The role of Protease-Activated Receptor-1

in stress-induced neuronal activity

and behavioural responses

Thesis submitted for the Degree of Doctor of Philosophy in the Faculty of Medicine and Biological Sciences at the University of Leicester

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Abstract

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Julie-Myrtille Bourgognon

Serine proteases and their target molecules have been implicated in numerous aspects of CNS function, inducing various forms of learning, memory and emotional responses. This work sought to elucidate the role of the protease-activated receptor-1 (PAR-1) in stress-induced neuronal plasticity and behavioural responses. PAR-1 gene and protein are present in most regions of the mouse brain with high levels in the thalamus, the hippocampus and the amygdala. Due to its localization in limbic areas, PAR-1 regulation in the amygdala was examined following restraint stress or fear conditioning (FC). The finding that PAR-1 protein but not gene levels are downregulated led to the hypothesis that PAR-1 must be cleaved by stress-related proteases. Indeed my results indicate that a limbic-serine protease neuropsin cleaves the receptor in an indirect manner. This led us to investigate behavioural phenotypes of PAR-1+/− and neuropsin−/− mice during fear, anxiety, and learning-related tests. PAR-1+/− and neuropsin−/− mice are characterised by an anxiogenic and an anxiolytic behaviour, respectively, both profiles being related to amygdala dysfunction. Indeed amygdala-related neuronal activity following FC appeared elevated based on abnormally elevated c-Fos and P-CREB levels in fear conditioned PAR-1+/− mice. This may underpin a higher susceptibility of these mice towards anxiety and suggest an inhibitory role of PAR-1+ during fear learning. Further electrophysiology data suggest that neuronal excitability and plasticity after FC are affected by PAR-1 through modulation of AMPA receptors. A role of the transmembrane AMPA receptor regulatory protein Stargazin in this process is excluded as its gene levels are not affected by FC. Following fear learning PAR-1+/− mice display differential phosphorylation levels of GluR2 subunit. Furthermore dissimilar regulation of AMPAR splice variant between PAR-1+/− and wild-type mice could account for PAR-1-related behaviour and electrophysiological characteristics. The results presented here reveal a previous uncharacterized inhibitory role of PAR-1 in the central nervous system.
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<tbody>
<tr>
<td>ABP</td>
<td>AMPA receptor binding protein</td>
</tr>
<tr>
<td>ACSF</td>
<td>Artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
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<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate</td>
</tr>
<tr>
<td>APC</td>
<td>Activated Protein C</td>
</tr>
<tr>
<td>BLA</td>
<td>Basolateral nucleus of the amygdala</td>
</tr>
<tr>
<td>CaMK</td>
<td>Ca$^{2+}$/calmodulin-dependent protein kinases</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CE</td>
<td>Central nucleus of the amygdala</td>
</tr>
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<td>CNⅢH</td>
<td>Cornichon homolog</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding</td>
</tr>
<tr>
<td>CS</td>
<td>Conditioned stimulus</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacyl glycerol</td>
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<td>eGFP</td>
<td>enhanced Green fluorescent protein</td>
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<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>EPCR</td>
<td>Endothelial protein C receptor</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
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<td>GABA</td>
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<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
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<td>G-protein coupled receptor</td>
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<td>Glutamate receptor-interacting protein</td>
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<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
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<td>LA</td>
<td>Lateral nucleus of the amygdala</td>
</tr>
<tr>
<td>LTD</td>
<td>Long term depression</td>
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<tr>
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<td>Long-term potentiation</td>
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<tr>
<td>mEPSCs</td>
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<td>Neuropsin</td>
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<td>Protein kinase C</td>
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<td>PTSD</td>
<td>Post traumatic stress disorder</td>
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<td>qRT-PCR</td>
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<tr>
<td>TARPs</td>
<td>Transmembrane AMPA receptor regulatory proteins</td>
</tr>
<tr>
<td>tPA</td>
<td>Tissue plasminogen activator</td>
</tr>
<tr>
<td>TRAP</td>
<td>Thrombin receptor agonist</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase-type plasminogen activator</td>
</tr>
<tr>
<td>US</td>
<td>Unconditioned stimulus</td>
</tr>
<tr>
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<td>Wild-type</td>
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Chapter I

Introduction
I.1. INTRODUCTION

Protease-activated receptors (PARs) belong to the seven transmembrane G-protein coupled receptors superfamily. Four members of the PARs family have been described. PAR-1, PAR-2 and PAR-3 are encoded by F2R, F2RL1 and F2RL2 genes localised on chromosomes 5q13 (human) or 13d2 (mouse) whereas PAR-4 gene F2RL3 is found on the human 19p12 or murine 8b3 chromosome (Xu, 1998; Kahn, 1998). Those receptors are expressed in a variety of cell types: vascular, immune, endothelial and epithelial cells, as well as myocytes, fibroblasts and cells of the nervous system. PAR-1, originally called the thrombin receptor, was first cloned by Rasmussen (1991) who described its amino acid composition (427 residues), structured domains and the thrombin cleavage site.

PAR-1 has a broad tissue distribution and is involved in a wide variety of cellular events. It is the primary thrombin receptor on human blood platelets (Coughlin, 2005) and thus has historically been associated with blood coagulation. On the surface of platelets, Factor Xa activates prothrombin into thrombin which, by cleaving PAR-1, promotes platelets aggregation. As the coagulation propagates the “thrombin burst” leads to the formation of fibrin clots (Adams, 2009; Chambers, 2002). Pro-coagulatory properties of thrombin are regulated by a cofactor thrombomodulin. Upon thrombomodulin binding thrombin activates coagulation inhibitor proteins C and S (Esmon, 1995) and thus acts as an anticoagulant.

Although PAR-1 was originally cloned as the receptor for thrombin it soon became apparent that this protease also cleaves other members of the PAR family, i.e. PAR-3, and -4.
Chapter I

Genetic disruption of PAR-1 in mice gave further insight into the role of this receptor in health and disease. Mice lacking PAR-1 were generated fifteen years ago by deleting the gene portion encoding the transmembrane domains 1-7 of the receptor (Connolly, 1996). Fifty per cent of PAR-1 knock-out mice die at embryonic days 9-10 (Connolly, 1996) revealing the importance of endothelial PAR-1 for a normal blood vessel development (Griffin, 2001). Viable PAR-1 deficient mice appear grossly normal but show altered responses to vascular injury characterised by an impaired thickening of vessel walls and a disturbed regulation of blood vessel diameter (Cheung, 1999).

In order to better understand the role of PAR-1 in the brain it is important to get familiar with its mode of action in other systems. Thus a brief description of the localization and the role of PAR-1 related to the cardiovascular system, inflammation, neuropathogenesis, neurodegenerative disorders and cancer, will follow. One striking feature of PAR-1 is its ability to generate contrasting effects depending on the dose and structure of the agonist as well as cofactors present in the system. This phenomenon can be explained by ligand-biased signalling whereupon stimulation with different ligands, G-protein-coupled receptors (GPCRs) associate with different G protein subunits or other effectors resulting in activation of different signal transduction pathways (Russo, 2009). Thus, different proteases or a different dose of the same protease would result in different PAR-1 G-protein coupling profiles triggering contrasting cellular responses. Several examples of such effects are described below.
I.1.1. PAR-1 and the cardiovascular system

PAR-1 expression in the vascular system has been extensively studied. High PAR-1 levels have been demonstrated in endothelial and smooth muscles cells (Nelken, 1992). The presence of PAR-1 in blood vessels suggests a role in regulation of vascular tone. Analysis of the literature reveals a complicated picture where different preparations or animal species often generate conflicting results. Thus PAR-1 or PAR-2 selective agonists trigger relaxation of human and porcine pulmonary and coronary arteries (Hamilton, 2001 and 2002). On the other hand, PAR-1 activation generates calcium-dependent contractile response in rat aortic rings (Antonaccio, 1994). Thrombin receptor-derived agonist peptides also trigger contraction of vascular smooth muscles and various blood vessel preparations (Tay-Uyboco, 1995; Ku, 1997). PAR-1 agonists trigger vasodilatation in isolated piglet lungs (Pinheiro, 1993) or vasoconstriction in guinea pig lungs (Lum, 1994). In mice, PAR-1 can cause hypotension (Cheung, 1998) although no vascular-related abnormalities have been noticed in adult PAR-1 knockout mice (Darrow, 1996).

In conclusion, the effects of PAR-1 activation on the vascular tone and blood pressure are often species- and tissue-specific with opposite responses in different experimental preparations or animals.

I.1.2. PAR-1 and inflammation

Thrombin-mediated activation of PAR-1 and PAR-4 (in human) or PAR-4 and PAR-3 (in mice) is known to play a role in haemostasis by eliciting platelet aggregation, granular secretion and procoagulant activity (Kahn, 1999; Nakanishi-Matsui, 2000). The role of PAR-1 in regulating thrombosis and blow flow is particularly prominent during
inflammation and sepsis, where PARs expression is regulated by inflammatory mediators (Nystedt, 1996; Hamilton, 2001; Ramachandran, 2007). In human platelets PAR-1 is activated by low concentrations of thrombin while high concentrations are necessary for PAR-2-mediated platelet activation (Kahn, 1999).

PAR-1 and PAR-2 are also present on a variety of white blood cells such as macrophages, monocytes (Colognato, 2003) and mast cells (D’Andrea, 2000). PAR-2 and PAR-3 have been described on eosinophils and neutrophils (Miike, 2001; Shpacovitch, 2004). Vergnolle (2002) reported the presence of PAR-4 on leucocytes. White blood cells together with mast cells are involved in inflammatory response and PARs are thought to play a role in chemotaxis (Colotta, 1994) and cytokine release (Deng, 2008) during this process. The above effects could play a role in the nervous system inflammation because PAR-1 is expressed in nodes of Ranvier on rat sciatic nerves and is involved in inflammatory demyelination (Shavit, 2008).

PARs are abundant in the gastrointestinal system where they are involved in the innate immune responses. PAR-1 and PAR-2 promote epithelial cell proliferation (Shi, 2004), disruption of the intestinal barrier (Cenac, 2002) and smooth muscle contractility in the gut (Kawabata 2001). Those two receptors, when carried by lymphocytes are involved in colon inflammatory response (Cenac, 2002). Finally, Buresi (2005) demonstrated that PAR-1 expressing enteric neurons were responsible for inhibition of the protective chloride secretion, therefore facilitating pathogen invasion.

Outside the gastrointestinal system PAR-1 plays an important role as a pro-inflammatory mediator transmitting nociceptive messages to the central nervous system to
mediate hyperalgesia (Yang, 2005; Vergnolle, 2001). Activation of PAR-1 in sensory neurons causes oedema (de Garavilla, 2001). However, PAR-1 also takes part in anti-inflammatory processes as demonstrated by Vergnolle (1999) who used low dose PAR-1 agonist to induce mechanical and thermal analgesia and decreased inflammation-induced hyperalgesia in the mouse. PAR-1-mediated analgesia has also been highlighted in a recent study demonstrating its ability to increase endogenous opioid peptide expression and control pain (Martin, 2009).

Sepsis is a serious medical condition in which the body fights generalized inflammation together with acute organ dysfunction. Most septic patients show a reduction in Activated Protein C (APC), a pro-fibrinolytic, anticoagulant and an anti-inflammatory agent (Bernard, 2001). APC is regulated both by thrombin and PAR-1. In physiological conditions, thrombin activates Endothelial Protein C Receptor-Protein C (EPCR-PC) to generate anti-inflammatory EPCR-activated PC (EPCR-APC) complex that cleaves PAR-1 (Riewald, 2002). However the role of thrombin and PAR-1 during sepsis appears more complex. Kaneider et al (2007) described contrasting roles of PAR-1 during the different stages of sepsis in mice. At early stages of sepsis thrombin-activated PAR-1 promotes vascular barrier disruption via a G_{12/13}-Rho pathway. As sepsis progresses PAR-1 becomes protective by transactivating PAR-2 and leading to G_{7}-Rac mediated barrier repair (Kaneider, 2007).
I.1.3. PAR-1 and cancer

PAR-1 plays a prominent role in cell invasiveness in cancer. High levels of PAR-1 expression have been reported in breast carcinoma cell lines (Even-Ram, 1998) leading to studies on the role of thrombin receptors in mitogenesis, cell growth, carcinogenesis, tumorigenesis, and metastasis (Tellez, 2003; Nierodzik, 2006). It has been shown that persistent signalling of epidermal growth factor receptor/ErbB2 in breast cancer is mediated by thrombin-activated PAR-1 and facilitates invasion (Arora, 2008). It has been suggested that PAR-1 activation plays a role in the regulation of genes promoting angiogenesis and tumor invasion such as interleukins, integrines and vascular growth factors (Nierodzik, 2006; Tellez, 2003). Boire (2005) reported that fibroblast-secreted matrix metalloprotease-1 (MMP-1) could activate PAR-1 and described PAR-1 as a potent tumorigenic and pro-invasive factor for human breast cancer. Thrombin and MMP-1 produce independent or synergistic effects on angiogenesis and tumor progression (Blackburn, 2008). Interestingly, Nierodzik suggested a role for thrombin in the maintenance of dormant tumor cells that would grow when thrombin reaches a critical concentration (Nierodzik, 2006).

I.1.4. PAR-1 and the central nervous system

So far, the reports are scarce probably because of a lack of an obvious phenotype.
1.1.4.a. Trauma and seizures

The role of PAR-1 is particularly pronounced in brain pathology. Trauma, ischemia or inflammation increases vascular permeability and causes brain blood barrier disruption allowing circulating molecules, such as thrombin to enter brain parenchyma. Endothelial cells of the neighbouring blood vessels can also release thrombin (Grammas, 2004) and contribute to this process. Both prothrombin (Dihanich, 1991) and all four PARs are expressed in the CNS. Although PARs expression can be region-specific, it is commonly accepted that they are present in both neurons and glia (Striggow, 2001). The role of thrombin and PARs in the brain was first studied by Citron et al. (2000). They found that prothrombin and PAR-1 mRNA were upregulated with similar kinetics after spinal cord injury. Later, different models of injury have been shown to elicit contrasting patterns of regulation of the PAR-1 gene. For instance, hippocampal PAR-1 mRNA levels increased in response to ischemia in organotypic slice cultures (Striggow, 2001) but not following two-vessel occlusion in rats (Riek-Burchardt, 2002).

It has been suggested that extravasation of thrombin and its diffusion into the nervous system following trauma, stroke or surgery, predisposes the brain to seizures (Lin, 2003). This hypothesis is supported by brain injections of thrombin triggering seizures and brain oedema, this effect being blocked by co-administration of thrombin inhibitors (Lee, 1997). Mice overexpressing or lacking the thrombin inhibitor protease-nexin-1 (PN-1) show a lower threshold of epileptic activity and the imbalance between thrombin and PN-1 leads to an increased neuronal excitability (Luthi, 1997). Moreover, it has been shown that PAR-1 activation by thrombin facilitates the appearance of spontaneous
seizure-like activity independently of NMDAR activation, in CA1 and CA3 region of the rat hippocampus (Maggio, 2008).

1.1.4.b. Calcium homeostasis

Calcium homeostasis is central to numerous biological processes within the cell and represents a critical convergent point of plasticity-related mechanisms in neurons. Calcium can regulate neurite outgrowth (Lankford, 1989) and is crucial for long-term potentiation (LTP) induction (Grover, 1990). Thrombin via PAR-1 cleavage elevates calcium levels in the neuronal cell line NSC19 (Smirnova, 1998). Kiseleva (2004) demonstrated that thrombin and PAR-1 agonists are able to induce calcium mobilization in cultured neurones of rat hippocampus. Moreover, PAR-1 together with PAR-2 have been shown to mediate calcium signalling in glial cells (Ubl, 1997 & 1998).

1.1.4.c. Neurite outgrowth and glial cells morphology

Numerous studies demonstrated that PAR-1 alters the morphology of neurones and astrocytes and therefore could be involved in structural remodelling of cellular connections. It appears however that PAR-1 could exert contrasting effects in different cell types. Several articles demonstrated that thrombin and PAR-1 agonists reverse astrocyte stellation and stimulate their proliferation (Beecher, 1994; Grabham, 1995; Nicole, 2005; Sorensen, 2003). For example, the PAR-1 role was studied by Miller et al. (1996) who observed an up-regulation of β-actin mRNA levels in astrocyte culture after thrombin or PAR-1 agonist treatment as well as the reversal of astrocyte stellation.
Thrombin and PAR-1 agonists could alter neuronal morphology by facilitating astrocytic secretion of a potent stimulator of neuronal growth, survival and axonal branching, nerve growth factor (Neveu, 1993; Debeir, 1996). Consistent with this, Debeir et al (1998) showed that low concentration of thrombin or a PAR-1 agonist TRAP-14 when added to differentiated neurone-glia co-cultures favours neurite elongation but inhibits sprouting. However Jalink et al (1992) demonstrated that thrombin and TRAP-14 induce cell rounding and neurite retraction in primary neuronal cultures.

1.1.4.d. Neuronal survival and neurodegeneration

It has been shown that PAR-1 -4 gene expressions are regulated by oxygen-glucose deprivation with distinct dynamics implicating the above receptors in pathophysiology of brain ischemia (Striggow, 2001). However, the extent and kinetics of these events vary depending on the experimental paradigm used (Striggow, 2001; Riek-Burchardt, 2002; Rohatgi, 2004).

PAR-1 has been implicated in both neuronal death and survival with species, region and dose-specific contrasting effects often observed. For example, Turgeon (1998) reported degeneration and death of avian spinal motorneurones after PAR-1 activation whereas rat primary astrocytes and hippocampal neurons were protected from hypoglycaemia-induced cell death by thrombin and PAR-1 agonists (Vaughan, 1995). Striggow et al (2000) proposed that activation of PAR-1 could have opposite effects on neuronal death depending on the concentration of the agonist. They showed that low
concentrations of either thrombin or a PAR-1 agonist protected organotypic slice cultures of rat hippocampus against experimental ischemia while high concentrations exacerbated neuronal loss.

The involvement of PAR-1 in neurodegeneration was also studied by either genetic disruption or pharmacological inhibition of the receptor. PAR-1 knockout mice display a smaller infarct volume and resulting motor deficits than wild-type mice following transient focal cerebral ischemia (Olson, 2004). A similar protection was achieved with a PAR-1 antagonist in a model of hypoxia/ischemia-induced cell death (Junge, 2003; Olson, 2004).

PAR-1 can be cleaved by a variety of proteases (Luo, 2007) and thus it is important to determine what proteolytic enzymes cleave PAR-1 during neurological insults. This issue is currently far from clear. During stroke, however, PAR-1 is likely to be activated by extravasated thrombin. In these conditions thrombin is harmful because inhibiting its formation with antithrombin reduces the infarct volume and increases animal survival (Cuomo, 2007).

PAR-1 neuroprotective role has been closely linked to the Activated Protein C pathway. For instance APC protects mouse cortical neurones from apoptosis through activation of PAR-1 and PAR-3 (Guo, 2004). Another study demonstrated that PAR-1 was required for APC-mediated postischemic neovascularization and neurogenesis (Thiyagarajan, 2008).

In keeping with the involvement of PAR-1 in regulating neuronal death and survival the role of the above receptor in human neurodegenerative disorders (or their
animal models) has also been reported. PAR-1 mRNA levels are upregulated in human post-mortem samples of patients diagnosed with Parkinson’s disease (PD; Ishida, 2006) suggesting the involvement of PAR-1 in this pathology. Analysis of the available literature revealed that activation of PAR-1 can be either protective or detrimental depending on the model, species or the agonist used. Hamill et al, (2007) demonstrated that PAR-1 activation contributes to the degeneration of dopaminergic neurones in the mouse 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of PD. However, the effect of thrombin may vary depending on the time of administration. Thrombin preconditioning (i.e. pre-treatment with a low dose of thrombin) is protective in a rat model of PD and this effect is mediated by PAR-1 (Cannon, 2005 & 2006). Co-administration of either thrombin or a PAR-1 agonist together with the neurotoxin 6-hydroxydopamine increases behavioural and neurochemical deficits in rats (Cannon, 2007). Delayed infusion of thrombin prevents behavioural deficits as well as dopamine depletion in the same model (Cannon, 2007).

Interestingly, in rat substantia nigra, it is PAR-4 but not PAR-1 that mediates thrombin-induced apoptosis of dopaminergic neurones (Choi, 2003; Herrera, 2008) leading to the hypothesis that some others proteases (e.g. MMP-1) upregulated in the MPTP model of Parkinson’s disease, are likely to contribute towards PAR-1 cleavage (Hamill, 2007).

The studies on the role of thrombin and PAR-1 in Alzheimer’s disease (AD) often yield contrasting results. In addition to blood vessels, pyramidal neurons and astrocytes, thrombin was found in extra- and intracellular neurofibrillary tangles, senile plaques, and
reactive microglial cells of AD brains (Arai, 2006). A thrombin-like enzyme is involved in
the microtubule-associated protein tau cleavage (Wang, 2007) and thrombin has been
found to cleave tau in vitro (Aria, 2005). PAR-1 and PAR-4-induced tau
hyperphosphorylation and aggregation lead to apoptosis of hippocampal neurons (Suo,
2003). The involvement of the thrombin receptor in AD is corroborated by the finding
that PAR-1 mRNA astrocytic levels are upregulated in rat hippocampus after injection of
the neurotoxicant trimethylin that mimics AD’s pathophysiology and symptomatology
(Pompili, 2004).

In contrast to the above studies Pike (1996) demonstrated that thrombin and
PAR-1 could counteract the pathological effects of Aβ. Thrombin or a PAR-1 agonist
SFLRN attenuated β-amyloid-induced neurodegeneration of hippocampal neurones, as
well as reversed astrocytic stellation (Pike, 1996). Interestingly these effects were blocked
by the thrombin inhibitor protease-nexin-1 that has been shown to be downregulated in a
model of AD (Choi, 1995). Thus, the disruption of a subtle balance between thrombin,
its receptors and inhibitors might be essential for the appearance/progression of AD.

I.1.5. Proteases and PAR-1 mode of activation

The PARs family differs from other GPCRs by its unique mechanism of
activation, first described by Vu et al (1991) for PAR-1. This process requires cleavage of
the N-terminal of the receptor by protease(s) to unmask a tethered ligand and in turn
activating the receptor. Thrombin, the central molecule of the coagulation cascade, can
cleave a limited number of substrates that fall within its substrate specificity (Brass, 1994)
including PAR-1, PAR-3 and PAR-4 (Macfarlane, 2001), whereas trypsin but not thrombin activates PAR-2 (Nystedt, 1994). Thrombin’s active site recognises the selective arginyl peptide bond VNPR (Figure I.1) in the amino-terminal domain of the receptor and PAR-1 hirudin-like domain (Vu, 1991) binds to thrombin’s anion-binding exosite (Figure I.1). PAR-1 is cleaved at Arg 41/ Ser 42 (Vu, 1991; Figure I.1; Table I.1). The tethered ligand (SFFLRN in mouse) is unmasked and with thrombin’s guidance (Brass, 1994) binds to the second extracellular loop of the receptor, leading to the activation of G-proteins bound to its cytoplasmic tail (Swift, 2000). In keeping with this theory, synthetic peptides corresponding to the tethered ligand also potently activate PAR-1 (Hollenberg, 2003).

Thrombin cleaves PAR-1 at two other sites (Arg 46 and Lys 76) albeit with 25000-fold slower kinetics than at Arg 41 (Kuliopulos, 1999). Efficient receptor cleavage at low thrombin concentration depends on the interaction between hirudin-like domain of PAR-1 and the thrombin anion binding sequence. That explains why PAR-4, which lacks a hirudin-like domain, responds only to high thrombin concentrations (Xu, 1998).
Figure I.1. Mouse protease-activated receptor-1 sequence and its thrombin interaction domains.
Thrombin binds to the hirudin-like domain before cleaving the receptor at position 41/42 therefore releasing the tethered ligand (sequence accession number P30558).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>PAR-1</th>
<th>PAR-2</th>
<th>PAR-3</th>
<th>PAR-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activating proteases</td>
<td>Thrombin&lt;sup&gt;1&lt;/sup&gt;, Factor Xa&lt;sup&gt;3&lt;/sup&gt;, Cathepsin G, CaPI&lt;sup&gt;4&lt;/sup&gt;</td>
<td>Trypsin, trypase, Factor VIIa, Factor X, MT-SP1, proteinase 3.</td>
<td>Thrombin, trypsin</td>
<td>Thrombin, trypsin, cathepsin G, Factor VIIa, Factor X</td>
</tr>
<tr>
<td>Inhibitors</td>
<td>Plasmin&lt;sup&gt;2&lt;/sup&gt;, protease G, calpain</td>
<td>Chymase, elastase, Cathepsin G</td>
<td>Cathepsin G</td>
<td>unknown</td>
</tr>
<tr>
<td>Cleavage site</td>
<td>R&lt;sub&gt;141-142&lt;/sub&gt;SFLRN (h), R&lt;sub&gt;414-42&lt;/sub&gt;SFLRN (r, m)</td>
<td>R&lt;sub&gt;364-365&lt;/sub&gt;SLIGKV (h), R&lt;sub&gt;364-365&lt;/sub&gt;SLIG RL(r), R&lt;sub&gt;545-546&lt;/sub&gt;SLIG RL(m)</td>
<td>K&lt;sub&gt;591-592&lt;/sub&gt;TFRGAP (h), K&lt;sub&gt;571-598&lt;/sub&gt;SFNGGP (m)</td>
<td>R&lt;sub&gt;571-592&lt;/sub&gt;GYPGQV (h), R&lt;sub&gt;583-592&lt;/sub&gt;GYPGQV (r), R&lt;sub&gt;594-605&lt;/sub&gt;GYPGQV (m)</td>
</tr>
<tr>
<td>Tethered ligand (h)</td>
<td>SFLRN</td>
<td>SLIGKV</td>
<td>TFRGAP</td>
<td>GYPQGQ</td>
</tr>
<tr>
<td>Example of peptides agonists</td>
<td>SFLRN, TFLLRN, GFIYF, Trg</td>
<td>SLIGKV, SFLRN</td>
<td>-</td>
<td>GYPQGQV, AYPGKF</td>
</tr>
<tr>
<td>Example of peptides antagonists</td>
<td>3-mercaptoepropionyl-F-Cha-Cha-RKPDKamide, SCH79797, RWJ56110&lt;sup&gt;6&lt;/sup&gt;</td>
<td>ENMD-1068&lt;sup&gt;6&lt;/sup&gt;</td>
<td>-</td>
<td>YD-3, trans-cinnamoyl-YPGKF-amide</td>
</tr>
</tbody>
</table>

Table I.1. Summary (non exhaustive) of PARs activators and inhibitors, cleavage sites, agonist and antagonist peptides.
<sup>1</sup> Kuliopulos, 1999; <sup>2</sup>Riewald, 2001; <sup>3</sup>Riewald, 2002; <sup>4</sup>Boire, 2005; <sup>5</sup>Kawabata, 1999. See also Andrade-Gordon, 1999; Ahn, 2000; Kelso, 2005. See also Ossovskaya and Bunnett, 2004; Saito and Bunnett, 2005; Luo, 2007. PAR: protease activated receptor; APC: activated protein C; MT-SP1: membrane-type serine protease 1; h: human; r: rat; m: mouse.
PARs can be cleaved by other proteases apart from thrombin (Table I.1); for instance, APC activates PAR-1 (Riewald, 2002) and triggers its phosphorylation upon binding to EPCR rendering the receptor irresponsible to thrombin (Russo, 2009). Plasmin cleaves PAR-1 both at the thrombin cleavage site and downstream, closer to the cell membrane. In the latter case, plasmin-mediated cleavage results in the removal of the tethered ligand and desensitization of PAR-1 to other proteases (Kulipoulos, 1999). Calpain and leukocyte elastase, cathepsin G, and proteinase 3 also inactivate PAR-1 (Loew, 2000).

Different PARs are often expressed in the same cell enabling the cross-talk between the receptors. Both PAR-1 and PAR-4 are expressed in human platelets (Xu, 1998), and PAR-3 together with PAR-4 in murine platelets (Kahn, 1998). PAR-1, PAR-2 and PAR-3 are co-expressed in human endothelial cells (Schmidt, 1998; O’Brien, 2000). The cross-talk between PARs may involve transactivation of an intact PAR-1 molecule by a cleaved PAR-1 receptor as shown by Chen (1994). Also, cleaved PAR-1 transactivates PAR-2 in endothelial cell lines (O’Brien, 2000; Kaneider, 2007). In murine platelets, PAR-3 acts as a cofactor to facilitate low-affinity PAR-4 cleavage and activation by guiding thrombin towards PAR-4 (Nakanishi-Matsui, 2000). In HEK cells, PAR-3 regulates PAR-1 signalling by forming heterodimers which favour coupling with Gα13 protein while PAR-1 homodimers signal through both Gαq and Gα13 (McLaughlin, 2007). It is worth mentioning that unlike the other PARs, PAR-3 lacks the C-terminal domain and synthetic ligands fail to activate the protein (Ishihara, 1997); it explains why PAR-3 acts usually as cofactor rather than a fully-signal-competent receptor.
**Parstatin** is a synthetic peptide corresponding to the 1- to 41- amino acid fragment released from PAR-1 cleavage. The name parstatin was first introduced by Zania (2009) in reference to the first 23 amino acids forming a hydrophobic segment allowing the peptide to cross the plasma membrane and reach intracellular targets (Vu, 1991; Furman 1998; Routhu, 2010). In fact, the effects of parstatin are mediated by the first 1-to 26 amino acids of PAR-1 (Zania, 2009; Routhu, 2009). Parstatin binds to the platelet surface to strongly induce platelet aggregation synergistically with other platelet activators (Furman, 1998).

Human or mouse parstatin inhibits angiogenesis in chick or rat tissues, formation of capillary-like structures by endothelial cells as well as endothelial cell growth (Zania, 2009). Parstatin mediates endothelial cell apoptosis via an increase in caspase-3 activity (Zania, 2009). DNA synthesis monitored by incorporation of tritiated thymidine was also inhibited with a more robust effect on stimulated dividing cells by growth factors. Parstatin was also found to block growth factor-induced ERK1/2 activation. These effects are dose-dependent (Zania, 2009).

PAR-1 activation is known to regulate vascular tone. In preconditioning and reperfusion experiments, parstatin manifests cardioprotective functions *in vivo* and *in vitro* if used at adequate concentrations by reducing the infarct size and promoting normalization of left ventricular pressure (Hamilton, 2001). Parstatin effects are mediated via Gi protein. The signalling pathways are not completely elucidated but involve p38-MAPK and ERK1/2 signalling, PI3K, Akt, nitric oxide synthase, guanylyl synthase and K<sub>ATP</sub> channels (Strande, 2009; Routhu, 2009). The mechanism by which parstatin
activates G proteins is unknown. It is likely that the peptide could accumulate extracellularly and interact with cell membrane receptor(s). It could also cross the cell membrane to reach its target. It is possible that parstatin might be further processed by a signal peptide peptidase, an intramembrane protease at position 24 and 26 (Vu, 1991).

I.2. PARs signalling pathways

I.2.1. PARs and G protein coupling

Upon ligand binding, the conformation of a GPCR is modified and induces the activation of the coupled G protein i.e. the GDP/GTP exchange. The $G\alpha$-GTP complex dissociates from $G\beta\gamma$ and then both G protein subunits stimulate or inhibit other downstream effectors (Oldham, 2006).

PARs are coupled to different G-proteins depending on the cell type. PAR-1 interacts with the $G\beta\gamma$ subunit and several $\alpha$-subunits, in particular $G\alpha_{q11}$, $G\alpha_{12/13}$ and $G\alpha_{q16}$ and therefore triggers different signalling cascades (Figure I.2) that influence cell differentiation, migration, proliferation and mitogenesis.
Figure 1.2. Activation of PAR-1 and its consequences.
Thrombin activates PAR-1 by cleaving the N-terminal subunit just before Ser42, releasing the peptide parsatin. The newly formed tethered ligand at the N-terminal binds to the second extracellular loop of PAR-1. This action triggers activation of G-proteins: the $G_{12/13}$ subunit inhibits adenyl cyclase in glial cells and thereof decreases AMPc production. The $G_{q/11}$ subunit activates the phospholipase C (PLC), which activates Protein Kinase C (PKC) and causes an increase in intracellular calcium, via dyacylglycerol (DAG) and Inositol triPhosphate (IP3), respectively. The $G_{12/13}$ subunit binds to PAR-1 as well, to activate Rho/Rock pathway. The $\beta_\gamma$ subunit couples PAR-1 to several other pathways such as phosphatidylinositol (PI)-3 Kinase and G-protein receptor kinases (GRKs).
1.2.1.a. Signalling via Ga\textsubscript{q}

The principal mechanism of PAR-1 signalling is through Ga\textsubscript{q} as demonstrated by Hung \textit{et al} (1992) in fibroblasts and platelets. Activation of Ga\textsubscript{q} triggers via phospholipase C-\(\beta\)1 (PLC-\(\beta\)1) the generation of inositol triphosphate (IP\(_3\)) thus mobilizing intracellular calcium and diacyl glycerol (DAG) to activate PKC and mitogen-activated protein (MAP) kinases (Figure I.2). In an attempt to clarify thrombin-activated pathways in rodent fibroblasts, Fee \textit{et al} (1994) demonstrated that IP\(_3\) and calcium responses were attenuated in fibroblasts expressing low PLC-\(\beta\)1 levels when incubated with thrombin. Other PLC isoforms can also be involved in PAR-1 signalling; for instance, in murine glial cells, the intracellular calcium increase triggered by thrombin or PAR-1 agonist peptide results from activation of PLC-\(\beta\)3 (Hwang, 2005).

The role of Ga\textsubscript{q} in PAR-1-mediated effects is further corroborated by the fact that thrombin signalling in rodent fibroblasts and human platelets that express PAR-1 is attenuated by microinjection of Ga\textsubscript{q} antibodies (Baffy, 1994; Benka, 1995). Moreover, thrombin-induced platelet aggregation and degranulation were impaired in Ga\textsubscript{q} -deficient mouse platelets, while the shape-change remained unaltered (Offermanns, 1997). PAR-1 signalling via Ga\textsubscript{q} might play a role in communication between different cell types in the brain as recently, Lee \textit{et al} (2007) have demonstrated that astrocytic Ga\textsubscript{q/11}-coupled PAR-1 stimulates calcium-dependent release of glutamate leading to the enhancement of synaptic NMDA receptor currents in CA1 neurons in rodent hippocampal slices. An astrocytic PAR-1 activation by plasmin, has also been shown to potentiate NMDA receptors in rat hippocampal slices (Mannaioni, 2008) probably via phosphoinositide
hydrolysis and calcium release as well as ERK1/2 phosphorylation. Inhibitory effects of PAR-1 on nociception and hyperalgesia in the CNS of mice are mediated by the NMDA receptor (Fang, 2003).

PAR-2 might be coupled to $\alpha_{q/11}$ since its activation leads to IP3 and DAG production and calcium elevation (Nystedt, 1995; Macfarlane, 2001). PKC, MAPKinases and NF-κB signalling have also been reported after PAR-2 activation in various cell types (Okamoto, 2001; Seatter, 2004; Belham, 1996; Macfarlane, 2005; Bretschneider, 1999).

PAR-3 is known for its ability to dimerize with other PARs (McLaughlin, 2007) and was thought to be unable to signal on its own due to its truncated C-terminal tail (Ishihara, 1997). However recent reports challenge this view. For instance, PAR-3 participates in inflammatory reactions through PAR-3-mediated ERK1/2 phosphorylation and interleukine 8 release in HEK and human lung cells that occurs independently of other PARs (Ostrowska, 2008). Another study shows that Rho and calcium signalling pathways are activated following thrombin action on PAR-3 but not PAR-1 or PAR-4 in lung epithelial cells (Seminario-Vidal, 2009). These results suggest that PAR-3 can signal autonomously via a still unknown signalling mechanism.

It has been demonstrated that PAR-4 activation generates $\alpha_q$-related calcium influx (Xu, 1998; Camerer, 2002) and MAPK signalling (Bretschneider, 2001).
1.2.1.b. Signalling via Gβγ

Gβγ subunit plays a role in PAR-1 signalling notably by activation of phosphatidylinositol (PI) 3-kinase linking PAR-1 to cytoskeletal structure, cell motility, survival, and mitogenesis (Figure I.2). In endothelial cells PAR-1 activation generates two signals, the first one through Gαq and PKC-δ and the second via the Gβγ dimer and PI 3-kinase. Both signals converge at Akt to induce NF-κB activation and ICAM-1 expression (Rahman, 2002) resulting in endothelial adhesivity. Proliferation of rat astrocytes is similarly mediated by two signalling pathways generated by PAR-1 activation: a Ras-dependent pathway mediated by Gβγ that involves ERK1/2 phosphorylation and, in addition, a Ras-independent pathway regulated by PKC (Wang, 2002). Production of transforming growth factor-β3 that controls proliferation and differentiation of cells occurs after incubation of cultured astrocytes with plasmin which, via PAR-1 induced PI3K–Akt signalling pathway (Maeda, 2009). In cerebral ischemia, thrombin-activated PAR-1 stimulates the JNKs pathway in CA1 neurones of rat hippocampal slices resulting in neuronal death (Price, 2009).

1.2.1.c. Signalling via Gα40

PARs can also signal through Gαi (Figure I.2). Klages (1999) reported that thrombin-induced decrease in cAMP production was not affected in Gαq knockout mice suggesting that PAR-1 could also signal via Gαi. Indeed Hung (1992) demonstrated that in fibroblasts PAR-1 activation inhibits adenylyl cyclase and suppresses formation of cAMP via activation of Gαi. In addition, thrombin-stimulated arachidonic acid release is
suppressed in CHO cells transfected with a mutant of Ga\textsubscript{2} protein, suggesting that G a\textsubscript{2} couples PAR-1 to cytoplasmic phospholipase A\textsubscript{2} (Winicz, 1994).

PAR-1 couples also to Go\textsubscript{6} protein in endothelial cells and regulates calcium signalling and cytoskeletal rearrangements (Vanhauwe, 2002).

1.2.1.d. Signalling via Ga\textsubscript{12/13}

PAR-1 and PAR4 are expressed by human platelets in which phosphorylation of Go\textsubscript{q11} and Ga\textsubscript{12/13} occurs following receptors activation by thrombin. Both receptors might be involved to a different degree as their activation by agonists resulted in different kinetics of intracellular calcium increase in platelets (Covic, 2000).

Cell shape and migration are controlled by PAR-1 via coupling to Ga\textsubscript{12/13} (Figure I.2) which interact with Rho guanine-nucleotide exchange factors known to regulate cell morphology and migration (Klages, 1999).

Upon stimulation, modification of the platelet shape is likely to be mediated by PAR-1 as Rasmussen (1993) observed this phenomenon after PAR-1 activation by agonist. Dorsam (2002) demonstrated that the above effect might be mediated through Ga\textsubscript{12/13} as a Rho kinase inhibitor blocked PAR-1-induced platelet shape-change. The same author also reported that a simultaneous activation of Ga\textsubscript{12/13} together with Ga\textsubscript{i} is necessary for platelet aggregation. Huang (2007) further corroborated the above result by showing, that in human platelets, a low dose of thrombin activates PAR-1 coupled to
Ga$_{13}$. This in turn stimulates a Rho kinase-independent downstream mechanism, promoting calcium mobilization and platelet aggregation.

PAR-1 has also been shown to influence the actin skeleton. In human umbilical vein endothelial cells, PAR-1 activators and Rho-GTPase-coupled mechanisms induce stress fiber formation, accumulation of cortical actin, and cell rounding (Vouret-Craviari, 1998). The brain isoform of creatine kinase involved in ATP homeostasis regulation has been shown to interact \textit{in vivo} and \textit{in vitro} with the carboxy-tail of PAR-1 in order to activate Ga$_{12/13}$ and the RhoA pathway with subsequent actin rearrangement and shape changes in astrocytes and retinoblasts (Mahajan, 2000). The chaperone molecule Hsp90 interacts \textit{in vivo} and \textit{in vitro} with PAR-1-Ct and mediate astrocytic morphology changes (Pai, 2001).

In sepsis high levels of thrombin are released in systemic blood circulation. PAR-1 is activated and promotes vascular damage via Ga$_{12/13}$ signalling and subsequent Rho-dependent cell contraction (Klarenbach, 2003). At later stage, PAR-1 signalling switches through PAR-2 transactivation and activation of Ga$_{7}$-Rac protective pathway (Kaneider, 2007).

I.2.2. G protein independent signalling

G proteins are not always involved in PAR-1 signalling. It has been recently found that cytoskeleton remodelling by thrombin-activated PAR-1 (Han, 2009) in endothelial cells involves a phosphoprotein zyxin implicated in the regulation of signalling and
cytoskeleton dynamics. Zyxin binds to PAR-1-Ct independently of G protein, leading to actin stress fiber formation and gene transcription (Han, 2009).

I.3. Objectives of the project

Extracellular proteolysis has been recently recognized as an important mechanism regulating numerous aspects of brain physiology. Serine proteases and their target molecules facilitate activity-dependent plasticity in neurons, both at morphological and functional levels. One important target for extracellular proteases is PAR-1. This work sought to elucidate PAR-1 role in stress-induced neuronal plasticity and behavioural responses. First localization and expression of the PAR-1 gene and protein in the mouse brain were investigated. Next the regulation of PAR-1 by stimuli producing either innate (restraint stress) or learned fear (Pavlovian conditioning) was examined. Then interaction of PAR-1 with the protease neuropsin was examined and the role of both proteins in animal behaviour was assessed. Finally, PAR-1-related signalling pathways during anxiety and fear were investigated, including the effect on AMPA receptors.
Chapter II

Material and Methods
II.1. Genotyping

Mice (C57/BL6J background) were housed three to five per cage in a colony room with a 12 hours light/dark cycle (lights on at 7 AM) with *ad libitum* access to commercial chow and tap water. Breeders as well as the first litter from each breeding pair were systematically genotyped. DNA was extracted from wild-type, PAR-1\(^{+/+}\) or neuropsin \(^{+/+}\) mice ear samples with the GenElute™ Mammalian Genomic DNA Miniprep kit (Sigma). Primers are described in Table II.1. For each reaction, 12 μL of MangoMix™ (Bioline) was mixed with 1 μL cDNA, 0.5 μL of each 1 μM primers and 10 μL of water. PCR was performed using Eppendorf Mastercycler. PCR conditions are described in Table II.2. PCR products were loaded on 1.5% agarose gel together with GeneRuler™ 1 kb Plus ladder (Fermentas) to assess correct band sizes (Table II.1; Figure II.1).

Table II.1. PCR primers for genotyping

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Origin</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAR-1(^{+/+}) for</td>
<td>CAGAGAGGACAGATGCTACGGT</td>
<td>Dr Coughlin</td>
<td>280</td>
</tr>
<tr>
<td>PAR-1(^{+/+}) rev</td>
<td>CCTTCATCCTCAAGACGAACA</td>
<td>Dr Coughlin</td>
<td></td>
</tr>
<tr>
<td>PAR-1(^{+/+}) for</td>
<td>GATTTGTGTTTCATTGTCAGCCTTC</td>
<td>Dr Connolly</td>
<td>545</td>
</tr>
<tr>
<td>PAR-1(^{+/+}) rev</td>
<td>ACGTGTCAGACAGCGTGGAAC</td>
<td>Dr Connolly</td>
<td></td>
</tr>
<tr>
<td>Neuropsin(^{+/+}) for</td>
<td>CAGAGAGGACAGATGCTACGGT</td>
<td>Pr Shiosaka</td>
<td></td>
</tr>
<tr>
<td>Neuropsin(^{+/+}) rev</td>
<td>CCTTCATCCTCAAGACGAACA</td>
<td>Pr Shiosaka</td>
<td>360</td>
</tr>
<tr>
<td>Neuropsin(^{+/+}) rev</td>
<td>GATTTGTGTTTCATTGTCAGCCTTC</td>
<td>Pr Shiosaka</td>
<td>1021</td>
</tr>
</tbody>
</table>
Figure II.1. Genotyping of PAR-1<sup>−/−</sup> and neuropsin<sup>−/−</sup> mice. Ear samples were taken and DNA extracted. PCR was performed with specific primers in order to differentiate PAR-1<sup>−/−</sup> mice from WT (A) and neuropsin<sup>−/−</sup> mice from WT (B).
Table II.2. PCR conditions used for genotyping

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Initial denaturation</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Amplification</th>
<th>Final amplification</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAR-1</td>
<td>95°C, 5 min</td>
<td>94°C, 45 s</td>
<td>59°C, 1 min</td>
<td>72°C, 45 s</td>
<td>72°C, 10 min</td>
<td>30</td>
</tr>
<tr>
<td>Neuropsin</td>
<td>95°C, 5 min</td>
<td>96°C, 30 s</td>
<td>55°C, 30 s</td>
<td>72°C, 1 min</td>
<td>72°C, 3 min</td>
<td>35</td>
</tr>
</tbody>
</table>

II.2. Quantitative reverse transcription- polymerase chain reaction

II.2.1. Sample collection

Experiments were performed on seven to nine weeks old male wild-type C57/BL6 J, PAR-1\(^{+/-}\) and neuropsin\(^{+/-}\) mice.

Prior to RNA extraction, equipment and bench are cleaned with RNAse Erase\(^\circledast\) (MP Biomedicals). Mice were anaesthetized with sodium pentobarbital (50mg/kg) and perfused transcardially (ice-cold PBS). Brain was removed and a coronal slice -0.58 to -2.3mm relative to Bregma was cut using a brain matrix (Stoelting); the amygdalae were dissected in ice-cold PBS and stored in “RNA later” buffer (Qiagen). When the basolateral complex of the amygdala was dissected, the protocol was modified in order to limit tissue degradation. Brain was removed, glued onto a ice-cooled brain holder and immediately immerged in iced-cold Ringer’s solution composed of (in mM): 25 glucose; 115 NaCl; 1.2 NaH2PO4; 3.3 KCl; 2 CaCl2; 1 MgSO4; 25.5 NaHCO3 (Matys, 2004). The solution was equilibrated with 95% O2-5% CO2 for 15 minutes before use. A coronal section (-0.58 to -2.3mm relative to Bregma) was cut using a vibratome and basolateral amygdalae were dissected under the microscope, collected in “RNA later” and stored at 4 degrees.

Total RNA was extracted from tissue samples according to the manufacturer’s instructions (RNeasy Lipid tissue mini kit, Qiagen). The RNA was treated with RNase-
Free DNase (Qiagen) to remove any genomic DNA and was quantified using a Nanodrop*1000 spectrophotometer (Labtech).

For each sample 2 µg of RNA were converted to cDNA according to Invitrogen recommendations for the use of SuperScript™III first-strand synthesis system. DNA-containing tubes were then stocked at -20°C until used.

II.2.2. qPCR

II.2.2.a. Primer design

Primers for PAR-1 and stargazing (Table II.3) were designed using Primer3 software. The amplified DNA region was designed to span one or more introns to avoid the presence of genomic DNA product. The following parameters were taken into account for the design: the primer length between 18 and 24 nucleotides, the melting temperature between 55 and 60 degrees, the GC content between 45 and 58%, a maximum self complementarity of 4 and a GC clamp of 1. Potential to form self-complementarity or cross-complementarity to other primer, or hairpins was verified with Operon software. The product size should ideally be between 65 and 250 bp when working with SYBR Green (manufacturer’s recommendation). A BLAST search against mouse genome with NCBI Blast was performed to insure that the primers recognize only the cDNA of interest. Primers were bought from Invitrogen.
Table II.3. Primers for qRT-PCR. Splice variant-specific AMPAR subunits primers were described by Namekata (2006). β-actin primers were described by Salter (2005).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Sequence accession number</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAR-1 for</td>
<td>TGAGCCAGCCAGAATCAGAGA</td>
<td>NM 010169.3</td>
<td>144</td>
</tr>
<tr>
<td>PAR-1 rev</td>
<td>CCTCCAGCAGGAGCTTTC</td>
<td>NM 010169.3</td>
<td></td>
</tr>
<tr>
<td>Stargazin for</td>
<td>AGACACGCCACAACATCATC</td>
<td>NM 007583.2</td>
<td>161</td>
</tr>
<tr>
<td>Stargazin rev</td>
<td>TAGAAGGACCAGCGTACAGGA</td>
<td>NM 007583.2</td>
<td></td>
</tr>
<tr>
<td>GluR1 for</td>
<td>ACACCATGAAATGGGAGGTAACCT</td>
<td>X 57497</td>
<td>200</td>
</tr>
<tr>
<td>GluR1 flip rev</td>
<td>ACTGGTCCTTGTCTTTACTCCGGA</td>
<td>X 57497</td>
<td></td>
</tr>
<tr>
<td>GluR1 flop rev</td>
<td>ACTGGTCCTTGTCTTTACTCCGGA</td>
<td>BC 056397</td>
<td></td>
</tr>
<tr>
<td>GluR2 for</td>
<td>ACACCATGAAATGGGCGGCAACC</td>
<td>AB 111957</td>
<td>200</td>
</tr>
<tr>
<td>GluR2 flip rev</td>
<td>ACTGGTCCTTGTCTTTACTCCGGA</td>
<td>AB 111957</td>
<td></td>
</tr>
<tr>
<td>GluR2 flop rev</td>
<td>ACTGGTCCTTGTCTTTACTCCGGA</td>
<td>AB 03540</td>
<td></td>
</tr>
<tr>
<td>GluR3 for</td>
<td>ATACGATGAAAGTGGGACCTATC</td>
<td>NM 016886</td>
<td>200</td>
</tr>
<tr>
<td>GluR3 flip rev</td>
<td>ACTGGTCCTGTCCTTTACTCCGGA</td>
<td>NM 016886</td>
<td></td>
</tr>
<tr>
<td>GluR3 flop rev</td>
<td>ACTGGTCCTGTCCTTTACTCCGGA</td>
<td>AB 102775</td>
<td></td>
</tr>
<tr>
<td>GluR4 for</td>
<td>ACACGATGAAATGGGAGGACCTATC</td>
<td>NM 019691</td>
<td>200</td>
</tr>
<tr>
<td>GluR4 flip rev</td>
<td>ACTCGTCTCTGTCTTTACTCCGGA</td>
<td>NM 019691</td>
<td></td>
</tr>
<tr>
<td>GluR4 flop rev</td>
<td>ACTCGTCTCTGTCTTTACTCCGGA</td>
<td>AB 022913</td>
<td></td>
</tr>
<tr>
<td>β-actin for*</td>
<td>TGCTCCTCCTGAGCGCAAGTACTCTC</td>
<td>NM 007393</td>
<td>107</td>
</tr>
<tr>
<td>β-actin rev*</td>
<td>CGGACTCATCGTACTCTGCTTGC</td>
<td>NM 007393</td>
<td></td>
</tr>
</tbody>
</table>

During the polymerase chain reaction the template DNA should double with each cycle when assuming that the efficiency of the reaction is 100%. In order to accurately
compare the expression of different genes in the same sample the reaction efficiency must be calculated for each primer pair. Hence the standard curve of the logarithm of the template concentration was plotted against the number of cycles necessary to reach the fluorescence threshold (Ct). The percentage efficiency of each reaction (Table II.4) was calculated using the equation:

\[
\% \text{ efficiency} = 100 \times (10^{-\left(\frac{1}{\text{slope}}\right)} - 1)
\]

where “\(10^{-\left(\frac{1}{\text{slope}}\right)}\)” is the efficiency coefficient and slope describes the slope of the standard curve plot. For a reliable measurement of gene expression a reaction efficiency of 100\%±10\% was required. Efficiency of AMPA receptor variant-specific qRT-PCR reactions were also assessed as it was not reported by the authors (Namekata, 2006; Table II.4).

**II.2.2.b. qPCR**

The PCR was performed using Chromo4/PTC-200 thermal cycler (MJ Research). For quantitative RT-PCR, SYBR Green Master Mix was used (Applied Biosystems). Triplicate wells contained 20 \(\mu\)L of SYBR Green Master Mix, 2 \(\mu\)L of cDNA, 10 \(\mu\)M of primers and water to a volume of 40 \(\mu\)L per reaction. Also, control reactions were run without DNA template or with unconverted RNA to check for possible contamination from the reagents or the mRNA.

PCR conditions for each reaction are described in Table II.5.
### Table II.4. Primer percentage efficiency

<table>
<thead>
<tr>
<th>Primers</th>
<th>% efficiency</th>
<th>Primers</th>
<th>% efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAR-1</td>
<td>104.30</td>
<td>GluR2 flop</td>
<td>97.60</td>
</tr>
<tr>
<td>Stargazin</td>
<td>102.57</td>
<td>GluR3 flip</td>
<td>95.67</td>
</tr>
<tr>
<td>GluR1 flip</td>
<td>108.68</td>
<td>GluR3 flop</td>
<td>95.41</td>
</tr>
<tr>
<td>GluR1 flop</td>
<td>94.51</td>
<td>GluR4 flip</td>
<td>100.50</td>
</tr>
<tr>
<td>GluR2 flop</td>
<td>101.20</td>
<td>GluR4 flop</td>
<td>90.50</td>
</tr>
</tbody>
</table>

### Table II.5. qRT-PCR conditions used for PAR-1, Stargazin and AMPAR subunits

<table>
<thead>
<tr>
<th>Target</th>
<th>Initial denaturation</th>
<th>40 cycles</th>
<th>Final amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>95°C, 15 min</td>
<td>Denaturation</td>
<td>Annealing</td>
</tr>
<tr>
<td>PAR-1</td>
<td>94°C, 15 s</td>
<td>55°C, 30 s</td>
<td>72°C, 30 s</td>
</tr>
<tr>
<td>Stargazin</td>
<td>94°C, 15 s</td>
<td>47°C, 30 s</td>
<td>72°C, 30 s</td>
</tr>
<tr>
<td>AMPAR</td>
<td>94°C, 15 s</td>
<td>55°C, 30 s</td>
<td>72°C, 30 s</td>
</tr>
</tbody>
</table>
II.2.2.c. qPCR specificity

As SYBR green binds to any double-stranded DNA including primer-dimers, and dissociate from it as the temperature reaches the melting point, resulting in a rapid drop of fluorescence (melting curve, Figure II.2). The melting point curve is obtained by plotting the negative first derivative of fluorescence against temperature. As a melting point is unique for each product, the presence of a single peak (Figure II.2) confirms the absence of primer-dimer artefact or other contamination. Melting curves were analysed after each qRT-PCR and only single peaks were produced. Also the PCR products were systematically run on a 1% agarose gel; the presence of a unique DNA band (sizes described in Table II.3) confirmed that the reactions were not contaminated.

II.2.2.d. Quantification

To calculate the relative expression of our gene of interest, qRT-PCR was performed in triplicates using gene-specific and actin-specific primers. The number of cycles after which the fluorescence intensity has reached Ct was measured. The Ct was set at the exponential phase of the reaction as illustrated on the amplification curve (Figure II.3). To compare the expression of different genes in the same sample or the same gene in different conditions or tissues, the gene expression was calculated using the $\Delta\Delta Ct$ method:

$$\text{Gene expression} = \text{efficiency coefficient} ^ {-\Delta Ct}$$

with $\Delta Ct = \text{average } Ct_{\text{gene of interest}} - \text{average } Ct_{\text{reference gene \beta-actin}}$
Figure II.2. Representative melting curves and melting points for PAR-1 qRT-PCR.
PAR-1 gene expression was quantified by qRT-PCR in thalamus samples (triplicate). When the melting temperature is reached, double-stranded DNA dissociates into single-stranded DNA and the SYBR green dye is released, leading to a drop of the fluorescence as illustrated by the melting curve (arrow in A). The reaction specificity is verified by the presence of only one melting point (in this example at 80°C, B) that confirms that only one DNA product was present in the qRT-PCR.
Figure II.3. Representative amplification curves of PAR-1 cDNA for thalamus and cortex samples (triplicate).

The threshold (dotted line) separates the background from the relevant amplification signals; the threshold cycle is used to calculate the initial DNA quantity in each sample. The more the sample is diluted, the higher its Ct value will be. The difference between cortex and thalamus Ct values is 3.58 times ($C_{t2} - C_{t1}$) meaning that the thalamus cDNA quantity is (PAR-1 primer efficiency $\text{number of cycles} = 2^{0.043.58}$) 12.83 fold higher than the cortical one.
II.3. Western blotting

II.3.1. Tissue dissection

Mice were anaesthetized with pentobarbital and transcardially perfused with ice-cold PBS. Their brains were removed and were placed on a brain matrix (Stoelting) to cut a coronal section at approximately -0.58 to -2.3 mm relative to Bregma. Five regions were dissected from the coronal section (cortex, amygdala, hippocampus, hypothalamus, and thalamus; Figure II.4). Samples from the hypothalamus, medulla and cerebellar cortex were taken from the remaining tissue. All samples were frozen in 2 mL tubes placed in dry ice and then stored at -80°C until further use. To measure fear conditioning-induced changes in PAR-1 levels, amygdala fragments (slice -0.58 to -2.3 mm relative to Bregma) containing the basolateral complex were collected using a vibratome and a dissecting microscope. Samples were homogenized in 0.1 M Tris, 0.1% Triton X-100, pH 7.4, containing phosphatase (10 mM NaF; 1 mM Na orthovanadate) and protease inhibitors (Complete®, Roche). When protein phosphorylation levels were assessed, the brain was removed and immediately immersed in iced-cold Ringer’s solution (described in II.2.1; Matys, 2004). The solution was equilibrated with 95% O2-5% CO2 for 15 minutes before use. This solution was used in order to preserve brain tissue during the dissection of the basolateral complex.
Figure II.4. Dissection of five different regions on a coronal mouse brain section.
Hippocampus is removed by cutting the brain slice with a blade just below the hippocampal area (1) and by detaching gently with tweezers the region of interest (2) from the cortex. The amygdala is separated from the rest of the section by cutting the slice further down through an imaginary line above the division of the external capsule (3), then bilaterally by following the internal capsule (IC, 4) on one side and through the piriform cortex (5) on the other side. Finally amygdala is isolated by removing posterolateral and posteromedial cortical nuclei (PCo, 6). The hypothalamus is separated from the thalamus by cutting the slice through a line passing above the 3rd ventricle (7). BLA: basolateral amygdaloid nucleus; CeA: central amygdaloid nucleus; LA: lateral amygdaloid nucleus; MeA: medial amygdaloid nucleus; IC: internal capsule; PCo: piriform cortex. The brain picture is from Allen Brain Atlas.
II.3.2. Tissue preparation

Individual samples were homogenized with an Ultra Turrax homogenizer (IKA) in 0.1 M Tris, 0.1% Triton X-100, pH 7.4, containing phosphatase and protease inhibitors (described in II.3.1). Protein concentration was adjusted to 2 mg/mL using the Bradford method (Pierce – the method is detailed below). Samples (40 µg per lane) were reduced in loading buffer (Table II.6) and denatured (100°C for 5 minutes) before being subjected to SDS-PAGE electrophoresis (120 V, 400 mA, 1 hr, Mini Protean apparatus, BioRad) in running buffer (Table II.6) and transferred onto nitrocellulose membrane (100 V, 400 mA, 1hr; the transfer buffer is detailed in Table II.6).

Table II.6. Western blotting buffers

<table>
<thead>
<tr>
<th>LOADING Buffer</th>
<th>RUNNING Buffer</th>
<th>TRANSFER Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>62.5 mM Tris-HCl</td>
<td>25 mM Tris Base</td>
<td>25 mM Tris Base</td>
</tr>
<tr>
<td>2% SDS</td>
<td>200 mM glycine</td>
<td>200 mM glycine</td>
</tr>
<tr>
<td>0.1% bromophenol blue</td>
<td>0.1% SDS</td>
<td>20% v/v methanol</td>
</tr>
<tr>
<td>10% glycerol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 mM dithitreol</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
II.3.3. Protein quantification by the Bradford method

A stock solution of 2 mg/mL bovine serum albumin (Sigma) was used to establish the calibration curve and its correlation coefficient was calculated to insure a good linear approximation ($R^2$ should be greater than 0.97). Five and ten times serial dilutions of brain samples were prepared in triplicate in homogenization buffer. Five microliters of each BSA and brain samples were loaded onto a 96-well plate before the addition of the Bradford reagent. Homogenization buffer alone was used as a reagent blank. Absorbance was measured at 595 nm with a spectrophotometer. The equation of the standard curve was used to determine the protein concentration of the brain samples.

II.3.4. Protein level detection

Samples (40 µg per lane) were reduced, denatured (100°C for 5 minutes) and subjected to SDS-PAGE electrophoresis. After transferring onto nitrocellulose membrane and blocking in TBST-milk (TBS, 0.1% Tween 20, 5% skim milk) for 1 hr at RT, the membrane was probed with a rabbit anti-PAR-1 (gift of Dr. M. Runge, 1:1000, 4°C, O/N), followed by a peroxidase labelled anti-rabbit secondary (1:2000, 1 hr at RT, Vector Labs). To normalize the results the membrane was stripped (Restore Western blot stripping buffer, PIERCE) and re-blotted using mouse anti-β-actin antibody (1:1000 in TBST-5% milk, 1 hr, RT, Sigma) followed by an anti-mouse HRP-conjugated secondary (1:1000, 1 hr, RT). Luminescence was detected using Western Blot luminal reagent (Santa Cruz) and Kodak biomaxXAR photographic film. The film was then scanned and
relative band intensities were measured using Scion Image (Scion Corporation). After background removal, the number of pixels/intensity of each band (for the protein of interest and β-actin) was measured by drawing a box around the largest band and dragging it over the remaining ones. The amount of protein of interest was then normalized to the amount of β-actin.

Total brain homogenate were probed with several anti-PAR-1 antibodies under conditions described in Table II.7 in order to determine which antibody had the best specificity. The polyclonal rabbit anti-rat-PAR-1 antibodies (clones 11 and 12) have been raised against the extracellular amino acid sequence SFFLRNPSEC (Wilcox, 1994). A decreased immunoreactivity of PAR-1 detected with this antibody means that the Nt of the protein has been cleaved in the region between Ser 42–Phe 43 and the first intracellular domain, hence removing PAR-1 tethered ligand.

Levels of phosphorylated proteins (P-GluR1, P-GluR2) were normalized to total protein levels. Western blotting conditions and antibodies are detailed in Table II.8.
Table II.7. Antibodies and conditions used to detect PAR-1 protein in Western blotting.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Dilution</th>
<th>Incubation conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAR-1 H111</td>
<td>Santa Cruz (sc-5605)</td>
<td>1/200 -1/1000</td>
<td>ON at 4°C in TBST -5% milk or TBST-1% BSA</td>
</tr>
<tr>
<td>PAR-1 C18</td>
<td>Santa Cruz (sc-8202)</td>
<td>1/200 - 1/1000</td>
<td>ON at 4°C in TBST -5% milk or TBST-1% BSA</td>
</tr>
<tr>
<td>PAR-1 S19</td>
<td>Santa Cruz (sc-8204)</td>
<td>1/200 - 1/1000</td>
<td>ON at 4°C in TBST -5% milk or TBST-1% BSA</td>
</tr>
<tr>
<td>PAR-1-Thrombin receptor</td>
<td>BD Bioscience (611522)</td>
<td>1/50 - 1/250</td>
<td>ON at 4°C in TBST, or TBST -5% milk or TBST-1% BSA</td>
</tr>
<tr>
<td>SPAN 12</td>
<td>ImmunoTech (2086)</td>
<td>1/1000</td>
<td>ON at 4°C in TBST-5% milk</td>
</tr>
<tr>
<td>PAR-1 clone 11</td>
<td>Dr Runge</td>
<td>1/1000</td>
<td>ON at 4°C in TBST-5% milk</td>
</tr>
<tr>
<td>PAR-1 clone 12</td>
<td>Dr Runge</td>
<td>1/1000</td>
<td>ON at 4°C in TBST-5% milk</td>
</tr>
</tbody>
</table>

Table II.8. Antibodies and conditions used to assess P-GluR1 and P-GluR2 protein levels.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Dilution</th>
<th>Incubation conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit anti-P-GluR1</td>
<td>Novus Biologicals</td>
<td>1/500</td>
<td>ON at 4°C in TBST-5% milk</td>
</tr>
<tr>
<td>Rabbit anti-P-GluR2</td>
<td>Millipore</td>
<td>1/500</td>
<td>ON at 4°C in TBST-5% milk</td>
</tr>
<tr>
<td>Mouse anti-total-GluR1</td>
<td>Santa Cruz</td>
<td>1/200</td>
<td>ON at 4°C in TBST-5% milk</td>
</tr>
<tr>
<td>Mouse anti-total-GluR2</td>
<td>Santa Cruz</td>
<td>1/500</td>
<td>ON at 4°C in TBST-5% milk</td>
</tr>
<tr>
<td>anti-Rabbit HRP secondary</td>
<td>Vector Labs</td>
<td>1/1000</td>
<td>1hr at RT</td>
</tr>
<tr>
<td>anti-Mouse HRP secondary</td>
<td>Vector Labs</td>
<td>1/1000</td>
<td>1hr at RT</td>
</tr>
</tbody>
</table>
II.4. Immunohistochemistry

To investigate the expression pattern of PAR-1 and to reveal the phenotype of PAR-1-positive cells, quadruple staining was performed. Mice were anesthetized with pentobarbital, perfused transcardially with ice-cold 4% paraformaldehyde in PBS containing phosphatase inhibitors (1 mM Na3VO4 and 10 mM NaF) and their brains removed. The brains were fixed in 4% paraformaldehyde overnight. 70 μm coronal brain sections were then cut using a vibrating microtome (Campden Instruments Vibroslice HA752) and stored in 4°C in PBS containing sodium azide until analyzed. Free-floating sections were blocked with goat serum in PBS (1:500, 4 hr, RT) and incubated with rabbit anti-PAR-1 (1:500, 4°C, 4 hr, gift of Dr. Marshall Runge), followed by the addition of mouse anti-NeuN (1:200, 4°C, O/N, Chemicon) and chicken anti-GFAP (1:1000, 4°C, O/N, Abcam) to the same wells. After several washes, the sections were incubated with the appropriate secondary antibodies (Table II.9). DAPI (pseudo-colored in grey) was used to visualize cell nuclei in conjunction with PAR-1, NeuN and GFAP labelling with a fluorescent microscope (Leica, magnification x80). Quantification was performed by counting the PAR-1-, NeuN- or GFAP-immunoreactive cells in each region, relative to the total number of cells in the same region.

To examine the level of c-Fos and P-CREB separate sections were incubated with anti-c-Fos (1:200, Cell Signalling) or anti-phospho-CREB antibodies (1:200, Cell Signalling), respectively. Brain slices were then washed before secondary antibody (Table II.9) together with TOTO-3 iodide (1 nM, Invitrogen) were applied. Sections in which the primary antibodies were omitted served as controls. The images were collected using Zeiss LSM510 confocal microscope, at magnification x63. Channel gains were 724 (c-
Fos) and 700 (P-CREB). Sections in which the primary antibodies were omitted served as control. For the quantification, immunopositive cells were manually counted within the basolateral amygdala area.

### Table II.9. Secondary antibodies used for immunohistochemistry.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Color</th>
<th>Source</th>
<th>Dilution</th>
<th>Incubation conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti-Rabbit AlexaFluor 488</td>
<td>Green</td>
<td>Invitrogen</td>
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<td>ON at 4 °C in TBST</td>
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<tr>
<td>Goat anti-Mouse AlexaFluor 546</td>
<td>Red</td>
<td>Invitrogen</td>
<td>1/500</td>
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<tr>
<td>Goat anti-Chicken AlexaFluor 647</td>
<td>Blue</td>
<td>Invitrogen</td>
<td>1/500</td>
<td>ON at 4 °C in TBST</td>
</tr>
<tr>
<td>Goat anti-Rabbit Cy3</td>
<td>Red</td>
<td>Abcam</td>
<td>1/500</td>
<td>ON at 4 °C in TBST</td>
</tr>
</tbody>
</table>

II.5. Proteases cleavage assays

II.5.1. PAR-1 protein levels cleavage by neuropsin and tissue Plasminogen Activator (tPA) in SH-SY5Y cells

SH-S5Y5 cells (gift from Professor Challiss) were grown to 80-90% confluence in 60 mm Petri dishes in MEM Earle’s (Invitrogen), 5% FBS (Sigma), 5% newborn calf serum (Sigma), 2 mM Glutamine, 1% fungizone (Sigma) and 1% penicillin/streptavidin (Sigma). Plates were rinsed twice with PBS heated at 37°C; eight of them were incubated with PBS alone as control and eight others with 500 nM recombinant human neuropsin (R&D Systems) or 1 μg/mL tPA (four plates; Alteplase, Genetech) for 15 min at 37°C.
Proteases action was stopped by cooling the plates on ice and adding 100 μL of PBS containing a protease inhibitor cocktail (Complete®, Roche) to each dish. Cells were harvested using cell scrapers and transferred to ice-cold tubes for centrifugation (3000 rpm for 5 min at 4°C). 200 μL of lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, 0.5 % NP40, 1 mM NaOV and 10 mM NaF) was mixed to the supernatant with a 25G syringe and tubes were left on ice for 30 min, with 3 more syringe-mixing sessions. After centrifugation (12000 rpm, 15 min, 4°C) and 30 μL of supernatant was used to quantify PAR-1 protein levels by Western blot as described previously.

II.5.2. PAR-1-HA cleavage by neuropsin: in vitro assay

The first step of this experiment was to create a pPAR-1-HA-IRES2-EGFP construct in which PAR-1 protein is tagged with HA. The HA-tag (YPYDVPDYA) is derived from the human influenza hemagglutinin and when fused to a protein facilitates its affinity purification. With this construct production of PAR-1-HA is concomitant to that of enhanced Green Fluorescent Protein (eGFP) thanks to the internal ribosome entry site (IRES2) and expression of eGFP eases transfection efficiency assessment with a fluorescent microscope.

II.5.2.a. Cloning

PCR was performed in order to add the HA tag to PAR-1 (Figure II.5).
cDNA primers are described in Table II.10. 500 ng of PAR-1 cDNA were added to 10 μL of 10x cloned Pfu DNA polymerase reaction buffer (Stratagene), 2 μL of dNTPs (100 mM), 20 pmol/μL of primers, 2 μM Pfu turbo (Stratagene- gift from Dr Tobin) and water up to 100 μL. Cycling conditions are described in Table II.11.

Table II.10. Primers for PAR-1-HA cloning. In the reverse primer, PAR-1 sequence is in italic, the HA sequence is underlined and the stop codon is in bold.

<table>
<thead>
<tr>
<th>Primer</th>
<th>5'→3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>for</td>
<td>GACTGAATTCAGAAGTGGCGACGCG</td>
</tr>
<tr>
<td>rev</td>
<td>GATCGGATCCACTAGCGTAATCTGGAACATCGTATGGTAAGCTAATAGCTTTTTGTATG</td>
</tr>
</tbody>
</table>

Table II.11. PCR conditions for PAR-1-HA cloning

<table>
<thead>
<tr>
<th>Target</th>
<th>Initial denaturation</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Amplification</th>
<th>Final amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAR-1</td>
<td>98°C, 2 min</td>
<td>98°C, 30 s</td>
<td>55°C, 30 s</td>
<td>72°C, 90 s</td>
<td>72°C, 5 min</td>
</tr>
</tbody>
</table>
Figure II.5. Cloning of PAR-1-HA into pIRES2-EGFP.

pPAR-1-HA-IRES2-EGFP construct (6.67 kb) is represented in A with the enzymes restriction sites of interest. To obtain this construct, pIRES2-EGF vector was linearized using EcoRI + BamHI (B, lane 1) and PAR-1 cDNA was amplified by PCR using a reverse primer containing the HA Tag sequence (lane 2). After purification, ligation was performed. Correct insertion of PAR-1-HA into pIRES2-EGFP was verified by digestion of the construct with appropriate enzymes (C).
PCR product was digested with EcoRI and BamHI for 1 hr, 37°C. The same enzyme mix was used in parallel to digest pIRES2-EGFP vector. Both digestion products were loaded onto a gel and bands corresponding to PAR-1-HA (1380 bp) and cleaved vector (5.3 kb) were extracted using QIAquick Gel Extraction kit (Qiagen). Migration of 2 μL of each DNA was performed onto an agarose gel to evaluate PAR-1-HA cDNA and vector amounts. Ligation was performed by mixing the products according to a molar ratio of 2:1 (insert:vector) in 3 μL buffer, 1 μL Taq DNA Ligase (New England Biolabs) and 9 μL water, for 30 min at 16°C. Bacteria (XL-10 Gold Ultracompetent Cells, Stratagene) were transformed with the ligation product (pPAR-1-HA-IRES2-EGFP) following manufacturer’s instructions, spread on neomycin (25 μg/mL) agarose plates and grew overnight. The next day random clones were picked up and grown in 5 mL neomycin LB medium at 37°C overnight with vigorous shaking. DNA was extracted using QIAGEN Plasmid Mini kit and 1 μL was tested for the presence and the direction of the PAR-1-HA insert by restriction analysis using either EcoRI and BamHI, or SacI. (Enzymes and buffers are from New England Biolabs; Figure II.5). Two clones were then selected and midiprep was performed (QIAGEN Plasmid Midi kit) to produce large amount of DNA. Previous digestions were done again and sequencing (primers showed in Table II.12) confirmed the presence of HA Tag in the N-terminal of PAR-1 and that PAR-1-HA had been amplified correctly. Sequencing is a service provided by the Protein Nucleic Acid Chemistry Laboratory (PNACL) of the University of Leicester.
Table II.12. Primers for PAR-1-HA sequencing.

<table>
<thead>
<tr>
<th>Primer</th>
<th>5'→3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>for</td>
<td>GGGACTCAACATCACCACC</td>
</tr>
<tr>
<td>rev</td>
<td>ATAGGGACGCGGGAAGAC</td>
</tr>
<tr>
<td>rev</td>
<td>GACAGGGACTGGATCGGATA</td>
</tr>
<tr>
<td>rev</td>
<td>GCAGACGATGAAGATGCAGA</td>
</tr>
</tbody>
</table>

II.5.2.b. Production of PAR-1-HA protein

HEK cells were chosen for their high transfection efficiency (Thomas, 2005). HEK cells (gift from Professor Hartell) were grown in DMEM without pyruvate and supplemented with L-Glutamine (Invitrogen), 10% horse serum (Sigma) and 1% penicillin/streptavidin (Sigma). 15 flasks of HEK cells were transfected with pPAR-1-HA-IRES2-EGFP vector using lipofectamine 2000 (Invitrogen). The day after, 5 flasks of non-transfected cells and those expressing eGFP together with PAR-1-HA were rinsed twice with PBS (warmed up in a 37°C water bath) and harvested in 1 mL/dish of PBS containing proteases inhibitors (Roche). They were centrifuged at 3000 rpm for 5 min at RT. The pellets were homogenised in 1.4 mL M-PER Mammalian Protein Extraction reagent (Pierce) containing 4 μL of DNase I. The mixture was left on ice for 30 min, with regular pipetting with a syringe bearing a 25 gauge needle in order to facilitate cellular and DNA breakdown. The supernatant was collected after centrifugation at 14000 rpm for 30 min at 4°C and was kept at -80°C until use. PAR-1-HA from control and transfected cells, as well as 50 μl of HA-tagged positive control lysate (GST-HA) from the kit were immunoprecipitated using ProFound Mammalian HA Tag IP/Co-IP kit
(Pierce) following manufacturer’s instructions. The last step consisted in eluting 30 µL of PAR-1-HA in Gentle Elution Buffer pH 6.6 (Pierce) followed by a 30 min dialysis at RT in 500 mL of 0.2 mM Tris-HCl, pH 8 using Slide-A-Lyzer MINI Dialysis Units (Pierce) so that PAR-1-HA protein is in a pH that allows further proteolytic cleavage.

II.5.2.c. Neuropsin activation and in vitro cleavage assay

Activation of neuropsin was performed as described by Shimizu (1998). 2.1 AU of Lysyl endopeptidase were bound to 600 µL of CNBr-activated Sepharose 4B according to manufacturer’s instructions (GE Healthcare). 5 µg of neuropsin were incubated with 200 µL beads that is 0.7 AU of lysyl endopeptidase in 100 µL of 50 mM Tris HCl pH8, for 30 min at 37°C. The same amount of protease was incubated alone in buffer as a control. Supernatants were collected after centrifugation.

PAR-1-HA and control were incubated with supernatants containing activated neuropsin or control, for 1h at 37°C. Western blot for PAR-1 was performed as described previously (50 µL of product per lane). After the membrane was stripped, washed with PBS and blotted with an anti-neuropsin antibody (11pAb, gift from Pr Shiosaka). Another membrane was blot with anti-HA High Affinity antibody (1:2000 in TBST-1% BSA, Roche), and after washing, blotted with anti-rat-HRP antibody (1:1000, Abcam) to reveal the presence of the HA tag.
II.6. Co-immunoprecipitation

Wild-type mice brains were homogenized in RIPA buffer (50 mM HEPES, 5 mM EDTA, 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, pH 7.4) with protease inhibitors (Roche) and protein concentration was adjusted to 2 mg/mL as described previously. Samples (400 µl) were pre-cleared with non-specific rat or rabbit IgGs (1 µg, 1hr, 4°C, Sigma) while Protein G-sepharose™ 4 fast flow beads (GE Healthcare) were gently washed 5 times with PBS containing phosphatase inhibitors (Roche) and recovered by centrifugation (2000 rpm, 2 min, 4°C). Then 40 µL of beads were added to each sample and mix gently on a rotator at 4°C for 30 min before being spun down (2000 rpm, 2 min, 4°C) to remove beads, non-specific IgGs and the attached proteins. Some new beads were added to the supernatant and the clearing step was repeated once. Immunoprecipitation was performed overnight at 4°C by adding to the samples 1 µg of rabbit anti-PAR-1 (gift of Dr. Marshall Runge) or rat anti-neuropsin (mAbF12, MBL International), followed by adsorption to protein G-Sepharose beads (40 µL per sample). In control experiments, the antibodies were replaced with non-specific rabbit or rat IgGs (Sigma). The next day beads were spun down (2000 rpm, 2 min, 4°C), supernatant discarded and pellet was resuspended in 1 mL of PBS containing phosphatase inhibitors and centrifuged (2000 rpm, 2 min, 4°C). The washing step was repeated 4 times on the pellet. The proteins were eluted in a loading electrophoresis buffer containing DTT, separated by SDS-PAGE and transferred onto nitrocellulose membrane. Western blotting was performed using either rabbit anti-PAR-1 antibody (1:1000, 1 hr, RT) or goat anti-neuropsin antibody (anti-KLK8, 1:200, 1 hr, RT, Santa Cruz Biotechnologies), followed by peroxidase labelled anti-rabbit or goat secondary (1:2000, 1hr, RT, Vector Labs).
II.7. Behaviour

Prior to each experiment the animals were left undisturbed for at least a week in their home cage. All behavioural experiments were performed in a quiet dedicated room during the light period of the circadian cycle (7am-7pm) as described (Pawlak, 2003).

Separate groups of animals were used for each experimental time-point in order to expose the animals to a behavioural test only once.

II.7.1. Restraint Stress

Control animals were left undisturbed, and stressed animals were subjected to a single six-hour restraint. The mice were placed in their home cage in wire mesh restrainers secured at the head and tail ends with clips. After restraint, animals were returned to their home cage.

II.7.2. Elevated plus-maze

The test was performed essentially as previously described (Malleret, 1999; Pawlak, 2003). The apparatus was made of four non-transparent white Plexiglas arms: two enclosed arms (50×10×30 cm) that formed a cross shape with the two open arms (50×10 cm) opposite each other. The maze was 55 cm above the floor and each open arm was illuminated with a spotlight. Animals were transferred to the behavioural room and left undisturbed for one hour prior testing to accommodate to the dimly illuminated room.
Behaviour was recorded by an overhead camera. Mice were tested the day after restraint and placed individually on the central platform, facing an open arm, and allowed to explore the apparatus for five minutes. The number of entries in open and closed arms was counted. Total number of entries was used as an indicator of locomotor activity. Anxiety index was calculated using the formula: entries in open arms/total entries. The lower the value, the higher the level of anxiety.

II.7.3. Open field

Wild-type, PAR-1$^+$ or neuropsin$^+$ mice were moved to the behavioural room one hour before the beginning of the experiment to familiarize the animals with the new environment (light and odour mainly). Then, the mice were placed in a 50x50x50 cm Plexiglas box and were left free to move during ten minutes. The box was cleaned with 70% alcohol after each session to avoid any odorant cues. A camera (Quickcam Sphere, Logitech) placed above the box recorded the session. Analysis with the ANY-MAZE software allowed us to quantify several parameters (time spent in the centre, distance travelled..) in order to evaluate anxiety-like behaviour.

II.7.4. Novel object recognition

The day after the open field experiment (which served as a habituation session), mice were moved back into the behavioural room for a 1 hour habituation period. Wild-type, PAR-1$^+$ or neuropsin$^+$ mice were placed in a 50x50x50 cm Plexiglas box (used the day
before for open field). They were presented two objects and left free to explore them for five minutes. Exploring the object was scored when the animal head was facing the object within three cm away from the object. The box and the objects were cleaned with 70% alcohol after each session to avoid any odorant cues. At the end of the session mice were put back in their home cage for three hours. One of the objects was replaced with an unfamiliar object and mice were retested (short-term retrieval). Another retention test was done 24 hours later (long-term memory). Time spent exploring both objects was recorded. The position of the two objects was counterbalanced and randomly permuted for both genotypes.

II.7.5. Fear Conditioning

II.7.5.a. Classic Pavlovian protocol

The mice were individually placed in the conditioning chamber for two minutes before they received three conditioned stimulus-unconditioned stimulus (CS-US) pairings (Figure II.6, A). The last two seconds of the tone (CS, 30 sec, 2.8 kHz, 85 dB) were paired
Figure II.6. Fear conditioning protocol.
During the training session (A), the mouse is placed in the conditioning chamber for 2 minutes before it received three pairings of CS-US (conditioned stimulus-unconditioned stimulus). The tone (2.8 kHz, 85dB) lasted for 30 seconds, the last 2 seconds were paired with the footshock (0.4mA). The mouse remained in the conditioning chamber for 1 more minute and was then moved to its home cage. The next day the mouse was placed back in the chamber used for the training (B) and the freezing time was recorded for 3 minutes to assess the context-dependent learning. Cued-conditioning was evaluated 48 hours after training (C). The mouse was allowed to explore a novel context for 2 minutes, after which the CS was played (2 minutes, 2.8 kHz, 85dB) and the time the mouse was frozen was recorded.
with the footshock (US, 2 sec, 0.4 mA) delivered through a grid floor. After training was completed mice remained in the conditioning chamber for one more minute and were then moved to their home cage. In order to eliminate the presence of a ceiling effect that might be observed in other studies (Almonte, 2007), WT mice were subjected to a strong conditioning protocol comprised of five footshock-tone pairings at 0.5 mA.

The next day the mice were placed back in the training chamber and freezing was monitored for three minutes to assess context-dependent learning (Figure II.6, B). Cued-conditioning was evaluated 48h after training. The mouse was placed in a novel context (chamber with flat plastic floor and walls) for two minutes, after which the CS was delivered (2 min, 2.8 kHz, 85 dB) and freezing was monitored (Figure II.6, C). Data were analysed using FreezeView software.

**II.7.5.b. Unpaired fear conditioning**

During the training the three presentations of the CS and US occur in a non-overlapping manner (Figure II.7) so that the animal does not associate the tone with the footshock. Context and cued retrieval procedures remain similar to those of the classic fear conditioning protocol.
Figure II.7. Unpaired fear conditionning.
During training (A) the tone and the footshock are separated so that the animal does not associate them. In this protocol only the context is associated with the electrical shock. Context (B) and Cued (C) testing remain similar to the classic fear conditioning protocol (Fig II.6.).
II.7.5.c. Pain threshold

The sensitivity of wild type mice, PAR-1$^+$ and neuropsin$^+$ animals to footshock was tested as described previously (Bourtchuladze, 1994). The mice were subjected to a series of mild footshocks of increasing intensities (in 0.05 mA increments) and behavioural reaction was measured. The lowest current intensity to elicit flinching, jumping and vocalization was determined.

II.8. Stereotaxic injections

Mice were intraperitoneally anaesthetized with ketamine/xylazine (100 and 10 mg/kg, respectively) placed in a stereotaxic apparatus and bilaterally implanted with stainless steel guide cannulae (26 gauge; Plastics One, Roanoke, VA; Picture II.1) aimed above the basolateral complex of the amygdala (1.5 mm posterior to Bregma, 3.0 mm lateral and 4.0 mm ventral). The cannulae were secured in place with dental cement. Dummy cannulae (Picture II.1) were inserted into all implanted cannulae to maintain patency. After one week dummy cannulae were replaced with the injection cannulae (33 gauge, projecting 0.75 mm from the tip of the guide cannulae to reach the basolateral complex of the amygdala) and the mice were injected with the PAR-1 antagonist SCH79797 (1 μM, 0.5 μL, NeoMPS) or vehicle [1:4 DMSO in ACSF composed of (in mM) 10 HEPES, 140 NaCl, 2.8 KCl, 1 MgCl2*6H2O, 2 CaCl2*2H2O, pH 7.3 and 10 glucose] followed by fear conditioning. After the experiment a small amount of bromophenol blue was injected to visualize the guide and injection cannulae tracks, the brains were sectioned and the cannulae placement was determined histologically (Picture II.2).
A guide cannula (A, a) sealed by an implanting dummy cannula (A, b) is implanted into the mouse brain. 15 minutes after surgery, the implanting cannula is replaced by a dummy cannula (A, c) that is screwed to the pedestal of the guide cannula. The injection cannula (A, d) connected to an Hamilton syringe (B, a) with PE50 tubing (B, b) is used for drug injection.
Picture II.2. Histological verification of stereotaxic amygdala injections. Bromophenol blue was injected along the guide and injection cannulae, brain dissected and fixed in 4% PFA. Later pictures of 200μm coronal sections were taken. Representative picture of the guide cannula penetrating towards the amygdala (A, white arrow) and the tip of the injection cannula (A, black arrow) inside the basolateral complex. Matching brain atlas plane in B. The positions of the injection cannula tips are shown on consecutive coronal brain atlas planes (C).
II.9. Statistical analysis

Statistical analysis was performed using SPSS18.0 software. Data are presented as mean ± sem. P values are from Student test when comparing two groups, or analysis of variance (ANOVA) and Tukey’s post-hoc test. Differences between conditions were considered significant at p<0.05 (*p<0.05, **p<0.01, ***p<0.001; ns: not significant).
Chapter III

Localization and expression of PAR-1 in the mouse brain
III.1. Introduction

III.1.1. PARs expression in rodent brain

All PARs are widely expressed throughout the rodent brain but their patterns of expression show some species-specificity. In the rat brain, PAR-1 has been detected using RT-PCR, immunohistochemistry (Striggow, 2001), in situ hybridization and northern blot (Weinstein, 1995; Niclou, 1998). At embryonic stages brain PAR-1 mRNA shows a diffuse pattern of expression with the exception of the cortex, striatum and dorsal root ganglia which are more intensely labelled. In adulthood, the faint signal disappears and some neuronal and astrocytic cell populations become strongly PAR-1 positive (Weinstein, 1995; Niclou, 1998). Strong PAR-1 immunoreactivity was present in the pyramidal cell layers of the CA1-4 regions of the hippocampus (Striggow, 2001), and PAR-1 transcript was found in immature neurons of the dentate gyrus and astrocytes of the strata radiate and oriens (Weinstein, 1995; Niclou, 1998). PAR-1 mRNA and protein were seen and in cell bodies and processes of cortical neurons (Weinstein, 1995; Niclou, 1998; Striggow, 2001). Immunolabelling is observed in the central and lateral nuclei of the amygdala, in the thalamus, hypothalamus and striatum (both caudate and putamen; Weinstein, 1995). Neurons of the cerebellum, the grey matter of the spinal cord, the optic tract and principal neuronal layers of the olfactory bulb as well as the ependymal cells of the lateral ventricule in rat brain are also immunoreactive (Weinstein, 1995; Niclou, 1998; Olianas, 2007; Henrich-Noack, 2006). PAR-1-positive neurons display diverse neurotransmitter phenotypes (monoaminergic-, cholinergic-, GABAergic-, glutaminergic cells; Weinstein, 1995).
PAR-2 and PAR-3 proteins are found in neuronal cell bodies of the hippocampal CA1 region, and in all cortical layers, amygdaloid nuclei, thalamus, hypothalamus and striatum (Striggow, 2001). PAR-4 is localized in distinct neuronal somata, axons and dendrites of the hippocampal formation, in all cortical layers, in the dentate area, in the thalamus, hypothalamus and amygdala (Striggow, 2001).

All four receptors are functional in rodent astrocytes (Wang, 2002) and microglia cells (Suo, 2002 & 2003a). Wang et al (2004) showed that intracellular calcium mobilization followed PAR-1 activation in oligodendrocytes.

III.1.2. PARs expression in human brain

In the human brain, PAR-1 mRNA and protein (Junge, 2004; Ishida, 2006) are mainly expressed in astrocytes localized in white and gray matter of the cerebellum, temporal lobe, frontal lobe, and striatum. They are also detected at moderate levels in cell bodies, dendritic and axonal processes of hippocampal, cortical and striatal neuron. Diffuse PAR-1 staining is seen in the neuropil where synapses stand (Junge, 2004). Another group, using a different antibody, has found PAR-1 immunoreactivity in astrocytes but neither in neurons, oligodendrocytes nor microglia (Ishida, 2006).

As for other PARs, PAR-2 and 3 are present in cultures primary human astrocytes (Luo, 2007). PAR-2 has been described in human brain neurons (D’Andrea, 1998) but PAR-4 has not been found in the human brain (Ishida, 2006).
III.2. Results

III.2.1. PAR-1 gene expression in the mouse brain

The expression of the PAR-1 gene was examined in various brain regions, using quantitative reverse-transcript PCR (qRT-PCR). It revealed that PAR-1 mRNA was expressed in all seven brain regions examined (amygdala, cerebellum, hippocampus, hypothalamus, thalamus, cortex and medulla). However, there were profound regional differences in this respect, with the amygdala-hippocampal and the thalamic mRNA levels 1.7-, 2.8 and 13-fold higher (respectively) than the cortical expression (Figure III.1). This result suggests that the PAR-1 gene is regulated in the mouse central nervous system.

III.2.2. PAR-1 protein expression levels in the mouse brain

To know whether PAR-1 protein levels followed the pattern of its gene expression, Western blot was performed. Commercially available anti-PAR-1 antibodies (from Santa Cruz, BD Biosciences and Immunotech) showed poor PAR-1 specificity (Figure III.2, A). However rabbit anti-PAR-1 antibody (Wilcox, 1994; kindly provided by Dr Marshall Runge) allowed specific detection of the PAR-1 protein using Western blotting (Figure III.2, B). Western blotting was performed using extracts from the above mentioned seven brain regions and normalized to the hypothalamus level. The highest levels of PAR-1 protein were found in the amygdala (381 ± 67.58%) and cortex (351 ± 13.61%), while the thalamic and medulla expression were moderate (244 ± 22.66% and 252 ± 37.42%; Figure III.3).
Figure III.1. Quantification of PAR-1 gene expression in different brain regions by qRT-PCR.
Indicated brain regions were dissected, homogenized, RNA extracted and qRT-PCR was performed with a set of PAR-1-specific primers. The highest PAR-1 gene expression was observed in the thalamus, and the lowest in the cortex (A). To confirm the specificity of the amplification the qRT-PCR products were electrophoresed on the agarose gel (B). A single band of 100 bp corresponding to the predicted size of the qRT-PCR product was observed. Results are showed as mean ±SEM, n=4 animals.
AMY: amygdala; CER: cerebellum; HIP: hippocampus; HYP: hypothalamus; THA: thalamus; COR: cortex; MED: medulla.
Figure III.2. PAR-1 antibody specificity.
Fifty micrograms of brain homogenate from WT mouse were loaded onto a gel for Western blot; PAR-1 was not detected when membranes were incubated with commercially available anti-PAR-1 antibodies (Santa Cruz H111, C18 and S19, 1:1000; BD Bioscience, 1:250; Immunotech SPAN 12, 1:1000) followed by the appropriate secondary antibody (A). Conversely anti-PAR-1 antibody clones number 11 and 12 (1:1000; gift of Dr M. Runge) allowed the detection of a 50 kDa band with brain homogenate from a WT but not from a PAR-1<sup>−/−</sup> mouse (B).
PAR-1 protein expression in the hippocampus, cerebellum and hypothalamus were the lowest (172 ± 68.42%; 162 ± 25.99%; 100 ± 10.74%; Figure III.3).

### III.2.3. PAR-1 localization in the mouse brain

To better understand the role of PAR-1 in the central nervous system the pattern of its expression was examined in the mouse brain as it has not been previously described in this species. Immunohistochemistry confirmed the presence of PAR-1 positive cells in most regions of a mouse brain, including limbic areas such as the hippocampus and the amygdala (Figures III.4 & 5). To determine the phenotype of PAR-1-positive cells, triple immunohistochemistry was performed together with PAR-1 antibody using astrocyte-specific (anti-GFAP), or neuron-specific (anti-NeuN) antibodies. The majority of PAR-1-positive cells co-localized with NeuN, indicative of their neuronal phenotype (Figures III.4 & 5). The phenotypic profile of PAR-1 positive cells was quantified in the amygdala, hippocampus and cortex. Depending on the region examined 92 to 99% of PAR-1-positive cells were neurons (92.85 ± 3.6% in the amygdala, 98.75 ± 1.3% in CA1 and 97.85 ± 1.1% in the cortex; Figure III.6). PAR-1 expression was most prominent in cell bodies and neuronal processes. Our immunohistochemistry revealed weaker expression of PAR-1 in astrocytes (Figure III.6 & 7) with less that 2% of this cell population being PAR-1-positive in the amygdala, CA1 and cortex. Because PAR-1 was found predominantly in neurones, PAR-1 expression in any particular neuronal sub-population was investigated. To this end the percentage of PAR-1-positive neurons was calculated. While 97.73 ± 2.3% and 99.6 ±
0.4% of neurons were PAR-1 positive in the hippocampus and cortex respectively, this proportion amounted to only 80.4 ± 8.3% in the amygdala (Figure III.7).
Figure III.3. Quantification of PAR-1 protein expression in different brain regions by Western blotting.

Brain regions were dissected, homogenized and after electrophoresis, the proteins were transferred onto a nitrocellulose membrane. After blocking, the membranes were probed with a primary antibody against PAR-1 (1:1000). To normalize the results, the membranes were stripped and re-blotted for actin (1:2000), followed by an appropriate HRP-conjugated secondary antibody (1:2000). The Western blot revealed the highest expression of PAR-1 protein in the amygdala, and the lowest in the hypothalamus. AMY: amygdala; CER: cerebellum; HIP: hippocampus; HYP: hypothalamus; THA: thalamus; COR: cortex; MED: medulla.
Figure III.4. The expression of PAR-1 in the CA1 region of the hippocampus. Laminin immunohistochemistry (A, courtesy of Dr Pawlak) or DAPI staining (B) was performed to highlight the hippocampus morphology. After blocking, mouse brain sections were incubated with a rabbit anti-PAR-1 antibody and detected using an Alexa Fluor 488-conjugated anti-rabbit secondary (E). To determine the phenotype of PAR-1-positive cells, multiple staining was performed using astrocyte-specific (anti-GFAP, C), or neuron-specific (anti-NeuN, D) antibodies. Triple staining revealed the majority of PAR-1-positive cells co-localizing with NeuN, which indicated their neuronal phenotype (arrows in D, E and F). PAR-1 expression was mostly confined to cell bodies and neuronal processes (arrowheads in E and F). A weaker expression was occasionally observed in astrocytes.
Figure III.5. The expression of PAR-1 in the amygdala.
Multiple staining for PAR-1 protein (A), neurons (B) and astrocytes (C) was performed as described in Fig III.4; DAPI (D) was used to visualize the amygdala morphology. PAR-1 positive cells in the amygdala follow the expression pattern observed in the CA1 of the hippocampus and mainly co-localized with neuronal cell bodies (E). However, PAR-1 expression in astrocytes (G) was even scarcer than in the CA1. Control (H) was obtained by omitting the primary anti-PAR-1 antibody.
Figure III.6. Percentage of PAR-1 positive cells identified as neurons or astrocytes.

Quantification of triple immunohistochemical labeling revealed that 92-99% of the PAR-1 positive cells in the amygdala, hippocampus and cortex co-expressed NeuN and were therefore identified as neurons. Less than 2% of the PAR-1 positive cells co-expressed GFAP, which indicated their astrocytic lineage. Cells not expressing either marker were classified as of the unknown lineage. Results are shown as mean ±SEM, n = 5 animals.
Figure III.7. Percentage of neurons (NeuN⁺) and astrocytes (GFAP⁺) expressing PAR-1.

To investigate whether the neuronal and astrocytic populations were diverse with respect to the PAR-1 expression, the percentage of NeuN⁺ and GFAP⁺ cells (neurons and astrocytes, respectively) co-expressing PAR-1 was calculated. In all the regions examined the majority of NeuN⁺ cells (80, 97 and 99% for the amygdala, hippocampus and cortex, respectively) expressed PAR-1. On the other hand only 8 to 23% percent of astrocytes were PAR-1-positive. Results are shown as mean ±SEM, n = 5 animals.
III.3. Discussion

PAR-1 has been implicated in several aspects of CNS physiology, including perception of pain and neuronal death in the hippocampus. However, there are no studies comparing the thrombin receptor protein and gene expressions in the mouse brain using Western blotting, qRT-PCR and immunohistochemistry.

The qRT-PCR revealed that the PAR-1 gene was expressed in all seven brain regions examined with profound regional differences. This result suggests that, in basal conditions, the PAR-1 gene is regulated in the central nervous system, and that its regulatory mechanisms may play an important role in brain physiology. Western blotting using extracts from the same brain regions showed the highest levels of PAR-1 protein in the amygdala and cortex. The observed differences between the gene and protein expression patterns may stem from either differential transcriptional regulation of PAR-1 expression in different brain regions, or the presence of translational/post-translational mechanisms regulating PAR-1 protein levels. The latter could include cleavage/inactivation of PAR-1 by different proteases, followed by different trafficking/internalization rates of the receptor in various areas of the brain.

PAR-1 expression was next examined using immunohistochemistry that confirmed the presence of PAR-1 positive cells in most regions of a mouse brain, with a prominent expression in limbic areas. This finding is in agreement with a previous study (Striggow, 2001) which showed a strong PAR-1 staining in the cortex, hippocampus and amygdala of a rat. The predominantly neuronal PAR-1 expression is not surprising, since various PAR-1 agonists have previously been shown to elicit neuronal responses in other species.
(Yang, 1997; Luo, 2005). Immunohistochemistry revealed weaker expression of PAR-1 in astrocytes. Previous studies have demonstrated species-specific differences in astrocytic expression of PAR-1 (the highest levels found in humans), and have shown that even this scarce astrocytic population produces physiologically significant responses (Junge, 2004).

PAR-1 might be expressed in a particular neuronal sub-population as 97-99% of neurons were PAR-1 positive in the hippocampus and cortex, while this proportion was reduced to 80% in the amygdala. This finding suggests that PAR-1 may be widely expressed in principal neurons in the limbic areas, but may be excluded from a particular cell type in the amygdala. Indeed, the lateral and basolateral nuclei of the amygdala contain 25% of inhibitory interneurons (McDonald & Augustine, 1993; Pare & Smith, 1993; Niehoff & Kuhar, 1983), while the neuronal population of the hippocampus contains only 10% of interneurons (Jones, 1999). Further immunohistochemistry experiment could clarify this point, using antibodies to detect PAR-1 together with neuronal type-specific markers (e.g. parvalbumin, somatostatin or other markers to stain inhibitory neurones or VGLUT1 to label excitatory neurones).

Altogether these experiments show high levels of PAR-1 protein in cell bodies and processes of the limbic system neurons. This localization suggests the involvement of PAR-1 in cognitive processes such as learning and memory. The expression of PAR-1 was particularly high in the amygdala, the emotional centre of the brain, which suggests the role of this receptor in neuronal responses associated with stress, fear and anxiety.
Chapter IV

Regulation of PAR-1 protein and gene expression by stressful stimuli and its relation to proteolytic cleavage
IV.1. Introduction

It has been demonstrated that the amygdala displays high levels of PAR-1 protein (Chapter III); The amygdala is part of the limbic system and is located within the medial temporal lobes of the brain. It plays a major role in the formation, storage and consolidation of memories associated to emotions. The presence of PAR-1 in this region raises a possibility that it could be regulated in response to noxious stimuli and play a role in stress and fear learning. This chapter provides a critical review of the available literature on the mechanisms of PAR-1 regulation (including its activation, inhibition and trafficking) along with experimental assessment of regulation of the PAR-1 gene and protein expression by stress and fear conditioning.

IV.1.1. PAR-1 mRNA regulation

Is PAR-1 regulated by cellular stress? Nguyen (2001) showed that arterial shear stress reduces PAR-1 mRNA and protein levels in endothelial cells, as described previously in human aortic and rat smooth muscle cells (Papadaki, 1998). In a rodent model of hypertension, angiotensin II triggers an elevation of PAR-1 mRNA levels via a superoxide-dependent mechanism (Capers, 1997).

In the central nervous system, upregulation of PAR-1 mRNA and protein expression in astrocytes have been reported in Parkinson’s disease (Ishida, 2006).

The promoter activity of the human PAR-1 gene is modulated by several regulatory elements among which the transcription factors Sp1 and Sp3. They bind to the promoter proximal sequence between bp -303 to -164 and generate opposite effects (i.e. Sp1
activating and Sp3 inhibiting) in human endothelial cell cultures (Wu, 1998). In the metastatic melanoma, Tellez et al (2003) showed that PAR-1 expression level is regulated by the ratio between the transcription factors activating-protein 2 (AP-2) and Sp1. This mechanism is further supported by their observation that the suppression of AP-2 in melanoma cells results in PAR-1 overexpression (Tellez, 2003).

IV.1.2. Mechanism of receptor activation, internalization and inhibition

IV.1.2.a. PAR-1 activation and internalization

PAR-1 is present at the surface of blood platelets with no intracellular reserve per se of the receptor available. However the receptor is also present in the membrane of the surface connecting system -formed by deep intracytoplasmic invaginations of the membrane- which serves as a storage and an exit route for products released during platelet activation. Upon activation, the surface connecting system transfers the receptors to the platelet membrane in order to amplify the response to thrombin. The fate of activated receptors is not well understood. Norton et al (1993) suggested that thrombin – activated PAR-1 receptors remain on the platelet surface in a desensitized state. However, a fraction might undergo internalization or be released in platelet microparticles (Molino, 1997). Platelets do not renew their PAR-1 protein reserve as once activated they are incorporated into the blood clot.

Unlike platelets, endothelial cells and fibroblasts have to repeatedly respond to thrombin over time by employing different PAR-1 activation/desensitization mechanisms. PAR-1 receptors are localized at the plasma membrane where they can be activated. PAR-
1 is also stored in an intracellular pool not accessible to proteases (Hein, 1994). The receptors undergo tonic cycling between the membrane and the intracellular pool due to a signal contained by the carboxy tail (Shapiro, 1996). Rapid cellular resensitization to thrombin signalling depends on this intracellular reserve of receptors.

Desensitization, internalization, degradation and downregulation are the three consecutive processes that terminate PAR-1 signalling. However when PAR-1 has been activated by APC, desensitization but not internalization occurs (Russo, 2009). Activation by proteases facilitates trafficking of new PAR-1 molecules to the plasma membrane to sustain cell’s ability to respond to thrombin (Trejo, 1998).

Phosphorylation of the carboxy tail is required for a rapid shut-off of the receptor signalling by uncoupling it from G-protein. Ishii (1994) has shown that in Xenopus oocytes the thrombin receptor is desensitized due to phosphorylation on Ser/Thr residues of the C-t by the G-protein coupled receptor kinase 3 (GRK-3). GRK5, but not GRK2 and 6, has been identified to phosphorylate PAR-1 and inhibit its signalling in cultured endothelial cells (Tirupathi, 2000). Phosphorylation represents a shut-off mechanism of the receptor’s actions but naïve receptors undergo internalization independently of phosphorylation (Hammes, 1999; Shapiro, 1996). Additionally β-arrestins play a role in the desensitization process probably by promoting the uncoupling of PAR-1 from the G proteins (Paing, 2002). However, arrestins do not target the receptor for internalization and are not necessary for either sorting PAR-1 to lysosomes or its degradation (Paing, 2002).
Chapter IV

Receptor activation by proteases is necessary for its internalization (Brass, 1994). In fibroblasts and endothelial cells upon cleavage by thrombin, PAR-1 is internalized in endosomes and directed to lysosomes (Hein, 1994). It seems that half of the activated receptors are internalized rapidly, whereas the remaining PAR-1 are cleared much slower (Woolkalis, 1995), 25 to 60% of the cleaved receptors remaining on the membrane for at least two hours (Ishii, 1993). Approximately 30 minutes after protease activation a fraction of the intracellular pool is directed to the plasma membrane, independently of protein synthesis (Hein, 1994; Woolkalis, 1995). Lysosomal sorting of the receptor is critical for termination of its thrombin-induced signalling as contrary to classic GPCRs, agonist-receptor dissociation is not a mechanism available to PAR-1 (Trejo, 1998).

The activation-dependent internalization and degradation signal is located within a YXXL (amino acids 383 to 386) motif on PAR-1 carboxy tail (Trejo, 1998; Paing, 2004). Activated-PAR-1 is rapidly recruited to clathrin-coated pits and internalized in a dynamin-dependent fashion (Trejo, 2000; Paing, 2002). Phosphorylation of the above motif is not necessary for constitutive internalization and recycling (Paing, 2004). The tonic cycling mechanism involves a distinct YXXL motif at the very end of the carboxy tail (amino acids 420 to 423) that facilitates the association with the membrane clathrin-adaptor AP-2 (Paing, 2006). Additionally ubiquitination might affect AP-2 binding to PAR-1 and thus negatively regulate its constitutive internalization (Wolfe, 2007). This mechanism would explain why most receptors, ubiquitinated in basal conditions, remain at the cell surface accessible to proteases.
The carboy tail of the thrombin receptor contains the lysosomal-sorting message whose recognition does not involve ubiquitination (Trejo, 1999). Rather, the endosomal sorting protein nexin-1 is necessary to direct agonist-activated PAR-1 to a lysosomal degradation pathway; nexin-1 can form heterodimer with nexin-2, which might play a role as a regulator of PAR-1 sorting to lysosomes as it has been shown to inhibit PAR-1 lysosomal trafficking in vitro (Wang, 2002; Gullapalli, 2006).

In human erythroleukemia and megakaryocyte cell lines, cleaved-PAR-1 receptors are mostly internalized into endosomes. A few are recycled back to the membrane surface but remain unresponsive to thrombin for several hours (Hoxie, 1993). However, those receptors can be activated by the PAR-1 agonist SFLLRN. A full response to thrombin recovers slowly and is due to newly synthesized PAR-1 receptors (Hoxie, 1993; Brass, 1994).

Overall a characteristic feature of the PAR-1 proteins is their sorting to lysosomes (Trejo, 1998). Termination of PAR-1 signalling in neurons has not been elucidated so far. However one would expect that neuronal activated PAR-1 is desensitized, internalized and degraded in lysosomes.

**IV.1.2.b. PAR-1 inhibition**

A number of proteases have been shown to cleave PAR-1 between the tethered ligand and the first intracellular portion of the protein rendering the receptor resistant to subsequent activation by thrombin. The neutrophil-derived cathepsin G inhibits thrombin’s action by cleaving PAR-1 both at the thrombin’s cleavage site and
downstream from the tethered ligand (Molino, 1995). Similar secondary cleavage sites are 
used by elastase and proteinase 3 (Renesto, 1997) to inhibit thrombin’s effect in 
endothelial cells and platelets. Pre-treatment of fibroblast with a mast cell protease 
chymase results in inability of thrombin to activate PAR-1 (Schechter, 1998). The 
mechanism of PAR-1 inhibition by the modified bradykinin-derived blocking peptides, 
i.e. thrombostatins remains unclear (Hasan, 1996; Derian, 2003).

IV.1.3. Regulation of protein and gene expression by fear conditioning and stress

Protein and gene expression are known to be modified by psychological stress. For 
instance chronic but not acute restraint downregulates NR1, NR2A, and NR2B subunits 
(Pawlak, 2005). Proteases and their inhibitors are also regulated by noxious stimuli. 
Tissue plasminogen-activator (tPA; Pawlak, 2003) and its main inhibitor plasminogen-
activator inhibitor-1 (PAI-1; Yamamoto, 2002) are both induced by restraint stress in the 
central and medial amygdala. Also, acute stress has been shown to increase mRNA 
expression of neuropsin in the mouse hippocampus (Harada, 2008).

Fear conditioning has been reported to modify gene expression in the amygdala 
(Lamprecht, 2009) as well as in the hippocampus (Mei, 2005; Huff, 2006). Ploski and 
colleagues (2010) clearly showed that fear conditioning itself and not exposure to a novel 
context, a tone or an electric shock was responsible for a significant increase of plasticity-
related genes (among those Arc, Fos, Atf3 Activating transcription factor 3) in the lateral 
amygdala of rats.
4.1.4. Potential activators

4.1.4.a. The tPA/plasmin system

Tissue plasminogen activator is well known for its role in breaking down blood clots. Plasminogen is secreted by the liver and released in the blood circulation where it can be cleaved by tPA or other activators like urokinase-type plasminogen activator (uPA) or factor XII. The resulting broad spectrum protease plasmin is responsible for cleaving several plasma proteins, among which fibrin is characterized best.

The extracellular serine protease tPA is present in the brain and serves as an immediate-early gene activated by neural activity such as seizure, kindling and LTP via activation of NMDA receptors (Quian, 1993; Huang, 1996; Baranes, 1998; Salles 2002;). Moreover, tPA is secreted extracellularly following depolarization of cortical neurons and cleaves the NR1 subunit of NMDA receptor (Nicole, 2001).

tPA and uPA do not cleave PAR-1 directly (Parry, 1996; Altrogge, 2000), but rather indirectly through activation of plasminogen to plasmin. Indeed, plasmin and thrombin have similar affinity for PAR-1 extracellular domain but cleave PAR-1 at different sites (Kuliopulos, 1999). When plasmin cleaves the receptor at Arg70, Lys76 or Lys82 and therefore removes the tethered ligand, PAR-1 is desensitized and the calcium response to thrombin is attenuated. Cleavage at Arg41 by high concentration of plasmin activates PAR-1 in a similar way as thrombin leading to platelet aggregation (Kuliopulos, 1999). In human platelets, aggregation can also be achieved via PAR-4 cleavage by plasmin (Quinton, 2004).
tPA as well as plasmin generate production of IP3 in hippocampal slices (Junge, 2003). As no detectable cleavage of PAR-1 exodomain by tPA was found (Kuliopulos, 1999) and IP3 generation was absent in plasminogen or PAR-1 knockout mice (Junge, 2003), it is likely that tPA activates plasminogen to plasmin which, via PAR-1, triggers IP3 formation.

Plasmin has been described in the central nervous system (Melchor, 2005; Salles, 2002; Basham, 2001) and might play a role in synaptic plasticity (Mizutani, 1996; Nagai, 2004). The role of the tPA/plasmin system in synaptic transmission was confirmed by Mannaioni et al who demonstrated that NMDA receptor responses of CA1 pyramidal cells are potentiated by plasmin, via activation of astrocytic PAR-1 (Mannaioni, 2008).

**IV.1.4.b. Trypsin**

Trypsin is a serine protease produced by the pancreas and is the key activator for PAR-2 (Nystedt, 1994). At elevated concentration, trypsin cleaves PAR-1 (Vu, 1991) and activates the receptor in epithelial cells and to a lesser extent in astrocytoma cells (Grishina, 2005). At lower concentration, trypsin disarms the receptor that is in turn unable to be activated by thrombin (Kawabata, 1999).

The human genome contains eight different trypsin genes (Paju, 2006) but only three of them are coding for zymogen proteases. Trypsinogen-4 is a brain-specific trypsin isoform widely expressed in the human species with high levels of mRNA found in the cerebellar cortex, olfactory bulb and hippocampus. Activation of the zymogen depends upon the brain area and is particularly elevated in the cerebellar cortex and the
hippocampus (Toth, 2007). Moreover, trypsinogen expression overlaps with PAR-1 as immunohistochemistry revealed the presence of the protease in neurons, glial cells and in the extracellular matrix (Toth, 2007). Mesotrypsin/trypsin-4 strongly activates PAR-1-mediated calcium signalling in rat astrocytes and rat dorsal root ganglions neurons (Wang, 2006; Knecht, 2007). High concentration of mesotrypsin triggers a significant increase of cytosolic calcium via PAR-1 in human astrocytoma cells but not in human epithelial cell lines (Grishina, 2005). Thus mesotrypsin might be involved in PAR-1-mediated signalling in brain cells.

**IV.1.4.c. Activated Protein C**

APC cleaves PAR-1 at Arg41 but with specificity constants much lower than thrombin (Kuliopulos, 1999; Parry, 1996) making it unlikely for APC to directly affect the receptor in vivo.

**IV.1.4.d. Tryptase**

Tryptase is secreted by mast cells during trauma and inflammation and cleaves human PAR-1 synthetic peptide at the thrombin’s activation site, as well as one more distal site, resulting in the removal of the tethered ligand domain (Molino, 1997). Tryptase triggers phosphoinositol hydrolysis in COS-1 cells via PAR-1 cleavage; however, the serine protease fails to activate the endogenous thrombin receptor, as it does not induce platelet aggregation nor intracellular calcium increase in the megakaryocytic CHRF-288 cells (Molino, 1997).
IV.1.4.e. Granzyme A

Granzyme A is produced by cytotoxic T and natural killer cells but not by brain cells. It cleaves PAR-1 at the thrombin cleavage site, induces neurite retraction and reverses astrocytes stellation but fails to trigger platelet aggregation (Suidan, 1994).

IV.1.4.f. Matrix metalloprotease-1

Matrix metalloproteases (MMP) are involved in inflammatory processes and tissue-remodelling events and studies have been focusing especially on their role in tumor invasion and metastasis. The extracellular matrix degrading enzyme MMP-1 cleaves PAR-1 (Goerge, 2006) at a different position (LD\textsuperscript{39}P\textsuperscript{40}RSFL) rather than the classic thrombin-cleavage site, creating a longer tethered ligand (Trivedi, 2009). A recent article described how MMP-1 activates the PAR-1-Akt pathway in breast carcinoma cells therefore activating the proliferation and survival of the tumor (Yang, 2009). Trivedi (2009) showed in thrombocytes that following collagen incubation PAR-1 is cleaved by MMP-1, causing calcium and p38 MAPK signalling, Rho activation, and platelet aggregation.

IV.1.5. Potential inactivators

IV.1.5.a. Elastase, Cathepsin G and proteinase 3

Elastase, cathepsin G and proteinase 3 are present in the hippocampal CA1 region, the cortex and the cerebellum of rodents (Davies, 1998). They are secreted by activated neutrophils and downregulate PAR-1 activity on endothelial cells and platelets (Selak, 1994; Weksler, 1989).
Pre-treatment of human platelets and endothelial cells with elastase inhibits their calcium signalling response to thrombin due to degradation of the N-terminus of the receptor (Val 72-Ser 73 and Ile 74-Asn 75; Renesto, 1997) but not their response to agonist (Greco, 1996; Parry, 1996).

Another protease cathepsin G described in the human cerebral cortex (Savage, 1994) cleaves PAR-1 at Arg$^{41}$, therefore enabling the receptor and causing an increase in intracellular calcium concentrations. Cathepsin G also disables PAR-1 receptor by cleavage at Phe$^{55}$ thus inhibiting PAR-1 response to thrombin but not agonist peptide in platelets, CHRF-288, HEL and endothelial cells (Molino, 1995; Renesto, 1997). Similar events are observed with cells treated in proteinase 3-treated cells which cleave PAR-1 at Val 72-Ser 73 (Renesto, 1997). Cathepsin G is known to activate PAR-4 on human platelets (Sambrano, 2000).

**IV.1.5.b. Chymotrypsin**

Chymotrypsin is a digestive enzyme synthetised by the pancreas and its mRNA has been detected in the hippocampus of the rodent brain (Davies, 1998). It abolished PAR-1-mediated phosphatidylinositol breakdown in transfected HEK-298 cells without modifying the response to the agonist SFFLRNP (Vouret-Craviari, 1995). Indeed, chymotrypsin cleaves a synthetic thrombin peptide corresponding to residues 38-60 at Phe 43, inactivating the extracellular domain (Parry, 1996).
IV.1.5.c. Kallikreins

Kallikreins are a family of secreted serine proteases that can be divided into two groups. First, plasma kallikrein is synthesized in the liver and cleaves kininogen to release the potent vasodilator peptide bradykinin. The human tissue kallikreins form the second group composed of fifteen members that are expressed in the central nervous system except for KLK3, 4, and 15, involved in prostate, ovarian or breast cancers (Yousef, 2003; Vandell, 2008).

Oikonomopoulou et al. (2006) demonstrated that PAR-1, -2 and -4 synthetic peptides are cleaved by hKLK-14, -5 and -6 in vitro. The same kallikreins activate calcium signalling in HEK cells, with hKLK-14 causing a strong signal via PAR-2. PAR-1 activation is responsible for only a moderate calcium increase (when compared to that triggered by thrombin or TFLLR-NH₂). Interestingly, at higher concentrations, hKLK-14 inhibits PAR-1 probably by removal of the tethered ligand (Oikonomopoulou; 2006). Kallikrein 1 promotes PAR-1-mediated keratinocyte migration (Gao, 2010).

The most abundant kallikrein in the brain is hKLK-6 or neurosin. It has been described in the CNS of rat and human including the hippocampus, substantia nigra, and cerebral cortex with the highest expression in the spinal cord and medulla oblongata (Scarisbrick, 1997). It is expressed both in glia and neurons of rodents (Yamanaka, 1999; Ogawa, 2000). hKLK-6 activates PAR-1 and PAR-2 in astrocytes but only PAR-1 in a neuronal cell line and induces calcium and ERK signalling (Vandell, 2008).

Kallikrein 4 is found in the prostate and its overexpression during prostate cancer is thought to play a role in increased cell proliferation and mobility (Klok, 2007).
Although hKLK-4-induced calcium mobilization via PAR-1 is more potent than via PAR-2, it is the latter receptor that signals more efficiently when stimulated by hKLK-4 in transfected mouse embryonic fibroblasts; hKLK-4-activated PAR-2 expression contributes to prostate cancer progression (Ramsay, 2008). PAR-1 mRNA and proteins levels are elevated during prostate cancer (Black, 2007) and it is not excluded that the receptor becomes a hKLK-4 target in such an abnormal environment (Ramsay, 2008).

The trypsin-like serine protease neuropsin, also called kallikrein 8, is highly expressed in limbic areas and is involved in synaptic plasticity (see Introduction of Chapter 6). Neuropsin was cloned from the mouse brain (Suzuki, 1995) and its mRNA is found in the brain limbic structures, especially in hippocampal pyramidal neurons and in the lateral amygdaloid nucleus, areas involved in learning, memory and cognitive function (Chen, 1995). Neuropsin-specific in situ hybridization signal is also found in the cortex (Suzuki, 1995).

Neuropsin is produced as a zymogen and is secreted extracellularly from axons of the Schaffer-collateral pathway (Chen, 1995) before being activated (Shimizu, 1998). Neuropsin brain-active form of 32 kDa is obtained by removal of 4 amino acids (QGSK) by endoproteases and hydrolyses Arg-X and Lys-X bonds (Shimizu, 1998).

Neuropsin is able to degrade extracellular matrix proteins like L1-cell adhesion molecule (Matsumoto-Miyai, 2003), fibronectin (Shimizu, 1998) and EphB2 (Attwood, 2011), whereby regulating plasticity-like changes.

Neuropsin has previously been shown to cleave the following peptides: Val-Pro-Arg, Phe-Ser-Arg, Val-Leu-Arg, Asp-Pro-Arg, Glu-Gly-Arg (Shimizu, 1998; Rajapakse, 2005;
list not exhaustive). Three of these sequences are found in the mouse PAR-1 sequence: Asp-Pro-Arg, Glu-Gly-Arg and Val-Leu-Arg (Figure IV.1). The triplet VLR is unlikely to be cleaved by neuropsin as it is situated in the cytoplasm. However, both NPR and EGR are on the extracellular portion of the receptor, therefore accessible to neuropsin.
Figure IV.1. Mouse thrombin receptor sequence and the putative neuropsin-cleavage sites.

Neuropsin has previously been shown to cleave amino acid triplets outlined in the PAR-1 sequence (accession number P30558). NPR and EGR are of interest for this study because of their extracellular location near the thrombin cleavage site (underlined).
IV.2. Results

IV.2.1. Regulation of PAR-1 protein and gene levels by stress

It has previously been demonstrated that proteases are regulated by stress in the amygdala, where they are critically involved in stress-induced neuronal plasticity and behavioural responses (Pawlak, 2003; Attrwood, 2011). Since PAR-1 is activated by extracellular proteolysis, its regulation by stress in the amygdala was studied. Mice were subjected to acute restraint stress (six hours) before amygdalae were removed and PAR-1 protein levels quantified by Western blotting. Acute restraint stress resulted in a significant decrease in PAR-1 levels (to 44.57 ± 8.7% of control values; p<0.01, Figure IV.2).

To investigate whether the decrease in PAR-1 protein levels was accompanied by changes in the PAR-1 gene expression qRT-PCR was performed. The expression of the PAR-1 gene in the amygdala remained unchanged after six hours of restraint stress (Figure IV.3).
Figure IV.2. PAR-1 protein level is downregulated by restraint stress in the amygdala.

The amygdalae were dissected, homogenized and after electrophoresis the proteins were transferred onto a nitrocellulose membrane. After blocking, the membranes were probed with an anti-PAR-1 antibody (1:1000, A). To normalize the results, the membranes were stripped and re-blotted for actin (1:2000, A). Quantification revealed that the expression of PAR-1 decreased after restraint stress, and this effect correlated with the duration of the restraint (B). Results are shown as mean ±SEM, **p<0.01, Student’s test. The number of animals is indicated in each bar (B).
Figure IV.3. Restraint stress does not affect PAR-1 gene expression in the amygdala. Mice were subjected to six hours of restraint stress, or left undisturbed in their home cages. The amygdalae were dissected, homogenized, RNA extracted, transcribed to cDNA and qRT-PCR was performed with a set of PAR-1-specific primers. PAR-1 gene expression in the amygdala was not affected by restraint stress. Results are shown as mean ±SEM. The number of animals is indicated in each bar.
IV.2.2. PAR-1 protein levels after fear conditioning

Because PAR-1 is expressed in limbic areas and particularly prominent in the amygdala - a region involved in emotional learning-, PAR-1 protein levels following fear conditioning (FC) were assessed. For this purpose, wild-type mice were sacrificed at different time points (five and fifteen minutes, and six hours) after three tone-footshock pairings. Western blots revealed that PAR-1 protein levels decreased significantly five minutes after fear conditioning (61.68±0.16% $F_{(1,13)}=5.14$, $p<0.05$; Tukey’s post-hoc test $p<0.05$, control compared to five minutes after FC; Figure IV.4).

The above experiments demonstrate that either stress or fear conditioning cause a significant and long-lasting decrease in the amygdala PAR-1 protein levels.
Figure IV.4. PAR-1 protein cleavage in WT mice after fear conditioning.

WT mice have been subjected to fear conditioning and sacrificed five minutes later. Amygdalae were dissected and processed for Western blot (A). Quantification revealed that PAR-1 protein levels were decreased by 38% after fear conditioning (B). Results are shown as mean ±SEM. *p<0.05 compared to control, Tukey’s post-hoc test. The number of animals is indicated in each bar (B).
IV.2.3. Contribution of neuropsin to stress/fear conditioning-induced downregulation of PAR-1

The above data indicate that PAR-1 protein levels are decreased after fear conditioning. One possibility is that PAR-1 may be cleaved by proteases such as thrombin, plasmin, tPA or kallikreins. Neuropsin is a good candidate due to its high expression in structures involved in learning, memory and emotion, and because PAR-1 contains putative neuropsin cleavage sites.

Therefore, the next objective was to examine PAR-1 protein cleavage after fear conditioning in neuropsin−/− mice. Animals were subjected to the behavioural test and sacrificed five, fifteen minutes or six hours after. Western blotting revealed that PAR-1 protein levels in the amygdala are comparable to control five minutes after stress (100 ± 8.69 and 119 ± 5.66, respectively; p>0.05; Figure IV.5, A and B). Two-way ANOVA analysis shows a main effect of FC with PAR-1 protein levels diminished at fifteen minutes and six hours (78.39 ± 8.18 and 56.46 ± 17.28, respectively;) compared to control (F(1,13)=5.14, p<0.02; Tukey’s post-hoc test p<0.05). This result suggests a possible physical interaction between neuropsin and PAR-1 proteins.

To investigate this hypothesis co-immunoprecipitation was performed. A band of 48 kDa corresponding to PAR-1 in material immunoprecipitated using anti-PAR-1 but not control antibody was visible (Figure IV.6, A). After the membrane was incubated with an
Figure IV.5. PAR-1 protein levels in neuropsin−/− mice after fear conditioning. Neuropsin−/− mice have been subjected to fear conditioning (FC) and sacrificed five, fifteen minutes or six hours after. Amygdalae were dissected and processed for Western blot using an anti-PAR-1 antibody (A). Quantification revealed that PAR-1 protein levels were decreased significantly fifteen minutes and six hours after FC (B). Results are shown as mean ±SEM; *p<0.05 compared to control, Tukey’s post-hoc test. The number of animals is indicated in each bar (B).
Figure IV.6. PAR-1 physically interacts with neuropsin.
Co-immunoprecipitation was performed on brain homogenates using an anti-PAR-1 antibody (A, B). In control experiments, the immunoprecipitating antibodies were replaced with irrelevant IgGs from the same species. Proteins were eluted, separated by SDS-PAGE and transferred onto the nitrocellulose membrane. Western blotting revealed a 50 kDa band corresponding to PAR-1 protein (A) when the membrane was blotted with an anti-PAR-1, but not a control antibody. When blotted with an anti-neuropsin antibody but not a control IgG, the membrane revealed a band of approximately 55 kDa (B).
anti-neuropsin antibody a band at approximately 55 kDa was detected (Figure IV.6, B) in material immunoprecipitated with anti-PAR-1 antibody, but not with control IgG.

To obtain direct evidence of a possible cleavage of PAR-1 by neuropsin, neuroblastoma cells known to express PAR-1 (Cheema, 2008) were incubated with PBS + neuropsin or PBS alone for 15 min. Then SH-SY5Y cells were harvested and prepared for Western blot. Incubation of SH-SY5Y cells with neuropsin resulted in a significant decrease in the density of the native PAR-1 band by 25.4 ±7.04% (p<0.05; Figure IV.7). In a control experiment, SH-SY5Y cells were incubated with tPA and a Western blot confirmed that tPA cleaves PAR-1 (Figure IV.7) as previously described in the literature (Junge, 2003). This result implies that neuropsin is able to cleave PAR-1 in an in vitro system but does not provide any indication about the nature (i.e. direct or indirect) of the cleavage.
Figure IV.7. Neuropsin causes a decrease in PAR-1 protein levels. SH-S5Y5 cells were incubated with 50 nM Neuropsin or tPA in PBS for fifteen minutes at 37°C. Protease inhibitors were added to the plates, cells were harvested and homogenised. Western blot was performed using anti-PAR-1 and anti-actin antibodies; PAR-1 levels were reduced after tPA or neuropsin incubation. (A). PAR-1 protein levels decreased by 25% after neuropsin incubation (B). Results are shown as mean ±SEM; *p<0.05, Student`s test. The number of observations is indicated in each bar (B).
To address this question, large amounts of PAR-1 protein were produced by transfected HEK cells with a vector containing the PAR-1-HA-IRES2-EGFP construct. The success of the transfection was confirmed by fluorescent microscopy (Figure IV.8, A), and further purification of PAR-1-HA protein using anti-HA-antibody bound columns (Pierce) was performed. Then the recombinant protein was incubated with activated neuropsin or PBS alone and Western blotting was performed using either an anti-HA or anti-PAR-1 antibody. 50 kDa bands corresponding to PAR-1 protein were visualized with both antibodies in controls and neuropsin-incubated samples (Figure IV.8, B). If cleavage of PAR-1 protein had occurred extra bands would have appeared in neuropsin-treated samples. This result suggests that neuropsin does not directly cleave PAR-1.
Figure IV.8. Neuropsin cleaves PAR-1 indirectly.
HEK cells were transfected with pPAR-1-HA-IRES2-EGFP construct. 48 h later, pictures were taken with a fluorescent microscope to visualize the transfected cells (A). Then cells were harvested, lysed and supernatant collected after centrifugation. PAR-1-HA was purified by pouring supernatant onto columns containing immobilized anti-HA Ab. Activated neuropsin was incubated with the purified PAR-1-HA. Western blot was performed using either an anti-HA antibody or a PAR-1 antibody (B). Only one band at 50kDa corresponding to the PAR-1-HA protein was present in both blots, with or without neuropsin incubation.
IV.3. Discussion

 Proteases and their inhibitors are known to play a role in restraint stress and fear conditioning (Pawlak, 2003; Norris, 2007; Horii, 2008). Downregulation of amygdala PAR-1 protein levels after restraint stress could either reflect PAR-1 cleavage by stress-related proteases followed by its internalization and degradation or could be caused by the decrease in the expression of the PAR-1 gene. The latter possibility was ruled out by demonstrating that PAR-1 gene expression remained unchanged after acute restraint stress. Because the decrease in PAR-1 protein levels upon restraint stress were not accompanied by comparable changes in the PAR-1 gene expression, the latter parameter was not studied further. Our results together with the literature suggest that PAR-1 cleavage by proteases was the primary mechanism responsible for the decrease in PAR-1 protein levels observed after stress/fear conditioning.

 The fate of the cleaved PAR-1 receptors after stress/fear conditioning was not examined in this study as the use of unfractionated amygdala homogenates did not allow separate measurement of the membrane and internal stock of PAR-1 levels. Once internalized and degraded, the membrane pool of PAR-1 is usually replenished by new receptor molecules coming from an intracellular pool. This trafficking is rapid and occurs within 30 minutes after PAR-1 activation (Hein, 1994; Woolkalis, 1995). However, in the present study PAR-1 protein levels were not restored to control levels after restraint stress or fear conditioning. This is consistent with prolonged, rather than acute, PAR-1 cleavage by proteases after exposure to noxious stimuli. Indeed, extracellular proteases have been shown to remain active for a considerable period following the restrain stress
(e.g. extracellular tPA activity is elevated at thirty minutes after the beginning of restraint stress, Pawlak, 2003), and could degrade the newly membrane-inserted PAR-1 molecules. While a six-hour restrain stress is likely to result in prolonged cellular changes, the temporal pattern of the decrease in PAR-1 protein levels after fear conditioning indicates that even short-lasting traumatic episodes can trigger a long-lasting physiological response.

Are there any examples in the literature pointing to experience-dependent proteolytic cleavage of a membrane receptor that regulates neuronal activity? The answer to such a question is “yes”. The tPA/plasmin system could serve as a proteolytic cascade model controlling this kind of responses. tPA is regulated by various forms of neuronal activity and is liberated into the extracellular space in the amygdala following stress or application of the stress hormone corticotropin-releasing factor (Pawlak, 2003; Matys, 2004). tPA has been shown to enhance the NMDA receptor activity by cleaving its NR1 subunit (Nicole, 2001), probably indirectly, through activation of plasmin (Matys & Strickland, 2003). Thus, PAR-1 represents another example of experience-specific regulation of neuronal activity and behaviour by highly specific and localized proteolytic cleavage.

What could be the identity of protease(s) that cleaves PAR-1 during restraint stress or fear conditioning? The experiments reported in this chapter demonstrate that neuropsin is likely one of them, contributing to the cleavage of PAR-1 early after fear conditioning. In neuropsin−/− animals, the reduction of PAR-1 protein levels in the amygdala following stress is delayed compared to wild-type. Neuronal release and activation of neuropsin in the extracellular milieu following emotional learning during fear conditioning is plausible as neuropsin is known to be involved in the early phase of LTP (Momota, 1998; Komai,
2000) and its mRNA is upregulated after stress in the hippocampus (Harada, 2008). This result raises the possibility that neuropsin might contribute to PAR-1 cleavage at an early stage after stress.

The co-immunoprecipitation experiment revealed that both neuropsin and PAR-1 might interact in the brain of wild-type animals in control conditions. The reason for the observed shift in neuropsin molecular weight from 32kDa (Shimizu, 1998) towards 55kDa (Figure IV.6, B) is not clear but could be due to neuropsin posttranslational modifications or putative neuropsin dimers, as observed for tPA (Nienaber, 1992).

Further evidence of neuropsin’s ability to cleave PAR-1 comes from SH-SY5Y cells, where PAR-1 degradation occurred within fifteen minutes of neuropsin incubation. Pre-activation of neuropsin prior to its addition to the cell medium was not necessary (Attwood, 2011) indicating that neuropsin is activated by a native protease(s) in the cell culture system. This protease(s) might be tissue-specific because Shimizu’s (1998) did not observe PAR-1 activation or platelet aggregation following application of recombinant neuropsin. In his study however, platelets were isolated by gel filtration prior to aggregation which might have resulted in elimination of putative extracellular cofactors or plasma proteases necessary to activate neuropsin.

However, PAR-1 cleavage, although delayed, still takes place in neuropsin+ mice. There are several possible explanations of this effect. First, neuropsin might not cleave PAR-1 directly but rather speed up the activation of another protease that ultimately cleaves PAR-1. This is supported by the in vitro experiments where cleavage of PAR-1-HA by neuropsin was examined in a purified system. Neuropsin was first activated in vitro
as described by Kato (2001) before incubation with PAR-1-HA protein. No cleavage of PAR-1-HA was observed. There are several factors that might have affected this experiment. First, higher concentrations of neuropsin might be necessary to cleave PAR-1 efficiently. Second, an isolated PAR-1-HA protein might have a different conformation than that of the transmembrane protein preventing the proper recognition and cleavage of the PAR-1-HA protein by neuropsin. Next, an unidentified cellular co-factor might be necessary to catalyze PAR-1 cleavage by neuropsin, similarly as shown for tPA and annexin II (Hajjar, 1994). This idea is supported by a previous report describing PAR-3 as a cofactor for PAR-4 activation (Nakanishi-Matsui; 2000). Most probably, however, neuropsin cleavage of PAR-1 is indirect through activation of an unknown downstream protease. Such a mechanism would be analogous to the mode of action of the tPA/plasmin system, where tPA activates the zymogen plasminogen to the broad-spectrum protease plasmin, which ultimately degrades a variety of substrates such as laminin, the NR1 subunit of the NMDA receptor or PAR-1 (Nagai, 2006 and this study).

Currently it is not known whether neuropsin could serve a similar function or which protease(s) it may activate. It is possible that neuropsin takes part in the initial steps of PAR-1 cleavage and in the absence of neuropsin other serine proteases are upregulated to compensate for its absence and, albeit less efficiently, cleave PAR-1. Such a hypothesis is supported by the presence of broad spectrum serine proteases in the amygdala that have overlapping proteolytic spectra (Gschwend, 1997; Pawlak, 2003; Krueger, 1997). Plasmin or tissue kallikreins could all partially compensate for the lack of neuropsin during fear conditioning in neuropsin-deficient mice.
However, neuropsin has a rather narrow proteolytic spectrum. Our knowledge of the structural basis of its proteolytic specificity is still incomplete, but it is known that it preferentially cleaves Arg-X and Lys-X bonds (Shimizu, 1998). Thus, other proteases are unlikely to fully compensate for the absence of neuropsin – their action would result in PAR-1 cleavage at alternative sites that would likely promote a different set of signalling events. It is also possible that the expression of membrane receptors, adhesion molecules and extracellular matrix molecules, that may normally serve as cofactors or intermediates during neuropsin-mediated cleavage of PAR-1, is altered in neuropsin-deficient animals. In fact, data from our laboratory indicate that in basal conditions 19 transcripts are differentially expressed in the amygdala of neuropsin<sup>−/−</sup> mice as compared to wild-type animals (Artwood, 2011). These differentially expressed transcripts include NCAM2 and transmembrane glycoproteins Tmem159 and Tmem181. It is tempting to speculate that some of these differences could contribute to the delay in PAR-1 cleavage and behavioural phenotype observed in neuropsin-deficient mice after stress and fear conditioning (Chapter V).

Overall, these results indicate that PAR-1 is cleaved in the amygdala following stress/fear conditioning and suggest an indirect involvement of neuropsin in this process.
Chapter V

Roles of PAR-1 and neuropsin in stress-related behaviour
V.1. Introduction

V.1.1. Stress

The term stress was introduced in the 1930s by Hans Selye who defined it as a non-specific response of the organism to the stressor, a stimulus that disrupts the natural dynamic homeostasis of the living organism. The body’s reaction to stress is described as the “general adaptation syndrome” which is divided into three stages. First, the alarm step is set off by a stressor and characterised by activation of the sympathetic neuronal system and adrenaline release from the adrenal medulla so that the body is ready for “fight or flight”. Then the resistance stage follows that involves the body mobilizing resources to cope with the stressor, by adopting cognitive, physiological and behavioural responses aimed to restore homeostasis. The term allostasis is used to define the adaptive mechanisms undertaken by the body to maintain homeostasis (Sterling & Eyer, 1988). However, persistent stressful situation exhausts the available resources and lead to organ dysfunctions (defined as allostatic load; McEwen, 2008) and if not remedied lead to death. In some extreme cases of physical and/or psychological trauma e.g. in life threatening situations (torture, major illness, natural disaster, traffic accident, war) the individual’s ability to cope with stress can be overwhelmed. The above situations can trigger an extreme form of anxiety called the post traumatic stress disorder (PTSD) that may eventually lead to suicide (McFarlane, 2002; Moores, 2008).

The brain is the organ that assigns “emotional value” to stressors, evaluate their significance according to past-experiences and engage the most appropriate response in order to maintain homeostasis. In response to a stressor prefrontal, cortex, hippocampus
and amygdala signal to the hypothalamus to activate the hypothalamo-pituitary-adrenal axis to stimulate ACTH (Van de Kar, 1991) and glucocorticoid secretion (Dunn, 1986). The anatomy and morphology of the responsive brain areas (Dedovic, 2009) are modified by stress leading to hippocampal dendritic atrophy (Magarinos, 1996), a reduction in hippocampal volume (Smith, 2005) whereas amygdala activation (Rauch, 2000) is translated by a persistent volume increase and dendritic outgrowth (Vyas, 2002 & 2004).

V.1.2. Behavioural tests of learning, fear and anxiety

A significant number of behavioural tests have been designed to evaluate learning, memory and anxiety in rodents. Various aspects of learning and memory can be measured by the Morris water maze (Horii, 2008), passive-avoidance tests (Almonte, 2007), novel object recognition (Ennaceur & Delacour, 1988), fear conditioning (Horii, 2008) and other, less commonly used paradigms (Sharma, 2010). The elevated-plus maze and open field are often used to assess anxiety. The above tests are designed to measure different aspects of cognition and distinct emotional traits. Due to their complex nature and ongoing discussions in the neuroscience community regarding their interpretation (Hogg, 1996; Crabbe, 1999; Kulesskaya, 2011; Prager 2011), their detailed description is beyond the scope of this chapter. Below is presented a short literature overview of the behavioural methods utilized in this study.
V.1.2.a. Restriment stress

Animal immobilization is a technique that induces a stress response from the body that involves both neurogenic and psychogenic elements. A large variety of protocols used for restraint make comparisons between different studies difficult. Several methods can be used to immobilize the animal (reviewed in Buynitsky, 2009) and the duration of the restraint used is highly variable, from as short as ten minutes (Jackson, 2006) up to six hours (Pawlak, 2003). Similarly, a number of protocols to induce chronic stress are in use (Benatti, 2003), with six hours of restraint repeated for 21 days being the most common (Pawlak, 2003).

Chronic stress induces anxiety-like behaviour (Shekhar 2005; Bondi, 2008) but no change in locomotor and exploratory behaviour (Pego, 2008). In the present study, wire mesh restrainers have been used. When applied chronically, this method has been shown to impair spatial memory, trigger hippocampal CA3 dendritic atrophy and amygdala dendritic outgrowth (Vyas, 2002); it facilitates fear conditioning and decreases open field exploration (Conrad, 1996 & 1999; Bennur, 2007; Magarinos, 1995). Acute physical restraint is known to increase c-Fos expression in the amygdala and promote anxiety (Mendonca-Netto, 1996; Hsu, 2007; Moller, 1997). Acute but not chronic restraint contributes to the reduction in the social conflict test in rodents (Wood, 2003).

V.1.2.b. Elevated-plus-maze

In this test the animal is placed in the centre of an elevated cross-shaped maze consisting of two enclosed and two open arms. The animals are left free to explore this
novel environment for five minutes. The test is based on the conflict created by the natural aversion of rodents for illuminated open areas and their natural tendency to explore novel places (Handley, 1984). Originally this task was created for testing drugs effect on anxiety (Pellow, 1985). This model of unconditioned aversion is nowadays more broadly used to assess general emotional behaviour/emotionality related to addiction and withdrawal, generalised-anxiety and PTSD (Jung, 2000; Walf, 2007; Imanaka, 2006). The ratio of open arm to total arm entries is considered a measure of relative aversiveness of the open arms (Handley, 1993) and is reported in this work as the anxiety index. Total or closed arm entries are used as a measure of locomotor activity (Lister, 1987; Pego, 2008) implying that exploratory behaviour is independent on the anxiety state of the animal. This view has transformed the original interpretation of the approach/avoidance conflict model into an anxiety/locomotor activity test.

V.1.2.c. Open field

Originally the test was designed to evaluate ambulation and defecation (Hall, 1934) two measures related to emotion: an animal experiencing anxiety would defecate more and would move less. Later, the use of this test for anxiety evaluation was strengthened as it was discovered that the more time the animal spent in the centre of the field, the less anxious it became (Royce, 1977). Anxiolytic drugs indeed increase the time spent by the animals in the centre of the open field (Simon, 1994; Prut, 2003). Total distance travelled is an indicator of general activity and exploratory behaviour (Domínguez, 2005; Matynia,
2010) and entries into and time spent in the inner area of the open field are measures of anxiety-like behaviour (Kung, 2010).

The elevated-plus maze and the open field tests are influenced by a number of environmental factors and animal strain's features and emotional state that can misled the interpretation of the results (Stanford, 2007). Therefore these tests should be complemented by other behavioural tests, for example the Morris water maze, or Pavlovian conditioning in order to discriminate between motor output, exploratory drive and anxiety state (Periera, 2005; Lewejohann, 2006; Stanford, 2007).

V.1.2.d. Novel object recognition

Novel object recognition is a non-spatial test of recognition memory (Ennaceur & Delacour, 1988) based on the natural tendency of rodents to explore a novel object instead of a familiar one. This learning and memory task is designed to test the ability of the animal to discriminate between objects. Using this test, the roles of glutamate, serotonin or acetylcholine receptors have been investigated on different stages of memory, i.e. encoding, formation and retrieval (Winters, 2005, Schiaparelli, 2006; Rutten, 2006). The perirhinal cortex is a region with a significant role in encoding objects and damaging this area impairs object recognition (Gaffan, 2004; Norman, 2005). The degree of impairment is increased when the fornix is also lesioned and it is known to impair spatial learning to the same extent as pure hippocampus lesions (Norman, 2005). Hippocampus involvement in the simple object recognition task (i.e. performed in a simple environment) is subject to question as different studies have produced contradictory
results. Almost complete damage of the hippocampus seems to be necessary to impair object recognition after a minimum retention delay of five minutes (Mumby, 2002; Clark, 2000; Broadbent, 2004). However, other studies showed that rats with hippocampal lesions performed well in simple object recognition (Langston, 2010; for review Mumby, 2001) and no hippocampal c-Fos activity has been detected during this task (Zhu, 1995; Wan, 1999). Therefore hippocampus is thought to be responsible for processing information that link the object, place and context (Langston, 20010) and its role in storage and/or retrieval of object recognition memory is under scrutiny (Langston, 2010).

V.1.2.e. Fear conditioning

Classical fear conditioning involves presenting a neutral conditioned stimulus (CS, tone, light) paired with an aversive unconditioned stimulus (US, electrical footshock) to the animal. After several pairings, the CS when presented alone elicits conditioned emotional responses such as freezing; similar behaviour is noticed when the animal is placed in the context (cage) where pairing occurred. For many years, the classic view was that hippocampus was responsible for associating the context to the aversive stimulus (Winocur, 1987), whereas the amygdala was the region where the tone was associated to the US. However, recent studies revealed a much more complicated picture as described below and illustrated by the Diagram V.1.
Diagram V.1. Fear conditioning circuits in the brain.
The cortex and the hippocampus both transmit contextual information to the amygdala whereas the auditory stimulus reaches the amygdala via the thalamus and the cortex. The lateral (LA) and basal (BA) nuclei of the amygdala receive contextual and spatial information from the hippocampus, and sensory inputs are transmitted via the thalamus and the auditory cortex. The basolateral nuclei are the main centre for the CS-US association and memory acquisition and storage. A direct connection between the central (CcA) and the lateral nuclei is also essential for memory acquisition. The central nucleus projects to the hypothalamus and the brainstem (midbrain, medulla) that in turn modulate hormonal, motor and sensory parameters involved in the fear response, such as freezing, respiration, blood pressure.
V.1.2.e.i. *The role of the hippocampus in fear conditioning.*

Maren *et al* demonstrated that damaging the dorsal hippocampus by NMDA infusion seven days before fear conditioning training did not block learning of context fear, suggesting that the hippocampus was not essential for context-dependent learning (Maren, 1997; see also Richmond, 1999). This led to the hypothesis that an extrahippocampal system might be exploited to process and link the contextual information to the aversive stimulus. Phillips & LeDoux (1992) hypothesised that the amygdala was the region where association between an aversive stimulus and a conditioned stimulus takes place. According to this model the hippocampus could serve as one of the structures that transmits context-related information to the amygdala where the CS-US association would occur. This idea was strengthened by experiments demonstrating that context and cued conditioning were both impaired after the amygdala was damaged by electrolytic lesions in rats (Phillips & LeDoux, 1992). However a lesion to the dorsal hippocampus only caused impairment of the context conditioning while the response to the tone was not affected (Phillips & LeDoux, 1992). Indeed, Biedenkapp *et al* (2009) have shown that in an intact system, the hippocampus supports pattern completion and relays the information through the CA1-ventral subiculum pathway to the amygdala. Thus, freezing to the context is impaired in rodents when the hippocampus or the ventral subiculum, connecting the hippocampus to the amygdala, are damaged before or after FC training (Maren, 1999; Biedenkapp, 2009).

The above experiment leads to the question of how contextual information is processed when the hippocampal function is injured before FC. Perirhinal and postrhinal
cortices of the medial temporal lobes are known to project to the hippocampus (Lavenex & Amaral, 2000) and the perirhinal cortex also projects to the amygdala (Faulkner, 1999). If these regions are damaged before or after FC, context-dependent FC is impaired (Buffalo, 1999; Bucci, 2000 & 2002; Linquist, 2004). The above data make perirhinal and postrhinal regions strong candidates to mediate the context information to the amygdala when the hippocampus or ventral subiculum are damaged (Biedenkapp, 2009).

These studies have positioned the hippocampus as a key region for context-dependent fear conditioning. In addition, several articles have described impairment in both context and cued conditioning when the amygdala was damaged (Phillips & LeDoux, 1992; Gentile, 1986; Iwata, 1986).

V.1.2.e.ii. The role of the amygdala in conditional fear memories

The amygdala is an almond-shape group of brain nuclei: lateral, basal, accessory basal (these areas form the basolateral (BLA) complex), central, medial and periamygdaloid cortical nuclei. The basolateral amygdala is the primary site used by an intact animal for the acquisition and storage of fear memories as pre- or post-training BLA lesions led to disappearance of conditional freezing (Maren, 1996). Additionally, acquisition of conditional fear is blocked by reversible inactivation of the BLA (Muller, 1997) or intra-BLA infusion of NMDA receptor antagonists (Maren, 1996; Lee & Kim, 1998). The BLA role in memory storage is illustrated by the recent work of Gale et al who performed two fear conditioning training sessions at several-month intervals. They then damaged the BLA of rats by NMDA infusion before testing the animals for the remote
(16-month-old) or recent (one-day-old) context and cued memory. Freezing behaviour was impaired in BLA-damaged rats for both recent and remote conditioned context and cued fear (Gale, 2004).

Plasticity in the BLA is important for acquisition and storage of fear memory. However, extensive over-training can overcome context- but not tone-related deficits created by pre- but not post-training BLA lesions (Maren, 1999 & 2001). Nevertheless, BLA-bypassed acquisition of fear-context memories can be formed although less efficiently (Poulos, 2009; Ponnusamy, 2007), and they do not allow permanent storage of fear memory as it fades away seven days after training (Poulos, 2009).

The central nucleus of the amygdala seems to be involved in the BLA-independent fear-learning pathway, since overtraining of BLA-lesioned animals did not result in freezing to context and tone when CeA was inactivated (Anglada-Figueroa, 2005; Zimmerman, 2007). Both lateral and central amygdalae are likely to receive a direct auditory input as they receive projections from the auditory cortex and thalamus (McDonald, 1998; Turner & Herkenham 1991; Li, 1996).

The direct connection between the central and lateral nuclei (LA and CE) is necessary for the acquisition of fear conditioning. Damage to the above nuclei abolished fear conditioning to the levels observed in the unpaired group (Nader, 2001). Lesions to other nuclei (basal, accessory basal and medial nuclei) did not affect fear conditioning (Nader, 2001). There is little consensus among investigators as for the roles of LA and CE in fear conditioning. LA is essential for fear memory acquisition (Wilensky, 1999) and consolidation (Schafe, 2000 & 2001) and is thought of being the site of learning-related
plasticity (Amorapanth, 2000). CE is also required for the above processes (Pare, 2004; Wilensky, 2006, Zimmerman, 2007; Kolber, 2008), but in addition relays the acquired information to the circuits that control freezing (Amorapanth, 2000).

V.2. Results

Since PAR-1 is present in amygdala and hippocampus, two regions implicated in behavioural responses to stress, a battery of behavioural tests were undertaken to investigate whether PAR-1<sup>+</sup> mice display any behavioural phenotype. Neuropsin<sup>−</sup> animals were also tested since previous work (Chapter IV) showed that neuropsin could interact with PAR-1.

V.2.1. Novel object recognition reveals normal hippocampal function in PAR-1<sup>+</sup> mice.

In this task, wild-type and PAR-1<sup>+</sup> mice were trained to familiarize with two objects (Figure V.1, A). During the training session when both objects were novel to the animals, the mice did not show any preference towards any of them as time spent exploring both objects was similar for wild-type and PAR-1<sup>+</sup> mice (Table IV.1, Figure V.1, B).
Table V.1. Average time (seconds) spent exploring each object during the training session (n= 10 animals).

<table>
<thead>
<tr>
<th></th>
<th>Red object</th>
<th>Grey object</th>
<th>P value (t test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>5.3±0.7</td>
<td>6.3±0.9</td>
<td>0.4131 (ns)</td>
</tr>
<tr>
<td>PAR-1&lt;sup&gt;+&lt;/sup&gt;</td>
<td>5.2±0.7</td>
<td>6.7±1.9</td>
<td>0.4633 (ns)</td>
</tr>
</tbody>
</table>

ns= not significant

Ninety minutes after the training session the animal was returned to the arena where one of the objects was replaced by a novel one. If the mouse spends more time exploring the novel object than the familiar one then one concludes that short-term memory is intact. This was the case in this experiment (Figure V.1, C) where both wild-type and PAR-1<sup>+</sup> mice spent comparable exploration time around the novel object (70.33 ± 2.4% and 74.77 ± 3.9%, respectively, p>0.05; Figure V.1, C). Twenty-four hours after the training session the animals were placed back in the arena. This long-term memory assessment revealed that both genotypes spent equal amounts of time interacting with a novel object (WT 63.44 ± 3.5 and PAR-1<sup>+</sup> 64.26 ± 9.2; p>0.05; Figure V.1, C).

The lack of differences in the exploration time of the novel objects between knock-out and wild-type mice indicates that hippocampal function is intact in PAR-1<sup>+</sup> mice.
Figure V.1. Hippocampus-dependent functions are intact in PAR-1\(^{-}\) mice.

In the novel object recognition task, the mice displayed similar exploratory behaviour when 2 objects (red and grey, A) were presented during a training session (B). When one of the objects was replaced with an unfamiliar one (yellow or blue, A). Both wild-type and PAR-1\(^{-}\) mice spent 70 - 74% of the exploration time around the novel object 1.5 hours after training, and 63-64% of the time one day after training (C). Lack of differences in the exploration time between genotypes indicates that hippocampal function is intact in PAR-1\(^{-}\) mice. Results are shown as mean ±SEM. The number of animals is indicated in each bar (B).
V.2.2. PAR-1$^{+/−}$ and neuropsin$^{−/−}$ mice display opposite stress-induced anxiety-like behaviour in the elevated plus maze

**V.2.2.a. PAR-1$^{+/−}$ animals show normal stress-induced anxiety**

Wild-type and PAR-1$^{+/−}$ mice were tested in the elevated plus maze in control conditions or one day after restraint stress. Two-way ANOVA revealed a main effect of treatment on the number of entries in the open arms ($F_{(1,20)}=15.55$, $p<0.01$; Figure V.2, A). ANOVA analysis did not detect any other effect for the other parameters (entries in closed arms, total entries and anxiety index; Figure V.2, B, C & D). The elevated plus-maze did not reveal any stress-related behavioural differences between PAR-1$^{+/−}$ and WT mice.
Figure V.2. PAR-1⁻/⁻ and WT mice display similar performances in the elevated plus maze after restraint stress.

WT and PAR-1⁻/⁻ mice were subjected to restraint stress for six hours; the day after they were tested in the elevated plus maze alongside control mice. Anova analysis revealed a main effect of the treatment but no effect of the genotype or genotype x treatment (A). Overall level of activity measured by closed arm entries (B) and total entries (C) was similar in both genotypes. The anxiety index was not modified by stress (D). Results are shown as mean ±SEM; *p < 0.05, Tukey’s post-hoc test. The number of animals in each group is indicated in the bars (A).
V.2.2.b. Neuropsin\textsuperscript{−}\textsuperscript{−} animals present an anxiolytic behaviour after restraint stress

To complete the study of a hypothetical interaction between PAR-1 and neuropsin, I tested whether neuropsin\textsuperscript{−}\textsuperscript{−} mice would show a similar pattern of behavioural responses to PAR-1\textsuperscript{−}\textsuperscript{−} animals, i.e. an anxiogenic phenotype.

Two-way ANOVA revealed a main effect of the genotype on the number of open arm entries ($F_{(1,20)=7.47, \ p<0.01}$; Figure V.3, A) and a significant genotype x treatment interaction ($F_{(1,20)=4.36, \ p<0.05}$). Indeed, the WT entered less in the open arms after stress compared to WT control (1.71 ± 0.35 and 4.12 ± 0.69, respectively; $p<0.05$) and to stressed NP\textsuperscript{−}\textsuperscript{−} (3.92 ± 0.38; $p<0.05$). A genotype effect and a genotype x treatment interaction were also found significant for the anxiety index ($F_{(1,20)=18.52, \ p<0.001}$ and $F_{(1,20)=11.25, \ p<0.01}$, respectively; Figure V.3, B). A subsequent Tukey’s post hoc test showed that the anxiety index of the stressed WT mice was more elevated than that of the WT control mice (10.14 ± 1.83 and 5.33 ± 1.36, respectively; $p<0.001$) and of the stressed NP\textsuperscript{−}\textsuperscript{−} (3.56 ± 0.46; $p<0.001$). These results indicate that the NP\textsuperscript{−}\textsuperscript{−} mice phenotype is anxiolytic. The number of closed arm entries and total entries were similar for both genotypes, indicating a normal locomotor activity of the NP\textsuperscript{−}\textsuperscript{−} mice.
Figure V.3. Anxiolytic phenotype of the neuropsin$^{-}$ mice in the elevated-plus maze after restraint stress.
Wild-type and NP$^{-}$ mice were subjected to restraint stress for six hours. The day after they were tested for the elevated plus maze alongside control mice. Unlike WT, NP$^{-}$ mice did not develop any anxiety-related behaviour as their entries into open arms (A) and anxiety index (D) remained unchanged after stress. General locomotor activity was not affected in neuropsin$^{-}$ mice since the number of entries in closed arms (B) and total entries (C) were similar to those of wild-type mice. Results are shown as mean ±SEM; $^* p < 0.05$, Tukey’s post-hoc test. The number of animals in each group is indicated in the bars (A).
V.2.3. Behavioural measure of anxiety in the open field

V.2.3.a. **PAR-1<sup>+</sup> animals present an anxiogenic behaviour after restraint stress**

The open field test measures a variety of functions including amygdala-dependent anxiety-like behaviour (time spent in the centre *versus* periphery). Two-way ANOVA reveals a main effect of the treatment and the genotype for the number of entries in the centre (F<sub>(1,41)=15.33, p<0.001</sub> and F<sub>(1,46)=9.95, p<0.01</sub>; Figure V.4, A). Further post-hoc analysis showed that stressed WT and PAR-1<sup>+</sup> mice entered the centre less (31.2 ± 3.75 and 9.9 ± 2.02, respectively) than their respective control counterparts (WT 50.6 ± 6.23 and PAR-1<sup>+</sup> 33.3 ± 4.21; p<0.05). A trend toward treatment effect was also found for the time spent in the centre (F<sub>(1,41)=5.78, p<0.05</sub>; Figure V.4, B) but this result was not further confirmed with the Tukey’s post-hoc test as the difference between PAR-1<sup>+</sup> control and stressed groups was not quite significant (51.2 ± 7.93 and 11.5 ± 2.54; p=0.055). Finally the distance travelled in the centre was characterised by a main effect of the treatment and the genotype (F<sub>(1,41)=6.98, p<0.05</sub> and F<sub>(1,41)=19.32, p<0.001</sub>, respectively; Figure V.4, C). Indeed, PAR-1<sup>+</sup> mice travelled less in the centre than the WT in control conditions (2.4 ± 0.43 and 4.4 ± 0.34, respectively; Tukey’s post-hoc test p<0.05) and after stress (0.8 ± 0.16 and 3.5 ± 0.67, respectively; Tukey’s post-hoc test p<0.01). These results indicate that PAR-1<sup>+</sup> mice display an anxiogenic behaviour.
Figure V.4. PAR-1<sup>−/−</sup> mice are characterised by an elevated anxiety-like behaviour in the open field.

Wild-type (WT) and PAR-1<sup>−/−</sup> mice were subjected to restraint stress for six hours. The day after they were tested (together with control mice) in the elevated plus maze. Stressed WT and PAR-1<sup>−/−</sup> mice entered the centre less often (A). The time spent in the centre was similarly affected but the differences did not reach statistical significance (B). Higher anxiety level in PAR-1<sup>−/−</sup> animals were illustrated by a shorter distance travelled in the centre before and after stress (C). Results are shown as mean ±SEM; *p<0.05 and **p<0.01, Tukey’s post-hoc test. The number of animals in each group is indicated in the bars in (A).
V.2.3.b. Neuropsin<sup>−</sup> mice present an anxiolytic behaviour after restraint stress

To complete the investigation of the behavioural phenotype with the elevated plus maze, the neuropsin<sup>−</sup> mice, were tested alongside wild-type mice in the open field arena. Analysis of the number of entries in the centre revealed a main treatment effect ($F_{(1,43)}=6.24$, $p<0.05$; Figure V.5, A) as the stressed WT entered less in the centre than the WT control group (31.2 ± 3.75 and 50.6 ± 6.23, respectively; Tukey’s post-hoc test $p<0.05$). A main genotype effect was also observed for the time and the distance travelled in the centre ($F_{(1,43)}=22.36$, $p<0.001$ and $F_{(1,43)}=33.82$, $p<0.001$, respectively; Figure V.5, B). Subsequent Tukey’s post-hoc test showed that NP<sup>−</sup> in control and stress conditions stayed for a longer time in the centre (293.87 ± 74.59 and 304.62 ± 90.63, respectively) compared to WT (control 76.8 ± 7.64, $p<0.05$ and stress 50 ± 6.56, $p<0.01$). The NP<sup>−</sup> mice also travelled more in the centre than the WT before (11.44 ± 1.74 and 4.4 ± 0.34, respectively; Tukey’s post-hoc test $p<0.01$) and after stress (10.54 ± 2.42 and 3.5 ± 0.67, respectively; Tukey’s post-hoc test $p<0.001$; Figure V.5, C). These results illustrate the anxiolytic phenotype of the NP<sup>−</sup> mice.
Figure V.5. Neuropsin\textsuperscript{-/-} mice exhibit an anxiolytic phenotype in the open field. Wild-type and NP\textsuperscript{-/-} mice were subjected to restraint stress for six hours; the day after they were tested for the open field alongside control mice. Stressed WT mice displayed anxiety-like behaviour as shown by a reduced number of entries into the centre (A) compared to WT control. This parameter remained unchanged after stress for the NP\textsuperscript{-/-} animals (A); moreover, NP\textsuperscript{-/-} mice made longer visits to the centre of the arena (B) and travelled longer distances in the centre (C) than the WT, irrespectively of the treatment. This result illustrates the anxiolytic phenotype of the NP\textsuperscript{-/-} mice. Results are shown as mean ±SEM; *p<0.05, **p<0.01 and ***0<0.001, Tukey's post-hoc test. The number of animals in each group is indicated in the bars in (A).
V.2.4. The role of PAR-1 and neuropsin in a fear-learning task

The novel object recognition task suggests normal hippocampal function in mice lacking PAR-1. The open field highlighted opposite anxiety-related behaviours of PAR-1<sup>+</sup> and neuropsin<sup>+</sup> animals pointing towards a role of those proteins in regulating emotion. The amygdala is the brain area where emotional responses are produced and as a result a third behavioural test fear conditioning was performed to investigate PAR-1 and neuropsin involvement in this hippocampus- and amygdala-dependent test.

V.2.4.a. Amygdala-dependent learning is enhanced in PAR-1<sup>+</sup> mice.

Wild-type and PAR-1<sup>+</sup> mice were subjected to fear conditioning. This test measures the function of the hippocampus (related to context conditioning) and the amygdala (cued conditioning) by association of the cage/tone with a footshock. Both PAR-1<sup>+</sup> and wild-type mice froze equally to the context in which the footshock was delivered (15.19 ± 2.27% and 15.61 ±2.9%, respectively; Figure V.6). However, after cued conditioning PAR1<sup>+</sup> animals displayed a two-fold increase in freezing compared to their wild-type counterparts (47.98 ± 6.68% and 26.85 ± 3.51%, respectively; p<0.01; Figure V.7), which reflects an increased anxiety and an enhanced amygdala-dependent learning. It was noticeable that basal freezing to a new context (the fear conditioning apparatus) although very low, was significantly higher for PAR-1<sup>+</sup> mice compared to WT (3.30 ± 1.2% and 0.30 ± 0.24%, respectively; p<0.05; Figure V.6).
Figure V.6. Hippocampus-dependent learning is not affected in PAR-1<sup>−/−</sup> mice. Mice were subjected to fear conditioning. The day after the training, they were placed in the same chamber and the percentage of freezing was recorded to evaluate hippocampus-dependant learning. Although PAR-1<sup>−/−</sup> mice froze more than WT during training, probably due to their higher anxiety towards new environment, both genotypes froze at the same extent during the context-dependant retrieval (representative movement traces recorded during fear retrieval are showed in A, with the yellow bars below each panel indicating lack of movement; quantified in B). This result suggests that hippocampus-dependent learning is intact in PAR-1<sup>−/−</sup> mice. Results are shown as mean ±SEM; *p<0.05, Student’s test. The number of animals in each group is indicated in the bars (B).
Figure V.7. Amygdala-dependent learning is enhanced in PAR-1<sup>−/−</sup>mice. Mice were placed in a compartment different from the conditioning chamber, and the tone was played (time indicated as the red line on the x-axis). The percentage of freezing, indicative of amygdala-dependent learning, was significantly higher for the PAR1<sup>−/−</sup> (representative movement traces recorded during fear retrieval are showed in A, with the yellow bars below each panel indicating lack of movement; quantified in B). Results are shown as mean ±SEM; *p < 0.01, Student’s test. The number of animals in each group is indicated in the bars (B).
To rule out the possibility that one genotype might be more sensitive to pain than the other, and assure that differences in freezing behaviour are unrelated to a dissimilar pain perception, the pain threshold was established for each genotype (for principle see Material & Methods). Wild-type and PAR-1<sup>−/−</sup> animals displayed the same pain threshold (Figure V.8). Therefore the observed differences between genotypes in fear conditioned-freezing levels were due to differences in fear-learning levels.

In order to verify that freezing in response to the tone was due to learning and not to a stress-related situation, an unpaired fear conditioning experiment was performed in which the cue was not concomitant with the footshock. No difference in either baselines, or context or tone retrieval was noticed when comparing WT and PAR-1<sup>−/−</sup> mice (p>0.05; Figure V.9). The level of freezing to the context (WT 15.42 ± 1.39 and PAR-1<sup>−/−</sup> 12.76 ± 1.85) was similar to the one obtained with the classic fear conditioning experiment for wild-type (15.19 ± 2.27; p>0.05) and PAR-1<sup>−/−</sup> animals (15.61 ± 2.0; p>0.05; Figure V.9, B). Freezing to the tone was significantly reduced for both groups (WT 11.32 ± 1.13%, p<0.05; PAR-1<sup>−/−</sup> 10.06 ± 1.78%, p<0.01) compared to classic conditioning (WT 26.85 ± 3.5%, PAR-1<sup>−/−</sup> 47.98 ± 6.67%) as the animals did not associate the tone with the footshock (Figure V.9, B).
Figure V.8. Pain threshold is not affected by the deletion of the PAR-1 gene. Electrical footshock stimuli of increasing intensity were applied to WT and PAR-1⁻/⁻ mice and pain-related behaviour (flinching, jumping and vocalizing in human frequencies) was assessed. No difference was found between WT and PAR-1⁻/⁻ mice. Results are shown as mean ±SEM. The number of animals in each group is indicated in the bars.

Figure V.9. Unpaired fear conditioning does not induce tone-dependent learning in PAR-1⁻/⁻ and WT mice. Following an unpaired fear conditioning protocol, WT and PAR-1⁻/⁻ animals froze at similar levels during context retrieval (A). As expected from an unpaired FC training, none of the genotypes associated the tone with the footshock as they did not freeze during tone retrieval (B). This experiment demonstrates that the enhanced auditory memory in PAR-1⁻/⁻ mice (Fig. V.7) is due to the CS-US association. Results are shown as mean ±SEM. The number of animals in each group is indicated in the bars (A, B).
In the classic fear conditioning, WT mice froze less than the PAR-1\(^{+/−}\) to the tone. One could argue that it might be because the WT mice have reached a freezing ceiling. To rule out this possibility another test was performed to establish whether the cued-freezing behaviour of WT could be raised further up to the level of cued-freezing of PAR-1\(^{+/−}\) mice. To achieve this, a stronger fear conditioning protocol was used (see Material & Methods). Using 0.5 mA footshock and five pairings tone-footshock, the freezing capacity of the WT mice to the context was elevated to 42.87±8.51% (Figure V.10) which constitutes a 2.8 fold increase compared to the mild fear conditioning protocol (15.2±2.3%; Figure V.6). The levels of freezing behaviour to the tone in WT mice (48.49±8.06%; Figure V.10) were similar to those of the PAR-1\(^{+/−}\) described previously (47.98 ± 6.68%; Figure V.7). This experiment shows that the cued-freezing difference between the genotypes observed using a mild protocol is due to the lack of PAR-1 and not to a ceiling effect that would occur in wild-type animals.

To confirm the involvement of PAR-1 in cued-fear memory, the PAR-1 antagonist SCH79797 (an inhibitor of the PAR-1 tethered ligand; Ahn, 1999) or vehicle were injected in the amygdala of wild-type animals prior their fear-conditioning training. Both wild-type control and those injected with the PAR-1 antagonist froze equally to the context in which they received the footshock (17.59 ± 3.46% and 25.14 ±2.72%, respectively; p<0.05; Figure V.11). However, after cued conditioning the mice that received the PAR-1 antagonist prior to training displayed a significant increase in freezing compared to the control mice (42.98 ± 5.08% and 25.61 ± 2.80%, respectively; p<0.01; Figure V.12). This freezing level is comparable to one
Figure V.10. Equating PAR-1\textsuperscript{-/+} fear levels in wild-type mice.
In order to eliminate the possibility that the difference in freezing to the tone between WT and PAR-1\textsuperscript{-/+} mice (Fig. V.7) is due to an inability of the WT mice to freeze beyond this level, WT mice were subjected to a strong fear conditioning protocol (5 pairings, 0.5mA footshock). Freezing to the context was enhanced compared to the classic fear conditioning (Fig. V.7) and WT mice reached a similar tone-freezing level than that displayed by the PAR-1\textsuperscript{-/+} mice under classic fear conditioning (Fig. V.7). This results confirm that the difference in freezing to the tone between WT and PAR-1\textsuperscript{-/+} mice is due to a learning deficit of the PAR-1\textsuperscript{-/+} mice. Results are shown as mean ±SEM. The number of animals is indicated in the bars.
Figure V.11. Hippocampus-dependent learning is unaffected in mice treated with a PAR-1 antagonist.
Prior to fear conditioning training, a PAR-1 antagonist, SCH79797, or vehicle, was injected bilaterally into the amygdalae. The day after training, mice were tested for context memory. Both control and PAR-1 antagonist treated animals showed a similar level of freezing behaviour to the context as shown by the representative movement traces (the yellow bars below each panel indicating lack of movement, A; quantified in B). Results are shown as mean ±SEM. The number of animals in each group is indicated in the bars (B).
Figure V.12. Amygdala-dependent learning is enhanced in mice treated with a PAR-1 antagonist.

Prior to fear conditioning training, a PAR-1 antagonist, SCH79797, or vehicle, was injected bilaterally into the amygdalae. Forty-eight hours after training, cued-memory was tested. Mice treated with a PAR-1 antagonist froze significantly more to the tone (time indicated as the red line on the x-axis, A) than control animals as shown by the representative movement traces (the yellow bars below each panel indicate lack of movement, A; quantified in B). Results are shown as mean ±SEM.; **p<0.01, Student’s test. The number of animals in each group is indicated in the bars (B).
displayed by fear conditioned-PAR-1 knockout animals (Figure V.7). This result strengthens the finding that PAR-1 plays a role in cued-fear memory.

V.2.4.b. Amygdala-dependent learning is impaired in neuropsin<sup>−/−</sup> mice

Fear conditioning was also performed on neuropsin<sup>−/−</sup> animals. The freezing baselines to a new context (Figure V.13) or a new tone (Figure V.14) were similar in both genotypes. Neuropsin<sup>−/−</sup> animals did not show any impairment of hippocampal functions as they froze at the same level than WT mice to the context (15.66±2.3% and 17.65±2.2%, respectively; Figure V.13). However, freezing levels to the tone were significantly lower in knockout compared to WT mice (26.84±4.1% and 41.19±3.2%, respectively; p<0.05, Figure V.14) revealing an amygdala-dependent deficit.

Sensitivity to pain was comparable in both genotypes (Figure V.15) confirming that during fear conditioning the observed differences between wild-type and neuropsin<sup>−/−</sup> mice were due to fear learning.
Figure V.13. Hippocampus-dependent learning is not affected in NP^+/− mice. WT and NP^+/− mice were subjected to fear conditioning; the day after the training, they were placed in the same chamber and the freezing behaviour was recorded. Both genotypes froze to the same extent during the context-dependant retrieval as shown by the representative movement traces (yellow bars below each panel indicate lack of movement, A; quantified in B), indicating that the hippocampus-dependent learning was intact in NP^+/− mice. Results are shown as mean ±SEM. The number of animals in each group is indicated in the bars (B).
Figure V.14. Amygdala-dependent learning is impaired in NP<sup>−/−</sup> mice.

WT and NP<sup>−/−</sup> mice were subjected to fear conditioning. Two days after the training, the mice were exposed to the tone (time indicated as the red line on the x-axis) and their freezing behaviour was recorded. NP<sup>−/−</sup> mice froze less than the WT mice as shown by the representative movement traces (yellow bars below each panel indicate lack of movement, A; quantified in B). This result points towards an impaired amygdala-dependent learning of the NP<sup>−/−</sup> mice. Results are shown as mean ±SEM; *p<0.05, Student’s test. The number of animals in each group is indicated in the bars (B).
Figure V.15. Pain threshold is not affected by the deletion of the neuropsin gene. Mice were subjected to electrical footshock of increasing intensity. No difference was found in the graded responses to pain (flinching, jumping and vocalizing in human frequencies) between WT and NP−/− mice. Results are shown as mean ±SEM. The number of animals in each group is indicated in the bars.
V.3. Discussion

Anxiety refers to a broad range of subjective emotional and somatic sensations, often perceived as unpleasant, that can be triggered by a variety of stimuli. Fear and anxiety can be formed as a part of an evolutionarily beneficial and contextually correct adaptive response allowing the organism to recognize, predict and avoid danger. However, severe or prolonged stress can result in maladaptive forms of neuronal plasticity leading to elevated, pathological anxiety that can be socially and evolutionarily detrimental. Although anxiety disorders are often associated with abnormally elevated fear responses to trigger cues, some forms of anxiety involve a generalized panic reaction to neutral cues that are perceived as potentially unsafe.

While the neuroanatomy of fear and anxiety has been extensively studied and is relatively well understood (Phillips & LeDoux, 1992; Amorapanth, 2000; Biedenkapp, 2009; Ponnusamy, 2007; Kolber, 2008; Poulos, 2009), the molecular aspects of emotional responses remain to be elucidated. In this chapter the role of PAR-1 and neuropsin in behavioural response to restraint stress and Pavlovian fear conditioning was investigated. Innate and learned fear were measured in wild-type, PAR-1mice and neuropsinmice using well-characterized behavioural tests such as the elevated-plus maze, open field as well as freezing behaviour during presentation of the conditioned stimulus in the fear conditioning paradigm. Taken together the results show that innate and learned fear levels are elevated in PAR-1mice whereas neuropsinmice display an anxiolytic phenotype compared to their wild-type counterparts.
The role of PAR-1 in behavioural responses has not been extensively studied so far. The only article dealing with the above issue showed, among other tests, normal exploratory and locomotor behaviour as well as unimpaired motor learning and balance in PAR-1<sup>−/−</sup> mice (Almonte, 2007). Also, Almonte et al did not see any differences between the knockout and wild-type animals in the elevated-plus-maze in the absence of stress. However Almonte et al (2007) found deficits in the passive avoidance and conditioned cued fear learning. In contrast, the current study shows that cued conditioning was enhanced in PAR-1<sup>−/−</sup> mice (Figure V.7). How to explain the above differences between the present study and Almonte’s? There are two major differences in the protocol used by Almonte and colleagues. First Almonte’s fear conditioning protocol was much stronger (five pairings, electric shock of 0.7 mA intensity) and much longer (>22 minutes) compared to the protocol used in this work (three pairings and 0.4 mA; duration of 6.5 minutes). Such a strong and long protocol results in a very strong freezing response to the conditioned stimulus in wild-type mice, representing a ceiling effect. Such a pronounced immobility reaction in wild-type animals makes it very difficult to observe any potential increase in the freezing response in simultaneously tested genetically modified mice. In this project a mild conditioning protocol was deliberately used to be able to detect possible bidirectional changes in freezing (increase or decrease) in PAR-1-deficient mice. The observed increase in freezing behaviour of the WT mice after performing a strong protocol (Figure V.10) confirms the absence of a ceiling effect during the mild protocol (Figures V.6 & V.7). Secondly Almonte calculated the percentage of freezing using a tone repeated three to five times for 30 seconds whereas in this work freezing was assessed
during a single one minute tone. The above methodological differences could at least partially explain different results obtained.

Setting methodological differences aside, the results of the present and Almonte’s studies could in fact be complementary. While a mild conditioning protocol would reveal an abnormally low threshold for cued-freezing behaviour in PAR-1\(^+\) mice, a stronger protocol would unmask the inability of the PAR-1\(^{-}\) mice to process the information appropriately and develop adequate coping strategies in response to high-intensity fear-conditioning training. Footshock of high intensity and duration likely resulted in a broad neuronal activation across a number of fear-related brain regions in wild-type mice, thus engaging strong adaptive plasticity-related mechanisms; it is possible that these mechanisms did not function properly in PAR-1\(^{-}\) mice. Thus, the results of the present and Almonte’s studies could show different aspects of a common mechanism, similar to those described by Costa-Mattioli et al (2005). Their study shows, using Morris water maze, that in GCN2\(^{-}\) mice memory is enhanced after weak training while after intense training it is inhibited.

How could PAR-1 accomplish this dual role in response to different stimulus intensities? There are several possible explanations for such a mechanism. First, PAR-1 is known to associate with and signal through different G-proteins in a tissue-specific manner. It is conceivable that PAR-1 could utilize different G-proteins and signal transduction pathways in response to stimuli of different intensity. Such stimuli could result in the liberation of distinct “protease cocktails” into the synapse, and as a consequence, in experience-specific cleavage of PAR-1 at different sites depending on the
stimulus potency. Such “experience-directed” signalling could be achieved either by changing PAR-1/G-protein coupling in response to PAR-1 cleavage or by interacting with a different set of binding partners that would affect PAR-1 conformation and change its signal transduction pathway. Such a hypothesis is supported by previous reports of ligand-biased signalling as a common mechanism regulating GPCR functioning and by studies showing that various ligands lock GPCRs in different conformational states (Rosenbaum, 2009). To my knowledge the hypothesis of “experience-directed” signalling has never been tested. In another set of experiments, not included in this dissertation, our lab demonstrated that PAR-1 G-protein coupling is indeed dynamic and experience-specific (Annexe A3).

Although PAR-1 expression in the cortex and the amygdala are similar as measured by Western blotting (Figure III.3) it is unlikely that cortical PAR-1 is involved in fear learning. First, contextual fear that requires medial prefrontal cortex (Runyan, 2004) is normal in PAR-1+/− mice (Figure V.6). Moreover, another form of learning that requires the hippocampus and cortex, the novel object recognition (Akirav, 2006), is not affected in PAR-1+/− animals. Additionally, selective inhibition of PAR-1 in the amygdala by acute bilateral injection of a PAR-1 antagonist disrupted the acquisition of fear pointing to the involvement of amygdalar PAR-1 in fear learning (Figure V.12).

In this study PAR-1 protein was also found in the hippocampus (Figure III.3). The role of hippocampal PAR-1 is not clear. Interestingly, contextual fear memory was not affected by the absence of PAR-1, even though it has previously been shown to be not only dependent on hippocampus but also on amygdala (Phillips, 1992; Gale, 2004). It is
possible that PAR-1 levels, or its function, may differ among discrete local amygdala
circuits modulating the input from the hippocampus versus those receiving the input from
the auditory thalamus. Unaltered contextual conditioning in PAR-1⁺ mice may reflect
pathway-specific differences in compensatory signalling components (e.g. the expression,
alternative splicing or posttranslational modifications of other PARs, their putative
activating proteases or protease inhibitors) resulting in pathway-specific differences in
signal buffering and/or amplification. This possibility is supported by the finding that
local inhibition of PAR-1 in the amygdala enhanced cued responses while leaving
contextual responses unaltered (Figures V.11 & 12).

The behavioural phenotype of neuropsin⁻/⁺ mouse has been described in several
studies. Tamura (2006) reported normal locomotor activity and an impairment of spatial
learning using the hippocampus-dependent Morris water maze and Y maze tests. This
result was in disagreement with other studies where neuropsin⁻/⁺ animals performed as well
as wild-type in the water maze spatial reference memory task (Davies, 2001). Differences
in experimental conditions or the strategy to knockout the neuropsin gene have been
suggested to be responsible for these discrepancies (Tamura, 2006).

In another study neuropsin⁻/⁺ mice have been subjected to a series of 11 consecutive
behavioural tests (Horii, 2008) that revealed higher anxiety levels by less time spent in the
open arms of the elevated-plus maze as well as an increased cued memory during fear
conditioning test. There are substantial methodological differences between the study by
Horii et al. (2008) and the present work. They sequentially subjected wild-type and
neuropsin⁻/⁺ mice to a comprehensive battery of behavioural tasks that likely have
influenced their performance in subsequent tests. In contrast, in this study the mice were subjected to the elevated-plus maze only once (in fact separate groups of animals were used for each experimental time-point in order to expose the animals to the maze only once) and therefore their performance was not biased by other tests. Moreover Horii et al. (2008) described neuropsin⁻ mice baseline behavioural parameters but did not test the effect of stress on the behaviour of those animals. Out of two measures of anxiety reported in that study only one was slightly affected in neuropsin⁻ mice (time spent in open arms) while the other was not (the number of entries into the open arms, similar to our observations). Thus, notwithstanding important methodological differences between the two studies, they do not report fundamentally different findings.

To confirm the general role of neuropsin in anxiety an additional test of anxiety (the open field) was performed in wild-type and neuropsin-deficient mice. The open field results show, like the elevated-plus maze data, an anxiolytic phenotype of neuropsin⁻ mice (particularly prominent after stress). Showing an anxiolytic phenotype of neuropsin-deficient mice in two different tests rules out nonspecific factors that might have biased this study. Finally the fear conditioning experiment revealed a deficit in cued-freezing and points directly towards a role of neuropsin in amygdala-associated fear responses.

The experiments reported here indicate that PAR-1 and neuropsin play roles in both anxiety and conditioned fear. What is the difference between fear and anxiety? Anxiety arises when the animal is presented with several response options that generate a potential emotional conflict (Barkus, 2010). For example in the elevated-plus maze the mouse can choose between approach or avoidance of the exploration of the open arms. The
difference between fear and anxiety is sometimes described in terms of differences in coping/defensive strategies (McNaughton & Gray, 2000). Fear is directed towards removing the animal from a hazardous environment (active avoidance strategy) or adapting a response that would allow to deal with stimuli previously associated with dangerous situations (freezing during acoustic fear conditioning). In contrast, the level of anxiety determines whether the animal will engage in a potentially dangerous activity (passive coping strategy). Thus, fear and anxiety deal with present and potential danger, respectively.
Chapter VI

PAR-1 signal transduction and regulation of AMPA receptors
VI.1. Introduction

Learning, including amygdala-based paradigms containing an emotional component, is often paralleled by plasticity-like changes in neuronal excitability and LTP. Electrophysiological data from our laboratory (see Annexes A1 & A2; courtesy of Dr. Emanuele Schiavon) indicate that the enhancement of conditioned fear in PAR-1⁺ mice is paralleled by an increase in the late phase of LTP, higher neuronal excitability (current clamp recordings) and frequency of AMPA-receptor dependent miniature excitatory postsynaptic currents (mEPSCs, whole cells recordings) of principal basal amygdala neurons. Indeed, the frequency of activation of AMPA receptors (time decay of the mEPSCs) was found to be significantly elevated in knockout compared to WT after FC. These results point towards a regulation of neuronal excitability and plasticity after fear conditioning by PAR-1-mediated modulation of AMPA receptors.

How PAR-1 could modify AMPA receptor properties? In this chapter the current state of knowledge regarding factors modulating AMPA receptor properties have been described. Moreover, the regulation of some of these modulators (stargazing gene expression, AMPA-receptor phosphorylation, expression and splice variants) by conditioned fear has been investigated experimentally.

VI.1.1. PAR-1 transduction pathways

PAR-1 coupled G-proteins activation triggers cellular signalling through diverse pathways. For example, the monomeric G protein RhoA has been implicated in hippocampal neuronal death after PAR-1 activation by thrombin (Donovan, 1997).
Neurite retraction and calcium mobilization are also downstream of RhoA activation (Chong, 1994; Smirnova, 2001). Activation of PARs initiates protein kinase signal-transduction pathways such as MAPK signalling cascade (Jiang, 2002), p38 and p44/42 mitogen-activated protein kinases (Choi, 2003). PAR-1 activation stimulates proliferation of astrocytes via Gβγ protein and phosphatidylinositol 3-kinase but also via Gαi and the PKC pathway (Wang, 2002). Activation of ERK1/2 leads to astroglial proliferation (Wang, 2002) and astrogliosis after brain injury in vivo (Nicole, 2005). MAPKs are also involved in PAR-1 mediated neuroprotection (Jiang, 2002; Xi, 2001).

PAR-1 activation by thrombin potentiates NMDAR responses in rodent hippocampal neurons or recombinant system (Gingrich, 2000). This effect is restricted to receptors containing NR1/NR2A or NR1/NR2B subunits. Leakage of thrombin into the brain during hemorrhagic stroke could then contribute to the NMDA receptor-dependent expansion of neuronal injury (Lee, 1999). Similar role might be played by other proteases, like plasmin, generated at high levels in the hemorrhagic brain (Tsirka, 1995 & 1997). Plasmin is known to activate PAR-1 (Ishihara, 1997) and to potentiate NMDA receptor current (Mannaioni, 2008).

PAR-1 signalling pathways have been described in detail in Chapter I.

VI.1.2. CREB and c-Fos as markers of synaptic plasticity

Neuronal stimulation triggers intracellular elevations of Ca²⁺ and cAMP that in turn activate the nuclear cAMP response element-binding protein CREB (Ghosh &
CREB binds to the promoter regions of early response genes including *c-fos* (Sheng, 1990) to regulate their expression. Phosphorylation of CREB is considered as a marker of synaptic plasticity because activation of NMDA receptors, postsynaptic calcium release and calcium-dependent CaM kinase activity are necessary for plasticity through activation of CREB (Deisseroth, 1996). CREB has also been studied in relation to learning and memory processes in rodents. This transcription factor has been shown in the contextual fear conditioning task to play a role in long- but not short-term memory in the hippocampus (Bourtchuladze, 1994). CREB overexpression in the basolateral complex of the amygdala of rats enhances the formation of long-term memory following sustained training (Josselyn, 2001). CREB phosphorylation/activation and c-Fos expression were induced in the basolateral and central nuclei of the amygdala after cued-retrieval; increased P-CREB immunoreactivity was also detected in the lateral amygdala. No changes were observed in the hippocampus and dentate gyrus (Hall, 2001).

The immediate early gene *c-fos* is a marker of neuronal activity (Sagar, 1988) and the protein is synthesized after fear conditioning (Milanovic, 1998). Hippocampal expression of c-Fos protein following LTP induction has been reported in connection with CREB phosphorylation by activated CaMKIV and MAPK (Kasahara, 2001; Kang, 2001). c-Fos protein expression is also triggered by novelty in the rat brain (Handa, 1993; Papa, 1993). The amygdala is required for fear conditioning and its sub-nuclei differentially express c-Fos in a stimulus-dependent manner. Indeed one hour after fear conditioning c-Fos levels increase in the central nucleus of the amygdala and to a lesser extent in the basolateral nucleus due to the shock itself, whereas exposure to a tone
resulted in higher c-Fos levels in the basolateral nucleus of the amygdala (Radulovic, 1998).

VI.1.3. AMPA receptors

AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors are localized in the central nervous system and are ionotropic transmembrane receptors for glutamate as well as cation channels mediating fast excitatory synaptic transmission (Mayer, 1987). Presynaptically released-glutamate binding to AMPA receptor leads to sodium influx into the postsynaptic cell and depolarization that contributes to the unblocking of NMDA receptor by magnesium. The resulting calcium influx through NMDA receptor contributes directly or via activation of CaMKII to AMPA receptor upregulation at the membrane and elevation of their single-channel conductance.

Four homologous subunits GluR1 to GluR4 (alias GluRA-D or GluA1-A4) can be combined together to form AMPA receptor; each subunit contains an extracellular Nt domain, three transmembrane domains and an intracellular Ct (Hollmann, 1994). In the hippocampus, GluR1-GluR2 and GluR2-GluR3 complexes (important for constitutive expression and cycling of AMPA receptor (Shi, 2001)) are predominant in the pyramidal cells.

Each AMPAR subunit exists as two forms, flip and flop (i/o) due to alternative splicing in the extracellular region that precedes the Ct (Sommer, 1990). Both isoforms are expressed throughout the development, the flip form being predominant in the whole
brain whereas the flop form is gradually recruited after birth especially in the cortex, hippocampus and caudate nucleus (Monyer, 1991). The two isoforms differ by only a few amino acids but present distinct desensitization kinetics (Mosbacher, 1994). For instance, although opening rate constants are comparable for both GluR1-2-3 flip and flop isoforms, GluR2-, 3- and 4-flop variants desensitize faster than their flip form by promoting a more rapid closure of the channel; the GluR1 variants display similar closing properties (Mosbacher, 1994; Quirk, 2004; Pei, 2007 & 2009). Similarly, heterodimers display distinct desensitization time constants according to their composition (Mosbacher, 1994).

AMPA receptor properties of calcium permeability are conferred by a post-transcriptional modification in the Q/R editing site of the GluR2 subunit that is present in most of the receptors (Sommer, 1991); thus in the CNS, a large majority of these subunits are under the GluR2(R) form so that AMPA receptors are impermeable to calcium (Geiger, 1995).

VI.1.4. AMPA receptor interaction with proteins

Protein-protein interactions are important to regulate the localization and trafficking of AMPA receptor subunits. Depending on the C-t domain subunit receptors can interact with different molecules. GluR2 and 3 can be bound to anchor proteins glutamate receptor-interacting protein (GRIP) and AMPA receptor binding protein (ABP) that maintain the receptors at the synapse (Osten, 2000). This anchorage terminates when the receptors are phosphorylated and consequently bound to protein interacting with C-
kinase-1 ( PICK-1) that acts as a calcium sensor involved in AMPAR internalization and NMDA-dependent LTD (Hanley, 2005; Seidenman, 2003). The interaction of GluR2 with PICK1 can be disrupted by the ATPase NSF ( N-ethylmaleimide-sensitive fusion protein) that interacts with GluR2 (Hanley, 2002).

VI.1.4.a. Neuronal activity-regulated pentraxin (Narp)

Synaptic activity regulates expression of Narp, an immediate-early gene found in rat neurons of the hippocampus, dentate gyrus and cortex (Tsui, 1996; O’Brien, 1999). In cultured rat hippocampal or spinal neurons, Narp colocalised at excitatory synapses with GluR1 in aspiny neuronal cells. This extracellular protein (Tsui, 1996) has been shown to interact with —directly or via Narp-Narp connections- and cluster glutamate receptors even on opposing cells (O’Brien, 1999 & 2002). This secreted protein is thought to facilitate the formation of new excitatory synapses and to directly aggregate AMPAR at postsynaptic dendrites thus increasing and/or strengthening excitatory synapses (O’Brien, 1999 & 2002).

VI.1.4.b. Cornichon proteins

Cornichons have been described as cargo proteins for trafficking of EGFR ligands (Bokel, 2006). Cornichon homolog 2 (CNIH-2) and CNIH-3 have been co-purified with all four AMPAR subunits from the rat brain (Schwenk, 2009). They are expressed in most brain regions – neocortex, hippocampus- on neurons and astrocytes but interestingly, not
in the cerebellar granule cells where TARPs are found (see paragraph VI.1.4.c). CNIIH-2 and 3 are localized at postsynaptic sites, dendritic shafts and spines in the CA1 region. Cornichon proteins increase the number of receptors at the plasma membrane and modulate gating properties of AMPAR leading to stabilization of the molecules in an open state (Schwenk, 2009). As described by Schwenk, CNIIH-2 and 3 considerably slow the deactivation and desensitization time decays of different AMPAR subunit assemblies without affecting their recovery from desensitization (Schwenk, 2009).

VI.1.4.c. Transmembrane AMPA receptor Regulatory Proteins

TARPs are composed of Stargazing or γ-2, γ-3, γ-4 and γ-8 that promote surface expression of mature AMPAR (Tomita, 2003); expression of these proteins is differentially regulated throughout development, γ-4 tending to decrease and others to increase after birth. In the rat brain, stargazin is found in cerebellum, cerebral cortex, hippocampus/CA3 and dentate gyrus, γ-3 in the forebrain and cortex. γ-4 localisation is diffused and slightly higher expressed in corpus striatum and in glia whereas γ-8 is enriched in the hippocampus, cortex and olfactory bulb (Tomita, 2003; Sharp, 2001; Fukaya, 2005). They are expressed in the dendritic cytoplasm before being localised at the post-synaptic side of excitatory synapses where they are bound to specific AMPAR subunits (Tomita, 2003).

Stargazin interacts with all AMPAR subunits and also binds to the synaptic scaffolding protein PSD-95 (Chen, 2000). This TARP is highly expressed in cerebellar granule cells and has spontaneously mutated in Stargazer mice which are epilepsy-free and
display cerebellar ataxia. Their cerebellum lacks any AMPA receptors (Letts, 1998; Noebels, 1990) and this absence can be reversed by transfection of cerebellar granule cells with stargazing (Chen, 2000). Stargazin mediates AMPA receptor export from the endoplasmic reticulum (ER; Tomita, 2003) to the plasma membrane (by obstructing some ER retention signals; Bedoukian, 2006) and then to the synapse (Chen, 2000; Cuadra, 2004) where thanks to its binding to PSD-95 it stabilizes and traps the receptor at the postsynaptic membrane (Priel, 2005; Bats, 2007). Stargazin seems also to interact in the ER with the flop isoform of the GluR4 subunit that is less efficiently exported in cells lacking TARPs; therefore stargazing transfection rescues flop-GluR4 levels at the plasma membrane (Coleman, 2006).

Stargazin is able to modulate the biophysical properties of AMPA receptor channels and therefore the synaptic responses. Indeed stargazin and γ-3, and to a lesser extent γ-4 and γ-8 potentiated responses to kainate for GluR1i/o and GluR1i/o-GluR2i/o dimers (Tomita, 2005). Following glutamate stimulation of Xenopus oocytes, HEK and tsA201 cells, stargazin slows GluR1i/o, GluR2i and GluR4-containing receptors desensitization and deactivation while increasing their recovery toward the open state (Priel, 2005; Turetsky, 2005; Tomita, 2005; Cho, 2007) thus enhancing glutamate-evoked current (Tomita, 2005). This effect makes the AMPA receptors significantly more responsive to glutamate when bound to stargazing and possibly other TARPs (Tomita, 2005).
VI.1.5. LTP and LTD-related AMPA receptor modulation

AMPA receptors are glutamate-gated channels that mediate fast excitatory synaptic transmission responsible for most of the postsynaptic depolarization that induce neuronal firing. Glutamate binding opens the receptor which rapidly desensitizes (with a time constant \( \tau = 1-15 \) ms) and closes upon glutamate removal with a \( \tau \) deactivation of 1-3 ms. They are regulated by phosphorylation/dephosphorylation by PKA, PKC and CaMKII.

Silent synapses contain NMDA- but not AMPA- receptors (Isaac, 1995; Liao, 1995); they acquire the latter following LTP and the receptors are primarily constituted of GluR1 and GluR4 (for review see Bredt, 2003). The apparent single-channel conductance of GluR1 is increased by phosphorylation of the receptor by CaMKII during LTP induction (Barria, 1997, Lee, 2000). Phosphorylation of GluR1 by PKA and CaMKII and of GluR4 by PKA drives these receptors to the synapse (Esteban, 2003).

Plant (2006) has shown that in the early phase of hippocampal LTP, GluR2-lacking calcium permeable AMPA receptors were incorporated at synapses of principal neurons to stabilize and conserve the increase in synaptic strength before being replaced by calcium-impermeable AMPA receptors containing GluR2 subunit. The primary role of GluR2/3 subunit is to stabilize synaptic activity. Indeed, basal synaptic transmission is reduced in mice lacking both GluR2/3 subunits; however their absence did not block expression of enhanced levels of NMDA receptor- dependent LTP and LTD in the hippocampus leading to the hypothesis that these subunits might play a role in restricting synaptic changes (Meng, 2003). As the above double knockout mice were able to express LTP and

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LTD due to the presence of GluR1-containing AMPA receptor, the latter subunit emerges as an indispensable molecule for expression of synaptic plasticity.

During LTD, removal of AMPA receptors from synapses occurred by either phosphorylation or dephosphorylation of AMPA receptor subunits (Chung, 2000; Lee, 2000). Phosphorylation of GluR2 subunit by PKC allows a rearrangement of this subunit with other proteins (GRIP, PICK-1) and is critical for induction of LTD in the cerebellum (Chung, 2003).

Constitutive cycling occurs at the synapse independently of synaptic activity in order to maintain and/or stabilize synaptic strength. It was originally thought that cycling of GluR1-lacking receptors in and out of the synapse underlies this process (Malinow, 2002) but GluR1 homomers are involved as well (Plant, 2006) in a region-dependant manner (Kessels, 2009). The AMPA receptor subunit composition in distinct amygdala nuclei is not known. However Humeau et al have demonstrated that the makeup of AMPAR varies according to the studied pathway (thalamo or cortico-amygdala pathways) and therefore influences the activity-dependent synaptic plasticity (Humeau, 2007). In the lateral amygdala, more than 80% of postsynaptic thalamic auditory spines were immunopositive for GluR2 and GluR3 subunits with a large GluR2 cytoplasmic pool (Radley, 2007).
VI.1.6. AMPA receptors and fear conditioning

Fear conditioning has been shown to induce a rapid expression of GluR1 (Yeh, 2006) and GluR1 delivery at specific thalamo-lateral amygdala synapses is essential for associative-learning processes (Rumpel, 2005). Freezing behaviour has been studied in GluR1- or GluR3 knockout mice after fear conditioning (Humeau, 2007) and only mice lacking the GluR1 receptor failed to acquire cued- and context-conditioned freezing. This behaviour was only delayed in GluR3 knockout animals. In both genotypes LTP was reduced at cortico-LA synapses whereas LTP was impaired at thalamo-LA synapses in GluR1/− but not GluR3/− animals (Humeau, 2007). Also, thalamo-amygdala LTP is blocked when GluR1 trafficking is disrupted and the acquisition of auditory fear conditioning is only partial (Rumpel, 2005).
VI.2. Results

VI.2.1. Role of PAR-1 in synaptic activity after stress

Wild-type and PAR-1\(^+\) mice were subjected to fear conditioning and sacrificed one hour later together with naïve mice of both genotypes. Immunohistochemistry was performed on free-floating brain sections to stain c-Fos and P-CREB positive cells in the basolateral amygdala (Figures VI.1 and 2, respectively).

Two-way ANOVA revealed a main effect of genotype, treatment and a significant genotype x treatment interaction for both c-Fos and P-CREB (Table VI.1).

---

### Table VI.1. c-Fos and P-CREB: two-way ANOVA results.

<table>
<thead>
<tr>
<th>Activity measure</th>
<th>Effect</th>
<th>F value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>genotype</td>
<td>F(1,27)=6.99</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>treatment</td>
<td>F(1,27)=106.89</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>genotype x</td>
<td>F(1,27)=6.72</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c-Fos</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>genotype</td>
<td>F(1,19)=10.55</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>treatment</td>
<td>F(1,19)=84.94</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>genotype x</td>
<td>F(1,19)=4.95</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-CREB</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(n= 5 to 10 animals)
Figure VI.1. c-Fos induction in the basolateral amygdala is higher in PAR-1⁻/⁻ mice than in WT after fear conditioning.

WT and PAR-1⁻/⁻ mice were subjected to fear conditioning and culled one hour later. Brains were removed, fixed and cut into 70μm thick sections. Immunohistochemistry was performed using an anti-c-Fos antibody (red, A and B) together with DNA marker Toto-3 (purple, A). Dotted lines delineate the basolateral amygdala. Squares demarcate the magnified zones seen in (B). LA: lateral amygdala; BLA: basolateral amygdala.
Figure VI.2. P-CREB induction in the basolateral amygdala is higher in PAR-1⁻/⁻ mice than in WT after fear conditioning. WT and PAR-1⁻/⁻ mice were subjected to fear conditioning and culled 1 hour later. Brains were removed, fixed and cut into 70μm thick sections. Immunohistochemistry was performed using an anti-P-CREB antibody (red, A and B) together with DNA marker Toto-3 (purple, A). Dotted lines delineate the basolateral amygdala. Squares demarcate the magnified zones seen in (B). LA lateral amygdala – BLA basolateral amygdala.
Both neuronal activity markers were significantly elevated following fear conditioning in both genotypes (Figures VI.3 and VI.4; Table VI.2). However, the fear-induced increase in both c-Fos and P-CREB levels were more pronounced in PAR-1−/− mice when compared to wild-type animals.

Table VI.2. c-Fos and P-CREB: Tukey’s post-hoc results.

<table>
<thead>
<tr>
<th>Synaptic marker</th>
<th>Compared groups</th>
<th>Mean ± SEM</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-Fos</td>
<td>PAR-1−/− control and FC</td>
<td>13.31 ± 1.44 and 75.72 ± 6.47</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>WT control and FC</td>
<td>13.05 ± 2.92 and 50.44 ± 6.74</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>PAR-1−/− FC and WT FC</td>
<td>75.72 ± 6.47 and 50.44 ± 6.74</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>P-CREB</td>
<td>PAR-1−/− control and FC</td>
<td>14.88 ± 1.53 and 39.95 ± 3.85</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>WT control and FC</td>
<td>12.63 ± 1.61 and 27.95 ± 1.37</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>PAR-1−/− FC and WT FC</td>
<td>39.95 ± 3.85 and 27.95 ± 1.37</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
Figure VI.3. *c-Fos* expression in the basolateral amygdala is higher in PAR-1−/− mice than in WT after fear conditioning. WT and PAR-1−/− mice were subjected to fear conditioning and culled one hour later. Immunohistochemistry was performed on thick brain sections and quantification revealed that *c-Fos* levels were significantly increased after fear conditioning in WT and PAR-1−/− mice. However, this increase was 50% higher in PAR-1−/− mice compared to WT. Results are shown as mean ±SEM; **p<0.01, ***p<0.001, Tukey’s post-hoc test. The number of animals in each group is indicated in the bars.
Figure VI.4. P-CREB expression is higher in the basolateral amygdala of PAR-1⁻/⁻ mice than of WT after fear conditioning. WT and PAR-1⁻/⁻ mice were subjected to fear conditioning and culled one hour later. Immunohistochemistry was performed on thick brain sections and quantification revealed that P-CREB levels were significantly increased after fear conditioning in WT and PAR-1⁻/⁻ mice. However, this increase was 50% higher in PAR-1⁻/⁻ mice compared to WT. Results are shown as mean ±SEM; **p<0.01, ***p<0.001, Tukey’s post-hoc test. The number of animals in each group is indicated in the bars.
VI.2.2. AMPA receptor subunit phosphorylation

AMPA receptors play a role in synaptic plasticity and LTP (Man, 2003; Watt, 2004). Synaptic and extrasynaptic trafficking of GluR2-containing AMPA receptors have been described during synaptic plasticity (Lu, 2005) that might result from altered protein-protein interactions due to AMPA receptor subunit phosphorylation. Electrophysiological studies conducted in our laboratory revealed unusually high levels of fear-induced L-LTP in PAR-1⁻ mice. GluR1 phosphorylation levels were similar in both genotypes in control and fear conditioned mice (two-way ANOVA p>0.05; Figure VI.5, A and B). A main effect of the treatment emerged for GluR2 subunit phosphorylation levels (F_{1,20}=7.93, p<0.014), with the PAR-1⁻ levels of P-GluR2 being decreased after fear conditioning compared to control (100 ± 8.46 and 62.45 ± 7.42 respectively; Tukey’s post-hoc, p<0.05; Figure VI.5, C).

VI.2.3. Stargazin gene expression

Since stargazing has been shown to influence the time decay of the AMPA receptors, its gene expression was examined by qRT-PCR in the amygdala of WT and PAR-1⁻ animals. Stargazin levels were similar in both genotypes in control conditions (Figure VI.6) and were not affected by fear conditioning (two-way Anova p>0.05; Figure VI.6).
Figure VI.5. Fear conditioning led to a significant decrease in GluR2 phosphorylation levels in PAR-1<sup>−/−</sup> mice. WT and PAR-1<sup>−/−</sup> mice were subjected to fear conditioning and sacrificed straight after. Amygdalae were removed and Western blots for total (T) or phosphorylated (P) forms of GluR1 and GluR2 were performed. GluR1-phosphorylation was not affected by fear conditioning in any of the genotypes (A, B) whereas P-GluR2 levels were decreased after fear conditioning by 24% in WT and significantly by 37% in PAR-1<sup>−/−</sup> mice (A, C). Results are shown as mean ±SEM; *p<0.05, Tukey’s post-hoc test. The number of animals in each group is indicated in the bars.
Figure VI.6. Stargazin mRNA levels are not affected by fear conditioning in both WT and PAR-1<sup>−/−</sup> mice. Mice were subjected to fear conditioning and culled two days later. Brains were removed, amygdalae were dissected and qRT-PCR was performed to quantify stargazin mRNA levels. Stargazin expression after fear conditioning amounted to 68 and 77% of the control levels in WT and PAR-1<sup>−/−</sup> mice, respectively (difference not significant). Results are shown as mean ±SEM. The number of animals is indicated in the bars.
VI.2.4. AMPA receptor subunit’s gene expression

Another mechanism that could be responsible for an adjustment of the time decay is a modification of the AMPA receptor subunit composition. Therefore, GluR1-4 gene expressions in the amygdala of wild-type and PAR-1\textsuperscript{-}/ mice were assessed by qRT-PCR in control conditions and two days after fear conditioning. Total expression of GluR1 and GluR3 were not affected by fear conditioning (Figure VI.7, A and C). Two-way ANOVA analysis of the levels of GluR2 total expression revealed a significant genotype x treatment interaction ($F_{0.20} = 7.49$, $p<0.05$) due to a decrease of GluR2 in WT mice after FC (control 100 ± 11.18 and FC 63.80 ± 6.47; Tukey’s post-hoc, $p<0.05$, Figure VI.7, B). As for GluR4 expression, ANOVA analysis showed a marked effect of genotype ($F_{0.20} = 5.02$, $p<0.05$), treatment ($F_{0.20} = 14.83$, $p<0.001$) and a genotype x treatment interaction ($F_{0.20} = 9.52$, $p<0.01$). More specifically, GluR4 expression was lower in PAR-1\textsuperscript{-}/ compared to WT in control conditions (55.53 ± 4.72 and 100 ± 13.16, respectively; Tukey’s post-hoc, $p<0.01$, Figure VI.7, D).

Total GluR4 expression was not affected by fear conditioning in PAR-1\textsuperscript{-}/, but was significantly reduced after FC in wild-type mice (100 ± 13.16 and 49.15 ± 8.17, respectively; $p<0.001$, Figure VI.7, D). Those results show that the total expression of both GluR2 and GluR4 is differentially regulated in PAR-1\textsuperscript{-}/ mice compared to WT in control conditions and/or after FC.
Figure VI.7. Genotype and fear conditioning effect on AMPA receptor subunit total expression in WT and PAR-1−/− mice.
Mice were subjected to fear conditioning and culled two days later. Brains were removed, amygdalae dissected and qRT-PCR was performed to quantify the expression of each AMPA receptor subunit. While GluR1 and GluR3 expression (A, C) were similar in WT and PAR-1−/− animals, GluR2 levels were reduced after FC in WT but not in PAR-1−/− mice (B). Differences were most pronounced for GluR4 levels; PAR-1−/− animals had lower levels of GluR4 (D) compared to WT in control condition. GluR4 expression remained unchanged after FC in PAR-1−/− mice while it was dramatically reduced in WT. Results are shown as mean ±SEM; *p<0.05, **p<0.01, ***p<0.001, Tukey’s post-hoc test. The number of observations is indicated in the bars.
Because the flip-flop isoforms have been implicated in modulating AMPA receptor
desensitization speed, resensitization and gate closing (Mosbacher, 1994; Sommer, 1990;
Pei, 2007), the flip-flop ratio of each subunit were analysed in control and fear
conditioned animals. No genotype- nor fear-related difference was seen for GluR1 and
GluR2 subunits (Figure VI.8, A and B). Concerning GluR3 i/o ratio, two-way ANOVA
reveals a significant genotype x treatment interaction ($F_{(1,20)}=13.98$, p<0.01). Post-hoc
comparison indicated lower GluR3 i/o ratio in PAR-1−/− mice than in WT in control
conditions ($3.67 \pm 0.58$ and $8.40 \pm 0.50$, respectively; p<0.01). Fear conditioning
triggered opposite effects in the flip-flop ratio between genotypes; it was reduced in WT
($8.40 \pm 0.50$ and $5.52 \pm 0.98$ respectively; ns) whereas it was increased in knockout
animals (control $3.67 \pm 0.58$ and FC 7.26 ±1.03; p<0.05, Figure VI.8, C). Finally for
GluR4 subunit, both genotypes display similar GluR4 i/o ratio in control conditions.
There was a treatment effect ($F_{(1,20)}=5.66$, p<0.05) together with a genotype x treatment
interaction ($F_{(1,20)}=4.29$, p<0.05) found by ANOVA, due to a decrease of GluR4 i/o ratio
after FC in wild-type mice (control $1.92 \pm 0.23$ and FC $0.99 \pm 0.09$; p<0.05, Figure
VI.8, D). The above results point up towards a role of the GluR3 and GluR4 subunit
alternative splicing in fear-induced PAR-1 signal transduction.
Figure VI.8. Genotype and fear conditioning effect on flip-flop ratio of AMPA receptor subunit in WT and PAR-1⁻/⁻ mice.
Mice were subjected to fear conditioning and culled two days later. Brains were removed, amygdalae dissected and qRT-PCR was performed to quantify the expression of each AMPA receptor subunit. GluR1 and GluR2 flip-flop isoforms (A and B, respectively) were similar in both genotypes and unaffected by fear conditioning. GluR3 flip-flop ratio of PAR-1⁻/⁻ mice was lower than that of WT in control conditions (C) and was increased after FC while diminished in WT (C). GluR4 flip-flop ratio was decreased in WT mice after FC but remained unchanged in PAR-1⁻/⁻ (D). Results are shown as mean ±SEM; *p<0.05, **p<0.01, Tukey’s post-hoc test. The number of observations is indicated in the bars.
VI.3. Discussion

In this chapter neuronal activation in the basolateral amygdala following fear conditioning using c-Fos expression and phosphorylation of cAMP regulatory element binding protein CREB was studied. These parameters were studied because both the increase in c-Fos expression and phosphorylation of CREB are widely accepted as markers of neuronal activation following various learning tasks and have been extensively studied in fear conditioning (Bourtchuladze, 1994; Josselyn, 2001; Hall, 2001).

The results showing that in wild-type mice, c-Fos and P-CREB levels were increased following fear conditioning are consistent with the literature (Bourtchuladze, 1994; Josselyn, 2001; Radulovic, 1998). PAR-1+/− mice were found to display higher P-CREB levels together with elevated FOS expression in the basolateral amygdala compared to wild-type after fear conditioning. This result suggests an increased susceptibility/sensitivity of knockout animals to the conditioning task and is consistent with enhanced fear learning in PAR-1+/− mice when compared to their wild-type counterparts.

The results from our laboratory indicate that the increase in fear-associated memory observed in PAR-1-deficient animals (Figure V.7) is accompanied by changes in a number of neuronal plasticity-related parameters in the basal nucleus of the amygdala. In particular, we found an increase in the late phase of LTP, higher neuronal excitability and frequency of AMPA-receptor dependent miniature excitatory post-synaptic currents of excitatory basal amygdala neurons. The frequency of activation of AMPA receptors (time decay of the mEPSCs) was found to be significantly elevated in knockout compared to
WT after FC (Annexe A2). These results suggest that neuronal excitability and plasticity after fear conditioning are affected by PAR-1 through modulation of AMPA receptors.

Stargazin is a modulator of AMPA receptor channel properties (Tomita, 2005) and could potentially contribute to the differences in plasticity-related changes and differences in the AMPA receptor currents observed after fear conditioning between PAR-1⁻/⁻ and wild-type mice. However, stargazing’s gene expression is similarly regulated by fear conditioning in both genotypes and therefore is unlikely to play a major role in modulation of AMPA receptors in PAR-1⁻/⁻ mice. It cannot however be excluded that stargazing protein levels could be altered in PAR-1⁻/⁻ animals as compared to wild-type mice and further experiments are needed to address such a possibility. The literature shows that stargazing’s expression in the amygdala is relatively low compared to other brain regions (Tomita, 2003) which indicates that it could play a relatively minor role in regulating electrophysiological properties of AMPA receptors in this region.

Thus the involvement of AMPA receptors regulatory proteins in the modification of electrophysiological properties of AMPA receptors in mice lacking PAR-1 remains to be elucidated; it might be caused by other AMPA receptor auxiliary proteins like CNIH-2 and 3 which are both expressed in the amygdala (Lein, 2007) and have been reported to slow desensitization (Schwenk, 2009), or by some other yet unidentified molecules.

Electrophysiological properties of AMPA receptors can also be regulated by phosphorylation (Song & Huganir, 2002). For example, it has been demonstrated that phosphorylation of serine 831 in the GluR1 subunit by CaMKII regulates the ion channel properties of GluR1-containing AMPA receptors (Derkach, 1999). In the present work a
decrease of GluR2 phosphorylation in PAR-1<sup>−/−</sup> mice could reduce synaptic inhibition because GluR2/R3 subunits are thought to play a role in restricting synaptic changes (Meng, 2003). The above finding supports the idea of an inhibitory role of PAR-1 in neuronal plasticity and behavioural responses associated with fear conditioning.

mRNA expression by qRT-PCR is a rough but informative estimate of the subunit composition of AMPA receptors; their mRNA levels correlate well with the channel properties of AMPA receptor (Jonas, 1994; Geiger, 1995). AMPAR desensitization time depends on the subunit combination (Mosbacher, 1994), which is unknown in the basolateral amygdala. In fear-naïve animals, the time decay of AMPAR was found to be similar in both genotypes (Annexe A1) suggesting that a lower expression of GluR4 subunits in PAR-1<sup>−/−</sup> mice does not play a major role in regulating this parameter or it is counterbalanced by another compensatory mechanism. After fear conditioning, total expression of GluR1 and 3 subunits was not affected by either genotype; this was not the case for the total amount of GluR2 and 4 that was significantly decreased by fear learning in wild-type but not PAR-1 knockout animals. A fear-learning-dependent redistribution of those two subunits might play a key role for maintaining time decay to a certain level in wild-type mice whereas the absence of these changes in PAR-1<sup>−/−</sup> mice could contribute to faster desensitization of the receptor.

The GluR1 and 2 flip-flop ratios were comparable for both genotypes and remained unchanged after fear conditioning therefore being unlikely to play a role in the time decay variation. Differences for GluR3- and 4- i/o ratio in control and/or after fear conditioning suggest that these subunits might be involved in PAR-1-related time decay
regulation. However, these results should be interpreted with caution. Lack of in vivo literature data describing electrophysiological properties of AMPA receptors of various subunit compositions and their flip-flop variants makes the interpretation of these results difficult and speculative. Moreover, it is unclear whether the observed changes were causal or compensatory in respect to fear-induced changes in electrophysiological properties of AMPA receptors.

The above data provide for the first time a global picture of fear-induced changes in the overall expression and splice variant regulation of AMPAR in the basolateral amygdala and its modulation by PAR-1. Further studies are required to clarify if the changes observed are cell-type specific and whether they are restricted to cells directly activated by fear conditioning or rather modulatory neurons within the fear circuit. Altogether these results show that AMPA receptors-related differences precipitated by the lack of PAR-1 might promote higher neuronal excitability and neuronal plasticity in PAR-1<sup>-</sup> mice.
Chapter VII

Conclusion
This work assembles molecular and behavioural data describing the role of the protease-activated receptor-1 in the mouse brain. A model for a PAR-1 role issued from the principal findings of this work is represented in Diagram VII.1. PAR-1 is found to be highly expressed in neuronal cells of limbic areas, particularly prominent in the amygdala, a brain region implicated in behavioural responses to stress. PAR-1 protein levels are decreased after stressful stimuli consistent with its cleavage by proteases. However, the exact site and consequences of this cleavage are not known. If neuropsin activates PAR-1 in the amygdala then genetic disruption of either molecule should have similar behavioural consequences in amygdala-dependent tasks. On the other hand, if neuropsin cleaves PAR-1 downstream of the thrombin cleavage site and inactivates the receptor then the behavioural phenotypes of PAR-1$^{-/-}$ and neuropsin$^{-/-}$ mice should be opposite. Indeed, mice lacking PAR-1 display opposite behavioural responses to restraint stress compared with neuropsin-deficient animals (increase and decrease in anxiety levels, respectively), raising a possibility that neuropsin may inactivate PAR-1.

Unconditioned (innate) and conditioned (learned) fear have both overlapping and distinct neuronal circuits and molecular mechanisms. To investigate the involvement of PAR-1 in learned fear and its possible modulation by neuropsin, mice were subjected to fear conditioning. Similarly to unconditioned fear, PAR-1$^{-/-}$ mice demonstrated an increase and neuropsin$^{-/-}$ animals a decrease in behavioural responses to this paradigm. Enhanced fear in PAR-1$^{-/-}$ animals was corroborated at a cellular level by an enhanced neuronal activity and excitability. These results indicate general and opposing roles of PAR-1 and neuropsin in regulating amygdala functions.
Diagram VII.1. PAR-1 plays an inhibitory role in the basolateral amygdala during a fear-learning event.
LA lateral amygdala; BA basal amygdala; CeA central amygdala; ▲ Excitatory principal neurons; ● Inhibitory interneurons. The amygdala inhibitory circuitry schema is simplified from Ehrlich (2009).
Diagram VII.1 PAR-1 plays an inhibitory role in the basolateral amygda during a fear-learning event.

In the current work PAR-1 was enriched in the mouse neurons of the amygdala, a brain region implicated in learning and emotion. Mice lacking PAR-1 are characterised by an increased neuronal activity as shown by elevated c-Fos and P-CREB immunoreactivity in the basolateral (BLA) nuclei (Figures VI.1-4), pointing to a lack of inhibition. This hypothesis was strengthened by the finding that the absence of PAR-1 in mice exacerbates tone- but not context- dependent fear learning (Figure V.7); this could reflect the free expression of some excitatory mechanisms that are not anymore under PAR-1-inhibitory regulation. In response to an aversive experience PAR-1 is cleaved (Figures IV.2-4). The findings that PAR-1 was cleaved in vitro by neutropsin (Figure IV.7) and that NP-1 animals display impaired-cued learning (Figure V.14) suggests a possible connection between the two molecules but further experiments are necessary to consolidate this idea.

AMAR-related differences were found (phosphorylation levels and subunits expression) between wild-type and PAR-I- mice (Figures VI.5, 7 & 8). This could be due to a compensatory mechanism due to the absence of the protein, or may reflect a close relationship between PAR-1 and the AMPAR (i.e. activation of PAR-1 would modify the pre-cited AMPAR features).

The inhibitory role of PAR-1 could be explained by its localisation on BLA inhibitory interneurones (and possibly in other nuclei of the amygdala- this has not been studied here). Immunohistochemistry revealed the presence of the PAR-1 protein in almost all neurons of the amygdala (Figure III.5). As specific activation of fear neurons by a given conditioned stimulus was shown recently by Herry (2008), it is possible that PAR-1 might be activated only in the subset or subsets of neuronal cells that are activated in response to fear conditioning. The extended c-Fos and P-CREB activation over the BLA suggests that PAR-1 inhibition targets a wide range of cells within these nuclei. PAR-1 inhibitory action would modulate memory and behaviour so that in response to a fearful event of mild intensity, the mice freezing levels are kept moderate.
In addition to its effect on PAR-1, neuropsin could use other substrates to modulate anxiety and fear. It has been demonstrated that neuropsin cleaves a presynaptic adhesion molecule L1-NCAM (Matsumoto-Miyai, 2003) or fibronectin (Shimizu, 1998), both of which are expressed in the limbic system (Munakata, 2003; Hoffman, 1998). Our lab have recently demonstrated that neuropsin cleaves a postsynaptic tyrosine kinase receptor EphB2 (this cleavage occurs upon stress in the amygdala and facilitates the dynamic interaction of EphB2 with the NMDA receptor, thus enhancing NMDA current and promoting anxiety; Attwood, 2011). PAR-1 has also been shown to regulate NMDA receptor-signalling (Lee, 2007; Mannaioni, 2008) raising a possibility that the PAR-1 and EphB2 pathways might interact, directly or indirectly. It is not yet known whether neuropsin-mediated cleavage of PAR-1 contributes to EphB2-dependent anxiogenic effect.

Fear conditioning results in adjustments of multiple receptor and neurotransmitter systems in the amygdala. The roles of these receptors are diverse, but collectively they contribute to the efficient transformation of a novel set of environmental signals into a memory trace of appropriate strength and specificity. The present experiments show that PAR-1 plays a prominent role in this process. In wild-type animals upon CS-US association (but not after the unpaired protocol) PAR-1 shifts its coupling from excitatory to inhibitory G-proteins (Annexe 3), demonstrating the selective involvement of a PAR-1 G-protein switch in learning. Consistent with this, deficient inhibitory tone in the amygdala of fear conditioned PAR-1−/− mice results in the enhancement of fear, demonstrating that the dynamic G-protein coupling - a new form of
experience-dependent plasticity - may be relevant to the pathological fear and emotional learning associated with anxiety disorders.

How could the elevated anxiety and enhancement in fear memory in mice lacking PAR-1 be explained? From the results presented in this work emerges the idea of PAR-1 playing an inhibitory role in wild-type mice; hence when absent, all the excitatory mechanism triggered by a stressful stimuli would be fully expressed. This hypothesis draws the attention onto the G proteins that couple the thrombin receptor and in particular onto the recently described “biaised agonism” mechanism. It defines the fact that agonists, proteases or peptides ligands, once bound to a same receptor, are able to trigger distinct signalling responses. This concept is well known for GPCRs and a large amount of work has already been dedicated to the description of 5-HT2 serotonin, β2-adrenergic, V2 vasopressin receptors functional selectivities (Kenakin, 2007; Urban, 2007; Yao, 2006). Other studies have revealed that this concept could be applied to PARs (Vouret-Craviari, 1992; Lasne, 1995; Russo, 2009). McLaughlin (2005) demonstrated that thrombin-activated PAR-1 signals essentially via G_{12/13} in human endothelial cells whereas agonist peptides elicit preferentially the Gα_{q}-related signalling. Indeed, mutations of the extracellular portion of the thrombin receptor revealed profound differences between thrombin and TRAP functional activation (Blackhart, 2000). The authors suspected that they were due to either a specific tethered ligand-related intramolecular mechanism or to differences in the mechanism of activation of the receptor.

Those elements led us to investigate the nature of the G proteins that are bound to PAR-1 before and after fear conditioning. In this experiment (conducted by Dr Uddin) PAR-1 agonist TRαg-stimulated [35S]GTPγS binding to G proteins in membrane
preparations of naïve and fear-conditioned mice basolateral amygdala was measured. In control conditions PAR-1 was found to be preferentially coupled to \( \text{G} \alpha_{q/11} \) protein and to a lesser extent to \( \text{G} \alpha_0 \) (Annexe A3). After fear conditioning PAR-1-coupling to the inhibitory \( \text{G} \alpha_0 \) protein was exacerbated to the detriment of excitatory \( \text{G} \alpha_{q/11} \) (Annexe A3). Fear conditioning would have triggered such a G-protein switch in order to keep AMPA-related responses, neuronal excitability, L-LTP and fear learning at moderate levels that characterise an adapted response to stress.

Proteases are activated following stress (Pawlak, 2003). In particular neuropsin mRNA expression is increased following acute stress (Harada, 2008) and is activated during LTP (Shimizu, 1998; Komai, 2000). A hypothesis is that following a fear conditioning protocol, neuropsin is released and activated in the extracellular milieu. The evidence for an interaction between neuropsin and PAR-1 needs to be strengthened, together with the consecutive contribution of neuropsin to moderate neuronal excitability and behavioural responses.

Previous studies reported as unlikely that neuropsin could activate PAR-1 but those experiments were conducted in an \textit{in vitro} system (Shimizu, 1998) and therefore neglect any cofactor intervention or the fact that the protease action on PAR-1 may not be an activation as such. Furthermore PAR-1 sequence contains some amino-acid triplets susceptible to be targeted by neuropsin (Shimizu, 1998; see Chapter IV).

If the thrombin receptor does exist in several active states (Russo, 2009) then neuropsin action could be that of modulating the equilibrium between two conformations of the PAR-1 receptor. The neuropsin-related allosteric receptor conformation change
would directly generate coupling to $\alpha_0$ or would modify PAR-1 response to agonist/tethered ligand in favour of $\alpha_0$ protein coupling. This mechanism has been described previously by Maillet (2007) about an allosteric modulation of another GPCR tachykinin type 2 receptor.

Another possible mechanism to explain the G-protein switch would be a specific phosphorylation of the thrombin receptor triggered by the fear-learning process. Indeed it has been reported previously (Daaka, 1997) that PKA-dependent $\beta_2$-adrenoreceptor phosphorylation induced the receptor to switch from $G_i$ to $G_i$ protein in a cell culture system. This finding has since been questioned (Friedman, 2002) but is worth considering for future work aiming to elucidate PAR-1 switch mechanism, for instance by examining if some specific sites of the thrombin receptor are phosphorylated due to a fear-learning process.
Annexes
Annexe A1. Reversal of amygdalar LTP and augmentation of fear following conditioning in PAR-1-deficient mice.

WT or PAR-1−/− mice were either left undisturbed (naïve) or subjected to fear. To record field potentials in the lateral–basal amygdala pathway, coronal slices containing the amygdala were prepared before or 48 hours after fear conditioning and LTP was induced by high frequency stimulation (electrode placement in A and timing shown by asterisks in B, C, D) of the lateral nucleus. (B) Tetanic stimulation resulted in a stable LTP in naïve PAR-1+/− (black symbols) which lasted more than two hours, which was depressed in PAR-1−/− mice (red symbols). (C) Fear conditioning caused a reversal of LTP in PAR-1−/− animals which was approximately 2-fold higher than in PAR-1+/− mice during the course of the experiment. (D) When during training the tone and footshock were delivered in a random manner the increase in the early phase of LTP (E-LTP) in PAR-1+/− mice still persisted, while the reversal in the late phase (L-LTP) was no longer observed, indicating that the latter phenomenon was fear learning-specific. Individual traces at the baseline (1), 30 seconds (2) and 2 hours post–tetanus (3) are shown as inserts in B, C and D. Data shown as mean ±SEM. LA: lateral amygdala, BLA: basal amygdala, MeA: medial amygdala, CA: central amygdala.

Annexe A2. Fear conditioning regulates miniature AMPA currents (mAMPACs) and neuronal excitability in the basal nucleus of the amygdala.

PAR-1\(^{-/-}\) and PAR-1\(^{+/-}\) mice were subjected to fear conditioning and amygdala slices prepared 2 days later. Continuous whole-cell voltage clamp recording of mAMPACs in principal neurons of the basal nucleus of the amygdala showed a similar amplitude in PAR-1\(^{-/-}\) and PAR-1\(^{+/-}\) mice (cumulative distribution and mean±SEM value in A; amplitude distribution in B and C). However, mAMPACs were ~2-fold more frequent in PAR-1\(^{-/-}\) animals (inter-event interval amounted to 211±46 ms in PAR1\(^{-/-}\) compared to 424±89 ms in PAR1\(^{+/-}\), p<0.05; cumulative distribution and mean±SEM in D, inter-event distribution in E and F). Please note that the absolute number of mAMPACs in PAR1\(^{-/-}\) (C) is higher than PAR1\(^{+/-}\) (B) as a consequence of their higher frequency. Moreover, AMPA receptors desensitize faster in PAR-1\(^{-/-}\) mice after fear conditioning, as decay time (cumulative probability, mean±SEM and representative traces in G; distribution shown in H and I) of mAMPACs show a significant decrease in PAR1\(^{-/-}\) mice (4.3±0.1 ms, n=600 events, N=12 cells) compared to PAR1\(^{+/-}\) (6.3±0.3 ms n=600, N=12; p<0.05). Best curve fittings for the decay traces are shown in the insert (green lines). (J, K, L, M) Current-clamp experiments revealed that neuronal firing rate is higher in PAR1\(^{-/-}\) mice after fear conditioning. Voltage responses (representative traces in J, K) were recorded by currents steps from -100 to +600 pA in 50 pA increments (L) from principal neurons of the basal nucleus of PAR1\(^{+/-}\) and PAR1\(^{-/-}\) mice 2 days after fear conditioning. Number of action potential spikes was counted as a function of depolarizing current injection (M). Fear conditioning significantly increased the action potential firing rate in PAR1\(^{-/-}\) (p<0.01 at 150 pA; p<0.05 at 200pA). Insert shows a significant increase in the mean input resistance in PAR1\(^{-/-}\) mice (189±23 MΩ; n=8) compared to PAR1\(^{+/-}\) (121±10 MΩ, n=7; p<0.05). *p<0.05, **p<0.01. Data shown as mean SEM.

Annexe A3. PAR-1 / Gα-protein coupling in the amygdala is dynamic and regulated by fear learning.

(A) Western blotting demonstrated a 40% decrease in the amygdala PAR-1 levels 5 min after fear conditioning consistent with its cleavage by proteases (n = 4 per group). PAR-1 levels were back to normal 48 h later. (B) Fear conditioning did not affect cellular Gαo or Gαq/11 levels. (C, D) To assess whether PAR-1 cleavage alters its Gα-protein binding we performed [35S]GTPγS binding and Gα-specific immunoprecipitation assay. (C) TRaγ-induced increases in [35S]GTPγS binding to Gαo and Gαq/11 were abolished in PAR-1−/− animals confirming assay specificity. (D) Stimulation of PAR-1 in PAR-1+/− mice with TRaγ revealed a fear conditioning-specific 2-fold increase in PAR-1 Gαo coupling while Gαq/11 coupling was unaffected. The unpaired protocol did not change PAR-1-G protein association.

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