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„Central amygdala control of emotional states via the
basal forebrain and bed nucleus of stria terminalis“

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1 Introduction

1.1 Emotions

Emotions are an inherent component of our mental self. They attribute value and valence to our environment, thereby representing a major driving force in our motivational behavior. An essential function of the so-called ‘affect system’ involves the instant categorization of environmental cues, based on the significance for animal survival. It triggers innate defense or approaching behavior, while at the same time providing the physiological support for engaging in these actions (LeDoux, 1995).

Studies on emotions date back to the ancient Greeks, who understood emotions as disrupters of human reason and rationality (Cacioppo and Gardner, 1999). However, this notion turned out to be a great misconception, as data from various lesion cases in the past illustrated the necessity of emotional tagging of higher cognitive processes, such as decision-making and attention (Adolphs et al., 1994; Pessoa, 2008; Anderson and Phelps, 2001).

Charles Darwin’s early contribution to the field of emotions manifested in his book, published in 1872, *The Expression of the Emotions in Man and Animals*, which seeks to trace back human characteristics of the expression of emotions to the animal world. He is now thought to be the first to suggest the presence of emotions throughout the animal kingdom, thereby legitimating animal subjects in this field (Darwin, 1872).

Yet, a distinction has to be made when dealing with the term ‘emotions’. Since simply not measureable in non-vocalizing species, it must be separated from the subjective experience upon encounter of an emotionally-charged event, which is hence referred to as a conscious ‘feeling’. In neuroscience ‘emotion’ is generally conceived as a mental state that elicits behavioral strategies and physiological responses in an animal, independent of what was actually perceived. Basically, this notion separates subjective mental states (‘basic emotions’), which are assumptions from a human perspective, from the fundamental survival functions of emotions (LeDoux, 2012a).

In psychology there have been various attempts to categorize the multitude of human basic emotions, thereof the most highly and continuously appreciated model was coined by James Russell in 1980, termed *The Circumplex Model of Affect* (Russell, 1980). Because a fundamental component of social interactions in the life of every individual is the recognition and interpretation of emotional expressions and behavior, Russell hypothesized that a cognitive map of principal emotion categories must exist in the human brain. Based on categorization tasks of emotionally tagged words, these results led him to the proposition of a model, where similar emotional categories would cluster in the same quadrant and where opposite emotions, such as fear and calmness, would also oppose each other, in a bipolar parameter space (Calder et al., 2001; Russell, 1980). Interestingly, in this model the vertical and horizontal axis very well correlate with two parameters of basal emotions, which are valence and arousal, respectively. In conclusion, any emotion can be assigned an intrinsic valence and degree of arousal, that can be elicited by environmental stimuli (fig.1.1a, taken from Calder et al., 2001).

In neuroscience the theory of emotions was further reduced to mental states that are evoked by stimuli, traditionally thought to be relevant for animal survival. According to Rolls, these stimuli comprise positive or negative reinforcers, which themselves are represented by innate or learned rewards or punishers. In graph 1.1b, ascending or descending from the origin on the vertical axis illustrate emotional states, evoked by rewarding (S^+) or fearful cues (S^-), respectively. Similarly, states that are elicited by the termination or omission of the positive ($\bar{S}^+$ or $S^+!$) or negative ($\bar{S}^-$ or $S^-!$) reinforcers are indicated along the horizontal axis (Calder et al., 2001). This conceptual framework seems more appropriate in meeting the assumptions that are inherently made when studying emotions in animals, as mentioned above.

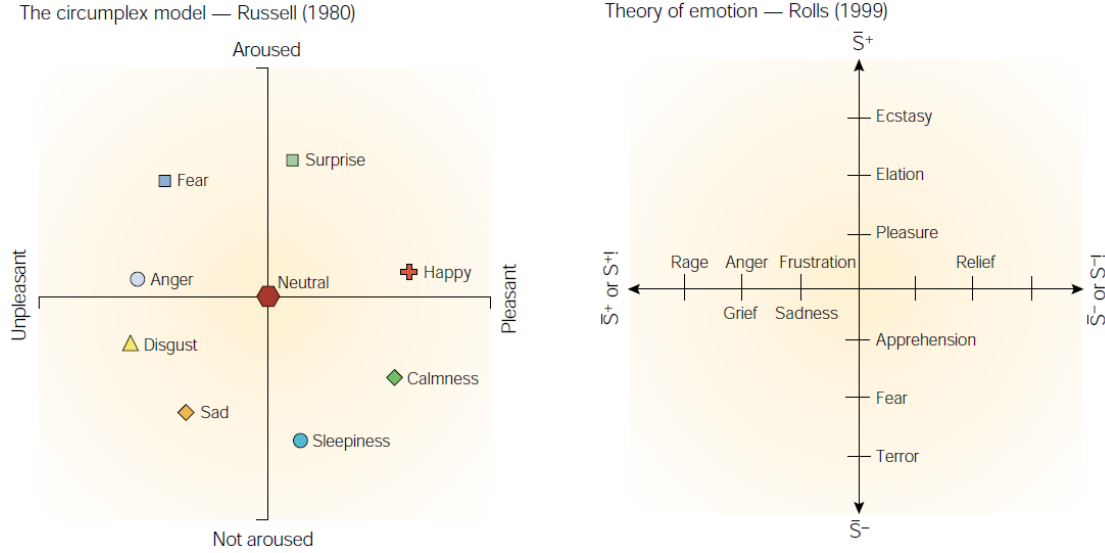


Figure 1.1: Theory of emotions in psychology and neuroscience by Russell (left) and Rolls (right), respectively. Figure taken from Calder et al. (2001)

A long-standing question in neuroscience concerns the neural substrates of emotions. In 1949, this issue seemed to be solved by the inception of the Limbic System Model by Paul MacLean (MacLean, 1949, 1952). After Paul Broca first coined the term ‘limbic’ in his work *Le grand lobe limbique* in 1889, MacLean formally introduced the Limbic System Concept as part of his triune brain theory, in 1952 (MacLean, 1952). Based on evolutionary and anatomical considerations the brain can be functionally divided into three major structures, which evolved sequentially: ‘The Reptilian Complex’ (basal ganglia and brainstem), the ‘Paleomammalian Complex’ (the Limbic System) and the ‘Neomammalian Complex’ (the Neocortex), which was believed to represent a mammalian singularity. According to MacLean, parts of the limbic system include subcortical structures like the septum, amygdala, hypothalamus, hippocampus and the cingulate cortex, which, due to their primordial origins in mammalian evolution, would be the key mediators of the ‘primitive’ emotions and function in isolation of other structures, such as the neocortex, which on its own was conceived as the core of cognition (LeDoux, 2012b). Nevertheless, soon upon its introduction the limbic system was repeatedly challenged as the sole mediator of emotions, which led to continuous patching of its obvious shortcomings. Major issues involved the hippocampus, the purported center of the limbic system, as it was shown to be essential in allegedly pure cognitive functions, such as memory. As well as the fact that rudimentary forms of cortices are present in birds and reptiles is refuting the

assumption of a mammalian peculiarity regarding the neomammalian complex. These and other findings rendered the concept obsolete in the functional sense, while still being appreciated in aspects of brain evolution (LeDoux, 2000).

Despite its persistence, the limbic system concept is slowly being eroded and major progress in the field of emotions, especially fear, has been made since then. This can be chiefly attributed to the advent of *Pavlovian Conditioning* as a means for mapping out specific structures and circuits underlying this paradigm (see section 1.2). The neural pathways of fear have been resolved in great detail, which makes it the most busy area of research on emotions. Much less is known about positively charged emotions, which was in part due to the lack of specific methods in the past (LeDoux, 1998). A classical paradigm in studying these is appetitive conditioning, which triggers innate approach behavior and is now applied in studying positively-charged emotions (LeDoux, 2012a). Not surprisingly, fear circuits partly correspond with the limbic system and one of the major hubs in fear and other emotion states, such as anxiety, is the amygdala. It is one of the most highly interconnected nuclei in the brain and was shown to be essential in the Pavlovian fear conditioning paradigm (Pessoa, 2008; LeDoux, 2000).

1.1.1 Fear and Anxiety

Fear and anxiety are conceived as emotions that support individual survival by avoiding threatful situations. Although the terms were often used ambiguously and interchangeably in the literature, a growing body of evidence suggests these emotions are fundamentally different. Strong indications for their separate neurobiological underpinnings come in part from varying sensitivity of different anxiety disorders to anxiolytic drugs, which points towards specificity of certain drug classes for fear- or anxiety-related disorders (Sartori et al., 2011). Adding to this, the impact on pain diverges, as fear blunts, whereas anxiety enhances self-reported pain perception. Nevertheless they share characteristics, such as their negative valence or autonomous arousal (see fig.1.8). Conceptually, both fear and anxiety can be further subclassified in a ‘trait’ and ‘state’ domain, depending on the conceptual framework. In clinical science, the primary focus is on trait emotions, as it describes a characteristic of a genetically predisposed individual that may display a

particular emotion to a pathological extent. Conversely, neuroscience is mostly concerned with ‘state emotions’, which describe the typical response to a particular event within a species (Sylvers et al., 2011).

Psychology and neuroscience have demarcated fear and anxiety similarly, although differences are more clearly defined in the latter. According to both concepts, fear is expressed to an acute environmental cue that elicits various behavioral responses, such as freezing or escape. Consequently, individuals perceive fear in situations, when actively coping with a threatful situation. Upon removal of the stimulus, fear dissipates rapidly.

In contrast, anxiety is future-oriented and emerges from the uncertainty of a diffuse threat, which is therefore not predictable. It is considered that anxiety may also result from unresolved fear, meaning the inability to avoid acute aversive events may lead to elevated states of vigilance (e.g. stress state). As a consequence, a major characteristic of anxiety is that it can appear over an extended period of time and is therefore synonymous with sustained or tonic fear, as opposed to phasic fear (Sylvers et al., 2011).

Experiments on healthy humans and patients, suffering from various trait anxiety disorders, took advantage of the differential vulnerability among these disorders to predictable (fear-related) and unpredictable (anxiety-related) aversive events. Based on this classification, Panic Disorder (PD) and phobias belong to the former, exhibiting elevated responses to a conditioned (cued) stimulus, whereas Post-Traumatic Stress Syndrome (PTSD) and generalized anxiety disorder (GAD) display elevated responses to diffuse, more context-related stimuli. Taken together, the predictability and general timing properties of a stimulus play an instructive role in which neural substrates are engaged in a given situation (Davis et al., 2010).

Probing Fear and Anxiety in Animals

Behavioral tests can be divided in two major groups: conditioning procedures rely on learning, whereas unconditioned tests harness ethological traits, such as the innate avoidance behavior towards brightly lit, open spaces in rodents. Classic examples for unconditioned tests are the elevated plus-maze and open-field test that specifically measure the anxiety component of an animal (see section 2.2.8). Learning procedures, such as Pavlovian fear conditioning (see 1.2), are more suitable for measuring acute fear responses to

a defined stimulus. Importantly, these tests rely on memory formation, thus a low fear response may also result from impaired fear memory, instead of low fear levels per se (Sartori et al., 2011).

1.2 Pavlovian Fear Conditioning

Pavlovian Conditioning is a training procedure first described by the Russian physician Ivan Pavlov in 1927 (Pavlov, 1927). Initially described as a form of appetitive conditioning as the basis for the salivation reflex of Pavlov's dogs, appearing upon presentation of the sound of a bell, it is now the crucial paradigm in deciphering the circuit interactions underlying (learned) fear. The procedure describes a principal mode of operation of the brain, trying to anticipate events in the environment, based on prior experience, thereby allowing for avoidance of potential danger or approaching a possible food source. The capability of identifying such cues as relevant is a major determinant in animal survival and is accordingly conserved across many different phyla of the animal kingdom, ranging from worms to humans. It has therefore markedly aided in understanding the fundamental processes of associative learning and memory formation (Fanselow and Poulos, 2005; LeDoux, 2000)

Fear conditioning is composed of two initially independent environmental cues, which become paired during the procedure. One, the conditioned stimulus (CS), is initially neutral and thus does not trigger any behavior in the first place. Usually the CS is presented as a sound, although in principle any cue perceivable, being visual, olfactory or contextual in nature, can serve the purpose. In contrast, the second, unconditioned stimulus (US), is emotionally charged. It elicits behavioral responses without any previous experience or learning. In fear conditioning this stimulus is typically presented as a mild footshock that is perceived as aversive pain. Upon successive presentations of these stimuli, the CS preceding the US, the CS gains access to the innate circuitry mediating the US responses, which renders it aversive (see fig.1.2).

Ultimately, the CS is associated with the noxious experience of the US and capable of triggering behavioral responses, such as freezing or escape, and autonomous responses, such as tachycardia, hypertonia and endocrine signaling (LeDoux, 2000).

Alongside conditioning to an explicit CS, contextual cues in the background (e.g. olfactory or visual stimuli) become inevitably incorporated into the aversive experience as well, which is termed (background) context conditioning. Thus the exposure to the mere context, in which the initial CS-US pairing or US alone occurred, is capable of triggering the same behavioral and autonomous responses as the actual US (Blanchard et al., 1970; Phillips and LeDoux, 1992). This requires caution in designing the conditioning and testing environment when testing an explicit CS.

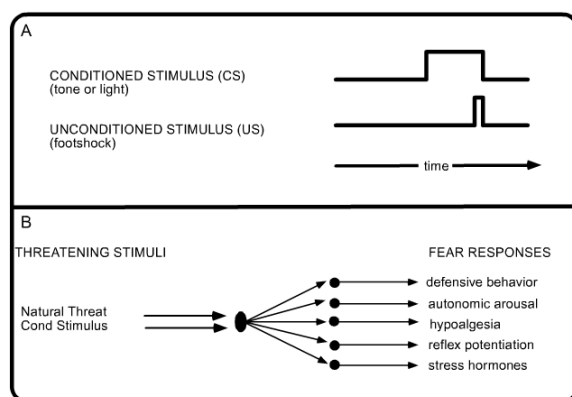


Figure 1.2: **a** Illustration of the fear conditioning paradigm. A neutral stimulus (CS) is followed by an aversive US, making innate behavioral and physiological responses accessible to the CS. **b** Summary of responses triggered by an intrinsically aversive cue. Figure taken from LeDoux (2000)

1.2.1 Fear Extinction

Extinction refers to a process, where the expression of a once acquired fear memory ceases. Repeated non-reinforced presentation of a CS, being contextual or explicit, renders the CS continuously less predictive for the US, thereby diminishing fear responses, a phenomenon first described by Pavlov (Pavlov, 1927). Importantly, different mechanisms of fear extinction may exist that are unrelated to an actual erasure or ‘unlearning’ of memory, but rather require an additional learning event, without destroying the previously acquired US-CS association (Herry et al., 2010). This conclusion was drawn because fearful memories usually persist for long periods of time, whereas extinction specifically requires the presence of the CS that rapidly blunts fear responses. Along these lines of evidence, an extinguished fear response can recover spontaneously and can be reinstated by re-presentation of the US alone, pointing towards the prolonged existence of the memory trace, masked by the mere prevention of its expression (Myers and Davis, 2007). Interestingly, extinction is cue-specific, meaning that an extinction procedure to

a specific CS does not generalize to a second CS, even though initially conditioned to the same US. This important feature also applies to contextual cues, meaning that the extinguished fear expression to a CS can be renewed by the context, in which the training occurred (fear renewal) (Bouton and Bolles, 1979). The neural substrates of extinction have been studied extensively and are discussed in section 1.3.1.

It is fear conditioning that paved the way for understanding the functional basis for facilitation of a conditioned fear response. A key role was thereby assigned to the amygdala. Representing the neural substrate of (learned) fear, where CS and US converge to form associative memory, the amygdala was shown to innervate virtually all structures that mediate the physiological and behavioral responses induced by a noxious stimulus (Fanselow, 1994; LeDoux, 2012b). This pivotal position in the circuit and the convincing findings in several model organisms also led to the implication of the amygdala in various psychological conditions in humans, such as fear- and anxiety-related disorders (Aggleton, 1993; Kim et al., 2011; Lederbogen et al., 2011).

1.3 The Amygdala

The amygdala is located in the medial temporal lobe of mammals and comprised of many heterogenous nuclei. These subnuclei have been defined based on histological criteria, such as connectivity, neurotransmitter content and cell/neurite morphology. The conception of the amygdala as a single anatomical unit has been questioned in the past on various grounds. This is also reflected by the conflicting and redundant nomenclature applied to partly overlapping subnuclei (LeDoux, 2007). Criticism was drawn by the historical definition of the structures that, based on anatomical characteristics, do not form a functional unit, but rather represent an arbitrary subsumption of sets of phylogenetically different nuclei. On the basis of histochemical properties, mostly immunohistochemistry for γ -aminobutyric acid (GABA), and circuit architecture, the amygdala can be seen as a collection of three developmentally differentiated subdivisions, which are of cortical, striatal and claustral origin. The latter can be seen as a cortical specialization and is hence subsumed into the cortical portion (Swanson and Petrovich, 1998).

The major cortical components of the amygdala include the Lateral Amygdala (LA),

Basal Amygdala (B), Accessory-Basal Amygdala (AB) and Cortical Amygdala (CO), of which the LA and B are often collectively termed the Basolateral Complex (BLA) (LeDoux, 2000). The striatal components are represented by the Central Amygdala (CE) and the Medial Amygdala (M), both being conceived as a differentiated striatal extension. This classification is backed by the neurotransmitter composition and cytoarchitecture, which shows predominant GABAergic projection neurons in the CE and M, akin to the striatum. In contrast, the LA is dominated by glutamatergic pyramidal neurons, resembling a cortical make-up. Neuropeptides are mainly concentrated in the striatal portion of the amygdala and are, apart from local interneurons, virtually absent in cortical structures, such as the presumed cortical portion of the amygdala (Swanson and Petrovich, 1998).

Every single aforementioned nucleus can be further resolved into multiple subdivisions, which are thought to represent individual processing units that are connected via inter-nuclear, intra-nuclear and intra-divisional circuits, which themselves again form unidirectional and reciprocally connected macrocircuits with cortical and subcortical structures outside of the amygdala (Ehrlich et al., 2009). This is exemplified within the LA, whose dorsolateral subnuclei receive fast-responding thalamic input upon an auditory stimulus, while the medial divisions of the LA receive higher-order cortical information (fig.1.3a *lower-right*; Pitkänen et al., 1997).

In figure 1.3a (*bottom-left*), the general scheme of inter-nuclear information flow within the amygdala is illustrated. As mainly revealed by tracing the auditory CS in fear conditioning, sensory information primarily enters the amygdala in the LA and is conveyed to nuclei B, AB and CE. B and AB themselves project to the CE, which qualifies the central amygdala as the major output structure (LeDoux, 2000). In addition, the BLA connects with the CE indirectly via the Intercalated Cell Masses (In) of the amygdala, emphasizing the general scheme of cortico-striatal directionality of information flow.

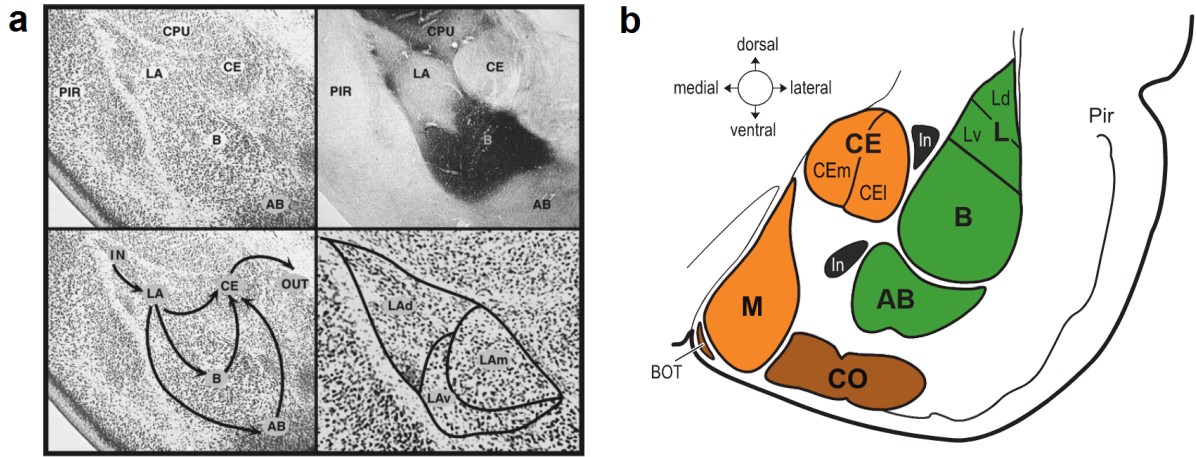


Figure 1.3: **a** Different stainings on adjacent coronal sections illustrate the nuclear composition of the amygdala. (*upper-left*, Nissl-staining; *upper-right*, Acetylcholinesterase-staining; *lower-left*, Connection schematic of major amygdala subnuclei; *lower right*, Magnification of LA, showing subdivisions dLA, vLA and mLA) Figure taken from LeDoux (2000) **b** Illustration of the amygdala subnuclei. The color code distinguishes three groups (see text; orange: striatal nuclei, green: claustral nuclei, brown: cortical nuclei). Figure adapted from Knapska et al. (2007). **Abbreviations:** L, LA, Lateral Amygdala; B, Basal Amygdala, AB; Accessory-Basal Amygdala; CO, cortical amygdala; CE, central Amygdala; M, medial amygdala; In, intercalated nuclei; BOT, bed nucleus of the olfactory tract (BOT), Pir, piriform cortex.

In contrast to the requirement of the LA for conditioning to an explicit tone, background context conditioning in addition requires the ventral hippocampus and the B and AB nuclei, which relay the context information to the amygdala output nucleus CE (Phillips and LeDoux, 1992; Goosens and Maren, 2001). This scheme of overall serial processing, from the LA to the CE, has been complemented by a study on appetitive conditioning, which demonstrated the potential for parallel encoding of different aspects of a stimulus. This further emphasizes differential functions of cortex- and striatum-like amygdalar nuclei (Balleine and Killcross, 2006).

Yet, a distinction has to be made between acquired and innate fear, as there are stimuli that are inherently aversive and do not require a learning event. These stimuli are processed along parallel pathways that are hard-wired in the brain and mainly include visual, olfactory and vomeronasal cues that are associated with potential predators or aggressive conspecifics. The amygdala can be seen as a control system that integrates distinct environmental cues and subsequently coordinates appropriate reactions. As opposed to the requirement of the LA-CE pathway for learned fear, cues intrinsically bearing emotional value, specifically require the medial amygdala (M) and are processed along

distinct pathways in the hypothalamus (Swanson and Petrovich, 1998). Subregions in the periaqueductal grey (PAG), in turn, mediate the expression of the proper behavioral strategies, depending on the imminent cue (see summary in fig.1.4). There is evidence for conservation of these parallel routes of processing across species, at least for mammals, although details that account for species-specific peculiarities must be kept in mind (Gross and Canteras, 2012).

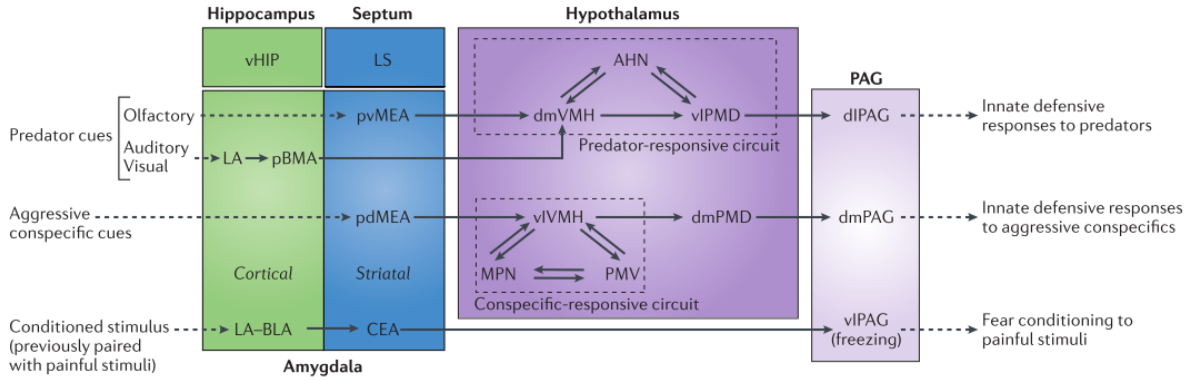


Figure 1.4: Summary of evidence for parallel processing pathways for learned and innate fear of various sensory cues. Olfactory and non-olfactory cues reach the amygdala at different sub-regions in the medial amygdala and require various hypothalamic nuclei for initiation of a fear response by the periaqueductal grey (PAG). In contrast, learned fear, as obtained in the Pavlovian fear conditioning paradigm, requires the lateral and central nucleus of the amygdala, targeting a distinct subregion in the PAG. **Abbreviations:** VMH, ventromedial hypothalamus; PMD, dorsal premammillary nucleus; PAG, periaqueductal grey; LA, lateral amygdala; BMA, basomedial amygdala; MEA, medial amygdala; AHN, hypothalamic nucleus; MPN, medial pre-optic nucleus; PMV, ventral premammillary nucleus; CEA, central amygdala; BLA, basolateral complex; LS, lateral septum; HIP, hippocampus. Figure taken from Gross and Canteras (2012).

1.3.1 Fear Learning in the Amygdala

In order for a CS to acquire functional properties of a US, learning has to occur. Although changes in the structure of synapses in various regions in the brain have been observed upon fear conditioning, most is known about the learning mechanisms in the LA (Letzkus et al., 2011). Responsive cells to nociceptive and auditory stimuli have been observed in the LA_{dl} , a widely accepted prerequisite for forming associations (Pitkänen et al., 1997; LeDoux, 2000). The fundamental view on learning mechanisms in neuroscience is represented by the Hebbian model of plasticity, a hypothesis initially proposed

by Donald Hebb in 1949 (Sejnowski, 1999). Temporally correlated weak presynaptic input to a strongly depolarized postsynaptic neuron results in molecular changes of the respective co-firing weak synapse, which in turn facilitates strengthening of this synapse, a process termed long-term potentiation (LTP). Accordingly, strong depolarization of LA pyramidal neurons by an impacting nociceptive US produces structural changes in concomitantly firing synapses, delivering the CS. Various receptors, cellular signaling cascades and neurotransmitter systems seem to be involved in mediating Hebbian plasticity (Johansen et al., 2010, 2011). Although a definite picture is still lacking, pharmacological studies have pointed toward a *N*-Methyl-D-Aspartate (NMDA)-receptor dependent mechanism for thalamo-LA synapses, as its blockade interferes with memory acquisition, but not expression (LeDoux, 2000). Cortico-LA synapses are functionally and morphologically different and were shown to express plasticity by a NMDA-independent, presynaptic mechanism (Humeau et al., 2005).

Despite the evidence for glutamatergic plasticity, the importance of inhibitory networks within the amygdala macrocircuitry, shaping the acquisition, maintenance and extinction of fear memories, has become apparent. GABAergic interneurons substantially impact LTP in other neurotransmitter systems, such as the thalamo- and cortico-LA synapses. These neurons provide feedback and feed-forward inhibition onto principal neurons and, being subject to neuromodulation, they constitute a major factor in modeling information processing in the amygdala, depending on the behavioral and physiological state of the animal (Ehrlich et al., 2009). Rhythmic activity, such as theta- and gamma-oscillations, are largely attributed to the function of interneurons. The amygdala was shown to be entrained with these rhythms with various other brain regions, depending on the memory task, being acquisition, retrieval or extinction (Paré et al., 2002; Pelletier and Paré, 2004). More specifically, entrainment of LA, IL and CA1 of the hippocampus correlates with fear retrieval, whereas theta-coupling decreases over the course of extinction training. Most importantly, artificial maintenance of entrainment of LA and CA1 throughout extinction training showed continuous fear expression, pointing towards sufficiency in trained animals. It is thought that coupling of LA, IL and CA1 does correlate with the success of fear conditioning, which is indicative of a role in memory consolidation. Notwithstanding the apparent necessity of these oscillations, their precise function remains undefined,

although speculations suggest the generation of a general spatiotemporal code for the coordination of long-range interactions by allocating time windows for communication (Lesting et al., 2011).

Neural Mechanisms of Extinction

The most important mediators of fear extinction are thought to be the hippocampus, prefrontal cortex and the amygdala, although the mechanism remains largely undefined. This can in part be attributed to the fact that differential mechanisms are employed depending on the time interval between training and extinction (Myers and Davis, 2007). Generally it is thought that extinction memory is distributed among different regions of the brain, while additionally, acquisition and consolidation require differential sets of these. Acquisition seems to depend on the basal amygdala (B), which reciprocally connects to the medial prefrontal cortex (mPFC) and hippocampus. There is evidence that B harbours two populations of neurons, which are active differentially, in a high or low fear state, suggesting distinct long-range extinction and fear-pathways to the mPFC and hippocampus (Herry et al., 2008). On the other hand, expression of extinction seems to require activity in the mPFC, specifically the infralimbic cortex (IL), which exerts excitatory drive to the intercalated cell masses (ITCs), cell clusters located between BLA and CE (Sotres-Bayon and Quirk, 2010; Herry et al., 2010). This picture is corroborated by the finding that ITC themselves are essential, as their ablation resulted in blunted expression of extinction (Likhtik et al., 2008).

In summary, changes within the BLA include glutamatergic plasticity during acquisition, followed by enhanced inhibition by interneurons during expression. Additionally, ITC activity is required for expression of extinction, probably by feed-forward inhibition of projections from the BLA to the central nucleus (CE) and by direct activation by the IL and probably also other structures (Ehrlich et al., 2009). Finally, IL activation also inhibits the medial division of the CE (CEm), the major output structure of the amygdala (Herry et al., 2010; Bienkowski and Rinaman, 2013).

1.3.2 The Central Amygdala

The Central Amygdala (CE) is part of the striatal portion of the amygdala and serves as the major output station of the amygdala circuitry. In the rat the CE can be subdivided into medial (CE_m), lateral, capsular and ventral divisions, while for functional reasons the latter three are commonly referred to as the lateral nucleus of the CE (CE_l) (Cassell et al., 1999). Particularly the CE_m seems to be the executive subdivision in eliciting the final motor and autonomous responses upon a CS, as suggested by its extensive projections to the brainstem and hypothalamus. CE is predominantly made up of GABAergic neurons showing medium spiny neuron-type morphology. In addition, numerous neuromodulators and neuropeptides are found especially in the CE_l , emphasizing its potency in modulating information processing within CE and in its target regions. Neuropeptides include the endogenous opioids dynorphin and enkephalin, somatostatin, substance P, neurotensin and corticotropin-releasing hormone (CRH; also known as corticotropin-releasing factor, CRF) (Cassell et al., 1986). Interestingly, another element of asymmetry within the CE is provided by differential innervation of the monoaminergic system. Whereas the CE_l is predominantly innervated by dopaminergic fibres, the CE_m is dominated by noradrenergic fibres (Freedman and Cassell, 1994). This innervation pattern is replicated in other basal forebrain structures, such as the lateral and ventrolateral Bed Nucleus of Stria Terminalis ($BNST_l$ and $BNST_{vl}$, respectively) and therefore represents a general scheme for the extended amygdala macrocircuitry (see section 1.4).

Electrophysiological data suggests that CE is primarily composed of four distinct cell types, based on firing pattern. The most abundant neurons in the rat CE seem to be low-threshold bursting neurons, followed by regular spiking, late-firing and fast-spiking neurons. Among different species, there is a large variation in the proportion of these cell types in the CE. This is considered causally related to the diverse autonomous and behavioral strategies observed in different species (Dumont et al., 2002).

The CE_m and CE_l differ markedly in their long-range afferent and efferent projections, indicated by unidirectional connectivity within the CE, running from CE_l to CE_m . Apart from the innervation of the parabrachial nucleus (PB) by the CE_l , only CE_m innervates brainstem regions intensely, such as the periaqueductal grey (PAG), a key region mediat-

ing the motor response for suppressing ongoing behaviors, resulting in immobility ('freezing') (De Oca et al., 1998). Instead, major projections of the CE_l run along the *ansa peduncularis* and *stria terminalis* to the antero-lateral Bed Nucleus of Stria Terminalis ($BNST_{al}$), the CE_m and most prominently to the Substantia Innominata (SI), a region in the basal forebrain harboring the Nucleus Basalis of Meynert (NBM). Interestingly, most sensory modalities, either thalamic or cortical, represent at least indirect afferents of the CE_l (Petrovich and Swanson, 1997). Adding to this fact, nociceptive information from the spino(trigemino)–parabrachio–amygdaloid pathway reaches the CE_l , which renders it another site of CS-US convergence for forming associations. Indeed, the CE was shown to be required for acquisition of conditioned fear and exhibits the potential for plasticity via NMDA-dependent mechanisms, as its reversible inactivation during training interfered with fear learning (Samson and Paré, 2005; Wilensky et al., 2006; Cioocchi et al., 2010).

In order to understand the mechanisms of fear processing within the CE, cellular interactions must be studied on the microcircuit level. Evidence for functional segregation of distinct cell types came from the discovery of antagonistic modulation of neuronal activity by the peptide hormones/neuromodulators oxytocin (OT) and vasopressin (Vp). Receptors are spatially segregated, the OT-receptor confined to the CE_l , whereas the VP-receptor is located in the CE_m . OT selectively increases firing frequency in 21%, but simultaneously decreases it in 50% of neurons specifically in the CE_l and in neurons of the CE_m . Conversely, Vp increases firing rate of neurons in the CE_m , not affecting neurons in CE_l , which suggests an inhibitory network within the CE (Huber et al., 2005). Endogenous OT in the CE was shown to originate from hypothalamic efferents and strikingly exerts its indirect, inhibitory effects on PAG-projecting CE_m output neurons only. Thus the effect of OT is restricted to the behavioral domain of a fear response, ignoring the autonomous component that is mediated by a distinct neuronal population in the CE_m (Viviani et al., 2011; Knobloch et al., 2012).

An Inhibitory Microcircuit in the CE_l Gates Conditioned Fear

The differential role of neurons in the CE_m and CE_l was confirmed by the discovery of a (dis)inhibitory microcircuit in the CE that is capable of switching fear responses in the mouse. The marker Protein Kinase C- δ (PKC- δ) is expressed in the thalamus, the CA3 of the hippocampus in addition to approximately 50% of GABAergic neurons that correspond to the late-firing population in the CE_l (fig.1.5, see above).

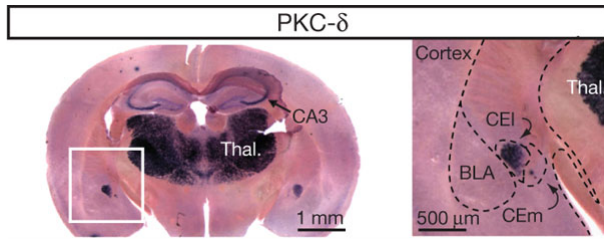


Figure 1.5: *In situ* hybridization for PKC- δ mRNA, showing expression in the CE_l , thalamus and CA3 of the hippocampus. Figure taken from Haubensak et al. (2010).

Strikingly, 65% of PKC- δ^+ neurons express the OT-receptor (see section 1.3.2) and are distinct from CRH-expressing cells. Optogenetic and pharmacogenetic experiments identified the wiring diagram, which shows reciprocal inhibitory connections between PKC- δ^+ and PKC- δ^- cells. In addition, PKC- δ^+ cells unidirectionally innervate the CE_m . Upon impact of a CS in fear conditioned animals, PKC- δ^+ are inhibited, while in contrast, PKC- δ^- are excited by the CS (Haubensak et al., 2010). Hence, they correspond to CS_{off} and CS_{on} units, respectively, which were described as distinct subpopulations based on their activity pattern upon a CS presentation (Ciocchi et al., 2010). Under basal conditions PKC- δ^+ neurons are active, tonically inhibiting the PAG-projecting output neurons in the CE_m and their functional counterpart in the CE_l , thereby preventing a fear response. An incoming CS from the BLA or the thalamus excites PKC- δ^- cells, which in turn inhibit PKC- δ^+ cells, which leads to disinhibition of CE_m output, triggering a fear response (fig.1.6). Thus, the relative activity of these subpopulations, determined by inputs and behavioral state, could trigger the ultimate behavioral output. The finding that lesioning of CE_l specifically leads to unconditioned freezing corroborates the model of inhibitory gating of CE_m by CE_l (Ciocchi et al., 2010). Paradoxically, GABAergic CS_{on} units project to CE_m as well, yet showing the inverse effect of PKC- δ^+ on CE_m neurons (Haubensak et al., 2010).

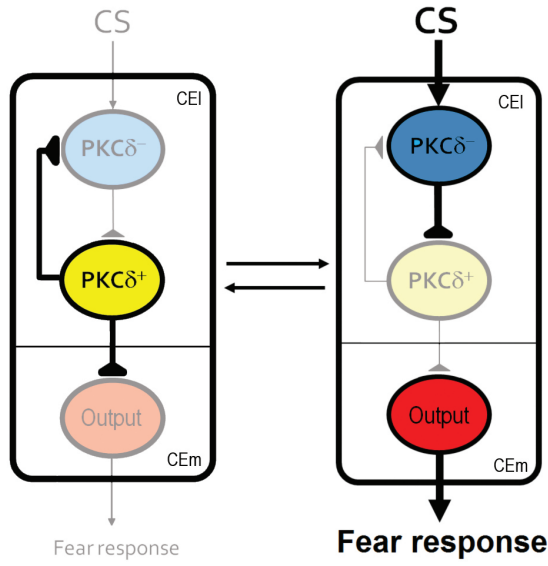


Figure 1.6: Illustration of the mechanism of inhibitory gating in the CE. An incoming CS selectively activates PKC- δ^- neurons that are tonically inhibited by PKC- δ^+ neurons in the absence of the CS. Activation of PKC- δ^- inhibits PKC- δ^+ neurons, thereby disinhibiting output neurons in the CE_m, eliciting a fear response.

1.4 The Extended Amygdala

The Extended Amygdala (EA) is a conceptual macrostructure, introduced by George Alheid and Lennart Heimer in 1988 (Alheid and Heimer, 1988; Olmos and Heimer, 1999). The pivotal discovery was the striking resemblance in connectivity, structural and neurochemical organization of the dorsal to the ventral striatopallidal system (striatum and globus pallidus) of the basal ganglia. This make-up is considered a general scheme of the genetic building blocks that construct the basal forebrain and has led to the functional extension of this concept to the hippocampal-septal-diagonal band complex and the EA. This view was corroborated by the finding that these individual systems show few interactions/overlaps and largely differ in the source of information they process. As well, the general information flow in the basal forebrain circuitry seems to be widely conserved across circuits. Generally, cortex(-like), excitatory projections reach spiny GABAergic neurons, which relay to leptodendritic, spine-poor GABAergic neurons that innervate brainstem and hypothalamic targets (Alheid, 2003).

The EA encompasses the striatal portion of the amygdala (CE and M), the bed nucleus of the stria terminalis (BNST) and sublenticular substantia innominata (SI). The fibre tract *stria terminalis* consists of at least two separate columns that connect the CE and

M with the BNST_l, SI and BNST_m, respectively (Cassell et al., 1999). This finding has led to the definition of a central and a medial portion of the EA (EA_c, EA_m). Although the concept of an EA_m is contested, the notion of an EA_c is widely accepted and includes the CE, BNST_l and the SI (Alheid, 2003, figure 1.7).

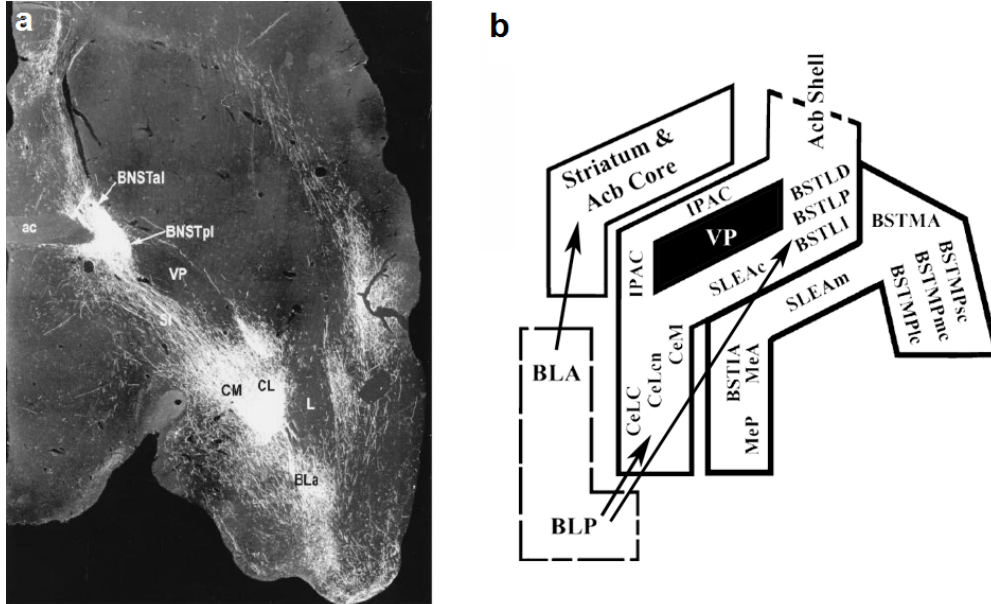


Figure 1.7: **a** Deposition of the anterograde tracer biotinylated dextran amine (BDA) into the BLA_p (not shown). The horizontal section shows fibres ascending through the BLA_a and terminating in the CE, whereas a subfraction proceeds through the CE and SI to innervate the BNST_l. Figure adapted from Walker et al. (2003); **b** Illustration of the Extended Amygdala macrocircuit. CE and M innervate the BNST in separate columns. The CE, M and BNST thereby form a continuum, giving rise to the SLEA and IPAC that surround the VP. The magnocellular BLA_a selectively innervates structures of the basal ganglia, whereas the BLA_p marks the origin of the central extended amygdala. Figure adapted from Alheid (2003). **Abbreviations:** BL/BLA, Basolateral Amygdala; C/Ce, Central Amygdala; SI, Substantia Innominata; L, Lateral Amygdala; BST/BNST, Bed Nucleus of stria terminalis; ac, anterior commissure; Acb, Nucleus Accumbens; SLEA, Extended Amygdala, sublenticular part; Me, Medial Amygdala; VP, Ventral Pallidum; IPAC, Interstitial Nucleus of the Posterior Limb of the anterior commissure;

The CE and BNST are highly related in terms of their anatomy, neurochemistry, development and cytoarchitecture and in addition share many of afferent and efferent projections (Walker et al., 2003; Bienkowski and Rinaman, 2013). As noted by Alheid (2003), a neuromarker found in the lateral or medial portion of the CE, can be readily found in the respective subnucleus of the BNST, which is also true for CRH. The basolateral amygdala (BLA), especially the parvocellular, posterior part (BLA_p), projects

to both the CE_l and to the $BNST_l$, marking the cortex-like origin of the EA (fig.1.7). Importantly, fibres from the BLA_p to the BNST proceed through the CE, questioning lesion/stimulation studies on the CE in the past, that might have also destroyed/activated fibres of passage (Tye et al., 2011). Despite their marked similarities, there is a compelling body of evidence that CE and BNST are involved in different aspects of a fear response (Davis et al., 2010).

1.4.1 Bed Nucleus of Stria Terminalis

The BNST is a large conglomerate of 30 subnuclei with heterogenous appearance. Developmentally and cytoarchitecturally it was divided into an anterior and posterior portion and due to highly topographic amygdalar innervation into a lateral and medial portion (BLA , CE and M, respectively). In accordance with the EA-concept, the posterior and antero-medial BNST are primarily innervated by the M, associated with the accessory olfactory system, whereas the $BNST_{al}$ is innervated by the CE_l primarily (Dong et al., 2001a). Of particular interest are the oval and the fusiform nuclei ($BNST_{ov}$ and $BNST_{fu}$) of the $BNST_l$, as they receive dense CRH-positive projections from the CE_l . In fact, together with CE subnuclei they form a highly interconnected GABAergic, CRH^+ (apart from CE_m) macrocircuit, as depicted in figure 1.8b. As described previously, CE and BNST share many of their efferent targets, which is, upon activation, reflected in their related autonomous and behavioral outcome, with the exception of the almost exclusive innervation of the CRH^+ paraventricular hypothalamus (PVH) by the $BNST_{ov/fu}$ (see fig.1.8a, Dong et al. (2001b)). The PVH is thought of as the entry point to the stress-reactive circuitry in the central nervous system (CNS), where it effects the physiological adaptations required to maintain homeostasis via direct central pathways in brainstem nuclei and spinal cord and indirectly by initiating an endocrine signaling cascade on the interface to the blood circulation, termed the hypothalamo-pituitary-adrenal axis (HPA axis, see section 1.4.1; Herman et al. (2005)). Nevertheless, CRH in the CNS, mediated by CRH receptors (CRH_1R and CRH_2R), is crucial for both pathways and was shown to have similar effects on memory as peripheral stress hormones (McGaugh, 2004).

The PVH itself is activated by distinct classes of stressors that can be categorized ac-

cording to their ‘limbic’ or ‘non-limbic’ origin. Non-limbic stressors include general interoceptive disruptions, such as visceral distress or infections that do not require further processing and therefore ascend from brainstem structures. Limbic stressors, on the contrary, relate to exteroceptive stressors/threats that require processing of various sensory modalities in order to determine the relevance of the stimulus (Hammack et al., 2010). Limbic structures that are known to regulate stress responses, such as the hippocampus, mPFC and the amygdala, have no direct access to the PVH-mediated stress circuitry, therefore the BNST represents the gateway to the stress effector system for the limbic system (Herman et al., 2005). However, BNST nuclei differentially impact the stress axis. The BNST_{al} was shown to activate the HPA axis, whereas the BNST_p is involved in its downregulation, thereby maintaining homeostasis (Choi et al., 2007).

The Hypothalamo-Pituitary-Adrenal Axis

The hypothalamo-pituitary-adrenal axis (HPA axis) is a neuro-endocrine interface, that mediates physiological adaptations to homeostatic challenges, as well as to the circadian rhythm. The HPA axis is initiated by the release of CRH and/or arginine vasopressin (AVP) by PVH neurons into the hypophyseal portal vein on the level of the median eminence. CRH in the portal circulation, in turn provokes release of adrenocorticotrophic hormone (ACTH) into the systemic circulation by the anterior pituitary gland. Consequently, ACTH initiates the synthesis of glucocorticoids (a class of steroid hormones, cortisol/corticosterone in humans/rodents, respectively) in the cortex of the adrenal gland and their subsequent release into the circulation (Herman et al., 2005).

Glucocorticoid feedback in the CNS

Glucocorticoids elicit myriad processes in the body. In the periphery they act as catabolic hormones that provide the metabolic support in order to cope with a potential stressor. But as steroid hormones, glucocorticoids readily cross the blood-brain-barrier, making them powerful messengers in the CNS. Because the two known receptors, mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) are expressed throughout the brain, although primarily in the limbic system, they also modulate limbic, as well as higher-order cognitive functions (Herman et al., 2005). Glucocorticoids were shown to favor

generalization of fearful memories on the level of the hippocampus, which led to the incorporation of non-predictive cues into the fearful experience (Kaouane et al., 2012). In the amygdala, corticosterone was shown to facilitate memory consolidation in the BLA, in concert with other neurotransmitter systems that probably also involve indirect mechanisms (McGaugh, 2004). In the CE and BNST, corticosterone exerts positive feedback by elevating mRNA levels for CRH, thereby boosting the behavioral and autonomous component of a fear response (Davis et al., 2010; Dong et al., 2001b).

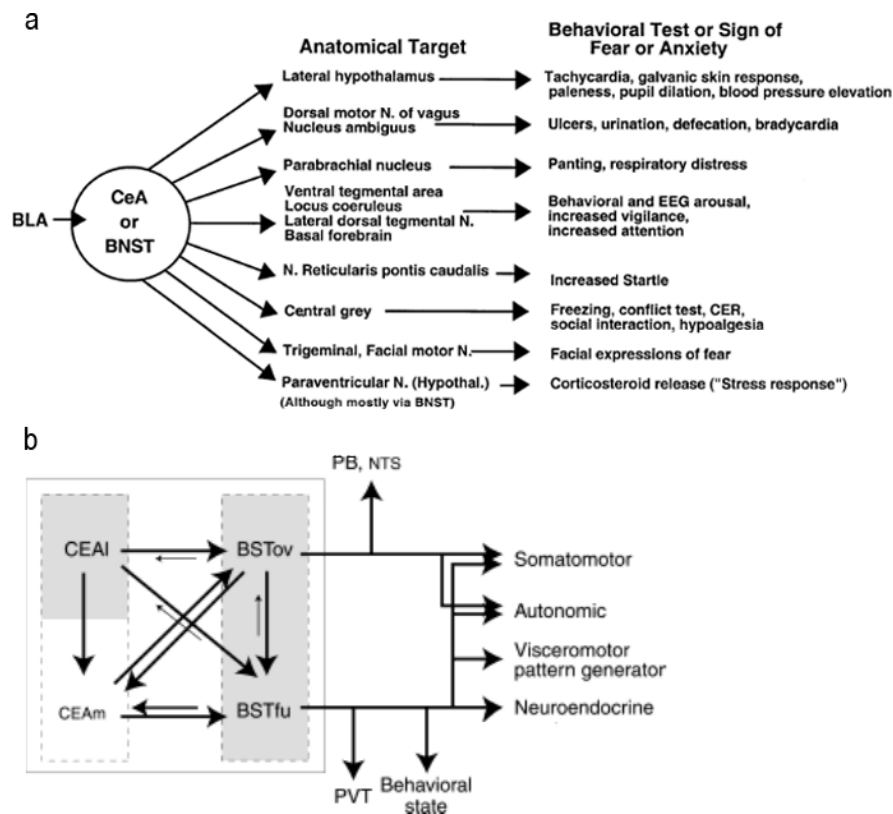


Figure 1.8: **a** Converging efferent projections of the CE and BNST are illustrated. With the exception of the paraventricular hypothalamus (PVH), anatomical targets are largely shared, leading to the typical behavioral and physiological outcomes of a fear response. Figure taken from Walker et al. (2003) **b** Overview of the interconnectivity of the BNST and CE. Relative size of arrows reflects the strength of innervation. CRH⁺ nuclei are indicated in grey. Figure adapted from Dong et al. (2001b). **Abbreviations:** PB, parabrachial nucleus; NTS, nucleus of the solitary tract; PVT, paraventricular thalamus; CEA, central amygdala; BST, bed nucleus of stria terminalis;

Fear and Anxiety in the Extended Amygdala

As noted above, the EA is a key hub in emotional processing. Nevertheless its embedded sub-components were shown to engage in phasic and sustained fear differentially (see section 1.1.1). Notably, as mainly concluded from the startle response paradigm, the function of CE and BNST could be dissociated. Inactivation of the CE selectively blunts responses to a conditioned fear stimulus, whereas the same procedure done for the BNST resulted in the unresponsiveness to the anxiety component of light-enhanced startle. Strikingly, inactivation of the BLA_p abrogated responses to both. This difference seems to be unrelated to conditioned versus unconditioned stimuli, but instead is dependent on the simple duration of stimuli, which has been demonstrated across different response measures (startle, freezing and conditioned suppression; Walker and Davis (2008)). In line with these experiments are observations that the response to a fear conditioned context, a typical long-lasting stimulus accompanied by uncertainty for the occurrence of a potential threat, is particularly sensitive to disruptions of BNST function. This is emphasized by the concomitant reduction in autonomous responses, as tachycardia and elevated blood pressure to an aversive context. A notable effect upon BNST inactivation is the actual potentiation of phasic fear, which strongly suggests that sustained fear in the BNST inhibits phasic fear responses mediated by the CE (Davis et al., 2010; Pape, 2010). This is backed by the anatomy of the EA macrocircuit, which shows strong innervation of the CE_m , the key nucleus in eliciting freezing responses, by the stress-responsive, CRH^+ $BNST_{ov}$ and $BNST_{fu}$ (fig.1.8b). The inverse seems plausible as well, as CE_m GABAergic but CRH^- fibres also strongly innervate the $BNST_{ov/fu}$ (Dong et al., 2001b).

Importantly, the effect of CRH, as measured by the CRH-enhanced startle response, seems to selectively exert its effect in the anxiety domain of the fear response, since intraventricular administration of CRH leaves phasic fear responses unaffected (Walker et al., 2003).

Conclusion and Hypothesis I

There is clear evidence that CE and BNST differentially process fear and anxiety. The former is responsible for eliciting immediate behavioral and autonomous responses upon encounter of an acute stressor/threat. The latter, although stimulating related autonomous responses, mediates the physiological adaptations to a more distant but long-lasting threat, that translates into an emotional state, related to chronic stress and anxiety. Therefore we hypothesize that the PKC- δ^- CRH $^+$ projection from the CE $_l$ to the BNST $_{ov/flu}$ serves as an integrator of phasic fear responses into the chronic stress state. Accordingly, repeated exposure to an acute stressor would add up over time and result in alteration of the general affective state of the animal.

1.4.2 Nucleus Basalis of Meynert

The CE has strong projections to the Nucleus Basalis of Meynert (NBM), which is part of the cholinergic system of the mammalian brain that consists of two major subsystems. One is located in the brainstem, innervating the thalamus, whereas the magnocellular basal forebrain cholinergic system innervates the neocortex and allocortical sites. The latter is formed by a continuum of four subregions termed medial septal nucleus (MS), vertical (vdB) and horizontal (hdB) limb nuclei of the diagonal band of Broca and the NBM. MS and vdB innervate the hippocampus and hdB the olfactory bulb, whereas the NBM diffusely innervates the entire neocortex and BLA (Everitt and Robbins, 1997).

The NBM is located in the SI, where the cholinergic neurons intersperse with GABAergic and minor glutamatergic neurons that are all represented in the efferent projection pattern of the NBM. Depleting the BLA of acetylcholine, originating from the NBM, is known to interfere with fear memories in diverse training paradigms in rodents, whereas other studies on rodents and primates indicated that the NBM corticopetal projections do not have profound impact on memory functions, instead it modulates attentional functions, arousal and to some degree perceptual plasticity in the cortex (Zaborszky et al., 1999; McGaugh, 2004).

The amygdala is capable of biasing the receptive field in the auditory cortex towards a CS, which was shown to depend on engagement of the cholinergic system of the basal

forebrain (Puckett et al., 2007). There is evidence that the amygdala responds rapidly to potential emotionally relevant stimuli prior to awareness, which indicates that cues could be pre-selected with respect to their significance, and in turn provide cortical arousal by cholinergic drive via the NBM. Indeed, desynchronization with neural oscillations in the cortex, a hallmark of cholinergic signaling, correlates with fluctuations in spontaneous activity of neurons in the CE, suggesting a causal relationship (Phelps and LeDoux, 2005; Kalmbach et al., 2012).

Conclusions and Hypothesis II

The projection of CE to the NBM may represent one route of many, by which the amygdala modulates memory, attention and other cognitive functions in the CNS (LeDoux, 1995). Activation of nicotinic acetylcholine receptors (nAChR) in the cortex block thalamo-cortical feed-forward inhibition and thereby enhance information flow into the cortex. In addition, activation of muscarinic AchR (mAChR) hyperpolarizes GABAergic interneurons which enforces this effect (Goard and Dan, 2009). Accordingly, we hypothesize that activation of the NBM by neurons in the CE_l might improve associative learning to salient events by either modulating memory consolidation in the BLA or attention and arousal processes in the neocortex.

1.5 Aim and Experimental Approach

The inhibitory microcircuit in the CE_l (see section 1.3.2) was chosen as an entry point into the EA macrocircuitry. This study sought to relate the well described microcircuit mechanisms within the CE_l to the efferent target structures BNST and NBM in the context of the phasic/sustained fear concept (see section 1.4.1) and cortical arousal (see section 1.4.2), respectively. Therefore CE_l efferents to NBM and $BNST_l$ were initially defined histologically in order to determine the individual contribution of each subpopulation. Thereafter, the projections were characterized functionally by optogenetic manipulation of terminal fields of CE_l projections in the NBM and $BNST_{al}$. The CS_{on} cells, which are $PKC-\delta^-$, are of particular importance, as they are activated by the CS and thereby are the potential mediators of the coordinated responses upon the impact of a fearful stimulus. We therefore applied an optogenetic strategy that selectively targets this population (see section 2.2.1). The modulatory impact of activity in the respective target nucleus on behavior was assessed in unconditioned anxiety- and conditioned place preference assays, as well as in the Pavlovian fear conditioning paradigm (see section 2.2.8 in methods). In addition, the autonomous component of these projections was assessed by implanting heart rate monitors into a sub-cohort of the respective groups after all behavioral experiments were completed.

2 Materials and Methods

2.1 Materials

2.1.1 Animals

For neuronal tracing studies wild-type C57BL/6N, whereas for all optogenetic experiments PKC- δ ::GluCl α -ires-Cre transgenic mice in the C57BL/6N background were used. The design of the transgene is depicted in figure 2.1 and described in Haubensak et al. (2010). The transgenic allele was maintained heterozygous in the population and genotyped after weaning (see section 2.2.2 for primer sequences). Mice were group-housed before and single-housed after surgical procedures at 21 °C in a 14h light and 10h dark circadian cycle, independent of daylight-saving time. Food and water were supplied ad libitum with antibiotics and analgesic provided via drinking water for 14 days after surgeries. (see section 2.1.2 for compounds and concentrations)

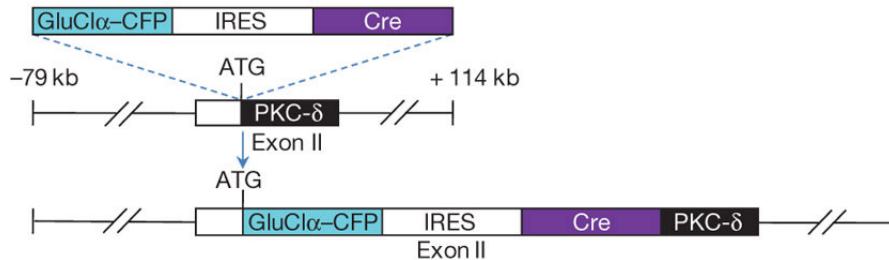


Figure 2.1: Scheme of the PKC- δ transgene locus. Figure taken from (Haubensak et al., 2010)

2.1.2 Anesthetics and Antibiotics

Ketamine/Xylazine

Ketamine and Xylazine were purchased from OGRIS Pharma Vertriebs-GmbH as Ketazol[®] ($100\frac{mg}{ml}$) and Xylazol[®] ($20\frac{mg}{ml}$), respectively. Both were mixed in Phosphate-buffered Saline (PBS) to achieve concentrations of $10\frac{mg}{ml}$ for Ketamine and $1\frac{mg}{ml}$ for Xylazine. This final solution was injected intraperitoneally at a volume of $10\frac{\mu l}{g}$ of bodyweight. Surgical procedures were delayed until plantar reflexes were absent.

Carprofen

The general anesthetic carprofen was obtained as Rimadyl[®] ($50\frac{mg}{ml}$) from Pfizer Corporation Austria. The volume of Carprofen that yields $4\frac{\mu g}{g}$ of bodyweight was diluted in PBS to a final volume of $200\mu l$ that was postoperatively injected intraperitoneally. Additionally Carprofen was supplied in the drinking water for 10 days after surgery at a concentration of $250\frac{mg}{l}$.

Enrofloxacin

Enrofloxacin was obtained as Baytril[®] ($100\frac{mg}{ml}$) from KVP Pharma + Veterinär Produkte GmbH. It was provided via drinking water for 10 days after surgery at a concentration of $400\frac{mg}{l}$.

Isoflurane

Isoflurane was obtained from Abbot Laboratories as Isoflo[®] (100% w/w). Animals were anesthetized with isoflurane prior to optic-fibre insertion into the implanted cannula. (see section 2.2.8 in Methods for procedural description)

Lidocaine

Lidocaine was obtained as Xylanaest[®] (1%) from Gebro Pharma. $100\mu l$ of Xylanaest[®] were injected subcutaneously on the skull prior to surgical incision for local anesthesia.

Ophthalmic Ointment

Animal eyes were lubricated with ointment in order to avoid drying of the open eye during anesthesia. It was obtained as Refobacin[®] from Merck GmbH and contained the broad-spectrum antibiotic gentamicin (3mg/g).

2.1.3 Chemicals and Reagents

Solutions

Sterile water and Phosphate-Buffered Saline (PBS) were provided by the in-house IMP media kitchen. 1% Bovine Serum Albumine/PBST (BSA) (m/V), used for blocking in immunohistochemistry (see section 2.2.7), was prepared from solid powder (Sigma Aldrich Handels-GmbH). 10U/ml Heparin/PBS was prepared from heparin sodium salt (≥ 180 USP units/mg, porcine intestinal mucosa, Sigma Aldrich Handels-GmbH). 4% paraformaldehyde (PFA)/PBS pH7.4 (m/V) was prepared from a local stock solid powder and filtered before use. 15% Sucrose/PBS (m/V) was prepared from sucrose crystals (Fluka Biochemika). Ingredients of the Tail-Buffer for DNA-isolation were 100mM Tris pH8, 200mM NaCl, 5mM EDTA pH8, 0,1% SDS. 0.1% PBST for immunohistochemistry was prepared from Triton[®] X-100 (Fluka/Sigma Aldrich) in PBS.

Mounting and Embedding Media

Fluorescence mounting medium was purchased from Pathology Products Dako Österreich GmbH and used for mounting fluorescently labelled tissue on slides. Embedding medium Tissue Tek[®] was obtained from Sakura Finitech Europe B.V. and used for embedding and cryoprotecting fixed brain tissue in preparation for further processing.

Adhesives

Dental adhesive resin cement (Superbond C&B, Sun Medical Co.,Ltd) and acrylic resin cement (Jet Set-4TM Liquid, Lang Dental Manufacturing Co., Inc.) were used to retain the implanted guide cannula on the skull of the animal.

Cholera Toxin Subunit B (CT-B) conjugates

Cholera toxin is an AB-toxin from the bacterium *Vibrio cholerae*, consisting of two subunits. Subunit A is an ADP-ribosyltransferase, interfering with G-protein coupled signaling of the cell, whereas subunit B is required for cellular uptake of the entire toxin by binding to the pentasaccharide chain of ganglioside GM₁ (Enomoto and Gill, 1980; Aman et al., 2001). Thus the recombinant and non-toxic subunit B represents a powerful tool for neuronal tracing experiments, since it is transported retrogradely from axon terminals to the soma of a neuronal cell (Luppi et al., 1990).

CT-B conjugates were obtained from Invitrogen as CTB-Alexa Fluor[®]-488 (C-22841) and Alexa Fluor[®]-555 (C-22843) as dry powder and dissolved in 8.7% glycerol/PBS to yield a final concentration of 1 μ g/ μ l for injection.

2.1.4 Viruses and Constructs

The Adeno-associated virus (AAV) belongs to the *Parvoviridae* family and has a packaging capacity of 4.7-5kb. AAV is replication-deficient, thus requires helper viruses such as adenovirus or herpesvirus. In the absence of these, the AAV-genome is stably maintained in the host as episomes or integrated into the genome Shin et al. (2012). Because it combines effective delivery of genetic material for long-lasting gene expression with very low levels of toxicity in the central nervous system, it became a powerful tool for transduction of post-mitotic neuronal cells (Betley and Sternson, 2011). The plasmid pAAV-dflox-ChR2(H134R)-mCherry-WPRE (fig.2.2 was cloned by inserting the ChR2(H134R)-mCherry fragment from the construct pAAV-DIO-ChR2(H134R)-mCherry-WPRE (not shown, obtained from Addgene 20297) inversely into the pBR233 backbone. Upon encounter with Cre-recombinase (Nagy, 2000) the ChR2(H134R)-mCherry fragment within two double lox-sites become irreversibly inverted and thereby switched off by a mechanism described by Fenno et al. (2011). Viral vector production was performed by Penn Vector Core (University of Pennsylvania). The sequence located within 2 inverted terminal repeats (ITRs), which form a T-shaped hairpin, essential for replication, is packaged into viral particles (Bohenzky et al., 1988). Viral titre obtained were 3.88×10^{12} gc/ml for the Cre-OFF virus (serotype AAV2/5).

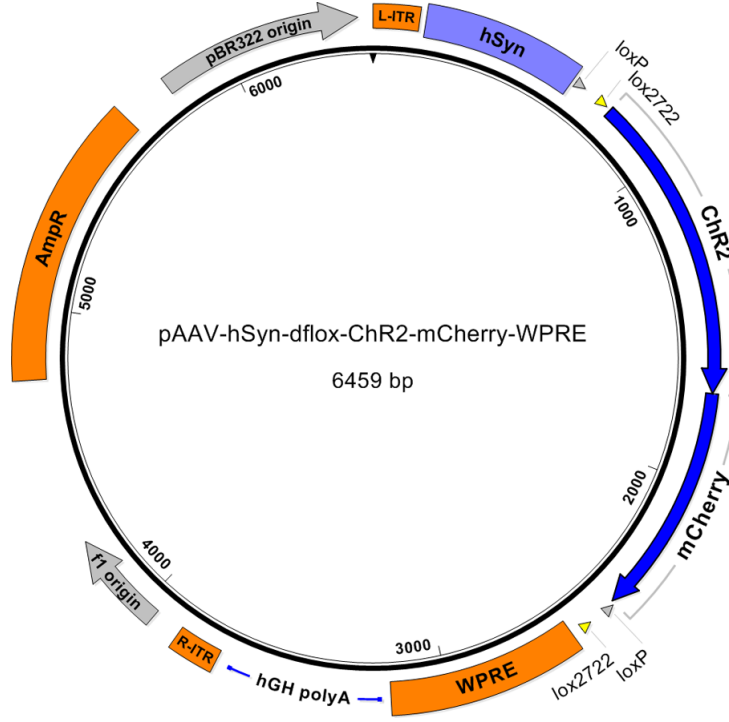


Figure 2.2: Chr2-mCherry is being expressed in neuronal cells, not expressing Cre-recombinase, which irreversibly switches the ChR2(H134R)-mCherry fragment in PKC- δ^+ cells. **Abbreviations:** L-ITR/R-ITR, Inverted Terminal Repeats; EF1a, elongation factor 1a promoter; hsyn, human synapsin promoter; ChR2, Channelrhodopsin 2 (H134R); WPRE, Woodchuck Hepatitis Post-Transcriptional Regulatory Element; AmpR, Ampicillin-resistance gene; dflox, double-floxed; DIO, Double-floxed Inverted Open reading frame; loxP/lox2722 - independent recognition sites for Cre-recombinase

2.1.5 Cryostat

Fixed and frozen brain tissue was sliced with MB DynaSharp[®] Microtome Blades (Thermo Scientific) in 14 μ m coronal sections on a Microm HM560 cryostat and put on microscopy slides (Thermo Scientific) for further processing.

2.1.6 Confocal Microscopy

All histological images of neuronal tracing experiments were acquired on the point laser scanning confocal microscope LSM 510 Axiovert 200M with a 25x/0.8 LD LCI plan-apochromat, multiimmersion objective. Lasers used were Laser Diode 405nm 25mW, Argon 458/488/514nm 30mW, DPSS 561nm 15mW, HeNe 633nm 5mW. Acquisition was performed in frame scanning mode in order to avoid bleed-through between channels. (see Smith, 2008 and Paddock, 1999)

2.1.7 Stereotaxic Surgery Equipment

Intracranial injections and cannulations were performed on a stereotactic alignment instrument (Model 1900, David Kopf Instruments) with an Anilam[®] Wizard 550 digital readout system for coordinates and Zeiss f170 tube binoculars.

Further stereotaxic instruments included non-rupture earbars, an Electrode Holder, a Micro Manipulator, an Alignment Indicator, a Stereotaxic Drill and a Centering Scope 40X (Models 1922, 1970, 1940, 1905, 1911 and 1915, respectively; Kopf[®]). For stereotaxic injections a Nanoliter 2000 injector, driven by a Micro4[™] MicroSyringe Pump Controller (World Precision Instruments), was used. Needles were pulled with a Micropipette Puller (Model P-97, Sutter Instruments) from 3.5in glass capillaries (World Precision Instruments).

2.1.8 Cannulas and Mounting Screws

Bilateral guide cannulas C232G-1.5/SP and C232G-3.0/SP (Doubles) were obtained from PlasticsOne in gauge 22 with a cannula center-to-center distance of 1.5 and 3mm, respectively, with corresponding dust caps 303DCFT. Internal cannulas C232I/SP were used for mounting of guide cannulas on the electrode holder for implantations and infusions of muscimol. Corresponding dust caps were 303DCFT.

Precision Stainless Steel 303 Machine Screws (B000FNOJ58, Antrin Miniature Specialties, Inc.) were used to retain the guide cannula on the skull of the animal.

2.1.9 Fibre Patch Cables

Fibre patch cables ($\varnothing 200\mu$) for delivery of light pulses through the implanted guide cannula were obtained from Thorlabs, Inc. The fibre was varnished in black prior to use, in order to prevent visual perception of the light pulses of the mouse during behavioral assays.

2.1.10 Antibodies

Table 2.1.10

type	species	supplier
Anti-PKC- δ (IgG _{2A})	mouse	BD Transduction Laboratories TM
Anti-mouse (IgG) - Alexa-633	goat	Invitrogen

2.2 Methods

2.2.1 Optogenetics

The term optogenetics encompasses a widely applied method in neuroscience, which allows for interference with neuronal function on the circuit level. It takes advantage of membrane-incorporated microbial opsins that are excitable by light, due to the covalently bound prosthetic chromophore all-*trans*-retinal. Upon absorption of photons of a defined wavelength spectrum, these proteins generate highly time-locked photocurrents, thereby depolarizing or hyperpolarizing cells, depending on the ion transported (Boyden et al., 2005). Three major classes of opsins exist in different microorganisms and were further adapted by codon optimization, point mutations and alterations in trafficking motifs, to accomplish the requirements for the application in neurons. These adaptations include improvements in photocurrents and kinetics and more efficient localization to the plasma membrane of cells. Schematic images of Channelrhodopsin and Halorhodopsin are shown in figure 2.3a (Zhang and Aravanis, 2007).

As opposed to Channelrhodopsin-1, which is permeable for protons, Channelrhodopsin-2 (ChR2) is selective for conducting mono- and divalent cations across the plasma membrane, allowing for effective depolarization of neurons. ChR2 is a 7-transmembrane protein, originally cloned from the green alga *Chlamydomonas reinhardtii*, where it is thought to mediate phototaxis and photophobic responses (Nagel et al., 2002, 2003). Site-specific mutagenesis yielded ChR2 isoforms with different channel properties. Substitution of algal with human codons gave rise to the humanized form of ChR2 (hChR2), a procedure called codon optimization. Introduction of the amino acid substitution H134R

resulted in a channel with increased photocurrent, while concomitantly decelerating the channel-closing kinetics (Off-kinetics) from 10 to 18ms (Nagel et al., 2005; Yizhar et al., 2011). Nevertheless, the version hChR2(H134R) was used in this study, since the Off-kinetics still suffice in the low-frequency stimulation regimen employed here.

Halorhodopsin (NpHR) is a light-gated chloride pump, obtained from the extremophile archaeon *Halobacterium pharaonis*. As being not a simple channel, NpHR requires constant light in order to complete the entire photocycle for actively transporting chloride ions across the plasma membrane, thereby effectively hyperpolarizing the cell. The initial version NpHR failed to produce sufficient currents due to retention of the protein in the Endoplasmatic Reticulum (ER) (Gradinaru et al., 2007). Addition of an ER-export signal from $K_{ir}2.1$ channel and a signal peptide for membrane insertion from the nAChR resulted in significantly higher photocurrents and was termed eNpHR2.0 (Gradinaru et al., 2008). Finally NpHR3.0 contained an additional neurite trafficking signal from the $K_{ir}2.1$ channel, what further enhanced ion conductance and which was coinjected with ChR2 in this study (Gradinaru et al., 2010).

Combined with genetic tools such as the Cre-lox system, opsins can be targeted to defined sets of neuronal populations (Luo et al., 2008). *In vivo* delivery by means of viral vectors, such as Adeno-Associated Virus (AAV) (Adamantidis et al., 2007; Haubensak et al., 2010) or transgenic animals (Madisen et al., 2012; Fenno et al., 2011) have been successfully accomplished. Absorption spectra of ChR2 and NpHR are shown in figure 2.3b. Peak activation of ChR2 and NpHR3.0 are at 470 and 590nm, respectively.

Stimulation of ChR2 was done at a wavelength of 455nm at a frequency of 10Hz with 20ms pulse length. Burst width varied according to the behavioral test (see section 2.2.8).

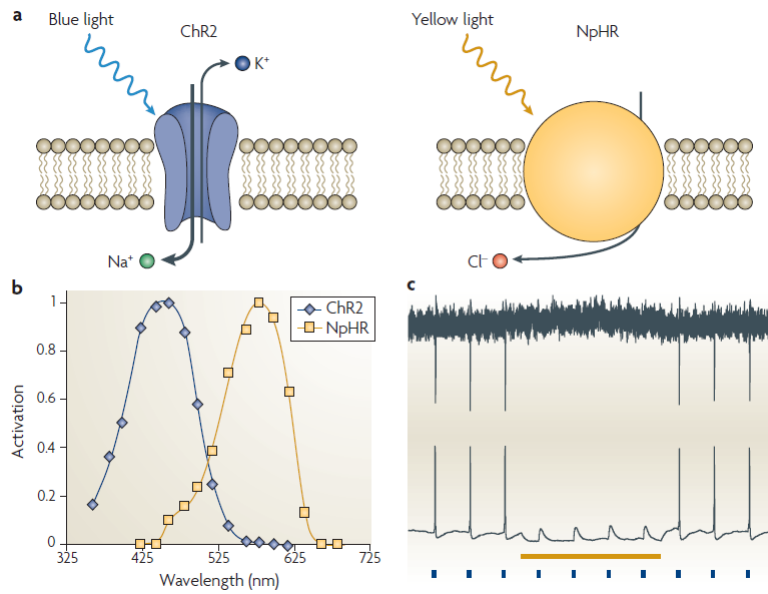


Figure 2.3: **a** Schematic function of the Channelrhodopsin and a Halorhodopsin family. Upon light absorption of the appropriate wavelength the channels are highly permeable for cations or chloride, respectively. **b** Absorption spectra of ChR2 and NpHR. **c** Illustrating traces of hippocampal neurons upon stimulation with light pulses of indicated color. Configuration: Cell-attached (top), whole-cell current clamp (bottom). Figure taken from Zhang and Aravanis (2007).

2.2.2 Genotyping

Transgenic PKC- δ ::GluCl α -ires-Cre mice were maintained in a heterozygous state, hence they were genotyped at weaning age (21 days). Clipped tissue from the ears was digested in 490 μ l Tail Buffer and 10 μ l Proteinase K (Fermentas) overnight at 55°C and genomic DNA was extracted as previously described (Wang and Storm, 2006). Genotyping primers are listed in table 2.2.2 and an exemplary result of genotyping is shown in figure 2.4.

Table 2.2.2

primer	sequence
ExII.f	5'-tctgcctgggtgtgtatcag-3'
ExII.r	5'-gcctccttcattcttcacagc-3'
GluCl.r	5'-gtaagcctgcttctcgttgg-3'

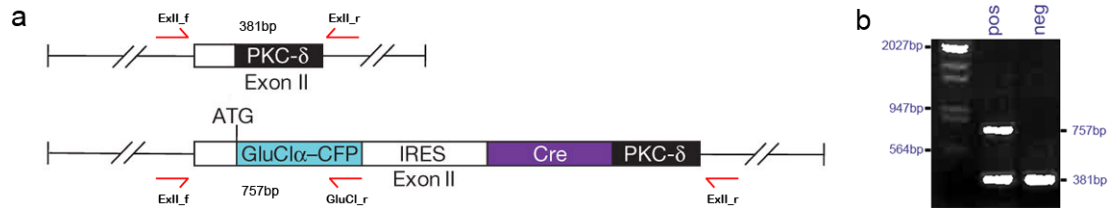


Figure 2.4: **a** Schematic of the genotyping design, adapted from Haubensak et al. (2010). **b** Exemplary genotyping result, showing a GluCl α -ires-Cre positive and negative mouse, respectively.

2.2.3 Stereotaxic Surgery

Mice were anesthetized with Ketamine/Xylazine as described above and left until the absence of plantar reflexes, indicating deep anesthesia. Eyes were covered with ointment and apical fur of the head was removed. After gently pulling the tongue out of the mouth to avoid blockage of airways, the mouse was placed into the stereotaxic apparatus on a heating pad, in order to maintain a constant core temperature of 35°C. The animal head was cautiously fixed in the apparatus between non-rupturing ear bars. After subcutaneous application of 100 μ l of local anesthetic (Xylanaest[®]) on the skull, a longitudinal incision along the sagittal suture was made on the scalp, slightly extending bregma and lambda (see fig.2.2.3). The cleaned and dry skull was then adjusted with a stereotaxic alignment indicator in the lateral and anterior-posterior axis (AP-axis; bregma and lambda being on the same horizontal plane), hereafter bregma representing the landmark where the AP- and lateral axis are zero. Stereotaxic coordinates were obtained from Paxinos and Franklin (2007) and bilateral holes were drilled ($\varnothing$ 0.8mm for cannula implantation, $\varnothing$ 0.6mm for mounting screws and $\varnothing$ 0.3mm for injections), minimizing any damage to the brain tissue underneath. After metal screws were shallowly inserted into the skull, a glass needle was filled with mineral oil and mounted on the injecting device, avoiding air bubbles. Injection solution was aspirated into the needle and positioned above the centre of the hole. The dorsal-ventral axis is set to zero, when it is level with the bone and subsequently inserted into the brain, according to the coordinates of the region of interest. The volume injected, was adjusted according to the size of the target region and are listed in table 2.2.3 below. In case of implantation, the cannula was mounted with an internal cannula onto an electrode holder, soaked in 70% ethanol for sterilization and in-

serted into the brain 0.5mm above the target region to be illuminated. After the cannula was glued to the skull using dental glue and left for 5 minutes for polymerization, then dental cement was brushed on the layer of glue, linking mounting screws and cannula and also left for 5 minutes for complete stabilization. Finally the scalp was re-sutured, minimizing the area of skull exposed, and the cannula capped, preventing infection.

Table 2.2.3

region	coordinates [mm]			volume [nl]
	bregma	lateral	ventral	
CE _l	-1.34	2.7	-4.5	100
NBM	-0.5	1.5	-4.8	20
BNST _{al}	+0.14	0.75	-4.0	30

Abbreviations: CE_l, Central Amygdala, lateral division; NBM, Nucleus Basalis of Meynert; BNST_{al}, Bed Nucleus of stria terminalis, anterolateral division;

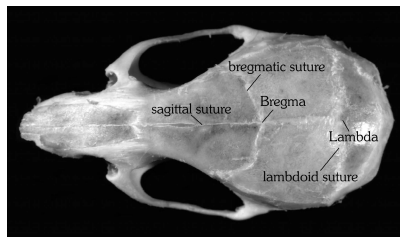


Figure 2.5: Diagram of a mouse skull taken from Paxinos and Franklin (2007). Sutures and landmarks are indicated and serve as reference points in stereotaxic surgeries.

2.2.4 Heart Rate Monitor Implantation

Implantation of G2 HR E-Mitters was performed according to Respironics® Mini Mitter® guidelines. Implantations were performed under sterile conditions on a heating pad to maintain a constant body temperature. Mice were anesthetized by isoflurane and the procedure was started upon the absence of plantar reflexes. After removing abdominal fur and disinfection of the skin, a 2cm longitudinal incision of the abdominal wall was made approximately 1cm below the diaphragm along the *linea alba*. A sterile E-mitter is inserted along the sagittal plane into the abdominal cavity, the leads pointing cranially. After the wires penetrated through the body wall, it was closed with sterile suture mate-

rial, while the skin remained open. Two small incisions were made on the skin, one medial to the right clavicle, the other left of the *xiphoid process* and cranial to the last rib on the left side of the animal, resulting in a 45-60° angle to the transverse plane of the heart. The longer negative lead is channelled subcutaneously to the hole next to the clavicle, resting on the *pectoralis superficialis* by means of a trochar sleeve. A metal ferrule is then crimped on the terminal of the lead, which is then fixed onto the muscle tissue by suture material. This procedure is repeated for the positive lead next to the *xiphoid process* on the chest muscle. Incisions were then closed with sterile suture material and the skin was disinfected. Mice were then returned to their home cage, put on a heating pad over night for recovery.

2.2.5 Heart Rate Measurement

Mice were tested 2 weeks after surgical procedure and were therefore briefly anesthetized with isoflurane (<1min) for optic fibre insertion and returned to their homecage thereafter. After 30min recovery from anesthesia, the home cage was placed on the Energizer/Receiver-4000 for heart rate recording with VitalViewTM software. The heart rate was recorded for a total of 90min, starting with 30min baseline, followed by 30min optogenetic stimulation and 30min post-stimulation period. Data was sampled once every second and the mean of the resulting 1800 values per period were taken for analysis.

2.2.6 Perfusions

Mice were deeply anesthetized with Ketamine/Xylazine. Once plantar reflexes were absent, paws were fixed to the surface and the thorax was opened with surgical scissors. A 21G butterfly needle was inserted 3mm into the left ventricle of the heart, avoiding piercing of the septum. After the right atrial auricle was incised, the perfusion valve was opened, letting 10U/ml Heparin/PBS streaming through the vasculature, driven by gravity flow. After 10ml Heparin, the valve was switched and the animal was perfused with 30ml of 4% PFA/PBS. The fixed brain could now be extracted by 3 strategical cuts from the posterior of the skull to the auditory canals and one approximately 4mm along the sagittal suture. For the purpose of freezing, the extracted brain was dehydrated by storing it in 15% sucrose/PBS at 4°C overnight. After embedding the brain in embedding medium for cryoprotection, it was deep frozen with dry ice, immersed in 96% ethanol and put to -80°C for long-term storage.

2.2.7 Immunohistochemistry

After cryostat sectioning, histological slices were dried for half an hour at room temperature. After washing with PBST for 10min, the blocking solution was applied for 30min at room temperature, followed by the incubation of the primary antibody in blocking solution (1:1000) overnight in a humidified chamber at 4°C. The slides were washed 3 times for 10min in PBST, followed by the incubation with the secondary antibody and 4',6-diamidino-2-phenylindole (DAPI; 1:1000), for labeling of nuclei, in blocking solution (1:1000) for 2 hours at room temperature in a humidified chamber. The slides were again washed 3 times for 10min with PBST and finally mounted with a fluorescent mounting medium.

2.2.8 Behavior

For insertion of the optogenetic fibre the guide cannula, mice were anesthetized with isoflurane (<1min) and put back to their home cage for recovery at least 30min before behavioral testing. Laser stimulation during all assays was done with rectangular 20ms

pulses at a frequency of 10Hz with a peak intensity of 3mW. The behavioral readouts of every assay were quantified with a video-tracking software (ANY-mazeTM, Stoelting Co.).

Results of separate control groups for the BNST and NBM were pooled for every behavioral test, as they were not statistically different.

Anxiety Assays

Standard behavioral testing of anxiety was performed in the Open Field Test (OFT) and an Elevated Plus Maze (EPM). The OFT is a square open box (60x60cm) with surrounding walls emerging 20cm from the ground. The open zone of it was considered a 30x30cm area in the very center of the arena. The EPM is elevated 50cm from the ground and consists of 4 arms, each 30cm long, arranged in a plus-shape. Two opposing arms are closed (encircled with 20cm walls) and the remaining 2 are open.

Animals performing these tests experience a conflict between their intrinsic exploratory drive and their innate aversion towards open and bright areas. Hence they preferentially stay close to the walls in the OFT and in the closed arms of the EPM. The time spent in open areas/arms is therefore a measure for anxiety in these assays. The capacity of these assays is emphasized by increased time in open compartments upon administration of anxiolytic drugs (Pellow and File, 1986; Prut and Belzung, 2003).

Each anxiety assay lasted 12.5 minutes, where 2.5 minutes bursting intervals of the laser alternated with 2.5min of no laser stimulation, starting with no stimulation. Mice underwent the OFT test first, upon a 5 minutes return to their homecage, the EPM was performed.

Data extracted from these assays were as listed below.

- Total Distance Travelled during stimulation [m]
- Time Spent in the Open Compartment during stimulation [percentage of total time]

Conditioned Place Preference Assay (CPP)

The CPP arena consisted of 3 compartments. The 2 square compartments (15x15cm) were arranged in a 120° v-shape, resulting in the third equilateral triangular compartment (neutral zone) between them (15cm). The walls of both square compartments were painted with white dots or stripes on a grey background, respectively. The assay was performed for 15min on each 7 consecutive days, starting with the pre-exposure trail on the first day, where no laser stimulation was applied, in order to detect any intrinsic preference for a compartment. On the 5 following days mice received laser stimulation exclusively during their stay in the dotted compartment, however only for a maximum of 1 minute. On the last day's trail, mice performed the assay again without laser stimulation. Data extracted from these assay was the place preference score, which was calculated as below.

$$\text{place preference score} = \frac{\text{time in conditioned zone}}{\text{total time of assay} - \text{time in the neutral zone}}$$

Pavlovian Fear Conditioning

The fear conditioning protocol was performed on 4 consecutive days. On the first day, mice were habituated to testing context A and to the CS⁺ and CS⁻, which were presented 4 times each. The two CS consisted of 50ms pips of either 7kHz or white noise, 80dB, 2ms rise and fall, repeated at 0.9Hz for 30s total.

The next day, mice underwent conditioning in context B of one CS (CS⁺) to the US, which was a 0.6mA, 1s foot-shock, whereas the second CS (CS⁻) was not reinforced. The two tones were counterbalanced within a group. After a 3min baseline period, 5 CS⁺-US associations and 5 CS⁻ were presented in a random fashion, separated by a 20-180s time interval, with constraints that a session should start with a CS⁺-US, end with a CS⁻ and a maximum of 2 identical CS should be presented consecutively. During both CS, the mice received light stimulation, which co-terminated with the US in the case of the CS⁺. On the third day testing and extinction was performed in the same session in context A, which consisted of 12 CS presentations each, both non-reinforced. No light stimulation was applied during the first 4 of each CS, which is referred to as the testing session. During

the remaining 8 CS presentations, light stimulation was applied as during conditioning, referred to as the extinction session.

On the last day, the extinction recall session, 4 presentations of each CS were presented without light stimulation.

Every session was performed without ambient light and was recorded with an infrared camera above the conditioning chamber. Resulting videos were analyzed with a custom-made MATLAB software that scored freezing by subtracting consecutive frames and counting significant motion pixels. Freezing was analyzed during the baseline period of each session (first 1min) and during CS presentations (30s).

2.2.9 Histological Control

In order to verify the correct positioning of the guide cannula on the terminal field of the respective area and viral expression in the CE_l , mice were sacrificed and brains sliced after behavioral testing. All mice in the results exhibited a highly stereotypic expression pattern in the CE_l and cannulas were on the correct spot. Mice that deceased prematurely, lacking histological control, were excluded from the analysis.

3 Results

3.1 Histology

3.1.1 Assessment of Projection-Specificity of Neuronal Subpopulations in the CE_l

In order to determine the contribution of each cell-type to the projections to the NBM and BNST_l, a CT-B retrograde tracer, conjugated with a fluorophore, was injected in comparable volumes into the respective area. After 7 days, mice were sacrificed and coronal sections of CE_l were stained for PKC- δ . CTB⁺ cell bodies identified projection neurons into the specific area, whereas the identity of the neuron was determined by the presence or absence of PKC- δ protein. Labeling and co-labeling was quantified by manual counting and revealed a highly asymmetric projection pattern in both areas. 69% of neurons projecting to the BNST were negative, whereas 72% of projection neurons to the NBM were positive for PKC- δ (fig.3.1). In addition the NBM is most densely innervated by the CE_l , as 13% of cells in it where CTB⁺ over DAPI, as opposed to 6% CTB⁺/DAPI for the BNST_l injections.

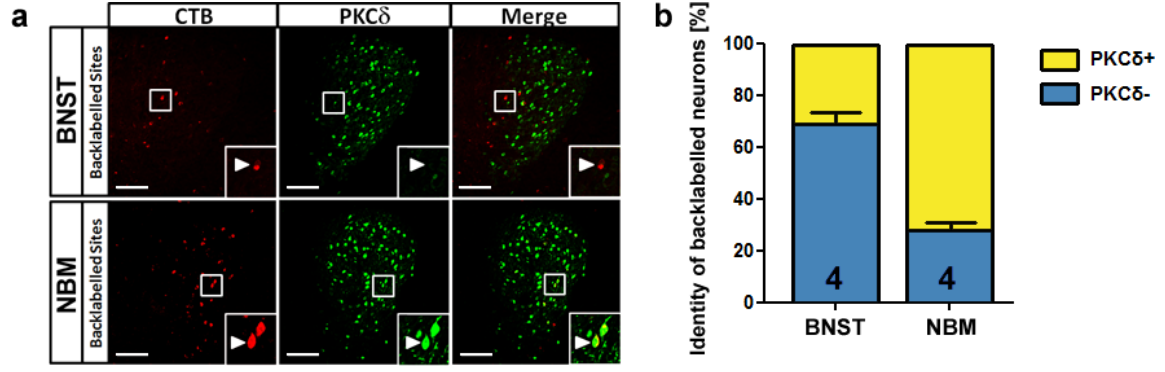


Figure 3.1: **a** Confocal images of coronal slices of the central amygdala (CE) with CTB-backlabelled neurons from the nucleus basalis (NBM) and bed nucleus of the stria terminalis (BNST), respectively. Slices were stained for PKC- δ protein. Arrows indicate representative CTB-backlabelled cells, positive (NBM) or negative (BNST) for PKC- δ . **b** Quantification of cell counts within the lateral CE showing backlabeling from NBM and BNST. Neurons projecting to the BNST and NBM are 31% and 72% positive for PKC- δ , respectively. The number in bars corresponds to the number of biological replicates. Data plotted as mean $\pm$ SEM. white scalars: 100 μ m.

3.2 Behavior

3.2.1 Effect of Projection-specific Activation of PKC- δ^- Neurons on Anxiety

For the assessment of effects on anxiety by each projection, animals underwent arena-based testing of anxiety measures in the open-field test (OFT) and elevated-plus maze (EPM) in this particular order. Every test lasted 12.5 minutes, during which the optogenetic stimulation lasted 2.5 minutes, starting with no stimulation. Quantified in figure 3.2 are aggregated measures during 2 episodes of light stimulation (5min total), in order to pick up stimulation-specific effects. The left panels in figure 3.2a/b quantify exploratory activity, expressed as total distance travelled, whereas the right panels show the anxiety measure, time spent in the anxiogenic (open) compartment (center in the OF/open arms in EPM; see section 2.2.8).

The OFT is more sensitive to measures for exploratory activity and shows a significant reduction in total distance travelled in PKC- δ_{CEl}^- terminal field stimulation in the BNST_{al}, compared to control animals (p=0.021), whereas only a non-significant trend can be observed in the EPM. Exploratory activity is unaffected in the NBM group, compared to controls. The time in the open compartment, the measure for anxiety, shows differences between groups in both the OFT and EPM, compared to controls.

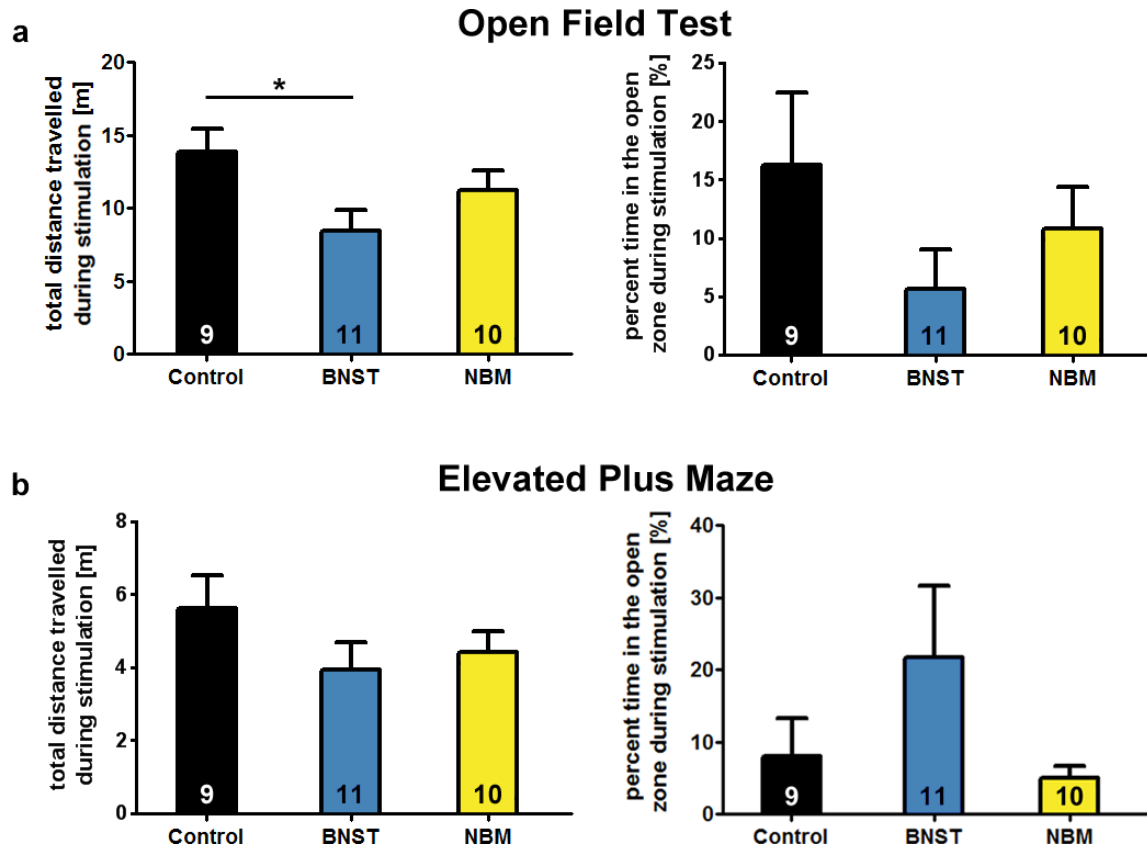


Figure 3.2: Graphs show total distance travelled and time in the open compartment during episodes of light stimulation in the OFT (**a**) and elevated plus maze (**b**). t-tests were performed between controls and BNST and between Controls and NBM. α -value was adjusted, accordingly. (* p<0.025). Numbers in bars correspond to number of subjects. Data plotted as mean $\pm$ SEM.

3.2.2 Projection-specific Activation of PKC- δ^- Neurons shows no Intrinsic Preference

The valence of the activity of each PKC δ^- projection in the respective area was tested in a Place-Preference (PP) assay. Results in figure 3.3 depict the place preference (PP) score before (pre-exposure) and after (testing) a 5 days training procedure, where projections were specifically stimulated in one compartment of the PP-arena see section 2.2.8. Non-significant changes in the testing session compared to the pre-exposure session, as well as non-significant differences between groups were observed, as analysed with two-way ANOVA with post-hoc Bonferroni t-test.

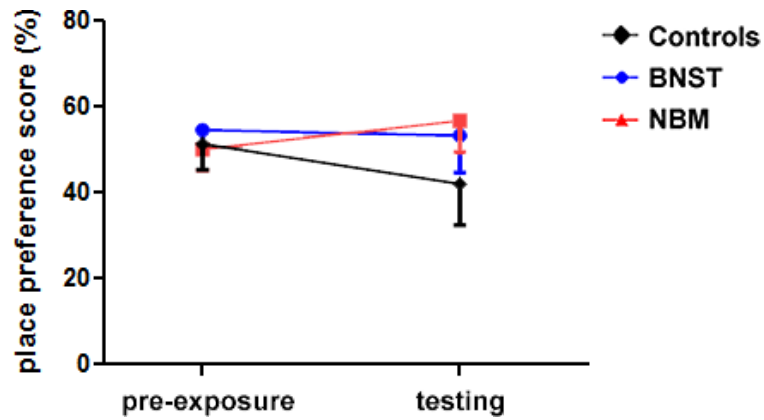


Figure 3.3: Ratio of time spent in the stimulated compartment versus neutral compartment is plotted for the pre-exposure and testing session. Training sessions non-significantly altered intrinsic preference for either compartment, as revealed by two-way ANOVA with post-hoc Bonferroni t-test.

3.2.3 Modulatory Effect of Projection-specific Stimulation in the Fear Conditioning Paradigm

In order to assess the functional impact of PKC- δ^- projections to the NBM and BNST_l in fear learning, terminal fields were stimulated in the respective area in the Pavlovian fear conditioning paradigm during all CS presentations, including the CS⁺-US pairings. Results in figure 3.4 depict freezing during baseline and averaged freezing scores during 4 CS^{+/-} presentations without stimulation in the testing session, after a one-day conditioning session (5 CS⁺-US pairings). Two-way ANOVA and Bonferroni post-hoc test (all $p < 0.001$) reveals clear conditioning to the CS⁺ in all three groups, compared to baseline freezing. Mice displayed significantly more freezing to the CS⁻ compared to baseline in the control and BNST group ($p < 0.05$ and $p < 0.01$, respectively). Conversely, NBM stimulated animals showed significantly higher freezing to the CS⁺ than to CS⁻ ($p < 0.05$), which is reflected in the CS⁺/CS⁻ mean ratio of 1.53, as opposed to 1.03 for the BNST and 1.26 for the control group.

Interaction of differential freezing to CS⁺ versus CS⁻ between the NBM and BNST group has been analyzed by two-way ANOVA and revealed a strong trend ($p = 0.064$).

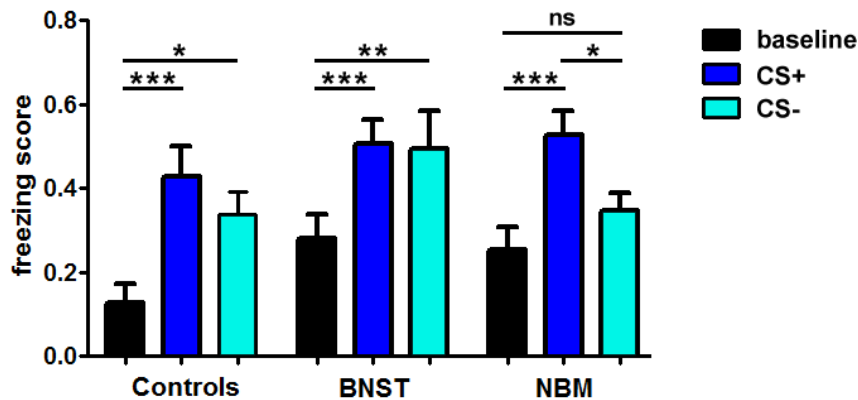


Figure 3.4: Freezing scores during baseline, CS⁺ and CS⁻ of the control, BNST and NBM group are plotted. Data are analysed by two-way ANOVA with post-hoc Bonferroni t-test, showing effective conditioning to the CS⁺ in all groups. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; plotted as mean $\pm$ SEM)

3.2.4 Modulatory Effect of Projection-specific Stimulation on Extinction

Testing was directly followed by the extinction session on the same day, which consisted of 8 CS^{+/−} presentations, which were accompanied by light stimulation, thereby assessing possible effects on acquisition of extinction. The extinction recall session followed the next day and is expressed as the mean of freezing scores during 5 CS without light stimulation. Results in figure 3.5a show insignificant extinction to conditioned stimuli in all groups, indicated by persistent freezing responses to the CS. Graph 3.5b depicts extinction performance, illustrated as freezing scores before and after the extinction session. In contrast to the other groups, the NBM group displays a slight, but insignificant rise in mean freezing score.

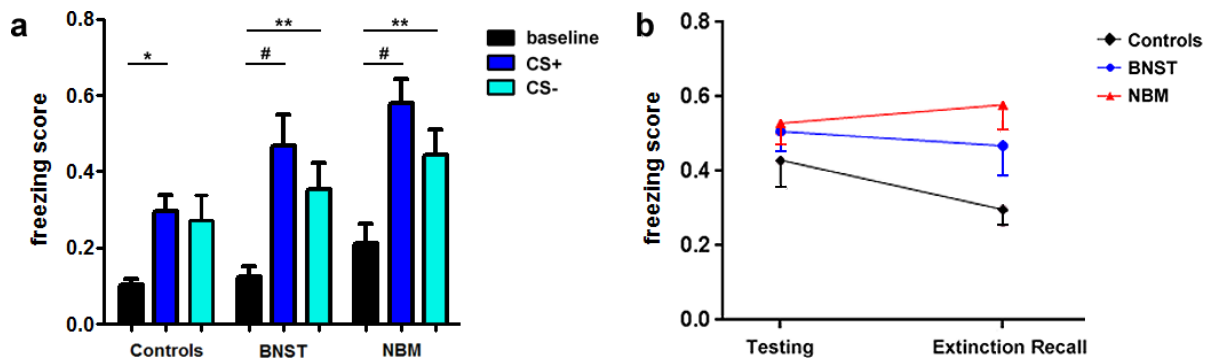


Figure 3.5: **a** Baseline and mean freezing score to CS^{+/−} are plotted for the control, BNST and NBM group. Mice showed no extinction to both CS (# p<0.0001, ** p<0.01, * p<0.05). Data plotted as mean ±SEM. **b** Change in mean freezing scores to 5 presentations of the CS⁺ after fear extinction is shown. Mice showed non-significant difference to the CS⁺ before and after the extinction session.

3.2.5 Autonomous Effect of Projection-specific Stimulation

The heart rate of 2 mice each of the BNST, NBM and control group was recorded for a total of 90min. 3 different time periods were defined: baseline, stimulation and post-stimulation period, each lasting 30min. In figure 3.6 the mean changes of heart rate upon optogenetic stimulation and post-stimulation period are plotted in absolute values with respect to the mean heart rate during the baseline period. Stimulation in the BNST group resulted in an increase compared to baseline, which again dropped after stimulation, whereas the heart rate of control animals remained constant (Absolute values in beats per minute [BPM $\pm$ SEM]; Control: 585 $\pm$ 18, 588 $\pm$ 18, 593 $\pm$ 18; BNST: 467 $\pm$ 147, 586 $\pm$ 83, 422 $\pm$ 167; NBM: 529 $\pm$ 97, 593 $\pm$ 17, 603 $\pm$ 11 for baseline, stimulation and post-stimulation, respectively). Due to the very low number of subjects, no significant changes in heart rate could be detected with two-way ANOVA ($p>0.05$), although a tendency for an increase upon stimulation can be observed in the BNST group compared to control animals. Nevertheless more subjects will be required for confirmation of these results. No conclusion can be drawn from the NBM group, as it shows very high variability among subjects, emphasizing the requirement for additional data points.

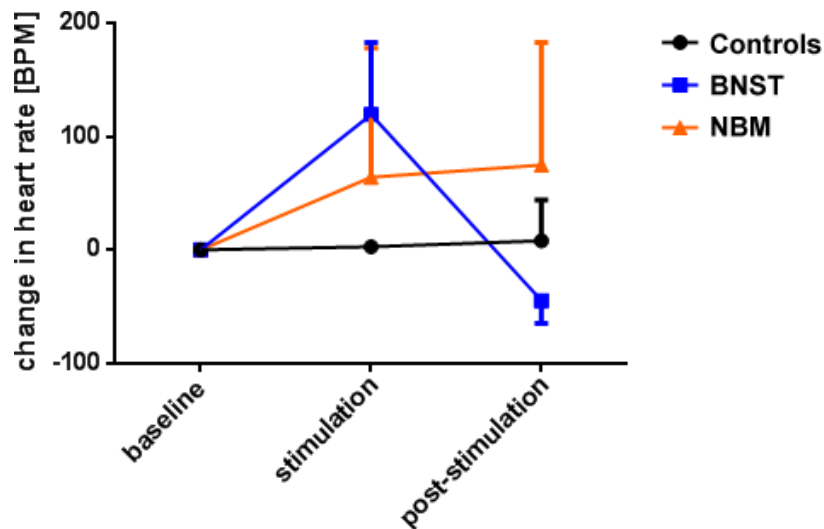


Figure 3.6: Changes of the mean heart rate during the stimulation and post-stimulation period compared to the mean of the baseline period are shown. The BNST group shows a response to optogenetic stimulation by an increase of the heart rate, which reverts to baseline level after stimulation. Data plotted as mean $\pm$ SEM.

4 Discussion

4.1 Neuronal Subpopulations in the CE_l show Projection-Specific Asymmetry

The neuronal subpopulation in the CE_l exhibit a highly asymmetric projection pattern, although PKC- δ^- neurons project to the BNST_{al} and NBM. In the context of the micro-circuit mechanisms, this could play a crucial role in coordinating the responses outside of the central amygdala. As revealed by Haubensak et al. (2010), PKC- δ^- neurons respond to the CS, which shifts the relative activity of these subpopulations towards PKC- δ^- . In parallel to orchestrating the actual behavioral outcomes in the brainstem, the CE_l might send efferent copies to pathways in the extended amygdala, where this information could be fed into the anxiety- and arousal-related circuitry.

The most substantial amygdaloid projections to the basal forebrain originate from the CE, followed by the accessory-basal nucleus and basolateral complex (Jolkkonen et al., 2002). This seems compatible with these histological results, that attribute the major target region of the CE_l to the nucleus basalis in the basal forebrain.

The BNST, the limbic gateway to the paraventricular hypothalamus, is preferentially innervated by the CS-responsive PKC- δ^- population, which is in line with our hypothesis, that this projection could serve to integrate phasic fear into the anxiety-related circuitry. As these neurons express CRH, elevated release of this neuromodulator in the BNST could then enhance the stress reactive cascade, terminating in glucocorticoid release (Herman et al., 2005).

4.2 Integration of Phasic Stimuli into Chronic States via the Central Amygdala

Apart from a significant reduction of exploratory activity in the open field test, no significant effect of PKC- $\delta_{CE_l}^-$ terminal field stimulation in the BNST on anxiety-related behavior could be observed. In line with these results, chronic overexpression of CRH in the CE_l CRH⁺ neurons, which enhanced HPA-axis activity, was shown to reduce total locomotion in rats performing the OFT. (Flandreau et al., 2012). However, this study demonstrated a marginal anxiogenic impact of elevated CRH levels in the BNST_{al} in the EPM. This discrepancy could be attributed to a possible floor-effect, resulting from already high levels of anxiety in control animals, which could occlude effects in the BNST group. Since housing conditions profoundly influence measures for anxiety (Sartori et al., 2011), isolation housing after surgery may have concealed possible effects. This was demonstrated in a study on the anxiolytic properties of BLA-CE signaling that showed marked differences on baseline anxiety in group-housed versus single-housed animals, the latter displaying elevated anxiety measures (Tye et al., 2011).

Although mild unconditioned effects for the CE_l to BNST projections have been observed, the modulatory influence on fear conditioning seems to be more profound. The BNST group showed generalization of the fear response to unspecific cues, as indicated by freezing levels to the CS⁻, whereas the NBM group significantly discriminated between CS⁺ and CS⁻, in contrast to the control group.

As corticosterone in the hippocampus was shown to favor memory consolidation for unpredictable cues (Kaouane et al., 2012), PKC δ^- and probably CRH⁺ signaling to the BNST could account for the incorporation of inaccurate explicit cues. Corticosterone was shown to enhance memory to fearful events in the BLA and as a circulating hormone, it is temporally uncoupled from both the predictive and unpredictable phasic stimuli in fear conditioning, which could in turn lead to the generalization effects observed here. Nevertheless, CRH was also shown to trigger very similar effects as corticosterone does, although in a more central and direct fashion (McGaugh, 2004).

The apparent discrepancy between the conditioned and unconditioned effects are in line

with a hypothesis phrased by Davis et al. (2010). Because fibres from both the CE_l and BLA_p reach the BNST to converge in the $BNST_{ov/ fu}$, these afferents might provide two independent components of fear that are important for a sustained fear response. Accordingly, the BLA_p as the major site of plasticity, might deliver the actual information, whereas CRH from GABAergic CE_l terminals (and within the $BNST_{ov/ fu}$) facilitates glutamate release of BLA terminals via presynaptic CRH-receptors and could therefore code for the ‘arousal’ component in the BNST. Artificial elevation of CRH, achieved by optogenetic stimulation of CRH^+ terminals, accompanied by omission of an actual information substrate could then simply amplify noise and other signals arriving in the BNST. Conversely, in the conditioned situation, CS information might arrive in the BNST from the BLA_p , which is in turn facilitated by concomitant CRH release of $PKC-\delta_{CE_l}^-$ neurons. CRH exerts its effect via CRH1- and CRH2-receptors, which are present on presynaptic terminals of GABAergic terminals in the CE. These receptors were shown to be required for ethanol-induced enhanced GABAergic neurotransmission (Gilpin, 2012). That CRH might act irrespective of valence, was also demonstrated by Lemos et al. (2012), who showed that the valence of CRH in the nucleus accumbens diametrically shifted from pleasant to aversive, depending on the emotional state of the animal, which is in accordance with the results of the place preference assay that showed no avoidance behavior toward putative elevated CRH upon stimulation in the BNST (fig.3.3).

CRH in the PVH triggers the HPA axis and CRH overexpression leads to its hyperactivity. Apart from endocrine signaling, CRH influences autonomous functions of the body. Global overexpression of CRH in the CNS was shown to persistently elevate the heart rate of transgenic animals, thereby mimicking a hallmark of chronic stress (Dirks et al., 2002). Evidence for a CRH effect on heart rate specifically via the BNST was also shown by direct infusion of CRH into the BNST, resulting in tachycardia (Nijsen et al., 2001). The results shown in figure 3.6 are in line with previous studies and implicate the $PKC-\delta_{CE_l}^-$ projection to the $BNST_{al}$ in triggering autonomous responses to a fear stimulus. Nevertheless, the low number of subjects in this experiment confounds any conclusive evidence, thus more animals need to be added.

4.3 Central Amygdala Control of Emotional Salience

Similarly as for the PKC- δ_{CEl}^- projection to the BNST, activation of PKC- δ_{CEl}^- terminals in the NBM did not show any significant effect on baseline behavior. This is in line with a previous study that pharmacologically activated this projection in a mutant background for a serotonin receptor and selectively restored it in oxytocin-responsive neuronal population of the CE. Systemic treatment with the agonist resulted in no change of exploratory activity. However, a marked switch in the qualitative response to a CS in the fear conditioning paradigm could be observed. Instead of freezing upon CS-presentation, mice chose active avoidance as a behavioral strategy to the imminent threat in the fear conditioning paradigm (Gozzi et al., 2010). This behavioral switch could not be observed here and may originate from both qualitative or quantitative differences. First, the high-frequency light train generated by this optogenetic strategy interferes with endogenous signaling in these neurons, whereas pharmacological activation of the G-protein coupled serotonin receptor generally depolarizes the cell to a certain extent, thereby increasing spiking probability. Second, this approach also allows for a broad manipulation of all cells of this type, irrespective of projection pattern, whereas the optogenetic approach here specifically targets PKC- δ^- neurons, that do not respond to OT, project to the NBM, in a non-quantitative manner.

However, fear conditioning revealed effective differentiation between the predictive and non-predictive cue for the shock in the PKC δ_{CEl}^- terminal field stimulation in the NBM. Interestingly, it was shown previously that NBM function is required in selecting salient cues, whereas it is dispensable for the rejection of non-signals, which is in line with the results observed here (Everitt and Robbins, 1997). Although the precise mechanism has to be resolved, two general pathways are conceivable. Modulation of memory could occur via a BLA-CE-NBM-BLA loop, as NBM cholinergic neurons innervate the BLA (Everitt and Robbins, 1997). Thereby the CE could shape its own input and elicit fear responses in a more precise manner. As cholinergic agonists effectively modulate memory consolidation in the BLA and acetylcholine is most probably released in a more precisely timed manner than corticosterone, this might represent a route by which the amygdala selects the correct, predictive cue (McGaugh, 2004). Selective lesioning of amygdalopetal cholin-

ergic neurons of the basal forebrain was shown to impair memories to aversive events, as assessed with an inhibitory avoidance task (Power and McGaugh, 2002).

Acetylcholine in the neocortex is less implicated in memory, instead it is strongly associated with various attentional functions and cortical arousal (Pessoa, 2008). The amygdala exerts its impact on many sensory cortical areas, however, the indirect projection from CE_l to the NBM is very powerful in that it changes cortical activity more globally (LeDoux, 1995). The microcircuit mechanism in the CE seems to have relevance for cortical processes, corroborated by the observation that spontaneous activity of cells in the CE correlate with fluctuations in cortical EEG (Kapp et al., 1994; Phelps and LeDoux, 2005). Especially in the visual system, it was demonstrated that NBM activity increases performance in visual tasks. Stimulation of the NBM increased the response reliability to presentations of natural scenes, which is thought to result in increased detection performance (Goard and Dan, 2009).

GABAergic neurons in the CE_l are known to primarily innervate GABAergic neurons in the magnocellular basal forebrain. As the NBM is composed of GABAergic interneurons and long-range GABAergic projection neurons, there are two possible ways of effecting cortical processes. Inhibition of local interneurons in the NBM by GABAergic innervation of the CE_l would result in disinhibition of cholinergic neurons in the NBM that in turn increase thalamo-cortical information flow and cortical desynchronization (Goard and Dan, 2009). The second, although not mutually exclusive scenario, is that CE_l neurons innervate GABAergic projection neurons in the NBM, which themselves exclusively innervate GABAergic interneurons in the cortex. Accordingly, very few neurons in the CE_l would be capable of exerting control on a wide-spread cortical area (Zaborszky et al., 1999).

4.4 The C57BL/6N Strain is Refractory to Extinction Training

Extinction training did not significantly change the response to both CS in any group, compared to the testing session. The entire extinction training was composed of only 12 CS presentations each, which could mean that more presentations are required for efficient extinction. Nevertheless, studies have suggested the C57BL/6N mouse strain as a model for PTSD, since one single shock is enough to elicit common symptoms of PTSD, such as social withdrawal in this strain (Sartori et al., 2011; Siegmund and Wotjak, 2007). A hallmark of PTSD is the persistent fear response to an event that was previously associated with trauma, that is failure to recall extinction memory, which could explain the behavioral phenotype observed here (Milad et al., 2009).

4.5 Conclusion and Outlook

Projection-specific activation of CS-responsive $\text{PKC-}\delta_{\text{CE}_l}^-$ neurons produced largely no changes in baseline behavior, whereas modulatory impact within the fear conditioning paradigm could be observed. Given the high content of neuromodulators in the CE_l , an interpretation of these findings could be that apart from gating conditioned fear, this nucleus generally functions in gating stimuli outside of the amygdala according to their significance. To accomplish this, the CE_l takes advantage of a broad repertoire of intrinsic and extrinsic neurotransmitter/neuromodulators, such as CRH and acetylcholine, respectively (fig.4.1). The BNST could integrate CRH levels provided by the CE_l , which would then facilitate concomitant (unprecise) inputs into the BNST. The activation of the NBM by the central amygdala alters cortical activity, which could prioritize significant events for processing. It is conceivable that fast thalamic bottom-up information reach the LA or CE_l directly, which could instantly fuel cortical arousal by cholinergic drive from the nucleus basalis and which would in turn trigger top-down control over emotional responses by slower cortical inputs onto the same structures.

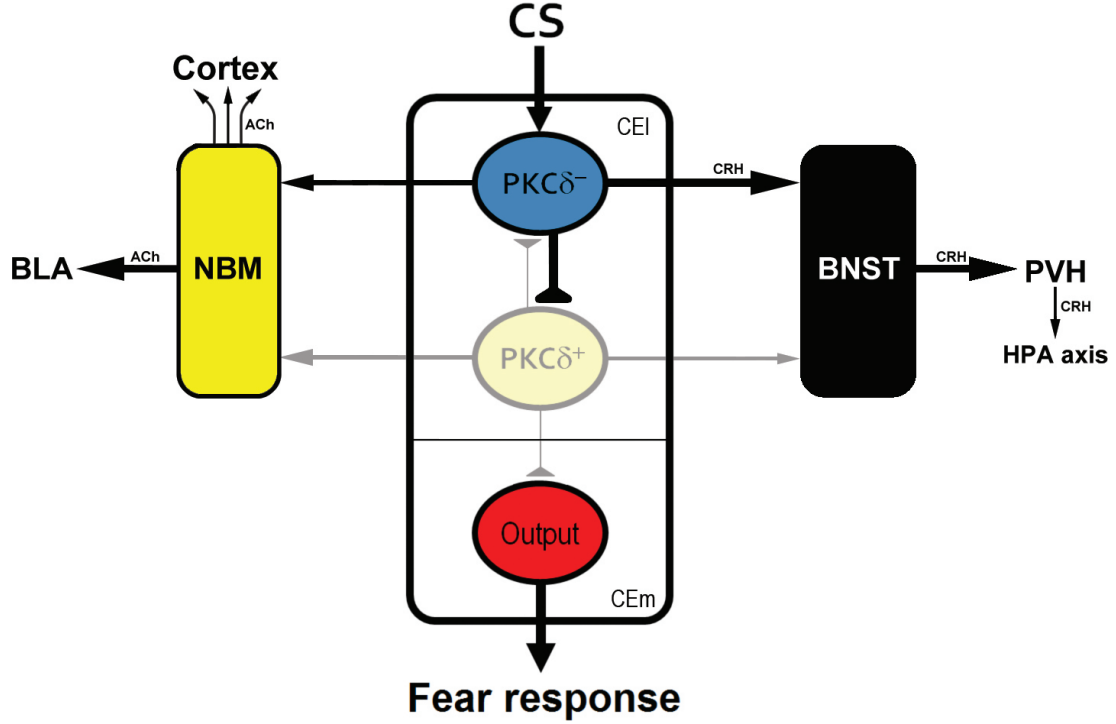


Figure 4.1: Proposed model on how PKC- δ^- neurons send efferent copies of a fear stimulus into anxiety- and cortical arousal-related circuitry, while at the same time disinhibiting neurons in the CE_m, thereby triggering a phasic fear response. Abbreviations: NBM, nucleus basalis of Meynert; BNST, bed nucleus of stria terminalis; CRH; corticotropin-releasing hormone; ACh, acetylcholine; CE, central amygdala; PVH, paraventricular hypothalamus; HPA axis, hypothalamo-pituitary-adrenal axis;

The role of PKC- δ^+ neurons with respect to the EA macrocircuitry remains to be elucidated. As the active population in basal conditions, NBM-projecting PKC- δ^+ neurons could sustain an arousal tone in the cortex, which could generate a general risk assessment state in the animal, as similarly proposed by Pape (2010). In accordance with the proposed model in section 4.2, where CRH provided by the PKC- δ^- population might facilitate glutamatergic afferent signals in the BNST, the minor CRH $^-$ PKC- δ^+ population may in contrast maintain homeostasis by their tonic GABAergic drive in the BNST in the absence of a fearful stimulus.

This thesis provides evidence for the relevance of a defined cell type in the CE_l, the PKC- δ^- neurons, that feed information into extra-amygdalar circuitry to mediate the diverse effects observed upon a fear stimulus. Although there is clear behavioral relevance, future effort has to be put into elucidating the precise mechanism by which these effects are realized in the target structures, notably the NBM and BNST.

5 References

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6 Appendix

6.1 List of Abbreviations

AB	accessory-basal amygdala
ac	anterior commissure
Acb	nucleus accumbens
AchR	acetylcholine receptor
ACTH	adrenocorticotrophic hormone
ADP	adenosine diphosphate
AHN	anterior hypothalamic nucleus
ARG	arginine vasopressin
B	basal amygdala
BDA	biotinylated dextran amine
BLA	basolateral complex
BMA	basomedial amygdala
BNST, BST	bed nucleus of stria terminalis
BOT	bed nucleus of the olfactory tract
BPM	beats per minute
BSA	bovine serum albumine
CA	cornu ammonis
CE, C, CEA	central amygdala
ChR	channelrhodopin
CNS	central nervous system
CO	cortical amygdala
CRH, CRF	corticotropin-releasing hormone
CS	conditioned stimulus
CT-B	cholera toxin B
DAPI	4',6-diamidino-2-phenylindole
DIO	double-floxed inverted open reading frame
dflox	double-floxed
EA	extended amygdala
EDTA	ethylenediaminetetraacetic acid
EEG	electroencephalography
EF	elongation factor
EPM	elevated plus maze
GABA	γ -amminobutyric acid
GAD	generalized anxiety disorder
GluCl	glutamate-gated chloride channel
GR	glucocorticoid receptor
hdB	horizontal limb nuclei of the diagonal band of Broca

HIP	hippocampus
HPA	hypothalamo-pituitary-adrenal axis
IL	infralimbic cortex
In, ITC	intercalated nuclei, intercalated cell masses
IPAC	Interstitial nucleus of the posterior limb of the anterior commissure
ires	internal ribosomal entry sequence
ITR	inverted terminal repeat
LA, L	lateral amygdala
LS	lateral septum
LTP	long-term potentiation
M, MEA	medial amygdala
MPN	medial preoptic nucleus
MR	mineralocorticoid receptor
NBM	nucleus basalis of meynert, nucleus basalis magnocellularis
NMDA	<i>N</i> -methyl-D-aspartate
MS	medial septal nucleus
NpHR	<i>Halobacterium pharaonis</i> halorhodopsin
NTS	nucleus of the solitary tract
OFT	open field test
OT	oxytocin
PAG	periaqueductal grey
PB	parabrachial nucleus
PBS	phosphate-buffered saline
PFC	prefrontal cortex
Pir	piriform cortex
PD	panic disorder
PKC- δ	protein kinase C- δ
PMD	dorsal premammillary nucleus
PMV	ventral premammillary nucleus
PTSD	posttraumatic stress disorder
PVH	paraventricular hypothalamic nucleus
PVT	paraventricular thalamic nucleus
RNA	ribonucleic acid
SEM	standard error of the mean
SI	substantia innominata
SLEA	sublenticular extended amygdala
US	unconditioned stimulus
vdB	vertical limb nuclei of the diagonal band of Broca
VMH	ventromedial hypothalamus
Vp	vasopressin
VP	ventral pallidum

6.2 Abstract

Emotions have been a major area of research over the last decade. In fact, fear is the emotion most heavily studied, which has led to a detailed understanding of the global neuroanatomy of fear. A key role is assigned to the amygdala, a central hub for emotion processing, located in the medial temporal lobe, which is known to mediate the behavioral and autonomous responses that we observe during the experience of fear. With the macrocircuitry in mind, knowledge of microcircuit mechanisms is just beginning to emerge, mostly due to the recent development of genetic and optogenetic techniques that allow for precise targeting of specific cell types and interfere with their function.

A microcircuit of distinct cell populations, marked by the presence or absence of protein kinase C- δ (PKC- δ), in the central amygdala (CE), the major output of the amygdalar complex, has been described in great detail. It has been shown that the relative activity of these cell populations is shifted upon the impact of a fear stimulus towards the PKC- δ^- neurons, as opposed to the tonic activity of PKC- δ^+ neurons in the resting state. This shift disinhibits neurons in the medial CE, thereby eliciting a fear response.

This study seeks to contextualize the knowledge of the microcircuit mechanism in the CE by embedding it in a conceptual macrostructure, the extended amygdala (EA). The EA includes the Bed Nucleus of Stria Terminalis (BNST) and the Nucleus Basalis of Meynert (NBM), structures known to be crucial for sustained fear responses and cortical arousal, respectively. In this thesis, the projections of PKC- δ^- neurons in the CE, that are activated by a fear-associated stimulus, were characterized histologically and functionally by optogenetic activation of their axon terminals in the BNST and NBM in a variety of behavioral paradigms that assess their impact on anxiety and fear.

We found that projections of PKC- δ^- and PKC- δ^+ neurons are asymmetrically represented in the NBM and BNST. Furthermore, activation of PKC- δ^- terminals from the lateral CE in the NBM and BNST exerted modulatory influence on fear learning in the Pavlovian fear conditioning paradigm. Stimulation in the BNST during training resulted in major generalization of fear that led to the incorporation of irrelevant stimuli into the fear experience. Conversely, stimulation of PKC- δ^- fibres in the NBM led to enhanced differentiation between predictive and non-predictive cues, probably due to enhanced at-

tentional functions in the cortex.

This study provides a first mechanistic framework for how a microcircuit in the CE_l coordinates behavioral and autonomous responses on the macrocircuit level. These findings could help in increasing the knowledge on how global macroanatomical principles are mediated on the microcircuit level.

6.3 Zusammenfassung

Die Forschung an Emotionen ist ein zunehmend bedeutendes Thema der Neurowissenschaften. Durch diese Anstrengungen konnte die grundsätzliche Neuroanatomie der Angst definiert werden, wobei die Amygdala im medialen Temporallappen eine zentrale Position einnimmt. Da sie sowohl die autonomen als auch die Verhaltensreaktionen, die ein Angststimulus induziert, vermittelt, wird die Amygdala nun als ein emotionales Zentrum des Gehirnes verstanden. Das neuroanatomische Wissen der Angst wird zunehmend durch neue Erkenntnisse über die Mechanismen in den neuronalen Netzwerken selbst komplementiert. Große Fortschritte gelangen nicht zuletzt durch methodische Neuerungen, wie das Aufkommen der Optogenetik, die eine örtlich und zeitlich präzise Kontrolle definierter neuronaler Populationen erlaubt.

Ein solcher Mikroschaltkreis wurde in der lateralen zentralen Amygdala (CE_l) beschrieben und von zwei unterschiedlichen Zellpopulationen, die sich durch die Expression der Proteinkinase C- δ (PKC- δ) unterscheiden, gebildet. Im Ruhezustand sind die PKC- δ^+ Zellen tonisch aktiv und inhibieren damit PKC- δ^- und Stammhirn-projizierende Zellen in der medialen CE. Die relative Aktivität dieser Zellpopulationen wird jedoch durch einen Angststimulus zu den PKC- δ^- Zellen hin verschoben, wodurch die PKC- δ^+ Zellen inhibiert und somit die Effektorzellen in der medialen CE disinhibiert werden, wodurch Angstverhalten ausgelöst wird. Im Zuge dieser Arbeit sollte das Wissen über die neuronalen Mechanismen in der CE_l auf einen konzeptionellen Makroschaltkreis, der Erweiterten Amygdala (EA), angewandt werden. Zur EA zählen der Bed Nucleus striae terminalis (BNST) und der Nucleus Basalis (NBM). Es wurde zuvor gezeigt, dass der BNST für chronische Angstzustände verantwortlich ist, wohingegen die Aktivität im NBM kortikale Erregung verursacht. Die Projektionen der CE_l zum BNST und NBM wurden im Zuge dessen histologisch und funktionell, anhand unterschiedlicher Verhaltenstests charakterisiert.

Diese Arbeit zeigt, dass der Beitrag der PKC δ^+ und PKC δ^- Zellen der CE_l zur jeweiligen Projektion zum BNST und NBM eine Asymmetrie aufweist. Ebenso, dass die optogenetische Aktivierung der Axone von PKC δ^- Zellen im BNST und NBM modulatorischen Einfluss auf die Angstkonditionierung nimmt. Die Aktivierung im BNST

bewirkte eine Generalisierung der Angst, sichtbar durch eine vergleichbare Angstreaktion zu einem unspezifischen Stimulus. Im Gegenteil dazu zeigte eine Aktivierung im NBM eine Verbesserung der Unterscheidungsfähigkeit zwischen den spezifischen und unspezifischen Stimuli, womöglich ausgelöst durch erhöhte kognitive Prozessierung in kortikalen Regionen.

Diese Arbeit zeigt, wie ein Mikroschaltkreis in der CE_l autonome, wie auch Verhaltensreaktionen der Angst im Kontext eines Makroschaltkreises koordiniert. Diese Resultate könnten zur Aufklärung beitragen, wie globale makroanatomische Prinzipien durch Mikro-schaltkreise vermittelt werden.

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	Elisa	Patch Clamp
	Elispot	Western Blot
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	Receptor Binding Studies	Cryosection

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PUBLICATIONS

*Malin Hedlund, Olga Nagaeva, Dominic Kargl, Vladimir Baranov, Lucia Mincheva-
Nilsson. Thermal- and oxidative stress cause enhanced release of NKG2D ligand-
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2011 Feb 25;6(2):e16899.*