Increased Transforming Growth Factor-β1 Modulates Hippocampal Glutamatergic Synaptic Protein Expression and Synaptic Transmission

by

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Abstract

Transforming growth factor-beta 1 (TGF-β1) is a multifunctional cytokine that orchestrates key events of development, disease and repair in the central nervous system (CNS). To investigate the effects of chronically producing TGF-β1 on synaptic structure and synaptic transmission, I performed immunohistochemistry and immunoblot of brain tissues from transgenic mice (TGF-β1 mice) that over-express active form of TGF-β1 from astrocytes in the CNS. Immunohistochemical assays showed that synaptophysin increased in the CA3 subfield whereas calbindin-D28K decreased in the mossy fibres. Immunoblot analysis revealed that several α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptor subunit proteins were up-regulated in the hippocampus of TGF-β1 mice. To examine the direct effect of TGF-β1 alone on glutamatergic synaptic activity, cultured hippocampal neurons were treated with or without TGF-β1. Electrophysiological recordings displayed that TGF-β1 significantly increased the amplitude of glutamate-evoked current (p<0.05). Taken together, these data suggest that TGF-β1 modulates hippocampal glutamatergic synaptic protein expression and regulates synaptic transmission.
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Chapter 1
Introduction

1.1 Overview of TGF-β

Transforming growth factor-beta (TGF-β) was discovered in the early 1980s for its capacity to cause phenotypic transformation of normal rat kidney cells in soft agar (Roberts et al., 1981). It is a prototypic molecule of a growth factor superfamily that is grouped into four subfamilies by virtue of sequence similarities, including the TGF-β family, the glial cell line-derived neurotrophic factor (GDNF) family, activins, and decapentaplegic (Dpp) and vegetal-1 (Vg1)-related (DVR) group, which is often referred to as the bone morphogenetic protein (BMP) family. The superfamily comprises as many as 100 distinct proteins and nearly 30 proteins in mammals (Bottner et al., 2000, Gomes et al., 2005). Soon after the discovery of TGF-β, it became apparent that the biological activities of TGF-β extend far beyond the context of its initially described transforming properties. The multiplicity of the roles of TGF-β as a regulatory cytokine was demonstrated early when it was shown to act differently in the same cells in which it was assayed. For example, in the presence of platelet-derived growth factor (PDGF), TGF-β stimulated cell growth, whereas in the presence of epidermal growth factor (EGF), it functioned as a growth inhibitor (Roberts et al., 1985). TGF-βs are now widely considered as multifunctional cytokines that play regulatory roles in key events of development, disease, and repair of many organs and systems (Bottner et al., 2000, Gomes et al., 2005, Vivien and Ali, 2006). They participate in a wide spectrum of cell and tissue functions, ranging from cell-cycle control to regulation of embryogenesis and organogenesis, extracellular matrix metabolism, hematopoiesis, and angiogenesis (Bottner et al., 2000, Gomes et al., 2005, Vivien and Ali, 2006, Massague et al., 2000, Pelton et al., 1991).

To date, five isoforms of the TGF-β family (also known as TGF-β proper) have been isolated, three of which are expressed in mammalian tissues: TGF-β1, -β2, and -β3. Expressed in numerous tissues such as mesenchyme, connective tissues, endothelium, platelets, and immune and bone cells (Bottner et al., 2000), all five TGF-βs have 64-82% homology and share
commonalities in their synthesis from a long precursor as well as in essential structural features such as conservation of all nine cysteine residues in the processed peptide. Notably, TGF-β1 is identical in human, monkey, pig, cow, and chicken, while human TGF-β3 has only one conservative amino acid substitution when compared with its chicken counterpart (Roberts et al., 1990).

Following the identification and characterization of TGF-β (Assoian et al., 1983), it began to be recognized as an active modulator of immune and inflammatory processes (Wahl, 1994, Sporn, 2006). At peripheral sites of injury, TGF-βs are released locally from platelet stores early in an inflammatory response and is then further generated by inflammatory cells themselves as part of the cytokine network. As one of the first agents on scene of trauma or infection, it instigates the ensuing response by orchestrating leukocyte recruitment and activation (Wahl, 2007). Upon activation of inflammatory cells, TGF-βs then down-regulate these processes by inhibiting their functions (Tsunawaki et al., 1988) and subsequently mediates healing through resolution of inflammation and promotes tissue repair by inducing fibroblast recruitment and extracellular matrix synthesis (Wahl, 1994). Therefore, TGF-β may act as both the instigator and an innate depressant of immune response (Wahl, 2007), and it would be a mistake to label this growth factor as a promoter or suppressor of one condition. This apparent contradiction in the influence of TGF-β on immune cells, both stimulatory and inhibitory, is accounted for, at least in part, by its varied effects on resting and activated cells. In general, the resting or immature immune cells receive stimulatory effects (McCartney-Francis et al., 1990), whereas activated representatives of the same cell populations may observe inhibitory effects (Tsunawaki et al., 1988). Perceivably, disrupting the balance of TGF-β that serves as a converting factor, switching an active inflammation site into one dominated by repair, may have pathological consequences (Wahl, 1994).

The need for maintaining TGF-β balance extends beyond the immune system into signalling networks that control the differentiation, growth and final state of cells. TGF-β is both a tumour-suppressor and promoter. Given that inhibition of cell growth in response to TGF-β is predominant in epithelial, endothelial, hematopoietic, neural, and certain types of mesenchymal cells (Massague et al., 2000), disruption of its signalling pathway contributes to tumourogenesis (Shi and Massague, 2003). Tumour-derived mutations have been observed in TGF-β family
receptors. In fact, TGF-β receptor is inactivated by mutation in most human gastrointestinal cancers with microsatellite instability (Grady et al., 1999), and a number of other somatic and hereditary disorders are a result of mutations or malfunctions in the signalling proteins involved TGF-β pathway (Massague et al., 2000).

In general, the effects of TGF-β1 and other isoforms are pleiotropic and embedded in their contextual environment, in which the extent of their influences depend on interactions with other cytokines and growth factors as well as the type and differentiation state of target cells.

1.1.1 Molecular Structure of TGF-β

TGF-β1, the first discovered member of the TGF-β superfamily (Roberts et al., 1981), is a 25 kDa homodimer. Comparable to the typical structure of secreted signalling molecules, TGF-βs are synthesized as latent preproproteins that undergo proteolytic cleavage for receptor recognition (Assoian et al., 1983). An amino-terminal signal sequence targets the molecule to the secretory pathway, and a carboxy-terminal fragment of 110-140 amino acids that contains seven invariant cystein residues is released as a result of processing at dibasic cleavage sites (RXXR motifs). These released carboxy-terminal domains then undergo homo or heterodimerization, which generates the biologically active molecules (Bottner et al., 2000). Prior to the dimerization, the monomers make up several extended β strands interlocked by three conserved disulfide bonds that form a tight structure known as the “cystein knot.” The dimeric active form of this cytokine is stabilized by hydrophobic interactions that are furthered fortified by an intersubunit disulfide bridge in most cases (Shi and Massague, 2003). This conversion from the latent to the active form is crucial for bioactivity, and it also represents an important mechanism by which TGF-β1 activity is regulated in vivo (Wyss-Coray et al., 1995).
1.1.2 TGF-β Receptors and TGF-β-mediated Signal Transduction

TGF-β isoforms elicit cell type-specific responses through the ligand-induced formation of a heteromeric receptor complex between two cell-surface proteins termed type I (TβR-I; 53 kDa) and type II (TβR-II; 70-100 kDa) receptors (Massague, 1990). TGF-βs bind to a third type of receptor (TβR-III; 200-400 kDa) that corresponds to two related proteins called betaglycan or endoglin, and it is believed to be involved in formation of receptor complexes and modulates ligand access to the signalling receptors (Cheifetz et al., 1992, Lopez-Casillas et al., 1993). In contrast, TβR-I and TβR-II proteins belong to the class of transmembrane protein serine/threonine kinase receptors, which, with the exception of GDNF and related factors, mediate signal transduction of all TGF-β family members. In the human genome, the serine/threonine receptor kinase family consists of 12 members – 7 TβR-I and 5 TβR-II receptors (Manning et al., 2002). They are glycoproteins comprised of a short extracellular ligand-binding region, a single transmembrane segment, and an intracellular region with kinase activity (Bottner et al., 2000, Shi and Massague, 2003). The binding of a TGF-β ligand first to the TβR-II receptor leads to a physical and functional interaction with the TβR-I receptor on the cell surface. The formation of this heteromeric complex triggers TβR-II to phosphorylate a conserved region located immediately upstream of the TβR-I kinase domain called the GS domain, which contains a characteristic glycine- and serine-rich sequence. Activated TβR-I then induces the propagation of signal transduction through transient recruitment and subsequent phosphorylation of Smad proteins, which carry the TGF-β signal into the cell nucleus (Bottner et al., 2000, Shi and Massague, 2003). The Smad proteins are vertebrate homologues of the drosophila protein, mothers against decapentaplegic (Mad), and the Caenorhabditis elegans protein Sma.

There are eight known distinct Smad proteins that are divided into three functional classes. The first class consists of receptor-mediated Smads (R-Smads), which are direct substrates of specific type I receptor kinases acting in a pathway-restricted fashion. Smad-1, -5, and -8 are associated with BMP type I receptors, while Smad-2 and -3 are linked with type I receptors of activin and TGF-β (Lagna et al., 1996, Zhang et al., 1996). The second class comprises the common-mediator Smads (Co-Smads), which form heteromeric complexes with activated R-Smads. The Smad complexes translocate to the nucleus and are required for the assembly of transcriptional apparatus with other nuclear cofactors to activate responses of target genes (Massague et al.,
Within the cytosol, released Smad-2 associates with Smad-4, the only known vertebrate Co-Smad member. Smad-4 is the central signalling component as it is capable of binding to different pathway-specific Smads (Lagna et al., 1996). The third class contains inhibitory Smad (I-Smad) that serves to counteract the activity of R-Smad/Co-Smad complexes (Bottner et al., 2000, Shi and Massague, 2003). Smad-6 and -7 are the most divergent mammalian Smad proteins that antagonize TGF-β and BMP signalling at multiple levels. Inhibition may occur by competing with R-Smads for type I receptor (Imamura et al., 1997, Nakao et al., 1997) or Smad-4 interaction (Massague, 1998) and by targeting the receptors for degradation (Shi and Massague, 2003). Thus a precise and complex repertoire of cell-specific transcription factors and recruitment of co-activators and co-repressors collectively dictate which target genes are activated upon stimulation with a given ligand (Hill, 1999).
1.2 TGF-β in the Central Nervous System

1.2.1 Expression of TGF-βs and TβRs

From early embryonic stages of mammals, mRNA and protein of all three TGF-β isoforms are expressed in the central nervous system (CNS) and peripheral nervous system (PNS) (Bottner et al., 2000, Miller et al., 1989, Flanders et al., 1991). However, each isoform has a different distribution in vivo (Pelton et al., 1991) and thus is considered to be an independent regulatory molecule. Immunohistochemical and in situ hybridization studies supplemented by northern blotting have indicated that in the healthy developing and adult brain, TGF-β2 and -β3 isoforms account for virtually all the TGF-β immunoreactivity while TGF-β1 is restricted to the meninges and choroid plexus (Bottner et al., 2000, Flanders et al., 1991, Unsicker et al., 1991). Recently, Miller reported that TGF-β1 ligand is also expressed by cells in the neocortical proliferative zones and neurons in the cortical plate. Unlike the protein, TGF-β1 mRNA is constitutively expressed in certain regions of the brain, such as the hippocampus, cortex and hypothalamus (Vivien and Ali, 2006, Plata-Salaman et al., 2000), suggesting that TGF-β1 protein expression may be spatially regulated. At the cellular level, both in vivo and in vitro evidence suggest that all CNS cell types can respond to and be a source of TGF-β (Vivien and Ali, 2006). Upon lesioning of the brain, TGF-β1 is induced in neurons, astrocytes and microglia (Flanders et al., 1998).

TβR-I and TβR-II expression is also widespread in the developing and mature CNS, and many studies have reported the presence of their mRNAs in specific regions of the brain, including the cortex and hippocampus (Bottner et al., 1996, Soderstrom et al., 1996, Tomoda et al., 1996, Slotkin et al., 1997, Vivien et al., 1998). Several neuronal populations present in the cortex, hippocampus, brainstem and spinal cord, as well as astrocytes and microglia express receptors for TGF-βs in vivo and in vitro, whereby their spatial and temporal expression is regulated during development and injury (Unsicker et al., 1991, Flanders et al., 1998, Vivien et al., 1998, De Groot et al., 1999, de Sampaio e Spohr et al., 2002, Buckwalter and Wyss-Coray, 2004, Sousa Vde et al., 2004).
1.2.2 TGF-β1 in Neural Functions

TGF-β1 is the best studied TGF-β isoform. Its role in the nervous system began to emerge almost two decades ago (Flanders et al., 1991, Finch et al., 1993, Krieglstein et al., 1995), and it is now widely recognized as an important component of nervous stem functions, including regulation of neuronal survival (Schober et al., 1999, Brionne et al., 2003), neuronal migration (Bottner et al., 2000, Siegenthaler and Miller, 2004), astrocyte development and differentiation (Gomes et al., 2005), cerebral gene expression (Lesne et al., 2002), and orchestration of brain’s response to injury (Vivien and Ali, 2006, Flanders et al., 1998).

Despite the similarities of TGF-β isoforms, TGF-β1 appears to exert distinct functions. In vivo distribution of TGF-β1 and the receptors point to its role in brain development. A recent study has indicated that TGF-β1 knockout mice show a significant increase in degenerating neurons with prevalent neuronal apoptosis, reduced neocortical presynaptic function, decreased ECM protein laminin, and microgliosis (Brionne et al., 2003). Thus, the endogenous level of this cytokine appears to modulate cortical development. Interestingly, TGF-β2 and -β3 are often co-expressed and subsequently act in conjunction. For instance, TGF-β2, -β3 and their receptors are expressed in early embryonic structures such as notochord and floor plate, as well as in the area where midbrain dopaminergic neurons are developing (Gomes et al., 2005). A study led by Krieglstein’s group reported that in addition to Sonic hedgehog, one of the inductive signals derived from the ventral midline that is required for the development of midbrain dopaminergic neurons, TGF-β2 and -β3 are essential mediators of the induction and survival of midbrain dopaminergic neurons (Farkas et al., 2003). Several other groups have also reported similar findings (Poulsen et al., 1994, Roussa et al., 2004). These studies point to the plausibility that TGF-βs are candidate molecules for therapeutic agents in neurological disorders, especially Parkinson’s disease.

Several groups have reported TGF-β1 expression in neuron and astrocyte cultures (Vivien et al., 1998, de Sampaio e Spohr et al., 2002, Sousa Vde et al., 2004, Lindholm et al., 1992), further supporting the role of TGF-β1 in the unlesioned brain. The immunoreactivity of TGF-β1 in neurons was described in different brain regions, albeit with slightly different pattern of labelling. Cerebellar and cortical neurons displayed punctuate labelling throughout the neuronal
processes whereas midbrain neurons were mainly stained in the soma (Sousa Vde et al., 2004). With this region-specific expression pattern of TGF-β1 in neurons, more work is needed to delineate the exact distribution of TGF-β1 in the different cell types of the brain.

Other studies have revealed a possible relationship between TGF-β1 and astrocytes. All three TGF-β isoforms are known inhibitors of astrocyte proliferation (Lindholm et al., 1992, Toru-Delbauffe et al., 1990, Baghdassarian et al., 1993, Hunter et al., 1993). They induce growth suppression either directly or in concert with other growth factors such as fibroblast growth factor (FGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), interleukin-alpha (ILα) and interleukin-2 (IL2), by enhancing or antagonizing their actions (Hunter et al., 1993, Vergeli et al., 1995, Krieglstein et al., 1998, Krieglstein et al., 1998). However, their effects on proliferation may be region-specific as TGF-β1 was shown to differentially regulate primary astrocytes isolated from forebrain and brainstem (Johns et al., 1992). TGF-β1 also affects morphology and motility of astrocytes. In the presence of FGF, in vitro treatment of TGF-β1 has shown to cause changes in cellular morphology, namely the development of branched processes that are characteristic of hypertrophic astrocytes and colony formation (Toru-Delbauffe et al., 1990, Labourdette et al., 1990). In addition, TGF-β1 alters astrocytic cell-cell interactions and induces cytoskeleton rearrangement associated with cell movement (Gagelin et al., 1995). More recently, TGF-β1 has emerged as a novel mediator of neuron-glia interactions, especially during astrocyte differentiation (de Sampaio e Spohr et al., 2002). In vivo administration of TGF-β1 induces several neuronal and astrocytic cytoskeleton genes such as glial fibrillary acidic protein (GFAP), an astrocyte marker, and tubulin (Laping et al., 1994). Also, TGF-β1 has an effect on the extracellular matrix (ECM) metabolism. It stimulates the production and the incorporation of laminin and fibronectin into the ECM of primary cultures of cerebellar astrocytes (Baghdassarian et al., 1993). Correspondingly, in vivo overproduction of TGF-β1 by astrocytes in transgenic mice resulted in increased production of laminin and fibronectin (Wyss-Coray et al., 1995) whereas TGF-β1 knockout mice presented a reduced expression of laminin (Brionne et al., 2003). Given the importance of ECM in CNS development, the various changes promoted by TGF-β1 in astrocyte morphology and growth as well as in cytoskeleton and ECM production further strengthen the role of this cytokine in brain development.
In the CNS, there is relatively little evidence about the Smad pathway (Gomes et al., 2005). A study by Zhou et al. showed that Smad-4 knockout mice with a specific disruption of this protein in the brain presented abnormal motor control and a decrease in the number of Purkinje cells in the cerebellum (Zhou et al., 2003). Another study by Burton et al. demonstrated one of the first evidences of TGF-β1-induced Smad nuclear translocation in astrocytes (Burton et al., 2002). They showed that TGF-β1 treatment induced the expression of Alzheimer β amyloid precursor protein (APP) gene in human astrocytes via a Smad-3/4 dependent pathway, and that this high level of APP and TGF-β1 was correlated with a significant increase in expression and nuclear localization of Smad-4 in Alzheimer cases (Burton et al., 2002). TGF-β/Smad activated pathways represent an emergent field (Gomes et al., 2005). Understanding the molecular events in this circuit will further elucidate the role of TGF-β in CNS development and disease.

1.2.3 TGF-β1 and Neuroprotection

TGF-β1 has been characterized as an injury-related cytokine in the CNS on the grounds that it is up-regulated in many acute or chronic disorders (Vivien and Ali, 2006). There are several lines of evidence suggesting that this up-regulation may exhibit neuroprotective functions. Chronic cerebral production of TGF-β1 has shown to protect neurons against both acute and chronic injury (Brionne et al., 2003). TGF-β1 also plays important beneficial roles in cerebral ischemia (Docagne et al., 2003, Lin et al., 2006). Following experimental hypoxia (McNeill et al., 1994), global (Lehrmann et al., 1995, Zhu et al., 2000) or focal (Lehrmann et al., 1998, Ruocco et al., 1999, Yamashita et al., 1999) ischemia, the expression of TGF-β1 mRNA and protein was shown to increase. Given the secretion of this cytokine in neurons, astrocytes, activated microglia and endothelial cells under ischemic conditions, a consensual answer to its cellular sources remain elusive (Vivien and Ali, 2006). The discovery of an endogenous production of TGF-β1 in response to ischemia instigated several studies that demonstrated its intrinsic neuroprotective role in the brain. Ruocco et al., found that administration of a soluble receptor to block the action of TGF-β1 production in response to ischemic insult significantly exacerbated transient focal ischemic damages in rats (Ruocco et al., 1999). Moreover, additional studies addressing exogenous application of TGF-β1 showed similar protective functions. Intra-
cerebroventricular injection of TGF-β1 before the induction of focal ischemia in mice (Prehn et al., 1993) or global ischemia in rats (Henrich-Noack et al., 1996) led to a moderate suppression of brain lesions. Also, adenovirus-mediated overproduction of TGF-β1 was found to be beneficial following transient ischemia in mice (Pang et al., 2001). There exists a concourse of studies that reveal beneficial activity of both the exogenous and endogenous TGF-β1 in ischemic conditions. However, the exact mechanisms involved in this neuroprotection remains inconclusive (Vivien and Ali, 2006). Perhaps the array of proposed mechanism is a testament to the multiplicity of the roles of this cytokine.

In vitro studies have further demonstrated the neuroprotective nature of TGF-β1. The cytokine exerts selective neuroprotection against N-methyl-D-aspartate (NMDA) receptor-mediated excitotoxicity, while remaining ineffective against α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) or kainite toxicity (Buisson et al., 1998, Docagne et al., 2002, Buisson et al., 2003). This protective activity is initiated at the astrogentic level through the binding of TGF-β1 to its receptors, which induces the recruitment of Smad3 and subsequently the synthesis and release of the type 1 plasminogen activator inhibitor (PAI-1) in astrocytes. PAI-1 inhibits the deleterious proteolytic activity of the serine protease tissue plasminogen activator (tPA) on NMDA receptors. Depolarized neurons release tPA, which potentiates NMDA receptor-mediated calcium influx and neuronal death by cleaving the amino terminal domain of the NMDA receptor NR1 subunit (Nicole et al., 2001, Fernandez-Monreal et al., 2004).

In addition to the protective role of TGF-β1 in excitotoxicity, there have been many investigations of its anti-apoptotic activity in the CNS, particularly in specific cell types and experimental conditions. Krieglstein’s group conducted in vitro studies that have demonstrated the ability of TGF-β1 to block staurosporin-mediated activation of caspase-3, a major inducer of apoptosis, in cultured hippocampal neurons (Zhu et al., 2001). They proposed that this anti-apoptotic effect depends on both the phosphatidylinositol-3-OH (PI3) kinase/Akt and extracellular-signal related kinases-1 and 2 (Erk1/2) pathways (Zhu et al., 2004). Generally, the mechanism of this anti-apoptotic activity involves a shift in the balance between pro- and anti-apoptotic members of the Bcl-2 family. In vivo adenovirus-mediated over-expression of TGF-β1 was shown to suppress ischemia-induced expression of the pro-apoptotic protein Bad, a member
of the Bcl-2 family, and activation of caspase-3, which consequently resulted in reduce neurological deficits and ischemic lesions (Zhu et al., 2002).

1.2.4 TGF-β1 and Neuropathology

In addition to the neuroprotective properties of TGF-β1, dysregulations in its signalling pathways are implicated in a number of neurological diseases (Vivien and Ali, 2006, Buckwalter and Wyss-Coray, 2004). In the CNS, TGF-β1 secretion is related to the control of inflammation and immune responses upon injury and aging (Gomes et al., 2005, Finch et al., 1993, By et al., 2001). All three isoforms are produced by both neurons and glial cells (Lindoholm et al., 1992, de Sampaio e Spohr et al., 2002, Sousa et al., 2004). In the injured brain, astrocytes and microglia are the major source of TGF-β1 (Finch et al., 1993), but neurons also possess the capacity for its production (Flanders et al., 1998).

Increased levels of TGF-β1 is associated with various forms of brain insults and autoimmune and neurodegenerative diseases such as AIDS, HIV-1 encephalitis and Alzheimer’s disease (AD) (Vivien and Ali, 2006, Flanders et al., 1998, Buckwalter and Wyss-Coray, 2004, Finch et al., 1993, Wahl et al., 1991). For instance, in brain tissues of AIDS patients, TGF-β1 protein expression was confined mainly to astrocytes and microglia (Wahl et al., 1991). AD is quickly emerging as a complication that is strongly implicated with TGF-β1 (Table 1). Two neuropathological hallmarks that characterize the AD brain are neurofibrillary tangles, which correspond to abnormal phosphorylation of the microtubule associated protein tau (Sergeant et al., 2005) and the accumulation of amyloid plaques. The amyloid plaques are primarily comprised of extracellular deposits of amyloid-β peptides (Aβ), which is derived from the processing of the transmembrane protein APP (Selkoe and Schenk, 2003, Tanzi and Bertram, 2005). Abnormal production or accumulation of Aβ is implicated in the pathogenesis of AD, but more cofactors are likely to modulate Aβ toxicity (Buckwalter and Wyss-Coray, 2004). There is strong evidence that indicate TGF-β1 as an important regulator of Aβ deposition in the brain. In AD patients, immunoreactivity of TGF-β was found in both amyloid plaques and tangles, with TGF-β1 staining primarily evident in plaques and TGF-β2 in tangles, microglia and glial cells
These results have also been observed in rodents. A line of transgenic mice that has been engineered to over-express the human APP carrying the familial AD mutations showed an astroglial overproduction of TGF-β1 and -β3 around amyloid deposits (Apelt and Schliebs, 2001). The effects of increased TGF-β1 on amyloid plaques have also been studied in a different transgenic mouse model. These mice, which chronically overproduce TGF-β1 in astrocytes (Wyss-Coray et al., 1995), showed AD-like microvascular degeneration in adult mice (Wyss-Coray et al., 2000) and amyloidosis in the cerebral vasculature (Wyss-Coray et al., 1997). Interestingly, transgenic mice that over-express both human APP and TGF-β1 displayed a significant reduction in parenchymal amyloid plaques and overall Aβ load in the hippocampus and neocortex when compared with singly transgenic human APP littermate controls (Wyss-Coray et al., 2001). This decrease in parenchymal plaques was associated with a strong activation of microglia and astrocytes in vivo, an observation strengthened by the demonstration of TGF-β1 stimulated Aβ clearance in microglial cultures (Wyss-Coray et al., 2001). Moreover, it was found that these bigenic human APP/TGF-β1 mice exhibit earlier Aβ deposits around cerebral vessels than single APP transgenic mice, further suggesting that TGF-β1 is capable of enhancing APP metabolism or processing (Wyss-Coray et al., 1997). This positive effect that TGF-β1 has on APP expression has been also reported in several in vitro studies, both in rodents (Lesne et al., 2003) and human astrocytes (Burton et al., 2002). Studying chronic over-expression of TGF-β1 in a mouse model for AD thus revealed that TGF-β1 has a key role in the development of cerebral amyloid angiopathy and also reduces amyloid deposition in the parenchyma, highlighting the opposing effect of TGF-β1. As such, therapeutic approaches blocking the effect of TGF-β1 on the vasculature or promoting its effect in the brain parenchyma can be explored (Buckwalter and Wyss-Coray, 2004).
Table 1: TGF-β1 in Alzheimer’s disease (AD)

<table>
<thead>
<tr>
<th>Condition/model</th>
<th>Changes Observed/Induced</th>
</tr>
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</table>
| Expression of TGF-β1 in AD | - Decreased TGF-β1 plasma levels (Mocali et al., 2004)  
- Increased levels of TGF-β1 in cerebrospinal fluid (Zetterberg et al., 2004), brain parenchyma and amyloid plaques (Peress and Perillo, 1995, van der Wal et al., 1993)  
- Increased TGF-β1 mRNA in post-mortem brain tissues of AD patients (correlated with amyloid-β deposition in damaged cerebral blood vessel (Wyss-Coray et al., 1997) |
| TGF-β1 mice | - Amyloid-β deposition in cerebral blood vessels and meninges (Wyss-Coray et al., 1997)  
- AD-like microvascular degeneration (Wyss-Coray et al., 2000) |
| hAPP/TGF-β1 bigenic mice | - TGF-β1 accelerated deposition of amyloid-beta peptide in cerebral blood vessels (Wyss-Coray et al., 1997)  
- TGF-β1 promotes microglial activation-associated clearance of parenchymal (but not cerebrovascular) amyloid-β peptide (Wyss-Coray et al., 2001)  
- TGF-β1 reduces overall cerebral amyloid-β peptide load and plaque burden in the brain parenchyma (Wyss-Coray et al., 2001) |

TGF-β1 mice = transgenic mice that over-express TGF-β1; hAPP/TGF-β1 bigenic mice = transgenic mice that over-express both TGF-β1 and human amyloid precursor protein (hAPP) and develops AD-like pathology
1.2.5 TGF-β1 and Behaviour

Despite the multifunctional nature of TGF-β1 in the central nervous system, its role in behaviour remains unknown. More than a decade ago, Hsiao and colleagues found a correlation between memory deficits and Aβ elevation in adult transgenic mice over-expressing an isoform of human APP (Hsiao et al., 1996). Given the extensively described functional association between TGF-β1 and Aβ, one can implicate the plausible effect that TGF-β1 may have on mammalian behaviour. However, there is a surprising lack of studies in the literature that report behavioural consequences as a direct function of TGF-β1 expression.

Very recently, our collaborators in the Frankland laboratory have made strides into this virtually untouched field. They have found convincing data on the consequences of chronic TGF-β1 overproduction on cognitive behaviour and adult neurogenesis using a transgenic mouse model (unpublished data, via personal communication). In all, the role of TGF-β1 on neuronal functions and properties in the hippocampus presents a novel field, and it promises to be a topic that presages further delineation of the widespread impact that TGF-β1 has in brain function.
1.3 The Hippocampus and Synaptic Transmission

1.3.1 Hippocampal Circuitry

The hippocampal formation comprises four cortical regions, all of which interconnect by largely unidirectional projections to form a continuous neuronal network. These regions include the entorhinal cortex, the dentate gyrus, the hippocampus proper (which can be divided into three general subfields, namely CA1, CA2 and CA3), and the subicular complex, which is subdivided into subiculum, presubiculum and parasubiculum. The general circuitry of the hippocampus can be summarized as follows. Synaptic input to the hippocampus proper network stems from axons originating from the dentate gyrus, which receives input from the entorhinal cortex, known as the perforant pathway. The dentate gyrus granule cells give rise to distinctive axons, collectively referred to as the mossy fibres (MF). MFs collateralize in the polymorphic layer of the dentate gyrus and provide a prominent input to the stratum lucidum where they form en passant synapses on the proximal dendrites of CA3 pyramidal cells. The CA3 pyramidal cells have highly collateralized axons that contribute to associational projections that terminate within CA3 and also give rise to the major projection to CA1 pyramidal cells known as the Schaffer collaterals. The axons of CA1 neurons connect back to the entorhinal cortex via the subicular complex, forming the hippocampal circuit (Figure 2) (Amaral and Witter, 1989).
1.3.2 Glutamate and Glutamate Receptors

1.3.2.1 Overview of Glutamate Receptors

Glutamate is the principal excitatory neurotransmitter in the mammalian CNS. It accounts for the synaptic transmission in approximately half of the synapses in the forebrain (Simeone et al., 2004). Upon release from the presynaptic terminals, glutamate binds to the various types of glutamate receptors (GluRs). GluRs are intimately involved in both the physiological and pathological functions of the brain. They mediate most of the excitatory transmission in the mammalian CNS, and they also play a central role in regulating plastic changes in synaptic transmission underlying memory and learning as well as the formation of neural networks during development (Ozawa et al., 1998, Santos et al., 2009). Excessive activation of GluRs during stress to the brain such as ischemia, head trauma and epileptic seizures leads to the death of central neurons, and glutamate neurotoxicity may be involved in the geneses of various neurodegenerative diseases (Ozawa et al., 1998).

GluRs are largely divided into two distinct classes: ionotropic and metabotropic receptors. The ionotropic receptors contain cation-specific ion channels, and the binding of glutamate leads to a conformational change that allows passage of Na\(^+\) and Ca\(^{2+}\) through a pore. They are further categorized into two subgroups: NMDA receptors and non-NMDA receptors, which are subdivided into AMPA and kainate receptors. Alternatively, metabotropic receptors are coupled to GTP-binding proteins (G-proteins) and modulate the signalling pathway of intracellular messengers, including the phosphorylation of voltage-gated and ligand-gated ion channels and gene transcription (Simeone et al., 2004, Ozawa et al., 1998). With the dramatic technical advancements made over the past couple of decades, such as the molecular cloning technology and manipulations of the GluR gene expression, the GluR system has been extensively investigated. This dissertation focuses on describing the properties of ionotropic glutamate receptors, primarily the AMPA receptors (AMPARs) and NMDA receptors (NMDARs), in the context of hippocampal and neocortical synaptic transmission, with a specific emphasis on the hippocampal mossy fibre-CA3 pyramidal synapses.
1.3.2.2 AMPA Receptors: Structure, Distribution, and Synaptic Transmission

AMPA receptors mediate the majority of fast excitatory neurotransmission in the CNS (Ozawa et al., 1998). AMPAR are either homomeric or heteromeric tetramers comprised of 4 subunits (GluR1-4 or A-D), which are of similar size (~900 amino acids with a molecular weight of about 105 kDa) and share approximately 70% amino acid sequence identity (Keinanen et al., 1990, Collingridge et al., 2004). Each subunit consists of a large extracellular N-terminus, four hydrophobic domains, M1-M4 (three of which transverse the membrane while one is a hairpin loop that contributes to the pore-lining region), and an intracellular C-terminus whose tail length varies between subunits (Simeone et al., 2004, Malinow and Malenka, 2002). GluR2, in particular, is arguably the most interesting subunit of the four in regard to receptor function. Its mRNA undergoes editing at the “Q/R site” (Burnashev et al., 1992) and it dictates AMPAR biophysical properties and interacts with various molecules associated with receptor trafficking (to be discussed below) (Collingridge et al., 2004, Isaac et al., 2007). GluR2-containing AMPARs are Ca\(^{2+}\)-impermeable (CI-), have low conductance, have relatively linear I-V relationships (Ho et al., 2007) and are less sensitive to voltage-dependent polyamine block (Hollmann et al., 1991, Donevan and Rogawski, 1995). The majority of AMPARs in mature principal neurons contain this critical subunit and thus CI-AMPARs dominate transmission between excitatory neurons, whereas CP-AMPARs participate at principal cell synapses early in development in various regions of the CNS (Isaac et al., 2007, Ho et al., 2007). However, in certain neuronal populations and under specific physiological and pathological conditions, GluR2-lacking AMPARs are expressed (Isaac et al., 2007, Terashima et al., 2004). These AMPARs lacking edited GluR2 are readily Ca\(^{2+}\)-permeable (CP-), have higher conductance, and exhibit inwardly rectifying I-V relationships caused by voltage-dependent channel block by intracellular polyamines (Donevan and Rogawski, 1995, Koh et al., 1995). As such, variations in the assemblies of these subunits account for remarkable differences in functional and kinetic properties of AMPARs and thus synaptic function.

Following the molecular identification of the receptor subunits, studies were conducted to examine the AMPAR distributions in the CNS. AMPA subunits have widespread expression profiles across brain regions and are present in neurons, interneurons and some populations of
glial cells, including astrocytes (Martin et al., 1993, Bahr et al., 1996, Seifert et al., 2003, Lin and Bergles, 2004). Using in situ hybridization histochemistry (Keinanen et al., 1990), it was revealed that there are regional differences in the distribution of AMPAR densities, but they are in general ubiquitously expressed throughout the CNS. In the hippocampus, the GluR1, GluR2 and GluR3 mRNAs are abundantly expressed in the pyramidal cell layer and dentate gyrus. In contrast, the expression of GluR4 mRNA is considerably less than that of GluR1-3 mRNAs, and is relatively higher in CA1 and dentate gyrus than in CA3 subfield. In the cerebral cortex, GluR2 mRNA is uniformly found in all layers, while the expression patterns of GluR1, GluR3 and GluR4 mRNAs differ among layers (Ozawa et al., 1998, Santos et al., 2009, Keinanen et al., 1990). Immunocytochemical findings indicate a prominence of heteromeric AMPARs composed of GluR1-GluR2 or GluR2-GluR3 subunits in the hippocampus, with a small population of homomeric GluR1 in the CA1/CA2 region (Wenthold et al., 1996). On the other hand, one study found that CA3 pyramidal neurons appear to primarily express GluR1 and GluR2 subunits (Geiger et al., 1995). Despite the difference in expression profiles within the hippocampus, patch clamp experiments demonstrated that the functional properties of dendritic glutamate receptors, both AMPARs and NMDARs, in CA1 and CA3 subfields appear to be similar (Spruston et al., 1995).

AMPARs play important roles in controlling synaptic function in the developing and mature CNS (Simeone et al., 2004). They are critical in the expression of different forms of long term plasticity such as long term potentiation (LTP) and long term depression (LTD) (Santos et al., 2009, Isaac et al., 2007). Briefly, LTP is characterized by a long lasting enhancement in the strength of synaptic transmission following a short period of repetitive high frequency stimuli, and LTD is reduction in the efficacy of synaptic transmission caused by a period of low-frequency synaptic stimulation (Santos et al., 2009). Chemical forms of LTP and LTD also exist, which is induced by pharmacological stimulation. Furthermore, postsynaptic AMPARs contribute to homeostatic plasticity, whereby chronically inhibiting glutamate receptors leads to an increase in surface AMPAR expression at synapses and in the amplitude of AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs) – mechanisms likely used by neurons to stabilize the loss of activity and synaptic strength (Santos et al., 2009). In addition to their role in neuronal plasticity, AMPARs regulate excitotoxicity, mediated largely by the influx of Ca$^{2+}$ (Choi et al., 1988). Their involvement has also been implicated in animal models of
ischemia and neurodegenerative disorders (Pellegrini-Giampietro et al., 1994). It was reported that prior to cell death, hippocampal CA1 pyramidal cells exhibit enhanced AMPAR-mediated CA\textsuperscript{2+} influx and decreased mRNA and protein levels of GluR2 and GluR3 (Pellegrini-Giampietro et al., 1992, Gorter et al., 1997).

1.3.2.3 NMDA receptors: Structure, Distribution, and Synaptic Transmission

To date, seven subunits have been identified in the NMDA receptor family: NR1, NR2A, NR2B, NR2C, NR2D, NR3A, and NR3B. NMDARs form a tetrameric complex, and most receptor complexes have a heteromeric composition of two NR1 and two NR2 subunits, including those in the hippocampus, while few receptor channels are homomerically assembled with only NR1 subunits. Receptors with this homomeric subunit composition show significantly less current responses than their heteromeric counterparts (Ozawa et al., 1998), as do those assembled with NR3 and NR1 or NR2 subunits (Wenthold et al., 2003). Similar to other ionotropic glutamate receptors, these NMDAR receptor subunits share the same basic structure, whereby each has a large extracellular NH\textsubscript{2}-terminal, four transmembrane domains (M1-M4), of which the M2 segment contributes to the lining of the channel pore, and an intracellular C-terminal. NR1 and NR2 subunits are only about 20% homologous to each other, however, the four NR2 isoforms are more homologous (55 to 70%) (Simeone et al., 2004). Likewise, NR3A and NR3B share approximately 50% homology with one another, but considerably less with the NR1 and NR2 subfamilies (Chatterton et al., 2002). NR1 functions as a fundamental subunit to form heteromeric NMDARs, as NR2 subunits can form functional receptor channels only when co-expressed with NR1. Thus, the NR2 subunits can be considered as modulatory subunits (Ozawa et al., 1998) that are critical for several biophysical and pharmacological properties of the NMDAR. This includes sensitivity to Zn\textsuperscript{2+}, protons, polyamines, the high affinity for glutamate, modulation by glycine, fractional Ca\textsuperscript{2+} current, single channel conductance, and channel kinetics such as open probability and deactivation time (Groc et al., 2009). NMDARs are activated by the binding of glutamate, and glycine also serves as an obligate co-agonist required for the receptor channel to enter the open state (Ozawa et al., 1998). The binding site of glutamate site is located
on the NR2 subunit, and glycine binds to a site located on the NR1 subunit (Simeone et al., 2004, Groc et al., 2009).

Numerous ligand binding and in situ hybridization studies examining the distribution of NMDA receptor subunits have shown that NMDA receptors are found throughout the brain but predominantly within the forebrain. The hippocampal CA1 region, in particular, display the highest levels. In the adult rodent, the NR1 mRNA is distributed ubiquitously across the brain. The four NR2 transcripts, on the other hand, have distinct regional patterns. The NR2A transcript has widespread distribution in the brain, but is concentrated in the cerebral cortex, hippocampus and cerebellum. NR2B mRNA is selectively present in the forebrain with high expression profiles in certain regions including the hippocampus, cerebral cortex, septum, caudate putamen and olfactory bulb. NR2C and NR2D transcripts were mainly detected in cerebellum and midbrain structures, respectively. In the adult hippocampus, NR2A and NR2B mRNAs are predominant in CA1 and CA3 pyramidal cells, and NR2C and NR2D mRNAs are found in a subset of hippocampal neurons, most likely interneurons (Ozawa et al., 1998, Monyer et al., 1994). The expression pattern of the synaptic NMDAR subunits is regulated developmentally, whereby the NR2A/NR2B ratio of synaptic NMDARs increase over time. NR2B-containing receptors predominate in the hippocampus and other brain regions early in the development, while NR2A expression is low shortly after birth but continues to increase at synapses (Wenthold et al., 2003, Monyer et al., 1994). This change in synaptic composition of NMDAR subtype can also be driven by activity (Barria and Malinow, 2002, Bellone and Nicoll, 2007), experience (Mierau et al., 2004), or learning (Quinlan et al., 2004). Interestingly, NMDAR binding in the stratum lucidum is much lower than in other areas of the hippocampus – an observation that initially set apart mossy fibre synapses to be different from other excitatory synapses in the hippocampus (Nicoll and Schmitz, 2005). Indeed, the relative contribution of NMDA responses to AMPA responses at mossy fibre synapses is approximately a third of that found simultaneously at neighbouring associational-commissural synapses (Weisskopf and Nicoll, 1995). Immunohistochemical experiments have substantiated these findings by showing reduced levels of NMDAR subunits at mossy fibre synapses (Watanabe et al., 1998). Conceivably, alterations in NMDAR expression can have unique implications in the hippocampal CA3.
There are a few characteristics that provide NMDARs molecular basis for their role in synaptic transmission and plasticity. With regard to ion permeation properties, Na\(^+\), K\(^+\) and Cs\(^+\) ions permeate through the channel with low selectivity, but the major differences from non-NMDARs is their high permeability to Ca\(^{2+}\) during the activation of the receptor (Mayer and Westbrook, 1987) and voltage-dependent block by Mg\(^{2+}\) within the channel pore (Mayer et al., 1984). At resting potentials, the channel remains blocked by Mg\(^{2+}\), and ionic currents pass only when the neuronal membrane is sufficiently depolarized (Ozawa et al., 1998). In synaptic plasticity, the activation of NMDAR initiates a set of events that leads to the rapid recruitment of AMPARs into the synapses (Santos et al., 2009). Although both receptor types are actively involved in the regulation of long term synaptic activity, NMDARs mediate excitatory synaptic transmission in ways that are distinct from AMPARs. NMDARs have an affinity for glutamate that is approximately 500 times higher than that of AMPAR, but they are thought to contribute less to basal synaptic transmission and are responsible for the delayed component of the excitatory postsynaptic current (EPSC) (Simeone et al., 2004). The NMDA-mediated EPSC has a much slower rise and decay times relative to the AMPA-EPSC, which is a result of slow gating kinetics of NMDARs (Lester et al., 1990). Not surprisingly, the pattern of subunit composition controls synaptic plasticity and dictates the nature of EPSCs produced (Barria and Malinow, 2005). The kinetics of NMDA EPSCs become faster during development in the CNS and correlate with an increase in expression of the NR2A subunit and a decrease in the sensitivity to NR2B-selective antagonists (Wenthold et al., 2003). This can be explained, at least in part, by the fact that NR2A-containing receptors produce faster (Monyer et al., 1994) and smaller EPSCs (Barria and Malinow, 2002) than NR2B-containing receptors, and thus allow less Ca\(^{2+}\) entry, which could affect LTP (Quinlan et al., 2004). The different patterns of NMDAR subunit assembly may also regulate synapse maturation. Expression of the NR2B subunit is correlated with a high release probability that decreases as neurons mature (Wenthold et al., 2003). In all, it is conceivable that differential spatial and temporal changes contribute to the fine tuning of NMDAR functions and their channel properties in both the developing and adult brains, which, in turn, may modify long-term synaptic activity.
1.3.2.4 Trafficking of Glutamate Receptors and their Interacting Proteins in Synaptic Plasticity

Phosphorylation is a post-translational modification that regulates AMPAR function, physiological properties of the channel and protein trafficking (Santos et al., 2009). There is growing evidence in the literature that endorses a critical role for the trafficking of AMPARs and NMDARs to and from synapses during forms of long term synaptic plasticity (Malinow and Malenka, 2002). It is an area of great interest as it provides light into the potential cellular mechanisms that underlie learning and memory. As mentioned above, the subunits show differences in their C-terminus tail, and AMPAR trafficking is mediated by several associated proteins that interact with specific C-terminal tail of the receptor subunits. Most of these proteins have single or multiple PDZ (postsynaptic density-95/discs large/zonula occludens-1) domains through which the C-terminus binds. The proteins that interact with GluR2/3 include glutamate receptor interacting protein (GRIP), AMPA receptor-binding protein (ABP), and protein interacting with C kinase 1 (PICK1) (Santos et al., 2009).

PICK1 is known to interact with GluR2 and GluR3 subunits and is involved in NMDA-induced AMPAR trafficking (Hanley and Henley, 2005, Terashima et al., 2008). Studies have demonstrated a role for PICK1 in regulating the GluR2 content transiently in MF-PYR synapses early in development (Ho et al., 2007) and also in hippocampal CA1 synapses (Terashima et al., 2004). Specifically, Terashima et al. reported that PICK1 causes a decrease in endogenous GluR2, but not GluR1, surface expression – resulting in GluR2-containing Cl-AMPAR being replaced by GluR2-lacking CP-AMPAR – and thus is involved in the regulation of synaptic strength and AMPAR subunit composition via its PDZ domain at synapses in the CA1 (Terashima et al., 2004). Furthermore, PICK1 is required for several forms of synaptic plasticity in diverse areas of the CNS (Isaac et al., 2007). In a recent study, Isaac and colleagues demonstrated a requirement for PICK1 in the hippocampal NMDAR-dependent bidirectional synaptic plasticity by proving that gain or loss of PICK1 function affects NMDAR-dependent LTP and LTD (Terashima et al., 2008). More recently, Haglerod et al. further substantiated the importance of PICK1 in the regulation of AMPARs in hippocampal excitatory synapses. The authors found that PICK1 and GluR2 are co-expressed in the presynaptic plasma membrane as well as in vesicles (Haglerod et al., 2009), which expands on the established role of PICK1 in
mediating Ca\(^{2+}\)-dependent recycling of GluR2 in the postsynaptic compartment (Hanley and Henley, 2005). However, the requirement of the GluR2 subunit in the trafficking of AMPAR appears debatable. A study using KO mice lacking GluR2/3 subunits provided evidence that GluR2 is not necessary for the activity-dependent recycling of AMPARs. This finding suggests that the more important role of GluR2 in the context of trafficking may be the effect of Ca\(^{2+}\) permeability on AMPAR endocytosis (Biou et al., 2008). The differences in the conclusions reached may be attributed to methodological differences and specific type or condition of the cell. In any case, more studies using multiple strategies are needed to delineate the cell-specific mechanism of AMPAR surface expression in the context of synaptic plasticity.

Like the AMPAR, NMDAR is not a static resident of the synapse. The number and composition of synaptic NMDARs across different surface compartments (synaptic, extrasynaptic) is modulated by several factors, and its trafficking mechanisms provide plausible molecular pathways for LTP and LTD and thus learning and memory (Wenthold et al., 2003). Compared to AMPARs, they are more stable components of the postsynaptic membrane, and their stability at synapses can be provided by interactions with various proteins. For instance, the basal rate of the surface AMPAR endocytosis in cultured cortical neurons is nearly threefold that of the NMDAR (Huh and Wenthold, 1999). Thus, synaptic plasticity is generally more associated with changes in distribution and numbers of surface AMPARs than it is with NMDARs. Out of more than 70 proteins that have been shown to interact with the NMDAR, one of the best characterized proteins is PSD-95, an abundantly expressed protein in the postsynaptic density. PSD-95 binds directly to the C-terminus of the NR2 subunit through its PDZ domain, and it also acts in conjunction with other related proteins to link NMDARs and AMPARs to influence targeting of synaptic receptors (Wenthold et al., 2003). Interestingly, a number of studies have shown that mice lacking different NR2 subunits retain the ability to form functional synapses, meaning while these subunits play important roles in long-term synaptic function, they are not crucial in regulating subunit trafficking to the synapse (Wenthold et al., 2003). In response to stimulation, induction of LTP in the immature CA1 synapses has shown to induce a rapid redistribution of synaptic NR2 subunits (Bellone and Nicoll, 2007). Thus, alterations in subunit composition can affect long-term synaptic transmission and vice versa. In general, there is still a relatively large gap of knowledge concerning how this protein and the likes exactly participates in NMDAR
trafficking. Further studies would better elucidate how NMDAR recycling affects long term synaptic function.

1.3.3 Mossy Fibre-CA3 Pyramidal Cell Synapses

The dentate gyrus is the main source of inputs to the hippocampus. It has two blades, an upper suprapyramidal blade closer to the CA1 and a lower infrapyramidal blade which are separated by the hilus region. These two blades converge adjacent to the entorhinal cortex (Amaral and Witter, 1989). The granule cell is the principal cell of the dentate gyrus. These cells have relative small somata when compared with other principal cells of the hippocampal formation, and its dendritic branches are highly variable in number and shorter than their CA1 and CA3 pyramidal neuron counterparts (McBain, 2008). Axons of these neurons are unique in that they form anatomically specialized synapses depending on the nature of their postsynaptic targets. They innervate numerous cell types within the hilar compartment via local collaterals and their MF projections to the stratum lucidum contain few branch points (Claiborne et al., 1986).

The MF synapse occurs between the granule cells of the dentate gyrus, targeting both within the hilar region and CA3 subfield of the hippocampus proper. A growing number of studies have revealed that MF synapses possess unique anatomical and physiological properties, such as the fact that MF-CA3 pyramidal (MF-PYR) connections mature entirely postnatally (Ho et al., 2007). In addition, they are a specific example of a synapse designed for target-specific compartmentalization of synaptic transmission (McBain, 2008). Three basic types of mossy fibre presynaptic terminals are present along the entire length of the main axon: large mossy fibre boutons (MFBs) that primarily target CA3 pyramidal cells and hilar mossy cells, filopodial extensions that project from these large mossy boutons, and small en passant varicosities (Nicoll and Schmitz, 2005, McBain, 2008, Claiborne et al., 1986). Interestingly, the latter two smaller terminals mainly innervate only γ-aminobutyric acid (GABA)-ergic inhibitory interneurons in the hilus and stratum lucidum, and they considerably outnumber the large MFBs in both the hilus and the CA3 subfield (McBain, 2008). Thus, if based solely based on the number of synapses, the primary targets of dentate gyrus granule cells are inhibitory interneurons (Acsady et al.,
Such segregation in the anatomy of MF terminal types is unique throughout the CNS, suggesting that it may serve as functional specialization of synaptic output. Indeed, several studies have highlighted that the parent MFB and the filopodial extensions function independently with regard to their respective transmitter release probabilities, mechanism of plasticity, and their differential sensitivity to high-frequency stimulation (McBain, 2008).

1.3.4 Synaptic Transmission of the Mossy Fibre Pathway

The dentate gyrus granule cell is known to secrete the neurotransmitter glutamate (McBain, 2008). In addition to glutamate release, excitatory transmission at the MF synapses depends on the activation of glutamate receptors on the postsynaptic neuron. There is remarkable target cell specificity at the MF synapse, and multiple receptor mechanisms as well as short-term and long-term responses to stimulation exist (Nicoll and Schmitz, 2005). Synapses can differ in their response to repetitive activation – some may show synaptic depression while others show facilitation. Compared to most other synapses in the CNS, the MF-PYR synapse exhibits large paired-pulse facilitation, a presynaptic phenomenon that interacts closely with neurotransmitter release (Salin et al., 1996). Also, a particularly unusual property at this synapse is its ability to undergo marked frequency facilitation, whereby increasing the frequency of stimulation from low to moderate rates can cause multiple increases in synaptic strength (Nicoll and Schmitz, 2005). In contrast to MF-PRY synapses, the neighbouring Association-Commissural Synapses and Schaffer Collateral Synapses in CA1 show little facilitation (Nicoll and Schmitz, 2005). Interestingly, the pronounced short-term facilitation is specific to the large MFB; MF terminals targeting interneurons show much less facilitation and can even undergo depression (Toth et al., 2000). Thus, the various forms of synaptic plasticity at MF synapses are target-specific and are distinctive from that found at typical cortical synapses and even within the hippocampal network.
1.3.5 Long Term Potentiation and Long Term Depression

Much of the current understanding of the hippocampal short- and long-term plasticity has been derived from studies that have focused on the Schaffer collateral-CA1 synapse (McBain 2008). The pace of studies being reported on the synaptic plasticity at MF synapses has been increasing in the last few decades, and both universal consensus and controversies on the topic have emerged. There are two areas of unanimous agreement. First, the induction of MF LTP is NMDAR-independent (Harris and Cotman, 1986). The exact mechanism involved in the induction of MF LTP, however, remains as one of the contentious debates. The controversies surround the involvement of postsynaptic Ca\(^{2+}\) and the various roles of glutamate receptor subtypes (Nicoll and Schmitz, 2005). The second universal consensus is that MF LTP is expressed presynaptically by a persistent increase in neurotransmitter release (Staubli et al., 1990, Kobayashi et al., 1996). Interestingly, despite the finding that the induction of MF LTP was independent of NMDAR activation, it has been revealed that MF synapses possess the ability to evoke NMDAR currents (Weisskopf and Nicoll, 1995). There are many factors that could have contributed to the disagreements, including technical difficulties of selectively recording from MF synapses (Nicoll and Schmitz, 2005).

In the CNS, many synapses show activity-dependent long-term changes in synaptic strength. LTP at the Schaffer collateral-CA1 synapse and most other synapses in the CNS requires the activation of NMDARs and is widely accepted to be expressed postsynaptically as an increase in AMPAR activities (Nicoll and Schmitz, 2005). However, as described above, plasticity at the MF synapse is fundamentally different from other excitatory synapses in the hippocampus. Cyclic AMP (cAMP) is a key factor that accounts for the presynaptic expression of LTP (Nicoll and Schmitz, 2005). MF-PRY LTP arises via an adenylyl cyclase-cAMP-dependent mechanism involving the activation of protein kinase A (PKA). Studies employing knockout mice have revealed that PKA substrates in MF terminals required to strengthen synaptic transmitter release include the synaptic vesicle protein Rab3A (Castillo et al., 1997) and the active zone protein Rim1a, which also binds to Rab3A (Castillo et al., 2002). It is also important to note that presynaptic Ca\(^{2+}\) entry is unchanged during MF LTP (Kamiya et al., 2002). Thus, the collective evidence indicates that a rise in postsynaptic Ca\(^{2+}\) has little involvement in MF LTP, whereas
considerable data supports a role for presynaptic Ca\textsuperscript{2+} channels, specifically, voltage-dependent R-type channels (Nicoll and Schmitz, 2005).

MF synapses also express LTD, and as is the case with LTP, the mechanisms are fundamentally distinct from those operating at others excitatory synapses in the CNS (Nicole et al., 2001). Synapses made by MFs onto interneurons have a gamut of short- and long-term response to synaptic stimulation, including pronounced depression to modest facilitation (Toth et al., 2000). There are two types of MF-interneuron synapses – synapses that are formed with either CP-AMPARs or CI-AMPARs, as occurs on CA3 pyramidal cells (Nicoll and Schmitz, 2005). Interestingly, the high-frequency induction protocol that is used to elicit LTP at MF-PYR synapses induces two different forms of LTD at MF-interneuron synapses. This differential distribution of plasticity along the same axon indicates that the divergent MF presynaptic terminals have distinct functional properties such as their ability to modify release independently (McBain, 2008). At the MF-interneuron synapses comprised of CI-AMPAR, LTD is NMDAR-dependent, has a postsynaptic induction and expression, and, similar to NMDAR-dependent LTD observed at other synapses, it involves down-regulation of surface AMPARs via endocytosis. In contrast, synapses that contain CP-AMPARs are NMDAR-independent, are expressed presynaptically, and their activation result in a reduction in transmitter release probability (McBain, 2008). One fascinating feature of the MF-interneuron synapses is the coordinated subunit composition of the postsynaptic AMPARs and NMDARs. Interestingly, CI-AMPARs generally occur at synapses together with NR2B-lacking NMDARs, whereas CP-AMPARs are typically found at synapses also populated with NR2B-containing NMDARs (Bischofberger and Jonas, 2002, Lei and McBain, 2002).
Chapter 2
Rationale, Hypothesis, and General Goals

2.1 Rationale

TGF-β1 is an injury-related cytokine and exerts multifaceted functions in the developing and mature brain. Specifically, it regulates development and differentiation, promotes cell survival or induces apoptosis, stimulates cell proliferation or inhibits growth, and initiates or resolves inflammation (Bottner et al., 2000, Gomes et al., 2005, Vivien and Ali, 2006, Buckwalter and Wyss-Coray, 2004). Its effects depend on the condition of the cell’s environment, the type of cell involved, and the level and duration of TGF-β1 production. In the mammalian CNS, TGF-β receptors are expressed in neurons, astrocytes and microglia; changes in the level and duration of TGF-β1 induction in these cells confer a broad range of effects on their functions (Flanders et al., 1998, Vivien et al., 1998).

TGF-β1 expression is up-regulated acutely after various forms of brain insults and chronically in neurodegenerative disorders such as Alzheimer’s disease (Buckwalter and Wyss-Coray, 2004), suggesting that TGF-β1 is an important component of brain’s response to injury. Transgenic mice over-expressing TGF-β1 have been helpful in understanding its implications in neurodegenerative diseases. Surprisingly, apart from the findings reported by Wyss-Coray and colleagues, few data is available concerning the effects of increased TGF-β1 on synaptic structure and transmission in the hippocampus, a brain structure that is critically associated with learning and memory. Different forms of synaptic plasticity at glutamatergic synapses are proposed as cellular mechanisms underlying learning and memory. Recent findings from Dr. P. Frankland’s laboratory suggest that chronically increasing the production of TGF-β1 impairs adult neurogenesis and cognition in mice (personal communication). The general goal of my thesis project is to examine whether increased TGF-β1 affects the expression of glutamatergic synaptic proteins in the hippocampus, and hence regulates synaptic transmission.
2.2 Hypothesis

I hypothesize that increased TGF-β1 modulates glutamatergic synaptic transmission and the expression levels of synaptic proteins, including ionotropic glutamate receptors, in the hippocampus and neocortex.

2.3 General Goals

1. To observe the consequences of chronically increased TGF-β1 production on the expression of synaptic proteins by using a transgenic mouse line that over-expresses active form of porcine TGF-β1 driven by a GFAP promoter.

2. To examine the effect of TGF-β1 alone on the morphology and functions of cultured glial and neuronal cells, in particular:
   a) Morphological and proliferative properties of astrocytes.
   b) Synaptic transmission assessed by measuring various transmembrane currents, including glutamate receptor and voltage-gated ion channel mediated currents.
Chapter 3
Materials and Methods

3.1 Transgenic mice

Transgenic mice (line T64), engineered to express constitutively active form of porcine TGF-β1 under control of a GFAP promoter, have been generated and described in the work of Wyss-Coray and colleagues (Wyss-Coray et al., 1995, Brionne et al., 2003, Wyss-Coray et al., 2000, Wyss-Coray et al., 1997, Wyss-Coray et al., 2001, Wyss-Coray et al., 1997, Buckwalter et al., 2006). These transgenic mice (TGF-β1 mice) express TGF-β1 protein in astrocytes throughout the brain, and primary astrocytes from TGF-β1 mice secrete about four times more bioactive TGF-β1 than normal non-transgenic astrocytes. Both the porcine (transgene) and murine (endogenous) TGF-β1 mRNA levels were increased in brains of TGF-β1 mice (Wyss-Coray et al., 1995), with transgenic TGF-β1 mRNA approximately twofold greater than endogenous mRNA (Wyss-Coray et al., 2000). In particular, TGF-β1 mice express TGF-β1 protein approximately twenty-fold greater in the hippocampus and to a much lesser extent in the cortex (personal communication with our collaborators). All transgenic mice used in the study were heterozygous for the TGF-β1 transgenes. In comparison with the mature murine peptide, the active mutant of porcine peptide differs only in one amino acid, and the mutated pTGF-β1 cDNA has been shown to be bioactive when expressed in the transgenic mice (Wyss-Coray et al., 1995). For the purpose of our study, we used F1 offspring from the pure C57B6 background TGF-β1 mice in our biochemical experiments. High-level TGF-β1 overexpression results in the development of communicating hydrocephalus (Wyss-Coray et al., 1995), whereas lower-level expression in mice, such as those used in this study, does not cause this complication. Non-transgenic litter-matched wild type (WT) mice served as controls. The mice were bred and genotyped in Dr. Paul Frankland’s laboratory at the Hospital for Sick Children in Toronto.
3.2 Dissection of the brain

Animal care was in accordance with institutional guidelines. Briefly, postnatal transgenic mouse pups were anesthetised using isofluorane and sacrificed by cervical dislocation. The dissection instruments were sterilized and the pup's neck was wiped with 70% ethanol. Using microdissecting scissors, the skin was opened at the midline of the head, cutting from the base of the skull to the mid-eye area. Skin flaps were folded back to expose the superior face of the brain. The skull was carefully opened at the midline fissure, and the two flaps were pulled apart to gain access to the brain without cutting into the brain tissue. The brain was scooped out of the skull with a microspatula underneath and along the length of the brain from the olfactory lobes to the beginning of the spinal cord. The brain was then immediately transferred to the tissue culture hood, gently placed into a 60 mm culture-dish and wash twice with 5ml of Phosphate Buffered-Saline (PBS) without Ca$^{2+}$ and Mg$^{2+}$. While steadying the brain with forceps, the cerebrum was separated from the cerebellum and brain stem. Cerebral hemispheres were separated from each other by gently teasing along the midline fissure with the sharp edge of a second pair of forceps. To reduce the number of contaminating fibroblasts, the cerebral hemispheres were staged under a dissecting microscope and the blood vessels and meningeal membranes were peeled from the cortical tissue. To isolate the hippocampus, a paramedial longitudinal cut was made through the length of the cortex superior to the borders of the hippocampus. The cortical layer was folded laterally and the hippocampal piece was dissected out from each hemisphere using a pair of curved forceps. For dissection of the embryonic rat brains, all steps were taken in the culture hood after removing the embryos from the maternal rat.

3.3 Cell culture

The general procedure for cell dissociation and culture has been previously described (Dong et al., 2004). Briefly, pregnant Wistar (Charles River, St. Constant, QC, Canada) rat was anesthetised using isofluorane and sacrificed by cervical dislocation. Whole brains of embryonic day 18 (E18) rat embryos were isolated and hippocampal and neocortical tissues were dissected
from the brain as described above. Primary neuronal cultures were prepared from the hippocampal tissues while primary astrocyte cultures were prepared from the neocortical tissues. Dissociated brain tissues were gently and mechanically triturated without enzymatic treatment in plating medium, consisting of Dulbecco’s MEM (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS and 1% penicillin-streptomycin glutamine (PSG). After allowing non-dispersed tissue to settle for 2 minutes, the supernatant containing neurons were initially seeded on poly-D-lysine (#P7280, Sigma, St. Louis, MO, USA) coated 35 mm Nunc culture dish (Fisher Scientific, Pittsburgh, PA, USA) or 18 mm-diameter round glass coverslips in plating buffer, which consists of Neurobasal medium (#21103, Invitrogen Corporation), 2% B-27 (1:50) supplement (#17504, Invitrogen Corporation/GIBCO, Grand Island, NY, USA), 0.5% fetal bovine serum (FBS), and (in mM) 0.5 L-glutamine, 0.025 glutamic acid, 0.5 Sodium pyruvate. After overnight incubation 37°C in a humidified incubator under an atmosphere of 95% air and 5% CO₂, approximately one-third of the medium was replaced with maintenance medium made up of Neurobasal medium (Invitrogen Corporation, Carlsbad, CA), 0.5 mM L-glutamine (#25030, Invitrogen Corporation, Carlsbad, CA) and 2% B-27 (1:50) supplement (Invitrogen Corporation). Cultured neurons were grown over the course of 14 days, and the medium was washed every 3–4 days. Under this condition, only a small number of glial cells were present in cultures (Dong et al., 2004). The cultured neurons were used at different time-points for electrophysiological and immunocytochemical experiments (to be discussed below). Similarly, astrocytes were cultured from mechanically triturated neocortical tissues and grown in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin glutamine (PSG) until full confluency. The absence of Neurobasal medium and supplements prevented neuronal cells from growing. Cultured astrocytes were maintained at 37°C in a humidified atmosphere of 5% CO₂, and the medium was washed every 3–4 days.

For primary astrocyte cultures prepared from transgenic mice, whole brains of both the WT and transgenic postnatal mice (1–2-month-old) were dissected as described above and placed in separate culture dishes. Neocortical and hippocampal tissues were washed five times in PBS without Ca²⁺ and Mg²⁺ and subsequently minced. Minced pieces of brain tissue were trypsinized (0.25%) in 37°C water bath for 5 minutes, shaken intermittently. Trypsinized cells were re-suspended three times in supplemented DMEM, filtered through 100 µm (#352360, BD Biosciences, Bedford, MA, USA) then 40 µm nylon cell strainers (#352340, BD Biosciences),
and centrifuged twice (1200 rpm, 5 min). The pelleted cells were re-suspended and seeded in tissue-culture dish (Nunc) or on glass coverslips in supplemented DMEM. The medium was changed once the next day. Cells were grown in the incubator set to 37°C in a humidified atmosphere of 5% CO₂. After the cells reached full confluency, they were re-seeded and the medium was washed every 3 days.

3.4 Immunohistochemistry and Confocal Microscopy

Immunohistochemical procedure was performed as the following. Briefly, 4% paraformaldehyde (PFA)-fixed brain specimens were cut using a 1000 Plus Vibratome (Pelco 102, Ted Pella Inc., Redding, CA, USA) into 40-µm free-floating sections to perform immunostaining. Tissue sections were permeabilized in 0.25% Triton X-100 for 5 minutes, blocked in 10% normal serum in 0.25% Triton X-100 for 2 hours, washed with Phosphate-Buffered Saline (PBS), and then incubated with primary antibodies overnight. Primary antibodies used included mouse monoclonal anti-synaptophysin-1 fluorescence-labelled with Oyster 550 (#101 011C3, 1:1,000, Synaptic Systems, Goettingen, Germany), rat monoclonal anti-CD11b (#MCA74G, 1:150, AbD Serotec, Oxford, UK), mouse monoclonal anti-glial fibrillary acidic protein (GFAP) Cy3 conjugate (#C9205, 1:1,000, Sigma-Aldrich, St. Louis, MO, USA), mouse monoclonal anti-neuronal nuclei (NeuN) Alexa Fluor 488 conjugated (#MAB377X, 1:200, Millipore, Billerica, MA, USA), rabbit polyclonal anti-calbindin-D28K (#AB1778, 1:1,000, Millipore), and rabbit polyclonal anti-microtubule-associated protein 2 (MAP2) (#AB5622, 1:400, Millipore). After three washes with PBS, Cy3- or FITC-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) in 0.25% Triton X-100 were added for incubation at room temperature for 2 hours. When necessary, the process was repeated for double labelling of another primary antibody. Glass coverslips were mounted on stage glass over the tissue sections using Fluoromount-G (#17984-25, Electron Microscopy Sciences, Hatfield, PA) for confocal microscopy. We repeated the immunohistochemical assays of each protein in brain tissue sections of 2–4 mice.
To examine the effect of TGF-β1 on the localization of AMPAR and NMDAR subunits in neurons, standard immunocytochemistry of cultured cells was performed as previously described (Dong et al., 2004). Briefly, the cultured hippocampal neurons on coverslips were randomly separated into two groups, one for permeabilized and the other for non-permeabilized staining. For cytosolic staining under permeabilizing conditions, cells were fixed with 3.7% paraformaldehyde and 4% sucrose in D-PBS for 30 min, permeabilized in 0.1% Triton X-100 for 15–30 min, blocked in 5% normal donkey serum (NDS) for 1 hr and then incubated with primary antibodies for 2 hr or overnight at 4°C. The primary antibodies used include rabbit polyclonal anti-GluR2/3 (1 µg/mL, #AB1506, Millipore) and rabbit polyclonal anti-NMDAR2A/B (0.5 µg/mL, #AB1548, Millipore). For cell surface staining, neurons were fixed under non-permeabilizing conditions using 3.7% paraformaldehyde and 4% sucrose in PBS for 15 minutes at room temperature, blocked with 1% NDS in D-PBS, and incubated (overnight at 4°C) with the same primary antibodies used under permeant conditions in 0.1% NDS in D-PBS. Incubation allowed for the antibodies to recognize extracellular epitopes of their respective receptor subunits. All cells were then incubated with appropriate FITC-conjugated secondary antibody (1:500; Jackson ImmunoResearch) in D-PBS at room temperature for 1-2 hours. After, the labelled cells in non-permeant conditions were permeabilized with 0.1% Triton X-100 in PBS (5 minutes at room temperature) and thoroughly rinsed, neurons in both groups were incubated (for 20 minutes at room temperature) with a probe for phalloidin made from a mushroom toxin conjugated to Alexa Fluor 546 dye (1:40, #A-22283, Molecular Probes, Eugene, OR, USA) to stain for F-actin in neurons. After 3 washes in D-PBS, the coverslips were mounted with Fluoro-Mount G (Electron Microscopy Sciences, Hatfield, PA) for confocal microscopy. In each experiment, at least 10 imaging fields of the control and treated neurons were analyzed.

Confocal images of stained brain tissues were imaged using a Deconvolution system with apotome on a Zeiss Axiovert 200 inverted microscope (Carl Zeiss, Göttingen, Germany) fitted with a ×5, ×20 or ×63 objective lens. The visual field was blindly moved to a random site on a cell-culture or tissue coverslip. To reduce photo-bleaching of the cells, the laser was minimally shuttered for the duration of less than 1 second per imaging field. The fluorescence intensity for a specific protein stain was set below the threshold for the negative control. Digital images of cell cultures and brain tissues, with a focus on the hippocampal structure, were obtained using Zeiss LSM510 scanning program software for analysis.
3.5 Western Blot

To confirm the results obtained through the immunohistochemical assays, immunoblotting assays were performed. All fresh hippocampal and neocortical tissue samples were taken from approximately 2-month-old WT and TGF-β1 mice, unless otherwise specified. The general procedure used for western blotting has been previously described (Dong et al., 2004). Briefly, tissue samples were homogenized in lysis buffer containing RIPA buffer (1% Nonidet P-40, 0.1% Sodium dodecyl sulphate (SDS), 0.5% Deoxycholic acid (DOC), PBS), and (in mM) 100 ethylenediaminetetraacetic acid (EDTA) (pH 8.0), 2 phenylmethylsulphonyl fluoride (PMSF), and 10 µg/ml Aprotinin (#A-3428, Sigma, St. Louis, MO) and 10 µg/ml Leupeptin (#L-2023, Sigma, St. Louis, MO, USA). Lysed tissue samples were incubated on ice for 30 minutes, and centrifuged at 12,000 rpm for 5 minutes at 4°C. Supernatant from each sample was used for quantification. Protein concentrations were assayed by the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA).

Protein samples (5–60 µg) were boiled for 5 minutes in reducing sample buffer and fractionated on 8, 10 or 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (#162-0115, Bio-Rad Laboratories) in transfer buffer at 4°C for 2 hours. Membranes were blocked with 5% skim milk powder in TBS-T (0.1% Tween 20, 1M Tris-HCl pH 8.0 and 5M NaCl) or 5% bovine serum albumin (BSA) in TBS-T and were incubated at 4°C overnight in primary antibodies. The commercially available primary antibodies used included mouse monoclonal anti-synaptophysin-1 (1:10,000, #101 011, Synaptic Systems, Goettingen, Germany), rabbit polyclonal anti-calbindin D-28K (1:10,000, #AB1778, Millipore, Billerica, MA, USA), rabbit polyclonal anti-GFAP (1:50,000, #AB7260, Abcam Inc., Cambridge, MA, USA), goat polyclonal anti-GluR2 (1:200, #sc-7611, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), rabbit polyclonal anti-GluR2/3 (0.8 µg/mL, #AB1506, Millipore), goat polyclonal anti-GluR4 (1:200, #sc-7614, Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-NMDAR2A/B (0.3 µg/mL, #AB1548, Millipore), rabbit polyclonal anti-NMDAR2A (1:500, #AB1555P, Millipore), rabbit polyclonal anti-NMDAR2B (1:1000, #AB1557P, Millipore), mouse monoclonal anti-NMDAR1 (1:500, #556308, BD Biosciences), rabbit polyclonal anti-porcine TGF-β1 (1:2,000, #CPT001, Cell Sciences, Inc., Canton, MA, USA), mouse monoclonal anti-β-actin (1:5,000, #A5441, Sigma), and horseradish peroxidase (HRP)-conjugated mouse monoclonal
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:5000, #AB9482, Abcam Inc.). β-actin or GAPDH were used as loading controls. After three washes in TBS-T, blots were incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 hours. Secondary antibodies used included rabbit anti-goat IgG HRP conjugate (#172-1034, 1:2000, Bio-Rad Laboratories), Goat anti-rabbit IgG HRP conjugate (#172-1019, 1:10,000, Bio-Rad Laboratories), and sheep anti-mouse IgG HRP conjugate (#NA 931, 1:5,000, Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). Following incubation with the secondary antibody, blots were washed three times in TBS-T and proteins of interest were detected with ECL Western Blotting Detection Reagent (GE Healthcare, Buckinghamshire, UK) in accordance with the manufacturer’s instructions. The blotting films were scanned using GeneSnap (Syngene, Synoptics Ltd., Cambridge, UK) acquisition software and band densities were quantified using the GeneTool (Syngene, Synoptics Ltd.) program. The assay was repeated at least 2-3 times for each antibody tested. Values of the band density were normalized to the level of respective β-actin or GAPDH.

3.6 Whole-cell Patch Clamp Recording

Cultured hippocampal neurons from E18 rats were treated with TGF-β1 (#240-B, R&D Systems, Minneapolis, MN, USA) at a concentration of 4 ng/ml beginning on the day the culture was made, and fresh TGF-β1 was added to the cultured medium every 3–4 days when changing the media. Cultured neurons were used for whole-cell patch-clamp recordings on the 11th and 12th day in vitro (DIV), unless otherwise specified. All electrophysiological measurements were taken at room temperature (~23°C) at a constant holding membrane potential of -60 mV and normalized to cell capacitance in the whole-cell configuration using a MultiClamp 200B Computer-Controlled Microelectrode Amplifier (Axon Instruments, Foster City, CA, USA) and a Digidata 1322 (Axon Instruments) data acquisition system controlled by pCLAMP ver10.2 software (Axon Instruments). Cells in treated and non-treated control dishes were randomly selected under conventional microscopy.
Patch electrodes were pulled from 1.5-mm thin-walled borosilicate glass tubes (TW150F-3, World Precision Instruments, Inc., Sarasota, FL, USA) on a PP-830 Microelectrode Puller (Narishige, East Meadow, NY, USA) and had typical resistances of approximately 3-4 MΩ when fire polished and filled with intracellular solution. Pipettes were filled with distinct intracellular solutions to measure different types of transmembrane currents. The intracellular solution (ICS) used to measure voltage-gated K⁺ channel currents and glutamate-evoked currents contained the following (in mM): 130 KCl, 2 MgCl₂, 1 CaCl₂, 11 EGTA, 10 HEPES, 4 MgATP (adenosine 5′-triphosphate), pH 7.3, adjusted with KOH (osmolarity, ~315 mOsm). The ICS used to measure voltage-gated Na⁺ (Naᵥ) channel currents and spontaneous excitatory postsynaptic currents (sEPSCs) consisted of the following (in mM): 120 CsCl, 2 MgCl₂, 1 CaCl₂, 2 K₂ATP, 11 EGTA, 10 HEPES, 10 tetraethylammonium (TEA)-Cl, pH 7.3, adjusted with CsOH (osmolarity, ~315 mOsm). The presence of K⁺ channel blocker TEA and low-K⁺ solution eliminated the otherwise apparent voltage-gated K⁺ outward currents. The voltage clamp protocol compensated for, at least in part, the application of TEA to measure spontaneous activity. During experiments measuring Naᵥ channel currents and sEPSCs, culture medium was replaced and bathed with an extracellular solution (ECS) composed of the following (in mM): 154 NaCl, 2 CaCl₂, 5 KCl, 5 HEPES, 10 Glucose, pH 7.4, adjusted with NaOH (osmolarity, ~315 mOsm). During experiments measuring Kᵥ channel and glutamate-evoked transmembrane currents, bath solutions contained 1 µM Tetrodotoxin (TTX) (#T-550, Alomone Labs Ltd., Jerusalem, Israel) to selectively and reversibly block excitable Na⁺ channels. Membrane patches were directly exposed to test solutions (ECS, 100 µM glutamate) for a duration of 2 seconds via a computer-controlled two-barrel perfusion system (SF-77B, Warner Instruments, Hamden, CT, USA) and transmembrane voltage-gated Na⁺ and K⁺ channel currents were elicited with a series of 500 ms depolarizing pulses in 10 mV increments to +50 mV from a clamped membrane potential of -60 mV. Electrical signals were recorded at a total of twelve fixed holding potentials, amplified, digitized and filtered (1–2 kHz). Inspection of the raw data was used to eliminate any false events, and the trigger level for the detection of events was set much higher than the baseline noise. We acquired transmembrane currents on-line using Clampex (Axon Instruments), and analyzed the data off-line using Clampfit (Axon Instruments).
3.7 Statistical Analyses

Statistical analyses were performed with Sigmaplot software (SPSS, Chicago, IL, USA). All data are presented as mean ± the standard error of mean (SEM) and were examined using Student’s paired t-tests (two-tailed) where appropriate. To examine the equality of variances between two groups, Levene’s test was also performed. All statistical tests were two-tailed and the statistical significance was considered at the p < 0.05 level.
Chapter 4
Results

4.1 Increased expression of TGF-β1 in transgenic mice

Homogenates of the hippocampus and neocortex from TGF-β1 mice were analyzed using Western blots with rabbit anti-porcine TGF-β1. Blotting results demonstrated the presence of the porcine TGF-β1 protein in the hippocampal and neocortical tissues of TGF-β1 mice, whereas virtually no porcine TGF-β1 protein was detected in the non-transgenic WT tissues (Figure 4-1A). The immunoblotting band was detected at approximately 45 kDa, which is representative of the unprocessed form of TGF-β1 (Wyss-Coray et al., 1995). Regional disparities between the hippocampal and neocortical expression of TGF-β1 within the TGF-β1 mice group were observed, in which the protein density in the hippocampal extract from TGF-β1 mice was approximately 40% greater than that of the cortical extract (Figure 4-1B).

Figure 4-1. Expression of TGF-β1 in brains of T64 and non-transgenic WT mice. (A) Hippocampal and neocortical protein extracts from 2-month-old WT and TGF-β1 mice were analyzed by immunoblot (40 g protein/lane). Extracts of TGF-β1 mice, but not those of WT mice, produced TGF-β1 immunoreactive bands corresponding to intact precursor form of porcine TGF-β1 (n = 1). (B) Quantification of the immunoblots as shown in A. The amount of TGF-β1 protein in hippocampal and neocortical extracts is normalized to GAPDH, which was used as the loading control. WTh: hippocampal protein extracts from WT mice; T64h: hippocampal protein extracts from TGF-β1 mice; WTc: cortical protein extracts from WT mice; T64c: cortical protein extracts from TGF-β1 mice.
4.2 Increased TGF-β1 leads to activation of glial cells

It was reported that aged transgenic mice (at least 12-month-old) over-expressing TGF-β1 display microgliosis (activation of microglia) and a characteristic pattern of perivascular astrocytosis (activation of astrocytes) (Buckwalter and Wyss-Coray, 2004). To examine whether glial cells are also activated in the hippocampus and neocortex of young mice (2-month-old), immunohistochemical assays were performed on free floating brain sections of TGF-β1 mice (n = 3) and non-transgenic WT littermates (n = 3) using antibodies against marker proteins of astrocyte and microglia, respectively. GFAP is an intermediate filament protein found specifically in astrocytes and its accumulation is an index of astrocyte activation (Reilly et al., 1998). Cluster of differentiation molecule 11B (CD11b, also known as integrin-αM) is a cell surface antigen. CD11b expression is restricted to microglia in the brain and increased CD11b is a hallmark of microglia activation (Block et al., 2007, Wakselman et al., 2008). Immunohistochemical analysis displayed that astrocytic over-expression of TGF-β1 in the transgenic mice led to astrocytosis and microgliosis, as indicated by a marked increase in the immunoreactivities of GFAP and CD11b in the neocortex as well as the hippocampus proper, particularly in the dentate gyrus, CA1, and CA3 subfields (Figure 4-2A,D). Specifically, the number of GFAP-immunopositive cells increased and Western blot analysis showed that the total GFAP protein level was significantly greater in TGF-β1 mice than that in WT littermates (p = 0.037, hippocampus; p = 0.041, neocortex) (Figure 4-2B). Expressed protein level in WT mice was approximately 40% and 25% of that in the hippocampus and neocortex, respectively, of TGF-β1 mice (Figure 4-2C). CD11b staining revealed that microglia in the hippocampus and neocortex of TGF-β1 mice are more numerous and have more processes and cytoplasm than those in WT littermates (Figure 4-2D). Taken together, my results indicate that astrocytic over-expression of TGF-β1 induces inflammatory responses in specific brain regions of young mice.
Figure 4.2. Over-expression of TGF-β1 leads to activation of glial cells. TGF-β1 mice demonstrate increased immunoreactivities of (A) GFAP, an astrocyte marker, and (D) CD11b, a microglia marker, in the hippocampus and neocortex. Immunohistochemistry revealed that more cells are immuno-positive for both markers in TGF-β1 mice. (B) Protein extracts from WT and TGF-β1 mice were analyzed by immunoblot (50 g protein/lane). Immunoreactive bands corresponding to GFAP (the lower band is thought to be a degradation product) were significantly higher in the hippocampal and neocortical protein extracts of TGF-β1 mice than those in WT mice (Mean ± SEM, * p < 0.05, n = 3). (C) Quantification of GFAP protein in WT mice is expressed as a percent of that in TGF-β1 mice after normalization to GAPDH, which was used as loading control. Scale bar = 100 µm (CA1, CA3, and DG Hilus); 50 µm (Neocortex).
4.3 Increased TGF-β1 modulates expression of synaptic proteins in the hippocampus

The specific role of TGF-β1 in the regulation of synaptic structure is largely uncertain. Thus, I next examined whether TGF-β1 over-expression affects glutamatergic synaptic protein expression. I first performed immunohistochemistry using an antibody against synaptophysin, a glycoprotein primarily expressed in presynaptic terminals. The immunochemical assay displayed that the immunoreactivity of synaptophysin clusters in the hilus of dentate gyrus and the stratum lucidum of CA3 region were increased in TGF-β1 mice (Figure 4-3A). This finding suggests that TGF-β1 may increase the number of mossy fibre synapses, or cause alterations in their terminals.

To test whether TGF-β1 over-expression indeed affects mossy fibres, I immuno-stained brain tissues using an antibody against calbindin-D28K (henceforth calbindin). Calbindin is an intracellular Ca$^{2+}$-binding protein that is selectively expressed in certain neuronal populations within the brain, including the dentate gyrus granule cells (Baimbridge and Miller, 1982). Thus, it serves as a marker for mossy fibre projections. My result showed that although calbindin was highly expressed in mossy fibres originating from the hilus of dentate gyrus, it did not form clusters in the stratum lucidum of the CA3 region. Interestingly, I observed a decrease in calbindin immunoreactivity in CA1 pyramidal cells as well as in the stratum lucidum layer, where the mossy fibre terminals synapse on to the dendrites of CA3 pyramidal neurons (Figure 4-3B). To determine whether synaptophysin and calbindin are exactly co-localized in the mossy fibre terminals, I performed double immuno-staining against these two proteins. My result showed that: 1) both synaptophysin and calbindin are expressed in mossy fibres, but calbindin only partially co-localized with the synaptophysin clusters; 2) increased TGF-β1 up-regulated synaptophysin but down-regulated calbindin in the hippocampus (Figure 4-3C). These immunohistochemical results were further verified by Western blot assay (Figure 4-3D).
Figure 4-3. Over-expression of TGF-β1 induces changes in the expression of synaptic proteins in mossy fibre synapses. Immunohistochemical assays in TGF-β1 mice demonstrated a decrease in the labelling of synaptophysin, a presynaptic marker (red) (A) and an increase in the labelling of calbindin, a calcium buffer and selective marker of the mossy fibre synapse (green) (B). (C) Overlay of these two markers in the dentate gyrus and CA3 subfield of the hippocampus as shown in A and B. The staining revealed a partial co-localization of synaptophysin and calbindin immuno-clusters in mossy fibre terminals. (D,E) Protein extracts from WT and TGF-β1 mice were analyzed by immunoblot (5–50 g protein/lane). Higher immunoreactive bands corresponded to synaptophysin (D) and lower immunoreactive bands to calbindin (E) in the protein extracts of TGF-β1 mice. Quantification of proteins synaptophysin and calbindin in hippocampal and neocortical extracts is normalized to GAPDH (n = 1). Yellow arrow = pyramidal neuron; White arrow = granule neuron; Scale bar = 100 µm (CA1, CA3 and DG Hilus); 50 µm (Neocortex).
4.4 Increased TGF-β1 causes up-regulation of ionotropic glutamate receptor subunits in the hippocampus

Altered expression of the presynaptic protein, synaptophysin, in TGF-β1 mice led me to evaluate whether over-expression of TGF-β1 affects glutamatergic postsynaptic proteins in the hippocampus and neocortex. I measured the expression level of AMPARs and NMDARs because these ionotropic glutamate receptors, in addition to mediating most excitatory transmission in the CNS, play key roles in developmental synaptogenesis and in synaptic plasticity. Specifically, I made Western blot assays of GluR2-4, as well as NR1 and NR2A/B subunits, which, excluding GluR4, are the predominant subunits of synaptic AMPARs and NMDARs, respectively, in the hippocampus (Ozawa et al., 1998, Santos et al., 2009). My data revealed that in comparison with WT littermate controls, there was a significant increase in the protein levels of AMPA receptor subunits GluR2 (p = 0.032), GluR2/3 (p = 0.006), but not GluR4, and NMDA receptor subunits NR1 (p = 0.012) and NR2A/B (p = 0.020) in the hippocampus of TGF-β1 mice (Figure 4-4). The expression of these tested subunits in the neocortex of TGF-β1 mice showed a trend of increase but was not significant. These results indicate that chronically increased production of TGF-β1 also modulates the expression of postsynaptic proteins in the hippocampus of TGF-β1 mice.
Figure 4-4. Over-expression of TGF-β1 modulates AMPA and NMDA receptor subunit expression. Total expression levels of various AMPA and NMDA receptor subunits were analyzed by immunoblot using protein extracts from the hippocampus and neocortex of WT and TGF-β1 mice (40–50 g protein/lane). Representative immunoblots of (A) GluR2/3 (** p < 0.01, n = 5), (B) GluR2 (* p < 0.05, n = 4), (C) GluR4 (n = 2), (D) NR1 (* p < 0.05, n = 3) and (E) NR2A/B (* p < 0.05, n = 5) proteins are shown, normalized to GAPDH levels. Hippocampal expression of subunits GluR2/3, GluR2, NR1, and NR2/B were significantly increased in TGF-β1 mice. Error bars denote ± SEM.
4.5 PICK1 is not changed in the brain of TGF-β1 mice

PICK1 is a calcium-sensing, PDZ domain-containing associated protein of the AMPA receptor that directly interacts with GluR2 and GluR3 subunits and regulates their trafficking. Specifically, it has been shown to interact with GluR2 at presynaptic and postsynaptic sites and is suggested to be involved in NMDAR-mediated synaptic plasticity (Hanley and Henley, 2005). To test whether the observed changes in the GluR2/3 protein expression is correlated to changes in PICK1 expression, Western blot was performed in hippocampal and neocortical protein extracts of WT and TGF-β1 mice (n = 2). I found no significant change in the expression of PICK1 in the hippocampus and neocortex. Thus, I speculate that the observed increase in the expression of GluR2/3 subunits may be a PICK1-independent phenomenon.

Figure 4-5. Expression of PICK1 in brains of T64 and WT mice show no changes. Hippocampal and neocortical protein extracts from WT and TGF-β1 mice (n = 2) were analyzed by immunoblot (40–50 g protein/lane). There was no significant difference in the density of immunoreactive bands corresponding to PICK1 between WT and TGF-β1 mice.
4.6 Changed morphology and proliferation of cultured primary astrocytes from TGF-β1 mice

Given the onset of astrogliosis in the hippocampus and neocortex of young TGF-β1 mice, I next examined whether these astrocytes display unique phenotypic properties in vitro. Cultured primary astrocytes from the hippocampus and neocortex of TGF-β1 mice showed morphological changes representative of that in activated astrocytes, with elongated cell bodies and processes after DIV14 and DIV21 (Figure 4-6A). Astrocytes from TGF-β1 mice also displayed increased survival when compared with that of the WT mice (Figure 4-6B). To test if these morphological changes are observed in astrocytes with prolonged exposure to TGF-β1, I treated primary embryonic rat astrocyte cultures with TGF-β1 (4 ng/ml). It was found that TGF-β1 treatment throughout cell proliferation did not induce the same changes in astrocytic morphology (data not shown). TGF-β1 is generally an inhibitor of astrocyte proliferation (Lindholm et al., 1992, Baghdassarian et al., 1993). Hence, as expected, the putative effect of TGF-β1 treatment (1 ng/ml and 4 ng/ml) on the proliferation of cultured astrocytes was inhibitory over the course of DIV7 (Figure 4-6C). This suggests that in vivo effects of TGF-β1 over-expression on the morphological and proliferative properties of astrocytes cannot be directly translated to in vitro conditions, likely due to a complex set of signalling pathways that involve actions of other growth factors and signals.
Figure 4-6. Increased TGF-β1 affects the morphology and proliferation of cultured primary astrocytes. Hippocampal and cortical astrocytes from postnatal WT and TGF-β1 mice (1–2-month-old) were cultured. Compared to the quiescent astrocytes from WT mice, astrocytes from TGF-β1 mice showed activated morphology (magnification ×400) (A) and increased survival (magnification ×50) (B) after DIV14 and DIV21. (C) Rat embryonic astrocytes were cultured over 7 days in the presence or absence of TGF-β1 (1 and 4 ng/mL). TGF-β1 treatment inhibited proliferation of astrocytes.
4.7 TGF-β1 up-regulates ionotropic glutamate receptor subunits in vitro

To examine whether the increased ionotropic glutamate receptor expression in the hippocampus of TGF-β1 mice is a direct consequence of increased TGF-β1, I treated cultured rat embryonic hippocampal and neocortical neurons at DIV11 with TGF-β1 (4 ng/ml) in the presence or absence of SB 431542 (10 µM). SB 431542 is a selective inhibitor of TGF-β type 1 (TβR-I) receptor kinases (including activin receptor-like kinase 5, which is a TβR-I that specifically mediates TGF-β1 signalling). I then performed immunocytochemical assays of GluR2/3 and NR2A/B subunits in control and treated neurons under non-permeabilized and permeabilized conditions. My results revealed a slight increase of surface GluR2/3 subunits (Figure 4-7A), and an increase in both the surface and internal NR2A/B subunits (Figures 4-7C and 4-7D) in TGF-β1 treated cells in comparison to the non-treated control cells.
Figure 4-7. Increased TGF-β1 modulates ionotropic glutamate receptor subunit expression. Rat embryonic hippocampal and neocortical neurons were cultured and analyzed for GluR2/3 and NR2A/B subunit localization using immunocytochemistry. Cell surface expression and internal expression levels were detected by non-permeabilized (A,C) and permeabilized (B,D) conditions, respectively. Red fluorescent-tagged phalloidin was used to probe for F-actin in neurons, and a FITC-secondary antibody was used to detect anti-GluR2/3 (A,B) and anti-NR2A/B (C,D). While the cell surface immunoreactivity of GluR2/3 showed a slight increase (A), its intracellular immunoreactivity in TGF-β1 treated neurons is comparable to that of the non-treated control cells (B). The immunoreactivity of NR2A/B was higher at the cell surface (C) and in the intracellular compartment (D) of neurons treated with TGF-β1 (4 ng/ml). SB 431542 treatment (10 μM) produced little effect. Scale bar = 20 μm.
4.8 TGF-β1 enhances glutamatergic activity and the function of voltage-gated channels in vitro

Genetically over-expressing TGF-β1 or treating cultured neurons with TGF-β1 increased the expression of some subunits of ionotropic glutamate receptors. To ascertain the direct effect of TGF-β1 on the regulation of glutamatergic transmission, I measured glutamate-evoked currents in control and TGF-β1 (4 ng/ml) treated rat embryonic hippocampal neurons at DIV11 or DIV12 using whole-cell voltage-clamp recordings. I found that treatment with TGF-β1 alone significantly increased glutamate-evoked transmembrane current (p = 0.016, n = 17) (Figure 4-8A,B). Additionally, I found that some control neurons (n = 4 out of 12 recorded cells) exhibited spontaneous excitatory postsynaptic currents (sEPSPs) at DIV11 and/or DIV12. The sEPSCs were displayed as short-lasting current events and long-duration current valleys (upper trace in the Figure 4-8C). In contrast, TGF-β1 treatment caused more neurons (n = 9 out of 14 tested cells) to display sEPSCs, most of which were current valleys with higher amplitude (lower trace in the Figure 4-8C). This result further indicates that TGF-β1 alone up-regulates hippocampal glutamatergic activities.

To broaden the evidence that TGF-β1 alone modulates neuronal functions, I also measured the amplitude of currents mediated by voltage-dependent ion channels in the presence of the K⁺ channel blocker tetraethylammonium (TEA, 10 mM) or the Na⁺ channel blocker tetrodotoxin (TTX, 1.0 μM), respectively. When compared with the control hippocampal neurons, the amplitudes of currents mediated by voltage-gated Na⁺ channels (p = 0.049, 0.038, 0.047 at membrane potentials of -30 mV, -20 mV, and -10 mV, respectively; n = 9) and by K⁺ channels (p < 0.005 at membrane potentials from -20 mV to 50 mV; n = 9) were significantly increased in TGF-β1 treated neurons.
Effect of TGF-β1 Treatment on Spontaneous EPSCs

A

![Bar graph showing the Amplitude of Glutamate Current (pA) with control and treatment conditions.](image)

B

![Graphs showing Glutamate 100 μM control and TGF-β1 treated conditions with 2000 ms and 100 pA scales.](image)

C

![Effect of TGF-β1 Treatment on Spontaneous EPSCs.](image)
D

**Amplitude of Na\(^+\) Current (pA)**

- **Control**
  - $V_M\colon -30 \text{ mV}$
  - $-20 \text{ mV}$
  - $-10 \text{ mV}$

- **TGF-\(\beta\1**
  - $-30 \text{ mV}$
  - $-20 \text{ mV}$
  - $-10 \text{ mV}$

E

**Control**

**TGF-\(\beta\1**

- $-30 \text{ mV}$
- $-20 \text{ mV}$
- $-10 \text{ mV}$
Figure 4-8. TGF-β1 enhances glutamatergic activity and voltage-gated channel function. Whole cell recordings were performed on cultured WT rat embryonic hippocampal neurons (DIV11-12) to measure synaptic transmission. (A) Glutamate-evoked currents were recorded in neurons in the presence or absence of TGF-β1 (p < 0.05; n=17, control; n=17, treated). (B) Quantification of the recorded amplitudes of the glutamate-evoked currents indicated a significant increase in TGF-β1 treated neurons. Treated neurons also displayed higher amplitudes of (C) sEPSCs (p < 0.05; n=9, control; n=9, treated) and significantly increased amplitudes of voltage-gated Na⁺ (D, E) (p < 0.05; n=9, control; n=9, treated) and K⁺ transmembrane currents (F, G) (p < 0.01; n=9, control; n=9, treated) when the cells were held at membrane potentials between -10 to -30 mV and -20 to 50 mV, respectively. Error bars = Mean ± SEM.
Chapter 5
Discussion

In this thesis study, I made the following findings: 1) genetically over-expressing TGF-β1 in the brain activates astrocytes and microglia in the hippocampus and neocortex; 2) over-expression of TGF-β1 augments the levels of pre- and postsynaptic proteins at glutamatergic synapses in the hippocampus; 3) treatment of cultured primary hippocampal neurons with TGF-β1 alone enhances glutamatergic activities. These findings further elucidate the role of TGF-β1 in the regulation of brain functions.

5.1 Effects of Chronically Increasing TGF-β1 Production on Gliosis in the Brain of Young Mice

A previous study showed that aged TGF-β1 mice (>12-month-old) of the T64 transgenic line display widespread astrocytosis with most prominence around cerebral blood vessels (Wyss-Coray et al., 1995). My results indicate that over-expression of TGF-β1 in the brain of the same transgenic mice also induces glial activation at a younger age, in which astrogliosis is extensively observed concomitantly with microgliosis in the hippocampus and neocortex. The increase in the number of GFAP- and CD11b-immunopositive cells is in line with a recent report that 8-week-old TGF-β1 mice exhibited higher fraction of survival of astrocytes and microglia relative to WT mice nearly a month after BrdU labelling (Buckwalter et al., 2006). Because activation of glial cells is considered a key causative factor that underlies inflammation in neurodegenerative diseases (Buckwalter and Wyss-Coray, 2004, Block et al., 2007), my work adds a piece of evidence to the role of TGF-β1 in mediating glial functions in the context of neuroinflammation.
5.1.1 Increased microgliosis in TGF-β1 mice

Within the CNS, microglial cells constitute a population of resident macrophages that may be activated by diverse injury-related cytokines. Given that microglia are involved in various immune functions including antigen presentation, phagocytosis, chemotaxis, and the production of cytokines, free radicals, and nitric oxide (Bottner et al., 2000), microgliosis is present in and is closely associated with the pathology of diverse brain diseases including neurodegenerative disorders and neuronal death (Block et al., 2007). For instance, microglia serve as a source of glutamate that can trigger neuronal apoptosis (Wakselman et al., 2008). Interestingly, it has been shown that reactive microglia produce TGF-β1 mRNA in the hippocampus in response to kainic acid-induced neurodegeneration (Morgan et al., 1993). Microglia can also synthesize and release TGF-β1 in response to pro-inflammatory cytokines such as interleukin (IL), interferon, and tumor necrosis factor families in vitro (da Cunha et al., 1993, Chao et al., 1995). In certain conditions, TGF-β1 can block microglial proliferation (Suzumura et al., 1993, Lodge and Sriram, 1996, Xiao et al., 1996) and selectively induce apoptosis, via a pathway independent of bcl-2 oncoprotein, without altering the survival of astrocytes and oligodendrocytes (Xiao et al., 1997). In addition, genetically modifying TGF-β1 production in vivo appears to insinuate diverse effects on microglial cells. Studies have reported that over-expression or under-expression/knock out of TGF-β1 results in microgliosis (Buckwalter and Wyss-Coray, 2004). For instance, widespread microgliosis (including in the neocortex and hippocampus) accompanied by increased apoptotic neurons and reduced neocortical presynaptic integrity was observed in neonatal mice lacking TGF-β1 (Brionne et al., 2003), suggesting that deviating from the homeostatic balance in the level of TGF-β1 may perpetuate a series of reactions that lead to inflammatory responses in the brain. On the other hand, TGF-β1 may stimulate microglia-mediated clearance of amyloid-β peptide (Wyss-Coray et al., 2001), suggesting that microglia can function to alleviate pathological factors. Thus, despite the fact that microglia can contribute to neuronal damage in neurodegenerative diseases, it appears TGF-β1 may also serve as an anti-inflammatory agent in the CNS by regulating the functions of microglia.

A study has found that the developmental death of neurons in the hippocampus around birth is triggered by microglial CD11b integrin and the immunoreceptor DNAX-activating protein of 12 kDa (DAP12) (Wakselman et al., 2008), further highlighting the profound roles microglial cells
play in the normal and diseased brain. In this regard, the observed increase in CD11b protein in the hippocampus of TGF-β1 mice may have functional implications, especially during early postnatal growth. However, exactly how TGF-β1 controls microglial function during development and in diseased brain remains inconclusive. Further tests are required to confirm whether the observed microgliosis in young mice is a consequence of solely increasing the effects of TGF-β1.

5.1.2 Increased astrogliosis in TGF-β1 mice

Astrocytes respond to various CNS injuries with morphological changes and variation in the expression of diverse substances including GFAP (Wyss-Coray et al., 1995). In addition, while astrocytic production of a wide spectrum of inflammatory mediators, cytokines, growth and neuroprotective factors may confer beneficial effects, astrogliosis is a major component of the inflammatory response and an indicator of injury and disease in the CNS (Buckwalter and Wyss-Coray, 2004). In fact, excessive astrogliosis is detrimental as it can result in the formation of glial scars that hinder axonal sprouting (Buckwalter and Wyss-Coray, 2004).

Induction of reactive astrocytes is one response to brain lesions that numerous molecules orchestrate (Bottner et al., 2000). Consistent with astrogliosis in TGF-β1 mice, I observed that cultured astrocytes from TGF-β1 mice assumed an activated morphology (with processes and elongated cell bodies) and displayed an increased proliferation rate, which were markedly different from those of the WT littermate controls. Previous reports show that TGF-β1 induces process and colony formation in the presence of FGF-1 or -2 (Toru-Delbauffe et al., 1990, Labourdette et al., 1990). In fact, TGF-β1 frequently acts together with other growth factors and cytokines, as members of the FGF, EGF, IL, and TGF-β families themselves are known to regulate astrocytic expression of TGF-β isoforms (Lindholm et al., 1992, da Cunha et al., 1993). Considering that the activated astrocytes produce and release multiple soluble factors such as chemokines, growth and inflammatory factors (Wyss-Coray and Mucke, 2002, Wyss-Coray et al., 2003), it is plausible that the observed changes in the morphology of astrocytes from a quiescent to an activated state may not be the sole effect of TGF-β1. Indeed, I found that TGF-β1
treatment alone did not induce morphological changes in cultured astrocytes, which is in line with a previous report that showed treating cultured astrocytes with TGF-β1 produced little morphological change, but resulted in a significant increase in GFAP mRNA and protein (Reilly et al., 1998). Interestingly, the same study revealed that co-application of TGF-β1 and FGF-2 produced a dramatic change in the morphology of astrocyte, and FGF-2 inhibited the TGF-β1-mediated increase in GFAP mRNA and protein (Reilly et al., 1998). It is therefore likely that TGF-β1 alone induced the enhancement of GFAP-immunoreactivity in TGF-β1 mice but is not required for changes in astrocytic morphology. Thus, it appears that TGF-β1 possesses the ability to cause differential effects on the phenotypic properties of astrocytes. Also, the possibility of astrogliosis being a consequence of the autocrine action of TGF-β1 cannot be discounted. Wyss-Coray and colleagues have shown that the endogenous murine TGF-β1 mRNA was increased in TGF-β1 mice over-expressing porcine TGF-β1 (Wyss-Coray et al., 1995, Wyss-Coray et al., 2000), suggesting that manipulating the porcine transgene influences the endogenous production of TGF-β1.
5.2 Effects of Chronically Increasing TGF-β1 Production on Glutamatergic Synapses

Despite numerous studies on the functions of TGF-β1 in the CNS, the current knowledge of its specific role in the regulation of synaptic biology and structure is in its infancy. There exists an intimate relationship between glial cells and neurons in synaptic signalling (Lin and Bergles, 2004, Haydon, 2001, Santello and Volterra, 2009). Indeed, astrocytic release of glutamate induces synaptic modifications including the activation of glutamate receptors at hippocampal synapses (Santello and Volterra, 2009). Interestingly, TGF-β1 has been shown to compromise astrocytic ability to metabolize glutamate (Brown, 1999). Considering these findings with the changes I observed in glial cells, I became interested in examining whether over-expressing TGF-β1 alters synaptic molecules and functions. Given that glutamate is the major excitatory neurotransmitters in the CNS, my study focused on observing the changes in glutamatergic synapses.

5.2.1 TGF-β1 over-expression modulates the expression of presynaptic proteins

My results show that TGF-β1 over-expression increased the immunoreactivity of synaptophysin in hippocampal synapses, particularly those within the dentate hilus and at the mossy fibre-CA3 synapses. In addition, I found a decrease in the immunoreactivity of calbindin in dentate gyrus granule and CA1 pyramidal cells. Synaptophysin is a glycoprotein primarily expressed in the presynaptic compartment, whereas calbindin is known to be highly expressed in mossy fibre projections of the dentate gyrus granule cells (Baimbridge and Miller, 1982). Interestingly, immuno-clusters of synaptophysin and calbindin in the CA3 subfield were only partially co-localized, suggesting that the two markers may not necessarily be indicative of the same structural component. Indeed, while synaptophysin is strictly a presynaptic marker, calbindin show immunoreactive labelling in soma, dendrites and presynaptic terminals of an individual pyramidal cell (Baimbridge and Miller, 1982, Chard et al., 1995). Its partial co-localization with synaptophysin can thus be explained, at least in part, by the fact that the two proteins are
differentially expressed. Hence, my results suggest that over-expressing TGF-β1 may induce alterations in the presynaptic compartment of hippocampal synapses.

My work is also closely pertinent to one particular study by Wyss-Coray and colleagues, in which the investigators described the effects of TGF-β1 on synaptic and neuronal degeneration by demonstrating changes in the immunoreactivities of synaptophysin and calbindin in different transgenic mouse lines (Brionne et al., 2003). The mice used in the study were a cross between transgenic mice over-expressing TGF-β1 at medium levels (T115) (Wyss-Coray et al., 1995, Wyss-Coray et al., 2000) with Apoe knockout mice (Apoe<sup>−/−</sup>), which show age-dependent loss of synaptophysin-positive presynaptic terminals and neuronal cytoskeletal abnormalities (Masliah et al., 1995). In T115 mice, which is a higher expresser line than the T64 mice I used, cerebral over-expression of TGF-β1 largely prevented the loss of synaptophysin caused by chronic injury and the reduction of pyramidal cells expressing calbindin after acute excitotoxicity in the neocortex. Thus, these findings suggest a role of TGF-β1 in protecting against both acute and/or chronic neuronal injury (Brionne et al., 2003). The finding that TGF-β1 over-expression prevents injury-induced reduction in calbindin expression seems to contradict the observation from my study, in which over-expression of TGF-β1 decreased calbindin immunoreactivity in mossy fibres of T64 transgenic mice. There are a few plausible explanations for these conflicting results. First, the changes in calbindin expression were observed in different brain regions. Second, I did not induce neuronal injury in the T64 transgenic mice. As such, region-specific and/or injury-induced effects on calbindin expression were not examined in my study. Third, given that Brionne et al. reported disparities in the pattern of calbindin immunoreactivity between the transgenic mice expressing TGF-β1 at low (T64) and medium (T115) levels, the possibility that these two transgenic mouse lines have critical differences cannot be discounted.

The TGF-β1-mediated changes in calbindin expression may have important implications in hippocampal synaptic transmission and behaviour. Calbindin (approximately 30 kDa) is a ubiquitous Ca<sup>2+</sup>-binding protein that plays diverse physiological functions in numerous cell types including neurons (Kojetin et al., 2006). As an intracellular Ca<sup>2+</sup> buffer and sensor, it is essential in neural function, regulating synaptic interactions in the hippocampus and modulating Ca<sup>2+</sup> channel activity, Ca<sup>2+</sup> transients and neuronal firing (Kojetin et al., 2006, Kohr et al., 1991). Many studies have delineated the functional consequences of manipulating calbindin expression
level. There are arguments for its role as a protective agent in the injured brain. For instance, it has been demonstrated that over-expression of calbindin protects neurons from transient ischemia (Yenari et al., 2001), and cultured hippocampal and neocortical astrocytes induced to express calbindin by injury-related cytokines were resistant to acidosis and calcium ionophore toxicity (Mattson et al., 1995). In addition to its neuroprotective properties, several groups have proposed that calbindin is a regulator of synaptic function. One recent study showed that increasing calbindin levels selectively in the dentate gyrus disrupts mossy fibre-CA3 presynaptic function and impairs spatial cognition (Dumas et al., 2004). The authors recorded hippocampal evoked synaptic responses in vivo and in vitro and found that calbindin over-expression increases transmitter release (thus presynaptic strength) but impairs induction of LTP and reduces post-tetanic potentiation (PTP) at mossy fibre synapses, thus implying potential changes in the presynaptic compartment (Dumas et al., 2004). On the other hand, calbindin knockout mice showed deficits in hippocampal LTP and spatial learning (Molinari et al., 1996, Jouventeau et al., 1999). These studies therefore support the role of calbindin in the regulation of excitatory transmission at hippocampal synapses, including its contribution to long-term synaptic plasticity (Chard et al., 1995). Interestingly, chronic over-expression of TGF-β1 leads to correlated deficits in spatial cognition (Dr. Frankland’s work, personal communication). Considering these findings, together with my observation of TGF-β1-induced reduction of mossy fibre calbindin immunoreactivity in the same transgenic mice, there may be a possible link between calbindin and cognition as a function of TGF-β1. Such a speculation is supported by previous works that have shown a reduction of calbindin expression in the brain of patients with neurodegenerative disorders including Alzheimer’s disease (Iacopino and Christakos, 1990, Hof and Morrison, 1991).

5.2.2 TGF-β1 over-expression alters the expression of postsynaptic proteins

Earlier work has demonstrated that TGF-β1 plays a key role in long-term synaptic facilitation in isolated Aplysia ganglia, suggesting that TGF-β1 is an important constituent within the synaptic signalling cascade for adult neuronal plasticity (Zhang et al., 1997). My study presents new
evidence that TGF-β1 affects hippocampal glutamatergic synapses in rodents. I found that TGF-β1 over-expression induced a significant increase in the hippocampal expression of AMPAR subunits GluR2/3, without concomitant alterations in GluR4 subunit protein levels. The lack of change in the expression of GluR4 subunit can be explained by the following reasons: first, GluR4 is primarily expressed in the hippocampus during the early postnatal period; second, its expression is generally lower throughout the CNS except in the reticular thalamic nuclei and the cerebellum (Santos et al., 2009); and third, the major species of AMPAR that predominate in the mature forebrain, including hippocampus and cerebral neocortex, are heteromers containing GluR2 (Wenthold et al., 1996). Selective up-regulation of GluR2 subunit, in particular, may have important functional implications, as it plays a critical role in determining AMPAR function, assembly and trafficking. In fact, it is the most tightly regulated of the glutamate receptor subunits (Isaac et al., 2007). The elevation in the level of GluR2 subunit may be indicative of increased GluR2-containing Ca\(^{2+}\)-impermeable AMPARs expression at the hippocampal synapses in young TGF-β1 mice. This, in turn, would allow less flux of Ca\(^{2+}\) and potentially lower the demand for intracellular Ca\(^{2+}\) buffering proteins such as calbindin. In addition, I found that the expression of PICK1, a Ca\(^{2+}\) binding protein that interacts with GluR2 subunit (Hanley and Henley, 2005), was not altered in the hippocampus of TGF-β1 mice. Thus, I propose that the up-regulation of GluR2 occurred independently of PICK1, or, alternatively, TGF-β1 could have interfered with the signalling pathway upstream of PICK1 activation.

I also demonstrated that NR2A/B subunit proteins significantly increased in the hippocampus of TGF-β1 mice. For the following key reasons, it would be of great importance to determine which of these two subunits show greater changes in their mRNA and protein levels. First, NR2A and NR2B possess distinct kinetic and pharmacological characteristics that are associated with synaptic development and activity (Wenthold et al., 2003, Monyer et al., 1994, Barria and Malinow, 2002). Second, there are mounting evidence to support the notion that NR2A and -2B subunits are critical factors in establishing the polarity of hippocampal synaptic plasticity. While a dominant role of the NR2A subunit in LTP is generally accepted, controversies exist regarding the role of the NR2B subunit. Liu et al. have argued that NR2A-containing receptor activation solely controls LTP induction while NR2B-containing receptor activation is required for LTD production in the hippocampus of young rats (Liu et al., 2004). Conversely, Tsien and colleagues have argued against the necessity of NR2B activation for LTD using genetically altered mice,
demonstrating that NR2B over-expression in forebrain of mice led to an increase in LTP but no LTD (Tang et al., 1999). Furthermore, another view is that hippocampal LTP induction can be generated by either NMDAR subtype (Berberich et al., 2005). Therefore, while the regulation of long-term synaptic plasticity by NMDAR subtypes remains a topic of extensive debate, the observed increase in the expression of the three major NMDAR subunits in the hippocampus of TGF-β1 mice endorses the idea that TGF-β1 regulates hippocampal synaptic plasticity in mammals.

Interestingly, the subunit composition of postsynaptic NMDARs and AMPARs appears to be regulated in a coordinated manner in certain populations of synapses, such as the mossy fibre-interneuron synapses. In these synapses, NMDAR subunits are associated with specific AMPAR types (McBain, 2008). Population of synapses that contain GluR2-containing Ca\(^{2+}\)-impermeable AMPAR receptors are typically present together with NR2B-lacking NMDARs, whereas GluR2-lacking Ca\(^{2+}\)-permeable AMPARs are often found at synapses populated with NR2B-containing NMDARs (Bischofberger and Jonas, 2002). Therefore, I speculate that TGF-β1-mediated increase in NMDAR subunits may be associated with the increment of AMPAR subunits, or vice versa.

In general, my study shows, for the first time, that astrocytic over-expression of TGF-β1 up-regulates AMPAR and NMDAR expression. Together with the finding that the presynaptic immunoreactivity of synaptophysin was enhanced in the dentate hilus and stratum lucidum, my results suggest that increased TGF-β1 causes fundamental changes in pre- and postsynaptic protein expression at hippocampal glutamatergic synapses. The functional consequences of these changes and how they precisely affect long-term hippocampal synaptic plasticity and thus learning and memory remain to be studied.
5.3 Increase in TGF-β1 Alone Alters Glutamatergic Synaptic Activities

I found that astrocytic over-expression of TGF-β1 leads to astrogliosis under in vivo and in vitro conditions. Therefore, the altered synaptic protein expression observed in the hippocampus of TGF-β1 mice could be due to indirect actions of gliosis, which results in production and release of multiple cytokines. To examine whether increased TGF-β1 alone regulates the expression of glutamate receptors and glutamatergic synaptic activities, I studied the effects of treating cultured hippocampal neurons with TGF-β1 on glutamatergic synaptic protein expression and glutamatergic function.

Activation of ionotropic receptors induces rapid ion flux and changes in the membrane potential that are essential for evoking action potentials and triggering changes in gene expression, which, in turn, lead to alterations in morphology, excitability, and plasticity (Lin and Bergles, 2004). Therefore, I first examined the expression of ionotropic glutamate receptors in the cultured neurons using immunocytochemistry. The goals of this experiment were to determine whether increasing TGF-β1 alone indeed enhances AMPAR and NMDAR subunit expression in neurons; and, if so, to examine whether this increase is observed at the cell surface level, extrasynaptic level and/or overall internal expression level. My preliminary results showed that treated neurons display an increase in NR2A/B immunoreactivity at the cell surface and in the intracellular compartment. The cell surface immunoreactivity of GluR2/3, however, displayed only a slight increase, rendering the idea of their up-regulation in glial cells more probable. Surprisingly, SB 431542 treatment did not appear to reverse the effect induced by TGF-β1 as both the immunoreactivities for GluR2/3 and NR2A/B showed little to no changes in comparison to the TGF-β1 treated cells. There are a few plausible explanations. First, the neuronal cultures were made from cortical tissue containing both the hippocampus and neocortex. Hence, the stained cells could have been neocortical neurons, whose AMPAR and NMDAR subunit expression was not significantly altered. Second, 10 µM of compound SB 431542 may not have been sufficient to completely block the effects of TGF-β1. Third, the changes in the expression level of the glutamate receptor subunits could have been due to a combined action of numerous cytokines, the conditions of which was not examined and thus cannot be counted for in our in vitro experiment. Interestingly, Tsuzu et al. reported developmental changes in AMPAR subunit
mRNAs in cultured embryonic rat hippocampal neurons. GluR1-4 mRNA harvested from single pyramidal-like cells showed that their sum mRNA expression reached maximal level at day 4-5 in vitro, and that the major subunits were GluR1 and GluR2. While GluR1 and GluR2 reached their day 9 levels and ratios at day 4-5, GluR3 and GluR4 expression levels, on the other hand, appeared to decrease between day 4-5 and 9 (Tsuzuki et al., 2001), suggesting that GluR2/3 heteromers are not a predominant subunit combination. Taking into account the results from this study, we can reason that the majority of AMPARs in our DIV11-12 hippocampal neurons were composed of GluR1 and GluR2 subunits, and thus do not reflect corresponding changes in GluR2/3 immunoreactivity.

Previous studies have shown that TGF-β1 regulates functional expression and stimulation of Ca²⁺-activated K⁺ channels in developing chick ciliary ganglion neurons (Cameron et al., 1998). My study revealed that TGF-β1 treatment significantly augments the amplitudes of glutamate-evoked currents in DIV11-12 hippocampal neurons. This increase in glutamate-evoked currents may be due, at least in part, to the observed up-regulation of the ionotropic glutamate receptor subunits. Given that the major AMPA receptors in cultured hippocampal neurons are composed of GluR1-GluR2 (Tsuzuki et al., 2001), it is likely that these receptors are involved in this glutamate-evoked response. In addition, hippocampal neurons treated with TGF-β1 displayed higher amplitudes of spontaneous EPSCs (sEPSCs) than the control cells. Since sEPSCs are induced by spontaneous action potentials or possibly residual Ca²⁺ from spontaneous and/or evoked action potentials (Cormier and Kelly, 1996), my findings indicate that TGF-β1 increases excitatory hippocampal glutamatergic synaptic transmission. Moreover, I found that cultured hippocampal neurons treated with TGF-β1 also showed significantly higher amplitudes of Na⁺ and K⁺ voltage-gated channel currents, further supporting the role of TGF-β1 as a modulator of neuronal activity. While in vitro analysis of synaptic transmission is insightful, it cannot be extrapolated to predict in vivo implications such as behavioural effects.
5.4 Future Studies

My thesis study serves as a starting point from which further studies can be carried out. In all, more imaging and probing experiments are needed to corroborate my findings. For instance, TGF-β1-mediated changes in AMPAR and NMDAR subunit expression can be examined in vitro by performing immunoblot assays in control and TGF-β1 treated hippocampal neurons cultured in the presence or absence of SB 431542. Also, the association between astrocytosis and microgliosis with synaptic structure and transmission in relation to TGF-β1 is a topic of interest since glial cells produce a variety of regulatory factors. As such, experiments using co-cultures of hippocampal neurons with astrocytes, microglia, or mixed glial cells may be particularly useful. Interestingly, it has been demonstrated that culturing neurons with astrocyte monolayers markedly increased TGF-β1 synthesis and secretion by astrocyte (de Sampaio e Spohr et al., 2002). Other co-cultures studies report different results. It was found that cerebellar neurons co-cultured with astrocytes become susceptible to TGF-β1-induced damage such as astrocyte-mediated glutamate toxicity (Brown, 1999). On the other hand, astrocytes also possess the ability to mediate neuroprotective activity of TGF-β1 against NMDA-induced excitotoxicity in cortical neurons (Docagne et al., 2002), suggesting that TGF-β1 may confer region- and condition-specific effects. However, conducting co-culture experiments may pose some technical challenges since preparing primary neuronal or glial cultures become progressively more difficult as mice age.

As an alternative, hippocampal slice recordings can be performed to corroborate my in vitro results. Considering my finding that TGF-β1 regulates synaptic transmission, together with the observations from Dr. Frankland’s laboratory that aged TGF-β1 mice show cognitive deficits compared to the WT littermates, future studies can be directed toward examining the potential role that TGF-β1 plays in regulating long-term synaptic plasticity in mammals. Furthermore, it would be of great interest to investigate whether the changes I observed in the expression of pre- and postsynaptic proteins in young TGF-β1 mice persist in the aged mice and/or show age-related modifications. Doing so would provide important evidence and help elucidate the mechanisms by which cognitive deficits occur in aged TGF-β1 mice.
5.5 Conclusion

Taken together, my study demonstrates that astrocytic over-expression of TGF-β1 elicits an inflammatory response in the hippocampus and neocortex of young mice, including the activation of astrocytes and microglia. Since the pleiotropic nature of TGF-β1 is often defined by complementary actions of various signalling molecules as well as the physiological or pathophysiological context in which it is expressed, the likelihood of other regulatory signals being involved in the TGF-β1-mediated gliosis cannot be entirely discounted. Increased TGF-β1 also appears to modulate hippocampal expression of pre- and post-synaptic proteins. How the effects of glial activation directly relate to the changes in synaptic protein expression as well as short- and long-term synaptic transmission remain to be studied. Finally, I propose that increased TGF-β1 alone enhances glutamatergic synaptic transmission, at least in culture conditions. Overall, the outcomes from this study further add to the list of the diverse roles that TGF-β1 play in the CNS. Furthermore, my study conjures various areas in which to institute further research, such as the mechanisms through which TGF-β1 mediates neuronal function during neuroinflammation and/or neurodegeneration. Better knowledge of its effect will likely lead to the discovery of interesting targets for the treatment of both acute and chronic CNS pathologies.
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