THE ROLE OF THE AMYGDALA IN EMOTIONAL MEMORIES: A MULTIDISCIPLINARY APPROACH

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PAR

Kamila MARKRAM

Diplom in Psychologie, Technische Universität Berlin, Allemagne
de nationalité allemande

acceptée sur proposition du jury:

Prof. M. Herzog, président du jury
Prof. M. D. C. Sandi Perez, directrice de thèse
Prof. O. Blanke, rapporteur
Prof. P. Vuilleumier, rapporteur
Prof. C. Regan, rapporteur

ÉCOLE POLYTECHNIQUE
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Abstract – English version

This thesis investigates the role of the amygdala for the establishment of fear memories with a multidisciplinary approach, including behavioural, psychopharmacological, genetic, molecular, and electrophysiological techniques in rats or mice, under healthy or pathological conditions. This research program aims to shed light on the acquisition and storage of emotional memories in the amygdala and closely interconnected brain areas.

In one line of experiments, the molecular mechanisms leading to the establishment of fear memory traces in the amygdala were investigated. For this purpose, the functional role of the polysialylated neural cell adhesion molecule PSA-NCAM, expressed in the synaptic junction, was assessed in the amygdala – and also prefrontal cortex and hippocampus – with psychopharmacological and genetic approaches and tasks that strongly rely on these brain areas. Two lines of studies were followed: 1) amygdala-targeted cleavage and enhancement of PSA-NCAM in rats and 2) general cleavage of PSA-NCAM throughout the brain using genetically modified mice. Taken together, both approaches show that amygdaloid PSA-NCAM plays no role in the acquisition and storage of fear memories, but is rather involved in their extinction. Furthermore, the results confirm the importance of PSA-NCAM in hippocampus mediated learning and for the first time show that prefrontal cortex mediated learning depends on PSA-NCAM. These results suggest that PSA-NCAM is selectively involved in some, but not all, synaptic plasticity processes in the brain.

In another line of experiments, the valproic acid (VPA) animal model of autism was used to investigate a possible contribution of the amygdala towards the autistic pathology. VPA was injected once at a specific time point during gestation, the time of neural tube closure. The offspring of such treated rats were first characterized in a broad set of behavioural tasks. It was found that VPA-treated offspring exhibited very specific behavioural anomalies closely resembling autistic symptomotology, such as impaired social interaction, exploration and recognition, enhanced repetitive behaviours, impaired sensorimotor gating and increased anxiety, while other behavioural parameters were left unharmed. Once the validity of the model was established, amygdala functionality was assessed. The results demonstrated that VPA-treated offspring exhibited highly enhanced conditioned fear memories, which generalized to other stimuli and were resistant to extinction. Electrophysiological *in vitro* recordings in the amygdala revealed hyper-reactivity towards stimulation and enhanced activity-induced synaptic plasticity. These results imply that enhanced activity and plasticity in the amygdala may underlie the exaggerated fear memories. Furthermore it is suggested in this thesis that a hyper-reactive amygdala may underlie some of the most basic symptoms observed in autism: reduced social interactions and resistance to rehabilitation.

Keywords:
Amygdala, anxiety, autism, emotions, fear conditioning, fear extinction, hippocampus, long-term potentiation, memory, consolidation, neural cell adhesion molecule, NCAM, polysialic acid, PSA, PSA-NCAM, prefrontal cortex, social behaviour, synaptic plasticity, valproic acid, VPA, valproic acid rodent model of autism

Abstract – German version

Thema dieser Doktorarbeit ist die Rolle der Amygala in der Entstehung von aversiven Gedächtnisinhalten. Dabei wird ein multidisziplinärer Ansatzes verfolgt, der psychopharmakologische, genetische, molekulare und elektrophysiologische Techniken, sowie Verhaltensstudien, in Ratten und Mäusen, unter normalen, und auch pathologischen Bedingungen, beinhaltet.

Einen Reihe von Experimenten untersucht die molekularen Mechanismen, die zur Bildung eines Furchtgedächtnisinhaltes in der Amygdala führen. Zu diesem Zweck wurde die Funktion von PSA-NCAM, eines „neural cell adhesion molecules“, welches an Synpasen exprimiert wird, in der Amygdala – und auch dem Hippocampus und prefrontalen Kortex – untersucht. Dies geschah unter Anwendung von psychopharmakologischen und genetischen Techniken. Zudem wurden Verhaltensparadigmen durchgeführt, welche Verarbeitung in diesen Gehirnregionen erfordern. Zwei Ansätze wuden verfolgt: Zum einem wurde PSA-NCAM gezielt aus der Amygdala entfernt. Zum anderen, wurde PSA-NCAM im gesamten Gehirn von genetisch modifizierten Mäusen entfernt. Beide Ansätze ergaben, dass PSA-NCAM nicht notwendig ist in der Amygdala für die Bildung und Speicherung eines konditionierten Furchtengrams. Zudem untermauern die Ergebnisse die Notwendigkeit von PSA-NCAM für vom Hippocampus abhängiges Lernen und zeigen zum ersten Mal, dass Lernen, welches dem prefrontalen Kortex unterliegt, ebenfalls PSA-NCAM erfordert. Aus diesen Ergebnisse wird geschlossen, dass PSA-NCAM selektiv in einigen, aber nicht allen plastischen Prozessen im Gehirn partizipiert.

Eine andere Reihe von Experimenten untersucht im Rahmen des Valproinsäure (VPA)-Autismus Tiermodells, inwiefern die Amygdala zur Pathologie von Autismus beiträgt. Hierzu wurde VPA einmalig zu einem bestimmten Zeitpunkt, nämlich während der Schliessung des Neuralrohres, in die schwangere Ratte injiziert. Die Jungen solcher mit VPA behandelten Muttertiere unterliefen dann einer weitgefächterten Verhaltenscharakterisierung zum Zweck der Modellvalidierung. Die Ergebnisse zeigten, dass VPA behandelte Rattenabkömmlinge ein spezifisches Muster abnormalen Verhaltens aufweisen, welches enge Parallen zu autischen Symptomen aufzeigt, darunter eingeschränkte soziale Interaktionen, erhöhtes repetitives Verhalten, eingeschränkte sensomotorische Verarbeitung, und erhöhte Ängstlichkeit. Weil diese Verhaltenscharakteristka zur Validierung des Modells beitrugen, wurden daraufhin die Funktionen der Amygdala untersucht. Die Ergebnisse zeigten, dass Abkömmlinge von VPA behandelten Muttertieren eine stark erhöhte Gedächtniskapazität für konditionierte Furcht aufwiesen, die auf andere Stimuli generalisiert wurde und eine hohe Extinktionsresistenz aufwies. Elektrophysiologische *in vitro* Ableitungen in der Amygdala ergaben eine erhöhte Erregbarkeit und Langzeitpotenzierung. Diese Ergebnisse implizieren, dass erhöhte Erregbarkeit und Plastizität in der Amygdala dem erhöhten Furchtgedächtnis zugrunde liegen könnte. Zudem wird in dieser Arbeit vorgeschlagen, dass eine hyper-aktive Amygdala einigen der fundamentalen autischen Symptome zugrunde liegen könnte: den reduzierten sozialen Interaktionen und der Resistenz gegenüber Behandlungsversuchen.

Amygdala, Ängstlichkeit, Autismus, Emotionen, Furchtkonditionierung, Furchtextinktion, Hippokampus, Langzeitpotenzierung, Gedächtnis, Konsolidierung, neural cell adhesion molecule, NCAM, polysialic acid, PSA, PSA-NCAM, Präfrontaler Kortex, Sozialverhalten, Synaptische Plastizität, Valproinsäure, VPA, Valproinsäure Tiermodell des Autismus

1. The amygdala

The amygdala is a structure located deep in the anterior inferior temporal lobe of the brain. The amygdala has been assigned many functional roles in the brain, including detecting and interpreting signs of emotional and social significance in the environment, modulating memory storage across multiple brain sites, establishing fear memories, anxiety and the regulation of autonomic and hormonal responses.

This chapter first reviews the anatomy and connectivity of the amygdala. Secondly, the functions and relevance for pathologies are discussed.

1.1. General anatomy and connectivity

The amygdala is an almond shaped structure located within the temporal lobe, just anterior to the hippocampus and consists of approximately 13 nuclei, all of which can be further divided into subdivisions. The subdivisions are based on cytoarchitectonics, histochemistry and connectivity patterns. Most anatomical studies focus on the rat amygdala, but there are also anatomical descriptions of cats and monkeys. This section briefly describes the rat amygdala anatomy with the nomenclature introduced by Price and others (Price et al., 1987).

1.1.1. Anatomy

The amygdala can be divided into three classes of nuclei: 1) the deep or basolateral group, including the lateral, the basal (lateral and basal are sometimes called the basolateral nucleus = BLA) and the accessory basal or basomedial nucleus; 2) the superficial or cortical-like group, including the cortical nuclei and the nucleus of the lateral olfactory tract; and 3) the centromedial group, including the central and medial nuclei. There is also a separate set of nuclei that does not easily fall into any of these groups, which are the intercalated cell masses and the amygdalohippocampal area. The intercalated cell masses contain solely GABAergic neurons which can inhibit the central and medial nucleus (Pare and Smith, 1993; Quirk et al., 2003). The nuclei and their abbreviations (which will be further used in the text) are summarized in table 1. Any of these nuclei has several subdivisions, which are shown in figure 1.

Although this classification has been adopted by many, some authors suggested a different classification. For example, it was argued that the centromedial amygdala should be extended rostrally and medially by including the BNST and caudodorsal regions of the substantia innominata and calling the whole complex the "extended amygdala" (Alheid and Heimer, 1988).

Table 1. Anatomical classification of amygdaloid nuclei.

Group	Nucleus	Abbreviation
Basolateral group	Lateral nucleus	LA
	Basal nucleus	B
	Accessory basal or basomedial nucleus	AB
Cortical-like group	Nucleus of the lateral olfactory tract	NLOT
	Bed nucleus of the accessory olfactory tract	BAOT
	Anterior cortical nucleus	CoA
	Posterior cortical nucleus	CoP
	Periamygdaloid complex	PAC
Centromedial group	Central nucleus	CeA
	Medial nucleus	M
	Amygdaloid part of the bed nucleus of the stria terminalis	BNST
Other nuclei	Anterior amygdaloid area	AAA
	Amygdalo-hippocampal area	AHA
	Intercalated cell masses	ICM

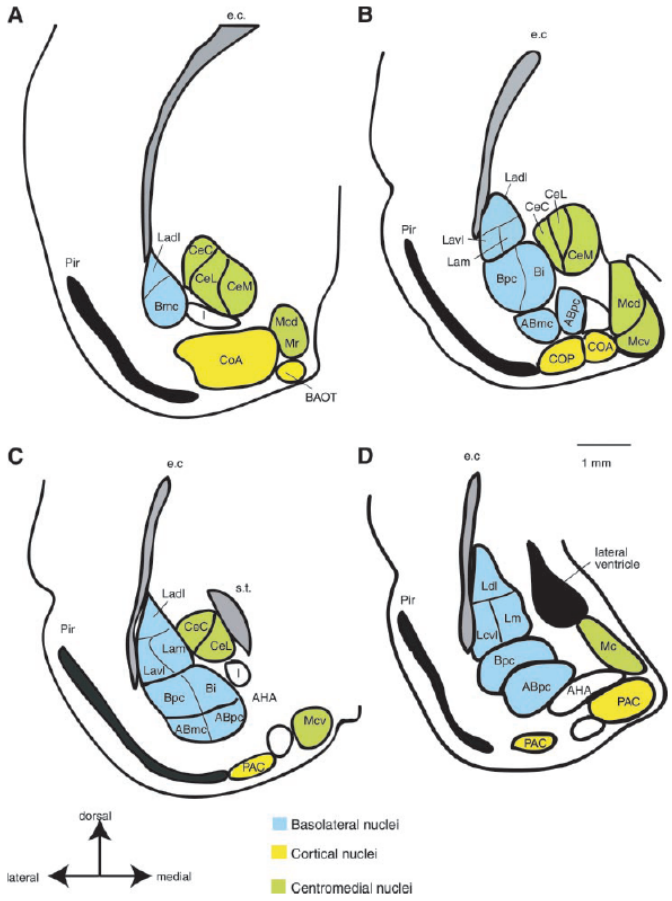

Figure 1. Nuclei of the rat amygdaloid complex.
Coronal sections are drawn from rostral (*A*) to caudal (*D*). The different nuclei are divided into three groups as described in text. Areas in blue form part of the basolateral group, areas in yellow are the cortical group, and areas in green form the centromedial group. ABmc, accessory basal magnocellular subdivision; ABpc, accessory basal parvicellular sub-division; Bpc, basal nucleus magnocellular subdivision; e.c., external capsule; Ladl, lateral amygdala medial sub-division; Lam, lateral amygdala medial subdivision; Lavl, lateral amygdala ventro-lateral subdivision; Mcd, medial amygdala dorsal sub-division; Mcv, medial amygdala ventral subdivision; Mr, medial amygdala rostral subdivision; Pir, piriform cortex; s.t., stria terminalis. Adopted from Sah et al., 2003.

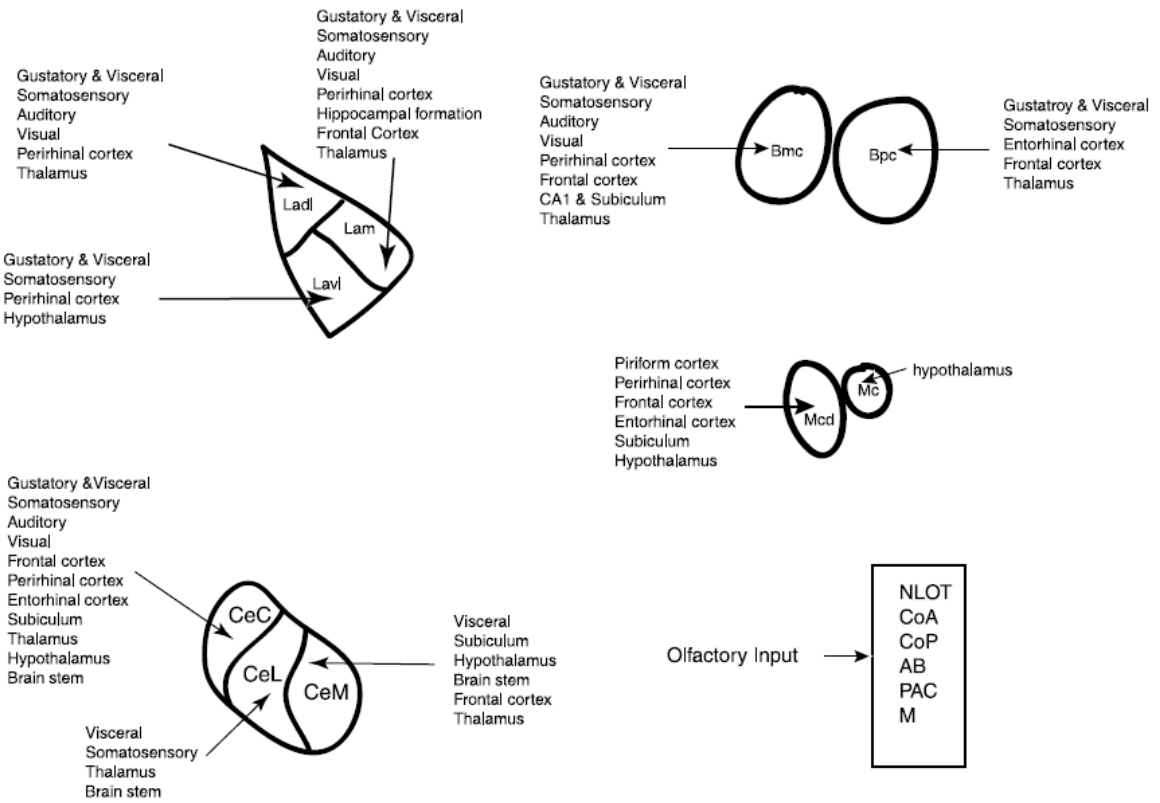

Figure 2. Sensory inputs to the amygdaloid nuclei. Neuromodulatory inputs (e.g. acetylcholine, serotonin, etc) have been omitted for clarity. Adopted from Sah et al., 2003.

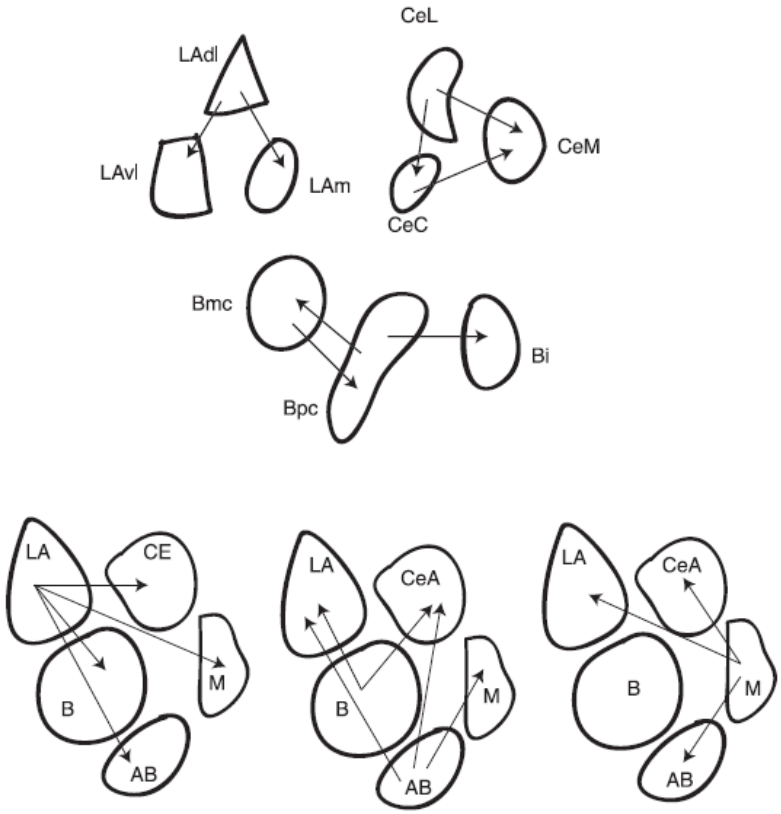

Figure 3. Intraamygdaloid connections. Most connections between nuclei within the amygdala are glutamatergic. Adopted from Sah et al.,

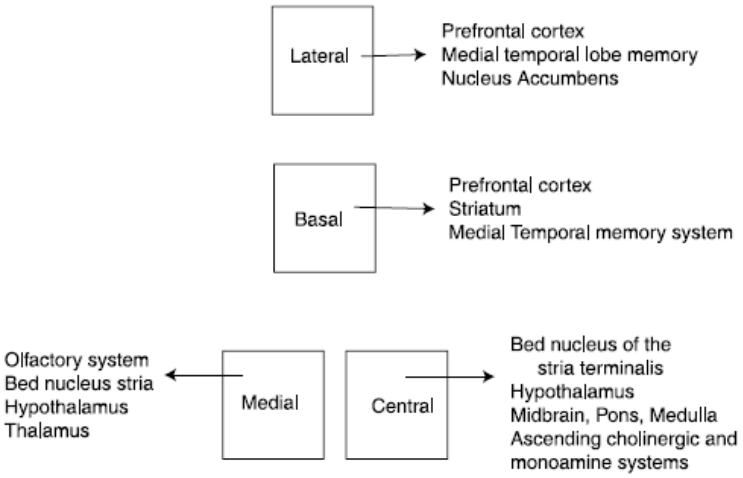

Figure 4. Main outputs from the amygdaloid nuclei.
Neuromodulatory inputs (e.g. acetylcholine, serotonin, etc) have been omitted for clarity. Adopted from Sah et al., 2003.

1.1.2. Connectivity

1.1.2.1. Sensory inputs

Inputs into the amygdala can de divided into those arising in cortical and thalamic and into those arising in the hypothalamus or brain stem. Glutamatergic projections from the cortex supply highly processed information from sensory association areas and structures related to memory systems, like the medial temporal lobe. Hypothalamic and brain stem afferents supply autonomic inputs (reviewed in Sah et al., 2003).

The amygdala receives information from all modalities: olfactory, somatosensory, gustatory, auditory, visual and visceral. The amygdaloid innervation pattern can be seen in figure 2. Here the most relevant input paths for fear conditioning, somatosensory and auditory inputs converging on polymodal neurons in the lateral amygdala (Romanski et al., 1993) are highlighted.

Only few connections reach the amygdala directly from the primary somatosensory cortex. Most afferents go through the dysgranular parietal insular cortex in the parietal lobe (Shi and Cassell, 1998a). These projections target the LA, B and CeA (McDonald and Jackson, 1987; Shi and Cassell, 1998a, 1998b). The pontine parabrachial nucleus and two thalamic nuclei, the geniculatum mediale and the posterior internuclear nucleus (PIN) also project to the amygdala. This pathway was suggested to transmit nociceptive information (Ledoux et al., 1987; Bernard et al., 1989; Bordi and LeDoux, 1994) relevant for fear conditioning. Projections arising in the PIN target all subdivisions of the LA, but also the AB and medial division of the CeA (Bernard et al., 1993; Linke et al., 2000).

Fast auditory inputs stem from the geniculatum mediale in the thalamus and somewhat slower auditory inputs stem from the auditory association area Te3.

The amygdala also receives highly processed polymodal information from the prefrontal cortex, the perirhinal cortex and the hippocampus.

The prefrontal cortex receives information from all sensory modalities and projects in an organized way to the amygdala, particularly to LA, but also to B, AB and CeA and the intercalated cell masses (Sesack et al., 1989; McDonald et al., 1996).

Areas such as the perirhinal cortex, enthorinal cortex, parahippocampal cortex and the hippocampus form part of the medial temporal lobe declarative memory system (Milner et al., 1998). Strong reciprocal connections exist between the amygdala and these areas. For example, hippocampal inputs originating in the subicular region project mainly to the basal nucleus, but most other nuclei are also sparsely innervated (Canteras and Swanson, 1992). This reciprocal pathway is believed to transmit the contextual information relevant for contextual fear conditioning (LeDoux, 2003).

1.1.2.2.Intramygdaloid connections

Amygdala nuclei exhibit extensive intra- and internuclear connectivity (fig. 3). For example, the unimodal sensory information enters the lateral subdivision of the LA, whereas polymodal information from the medial temporal lobe memory system enters the medial part of the LA (Pitkanen, 2000). Strong projections from the lateral to medial part suggest that the medial LA might be a site of integrating sensory information with past experiences. The LA sends extensive connections to the basal and accessory basal, central and periamygdaloid nuclei (Pitkanen et al., 1995). All these nuclei, except the central, project back to the LA (Savander et al., 1995; 1996b; Savander et al., 1996a). Most of these projections terminate in

the medial and ventrolateral LA and are glutamatergic, while some are inhibitory (Savander et al., 1997).

The basal and accessory basal nuclei, receive strong cortical inputs. The largest projection from B is to the medial CeA and since these afferents form asymmetric synapses they are thought to be glutamatergic (Pare et al., 1995). The AB sends projections to the LA, CeA, and M.

The central nucleus is the major output station of the amygdala. It receives inputs from all other amygdaloid nuclei, but sends very few afferents back to these nuclei (Jolkkonen and Pitkanen, 1998).

1.1.2.3. Efferent connections

The amygdala has widespread projections to cortical, hypothalamic and brain stem regions (fig.4).

The basolateral group sends extensive glutamatergic projections to the medial temporal lobe memory system (Petrovich et al., 2001). The B stands in a strong reciprocal connection with the hippocampus, but also projects to the prefrontal cortex, nucleus accumbens and the thalamus.

The most relevant output nucleus for conditioned fear responses is the CeA. Several nuclei in the midbrain, pons, and medulla regulate autonomic responses, whereas the hypothalamus in conjunction with the BNST may modulate autonomic responses. The CeA innervates all of these areas. Its activation evokes fearful and defensive behaviours, including freezing, potentiated startle, release of stress hormones, and changes in blood pressure, which are mediated by former brain regions. For example, the CeA projects to the brainstem in three main areas: the periaqueductal gray (PAG), which leads to freezing, vocalizations, startle, analgesia and cardiovascular changes (Rizvi et al., 1991; Bellgowan and Helmstetter, 1996); the parabrachial nucleus, which is involved in pain pathways (Gauriau and Bernard, 2002) and the nucleus of the solitary tract, which is connected with the vagal system (van der Kooy et al., 1984). Projections to the BNST control stress hormone levels (Van de Kar et al., 1991) and those to the lateral hypothalamus the blood pressure (LeDoux et al., 1988).

Further projections lead from the central and other amygdaloid nuclei to hypothalamic dorsolateral, caudolateral and ventromedial nuclei involved in regulating ingestive, reproductive and defensive behaviours (Petrovich et al., 2001).

In addition to these direct connections to the hypothalamus, the CeA also strongly innervates the BNST, which in turn innervates the hypothalamus. Both CeA and BNST project strongly to ascending monoaminergic and cholinergic nuclei, such as the noradrenergic locus coeruleus, the dopaminergic substantia nigra and ventral tegmental area, the serotonergic raphae nucleus and the cholinergic nucleus basalis (Price et al., 1987; Davis and Whalen, 2001). These nuclei not only have reciprocal connections to the amygdala, but innervate large areas of the forebrain and temporal lobe memory system, where they can modulate information processing related to perception, attention, reward expectancy and memory.

In summary, the amygdala not only receives information from all sensory modalities through fast thalamic connections and more processed channels from the association cortices, the medial temporal lobe memory system and prefrontal cortex, it also projects back to many brain areas. Through connections to the hypothalamus, the brain stem nuclei and forebrain it can rapidly modulate both peripheral emotional behaviour and information processing in the neocortex.

1.2. The function of the amygdala in the brain

The interest in the involvement of the amygdala in emotional processing stems from the now classical studies conducted by Klüver and Bucy in 1937. They found that bilateral ablations of the temporal lobes led to: (a) psychic blindness, i.e. the loss of ability to understand the meaning of objects by vision alone in the presence of normal visual discrimination skills, (b) oral tendencies, i.e. the use of the mouth rather than the hands to explore objects, (c) hyper-metamorphosis, i.e. a compulsion to react to every stimulus and (d) emotional changes, including changes or absence of anger and fear, lack of social behaviour, and abnormal sexual behaviours (Kluver and Bucy, 1937). These symptoms are now well known as the Klüver-Bucy syndrome and later studies have established that the same or similar changes in socio-emotional behaviours may be observed after more restricted bilateral damage to the amygdala (Rosvold et al., 1954; Schreiner and Kling, 1956; Weiskrantz, 1956; Aggleton and Passingham, 1981; Zola-Morgan et al., 1991) or the inferior temporal cortex alone (Horel et al., 1975). These studies demonstrated that the amygdala is essential for assigning emotional significance and producing appropriate behaviours towards sensory and social stimuli.

Since those pioneering studies the amygdala has been studied from many perspectives. In humans, amygdala function was investigated with imaging techniques during task processing, in patients with focal amygdaloid lesions or with abnormalities due to pathological states. In animals, lesioning studies, electrophysiological recordings and stimulations, neuro- and psychopharmacological studies were conducted. This produced a vast amount of data, and depending on the species studied and techniques used, different foci and theories on the function of the amygdala. The following chapters give a brief overview on the response characteristics and functions of the amygdala in the brain.

1.2.1. Responses to emotionally salient stimuli

As described in the previous chapter the amygdala receives a great amount of sensory input from all modalities. Indeed, there is a vast amount of human studies showing that the amygdala responds to both aversive, or negatively valenced, and to positively valenced stimuli as opposed to neutral stimuli (reviewed in Zald, 2003). Thus, an increase in amygdaloid activation can be observed to stimuli with an emotional value. The stimuli used in these studies were pretty broad. For example, on the aversive side stimuli included watching unpleasant pictures, tasting bitter solutions, smelling bad odours, experiencing pain, listing to or viewing aversive/threatening words or sounds and viewing fearful or angry faces. Positive stimuli included watching movies with happy or sexual contents, viewing beautiful faces, listening or viewing positive words, anticipating rewards or watching drug related videos by drug addicted people (reviewed in Zald 2003).

Overall, amygdala activation can be induced more reliably with aversive stimuli than with positively valenced stimuli. A metanalysis of neuroimaging studies on emotion reported 38 activation foci in the amygdala in response to negatively valenced stimuli as opposed to only 5 foci to positively valenced stimuli (Phan et al., 2002). Some authors suggest that the amygdala may be particularly biased towards evaluating threatening and dangerous stimuli in the environment (Adolphs et al., 1998; Adolphs et al., 2005). However, it has also been argued that this may be due to the different intensity and arousal levels evoked by negative and postitive valenced stimuli. It has been shown that the amygdala is activated more when the stimuli were more arousing, regardless of whether they were pleasant or aversive (Lane et al., 1999; Garavan et al., 2001). Commonly, aversive stimuli are more arousing and produce

more intense behavioural responses. Thus, it may be that this bias towards more reliable amygdala activation with negative stimuli may be due to their greater arousal evoking qualities.

In summary, neuroimaging data with human subjects showed that the amygdala is activated when confronted with emotionally relevant stimuli.

1.2.2. Attention

One of the consequences of increased amygdala activation to emotionally salient stimuli may lie in directing attention towards these stimuli. Indeed, we attend emotional information to a far greater degree than neutral, inexpressive stimuli. Due to its' extensive efferent projections, the amygdala is particularly well equipped to mediate this phenomenon. Two separate pathways are of particular importance: First, the central nucleus sends extensive projections to cholinergic cells in the nucleus basalis and to noradrenergic cells in the locus coeruleus. Both these centres innervate extensive parts of the neocortex and play well established roles in emotional arousal and attention processes (Aston-Jones et al., 1996; Holland and Gallagher, 1999). Second, the amygdala projects to primary sensory cortices, where it can directly modulate neural processing in a way that in particular emotionally relevant stimuli experience enhanced processing (Quirk et al., 1997; Morris et al., 1998c; Morris et al., 1998b; Tabert et al., 2001). Neuroimaging studies showed that the enhancement of emotionally salient stimuli in sensory regions is correlated with the amount of amygdala activation (Morris et al., 1998c; Morris et al., 1998b; Tabert et al., 2001).

1.2.3. Modulation of memory processes

Emotions act as enhancers for memory processes, can strengthen the memory trace and thus emotionally loaded incidents are remembered better than unemotional ones (McGaugh, 2004). Emotional arousal leads to an activation of the amygdala, which in turn may be the key structure to modulate memory storage in other brain areas. From this point of view the amygdala is not the place of memory storage itself, but has an influence on the acquisition and consolidation of memories in a divers sets of learning tasks, such as reward expectancy (Holland and Gallagher, 2004), passive and active avoidance (Liang et al., 1982; Liang and McGaugh, 1983a) and spatial learning (Roozendaal et al., 2004) and therefore affects memory contents in multiple brain areas. This view is supported by both human and animal research.

Rodent studies supported this view by several lines of evidence with a long tradition: early studies by Goddard showed that electrical stimulation of the amygdala shortly after aversive training impaired memory consolidation (Goddard, 1964). Later studies showed that depending on the stimulus intensity of electrical stimulation and the training conditioning (e.g. passive avoidance) the effects can be either facilitating or impairing (Gold et al., 1975).

Psychopharmacological experiments provided further evidence for the view that emotional arousal activates the amygdala and exerts modulatory effects on memory. Firstly, systemic post-training injections of epinephrine and glucocorticoids, hormones secreted by the adrenal glands during stressful or exciting experiences, or drugs affecting its receptors have modulatory influences on memory, either enhancing or impairing memory in a dose-dependent and time-dependent manner (McGaugh et al., 1975; Gold et al., 1977; Izquierdo and Dias, 1983; McGaugh, 1983; Bohus, 1994; Sandi and Rose, 1994; McEwen and

Sapolsky, 1995; Lupien and McEwen, 1997). Lesions of the amygdala (Cahill and McGaugh, 1990; Roozendaal and McGaugh, 1996b) or stria terminalis (Roozendaal and McGaugh, 1996a) block these modulatory effects, strongly suggesting that they are mediated by the amygdala. Secondly, intra-amygdalar infusions of generally arousing substances modulate memory storage in a diverse set of tasks. Infusions of β-adrenergic antagonists into the amygdala block the effects of systemically administered epinephrine, suggesting that peripheral effects are mediated through norepinephrine activation in the amygdala (Liang et al., 1986). Indeed immediate post-training intra-amygdalar administration of norepinephrine can enhance and impair memory (Liang et al., 1990). Glucocorticoid effects are also mediated through the activation of steroid receptors in the amygdala. Infusion of glucocorticoid agonists or antagonists into the amygdala modulate memory in a dose-dependent manner in a divers set of tasks, such as passive avoidance (Roozendaal and McGaugh, 1997) or water maze learning (Roozendaal & McGaugh,1997).

An important indicator for the hypothesis that the amygdala is not necessary for the storage of emotional information, but rather involved in mediating its' arousing effects on memory consolidation in other brain areas, is, that neither lesions of the amygdala itself (Cahill & McGaugh, 1990; Roozendaal & McGaugh, 1996b) nor its efferent projections via the stria terminalis (Liang and McGaugh, 1983b; Roozendaal and McGaugh, 1996a) block memory itself, but only the modulating effects of electrical stimulation and neuromodulatory drugs.

Since the pioneering psychopharmacological studies, intensive research has been undertaken in rodents to uncover the neuromodulatory interactions exerted by the amygdala (especially the basolateral complex) to influence memory consolidation in other brain regions (reviewed in McGaugh, 2004).

Studies with humans also support the view of memory modulatory role of the amygdala. Neuroimaging studies report a positive correlation between the amount of amygdala activation during learning and emotionally influenced memory retention (Cahill et al., 1996; Hamann et al., 1999; Canli et al., 2000; Cahill et al., 2001; Hamann, 2001; Canli et al., 2002). On the other hand, patients with amygdala lesions loose the better memory retention for emotionally loaded material (Markowitsch et al., 1994; Cahill et al., 1995; Adolphs et al., 1997).

In summary, both animal and human studies provide evidence for a role of the amygdala in modulating memory storage across multiple brain sites.

1.2.4. Fear conditioning

Another theory on the function of the amygdala in the brain stresses its' role in the acquisition of fear memories and states that some amygdaloid nuclei are indispensable for the acquisition as well as storage of conditioned fear (LeDoux, 2003).

Under the umbrella of this theory, research is typically undertaken with rodents using the classical cued and contextual fear conditioning paradigms. During the training, either a tone or the conditioning context (the conditioning chamber) are paired with an aversive stimulus, such as an electrical foot shock (unconditioned stimulus = US). After several pairings (varying from one to several dozens), the memory for the tone (cued) or the context (both: conditioned stimuli = CS) is measured in memory tests applied at different time points after training (minutes to days, months or even years). In the memory test, the animal is either exposed to the tone in a different context (memory test for the tone) or the same context as

during training. In neither test shocks are applied. Common dependent variables to measure memory are the time spent freezing, defecation, heart rate, vocalizations and startle response.

It is becoming more and more evident that different amygdaloid nuclei have different functions in the acquisition, storage and expression of conditioned fear. Two areas already mentioned in chapter 1.1.2 are of particular interest: the basolateral complex (BLA) and the central nucleus (CeA). The BLA is the sensory relay and integration station for information coming in from the thalamus and the cortex. The CeA controls the expression of fear responses to conditioned stimuli. It has extensive projections to various nuclei in the midbrain and brain stem to control rapid behavioral, autonomous and hormonal responses to threat and danger (Davis, 1992). Lesions of the CeA erase the whole spectrum of conditioned fear responses (Kapp et al., 1979; Gentile et al., 1986; Hitchcock and Davis, 1986), thus indicating its important role in fear expression.

Strong evidence supports the hypothesis that the association between the conditioned and unconditioned stimulus is acquired and permanently stored in the BLA.

First, lesion studies and psychopharmacological inactivation experiments showed that the BLA is necessary for the acquisition of conditioned fear to both, auditory and contextual stimuli (LeDoux et al., 1990; Phillips and LeDoux, 1992; Wilensky et al., 1999). The BLA was also shown to be a permanent storage place for this memory trace, since lesioning the amygdala even up to 16 months after auditory fear conditioning was able to erase the memory trace (Gale et al., 2004).

Second, single unit recordings showed that information about CS and US converge on single multimodal neurons in the LA (Romanski et al., 1993). Fear-conditioning induces LTP-like enhancement of responses in neurons in the LA *in vivo* (Rogan et al., 1997), in a similar way as the pairing of thalamic afferent stimulation with electric current injection into LA multimodal neurons does *in vitro* (Clugnet and LeDoux, 1990).

Third, psychopharmacological experiments revealed great details about the molecular cascades involved in the acquisition and stabilization of fear memories (reviewed in Blair et al., 2001; Schafe et al., 2001; Lamprecht and LeDoux, 2004; Rodrigues et al., 2004). These studies showed that the activation of receptors such as NMDA (Rodrigues et al., 2001) and voltage-gated calcium channels (Bauer et al., 2002), calcium-dependent kinases, including Ca^{2+}/Calmodulin-dpendent protein kinase II (Rodrigues et al., 2004), protein kinase A (Goosens et al., 2000; Schafe and LeDoux, 2000; Moita et al., 2002), protein kinase C (Goosens et al, 2000), extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) (Schafe et al., 2000) are all necessary for the formation of conditioned fear memories. In all of these studies specific blockers or antibodies to these molecules, infused directly into the amygdala interfered with the consolidation of auditory and/or contextual fear memories. Ultimately, these cascades induce gene activation and the production of proteins that may stabilize or enable changes at the synapse to consolidate memories. According with this view is the finding that the blockade of protein synthesis with anisomycin infused into the amygdala interferes with fear conditioning *in vivo* (Schafe et al., 1999; Schafe and LeDoux, 2000; Maren et al., 2003) and also with amygdaloid LTP *in vitro* (Huang et al., 2000).

Human studies also provide evidence for the importance of the amygdala in fear conditioning. For example, damage to the amygdala (Bechara et al., 1995) results in fear conditioning impairments. On the other hand, neuroimaging studies revealed that the amygdala activates during fear conditioning (Buchel et al., 1998; LaBar et al., 1998; Morris et al., 1998a).

Furthermore, the perception of particularly fearful faces also relies on the amygdala as revealed in both lesion (Adolphs et al., 1994; Adolphs et al., 1995) and neuroimaging (Morris et al., 1996; Breiter et al., 1996; Morris et al., 1998c; Whalen et al., 1998) studies.

Even though there is a great amount of data implicating the amygdala in not only the acquisition and expression, but also storage of conditioned fear, this topic is also highly controversial, since some studies question the BLA as an ultimate storage place for fear memories (Vazdarjanova and McGaugh, 1998; see previous subsection).

In summary, both human and animal studies suggest that the function of the amygdala might lie in acquiring, storing and controlling the expression of conditioned fear memories. Based on these studies it has been proposed that the amygdala may be the primary brain region in evaluating the environment for potentially threatening and dangerous cues.

1.2.5. Anxiety

It has been suggested that the amygdala may also mediate anxiety states (Davis and Shi, 1999; Lang et al., 2000). In contrast to conditioned fear, where an explicit conditioned cue activates the fear responses, anxiety is usually considered to be a more general state of distress, longer lasting and evoked by more generalized cues, which are not necessarily conditioned.

In animals anxiety has been studied using a variety of paradigms, including the light-potentiated startle paradigm (Davis and Shi, 1999; Lang et al., 2000). In nocturnal animals, such as the rat, it has been observed that exposure to bright light induces anxiety–like behaviours (File, 1980; Crawley, 1981), including a potentiated startle response to a tone (Walker and Davis, 1997). The enhancement of the startle response could be blocked by injection of anxiolytic drugs, suggesting that the effect was due to the anxiogenic properties of the light (Walker and Davis, 1997). Pharmacological inactivation of the basolateral amygdala and bed nucleus stria terminalis, part of the extended amygdala, impair light-enhanced startle (Lang et al., 2000). This is particularly interesting, because lesions of the BNST fail to block either fear-potentiated startle (Hitchcock and Davis, 1991) or conditioned freezing to an explicit cue (LeDoux et al., 1988), but they do block long-term sensitization of the startle response (Gewirtz et al., 1998) and the startle enhancing effect of the stress-hormone corticotropin (Lee and Davis, 1997). Corticotropin-releasing hormone is synthesized, among other brain regions, in the central amygdala, and corticotropin positive cells project to and act on receptors in the BNST (Sakanaka et al., 1986). Thus, it is has been suggested that phasic periods of fear activate the central nucleus, i.e. when confronted with a fear conditioned stimulus, which in turn may lead to long-term activation of the BNST via corticotrophin-releasing hormone and thus induce states of anxiety (Lang et al., 2000). It is conceivable that malfunctions in these circuits may lead to pathological states (see chapter 1.2.5).

1.2.6. Regulation of hormonal and autonomic responses

From the data reviewed in previous chapters, it is becoming increasingly obvious that through projections to many brain stem and hypothalamic regions the amygdala exerts a powerful control over motor, visceromotor and hormonal responses. Basically, this is accomplished through two pathways: the CeA and the BNST (Lang et al., 2000). The CeA pathway controls fear responses to explicit and conditioned cues and provokes a short term activation, whereas the BNST may respond to more diffuse and unconditioned cues, which initiate a rather long-term activation of the autonomic and hormonal responses. Thus, the CeA

pathway provokes short states of fear, whereas the BNST pathway may lead to general anxiety states (Lang et al., 2000). Figure 5 illustrates this point.

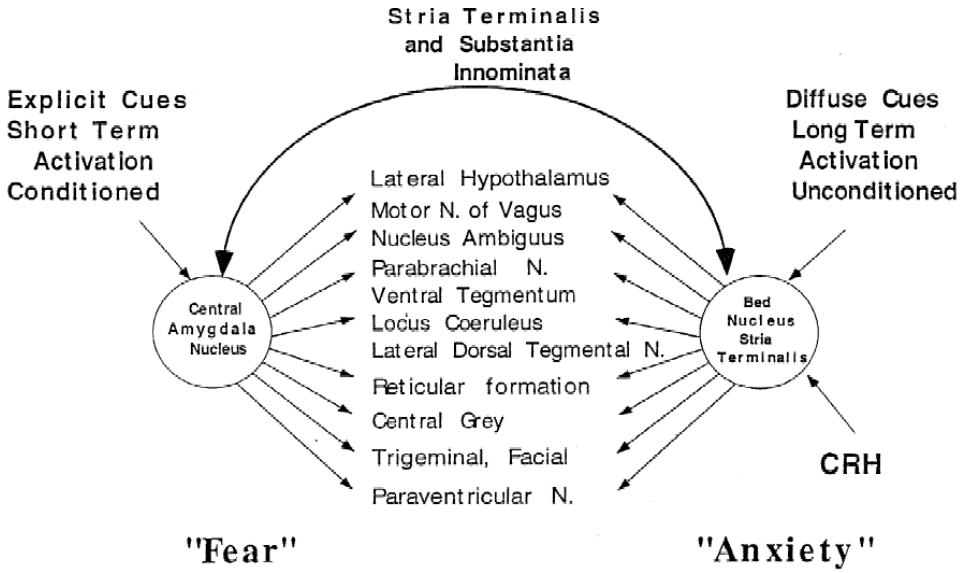

Figure 5. Differential involvement of the central nucleus and bed nucleus of the stria terminalis in regulating hormonal and autonomic responses in states of fear and anxiety. Note that both nuclei act on the same target structures, but lead to a differential activation pattern. The central nucleus provokes short-term activation of these systems and is accompanied by the sensation of fear. The bed nucleus of the stria terminalis may lead to long-term activation and a general feeling of anxiety. Adopted from Lang et al., 2000.

1.2.7. Socio-emotional behaviour

Finally, another function of the amygdala lies in processing and generating socio-emotional behaviours (Adolphs, 1999; 2006; Phelps, 2006). The studies that provide evidence for this facet of amygdala function generally stem from non-human and human primate lesion studies, as well as neuroimaging.

First indication that the amygdala might be involved in generating social behaviours stems from afore mentioned Klüver and Bucy (1937). In 1990 Brothers suggested that socio-emotional behaviour may be mediated by a network of three structures: the amygdala, prefrontal (orbitofrontal cortex and cingulate gyrus) and temporal (inferotemporal and superior temporal sulcus) regions (Brothers, 1990). This hypothesis was based mainly on neuropsychological and animal lesion data. For example, patients with implanted electrodes in the amygdala reported upon electrical stimulation to remember social encounters from their past which were accompanied by strong fearful, guilty, but also erotic feelings (Brothers, 1990).

In humans and non-human primates, damage to the amygdala primarily results in disinhibited approach behaviour and an unusual friendliness towards others (Adolphs, 1999; Emery et al., 2001).

In humans, amygdala damage also goes along with an impaired processing of emotionally and socially salient stimuli from faces. For example, these patients were impaired when judging particularly negative emotions, such as fear and sadness, but not happiness

(Adolphs et al., 1995; Adolphs and Tranel, 2004). Moreover, when asked to judge the trustworthiness from other peoples eyes, they judged those faces abnormally trustworthy and approachable who to normal subjects looked the most untrustworthy and unapproachable (Adolphs et al., 1998). In a recent study, Adolphs and colleagues found that a patient with amygdala damage failed to fixate the eye region of another person, indicating that information about the eye region was not used at all during this task. However, when instructed to attend the eye region, the patient was able to make correct judgments (Adolphs et al., 2005). These results indicate that the amygdala may process socio-emotional information from the eye regions of faces. While these finding were initially explained by assigning the amygdala a particular role in processing cues of danger and threat from faces, it was later discovered that it may play a broader role in perceiving complex social and emotional signals from both social stimuli such as faces and also non-social stimuli. For example, the amygdala is activated when a broader range of mental states has to be interfered from faces and eyes, such as concern, sympathy, flirtatiousness, etc. (Baron-Cohen et al., 1999). Furthermore, in a now classical study, Heider and Simmel (1944) demonstrated that normal subjects, when viewing videos that depict geometrical shapes on a plain, white background, spontaneously attribute social significance to the shapes. In contrast, patients with amygdala lesions do not make such attributions, but describe the shapes in purely geometric terms (Heberlein and Adolphs, 2004). Thus, the amygdala seems to be particularly involved in detecting and attributing social meaning to social as well as non-social stimuli.

It still needs to be elucidated, whether the same nuclei that contribute to fear conditioning, anxiety or memory modulation also process social information. Since neuroimaging studies do not have enough resolution to distinguish between amygdaloid nuclei (Zald, 2003), this question remains to be resolved. However, recent animal studies indicate that particularly the medial nucleus might be involved in processing social information (Ferguson et al., 2001). However, further studies will have to be conducted to elucidate this issue.

In summary, the amygdala participates in a network that processes social information and generates socio-emotional behaviour. Its' particular function might be to attribute social meaning to faces and environmental situations.

1.2.8. Implications for pathological states

Given the extensive connectivity of the amygdala with other brain areas and its important role in processing emotional stimuli, exerting control over attentive processes, modulating memory storage in multiple brain areas, processing, storing and controlling the expression of aversive fear conditioned memories, its involvement in controlling anxiety states, the regulation of hormonal and autonomic responses and also attributing social significance to stimuli, it is only self-evident that a dysfunction in this system may have severe consequences on our physical and particularly mental health.

Disorders associated with abnormal fear processing and anxiety, but also disorders associated with abnormal social behaviour have all been linked to structural or functional abnormalities in the amygdala (for reviews see Cottraux, 2005; Damsa et al., 2005; Hajek et al., 2005; Shayegan and Stahl, 2005; Blair et al., 2006).

For example, post-traumatic stress disorder (PTSD) is characterized by intrusive recollections of a traumatic event, hyper-arousal and avoidance associated with the traumatic experience. Recent neuroimaging evidence indicates that in these patients the amygdala might be hyper-reactive when exposed to trauma-triggering stimuli, but probably also to neutral stimuli (Damsa et al., 2005). A similar pattern of a hyper-reactive/active amygdala was found

in social phobia (Stein et al., 2002; Tillfors et al., 2002). Furthermore, volumetric abnormalities were found in patients with bipolar disorder (Hajek et al., 2005) and depression (Leppanen, 2006), another two anxiety-linked disorder.

Schizophrenia is a highly complex disorder characterized by multiple independent symptom domains, including cognitive and affective clusters. On the affective site schizophrenic patients have deficits in emotion recognition and interpretation. Interestingly, in these patients the amygdala seems to fail to activate in many situations (Shayegan and Stahl, 2005).

Finally, abnormalities in amygdala processing have also been reported in autism, which is characterized by abnormal social interactions, communication and increased repetitive behaviours. Chapter 3 deals in detail with the observations made in this domain.

In summary, abnormalities in amygdala volume and functionality have been observed in many psychiatric disorders. In this context it is worthy to mention that the amygdala is not the only structure contributing to the symptomotology of these disorders, but acts in conjunction with many other brain structures. In many cases, abnormalities in circuits encompassing the amygdala, the prefrontal and medial temporal lobe system are observed.

2. Aversive memories, the amygdala and PSA-NCAM

In this chapter some of the molecular mechanisms underlying the formation of conditioned fear in the amygdala are investigated. The neural cell adhesion molecule (NCAM) and its' polysialylated form (PSA-NCAM) are known to be involved in activity-induced synaptic plasticity processes and synaptic remodelling in the hippocampus thought to underlie the formation of memories (Fields and Itoh, 1996; Benson et al., 2000; Cremer et al., 2000; Ronn et al., 2000). Here, the question addressed is whether PSA-NCAM also mediates fear memory formation in the amygdala. Much to our surprise and at difference the vast amount of data implicating PSA-NCAM in hippocampus-dependent memory formation, we found that PSA-NCAM is not involved in aversive memory formation in the amygdala. These results suggest that PSA-NCAM might play very specific, brain region-dependent, and possibly synapto-specific roles in memory formation. Thus, our data indicate that not all mechanisms of memory consolidation involve this pathway.

This chapter introduces general concepts of cell adhesion molecules. The roles of NCAM and PSA-NCAM in activity-induced synaptic plasticity and learning and memory paradigms are reviewed. Finally, two studies summarizing our results are presented and the results discussed.

2.1. Structure and function of neural cell adhesion molecules

Cell adhesion molecules (CAMs) are cell-surface macromolecules that can hold the membranes of two cells together. CAMs are found in the CNS and are crucially involved in the development of the nervous system by regulating processes such as neuronal adhesion and migration, neurite outgrowth, fasciculation, synaptogenesis and intracellular signaling (Benson et al., 2000; Yamagata et al., 2003; Gerrow and El-Husseini, 2006). Since the expression of many CAMs persists into adulthood it has been suggested that they play a role in the activity-dependent synaptic plasticity leading to strengthening or weakening of existing synapses, rearrangement or even establishment of new synaptic contacts (Fields and Itoh, 1996; Benson et al., 2000; Yamagata et al., 2003; Washbourne et al., 2004). CAMs may have four distinct functions at the synapse (Yamagata et al., 2003). Firstly, as the name states, they are involved in linking pre- and post-synaptic membranes and thus ensure the *integrity of the junction and stability of synapses*. Secondly, CAMs may be involved in *target recognition*, an important aspect of synaptogenesis. Axons grow to their target fields and form synaptic contacts with the correct postsynaptic cell type. Given the huge number of specific synapses in the CNS, is it has been argued that specific markers or "tags" expressed pre- and postsynaptically signal the correct target. Already in 1963 Sperry suggested the influential "chemoaffinity hypothesis" in which the existence of specific adhesion molecules underlies a "lock and key" type mechanism (Sperry, 1963). However, it was not until recently, that CAMs were shown to play a crucial role in target recognition (Yamagata et al., 2002; Shen and Bargmann, 2003). Thirdly, CAMs can be involved in the *differentiation of pre- and postsynaptic specializations*. After contact initialization, the synapse has to become functional. At the presynaptic side, this requires the establishment of an active zone with a transmitter release machinery. At the postsynaptic side, the postsynaptic density (PSD) has to be built including the transmitter receptors and associated signalling molecules. The recruitment of different molecules to pre- and postsynaptic sites suggests the involvement of transmembrane adhesion molecules which may interact with distinct scaffolding and signalling proteins. Finally, CAMs could play a role in the *regulation of synaptic strength at mature synapses*. After the synapse is built, it may be stabilized or lost, depending, among other factors, on its usage and the amount of activation. For example, glutamatergic synapse maturation involves the transformation of an immature postsynaptic compartment into a mature spine. Spines develop from dendritic filopodia into actin-based shafts with a bulbous head. These morphological changes in the actin cytoskeleton involve many molecules, including CAMs (Ethell and Pasquale, 2005).

There are five different groups of adhesion molecules (fig.6), of which some members are expressed at the synaptic junction. The main groups are:

i) integrins
ii) cadherins
iii) neurexins
iv) neuroligins
v) members of the immunoglobulin (Ig) superfamily

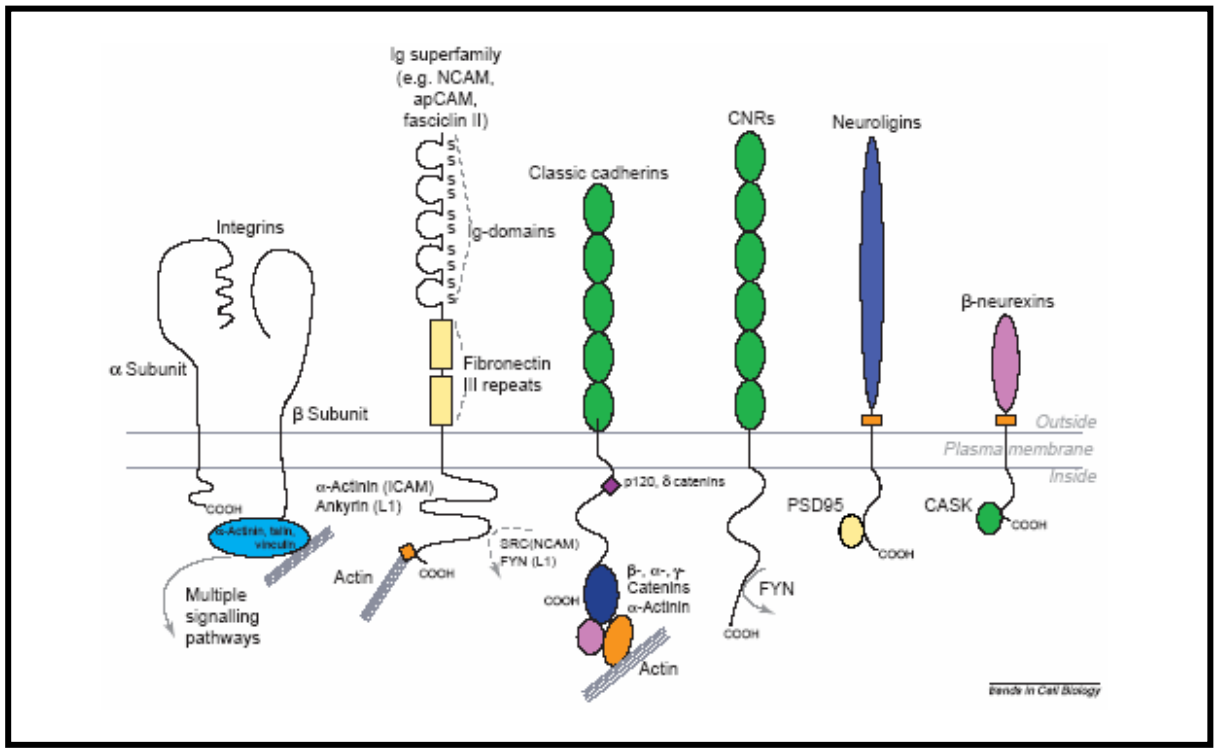

Figure 6. Schematic structure of cell adhesion molecules. For explanation on structure and function see text. From (Benson et al., 2000).

Integrins are heterodimeric glycoproteins consisting of two subunits, α and β, which can be expressed and localized differently. The cytoplasmic domain contains binding sites to interact with the actin-cytoskeleton and numerous other cytoplasmic molecules. The classic integrin function is to join cells with extracellular substrates. However, they also interact with Ig-superfamily members and cadherins in cell adhesion processes. Electron microscopy studies have shown that α8 and β9 subunits can be concentrated at postsynaptic densities (PSD) (Einheber et al., 1996), where they play an important role in activity-induced synaptic plasticity (Staubli et al., 1998).

The ***cadherin*** family includes classic cadherins (N-cadherin and E-cadherin), cadherin-like neuronal receptors (CNRs), and protocadherins. Cadherins are proteins with a single transmembrane domain mediating strong, Ca^{2+} dependent cell-cell adhesion. Most of the synapses in the CNS contain cadherins (Uchida et al., 1996). Their function may lie in controlling spine structure during synapse formation (Murase et al., 2002; Togashi et al., 2002). Due to the high amount of genes that encode protocadherin and their mutually exclusive loci in the CNS, their involvement in specific target recognition has been postulated (Kohmura et al., 1998).

Neurexins are expressed presynaptically and comprise an enormous variety of brain-specific molecules through differential splicing. β-neurexin in interaction with neuroligin has been mainly associated with the recruitment of postsynaptic molecules (Graf et al., 2004; Nam and Chen, 2005).

Neuroligins are type 1 membrane proteins expressed postsynaptically. They bind to neurexins in a Ca^{2+} dependent manner. Interactions between neuroligin and β-neurexin increases the size and number of presynaptic terminals (Levinson et al., 2005) and triggers the recruitment of presynaptic molecules (Scheiffele et al., 2000).

Members of the Ig- superfamily, such as the neural cell adhesion molecule (NCAM), apCAM (Aplysia homologue of NCAM present at sensory synapses), fasciliclin (Drosophila homologue of NCAM present at the neuromuscular junction), L1 or neuroplastin are either type I transmembrane or glycosylphosphatidylinositol (GPI)-linked proteins. They have up to five Ig-like domains that mediate recognition and adhesion. At least one NCAM isoform (NCAM-180, Persohn et al., 1989) and neuroplastin-65 (Smalla et al., 2000) were observed in dendritic spines in the adult brain.

2.1.1. NCAM

Genes

Three major NCAM isoforms can be generated by alternative splicing from a single-copy gene, which is composed of 19 exons and six smaller exons (Cunningham et al., 1987; Walmod et al., 2004) and can be further modified by alternative splicing in exons encoding the extracellular domain. NCAM differ in their molecular weight and their attachment to the membrane: NCAM-120, NCAM-140 and NCAM-180. NCAM expression can be regulated by several transcription factors coded, among others, by the *Hox* and *Pax* genes (Edelman and Jones, 1995).

Isoforms

NCAM-180 is preferentially localized to sites of cell-cell contact, including post-synaptic densities and has an extensive intracellular domain. NCAM-140 has a shorter cytoplasmic domain and is mobile in the membrane plane. NCAM-120 is attached to the membrane via GPI. The extracellular part of NCAM consists of five Ig-modules and two fibronectin type III (FnIII) homology modules and can interact with extracellular matrix molecules, such as heparin, heparin sulphate and collagens (Walmod et al., 2004). The cytoplasmic domain can be tethered to the cytoskeletal component actin, which has been implicated in the movement of molecules and vesicles, as well as morphological restructuring.

Expression pattern

All NCAM isoforms are expressed on neurons. They are found at pre- and postsynaptic membranes, but NCAM-180 is predominat only at postsynaptic densities (Persohn and Schachner, 1987; Persohn et al., 1989; Persohn and Schachner, 1990; Schuster et al., 2001). NCAM-140 and NCAM-120 are not only specific to neurons, but have also been found in astrocytes (Keilhauer et al., 1985), in oligodendrocytes (Bhatnagar et al., 2004), in Schwann cells (Seilheimer et al., 1989) an in other tissues, such as muscles (Covault and Sanes, 1986), endocrine cells (Langley et al., 1989) and reproductive organs (Moller et al., 1991).

Interactions

NCAMs bind trough homophilic (to another NCAM) or heterophilic (to another CAM, e.g. L1 or extracellular matrix molecules) interactions. The exact binding mechanism is not yet completely understood. It has been proposed for homophilic interactions to be mediated via reciprocal interactions of the third Ig domain between two NCAM molecules at opposing membranes (Rao et al., 1992). Alternative suggestions include reciprocal interactions between all five Ig domains, the third Ig domain with the fourth Ig domain, the fifth with the first Ig domain, the second with the fourth Ig domain or the third with the third Ig domain (Ranheim et al., 1996).

Function

The primary function of NCAM is to mediate neuron-to-neuron and neuron-to-glia adhesion (Keilhauer et al., 1985). NCAM is crucial for developmental processes such as cell migration and axonal guidance (Edelman and Jones, 1997), and neurite outgrowth (Rutishauser, 1985; Doherty et al., 1992; Hall et al., 1996). Antibodies against NCAM disrupt neuronal migration and arrangement of layers (Buskirk et al., 1980). Inactivation of the NCAM gene in mice results in reduced size of the olfactory bulb (Cremer et al., 1994) and abnormal lamination of mossy fibers innervating the CA3 region of the hippocampus (Cremer et al., 1997). In the adult brain, NCAM was linked to synaptic plasticity (see chapter 2.2). However, it has been proposed that this function is primarily mediated by polysialic acid (Eckhardt et al., 2000).

Signaling mechanisms

Besides of their adhesive function, it is now recognized that NCAMs are also primarily signaling molecules (Juliano, 2002; Rougon and Hobert, 2003; Walmod et al., 2004). Figure 7 describes several of these signaling pathways.

Several growth factor receptors and extracellular matrix molecules (ECM) have been identified to interact with the extracellular NCAM domain, such as the fibroblast growth factor receptor (FGFR; Doherty and Walsh, 1996), the glial cell derived neurotrophic factor (GDNF) (Paratcha et al., 2003), the brain derived neurotrophic factor (BDNF) (Muller et al., 2000), heparin (Cole and Glaser, 1986), a number of chondroitin sulfate proteoglycans (Grumet et al., 1993), heparan sulphate proteoglycans (Burg et al., 1995; Dityatev et al., 2004) and collagens (Probstmeier et al., 1992), all of which can trigger intracellular signalling cascades and lead to neurite outgrowth.

For example, it is established that NCAM can interact with the FGFR, which is an IgSF receptor tyrosine kinase. Homophilic NCAM binding leads to autophosphorylation of the FGFR (Saffell et al., 1997). This interaction is mediated by binding of the second NCAM FnIII module with FGFR Ig-modules D2 and D3 via the so called FG loop (FGL), which can induce neurite outgrowth (Doherty and Walsh, 1996; Kiselyov et al., 2003). The exact mechanism has to be established yet, but it is known that FGFR phosphorylation induces several intracellular cascades, including activation of the phospholipase Cγ (PCγ). The substrate of PCγ is PIP_2 (phosphatidylinositol 4,5-bisphosphate), which is cleaved to generate the two second messengers IP_3 (inositol 1,4,5-trisphosphate) and DAG (diacylglycerol). IP_3 diffuses through the cytosol and activates intracellular Ca^{2+} channels to release Ca^{2+} from intracellular stores (Kolkova et al., 2000b). DAG remains a part of the plasma membrane, and can either activate protein kinase C (PKC) or be converted by DAG lipase to 2-arachidonylglcerol (2-AG) or arachidonic acid (AA), which in turn controls several downstream signaling events, such as activation of voltage gated calcium channels (Williams et al., 1994).

An alternative concept for NCAM signaling has linked NCAM to the intracellular tyrosine kinases, p59Fyn and the focal adhesion kinase (FAK) (Beggs et al., 1997). Fyn, a member of the Scr-family of non-receptor tyrosine kinases, and FAK have been associated with NCAM-140. While it s not clear whether Fyn interacts directly with NCAM, FAK is believed to interact indirectly with NCAM by binding to the SH2 domain of Fyn (Beggs et al., 1997). Recruitment of the tyrosine kinases then leads to activation of the Erk/MAP kinase cascade and CREB phosphorylation (Schmid et al., 1999) and has been associated with neurite outgrowth (Beggs et al., 1994).

It is of interest to note, that the FGFR and Fyn/FAK pathways may be complementing each other. The FGFR is not found in cholesterol-rich membrane microdomains, the so-called lipid rafts (Davy et al., 2000), whereas NCAM-180 and NCAM-140 are localized both within

and outside lipid rafts. Outside of lipid rafts NCAM might trigger the FGFR pathway, whereas inside it might activate the Fyn/FAS pathway (Rougon and Hobert, 2003).

Furthermore there is a link between NMDA receptor (NMDAR) activation, increased NCAM-180 expression (Hoffman et al., 1998; 2001) and NCAM mediated synaptogenesis (Dityatev et al., 2004). This may be of particular relevance to activity-induced synaptic plasticity processes, since the NCAM associated increase in hippocampal spine synapses after LTP induction can be blocked by NMDAR antagonists (Schuster et al., 1998). In synaptic membranes of non-stimulated spine synapses, NCAM-180 and the NR2A subunit of NMDA receptors are accumulated in the centre of the postsynaptic density and co-redistribute to the edges of the postsynaptic densities 24 h after induction of LTP (Fux et al., 2003). The exact pathways, leading from NMDAR activation to an enhanced NCAM-180 expression are not known. However, it is conceivable that NCAM and NMDA receptors are cross-linked by the membrane-cytoskeletal linker protein spectrin, which binds to both molecules (Wechsler and Teichberg, 1998; Sytnyk et al., 2002). NCAM may then promote the postsynaptic accumulation of NMDA receptors and/or modify their activity.

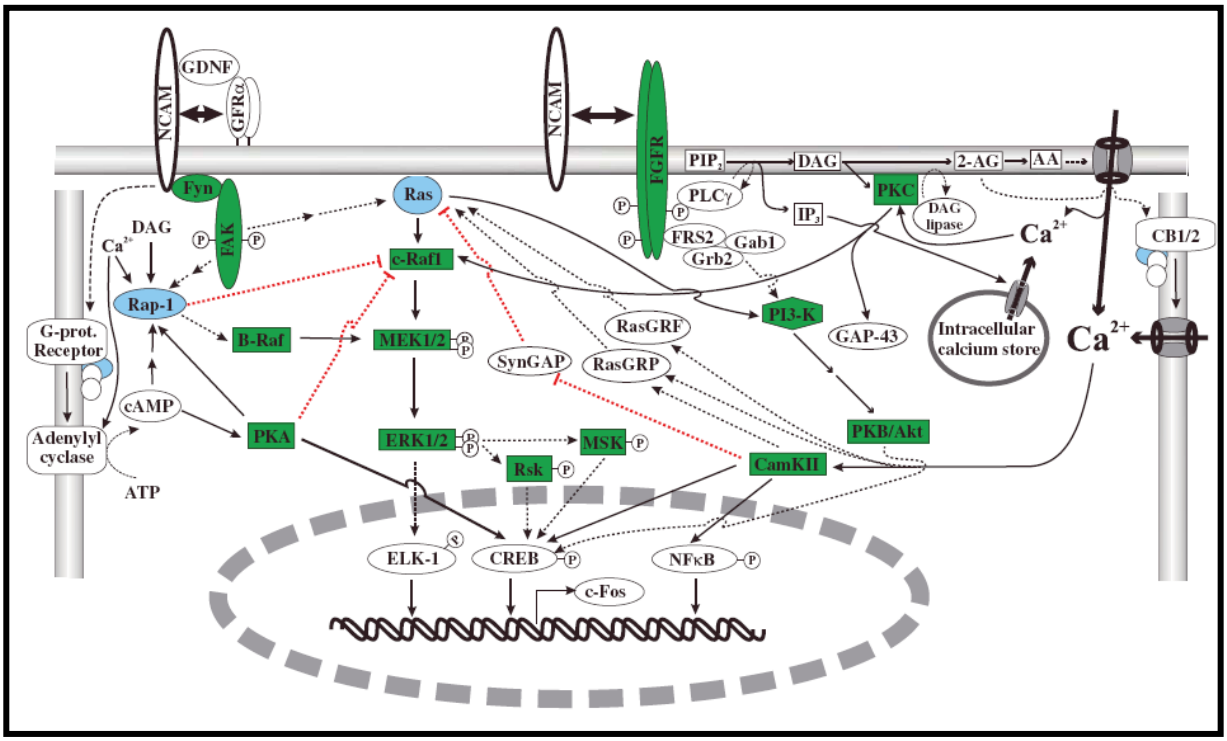

Figure 7. Intracellular signalling cascades associated with NCAM and PSA-NCAM. The figure is highly schematic and does not necessarily reflect the correct cellular localization of the respective proteins. Pathways, whose role in NCAM-mediated signalling remains to be determined, are indicated with dashed lines. Kinases are shown in green, Ser/Thr-kinases or dual-kinases as squares, Tyr-kinases as ellipses. GTPases are shown in blue. Inhibitory pathways are shown red. From Walmod et al., 2004.

2.1.2. PSA-NCAM

Structure

After translation NCAMs can be still modified by a variety of posttranslational modifications, including the addition of polysialic acid (PSA), a long homopolymer of α2,8-linked sialic acids.

Synthesis and Degradation

PSA is attached to NCAM in the trans-Golgi compartment via typical N-linked core glycosylation of the 5th Ig domain (fig. 8). This process is catalyzed by two sialyl transferases: sialyltransferase-X (STX or St8SiaII) and polysialyltransferase (PST or St8SiaIV), which are differently regulated during development (Eckhardt et al., 1995; Kojima et al., 1995; Nakayama et al., 1995; Scheidegger et al., 1995): St8SiaII/STX regulates PSA-NCAM expression during embryonic, perinatal and early postnatal development, whereas St8SiaIV/PST is predominant in the postnatal brain (Hildebrandt et al., 1998).

PSA can be degraded by a neural sialidase, which has been found in synaptosome-enriched fractions (Moran et al., 1986). During the postnatal period, when PSA expression decreases, the activity of the neural sialidase increases (Moran et al., 1986; Regan, 1991).

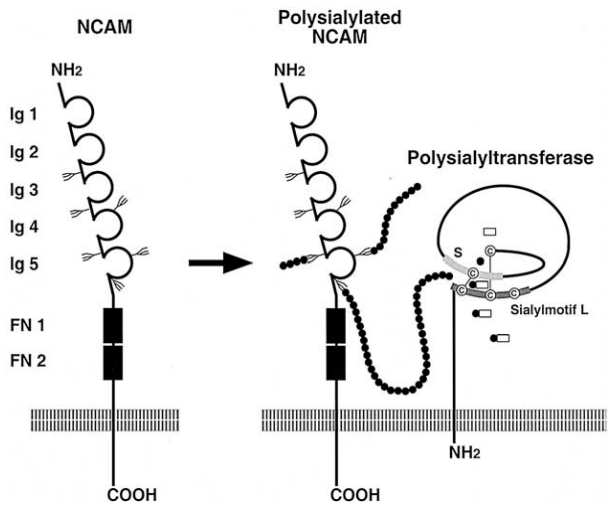

Figure 8. Schematic structure of NCAM and polysialyltransferase. NCAM has five immunoglobulin-like domains (Ig 1 to Ig 5) and two fibronectin type III repeats (FN 1 and FN 2). Polysialic acid is synthesized by polysialyl-transferases on *N*-glycans attached to the Ig 5 domain of NCAM. St8SiaIV, shown in the right, contains two disulfide bridges, denoted by thin lines with cysteines (©), and the COOH-terminal is close to sialylmotifs L and S. Sialylmotifs L and S are boxed. •, •• and • denote sialic acid, CMPNeuNAc and CMP, respectively. *N*-glycosylation sites in NCAM are shown by ψ. From (Angata and Fukuda, 2003).

Expression pattern

PSA is found almost exclusively in vertebrates and there almost exclusively in association with NCAM. However, PSA was also found to be expressed by St8SiaIV/PST (Muhlenhoff et al., 1996), one of the two enzymes known to synthesise PSA on NCAM, as well as by the α-subunit of a sodium channel (Zuber et al., 1992) and an intracellular protein of specific cancer-cells (Martersteck et al., 1996). During development, PSA-NCAM is widely expressed throughout the whole brain and crucially involved in cell migration, axonal outgrowth, fasciculation and pathfinding (for review see Kiss and Rougon, 1997; Ronn et al, 2000). In the adult brain, PSA-NCAM expression decreases dramatically, yet remains high in some areas known to be involved in structural remodelling and neurogenesis. Those include predominantly the rostral migratory stream in the olfactory system, the dentate gyrus, which continuously undergo neurogenesis, and the hypothalamus (Benson et al., 2000). However, in the rat, PSA-NCAM expression was also described in other brain regions in adulthood, such as various other subfields of the hippocampus (Nacher et al., 2002a), the amygdala (Nacher et al., 2002b), the prefrontal cortex (Varea et al., 2005), the piriform cortex and other neocortical regions (Seki and Arai, 1991; Nacher et al., 2002c).

PSA can be expressed at both, pre- and postsynaptic membranes (Schuster et al., 2001; Arellano et al., 2002).

Pharmacology against PSA-NCAM

PSA can be cleaved form NCAMs by means of endoneuraminidase (endoN). EndoN is an enzyme isolated from bacteriophages, which specifically recognizes the three-dimensional structure of polymers of sialic acid in a α2-8-linkage and cleaves units of eight sugar residues. It is highly specific such that no other sialic acid residues are degraded (Finne and Mäkelä, 1985).

Function

The attachment of PSA to NCAM has been proposed to attenuate its adhesive forces and shown to decrease homophilic as well as heterophilic NCAM binding (Yang et al., 1992; Yang et al., 1994; Fujimoto et al., 2001). Although this mechanism is not yet completely understood, there is evidence that this might be due to the large hydrated volume and negative charge of PSA. Therefore PSA-NCAM represents a less adhesive form of NCAM. This characteristic of PSA-NCAM might enable structural remodelling by eliminating some synaptic connections among multiple synaptic junctions, thereby possibly representing a segregation mechanism through which strong synaptic connections are established and weak ones eliminated.

In development, PSA-NCAM has been implicated in cell migration, axonal guidance and synapse formation (for review see Bruses and Rutishauser, 2001).

PSA-NCAM is involved in two of three modes of cell migration: axophilic and cooperative cell migration (but not gliophilic or radial migration). Studies with either NCAM knockout (which *de facto* also lack PSA) or endoN-treated mice revealed that both treatments result in smaller olfactory bulbs accompanied by an accumulation of olfactory interneuron precursors in the subventricular zone (Cremer et al., 1994; Ono et al., 1994) (fig. 9). Neuronal precursors migrate to the olfactory bulb in a stream using each other as substrate (cooperative migration). In this processes PSA might enhance the cycles of adhesion and deadhesion that would be required by such a mode of motility (Ono et al., 1994).

PSA-NCAM is important for axon guidance and synaptogenesis by interacting with multiple cues in the surrounding environment. During development, cycles of fasciculation (when axons travel in bundles) are followed by defasciculation (when the axons separate branch out and rearrange in the target region). PSA seems to reduce fasciculation thereby allowing axons to defasciculate. For example, in the peripheral motor system, motoneuron axon bundles up-regulate PSA expression when they enter the plexus region. During this time, they branch out and rearrange into muscle-specific nerves. Removal of PSA with endoN during this time prevents branching and results in pathfinding errors (Landmesser et al., 1990). Similarly pathfinding errors were observed in mossy fiber axons innervating the CA3 region of the hippocampus. Usually, mossy fibers originating in granule cells in the dentate gyrus form *en passant* synapses at proximal apical dendrites of pyramidal cells in the dendritic CA3 layer, a narrow band called stratum lucidum. After enzymatic or genetic removal of PSA-NCAM (or NCAM) mossy fiber axons exhibited ectopic synapses in the CA3 region, forming synapses in the pyramidal cell layer rather than stratum lucidum (Cremer et al., 1997; Cremer et al., 1998; Seki and Rutishauser, 1998). These data suggest that PSA-NCAM plays a crucial role for axonal pathfinding and synaptogenesis on the correct target.

In the adult brain, polysialylation of NCAM has been implicated in activity-induced plasticity, synapse formation and learning and memory processes (reviewed in the following chapters).

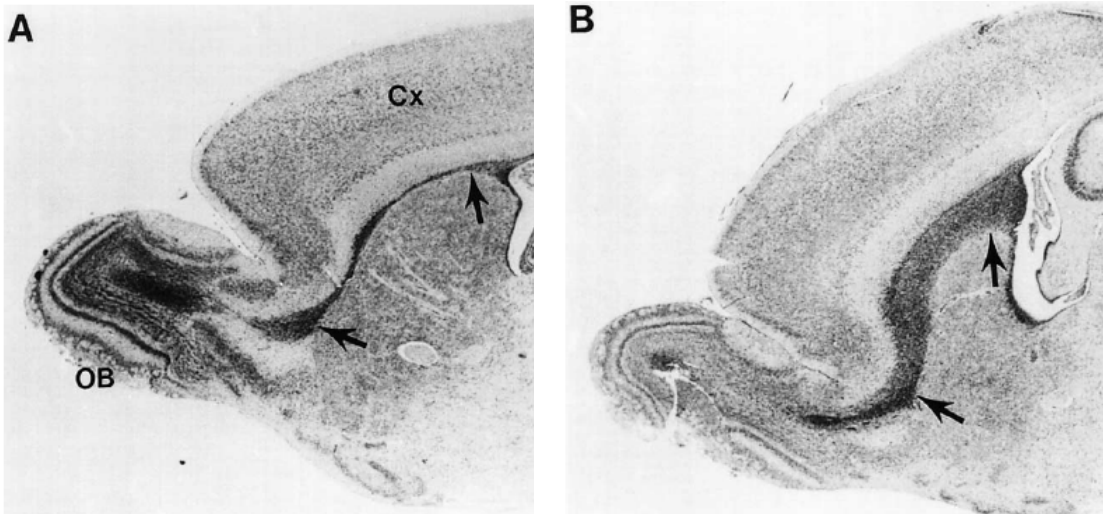

Figure 9. Effect of PSA on migration of olfactory bulb neural precursors in the subventricular zone of the neonatal mouse. (A) Precursors born in the subventricular zone below the cortex (Cx) follow a migratory path (arrows) toward the olfactory bulb (OB), where they become part of the characteristic layered structure of the OB. (B) When PSA is absent, either as a result of treatment with endo N or genetic deletion of NCAM, the precursors have difficulty migrating and thus overpopulate the subventricular zone. Note that as a result the olfactory bulb is smaller. From (Ono et al., 1994).

Signalling mechanisms

Several studies established links between PSA-NCAM and AMPA, NMDA and BDNF signalling.

While PSA-NCAM cleavage increased AMPA binding to AMPA receptors (AMPARs) (Hoffman et al., 1997), treatment with colominic acid, a bacterially derived PSA, prolonged the opening time of AMPAR-mediated currents in the young, but not old, hippocampus (Vaithianathan et al., 2004). These studies suggest that PSA-NCAM may have a direct or indirect influence on AMPARs, which may provide a mechanistic explanation for the PSA-NCAM involvement in activity-induced synaptic plasticity (Becker et al., 1996; Muller et al., 1996). Since expression of PSA on NCAM changes its adhesive forces, it has been suggested that conformational changes of the synapse may account for the observed effects.

NMDARs are also closely associated with PSA-NCAM expression (Bouzioukh et al., 2001; Nacher et al, 2001, Nacher et al., 2002c; Fux, 2003) and PSA-NCAM mediated activity-induced synaptic plasticity (Bouzioukh et al., 2001; Fux et al., 2003). For example, activation of NMDARs results in rapid changes in expression of PSA-NCAM. Electrical stimulation of the vagal nerve causes a rapid decrease of postsynaptic PSA-NCAM expression in the adult vagal nucleus both *in vivo* and *in vitro.* Inhibition of NMDAR and the downstream NO/cGMP pathway completely prevent PSA-NCAM internalization and concomitant LTD (Bouzioukh et al., 2001).

BDNF activates the receptor tyrosine kinase TrkB, which influences axon guidance and synaptic plasticity (Paves and Saarma, 1997; Thoenen, 2000). Muller et al. (2000) showed that impaired LTP usually observed in NCAM deficient mice and in organotypic slices lacking PSA-NCAM can be totally restored by application of BDNF. Furthermore, cleavage of PSA-NCAM resulted in a reduction of BDNF receptor TrkB activation (Muller et

al., 2000). This suggests that PSA-NCAM may enhance or facilitate BDNF signalling and thus influence axon guidance and BDNF dependent synaptic plasticity.

2.1.3. Models of neural cell adhesion molecule functioning

The mechanisms leading to a potentiation of synaptic strength were usually discussed in the context of an increase in transmitter release at the presynaptic site or an increase in sensitivity, number of active synapses and recruitment of silent synapses at the postsynaptic site. Recently, the focus of the discussion took a turn into the direction of the formation of new synapses and structural remodelling of the synaptic network. Several models may depict the way NCAMs and their polysialylation might be contributing to the strengthening of existing and/or to the formation of new synapses (for review see Benson, 2000). Those are:

1. NCAM/PSA-NCAM might directly modulate glutamate receptor properties. Therefore they might be directly responsible for the molecular events leading to the establishment of a potentiated response following LTP. Evidence indicates that both NCAM and PSA-NCAM expression are tightly linked to AMPAR and NMDAR functioning (Hoffman et al., 1997, 1998, 2001; Vaithianathan et al., 2004)
2. The presynaptic active zones and postsynaptic densities could be altered by the action of NCAMs. This would affect the density, compartmentation and position of glutamatergic receptors (i.e. Fux et al., 2003).
3. NCAMs could also affect the distance between pre-and postsynaptic membrane. Intracellular signalling cascades evoked by Ca^{2+} entry through NMDARs might increase the adhesive force between NCAMs or other CAMs expressed at the pre- and postsynaptic membrane and therefore shorten the synaptic cleft. This in turn would increase the glutamate concentration in the cleft and keep the glutamate receptors activated longer.
4. On a long-term scale NCAMs might also alter the interactions between neurons and the astroglia that usually surround the synaptic junction (Theodosis et al., 2004). Adhesion between neuronal and astroglia membrane might be altered, either by wrapping the astroglia closer around the cell and therefore introducing changes in glutamate re-uptake or by preventing pre- and postsynaptic communication by sliding into the synaptic cleft. For example, in hypothalamus glial cells were found to form a reversible barrier to synaptic communication.
5. NCAM and PSA-NCAM might be involved in the growth and remodelling of synapses through sophisticated homophilic or heterophilic interactions: while at some sites adhesion molecules might be internalized from the surface of the synaptic membrane (Mayford et al, 1992) or a less adhesive form might be acquired through attachment of PSA, at other sites NCAM or other CAM expression might be enhanced and adhesive forces therefore increased, a process which would result in a selective restructuring of the synaptic architecture.

2.2. NCAM and PSA-NCAM in activity-induced synaptic plasticity

The concept of activity-induced synaptic plasticity was raised on purely theoretical grounds by the psychobiologist Donald Hebb in his famous book "The Organization of Behaviour" published in 1949. Basically, he stated that if two cells interconnected by a synapse fire at the same time the synaptic connection between them should become stronger. About 20 years later, in 1973, this concept was empirically undermined with the discovery of long-term potentiation (LTP) in the rabbit hippocampus (Bliss and Lomo, 1973) and has since then inspired numerous studies. LTP can be induced by brief periods of high-frequency stimulation leading to potentiation of responses at the postsynaptic side which can last between minutes to hours and even days. LTP has been most extensively studied in the hippocampus, but can also be induced in any other region expressing glutamatergic synapses, including the neocortex (Lee et al., 1991), the amygdala (Clugnet and LeDoux, 1990), the optic tectum (Lewis and Teyler, 1986) or olfactory bulb (Patneau and Stripling, 1992). LTP is widely believed to be a substrate for memory.

The mechanisms underlying the establishment of an increase and maintenance of synaptic strength are not completely understood, yet there is a huge pool of evidence focusing on modifications of intracellular signaling cascades leading to an increase of transmitter release at the pre-synaptic side or the recruitment and regulation of receptors at the postsynaptic side. Moreover, long lasting changes in synaptic strength are thought to be accompanied by structural changes in neuronal architecture (Luscher et al., 2000; Carlisle and Kennedy, 2005). In accordance with this idea is that LTP can lead to the formation of new synapses, as it has been associated with the growth of new dendritic spines (Engert and Bonhoeffer, 1999) and presynaptic boutons (Antonova et al., 2001), the appearance of perforated synapses, and the formation of multiple synapse boutons (Toni et al., 1999).

Evidence indicates that activity-induced expression and modulation of CAMs in general and NCAM/PSA-NCAM in particular may be one of the crucial processes translating synaptic activity into structural changes of the synapse in the mature brain (Fields and Itoh, 1996; Ronn et al., 1998; Benson et al., 2000; Cremer et al., 2000; Ronn et al., 2000).

All studies examining the role of NCAMs and PSA-NCAMs on activity-induced synaptic plasticity were conducted almost exclusively in the rodent hippocampus (excluding studies on apCAM in Aplysia and Fas II in Drosophila reviewed separately) and can be classified as belonging to one of three main streams:

a. NCAM expression after induction of LTP (Fazeli et al., 1994; Schuster et al., 1998),
b. The use of antibodies against NCAM (Luthl et al., 1994; Ronn et al., 1995) or PSA-NCAM (Becker et al., 1996; Muller et al., 1996),
c. The use of knockout mice with deactivated genes for the NCAM isoforms (Muller et al., 1996; Cremer et al., 1998; Muller et al., 2000; Bukalo et al., 2004; Stoenica et al., 2006) or the sialyl transferases (Eckhardt et al., 2000; Angata et al., 2004; Stoenica et al., 2006).

A complex hippocampal subfield specific pattern of NCAM or PSA-NCAM dependency (or independency) emerged from these studies. It seems that both NCAM and PSA-NCAM are not involved in all synaptic plasticity processes, but exhibit a high degree of specificity, depending on the morphological context and receptors involved in these processes. The following sections discuss NCAM/PSA-NCAM involvement in activity-induced synaptic plasticity considering each of the hippocampal subfields. Tables 2 and 3 summarize the results and figure 10 depicts the hippocampal organization.

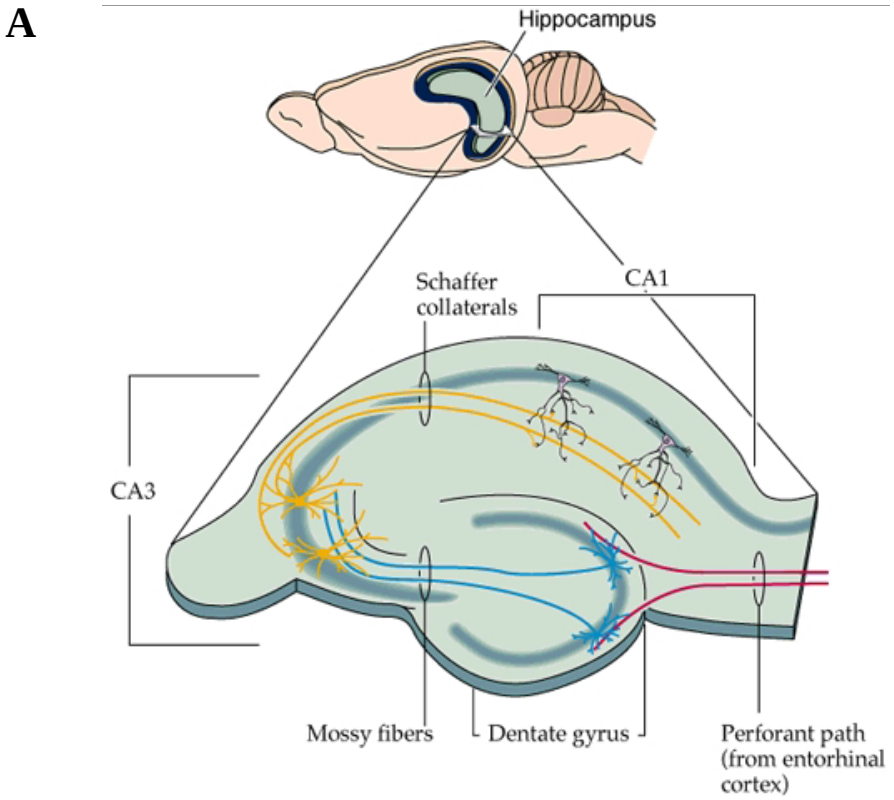

Figure 10. Scheme of the rat hippocampus. (A) The hippocampus is located in the temporal lobe and consists of the hippocampus proper (CA1-CA4) and dentate gyrus (GD). Information enters the dentate gyrus via the perforant path from the enthorinal cortex. Granule cells in GD project via mossy fibers to areas CA3/CA4. From there information propagates via the Schaffer collaterals to area CA1 and leaves the hippocampus via the the fornix in the subiculum. (B) Laminar organization of the hippocampus. Black triangles mark the stratum pyramidale. Stratum lucidum, grey; F, fimbria. Stratum granulosum of the GD is marked by large dots. The stratum moleculare is subdivided into thirds (1–3), the outer one (3) is in grey. The two rhomboids represent the areas trimmed for CA3/CA4 (strati pyramidale, lucidum, and radiatum) and for the dentate gyrus (strata granulosum and moleculare). Adopted from (Schuster et al., 2001).

2.2.1. Role of NCAM/PSA-NCAM in dentate gyrus synaptic plasticity

In the dentate gyrus, cumulative electrophysiological data suggest that activity-induced synaptic plasticity processes are mediated by NCAM alone and do not depend on PSA-NCAM.

The very first studies suggesting an involvement of NCAM in activity-induced synaptic plasticity processes stem from early studies monitoring the expression of NCAM after LTP induction. Fazeli and colleagues showed that extracellular NCAM expression is increased in the dentate gyrus 90 minutes after LTP induction in vivo (Fazeli et al., 1994). Schuster and colleagues elicited LTP in the perforant path of the hippocampus and checked with immunoelectron microscopy for the expression of NCAM-180 at spine synapses in the molecular layer of the dentate gyrus, since previous studies already revealed that this isoform is predominantly expressed at postsynaptic densities (Persohn et al., 1989; Persohn and Schachner, 1990). They showed that the expression of NCAM-180 increased from 37% in passive controls to 70% 24 hours following LTP. Furthermore, this elevation of NCAM-180 positive PSDs could be prevented by blocking NMDA receptors, indicating that NCAM expression depends on NMDA receptor activity (Schuster et al., 1998). This study also shows that NCAM is crucial not only in the initial induction and stabilization of LTP but also at later phases, suggesting a role in long-term structural remodelling of the synapse. Although fundamental, these two studies are the only ones throughout the whole hippocampus, which ever checked for NCAM expression after induction of LTP. They show that NCAMs are expressed in an activity-dependent manner at the synaptic junction where they might be crucially involved in the strengthening of the synaptic connection or the formation of new synapses.

Recently, an *in vivo* study with constitutive NCAM, St8SiaII/STX and St8SiaIV/PST knockout mice revealed the unique contributions of NCAM and PSA-NCAM to activity-induced synaptic plasticity in the dentate gyrus (Stoenica et al., 2006). Mice, which lacked NCAM throughout the whole development, exhibited impaired LTP, whereas mice deficient for either of the two polysialyltransferases had normal levels of LTP. However, St8SiaII/STX deficient mice exhibited impaired levels of basal synaptic transmission in the perforant – dentate gyrus pathway and St8SiaIV/PST deficient mice exhibited abnormalities in paired-pulse facilitation of transmitter release. These results suggest that in the dentate gyrus activity-induced synaptic plasticity requires only NCAM, but not the polysialylated form, PSA-NCAM. However, polysialylation of NCAM expressing immature granule neurons or synapses may support the development of basal excitatory synaptic transmission in the dentate gyrus.

2.2.2. Role of NCAM/PSA-NCAM in CA3 synaptic plasticity

In the CA3 subfield, LTP at mossy fiber synapses relies neither on NCAM nor on its polysialylated form. However, PSA-NCAM during development is important for axonal pathfinding and establishment of correct synaptic contacts.

Evidence undermining the role of NCAMs CA3 in synaptic plasticity stems from studies with constitutive NCAM deficient mice (Cremer et al., 1998). These animals exhibit basal synaptic transmission and maintain two forms of short-term synaptic plasticity, i.e. paired-pulse facilitation and frequency facilitation. However, when the mossy fibers of NCAM deficient mice receive several high-frequency pulses, which usually lead to long-lasting LTP at mossy fiber synapses in the CA3 region in the wildtype, LTP can in fact be induced, but not maintained. It decays to baseline levels within 40 min. Even if tempting to

conclude that NCAMs are necessary for stabilization of LTP in the CA3 subfield, it has to be taken into account that fasciculation and laminar growth of the mossy fibers are strongly affected in constitutive NCAM deficient mice, thus leading to ectopic synapses. In the NCAM knockout mice mossy fibers form synapses in the stratum pyramidale, rather than the stratum lucidum as observed in wildtype mice (Cremer et al., 1997; 1998). Therefore it is not clear whether the deficient LTP was due to the morphological changes or to a lack of NCAM. Cremer and colleagues argued for the second option, because LTP at mossy fibers is NMDA-independent and relies on a purely presynaptic increase in transmitter release. Moreover, since mossy fibers in NCAM knockout mice seem to be morphologically intact, Cremer et al. (1998) concluded that the impairment in LTP must be due to the NCAM deficiency rather than ectopic synapse formation. Nethertheless, the abnormal morphological development in the mossy fiber system cannot be completely excluded as an underlying cause.

To address this issue studies with conditional NCAM knockout mice were undertaken (Bukalo et al., 2004). In contrast to constitutive NCAM knockout mice, which lack NCAM throughout the whole development and the whole brain, in conditional NCAM mutants the NCAM gene can be ablated under the control of the αCaMKII promoter in hippocampal neurons after major developmental processes are completed. NCAM deficiency is usually observed from P22 onwards. In conditional NCAM deficient mice, mossy fiber projections into the CA3 region developed normally and LTP in the CA3 region was not affected.

This suggests that LTP impairments in adult constitutive NCAM knockouts might have been due to morphological changes, rather than NCAM deficiency itself. This view is supported by an immunogold electron microscopy study, showing that no NCAM isoforms are expressed at mossy fiber synapses in the CA3 subfield of an adult rat hippocampus (Schuster et al., 2001). Thus, NMDA independent LTP at mature mossy fiber synapses in the CA3 region seems to also be NCAM independent. However, this conclusion does not exclude that NCAM is very important for the correct establishment of synaptic contacts during development.

Studies with St8SiaII/STX and St8SiaIV/PST knockout mice investigated the involvement of PSA-NCAM in synapse formation and activity-induced synaptic plasticity in the CA3 region. Interestingly, the St8SiaII/STX mice exhibit similar morphological changes in the CA3 region as constitutive NCAM deficient mice (Angata et al., 2004), whereas St8SiaIV/PST mice exhibit normal laminar organization and synapse formation in CA3 (Eckhardt et al., 2000). This result suggests that polysialylation of NCAM probably in early development is crucial for the correct establishment of the CA3 mossy fiber innervation and synaptic architecture. Thus, it seems that only polysialylated NCAM, and not NCAM, alone mediates correct pathfinding and target recognition in the CA3 region. On the other hand, in none of the two sialyl transferase deficient mice LTP seemed to be affected (Eckhardt et al., 2000; Angata et al., 2004). This is in accordance with the results obtained from conditional NCAM deficient mice, which as a matter of fact also lack PSA-NCAM. Thus, PSA-NCAM seems not to contribute to synaptic plasticity mechanism at the mature mossy fiber synapse, but rather seems to play an important role in early pathfinding and axon targeting mechanisms. This is in accordance with immunogold electron microscopy data, which suggest no expression of PSA at mature mossy fiber synapses in the CA3 region of the hippocampus (Seki and Rutishauser, 1998; Seki and Arai, 1999; Schuster et al., 2001).

2.2.3. Role of NCAM/PSA-NCAM in CA1 synaptic plasticity

In the CA1 subfield, activity-induced synaptic plasticity seems to rely on the presence of PSA-NCAM, synthesized by the predominantly postnatally expressed St8SiaIV polysialyltransferase.

The first experiments conducted to investigate the role of NCAM in activity-induced synaptic plasticity in the CA1 subfield used antibodies applied *in vitro*. This treatment attenuated LTP induction, whereas it affected neither normal synaptic transmission nor the maintenance of LTP when antibodies were applied after the induction phase (Luthl et al., 1994; Ronn et al., 1995). This suggests a role for NCAMs in the early mechanisms leading to LTP.

Experiments with constitutive NCAM knockout mice as well as conditional NCAM deficient mice provided similar results: both mouse strains exhibited deficits in LTP (Muller et al., 1996; Muller et al., 2000; Bukalo et al., 2004) and the conditional NCAM deficient mice also in long-term depression (LTD; Bukalo et al., 2004).

However, as stated above, deficits in NCAM are accompanied by a loss of PSA as well. Thus, it is not clear whether possible plasticity impairments are mediated by NCAM alone or rather by its polysialylated form. Interestingly, cleavage of PSA-NCAM with endoN or lack of PSA in polysialyltransferase-deficient mice provided evidence that the LTP deficits observed above might actually be due to a PSA loss. Becker and colleagues applied endoN and observed severe deficits in the induction phase of LTP in the CA1 region (Becker et al., 1996). Mice deficient for the predominantly postnatally expressed St8SiaIV/PST enzyme also exhibited impaired LTP as well as impaired LTD (Eckhardt et al., 2000). Interestingly, mice deficient for the predominantly pre- and peri-natally expressed St8SiaII/STX were not impaired in LTP in the CA1 subfield (Angata et al., 2004). This suggests that in the CA1 subfield of an adult hippocampus, activity-induced synaptic plasticity relies on PSA-NCAM rather than NCAM alone. More specifically the synthesis of PSA on NCAM must be catalyzed by the predominantly postnatally expressed St8SiaIV/PST.

2.2.4. Conclusions on the hippocampus

In summary, the following complex picture emerges from above studies: NCAM alone seems to mediate activity-induced synaptic plasticity in the mature dentate gyrus. However, this is only the case for this region, as in any other subfield PSA-NCAM is required either for synaptic plasticity (CA1) in the mature brain or for correct synaptogenesis (CA3) during development. In the CA3 region, neither NCAM nor PSA-NCAM are required for LTP processes in the adult rat, but PSA-NCAM synthesized by St8SiaII/STX is required for correct pathfinding and axonal targeting during development. This is paralleled by the finding that neither NCAM nor PSA-NCAM are expressed at mature mossy fiber synapses (Schuster et al., 2001)**.** On the other hand, PSA-NCAM is expressed in a subpopulation of synapses in the CA1 region (Schuster et al, unpublished data cited in Schuster et al., 2001) and activity-induced plasticity processes at these synapses require the presence of NCAM polysialylated by St8SiaIV.

Thus, it seems that the two polysialyltransferases, St8SiaII/STX and St8SiaIV/PST, adopt differential roles: the predominantly prenatally expressed St8SiaII/STX seems to be involved in developmental processes, such as pathfinding, axonal targeting and correct synaptogenesis. Its cleavage does not necessarily have consequences for activity-induced synaptic plasticity in the adult hippocampus. On the other hand, the predominantly peri- and post-natally expressed St8SiaIV/PST plays a crucial role for activity-induced synaptic

plasticity in the mature brain, suggesting that polysialylation of NCAM at later stages in life may be primarily necessary for translating activity into dynamical changes of synaptic strength.

Whether NCAM and/or PSA-NCAM are involved in activity-induced synaptic plasticity is additionally determined by the receptors expressed at the synapse. Interestingly, in striking contrast to synapses in CA1, the dentate gyrus and associational and commissural synapses in CA3 itself, mossy fiber synapses in the CA3 region express a low level of NMDA receptor binding and consequently LTP at mossy fiber synapses is NMDAR-independent (Harris and Cotman, 1986). This form of LTP is initiated by calcium entry into presynaptic terminals and increases in transmitter release (Castillo et al., 1994). However, NCAM as well as PSA-NCAM expression is closely associated with NMDA receptors expression and their activation or deactivation (Hoffman et al., 1998; Bouzioukh et al., 2001; Nacher et al., 2001; Nacher et al., 2002c; Fux et al., 2003). Moreover, NCAM and PSA-NCAM mediated activity-induced synaptic plasticity processes require NMDA receptor activation (Schuster et al., 1998; Dityatev et al., 2004). Thus it is conceivable that NCAM and/or PSA-NCAM associated activity-induced synaptic plasticity occurs only at NMDA expressing synapses. Tables 2 and 3 summarize the results.

2.2.5. Role of apCAM in synaptic plasticity in Aplysia

Studies on the mechanisms of memory storage in the invertebrate Aplysia (and also Drosophila, see below) show that the stabilization of memories goes along with growth of new synaptic contacts (Bailey and Kandel, 1993). One of the systems to study the functional and structural changes of synaptic connectivity following experience or activity is the gill-withdrawal reflex of Aplysia. Application of serotonin *in vitro* mimics sensory stimulation and produces sensitization of the reflex, which is associated with increased synaptogenesis. More than 20 proteins have been identified to be increased by application of serotonin, whereas 5 proteins are decreased. Four of these molecules are cell adhesion molecules from the immunoglobulin superfamily, among them apCAM, the Aplysia homolog to NCAM. Application of serotonin resulted in a decrease of apCAM from presynaptic terminals of sensory neurons within 1 hour, whereas postsynaptic expression of apCAM in motor neurons remained unaffected (Bailey et al., 1992; Mayford et al., 1992). This synaptic apCAM down-regulation was due to cAMP and protein-synthesis dependent internalization (Bailey et al., 1992). Moreover, an antibody against apCAM caused defasciculation in a sensory neuron culture (Keller and Schacher, 1990). Taken together, these studies suggest that the presence of apCAM impairs growth of new synapses, whereas the internalization of apCAM after serotonin application may be necessary to allow defasciculation and thus be a first step towards the growth of new synapses.

2.2.6. Role of Fas II in synaptic plasticity in Drosophila

Findings in Drosophila closely resemble those of Aplysia. Here the NCAM homolog Fascillin II (Fas II) was investigated at the neuromuscular junction. During development Fas II was shown to control fasciculation and axonal pathfinding (Lin et al., 1994). Later onwards, Fas II is expressed pre-and postsynaptically and controls growth and stabilization of the neuromuscular synapse (Schuster et al., 1996a; Davis et al., 1997). Furthermore, the levels of Fas II play a critical role in controlling structural, and in conjunction with the activator of the transcription factor CREB, functional plasticity (Davis et al., 1996; Schuster et al., 1996b).

Similarly to apCAM in Aplysia, Fas II down-regulation at the synaptic junction is necessary to allow presynaptic pruning and thus the formation of new synapses (Schuster et al., 1996b).

Taken together, studies with the NCAM homologs apCAM and Fas II indicate that a down-regulation of these adhesion molecules may be a general mechanism allowing defasciculation. This may resemble a necessary step towards structural remodeling of the synaptic architecture following learning experiences. Internalization of adhesive molecules may resemble closely the polysialylation of the vertebrate homolog, NCAM, since PSA-NCAM may reduce the adhesive forces of NCAM. Thus, PSA-NCAM may produce de-adhesion of existing synaptic contacts necessary for the formation of new synapses and remodeling of the network in activity-induced synaptic plasticity processes or after learning experiences.

Table 2. Roles of NCAM, PSA-NCAM and polysialyltransferases in activity-induced synaptic plasticity in the adult rodent hippocampus

	Dentate Gyrus	CA3	CA1	Organotypic hippocampal culture
NCAM expression	Increase 90min [6] and 24h [11] after LTP induction	--	--	--
NCAM antibodies	--	--	Impaired LTP [7, 10]	--
Constitutively NCAM deficient mice	Impaired LTP [12]	Impaired LTP [4] (but abnormal mossy fiber innervation and ectopic synapses in CA3)	Impaired LTP [8, 9]	--
Conditionally NCAM deficient mice	--	Normal LTP [3]	Impaired LTP [3] Impaired LTD [3]	--
PSA-NCAM expression	--	--	--	--
EndoN	--	--	Impaired LTP [2]	Impaired LTP [8, 9, 13]
St8SiaII/STX deficient mice	Normal LTP [12]	Normal LTP [1] (but abnormal mossy fiber innervation and ectopic synapses in CA3)	Normal LTP [1]	--
St8SiaIV/PST deficient mice	Normal LTP [12]	Normal LTP [5]	Impaired LTP [5] Impaired LTD [5]	--
Putative molecular mediator	*NCAM dependent*	*PSA and NCAM independent Lamination of mossy fibers depends on St8SiaII*	*PSA (St8SiaIV) dependent*	

[1]Angata et al., 2004, [2]Becker et al., 1996 , [3]Bukalo et al., 2004, [4]Cremer et al., 1998, [5]Eckhardt et al., 2000, [6]Fazeli et al., 1994, [7]Luthi et al., 1994, [8]Muller et al., 1996, [9]Muller et al., 2000, [10]Ronn et al., 1995, [11]Schuster et al., 1998, [12]Stoenica et al., 2006, [13]Dityatev et al., 2004

Table 3. NCAM and PSA-NCAM expression at synapses in the hippocampus.

	Dentate Gyrus	CA3/CA4	CA1
NCAM	--	Not present at mossy fiber synapse. [1, 3] Present at mossy fibers in stratum lucidum. [3, 6]	--
NCAM-180	Present postsynaptically at 37% of spine synapses. [3, 5]	Not present at mossy fiber synapses. [3]	--
PSA	Present pre- and postsynaptically in at 38% of spine synapses. [3]	Not present at mature mossy fiber synapses. [1, 2, 3] Present at some immature mossy fiber boutons making contact with CA3 pyramidal cells. [1]	Present in a subpopulation of spine synapses. [4] Present in the CA1 cell layer and at Schaffer collaterals. [6]

[1] Seki and Arai, 1999; [2] Seki and Rutishauser, 1989; [3] Schuster et al., 2001; [4] Schuster et al., unpublished data; [5] Schuster et al., 1998; [6] O'Connell et al., 1997

2.3. NCAM and PSA-NCAM in learning and memory

NCAMs are among the most widely studied cell adhesion molecules in learning and memory paradigms. At the beginning of behavioural research with NCAMs stood the search for possible molecules involved in synapse formation and selection as observed after periods of enhanced neural activity (Chang and Greenough, 1984) or after *in vivo* learning (Wenzel et al., 1980). Since the expression of a number of synaptic plasma membrane proteins was found to be increased in the hours following a learning experience (Burgoyne and Rose, 1980; Sukumar et al., 1980; Bullock and Rose, 1992; Doyle et al., 1992b) it was suggested that they may play a role in the synaptic plasticity processes that underlie memory formation. NCAM, a member of the glycoprotein family and previously rather associated with developmental processes, was suggested as a possible candidate. Thus, the very same mechanisms that are responsible for neurite outgrowth, pathfinding, and fasciculation were hypothesized to be involved in synaptic plasticity and therefore underlie memory formation.

Studies exploring the role of NCAM and/or PSA-NCAM in learning and memory can be classified as belonging to one of the following fields:

a. Correlational: Evaluation of the expression of NCAM or PSA-NCAM after learning.
b. Interventive: Evaluation of the cognitive outcomes of infusion of agents that either impair of enhance NCAM or PSA-NCAM function.
c. Behavioural genetics: Evaluation of the cognitive outcomes of genetically modified mice with deactivated genes for the NCAM isoforms or the sialyl transferases.

The vast majority of the behavioural studies focus on the hippocampus – very much alike to plasticity studies which so far in rodents were all conducted in the hippocampus (see previous chapter).

The following sections briefly review the major insights gained into the roles of NCAM and its polysialylated form into learning and memory in the hippocampus followed by a review of data evaluating the role of NCAM and PSA-NCAM in fear associated learning – a form of learning which also relies on the amygdala. A much more complete summary of the behavioural data dealing with NCAM and PSA-NCAM can be found in Table 4.

Table 4. Summary of behavioural studies with NCAM and PSA-NCAM.

Approach	Task	Effect	Location	Authors
NCAM expression	Passive avoidance	Increase in synaptic active zones 5-6 h post-training	Lobus parolfactorius (chick striatum)	Skibo et al., 1998
	Passive avoidance	Enhanced NCAM-180 internalization 3-4 h post-training. Can be prevented by C3 infusion.	Dentate gyrus	Foley et al., 2000
	Active avoidance	Increase 3 h post-training	Optic tectum (zebrafish)	Pradel et al., 2000
	Water maze	No effect immediately post-training	Hippocampus Thalamus Striatum PFC Frontal cortex	Venero et al., 2004)
	Water maze	Increase 24 h post-training	Hippocampus	Venero et al., 2006
	Olfactory discrimination	No effect at any time point of measurement (3rd, 5th day of learning, 3 days post-training). Positive correlation between learning performance and NCAM expression.	Hippocampus Piriform cortex	Knafo et al., 2005
	Contextual fear conditioning	Decrease 12 h post-training after 0.2, 0.4 and 1 mA. Increase 24 h post-training after 1 mA.	Hippocampus	Merino et al., 2000
	Acute predator stress, Water maze	*Effect 30 min post-WM training:* No effect in hippo. No effect amygdala. Decrease in PFC. *Effect of acute stress before WM:* Decrease NCAM 180 in hippo. Decrease in PFC. No effect in amygdala.	Hippocampus Amygdala Prefrontal cortex Cerebellum	Sandi et al., 2005
	Chronic restraint stress (contextual fear conditioning)	Decrease	Hippocampus	Sandi et al., 2001
	Chronic restraint stress (water maze)	Decrease throughout the brain. Decrease in hippo. No effect in PFC.	Hippocampus Thalamus PFC Striatum	Venero et al., 2002
	Chronic restraint stress	Decrease of NCAM-140 (investigated NCAM 120, 140, 180)	Hippocampus	Touyarot and Sandi, 2002
	Chronic restraint stress (water maze) Individual differences.	Decrease of NCAM- 140 (investigated NCAM 120, 140, 180)	Hippocampus	Touyarot et al., 2004
	Chronic unpredictable stress in adulthood, test in old rats. Individual differences.	Decrease of NCAM-180	Hippocampus	Sandi and Touyarot, 2006
	CORT	Increase	Forebrain (chick)	Sandi et al., 1995
	Acute and chronic CORT	Increase after acute CORT in PFC. Decrease after chronic CORT in PFC. Decrease after chronic CORT in hypothalamus. No effect in other brain areas.	Hippocampus Prefrontal cortex Striatum Hypothalamus	Sandi and Loscertales, 1999

NCAM antibodies				
Anti-NCAM	Passive avoidance	Impaired when infused 5-6 h and tested 48 h post-training.	Ventricle	Doyle et al., 1992a
Polyclonal anti-NCAM (1 and 2)	Passive avoidance	Impaired when injected 6-8 h post-training and tested 24 h post-training. No effect at any other time point.	Intermediate medial hyperstriatum ventrale (IMHV) of chick.	Scholey et al., 1993
anti-NCAM?	Weak passive avoidance, CORT admin	Impaired the facilatory effect of CORT on learning	Forebrain (chick)	Sandi et al., 1995
Polyclonal anti-NCAM R1 and R2	Passive avoidance	Impaired when injected 5.5 h post-training and tested 24 h post-training,	IMHV (chick)	Mileusnic et al., 1995
Polyclonal R1	Passive avoidance	Impaired when injected 5.5 h post-training and tested 48 h later. No effect when tested 24 h later.	Ventricle	Alexinsky et al., 1997
Polyclonal anti-NCAM	Water maze	Impaired	Ventricle	Arami et al., 1996
Polyclonal R1	Odour discrimination task	Impaired when injected 5.5 h post-training and tested 48 h later.	Ventricle	Roullet et al., 1997
NCAM antisense oligonucleo-tides	Passive avoidance	Impaired when injected twice: both 12h before training and immediately afterwards and tested 3 h and 24 h post-training.	IMHV (chick)	Mileusnic et al., 1999
Synthetic peptides				
C3	Passive avoidance	Impaired when infused during training and 6-8 post-training and tested 48 h later, but not 24 h post-training. Prevents NCAM-180 internalization 3-4 post-training in dentate gyrus.	Ventricle	Foley et al., 2000
C3d	Contextual fear conditioning (0.4 mA)	Impaired when injected 5.5 h post-training and tested 2-3 and 7 days later. No effect when injected 2 days pre-training.	Ventricle	Cambon et al., 2003
C3d	Open Field	No effect	Ventricle	Cambon et al., 2003
C3	Open field	Impaired exploratory behaviour when infused pre-training.	Ventricle	Hartz et al., 2003
C3	Rotarod test	No effect on sensorimotor function when infused pre-training	Ventricle	Hartz et al., 2003
C3	Motility	No effect when infused pre-training.	Ventricle	Hartz et al., 2003
C3	Approach avoidance	Impaired when infused pre-training and tested 48 h post-training.	Ventricle	Hartz et al., 2003
C3	Water maze	No effect when averaged over all days. Impaired on second trial on day one (pre-training infusions).	Ventricle	Hartz et al., 2003
C3d	Water maze	Impaired in learning. Impaired in probe test.	Ventricle	Venero et al., 2006
FGL	Open field	No effect	Ventricle	Cambon et al., 2004
FGL	Contextual fear	Enhanced contextual fear 24	Ventricle	Cambon et al., 2004

	conditioning	h, 7 d and 28 d post-training	(post-training)	
FGL	Auditory fear conditioning	No effect 24 h and 7 d post-training, but: Enhanced 28 d post-training	Ventricle (post-training)	Cambon et al., 2004
FGL	Water maze	Enhanced on 2nd training session in first trial, Enhanced 24 h, 7 d and 14 post-training. No effect in probe trials. Enhanced reversal learning.	Ventricle Infusion after 1st and 2nd training session.	Cambon et al., 2004
Constitutive NCAM ko mice	Open field emergence test	Enhanced anxiety		Cremer et al., 1994
	Open field	No effect		Cremer et al., 1994
	Water maze	Impaired during acquisition. Impaired in probe trial.		Cremer et al., 1994
	Water maze	Enhanced anxiety (increased tendency to swim next to walls)		Cremer et al, unpublished, stated in Stork et al., 1999
	Inter-male aggression	Enhanced		Stork et al., 1997
	Light/Dark Avoidance Test	Enhanced anxiety		Stork et al., 1999
	EPM	Reduced anxiety due to increased locomotion		Stork et al., 1999
	Odour discrimination	Impaired odour discriminiation No effect short-term memory No effect odour detection thresholds		Gheusi et al., 2000
	Novel odour presentation	Abnormal activity in various brain regions		Montag-Sallaz et al., 2003
	Pre-pulse inhibition and facilitation	No effect		Plappert et al., 2005
	Habituation of startle response	No effect		Plappert et al., 2006
	Footshock sensitization of startle response	Impaired		Plappert et al., 2006
NCAM-180 ko mice: $NCAM^{-/-180-}$	Inter-male aggression	Enhanced		Stork et al., 2000
	Light dark test	Enhanced anxiety		Stork et al., 2000
	Elevated plus maze	Reduced anxiety due to increased locomotion		Stork et al., 2000
	Forced Swim	Reduced immobility. Enhanced locomotion.		Stork et al., 2000
	Water maze	Impaired acquisition. Impaired probe trial memory. Increased floating		Stork et al., 2000
	Auditory fear conditioning	Impaired tone memory (moderate)		Stork et al., 2000
	Contextual fear conditioning	Impaired context memory (strongly)		Stork et al., 2000
NCAM-180 ko mice: $NCAM^{-/-180+}$	Inter-male aggression	Restored to normal		Stork et al., 2000
	Light dark test	Restored to normal		Stork et al., 2000
	Elevated plus maze	Restored to normal		Stork et al., 2000
	Forced Swim	Enhanced immobility as compared to total mutants, but below wt. Reduced locomotion as compared to total mutants,		Stork et al., 2000

		but above wt.		
	Water maze	No effect		Stork et al., 2000
	Auditory fear conditioning	Impaired.		Stork et al., 2000
	Contextual fear conditioning	Impaired.		Stork et al., 2000
Conditional hippocampus ko mice	Water maze	Impaired (day 3)	Hippocampus	Bukalo et al., 2004
PSA-NCAM expression	Passive avoidance	Increase 12 and 24 h post-training	Hippocampus	Doyle et al., 1992b
	Passive avoidance	Increase 12-24 h post-training, can be abolish with amnesia inducing agents.	Hippocampus	Doyle and Regan, 1993
	Passive avoidance	Increase 10-12 h post-training	Dentate gyrus	Fox et al., 1995
	Passive avoidance	Increase 10-12 h, persisting up to 24-48 h post-training	Layer II of the enthorinal, perirhinal and piriform cortex	Fox et al., 2000
	Passive avoidance	Increase 12 h post-training at subtriangular septal zone in GABAergic interneurons	Septal nuclei and septohippocampal pathway	Foley et al., 2003a
	Water maze	Increase 12 h post-training at subtriangular septal zone in GABAergic interneurons	Septal nuclei and septohippocampal pathway	Foley et al., 2003a
	Water maze followed by passive avoidance	Increase 10-12 h post-training	Dentate gyrus (granular cells)	Murphy et al., 1996
	Water maze	Increase 10-12 h post-training	Enthorinal cortex	O'Connell et al., 1997
	Water maze followed by passive avoidance	Increase 12 h post-training	Dentate gyrus	Murphy and Regan, 1999
	Water maze	Increase 12 h post-training Negative correlation between PSA-NCAM expression and performance in WM	Dentate gyrus	Sandi et al., 2004
	Water maze	Increase 12 h post-training, no effect on neurogenesis	Dentate gyrus	Van der Borght et al., 2005
	Water maze	Increase 24 post-training in synaptosomes	Hippocampus	Venero et al., 2006
	Odour discrimination	Increase 12 h post-training	Dentate gyrus	Foley et al., 2003b
	Odour discrimination	Increase 24 h post-training in the hippo, but not piriform cortex	Piriform cortex Hippocampus	Knafo et al., 2005
	Contextual fear conditioning	No effect 12 and 24 h post-training after 0.2 and 0.4 mA Decrease 12 and 24 h post-training after 1mA	Hippocampus	Merino et al., 2000
	Contextual fear conditioning (Water maze)	Increase 12 h post-training after moderate shock intensities (0.4mA); Decrease 12 h post-training after traumatic shock intensity (1mA)	Dentate gyrus	Sandi et al., 2003
	Contextual fear conditioning	Increase 24 h post-training only after 1 mA	Dentate gyrus	Lopez Fernandez et al., unpublished data
	Contextual fear conditioning	No effect at any time point (30 min, 24 h) and intensity (0.4, 1 mA)	Amygdala	Lopez Fernandez et al., unpublished dat
	Auditory fear conditioning	No effect	Dentate gyrus	Lopez Fernandez et al., unpublished data
	Auditory fear conditioning	Increase in LA and CE after 1 mA, not 0.4 mA, 24 h post-training.	Amygdala	Markram et al., submitted-b
	Chronic restraint	Increase	Hippocampus	Sandi et al., 2001

	stress, (contextual fear conditioning)			
	Chronic restraint stress	Increase after 3 weeks of restraint. Return to baseline after 6 weeks of restraint.	Dentate gyrus	Pham et al., 2003
	Chronic restraint stress	Decrease in ME and CE	Amygdala	Cordero et al., 2005
	Chronic restraint stress	Increase	Piriform cortex	Nacher et al., 2004b
	Chronic CORT	Decrease	Piriform cortex	Nacher et al., 2004b
	Chronic CORT	Decrease	Dentate Gyrus	Nacher et al., 2004a
	Chronic stress during adulthood, test in old rats	No effect	Hippocampus	Sandi and Touyarot, 2006
Cleavage of PSA-NCAM by endoN	Water maze	Impaired on learning day 2 Impaired in probe test	Dorsal hippocampus	Becker et al., 1996
	Water maze	Impaired	Ventricle	Venero et al., 2006
	Contextual fear conditioning	Impaired	Dorsal hippocampus	Lopez Fernandez et al., unpublished data
	Contextual fear conditioning	No effect	Amygdala	Markram et al., unpublished data
	Auditory fear conditioning	No effect	Amygdala	Markram et al., submitted-b
Synthetic peptides				
pr2	Water maze	Enhanced	Hippocampus	Florian et al., 2006
pr2	Auditory fear conditioning	No effect 24 h and 28 d post-training	Amygdala	Markram et al., submitted-b
St8Sia-II ko mice	Auditory fear conditioning	Impaired		Angata et al., 2004
	Contextual fear conditioning	Impaired		Angata et al., 2004
	Passive avoidance	Impaired		Angata et al., 2004
	Open Field	Moved more in total and in the centre of the OF. Increased rearing.		Angata et al., 2004
	Water maze	No effect		Angata et al., 2004
	Startle response	No effect		Angata et al., 2004
	Prepulse Inhibition	No effect		Angata et al., 2004
	Motor activity	No effect		Angata et al., 2004
	Nociception	No effect		Angata et al., 2004
St8Sia-IV ko mice	Open field	No effect		Markram et al., unpublished data
	Novel object recognition test	No effect		Markram et al., unpublished data
	EPM	Reduced anxiety		Markram et al., submitted-a
	Water maze	Impaired learning (day 3) Impaired reversal learning		Markram et al., submitted-a
	Auditory fear conditioning	No effect		Markram et al., submitted-a
	Contextual fear conditioning	No effect		Markram et al., submitted-a

2.3.1. Time windows of action

Cumulative evidence over more than a decade of research indicates that NCAM and its polysialylation state are implicated in memory consolidation processes occurring during very specific and tight time windows and interference during these time periods can disrupt memory consolidation.

Pioneering experiments stem from C. Regans' lab. For example, Doyle et al. (1992b) infused antibodies against NCAM intraventricularly into rats at specific time points after the acquisition of a passive avoidance task. They found that infusions 6-8 h, but not immediately, 4 or 10 h after training impaired memory performance when tested 48 h after training. This result was confirmed in other studies using NCAM antibodies or substances that interfere with NCAM function, such as the synthetic peptide C3 (Foley et al., 2000; Cambon et al., 2003), and seems to be applicable to other species (Scholey et al., 1993; Mileusnic et al., 1995) and to divers learning paradigms, such as olfactory learning (Roullet et al., 1997) and contextual fear conditioning (Cambon et al., 2003). In all rodent studies substances were infused into the ventricles.

The conclusions drawn from these experiments stated two different time windows for glycoprotein synthesis and action during memory consolidation: one directly after task acquisition and one 5-8 h later (Scholey et al., 1993). The first phase was found to be NCAM-independent (Scholey et al., 1993) and could be blocked by antibodies to another member of the immunoglobulin superfamily, L1 (Scholey et al., 1995). The second phase relied on NCAM. A possible role NCAM might play during this phase could be related to synaptic remodelling occurring at this time and antibodies against NCAM might interfere with this process (Murphy and Regan, 1998). A process of deadherence might be occurring in this time, thus allowing an antibody to dock onto the exposed NCAM. Alternatively, NCAM might be newly synthesized and exported to the membrane in order to stabilize existing or newly forming synapses (Dityatev et al., 2000). Interestingly, in rats NCAM-180 was found to be internalized 3 – 4 h post-training in a passive avoidance task in the dentate gyrus (Foley et al., 2000) and in chicks NCAM expression was found to be increased at synaptic sites in the 6 – 8 h time window following a passive avoidance task (Skibo et al., 1998). This may suggest that NCAM is initially internalized in order to allow synaptic remodelling to occur and then re-expressed to either strengthen existing synapses or to participate in the formation of new synapses. A second possibility might be that NCAM-mediated intracellular signalling might be disrupted at this time point (Kolkova et al., 2000a; Ronn et al., 2002; Hofmann et al., 2004).

Polysialylation of NCAMs was also found to be precisely regulated during the consolidation processes in a variety of learning tasks. PSA-NCAM levels in the hippocampus or in areas feeding into the hippocampus, such as the enthorinal and piriform cortex, were increased 10 – 12 h after passive avoidance (Doyle et al., 1992b; Doyle and Regan, 1993; Fox et al., 1995; Fox et al., 2000; Foley et al., 2003a), water maze learning (Murphy et al., 1996; Murphy and Regan, 1999; Foley et al., 2003a; Sandi et al., 2004; Van der Borght et al., 2005; Venero et al., 2006), odour discrimination (Foley et al., 2003b; Knafo et al., 2005) and contextual conditioning (Sandi et al., 2003) and can be abolished with amnesia inducing agents (Doyle and Regan, 1993). Increased levels were also found 24 h post-training (Doyle et al., 1992b; Doyle and Regan, 1993; Fox et al., 2000; Knafo et al., 2005; Venero et al., 2006). It has been suggested that the polysialylation process might not be completed until even later (Fox et al., 2000), because in rodents (but not chicks) memory impairing effects of NCAM antibodies are normally not observed until 48 h post-training (Doyle et al., 1992a; Alexinsky et al., 1997).

Furthermore, interference with NCAM polysialylation by infusing endoN directly into the hippocampus (Becker et al., 1996) or into the ventricles (Venero et al., 2006) led to memory impairments in the hippocampus dependent water maze task.

Several functional roles have been suggested for PSA-NCAM in learning and memory. First, on the basis of the suggested unique property of PSA to make NCAM less adhesive, it was proposed that a learning-induced increase of PSA-NCAM serves as a selection mechanism between synaptic connections transiently overproduced in the learning process (Doyle et al., 1992b). The fact that PSA-NCAM expression levels increase several hours after NCAM antibodies are memory disruptive argues for such a hypothesis. It is conceivable that an over-production of synapses after the learning experience occurs in the 5-8 h time window, which is followed by an elimination process mediated by PSA-NCAM in the 10-12 h time window or even later. Interestingly, passive avoidance induced synaptogenesis in the dentate gyrus coincides with the period during which anti-NCAM is effective (O'Malley et al., 1998). While the phenomenon of synapse elimination in the mature brain is well established (Knott et al., 2002; Trachtenberg et al., 2002), there are not yet data proving a learning related PSA-NCAM involvement in synapse elimination. A second possible function of PSA-NCAM might be in directly contributing to synaptogenesis possibly by interacting with intercellular signalling cascades since elimination of PSA with endoN in cultured neurons prevents synaptogenesis (Dityatev et al., 2004).

In summary, these studies demonstrate a species, task and age unspecific role of NCAMs and its polysialylated state in memory consolidation. Also, they show that proper NCAM functioning 5–8 h post-training is critical for the establishment of long-term memories, possibly reflecting adhesion processes at newly synthesized synapses or de-adhesion processes to allow for network restructuring. Polysialylation at a later time point, starting 10 h after learning and continuing at least until 24 h, but possibly longer, may be crucial as a preliminary step towards remodelling and/or in the formation of new synapses.

2.3.2. Fear conditioning

Surprisingly, in rodents NCAM and PSA-NCAM mediated actions in memory consolidation have not been investigated in other brain areas than the hippocampus (or enthorinal and piriform cortex) and in other than hippocampus dependent memory tasks. Therefore, one of the main goals of this doctoral work was to investigate the role of amygdaloid PSA-NCAM in memory processes. In order to do so we used an amygdala-dependent memory tasks, auditory and contextual fear conditioning. This chapter gives an overview on the role of NCAM and PSA-NCAM in fear conditioning in the hippocampus.

Across the different studies addressing aversive memories, three types of "fear conditioning" tasks were used. 1) In the *step-through passive avoidance task* a footshock is administered in one part of the chamber and the latency to re-enter this part of the chamber is measured at later points. 2) In *contextual fear conditioning* footshocks are administered in a particular context and the freezing response is measured upon reinsertion into this context at later time points. 3) In *auditory fear conditioning* a tone coterminates with a shock and the freezing response to the tone alone (in a different context) is measured at later time points. Whereas in the first two tasks the context is learned to be associated with the aversive stimulus, in the last task a discrete tone is associated with the shock. Acquisition and consolidation of all of these tasks were shown to critically rely on the basolateral complex of the amygdala (Liang et al., 1982; Parent and McGaugh, 1994; Wilensky et al., 1999; Goosens

and Maren, 2001) and in case of the contextual association, the hippocampus is required as well (Phillips and LeDoux, 1992).

Both NCAM and PSA-NCAM play a role in fear conditioning. For example, in the hippocampus, NCAM expression is decreased 3–4 h after passive avoidance training (Foley et al., 2000) and 12 h following contextual fear conditioning, when moderate shock intensities were applied (Merino et al., 2000). However, application of a strong shock (1 mA) results in a NCAM up-regulation 24 h post-conditioning (Merino et al., 2000). In this context it is interesting to note, that stronger shock intensities usually lead to stronger and longer-lasting fear memories (Cordero et al., 1998). Both passive avoidance and contextual fear conditioning can be disrupted upon intra-ventricular infusion of C3, a synthetic peptide that interferes with NCAM function when infused after training (Foley et al., 2000) and in the 5 – 8 h time window post-training (Foley et al., 2000; Cambon et al., 2003). Thus, NCAMs play an important role in memory consolidation of aversive contents.

Both auditory as well as contextual fear memories can also be enhanced by means of a NCAM mimetic peptide, that acts in the so called FG loop (FGL) (Cambon et al., 2004). FGL is a 15 amino acid sequence in the second FnIII module of NCAM that forms part of the binding site of NCAM to the fibroblast growth factor receptor 1 (FGFR1). Upon homophilic binding, NCAM promotes neurite outgrowth through interactions involving FGL and the FGFR1 (Niethammer et al., 2002; Kiselyov et al., 2003). Intraventricular infusion of FGL just after fear conditioning improved contextual memory performance when tested 24 h, 7 and 28 days later. However, auditory fear memories were only enhanced when tested 28 days later, but not earlier, suggesting different consolidation mechanisms for conditioned fear to tones, which might become apparent only after longer time periods. FGL was also shown to enhance presynaptic function by short-term and long-term (48 h) facilitation of transmitter release and increased synaptogenesis in hippocampal cell cultures, which might resemble the underlying mechanism for the memory enhancing effects. However, neither in this nor in the two studies using C3 it can be excluded that the mnemonic effects were not also mediated by the amygdala, because the substances were infused into the ventricles rather than the hippocampus.

Further strong indication that NCAM or PSA-NCAM mediated processes contribute to fear memories stem from NCAM-deficient mice. In these mice both auditory and contextual fear memories are impaired (Stork et al., 2000), suggesting that NCAM-mediated consolidation processes might also be implicated in other brain regions than the hippocampus, because auditory fear conditioning relies on the amygdala. Furthermore, NCAM deficient mice also exhibit impaired footshock sensitization of a startle response to a tone (Plappert et al., 2006). Footshock sensitization is a form of contextual conditioning, during which the context becomes a fear inducing stimulus, leading to an increase in startle response to a tone (Richardson and Elsayed, 1998). This type of learning relies not only on the hippocampus, but also the amygdala (Hitchcock et al., 1989; Fendt et al., 1994), thus providing further indication of a possible amygdala involvement in NCAM mediated aversive learning.

A somewhat more complex picture emerges from studies focusing on PSA-NCAM involvement in fear conditioning. Ten to twelve hours after passive avoidance training PSA-NCAM is increased in the hippocampus (Doyle et al., 1992b; Doyle and Regan, 1993; Fox et al., 1995; Fox et al., 2000). In contextual fear conditioning on the other hand, PSA-NCAM expression in hippocampal synaptosomes is either not affected 12 h or 24 h after using moderate shock intensities (0.2 and 0.4 mA) or even down-regulated 24 h (but not 12 h) after applying high shock intensities (1 mA; Merino et al., 2000). A decrease in PSA-NCAM expression is rather surprising taking into account the consistent expression level increases over several memory tasks (see previous chapter). One possible explanation might lie in the different nature of the behavioural task. In fear conditioning and memory testing the rat is

exposed to a rather inescapable situation, whereas in any other task it has a behavioural choice. However, recent experiments in our lab suggest an increase in PSA-NCAM expression in the dentate gyrus 24 h after contextual fear conditioning using 1 mA shocks in an immunohistochemical preparation (M. Lopez Fernandez et al., unpublished data). Thus, the method applied to assess PSA-NCAM levels might be of importance. While there is no published data relating PSA-NCAM cleavage with fear conditioning, recent yet unpublished data in our lab shows, that infusion of endoN in the hippocampus interferes with the consolidation of contextual fear memories, but spares the consolidation of auditory fear memories (M. Lopez Fernandez et al., unpublished data). This suggests that PSA-NCAM expression in the hippocampus is necessary for the establishment of contextual fear memories, but not auditory fear memories.

Furthermore, adult mice lacking the predominantly prenatally expressed St8SiaII/STX exhibit impaired memories (but not acquisition) in all three fear learning related paradigms, auditory and contextual fear conditioning and passive avoidance (Angata et al., 2004), thus further suggesting an involvement of PSA-NCAM in fear conditioning. However, since PSA-NCAM expression levels in the amygdala of adult St8SiaII/STX knockout mice are normal it still remains unclear whether some undetected defect in the amygdala might be responsible for these deficits of rather other mechanisms, such as the ectopic synaptic architecture observed in the CA3 field of the hippocampus (Angata et al., 2004).

In summary, the above data suggest that both NCAM and PSA-NCAM might play crucial roles in establishing aversive memories. However, the exact locus of where NCAM and PSA-NCAM might contribute to synaptic remodelling processes underlying aversive memories remains elusive. There is evidence that remodelling processes take place in the hippocampus, since both NCAM and PSA-NCAM expression are modulated in the hippocampus following aversive learning, and PSA-NCAM cleavage directly into the hippocampus impairs contextual fear memory consolidation. However, taking into account a) the importance of the amygdala in fear-associated learning, b) the continued expression of PSA-NCAM in the amygdala of adult rats, and c) the importance of NCAM and PSA-NCAM for memory consolidation, it remains to be established, whether PSA-NCAM might also mediate fear memory consolidation in the amygdala. The following two papers address this hypothesis.

2.4. Introduction to studies and goals

The goal was to assess whether conditioned fear memories rely on PSA-NCAM mediated mechanism in the amygdala. In order to answer this question we pursued two approaches: In the first study, hand PSA-NCAM was either cleaved or enhanced directly in the basolateral amygdala by infusions of endoN or the peptide p2 into adult rats (Markram et al., submitted-b). In the second study, we used mice deficient for St8SiaIV, the polysialyltransferase preferentially expressed during the post-natal period and in adulthood (Hildebrandt et al., 1998). In contrast to St8SiaII/STX deficient mice, these mice express no PSA-NCAM throughout almost the entire adult brain as shown by Eckhardt et al. (2000) and confirmed in our own study (Markram et al., submitted-a). More than 230 rats (excluding pilot studies) and 70 mice underwent auditory fear conditioning, a task which crucially relies on the amygdala (LeDoux, 2003), and various other learning paradigms. Pilot studies evaluated the correct placement of infusion cannulae and verified the effectiveness of substance administration. The following two papers summarize our results.

Paper 1: Amygdala upregulation of NCAM polysialylation induced by fear conditioning is not required for fear memory processes

Amygdala Upregulation of NCAM Polysialylation Induced by Fear Conditioning is not Required for Fear Memory Processes

Kamila Markram[1], Miguel Angel Lopez Fernandez[1], Djoher Nora Abrous[2] and Carmen Sandi[1]

[1]*Brain Mind Institute, Ecole Polytechnique Fédérale de Lausanne (EPFL), Switzerland*
[2] *Domaine de Carreire, INSERM U588 146 rue Léo Saignât, Bordeaux Cedex, 33077, France*

Correspondence:
Prof. Carmen Sandi
EPFL SV BMI LNMC
AAB 1 19 (Bâtiment AAB)
Station 15
CH – 1015 Lausanne
Switzerland
Tel: +41-21 693 9534
Fax: +41-21 693 9636
carmen.sandi@epfl.ch

There is much interest to understand the mechanisms leading to the establishment, maintenance and extinction of fear memories. The amygdala has been critically involved in the processing of fear memories and a number of molecular changes have been implicated in this brain region in relation to fear learning. Although neural cell adhesion molecules (NCAMs) have been hypothesized to play a role, information available about their contribution to fear memories is scarce. We investigate here whether polysialylated NCAM (PSA-NCAM) contributes to auditory fear conditioning in the amygdala. First, PSA-NCAM expression was evaluated in different amygdala nuclei after auditory fear conditioning at two different shock intensities. Results showed that PSA-NCAM expression was increased 24 h post-training only in animals subjected to the highest shock intensity (1 mA). Second, PSA-NCAM was cleaved in the basolateral amygdaloid complex through micro-infusions of the enzyme endoneuraminidase N, and the consequences of such treatment were investigated on the acquisition, consolidation, remote memory expression and extinction of conditioned fear memories. Intra-amygdaloid cleavage of PSA-NCAM did not affect acquisition, consolidation or expression of remote fear memories. However, intra-amygdaloid PSA-NCAM cleavage enhanced fear extinction processes. These results suggest that upregulation of PSA-NCAM is a correlate of fear conditioning that is not necessary for the establishment of fear memory in the amygdala, but participates in mechanisms precluding fear extinction. These findings point out PSA-NCAM as a potential target for the treatment of psychopathologies that involve impairment in fear extinction.

The neural cell adhesion molecule (NCAM), a glycoprotein of the immunoglobulin superfamily, plays key roles in synaptic plasticity through a variety of mechanisms, including cell-cell adhesion, activation of intracellular signalling pathways, interaction with growth factor receptors, and posttranslational modifications (Benson, 2000; Fields, 1996; Sandi, 2004; Walmod, 2004). One major posttranslational modification of NCAM consists on the attachment of long chains of α2,8-linked polysialic acid (PSA) homopolymers. PSA-NCAM was shown to reduce NCAM-mediated cell adhesion (Rougon, 1993) and to be involved in cell migration, fasciculation, pathfinding and synaptogenesis (Landmesser et al., 1990; Kiss and Rougon, 1997; Ronn et al., 2000; Dityatev et al., 2004).

In the adult brain, PSA-NCAM was shown to critically contribute to activity-induced synaptic plasticity and memory formation. Drastic deficits in activity-induced synaptic plasticity were observed both after removal of PSA from NCAM with the enzyme endoneuraminidase N (endoN) (Becker et al., 1996; Muller et al., 1996; Stoenica et al., 2006) and in knock-out mice lacking St8SialV, the enzyme responsible for most of the post-natal polysialylation of NCAM (Eckhardt et al., 2000). Moreover, PSA-NCAM expression shows transient increases in the hippocampus 10-24 h after training (Murphy and Regan, 1998; Sandi et al., 2003, 2004; Venero et al., 2006), and its cleavage with endoN impairs spatial learning in the water maze (Becker et al., 1996; Venero et al., 2006).

Most of the existing evidence for the implication of PSA-NCAM in memory function (reviewed above) was obtained in the rodent hippocampus. However, NCAM polysialylation also occurs in other brain regions (Seki and Arai, 1991; Nacher et al., 2004; Varea et al., 2005), including the amygdala (Nacher et al., 2002), where its functional implications are still unknown. Since the amygdala is known to play a prominent role in emotional processing and fear conditioning, we hypothesized that PSA-NCAM mediated mechanisms occurring in the amygdala might play a role in fear learning. The molecular cascades that have been involved, so far, in the acquisition and stabilization of fear memories in the amygdala include a number of synaptic transients, followed by a variety of calcium-dependent kinases (for reviews see Lamprecht and LeDoux, 2004; Rodrigues et al., 2004) and the activation of proteins linked to cytoskeleton organization (Lamprecht et al., 2002, 2006; Shumyastsky et al., 2005). Ultimately, these cascades induce gene activation and the production of proteins in the amygdala (Schafe and LeDoux, 2000; Maren et al., 2003) that are hypothesized to stabilize or enable changes at the synapse to consolidate fear memories. Although cell adhesion molecules have been hypothesized to play a role in fear memories (Stork et al., 2000; Nacher et al., 2002; Lamprecht and LeDoux, 2004), available information for their implication is scarce.

Our goal here was to assess whether PSA-NCAM plays a functional role in the memory processing of conditioned fear traces in the amygdala. We show that fear conditioning induces an upregulation of PSA-NCAM in the amygdala that is not necessary for the acquisition, consolidation and recall of fear memories, but plays a role in extinction of fear-related memories.

MATERIALS AND METHODS

Animals

The subjects were 182 adult male Wistar rats (260–360 g) obtained from a commercial supplier (Charles River Laboratories, France) and housed as groups of three in standard plastic cages on a 12h light – dark cycle (lights on at 7:00 am). Water and food was provided *ad libitum*. Prior to behavioral experiments, rats were handled daily for 5 min during 3 consecutive days. All the procedures described were conducted in conformity with the Swiss National Institutional Guidelines on Animal Experimentation, and approved by the Swiss Cantonal Veterinary Office Committee for Animal Experimentation.

Surgery

In a set of experiments, prior to behavioral procedures rats were anesthetized with Isoflurane vaporizer (Provet, Switzerland) and mounted on a stereotaxic apparatus (Stoelting, USA). The scalp was incised and retracted and lambda and bregma were adjusted into the same horizontal plane. Small holes were drilled for bilateral placement of guide cannulae (22 gauge; Plastic One, Roanoke, VA) aimed at the lateral and basolateral nuclei of the amygdala (LA and BLA; 2.8 mm posterior to bregma, 5.0 mm lateral to midline, 8.0 ventral to the scalp) and two small stainless steel screws. Guide cannulae were then lowered, and dental acrylic was applied to keep them in place. After surgery, dummy cannulae (28 gauge, Plastic One, Roanoke, VA) were inserted in the guides to prevent intrusion of dirt. After the surgery, animals were returned to their home cages and allowed 1 to 2 days of recovery before intra-amygdala infusions. Several series of pilot experiments determined the need to have this short period of recovery. These pilot studies revealed that endoN effects following longer recovery periods (most probably due to the formation of scar tissue around the cannula) were not restricted to the amygdala, but largely diffused to other dorsal areas such as the striatum and hippocampus. In all cases, the health and general behaviour of experimental animals was checked and only those who presented signs of normal general behaviour were included in the study. Moreover, no differences in fear conditioning levels were observed between operated vehicle-injected animals that were allowed long (at least 5 days) versus short (1-2 days) recovery periods (data not shown).

Infusions

Rats were held gently, the dummies were removed from the guides and exchanged with internal infusion cannulae (28 gauge, Plastic One, Roanoke, VA), which extended 1 mm below the guide cannulae. The infusion cannulae were connected via polyurethane tubing to a microinjection pump (Harvard apparatus, Cambridge, MA) set for an infusion rate of 0.5 µl/min. Experimental groups were infused with either endo-N (0.2 units/hemisphere, 0.2 µl/hemisphere; Abcys, France), the unspecific N-methyl-D-aspartate (NMDA) receptor antagonist DL-2-amino-5-phosphonovaleric acid (APV, 10 µg/µl, 0.5 µl/hemisphere; Anawa, Switzerland) or the PSA-NCAM function enhancing synthetic peptide pr2 (0.002M, 0.2 µl/hemisphere; Schaffer Peptides, DK; Experiment 4). While endoN was diluted directly in artificial cerebrospinal fluid (ACSF, pH 7.4), both APV and pr2 were diluted in 10% demythyl sulfoxide (DMSO) in ACSF. Control groups received the corresponding vehicle injections, and 10% DMSO when appropriated. After infusion the cannulae were left in place for an additional minute to prevent back-leakage of the solutions and were then replaced with dummies. Animals were then returned to their home cages.

Fear conditioning

Training and testing took place in three identical rodent observation cages (30 x 37 x 25 cm) placed into a sound-attenuating chamber, illuminated by a 20W bulb. The side walls

of the observation cages were constructed of stainless steel, and the door of Plexiglas. The floor consisted of 20 steel rods wired to a shock source and solid-state scrambler for the delivery of footshock unconditioned stimuli (US). Ventilation fans provided a background noise of 68 dB (whole system: Panlab, Spain).

Animals were transported from the colony room to the adjacent fear conditioning room where training and testing occurred. After each testing session, animals were returned to their home cages (except in experiments 3 and 4, in which animals received a post-training infusion before being returned to their home cages).

In auditory fear conditioning (AFC) sessions rats were exposed to Context A (black walls of smooth texture, steel grid floor, cleaned with 2% ethanol) during 160 sec, followed by three presentations of tone-shock pairings in which the tone (20 sec, 80dB sound at 1000Hz) co-terminated with a foot shock (1 mA, 1 sec). The inter-tone interval was 40 sec and the conditioning session lasted 5.5 min in total.

In long-term and remote tone memory test rats were put into Context B (green walls of rough texture, grey plastic floor covered with flocks, cleaned with 4% chlorine) for 8 min in total and confronted with the same tone as in training during the last 5 min. In all long-term and remote contextual memory tests rats were exposed to Context A and left undisturbed for 8 min. Extinction was conducted in Context B and lasted 30 min in total during which 40 sec no-tone periods alternated with 20 sec tone periods (same tone as in training). Thus, the tone was presented 30 times during the extinction session. For data analysis, trials were averaged into 5 blocks each consisting of 6 trials. A tone memory test applied to measure extinction retention 24 h later was conducted equally to the extinction session, but consisted of only 6 trials, which were averaged into one value. Neither in the auditory and contextual memory tests nor during extinction, shocks were delivered.

The animals' behaviour was recorded and later scored with in house made behaviour observation software by an observer blind to the genotype. Indicator of fear was freezing, which is defined as behavioural immobility except for respiration movements for at least 2 sec. Freezing times were automatically transformed to percentage freezing values.

All experiments were conducted between 8.30 A.M. and 15.00 P.M.

General behavioural procedures

In the experiment aimed to determine PSA-NCAM expression in the amygdala rats were either left undisturbed or submitted to AFC with 0.4 mA or ACF 1 mA shock intensity. Twenty-four hours later, tone memory tests were conducted, after which animals were perfused and brains were processed for immunohistochemistry.

In all experiments involving infusions of either endoN, pr2 or APV rats received bilateral cannulae implants aimed at LA/BLA and were allowed 1–2 days of recovery from surgery before infusions or fear conditioning were performed. Usually, endoN was infused 48 h before AFC. Long-term memory tests for the tone were conducted 24 h and for the context 48 h after training and remote memory test were conducted 28 and 29 days post-training, respectively. Pre-training infusions of APV were administered 10 min before AFC. In experiments involving post-training infusions of either endoN or pr2, these were performed immediately after rats received AFC.

To test the effect of PSA-NCAM cleavage on fear extinction rats were submitted to a tone memory test 24 h after AFC. Based on their freezing values rats were assigned to either the vehicle or endoN group. Again one day later, surgery was performed and animals were infused directly with either vehicle or endoN aimed at LA/BLA and then returned to their home cages. The reason for the change in procedure (surgery and simultaneous infusion after AFC) was to assure optimal endoN diffusion into the amygdala. Pre-training surgery would have led to a too long interval between surgery and infusion, thus allowing the formation of

scar tissue around the cannula tip and prevention of optimal endoN diffusion (see above surgery). One day after surgery rats received a tone extinction session and 24h later tone memory was measured.

Histology

After infusion experiments, the accurateness of cannulae placement was obtained through histological verification. For this purpose animals were perfused, brains were removed and cut as described above. Sections were processed with immunohistochemical procedures for PSA-NCAM staining and served to verify both, cannula placement and accurateness of endoN infusions. Cannula placement was checked by mounting the wet slices on glass microscope slides and staining them with 0.5 % cresyl violet. Cannula placements were reconstructed on stereotaxic atlas templates from Paxinos and Watson (1998). Fourteen animals with inaccurate cannulae placements or endoN infusions were excluded from the study.

Immunohistochemistry

Rats were deeply anaesthetised (120 mg /kg pentobarbital) and transcardially perfused with 200 ml of phosphate buffered saline (PBS; pH=7.4) containing heparin (5 x 10^4 IU/ml), followed by 400 ml of 4% paraformaldehyde (PAF) in 0.1M phosphate buffer (pH=7.4). Brains were postfixed in PAF, then coronal sections (50µm thick) were cut on a vibratome (Leica VT 1000S) and collected in PBS (pH=7.4). For PSA-NCAM labelling free-floating sections were incubated with a mouse anti-Men B monoclonal anti-PSA-NCAM antibody (1:250; Abcys SA, France) overnight at room temperature, and another 24 h at 4°C. The slices were then treated with a biotin-labelled rabbit anti-mouse IgM (1:125; Abcys SA, France) and immunoreactivity was visualized with the biotin-streptavidin technique (ABC kit, Vectastain) using 3,3'-diaminobenzidine (DAB, Vector) as chromogen. The slices were then mounted, dehydrated and coverslipped.

Quantitative evaluation of staining

To evaluate variations in PSA-NCAM-IR within the amygdala, the immunohistochemical reaction was quantified using optical density (O.D.) on three sections (between 1.80 and 3.30 mm posterior to the bregma according to the atlas of Paxinos and Watson) from each animal (Manier et al., 1991). For this purpose a colour camera (ColorView I, Olympus) and image analysis software (analySIS FIVE, Olympus) coupled with the microscope were used. Studied regions were delineated using a computer mouse and mean optical densities and surface areas were measured on three different amygdaloid nuclei (lateral, basolateral and central). Results are expressed as relative density (OD/arbitrary area unit) subtracting systematically the background for each O.D. value.

Data analysis

Data are represented as means ± the standard errors of the means (s.e.m.). Anatomical and behavioural data were analysed with ANOVA (with treatment as between factor and block as a within factor) followed when necessary by Tukey's post hoc test. Whenever two groups were compared, an unpaired t-test was applied. Significance of results was accepted at $p \leq 0.05$.

RESULTS

Increased PSA-NCAM expression after auditory fear conditioning in the amygdala

The first experiment evaluated whether PSA-NCAM expression was modulated after auditory fear conditioning at two different shock intensities: 0.4 mA and 1 mA. As shown in Figure 1, a significant shock-intensity effect was encountered 24 h post-training in BLA and CE and a tendency in LA (BLA: $F_{2, 32} = 5.90$, $p = 0.009$; CE: $F_{2, 32} = 5.42$, $p = 0.013$; total: $F_{2, 32} = 5.28$, $p = 0.014$; LA: $F_{2, 32} = 2.97$, $p = 0.073$). Post-hoc Tukey tests revealed that animals subjected to the highest shock intensity (1mA; n = 9) exhibited significantly higher PSA-NCAM immunoreactivity than both, naïve (n = 6) and moderately (0.4 mA; n = 9) shocked animals in BLA and CE (BLA: *1mA vs. naive*: $p = 0.03$; *0.4 mA vs. 1 mA*: $p = 0.01$; CE: *1mA vs. naïve:* $p = 0.02$, *0.4 mA vs. 1 mA*: $p = 0.03$) and in tendency also in LA (LA: *1 mA vs. naive*: $p = 0.09$; *0.4 mA vs. 1 mA*: $p = 0.1$). No differences were observed between moderately shocked and naïve animals in any nuclei (all $p > 0.05$)

In summary, PSA-NCAM expression was significantly increased in BLA and CE 24 h post-training in animals subjected to the highest shock intensity.

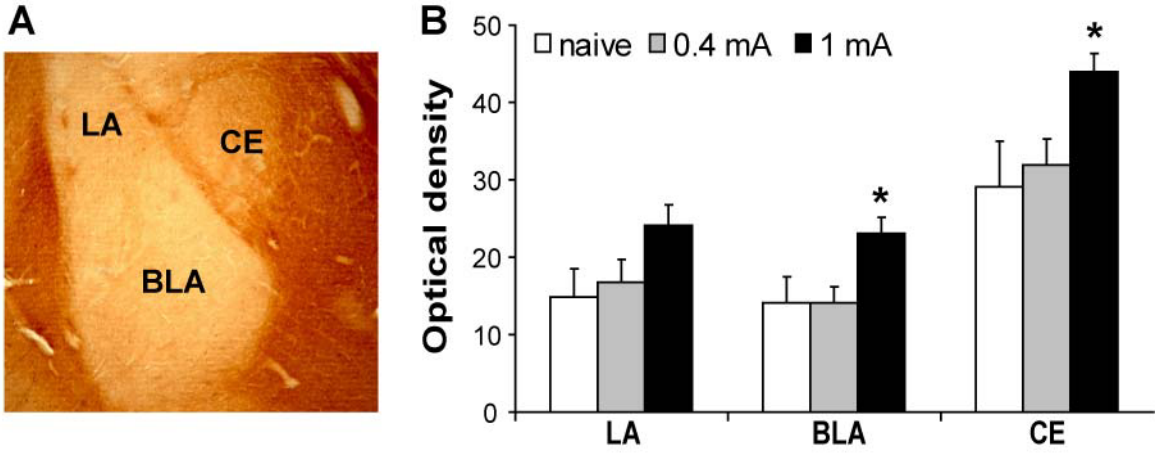

Figure 1. Enhanced PSA-NCAM expression in the amygdala 24 h after tone fear conditioning. (A) Brightfield photomicrograph of the amygdala showing PSA-NCAM expression in the amygdala of a naïve animal. (B) Optical density measurements of PSA-NCAM expression in LA, BLA and CE 24 h after auditory fear conditioning. PSA-NCAM is enhanced in BLA and CE when rats received a 1 mA shock, but not 0.4 mA shock. Naïve (n=6), 0.4 mA (n=9), 1mA (n=9). *, $p < 0.05$.

As a next step we investigated whether such upregulation of PSA-NCAM is necessary for long-term memory formation. Rats were bilaterally implanted with cannulae aimed at LA/BLA and endoN, a PSA-NCAM cleaving enzyme, was infused to study its' effects on various aspects of fear memory acquisition and consolidation. Since the enhancement of PSA-NCAM expression in the amygdala was obtained only with the highest shock intensities, all further experiments were conducted with 1 mA foot shocks.

Verification of the infusion

Cannulae placement. Only rats with cannulae tips at or within the boundaries of LA/BLA were included in the data analysis. Cannulae placement histology was performed for all experiments and exemplary cannulae tips are shown for experiment 2 (Fig. 2a).

Immunohistochemical PSA-NCAM staining. PSA-NCAM-IR was clearly reduced below the endoN infusion sites, yielding characteristic "white balls", which indicated a lack of PSA-NCAM expression that spread throughout LA and BLA and in many cases also to the CE, but spared the medial nucleus (ME; Fig. 2b,c).

Effects of pre-training intra-amygdala endoN infusions on fear memory formation

EndoN infusions (48 h pre-training) did not affect freezing behaviour during the training session (data not shown). Likewise, no differences between vehicle (n = 20) and endoN (n = 18) infused animals were detectable in the auditory and contextual memory tests (*tone memory*: $t_{36} = -0.62$, $p = 0.54$; *context memory*: $t_{36} = 0.57$, $p = 0.57$; Fig. 2e, f).

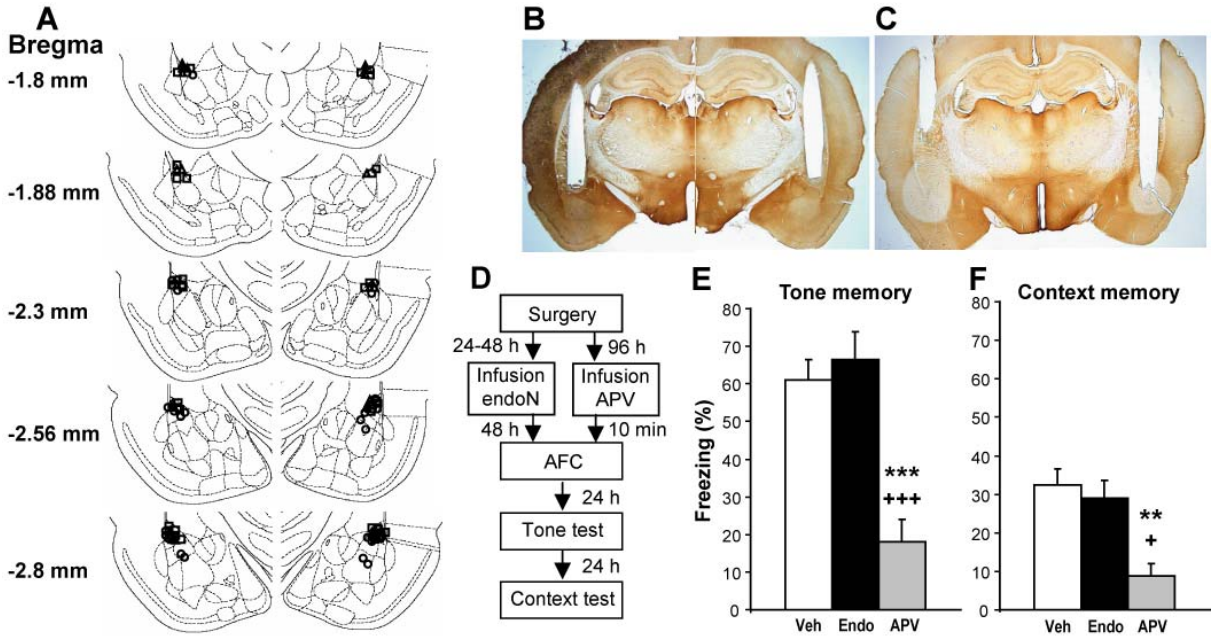

Figure 2. Pre-training intra-amygdala administration of endoN does not affect tone fear memory acquisition. (A) Exemplary cannula tip placement throughout the amygdala verified by histological procedure (squares = vehicle, circles = endoN, triangles = APV). (B-C) Brightfield photomicrograph showing immunohistochemical stainings for PSA-NCAM immunoreactivity. PSA-NCAM is expressed in the amygdala of a vehicle injected rat (B), but completely cleaved in an endoN injected rat (C). (D) Schematic of experimental protocol. (E-F) Percentage of total time spent freezing during the auditory memory test (D) and contextual memory test (E) in rats given intra-amygdala vehicle (n = 20), endoN (n = 18) and APV (n = 8) infusions. **, $p < 0.01$, ***, $p < 0.001$ relative to vehicle group, +, $p < 0.05$, +++ $p < 0.001$ relative to endoN group.

Effects of pre-training intra-amygdala APV infusions on fear memory formation

Since endoN injections did not significantly alter fear conditioning, we next performed a control experiment in order to verify the validity of our experimental conditions to target plasticity processes in the amygdala. To this end we compared the effects of endoN to that of the non-specific NMDA antagonist APV, known to disrupt fear conditioning when infused before training (Maren et al., 1996; Bauer et al., 2002). As previously described**,** APV (n = 8) markedly interfered with fear memory formation whereas endoN was inefficient (Fig. 2e, f). A one-way ANOVA with three groups (vehicle, endoN and APV) yielded a significant group effect in both, tone and context memory tests (*tone memory*: $F_{2, 43} = 10.16$; $p < 0.001$; *context memory*: $F_{2, 43} = 5.27$; $p < 0.01$). *Post-hoc* Tukey tests revealed significant differences between vehicle and APV, and between endoN and APV groups in both tests (all $p < 0.05$).

Effects of post-training intra-amygdala endoN infusions on fear memory consolidation

Since pre-training EndoN infusion did not disrupt fear memory formation when given pre-training, we tested whether endoN would impair fear memory consolidation when infused immediately after training rats in AFC. No differences in freezing were observed between

vehicle (n = 11) and endoN (n = 12) treated groups neither in the auditory nor contextual fear memory tests performed 24 hours after infusion (*tone memory*: $t_{21} = 0.69$, $p = 0.47$; *context memory*: $t_{21} = 0.44$, $p = 0.66$; Fig. 3).

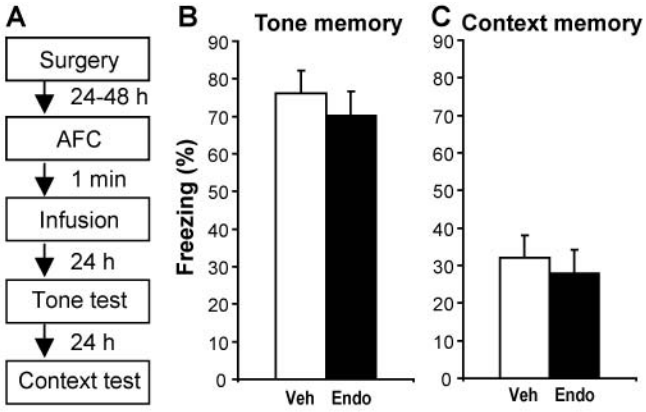

Figure 3. Post-training intra-amygdala administration of endoN does not affect tone fear memory consolidation. (A) Schematic of experimental protocol. Percentage of total time spent freezing during the auditory memory test (B) and contextual memory test (C) in rats given intra-LA/BLA vehicle (n = 11) or endoN (n = 12) infusions immediately after fear conditioning.

Effects of pre-training intra-amygdala infusions of endoN on remote memories

Commonly memory consolidation is studied up to one or two days after learning, which is referred to as "long-term" memory. However, systems consolidation spans over much longer periods of time and can even be disrupted months or years after the initial learning experience leading to memory impairments (for review see Dudai, 2004). The molecular mechanisms underlying the consolidation of so called "remote" memories are – if at all – poorly understood. Since PSA-NCAM up-regulation did not seem to be related to a successful establishment of fear memories when tested 24 hours later, we explore the possibility that it might be involved in remote memory. EndoN was infused 48 h prior to AFC and its effect on fear memories was assessed 28 days post-training. Yet again, vehicle (n = 9) and endoN (n = 10) treated groups expressed similar levels of fear in both, the tone and contextual memory test (*tone memory*: $t_{17} = -0.62$, $p = 0.54$; *context memory*: $t_{17} = 0.66$, $p = 0.52$; Fig. 4).

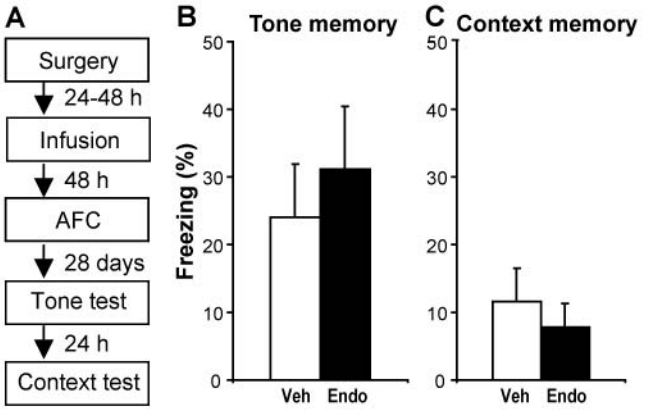

Figure 4. Pre-training intra-amygdala administration of endoN does not affect remote tone fear memories. (A) Schematic of experimental protocol. Percentage of total time spent freezing during the auditory memory test (B) and contextual memory test (C) in rats given intra-LA/BLA vehicle (n = 9) or endoN (n = 10) infusions 28 and 29 days post-training, respectively.

Effects of post-training intra-amygdala infusions of pr2

Rather than abolishing PSA-NCAM in the amygdala with endoN, the converse, the effects of the PSA mimetic peptide pr2 (Torregrossa et al., 2004) were investigated on the establishment of long-term fear memories. Rats received intra-amygdala pr2 infusions of a single dose immediately after AFC and were tested for their tone memory 24 h and 28 days post-training. No differences were detected between vehicle (n = 8) and pr2 (n = 10) treated

groups at both testing times (*24 hours*: $t_{16} = 0.69$, $p = 0.89$; *28 days*: $t_{16} = 0.68$, $p = 0.68$; Fig. 5).

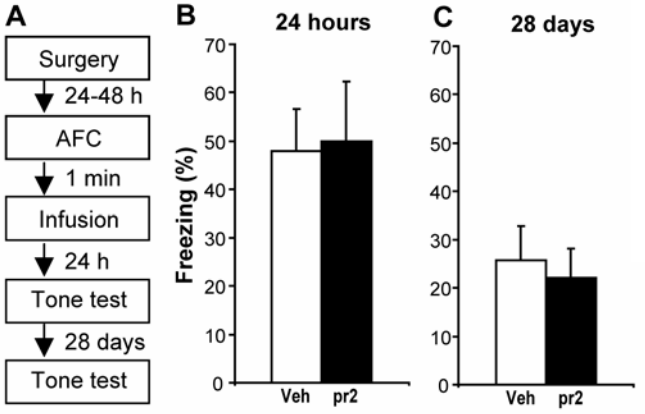

Figure 5. Post-training intra-amygdala administration of pr2 does not affect long-term tone fear memories. (A) Schematic of experimental protocol. Percentage of total time spent freezing during auditory memory tests performed 24 hours (B) and 28 days (C) post-training in rats given intra-LA/BLA vehicle (n = 8) or endoN (n = 10).

Effects of intra-amygdala infusions of endoN on fear extinction

Finally, the possibility that PSA-NCAM expression in the amygdala might be related to the extinction of fear memories was examined. Extinction refers to the decrease in freezing as a consequence of repeated tone exposure in absence of the shock. Animals were infused 24 h before receiving an extinction session. During extinction no differences were encountered between vehicle (n = 19) and endoN (n = 20) infused rats ($F_{1, 37} = .35$; $p = 0.55$). However, when memory for the tone was tested 24 h later, endoN infused rats froze significantly less, indicating a better extinction retention ($t_{37} = 2.03$; $p = 0.05$).

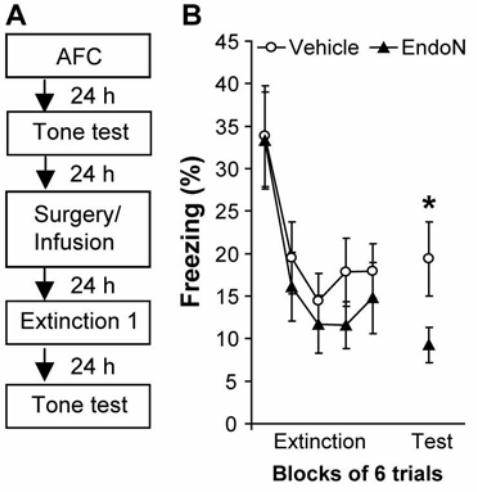

Figure 6. Pre-extinction intra-amygdala administration of endoN affects extinction memory. (A) Schematic of experimental protocol. (B) Average percentage freezing is shown in blocks of 6 trials (± s.e.m.) during two days of extinction in rats given intra-LA/BLA vehicle (n = 19) or endoN (n = 20) 24 h before the first extinction session. *, $p < 0.05$.

DISCUSSION

In the present study, the relevance of amygdaloid PSA-NCAM for the acquisition, consolidation and extinction of fear memories was evaluated. We found that its expression was increased most reliably in the BLA and CE 24 h post-training, under training conditions involving high, but not moderate, shock intensities. We then designed a series of experiments to assess the consequences of intra-amygdaloid cleavage of PSA from NCAM in fear conditioning. Surprisingly, pre- and post-training PSA cleavage with endoN did not interfere with neither the acquisition nor the consolidation of both, auditory and contextual fear memories when tested 1-2 days or 4 weeks after training. Post-training intra-amygdaloid infusions of the PSA mimotope peptide pr2, aimed to enhance PSA-NCAM activity, did not either influence auditory or contextual fear memories when tested 1 or 28 days post-training. In contrast, intra-amygdaloid PSA-NCAM cleavage facilitated fear extinction processes, thus accelerating forgetting of the fear memory traces.

One of the prevailing ideas about the role of PSA is that, due to its large hydrated volume and negative charge, it could act as a steric regulator of cell-cell surface apposition, reducing cell adhesion and, hence, allowing movement and thereby plasticity (Rutishauser and Landmesser, 1996). Cumulative evidence has shown that removing PSA from NCAM by endoN impairs synapse formation both in the developing brain (Landmesser et al., 1990) and in adulthood (Theodosis et al., 1999), including LTP-induced formation of perforated synapses in the CA1 hippocampal region (Dityatev et al., 2004).

Given (i) the central role of the amygdala in the encoding of fear conditioning (LeDoux, 2003), (ii) the role of PSA-NCAM in plasticity (see above) and memory formation (Murphy and Regan, 1998), and (iii) the upregulation of PSA-NCAM levels induced by fear conditioning observed in different amygdala nuclei in our study, it seems striking that elimination of amygdaloid PSA around the time of training did not have any consequence in either the acquisition, consolidation or later retrieval of the task. However, we propose below a number of non-mutually exclusive explanations that could account for such apparently paradoxical findings.

First, PSA-NCAM might participate in fear conditioning without being an essential step for memory formation. There are other examples in the literature in which specific activity-associated plasticity processes were shown to be dispensable for the related physiological mechanism. A paradigmatic one is the non-essential involvement of the hippocampus in delay eyeblink conditioning (as indicated by lesion studies; Solomon and Moore, 1975; Weiskrantz and Warrington, 1979), despite increased neuronal activity that is observed in this brain area during training (for a review see Christian and Thomson, 2003). A second, more relevant example in the context of the current study, is the lack of impact on parturition and lactation of PSA removal by endoN in the hypothalamus (Catheline and Theodosis, 2006), despite the requirement of PSA for a remarkable morphological plasticity (including synaptogenesis) that occurs in the hypothalamic oxytocin-system during both parturition and lactation (Theodosis et al., 1999). In the light of this latter and our own results, it is tempting to speculate that PSA-dependent synaptic remodelling (although it might play modulatory functions) might not be a necessary requirement for the basic functioning of those physiological systems whose activity is somehow closely linked to survival.

In questioning the implications of the lack of effect of amygdala PSA cleavage on the formation of the fear memory, we also speculate that synaptogenesis might not be a necessary step in the amygdala for the acquisition and/or (permanent/temporal) storage of fear memories. Although changes in synaptic efficacy in relevant circuits are believed to underlie memory storage (Martin et al., 2000), they can be achieved not only by new synapse formation, but also by changes in the strength of existing synapses (Moser, 1999). Whereas a

number of studies found evidence of learning-induced increased synaptic density in the hippocampus following, for example, spatial learning (Moser et al., 1994; O'Malley et al., 2000; Ramirez-Amaya et al., 2001; Eyre et al., 2003), there is not as yet indication of new synapse formation induced in the amygdala by fear conditioning (Lamprecht and LeDoux, 2004). Rather, it seems that this rapid defensive learning operate through the relatively more rapid and 'safe' mechanism (as opposed to synaptic pruning and /or elimination) of altering synaptic efficacy (Lamprecht et al., 2006). Thus, recent work has implicated an enlargement of postsynaptic densities (PSDs) associated to fear conditioning-induced translocation of profilin (an actin polymerization-regulatory protein) into dendritic spines in the lateral amygdala, as a mechanism contributing to the enhancement of associatively induced synaptic responses in the lateral amygdala following fear learning. Increased PSDs might reflect the incorporation of AMPA receptors into the synapse that was shown to occur in a large fraction of postsynaptic neurons in the lateral amygdala following fear conditioning and to be required for memory formation (Rumpel et al., 2005).

Therefore, these findings fit with the idea that, despite its hypothesized key role for activity-induced plasticity and long-term memory, PSA-NCAM might in fact not be a 'universal' requirement for every neuronal circuit engaged in information processing and storage. At difference to the complex computations believed to occur in hippocampus-dependent learning (Martin and Morris, 2002), fear is a defensive mechanism that, depending on the nature of the fear-eliciting stimuli, is either innately hardwired or can be acquired extremely rapidly (and therefore, be to a large extent pre-wired; Kim and Jung, 2006).

A second explanation for the role of PSA-NCAM in fear learning processes is that PSA-NCAM participates in resistance to extinction of the learned auditory fear memories, since cleavage of PSA by endoN injections facilitated extinction. The current view is that fear extinction is not just unlearning the US-CS association, but involves new learning implying that the CS does not longer predict danger (Quirk, 2002; Rescorla, 2004). Extinction training has been shown to involve a network of interactive brain regions, with connections between the medial prefrontal cortex and the amygdala playing a prominent role (for reviews, see Sotres-Bayon et al., 2004; Quirk et al., 2006). A number of molecular correlates of extinction have been identified (Myers and Davis, 2002), with data from micro-infusion studies having implied NMDA receptors, protein kinases and protein synthesis in the amygdala (Lin et al., 2003; Myers and Davis, 2002). Interestingly, amygdala BDNF signalling has been recently shown to play a key role in extinction of conditioned fear (Chhatwal et al., 2006) and PSA-NCAM, has been suggested to play a role in the sensitivity of neurons to BDNF (Vutskits et al., 2001). Although some molecular mechanisms involved in the acquisition and extinction of fear conditioning are common, a number of mechanisms were reported to differ between the two processes (Lin et al., 2003). Our findings add PSA-NCAM as one more molecular process that differs in the role played in the acquisition and extinction of auditory fear memories.

Finally, we propose that an alteration of PSA-NCAM expression may lead to fear sensitization and, potentially participate in pathologies**,** like phobias, post-traumatic stress disorders or anxiety, characterized by an impairment to extinguish fear. In fact, a variety of stress experiences have been shown to alter PSA-NCAM expression in different brain regions (Sandi et al., 2001; Pham et al., 2003; Nacher et al., 2004; Cordero et al., 2005; Lemaire et al., 2006). In line with this view is recent evidence showing increased synaptic density and/or dendritic arborization, in association with increased anxiety, following chronic immobilization stress (Vyas et al., 2002, 2004; Mitra et al., 2005a). Strikingly, a single stress experience is also able to induce a delayed spinogenesis in the BLA (as observed 10 days post-stress) (Mitra et al., 2005b). Given the reported role of PSA-NCAM in neuritogenesis and synaptogenesis (Bruses and Rutishauser, 2001; Dityatev et al., 2004), our findings point

out molecules involved in neuroplasticity, such as PSA-NCAM, as potential targets for the development of new treatments for fear-related and anxiety disorders.

In conclusion, we present evidence that amygdaloid up-regulation of PSA-NCAM as a consequence of fear conditioning is not necessary for the establishment of fear memories. However, amygdaloid PSA-NCAM seems to be implicated in precluding fear extinction processes. We suggest its involvement in neurobiological processes underlying anxiety-related pathologies.

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Paper 2: Selective learning and memory impairments in St8SiaIV/Polysialyltransferase deficient mice

Selective Learning and Memory Impairments in ST8SiaIV/Polysialyltransferase Deficient Mice

Kamila Markram[1], Rita Gerardy-Schahn[2] and Carmen Sandi[1]

[1]*Brain Mind Institute, Ecole Polytechnique Fédérale de Lausanne (EPFL), Switzerland*
[2] *Abteilung Zelluläre Chemie, Medizinische Hochschule Hannover, Germany*

Correspondence:
Prof. Carmen Sandi
EPFL SV BMI LNMC
AAB 1 19 (Bâtiment AAB)
Station 15
CH – 1015 Lausanne
Switzerland
Tel: +41-21 693 9534
Fax: +41-21 693 9636
carmen.sandi@epfl.ch

List of abbreviations:
AFC – auditory fear conditioning
EndoN – endoneuraminidase N
Ig – immunoglobulin
LA – lateral nucleus of the amygdala
LTD – long-term depression
LTP – long-term potentiation
mPFC – medial PFC
NCAM – neural cell adhesion molecule
PFC – prefrontal cortex
PSA – polysialic acid
PSA-NCAM – polysialylated neural cell adhesion molecule
ST8SialV or PST-1 – polysialyltransferase-1
STX or St8SiaII – sialyltransferase-X

The neural cell adhesion molecule (NCAM) has been implicated in regulating synaptic plasticity mechanisms as well as memory consolidation processes. Attachment of polysialic acid to NCAM (PSA-NCAM) has been reported to down-regulate its adhesive forces, a process hypothesized to be implicated in synapse selection after learning experiences. PSA-NCAM has been critically implicated in hippocampus-related synaptic plasticity and memory storage, but information about its functional role in other brain areas remains scarce. Here, we studied mice deficient for polysialyltransferase-1 (ST8SialV/PST-1), an enzyme which attaches PSA to NCAM during postnatal development and adulthood, and whose deficiency results in a drastic reduction of PSA-NCAM expression throughout the brain in adulthood. Mice were tested for their performance in the water maze and auditory fear conditioning (AFC). We report that ST8SiaIV knockout mice were impaired in spatial as well as reversal learning in the water maze. On the other hand, AFC was intact and ST8SiaIV mice exhibited no impairments in the acquisition or retention of cued fear memories. Spatial orientation learning and reversal learning require complex neural computations involving the hippocampus and prefrontal cortex, whereas cued fear conditioning is an associative type of emotional memory that relies on the amygdala. Therefore, our results indicate that PSA-NCAM contributes essentially to learning mediated by the hippocampus and prefrontal cortex, whereas it is not necessary for aversive emotional learning mediated by the amygdala.

Keywords: PSA-NCAM, memory, amygdala, hippocampus, prefrontal cortex

Learning experiences are believed to translate into changes of the synaptic connectivity between neurons, a process which may involve the formation of new synapses, dynamical changes in synaptic strength of existing synapses and/or elimination of unnecessary or weak synapses (Hebb, 1949; Kandel, 2001). One of the molecules identified to be involved in these processes is the neural cell adhesion molecule (NCAM), a member of the immunoglobulin (Ig) superfamily, expressed at pre-and post-synaptic zones (Persohn and Schachner, 1987; Schuster et al., 2001; Fux et al., 2003). Through homo- and heterophilic interactions, NCAM determines the binding forces between pre- and postsynaptic membrane. At the post-translational level, NCAM can be modified through attachment of polysialic acid (PSA-NCAM). PSA is a long homopolymer of α2,8-linked sialic acids. It is attached to NCAM in the trans-Golgi compartment via typical N-linked core glycosylation on the 5th Ig domain. This process is catalyzed by two sialyltransferases: sialyltransferase-X (STX or St8SiaII) and polysialyltransferase (PST or ST8SiaIV), which are differently regulated during development. STX regulates PSA-NCAM expression during embryonic, peri-natal and early post-natal development, whereas PST is predominant in the post-natal brain (Hildebrandt et al., 1998; Ong et al., 1998). During development, PSA-NCAM is widely expressed throughout the whole brain and crucially involved in cell migration, axonal outgrowth, fasciculation and pathfinding (for reviews see Kiss and Rougon, 1997; Ronn et al., 2000). In the adult brain, PSA-NCAM expression decreases dramatically, but remains high where neurogenesis and plasticity persists (Seki and Arai, 1991). Numerous studies demonstrated that PSA-NCAM expression is modulated in the hippocampus and other areas 10-24 h following training in a variety of tasks, including passive avoidance (Doyle et al., 1992a, Doyle et al., 1993, Fox et al., 1995, Fox et al., 2000; Foley et al., 2003), water maze (Murphy et al., 1996; O'Connell et al., 1997; Murphy and Regan, 1999; Sandi et al., 2004; Van der Borght et al., 2005; Venero et al., 2006), odour discrimination (Foley et al., 2003; Knafo et al., 2005) or contextual fear conditioning (Merino et al., 2000; Sandi et al., 2003). On the other hand, hippocampal cleavage of PSA from NCAM with the enzyme endoneuraminidase N (endoN) impairs spatial learning (Becker et al., 1996; Venero et al., 2006), blocks the maintenance of long-term potentiation (LTP; Muller et al., 1996; Becker et al., 1996) – a cellular model of learning and memory (Malenka and Nicoll, 1999) – and synaptogenesis on NCAM-expressing neurons (Dityatev et al., 2004).

In the present study we examined mice deficient for the polysialyltransferase ST8SiaIV or PST. These mice exhibit normal PSA-NCAM expression throughout early development and preserve normal morphological features (Eckhardt et al., 2000), in contrast to complete NCAM (Cremer et al., 1994, 1997; Chazal et al., 2000) or St8SiaII (Angata et al., 2004) knockout mice, who present significant developmental alterations. In adulthood, ST8SiaIV-deficient mice show unaltered NCAM expression, but decreased PSA-NCAM expression throughout the brain, and complete loss of PSA-NCAM in the hippocampus accompanied by an impairment of LTP and long-term depression (LTD; Eckhardt et al., 2000). These features of normal development, intact morphology and no loss in NCAM expression make these mice ideal for studying the unique contribution of PSA-NCAM to behaviour and cognition. Mice were tested for their performance in learning tasks with a major load of the hippocampus, PFC or amygdala. In the water maze, animals were trained to find a fixed platform location, a learning type that is mediated by the hippocampus (Morris et al., 1982) and on a reversal learning task that requires cognitive flexibility and critically depends on neural processing in the medial PFC (mPFC; Wolf et al., 1987; De Bruin et al., 1994; Lacroix et al., 2002). On its turn, AFC has been shown to require a functional amygdala (LeDoux et al., 1990). We report that ST8SiaIV knockout mice show impairments in spatial and reversal learning, but exhibited no deficits in the aversive emotional learning form of conditioned fear.

EXPERIMENTAL PROCEDURES

Animals

All experiments were conducted on age-matched wild-type (31±5 g of mean weight±S.D.) and ST8SiaIV knockout (30±6 g) mice. The generation of the ST8SiaIVmutation has been described previously (Eckhardt et al. 2000). The mice were backcrossed for 7 generations onto the C57BL/6J background. Heterozygous mice were then intercrossed to obtain homozygous mice. All mice were approximately 6 months old at the onset of behavioral testing. Mice were housed as groups of two to six in standard plastic cages on a 12h light – dark cycle (lights on at 7:00 am). Water and food was provided *ad libitum*. All experiments were conducted between 8.30 A.M. and 15.00 P.M. to avoid the influence of circadian hormonal fluctuations.

All the procedures described were conducted in conformity with the Swiss National Institutional Guidelines on Animal Experimentation, and approved by the Swiss Cantonal Veterinary Office Committee for Animal Experimentation.

Immunohistochemistry

Immunohistochemical procedures were performed at the age of 6 months to confirm that PSA-NCAM was depleted in the brain of ST8SiaIV knockout mice at the start of behavioral testing. In order to compare PSA-NCAM reductions with those occurring naturally in mice as a consequence of aging, immunohistochemical staining for PSA-NCAM in wild-type mice was done at two additional ages: 6 weeks and 13.5 months. The mice were deeply anaesthetised (120 mg/kg pentobarbital) and transcardially perfused with 100 ml of phosphate buffered saline (PBS; pH=7.4) containing heparine (5 x 10^4 IU/ml), followed by 120 ml of 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (pH=7.4). Brains were postfixed overnight in PFA, then coronal sections (50µm thick) were cut on a vibratome (Leica VT 1000S) and collected in PBS (pH=7.3). For PSA-NCAM labelling free-floating sections were incubated with a mouse anti-Men B monoclonal anti-PSA-NCAM antibody (1:250; Abcys SA, France) overnight at room temperature and another 24 h at 4°C. The slices were then treated with a biotin-labeled rabbit anti-mouse IgM (1:125; Abcys SA, France) and immunoreactivity was visualized with the biotin-streptavidin technique (ABC kit, Vectastain) using 3,3'-diaminobenzidine (DAB, Vector) as chromogen. The slices were then mounted, dehydrated and coverslipped.

Quantitative evaluation of staining

PSA-NCAM immunoreactivity was quantitatively compared between wildtype (n = 4) and ST8SiaIV knockout mice (n = 3) within the basolateral amygdala, the dorsal hippocampus and the medial prefrontal cortex by determining the optical density (O.D.) on brain slices. A colour camera (ColorView I, Olympus) and image analysis software (analySIS FIVE, Olympus) coupled with the microscope were used. Regions of interest were delineated using a computer mouse and mean optical densities and surface areas were measured. Results are expressed as relative density (O.D./arbitrary area unit) subtracting systematically the background for each O.D. value.

Water Maze

The water maze apparatus consisted of a large white circular pool (1.40 m in diameter) filled with opaque colored water (25 ± 1°C). A platform (10 cm diameter) was submerged 1 cm under the water surface. Both pool and platform were made of white polyvinyl plastic and offered no intra-maze cues to guide escape behavior. The water maze was surrounded with curtains containing several extra-maze visual cues.

The experimental procedure was organized as follows: 3 days of spatial training, followed by 2 days of reversal training. On the first day, all mice were habituated to the apparatus and water by swimming in the pool for 2 minutes without any platform, which was inserted only at the end of the habituation trial and mice were allowed to rest there for 15 sec before they were removed from the pool. This session was later evaluated for locomotion. Spatial learning sessions were conducted on three consecutive days (days 2 - 4) and each session consisted of 6 trials (intertrial interval (ITI): 20 min). Each trial started with the mouse facing the wall at one of 8 possible positions. The latency to find the platform was measured. If a mouse did not find the platform within 60 sec, it was guided towards it. Each mouse remained on the platform for 15 sec before it would be taken out. During learning sessions the platform remained in the same position. On day 5 a probe test was conducted, followed by a reversal learning session. For the probe test, the platform was removed from the pool and animals were released to the pool for a 60 sec period from the quadrant opposite to the one where the platform had been previously located. At the end of the probe trial the platform was re-inserted into the pool and mice remained on it for 15 sec. For the reversal learning session the platform position was changed to the opposite quadrant and remained there for two reversal learning sessions (days 5 and 6). As above, each reversal session contained 6 trials with an ITI of approximately 20 min. On day 7 a reversal probe trial was conducted, which differed from the one above only in that the mice were inserted into the pool form the quadrant opposite to the one where the platform had been during the previous reversal learning.

Video tracking software (Ethovision, Noldus, Netherlands) was used for automatic recording and analysis of escape latencies, distances swum and velocities.

Auditory fear conditioning

Training and testing took place in a rodent observation cage (20 x 20 x 27.5 cm) placed into a sound-attenuating chamber, illuminated by a 20W bulb. The side walls of the observation cage were constructed of stainless steel, and the door of Plexiglas. The floor consisted of 20 steel rods through which a scrambled shock from a shock generator could be delivered. Ventilation fans provided a background noise of 68 dB (whole system: Panlab, Spain).

Fear conditioning to the tone and fear conditioning to the context was performed on the same batch of animals (n=20/group), with a time span of at least 5 weeks in between. On the day of auditory fear conditioning, mice was transported from the colony room to the behavioural laboratory (situated in the adjacent room) and placed in the conditioning chamber. Mice were exposed to the conditioning chamber during 160 sec, followed by three presentations of tone-shock pairings in which the tone (20 sec, 80dB sound at 1000Hz) co-terminated with a foot shock (0.9 mA, 2 sec). The inter-tone interval was 40 sec and the conditioning session lasted 5.5 min in total. Chambers were cleaned with 2% ethanol between training sessions. Two days (long-term memory) and again 2 months later (remote memory), the mice were put in a novel context for 8 min in which they were re-exposed to the same tone, but no shocks, continuously during the last 5 minutes of the test. Change of the context was accomplished by exchanging visual (green plastic walls of rough texture) and odour (2% chlorine) cues. 4 days after AFC a fear generalization test to a different tone (400Hz, 80dB) was conducted with a subset of animals (n=23) in the same environment and with the same specifications as previously.

Animals' behaviour was recorded and later scored with in house made behaviour observation software by an observer blind to the genotype. Indicator of fear memory was freezing, which is defined as behavioural immobility except for respiration movements for at least 2 seconds. Freezing times were transformed to percentage freezing values.

Open field

The open field test was conducted under dim and dispersed light conditions in a white quadratic box (100 x 100 x 37 cm), divided into 4 square compartments (50 x 50 x 37 cm), allowing the evaluation of locomotor and exploratory activity of up to 4 mice at the same time. A camera was mounted vertically over it and connected to a data processing computer. The time spent in the centre and the periphery of the arena, the total distance moved and the mean velocity were recorded by a video tracking software (Ethovision, Noldus, Netherlands). Each mouse was placed into the centre of the arena and allowed to move freely for 30 minutes, while its behaviour was recorded on video. Between sessions, the box was carefully cleaned with soap water. The distance moved and mean velocity were taken as markers of spontaneous locomotor activity and exploratory behaviours.

Elevated plus-maze

The elevated-plus-maze consisted of two opposite open arms and two opposite closed arms (30 × 5 x 40 cm) arranged at right angles. The arms extended from a common central platform (5 × 5 cm) that gave access to all arms. The maze was elevated 70 cm above the floor under dim and dispersed light conditions. All sessions were videotaped by a camera positioned above the apparatus and connected to a computer. The total distance moved, time spent in the open and closed arms, number and latency of entries to the open and closed arms were automatically recorded by the video tracking software. The mice were placed on the central platform facing a closed arm. The experiment lasted 5 minutes. Between sessions the maze was cleaned with soap water. The distance moved and frequency of arm entries served as indicators of spontaneous locomotor activity, while differences in the proportions of time spent in the open and closed arms and latencies to enter the open arms were taken as indicators of anxiety.

Statistics

All results were expressed as mean ± SEM and analyzed with Student's t-tests or ANOVA for repeated measurements were appropriate. Significance of results was accepted at $p \leq 0.05$.

RESULTS

PSA-NCAM expression

Immunohistochemical analyses indicated that PSA-NCAM was clearly expressed in all three areas of interest, the PFC, hippocampus and amygdala, in 6 month-old wildtype mice. Moreover, ST8SiaIV knockout mice exhibited a drastic to almost complete loss of PSA-NCAM expression in all of these areas (Fig. 1). Quantitative comparisons with optical density measurements revealed that ST8SiaIV knockout mice exhibited a reduction of PSA-NCAM expression of 94 % in the medial prefrontal cortex, 75 % in the dorsal hippocampus and of 67 % in the basolateral amygdala (all Student's t-tests: $p < 0.0001$; Fig. 1F).

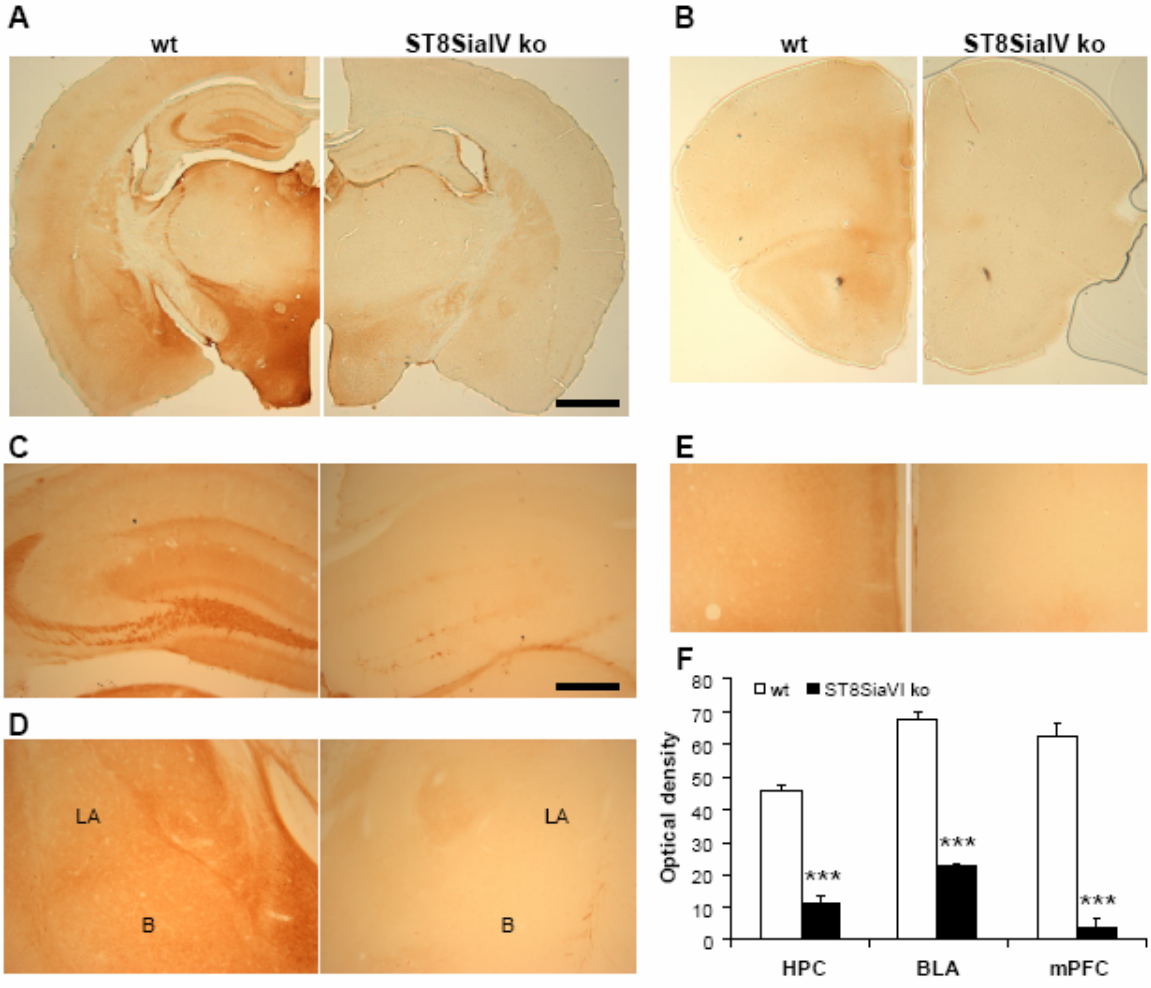

Fig. 1. Immunohistochemical comparison of PSA-NCAM expression. (A-E) Brightfield photomicrographs showing the expression of PSA-NCAM throughout the brain in wildtype (wt) and ST8SiaIV deficient mice (left and right column, respectively) obtained with anti-PSA-NCAM staining. PSA-NCAM expression in ST8SiaIV knockout mice is drastically reduced in (A) the hippocampus and amygdala and (B) the prefrontal cortex. Scale bar: 1 mm. (C-E) Higher magnification (x 10,000, scale bar: 200 µm) of (C) dorsal hippocampus, (D) lateral (LA) and basal (B) amygdala and (E) infralimbic prefrontal cortex. (F) Optical density measurements obtained from the the dorsal hippocampus (HPC), basolateral amygdaloid complex (BLA), and the medial prefrontal cortex (mPFC) confirm reduced PSA-NCAM expression in ST8SiaIV ko mice. ***, $p < 0.0001$.

Impaired learning in the water maze

Both wildtype and ST8SiaIV knockout mice learned to find the platform over the three spatial learning sessions (ANOVA repeated measurements; $F_{17,22} = 5.79$, $p = 0.03$) and did not differ from each other in their learning levels during the first 2 days (ANOVA repeated

measurements; day 2: $F_{1,22} = 0.032$, $p = 0.86$; day 3: $F_{1,22} = 0.018$, $p = 0.89$). However, on the third training day, PSA-NCAM deficient mice exhibited significantly longer escape latencies to find the platform during the first three trials (13-15) when compared to wildtype mice (ANOVA repeated measurements; day 4, trials 13 – 15: $F_{1,22} = 7.4$, $p = 0.01$; Fig. 2a). Following spatial learning, mice were submitted to reversal learning by changing the platform position to the opposite quadrant (days 5 and 6). ST8SiaIV knockout mice were clearly impaired in performing this task, as indicated by their much longer latencies to reach the platform (ANOVA repeated measurements; day 5, trials 22 – 23: $F_{1,22} = 7.56$, $p = 0.01$; day 6, trials 26 – 29: $F_{1,22} = 7.2$, $p = 0.01$).

Two probe trials were performed: one after the first three days of spatial habit learning and another one after the two days of reversal learning. In the first probe trial no differences were encountered between wildtype and ST8SiaIV knockout mice concerning the time spent in the target quadrant, where the platform was located during the three days of spatial learning (Student's t-test; $p = 0.3$). Similarly, in the second probe trial groups did not differ in the time spent either in first (Student's t-test; $p = 0.4$) or second target quadrant (platform located in the opposite quadrant) (Student's t-test; $p = 0.3$). No differences in swim speed during all spatial learning, reversal learning and probe tests were found (data not shown). Therefore, the deficits displayed by these animals were mainly related to learning the task and to adapt learning strategies to updated environmental conditions. Eventually, and provided enough training was given, they were able to learn to locate the platform. Under those circumstances, no retrieval deficits were observed, indicating that a PSA-NCAM deficit, although impairing learning, might not disrupt retrieval processes.

Taken together, PSA-NCAM deficient mice showed mild impairments during spatial training and progressively more severe impairments when they had to detach from the previously learned escape location and adapt a new learning strategy towards the re-located platform.

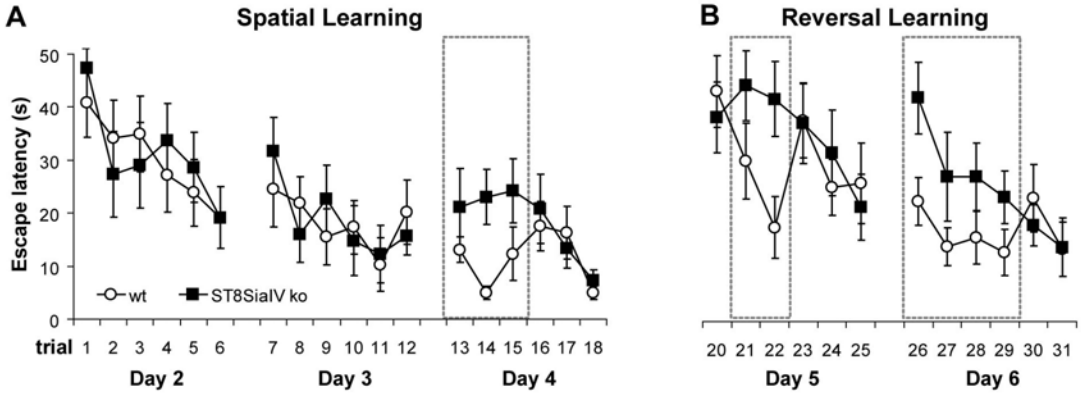

Fig. 2. Impaired spatial and reversal learning. (A) Escape latencies during the first three days of spatial habit learning in wild-type (wt) and ST8SiaIV deficient mice. Data points are group means per trial. There are no differences between wild-type (wt) and ST8SiaIV knockout (ko) mice during the first two days of spatial learning. On the third day, ST8SiaIV ko mice are impaired during the first three learning trials. (B) ST8SiaIV ko mice display impaired reversal learning on both of both of two reversal training sessions. N = 12/group. Dashed lines: $p < 0.05$.

Intact fear conditioned memories

During the habituation period before shock administration mice from both groups explored the new environment and exhibited no freezing (Student's t-test: $p = 0.5$; data not shown). After the onset of the shocking period, both groups developed the characteristic freezing response without any differences between each other (Student's t-test: $p = 0.3$; data

not shown). Likewise, both groups developed freezing when exposed to the tone in the auditory memory test 48 h after AFC, but did not differ significantly from each other (Student's t-test: $p = 0.8$; Fig. 3).

We speculated that this lack of effect might be due to the possibility that PSA-NCAM dependent synaptic plasticity processes might involve much longer consolidation periods (see, for example, Cambon et al., 2004). Therefore, we tested remote fear memories to the tone. However, when tested 2 months after auditory fear conditioning, no remote effect of PSA-NCAM deficiency was observed (wt: 8 ± 2% freezing, ST8SiaIV ko: 5 ± 2% freezing; Student's t-test: $p = 0.6$).

Furthermore, possible fear generalization processes were explored. Both groups exhibited virtually no freezing to a different tone and did not differ from each other (Student's t-test: $p = 0.3$; data not shown). Thus, PSA-NCAM deficiency in the brain does neither affect conditioned fear acquisition nor the formation of long-term and remote fear memories nor does it contribute to fear generalization mechanisms.

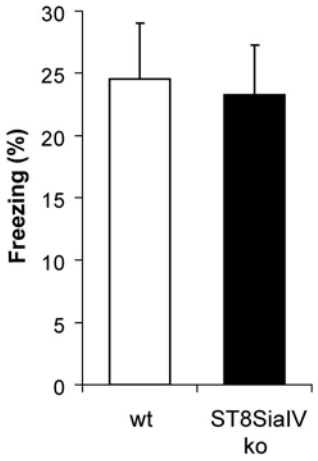

Fig. 3. Intact auditory fear memories. Percentage of time spent freezing in the fear memory test. Data are group means (±SEM). ST8SiaIV knockout (ko) and wild-type (wt) mice exhibit no differences in the memory test. N = 20/group.

Intact locomotor and exploratory activity

Locomotor and exploratory activity was measured across three different tests: the water maze, open field and elevated plus maze. In none of the tests we found any significant differences neither in the distance swum or moved, nor in speed levels (Student's t-test, all $p > .05$). Representative results for the open field are depicted in Fig. 4. Thus, PSA-NCAM deficiency does not impact locomotor and exploratory activity.

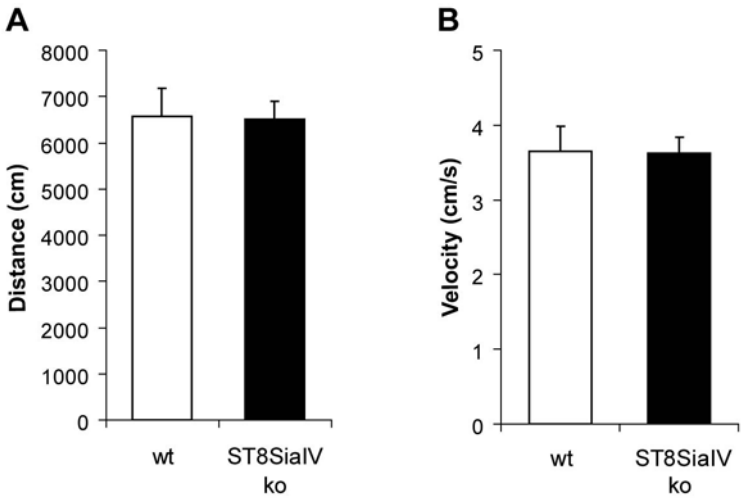

Fig. 4. Intact locomotion and exploration. Locomotor and exploratory activity in the open field test during a 30 min time period. Data are group means (±SEM). (A) Total distance moved (cm) in the open field. (B) Velocity of movement. There are no differences between ST8SiaIV knockout (ko) and wild-type (wt) on both variables. N = 12/group.

Decreased anxiety

In the EPM, ST8SiaIV deficient mice spent more time in the open arms (Student's t-test: $p = 0.04$; Fig. 5) and in tendency moved more in the open arms than wildtype mice (Student's t-test: $p = 0.07$). These behavioral changes are conventionally interpreted as indicative of lower anxiety levels.

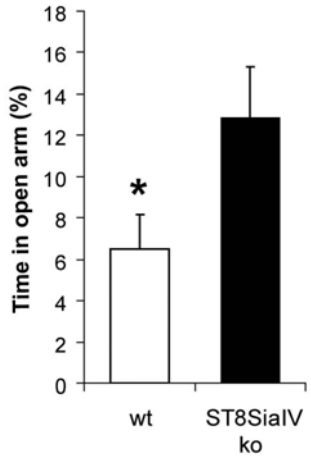

Fig. 5. Reduced anxiety. Percentage time spent in the open arms of the elevated plus maze during a 5 min time period. Data are group means (±SEM). ST8SiaIV knockout (ko) mice spend significantly more time in the open arms than wild-type (wt) mice. N = 12/group. *, $p < 0.05$.

DISCUSSION

The expression of PSA-NCAM in the adult brain relies critically on the presence of ST8SiaIV polysialyltransferase. In agreement with previous observations (Eckhard et al., 2000), we found that deficiency in ST8SiaIV leads to reduced PSA-NCAM levels throughout many forebrain areas. Our immunohistochemistry analyses showed a drastic reduction, though not total abolishment, of PSA-NCAM in the PFC, hippocampus and amygdala, among other brain areas. For the hippocampus it has been shown, and for the other two brain areas speculated, that PSA-NCAM expression is involved in synaptic plasticity and learning and memory processes. In the present study, we show that PSA-NCAM deficient mice are impaired in spatial learning in the Morris water maze and exhibit even greater difficulties in reversal learning (when the platform is reversed to the opposite quadrant). On the other hand, ST8SiaIV knockout mice apparently preserve normal auditory fear conditioning. Spatial learning and reversal learning are considered explicit types of learning and were shown to rely on the hippocampus and mPFC (Morris et al., 1982, Wolf et al., 1987; de Bruin et al., 1994; Lacroix et al., 2002) whereas fear conditioning is an implicit form of emotional memory that depends upon the amygdala (LeDoux et al., 1990). These results suggest that PSA-NCAM contributes to processes involved in explicit learning probably involving the hippocampus and PFC, whereas it is not necessary for the formation of cued fear memories occurring in the amygdala.

The role of PSA-NCAM in spatial learning

In order to navigate through the water maze and find the submerged platform, rats use extra-maze visual cues to form a spatial map of the environment, a form of learning which critically relies on the hippocampus (Morris et al., 1982). Evidence indicates that NCAM contributes significantly to spatial learning (Cremer et al., 1994; Arami et al., 1996; Cambon et al., 2004; Venero et al., 2006). Furthermore, several studies indicate that the polysialylation of NCAM is also involved in spatial learning. PSA-NCAM expression in the hippocampus is increased 10-24 h post-training in the water maze (Murphy et al., 1996; Murphy and Regan, 1999; Van der Borght et al., 2005; Venero et al., 2006), while intra-hippocampal enzymatic removal of PSA-NCAM yields spatial learning and memory deficits (Becker et al., 1996; Venero et al., 2006). LTP impairments in the CA1 region of the hippocampus have been observed after application of endoN (Muller et al., 1996; Becker et al., 1996) as well as in ST8SiaIV knockout mice (Eckhardt et la., 2000), further suggesting the implication of PSA-NCAM in hippocampal plasticity. Our results showing a learning impairment that becomes evident on the third day of water maze training (when animals mainly fine-tune their spatial learning about the platform location, as opposed to earlier training phases, when the task requires to learn many non-spatial related information) further emphasize the importance of PSA-NCAM for spatial learning. It is also important to note that NCAM levels and morphological development are unaltered in ST8SiaIV knockout mice (Eckhard et al., 2000), and therefore the learning impairment cannot be explained by structural deficits. Moreover, the possibility that the impairments could be due to abnormalities in locomotion or anxiety can be excluded, because no differences were found between wildtype and ST8SiaIV knockout mice in locomotor behavior in the open field, and ST8SiaIV knockout mice showed indexes of lower anxiety levels in the elevated plus maze. Low anxiety has been related to an advantage over high anxiety, for water maze learning (Herrero et al., 2006), and therefore current evidence does not support the view that such emotional trait might account for learning impairments observed in the ST8SiaIV knockout mice. Since PSA-NCAM deficiency was not accompanied by retrieval deficits once the animals had learnt the platform location, our results further reinforce the view for PSA-NCAM involvement in hippocampus-related spatial learning processes.

The role of PSA-NCAM in cognitive flexibility and adaptability

Spatial position reversal learning requires the capability to change the response rapidly as a function of altered stimulus contingencies and across species depends on the prefrontal cortex, since damage to this area may result in perseveration tendencies and a resistance to change (Eichenbaum et al., 1983; Wolf et al., 1987; De Bruin et al., 1994; LaCroix et al., 2002). The rat PFC consists mainly of two areas, the medial and lateral (orbitofrontal) part (Leonard, 1969), which are anatomically and functionally heterogeneous (for review see Dalley et al., 2004). For reversal learning in the water maze the medial prefrontal cortex (mPFC) turned out to be important as revealed by lesion studies (Wolf et al., 1987; De Bruin et al., 1994; LaCroix et al., 2002). In the present study we show, for the first time, that PSA-NCAM deficiency resulted in a markedly reduced capacity for reversal learning and an even greater impairment in retaining this new position until the subsequent day, suggesting a deficit in behavioral and cognitive flexibility. Such abilities are required for the successful adaptation to an ever-changing environment, which is one of the main features defining animal intelligence. Thus PSA-NCAM may be crucially involved in higher cognitive functioning.

The role of PSA-NCAM in establishing fear memories

The association between a discrete stimulus, such as a tone, and an aversive electrical shock is believed to be processed and stored in several nuclei of the amygdala (LeDoux et al., 1990). Damage to the lateral nucleus of the amygdala (LA), which receives information about the shock and tone from the thalamus, interferes with auditory fear conditioning (LeDoux et al., 1990) and several lines of studies suggest that the LA is a key locus for the acquisition and storage of conditioned fear (for reviews see LeDoux, 2003; Blair et al., 2001). For example, electrophysiological data demonstrates that fear conditioning is accompanied by synaptic plasticity processes in the LA (Quirk et al., 1995, 1997; Collins and Pare, 2000; Maren, 2000; Repa et al., 2001) and the disruption of protein synthesis in LA interferes with fear memory consolidation (Schafe et al., 1999; Maren et al., 2003) as well as LTP (Huang et al., 2000).

Nacher et al. (2002) recently demonstrated that PSA-NCAM is expressed in the amygdala and suggested a possible involvement in fear memory consolidation. Indeed Angata et al. (2004) showed that ST8SiaII deficient mice exhibit impaired auditory fear memories. Therefore, we hypothesized that PSA-NCAM would participate in amygdaloid synaptic process underlying fear conditioning. However, to our surprise, ST8SiaIV knockout mice exhibited normal auditory fear memories as compared to wildtype mice. Thus, we suggest that the polysialylated form of NCAM might not contribute to synaptic plasticity processes in the amygdala as it does in the hippocampus. In other words, PSA-NCAM might not necessarily be (equally) involved in all synaptic plasticity processes in the brain. Consistent with this idea is the observation that PSA-NCAM contributes to LTP in the CA1 region, but not CA3 region of the hippocampus (Eckhardt et al., 2000). However, no involvement of PSA-NCAM in amygdaloid synaptic plasticity does not exclude that NCAM *per se* is relevant for fear conditioning in the amygdala since NCAM deficient mice exhibit impaired auditory fear memories (Stork et al., 2000).

It is rather peculiar that adult mice deficient for St8SiaII/STX were impaired in auditory fear conditioning, even though they exhibit normal levels of PSA-NCAM in the amygdala (Angata et al., 2004), whereas adult St8SiaIV/PST deficient mice with abolished PSA-NCAM in the amygdala exhibit intact fear conditioned tone memories. While the previous study by Angata and colleagues (2004) implied an involvement of PSA-NCAM in auditory fear conditioning, our study does not support a role for PSA-NCAM in the acquisition and consolidation of fear memories. A possible explanation for this diverging data might be that St8SiaII/STX deficiency might have caused some subtle morphological

connectivity abnormalities in the amygdala during embryonic development which went undetected in the Angata study, but may underlie the deficits in fear memory.

Conclusion

PSA-NCAM deficient mice were impaired in spatial learning and reversal learning in the water maze, while auditory fear conditioning and memories remained intact. Since alterations in locomotion or increased anxiety levels could be excluded to explain the observed impairments, we conclude that the explicit learning forms of spatial and reversal learning involve PSA-NCAM mediated synaptic plasticity processes (most probably in the hippocampus and mPFC), while implicit aversive learning processes occurring in the amygdala do not require PSA-NCAM.

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2.5. Discussion and Perspectives

The surprising result of this work was that auditory fear conditioning, an amygdala-processing dependent task, led to an up-regulation of PSA-NCAM in the amygdala, but memory formation did not rely on the presence of this molecule. We found this by either cleaving PSA-NCAM directly from the amygdala or in PSA-NCAM deficient mice, which lack PSA-NCAM throughout the adult brain. This finding is in striking contrast to the involvement of PSA-NCAM in hippocampus-dependent learning, for which PSA-NCAM was shown to play a crucial role (Becker et al., 1996; Venero et al., 2006).

PSA-NCAM in fear extinction

Even though PSA-NCAM was not essential for the formation of an aversive memory trace in the amygdala, our results obtained from rats with amygdaloid PSA-NCAM deficiency, indicate that it contributed to the extinction of this memory trace. We observed a tendency to improved extinction retention also in adult St8SiaIV/PST knockout mice (unpublished data). However, more experiments need to be done in order to verify this trend.

Extinction is usually defined not as a forgetting, but as a new learning process in which a new association is build, namely that the conditioned stimulus no longer predicts danger. However, electrophysiological studies in the lateral amygdala also show that cells which exhibited enhanced responses to a conditioned tone become less responsive during extinction of the tone (Quirk et al., 1995). This implies that extinction might also resemble a forgetting like processes, in which the initial memory trace is erased or weakened. At this stage, it results difficult to establish in which of the two possible processes PSA-NCAM might be involved. However, the absence of PSA-NCAM enhances the retention of a previous extinction session. Taking this into account and the fact that polysialylation of PSA-NCAM results in deadhesion (Yang et al., 1992; Yang et al., 1994; Fujimoto et al., 2001), and probably the uncoupling of synaptic contacts, it is rather unlikely that the absence of PSA-NCAM might facilitate a forgetting process, since this would most probably require weakening or even loss of synapses. Whereas previous studies showed the requirement of PSA-NCAM for the induction of novel synaptic processes (Dityatev et al., 2004), no evidence has as yet been presented for a requirement of PSA-NCAM for the maintenance of already existing synapses. Thus, PSA-NCAM might be rather involved in the establishment of a new association between the tone and a no-danger signal. This new memory trace should be embedded in a new set of synapses with distinctive characteristics than the ones where the initial conditioned fear memory trace was stored, because the potential synapses encoding for the fear memory trace seem to rely on PSA-NCAM independent consolidation mechanisms. PSA-NCAM immunoreactivity in the adult hippocampus and the septohippocampal pathway was shown to be mainly stemming from GABAergic interneurons (Nacher et al., 2002a; Foley et al., 2003a). Thus, a possibility to test in future studies is that PSA-NCAM-mediated consolidation of the extinction engram might be encoded in GABAergic interneurons in the amygdala, which in turn might contribute to inhibiting the former fear memory.

In this context, it is interesting to note that fear extinction is mediated by a circuit involving the amygdala, hippocampus and prefrontal cortex (reviewed in Sotres-Bayon et al., 2004). In particular the medial prefrontal cortex (mPFC) was found to be involved in the retention of fear extinction training, since lesions of this area do not impair extinction learning *per se*, but do lead to impairments of extinction memory (Quirk et al., 2000). The amygdala and mPFC are strongly interconnected (reviewed in Sotres-Bayon et al., 2004). Neurons in the mPFC send excitatory inputs to the various regions of the amygdala, the lateral, basal and central nuclei, and also a region consistent of only GABAergic interneurons, the intercalated cell masses (ITC), which in turn inhibit output from the central nucleus (Quirk et al., 2003).

The excitatory projections to the lateral, basal and central nuclei are believed to form contacts with GABAergic interneurons since stimulation of the mPFC induces strongly inhibitory responses in the amygdala (Rosenkranz and Grace, 2001; 2002; Rosenkranz et al., 2003). Thus, PSA-NCAM mediated consolidation of fear extinction memory might take place at these synapses between mPFC projections and amygdaloid interneurons.

Although this is only a hypothesis, one of the main messages of this work is that PSA-NCAM mediated plasticity processes might be occurring only at very specific synapses and not in a homogeneous way at just any plastic synapse participating in learning. Whether PSA-NCAM contributes to synaptic plasticity processes may depend on the morphological and molecular context, i.e. where the synapse is located and which receptors are expressed. This view is supported by the observation that LTP at ectopic mossy fiber synapses in the CA3 region of the hippocampus, even though functionally normal, is impaired in constitutive NCAM deficient mice, but not in conditional NCAM deficient mice, in which synaptic morphology is normal.

The fact that cleavage of PSA-NCAM enhances extinction retention might be due to NCAM-mediated strengthening processes of GABAergic synapses. It is conceivable that adhesion forces are increased, and thus the synaptic cleft shortened. Alternatively, the pre- and/or postsynaptic machinery might be modified by NCAM dependent mechanisms (see chapter 2.1.3).

Roles of St8SiaII/STX and St8SiaIV/PST in auditory fear conditioning

It is peculiar that adult mice deficient for St8SiaII/STX are impaired in auditory fear conditioning, even though they exhibit normal levels of PSA-NCAM in the amygdala (Angata et al., 2004), whereas adult St8SiaIV/PST deficient mice with abolished PSA-NCAM in the amygdala exhibit intact fear conditioned tone memories. Thus, the Angata study implies an involvement of PSA-NCAM in auditory fear conditioning, whereas our two studies do not support a role for PSA-NCAM in the acquisition and consolidation of fear memories. The only explanation for this pattern I can conceive is that St8SiaII/STX deficiency might have caused some subtle morphological connectivity abnormalities in the amygdala during embryonic development which went undetected in the Angata study.

The role of NCAM per se in auditory fear conditioning

Taking into account that NCAM deficient mice do exhibit deficits in auditory as well as contextual fear memories (Stork et al., 2000), but mice and rats with amygdaloid PSA-NCAM deficiency do not, we suggest that this type of learning in the amygdala may rely solely on NCAM, but nor PSA-NCAM, mediated mechanisms.

Synaptic plasticity in the amygdala

In order to finally verify our conclusions that auditory fear memory consolidation is not mediated by PSA-NCAM-mediated mechanism, it would be important to study synaptic plasticity, i.e. in a LTP paradigm. Our prediction is that amygdaloid LTP in PSA-NCAM deficient slices could nethertheless be induced and maintained.

3. The amygdala in autism

This section deals with the amygdala under pathological circumstances. A rodent model of autism (Rodier et al., 1996; Rodier et al., 1997) was used to study behavioural and electrophysiological alterations in the "autistic" rat brain and a special emphasis was put on possible amygdaloid dysfunctions. At the behavioural level, we found strong evidence for an amygdala dysfunction in this model of autism, since fear memories were greatly enhanced, fear extinction impaired and social interactions reduced – all behaviours largely related to the function of the amygdala and associated brain structures. At the electrophysiological level, we found a hyper-reactive amygdala with enhanced synaptic plasticity, which may be a potential mechanisms underlying the enhanced fear memories.

In the following chapters a general introduction to autism is given, including a discussion on brain regions possibly affected in autism. The current "amygdala theory of autism" focuses on the involvement of the amygdala in socio-emotional behaviour. Evidence for this theory is discussed. Subsequently, an alternative and novel theory on the involvement of the amygdala in autism is suggested in this thesis – abnormal fear as a core pathology. Supporting evidence for this theory is presented in the attached paper and in an additional results section.

3.1. Autism – a heterogeneous neurodevelopmental disorder

Autism is recognized as a neurodevelopmental disorder which manifests within the first 3 years of age and progressively aggravates in the course of life. The prevalence of autism has been estimated from 1.2 per 10'000 to 30.8 per 10'000 (Bryson, 1996; Madsen et al., 2002) and recent estimates indicate an increasing prevalence which cannot be explained solely by increased public awareness (Byrd, 2002).

The exact underlying causes of autism are still unclear. It is however known that genetic and environmental factors interact in complex ways yielding the broad heterogeneity in phenotypes and severities.

Twin and family studies revealed a strong genetic predisposition. An identical twin to a child with autism has a 60 – 90% chance of being affected as well (Kotsopoulos, 1976; Folstein and Rutter, 1977; Geddes, 1977; Sloan, 1978; Gillberg, 1983; Spiker et al., 1994; Bailey et al., 1995; Le Couteur et al., 1996; Rutter, 2000; Hallmayer et al., 2002; Constantino and Todd, 2003; Kolevzon et al., 2004; Ho et al., 2005). Considerable effort has been invested in identifying possible genes and chromosomal loci affected in autism (Bacchelli and Maestrini, 2006).

On the environmental side, evidence has accumulated to indicate that autism can be caused by chemical insults during early embryogenesis and up to date, five environmental risk factors have been identified in epidemiological studies, all of which are potent teratogens: maternal rubella infection (Chess, 1971), ethanol (Nanson, 1992), misoprostol (Bandim et al., 2003), thalidomide (Stromland et al., 1994) and valproic acid (Moore et al., 2000; see chapter 3.6). Exposure to these teratogens during the first trimester of gestation increases the risk of autism (Arndt et al., 2005).

3.2. The autistic symptomotology

Autism was first described by Leo Kanner, a child psychologist, in 1943. His initial description of the syndrome, based on 11 case studies emphasized "…an innate inability to form the usual, biologically provided affective contact with other people."

According to the DSM-IV, autism is characterized by a triad of symptoms – impaired social interactions, communication deficits and stereotypic, restricted and repetitive behaviours, all of which are defined as follows:

Impaired social interactions

- Deviant eye gaze (no eye contact)
- Reduced facial and body expression to regulate social interactions
- Reduced or lack of peer relationships (few or no friendships, lack of interest in social interactions)
- Lack of spontaneous sharing of enjoyments, interests or achievements with other people (manifested in lack of showing, bringing or pointing out objects of interest)
- Failure to respond to other peoples emotions or attempts at socializing.

Impaired communication

- Delay or complete lack of the development of spoken language
- No initiation or sustainment of a conversation with others
- Stereotyped, repetitive or idiosyncratic use of language
- Lack of varied spontaneous make-believe play or social imitative play.

Restricted repetitive and stereotyped patterns of behaviour, interest and activities

- Obsessive interest in something to an abnormal range (e.g. dates, phone numbers, radio station call letters, etc.).
- Inflexible adherence to specific, non-functional routines or rituals (e.g. watching water rinse from a tap)
- Preference of sameness (e.g. same type of clothing, same type of food, same day structure, etc.)
- Stereotyped and repetitive motor habits (e.g. hand or finger flipping or twisting, or complex whole-body movements, such as rocking back and forth).
- Persistent preoccupation with parts of objects.

Apart from these three core domains of the autistic symptomotology described in the DMS-IV, autistic people exhibit many other characteristic features. Many cannot tolerate sensory stimulation, like loud noises, touches, lights and react in anxious and aggressive ways, when over-stimulated. Abnormal motor development is also a common feature. Enhanced levels of anxiety and an increased incidence of phobias were also reported (Evans et al., 2005).

Cognitive theories have been built around some of the autistic symptoms such as the inability to empathize with other peoples minds (Frith and Happe, 1994), deficits in executive function (Russell, 1997), deficits in holistic (or Gestalt processing) with a simultaneous preference for details (Frith, 1989; Happe and Frith, 2006), and deficits in face perception and evaluation of social cues from facial expressions (Baron-Cohen, 2004; Schultz, 2005).

Autistic people are severely impaired in empathising with other people and "reading their mind" as has been shown in over 30 experimental tests (see figure 11 for example). This deficit is captured in the "theory of mind" or "mind-blindness theory" of autism (Baron-

Cohen et al., 1985; Frith and Happe, 1994), which involves two elements: 1) the ability to attribute mental states to oneself and others, to be able to distinguish between oneself and others and realize that others have independent minds and may pursue different goals from oneself; 2) the ability to express an appropriate emotional reaction to the other person's mental state, thus to be able to empathize with the others' mind. Being able to read other peoples' minds involves propositional thought of the like "if I do such and such, he will likely do such and such". These capabilities seem to be disturbed in the autistic population.

Based on the observation that autistic people develop strong repetitive routines and have a preference for sameness, it has been argued that they exhibit a deficit in executive function (Russell, 1997), much of the like as patients with frontal lobe deficits exhibit symptoms of perversation and the inability to shift attention. The term "executive functions" encompass many kinds of mental operations which enable an individual to disengage from the immediate context in order to guide behaviour based on mental models or future goals, a function which is highly dependent on the integrity of the prefrontal lobes. There is evidence that autistic subjects perform badly on tests of executive function, such as for example the classic Wisconsin card sorting test (Sandson and Albert, 1984; Rumsey and Hamburger, 1990; Ozonoff et al., 1991) or the Tower of Hanoi planning task (Ozonoff et al., 1991), which has led to the "executive function theory of autism" (Russell, 1997).

Autistic individuals also display abnormally weak central coherence; that is to integrate sensory information in a holistic (Gestalt) manner (Frith, 1989; Happe and Frith, 2006). In the normal brain, the sensory system is predisposed to perceive information in an integrative and coherent way, such that individual features are automatically bound into a meaningful Gestalt. Autistic individuals, however, exhibit a preference for details. Consequently, they fail to process meaningful and patterned stimuli more effectively than random and devoid of structure stimuli (Frith, 1970b, 1970a) and tend to deal with information in a detailistic (piece-meal) manner. These assumptions are theoretically summarized in the "weak central coherence theory of autism" (Frith, 1989; Happe and Frith, 2006) and have been supported by experimental tasks, in which weak central coherence would be expected to have a task advantage over integrative, Gestalt perception or tasks in which integrative information processing would give an advantage over detailistic feature processing. For example, autistic subjects performed better than controls on the Wechsler Block Design task, which was due to a greater ability to segment the whole design into its component parts (Shah and Frith, 1993) and in the Embedded Figures Test, in which a hidden figure must be detected within a bigger picture (Shah and Frith, 1983). On the contrary, in a homograph disambiguation task which specifically requires the processing of information in context for its solution, autistic individuals failed to use preceding sentence context to determine the correct pronunciation of the homographs (Happe and Frith, 1997).

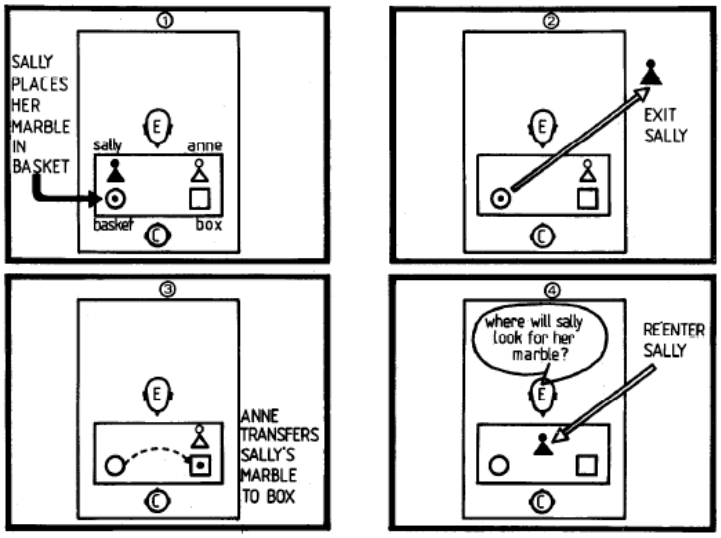

Figure 11. Classical theory of mind test from Baron-Cohen et al., 1985. The procedure was as following: There were two doll protagonists, Sally and Anne. First, it was checked that the children knew which doll was which (Naming Question). Sally first placed a marble into her basket. Then she left the scene, and the marble was transferred by Anne and hidden in her box. Then, when Sally returned, the experimenter asked the critical Belief Question: "Where will Sally look for her marble? ". If the children point to the previous location of the marble, then they pass the Belief Question by appreciating the doll's now false belief. If however, they point to the marble's current location, then they fail the question by not taking into account the doll's belief. These conclusions are warranted if two control questions are answered correctly: "Where is the marble really?" (Reality Question); "Where was the marble in the beginning?" (Memory Question). The control questions are crucial to ensure that the child has both knowledge of the real current location of the object and an accurate memory of the previous location. In this study 23 out of 27 normal children (85%) passed the Belief question, whereas only 4 out of 20 autistic children (20%) gave the correct answer to the Belief question.

Perception of faces and the meaning expressed in facial expressions has also consistently been found to be impaired in autism (Schultz, 2005). The correct perception of faces and interpretation of the emotions and mental states conveyed through expression are truly important in navigating through the social environment and establishing relationships. Humans have a perceptual bias for faces and virtually all adults are experts in the holistic recognition of faces (Diamond and Carey, 1986; Carey, 1992). Autistic infants, on the other hand, do not exhibit this preference for faces. In fact, the lack of interest in faces within the first 6 months of life is one of the best predictors of a later diagnosis of autism (Maestro et al., 2002). Consequently, autistic individuals do not develop this kind of face recognition expertise and are impaired in face recognition tasks (Langdell, 1978; Hobson, 1986a; Hobson et al., 1988a; Braverman et al., 1989; Boucher and Lewis, 1992; Davies et al., 1994; Klin et al., 1999) as well as in the correct interpretation of facial expressions (Hobson, 1986b; Weeks and Hobson, 1987; Hobson et al., 1988b, 1988a; Braverman et al., 1989; Tantam et al., 1989; Adolphs et al., 2001).

Autism is a heterogeneous disorder regarded as a continuum including Asperger Syndrome (AS) and Pervasive Developmental Disorder Not Otherwise Specified (PDD NOS). Collectively these syndromes form the Autistic Spectrum Disorders (ASD), with the underlying assumptions that the underlying neurobiological manifestations are shared. Asperger syndrome – almost isochronically to Kanner described by the child psychologist Hans Asperger in 1944 – also includes disturbances in social reciprocity, but does not include language delays. PDD NOS is a subsyndromal manifestation of autism.

Heterogeneity manifests itself within each of the three core symptoms. In the social domain, inter-individual variability may range from a complete absence of interest in

interacting with others, to more subtle dysfunctions in managing complex social interactions, in which other peoples intentions or the social context need to be taken into account. Communication impairments may range from a complete absence of spoken language over mild impairment, with the use of idiosyncratic vocabulary, to highly elaborate as can be the case in some individuals with Asperger's syndrome. Equally, stereotyped behaviours may range from simple motor stereotypies and a preference for sameness to more complex rituals, which may be accompanied by considerable distress and aggression when they cannot be fulfilled. The same accounts for intellectual capabilities. While the IQ of the majority of autistic individuals is low and ranges on the level of mental retardation, IQs can also be within the normal range and in some cases even highly above average. The extent to which the communication handicap prevents an accurate diagnosis of autism is unclear. Anecdotal reports indicate that in some cases, where the communication deficits were solved in some way, unusually high IQ's were revealed. Moreover, Asperger individuals without severe language and communication problems can exhibit truly high intellectual capabilities and excellent achievements in specific fields of interest as already noted by Asperger himself (Asperger, 1944).

3.3. Brain pathologies in autism

Since the amygdala is discussed in greater detail in chapter 3.4, the emphasis lies on other brain regions in this section.

3.3.1. Macroscopic alterations

The human brain continues to mature considerable throughout the first two years of life: dendritic arbors grow massively and are myelinated, synapses are formed and selected, and circuits are built and refined. Brain regions mature according to a specific hierarchical scheme: basic sensory areas mediating perceptual function mature earlier than higher order associative areas, such as the frontal cortex. This goes along with later development of refined skills and higher cognitive, emotional, social and communication functions. This process continues throughout childhood, and in case of synapse building and selection may be a lifelong processes, enabling continuous learning and memory experiences.

One of the most striking and reliable observations in the autistic brain is its abnormal development throughout childhood. Newborn autistic infants usually exhibit a normal (Lainhart et al., 1997; Stevenson et al., 1997; Gillberg and de Souza, 2002) or even slightly smaller than normal (Courchesne et al., 2003) brain size as measured by head circumference (HC), which reliably indicates brain size in young kids. However, within the first year of life there is an accelerated growth (Dementieva et al., 2005), such that by the age of 2-3 years the overall volume is about 10% higher than normal (Courchesne et al., 2001; Sparks et al., 2002). This pattern of accelerated growth can be observed in higher order cortical areas, namely the frontal lobe, the temporal lobe, in the limbic system and in the cerebellum. Magnet resonance imaging (MRI) unfolds the following picture in terms of white (axons) and gray (neurons) matter: white matter volumes are increased in the neocortex (18%) and in the cerebellum (39%), whereas gray matter is increases in the cerebrum (12%), but not cerebellum (Courchesne et al., 2001). In the neocortex, white matter increase is not uniform, but is most pronounced in the frontal, followed by the temporal and parietal lobes, whereas occipital lobes remain normal (Carper et al., 2002). In 2-3 year old autistic kids, gray matter is increased most pronounced in the frontal followed by the temporal lobes (Carper et al., 2002). Furthermore, there is evidence for an enlargement of the hippocampus in children ranging from 3 to 12.5 years of age (Sparks et al., 2002; Schumann et al., 2004). This abnormally accelerated growth early in childhood is followed by an abnormally slow or arrested growth in later childhood. In other words: the autistic brain outruns the normal brain within the first 4 years of life, reaches mature levels much faster and then ceases to develop further. Thus, throughout childhood the outgrowth declines and the normal brain catches up until the size of the autistic brain is only 1-2% above normal in adolescence (Redcay and Courchesne, 2005). In the normal brain gray matter increases by 20% between 2-4 and 6-8 years of age, but changes only by 1% in autistic brains (Carper et al., 2002). Likewise, in the normal brain white matter in the frontal lobes increases by 45% from 2-5 years to 5-9 years, but only by 7% in autistic brains (Carper et al., 2002). In the cerebellum, white matter volume increases by 50% in normal children from 2-3 years of age until adolescence, but only by 7% in autistic children (Courchesne et al., 2001).

In contrast to accelerated early growth of white matter in the cerebellum in early years, gray matter volumes in the cerebellum seem to be reduced, particularly in the cerebellar vermis (Hashimoto et al., 1995).

In older autistic subjects overall brain volume might be comparable to normal people or even exhibit some atrophy due to a developmental stop in childhood and probably due to

compensation mechanisms as a consequence of altered circuitry (see next chapter). Supporting evidence comes from autopsied autistic brains. During the period of 5 – 13 years of age autistic brains weight on average 100-200 g more than normal, whereas in adulthood (18-54 years) their weight is decreased by 100-200 g (Bauman and Kemper, 2003).

In summary, the development of autistic brains is abnormal, exhibiting accelerated growth during early childhood, which comes to a sudden stop and ceases to develop any further. The most pronounced abnormalities are observed in the frontal lobes.

3.3.2. Microscopic alterations

Post-mortem neuropathology on approximately 40 autopsied autistic brains (Williams et al., 1980; Coleman et al., 1985; Rodier et al., 1996; Bailey et al., 1998; Kemper and Bauman, 1998) revealed alterations in neuronal anatomy within frontal (Bailey et al., 1998; Kemper and Bauman, 1998), temporal (Bailey et al., 1998), parietal (Bailey et al., 1998), limbic (Raymond et al., 1996; Bailey et al., 1998; Kemper and Bauman, 1998) and cerebellar (Ritvo et al., 1986; Rodier et al., 1996; Bailey et al., 1998; Kemper and Bauman, 1998) regions. Bailey et al. (1998) reported irregular laminar pattern in the frontal lobe, ectopic neurons in the white matter, thickened areas in the parietal, temporal, frontal and cingulate lobes, and furthermore regions of increased neuronal density and subplial gliosis in the right cerebral hemisphere in 4 out of 6 autistic subjects with low IQs. Kemper and Bauman (1998) investigated the brains of 9 autistic subjects. In 8 out of nine subjects they found abnormally small neurons and increased cell packing in the anterior cingulated gyrus, amygdala, hippocampus, subiculum, enthorinal cortex, mamillary body and medial septum. Neurons in the CA1 and CA4 region of the hippocampus exhibited reduced complexity and less extensive dendritic arbors (Raymond et al., 1996). Consistently over several studies the number of Purkinje cells in the cerebellum was found to be reduced (Ritvo et al., 1986; Rodier et al., 1996; Kemper and Bauman, 1998). Modern stereological counts of neuron number confirm above studies and reveal an excess number of neurons in the cerebrum and a decreased amount of neurons in the cerebellum (Courchesne et al., 2005).

Further evidence for altered neuronal anatomy and circuitry stems from recent studies on minicolumnar arrangements in the neocortex (Casanova et al., 2002). The minicolumn is the smallest computational circuit in the brain (Mountcastle, 1997). It consists of a core line of vertically, between layers VI and II, ascending pyramidal and inhibitory neurons, their connections and input/output axons (fig. 12). A cell-poor area, the neurophil, surrounds the column core. The neurophil contains unmyelinated axon fibers, dendritic arborisations and synapses. A minicolumn is 30 – 60 μm in diameter and contains 80 – 100 pyramidal neurons. The size of minicolumns varies between cortical areas. In the frontal cortex minicolumns and pyramidal neurons are nearly twice as big as in the primary visual cortex (Casanova et al., 2002). Minicolumns in 9 autistic brains were abnormally narrow, both in the column core as well as in the neurophil, in the frontal and temporal lobes (fig. 12). This means that the autistic brain exhibits an increased number of minicolumns, thus more processing units and increased complexity (Casanova et al., 2002).

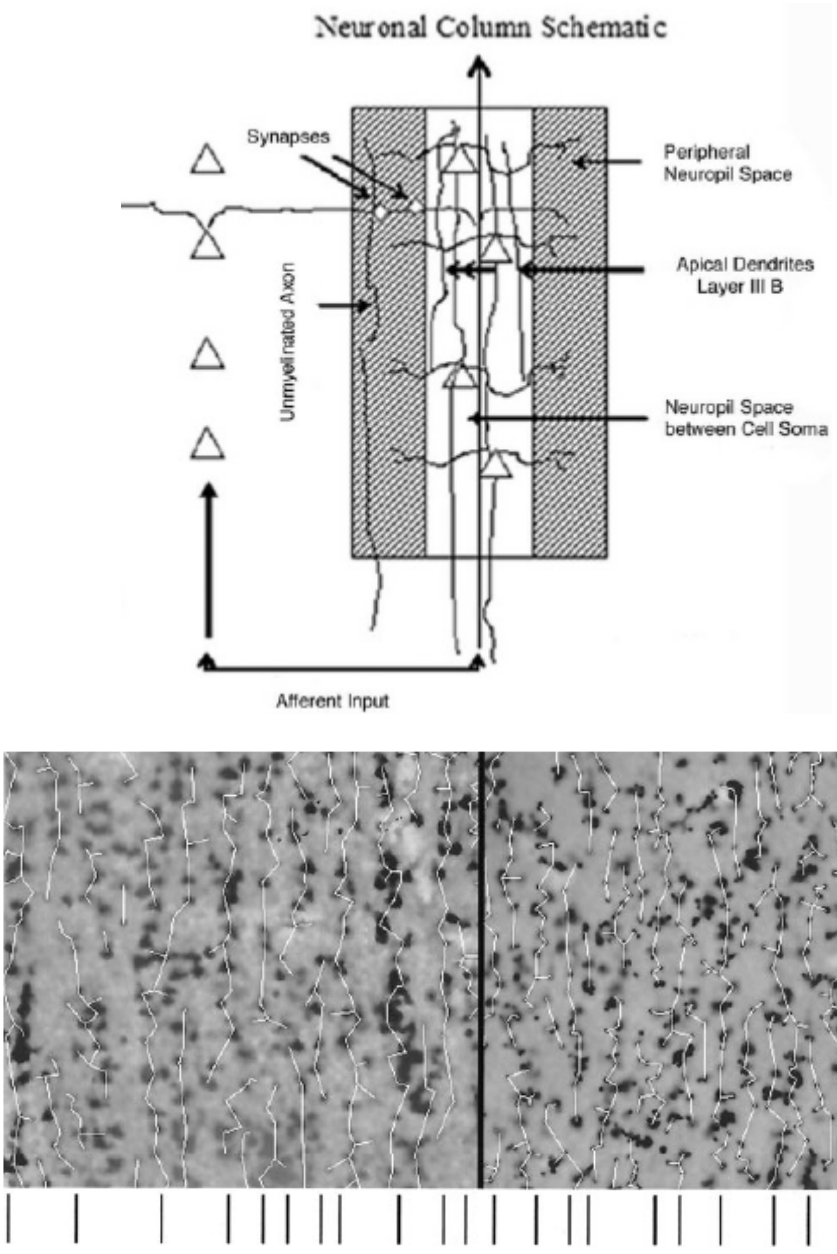

Figure 12. Minicolumnar organization in control and autistic brain. Microscopic fields (original magnification x100) of layer III of temporoparietal auditory area from the brain of an autistic patient (right) and an age-matched control (left). The superimposed Euclidean minimum spanning tree indicates the cell core of the minicolumn. Lines at the bottom of each figure define the boundaries of each minicolumn, that is, 10 in the control brain and 12 in the brain of the autistic patient. Computerized analysis of the series reveals greater neuronal dispersion, normal cell density and narrower minicolumns in the brains of autistic patients. From (Casanova et al., 2002). Scheme from (Courchesne et al., 2005).

Minicolumnar comparisons of an autistic 3-year old, autistic 41 year old and control adult brain in the frontal and occipital cortex confirmed Casanovas' initial finding of reduced minicolumn and neurophil size in the frontal lobes in autism in adulthood. Furthermore, frontal lobe minicolumns in the 3-year old autistic brain exhibited the same size as in the 41-year old brain, indicating arrested minicolumn growth in autism (in the same way macroscopic growth is arrested during development). Minicolumns in the occipital lobes, on

the other hand, did not differ from normal brains (Courchesne et al., 2005). It seems that microscopic studies parallel the developmental macroscopic findings. For example, in autistic children cells in some brain areas, like the cerebellum, were enlarged, whereas in autistic adults the same cells were small and pale and reduced in number (Bauman and Kemper, 2003). Taken together, these studies suggest progressive pathology, which changes with age. Clinically, this process might not be degenerative, but rather reflect the brains attempt to compensate for an abnormal circuitry.

Very interesting implications arise from the finding that the neurophil space around the column core is reduced (Casanova et al., 2002). This compartment holds the unmyelinated projects form interneurons and thus ensures the lateral and vertical inhibition of the local circuit. A defect in these GABAergic projections may – most obviously – correlate with the prevalence of seizures among the autistic population. However, it may also be related to more subtle features observed in autism, such as hyper-sensitivity to sensory stimulation (Casanova et al., 2003). It has been argued that a deficit in inhibition may disrupt the normal balance between excitation and inhibition in the columnar organization and lead to autism (Casanova et al., 2003). First evidence for this hypothesis stems from studies conducted within the VPA model of autism (see chapter 3.6.5 for details). *In vitro* electrophysiological recordings in the neocortex from brain slices of VPA-treated rats exhibit excessive reactivity to standard stimulation, thus providing first real evidence for an excitation/inhibition imbalance hypothesis of autism (Rinaldi et al., 2006a).

It has been speculated that abnormal development and early stage overgrowth may change the connectivity pattern between short- and long-range neuronal sites (Courchesne and Pierce, 2005; Courchesne et al., 2005). Courchesne et al., 2005 write:

> …the excess of frontal cortical neurons after the normal stage of apoptosis, which is normally largely completed prenatal, might impede the refinement of within minicolumn circuits, tip the excitatory–inhibitory balance in minicolumns towards excess excitation, and abnormally increase the target size for long-distance axons from posterior lower level systems which would effectively dilute their impact on frontal neural functioning. Further, following the simple principle that neurons that fire together wire together, the abnormal excess of frontal neurons, in the absence of normal local inhibitory modulation, might be predicted to create local and very short distance eddies of excitation that develop into excessively overconnected but dysfunctional local and short distance circuits. Conversely, long-distance cortical–cortical connectivity would be decreased because its development depends on spatiotemporally coherent bursts of activity. The net functional result is diminished impact of low-level information on frontal activity and diminished impact of frontal activity on posterior systems. In effect, then, frontal cortex is, relative to normal, "disconnected" from other cortical and subcortical structures, and instead frontal cortex mainly "talks with itself" …. The central function of frontal cortex, to integrate diverse information from multiple systems and provide directive and adaptive feedback, does not develop in autism….

Under-developed long-distance and over-developed short-distance connectivity may profoundly alter the way the brain processes information. This altered connectivity hypothesis of autism fits well with cognitive theories like the "weak central coherence theory" or "weak executive functions theory" and may account for decreased contextual sensory and social processing in favour of detail-driven and fragmented processing, as well as poor goal-directed behaviour and planning skills. Reduced top-down feedback from higher order associative cortices as observed in many functional neuroimaging studies (see next chapter) further supports this hypothesis. However, it should be noted that up to date this has been merely a hypothesis, with no direct evidence. The very first evidence comes again from studies with

VPA-treated rats. Pair-wise electrophysiological recordings between neurons revealed that the number of connections was increased by more than 50%. This hyper-connectivity was found only for neurons closely spaced to each other, typically within the dimensions of a minicolumn (~50 μm somatic distance), but not between minicolumns (100-200 μm apart). It was suggested that this pattern of very local hyper-connectivity may provoke more autonomous and isolated activity, which is more difficult to command (Rinaldi et al., 2006) – specially if the hypothesis of weak long-distance connections proves to be true.

In summary, on the microscopic level, the most common observation is increased cell packing in various brain regions. Moreover, the number of minicolumns, the most basic processing units of the neocortex, was found to be increased in the frontal and temporal lobes.

3.3.3. Functional alterations

Advances made with functional neuro-imaging techniques, such as positron emission tomography (PET), single-photon emission computed tomography (SPECT), magnetic resonance spectroscopy (MRS), and functional MRI (fMRI), all contributed greatly to the understanding of the autistic brain. The most pronounced finding was that activity in higher order associative cortices, the frontal and temporal regions, as well as in the cerebellum is reduced, whereas activity in lower order visual regions is normal or even slightly increased (Frith, 2003; Belmonte et al., 2004; Courchesne et al., 2005). These findings suggest a lack of integration of sensory information with cognitive evaluation and reduced top-down feed-back. As discussed above these findings may be due to altered connectivity, in particular poor long-range connections necessary for proper integration and modulation of information in higher brain areas.

Courchesne et al. (2005) summarized the functional neuroimaging data and report in their review that reduced activation of the frontal cortex was observed in a theory of mind task (Castelli et al., 2002), in response to socially familiar faces (Pierce et al., 2004), in face recognition (Hubl et al., 2003), in a working memory task (Luna et al., 2002), in an embedded figures task (Ring et al., 1999), in visual spatial attention tasks (Belmonte and Yurgelun-Todd, 2003) and during sentence comprehension (Muller et al., 1998; Just et al., 2004). Additionally, EEG studies consistently found reduced or absent electrical responses from the frontal cortex in several auditory and visual attention and orienting tasks (Courchesne et al., 1984; Ciesielski et al., 1990; Townsend et al., 1999).

Reduced activation of the temporal lobes was found during processing of vocal sounds (Gervais et al., 2004), speech sounds (Muller et al., 1999; Boddaert et al., 2003), face recognition (Schultz et al., 2000; Pierce et al., 2001; Pierce et al., 2004), evaluation of facial expressions (Critchley et al., 2000) and theory of mind tasks (Castelli et al., 2002).

In striking contrast to the hypoactivation of the frontal and temporal lobes stand normal or even hyper-activation of the occipital lobe in response to visual stimulation (Ring et al., 1999; Belmonte and Yurgelun-Todd, 2003; Hubl et al., 2003; Hadjikhani et al., 2004).

Functional connectivity studies revealed reduced functional connectivity between occipital and frontal or temporal lobes (Castelli et al., 2002), superior temporal to inferior frontal lobes (Just et al., 2004) and parietal to frontal lobes (Horwitz et al., 1988), thus confirming the reduced long-distance connectivity hypothesis.

In the cerebellum, reduced and normal to increased activity has been observed depending on the task type. Reduced activation was reported in attention tasks (Allen and Courchesne, 2003), speech recognition and generation (Muller et al., 1998; Muller et al.,

1999) and judgement of facial expressions. Normal to increased activation was observed during motor tasks (Muller et al., 2001; Allen and Courchesne, 2003; Allen et al., 2004).

Taken together, in the autistic brain higher order areas seem to be not fully activated and furthermore disconnected from lower order sensory areas during task processing. Lower order sensory areas seem to function normally or can even be hyper-activated (which would be in accordance with hyper-sensitivity to sensory stimulation). As a consequence, information from one area might not be integrated with information from another area, thus leaving the autistic person in a world with bits of isolated information that may seem chaotic and confusing. Striking support for this assumption comes from a fMRI study on face perception. In normal subjects faces activate the fusiform face area in the fusiform gyrus with 100% accuracy (Schultz et al., 2003). In autistic subjects, these regions exhibited abnormally weak or no activation at all. However, all autistic subjects were able to judge the gender of the face, indicating normal perceptual levels. Strikingly, the activation pattern evoked by the faces was distributed over several cortical regions (e.g. frontal, primary visual, cerebellum, etc.) and from subject to subject different. This suggests that autistic individuals "see" faces with different distributed neural system and each patient with a unique neural circuitry (Pierce et al., 2001). This finding may provide support for the altered wiring in autistic brains.

3.4. The amygdala theory of autism

The amygdala theory of autism has several deputies (Baron-Cohen et al., 2000; Sweeten et al., 2002; Amaral et al., 2003; Bachevalier and Loveland, 2005; Schultz, 2005). All of them emphasize the amygdalas' role in the regulation of social-emotional behaviours and all are based on the "social brain" theory suggested by Brothers (1990), which assigns the amygdala, the orbito-frontal cortex (OFC) and the superior temporal sulcus and gyrus (STG) a specific role in social intelligence. It is argued that since these regions underlie socio-emotional behaviour, they are also the most likely to be disturbed in autism, which is mainly an impaired social interaction syndrome. The particular interest for the amygdala in autism rose, because studies in non-human primates had shown that lesions of the amygdala provoked many socio-emotional impairments comparable to autistic symptoms (see below Chapter 3.4.3. Lesion studies in primates), which led Bachevalier (1994) to propose that amygdala lesions in non-human primates might be a suitable model to study autism. The term "amygdala theory of autism" was introduced by Baron-Cohen et al. (2000) after they set out to test the social brain hypothesis of Brothers (1990). The task involved judging from other peoples' eyes what the other person might be feeling or thinking. With fMRI they showed that indeed in normal subjects all the postulated social brain areas were activated. Autistic patients, on the other hand, activated orbito-frontal and temporal regions, but failed to activate the amygdala, thus giving rise to the term "amygdala theory of autism" (Baron-Cohen et al., 1999; Baron-Cohen et al., 2000).

It was argued that early developmental insults to the amygdala might have severe consequences for the subsequent development of social networks elsewhere in the brain (Bachevalier and Loveland, 2005; Schultz, 2005). The amygdala guides attention to socially and emotionally relevant stimuli, such as faces, and has been extensively linked to the processing of emotionally salient stimuli, including facial expressions (Breiter et al., 1996; Morris et al., 1996; Morris et al., 1998c; Morris et al., 1998a; Calder et al., 2001; Zald, 2003). A modulatory role of the amygdala on other brain regions when emotionally salient stimuli are present has long been postulated (see first chapter). A brain area consistently active when viewing faces is the fusiform face area (FFA) in the fusiform gyrus (FG), and it has been argued that the amygdala might amplify face perception in this area when emotions are expressed. Indeed Schultz (2005) showed that activity in the FFA is enhanced when subjects viewed emotional in contrast to neutral faces. This view is supported by a fMRI study on 26 patients with varying degrees of lesions in the amygdala, hippocampus or both and 13 matched normal subjects. Subjects were presented fearful or neutral faces. All subjects exhibit FFA and occipital lobe activation when viewing faces; healthy and subjects with hippocampus lesions also exhibited enhanced activity in those areas when viewing fearful faces. The amygdala lesioned patients, on the other hand, did not show this amplification effect when viewing fear expressing faces (Vuilleumier et al., 2004). These data provide evidence for the amplification function of the amygdala in the fusiform face area when emotionally salient stimuli are present. It implies that a functional failure in this system might severely disrupt the emotional evaluation of a social situation. This can be devastating if the failure occurred early in life. The preference for faces typically shown by newborn infants has been postulated to be mediated by a subcortical visual system that passes information from the retina to the superior colliculus, the pulvinar nucleus of the thalamus, and from there to the amygdala (Pasley et al., 2004). Schultz (2005) proposed that an insult to this system, and maybe even to the amygdala alone, may profoundly interfere with the development of socio-emotional behaviours. For example, a dysfunctional amygdala in early childhood may be responsible for the diminished attention to faces as observed in autistic infants as early as the first 6 month of life (Maestro et al., 2002). The failure to attend facial stimuli and assign

emotional value to social stimuli may then trigger a whole cascade of abnormal social development. Autistic individuals may never acquire normal face perception expertise, as indicated in several studies by a failure to activate the FFA (Critchley et al., 2000; Schultz et al., 2000; Pierce et al., 2001; Hall et al., 2003; Hubl et al., 2003; Piggot et al., 2004; Wang et al., 2004). A failure to perceive faces properly is a prerequisite in learning to interpret social signs expressed in facial expressions. The correct interpretation of facial expressions (i.e., Is the person friendly or angry towards me?) is certainly crucial for a successful navigation through the social world (see figure 13 for more details on this model).

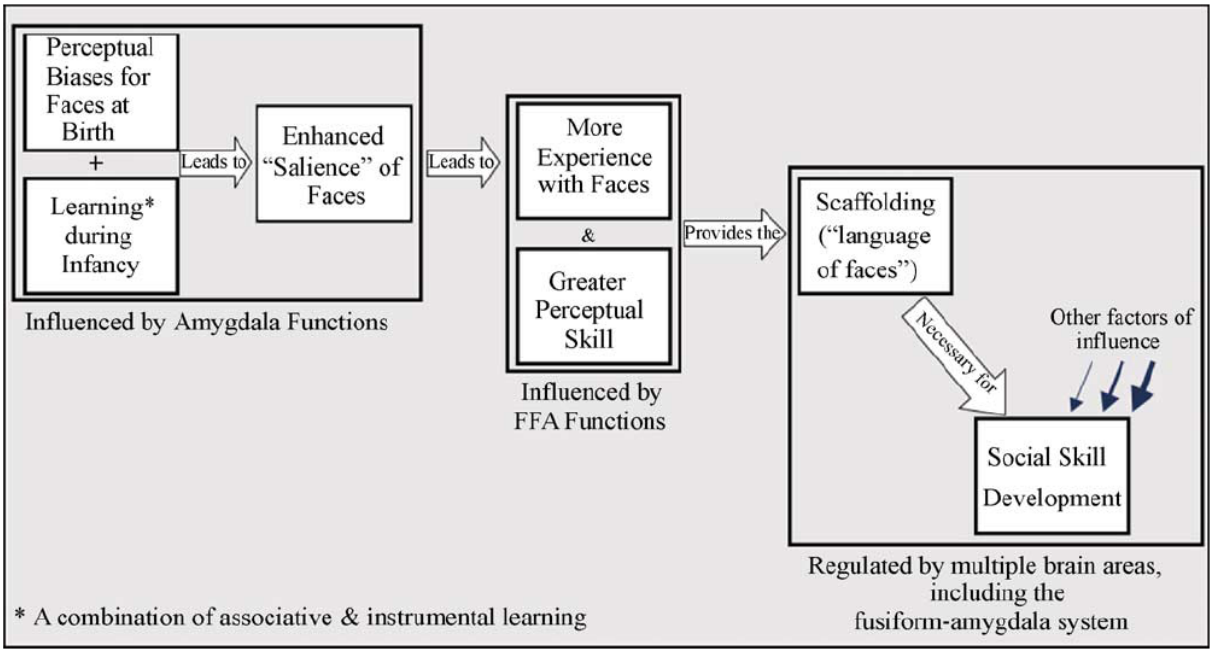

Figure 13. Model of the relationship between the development of face perceptual skills and social skills. The amygdala is hypothesized to have a crucial role in early development by guiding attention towards faces and evoking a perceptual bias for faces, which leads to enhanced salience for faces. Thus, expertise in the perception of faces is acquired, which leads to consistent activation of the fusiform face area, the face recognition and expertise area of the brain. Enhanced perceptual skills provide the basis for correct interpretation of facial expressions, most critical in the development of advanced social skills and for navigation through a social world. The function of the amygdala in this system is to signal other brain areas emotionally relevant stimuli and thus the salience of an event. A functional failure of the amygdala early in development may be deteriorating for social skill development in other brain areas (e.g. advanced interpretations of facial expression mediated by the superior temporal gyrus). Adapted form (Schultz, 2005).

Whereas Schultz (2005) proposes that autism might be caused by disruption of amygdala function alone early in childhood or even during embryogenesis, Bachevalier and Loveland (2005) argue that disruption of essentially two structures, the amygdala and/or the orbitofrontal cortex during early development may lead to a dysfunctional regulation of socio-emotional behaviours and thus autism. While a dysfunction of the amygdala might yield problems in detecting information relevant to the mental states, emotions and intentions of other people and their relevance to the self, a dysfunction of the orbitofrontal cortex would rather result in difficulty in modifying one's own behaviour appropriately in response to the social environment. Therefore an early insult to either of the two structures will have different developmental outcomes, which could account for the great heterogeneity observed in autism. It might also account for the different time courses of the disorder onset. For example, some children with autism exhibit symptoms, which can be recognized as early as the first year of life, such as deviant eye gaze, poor interest in faces and other people, less pointing to objects or orienting towards a person calling their name (Osterling and Dawson, 1994; Maestro et al.,

2002). Other children seem to initially develop normally, are attentive, display affect towards their parents and siblings, but suddenly around the second to third year of life regress in their development, lose acquired language skills, become remote, lose interest in others, develop unexpressive faces and fall into the autistic world. Bachevalier and Love (2005) reason that these different onsets of autism might be due to the differential time course of dysfunctional development of either the amygdala or the orbitofrontal cortex. The amygdala seems functional at birth (Kling, 1966; Humphrey, 1968; Kordower et al., 1992), whereas the prefrontal cortex is immature at birth and develops gradually over the postnatal period. For example, pyramidal cell dendritic arbors in layer 3 of the prefrontal cortex are only 3% of full mature size at birth, only 48% at 2 years of age and still need many more years until mature levels are reached (Courchesne et al., 2005). Likewise, functions that are mediated by the prefrontal cortex do not show within the first years of life and only emerge simultaneously with maturation of the prefrontal cortex. Thus, in autism abnormalities in social behaviour present at birth might be mediated by a dysfunctional amygdala, whereas abnormalities with a later onset might be related to a dysfunction of the orbitofrontal cortex, which would only show around the second year of life, when the prefrontal cortex starts to function.

Regardless of whether an early insult to the amygdala alone and/or to the orbitofrontal cortex might be the initial step towards impaired socio-emotional development and thus autism – and even regardless whether these theories are really true – autism is likely to be a function of many abnormally functioning brain regions. Both theories presented above have in common to postulate severe consequences on other closely interconnected brain regions, likewise involved in the processing of social information, such as the cingulate cortex, the superior temporal sulcus and the fusiform gyrus.

The following chapters review the many supporting evidence for the amygdala theory of autism (which made this theory so popular) stemming from structural neuroimaging data of autistic patients, post-mortem studies, lesion studies in non-human primates, similarities between amygdala-lesioned human patients and autistic people, and finally functional neuroimaging studies, all of which are reviewed below. Subsequently, an alternative role for the amygdala in autism than the one presented thus far will be introduced.

3.4.1. Structural alterations

Results from magnet resonance imaging (MRI) studies comparing autistic subjects with age, gender and IQ-matched subjects yielded a complicated and not always consistent picture about structural changes in the amygdala (summarized in table 5). While two studies indicated an increase in amygdala volume in older subjects (aged over 15 years; Abell et al., 1999; Howard et al., 2000), another two studies reported reduced amygdala volume in autistic patients (aged over 11 years; Aylward et al., 1999; Pierce et al., 2001). While above studies focused on older subjects, two studies explicitly turned to young autistic children (<12.5 years old) and found increased amygdala volumes (Sparks et al., 2002; Schumann et al., 2004). Other MRI studies looked at subjects with mean ages ranging from 7 to 41.4 years and failed to find any abnormalities in medial temporal lobe structures including the amygdala (Nowell et al., 1990; Courchesne et al., 1993; Haznedar et al., 2000; Schumann et al., 2004; Dziobek et al., 2006; Palmen et al., 2006).

It becomes apparent that there are high discrepancies in MRI results. These may be due to the high variability regarding the severity of psychopathology and the inclusion of patients from all age groups in the studies. Schuman et al., 2004 distinguish in their study between young (7.5 – 12.5 years) and older kids (12.75 – 18.5 years) and observe increased

Table 5. Structural Neuroimaging Studies Implicating the Amygdala and Other Brain Structures in Autism.

Study	Tested Group	Age	Structure	Volume
Schumann et al., 2004	53 Autists	7.5-12.5	Amygdala	Increased
Sparks et al., 2002	45 Autists	3-4	Amygdala	Increased
Howard et al., 2000	10 high functioning autists or Aspergers	15-40	Amygdala	Increased
Abell et al., 1999	15 high functioning Autists	Avg: 28	Amygdala / periamygdaloid complex	Increased
Aylward et al., 1999	14 non-mentally retarded autistic males	11-37 Avg: 21	Amygdala	Decreased
Pierce et al., 2001	7 autistic males	21-41	Amygdala	Decreased
Dziobek et al., 2006	17 Aspergers	41.4	Amygdala	No difference
Haznedar et al., 2000	10 Autists 7 Aspergers	Avg: 28	Amygdala	No difference (however, subjects with Asperger's had greater left amygdala volume than subjects with autism)
Palmen et al., 2006	42autistic or Asperger subjects	7-25	Amygdala	No difference
Palmen et al., 2006	42autistic or Asperger subjects	7-25	Hippocampus	Increased
Palmen et al., 2006	42autistic or Asperger subjects	7-25	Whole brain	Increased
Nowell et al., 1990	53 Autists	2-22 avg: 9	Amygdala and other limbic structures	No difference
Schumann et al., 2004	53 Autists	12.75-18.5	Amygdala	No difference
Howard et al., 2000	10 high functioning or Aspergers		Hippocampus Parahippocampus	Decreased (marginally)
Schumann et al., 2004	53 Autists	7.5-18.5	Hippocampus	Increased
Aylward et al., 1999	14 non-mentally retarded autistic males	11-37	Hippocampus	No difference
Dziobek et al., 2006	17 Aspergers	41.4	Hippocampus	No difference
Haznedar et al., 2000	10 Autists 7 Aspergers	Avg: 28	Hippocampus	No difference
Courchesne et al., 1993	21 Autists	6-32	Limbic structures	No difference
Courchesne et al., 1993	21 Autists	6-32	Temporal lobes	No difference
Abell et al., 1999	15 high functioning Autists	Avg: 28	Left middle temporal gyrus	Increased
Abell et al., 1999	15 high functioning Autists	Avg: 28	Left occipito-temporal cortex	Decreased
Abell et al., 1999	15 high functioning Autists	Avg: 28	Right inferior temporal gyrus	Increased
Courchesne et al., 1993	21 Autists	6-32	Frontal lobes	No difference
Abell et al., 1999	15 high functioning Autists	Avg: 28	Left inferior frontal sulcus	Decreased
Abell et al., 1999	15 high functioning Autists	Avg: 28	Right paracingulate sulcus	Decreased
Courchesne et al., 1993	21 Autists	6-32	Parietal lobes	Decreased
Courchesne et al., 1993	21 Autists	6-32	Basal ganglia	No difference
Courchesne et al., 1993	21 Autists	6-32	Diencephalon	No difference
Courchesne et al., 1993	21 Autists	6-32	Brain stem	No difference
Sparks et al., 2002	45 Autists	3-4	Cerebellum	Increased
Courchesne et al., 2001	60 autistc boys	2-16	Whole brain	Increased (2-3 years) No difference at older ages
Sparks et al., 2002	45 Autists	3-4	Whole brain	Increased
Aylward et al., 1999	14 non-mentally retarded autistic males	11-37	Whole brain	No difference
Dziobek et al., 2006	17 Aspergers	41.4	Whole brain	No difference

amygdala volumes only in the younger kids with autism as compared to controls, but no differences in the older groups. These findings suggest an abnormal amygdala development, in which the amygdala of autistic children reaches adult size before adolescence, whereas the amygdala of typically developing children undergoes a progressive growth throughout adolescence. This abnormal pattern of amygdala development resembles closely that encountered in the overall brain volume (see chapter 3.3.1). Thus, taking into account age differences may help to explain the inconsistent results. Furthermore, the data implicate that at least some subpopulations of autistic subjects exhibit a pathological amygdala.

3.4.2. Post-mortem microscopic alterations

In the largest *post-mortem* study conducted to date several abnormalities were found in the amygdala. These consisted of unusually small neurons and increased cell packing and were most pronounced in the cortical, medial and central nuclei, whereas the lateral nucleus was unaffected in 8 out of 9 brains. The one exception to this pattern was observed in a 12-year old autistic boy, whose amygdala was diffusely abnormal (Kemper and Bauman, 1998). Bailey and colleagues investigated 7 autistic brains and found increased neural density in the amygdala in one of them (Bailey et al., 1998). However, there are also a number of studies where no changes in the amygdala and related structures were reported (Williams et al., 1980; Coleman et al., 1985; Guerin et al., 1996; Rodier et al., 1996).

3.4.3. Lesion studies in primates

Much of the interest in the involvement of the amygdala in autism stems from lesions studies with non-human primates, which investigate the contribution of the amygdala to socio-emotional behaviours. Indeed lesioning the medial temporal lobe and specifically the amygdala was the first proposed animal model of autism (Bachevalier, 1994).

The interest in the amygdala in social behaviours originates in the afore mentioned Klüver-Bucy syndrome (see chapter 1), which can be evoked by bilateral damage to the amygdala (Rosvold et al., 1954; Schreiner and Kling, 1956; Weiskrantz, 1956; Aggleton and Passingham, 1981; Zola-Morgan et al., 1991) or the inferior temporal cortex (Horel et al., 1975). The first, now classical study, to show the involvement of the amygdala in social behaviour was conducted by Rosvold et al., 1954. Here the amygdala of the most dominant members of a group of eight male adult rhesus monkeys was bilaterally lesioned. Two of the three formerly dominant monkeys developed submissive behaviour and fell to the bottom of the hierarchy. Kling and colleagues further examined the involvement of the amygdala in social behaviour in a variety of non-human primates under both laboratory and wild-life conditions (Kling and Brothers, 1992). The cumulated results from these studies indicated that bilateral amygdala lesions disrupt species-specific social behaviour and lead to social isolation. For example, a group of vervets with bilateral amygdala lesions was studied in the wild. These animals were unresponsive to their group, failed to display appropriate social signals, withdrew from social interactions and were frequently killed by other members of the group (Kling et al., 1970). Studies by Thompson and colleagues also showed that bilateral amygdala lesions shortly after birth or in adulthood lead to persisting social disturbances and reduced fear of aggressive peers (Thompson and Towfighi, 1976; Thompson et al., 1977). These early lesion studies led Brothers (1990) to propose that the amygdala is essential in forming part of the brain network underlying social behaviour.

The striking similarities in behaviour between temporal lobe lesions in monkeys and human autism led to the theory that dysfunction in the medial temporal lobe and most probably in the amygdala may underlie autistic behaviours (Bachevalier, 1994). Consequently lesioning the amygdala and related structures was suggested as an animal model of autism (Bachevalier, 1994). Several studies by the group of Jocelyn Bachevalier were conducted under the umbrella of this theory. Newborn rhesus monkeys received medial temporal lobe lesions (including both, amygdala and hippocampus) and were raised with age-matched unoperated infant monkeys. The results showed that, at 2 months of age, the amygdala lesioned monkeys were more passive and irritable when confronted with a new situation and initiated less social interactions. At the age of 6 months the operated monkeys interacted even less with their peers, tried to actively avoid social contact, displayed blank, inexpressive faces, poor body expression, lack of eye contact and developed locomotor stereotypies and self-directed activities. Similar experiments lesioning just the amygdala yielded a very similar pattern of socio-emotional disturbances, but in an alleviated form. However, unlike monkeys with complete medial temporal lobe lesions, amygdala-lesioned monkeys did not display less acceptance of approach, stereotypic behaviours, or loss of facial and body expression, but were simply more passive (Bachevalier, 1994). Taking into account that impaired communication and social interaction are hallmark symptoms of autism, Bachevalier suggested that amygdala disturbances may underlie autisms and amygdala lesions may provide an animal model for studying autism.

Recently, the role of the amygdala as an essential part of the "social brain" has been questioned (Amaral et al., 2003; Amaral and Corbett, 2003). A study conducted on adult rhesus monkeys living in a semi-naturalistic cage environment found that amygdala lesioned monkeys were well capable to engage in social interactions and exhibited greater amounts of the latter, much to the contrary of above studies. The interactions were particularly increased in the early phase of the social encounter and the authors attributed this to a lack of fear or reluctance (Emery et al., 2001). Amaral points out that in the early studies almost all animals were reared without their mothers, which by itself may lead to profound effects on socio-emotional behaviour and may be confounded with the effect of amygdala lesions (Amaral et al., 2003). For this reason, two-week old amygdala lesioned monkeys were reared with their mothers and had daily access to a social group. Again, unlike in previous studies, maternally reared monkeys acquired the whole repertoire of social behaviours, including facial and body expressions and vocalizations. They were clearly attentive to the control peer, but surprisingly displayed increased fear in social interactions. This led the authors to propose that the amygdala might rather be involved in evaluating the danger in a social encounter rather than generating social behaviour *per se* (Amaral et al., 2003).

In summary, ablation of the temporal lobe and specifically the amygdala produced a pattern of impaired social communication and interaction. This led to the amygdala theory of autism, which states that an amygdala (and associated structures) dysfunction underlies the hallmark autistic symptoms of impaired social interactions and communication. However, recent findings with maternally reared monkeys question this theory, since amygdala lesioned neonates, who are returned to their mothers and exposed to sufficient social stimulation, do not display impaired social behaviour, but rather increased fear in the social encounter. Thus, for the moment it remains unclear whether the amygdala is indeed involved in the generation of social behaviour *per se* or rather in the correct evaluation of dangerous vs. benign signs in a social encounter.

3.4.4. Similarities between autism and patients following amygdalotomy

To answer the question whether an amygdala dysfunction underlies some of the symptoms observed in autism, it has to be addressed whether patients with amygdala lesions display similar impairments in social communication, interaction and increased stereotypic behaviour as observed in some of the monkey lesion studies.

A review by Sweeten et al., (2002) presents evidence that temporal lobe damage in humans has been associated with the development of autistic symptomotology and cites several case reports stating that children with severe temporal lobe damage due to viral encephalitis (DeLong et al., 1981; Gillberg, 1986), tumors (Hoon and Reiss, 1992; Taylor et al., 1999), tuberous sclerosis, where the presence of tubers was strongly related to temporal lobes (Gillberg et al., 1994; Bolton and Griffiths, 1997), or other causes (White and Rosenbloom, 1992; Deonna et al., 1993; DeLong and Heinz, 1997) have developed autistic symptoms.

However, patients with circumscribed bilateral amygdala lesions are rare. Amaral (2003) argues that patients with amygdala lesions do not exhibit the core autistic symptoms as required by the DMS-IV. He reports the case of patient S.M. who acquired a complete bilateral amygdala lesion due to Urbach-Wiethe disease during adolescence. This patient does not display any autistic symptoms, such as impaired communication or social interactions or stereotypic behaviours and is capable to lead a fairly normal life, since she obtained a high-school degree, is married, has kids and holds a job. However, she does exhibit more subtle deficits in recognizing facial expression (mostly fear) (Adolphs et al., 1994) and impaired conditioning (Bechara et al., 1995). Amaral also refers to the famous H.M. who had bilateral temporal lobotomy to erase the focal herd of his severe Grand Mal seizures. Even though H.M. suffered from severe amnesia ever after the surgery, he was not impaired in reciprocal social interactions nor did he display any other core autistic symptoms.

Thus, looking only at the core symptoms of autism bilateral damage to the amygdala does neither resemble the autistic symptomotology fully nor does it match characteristic socio-emotional disturbances observed after the early amygdala lesion studies in non-human primates. However, it cannot be excluded that a dysfunction of the amygdala does not contribute to more subtle features encountered in autism, such as the impaired recognition of certain emotions on facial expression or the knowledge about other people's mental states (Baron-Cohen et al., 1997; Baron-Cohen et al., 1999; Adolphs et al., 2001).

Adolphs and colleagues compared in a series of experiments brain-damaged, amygdala lesioned and control subjects in terms of their ability to recognize emotions conveyed through facial expressions (Adolphs et al., 1994; Adolphs et al., 1998; Adolphs et al., 1999; Adolphs et al., 2001; Adolphs et al., 2002; Adolphs, 2003; Adolphs and Tranel, 2003; Adolphs et al., 2005). They found that patients with either bilateral or unilateral damage to the amygdala exhibited impaired recognition of fear and some patients also of other negative emotions such as anger or disgust when compared to controls or other brain-damaged subjects. The recognition of happy emotions was never impaired (Adolphs et al., 1994; Adolphs et al., 1999; Adolphs and Tranel, 2003). These patients were also impaired when they had to judge the trustworthiness of faces or when they had to identify more complex social emotions from facial expressions or merely the eye region, such as arrogance, guilt, admiration or flirtatiousness (Adolphs et al., 1998; Adolphs et al., 2002). Moreover, it turned out that amygdala lesioned patients have deficits in gazing at the eyes of another person (Adolphs et al., 2005). This implies that they simply do not use the socio-emotional information conveyed through the eye region to read the other person's mental or emotional state. Furthermore, amygdala lesioned patients exhibit severe deficits in attributing mental

states to others particularly when they acquired the lesion early in life, but not during adulthood (Shaw et al., 2004).

In order to evaluate a possible contribution of the amygdala to autism, the same kind or similar experiments were conducted on autistic patients. Interestingly, most autistic patients displayed normal recognition of simple emotions including states of fear, but had severe problems in interpreting more complex social information such as judging the trustworthiness of others or interpreting the mental states of other people conveyed through both, the whole face or only the eyes (fig. 14) (Baron-Cohen et al., 1997; Adolphs et al., 2001). However, one autistic subject was also impaired when rating faces expressing fear, disgust, and surprise, a pattern of impairment resembling amygdala damage (Adolphs et al., 2001). Another study with high-functioning autistic subjects also observed a clear impairment in the recognition of fear and perception of eye-gaze direction (Howard et al., 2000), thus resembling the observations made in amygdala damaged subjects. However, in the latter study no other emotionally valenced stimuli were evaluated, making it difficult to conclude that autistic people have selective impairments in fear perception. Nethertheless, these results suggest that amygdala damaged and autistic people may share some common features.

In summary, although people with amygdala lesions are not autistic in the classical sense of the DMS-IV, they do display impairments also observed in autism, particularly inferring other people's state of mind, reading complex social cues from facial expressions and interpreting signals of threat and danger in faces. Therefore, some of the deficits observed in autism may be attributable in part to dysfunction in circuits including the amygdala.

3.4.5. Functional neuro-imaging studies

Up to date, three fMRI studies evaluated the involvement of the amygdala in autism focussing on face perception and evaluation of facial expressions. All of these studies reported a hypoactivation of the amygdala (Baron-Cohen et al., 1999; Critchley et al., 2000; Pierce et al., 2001; Schultz et al., 2003).

Pierce et al. (2001) evaluated basic face perception in 7 adult male autistic subjects, which had to judge the gender of non-emotional (neutral) faces. In contrast to many other studies, which report a 100% consistency in activating the fusiform face area (FFA) in the fusiform gyrus when normal subjects view faces (Haxby et al., 1994; Puce et al., 1995; Clark et al., 1996; Kanwisher, 2000), autistic subjects had an abnormally weak or no activation at all in the FFA. They also had reduced activity in the inferior occipital gyrus, superior temporal sulcus and amygdala. Thus, none of the regions usually supporting face perception was consistently activated in autistic subjects. On the other hand autistic subjects did not perform worse on the behavioural level. In the brain this performance was sustained by maximally activated aberrant and individual-specific neural sites, such as the frontal cortex, primary visual cortex and cerebellum. Thus it seems that in contrast to normal subjects, who exhibit clearly specialised face processing regions, autistic people perceive faces with unique and from individual to individual varying neural circuits.

Baron-Cohen et al. (1999) examined 6 subjects with autism on a test of judging from the eye expression what another person might be feeling or thinking (fig. 14). While normal subjects showed increased activity in the prefrontal cortex, superior temporal temporal gyrus and amygdala (the "social brain" as proposed by Brothers, 1990) on this task, autistic subjects did also activate fronto-temporal regions, but failed to activate the amygdala.

Critchley et al. (2000) investigated 9 autistic subjects, which had to distinguish between happy and angry faces under explicit (pressing a button indicating either happy/angry

or neutral face expression) or implicit (responding according to male-female) conditions. In contrast to controls, autistic subjects failed to activate the fusiform face area when explicitly judging facial expressions, and the left amygdala and left cerebellum when implicitly evaluating facial expressions.

In summary, fMRI data on autistic subjects indicate a hypo-activation of the amygdala in socially demanding task, such as the evaluation of facial expression or attributing mental states to other people.

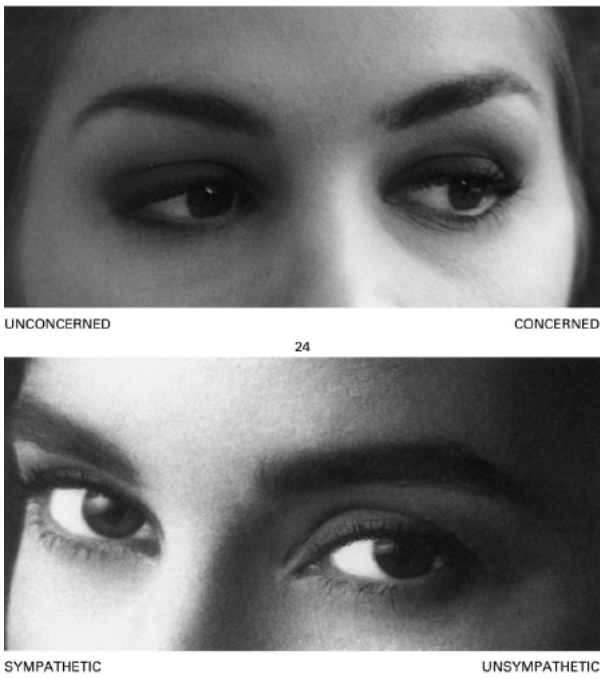

Figure 14. Attributing feelings and thoughts to other people's minds. Photographs of eyes were presented with a choice of mental state words (examples as shown). Top example: correct answer: Concerned; Bottom example: correct answer: Sympathetic. Autistic subjects were impaired on this task and failed to show an amygdala activation. Adopted from (Baron-Cohen et al., 2000).

3.5. An alternative amygdala theory of autism

Thus far, the current amygdala theory of autism emphasizes the role of the amygdala as an emotional salience detector, which tells other brain regions where the salience of an event lies, and thus has an important role in the regulation of socio-emotional behaviour and the affective evaluation of events. Even though the amygdalas' involvement in the regulation of socio-emotional behaviour seems indisputable, there might be an alternative view on how the amygdala contributes to the autistic pathology. Kanners' original case studies (1943) suggested that some of the autistic children he observed exhibited abnormal anxiety levels and fears. Some examples (Kanner, 1943):

> **Case 2**
>
> "Frederick W. was referred on May 27, 1942, at the age of 6 years, with the physician's complaint that his "adaptive behavior in a social setting is characterized by attacking as well as withdrawing behavior." …
>
> He is afraid of mechanical things; he runs from them. He used to be afraid of my eggbeater, is perfectly petrified of my vacuum cleaner. Elevators are simply a terrifying experience to him. He is afraid of spinning tops…."
>
> **Case 8**
>
> **"**Alfred L. was brought by his mother in November, 1935, at 3½ years of age with this complaint: He has gradually shown a marked tendency toward developing one special interest which will completely dominate his day's activities. He talks of little else while the interest exists, he frets when he is not able to indulge in it (by seeing it, coming in contact with it, drawing pictures of it), and it is difficult to get his attention because of his preoccupation.... there has also been the problem of an overattachment to the world of objects and failure to develop the usual amount of social awareness. …
>
> He is very fearful of being hurt, talks a great deal about the use of the electric chair. He is thrown into a panic when anyone accidentally covers his face. … He had many fears, almost always connected with mechanical noise (meat grinders, vacuum cleaners, streetcars, trains, etc.). Usually he winds up with an obsessed interest in the things he was afraid of. Now he is afraid of the shrillness of the dog's barking. …"
>
> **Case 11**
>
> **"**Elaine C. was brought by her parents on April 12, 1939, at the age of 7 years, 2 months, because of "unusual development": "She doesn't adjust. She stops at all abstractions. She doesn't understand other children's games, doesn't retain in stories read to her, wanders off and walks by herself, is especially fond of animals of all kinds, occasionally mimics them by walking on all fours and making strange noises." …
>
> She was "frightened" by noises and anything moving toward her. She was so afraid of the vacuum cleaner that she would not even go near the closet where it was kept, and when it was used, ran out into the garage, covering her ears with her hands…."

More recent studies have also suggested abnormally high anxiety levels and phobias (Muris et al., 1998; Gillott et al., 2001; Evans et al., 2005) in children with ASD and their relatives (Micali et al., 2004). For example, Muris et al. (1998) investigated 44 children diagnosed with ASD and found 84.1% of the children met the criteria for at least one anxiety disorder, such as simple phobia (28%), social phobia (9%), agoraphobia (20%), panic disorder (4%), separation anxiety disorder (12%), avoidant disorder (8%), overanxious disorder (10%) and obsessive-compulsive disorder (5%).

Anxiety, conditioned fear acquisition and recognition of fear expressed on faces have been strongly associated with the amygdala (Davis, 1992; LeDoux, 2003; Adolphs et al., 2005) as reviewed extensively in earlier chapters. Thus, it is conceivable that a dysfunctional amygdala contributes to the generation of increased anxiety and fears in autism. Amaral and

colleagues support this view (Amaral et al., 2003; Amaral and Corbett, 2003) based on two studies conducted in this group. First, contrary to all previous lesion studies, amygdala lesions in adult monkeys which were reared with their social group facilitated social behaviour rather than impairing it (Emery et al., 2001). Second, neonatal amygdala lesions in maternally reared monkeys failed to produce the expected pattern of social impairment as observed in neonatally lesioned monkeys, which were reared without their mothers in an impoverished social environment (Prather et al., 2001), suggesting that the supposedly lesion-mediated effects on social behaviour might be an artefact of abnormal up-bringing. Harlow's studies on monkeys reared in isolation and Spitz' studies on hospitalized children show that many symptoms observed in autism, such as impoverished social interaction, communication impairments and repetitive behaviours, may be caused by social and in particular maternal deprivation. Amaral goes as far as to question the involvement of the amygdala in social interaction (Amaral et al., 2003). In this thesis it is argued, that both amygdala theories of autism might complement, rather than exclude each other.

However, one apparent contradiction needs further consideration. Increased anxiety and fear would rather argue for a hyper-responsive amygdala in autism, as opposed to the hypo-responsive amygdala observed in fMRI studies in theory of mind tasks and face expression evaluation tasks. How can this apparent contradiction be explained? First, it is conceivable that socially relevant information processing might be mediated by other nuclei than the acquisition and storage of conditioned fear; that is the pairing between an aversive and a neutral stimulus. The latter is well studied anatomically and is mediated by the triad of lateral, basal and central nuclei. For the former it is less clear which nuclei participate, since most studies involving the amygdala in social behaviour, such as lesion and fMRI studies, refer to the amygdala as a whole and do not discriminate between nuclei. However, there is indirect evidence from rodent studies that differential nuclei might be involved in the mediation of social behaviours.

For example, it is long established that oxytocin plays an important role in social behaviours, such as processing of social cues, social recognition and social bonding (for review see Lim et al., 2005). Social recognition has been shown to be modulated by oxytocin in a dose-dependent way: low doses enhance social recognition memory whereas high doses have an impairing effect (Popik et al., 1992; Benelli et al., 1995). In order to establish the neural circuits underlying social recognition modulated by oxytocin, Fos activity was evaluated in oxytocin knockout mice and compared to wildtype mice. In wildtype mice the olfactory bulb, piriform cortex and medial amygdala were activated after a social encounter. Oxytocin knockout mice, on the other hand, also activated the olfactory bulb and the piriform cortex, but failed to activate the medial amygdala (Ferguson et al., 2001). Instead, knockout mice showed a massive induction of Fos in the somatosensory cortex and hippocampus, regions not activated in wildtypes. Furthermore, bilateral pre-testing injections of oxytocin into the medial amygdala of oxytocin knockout mice could rescue the social recognition deficit observed in these mice. These data suggest that oxytocin acts on the medial amygdala during a social encounter for the normal processing of social information and subsequent social recognition (Ferguson et al., 2001). To my knowledge, this study is the first to involve a specific amygdaloid nucleus in the processing of social information and it suggests that the medial amygdala might be particularly involved. Thus, it is conceivable that distinct amygdaloid nuclei mediate social information versus fear-related information. Consequently, it is also feasible to argue that a hypo-activation observed in fMRI studies in the evaluation of facial expressions and "theory of mind" tasks are due to a hypo-activation of the medial nucleus, whereas lateral, basal and/or central nuclei could be still hyper-responsive when activated with the right danger signalling triggers.

A second possibility might be that the amygdala in autistic individuals is chronically hyper-active leading to elevated baseline levels. Functional MRI measures the relative increase or decrease of cerebral blood flow in reference to baseline levels. If the amygdala of autistic people exhibits high baseline levels, the margin of how much a task could further activate it would be much lower than in normal people. Thus, activity evoked after task onset would appear low, because baseline levels are already high, whereas with moderate baseline levels event-evoked activity could be much higher. Therefore, hypo-activation observed after task onset might rather be due to increased baseline levels than to a response failure and what seems hypo-active could in reality be hyper-active.

In summary, in this thesis it is suggested that the amygdala might contribute to the autistic pathology in a different way than usually conceived. Rather than solely mediating the social deficits observed in autistic individuals, dysfunctional and excessive processing in the amygdala might be causal for the enhanced anxiety and fear so often reported in the autistic population. Further, it is suggested that enhanced fear and anxiety levels might underlie some of the core symptoms observed in autism, such as impaired social interactions. A person daunted by fears will normally not tend to interact with other people and will not dare to explore new situations and environments equally to a normal person. A recent study screening for autism-like symptoms in children with mood and anxiety disorders found that up to 62% of these kids fall into the autistic spectrum and might qualify for a possible ASD diagnosis (Towbin et al., 2005). Thus, increased fear processing might cause some of the autistic impairments in social and non-social situations.

3.6. Valproic acid and autism

Valproic acid (VPA) is a chemical compound whose mechanisms of therapeutic actions are not well understood. It may act by increasing GABA levels in the brain or by altering the properties of voltage-dependent sodium channels. Clinically, VPA was first introduced in 1964 in France as an anticonvulsant and later mood-stabilizing drug, primarily in the treatment of epilepsy and bipolar disorder; but also to treat migraine headaches and schizophrenia. In epileptics, VPA is used to control absence seizures, tonic-clonic seizures (grand mal), complex partial seizures, and the seizures associated with Lennox-Gastaut syndrome. Related drugs include the sodium salt – Sodium valproate, and a combined formulation – Valproate semisodium. VPA can cross the placenta or even into the breast milk. The most common defect observed after prenatal exposure to VPA is a neural tube defect, spina bifida, estimated to occur in 1% of pregnancies in which VPA was taken (Bjerkedal et al., 1982). Exposure to VPA may also lead to the fetal valproate syndrome, which is characterized by dysmorphic facial features (epicanthal folds, broad nasal bridge, short nose with antiverted nares, long upper lip, flattened philtrum, thin upper vermillion border, downturned mouth and low set posteriorly rotated ears; see fig. 15), hypospadias, strabismus, nystagistmus, low birth weight, and psychomotor delay (DiLiberti et al., 1984; Jager-Roman et al., 1986; Ardinger et al., 1988; Kozma, 2001).

3.6.1. Studies implicating VPA in autism

The first indications for VPA to cause autism stem from 7 case studies of kids with Fetal Valproate Syndrome (Christianson et al., 1994; Williams and Hersh, 1997; Williams et al., 2001), of which all exhibited a full diagnosis of autism. Moore and colleagues conducted a study on 57 children with various Fetal Anticonvulsant Syndromes (caused by a variety of anticonvulsant drugs) in Scotland (Moore et al., 2000). These children had all been exposed to either VPA alone (60%), VPA in combination with another anticonvulsant drug (21%) or another anticonvulsant drug (Carbamezepine or Phenytoin) alone or in combination with each other (19%). Shockingly, they reported 46 (81%) kids with speech delays and 34 (60%) kids with two or more autistic features, of whom 6 (11%) had a diagnosis of ASD. Furthermore, 46 (81%) had behavioural problems, 22 (39%) displayed hyper-activity or poor concentration, of whom 4 (7%) had a diagnosis of attention deficit/hyper-activity disorder. Forty-four (77%) kids had learning difficulties, 34 (60%) had gross motor delay, and 24 (42%) had fine motor delay. These finding confirmed the association between Fetal Valproate Syndrome and autism as suggested in the prior case reports. It furthermore suggested that behavioural problems might not be only associated with VPA but also other anticonvulsant drugs.

3.6.2. VPA, thalidomide and the early brain stem injury hypothesis

The malformations caused by valproic acid and another autism causing teratogen, thalidomide, indicate an early insult during embryogenesis and, more specifically, around the time of neural tube closure (Kozma, 2001; Arndt et al., 2005), which led to the hypothesis that autism may be caused by a brain stem injury during embryonic development (Stromland et al., 1994; Arndt et al., 2005).

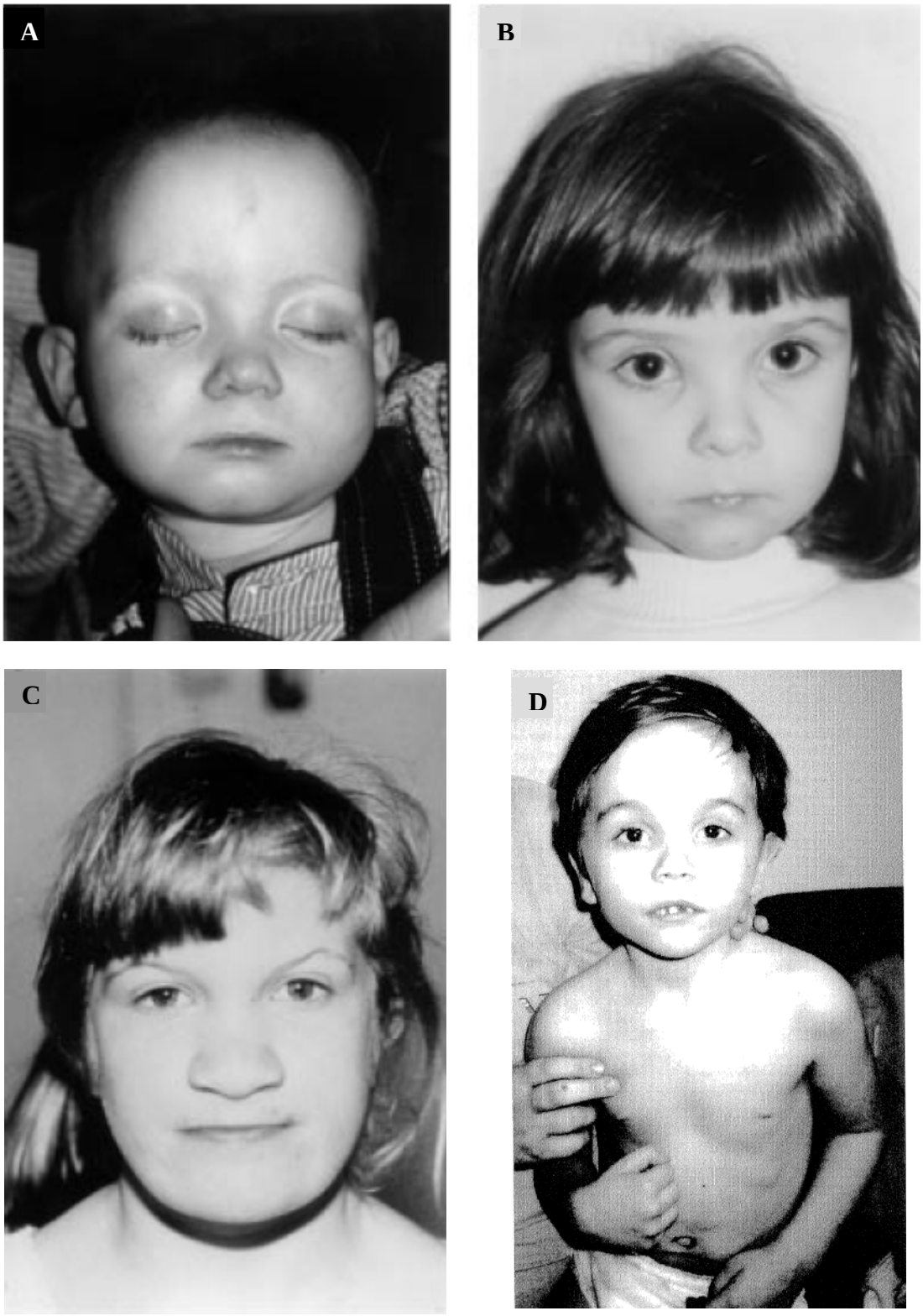

Figure 15. Children exposed to valproic acid. (A) a boy aged 22 months. Note epicanthic folds, infraorbital grooves, long, shallow philtrum, and thin upper lip. (B) A girl aged 3 years, 10 months, older sister of (A). Note medial deficiency of eyebrows, infraorbital grooves, short nose with antiverted nares, long, shallow philtrum, and thin upper lip. (C) A girl aged 11 years and 4 months. Note thin eyebrows, medial deficiency of eyebrows, flattened nasal tip, shallow philtrum, and thin upper lip. Adopted from (Moore et al., 2000). (D) Boy aged 5 years, 6 months. Note the facial anomalies and minor limb defects. Adopted from (Williams and Hersh, 1997).

First indications for the development of this hypothesis stem form a Swedish thalidomide study (Stromland et al., 1994). In this study 87 patients were included with the initial purpose to evaluated possible ophthalmologic effects, but a psychiatric evaluation was also performed. Five cases with autism were found in the thalidomide study. All these cases were from a group of 15 patients, of which it was known that the thalidomide exposure occurred between the 20th to 24th days of gestation. This indicated that the rate of autism after thalidomide exposure during this time period was extremely high, namely 1:3. No autistic cases were reported at any other exposure time. Since autism occurred only during this time period the brain areas which could have been directly affected were very restricted, because at this time point only few brain structures are present. During days 20 to 24 of gestation the neural tube closes and the first neurons are produced. These neurons are part of the motor nuclei of the cranial nerves. Thus, an injury to these neurons would come along with abnormalities in facial features, which was indeed observed in all of the five autistic thalidomide cases. Three patients had Duane syndrome (failure of the VIth/abducens cranial nerve to innervate the lateral rectus muscle by the eye with subsequent reinnervation of the muscle by the IIIrd/oculomotor cranial nerve); one patient had face paresis (oculomotor palsy); four had Möbius syndrome (failure of the VIIth/facial cranial nerve to innervate the facial muscles); two had abnormal lacrimation (due to a failure of the neurons of the superior salivatory nucleus (cranial nerve VII) to innervate the lacrimal apparatus). All 5 patients had ear malformations and hearing deficits. Ear malformations (Walker, 1977), eye motility problems (Scharre and Creedon, 1992) and Möbius syndrome (Gillberg and Steffenburg, 1989) had previously been associated with autism. In fact, external ear malformation is the most common physical abnormality observed in autism and the one which best distinguishes between autism and mental retardation (Walker, 1977). The new conclusion made in the thalidomide study was then to link all these symptoms to a brainstem injury at a very specific time during embryogenesis and to suggest, that the same brainstem injury can cause not only cranial nerve symptoms, but also autism.

How are the thalidomide cases related to autism associated with VPA? Some of the teratogentic effects of VPA resemble those of thalidomide. These include the above mentioned neural tube closure defects associated with cranial nerve injuries, such as facial dysmorphy and ear abnormalities. Even though VPA, as a remedy for epilepsy, is usually taken throughout the whole pregnancy, the time point of injury can be deduced on the basis of these physical malformations. Since these are very similar to the thalidomide-induced autistic cases and the exact time period for thalidomide to cause autism is known to be between embryonic days 20-24 (Stromland et al., 1994), it has been argued that the time point of VPA to cause autism has to be the same one (Rodier et al., 1996; Rodier et al., 1997).

The early brain stem hypothesis of autism states that all other brain defects observed in autism (see earlier chapters) must be a consequence of this one early brainstem injury, since at the time of insult no other brain regions are yet developed. Thus, one can imagine the early insult to the brain stem in terms of a big-bang that may alter the development of the nervous system and lead to malformation of other brain structures (Rodier et al., 1996).

Evidence for the hypothesis that autism is initiated at the time when cranial nerve motor nuclei are forming was then gathered on two fronts: microstructural examinations of brainstems of autopsied autistic brains and of rodents exposed to VPA during during neural tube closure.

3.6.3. Postmortem evidence for the early brain stem injury hypothesis of autism

The brain of an autistic person, never in touch with thalidomide or VPA, was examined for brain stem injuries and compared to a healthy brain (Rodier et al., 1996). It turned out that the brain stem of the autistic brain was indeed abnormal (fig. 16). Most striking was a loss of motor neurons in the facial nucleus. Whereas the facial nucleus in the healthy brain contained more than 9000 neurons, in the autistic brain only 400 neurons were present in this area. The superior olive, an auditory relay nucleus, was also missing completely. These results indicated that indeed brain stem injuries do occur in autism.

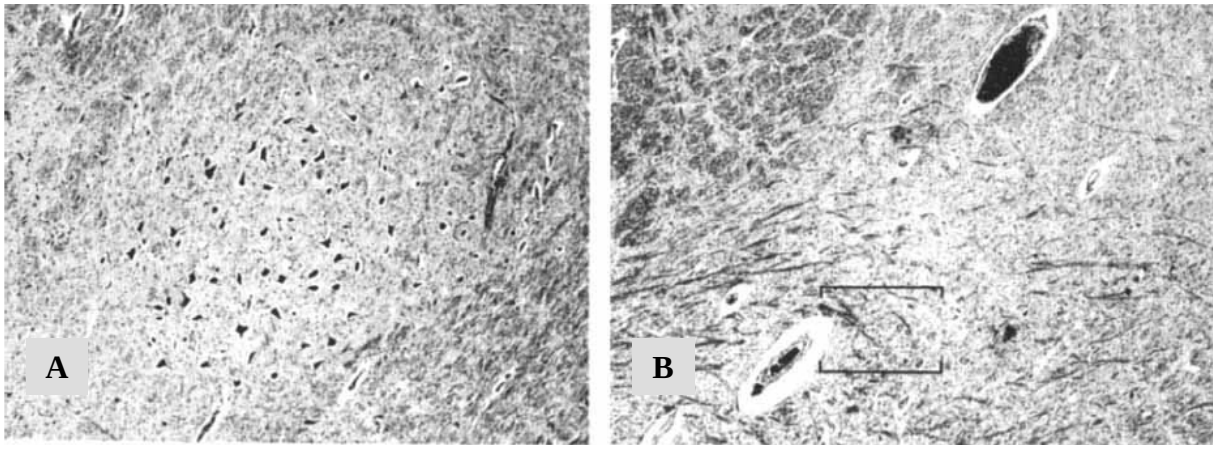

Figure 16. Loss of cranial nerve motor neurons in autism. These photographs contrast the facial nucleus of a control (A) and an austistic subject (B). The facial nucleus of the control is distinguished by the absence of myelinated fibers and by the presence of motor neurons. In contrast, the region in the autistic case not only lacks motor neurons but has many fibers passing in all directions. This suggests that the motor neurons were lost before the architecture of the region was established. Adopted from Rodier et al., 1996.

3.6.4. Development of the VPA rodent model of autism

In order to prove the hypothesis that an early brain stem injury may provoke the same pattern of overall brain anomalies as observed in autism, an animal model was developed by Patricia Rodier in 1996. VPA was the drug of choice, since thalidomide has different effects in rodents than in humans (Schumacher et al., 1972). VPA, on the other hand, is a powerful teratogen in rodents and produces many of the same external malformations as observed in humans (Binkerd et al., 1988; Collins et al., 1991; Ehlers et al., 1992). The time of neural tube closure in the rat occurs on day 11.5 and within the 12^{th} day of gestation, production of the motor nuclei of trigeminal, abducens, and hypoglossal nerves is completed (Altman and Bayer, 1980). Thus, a single dose of VPA (350 mg/kg) was administered to the pregnant dam on days 11.5, 12 and 12.5 post-conception. VPA administered on day 11.5 resulted in a reduction of the trigeminal and hypoglossal motor nuclei. Administration on day 12 caused an additional loss of neurons in the abducens nucleus (fig. 17) and on day 12.5 in all previous and additionally in the oculomotor nucleus.

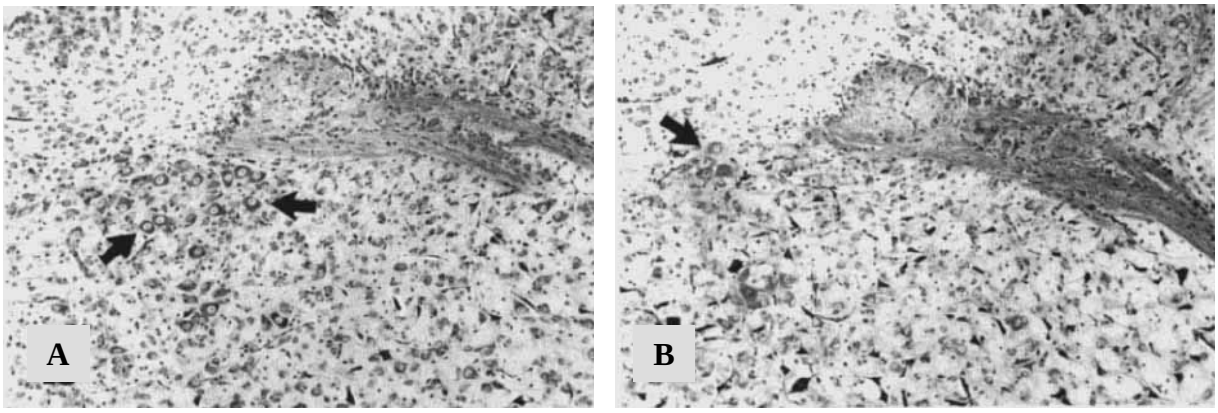

Figure 17. Loss of cranial nerve motor neurons in VPA treated rats. These photographs contrast the abducens nuclei of a control rat (A), pictured at the level of the genu (g) of the facial nerve, and the same level of the nucleus in a rat treated with valproic acid on the twelfth day of gestation (B). Arrows indicate examples of the large motor neurons of the abducens nucleus. Counts of serial sections from the treated brain showed no motor neurons on the left side and only a few on the right, while numerous motor neurons are seen in all control brains. Adopted from Rodier et al., 1996.

3.6.5. Validation of the VPA rodent model of autism

Follow-up anatomical studies in Rodiers' lab showed that VPA administration on day 12.5 results in a loss of cerebellar neurons (Rodier et al., 1997; Ingram et al., 2000), a feature present in the autistic brain as well (Ritvo et al., 1986; Kemper and Bauman, 1998). Purkinje cells were particularly reduced in the lobules VI-VIII and IX, but not the anterior lobules (IV-V) of the vermis. Moreover, the nucleus interpositus (corresponding to the globose and emboliform nuclei in humans) was smaller.

Thus, these experimental studies were able to prove that a single dose of VPA may cause the same neural tube closure injuries as observed in thalidomide, VPA and autistic cases (Rodier et al., 1996). Moreover, what made these findings so particularly interesting was that early brainstem injuries can trigger further brain malformations also observed in autism, such as in the cerebellum. Since the cerebellum is not yet present at the time of drug administration, the cell loss must be a direct or indirect consequence of the early brain stem injury.

On the behavioural level, it was already known for some time that VPA may cause severe and selective alterations in the offspring when administered during days 7-18 of pregnancy (Vorhees, 1987). However, selective VPA administration around the time of neural tube closure and with the explicit aim to screen for symptoms of autism was not undertaken until recently (Schneider et al., 2001; Schneider and Przewlocki, 2005; Schneider et al., 2006). Offspring of VPA-treated rats exhibited decreased social interactions, increased repetitive behaviours, locomotor hyper-activity, increased anxiety, lower sensitivity to pain, higher sensitivity to non-painful sensory stimulation and impaired pre-pulse inhibition. This distinctive pattern of behavioural impairments and enhancements resembles closely the autistic symptomotology and thus undermines the strength of the model.

Recently, the VPA model of autism was related to neurochemical alterations encountered in autism (Narita et al., 2002; Miyazaki et al., 2005). Administration of a single dose VPA on embryonic day 9 (which still falls into the critical time of neural tube closure in the rat) dramatically increased the serotonin levels in the blood and hippocampus postnatally (Narita et al., 2002). VPA administration also irreversibly altered serotonergic neuronal differentiation and migration (Miyazaki et al., 2005). Abnormalities in the serotonergic

system have been extensively linked to autism. Serotonin is increased in blood platelets in autistic people (Anderson, 1987; Cook et al., 1993; Betancur et al., 2002). PET imaging shows that radiolabelled $_{L}$-tryptophan is asymmetrically distributed in the dentato-thalamo-cortical pathway (Chugani et al., 1997). Also, while serotonin-synthesis is usually high in young kids and then gradually declines, autistic children exhibit abnormally high serotonin levels in some brain regions throughout development (Chugani et al., 1999b; Chugani et al., 1999a; Chugani, 2002). Thus, the results encountered in the VPA model of autism have striking similarities with the human data.

Electrophysiological and molecular studies on the VPA rat model of autism conducted by T. Rinaldi within her doctoral work in H. Markrams' lab for the first time reveal that neurons in the neocortex of VPA treated rats are hyper-connected within a minicolumn range, hyper-reactive to electrical stimulation, hyper-plastic and express an excessive amount of NMDA receptors (Rinaldi et al., 2006b; Rinaldi et al., 2006a). These data for the first time gives new insight into the wiring pattern, response characteristics and underlying molecular alterations of a potentially autistic brain and may provide the basis for the development of adequate drug therapies.

In summary, anatomical, behavioural and neurochemical data indicate that the VPA rat model of autism seems to closely parallel autistic pathology. This confirms the validity of the model and was the reason why we chose it for our own studies. Surely an animal model cannot entirely capture a complex neuro-and psychopathological disorder as autism, but it can provide a starting point for developing and testing hypothesis regarding network, electrical, molecular or behavioural alterations which cannot be tested in human subjects.

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3.7. Introduction to study

Apart from studies performed by Schneider and colleagues (Schneider et al., 2001; Schneider and Przewlocki, 2005; Schneider et al., 2006), no other publications exist on the behavioural alterations in the VPA animal model of autism. Therefore, we decided to do our own validation of the model by performing a vast behavioural characterization to screen for autism-like symptoms in the offspring of VPA-treated dams. Approximately 200 animals were tested for the following behaviours:

- Locomotion (open field, elevated plus maze, hole board, water maze)
- Anxiety (elevated plus-maze, open field)
- Social interaction (two different kinds of tests)
- Repetitive behaviour (Y-maze)
- Perception (startle response, pre-pulse inhibition)

Furthermore, several kinds of memory paradigms were evaluated with the animals:

- Fear memory
- Spatial memory
- Social recognition memory
- Object recognition memory

Additionally, electrical activity in the lateral nucleus (LA) of the amygdala in an *in vitro* slice preparation was recorded in collaboration with T. Rinaldi from the Laboratory of Neural Microcircuits. Selected results are presented in the paper and in the supplementary results section.

Paper 3: Abnormal fear conditioning and amygdala neural processing caused by prenatal exposure to valproic acid

Abnormal fear conditioning and amygdala processing caused by prenatal exposure to valproic acid

Kamila Markram[1], Tania Rinaldi[2], Deborah La Mendola[2], Carmen Sandi[1] and Henry Markram[2]
[1]Laboratory of Behavioral Genetics, [2]Laboratory of Neural Microcircuits, Brain Mind Institute; Ecole Polytechnique Federal de Lausanne (EPFL), Switzerland

Correspondence:
Henry Markram
EPFL SV BMI LNMC
AAB 1 19 (Bâtiment AAB)
Station 15
CH – 1015 Lausanne
Switzerland
Tel: +41 21 6939569
Fax: +41 21 6935350
Email: henry.markram@epfl.ch

A core feature of autism spectrum disorders is the impairment in social interactions. Among other brain regions, a deficit in amygdala processing has been suggested to underlie this impairment [1-3], but whether the amygdala is processing fear abnormally in autism, is yet not clear [4]. We used the valproic acid (VPA) rat model of autism to test for alterations in fear processing. VPA treated animals displayed increased anxiety and impairments in social interactions as previously shown, as well as abnormally high and longer lasting fear memories, which were over generalized and harder to extinguish. Multi-electrode array stimulation of slices from the lateral nucleus (LA) of the amygdala revealed a hyper-reactive amygdala with boosted synaptic plasticity. We therefore propose that a hyper-reactive and hyper-plastic amygdala underlies abnormal fear processing in an animal model of autism. We further speculate that abnormal fear processing could be a core pathology in autism which may underlie behavioural symptoms, such as impairments in social interactions, inability to unlearn past associations, and resistance to rehabilitation.

Autism is one of the fastest growing developmental disorders characterized by inhibited reciprocal social interactions, communication deficits and marked inflexibility to environmental changes, which makes rehabilitation exceedingly difficult. Several brain structures and pathways have been suggested to underlie this disorder, among them the amygdala, which exhibits a number of abnormalities. In autistic brains, the number of amygdaloid cells is increased and cell size is reduced [5]. The total amygdala volume in enlarged during early infancy [6,7], but can be reduced in adulthood [8]. Functionally, the amygdala has been linked to autism through its involvement in socio-emotional behaviour. Amygdala lesioned monkeys withdraw socially, fail to initiate and respond to social behaviours [9] and exhibit flat vocalizations that lack affect [10]. Consequently, the social deficits observed in autism have been attributed to weak amygdala functioning. However, there might be an alternative view to the involvement of the amygdala in autism. Kanner's original [11] and other more recent studies [12-14] suggest that abnormal anxiety, fears and phobias are also traits of autism and the amygdala has been extensively linked to both anxiety and conditioned fear, in humans and in animals [15,16]. Thus it may also be that some of the symptoms of autism are due to excessive amygdala activity. To test which of these alternatives are more likely, we performed behavioural tests on an animal model of autism aimed at evaluating amygdala function.

While any animal model of a human disorder is likely to fall short of replicating the disorder entirely, such models can provide a starting point and allow a spectrum of experiments that are impossible to perform on humans, and provide some important clues about the nature of the disorder. We therefore explored whether fear processing is affected in the valproic acid (VPA) rat model of autism [17]. VPA is one of the teratogens implicated in causing autism in humans [18-22] when administered around the critical time period of neural tube closure (E20-24) [23]. In the rat, a single intra-peritoneal injection of VPA to the pregnant dam at a corresponding time of E12.5 (see Methods) mimics several of the features observed in autism. Offspring of treated dams exhibit brain stem injuries, diminished number of Purkinje cells in the cerebellum, impaired social interaction, increased repetitive behaviour, decreased nociceptive thresholds, increased sensitivity to sensory stimulation and enhanced anxiety [17,24-26].

In order to further validate this model, we first performed several behavioural experiments (see Methods) aiming at the core symptoms of autism. For social interaction, we evaluated how pairs of unfamiliar VPA treated or control animals interacted freely for 20 min in a box containing an escape tube large enough for a rat. Treated animals exhibited less play behaviour (pinning, $P = .02$), explored each other less, as indicated by sniffing ($P < .000$) and touching each other ($P = .03$), and avoided interaction by hiding more in the tube than control animals ($P = .01$), but did not differ from control animals in their grooming ($P = .11$) and anogenital inspections ($P = .58$; $n = 10$ pairs/group; Fig. 1a) of each other. VPA treated rats therefore show deficits in some, but not all of the social interaction behaviours performed by rats.

Repetitive behaviour was also tested in a spontaneous alternation task in the Y-maze [27], which is thought to reveal obsessive-compulsive like behaviours [28]. Once the rat entered and briefly explored one of the two possible arms in the first trial, it was put again into the starting position for the second trial and the entrance into either a novel or the same arm previously entered was monitored. Normally, rats prefer to explore a novel arm in the second trial. In accordance with this, only 24 % of the control rats ($n = 41$) entered the same arm again. However, 51% of treated rats re-entered the same arm again ($n = 37$; $P = .01$; Fig. 1b), suggestive of repetitive tendencies.

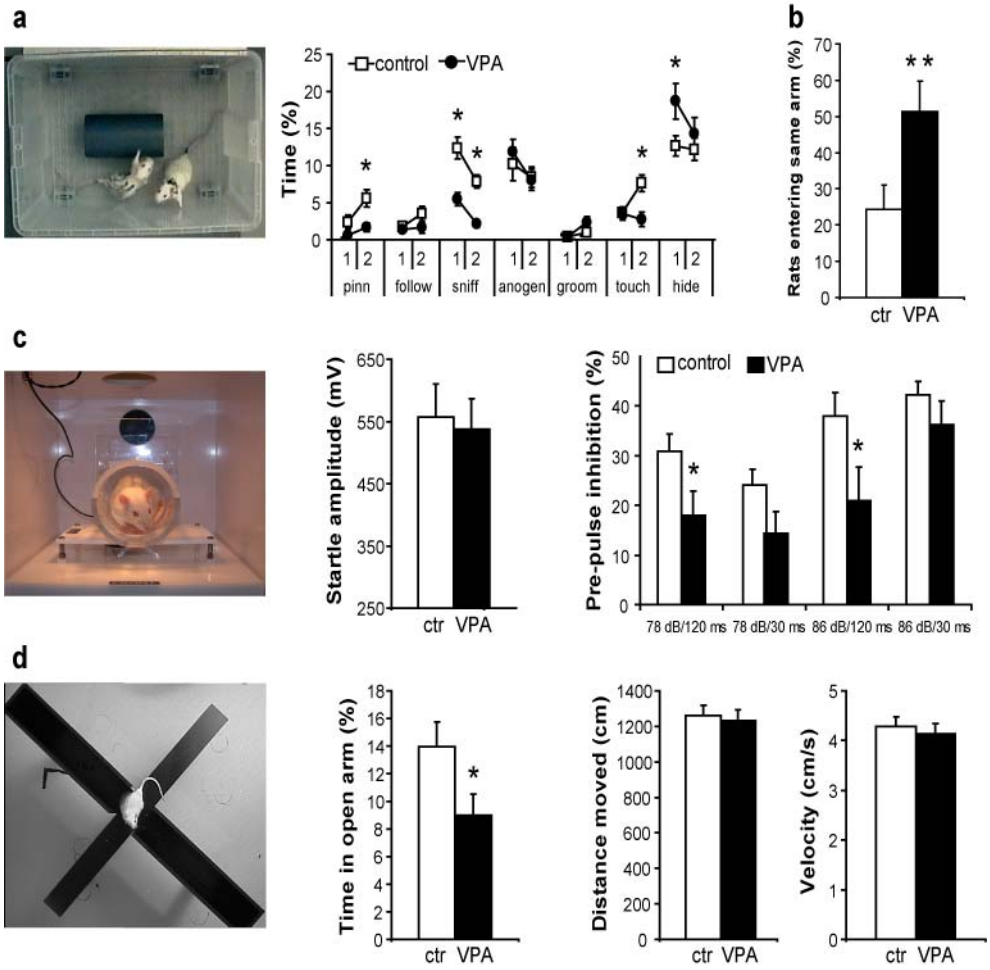

Figure 1 | Behavioral characterization of the VPA model of autism. Offspring of VPA-treated dams exhibit **a**, impaired social interactions (n = 10 pairs/group) **b,** enhanced repetitive behavior in the Y-maze (n = 41 control; n = 37 treated) **c,** normal startle response to a tone, but impaired pre-pulse inhibition (n = 58 control; n = 56 treated) **d,** increased anxiety in the EPM, but normal broad locomotion (distance moved and velocity) (n = 59 control; n = 52 treated). Data show mean ± s.e.m. (*, P < .05; **, P <.01).

Since increased sensitivity to sensory stimulation is also symptomatic of autism, we measured the startle response to a tone alone (115 dB, the startle tone), and when the tone was preceded by another, less intense tone (a prepulse tone). There was no difference in response to the startle tone between control and treated animals (2777 ± 301 mV, n = 58 for control; 2782 ± 304 mV, n = 56 for treated; P = .99; Fig. 1c), but prepulse inhibition was decreased in all four conditions (two prepulse intensities, 78 or 86 dB and two prepulse–startle tone intervals, 30 or 120 ms) and reached significance in two conditions (78 dB, 120 ms prepulse – startle tone interval, 31% PPI for control, 18% PPI for treated, P = .03; 86 dB, 120 ms prepulse – startle tone interval, 38% PPI for control, 21% PPI for treated, P = .04; Fig 1c).

We next examined anxiety levels in a standard elevated plus-maze (EPM). Treated animals spent 35% less time in the open arms (14% ± 1.8, n = 59 control; 9% ± 1.5 n = 52 treated; P = .038; Fig. 1c) and 14% more time in the closed arms (67% ± 2.8 for control; 77% ± 2.7 for treated; P = .01; data not shown) than controls, indicating increased anxiety in the VPA animals. This effect was not due to broad locomotor deficits since both total distances moved and average velocities were the same (distance, 1261 ± 56 cm for control, 1233 ± 60 cm for treated, P = .74; velocity, 4.3 ± 0.2 cm/s for control, 4 ± 0.2 cm/s for treated, P = .58; Fig 1d).

Offspring of VPA-treated dams therefore exhibited less social interactions and increased stereotypic and repetitive behaviour, two of the hallmark features of autism. Locomotion levels and reactions to simple auditory stimulation were normal in treated animals, suggesting no motor or simple sensory impairments. However, more complex auditory stimulation revealed impaired habituation, suggesting deficits also in sensorimotor gating, which may lead to sensory overload and the strong reactions to sensory stimulation, commonly observed in autism.

It is therefore not clear whether the increased anxiety is due to an over-exciting environment or whether it is a core deficit, which underlies avoidance of novel social or environmental situations. In either case, increased anxiety levels may indicate abnormal amygdala processing [29]. The amygdala is also considered to be the primary brain structure involved in fear processing [16] and we therefore explored whether fear processing was affected. Rats were exposed to three electric shocks (1mA, 1 sec every 60 sec) preceded by a tone (20 sec, 800 Hz, 80 dB), which co-terminated with the shock. The percent of time spent freezing was measured to quantify fear. Treated and control animals exhibited the same freezing levels in the shock period during training (65% ± 2 for control; 68% ± 2, for treated; n = 78 animals/group; P = .19; Fig. 2a) indicating normal fear acquisition and no differences in sensitivity to the shocks.

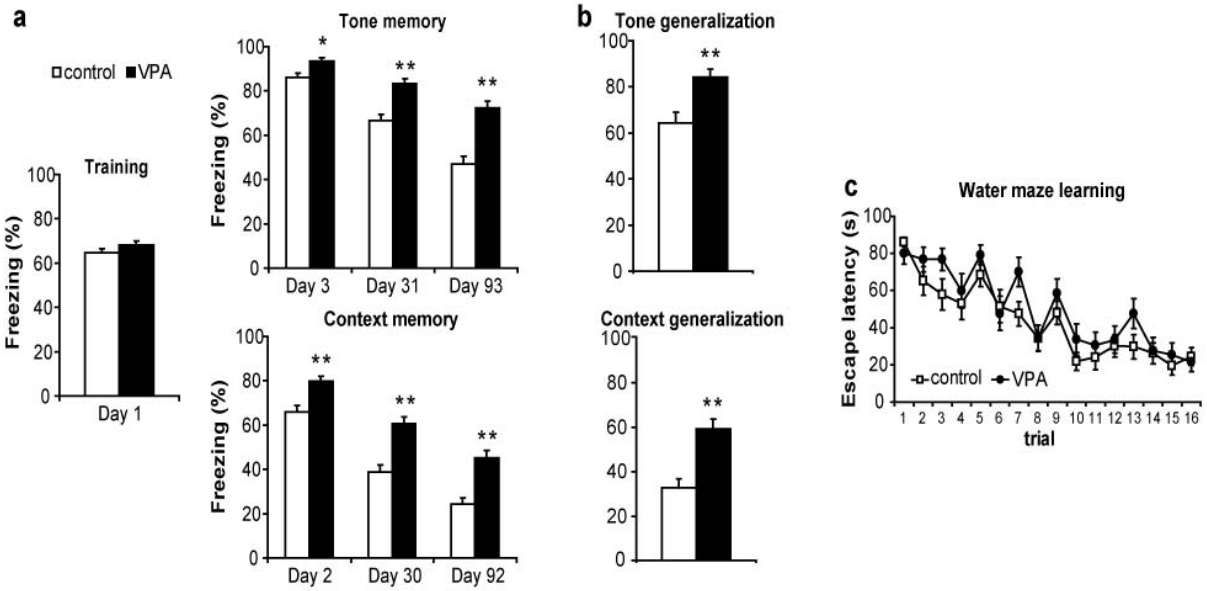

Figure 2 | Enhanced fear memories and fear generalization. Offspring of VPA-treated dams exhibits **a**, normal fear conditioning during training, but enhanced fear memories to the tone and context 1, 30 and 90 days after training, with the differences becoming more pronounced with time (n = 78/group) **b,** enhanced fear generalization to a different tone and different context (n = 36 control; n = 39 treated) **c,** normal to slightly impaired spatial learning in the Morris Water Maze (n = 20 control; n = 18 treated). Data show mean ± s.e.m. (*, P < .01; **, P <.001).

Fear memories were measured on two subsequent days starting 1, 30 and 90 days after fear conditioning for the context in which the fear was acquired, and the tone alone when presented in a novel context (see Methods). Conditioned fear memory to both, tone and context, was highly elevated in VPA-treated animals at all time points measured, with the differences between groups becoming more striking with time, reaching up to 46% higher freezing after 3 months (tone: day 3, 86 ± 2% for control, 93 ± 1% for treated, P = .004; day 31, 67 ± 3% for control, 83 ± 2% for treated, P < .0000; day 91, 47 ± 3% for control, 72 ± 3%

for treated, $P < .0000$; Context: day 2, 66 ± 3% for control, 80 ± 2% for treated, $P = .0001$; day 30, 39 ± 3% for control, 60 ± 3% for treated, $P < .0000$; day 90, 24 ± 3% for control, 45 ± 3% for treated, $P = .0002$; $n = 78$ animals/group; Fig. 2a).

We also examined how these conditioned fear memories generalized to related stimuli. The fear response to a different tone (400 Hz, amplitude 1, 80dB) or context (change of visual and odour cues) in treated animals was measured. We found that both groups expressed some fear when confronted with a different tone or a different context, but fear generalization was 24% and 45% greater in treated animals than controls, respectively (tone: 64 ± 5%, $n = 36$ for control, 84 ± 4%, $n = 39$ for treated, $P = .0008$; context: 33 ± 4% for control, 59 ± 5% for treated, $P < .0000$; Fig. 2b). Thus, offspring of VPA treated rats not only exhibited hyper-fear memories, but also once conditioned, over-generalized fear to other situations.

To check whether the augmented memory associated with the amygdala was perhaps part of a general augmentation of memory mechanisms in the brain, we tested the rats on another memory task, the Morris water maze which is more dependent on the hippocampus [30]. Rats were trained to find a submerged platform within 4 trails on each of 4 consecutive days. We found no differences between treated and control animals on any of the 4 training days or in a subsequent probe trial (all: $P > .05$, $n = 20$ for control and 18 for treated). This may indicate that amygdala-dependent fear memories are particularly enhanced in VPA animals. This does not exclude enhancement of other forms of memories since reward-based, rather than punishment-based, conditioning experiments, will also have to be performed. Recent studies also indicate that synaptic plasticity is greatly enhanced in the neocortex of these VPA rats [31], thus it is possible that memory tasks which depend more on the neocortex are also enhanced.

We next asked whether the enhanced fear memories could be extinguished in VPA treated rats as readily as in controls. For this purpose subgroups of animals underwent several kinds of fear extinction trainings. First, we applied contextual extinction training without any tone presentation. We found that in a post-extinction test, treated animals still preserved 57% of the pre-extinction freezing levels, whereas control animals exhibited only 16% of the initial freezing levels, thus indicating a severe impairment of treated animals to extinguish the conditioning context ($n = 17$ for control, $n = 20$ for treated; $P = .03$). Second, we attempted to extinguish the fear to the tone, by presenting the tone repeatedly in the same context as during the conditioning. In this configuration, treated animals were again severely impaired in extinguishing fear responses to the tone (45 ± 9% of pre-extinction freezing to tone, $n = 20$ for control, 79 ± 14% of pre-extinction freezing to tone, $n = 20$ for treated, $P = .04$). There are therefore strong preservation tendencies in the offspring of VPA-treated rats, which may underlie the highly stereotypic, ritualistic and inflexible behaviour as well as the resistance to rehabilitation. Surprisingly however, when we attempted to extinguish the tone in a novel context, the treated animals were able to unlearn fear responses to the tone almost as well as controls and exhibited only a tendency to impaired extinction (43 ± 4% of pre-extinction freezing to tone, $n = 36$ for control, 53 ± 4% of pre-extinction freezing to tone, $n = 39$ for treated, $P = .09$). Extinguishing fears in a different context from the one in which the fears were acquired, may therefore represent a novel potential approach in rehabilitation.

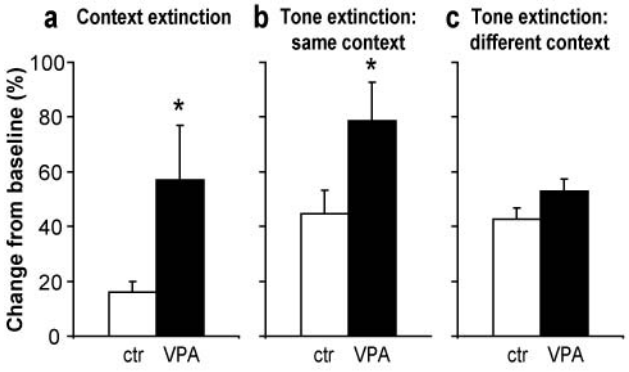

Figure 3 | Impaired fear extinction. Offspring of VPA-treated dams exhibits impaired fear extinction to **a**, the conditioning context (n = 17 control; n = 20 treated) **b,** a tone extinguished in the same context as during conditioning (n = 20/group), but almost normal extinction when **c**, the tone is extinguished in a novel context (n = 36 control; n = 39 treated)**.** Data show mean ± s.e.m. (*, $P < .05$).

These data strongly suggest a malfunction in the amygdala of the treated rats. To determine the nature of this malfunction, we performed electrophysiological experiments on the *in-vitro* slice of the lateral nucleus of the amygdala (LA) and examined how principal neurons (Fig. 4a) responded to stimulation of the amygdala and how synaptic connections reacted to a Hebbian pairing protocol. We found that strong stimulation using a multi-electrode array (MEA) can activate the circuit to produce reverberant activity that can persist for seconds without any further stimulation (Fig. 4b). We therefore compared these evoked activity states in control and VPA treated animals. For the equivalent stimulation (see Methods), we found nearly double the fraction of cells (83%) from VPA treated animals displayed this reverberant activity compared to 47% of cells from control animals (control, 10 out of 21 cells; treated, 15 out of 18 cells, P = 0.018). Furthermore, among the cells displaying this network state, the frequency of the activity was significantly higher in treated rats (n = 10 for control, 1.8 ± 0.3 Hz; n = 15 for treated, 3.0 ± 0.3 Hz; P = 0.022). The LA network is therefore more easily activated in VPA-treated rats (Fig 4b) which alone may contribute to augmentation of fear memories [32]. As synaptic plasticity in the LA is also thought to underlie fear memories [33-36], we tested long-term potentiation (LTP) in the VPA-treated rats. We found that the potentiation caused by a classical pairing protocol with an extracellular electrode placed just medial to the LA (see Methods) was significantly increased in treated rats (n = 15 for control, 45 ± 8 % increase; n = 10 for treated, 106 ± 18 % increase; P = 0.009; Fig. 4c). The amygdala of VPA treated animals is thus hyper-reactive and hyper-plastic. Hyper-reactivity and hyper-plasticity has also been observed in the neocortex of this animal model [31,37], indicating that this is not unique to the amygdala, but probably part of a more generalized phenomenon. Such changes in the amygdala however may underlie the augmented fear memory formation, over generalization and resistance to extinction in the VPA animals.

In summary, these experiments provide the first clue that abnormal fear processing could be a major factor shaping behaviour in autism. Fear memories were not only stronger and longer lasting, but generalized more and were harder to extinguish. Enhanced fear processing could perhaps underlie what has been considered to be core symptoms of autism, such as impaired social interactions, stereotypical and repetitive behaviours, avoidance of novel environments, as well as resistance to rehabilitation. These data also support the view that enhanced, rather than weak amygdala function may be part of the pathology in autism. The evidence that the amygdala is indeed not only processing information with exaggeration, but also seems to produce greater synaptic changes when activated, further supports the view of a hyper-functioning amygdala since this form of synaptic plasticity in the amygdala is

known to be required for the formation of fear memories [33-36]. Targeting these amygdala abnormalities may therefore offer an interesting possible treatment for autism. It now remains to be determined whether fear memories are amplified in humans with autism and to explore the possibility raised by these animal experiments that abnormal fear processing may underlie one of the most debilitating core symptom in autisms, the impairments in social interaction.

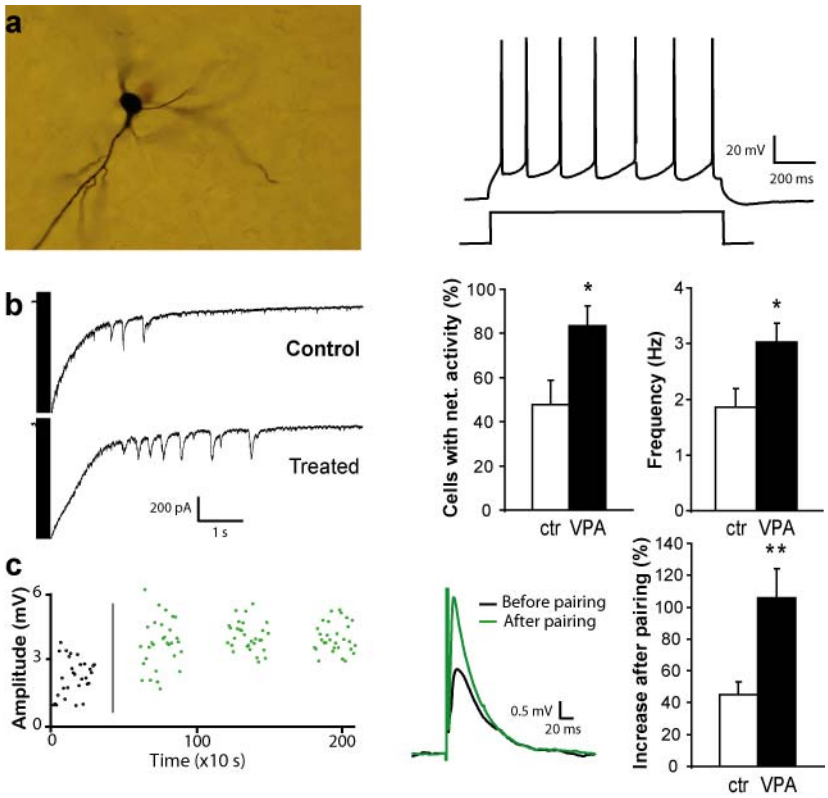

Figure 4 | Increased network excitability and enhanced LTP in the LA. a, Example of a principal cell of the LA and corresponding firing pattern. **b,** Response to the multi-electrode array (MEA) network stimulation in a voltage-clamped (-80 mV) principal cell, illustrating the typical network activity observed with this stimulation in control and VPA treated rats. The percentage of cells showing this type of activity was significantly higher in treated (15 out of 18 cells) than in control rats (10 out of 21 cells). Among the cells showing this activity, the frequency of network events was significantly higher in treated (n=15) than in control rats (n = 10). **c**, Example of the amplitude of the responses to extracellular stimulation before and after the pairing protocol (represented by the grey bar), and mean amplitudes before and after pairing. The bar graph represents the percentage increase in the response amplitude after the pairing for control (n = 15) and treated (n=10) rats. Data show mean ± s.e.m. (*, $P < 0.05$; **, $P < 0.01$).

METHODS

Valproic acid model of autism. Wistar Han rats (Charles River Laboratories, L'Arbresle, France) were mated, with pregnancy determined by the presence of a vaginal plug on embryonic day 1 (E1). The sodium salt of valproic acid (NaVPA, Sigma) was dissolved in 0.9% saline for a concentration of 150 mg/ml, pH 7.3. The dosing volume was 3.3 ml/kg; the dosage was adjusted according to the body weight of the dam on the day of injection. Treated dams received a single intraperitonal (ip) injection of 500 mg/kg NaVPA and control dams a single ip injection of saline on gestational day (GD) 12.5. Delivery of this dose to rats during embryogenesis has been shown to result in maximum levels of total valproic acid in maternal plasma in less than 1 hour, with a mean plasma elimination half life of 2.3 hours [38]. Dams were housed individually and were allowed to raise their own litters until weaning (P23). The offspring was then separated and housed in cages of 3-4 rats until the start of behavioral experiments (approximately 3 months after birth). Electrophysiological experiments were conducted on P12 – P16.

All experimental procedures were carried out according to the Swiss federation rules for animal experiments.

Social interaction. Rats were separated and housed individually the night before the experiment to enhance later social interactions. The apparatus was a white plastic box (50 cm x 40 cm x 40 cm) containing a tube big enough for a rat to hide inside. Rats were matched for their gender and weight. Pairs of either treated or control rats were put into the apparatus over a period of 20 min. The percent time spent pinning (one rat lies on its back, the other stands with two paws on top of it), following, touching, grooming each other, sniffing of any body part besides of anogenital parts, sniffing of the anogenital body parts and hiding inside the tube during the first and second 10 min were taken as indicators of social engagement.

Repetitive Behavior. Rats were tested in the spontaneous alteration paradigm. Rats were inserted individually into the start arm of a standard Y-maze (grey polyvinyl plastic) and allowed to move freely on each of two trials. On Trial 1, the animal was allowed to enter either arm, neither of which was rewarded, and remain there for 5 s. The rat was then returned to the start arm and the subsequent arm choice was recorded. The percentage of rats per group re-entering the same arm was taken as an indicator for repetitive behavior.

Startle response and prepulse inhibition (PPI). Acoustic startle reflex amplitude and PPI were measured with the SR-Lab System (San Diego Instruments, CA, USA). The equipment included response platforms that were individually calibrated and placed in sound attenuating chambers. Each chamber was equipped with an internal light, fan and sound generation system. Plexiglas cylinders large enough to hold adult rats with minimal restraint were mounted on the platforms. The white background noise was adjusted to a constant 70 dB.

The test session was conducted in 3 blocks and started with a 5 min acclimation period. Block 1 consisted of 5 startle only trials with a 115 dB acoustic stimulus pulse (startle tone). Block 2 had a total of 36 trials: 12 startle only trials as in Block 1 and 24 prepulse plus startle trials, conducted under 4 different conditions: the intensity of the prepulse was either 78 or 86 dB and the prepulse to startle tone interval was either 30 or 120 ms. The trials were presented in random order with the inter-trial duration ranging from 10 to 20 s. Block 3 had an additional 5 startle only trials. Each stimulus had a 2 ms rise/fall time. The entire test period lasted approximately 25 min.

Anxiety. Rats were inserted for 5 min in a standard elevated plus-maze (EPM), which consisted of two opposite open arms and two opposite closed arms (49 cm × 10 cm x 42 cm) arranged at right angles. The percent time spent in the open and closed arms indicated anxiety levels and the total distance moved and velocity indicated locomotor and exploration capabilities.

Fear conditioning. Fear conditioning occurred in a standard fear conditioning apparatus (Panlab, Barcelona, Spain). The floor consisted of 20 steel rods through which a scrambled shock could be delivered. The sidewalls of the observation cage were of black stainless steel and the door of Plexiglas. Auditory fear conditioning (AFC) training started with a 160 s habituation period, followed by three 1 mA shocks (inter-shock interval: 60 sec), each paired with a 20 sec tone (800 Hz, amplitude 3, 80 dB). Ethanol (4%) was used for cleaning between training sessions. Fear memories to the context were assessed by putting the rats for 8 min into exactly the same context as during training, but without shocks. To measure fear memories to the tone visual (green plastic walls) and odour (4% clorine) cues in the conditioning chamber were exchanged and rats remained in the chamber for 8 min, during which the tone was continuously on for the last 5 min. The percent time spent freezing during the 8 min context and 5 min tone memory test were taken as indicators of fear. Fear memories to the context and tone were assessed on two successive days, starting 1 day, 30 and 90 days post-training. Fear generalization to a novel context and tone was assessed as in the tone memory test, just that the tone was additionally changed as well (400 Hz, 80dB). Freezing during the first 3 min indicated novel context and during the last 5 min novel tone generalization. To assess fear extinction, animals were first re-conditioned (3 shocks, 0.5 mA) either to the context alone (contextual fear conditioning, CFC) or paired with a tone (as above) 1 day after he last memory test. Fear extinction was conducted under 3 conditions: the group that received contextual fear conditioning was extinguished to the same context on 4 subsequent days, starting with a 8 min pre-extinction context test, followed by two days of 30 min further context exposure and ending with another 8 min post-extinction context test. Rats which received AFC were extinguished in either the same or a different context as during training. The procedure was as above: first a 5 min pre-extinction tone memory test was conduced, followed by two extinction sessions (each 30 min in which a 20 s tone alternated with 40 s silence) and finally a 5 min post-extinction tone test. The percentual decrease in freezing from pre- to post extinction test was computed for each animal.

Morris water maze. The water maze apparatus consisted of a large circular pool (2.05 m in diameter) filled with water (25 ± 1°C). A platform (11 cm diameter) was submerged 1 cm under the water surface. Both pool and platform were made of black polyvinyl plastic and offered no intra-maze cues to guide escape behavior. The water maze was surrounded with curtains containing several extra-maze visual cues.

Rats received 4 days of spatial training, each session consisting of 4 trials (intertrial interval, ITI: 30 sec). Each trial started with the rat facing the wall at one of 4 possible positions. The latency to find the platform was measured. If a rat did not find the platform within 90 sec, it was guided towards it. Each rat remained on the platform for 15 sec before it would be taken out. On day 5 a probe test was conducted, in which the platform was removed from the pool and animals were released to the pool for a 60 sec period from the quadrant opposite to the one where the platform had been previously located. At the end of the probe trial the platform was re-inserted into the pool and rats remained on it for 15 sec.

Video tracking software (Ethovision, Noldus, Netherlands) was used for automatic recording and analysis of escape latencies, distances swum and velocities in case of the spatial training sessions and % time spent and distance swum in the target quadrant, number of virtual platform crossings and latency to reach the virtual platform in case of the probe trial.

Acute slice preparation. Rats (PN12 to PN16) were rapidly decapitated and coronal amygdala slices (300 μm thick) were sectioned on a vibratome (HR2, Sigmann Elektronik) in iced artificial cerebral spinal fluid (ACSF, contained (mM): 125 NaCl, 2.5 KCl, 25 glucose, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$ and 1 $MgCl_2$). Slices were incubated for 30 minutes at 35°C and then at room temperature until transferred to the recording chamber (room temperature or 34°C). Neurons in LA and B were identified using IR-DIC microscopy, with

an upright microscope (Olympus BX51WI, fitted with a 60x/0.90 W objective, Olympus, Switzerland). Recorded neurons were selected up to 70 μm below the surface of the slice.

Electrophysiological recording. Simultaneous whole-cell recordings from clusters of up to four neurons (pipette resistance 4 to 10 MΩ) were made and signals were amplified using Axopatch 200B amplifiers (Axon Instruments). Voltages (in current-clamp mode) or current (in voltage-clamp mode) were recorded with pipettes containing (mM): 100 potassium gluconate, 20 KCl, 4 ATP-Mg, 10 phosphocreatine, 0.3 GTP, 10 Hepes and 0.5% biocytin (pH 7.3, 270-300 mOsm). Membrane potentials were not corrected for the junction potentials between pipette and bath solution (~10 mV).

MEA stimulation. Multi-site extracellular stimulations were performed using a multi-electrode array (MEA) made of 60 3D platinum electrodes (Ayanda Biosystems, EPFL, Switzerland), on top of which acute brain slices were glued with a solution of nitrocellulose (0.14 mg/ml in ethanol). The responses of these stimulations were recorded in whole-cell patched PCs. For the study of network stimulation, 16 MEA electrodes were stimulated simultaneously with a Poisson train (50 Hz, 300 ms) at increasing stimulation amplitude (0.1 to 2V). Whole-cell voltage-clamped cells were then recorded at -80 mV.

Long-term potentiation. An extracellular electrode was placed 100-300 μm away (in the ventral striatum, just medial to LA [33]) from the whole-cell patched PCs. A stimulation pulse of amplitude producing a response of a few millivolts in the I-clamped patched cell was given before the pairing protocol, and the maximum amplitude of the mean of 30 traces was measured. The 3 second long pairing protocol consisted of a 30 Hz regular train stimulation to the extracellular electrode simultaneously with a depolarization above threshold of the patched cell, applied three times at 30 seconds interval. The response to the stimulation pulse was then monitored up to 30 minutes after the initial recording (3 sets of 30 stimulation pulses every 10 seconds, with 5 minutes interval between each set). The percent increase in the amplitude of response to the stimulation pulse after pairing compared to before pairing was measured. The total number of pyramidal neurons studied was 15 for control, 10 for VPA-treated rats.

Statistical Analysis. For comparison of rates and means two-sided Chi-square and Student's *t*-tests were used, respectively. Social interaction and spatial learning sessions one each of the 4 training days were evaluated with ANOVA for repeated measurements. Statistics reported in the text and figures represent the mean ± s.e.m.

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3.8. Impaired social recognition, but intact object recognition

We found that offspring of VPA-treated dams exhibited impaired social recognition, but intact object recognition, thus indicating a selective impairment in the social memory system. A task to assess sociability and the preferences for social novelty/recognition (fig. 18) was adopted from Nancy Crawley's group (Crawley, 2004; Moy et al., 2004). Briefly, in the first phase, sociability was assessed for 10 min by scoring each rat on measures of exploration in a central habituated area, a side chamber containing an unfamiliar younger conspecific (stranger 1) in a plastic cage, or a side chamber containing an unanimated object. After approximately 5 min, a second phase (also 10 min) was conducted, in which social recognition was evaluated by presenting the test rat with a choice between the first, now familiar, conspecific (stranger 1) in one side chamber, and a second unfamiliar rat (stranger 2) in the other side chamber. The time spent by the rat sniffing at the plastic cages was indicative of sociability in the first phase and preference for social novelty/social recognition in the second phase. We found that in the first phase of the experiment, control animals clearly preferred the rat (stranger 1) over the unanimated object. Offspring of VPA-treated dams also explored stranger 1 more than the object, but clearly to a much lower extent than control animals, thus indicating lower levels of sociability (fig.16a). In the second phase, control animals clearly preferred the novel rat (stranger 2) over the old rat (stranger 1). This indicated that normal rats exhibit a natural preference to explore a novel conspecific and are capable of discriminating between old and new rat based on an established memory trace of stranger 1. Offspring of VPA-treated rats, on the other hand, explored novel and old rat to the same extent (and again much below the exploration levels of control rats, fig.16a). VPA-treated rats were far worse in discriminating between old and new rat than controls (fig. 18c). This suggests two conclusions: either VPA-treated animals have no preference for social novelty as have normal rats or they cannot discriminate between old and new rats. The latter implies an impairment in social recognition memory in VPA-treated animals.

In order to test whether these impairments were unique to the social domain, the same animals underwent an object recognition task, as described elsewhere (Ennaceur and Delacour, 1988), with the only difference that the test was conducted in the same chamber and configurations as above social interaction and recognition task (fig. 18). Briefly, in the exploration phase two identical objects were exposed for 10 min. After a delay of approximately 5 min, one object was exchanged for a novel object and rats were allowed to explore both objects for another 10 min (recognition phase). Exploration was assessed as the time spent sniffing either object. In the exploration phase, both control and VPA-treated rats spent the same amount of time exploring the two identical objects and no preference for either object (fig. 18b). In the recognition phase, both groups preferred the novel object over the old object and there were no differences between groups (fig. 18b). Comparision of the discrimation capabilites revealed no differences between groups (fig. 18c). These results indicate that offspring of VPA-treated rats has no deficits in object recognition memory.

Thus, VPA treated rats approach unanimated objects in the same way as control rats do and have intact object recognition memory, but have selective impairments in social exploration and social recognition.

Since social recognition is mediated by oxytocin signalling in the medial amygdala (Ferguson et al., 2001) and oxytocin was found to be deregulated in autism (Modahl et al., 1992; Panksepp, 1993; Modahl et al., 1998; Hollander et al., 2003; Lam et al., 2005; Wu et al., 2005) as well as in offspring of VPA-treated rats (M. Toledo-Rodriguez, unpublished data), we are currently pursuing further molecular, electrophysiological and behavioural experiments to investigate whether and how oxytocin signalling in the amygdala contributes to overall symptomotology encountered in the VPA model of autism.

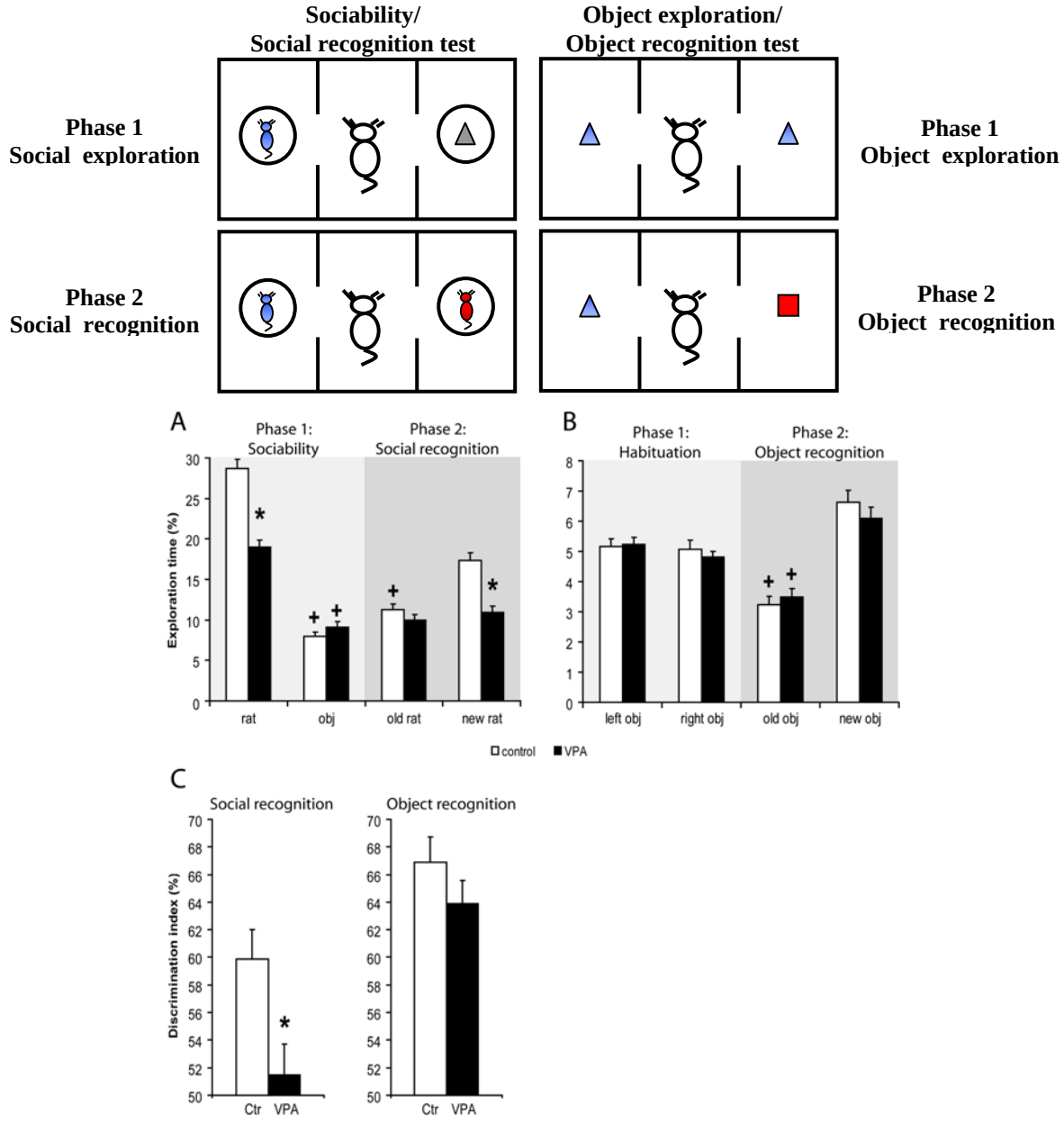

Figure 18. Impaired social interaction and recognition, but intact object exploration and recognition. Data are mean ± SEM. (A) Sociability and social recognition test. During Phase 1, the test for sociability, both control and VPA treated rats preferred to explore a rat over an unanimated object, but VPA treated rats explore the rat much less than controls. During the social recognition phase, only control rats show a social novelty preference. VPA treated rats explore both new and old rat to the same extent, and much less than control rats. (B) Object recognition test. During the habituation phase both control and VPA treated rats explore the two identical objects to the same extent. Equally, during the object recognition test, both groups prefer the new objects and there are no differences between groups. (C) Discrimination ratios (% time exploring the new rat or object as a proportion of total time exploring both rats or both objects). VPA treated rats exhibit impaired social, but intact object recognition. N=74 for control and 76 for VPA treated. *, p <.01, control vs. treated; +, p<.001, within group comparison. All Student's t-tests.

3.9. Altered NCAM expression in the VPA rat model of autism

In order to evaluate whether the hyper-plasticity encountered in the amygdala was paralleled by alterations in molecules involved in synaptic plasticity, we examined NCAM expression in 2 week old VPA-treated offspring.

Western blots were performed to evaluate the expression of NCAM major isoforms (NCAM-120, -140, and -180 kd) in crude synaptosomal preparations. In brief, equal amounts of protein (6 _g) were applied in each lane, separated on 7% (wt/vol) sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred (1 A, 1.5 hours) to an Immobilon-P membrane. After saturation of the nonspecific sites with 5% (wt/vol) skimmed milk in 50 mmol/L Tris-HCl, pH 8, 138 mmol/L NaCl, .05% Tween 20 (TBST), the blots were incubated for 1.5 hours at room temperature with a polyclonal rabbit anti-rat NCAM immunoserum (diluted 1:15,000). The blots were washed with TBST, incubated for 1 hour with a secondary antibody, an anti-rabbit immunoglobulin peroxidase conjugate (whole molecule conjugate; diluted 1:20,000), and finally developed with the enhanced chemiluminescence system. For the quantification of autoradiographic films, images were captured by high-resolution microdensitometry with a flat-bed scanner. Video images of the autoradiographs were converted to grey values and analyzed for optical density measurements with image analysis software. The integrated measures of band optical density multiplied by the area in number of pixels were recorded.

We found that of the three NCAM isoforms, NCAM-180 was significantly increased in the amygdala in VPA-treated offspring (fig. 19). This suggests that increased NCAM-180 expression may be one of possibly several altered molecular processes in the amygdala underlying enhanced synaptic plasticity in VPA-treated offspring.

In order to establish whether this pattern of altered NCAM expression was unique to the amygdala, NCAM expression was also determined in the hippocampus and prefrontal cortex (PFC). Interestingly, NCAM (NCAM-140 and -180) expression was also increased in the PFC, but downregulated in the hippocampus (NCAM-180) in VPA-treated offspring (fig. 19). This data indicates highly region-specific molecular alterations and implies differential prognosis for synaptic plasticity processes in these regions. For example, we would expect to find rather impaired activity-induced synaptic plasticity in the hippocampus, whereas quite the opposite would be predicted for the prefrontal cortex. *In vitro* electrophysiology data indeed indicates an increase in synaptic connectivity and plasticity in prefrontal cortex slices of VPA-treated rats (T. Rinaldi et al., in preparation).

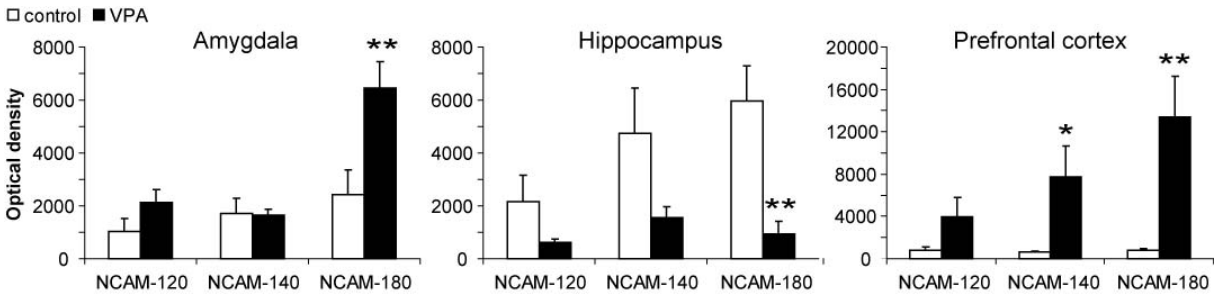

Figure 19. Altered NCAM expression in VPA-treated rats. Optical density measurments of synaptosomal NCAM in the amygdala, hippocampus and prefrontal cortex. Note the differential NCAM enhancements and decreases in VPA-treated offspring. *, $p < 0.05$; **, $p < 0.01$. All Student's t-tests.

These results for the first time indicate that neural cell adhesion molecules might contribute the altered circuitry and neural processing in autism. Genetic studies in humans suggest (even though controversial) that a mutation in the cell adhesion molecule neuroligin may contribute to autism by disturbing synaptogenesis (Jamain et al., 2003; Laumonnier et al., 2004). Based on the results obtained from the VPA rat model it would be certainly interesting to determine whether NCAMs are also associated with autism. Taking into account the synaptogenetic effects of NCAM and particularly PSA-NCAM (Dityatev et al., 2004) and the differential NCAM alterations observed in VPA-treated rats, we suggest that this molecule might contribute to the altered circuitry and hyper-connectivity observed in particular brain areas, such as the prefrontal cortex, but not others (Courchesne et al., 2005; see chapters 3.3.1 and 3.3.2).

3.10. Discussion and Perspectives

The key insight obtained from these studies is that the amygdala might be particularly strongly affected in autism and this effect may underlie many symptoms in autism. Several findings support this view: First, fear memories are greatly enhanced in VPA-treated animals. Second, other memories mediated by other brain areas, such as spatial memory and object recognition memory, remain unaffected in VPA-treated animals. Third, fear extinction is impaired in VPA-treated rats. Fourth, social interaction and recognition are reduced in VPA-treated animals. Fifth, amygdaloid neurons are abnormally responsive to electrical stimulation and synaptic plasticity is enhanced in VPA-treated rats. Sixth, neural cell adhesion molecules are over-expressed in the amygdala in VPA-treated rats. All these findings indicate a hyper-active and -responsive amygdala, which is capable of forming abnormally strong memories. We suggest that this abnormal processing in the amygdala may underlie some of the core symptoms of autism.

Enhanced amygdala activity may underlie core symptoms of autism

Several arguments support this novel view of a hyper-active amygdala underlying impaired social interactions and possibly communication. First, some structural MRI studies have shown that in the young and older autistic brains amygdala volumes can be enhanced (Abell et al., 1999; Howard et al., 2000; Sparks et al., 2002; Schumann et al., 2004). Enhanced amygdala volumes were also observed in post-traumatic stress disorder, in which the amygdala is hyper-active and –responsive to traumatic triggers (Damsa et al., 2005). Second, enhanced levels of amygdala activation due to reduced inhibition have been associated with increased anxiety and fear conditioning (Rodriguez Manzanares et al., 2005). It is conceivable that people with a highly responsive amygdala and consequently increased anxiety and fear levels might not be the most sociable people. In support of this view is that decreased amygdala activation has been linked to genetic hyper-sociability (Meyer-Lindenberg et al., 2005), whereas increased activation is observed in social avoidance and phobia (Stein et al., 2002).

A link to stress

Curiously the pattern of increased amygdala activity, enhanced plasticity and fear conditioning we observed in the VPA model of autism very much resembles the phenotype evoked by stress (Cordero et al., 1998; Rodriguez Manzanares et al., 2005). It is therefore conceivable that either the autistic individual is born with a "stressed amygdala" or that the amygdala is particularly vulnerable to stress in autism and throughout life acquires the stressed phenotype. Further studies, within the VPA model and particularly in autistic individuals, will need to elucidate this issue. A first study in this direction provides some indication that the cortisol circadian rhythm is indeed deregulated in autistic subjects (Corbett et al., 2006), thus indicating alterations in the stress system in autism which may be related to the abnormal amygdala functioning.

Possible mechanisms underlying failed extinction

A possible explanation for the finding that fear extinction is strongly impaired may lie in a weakened functional connection between the prefrontal cortex and amygdala. It has been suggested that the amygdala might control the mPFC during conditioning and as long as the CS predicts danger, inhibit mPFC activity. During extinction this process is reverted and eventually mPFC activity may be potentiated and in turn now inhibit amygdaloid output (Vouimba et al., 2000; Herry and Garcia, 2002; Quirk et al., 2003; Rosenkranz and Grace, 2003). As discussed in chapter 3.3.2 Courchesne and Pierce (2005) proposed that the

prefrontal cortex might be disconnected from other brain areas in the autistic brain. Our data may provide evidence for this hypothesis. However, we cannot be sure that there is indeed a lack of prefrontal inputs to the amygdala unless electrophysiological recordings are undertaken in the intact brain. Thus for the future, we plan to record in awake rats during task performance to investigate whether this hypothesis can be substantiated.

Advantages of using an animal model

Clearly the use of an animal model has many advantages; most obviously it allows studies that would be unethical in the human subject and in many cases also impossible. It would be unethical to fear condition autistic subjects, since this kind of treatment could be traumatizing for unknown periods of life. It would result impossible to obtain electrophysiological data on electrical discharge patterns from individual neurons and networks and the connections among them. Even though fMRI studies do focus on brain activity in autistic patients and it is possible – to some extent – to obtain data on connectivity between brain areas, it still remains impossible to derive the detail-rich electrophysiological information about the autistic network.

It seems natural to study a disorder as autism in a closely related animal that displays many "human" behaviours and that we can somehow relate to. Thus, it seems most natural to use monkeys as the model animal *par excellence*, because they are our closest relatives. They exhibit complex social hierarchical systems and a high degree of communication. Many behaviours can be studied in monkeys and related to humans. In autism research rodents seem out of place at the first sight, since human language and communication skills cannot be paralleled in mice or rats. However, rodents have a long and rich history of being studied in the neuropsychiatric context and have brought many new insights into fields like for example anxiety disorders, Alzheimer's research, Parkinson's and Huntington's disease, drug abuse research, etc. Further, rodents exhibit complex social systems and rich social interactions, and many behaviours, including memory, attention and emotion are well studied and understood in these animals. Clearly, rodents are much easier to breed and maintain than primates. The life span of a rat is much shorter than of a primate, allowing studying behaviours at different developmental stages. Faster results in a more efficient manner can be obtained when studying neural circuitry and possible molecular pathways or markers for a disorder. Screening for a variety of drugs can likewise be done much faster and more efficiently in the rodent model. I also believe it is ethically more justifiable to screen lower animals for possible electrophysiological, molecular and behavioural alterations and test new drug therapies on them rather than higher animals. Once there is a theory about possible alterations and mechanism at work on these three levels, the accumulated knowledge can be transferred to and tested in higher animals or even humans at later stages.

What is all of this for?

One does not just want to study a VPA-treated rat, and characterize its behaviour and brain activity as profoundly as possible. Ultimately, the goal is to understand autism better and to develop treatments that might alleviate the devastating and life-impairing symptoms for the affected individual and the family. Thus, what novel insights have we gained about autism from the VPA model? First, for the first time it could be shown that local microcircuits are hyper-connected and hyper-excitable (Rinaldi et al., 2006a), as has been previously hypothesized (Rubenstein and Merzenich, 2003; Casanova et al., 2002; Courchesne et al., 2005), but was improvable with human data. Second, *in vitro* recordings from VPA-treated animals revealed a hyper-plastic circuit in the somatosensory cortex as well as the amygdala (Markram et al., submitted-c; Rinaldi et al., 2006b). Enhanced plasticity was due to increased expression of NR2A and NR2B subunits. CamKII, an enzyme phosphorylated downstream of

NMDA receptor activation, was also increased (Rinaldi et al., 2006b). These data indicate that autism might be due to enhanced levels of NMDA receptors in the neocortex, thus leading to enhanced plasticity and local hyper-connectivity. Knowledge about micro-structural alterations and underlying molecular mechanism opens the promising opportunity for the development of new drug therapies, which can initially be tested for their efficacy in the rat model. Third, for the first time we show that general anxiety levels and fear memories are enhanced in VPA-treated animals and extinction of once acquired fear memories is greatly aggravated. Enhanced fear memories are accompanied by enhanced synaptic plasticity in the amygdala (Markram et al., submitted-c). Contrary to the neocortex, in the amygdala these changes are not accompanied by alterations in NMDA receptor expression (unpublished data). Currently we investigate possible alternative molecular mechanisms, such as alterations in oxytocin and vasopressin signalling in the amygdala (see below). These data may provide new insight into the functioning of the amygdala in autistic individuals, suggesting that it is hyper-active rather than hypo-active as currently assumed. Knowledge that some autistic individuals exhibit a hyper-active and –responsive amygdala may provide new approaches for the treatment of these individuals: Anxiolytic drugs may provide alleviation in some situations, perhaps when exposed to new environments. We plan to check whether the administration of anxiolytic drugs, such as benzodiazepines, before fear conditioning may alleviate the exaggerated fear memories in VPA-treated animals.

Another candidate linked to anxiety and social behaviour is oxytocin (Wheal et al., 1998). Oxytocin is known to reduce fear by acting on the amygdala (McCarthy et al., 1996) and more specifically the central amygdala, where it may inhibit the excitatory flow from the amygdala to brainstem sites mediating fear responses (Huber et al., 2005). Further, it mediates social recognition by acting on the medial amygdala (Ferguson et al., 2001). Furthermore, oxytocin expression is altered in some autistic individuals (Modahl et al., 1992; Green et al., 2001; Lam et al., 2005). Preliminary data in our group suggest that oxytocin expression is reduced in the offspring of VPA-treated animals (M. Toledo-Rodriguez, unpublished data). Several further studies within the VPA model are either currently undertaken or planned for the near future: a) evaluation of oxytocin and vasopressin, a closely related molecule also related to social behaviour, expressions in the amygdala; b) in vitro electrophysiological recordings to determine whether oxytocin application on slices from VPA-treated rats might alter and normalize the physiological response to stimulation and synaptic plasticity in the amygdala; c) application of oxytocin before social recognition and fear conditioning tasks to check whether behavioural responses can be normalized in affected animals.

In summary, the purpose of studying an animal model of autism, and not the affected human being himself, is to gain novel insights into structural, electrical, molecular and behavioural alterations that might not – or at least not as easily – be obtained from human experiments. These studies may elucidate which areas are particularly affected and how they are affected and finally provide an invaluable basis for the development of new drug therapies for autism.

Conclusions

What does this thesis contribute to the understanding of aversive memory formation in the amygdala?

One contribution lies in disentangling some of the molecular mechanisms underling fear memory formation it the amygdala. PSA-NCAM, a molecule widely implicated in synaptic plasticity processes underlying learning and memory in the hippocampus, was suggested to also contribute to aversive memory formation in the amygdala. This thesis presents strong evidence to conclude that this is not the case. Rather than in the formation, PSA-NCAM is involved in the extinction of fear memories in the amygdala. Thus, we conclude that even though PSA-NCAM may be expressed in a brain region and even modulated after learning, it does not necessarily mediate the learning process. Thus, PSA-NCAM mediated learning may exhibit strong specificity in terms of what learning task is used, which is the learning mediating brain region and which synapses are involved in the learning processes.

Another contribution of this work lies in providing a deeper knowledge about the potential underlying causes of autism. In this thesis it is shown that fear memories are greatly enhanced, generalized and resistant to extinction in the VPA rat model of autism. The amygdala is hyper-responsive and hyper-plastic in VPA-treated rats. Thus, we suggest that an excessively active amygdala forming strong fear associations may lie at the heart of the autistic pathology and lead to many of the symptoms observed in autism, such as impaired social interaction and resistance to rehabilitation.

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Curriculum vitae

Kamila Markram

Address
Rue de Valentin 34
1004 Lausanne
Switzerland
kamila.markram@epfl.ch

Personal Data
Born Kamila Senderek on Dec. 23rd, 1975 in Wroclaw, Poland
Citizenship: German

Languages
Bilingual in Polish and German
Fluent in Spanish
Fluent in English
Beginner in Portuguese
Beginner in French

Education

Since 2006	Post-doctorate, Swiss Federal Institute of Technology (EPFL), Lausanne, Switzerland Laboratory of Neural Ensemble Physiology, Prof. Dr. Miguel Nicolelis
2003 – 2006	Doctorate in *Neuroscience and Developmental Neurobiology*, Swiss Federal Institute of Technology (EPFL), Lausanne, Switzerland Laboratory of Behavioural Genetics, Prof. Dr. Carmen Sandi
1998 – 2003	Diploma in Psychology, Technische Universität, Berlin, Germany
1995	Graduated from Highschool (*Abitur*), Gymnasium Lünen-Altlünen, Lünen, Germany

Stipends and Awards

1992	Associated Field Service (AFS) school exchange program stipend
1999	ERASMUS university exchange program stipend
2004	Brain & Mind Award for Outstanding Progress in a PhD

Personal record

1986	Graduation from elementary school Lemförde, beginning of studies at the Gymnasium
July '92- July 1993	Exchange program with intercultural organisation (AFS), living with a Mexican family in Oaxaca, Mexico Attended secondary school: URSE of Oaxaca
1993	Return to Germany and continuation of former program of studies Honorary collaboration with AFS (1993 – 1995)
June 1995	Abitur (final examination at Gymnasium Lünen-Altlünen)
June '95-Aug. 1996	Stay in Mexico German teacher at a private language institute in Mexico City
Sept. '96-Jan. 1998	Studies in Philosophy, Theatre and Latin American studies at the Free University of Berlin
Since Jan. 1998	Studies in Psychology at the Technical University Berlin (TUB), Germany

Curriculum vitae

1998	Volunteer with Amnesty International in Berlin, Germany
July/Aug. 1998	Research internship in the Department of Psychobiology at TUB, Germany Topic: *Psychophysiology of Emotions: The Paradigm Public Speech* Supervisor: Dr. Susanne Baumann
March/April 1999	Internship at *Westfälisches Zentrum für Psychatrie, Psychotheraphie und Psychosomatik* (psychiatric clinic) in Dortmund, Germany
July-Sept. 1999	Vordiplom exams in psychology, overall grade: excellent
Oct. '99- July 2000	ERASMUS student exchange program in Madrid, Spain University: Complutense de Madrid (UCM)
Nov. '99-July 2000	Internship at *DATA* (rehabilitation clinic for patients with cerebral lesions) in Madrid, Spain
Feb. '00-July 2000	Research internship in the neuroscience laboratories of the UCM, Spain Topic: *Effects of Chronic Alcohol Consumption and Acute Alcohol Intoxication on Neuronal and Behavioral Parameters in the Rat* Supervisor: Dr. Luis Miguel Garcia Moreno
Oct. 2000	Return to former studies at Technical University Berlin, Germany
Oct.'00-March 2001	Student Research Assistant at the Department of Biological Psychology and Psychophysiology at the Humboldt-University at Berlin Research area: conduction of EEG experiments on the microstructure of cognitive information processing in the research project "*Group interactions in high risk environments*" Supervisors: Prof. Dr. Werner Sommer and Dr. Jörg Sangals
March- April 2001	Internship at the Max-Planck-Institute for Brain Research, Frankfurt, Germany
June '01-June 2002	Diploma thesis student at the Department of Neurophysiology at the Max-Planck-Institute for Brain Research, Frankfurt, Germany Topic: *Response coordination: does it depend on neuronal spatio-temporal dynamics?* Grade: excellent Supervisors: Prof. Dr. Wolf Singer and Dr. Sergio Neuenschwander at the MPI and Prof. Dr. Klaus Eyferth at the Technical University Berlin
Dec. 09-16, 2001	Participation in the FENS Winter School on the "*Structure and formation of neuronal representations",* Kitzbuehel, Austria
March '03	Graduated in Psychology; overall grade: excellent
Since Oct. 2003	PhD student at the Brain Mind Institute, EPFL, Lausanne, Switzerland Supervisor: Prof. Carmen Sandi, Laboratory of Behavioural Genetics
July 2004	Participation in the Riken Summer School Program "*Learning and Memory*", Japan
November 2004	Brain & Mind Award for Outstanding Progress in a PhD
July 2005	Participation in the CSHL Summer School "*Neurobiology of Memory*", USA
Aug. 2006	Doctorate in *Neuroscience and Developmental Neurobiology*
Since Aug. 2006	Post-Doctorate at the Laboratory of Neural Ensemble Physiology, Brain Mind Institute, EPFL, Lausanne, Switzerland Supervisor: Prof. Dr. Miguel Nicolelis

Research experience

~ with humans

Department of Biopsychology and Neuropsychology, Institute of Psychology TU Berlin

- Psychophysiology of emotions (fear): acquisition and evaluation of ECG data

Department of Biological Psychology and Psychophysiology at the Humboldt-University at Berlin

- Psychophysiology of cognition (cognitive microstructure of motor preparation): acquisition and evaluation of EEG data

Westfälisches Zentrum für Psychatrie, Psychotheraphie und Psychosomatik (psychiatric clinic) in Dortmund, Germany

- Participation in various therapy forms for psychiatric patients (*Einzel-, Gruppen-, Beschäftigungs-, Entspannungs-, Arbeitstheraphie and Kreative Theraphie)*
- Participation in application of psychological tests: general intelligence tests, specific tests (attention, memory)

Departamento Laboral del Centro de Educación y Rehabilitación de Personas con Discapacidad Fìsica (DATO), Madrid, Spain

- Cared for a patient with cerebral paralysis
- Participated in developing a neuropsychological rehabilitation program for the patient

~ with animals

Department of Biological Psychology, Institute of Psychology, Universidad Complutense de Madrid, Spain

- Behavioural experiments with rats (different learning and memory paradigms)
- Neuroanatomical staining
- Data analysis and statistics

Department of Neurophysiology, Max-Planck-Institute for Brain Research, Frankfurt, Germany

- *In vivo* electrophysiology (acquisition and evaluation of multi-unit recordings, field potentials, EEGs) in anaesthetised cats
- Neurosurgery on cats
- Data analysis and statistics

Brain Mind Institute, Swiss Federal Institute of Technology, Lausanne, Switzerland

- *In vitro* electrophysiology (acquisition of intra- and extracellular activity) on rat hippocampal brain slices
- *In vivo* behavioural experiments with rats (different learning and memory paradigms)
- Surgery and microinjections of drugs into specific brain areas of the rat
- Immunohistochemical techniques

Publications

- Garcia-Moreno, LM, Conejo, NM, Capilla, A, Garcia-Sanchez, O, **Senderek, K** and Arias, JL. (2002). Chronic alcohol intake and object recognition in young and adult rats. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, 26: 831-837.
- Garcia-Moreno, LM, Capilla, A, Garcia-Sanchez, O, Luque, J, **Senderek, K**, Conejo, NM and Arias, JL. (2004). Alcohol tolerance in rats submitted to different periods of chronic an acute ethanol intake. *Psicothema*, 16: 211-216.
- **Markram, K**., Gerardy-Schahn, R, Sandi C (submitted). Selective Learning and Memory Impairments in ST8SiaIV/Polysialyltransferase Deficient Mice.
- **Markram, K**., Lopez-Fernandez, MA, Abrous, DN; Sandi, C (submitted). Amygdala Upregulation of NCAM Polysialylation Induced by Fear Conditioning is not Required for Fear Memory Processes.
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- Garcia-Moreno, LM; Capilla, A.; Garcia-Sanchez, O; **Senderek, K**. (2000). Dependencia alcohólica en alcoholismo crónico y agudo en ratas. *Congreso national de Pscicobiologia*, Oviedo, Spain (abstract and poster).

- **Senderek, K**; Baron, J; Singer, W and Neuenschwander S (2001). Response coordination: Does it depend on neuronal spatio-temporal dynamics? FENS Winterschool, Kitzbühel, Austria (abstract and poster).
- **Markram K**, Sandi C (2005). The Role of PSA-NCAM in the Amygdala for the Formation of Emotional Memories. Cold Spring Harbor Summer School on "Neurobiology of Learning", USA.
- **Markram K**, Sandi C (2005). The Role of PSA-NCAM in the Amygdala for the Formation and Extinction of Long-Term and Remote Fear- Memories. Arc Lemanique Neuroscience Conference, Les Diablerets, Switzerland.
- **Markram, K**, La Mendola, D, Rinaldi, T, Sandi, C, Markram, H (2005). Enhanced Fear and Reduced Fear Extinction in an Animal Model of Autism. Society for Neuroscience Meeting, Washington, USA.
- **Markram, K**, Sandi, C (2005). Selective Learning and Memory Deficits in PSA-NCAM Deficient Mice. FENS, Vienna, Austria.
- La Mendola, D, **Markram, K**, Rinaldi, T, Sandi, C, Markram H (2006). Hyper-fear, reduced fear extinction and weakened inhibition in the amygdala in an animal model of autism. FENS, Vienna, Austria.