Glia going emotional: The impact of acute and repeated neonatal separations on astrocytes in the medial prefrontal cortex.

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This is dedicated to my grandfather,
the late **Gil E. Reyno Sr.** who taught me the
art of stubborn diligence and the secret part of industry.
His unshakable confidence in God and trust in His Word remain
to be his living legacy.

A C K N O W L E D G M E N T

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BE THE GLORY! GREAT THINGS HE HAS DONE!

A B S T R A C T

Astrocytes, once considered as merely supporting cells in the brain by only assisting neuronal functions are now implicated to play crucial roles in neuronal migration, establishment and maturation of synaptic contacts during early development. Relatively, only few reports have shown the impact of the neonatal environment on glial plasticity in higher associative brain regions as the medial prefrontal cortex (mPFC) that process, integrate and evaluate memories of learning and experiences. The present work tests the hypothesis if glial plasticity is affected by neonatal separation that altered the neuronal spine density of the mPFC in our previous findings. Neonatal separation was applied during the first three postnatal weeks, a critical period for synaptic plasticity in rodents. The expressions of two astrocytic markers, S100ß and GFAP (glial fibrillary acidic protein) were used to determine the impact of acute and repeated separation on five experimental groups of *Octodon degus*: 1) control, n=5 (CON): undisturbed in the home cage with parents and siblings from postnatal day (PND) 1-21; 2) acute separation+short reunion, n=6 (Group 2): 6 hr separation from parents and siblings on PND 21, returned to the home cage for 1 hr; 3) acute separation+extended reunion, n=4 (Group 3): 6 hr separation from parents and siblings on PND 19, returned to the home cage until PND 21; 4) repeated separation+short reunion, n=6 (Group 4): 1 hr/day separation from parents and siblings on from PND 1-21, returned to the home cage for 1 hr after the last separation; 5) repeated separation+extended reunion, n=4 (Group 5): 1 hr/day separation from parents and siblings on PND 1-14, returned to the home cage from PND 14-21. The density of S100ß-IR and GFAP-IR astrocytes was quantified in the subregions of mPFC including anterior cingulate (ACd), precentral medial (PrCm), prelimbic (PL) and infralimbic (IL) cortices. The somatosensory cortex (SSC) was used as a nonlimbic control region.

Both acute and repeated neonatal separation altered the density of S100ß-IR and GFAP-IR astrocytes in the mPFC showing increases in density of S100ß-IR

astrocytes in a region and layer-specific manner but decreases in density of GFAP-IR counterparts. Acute separation stress affected both the density and morphology of S100ß-IR and GFAP-IR astrocytes in the mPFC but repeated separation stress affected only the density but not the morphology of astrocytes. Extended reunion restored the branching complexity of GFAP-IR astrocytes similar to controls after acute separation stress but reduced the branching complexity after repeated separation stress. In the SSC, acute separation stress did not affect the S100ß-IR astrocytes but increased the GFAP-IR counterparts. Repeated separation+extended reunion increased the density of S100ß-IR astrocytes tremendously as well as the GFAP-IR counterparts.

Taking these findings together, the stress-induced alterations may have consequences in neuron-glia interaction thereby affecting the participation of astrocytes in modulating the synaptic plasticity particularly during the early period of postnatal development. These findings also provide evidence of uniqueness in spatial and temporal specificity of glial response towards a particular environmental stimulation.

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1.1 The dual nature of stress. In any given day, an individual is faced with overcoming various challenges that inevitably brings about a number of physiological changes. These changes have been generally identified as the stress response which was first described by Hans Selye in the 1930s. Selye pointed out that the body manifests an integrated set of responses in an effort to adapt and cope with stressors. The stress response, or simply stress, facilitates the motor execution of a behavioral response appropriate to the situation such as the fight or flight response in times of danger. Information from the external environment and the internal state or drive of an organism is finally integrated in the central nervous system (CNS), specifically in the brain. This defines the brain as the key organ of stress since it interprets what is threatening and stressful and therefore it also determines the physiological and behavioral responses (see review by McEwen, 2006). Adrenocorticotropic hormone (ACTH), the major stress hormone from the pituitary gland stimulates production of glucocorticoids from the adrenal cortex that triggers release of pro- and anti-inflammatory cytokines to cope with stress but at the same time the chronic increase of these mediators may have long-term adverse effects.

Exposure to stress is not always detrimental but in fact can enhance performance. The overall effects of stress on the individual may be determined by the amount of exposure to the stressors. Short term exposure produces adaptive changes such as inhibition of inflammation, resistance to infection and even memory enhancement. Long term exposure however, can bring about maladaptive changes such as enlargement of adrenal glands (Pinel, 2007).

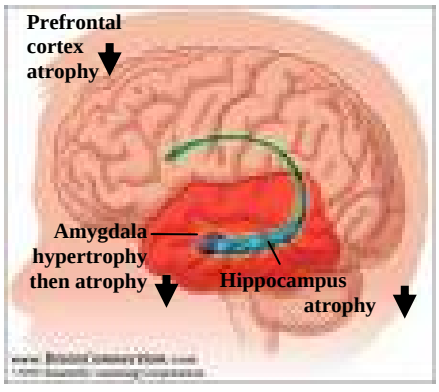

Fig. 1.2 Limbic regions that are involved in perception and response to stress. (McEwen, 2006)

1.2 Impact of stress on limbic regions. It has been postulated that the brain regions including the prefrontal cortex (PFC), amygdala and hippocampus respond to stress by structural remodeling to protect against permanent damage (McEwen, 2006). For example, chronic stress induces atrophy in the rat PFC (Radley et al.,

2006; 2005; Cook and Wellman, 2004) but on the other hand produces dendritic proliferation in neurons of the basolateral amygdala as well as in the orbitofrontal cortex (Vyas et al., 2002). In the hippocampus, acute stress increases spine synapses in the CA1 region (Shors et al., 2001) whereas chronic stress induces dendritic shortening (Pawlak et al., 2005) that occurs as well in the PFC due to neuronal death (Radley et al., 2006; Cook and Wellman, 2004) (Fig. 1.2). In the basolateral amygdala, both acute and chronic stress increase synapse formation (Mitra et al., 2005). The behavioral correlates of these observations were proposed to increase unlearned fear and conditioning and impairment of attention stability (Vyas et al., 2002).

1.3 The medial prefrontal cortex (mPFC) as a limbic region. It is widely accepted that PFC is a brain region involved in higher-order cognition including executive functions, memory, and socio-emotional processes which is important in processing, evaluating, and filtering (inhibiting) social and emotional information (Heilman and Gilmore, 1998). This region is most elaborated and the largest in primates and is proposed to inflexible which does not automatically orient to a novel stimuli (Miller and Cohen, 2001). The major subdivisions of PFC include: a) orbitofrontal which is proposed to the enhance motivations by smell, taste touch (Rolls, 2004); b) dorsolateral that processes sense of navigation or spatial information, evaluation and verification of experiences (Rugg et al., 1998); and c) medial prefrontal which is involved in judgement and selection (review by Petrides, 2000) as well as emotional learning processes. While the dorsolateral PFC has connections with the structures in the motor areas in the frontal lobe (Lu et al., 1994), the orbital and the medial PFC and associated with the limbic structures including hippocampus and amygdala and hypothalamus that process emotions and motivation (Barbas and Pandya, 1989).

Limbic, from a Latin word *limbus* for border was first used by Willis in 1667 to describe the area around the brainstem, Broca in 1878 added more areas including cingulate gyrus, parahippocampal and hippocampal formation. One of the major pathways in the limbic system that is involved in the cortical control of emotion is the papez circuit. Papez proposed that emotions develop in hippocampus,

transmitted to the mammiliary bodies, anterior nuclei of the thalamus and the cingulate cortex, is the reception area for emotional impulses (Fig. 1.3A). MacLean in 1970 emphasized that limbic system in mammals are more complex than lower animals, thus more structures were added in the model including amygdala, thalamic nuclei and mammillo-thalamic tract to mention few of them. Nauta and Domenick in 1980's added more structures to the circuit called the mesolimbic system composing of the posterior orbitofrontal cortex, nucleus accumbens, ventral tegmental area, raphe nuclei, and the locus coeruleus as some of them (Heilman and Gilmore, 1998).

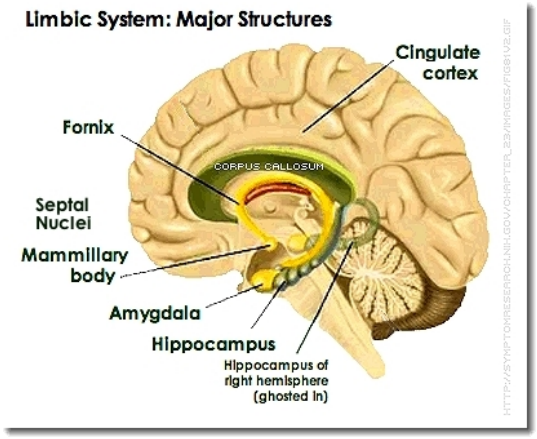

Fig. 1.3A. Schematic illustration of a midsagittal view of a human brain. The limbic system includes, but not limited to the fornix, septal nuclei, mammillary body, amygdala, hipocampus and the cingulate gyrus. Neural substrates may vary depending on the source being referenced. http://web.lemoyne.edu/~hevern/psy340/graphics/

The most prominent cytoarchitecture of rat prefrontal cortex is the absence of layer IV thereby is composed exclusively of agranular cortical areas. Most of the fibers in the rat PFC come from the cortex including somatosensory and limbic cortical areas similar to monkeys (Barbas, 1992). The medial prefrontal cortex is divided into anterior cingulate (ACd), medial precentral (PrCm), prelimbic (PL) and infralimbic (IL) (Krettek and Price, 1977). (Fig. 1.3B). The IL projects strongly to the shell of the nucleus accumbens, while the prelimbic area projects to the core of nucleus accumbens (Ongür and Price, 2000). In rodents, the anterior cingulate cortex is involved in communication and interaction between the pups and the dam. The mPFC along with the OFC networks project extensively to the limbic structures, e.g. the mPFC to the ventromedial caudate and putamen. Studies in monkeys and humans showed connections between mPFC and amygdala suggesting that these areas are

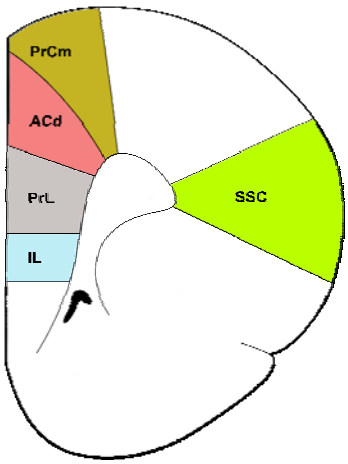

Fig. 1.3B. Schematic drawing of a coronal section of the rat PFC, AP +2.7mm from bregma. SSC = somatosensory cortex, PrCm = precentral Medial, ACd= anterior cingulate dorsal, PrL = prelimbic, IL = infralimbic.

closely connected in anxiety-like responses (Ghashghaei et al., 2002). In fMRI study, patients with chronic PTSD after being presented by fearful and happy faces facial expression showed inversely proportional cerebral blood flow between mPFC and amygdala (Shin et al., 2005). As the cerebral blood in the mPFC decreased, it increased in amydala. Furthermore, PTSD patients also showed reduced volume of the anterior cingulate. Anterior cingulate receives input from hippocampus, infralimbic cortex and also basolateral amygdala (Carr and Sesack, 1996, Hurley et al., 1991; Bacon et al., 1996). On the other hand, the prelimbic (PL) projects extensively to the striatum, while the infralimbic (IL) part projects to the restricted portions of the shell and core of nucleus accumbens (Acb) (Nakano et al., 1999). It has also been suggested that PL and IL of the mPFC are the autonomic motor areas due to their connections with most central autonomic nuclei including the spinal cord (Azuma and Chiba, 1995). Histological and imaging studies on human brains showed that clinical depressive disorders are associated with specific functional and cellular changes in the mPFC including activity and volume changes and in the number of glial cells (Ongür and Price, 2000).

1.4 Impact of early emotional experience on development. Early postnatal experience has a dramatic and lasting impact on the shaping of the individual's behavior at adolescence and adulthood. While genes rule before birth, starting at birth onwards, the environment takes over and shapes the sensory, motor systems and emotions of an individual and carves his adult life (Sullivan et al., 2000; Morriceau and Sullivan, 2006). The earliest external environment that manipulates the infant's physiological responses is the parent, particularly the mother. This very first emotional learning event called *filial printing* or formation of bond to a mother or caregiver which when disturbed will later result in adult deficiencies including deficits in speech behavior, intellectual and social incapacities (Skeels, 1966). It has been shown that childhood emotional trauma is predominantly associated with higher prevalence of both mood and anxiety disorders, particularly depression and post traumatic stress disorder (PTSD) (Maughan and McCarthy 1997; Post et al. 2001). One of the earliest studies on infant monkeys that underwent one or two 6-day separations from the mother at 30 or 32 weeks of age had less explorative

capacity and less likely to approach strange objects years later (Hinde and Mcginnis 1977). In humans, Gilmer and McKinney (2003) showed correlation between separation from or the loss of one or both parents and the chance of developing affective disorders and the separation from parents due to divorce has more impact than parental death. Maternal loss at or before 11 of age is a vulnerability factor and increased the risk of later depression (Harris et al., 1986; Brown, 1988). In a variety of animal models it has also been shown that neonatal emotional experience interferes with the establishment and maintenance of neuronal networks by leaving a footprint that carves the limbic synaptic wiring patterns throughout life (Sullivan et al., 2006). In rodents, the first two weeks of life are a critical period for neuronal development particularly in rats. The lasting impact of neonatal experiences may be due to neuroanatomical and neurochemical changes of limbic circuits in response to the emotional environment (Hall, 1998). In particular, social isolation of the developing neonate can have long-term consequences for adulthood, including alterations of learning and memory, eating, anxiety behaviors, and immune system (Boccia and Pedersenn, 2001; Francis et al., 1999). For example, 3 hr or longer duration of maternal separation increases stressor reactivity adulthood, including increased anxiety-like behavior (Neumann et al., 2005). Stressed neonatal rats showed poor avoidance learning in adulthood indicating that coping response is compromised (Tsoory and Richter-Levin, 2006). The acoustic communication with the mother`s voice is a strong emotional stimulus against separation induced-upregulation of N-methyl-D-aspartate (NMDA) receptors (Ziabreva et al., 2003). It was also observed that postnatal handling as short as 15 min is not stressful and dampens HPA responsivity as well as lessens anxiety-like behavior. Social isolation between during the first three weeks of life altered spatial learning in juvenile and adult rats (Frisone et al., 2002). Maternal separation enhanced synaptic connections in the PFC and hippocampus of preweaning rodents (Helmeke et al., 2001; Ovtscharoff and Braun, 2001; Bock et al., 2005). Cell damage was observed in the CA3 of the hippocampus at four days post-stress of restraint stress, CA3 pyramidal neurons decreased in apical dendrite and total dendritic length (Conrad et al., 1999). Degus raised without a father have reduced spine densities than biparentally-raised counterparts (Ovtscharoff et al., 2006). On the neurochemical level, neonatal

separation induces changes in catecholaminergic fibers in rat limbic system (Holson et al., 1991), activates HPA axis in degus (Gruss et al., 2006) and modulates monoaminergic systems both in neonates and adult tree shrews (Fuchs and Flugge, 2002). Socially-isolated rats show decrease in dopamine metabolites (Miura et al., 2002). These observations show that early emotional experience shapes the synaptic plasticity within the limbic system.

1.5 Astrocytes, the indispensable partner in tripartite synapse. Besides the neuron, the other type of cell in the nervous system is the glia which makes up 90% of the mammalian central nervous system. The name *glia,* a latin word for nerve glue, was coined by its discoverer, Rudolf Virchow (1856) but was first described and studied in length by Ramon y Cajal (1913), subsequently followed by del Rio Hortega (1928) and Penfield (1932) (Kettenman and Ransom, 1995). There are four types of glial cells (Fields & Stevens-Graham, 2002): oligodendrocytes, Schwann cells, microglia and astrocytes. Oligodendrocytes are rich in myelin that forms the sheaths that wraps around the axons, thereby increasing the speed and efficiency of axonal conduction. Schwann cells perform a similar function but in the peripheral nervous system (PNS). However, unlike the oligodendrocytes, Schwann cells are able to guide axonal regeneration after injury, allowing effective cell recovery which so far, is exclusive to the PNS. Microglia cells are the vaccuum cleaners in the sense that they engulf cellular debris in response to injury and disease. Finally, astrocytes, as the name suggests, are star shaped cells whose armlike extensions end in bulbous swellings that cover the outer surfaces of blood vessels (Nishiyama et al., 2005). They have thick primary processes and smaller branching second processes, giving them a bushy appearance (Bushong et al., 2002) (Fig. 1.5A). They make contact with neurons, thus synapses are referred to as TRIPARTITE synapses (Halassa et al., 2007, Araque et al., 1999), indicating that synaptic function is a homeostatic relationship between neurons and glial cells (Fig. 1.5B).

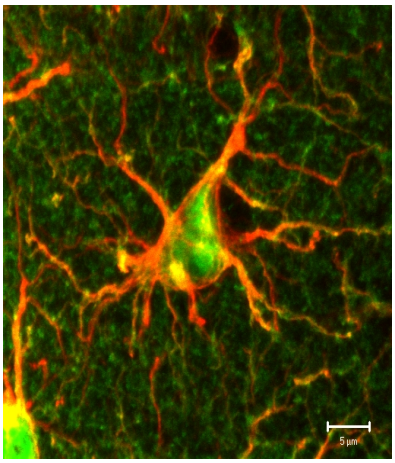

Fig. 1.5A. The astrocyte

Astrocytes, popularly known as intimate partners of neurons in the so called tripartite synapse are considered as important as neurons in the maintenance of cerebral cortical architecture and physiology during the entire development. During the early development, astrocytes control the neuronal development by releasing gliotrophic and neurotrophic factors for neurite growth and extension of serotonergic neurons (review by Azmitia, 2002), for sculpting, or pruning the neurons and establishing synaptic networks in mammalian retinal ganglion cells (review by Freeman, 2006) and guiding migrating neurons in the cerebral cortex (Levitt and Rakic, 1980; Nadarajah, 2003). Astrocyte-derived synaptogenic factor, thrombospondin (Christopherson et al., 2005) is increased during the early postnatal development when synapses form in large numbers but is significantly lower in adult brain. In the mature brain, astrocytes are active partners in synaptic functions. They release gliotransmitters that modulate or control synaptic transmission by acting on themselves or neuron partners (Reviews by Fellin et al., 2006; Volterra and Meldolesi, 2005; Allen and Barres 2005; Haydon, 2001; Vernadakis 1996). Astrocytes-derived glutamate binds to neuronal NMDA receptors (Pasti et al., 1997) or evokes Ca^{2+} waves within neighboring astrocytes and thereby further release of glial glutamate (Newman, 2001) and depolarize groups of neurons resulting in synaptic activity. In the limbic system, ATP released by astrocytes stimulates hippocampal interneurons through P2Y1 receptors that enhanced synaptic activity in CA3 and CA1 (Bowser and Khakh, 2004), but when degraded to adenosine by extracellular ectonucleotidase, it triggers heterosynaptic depression thereby balances the synaptic network between excitation and depression (Fellin et al., 2006). Finally, to maintain the integrity of the brain, astrocytes are part of the blood-brain barrier that controls blood-CNS interface (review by Takahashi and Macdonald, 2004). In an injured brain, astrocytes respond to protect by forming a jacket around the damaged space which is called astrocytic scar (Hamill et al., 2005).

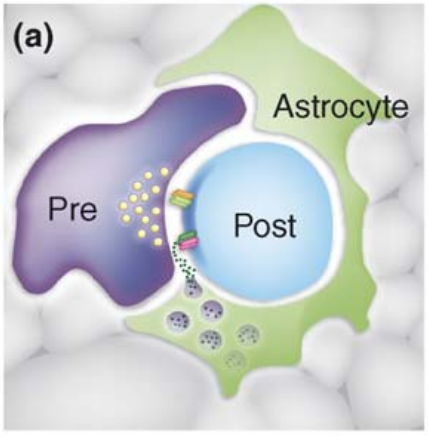

Fig. I.5B. The tripartite synapse (Halassa et al., 2007) refers to the formation of synapse between the presynaptic neuron, the postsynaptic neuron and the astrocyte.

1.6 Glial factors control of synaptic plasticity. For many years, astrocytes were treated as *nursing* or *kitchen cells* suggesting less functional significance for brain function (Haydon, 2001). But due to their consistent appearance particularly during development and development of neuropathological disorders, they tremendously caught the attention of neuroscientists in recent years. It has been demonstrated that the appearance of astrocytes coincides the time-window for neuronal plasticity indicating that neurons start to be functional only after the appearance of astrocytes (Pfrieger and Barres, 1997). In recent years, aside from neuropathological implications of astrocytes, the most interesting advances include their active participation in synaptogenesis during neuronal development via different soluble factors they produce (reviews by Turrigiano, 2006, Ullian et al., 2001; 2004). Astrocytes appear to control synaptic transmission which is favored by their strategic position, i.e. the processes of a single astrocyte may contact tens of thousands of synapses (review by Haydon and Carmignoto, 2006). For instance, *thrombospondins* are discovered as glial-derived factors that promote the formation of functional and stable synapses in retinal ganglion cells 7-fold indicating that astrocytes increase synapse number and synapse stability and maintenance (Christopherson et al., 2005). Glial cells also influences synaptic structure and sculpting by secreting *cholesterol* and imported by neurons to form synapses (Ullian et al., 2001). *Ephrin A3*, a protein expressed in astrocytes processes complemented with ephrin receptor ephA4 in neurons regulates spine shape by inhibiting spine extension (Murai et al., 2003). The removal of EphA4 enhances spine extension indicating that this receptor is required to maintain spine morphology. Interestingly, astrocytes have the same fundamental mechanisms as neurons. They express the same molecules as glutamate, ion channels and neurotransmitter receptors specific for glial-derived molecules and they also respond similar to neurons. However, glial Ca^{2+} signals propagate as calcium wave at rates of micrometers per second, while neuronal action potentials at metres per second (Haydon, 2001).

1.7 S100ß, a multi-faceted glial factor. S100ß is one among the 20 members of S100 Ca^{2+}-binding family of proteins which is exclusively found in vertebrates and most characterized than other members due to wide scope of biological function

including cell motility, proliferation, inhibition of phosphorylation, transcription to mention a few. The name S100ß was given when by Moore (1965) when the protein was discovered to dissolve in 100% ammonium sulphate. They are small (Mr = 10kD) molecules that usually exist in dimers, each of which is flanked by alpha-helices for a total of four helices (Isobe et al., 1978), both the N and C terminal domains binds Ca2+ (review by Heizmann et al., 2002) (Fig. 1.7A). S100ß is coined as a glial marker because it is localized in intermediate filaments (IFs), cytoplasmic microtubules (MTs), axonemal MTs and intracellular membranes in glial cells and several cell lines (review by Donato, 1999). However, it is expressed by few subpopulation of neurons and other cells in the peripheral nervous system including adipocytes, melanocytes, chondrocytes, oligodendrocytes and Schwann cells of the spinal cord (Garbuglia, et al., 1999). The S100ß gene is found on chromosome 21 and gene imbalance is a major contributor to the abnormalities of brain like Alzheimer's Disease (AD) and Down Syndrome (DS) (Reeves et al., 1994). Placed on a strategic position along IFs and MTs, S100ß is designed to crossbridge and or take part in the regulation of the dynamics of cytoskeleton constituent by inhibiting GFAP (glial fibrillary protein) and desmin subunits, vimentin (Ziegler et al., 1998), GAP-43 (Lin et al., 1994) and microtubule-associated protein tau (Baudier and Cole, 1988). The binding of S100ß with the N-terminal (head) domain of GFAP blocks the head-to-tail process of intermediate filament elongation and sequestration of IFs subunits (Fig. 1.7B) influencing the integrity of the cytoskeleton (Garbuglia et al., 1999), thereby defining the shape and morphology of the astrocyte. Double-immunostaining showed that S100 immunoreactivity is mainly colocalized with GFAP in astrocytes and oligodendrocytes (Richter-Landsberg and Heirich, 1995). Cytoskeletal preparation from rat hippocampal astrocytes showed S100ß inhibits the *in vitro* phosphorylation of GFAP and of vimentin (Frizzo et al., 2004). It is suggested that S100ß plays a role in remodelling the cytoskeleton by avoiding excess

Fig. 1.7A. Schematic illustration of S100B protein.
(www.biochem.uwo.ca/fac/shaw/structures.html)

IF assembly (Garbuglia et al., 1999). S100ß also binds to Zn^{2+} and zinc-binding increases S100ß affinity for Ca^{2+} and trigger a myriad of biological effects including cell motility, proliferation, transcription, regulation of nuclear kinase and neurite extension (review by Heizman, 2002; Fritz and Heizmann, 2004).

S100ß have actions that produce conflicting effects, that is, being both neurotrophic and neurotoxic. It is noted to be one of the most important trophic factors in the neuroplastic mechanisms of serotonergic neurons (Rothermundt et al., 2003, review by Azmitia, 2007), and shown to act via an extracellular domain of the receptor for advanced glycation end products (RAGE) in neurons (Huttunen et al., 2000) and astrocytes (Ponath et al., 2007) and microglia (Bianchi et al. 2007). On the other hand, it induces neuronal cell death when expressed in the extracellular space in high nanomolar or low micromolar levels by acting in cytokine-like manner that activates the astrocytes leading to the induction of inflammatory responses which is potentially detrimental (Hu and Van Eldik, 1996). For instance, glial activation leads to induction of pro-inflammatory cytokines including inteleukin-1ß (IL-1ß) and tumor necrosis factor alpha (TNF-∝) and stress-related enzymes including nitric oxide synthase (iNOS) followed by production of nitric oxide (Hu et al., 1997).

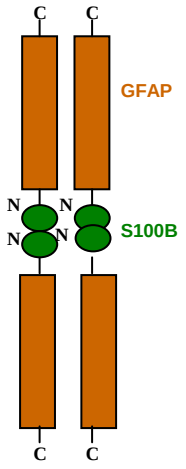

Fig.1.7B. The schematic representation of S100B-GFAP interaction (Garbuglia et al., 1999)

S100ß is a marker of both, immature and mature astrocytes (Tiu et al., 2000). It seems to imply that while S100ß serves as a neuronal growth factor during early development, it is a "gliotransmitter" that modulates synaptic plasticity in the mature brain. *In vitro* studies have shown that in high picolmolar levels, S100ß is a neurite extension factor during development by enhancing neuronal survival (Kligman and Marshak, 1985; Winningham-Major et al., 1989). In humans, S100ß appears during late gestation period and tends to increase during the postnatal period and remains stable for a lifetime. In rodents, the expression of astrocytic S100ß peaked at postnatal day 7 in the ventral posterior nucleus of the thalamus and in layer IV of the parietal cortex from postnatal 7-21 (Muneoka et al., 2003). In

hippocampus, cerebral cortex and cerebellum, astrocytic S100ß content was found similar between the second and fourth postnatal weeks (Tramontina et al., 2002) and astrocytic S100ß from neonatal hippocampus and cerebral cortex were expressed at a slower rate than cerebellar ones (Pinto et al., 2000).

S100ß plays an essential role for the maintenance of the synaptic network in the adult brain. It has been suggested that S100ß has an important role in modulating neuronal synaptic plasticity rather than in brain development (Reeves et al., 1994). For example, studies on transgenic mice which overexpressed the 100ß gene have enhanced generation of astrocytes or astrocytosis and axonal sprouting in the hippocampus indicating that increased expression in the brain has a positive effect on astrocytes and neurite proliferation. (Friend et al., 1992; Reeves et al., 1994). In adult cortex, lesion of serotonergic terminals decreased S100ß levels but treatment with 5-HT1A agonist reverted this loss and promoted recovery of the lesioned serotonin terminal (Yan et al., 1997; Haring et al., 1994). Thus, the decrease of serotonin levels in the adult rat may involve S100ß since it stabilizes microtubule proteins that are found in the dendrites (Meichsner et al., 1993). Treatment of unlesioned adult animals with a 5-HT1A agonist does not increase S100ß release or promote overgrowth of serotonin terminals, suggesting that this sequence of events is most robustly operative when the brain has been damaged (Haring et al., 1994). In vivo, mice with inactivated S100ß gene exhibited no avert abnormalities except for a higher sensitivity of cerebellar astrocytes to treatment with KCl or caffeine (Nishiyama et al., 2002) whereas *in vitro* the lack of S100ß resulted in decreased Ca^{2+}-handling capacity in astrocytes (Xiong et al., 2002). Recently, it was reported that increased glutamate in the synaptic cleft decrease the secretion of S100ß indicating excitotoxic damage (Tramontina et al., 2006).

1.8 GFAP consists the fibrils of astrocytes. GFAP (glial fibrillary acidic protein) is a major intermediate filament (IF) protein in astrocyte that maintains shape and organization of cytoplasm (Herrmann and Aebi, 2004). In particular, it is important in the motility and shape of astrocyte and in providing structural stability to astrocytic processes. The IFs together with actin filaments and microtubules are the constituent of the cytoskeleton which provides the astrocytes their bushy

appearance. GFAP is a type III 8-9 nm intermediate filaments (IF) first discovered by Eng and colleagues in 1969 (Eng et al., 2000) in mature astrocytes. In humans, 65 different IF proteins are presently identified (Herrmann and Aebi, 2004).

The GFAP subunit consists of an N-terminal head, central rod and C-terminal tail domains (Fig. 1.8). The rod domain is composed of two helical regions separated by short, non-helical stretches. The N-terminal head is critical for filament elongation, whereas the C- terminal tail appears less critical (review by Inagaki et al., 1994). The

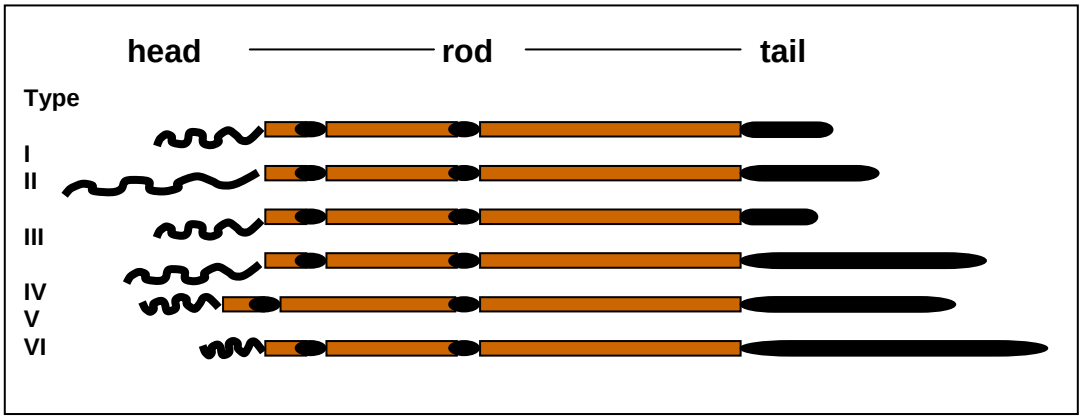

Fig. I.8. Schematic illustration of the six subclasses of intermediate filament proteins, type I-VI. GFAP belongs to type III to which vimentin, desmin and alpha-internexin also belong. (Inagaki et al., 1994)

central rod appears important for the lateral association of the subunits into dimers/ tetramers/octamers (Chou et al., 2007). Phosphorylation of the preformed GFAPs will result to their disassembly and phosphorylation of unassembled subunits make them incompetent to assemble. GFAP is the most common used marker along with vimentin for glial cells (Tiu et al. 2000). GFAP is recognized as a marker for mature astrocytes (Levitt and Rakic, 1980; Sarnat, 1992), while vimentin is present earlier in development in epithelial cells and radial glia indicating that it is expressed in the immature astrocytes (Sasaki et al., 1988). In the human brain, GFAP expression starts during late embryonic stages, increases to early postnatal period and declines in adulthood (Fox et al., 2004). GFAP progressively increase in the occipital and temporal cortices between 14-17 embryonic weeks, then decreased until 32 weeks before increasing again towards term (Tiu et al., 2000). In the rat cochlear nucleus, there is a progressive increase of GFAP-IR astrocytes, the expression becomes stable at one month (Burette et al., 1998). In monkey visual cortex, GFAP-IR astrocytes decrease postnatally until three months (Missler et al., 1994), while in cat

visual cortex, GFAP expression progresses from white matter on the first postnatal month and to the visual cortex area 17 and 18 at adulthood (Rochefort et al., 2005). Increased GFAP expression during the gestation period has also been documented in monkey (Levitt and Rakic, 1980) and fetal frontal lobe (Aquino et al., 1996, Honig et al., 1996) reflecting a pattern that GFAP increases as the astrocytes mature. Astrocytes in postnatal monkey frontal and temporal cortices were described to develop from displaying scarce and short processes at one month to abundant and extended processes at three years of age (Colombo and Bentham, 2006). GFAP is colocalized with two other glial cell markers, S100ß and glutamate transporter 1 (GLT-1) both in the cell bodies and processes but not with other neurotransmitter markers including dopamine receptor 1 (DP1) or synaptophysin (Tiu et al., 2000).

The expression of GFAP varies among astrocytes. For example, GFAP is expressed more abundantly in fibrous astrocytes within the white matter than in protoplasmic astrocytes located within the grey matter (review by Walz, 2000). Moreover, within a single astrocyte GFAP expression can also vary within different processes, i.e. astrocytic end-feet interacting with blood vessels or the pial surface have strong immunoreaction for GFAP, whereas the fine processes that enwrap the synapses or nodes of Ranvier are immunonegative for GFAP. The expression can be altered by environmental factors (see chapter 1.8). For instance, protoplasmic astrocytes located in the grey matter when they become reactive in response to neuronal damage (Vos et al., 2006; Swanson et al. 2004), they increase the expression of GFAP in the processes. Trauma, ischemia, infectious and neurological diseases and most recently, neurotoxicants including cadmium, trimehtyltin, ketamin, kainic acid, methylmercury transform microglia and astrocytes into activated phenotypes, a progressing event which is referred to as gliosis (O'Callaghan and Sriram, 2005).

1.9 Impact of early experience on astrocytes. Little is known about the involvement of astrocytes in learning and experience-induced plasticity in the adult or developing prefrontal cortex. To our knowledge only one study described the influence of early emotional experience on glial development in a limbic region. Bredy et al. (2003) found increased GFAP expression in the hippocampus of rats reared by mothers which provided lower maternal care than ones with high maternal

care. Previous studies investigating learning/experience-induced aspects of glial development were restricted to sensory regions. Astrocytes in the rat visual cortex and cerebellum dramatically respond to challenging experiences and learning opportunities by displaying increased cell number (hyperplasia) and volume fraction (hypertrophy), increased surface density and proliferation of astrocytic processes (Szeligo and Leblond, 1977; Sirevaag and Greenough, 1987, 1991). In the rat visual cortex, monocular deprivation decreased the density of GFAP-IR astrocytes in la layer-specific manner (Hawrylak & Greenough, 1995), and dark rearing decreases the levels of GFAP (Stewart et al., 1986), whereas in the mouse visual cortex, hippocampus and motor cortex, GFAP-IR astrocytes were not affected by monocular deprivation (Corvetti et al., 2003; 2006). These observations are paralleled by experiments, which induced moderate and more natural environmental alterations. For instance, increased densities of GFAP-IR processes and increased astrocyte-synapse contacts were detected in the visual cortex of weanling rats few days after rearing in an enriched environment (Jones et al., 1996; Jones & Greenough, 1996). Furthermore, it has been shown that early learning induces elongation of glial cell processes in the rat olfactory bulb, although the number of GFAP-positive cells between control and trained pups did not change (Matsutani & Leon, 1993). These observations reveal that astrocytes are factors which significantly influence neuronal and synaptic development in sensory and motor regions as well as in limbic cortical and subcortical areas.

1.10 Aims of the study. Pronounced synaptic plasticity including increase in dendritic spines in the PFC particularly in the ACd of *Octodon degus* and White-Wistar rats was described in response to repeated separation stress (Helmeke et al., 2001; Ovtscharoff and Braun, 2001; Poeggel et al., 2003; Bock et al., 2005). The present work is the extension of the previous work to determine whether single and repeated early separations on preweaning stage also affects the astrocytes, the partner of neurons in synaptic plasticity. This Dissertation presents the impact of acute and repeated neonatal separations on astrocytes and the probable implications of stress-induced changes in the developing mPFC.

The impact of preweaning 6 hr acute isolation stress and 1 hr repeated separation stress on *O. degus* was investigated to find out if:

1) S100ß and GFAP expressions of the juvenile mPFC are altered by stress;
2) stress-induced changes of the astrocytic markers are paralleled by changes in the morphology of astrocytes;
3) the stress-induced astrocytic changes are transient or lasting;
4) the prefrontal cortex is specifically and more sensitive to neonatal stress than the somatosensory cortex.

2.1 Animal Model. *Octodon degus* is a semi-precocial rodent (or according to other literature lagomorph). This animal was chosen as a model system opposed to the usual altricial laboratory rat or mouse because among many features, its sensory and motor systems are relatively mature at birth which resemble the situation in human newborns. The functional maturity of the sensory systems allows the neonate degu pups to recognize, learn and interpret acoustic signals and enable them to discriminate the difference between familiar and novel environment immediately after birth (Braun and Poeggel, 2001, Poeggel and Braun, 1996).

Fig. 2.1. *Octodon degus* pups have functional sensory systems at birth that resemble the human newborns.

The animal colony is bred and kept at the Leibniz Institute for Neurobiology, Magdeburg. Family groups consisting of an adult couple and their offspring were housed in wire cages (length x height x depth: 53 cm x 70 cm x 43 cm) equipped with little burrows and climbing scaffolds. The animals were exposed to a light/dark cycle with 12 hrs light (6:00 a.m./6 p.m.). Fresh drinking water and rat diet pellets were available ad libitum, vegetables were also fed. The rooms were air-conditioned with an average temperature of 22°C. A total of 25 male pups were analyzed on postnatal day (PND) 21. To prevent litter effects, a maximum of two male pups from each litter was tested.

2.2 Separation Procedure. In this study, separation was used to as stress paradigm. To induce stress, the pups were removed from their parents in the home cage and individually isolated in small and shallow plastic cages (37cm x 11cm x 8.5cm) with fresh wood shavings as bedding material. During separation, the pups could hear and smell their siblings but no visual and body contact with the siblings and no sensory contact with the parents were allowed. The following animal groups were compared (Fig. 2.2):

1) Group 1/ Controls (n=5): pups were reared undisturbed with family members from birth until postnatal day (PND) 21.

2) Group 2/ Acute separation stress + short reunion (n=6): pups were exposed to 6 hr separation on PND 21. After the separation, the pups were returned to the home cage and reunited with the family members for 1 hr (short reunion) before perfusion.

3) Group 3/ Acute reunion stress + extended reunion (n=4): pups were exposed to 6 hr separation stress on PND 19 and returned to the home cage and reunited with the family for 48 hr (extended reunion) until PND 21.

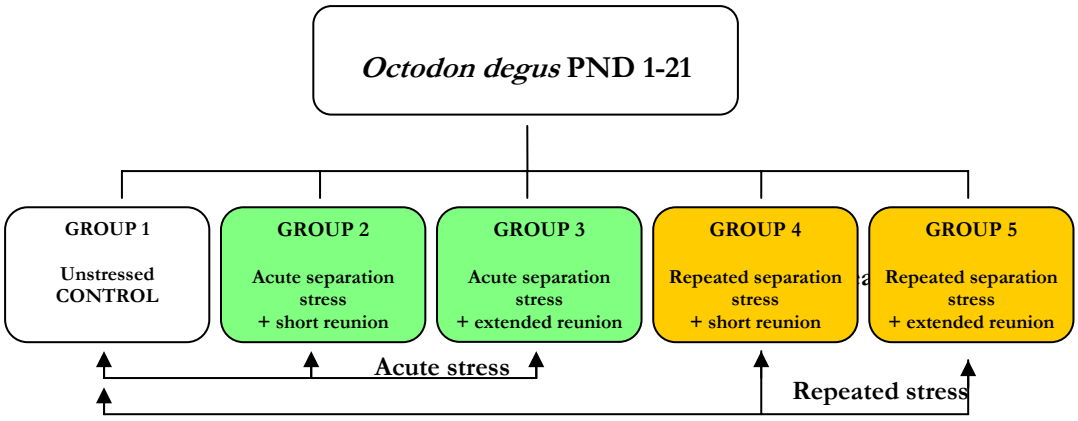

Fig. 2.2. Schematic diagram of acute and repeated neonatal separation paradigms.

4) Group 4/ Repeated separation stress + short reunion (n=6): pups were exposed to 1 hr daily separation stress from PND 1-21. After the last separation, the pups were returned to the home cage and reunited with the family for 1 hr before perfusion.

5) Group 5/ Repeated separation stress + extended reunion (n=4): pups were exposed to 1 hr daily separation stress from PND 1-14. Afterwards, the pups were returned to the home cage and reunited with the family and left undisturbed until PND 21.

2.3 Perfusion and Fixation Procedures. Brain fixation and preparation were done as previously described (Braun et al., 2000). The pups were weighed and deeply anesthesized by an intramuscular injection 1:4 Ketanest/Rompun (0.5 ml/100g body weight) (Ketanest: Parke-Davis, Berlin, Germany; Rompun: Bayer, Leverkusen, Germany). Transcardial perfusion was conducted with 50 ml Tyrode's buffer (pH=6.8) containing 1% Liquemin, an anticoagulant (Roche, Grenzach-Wyhlen, Germany) followed by 150 ml of fresh 4% paraformaldehyde in 0.1 M sodium acetate buffer (pH=6.5), followed by 300-400 ml of fresh 4% paraformaldehyde in 0.1 M sodium tetraborate buffer (pH=9.3). Brains were removed, weighed and postfixed in the last fixative for 1 hr at 4°C. Four alternating series of frontal brain sections at 50 μm-thick were cut with a vibratome (VT1000E, Leica, Wetzlar, Company) and collected in 24-well plates filled with 0.1 M PBS (phosphate buffered saline) (pH=7.4). The first and second alternating series of sections were used for S100β and GFAP immunoreactivity, the third was Nissl stained for layer identification and the fourth was used for double-labeling of GFAP and S100β to confirm the colocalization of both proteins. (Supplementary materials in Appendix 4).

2.4 Immunohistochemistry. This study involved colorimetric immunohistochemistry to: 1) quantify the density of S100ß and GFAP-IR astrocytes in four subregions of the mPFC and SSC (See Fig. 1.3B); 2) quantify structural integrity of GFAP-IR astrocytes; and 3) observe the morphological changes in S100ß-IR astrocytes.

Antibodies against the glial markers, S100β (an astroglial-derived neurotrophic factor) and GFAP (the main cytoskeletal astroglial protein) were applied for cellular identification of astrocytes. The sections were rinsed with phosphate buffered saline (PBS) and pretreated with 1% H_2O_2 in PBS containing 10% methanol for 45 minutes to block intrinsic peroxidase activity. Sections were rinsed 3-4 times in PBS and pre-incubated with 2% normal goat serum (NGS) to block non-specific binding (DAKO, Hamburg, Germany) and 0.1% Triton X-100 (Merck, Darmstadt, Germany) in PBS for 1 hr for membrane permeabilization. The pre-incubation solution was removed and sections were incubated with anti-S100β (1:2000; Sigma-Aldrich, Germany) or anti-GFAP (1:400; Sigma Aldrich, Germany) mouse

antibodies in PBS containing 1% NGS and 0.1% Triton X-100 for 48 hrs at 4°C. Primary antibodies were omitted from sections that served as controls to determine the specificity of the reaction. After 48 hrs, sections were rinsed thoroughly in PBS and incubated for 1.5 h with biotinylated goat-anti-mouse-IgG secondary antibody (Amersham, U.K.) diluted at 1:200 in PBS containing 1% NGS. Sections were rinsed in PB (Phosphate buffer) and incubated in Extravidin peroxidase complex (Sigma-Aldrich, Germany) diluted at 1:200 with PB for 2 hrs. The reaction was visualized by incubating the brain sections with 0.05% 3,3'-diaminobenzidine–HCl (DAB; Sigma-Aldrich, Germany), 0.2% ß-D-glucose, 0.003% glucose oxidase and 0.04% NH_4Cl in 50 mM Tris–HCl buffer for 15-20 min until optimal color reaction was achieved. Sections were mounted on gelatin-coated slides, dehydrated in ascending grades of ethanol and xylene and cover slipped with Histomount (Life Sciences International, Germany).

2.4.1. Astrocyte Density Quantification. The density of S100ß and GFAP-IR astrocytes were counted in both hemispheres of mPFC subregions: anterior cingulate cortex, the precentral medial cortex prelimbic cortex and infralimbic cortex, also in the somatosensory cortex for nonlimbic control. Four replicates per hemisphere for each animal was examined in images captured at 20x objective of an Olympus BH-2 fluorescence microscope, equipped with a video camera and a computer system. Using NIH-Image software, sequential counting frames were taken throughout each subregion covering the cortical layers I-VI (Fig. 2.4.1) and the density distribution of cells in each region was plotted. Layer extensions and margins were compared with adjacent Nissl stained sections and marked to calculate areas for layer-specific analysis of cell number distribution. Only S100ß and GFAP-IR cell bodies were counted and their density was calculated for each cortical layer.

2.4.2. Astrocyte Morphology Quantification. A total of 200 GFAP-IR astrocytes in the ACd were traced to determine differences in soma size, in the number and length of processes and in the number of branching points (nodes) between groups. Only astrocytes displaying their entirety, having intact cell body and elaborate branching were considered. Astrocytes were traced and reconstructed at 1000x using

Olympus BH-2 microscope equipped with image analysis system and Neurolucida morphometry software (MicroBrightfield, Colchester, VT).

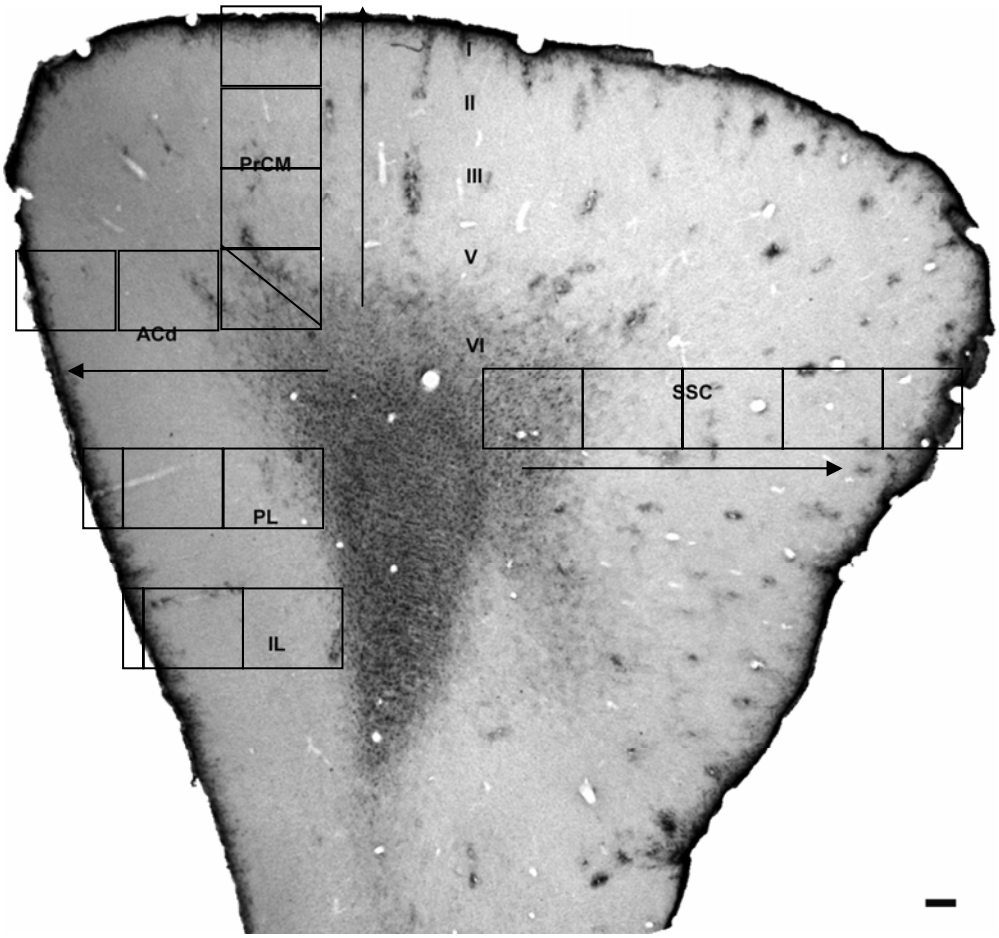

Fig. 2.4.1. Schematic illustration of section captured by sequential counting frames at 200x magnification covering the cortical layers I-VI of each mPFC subregion and SSC.

2.4.3. Fluorescent immunohistochemistry. Double-immunofluorescent labeling followed the same protocol as described above, but pretreatment of the sections with H_2O_2 and methanol were omitted. The sections were incubated with primary antibodies of rabbit-anti-GFAP (1:500; Sigma-Aldrich) combined with mouse-anti-S100ß (1:2000; Sigma-Aldrich) in a solution containing 1% NGS, 0.05% Triton-X-100 at 4°C for two days. After incubation with the primary antibodies, the sections were rinsed with PBS and incubated with the first secondary antibody anti-mouse Alexa 488 (1:250; Mobitec, Germany) for 30 minutes in the dark and followed by subsequent washes. Sections were then incubated with the second secondary

antibody anti-rabbit Alexa 546 (Mobitec, Germany) for 30 minutes and were rinsed thoroughly in PBS. The sections were mounted on chrome alum-coated slides, allowed to dry and cover slipped with Histomount. Images of astrocytes in the mPFC were taken using a confocal laser scanning microscope (Zeiss LSM 510 Meta).

2.5. Statistical analyses. Control and two separation groups (Acute: Group 2 and Group 3) or repeated groups (Repeated: Group 4 and Group 5) were compared using SigmaStat software (version 3.5; Jandel Scientific, Erkrath, Germany) (Fig. 2.2). In this study, acute versus repeated separation groups were not compared due to different reunion procedures that were employed. The values are represented as the mean±SEM with a significance level set at P=0.05. Significant differences (between different rearing and hemisphere) were determined with 2-way ANOVA (analysis of variance) and Tukey test as post hoc test (if data was parametric) and Holm-Sidak method (if data was non-parametric).

The results of this study showed that acute separation stress affects expression of astrocytic marker proteins both in density and/ or in the structure of astrocytes. Repeated separation stress affects the expression of astrocytic marker proteins in density but not the structure of astrocytes in the mPFC. The major findings of this study are the following:

1) the density of S100B-IR astrocytes in the mPFC are increased after acute or repeated separation stress;
2) the density of GFAP-IR astrocytes in the mPFC are decreased after acute or repeated separation stress;
3) The structural integrity of astrocytes in the mPFC is decreased by acute separation but not affected by repeated separation;
4) S100ß-IR astrocytes in the SSC are not affected by acute separation stress while GFAP-IR astrocytes in the same region are increased by it;
5) The structural integrity of astrocytes is enhanced by extended reunion after acute separation stress but diminished by extended reunion after repeated separation stress.

3.1. General observations of S100ß and GFAP expressions in the mPFC

3.1.1 Distribution of S100ß-IR and GFAP-IR astrocytes (Fig. 1). The initial observation in this study was the distribution of S100β and GFAP-IR astrocytes in the cerebral cortex. S100ß-IR astrocytes were homogenously distributed (Fig. 1A) while the GFAP-IR counterparts were mainly localized in layer I-II (Fig. 1B) and layer V/VI (except PL and IL) of the mPFC (Fig. 1C,D) and SSC (Fig. 1C). Long astrocytic processes in layer VI of PL and IL extending towards layer I were observed in Group IV but not in other groups (Fig. 1E). Astrocytic processes surrounding the blood vessels were also observed (Fig. 1F).

3.1.2 S100ß-GFAP Colocalization (Fig. 2). The immunofluorescence double-labeling showed that S100ß and GFAP are colocalized particularly in layer I-II of the cortex (Fig. 2A-C). GFAP-IR astrocytes were also present in layer V-VI but with undetectable S100ß but astrocytes in the middle layers were exclusively S100ß-IR. Within astrocytes, S100ß was seen in the soma and nucleus (Fig. 2D,F) while GFAP was more intense in the processes (Fig. 2E,F).

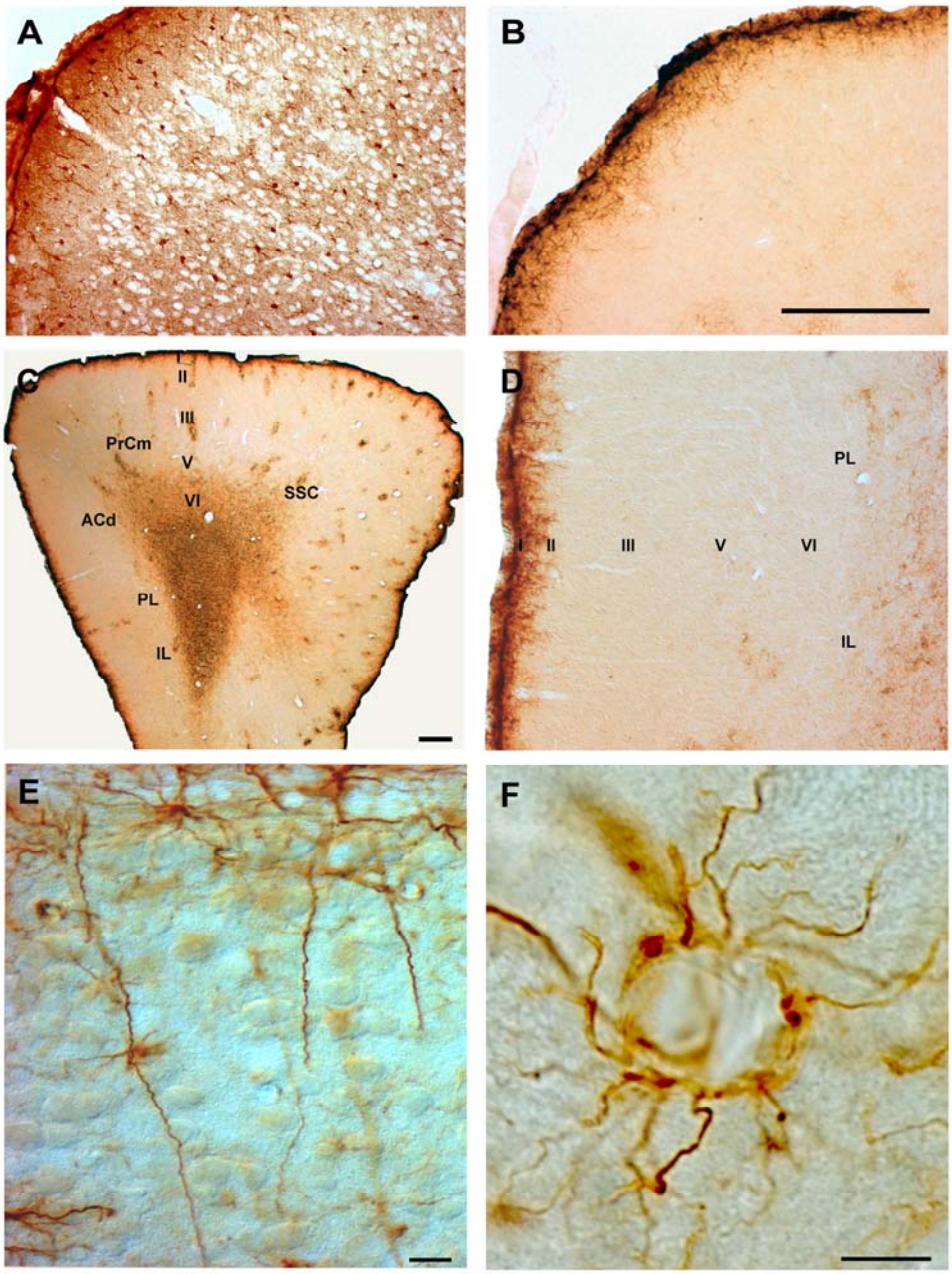

Fig. 1. Expressions of S100ß and GFAP in the cerebral cortex. S100ß-IR astrocytes (A) were homogenously distributed in all cortical layers while GFAP-IR astrocytes (B) were localized at the borders. PL and IL layer V-VI were completely devoid of GFAP-IR astrocytes (C,D) but long astrocytic processes were abundant in these subregions after repeated separation followed by short reunion (E). Blood vessels were usually surrounded by astrocytic processes (F). Scale bars: in A, B and D is 100 μm; in C is 200μm; in E and F is 10 μm.

3.1.3 Morphology of S100ß-IR and GFAP-IR astrocytes after acute separation stress (Fig. 3). In the control (Group I), intact and evenly-stained cell bodies and profused branches were observed in S100ß-IR astrocytes (Fig. 3A), even distribution of GFAP in the soma and processes (Fig. 3B). In Group II, S100ß-IR astrocytes were ruptured, cell bodies were broken (Fig. 3C) and GFAP-IR astrocytic processes were thickened (Fig. 3D). After extended reunion, S100ß-IR astrocytes in Group 3 were similar to Group 2 (Fig.3E) but in contrast, the GFAP-IR counterparts in the same group showed increased in astrocytic processes (Fig.3F).

3.1.4. Morphology of S100ß-IR and GFAP-IR astrocytes after repeated separation stress (Fig. 4). In controls (Group 1), intact and evenly-stained cell bodies and profused branches were observed in S100ß-IR astrocytes (Fig. 4A) and even distribution of GFAP in the soma and processes (Fig.4B). In Group 4, S100ß-IR astrocytes (Fig. 4C) and GFAP-IR counterparts (Fig. 4D) were similar to controls. After extended reunion, S100ß-IR (Fig. 4E) and GFAP-IR (Fig. 4F) astrocytes in Group 5 both showed reduced branching complexity.

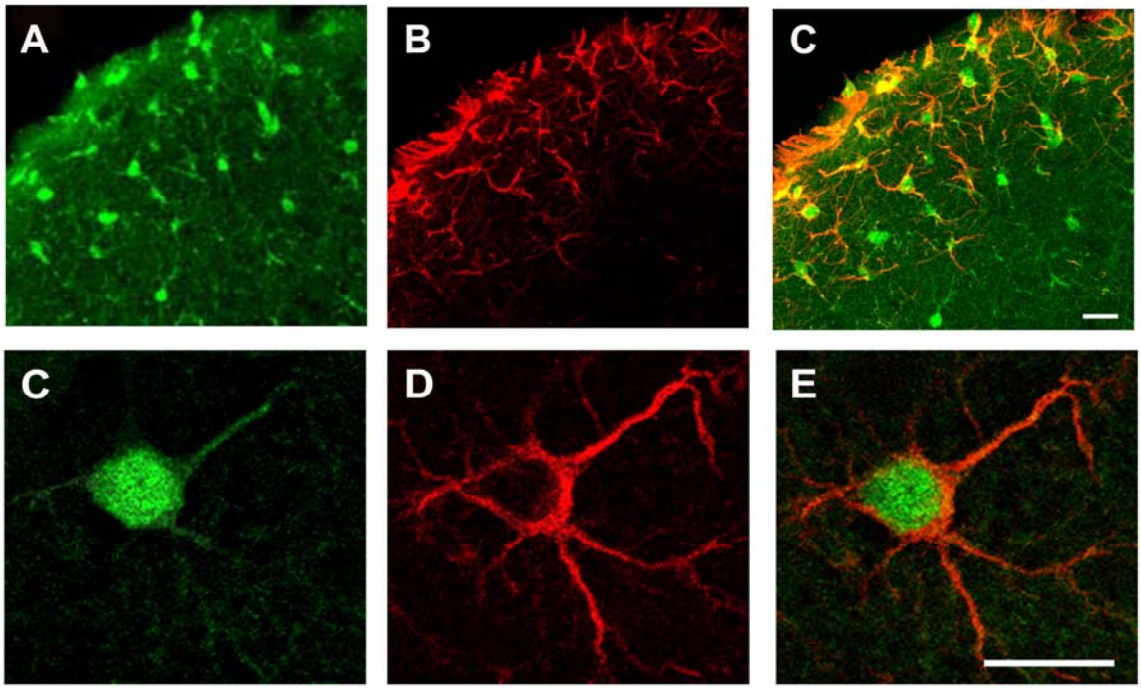

Fig. 2. Double immunostaining of S100ß and GFAP-IR astrocytes in the mPFC. S100ß-IR (green) and GFAP-IR (red) astrocytes are colocalized in layer I (A,B,C) of the cortex but not in other layers. In individual astrocytes, S100ß defines the cell body while GFAP is prominent in the processes (C,D,E). Scale bars: in A,B,C is 20 μm; in C,D,E is 10μm.

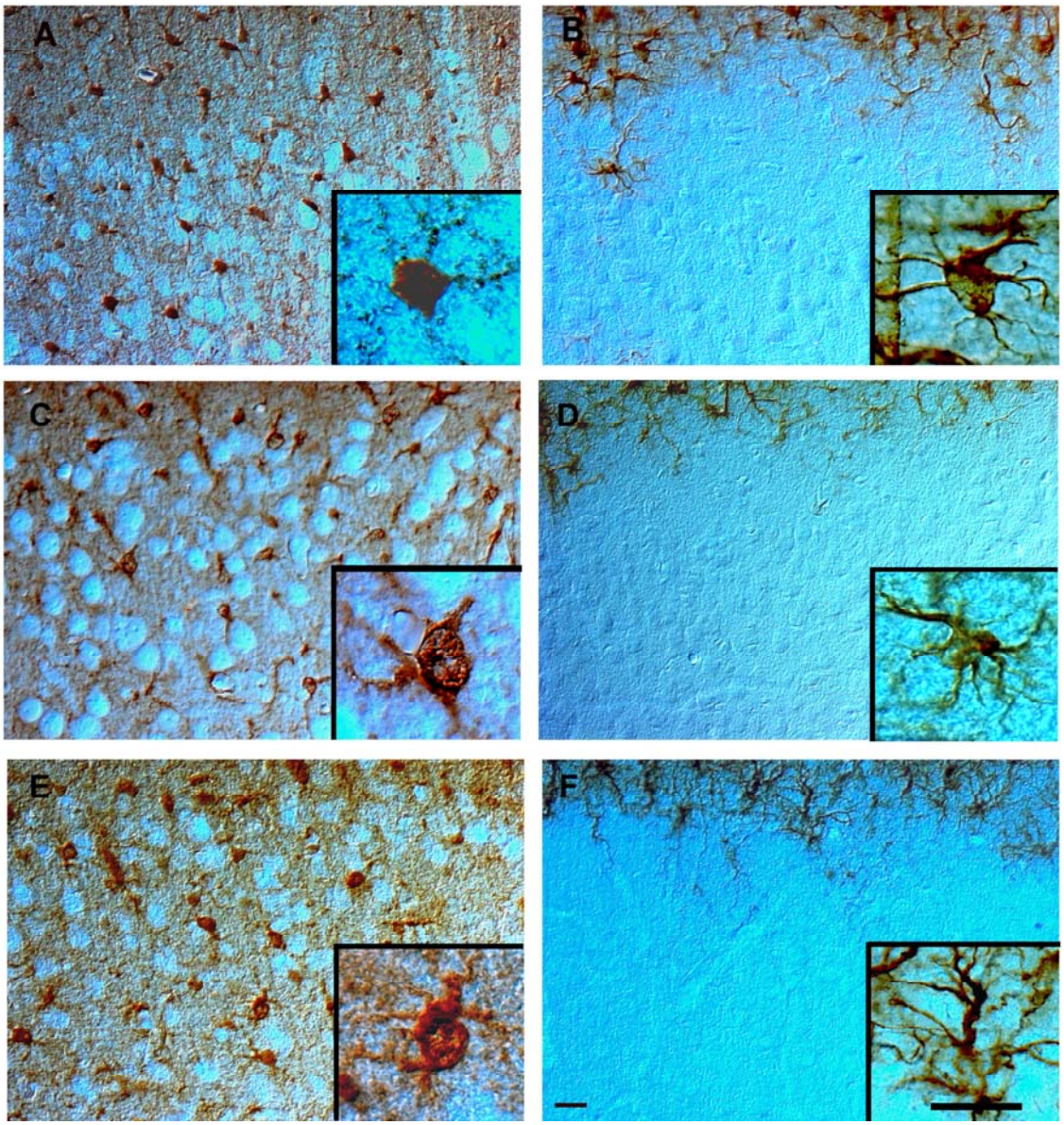

Fig. 3. Morphology of S100B-IR and GFAP-IR astrocytes in the mPFC after acute separation stress. Intact cell bodies in control S100ß-IR astrocytes (A) but "broken" cells and damaged processes were observed after acute separation stress whether it is followed by short (C) or extended reunion (E). Well-defined soma and profused branching in control GFAP-IR astrocytes (B) but shortened and shrivelled processes were observed after acute separation followed by short reunion (D) and restored by extended reunion (F). Scale bars: 50μm; 10 for inset.

3.2. Changes in S100ß and GFAP expressions in response to acute separation stress: comparison between short (Group 2) and extended (Group 3) reunion.

Region and layer-specific increases in density of S100ß-IR astrocytes but decreases of GFAP-IR counterparts were observed in the mPFC after acute separation. The numbers are represented by mean and SEM of left and right hemispheres per

group in an area of 0.159 mm², 4-5 counting fields of 60 sections (four replicates/ hemisphere/ region/ animal) at × 200 magnification. (Supplementary data in Appendix 1 and 2)

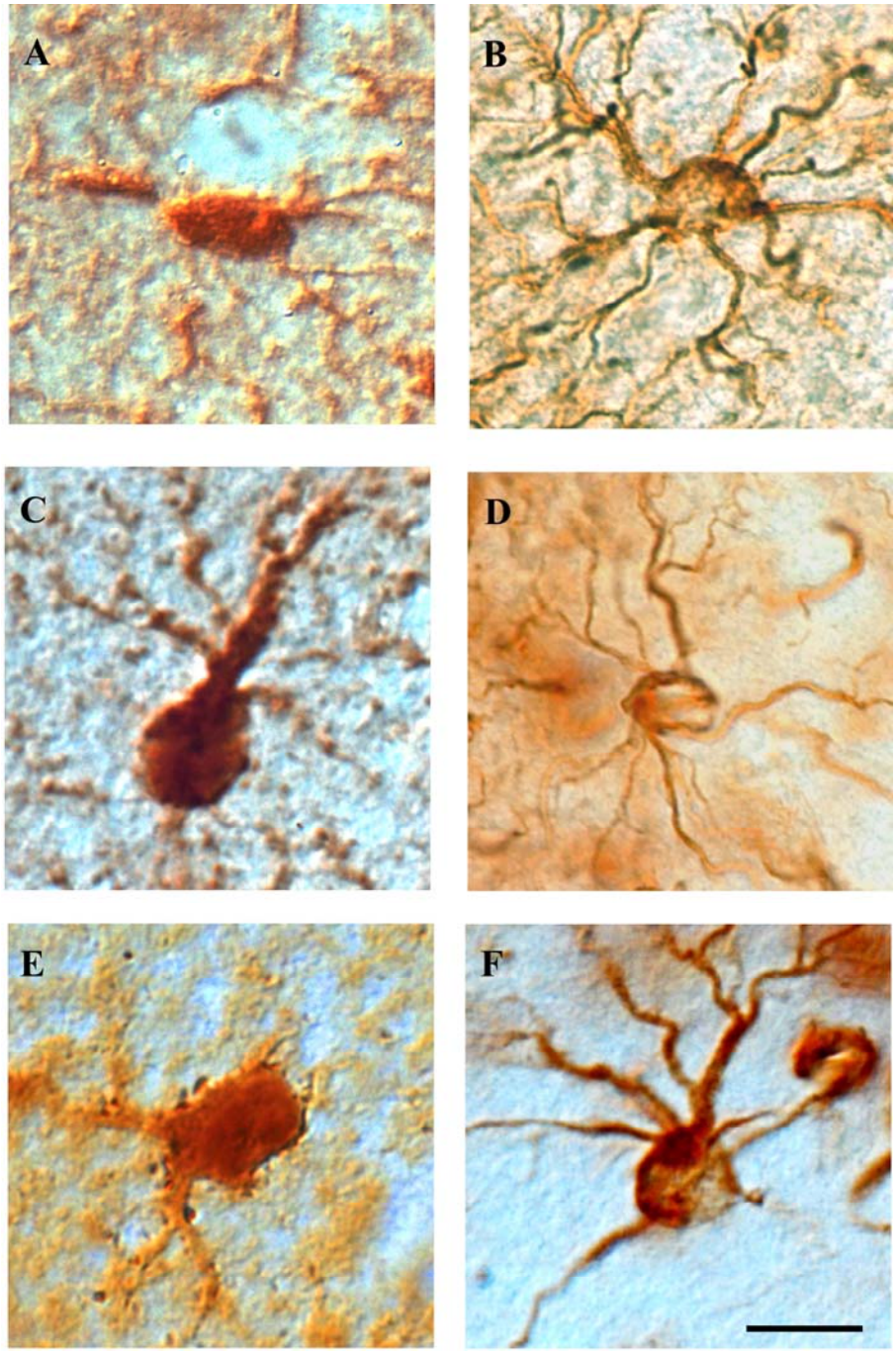

Fig. 4. Morphology of S100B-IR and GFAP-IR astrocytes in the mPFC after repeated separation stress. The cell body of S100ß-IR astrocytes in control (A) and in stressed counterparts remained intact whether followed by short (C) or extended reunion (E) although S100ß expression was more intense in stressed astrocytes. GFAP-IR astrocytes in control (B) and repeated separation followed by short reunion (D) showed similar profused branches which diminished after extended reunion (F). Scale bar is 10 μm.

3.2.1 The cell density of S100ß-IR astrocytes in the mPFC increases after acute separation stress (Fig. 5). Taking the values for layers I-VI (Fig. 5A), significant increases of S100ß-IR astrocytes were observed in the PrCM of Group 2.

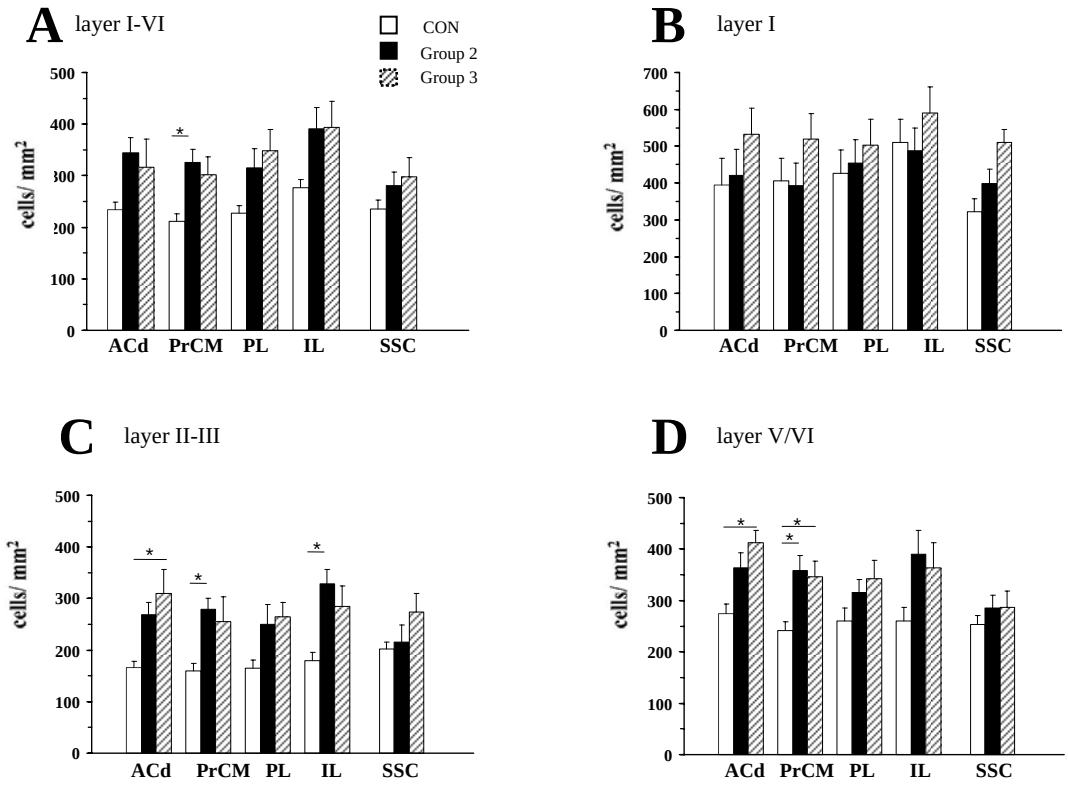

Fig. 5. Cell density of S100B-IR astrocytes in the mPFC and SSC of *O. degus* following acute separation. Cell density of all cortical layers I-VI (A); layer I (B); layer II-III (C) and layer V/VI (D). CON=control; Group 2= acute separation+short reunion; Group 3=acute separation+extended reunion.

with a strong trend of increase in Group 3 (P=0.07) compared to controls. In Group 2, S100ß-IR astrocytes were increased in the PrCM up to 153% with trend of increase in other subregions including ACd, PL, and IL. In Group 3, the density of S100ß-IR astrocytes showed a non-significant trend towards increase. No significant difference in the SSC was observed between the groups.

In layer-specific analyses, Layer I did not show a significant difference in density of S100ß-IR astrocytes in all the mPFC subregions between the groups although the cell density was highest in this layer (Fig. 5B). In layer II-III (Fig. 5C), a significant increase in density of S100ß-IR astrocytes were observed in the mPFC of both Groups 2 and 3 compared to controls. In Group 2, increased density of S100ß-IR

astrocytes was observed in PrCM (up to 174%) and IL (up to 182%) compared to controls with similar yet non-significant trend in PL. After reunion, Group 3 showed increase in ACd (up to 186%) compared to control with strong but non-significant trend in PrCM (P=0.09) and IL (P=0.06). No significant difference in PL between the groups was observed. In layer V/VI of the mPFC (Fig.5D), Group 2 showed significant increase in density of S100ß-IR astrocytes only in the PrCM (up to 148%) but not in PL and IL. After reunion, Group 3 showed increase in ACd (up to 150%) and PrCM (up to 143%) but not in other subregions. In SSC, no significant changes in density of S100B-IR astrocytes between the groups were observed.

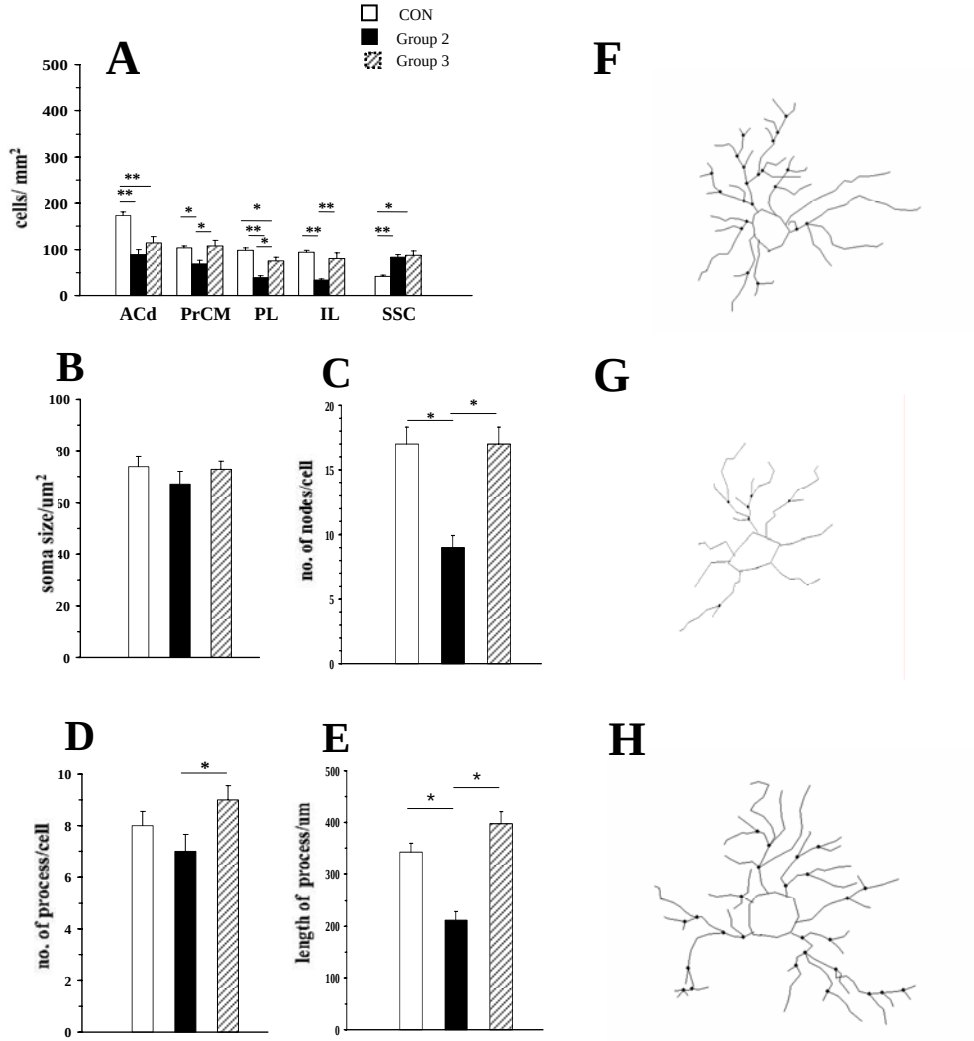

Fig. 6. Cell density of GFAP-IR astrocytes in the mPFC and SSC of *O. degus* following acute separation stress (A). Structural integrity of GFAP-IR astrocytes in the ACd including soma size (B), number of nodes (C), number of process (D), and length of process (E). Morphology of astrocytes in the ACd: F is CON, G is Group 2 and H is Group 3. CON=control; Group 2= acute separation+short reunion; Group 3=acute separation+extended reunion.

3.2.2. The cell density of GFAP-IR astrocytes in the mPFC is decreased after acute separation stress, but partly restored by extended reunion (Fig. 6). Stress-related analysis in GFAP-IR astrocytes was not analyzed in a layer-specific manner because they almost exclusively occupied the layer I-II. Few GFAP-IR astrocytes were present in layer V-VI of the ACd and PrCM but the same layer of PL and IL were completely devoid of them (Fig.1C, D). The density of GFAP-IR astrocytes in ths study can be presumed to be consisted mostly of those occupying the layer I-II.

Unlike the S100ß-IR astrocytes, a significant decrease in density of GFAP-IR astrocytes was observed in all mPFC subregions of Groups 2 and to a lesser extent in ACd and PL of Group 3 compared to controls (Fig. 6A): (ACd down to 51 %, PrCm down to 65 %, PL down to 40 %, IL down to 35 %). However, this decrease was restored similar to controls by extended reunion in Group 3 particularly in PrCm (up to 102%) and IL (back to 85%) but not in ACd (down to 65%) and PL (down to 76%) which remained significantly decreased when compared to control. In the SSC, a significant increase in density of GFAP-IR astrocytes were observed both in Group 2 (up to 204%) and Group 3 (212%) when compared to controls with no significant difference between them.

3.2.3. Morphological changes in GFAP-IR astrocytes after acute separation stress (Fig. 6). Changes of structural integrity of GFAP-IR astrocytes in the ACd were transient, reduced by acute separation stress but restored after 48 hr extended reunion (Fig. 6F,G,H). The morphology of GFAP-IR astrocytes in layer I-II including soma size and branching complexity were measured to assess whether the stress-induced decrease in density is paralleled with changes in structural integrity. The analysis of these morphological features was done in ACd because this subregion is involved in communication and emotional attachment between the pup and the dam.

No significant difference in soma size was observed between the groups (Fig 6B). A significant decrease in the number of astrocytic nodes was observed in Group 2

(down to 57%) when compared to control but this decrease was restored at 100% similar to controls after extended reunion in Group 3 (Fig. 6C). Similarly, the total length of processes was also reduced in Group 2 (down to 61%) than controls but was also restored up to 115% similar to controls after extended reunion in Group 3 (Fig. 6E). Furthermore, the number of processes per cell increased after extended reunion in Group 3 (up to 132%) when compared to Group 2 but not when compared to controls (Fig. 6D).

3.3. Changes in S100ß and GFAP expressions after repeated separation stress: comparison between short (Group 4) and extended (Group 5) reunion.

3.3.1. The cell density of S100ß-IR astrocytes in the mPFC also increases after repeated separation stress (Fig 7). Region and layer-specific increases in density of S100ß-IR astrocytes but decreases in GFAP-IR counterparts were observed in the mPFC after repeated separation stress. Taking the layers I-VI together (Fig. 7A), Group 4 showed significant increases in cell density of S100ß-IR astrocytes in PL (up to 136%) with strong trend of increase in ACd (P=0.07) compared to controls. After 7-day extended reunion, Group 5 showed a sharp increase in density of the S100ß-IR astrocytes in all mPFC subregions compared to controls (ACd up to 237%, PrCM up to 260%, PL up to 242%, IL 202%) and Group 4. Similarly, SSC showed a sharp increase in density of S100ß-IR astrocytes in Group 5 when compared to controls (up to 222%) and Group 4 (up to 224%).

In layer specific analyses of mPFC subregions, layer I (Fig. 7B) did not show a significant difference in density of S100ß-IR astrocytes in all mPFC subregions of Group 4 compared to controls. After the 7-day extended reunion, Group 5 showed increases in the ACd (up to 137%) and PL (up to 130%) when compared to control and increases in ACd (127%) and PrCM (143%) with a strong trend in PL (P=0.05) when compared to Group 4. The IL of both stressed groups was similar to control. Similarly, the SSC showed similar increase in Group 5 when compared to control (162%) and Group 4 (189%).

In layer II-III (Fig. 7C), a significant increase in density of S100ß-IR astrocytes was observed in three mPFC subregions of Group 4 (PrCM up to 156%, PL up to 168%, IL up to 169%) with a strong trend of increase in the ACd (P=0.06). Further increases in density of S100ß-IR astrocytes were observed after extended reunion in Group 5 when compared to controls (ACd up to 278%, PrCM up to 346%, PL up to 295%, IL up to 275%) and Group 4 (ACd up to 197%, PrCM up to 221%, PL up to 161%, IL up to 162%). In SSC, increase in density of S100ß-IR astrocytes was observed in Group 5 when compared to controls (259%) and Group 4 (222%).

In layer V/VI of the mPFC subregions (Fig. 7D), Group 4 showed a non-significant increase in density of S100ß-IR astrocytes compared to controls but with strong trend towards increase in ACd (P=0.07). After the extended reunion, Group 5 showed significant increase in density of S100ß-IR astrocytes in all mPFC subregions (ACd up to 208%, PrCM up to 263%, PL up to 213%, IL up to 213%)

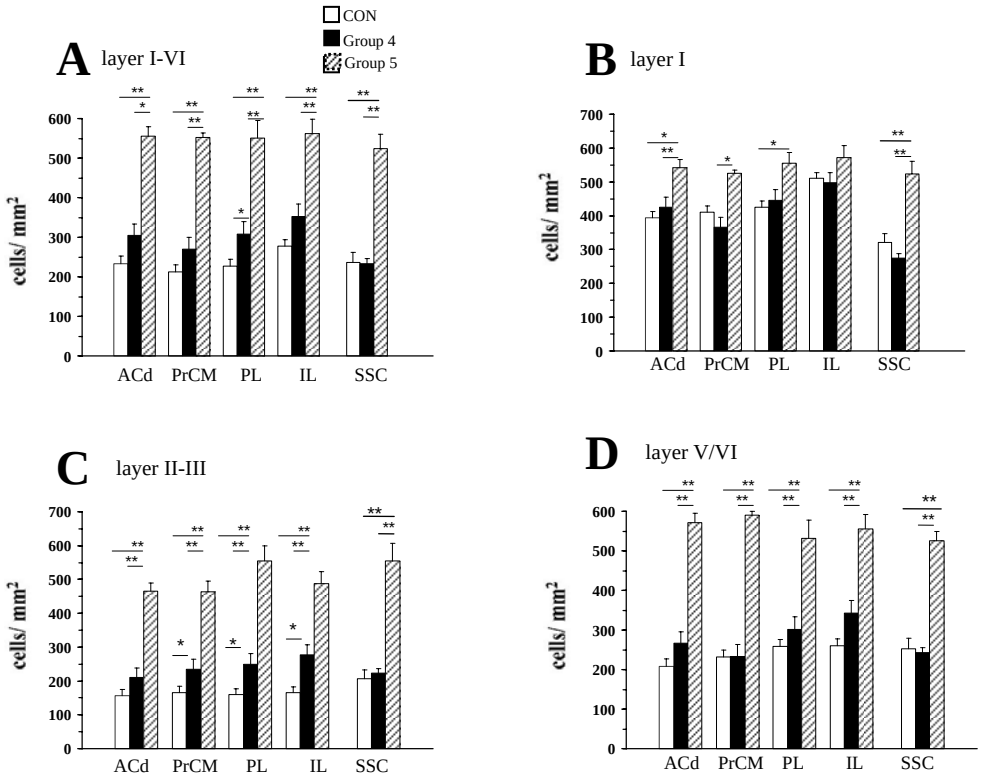

Fig. 7. Cell density of S100ß-IR astrocytes in the mPFC and SSC of *O. degus* following repeated separation. Cell density of layers I-VI (A); layer I (B); layer II-III (C); and layer V-VI (D). CON is control; Group 4= repeated separation+short reunion; Group 5=repeated separation+extended reunion.

compared to controls, also when compared to Group 4 (ACd up to 166%, PrCM up to 299%, PL up to 182%, IL up to 162%). In SSC, significant changes in density of S100B-IR astrocytes were observed in Group 5 when compared to control (207%) and Group 4 (215%).

3.3.2. The cell density of GFAP-IR astrocytes decreases after repeated separation stress and not restored after extended reunion (Fig. 8). Significant decreases of GFAP-IR astrocytes were observed in all mPFC subregions of Group 5 when compared to controls (Fig. 8A). In Group 4, decreases in density of GFAP-IR astrocytes were observed in three mPFC subregions (ACd down to 69%, PL down to 61%, IL down to 68%) but not in PrCM compared to controls. In Group 5, extended reunion induced a further decrease in density of GFAP-IR astrocytes in all the mPFC subregions (ACd down to 51%, PrCM down to 72%, PL down to 58%, IL down to 63%) compared to controls with no significant difference compared to Group 4 except in ACd ($P=0.027$). In SSC, the density of GFAP-IR astrocytes increased in Group 5 when compared to controls (up to 187%) and Group 4 (up to 187%).

3.3.3 Morphological changes of GFAP-IR astrocytes after repeated separation stress (Fig. 8). Structural integrity of GFAP-IR astrocytes in the ACd were not affected by repeated separation but altered by extended reunion (Fig. 8F,G,H). Similarly, the morphology of GFAP-IR astrocytes in layer I-II including soma size and branching complexity were measured to assess the prevailing chronic effects of short but repeated separation stress.

The soma soma size was similar between groups with a strong but non-significant trend towards decrease in Group 5 when compared to controls and Group 4 (Fig 8B). Furthermore, significant decrease in the number of astrocytic nodes was observed in Group 5 compared to controls (down to 61%) and Group 4 (down to 59%) (Fig 8C). Although no significant difference was observed in the number of processes ($P=0.16$) and length of processes ($P=0.06$) between the groups (Fig. 8D),

there was a strong trend towards decrease in total length of processes in Group 5 compared to other two groups (Fig.8E).

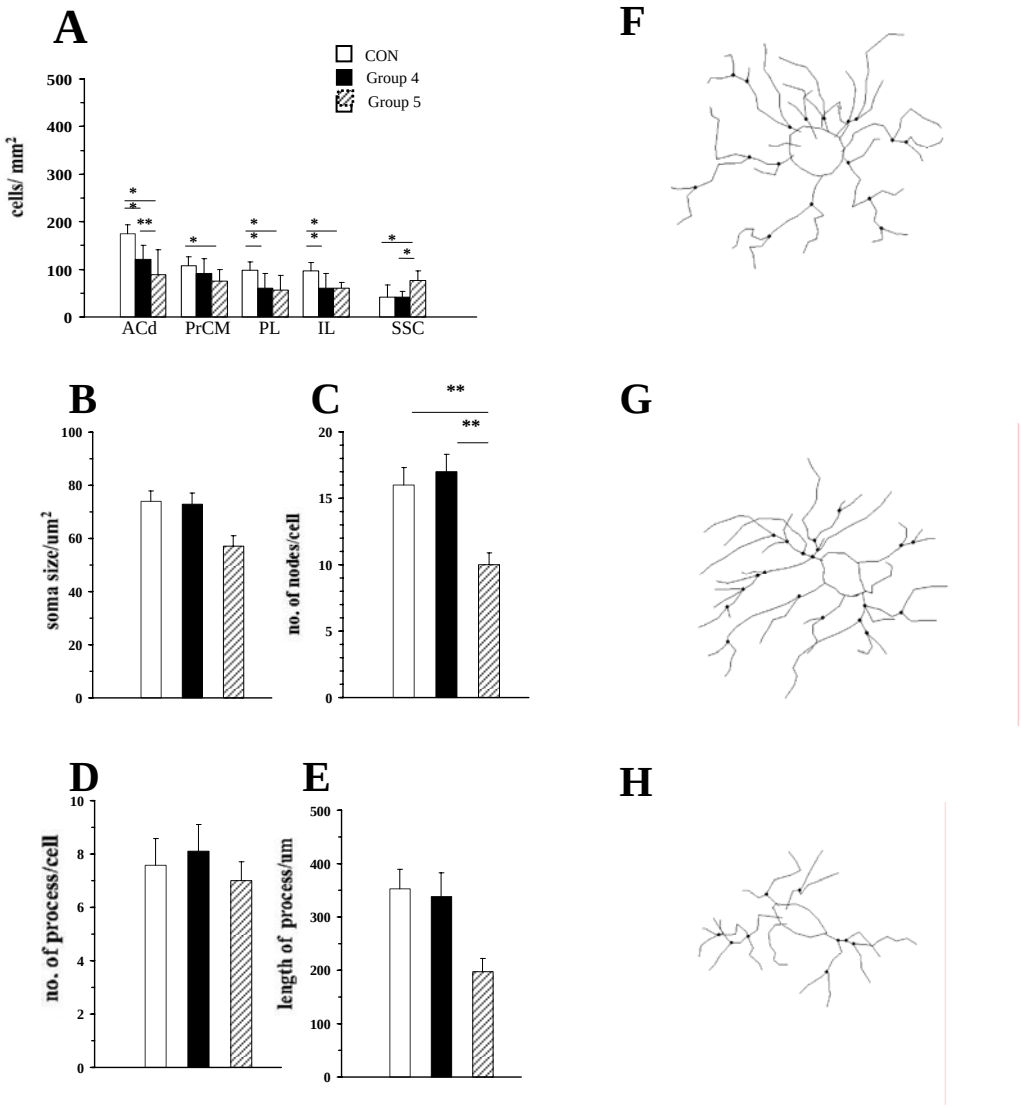

Fig. 8. Cell density of GFAP-IR astrocytes in the mPFC and SSC of *O. degus* after repeated separation stress (A). Structural integrity of GFAP-IR astrocytes in the ACd including soma size (B), number of nodes (C), number of processes (D), and length of processes (E) after repeated separation stress. Morphology of astrocytes in the ACd: F is CON, G is Group 4 and H is Group 5. CON=control; Group 4= repeated separation+short reunion; Group 5=repeated separation+extended reunion.

3.4 The impact of stress is similar on both cortical hemispheres. Although neonatal separation stress had an impact on the density of S100ß and GFAP–IR astrocytes in the mPFC, the mean density for left and right hemispheres were similar.

3.5 The brain and body weights are not altered by neonatal separations. No significant differences in the body weights (P=0.51) and brain weights (P=0.27) were observed between the groups after acute separation stress (Table 3.5A).

weight	CON	Group 2	Group 3	P ANOVA
body	56.36 g ±2.22	52.12 g ±3.96	57.59 g ±3.74	NS
brain	1.28 g ±0.09	1.29 g ±0.009	1.45 g ±0.08	NS

Table 3.5A. The brain and body weights after acute separation stress.

No significant differences in the body weights (P=0.06) and brain weights (P=0.49) were observed between the groups after repeated separation stress (Table 3.5B).

weight	CON	Group 4	Group 5	P ANOVA
body	56.36 g ±2.22	49.84 g ±1.05	46.9 g ±4.4	NS
brain	1.28 g ±0.09	1.14 g ±1.05	1.25 g ±4.4	NS

Table 3.5B. The brain and body weights after repeated separation stress.

4.1 Astrocytic response towards a stimulation. It was postulated that gliosis or astrocytic response towards stimulation occurs in two stages: first by somatic hypertrophy matched with process elongation, followed either by hyperplasia / cell proliferation or somatic cell death (Sirevaag and Greenough, 1991). The reaction time could either be slow or fast that predicts whether the astrocytes will survive or die after stimulation, respectively. It has been observed that astrocytic response occurs 30 min after traumatic brain injury (Miller et al., 1986), but it takes at least 30 days when astrocytes are exposed to enriched environment (Sirevaag and Greenough, 1991). This observation prompted the authors to suggest that an elevated level of stimulation that disrupts the membrane such as in brain injury induces the rapid astrocytic response while the constant, sub-disruptive stimulation such as exposure to enriched environment induces a very slow glial response (Sirevaag and Greenough, 1991).

Early life experiences, particularly those provided by the mother have been shown to play a critical role in physiological and behavioural development in non-human species (Kuhn et al., 1978, Sucheki et al., 1993, Hofer, 1994) and the first three postnatal weeks are critical period for neural development in rodents. The acute response of astrocytes may either reduce or enhance the damage to neurons depending on the timepoint of exposure (Swanson et al., 2004). The present study demonstrates that the durations of separation employed on early postnatal stage results in density increases of the S100ß-IR astrocytes in the mPFC, but decreases the density of GFAP-IR astrocytes in the mPFC. When the separation is acute, then changes in the structural integrity of GFAP-IR astrocytes in the ACd occur. Extended reunion with the family, after acute or repeated exposure to neonatal separation induces changes in the structural integrity of GFAP-IR astrocytes which remains to be determined whether these changes are positive or negative.

4.2 Cortical distribution and morphology of S100ß-IR and GFAP-IR astrocytes in the mPFC. Reports on immunohistochemical distribution of S100ß in the cerebral cortex are very few and are focused mostly on humans. These investigations include the spatio-temporal expression of S100ß between the human fetal and adult occipital cortex in which the number of S100ß-positive cells in aged

specimens remained similar to the late embryonic stage fetus (Tiu et al., 2000) indicating that S100ß is vital throughout the entire lifetime. In adult human cerebral cortex, S100ß immunohistochemistry was used to evaluate its relationship to serum levels as a cause of death in fatal head injuries and other fatalities (Li et al., 2006). It was also used to evaluate neural or glial cell specific types in prefrontal and temporal cortices (Steiner et al., 2007) where S100ß was found to be a less specific marker for astrocytes compared to GFAP. In other words, S100ß but not GFAP is also found in other glial cells including oligodendrocytes and other neuronal populations. Postnatal expression of S100ß-IR astrocytes were observed in young and adult rat visual cortex (Argandona et al., 2003), thalamus, parietal cortex (Muneoka et al., 2003), hippocampus (Reeves, 1994) having a homogenous distribution. On one hand, GFAP expression during early development is primarily investigated in the sensory regions. So far, this study is the first to show the distribution of S100ß and GFAP-IR astrocytes in the mPFC during the early postnatal development.

In the present study, the mPFC at P21 showed a striking contrast in the spatial pattern between S100ß and GFAP-IR astrocytes. While S100ß-IR astrocytes were homogenously distributed in all cortical layers, the GFAP-IR astrocytes were localized in layers I to II and also few were observed in layer VI similar to descriptions of mouse visual cortex at PND 24 (Corvetti et al., 2003). It can be presumed that GFAP is expressed by reactive astrocytes in the periphery of the cortex that actively participate in cortical expansion. It was suggested that the GFAP-IR astrocytes in layer I represent the pial-glial astrocytes that form the boundary at the surface of the brain, having the ability to attempt repopulation or increase in number due to their reactive nature (Steindler and Laywell, 2003). Thus, it was postulated that GFAP-IR astrocytes in layer I marks the immature part of the cortex and the middle layers consist the mature cortex due to the absence of GFAP-IR astrocytes (Hafidi and Galifianakis, 2003). Although astrocytes that inhabit the gray matter are protoplasmic astrocytes characterized mainly by their polygonal feathery-like processes (Bignami et al., 1972), GFAP-IR astrocytes in layer I had halo transparent cell bodies and dense processes while in contrast, the S100ß counterparts in the central layers had prominent cell bodies and profused branches

suggesting the presence of two-subtypes of astrocytes (Bignami et al., 1972). It was reported that the transformation of GFAP-expressing astrocytes to S100ß-IR characterizes the onset of astrocytic maturation (Raponi et al., 2007) and the GFAP negative astrocytes in the central layers may be considered as parenchymal astrocytes in the vascular neurogenic environment that perform the bulk of the astrocytic functions (Palmer et al., 2000). In this study, although GFAP-IR astrocytes were observed in layers V to VI of the mPFC, layer V-VI of PL and IL were devoid of them indicating that GFAP-IR astrocytes in the output layers of these subregions may be less responsive than other.

Aside from astrocytes occupying specific domains in the cerebral cortex, astrocytic proteins also occupy distinct sites within astrocytes suggesting that their position may be designed according to their function. In this study, the double-immunolabeling showed S100ß prominently in cell body and nucleus while GFAP defines the processes which may indicate that both proteins play equally important roles in regulating the cell shape and motility for maturation of cortical wiring. S100ß immunoreactivity has been observed both in the cytoplasm and nucleus of mature glial cells suggesting that S100ß participates in Ca^{2+} and Zn^{2+} cell regulation processes in both sites (Deloulme et al., 2004). In oligodendroglial progenitor cells (OPC), it has been observed that nuclear S100ß accumulation marks the immature, fast, multipotent OPC although it also persists in the adult OPCs but in reduced level (Deloulme et al. 2004). Other roles of the close interaction of S100ß-GFAP also include cell-cycle regulation, cell growth and cell differentiation.

4.3 Stress-induced changes of S100ß and GFAP expressions in the mPFC.

One aim of this study was to answer the question whether S100ß and GFAP expressions in astrocytes of the juvenile mPFC are altered by stress. One highlight of this study showed the increase of S100ß-IR astrocytes in the mPFC but not in the SSC following neotanal stress implying that emotional stimulation dramatically affects in particular the limbic region while nonlimbic area does not appear to be sensitive to it. Although S100ß expression naturally increases particularly during the early postnatal development, the present findings suggest that neonatal stress intensifies the increase perhaps via autocrine and paracrine effects. Astrocytes have

been proposed to monitor changes in the CNS so that when activated, they participate in the various cellular activities to cope in favor of the new environment (Sirevaag and Greenough, 1991). It was postulated that during activation, the intermediate filament network is altered particularly in the main processes which is presumed to be accompanied by dysregulation of many protein exressions (Bushong et al., 2002; 2004). In the context of stress-induced changes in neonatal astrocytes, retraction or shortening of processes may induce vulnerability to dysregulation of the developing brain circuitry.

In this study, both acute and repeated separations affect the number of astrocytes. Notably, the density of S100ß-IR astrocytes was increased after both acute and repeated separations, suggesting that astrocytes are highly responsive to a novel situation. Furthermore, the stress-induced increase in the density of S100ß-IR astrocytes remained high even after 48 hr extended reunion indicating that S100ß might participate not only during stress but as well as in the course of recovery periods. In other words, the overproduction of S100ß during stress may induce damage particularly in astrocytes if unable to cope but the S100ß secreted during the reunion period may enhance repair as previously suggested when the presence of S100ß was detected after acute brain injury (Kleindienst and Ross Bullock, 2006). In this study, the increase in density of S100B-IR astrocytes showed a layer-specific pattern, suggesting that the observed astrocytic changes might be induced by the synaptic (input) activity and also may be involved in experience-induced synaptic reorganization as consequence of stress exposure. It was previously reported in degu pups that repeated separation stress increases spine density in layer II-III of the ACd (Poeggel et al., 2003; Helmeke et al., 2001). This coincidence might indicate that the upregulation of neurotrophic factors such as S100ß might be involved in the observed spine increase. So far, no reports are available on layer-specific changes of S100B-IR astrocytes, but it was reported that S100ß upregulation may mean increased expression of this neurotrophic factor, which might stimulate both, glial or spine proliferation (Marshak , 1990; Whitaker-Azmitia and Azmitia, 1994; Wilson et al., 1988; Zimmer et al., 1995). This interpretation is further supported by the finding that the experimental removal of S100ß resulted in a 30-50% loss of cortical

synaptic density (Cheng et al., 1994). However, this speculation in our stress paradigm calls for further investigation.

Another highlight in this study is the downregulation of GFAP-IR astrocytes in the mPFC following neonatal separation stress. In contrast to the observation for the S100ß IR astrocytes, the decrease in the density of GFAP-IR astrocytes induced by acute stress was restored by extended reunion indicating that it reflects a more transient response and perhaps also is indicative of repair mechanisms in astrocytic cytoskeleton. On the other hand, the density of GFAP-IR astrocytes after repeated separation stress was further decreased by extended reunion indicating that cell death may result from the chronic effect of repeated stress.

The difference in the density of S100ß and GFAP-IR astrocytes may be due to the intracellular interaction of these two astrocytic markers. GFAP regulates cell motility (Lepekhin et al., 2001) and maintains structural stability (Trimmer et al., 1982) and is regulated by S100B inside the astrocytes (Sorci et al., 1998; Zimmer 2003). As mentioned before, S100ß inhibits GFAP expression to regulate the astrocytic cytoskeleton (Ziegler et al., 1998). And even though we cannot provide direct evidence for this interaction, it could be speculated that the increase in the density of S100ß-IR in the mPFC might be causally related to the decrease in the density of GFAP-IR. It was reported that intracellular overexpression of S100ß in transgenic mice show neural cytoskeletal change and decreased levels in cell cultures showed microfilament reorganization and altered cell morphology (Shapiro and Whitaker-Azmitia 2004). In contrast, in the SSC where the density of S100ß-IR astrocytes remained unaffected, an increase in the density of GFAP-IR astrocytes was observed.

4.4 Stress-induced morphological changes in GFAP-IR and S100ß-IR astrocytes. In this study, acute but not repeated exposure to separation stress was shown to affect the structure of astrocytes. It was observed that acute separation stress decreased the branching complexity of GFAP-IR astrocyte, indicating the impact of a single but long (6 hr) stress exposure. On one hand, repeated stress (1 hr, daily) did not affect the structural integrity of GFAP-IR astrocytes, indicative of

the favorable impact of short but repeated stress exposure. It also may indicate that the short but repeated stress may affect the cell proliferation but not the survival of individual astrocytes. The degenerated features of GFAP-IR astrocytes after acute separation stress in Group 2 were paralleled with retarded features of S100ß-IR astrocytes, including broken cell bodies, damaged and stunted branches. Although the study did not investigate apoptosis, the decrease in the number of GFAP-IR astrocytes might be due to cell death induced by S100ß overexpression. It was reported that social isolation induces astrocytic cell death (Takuma et al., 2004) by inducing cytosolic Ca^{2+} elevation, oxidative stress, nitric oxide secretion, mitochondrial dysfunction due to increased secretion of neurotrophic or neuroprotective substances as S100ß which could be detrimental to a juvenile brain.

4.5 Dual impact of reunion on stress-induced GFAP-IR astrocytes. Another highlight of this study is the reversal of both, in the number of GFAP-IR astrocytes as well as the morphology of astrocytic processes, after extended reunion of 48 hr following acute separation stress, suggesting a biphasic response of astrocytes (Margis et al., 2004, Nishio et al., 2003). This observation implies the ability of fast and efficient repair mechanisms mediated by the astrocytic machinery to reconstruct its cytoskeleton and proliferate. However, it was observed that after extended reunion following acute stress in Group 2, the density of GFAP-IR astrocytes in some mPFC subregions was significantly lower than controls indicating that for some brain regions more time may be required for the complete restoration of the astrocytic population. Morphologically, the extended post-stress reunion did not restore the degenerated morphological features of S100ß-IR astrocytes in the mPFC, indicating the lasting effects of acute neonatal stress on the fine structure of this astrocytic subtype.

One major observation on repeated separation stress was the trend of decreased branches in GFAP-IR after extended reunion, indicating atrophy of astrocytes in the mPFC following repeated stress. While the effects of acute stress may be transient, it was reported that repeated stress causes neuronal hypertrophy followed by atrophy of neurons in the PFC (McEwen and Chattarji 2004). This structural

remodeling of astrocytes in the mPFC may be presumed to have crucial effects in the maturation process of a neonatal brain when the brain circuitry is delicate.

4.6 Functional implications of changes in glial proteins. The observed stress-induced changes of glial proteins could be interpreted by different underlying mechanisms. The elevated expression of intracellular S100ß after stress induction could be due to increased protein synthesis, downregulation of protein degradation, decreased release of the protein into the extracellular space. Stress might activate these intracellular regulatory mechanisms, which should raise protein concentrations in astrocytes without disrupting the cell membrane in the case of repeated separation stress, where astrocytes remained intact after stress exposure. Alternatively, the increased density of S100ß-IR astrocytes might be the result of astrocytic proliferation due to increased secretion of S100ß protein, which has been shown to stimulate glial proliferation (see below). Along the same line, the decreased expression of GFAP in the stressed animals could reflect a downregulation of protein synthesis, degradation of this protein or cell death of astrocytes due to stress.

S100ß may be gliotrophic and neurotrophic or cytotoxic in function depending on its concentration. For years, S100ß has been known to be a potent glia-derived neurotrophic factor promoting neurite outgrowth and cell survival in nanomolar levels (Fig. 4.6). It promotes proliferation of astrocytes as well as survival and extension of neurites in neuronal cultures (Winningham-Major et al., 1989; Whitaker-Azmitia and Azmitia, 1994) and the absence of it decreased the number of synapses (Wilson et al., 1988). It increases neurite branching by promoting tubulin polymerization (Deinum et al., 1983). However, even in low micromolar levels, S100ß stimulates the expression of pro-inflammatory cytokines and induce apoptosis (Donato, 2001; 2003). S100ß activates NFkB and induces nitric oxide production resulting in cell death in rat glial cells (Takuma et al., 2004). Increased S100ß levels are found in cerebrospinal fluid (CSF) and/or serum of several acute and chronic injuries, including traumatic brain injury, stroke, Down syndrome, schizophrenia (Rothermundt et al., 2003; Zimmer et al., 2003) and other psychiatric disorders including PTSD (Diehl et al., 2007). Extra copies of S100ß genes showed

evidence of accelerated maturation and premature degeneration throughout the brain, indicating an early onset of Alzheimer's disease (Whitaker-Azmitia, 1997). S100ß is upregulated in tumors and malignant diseases (Davey et al., 2001; Lin et al., 2004).

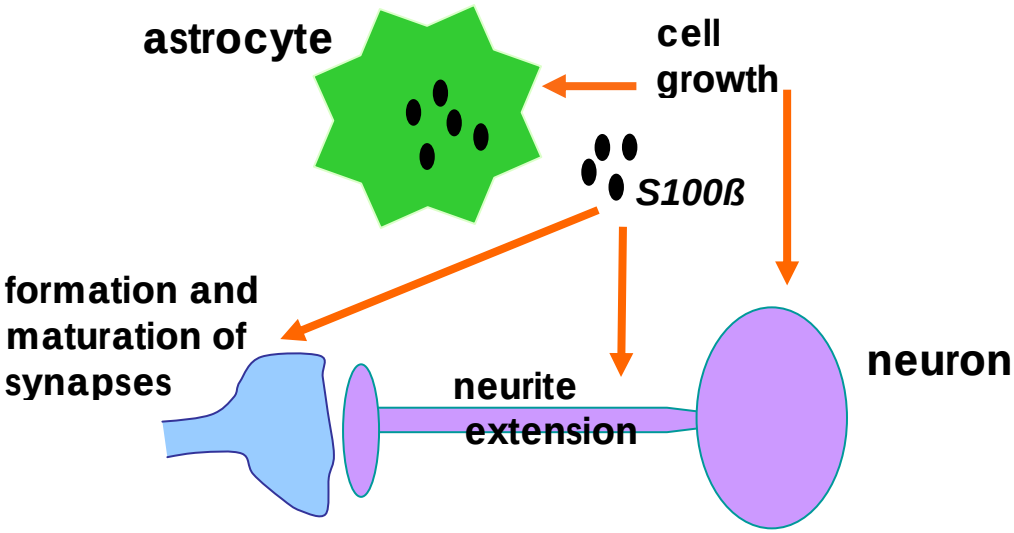

Fig. 4.6 Schematic illustration of S100ß as a gliotrophic and neurotrophic factor that enhances glial growth and differentiation as well as neurite extension. Modified from Zimmer et al., 1995

What are the probable mechanisms of S100ß expressions? Increased S100ß expression has been described using different stress paradigms in adult animals. In vitro, S100ß serum levels were elevated 120 mins after immobilization stress, but pharmacological stimulation by corticosterone failed to affect S100ß serum concentration. This suggests that the mechanism of S100ß increase is independent from corticosterone (Scaccianoce et al., 2004) although S100ß secretion appears to be modulated by several factors including ACTH, a mediator of stress (Suzuki et al., 1987). Release of S100ß occurs after 6 hr metabolic stress, implying an active, stress-triggered mechanism of S100ß (Gerlach et al., 2006). Serum deprivation in cell cultures induced an increase in S100ß secretion (Tramontina et al., 2006) and secretion of S100ß is influenced by a number of molecules like 5HT, DCG-IV (2',3'-dicarboxycyclopropyl glycine), glutamate and serum. Although the mechanism is not fully understood, it has been proposed that S100ß is influenced by c-AMP, so that DCG-IV that decreased c-AMP also decreases S100ß (Gillet 2004).

S100ß is secreted via a non-conventional export mechanism, the conventional way being via endoplasmic reticulum (ER)- Golgi trafficking, the pathway which some cytokines, such as interleukin (IL) 1 alpha and 1 beta, as well as fibroblast growth factor-2 (FGF-2) pursue (Davey et al., 2001). Although intermediary cellular and molecular events that link changes to function is still hardly understood, the secretion of S100ß is dependent on 5-HT1A receptor agonists, glutamate, adenosine and lysophosphatidic acid (Nishi et al., 1996; Donato, 2003, Tramontina et al., 2006). It has been suggested that S100ß is one of the most important neurotrophic factors in neuroplastic mechanisms of serotonergic neurons (Lin et al., 2004) and exerts multiple neurotrophic functions in the serotonergic system (Azmitia et al., 2007). S100ß increase is also through interaction with the extracellular domain of receptor for advanced glycation end products (RAGE) and activating a series of intracellular transduction pathways including NFkB pathway (Huttunen, 2000; Bucciarelli, 2006). In rat glial cells transcriptional activity, S100ß activates NFkB in the cytoplasm by translocating the p65 NFkB subunit into the nucleus, followed by stimulation of the NFkB specific DNA-binding activity and stimulation of NFkB dependent transcriptional activity but also inducing iNOS promoter activation and nitric oxide production (Lam 2001). S100ß also interacts with the transcription factor p53 and controls transcription (review by Ikura et al., 2002).

Another alternative explanation by which glial cells get activated may be through impulse activity in which neurotransmitter receptors on glial cells allow them to detect spillover of neurotransmitter from synaptic cleft similar how neurons do it (Fields and Stevens, 2000). These investigators proposed that during action potential, ATP is released via an unknown mechanism and glial cells are able to detect and respond to this neuronal activity via their membrane receptors for ATP or derivatives, i.e. adenosine. However, accumulation of neurotransmitters or gliotransmitters in the synaptic cleft that induces overstimulation of receptors may stimulate expression of proinflammatory cytokines and induce apoptosis.

GFAP decrease caused by early separation stress may reflect an indirect, glia-derived mechanism mediating stress-induced dendritic atrophy and spine loss as described in adult rat prefrontal and hippocampal neurons (McEwen, 2001; Radley et al.,

2005) which is causally linked to endocrine activation. It has been shown that stress exposure during the first three postnatal weeks induces a significant elevation of plasma cortisol levels in *Octodon degus* (Gruss et al., 2006). Isolation on the third week of life PND 15-21, i.e. consisting of 6-hour chronic isolation everyday for five days showed increased corticosterone in rat hippocampus and it is more detrimental than on the first week (Frisone et al., 2002). A variety of studies using different stress paradigms also point to a glucocorticoid-mediated regulation of GFAP expression. *In vivo,* it has been shown in adult rats that chronic stress, which comprised 6 days wheel activity and restricted access (1 hour/day) to food, induces increased numbers of GFAP-IR cells in the hippocampal CA-3 region but structure of GFAP-IR cells were not affected (Lambert et al., 2000). *In vitro,* GFAP mRNA and GFAP expression is downregulated in adult rat hippocampus and cortex in response to glucocorticoid application and stress (O'Callaghan et al., 1991; Nichols and Finch, 1994). Astrocytes when cocultured with neurons for a long period, i.e. three months, downregulates GFAP mRNA coupled with increased corticosterone levels (Rozovsky et al., 1995). The investigators hypothesize that the switch in the direction of GFAP in response to corticosterone is mediated by increased levels of glucocorticoid receptors which are localized both in astrocytes and neurons. Based on these findings, GFAP reduction in astrocytes may also be linked to the participation of HPA axis during this period of development.

There is an ongoing debate whether GFAP downregulation during development does not necessarily have adverse effects. Decreased GFAP during development and in maturity was characterized as quiescent astrocytes that enhanced neurite outgrowth (Holley et al., 2005) and absence or reduction of GFAP had no detrimental effect during development. For examples, GFAP null-mice have normal life span, reproduction (Pekny et al., 1995) and motor behavior (Messing and Brenner, 2003). They have normal responses to scrapie infection (Tatzelt et al., 1996) or kainic acid injection (Gomi et. al., 1995). However, severe GFAP decrease may induce dramatic changes in the brain circuitry since GFAP-expressing astrocytes play a role in regulating synaptic efficacy (McCall et al., 1996). Astrocytes devoid of IFs exhibits changes in morphology and motility severely compromised

which may impair formation of glial scars after brain or spinal cord injury (Lepekhin et al., 2001). GFAP-null mice suffer from blunt head trauma involving a whiplash-type injury (Nawashiro et al., 2000) ischemia (Nawashiro et al., 2000), hypotonic stress (Anderova et al., 2001) and severe traumatic injury (Pekny et al., 1999). It was shown that GFAP upregulation during development and regeneration protect neuronal dysfunctions, e.g. after injury, by increasing the expression of neurotrophic factors, cytokines, early response genes and transporter molecules (Eddleston & Mucke, 1993). GFAP increase is correlated with neuronal growth and reduction may decrease the ability of astrocytes to induce or maintain blood-brain barrier in the endothelium (Liedtke et al., 1996; Pekny et al., 1998; Reuss et al., 2003). Severe GFAP increase has been implicated in neuropathological disorders including autism, epilepsy, Alzheimer's Disease (AD), Down Syndrome (DS), traumatic brain injury (TBI). The aged brain is characterized by hypertrophy of astrocytes with a regular decrease in the number of neurons throughout the hippocampus, caudate nucleus and a number of major myelinated fibre tracts (Björklund et al., 1985; Soffie et al., 1999). Whether GFAP decrease in the PFC is detrimental or not during the neonatal stage and or carried on through adult life requires further investigation.

4.7 Functional implications of cortical astrocytes. Astrocytes that predominate the gray matter are of protoplasmic type (Bignami et al., 1972) and are noteworthy for their intimate associations with synapses particularly of the excitatory, spiny variety (Murai et al., 2003). Interestingly, neighboring protoplasmic astrocytes limit overlap or crossings between them to maintain individual domains/ areas creating exclusive territories within the neuropil (Bushong et al., 2002). In other words, astrocytes have their own specific territories as to where and which particular synapse to cover. Ultrastructure investigations suggest that astrocytes maintain a position near neuronal somata, dendrites and synapses to participate in synaptic function and plasticity providing neurotransmitters and energy substrates to neurons (Bushong et al. 2004). Therefore, the stressed-induced GFAP-IR astrocytes showing diminished in their branching complexity may reduce the efficacy of synapse due to the alterations of these mechanisms.

Astrocytes through secreting glial factors like S100ß, stimulate neurite outgrowth and their plasticity is linked to synaptogenesis. The increase of S100ß expression in the present study may imply the same idea which could have been the reason of spine increase in the mPFC. S100β was upregulated during lesion-induced sprouting and reactive synaptogenesis in dentate gyrus of rats (McAdory et al., 1998). It has been shown that developing neurons in culture do not achieve fully functional synapses until astrocytes are introduced (Pfrieger and Barres, 1997) indicating that the presence of astrocytes is important for synaptic function. (Jones and Greenough, 1996) reported that contact of astrocytes and interaction or envelopment between synaptic processes explains the mechanism by which astrocytes can optimally regulate the synaptic microenvironment (Anerson et al., 1994; Jones and Greenough, 1996). Glial factors including glutamate and ATP cause neuronal excitation and inhibition respectively which appoints the astrocytes for its heterosynaptic control (Fellin et al., 2006). For instance, glutamate from astrocytes triggers Ca^{2+} waves that in turn release Ca^{2+}-dependent release of glutamate in the extrasynaptic area and excites neuronal NMDA receptors (see review by Fellin et al., 2006). On one hand, ATP regulates extracellular signaling including calcium wave propagation and in delayed conversion to adenosine, it causes neuronal suppression (see review Fellin et al., 2006). Furthermore, astrocytes also secrete D-serine that modulates action of glutamatergic neurotransmission, neuronal migration, and long-term potentiation (Scolari and Acosta, 2007). From these observations, it is convincing that morphological changes in astrocytes can lead to serious changes in neuronal plasticity and overall synaptic functions in the brain of stressed animals.

Factors that are neurotrophic in the immature brain are essential for the maintenance of the adult brain, and S100ß may also be one of these factors. This could be the role of the astroglia 5-TH1A receptors to regulate the release of S100ß and thus maintain cortical integrity. In the adult, lesion of serotonergic terminals in the cortex decreased the S100ß levels. Withdrawal of S100ß or serotonin not only blocks growth but also retracts neurite extension (Azmitia et al., 1990). Treatment with 5-HT1A agonist can reverse this loss of S100ß and promote recovery of the lesioned serotonin terminal (Yan et al., 1997). Decrease of serotonin levels in the

adult rat, may possibly involved S100ß since it stabilizes microtubule proteins that are found in the dendrites (Meichsner, et al., 1993). Interestingly, treatment of unlesioned adult animals with a 5-HT1A agonist does not increase S100ß release or promote overgrowth of serotonin terminals, suggesting that this sequence of events is most robustly operative when the brain has been damaged. This may indicate that changes in astrocytes must take place in order for an increases release of S100ß to take place.In mature adult rat brain, removal of serotonin terminals by a 5,7-dihydroxytryptamine lesion, causes an increase in GFAP expression (Frankfurt et al., 1991).

Going back to the questions, the present findings show that: 1) S100ß and GFAP expressions in the juvenile mPFC are altered by stress; 2) stress-induced changes in S100ß and GFAP expressions are paralelled by changes in morphology of astrocytes; 3) stress-induced morphological changes are transient but their effects could be lasting and; 4) both the mPFC and SSC respond to stress but towards the opposite directions.

In conclusion, a single but long separation stress during preweaning period showed dramatic effects in density and structure of astrocytes and repeated but short exposures affect the number but not the astrocytic structure. The acute stress may exert a severe impact on the number and structural integrity of glial population in the limbic mPFC but reverted by extended reunion following stress. In contrast, repeated but short neonatal stress enhanced glial plasticity but astrocytic response fades once the emotional stimulation stops. Neonatal separation increases the expression of S100B-IR astrocytes that in turn suppressed the expression of the GFAP-IR astrocytes due to its intracellular inhibitory effects towards GFAP. Based from our observations, this study suggests that neonatal stress, acute or repeated showed adverse effects on astrocytes in the mPFC and therefore can be assumed to interfere in glial, neuronal and glial-neural synaptic plasticity during the prefrontal cortical development. Having known the impact of neonatal stress in the PFC on postnatal development, it is tempting to speculate that alterations incurred by stress during this period will be carried over in adult life. The memory of a stressful event

on juvenile stage may increase the anxiety-like behavior or lessen the exploratory activity or affect the solving-problem capacity of the organism in adulthood.

4.8 Future directions. We focused this present work on estimating the glial density and branching complexity of neonatal astrocytes. However, future investigations will track down molecular mechanisms of glial activation in relation to experience-induced neuronal plasticity and find out behavioural correlates of the stressed pups both on early development as well as in adulthood. It would be interesting to find out the behavioural equivalent of extension and retraction in astrocytes in the mPFC. Another consideration would be to modify the repeated separation from routinary into unpredictable paradigm and see if the glial response will be similar to the present findings. Given that the animals were only subjected to a one-time 6-hr separation treatment versus the repeated separation treatment of 1-hour separation for 21 days, it was most likely to have a greater impact than that of repeated separation. One can speculate that the animals eventually could not perceive the repeated separation as a stressor unlike the single episode since it occurred consistently on the same hour, with the same duration everyday. This provided a sense of predictability that the acute separation group was not afforded. It would be interesting to investigate if this repeated separation paradigm would prove adaptive behaviourally or not; if the pups grow to have better social skills or better learning capacity, for example. Furthermore, it will be interesting to find out the effects on S100ß-IR and GFAP-IR astrocytes in the mPFC when pups are subjected to the same repeated separation having the same total number of hours of exposure at various times of the day with different duration of exposure.

Lastly, with regards to the regional specificity of S100ß-IR and GFAP-IR astrocytes response in the mPFC, the morphology and structure of astrocytes could be quantified in other mPFC subregions to see if the response of astrocytes in those regions coincides with the present findings on the ACd. The changed in astrocytic morphology maybe assessed whether it is follows the pathway of apoptosis or necrosis. It would also be interesting to find out the correlation between neuronal plasticity and increase in the astrocytes in the present study.

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APPENDIX 1

Supplementary Data on Acute Separation Stress

Cell density of S100ß-IR astrocytes in the mPFC after acute separation stress (Group 2 and Group 3). The values are mean±standard error of S100ß-IR astrocytes in the mPFC subregions: [anterior cingulate (ACd), precentral medial (PrCM), prelimbic (PL), infralimbic (IL)] and somatosensory cortex (SSC). Significant differences (between different rearing and hemisphere) were determined with 2-way ANOVA and Tukey test as post hoc test. P= p values ANOVA; P1= p values Group 2 vs. control; P2 = p value Group 3 vs. control; P3= p values Group 2 vs. Group 3. *The group is significantly different at P=0.05.

Region	Control	Group 2	Group 3	P	P1	P2	P3
ACd	234±34.16	345±34.16	317±38.19	0.08	-	-	-
PrCM	212±13.72	326±24.74	302±33.90	0.01	0.009*	0.07	0.80
PL	227±34.67	315±31.65	348±38.77	0.64	-	-	-
IL	277±39.75	391±36.29	394±44.44	0.08	-	-	-
SSC	236±27.19	281±24.82	305±31.40	0.24	-	-	-

Table 1.1. All layers: Cell density of S100ß-IR astrocytes in the mPFC after acute separation stress.

Region	Control	Group 2	Group 3	P	P1	P2	P3
ACd	394±74.26	421±71.10	533±74.26	0.38	-	-	-
PrCM	406±62.53	393±62.53	520±69.91	0.36	-	-	-
PL	426±63.14	455±63.14	503±70.59	0.72	-	-	-
IL	511±63.38	487±63.38	590±70.87	0.54	-	-	-
SSC	322±67.61	398±61.72	510±75.60	0.19	-	-	-

Table 1.2. Layer I: Cell density of S100ß-IR astrocytes in the mPFC after acute separation stress.

Region	Control	Group 2	Group 3	P	P1	P2	P3
ACd	166±27.62	233±27.06	310±31.24	0.009	0.22	0.006*	0.17
PrCM	160±28.58	279±28.58	255±31.95	0.019	0.02*	0.09	0.84
PL	165±30.55	250±30.55	264±34.16	0.076	-	-	-
IL	180±28.97	328±28.97	284±32.38	0.005	0.004*	0.06	0.56
SSC	202±30.52	216±27.86	274±34.12	0.27	-	-	-

Table 1.3. Layer II-III: Cell density of S100ß-IR astrocytes in the mPFC after acute separation stress.

Region	Control	Group 2	Group 3	P	P1	P2	P3
ACd	274±29.49	363±26.92	412±32.97	0.01	0.08	0.013*	0.50
PrCM	241±27.22	358±24.85	346±30.44	0.01	0.01*	0.04*	0.95
PL	259±28.58	316±26.09	341±31.96	0.158	-	-	-
IL	260±44.08	390±40.24	364±49.29	0.098	-	-	-
SSC	253±26.37	285±24.07	286±29.48	0.62	-	-	-

Table 1.4. Layer V-VI: Cell density of S100ß-IR astrocytes in the mPFC after acute separation stress.

Cell density of GFAP-IR astrocytes in the mPFC after acute separation stress (Group 2 and Group 3). The values are mean±standard error of GFAP-IR astrocytes covering layer I-VI of the mPFC subregions: [anterior cingulate (ACd), precentral medial (PrCM), prelimbic (PL), infralimbic (IL)] and somatosensory cortex (SSC). Significant differences (between different rearing and hemisphere) were determined with 2-way ANOVA (two-way analysis of variance) and Tukey test as post hoc test except PL and IL that employed Holm-Sidak as posthoc test due non-parametric data. P= p values ANOVA; P1= p values Group 2 vs. control; P2 = p value Group 3 vs. control; P3= p values Group 2 vs. Group 3. *The group is significantly different at P=0.05.

Region	Control	Group 2	Group 3	P	P1	P2	P3
ACd	174±10.44	88 ±10.59	114±11.44	<0.001	<0.001*	0.002*	0.24
PrCM	104±9.53	68±8.82	107±9.53	0.01	0.03*	0.96	0.017*
PL	98±6.20	39± 6.20	75±6.79	<0.001	<0.001*	0.02*	<0.001*
IL	94±6.8	33± 6.87	80±7.53	<0.001	<0.001*	0.16	<0.001*
SSC	41±5.93	84 ±5.42	87± 6.64	<0.001	<0.001*	<0.001*	0.95

Table 1.5. Cell density of GFAP-IR astrocytes in the mPFC after acute separation stress.

Morphological measurements of GFAP-IR astrocytes in the ACd after acute separation stress. The values are mean±standard error of measurements in a total of 110 GFAP-IR astrocytes. Significant differences (between different rearing and hemisphere) were determined with 2-way ANOVA (two-way analysis of variance) and Tukey test. P= p values ANOVA; P1= p values Group 2 vs. control; P2 = p value Group 3 vs. control; P3= p values Group 2 vs. Group 3. *The group is significantly different at P=0.05.

GFAP-IR astrocytes	Control	Group 2	Group 3	P	P1	P2	P3
Soma size (μm²)	73.66 ± 3.92 μm²	66.89± 4.38 μm²	73.34± 3.58 μm²	0.45	-	-	-
No. of nodes	16.60± 1.27	9.59± 1.42*	17.08± 1.16*	<0.001	0.003*	0.95	0.001*
No. of processes	8.2± 0.56	7.1± 0.63	9.4± 0.51*	0.018	0.23	0.34	0.01*
Length of processes (μm)	343.75± 20.24 μm	211.04± 22.63 μm *	397.01± 18.47 μm*	<0.001	<0.001*	0.15	<0.001*

Table 1.6. Morphological measurements of GFAP-IR astrocytes in the ACd after acute separation stress.

APPENDIX 2

Supplementary Data on Repeated Separation Stress

2. Cell density of S100ß-IR astrocytes in the mPFC after repeated separation stress (Group 4 and Group 5). The values are mean±standard error of S100ß-IR astrocytes in the mPFC subregions: [anterior cingulate (ACd), precentral medial (PrCM), prelimbic (PL), infralimbic (IL)] and somatosensory cortex (SSC). Significant differences (between different rearing and hemisphere) were determined with 2-way ANOVA and Tukey test as post hoc test. P= p values ANOVA; P1= p values Group 4 vs. control; P2 = p value Group 5 vs. control; P3= p values Group 4 vs. Group 5. *The group is significantly different at P=0.05.

Region	Control	Group 4	Group 5	P	P1	P2	P3
ACd	234±22.65	304±20.67	556±25.32	<0.001	0.07	<0.001*	<0.001*
PrCM	212±22.39	270±20.43	553±25.03	<0.001	0.15	<0.001*	<0.001*
PL	227±23.68	308±21.62	551±26.48	<0.001	0.046*	<0.001*	<0.001*
IL	277±33.25	353±30.35	562±37.17	<0.001	0.23	<0.001*	<0.001*
SSC	236±20.45	233±20.45	524±22.86	<0.001	0.99	<0.001*	<0.001*

Table 2.1. All Layers: Cell density of S100ß-IR astrocytes in the mPFC after repeated separation stress.

Region	Control	Group 4	Group 5	P	P1	P2	P3
ACd	394±29.49	425±26.92	543±32.97	0.007	0.72	0.007*	0.028*
PrCM	410±37.78	366±33.79	525±41.38	0.021	0.66	0.12	0.017*
PL	426±30.36	446±27.71	555±33.94	0.021	0.87	0.02*	0.05
IL	511±29.28	497±26.73	572±32.73	0.19	-	-	-
SSC	322±35.48	276±35.48	524±39.66	<0.001*	0.64	0.003	<0.001*

Table 2.2 Layer I: Cell density of S100ß-IR astrocytes in the mPFC after repeated separation stress.

Region	Control	Group 4	Group 5	P	P1	P2	P3
ACd	166±21.50	235±19.63	463±24.04	<0.001	0.06	<0.001**	<0.001**
PrCM	160±34.77	250±31.74	554±38.87	<0.001	0.002*	<0.001**	<0.001**
PL	165±26.73	277±24.40	488±29.89	<0.001	0.013*	<0.001*	<0.001*
IL	180±34.26	304±31.27	495±38.30	<0.001	0.034*	0.002*	<0.001*
SSC	202±26.36	236±26.91	524±29.48	<0.001	0.63	<0.001*	<0.001*

Table 2.3. Layer II-III: Cell density of S100ß-IR astrocytes in the mPFC after repeated separation stress.

Region	Control	Group 4	Group 5	P	P1	P2	P3
ACd	274±22.45	344±20.49	571±25.10	<0.001	0.07*	<0.001**	<0.001**
PrCM	241±38.59	276±35.23	634±43.15	<0.001	0.78	<0.001**	<0.001**
PL	259±25.55	302±23.32	552±28.57	<0.001	0.44	<0.001**	<0.001**
IL	260±37.59	343±34.31	556±42.03	<0.001	0.24	<0.001**	<0.001**
SSC	253±21.21	243±24.12	524±24.49	<0.001	0.94	<0.001**	<0.001**

Table 2.4. Layer V-VI: Cell density of S100ß-IR astrocytes in the mPFC after repeated separation stress.

Cell density of GFAP-IR astrocytes in the mPFC after repeated separation stress (Group 4 and Group 5). The values are mean±standard error of GFAP-IR astrocytes covering the layers I-VI of the mPFC subregions: [anterior cingulate (ACd), precentral medial (PrCM), prelimbic (PL), infralimbic (IL)] and somatosensory cortex (SSC). Significant differences (between different rearing and hemisphere) were determined with 2-way ANOVA (two-way analysis of variance) and Tukey test as post hoc test except PrCM and SSC that employed Holm-Sidak method as posthoc test for non-parametric data. P= p values ANOVA; P1= p values Group 4 vs. control; P2 = p value Group 5 vs. control; P3= p values Group 5 vs. Group 4. *The group is significantly different at P=0.05.

Region	Control	Group 4	Group 5	P	P1	P2	P3
ACd	174±7.19	120± 7.19	89± 8.81	<0.001	<0.001*	<0.001*	0.027*
PrCM	103±6.69	92± 5.98	75± 7.33*	0.03	0.21	0.009*	0.09
PL	96±5.19	59± 5.12*	57± 6.27*	<0.001	<0.001*	<0.001*	0.95
IL	95±5.03	65± 4.95*	60± 6.07*	<0.001	<0.001*	<0.001*	0.79
SSC	41±3.20	41±3.8	77±12.16**	0.003	0.99	0.009*	0.006*

Table 2.5 Cell density of GFAP-IR astrocytes in the mPFC after repeated separation stress.

Morphological measurements of GFAP-IR astrocytes in the ACd after repeated separation stress. The values are mean±standard error of measurements in a total of 110 GFAP-IR astrocytes. Significant differences (between different rearing and hemisphere) were determined with 2-way ANOVA (two-way analysis of variance) and Tukey test. P= p values ANOVA; P1= p values Group 4 vs. control; P2 = p value Group 5 vs. control; P3= p values Group 5 vs. Group 4. *The group is significantly different at P=0.05.

GFAP-IR astrocytes	Control	Group 4	Group 5	P	P1	P2	P3
Soma size (μm²)	73.66 ±4.40 μm²	73.10±4.4 μm²	57.87±4.92 μm²	0.064	0.99	0.064	0.07
No. of nodes	16.60±1.5	17.00±1.51	10.06±1.69**	0.01	0.98	0.016*	0.023*
No. of process	8.26±0.49	8.3±0.4	7.0±0.55	0.16	-	-	-
Length process (μm)	343.75±43.92 μm	410.99±43.9 μm **	246.56±49.10 μm	0.06	-	-	-

Table 2.6. Morphological measurements of GFAP-IR astrocytes in the ACd after repeated separation stress.

APPENDIX 3

COMPARISON OF HEMISPHERES

3. Left vs. right hemispheres: cell density of S100ß-IR astrocytes in the mPFC after acute separation stress (Group 2 and Group 3). The values are mean±standard error of S100ß-IR astrocytes in the mPFC subregions showing non-significant differences between the groups: [anterior cingulate (ACd), precentral medial (PrCM), prelimbic (PL), infralimbic (IL)] and somatosensory cortex (SSC). Significant differences (between different rearing and hemisphere) were determined with 2-way ANOVA and Tukey test as post hoc test. P= p values ANOVA; P1= p values Group 2 vs. control; P2 = p value Group 3 vs. control; P3= p values Group 2 vs. Group 3. *The group is significantly different at P=0.05.

All Layer s				Layer I			
	Left	**Right**	**P**		**Left**	**Right**	**P**
ACd	295±29.03	302±29.03	0.81	**ACd**	400±68.63	499±68.63	0.25
PrCM	277±21.38	283±21.38	0.82	**PrCM**	437±53.14	443±53.14	0.94
PL	293±28.70	300±28.70	0.94	**PL**	449±53.66	473±53.66	0.75
IL	356±32.90	352±32.90	0.94	**IL**	519±53.87	540±53.87	0.95
SSC	256±21.58	291±23.98	0.28	**SSC**	424±55.97	396±55.97	0.83

Table 3.1. ALL LAYERS and LAYER I: Cell density of S100ß-IR astrocytes in the left and right hemispheres of mPFC subregions and SSC after acute separation stress:

Layer II-III				Layer V-VI			
	Left	**Right**	**P**		**Left**	**Right**	**P**
ACd	218±21.81	255±25.24	0.28	**ACd**	334±24.41	365±24.41	0.38
PrCM	229±24.28	234±24.28	0.90	**PrCM**	308±22.53	321±22.53	0.69
PL	226±25.97	226±25.97	0.98	**PL**	287±23.66	323±23.66	0.29
IL	263±24.61	265±24.61	0.95	**IL**	315±36.49	361±36.49	0.38
SSC	223±25.26	238±25.26	0.68	**SSC**	267±21.83	282±21.83	0.63

Table 3.2. LAYER II-III and LAYER V-VI. Cell density of S100ß-IR astrocytes in the left and right hemispheres of mPFC subregions and SSC after acute separation stress:

GFAP				Morphology of GFAP-IR astrocytes			
	Left	Right	P		Left	Right	P
ACd	120±9.08	130±8.61	0.42	**Soma size (µm)**	74.02±3.24	68.56	0.24
PrCM	92.01±7.78	94.38±7.40	0.82	**No. of nodes**	14.82±1.05	14.03	0.60
PL	72.81±5.23	69.01±5.23	0.61	**No. of process**	8.9±0.47	7.5	0.06
IL	63.08±5.79	75.18±5.79	0.95	**Length process (µm)**	316.29±16.75	318.23	0.93
SSC	72.39±4.91	69.61±4.91	0.68				

Table 3.3. LAYER V-VI: Cell density of S100ß-IR astrocytes in the left and right hemispheres of mPFC subregions and SSC after acute separation stress; and morphological changes of GFAP-IR astrocytes in the ACd.

Comparison of left hemispheres. There was no significant difference between the left hemispheres after acute separation stress.

All layers	Control	Group2	Group 3	P
ACd	222±48.31	329±48.31	335±54.01	0.78
PrCM	204±36.53	317±33.34	309±40.84	0.89
PL	230±49.04	306±44.76	344±54.28	0.96
IL	288±56.22	388±51.32	390±62.86	0.96
SSC	232±39.05	261±35.65	290±43.66	0.90

Table 3.4. All layers: Cell density of S100ß-IR astrocytes in the left hemisphere of mPFC subregions and SSC after acute separation stress.

Layer I	Control	Group 2	Group 3	P
ACd	421±15.12	459±29.08	320±54.52	0.43
PrCM	363±13.72	539±24.74	409±33.90	0.74
PL	432±14.81	463±36.66	452±40.70*	0.79
IL	518±15.41	467±40.80	572±49.92*	0.94
SSC	413±16.42	400±16.42	457±37.31	0.75

Table 3.5. Layer I. Cell density of S100ß-IR astrocytes in the left hemisphere of mPFC subregions and SSC after acute separation stress.

Layer II-III	Control	Group 2	Group 3	P
ACd	154±40.48	238±40.48	278±45.26	0.85
PrCM	149±40.42	301±40.42	238±45.19	0.63
PL	176±43.21	232±43.21	272±48.31	0.77
IL	178±40.96	339±40.96	272±45.80	0.87
SSC	213±43.16	200±39.40	257±48.26	0.75

Table 3.6. Layer II-III: Cell density of S100ß-IR astrocytes in the left hemisphere of mPFC subregions and SSC after acute separation stress.

Layer V-VI	Control	Group2	Group 3	P
ACd	247±41.71	337±38.07	418±46.63	0.69
PrCM	242±38.50	335±35.15	349±43.05	0.74
PL	222±40.42	294±36.90	347±45.19	0.63
IL	222±62.35	377±56.91	347±69.71	0.91
SSC	249±37.00	272±33.78	271±37.00	0.93

Table 3.7. Layer V-VI: Cell density of S100ß-IR astrocytes in the left hemisphere of mPFC subregions and SSC after acute separation stress:

Comparison of right hemispheres. There was no significant difference between the right hemispheres of Group 2 and Group 3 when compared to controls.

Layer I	Control	Group2	Group 3	P
ACd	367±15.12	608±29.08	522±54.52	0.43
PrCM	450±13.72	502±24.74	378±33.90	0.74
PL	420±14.81	447±89.66	553±40.70	0.79
IL	505±15.41	5071±40.80	609±49.92	0.94
SSC	290±16.42	426±16.42	491±37.31	0.75

Table 3.8. Layer I: Cell density of S100ß-IR astrocytes in the right hemisphere of mPFC subregions and SSC after acute separation stress.

Layer II-III	Control	Group2	Group 3	P
ACd	177±40.48	228±40.48	315±45.26	0.85
PrCM	171±40.42	257±40.42	272±45.19	0.63
PL	154±43.21	268±43.21	256±48.31	0.77
IL	181±40.96	318±40.96	296±45.80	0.87
SSC	190±43.16	226±39.42	291±48.26	0.75

Table 3.9. Layer II-III: Cell density of S100ß-IR astrocytes in the right hemisphere of mPFC subregions and SSC after acute separation stress.

Layer V-VI	Control	Group 2	Group 3	P
ACd	301±41.71	389±38.07	405±46.63	0.69
PrCM	240±38.50	380±35.15	343±43.05	0.74
PL	297±40.42	337±36.90	336±45.19	0.63
IL	297±62.35	403±56.91	381±69.72	0.91
SSC	258±37.00	298±33.78	310±47.77	0.93

Table 3.10. Layer V-VI: Cell density of S100ß-IR astrocytes in the right hemisphere of mPFC subregions and SSC after acute separation stress:

3. **Left vs. right hemispheres: Cell density of S100ß-IR and GFAP-IR astrocytes in the mPFC after repeated separation stress (Group 4 and Group 5).** The values are mean±standard error of S100ß-IR astrocytes in the mPFC subregions: [anterior cingulate (ACd), precentral medial (PrCM), prelimbic (PL), infralimbic (IL)] and somatosensory cortex (SSC). Significant differences (between different rearing and hemisphere) were determined with 2-way ANOVA and Tukey test as post hoc test. P= p values ANOVA; P1= p values Group 4 vs. control; P2 = p value Group 5 vs. control; P3= p values Group 4 vs. Group 5. *The group is significantly different at P=0.05.

All Layers				Layer I			
	Left	**Right**	**P**		**Left**	**Right**	**P**
ACd	371±18.74	358±18.74	0.62	**ACd**	472±24.41	436±24.41	0.30
PrCM	348±18.53	342±18.53	0.81	**PrCM**	442±29.80	426±31.86	0.30
PL	375±19.60	350±19.60	0.37	**PL**	491±25.13	460±25.13	0.71
IL	414±27.52	381±27.52	0.40	**IL**	541±24.23	512±24.23	0.39
SSC	325±17.38	337±17.38	0.62	**SSC**	379±32.05	369±32.05	0.97

Table 3.11. All layers and Layer I: Cell density of S100ß-IR astrocytes in the mPFC subregions and SSC after repeated separation stress:

Layer II-III				Layer V-VI			
	Left	**Right**	**P**		**Left**	**Right**	**P**
ACd	286±17.80	290±17.80	0.86	**ACd**	399±18.58	394±18.58	0.84
PrCM	333±28.78	310±28.78	0.58	**PrCM**	400±31.95	368±31.95	0.48
PL	325±22.13	295±22.13	0.32	**PL**	369±21.15	373±21.15	0.88
IL	343±28.36	309±28.36	0.41	**IL**	387±31.11	385±31.11	0.96
SSC	337±23.25	304±23.25	0.31	**SSC**	335±19.14	345±19.14	0.64

Table 3.12. Layer II-III and Layer V-VI: Cell density of S100ß-IR astrocytes in the mPFC subregions and SSC after repeated separation stress.

GFAP				Morphology of GFAP-IR astrocytes in the ACd			
	Left	Right	P		Left	Right	P
ACd	119.53±6.34	136.34±6.34	0.07	**Soma size (µm2)**	67.71±23.32	68.71±23.32	0.85
PrCM	92.98±5.64	87.01±5.64	0.44	**No. of nodes**	14.39±1.2	14.71±1.2	0.86
PL	70.27±4.64	72.36±4.64	0.74	**No. of process**	7.73±0.42	7.99±0.42	0.67
IL	70.61±4.49	76.47±4.49	0.35	**Length of process (µm)**	346.44±37.32	321.08±37.32	0.63
SSC	48±5.94	57±6.30	0.29				

Table 3.13. Cell density of GFAP-IR astrocytes in the mPFC subregions and morphological changes of GFAP-IR astrocytes after repeated separation stress.

Comparison of left hemispheres. There was no significant difference between the left hemispheres of Control, Group 4 and Group 5.

All layers	Control	Group 4	Group 5	P
ACd	222±32.03	308±29.24	583±35.81	0.52
PrCM	204±31.66	279±28.90	561±35.40	0.83
PL	230±33.49	296±30.57	573±37.45	0.87
IL	288±47.02	370±42.92	583±52.57	0.97
SSC	232±28.92	227.80±28.92	516.00±32.34	0.62

Table 3.14. All Layers: Cell density of S100ß-IR astrocytes in the left hemisphere of mPFC subregions and SSC after repeated separation stress.

Layer I	Control	Group 4	Group 5	P
ACd	421.80± 41.71	435.66± 38.07	561.25±46.63	0.30
PrCM	391.50±47.79	396.50±47.79	538.75±58.53	0.71
PL	432.40±41.41	469.66±39.19	573.25±48.00	0.38
IL	518.00± 41.41	511.83±37.80	595.25±46.30	0.39
SSC	362.00±50.17	282.00±50.17	516.00±56.09	0.97

Table 3.15. All Layers: Cell density of S100ß-IR astrocytes in the left hemisphere of mPFC subregions and SSC after repeated separation stress.

Layer II-III	Control	Group 4	Group 5	P
ACd	154±30.41	239±27.76	464±34.00	0.86
PrCM	149±49.17	251±44.88	599±54.97	0.54
PL	176±37.80	292±34.51	508±34.51	0.97
IL	288±47.02	370±42.92	583±52.57	0.97
SSC	213.60±37.29	282.83±34.04	516.00±41.69	0.31

Table 3.16. Layer II-III: Cell density of S100ß-IR astrocytes in the left hemispheres of mPFC subregions and SSC after repeated separation stress.

Layer V-VI	Control	Group 4	Group 5	P
ACd	247±31.75	341±28.98	609±35.50	0.18
PrCM	242±54.58	289±49.83	669±61.03	0.85
PL	230±36.13	308±32.99	577±40.40	0.88
IL	288±47.02	366±48.53	574±59.44	0.45
SSC	249.40±30.00	229.00±30.00	528.33±38.72	0.69

Table 3.17. Layer V-VI: Cell density of S100ß-IR astrocytes in the left hemispheres of mPFC subregions and SSC after repeated separation stress.

3. Comparison of right hemispheres. There was no significant difference between the hemispheres of Control, Group 4 and Group 5.

All layers	Control	Group 4	Group 5	P
ACd	245±32.02	301±29.24	528±35.81	0.51
PrCM	220±31.66	262±28.90	544±35.40	0.83
PL	224±33.49	296±30.57	529±37.45	0.87
IL	266±47.02	370±42.92	541±52.57	0.97
SSC	240.20±28.92	239.80±28.92	532.00±32.34	0.31

Table 3.18. All layers. Cell density of S100ß-IR astrocytes in the right hemispheres of mPFC subregions and SSC after repeated separation stress.

Layer I	Control	Group 4	Group 5	P
ACd	367.20±41.71	416.00±38.07	526.50± 46.63	0.30
PrCM	429.75±58.53	336.00±47.79	512.50± 58.53	0.71
PL	420.20±42.93	423.00±39.19	538.00± 48.00	0.38
IL	505.20±41.41	482.16±37.80	549.50± 46.30	0.39
SSC	282.00±50.17	306.20±50.17	532.00± 56.09	0.97

Table 3.19. Layer I: Cell density of S100ß-IR astrocytes in the right hemispheres of mPFC subregions and SSC after repeated separation stress.

Layer II-III	Control	Group 4	Group 5	P
ACd	177±30.41	231±27.76	426±34.00	0.86
PrCM	171±49.17	250±44.88	510±54.97	0.54
PL	154±37.80	263±34.51	468±42.71	0.97
IL	171±49.17	250±44.88	510±54.97	0.87
SSC	191.40±37.29	191.00±41.69	532.00±41.61	0.31

Table 3.20. Layer II-III: Cell density of S100ß-IR astrocytes in the right hemispheres of mPFC subregions and SSC after repeated separation stress.

Layer V-VI	Control	Group 4	Group 5	P
ACd	301±31.75	347±28.98	534±35.50	0.18
PrCM	240±54.58	262±49.83	600±61.03	0.85
PL	297±36.13	296±32.99	528±40.40	0.24
IL	297±53.16	321±48.53	538±59.44	0.45
SSC	258.00±30.00	258.20±30.00	521.40±30.00	0.69

Table 3.21. All Layers and Layer I: Cell density of S100ß-IR astrocytes in the right hemispheres of mPFC subregions and SSC after repeated separation stress.

APPENDIX 4

Supplementary Materials

PB-Phosphate buffer
stock solution 1 – (13.8 g/L) 0.1 M Na_2HPO_4. H2O
stock solution 2 – (14.2 g/L) 0.1 M Na_2HPO_4

stock solution 1 = 200 ml
stock solution 2 = 800 ml (1 L)
mix stock solution 1and 2 and set pH = 7.4

PBS-Phosphate buffer
stock solution 1 = 200 ml
stock solution 2 = 800 ml
+ 8.5 g NaCl (1 L), set pH = 7.4 with NaOH/ or HCl

Tris-HCl buffer
Tris-HCl 50 mM = 6.01 g dissolve in 2/3 distilled water
set pH = 7.6 with conc. HCl, adjust final volume to 1L

0.1M Tyrodebuffer (1L)
NaCl = 8g
KCl = 200 mg (M = 74. 56)
$CaCl_2$. $2H_20$ = 1g (M = 84.0)
NaHCO3 = 1g (M = 84.0)
NaH_2PO_4 x $2H_2O$ = 40 mg (M = 156.01)

- dissolve in distilled water, set the pH = 7.6 with NaCl or HCl, cool.

4% paraformaldehyde (PFA) in 0.1 M Sodium acetate buffer (pH = 6.5) (1L)

- weigh 8.2 g of sodium acetate and dissolve it in 800 ml distilled water
- mix and set pH = 6.5 with con. HCl. Adjust final volume to 1L.
- weigh 40 g PFA and dissolve it in 1L of Sodium acetate buffer
- mix, filter and keep it cool.

4% paraformaldehyde (PFA) in 0.1 M Sodium borate buffer (pH = 9.3) (1L)

- weigh 20.1 g of Sodium borate and dissolve in 800 ml distilled water
- mix and set the pH = 9.3 with NaOH. Adjust the volume to 1L.
- weigh 40 g PFA and dissolve it in 1L of Sodium acetate buffer
- mix, filter and keep it cool.

APPENDIX 5

Zusammenfassung

Der Einfluss von akuter und wiederholter neonataler Separation auf die S100ß- und GFAP-Immunoreaktivität im medialen Präfrontalcortex

Astrozyten wurde lange Zeit lediglich eine unterstützende Funktion für die Nervenzellen im Gehirn zugesprochen. Heute ist bekannt, dass Astrozyten wichtige Aufgaben bei der neuronalen Migration sowie bei der Bildung und Reifung synaptischer Kontakte während der frühen Hirnentwicklung erfüllen. Es gibt jedoch nur wenige Studien, welche die Bedeutung der neonatalen Umgebung auf die Plastizität und Reifung von Astrozyten in assoziativen Hirnregionen wie dem medialen Präfrontalcortex (mPFC) untersucht haben. Die vorliegende Arbeit überprüft die Hypothese, ob die Plastizität von Gliazellen im mPFC durch die frühe Separation von der Familie beeinflusst wird. Die Expression der astrozytischen Markerproteine S100ß und GFAP wurde untersucht, um den Einfluss von akuter und wiederholter Separation während der ersten drei Lebenswochen, einer kritischen Phase in der Entwicklung, zu überprüfen. Fünf experimentelle Gruppen der Strauchratte *Octodon degus* wurden verglichen: 1) Kontrolltiere (CON), welche ungestört bis Postnataltag 21 (PND 21) mit den Eltern und Geschwistern im Heimkäfig aufwuchsen; 2) Akute Separation (Group 2), diese Jungtiere wurden am PND 21 für 6 h von den Eltern und Geschwistern getrennt und danach 1h in den Heimkäfig zurückgesetzt; 3) Akute Separation + Reunion (Group 3) diese Tiere wurden am PND 19 für 6 h von den Eltern und Geschwistern getrennt und verblieben anschließend bis PND 21 im Heimkäfig; ; 4) Wiederholte Separation (Group 4), diese Jungtiere wurden von PND 1-21 täglich 1 h von den Eltern und Geschwistern getrennt und nach der letzten Separation für 1 h in den Heimkäfig zurückgesetzt; 5) Wiederholte Separation + Reunion (Group 5), diese Jungtiere wurden von PND 1-14 täglich 1 h von den Eltern und Geschwistern getrennt und verblieben danach bis PND 21 ungestört im Heimkäfig.

Die Dichte S100ß- und GFAP-positiver Astrozyten wurde in vier Subregionen des mPFC quantifiziert: anteriorer cingulärer Cortex (ACd), präcentraler medialer Cortex (PrCM), prälimbischer (PL) und infralimbischer (IL) Cortex. Als nichtlimbische Kontrollregion wurde außerdem der somatosensorische Cortex (SSC) untersucht.

Es zeigte sich, dass akute und wiederholte Separation die Dichte S100ß- und GFAP-positiver Astrozyten im mPFC ändert. Dabei erhöht sich die Dichte von S100ß schicht- und regionenspezifisch, während die Dichte von GFAP sinkt. Akute Separation führte außerdem zu einer veränderten Morphologie der Gliazellen, wiederholte Separation beeinflusste die Morphologie hingegen nicht. Reunion, also längeres Zurücksetzen in den Familienverband nach der Separation beseitigte die Veränderungen in der Morphologie nach akuter Separation und minimierte sie nach wiederholter Separation. Im SSC führte die akute und wiederholte Separation von der Familie nicht zu Veränderungen der S100ß-positiven Astrozyten, erhöhte jedoch die Dichte GFAP-positiver Astrozyten.

Diese Veränderungen in der frühen Entwicklung könnten Konsequenzen für die Interaktion von Neuronen und Gliazellen bei der Modulation der synaptischen Plastizität nach sich ziehen. Die Ergebnisse verstärken die anfänglich gestellte Hypothese, dass Gliazellen in assoziativen Hirnregionen durch neonatale Umweltveränderungen, wie die Separation von der Familie, beeinflusst werden.

APPENDIX 6

Erklärung

Antemano, Rowena R.
Gästehaus des IfN
Brenneckestr. 6
39118 Magdeburg

Hiermit erkläre ich, dass ich die von mir eingereichte Dissertation zu dem Thema:

Glia going emotional: The impact of acute and repeated neonatal separations on astrocytes in the medial prefrontal cortex.

selbständig verfasst, nicht schon als Dissertation verwendet habe und die benutzten Hilfsmittel und Quellen vollständig angegeben wurden.

Weiterhin erkläre ich, dass ich weder diese noch eine andere Arbeit zur Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. nat.) an anderen Einrichtungen eingereicht habe.

Magdeburg, 29. 05. 2007

APPENDIX 7

CURRICULUM VITAE

ROWENA REYNO ANTEMANO

Personal Data

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Education

2003-2007 PhD in Natural Science, major in Neurobiology
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1994-2002 MS Biology, major in Genetics
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1989-1993 BS Biology
College of Arts and Sciences
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Previous Employment

2000-2002	Research Associate, Marine Science Institute, University of the Philippines at Diliman
1999-2002	Project Group Secretary, Discovery and Development of Anticancer Drug and Other Agents for Immunoliposome-Mediated Drug Delivery to Breast Cancer, University of the Philippines
1999-2000	Graduate Assistant, Marine Science Institute, University of the Philippines
1988	Research Associate, Department of Environmental and Natural Resources, Quezon City

Awards and Research Fellowships

2003-2007	*Scholarship*, German Academic Exchange Service (DAAD)
2006	*Co-awardee, National Award* "Outstanding Research in Tropical Medicine in 2006" National Academy of Science and Technology (NAST), Manila, Philippines
2006	*Travel Award,* National Institutes of Health International Society of Developmental Psychobiology Meeting. Atlanta, Georgia
2000	*Thesis Grant,* Department of Science and Technology, Philippines
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Publication

Davis RA, Mangalindan GC, Bojo ZP, **Antemano RR**, Rodriguez NO, Concepcion GP, Samson SC, de Guzman D, Cruz LJ, Tasdemir D, Harper MK, Feng X, Carter GT, and Ireland CM. 2004. **Microcionamides A and B, Bioactive Peptides from the Philippine Sponge *Clathria (Thalysias) abietin.*** *The Journal of Organic Chemistry.* 69: 4170-4176.

Published /Electronic Abstracts

Antemano R, Helmeke C, Braun K. *2006*. Stress-induced changes of S100b- and GFAP-immunoreactive astrocytes in the rodent prefrontal cortex. Neural Plasticity 2007. 15th Annual Meeting of the Israel Society for Neuroscience. Abstracts.

Antemano R, Helmeke C, Braun K. *2006*. Stress-induced changes of S100b- and GFAP-immunoreactive astrocytes in the rodent prefrontal cortex." 36th Annual Meeting. Society for Neuroscience. http://www.abstractsonline.com/viewer/SearchResults.asp

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Conferences attended and Presentations

2006 36th Society for Neuroscience Meeting. Atlanta, Georgia
Poster: "S100ß and GFAP-IR astrocytes..."

2006 International Society for Developmental Psychobiology. Atlanta, Georgia
Oral: "S100ß and GFAP-IR astrocytes..."

2006 5th Forum of European Neuroscience Convention. Vienna, Austria
Poster: Stress-induced changes… in medial prefrontal cortex…

2005 35th Society for Neuroscience Meeting. Washington, DC
Poster: Astrocytes going emotional…"

2005 38th Annual Meeting, International Society for Developmental Psychobiology. Washington, DC
Poster: astrocytes going emotional…"

2001 Philippine Society of Biochemistry and Molecular Biology Convention. Manila, Philippines
Poster: Heptyl Prodigiosin Induces DNA Fragmentation…

2001 World Conference on Science and Technology. Manila, Philippines
Oral: Cytotoxicity… Against Various Human Cancer.

2000 Symposium on Scientific Research for Sustained Use and Efficient Management of Natural Resources.
Oral: In vitro Tumor Cytotoxicty …

2000 Philippine Society of Biochemistry and Molecular Biology Convention. Iloilo, Philippines
Oral: Adociaquinone B Induces Apoptosis...
Poster: Cytotoxicity of Marine Natural Products…