on the presynaptic membrane that reduce the available amounts of neurotransmitter in the synaptic cleft by blocking vesicle release. Inhibition of voltage-dependent calcium channels, activation of potassium channels and direct interference with the synaptic vesicle release mechanism are all implicated in the cannabinoid-evoked inhibition of transmitter release. Recent studies show that depolarization-dependent postsynaptic release of BDNF induces synaptic strengthening through TrkB mediated vesicle release in hippocampal neurons in vitro (Magby et al., 2006; Schinder et al., 2000; Schinder and Poo, 2000). Similar to the endocannabinoid system, post-synaptic Ca^{2+} influx stimulates the synaptic release of BDNF that subsequently binds to presynaptic TrkB receptors. TrkB receptor activation causes the presynaptic vesicles, charged with neurotransmitter, to fuse with the membrane and release their content. Retrograde signaling by endocannabinoids and BDNF shows remarkable similarities at mature synapses with their developmental effects during synaptogenesis. As shown in this thesis, endocannabinoids and phytocannabinoids reduce morphogenetic development of interneurons and act as a repellent cues for axonal growth cones. In contrast, BDNF is required for cortical interneuron differentiation and accelerates the formation of functional synapses and networks. The retrograde effects of endocannabinoids and BDNF in a functional circuit thus appear to reflect developmental programs, overarching the pattern and functional maintenance of central synapses.
Endocannabinoids influence the differentiation of neural progenitors either directly or by affecting the production of intermediary mediators in neighboring cells (Aguado et al., 2006; Galve-Roperh et al., 2006). In neural progenitors, endocannabinoids inhibit sustained ERK1/2 activation via attenuation of Rap1 and B-Raf signaling. A decrease in endocannabinoid-induced neuronal differentiation is accompanied by a reduced neurotrophin-induced activation of Erk1/2 (Rueda et al., 2002). The control of Erk1/2 activity might explain the different effects of endocannabinoids on neuro- vs. gliogenesis, since glial cells do not express B-Raf (Galve-Roperh et al., 2006). Data from NG108-15 neuroblastoma cells indicate that AEA, but not 2-AG or WIN55,212-2, may also inhibit neuronal differentiation in a CB1R-independent manner (Galve-Roperh et al., 2006). CB1R-independent regulation of neurogenesis may be specific for AEA, given its propensity to allosterically regulate the activity of a broad variety of receptors and ion channels affecting neuronal fate. Defining the cellular identities of neuronal precursors in conjunction with the identification of the molecular composition of the endocannabinoid pathways are essential to our understanding of how cannabinoids influence the developmental of neural progenitors.

### 3.4 ENDOCANNABINOIDS IN NEURONAL DIFFERENTIATION

The role of cannabinoids in neuronal progenitor development raises the question whether their effects are restricted to early progenitors or could be extended to later stages in neuronal differentiation. Knowledge on the effects exerted by cannabinoids and CB1Rs in terminal neuronal differentiation and the establishment of circuitries is sparse. So far, cannabinoids have been shown to counteract the forskolin-induced synaptogenesis of cultured hippocampal neurons (Kim and Thayer, 2001). In CB1R overexpressing B103 neuroblastoma cells AEA induced cell rounding, as well as neurite remodeling and retraction (Ishii and Chun, 2002). These morphogenic changes are linked to the activation of the Rho family of GTPases. In contrast, HU-210 promotes neurite outgrowth in Neuro 2A cells by the Gαo/i-mediated degradation of RapGAP1 and subsequent activation of Rap1 (Jordan et al., 2005).

A new hypothesis is that endocannabinoid signaling regulates aspects of growth cone motility and axon guidance. This is based on studies indicating that 2-AG stimulates neurite outgrowth of cerebellar neurons via a mechanism dependent on intrinsic DAGL activity within axonal growth cones. In contrast, CB1R antagonists abolish the N-cadherin and Fgf8-induced neurite extension (Williams et al., 2003). Further support for the regulation of neuritogenesis derives from the similar functional effects of other lipid mediators, such as lysophosphatidic acid and sphingosine-1-phosphate, as reviewed recently elsewhere (Galve-Roperh et al., 2006). The question emerges whether endocannabinoids can influence axonal growth and direction in developing neurons in a target derived fashion and are required for proper network formation.

### 3.5 CROSS-TALK BETWEEN CB1R AND OTHER RECEPTORS

The phylogenetically ancient endocannabinoid system has developed simultaneously with other signaling systems and interactions with cell surface and cytoplasmic proteins have evolved (McPartland, 2004). As most GPCRs (Devi, 2000), CB1Rs signaling occurs via the formation of homodimers (Wager-Miller et al., 2002). The previous
discussion about receptor interactions between GPCRs and tyrosine kinase receptors also holds true for cannabinoids receptors. Recent experiments have revealed multiple receptor interactions between cannabinoids and other receptor types as an essential mean to coordinate the availability of multiple ligands.

The first example of CB1R interaction is based on the notion that its effects in cancer cells are dependent on the cellular context. μM concentrations of cannabinoids commonly act as immunosuppressive and anti-mitotic agents for certain cancer types (Bifulco and Di Marzo, 2002; Guzman, 2003). Endocannabinoids and synthetic cannabinoids stimulate the proliferation and survival of cancer cells expressing EGFRs (Hart et al., 2004; Zhao et al., 2005) through Akt/Erk activation, but via upstream signaling steps different from those evoked by direct Gαi/o protein coupling. CB1Rs activate tumor necrosis factor ς-converting enzyme (TACE/ADAM17), a member of the disintegrin-metalloprotease family, through the cytoplasmic tyrosine kinases Src and Fyn (Hart et al., 2004). Ligand activation at the cell surface, by proteolytic ectodomain shedding of EGF precursors, induces rapid EGFR phosphorylation. EGFR transactivation elicits phosphorylation of its Src homology 2 domain-containing adaptor (Shc) with downstream activation of mitogenic protein kinase pathways.

Besides interaction with tyrosine kinase receptors, CB1R have been shown to dimerize and interact with different GPCRs. One example is provided the interaction between the CB1R and μ opioid receptors in Neuro 2A cells. In these cells, CB1Rs regulate neurite elongation through a Gαi/o signaling involving downstream proteasomal degradation of Rap1-GAPII and activation of the Src/Stat3 pathway (He et al., 2005; Jordan et al., 2005). Co-expression of CB1Rs and μ opioid receptors leads to the close association of these receptors. Simultaneous activation of μ opioid and CB1 cannabinoid receptors leads to a significant attenuation of Erk1/2 phosphorylation and reduced signaling via Src/Stat3 (Rios et al., 2006). Co-activation of the two receptors has further been implicated in neuritogenesis.

Interactions between CB1Rs and D2Rs provide another example of cross-talk and are involved in the control of synaptic activity within nigro-striatal pathway. CB1Rs preferentially localize to the nigro-striatal circuitry where their ability to tune GABAergic neurotransmission coincides with the capacity of GABAergic and dopaminergic neurons to produce endocannabinoids (Kofalvi et al., 2005). A mechanistic link between endocannabinoid and dopamine signaling is facilitated by the propensity of CB1Rs and D2Rs to dimerize (Kearn et al., 2005). CB1R/D2R complex formation is favored when both receptors are stimulated with subsaturating agonist concentrations, likely through interactions of transmembrane regions (Ng et al., 1996). Unlike the individual receptors, CB1R/D2R heterodimers couple to Gαs proteins to stimulate cAMP production and further enhance Erk1/2 activity. Activity-dependent elevations in dopamine and endocannabinoid levels at active synapses could trigger CB1R/D2R dimers and influence the control of motor behavior. Downstream heteromer signaling is induces a switch from Gαi/o to Gαs coupling. This might stimulate phosphorylation of the dopamine- and cAMP-regulated phosphoprotein of 32 kDa ( DARPP-32) (Andersson et al., 2005). Phospho-DARPP-32 would then enhance Ca2+ influx through L-type voltage-dependent Ca2+ channels by inhibiting protein phosphatase-1 thus promoting Ca2+-dependent endocannabinoid production and
controlling neuronal activity in the basal ganglia. Thus, cross-talk of CB1Rs with other GPCRs and receptor tyrosine kinases specify the downstream signaling machinery and determine the evoked physiological response. Further identification of endocannabinoid ligands, metabolic enzymes, and receptors together with defining second messenger cascades and affected genes will allow us to understand the cellular context and microenvironmental requirements necessary for physiological endocannabinoid signaling to occur and will reveal the neural basis of developmental defects imposed by prenatal drug abuse.
4 METHODOLOGICAL CONSIDERATIONS

4.1 IMMUNOMAGNETIC INTERNEURON SORTING

The availability of cell surface markers, which are specifically expressed on identified subsets of neurons, is the basis for immunomagnetic cell-sorting (IMCS). IMCS allows for the purification of desired neuronal subtypes and for the establishment of subpopulation enriched cultures. IMCS takes advantage of the selective expression of cell-surface receptors, e.g. CB\textsubscript{1}Rs (Katona et al., 2006), channels like Kv3.1b (Du et al., 1996), extracellular matrix components (Kobayashi et al., 2002), and neuropeptides/Ca\textsuperscript{2+}-binding proteins in morphologically or functionally distinct interneuron subsets. The target repertoire is far from being exhausted and recent findings revealing substantial temporal differences in the expression of various ion channels (Antonucci et al., 2001) between interneurons and pyramidal cells during development may present new candidates.

It must be noted that antibody-based isolation methods have several limitations: interneuron differentiation continues from midgestation and persists during the early postnatal period. \textit{In vitro} maintenance of isolated interneurons has proven difficult, as otherwise required supporting factors released by astroglia and other neurons must be exogenously replenished. Therefore, embryonic, partially-specified interneurons with greater survival and plasticity appear as preferred targets for isolation technologies. Isolation methods imply enzymatic digestion of tissues to yield single cell suspensions. Enzymatic treatments affect neuronal survival, eliminate neuronal processes, and effectively cleave extracellular protein epitopes that may hamper subsequent antibody binding. However, enzymatic digestion of embryonic tissues may also be a tool to allow partial penetration of antibodies through the cell membrane and designate intracellular protein epitopes as IMCS markers (Berghuis et al., 2004; Galoppin and Saurat, 1981)
5 RESULTS AND DISCUSSION

5.1 PAPER I

Brain-derived neurotrophic factor controls functional differentiation and microcircuit formation of selectively isolated fast-spiking GABAergic interneurons

In the neocortex and hippocampus, GABAergic interneurons control the output of pyramidal neurons via the formation of inhibitory synapses on different cellular compartments. Ensembles of fast-spiking basket cells (FS cells) mainly provide perisomatic inhibition on pyramidal cells. According to the classical definition, FS cells appear as a large syncytium of electrically and chemically-coupled neurons in cortical areas and express the Ca^{2+}-binding protein PV, share the ability of rapid changes in ion homeostasis by chondroitin sulfate proteoglycan-rich polyanionic perineuronal nets, and possess the ability to maintain high-discharge firing patterns (>100 Hz). The perinatal period is critical for the development of functional GABAergic interneurons. Activity-dependent release of differentiation factors, such as BDNF, from principal cells and the activity of neonatal neuronal networks regulate the degree of synaptogenesis, synapse selection and maintenance, and synaptic plasticity. However, as the cortical interneurons represent a vastly diverse neuronal population, their subtype-specific differentiation mechanisms are largely unknown. In this paper, we developed tools to purify subsets of FS cells interneurons and study the effects of BDNF and KCl-induced sustained membrane depolarization on this particular type of neurons.

5.1.1 Isolating fast-spiking interneurons

The lack of a protocol enabling the isolation of interneuron subpopulations has made the reliable identification of specified differentiation factors difficult. In this study, we therefore developed an IMCS assay that allows the purification of neurochemically-identified interneurons. We took advantage of the unique expression of the 3.1 subtype of Kv channels in FS cells in the hippocampus and neocortex from E17 and onwards (Perney et al., 1992). Custom-made rabbit anti-Kv3.1b antibodies were linked to sheep-anti-rabbit IgG-coated superparamagnetic beads and were incubated with heterogeneous suspensions of cortical cell suspensions from E18/19 rat embryos. The subsequent isolation of cells recognized by the antibody-bound magnetic beads via a magnetic field yielded ~3.5 x 10^5 cells per embryo. Reverse transcriptase PCR analysis of the isolated cell fraction confirmed the presence of mRNA transcripts characteristic for GABAergic interneurons, including GAD65, calbindin, calretinin, neuropeptide Y, cholecystokinin-8 and somatostatin. We further confirmed the efficacy of our system by determining that over ~85% percent of the total isolated neurons were immunopositive for Kv3.1b, as compared to ~5% in heterogeneous cortical samples.

We then followed characterized the in vitro morphogenesis of isolated Kv3.1b cells by studying their neurochemical and synaptic characteristics by means of immunohistochemistry and whole-cell patch clamp electrophysiology, respectively. Current-clamp recordings revealed progressive acquiring of intrinsic discharge...
properties. Electrical maturation paralleled the onset of inhibitory synapse formation with increasing sIPSC frequencies after 12 days in vitro (DIV). Immunocytochemical analysis revealed that the isolated Kv3.1b-positive cells acquired prominent morphological and molecular markers of FS cells. At 6 DIV, cultured interneurons were surrounded by perineuronal nets and expressed, though infrequently, calbindin D28k. Their GABAergic phenotype was verified by the histochemical presence of the GABA synthesizing enzymes GAD65/67 and of the vesicular GABA-transporter (VGAT), required to shuttle inhibitory neurotransmitters in synaptic vesicles. PV immunoreactivity was only found after prolonged periods in culture (12-16 DIV), reminiscent of its delayed expression in vivo (Seto-Ohshima et al., 1990).

In conclusion, the selective expression of Kv3.1b in the embryonic neocortex and hippocampus allows the efficient and highly selective isolation of immature FS cells by an immunomagnetic protocol. The heterogeneity of interneuron subtypes and the expression of different cell-surface proteins presumably also permit for the selective targeting of cell-surface markers on other types of cortical neurons. Therefore, we propose that IMCS is a valuable tool for the identification of signaling mechanisms of specified neuronal subpopulations and their roles in the morphological differentiation and formation of neuronal networks.

5.1.2 BDNF and membrane depolarization promote the differentiation of cultured fast-spiking interneurons

Although the isolated Kv3.1b cells expressed the morphological and molecular markers commonly observed in adult FS cells in vivo, the moderate mean discharge frequency (25 ± 18 Hz) indicated improper electric maturation as compared to their in vivo counterparts (>100 Hz). Developing interneurons have been shown to be dependent on the availability of BDNF for the establishment and maturation of synapses (Rutherford et al., 1997; Wardle and Poo, 2003). However, the role of BDNF in the maturation of functional networks of FS-cells is still unclear. Functional neuronal development can further be stimulated by electrical activity (Murphy, 2003). Therefore, we studied the cooperativity of BDNF signaling and electrical stimulation in FS cell development.

We found that BDNF treatment accelerated the morphological differentiation of isolated FS cells in vitro by increasing both the somatic diameter and the extension of neurites. Significantly, the combined treatment of KCl and BDNF was superior in promoting neurite branching. Prolonged depolarization appeared detrimental to Kv3.1b-isolated cells, as indicated by the shrinkage of their neurites. In accordance with the electrophysiological maturation of FS cells, the amounts of the synaptic proteins synaptophysin and vesicular GABA transporter progressively increase over time in culture. While BDNF stimulated the expression of both above markers, sustained membrane depolarization did not have an effect on the expression of synaptic proteins. Strikingly, detectable levels of VGAT and GAD65/67 preceded those of synaptophysin, which could suggest that developing FS cells harbor the capacity to synthesize and store GABA into synaptic vesicle before functional synapses become formed. However, the lack of detectable levels of synaptophysin could be attributed to a limited sensitivity of our analysis methods, and does not necessarily prove a complete absence of the protein.
We further ventured in the assessment whether BDNF and/or sustained membrane depolarization facilitated the intrinsic discharge properties and establishment of functional inhibitory synapses. Isolated interneurons gradually acquired repetitive firing, which was accelerated by the application of both BDNF and KCl at a frequency of 80 Hz at 12 DIV. However, in parallel to the detrimental effects of KCl on neurite growth, the stimulatory effects on the electrical maturation disappeared after 16 DIV. Since adult FS cells appear as a large syncytium, we checked whether cultured FS cells form frequent connections. Dual whole-cell recordings on cell pairs located 40-70 μm apart at 12 DIV formed an extensive interconnected neuronal network when exposed to BDNF and KCl. In all experiments, cultured FS cells were reciprocally connected and also generated autaptic synapses.

In conclusion, we show that fast-spiking basket cells can be isolated from late embryonic (E18-19) cortical tissues. Furthermore, our data indicates that membrane depolarization promotes interneuron differentiation at early developmental periods, but has a detrimental effect on the formation of neurites and synapses at later stages. The combined effects of BDNF and depolarization enabled cultured FS cells to mature, suggesting that the specification of these interneurons is regulated by their activity and the release of BDNF from the neighbouring, functionally active pyramidal cells.

5.2 PAPER II

Brain-derived neurotrophic factor selectively regulates dendritogenesis of parvalbumin-containing interneurons in the main olfactory bulb through the PLCγ pathway

Similar to the neocortex, the main olfactory bulb (OB) possesses GABAergic interneurons that play a major role in the local coordination of olfactory processing. Most interneurons are located in the EPL or surrounding the glomeruli, and act to fine tune the output of the mitral/tufted cells. The previous paper established a role for BDNF in the micro-circuit formation of PV-expressing FS cells in the neocortex. Subsequently, we have addressed whether BDNF plays, and through which signaling mechanisms, a similar role in the acquiring of synaptic connectivity and phenotypic interneuron differentiation in the main olfactory bulb.

5.2.1 Interneuron differentiation is impaired in BDNF−/− mice

To address the role of BDNF in olfactory bulb development, we studied mice lacking BDNF (BDNF−/−) (Ernfors et al., 1994). Macroscopic morphology of the OB was affected by BDNF as their size in BDNF−/− mice were 30-50% of wild type littermates. These changes were due to a decreased cell density in the cell-compacted zone (CCZ), the EPL and ML.

Specific populations of GABAergic interneurons in the OB can be distinguished by the expression of Ca²⁺-binding proteins (Celio, 1990; Kosaka et al., 1987). PV-expressing neurons are predominantly present in the EPL, whereas the glomeruli are surrounded by CB and CR-immunoreactive neurons. At postnatal day 6, BDNF−/− mice showed a reduced density of PV-expressing cells in the EPL, which was accompanied by the pronounced decrease in the immunoreactivity of PV-labeled dendrites. The detrimental
effects of BDNF removal on neuronal morphology appeared confined to the PV-expressing cell population, since prominent effects on CB and CR containing interneurons were not observed. The suppressed number of PV-ir cells and dendrites endured throughout the early postnatal period (up to day 30), thus excluding a mere delay in PV expression in BDNF

We addressed whether the above effects on dendritic morphology and cell numbers were specific to BDNF, or could be replaced by the actions of NT-3. For this purpose, we generated mice that express NT3 instead of BDNF (BDNFNT3/NT3) (Agerman et al., 2003). NT3 replacement led to a partial recovery of lost functions, as shown by the increased neuronal densities in the CCZ and the reversed compression of the EPL. Furthermore, the number of PV-ir neurons and cells bearing putative dendrites were increased in BDNFNT3/NT3 relative to BDNF

5.2.2 Specification of TrkB receptor signaling regulates interneuron survival and growth

BDNF signals are translated into a cellular response through the TrkB receptor by recruiting the adaptor molecules Shc/Frs2 or by activating PLCγ. To analyze whether Shc/Frs2 signaling is involved in the proper development of PV-ir interneurons, we used mice carrying a targeted single point mutation in the Shc/Frs2 binding site of the TrkB receptor (TrkBShc/Shc) (Minichiello et al., 1998). Similar to our results obtained in the BDNF-/- mice, we found a reduced density of PV-ir neurons in the TrkBShc/Shc signaling mutants. However, the number of cells with extensive dendritic trees was not affected, indicating that Shc/Frs2 is critical for interneuron survival but not for the regulation of dendrite growth in vivo.

We corroborated our findings by studying the effects of specific TrkB signaling pathway inhibitors on the survival and dendrite growth of cultured neurons derived from the olfactory bulb. BDNF significantly enhanced the neurite branching and elongation of cultured olfactory neurons. Inhibition of PLCγ and MAPK kinase signaling cascades suppressed the BDNF-induced neurite outgrowth without affecting the density of GABAergic neurons in the culture. Therefore, PLCγ and MAPK signaling appears to be critical for the differentiation, but not survival. In contrast, inhibition of PI3K signaling significantly reduced the density of BDNF-treated GABAergic interneurons, thus highlighting its role in regulating survival decisions.

The continuous addition of new interneurons in the olfactory bulb, persisting throughout postnatal life, assumes a high degree of adaptation and plastic reorganization of replenished GABAergic olfactory neurons together with their continuous ability to integrate into existing neuronal microcircuitries. In this study we have identified BDNF and its downstream signaling components as crucial regulators of the survival and differentiation programs of PV-expressing olfactory interneurons. Consequently, episodic impairments to BDNF/TrkB signaling may affect the proper establishment and continuous turnover of inhibitory components in olfactory neuronal networks and thereby affect sensory information.
5.3 PAPER III

Endocannabinoids regulate interneuron migration and morphogenesis by transactivating the TrkB receptor

The endogenous cannabinoids AEA and 2-AG have originally been identified as retrograde messengers, capable of modulating synaptic plasticity by inhibiting presynaptic neurotransmitter release through presynaptic CB₁Rs (Wilson and Nicoll, 2001). So far, studies addressing the roles of endocannabinoids primarily focused on understanding their modulatory actions on synaptic functions in the adult nervous system. However, mounting evidence indicates the existence of functional CB₁Rs in the embryonic nervous system (Mato et al., 2003), suggesting additional, yet unidentified roles endocannabinoids may subserve in the developing CNS.

Recent studies have shown that endocannabinoids promote neuronal progenitor proliferation and dictate progenitor differentiation into an astroglial lineage (Aguado et al., 2006). Contradicting effects of endocannabinoids and synthetic CB₁R agonists on neuronal proliferation in the adult dentate gyrus have been reported, however (Galve-Roperh et al., 2006; Jiang et al., 2005). Our caveat of knowledge on endocannabinoid actions on the positioning and phenotypic differentiation of neurochemically-identified neuron populations in the cerebral cortex, prompted us to study whether endocannabinoids are involved in controlling interneuron specification during corticogenesis.

5.3.1 Endocannabinoids as regulators of interneuron migration and differentiation

Since CB₁R-expressing interneurons represent a small percentage of the total neuronal mass in the neocortex and hippocampus, we established an IMCS protocol selectively isolate CB₁R-bearing neurons. Similar to the method described in Paper I, magnetic beads were linked to rabbit anti-CB₁R antibodies against an epitope on the extracellular N-terminal domain. Isolated neurons, (72,000 ± 16,000 cells per embryo) were immunoreactive for GAD65/67 (96%) and contained the vesicular GABA transporter, identifying that isolated cells belonged to the group of GABAergic interneurons. CB₁Rs and fatty-acid amide hydrolase (FAAH) were localized to axonal varicosities and growth cones in isolated interneurons. From an electrophysiological standpoint, cultured interneurons were predominantly regular spiking and formed inhibitory connections.

Our studies demonstrated that BDNF is required to fully differentiate cortical PV-containing interneurons. Similarly, BDNF triggered the extension of neurites of isolated CB₁R-expressing neurons in vitro. In contrast, AEA inhibited neuronal growth and even suppressed the BDNF-induced neurite branching and elongation in a competition assay. The restraining effects of AEA on interneuron differentiation were further supported by a reduced hyperpolarization of interneuron membranes. We concluded that endocannabinoids may direct the differentiation program of interneurons by primarily suppressing neurite elongation and synaptogenesis.
Cortical interneurons are derived from extracortical proliferative zones and undergo both long-distance and tangential migration and short-distance intracortical migration to achieve their final positions in specific cortical layers (Anderson et al., 2001; Marin and Rubenstein, 2003). Neuronal migration is regulated by a wide variety of diffusible factors (Marin and Rubenstein, 2003). Endocannabinoids have been shown to have chemotactic effects on microglial cells (Walter et al., 2003). Therefore, we tested whether endocannabinoids could modulate interneuron migration. Using a Boyden chamber assay, we identified the endocannabinoid AEA as a chemotaxic agent for CB₁R-expressing interneurons, an effect that was entirely dependent on CB₁R activation. The specificity of AEA actions on CB₁R-bearing interneurons is illustrated by the finding that isolated FS-cells, which lack CB₁Rs, do not respond to extracellular AEA signals. In contrast, BDNF exerted chemotactic effects on both types of interneurons.

5.3.2 Cannabinoids activate TrkB receptors

Recent data demonstrated that Trk receptors can be trans-activated by GPCRs (see Chapter 2.1). CB₁Rs have been shown to dimerize and interact with other classes of GPCRs (Kearn et al., 2005). Notably, CB₁R activation leads to rapid transactivation of the EGF receptor in cancer cells, via proteolytic ectodomain shedding of EGF precursors (Hart et al., 2004). As TrkB and CB₁Rs are targeted to the same cellular compartments during neuronal differentiation, we hypothesized that the CB₁R could exert some of its cellular effects through TrkB receptor transactivation. Stimulation of CB₁R-expressing interneurons with AEA induced a transient increase (peaking at 15-30 minutes) in the phosphorylation of membrane-targeted TrkB receptors. Immunoprecipitation assays, using transfected PC12 cells over-expressing both the CB₁R and TrkB receptors revealed a close interaction between the two receptors after AEA exposure. The assembly of CB₁R/TrkB complexes was also determined during 2-AG stimulation and was inhibited by AM251. The transactivation event was partly mediated by Src-kinases, as indicated by a reduced TrkB activation via AEA in the presence of the Src-inhibitor PP2. The physiological function of CB₁R-mediated TrkB activation is illustrated by the reduced chemotraction of GABAergic interneurons to AEA upon selective inhibition of TrkB receptor phosphorylation.

Our results identify a novel role for cannabinoids and the CB₁R during cortical development. AEA emerges as a chemo-attractive and morphogenic cue for CCK/CB₁R expressing neurons. Our hypothesis is supported by the finding that Δ⁹-THC treatment in doses mimicking marijuana intake in humans, increased the density of CCK⁺ expressing interneurons in the strata radiatum, lacunosum moleculare of the CA1-CA3 subfields of the hippocampus. We propose that the placement and connectivity patterns of particular cortical interneuron subclasses is defined by the availability and interplay of target-derived endocannabinoids and BDNF.

5.4 PAPER IV

Hardwiring the brain: Endocannabinoids control axon guidance

Growing axons are guided through the ligand-dependent activation of cell-surface receptors in their growth cones. Axonal growth cones are generally considered as the
cellular compartments to translate cues from various diffusible factors to a directional response. In the immature CNS, active CB1Rs were associated with developing axonal tracts in the white matter of mouse and human fetus (Bisogno et al., 2003). We previously identified the presence of CB1Rs in growth cones of developing cortical interneurons in vitro (paper III in this thesis) that was linked to a reduced neurite growth in response to endocannabinoids and synthetic CB1R ligands. In addition, the expression patterns of the CB1Rs coincide with increasing levels of AEA and 2-AG during the period of cortical network formation (Berrendero et al., 1999). These data lend support to the concept that endocannabinoids could serve as diffusible guidance factors, involved in axon guidance and synaptogenesis.

5.4.1 CB1Rs are associated with elongating axons in vivo

To confirm our hypothesis, we first characterized the subcellular localization of CB1Rs in the developing telencephalon. By using GAD67*GFP/+ mice, which express GFP in most GABAergic neurons, we localized CB1Rs to axons targeted to the cortex between the subplate and the intermediate zone at E13.5. CB1R-immunoreactivity was also observed in pyramidal cells that populate the forming hippocampus. At later stages developmental stages, white matter regions including the cingular blade, fimbria-fornix and corpus callosum exhibited prominent CB1R immunoreactivity. The presence of CB1Rs in major forebrain axonal tracts during the period of target-innervation and synaptogenesis supports our notion that endocannabinoids play a role in axon guidance. CB1Rs were first observed in tangentially-migrating GABAergic neurons from E12, at which the first wave of cortical interneurons leaves from the ganglionic eminence. However, CB1Rs became clearly associated with axonal growth cones only during the peak of axonal and dendritic growth and the synapse formation in GABAergic interneurons. This was further verified by the significant enrichment of CB1Rs in isolated growth cone particles from late-gestation embryonic cortices. We concluded that the combined presence of endocannabinoid (Berrendero et al., 1999) together with the targeting of the CB1Rs to axonal growth cones strongly suggest the involvement of endocannabinoid signaling in axonal guidance mechanisms in vivo.

We further identified the cellular localization of CB1R in axonal growth cones of isolated CB1R containing interneurons from E18/19 cortices. CB1Rs were distributed across the entire growth cone, including its actin-rich filopodia and in the tubulin-filled central domain and thus were ideally positioned to convert extracellular signals to changes in cytoskeletal dynamics. Activation of Erk1/2 by AEA in isolated growth cone particles and primary cultures revealed functionally active CB1Rs in axonal growth cones. In summary, CB1Rs are preferentially targeted to axonal growth cones of differentiating cortical interneurons with their subcellular ideally suiting the sensing of extracellular diffusible endocannabinoid guidance cues.

5.4.2 Endocannabinoids guide axonal growth cones

To test the effects of endocannabinoids on axon guidance, we adopted a growth cone turning assay (Ming et al., 1997), during which we applied continuous microgradients of BDNF and WIN55,212-2, a synthetic CB1R agonist, at growing axons of isolated CB1R-expressing neurons. We found that axonal growth cones turned towards a BDNF
point-source, in agreement with previous results of ours and others (Ming et al., 1999; Yuan et al., 2003) showing that BDNF promotes neurite growth. In contrast, a local gradient of WIN55,212-2 induced growth cone collapse and neurite retraction, or elicited growth cone repulsion, when compared to control gradients. We confirmed that these repellent effects were mediated by CB1Rs as the selective CB1R inverse agonist AM251 reversed chemorepulsion to chemotraction. We corroborated our findings by studying the effects of the cannabinoids AEA and WIN55,212-2 on directional growth cone steering of *Xenopus laevis* spinal neurons (that endogenously express CB1Rs) in a physiological electric field (Rajnicek et al., 2006a; Rajnicek et al., 2006b). *Xenopus* neurons steadily turned towards the cathode under control conditions. In contrast, application of AEA or WIN55,212-2 reduced the turning angle and the frequency of axons turning towards the cathode in a dose-dependent manner.

A growth cone turning response is established by a polarized dysbalance between polymerizing actin filaments and contracting myosin filaments (Yuan et al., 2003). Cytoskeletal dynamics are regulated by members of the Rho family of small GTPases, including Rho, Rac and Cdc42 (Jaffe and Hall, 2005). GTPases can be activated by a multitude of receptors, including Trk receptors and GPCRs, but their association with CB1R activation has not been described yet. We therefore studied whether Rho GTPases were activated in response to CB1R stimulation in primary cortical neurons. We found that RhoA was rapidly activated by both AEA and WIN55,212-2. Growth cone repulsion is commonly mediated by activation of RhoA (Jin et al., 2005; Yuan et al., 2003), which subsequently activates ROCK and the phosphorylation of myosin light chains. Based on our finding that agonist stimulation of CB1Rs activates RhoA, we tested the involvement of RhoA in the repulsive turning induced by WIN55,212-2. Pretreatment of our interneuron cultures with the ROCK-inhibitor Y-27632 (Yuan et al., 2003) converted WIN55,212-2 induced repulsive growth cone turning to chemotraction without affecting the rate of neurite elongation. Overall, we identified that CB1Rs couple to Rho GTPases and linked CB1Rs to growth cone repulsion via remodeling of the actin-cytoskeleton.

The in vivo relevance of our findings was studied by determining the density of CB1R-containing hippocampal and cortical afferents in adult mice lacking CB1Rs in forebrain GABAergic neurons (CB1R<sup>fl<sup>DLX5/6-Cre</sup></sup> mice) (Monory et al., 2006). We studied axonal arbors that otherwise have expressed CB1Rs by localizing vesicular glutamate transporter 3 (VGLUT3), a selective synaptic marker of CB1R-containing inhibitory terminals in the adult (Hioki et al., 2004). We found a significant increase in the density of VGLUT3-ir neuronal processes in the CA1 and dentate gyrus of the hippocampus, and layer 2/3 of the somatosensory cortex. Our in vivo data indicates that genetic ablation of CB1Rs may relieve the inhibition of axonal elongation posed by endocannabinoid signaling through CB1Rs during corticogenesis.

Overall, we defined a novel developmental role for endocannabinoid signaling in controlling the directional guidance of neuronal growth cones. CB1R-mediated axon guidance is evident in diverse neuronal populations as demonstrated in vitro by growth cone turning assays on GABAergic interneurons in rodents and *Xenopus* spinal neurons. Our evidence thus suggests that endocannabinoids play an unexpected role in axonal pathfinding.
6 CONCLUSIONS

Cortical network formation, and in particular the differentiation of local GABAergic interneurons is regulated by a vast array of diffusible factors. In this thesis we provided evidence showing that;

- immuno-magnetic cell sorting allows for the isolation of selected interneuron subtypes

- BDNF, in combination with sustained membrane depolarization, promotes the differentiation and functional microcircuit formation of cortical FS cells

- in the main olfactory bulb, BDNF and TrkB regulate PV expression and dendrite growth in GABAergic interneurons via activation of the PLCγ pathway

- endocannabinoids suppress BDNF-induced interneuron differentiation

- endocannabinoids act as chemo-attractants for migrating CB1R-containing GABAergic interneurons and accordingly affect the density of CCK-expressing interneurons in the hippocampus

- endocannabinoids transactivate the TrkB receptors through a signaling mechanism involving Src activation

- endocannabinoids function as extracellular growth cone repellent factors during the perinatal period corticogenesis
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