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A Connection for Object Valence“

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CONTENTS

1. INTRODUCTION

1.1 INVESTIGATING THE BRAIN

Modern neuroscience combines molecular biology, neurophysiology, anatomy, embryology, cell biology, and psychology - ultimately not only to understand how the brain works, but also how its activity is connected to our conscious and subjective experiences. In the last decades, huge progress has been made in exploring the anatomy and function at the level of single neurons and neuronal circuits. Together with improvements in imaging techniques it is now possible to start to decipher circuit level brain activity, and connect it to feelings and behavior. The assembly of information, from local molecular to brain wide activities, brings us closer to our eternal quest for understanding what and who we are.

1.1.1 THE BRAIN

The human brain is a network of more than 100 billion individual nerve cells, where each individual cell communicates with thousands of other cells in every moment of our lives. This vast flow of information ultimately constructs our perception, fixes our attention, and controls the machinery of our actions (Kandel 2000). Investigating the brain began centuries ago, but did not become the subject of distinct science until the late 1800s, when the first detailed descriptions of nerve cells were performed by Golgi and Santiago Ramon y Cajal. They identified the neuron as the elementary signal element of the nervous system (neuron doctrine) and recognized that processes of these building blocks are essential parts for the flow of information (Bear et al. 2007). Already before, Franz Joseph Gall, a German physician and neuroanatomist introduced three radical and pioneering ideas. First, that all behavior derives from the brain. Second, that specific regions in the cerebral cortex control specific functions and third, that mental function grows with use (Kandel 2000). Today much more is known about the transaction of the brain, but still several concepts remain largely valid.

1.1.2 MAIN FUNCTION

Why has the nervous system evolved and what is it for? Obviously the most important function of the nervous system is to promote survival and prevent death in a constantly changing environment. This requires adaptive decision making and behavioral programs that keep us away from danger and guide us to reward (Moser et al. 2014). At the heart of these actions are basic emotions, like fear and reward. Fear behaviors prevent negative experiences and reward behaviors help in seizing of opportunities. What is experienced as "bad" will be avoided and what is experienced as "good" will be repeated. Emotions are the driving force for reacting and remembering significant events and therefore an essential feature of the nervous system for survival.

1.2 EMOTIONS

Responses that occur when we defend against danger, interact with sexual partners, fight with an enemy, or have a tasty bite to eat promote the survival of individuals and their species (LeDoux 2000).

Studies of the neuronal basis of emotion already have a very long history in the field of neuroscience (Figure 1) (LeDoux 1987; LeDoux 1991). Although emotions have such a strong influence on our everyday lives, the definition of what an emotion actually is still remains a topic of lively discussion.

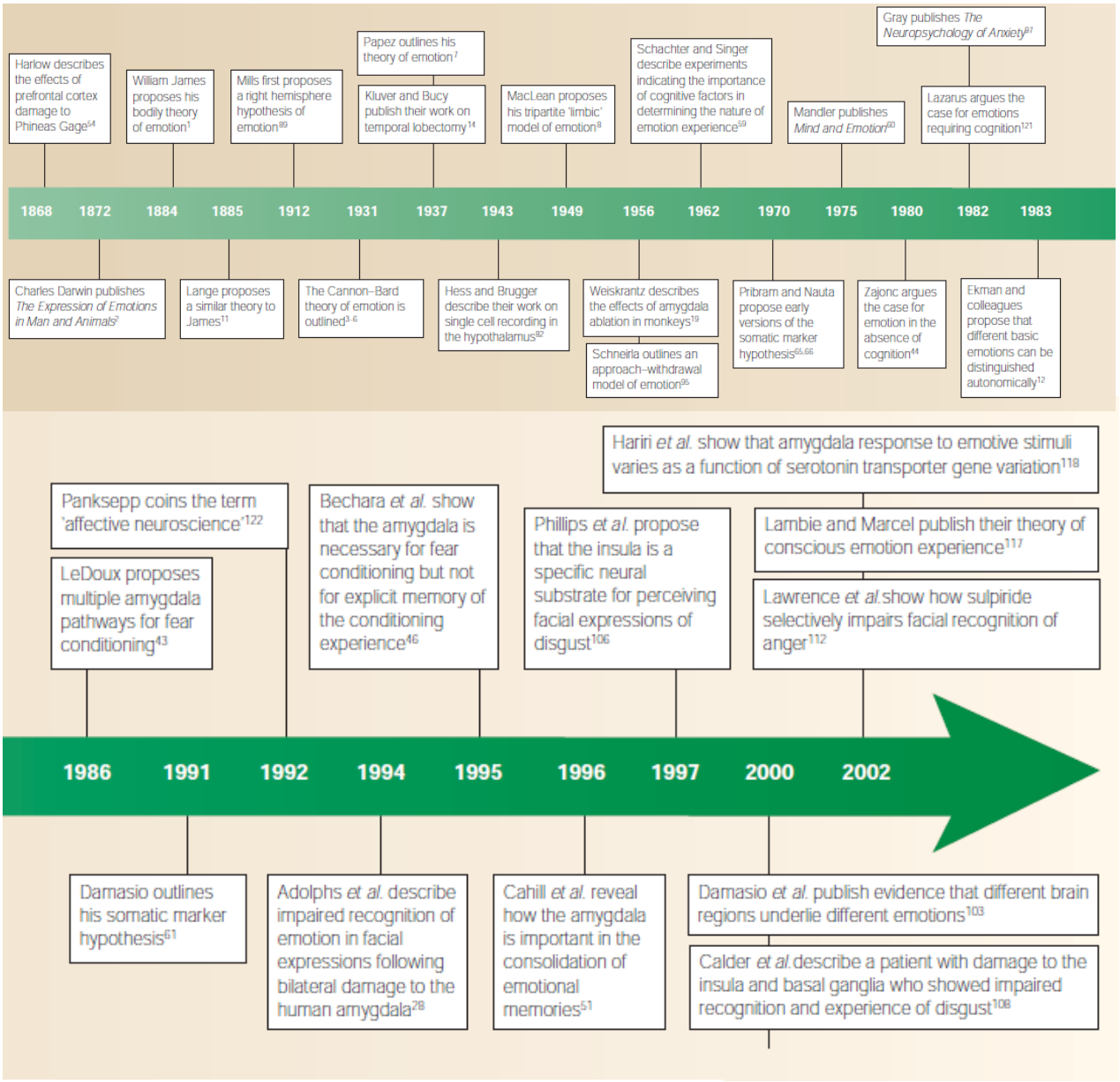


Figure 1. History of emotional research (Dalgleish 2004).

1.2.1 HISTORY OF EMOTION RESEARCH

First ideas about the nature of emotions probably arose from the work of Charles Darwin. In his book "The Expression of the Emotions in Man and Animals", published in 1872, he compared pictures and photographs of animals and people in different emotional states (Figure 2) (Darwin 1965). Two of his observations, that contributed predominantly to the field, were that at least some animal and human emotions are homologues and that a limited set of fundamental or basic emotions are present across species (Dalgleish 2004). The idea of basic emotions is also discussed thoroughly in a recent publication, where the authors support the notion that "emotional primitives" exist across phylogeny and can therefore be used to study emotions even in evolutionary very distant species (Anderson & Adolphs 2014). About 10 years after Darwin's disclosure William James proposed in his paper "What is Emotion" that emotions are simple expressions of bodily changes we have experienced (James 1884). Carl Lange also introduced similar ideas which led to the James-Lange theory (Lange 1885). This view was first challenged by Walter B. Cannon in the 1920s (Cannon 1927; Cannon 1931). Together with Philip Bard he showed that separation of the viscera from the brain in animals did not impair emotional behavior. In addition they performed cortical lesions in cats and observed inappropriate anger attacks, named sham rage (Dalgleish 2004). Because the separation of body perception from the brain, as well as the lesion of motor and somatosensory cortices had not led to a complete loss of emotional behavior, Cannon and Bard argued that the James-Lange theory was wrong. On the basis of their research, Cannon and Bard introduced the first theory for the brain mechanisms of emotion (Bard 1928; Bard & Rioch 1937). In their theory they argued that the hypothalamus is involved in emotional responses to stimuli. Normally the hypothalamus is inhibited by

neocortical regions, but removal of these regions frees the hypothalamic circuit from top-down control and allows for uncontrolled emotional behavior (Dalgleish 2004).

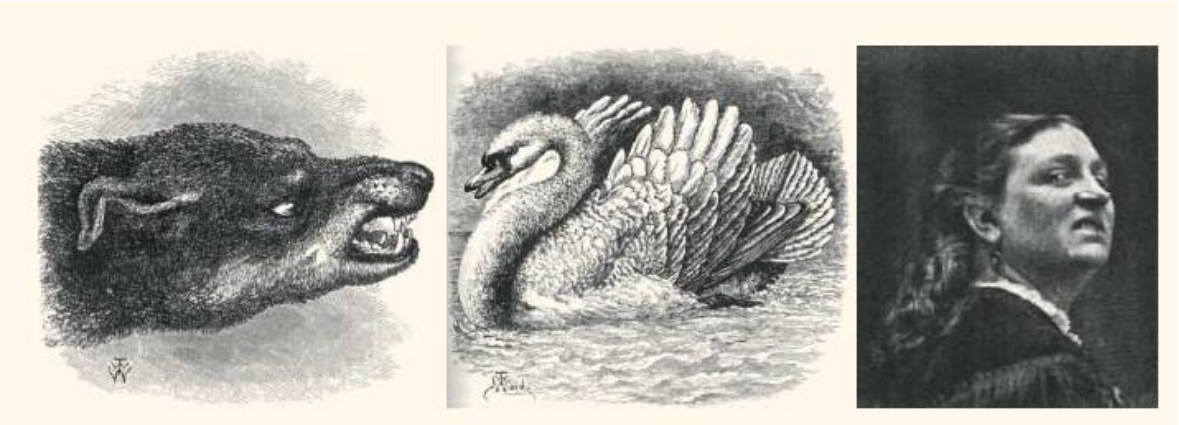


Figure 2. Expression of anger/aggression across different species Drawings from Charles Darwin's "The Expression of the Emotions in Man and Animals". **Taken from** (Dalgleish 2004).

In 1937, James Papez proposed several structures to be involved in the central neural circuit of emotions (Papez 1937). He thought of the thalamus as a junction where sensory information diverges into an upstream "thought" and downstream "feeling" pathway. He proposed that the cingulate cortex is a very important structure for the thought stream, where sensation becomes perception and memories get formed. The feeling route goes directly from the thalamus to the mammillary bodies and down to bodily systems. In his theory, the top-down control would arise from the cingulate cortex (Dalgleish 2004). Further information about important systems for emotion came from the seminal work of Klüver and Bucy. When they bilaterally removed the temporal lobe in monkeys they observed a very typical set of behaviors (Kluver & Bucy 1937). These included the loss of fear to previously threatening stimuli, an increased exploratory behavior, hypersexuality, and the attempt to eat a variety of things considered repulsive (feces, meat, rocks) (LeDoux & Phelps 1982). Paul MacLean in his attempt to build on these findings categorized the brain into three subareas, namely an ancient reptilian, an old mammalian and a new mammalian region and allocated different brain structures to these regions. He included many of the components of the Papez circuit, but also integrated two new and very important structures into the emotional brain system: the prefrontal cortex (PFC) and the amygdala (MacLean 1970). He also introduced the term "limbic system" for what he believed to be the visceral brain (MacLean 1952). The concept

of the limbic system was challenged when it was shown that damage to the hippocampus led to a distinct cognitive deficit in long term memory (Scoville & Milner 1957). The theory suggested that the limbic system would have a primitive architecture, not suited for cognitive functions and the hippocampus was believed to be an important part of this system. Although several ideas of MacLean's system turned out to be incorrect, his theory that emotional experiences are formed from the integration of sensations from the world with information from the body (Dalgleish 2004) still remains valid. Taken together, the interplay between perception of the outside world, which leads to physiological changes, and the integration of this into ongoing sensation, should lead to the emotional experience.

Today there is broad agreement that emotional states are experienced consciously as well as unconsciously. The cognitive processes run through pathways that connect the amygdala with the cerebral cortex. Especially subcortical parts of the nervous system, like the amygdaloid nuclei, the hypothalamus and the brain stem are principle structures for mediating the unconscious autonomic, endocrine and motor responses (Kandel et al. 2000). In contrast, conscious feelings are mediated by the cerebral cortex, where the cingulate cortex and the frontal cortices play essential roles.

The current challenge is to bring this gross functional neuroanatomy of emotions down to circuit level mechanisms of emotional processing. A good point of entry is to first look at circuits that are directly connected to functions allowing an organism to survive and thrive by detecting and reacting to challenges and opportunities (LeDoux 2012). As these basic functions should be conserved throughout evolution, new insights from simple organisms could potentially help in deciphering the blueprints for emotions also in humans.

1.2.2 EMOTIONAL VALENCE

At the heart of emotions, standard models assumed a bipolar spectrum ranging from negative to positive states (Briesemeister et al. 2012). But this very simplified model harbors several problems in interpreting various emotional states. In addition, widely used terms such as fear or happiness can obviously be interpreted in different ways. Fear of the bear approaching or fear experienced during a horror movie, though having probably overlapping experiences of fear would not be described as the same "feeling" or initiate the same reactions in the end (Russell 2003). Therefore, emotional states have to be more than simple extremes of two

opposing options. Emotional valence can be seen as the continuum of possible states along the line. Positive valence means a shift to rather positive association for e.g. a place, an object or an experience. Negative valence describes a more negative association to various stimuli. So, positive valence can also be associated with pleasantness and negative valence with unpleasantness (Viinikainen et al. 2010). But still, the question remains, how these various states can be explained psychologically and physiologically.

Two theoretical approaches have gained major attention in this discussion. The first assumes a limited number of discrete emotions with specific characteristics as well as physiological correlates and behavioral action tendencies (Briesemeister et al. 2012). These converge to emotional experiences, where at least 6 emotions are accepted: happiness, anger, disgust, fear, surprise and sadness (Viinikainen et al. 2010).

The second model builds upon a dimensional space. It assumes that a limited number of independent affective dimensions account for the emotional experience (Briesemeister et al. 2012).

The dimensional theory was especially refined by James A. Russell. He followed the concept of Oatley and Johnson-Laird (1987) who thought of two different dimensions, namely pleasure-displeasure (valence) and activation-deactivation (arousal). The arousal level can also be seen as the intensity of an emotion. Combining these two into a two dimensional space creates what Russell termed core affect (Russell 2003).

A feeling is therefore a single integral blend of two dimensions, with pleasure-displeasure as the horizontal dimension and arousal as the vertical dimension (Figure 3).

The attractiveness of this model comes from its broad application to different situations. Very subtle feelings could stay unconscious while very strong emotions will come into consciousness, are interpreted further and if necessary adapted to the present needs (Russell 2003).

In addition to the core affect, Russell introduced the affective quality. He described it as the property of the stimulus to change core affect. So, a negative experience with e.g. a person will lead to a change in core affect and therefore elicit behavior appropriate for reacting to that person.

Another very important concept is the attributed affect. It is defined by three features: a change in core affect, an object, and attribution of the core affect to the object. So a negative

experience to an object will attribute the core affect to this object and update the affective quality.

As a psychological model, Russell's ideas contributed a lot to our understanding of the variety of emotional states and the continuum connecting cursorily viewed similar emotions. Depending on the positive or negative valence as well as the arousal associated with a given experience, this model can provide very precise information of the emotional state appearing. A psychological concept of emotional valence is therefore at hand, but still very little is known about the physiological correlates in the brain. One recent study, using fMRI was able to find separable correlates for negative and positive valence when showing pictures of different emotional associations to human subjects (Colibazzi et al. 2010). They found higher BOLD signal associated to negative valence in the dorsolateral prefrontal cortex, anterior medcingulate cortex, frontal pole and inferior parietal cortex. By contrast, higher activation of the substantia nigra, ventral striatum and right caudate nucleus was seen for positive valence. It seems that within the brain several structures encode for different valence. The interplay of several components probably leads to the representation of a specific valence,

Figure 3. Russell's Dimension Model
According to Russell, core affects can be depicted on a two dimensional space. The horizontal axis illustrated the valence and the vertical axis the arousal level of a core affect. Depending on where on the chart the specific parameters come together, different core affects will be elicited. Several affects are illustrated. **Taken from** (Calder et al. 2001).

that together with the arousal level, can form the actual emotional feeling or in Russells' words, the core affect. However, there is still little known about the circuits which can actually trigger these responses.

One of the most basic and probably best studied emotions is fear. Psychological models as well as substantial knowledge about brain structures involved in fear learning and expression are already established. Therefore, to learn more about emotional valence it makes sense to first understand the mechanisms of fear.

1.2.3 FEAR AND ANXIETY

First of all it is deemed necessary to specify the terms, as “fear” and “anxiety” are often used imprecisely and several definitions have been set down for their description. According to Blanchard, fear is the motivation associated with behaviors that normally occur on exposure to clearly threatening stimuli (Blanchard et al. 2008). In contrast, anxiety can be seen as the motivation, associated with behaviors that occur to potential, signaled or ambiguous threat (Blanchard et al. 2008). Hence, anxiety is used when the source of threat is uncertain, or even unrealistic. It is the response to potential, rather than clear and present threat. Fear, in contrast, is the response to a realistic, often imminent threat which also elicits quite clear responses such as flight, avoidance or freezing (Blanchard & Blanchard 2008).

What makes fear an especially good model for studying emotions is firstly its strong conservation across many different species. There are several behaviors which have been characterized in a variety of species like flight, avoidance, freezing etc. (see e.g. (Blanchard 1997)). Secondly, there are well defined experimental procedures available for investigating fear, which can broadly be used both in animals and humans. Thirdly, fear is connected to many psychological pathologies and therefore a very important field for a profound mechanistic understanding of these disorders (LeDoux 1995).

CLASSICAL FEAR CONDITIONING

Pavlovian conditioning is one of the standard methods for fast and reliable connection of a neutral with a relevant stimulus. In emotional research one of the most widely used paradigms is classical fear conditioning.

In the classical fear conditioning paradigm, a biologically neutral stimulus, called the conditioned stimulus (CS), is temporally paired with a biologically relevant event, the unconditioned stimulus (US). This CS-US pairing initiates an associative learning process. The informational relationship between the CS and US forms the essential determinant of classical conditioning (Kim & Jung 2006). Therefore, the CS acquires the aversive properties of the US. After learning, the CS alone will lead to similar behavioral and physiological responses (the conditioned response (CR)) which would be normally elicited by the unconditioned stimulus (unconditioned response (UR)) (LeDoux 2000). The very effective tone-footshock pairings are one of the most widely used protocols for investigating fear and anxiety (Figure 4). In this setting, a tone is used as the CS and the footshock represents the US. Already after one to a few tone-footshock pairings, the sound presentation alone will produce typical defensive behaviors and physiological changes, normally elicited by the presence of the footshock (Blanchard & Blanchard 1969; Schneiderman et al. 1974). Also a variety of other stimuli can be used as the CS, such as visual or olfactory stimuli as well as the environment in which the US is experienced (i.e. the context).

One behavioral response which turned out to be a very informative measurement for fear stimuli and was extensively studied in rodents is freezing behavior (Fanselow & Bolles 1979; Sigmundi et al. 1980). Freezing is defined as the complete suppression of all movements, except those which are needed for respiration (Anagnostaras et al. 2010). The discovery that freezing is very sensitive to parametric manipulations which, as a result was expected to influence the level of learning and fear, was especially useful. It was shown for instance that freezing level increases with the number of trials and importantly also the intensity of a footshock in paired paradigms (Fanselow 1979; Young & Fanselow 1992).

1.2.4 FEAR AND THE AMYGDALA

Probably the earliest hints that the amygdala is involved in emotional processing came from studies by Klüver and Bucy, who showed that lesions to the temporal lobe in primates led, amongst many other symptoms, to a loss of fear response to previously threatening stimuli

(Kluver & Bucy 1937). Weiskrantz and others determined that confined lesions to the amygdala were responsible for the emotional components of the Klüver-Bucy syndrome (Weiskrantz 1956). Following studies have demonstrated reduced fear after amygdala damage in different mammalian species (Goddard 1964).

Having observed an important role of the amygdala in fear, scientists started to further investigate its involvement in learning and memory. Fuster and Uyeda found, after light-shock pairings that light alone evoked activity in the amygdala (Fuster & Uyeda 1971). One year later it was shown that extensive amygdaloid lesion dramatically reduced freezing to a context that was paired with a shock before (Blanchard & Blanchard 1972). Today there is already a large body of evidence from lesion, neurophysiology and pharmacology studies that the amygdala is an essential brain system in processing fear conditioning (Davis 1997; Fendt & Fanselow 1999; LeDoux 2014).

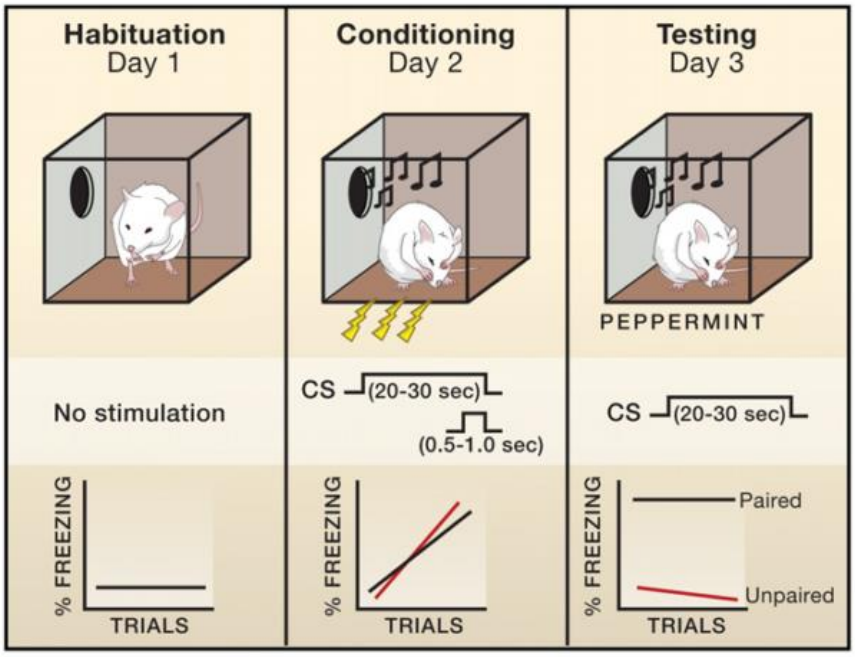


Figure 4. Pavlovian Fear-conditioning protocol typically used in rodents (**Top panel):** Shows schematic of fear conditioning. (**Middle panel):** The cues presented and (**Lower panel):** The percentage of freezing to the sounds. In this paradigm, two sounds were used and freezing values to both sounds are depicted. On the first day, the animal was placed in the experimental chamber to habituate to the environment **(Habituation)**. On the next day, the paired sound was played in temporal relationship with the footshock (US) and therefore represented the CS (Paired) **(Conditioning)**. A dissociable unpaired sound was played in random order and would therefore not be associated with the footshock (Unpaired). The effect was seen on the third day, where freezing was high to the paired tone but not the unpaired sound **(Testing)**. The testing session was performed in another environment, which could be another box shape, but also a different odor like peppermint, used in this setting. **Taken from** (Johansen et al. 2011).

1.3 THE AMYGDALA

The term amygdala (Greek for almond) was first used by the anatomist K. F. Burdach in 1819 to describe an almond shaped, subcortical cell mass located deeply in the medial temporal lobe, just rostral to the hippocampus (Davis & Whalen 2001). What Burdach described was probably the part of the amygdala referred today as the basolateral division of the amygdala.

In the 1920s J.B. Johnstone further investigated the amygdala and is credited for the partition of this structure into the basolateral, centromedial and cortical division (Whalen & Phelps 2009). He assigned the central, medial, and cortical nuclei and the nucleus of the lateral olfactory tract to a phylogenetically older corticomedial group and the lateral, basal and basomedial nuclei to a phylogenetically newer basolateral group (Knapska et al. 2007).

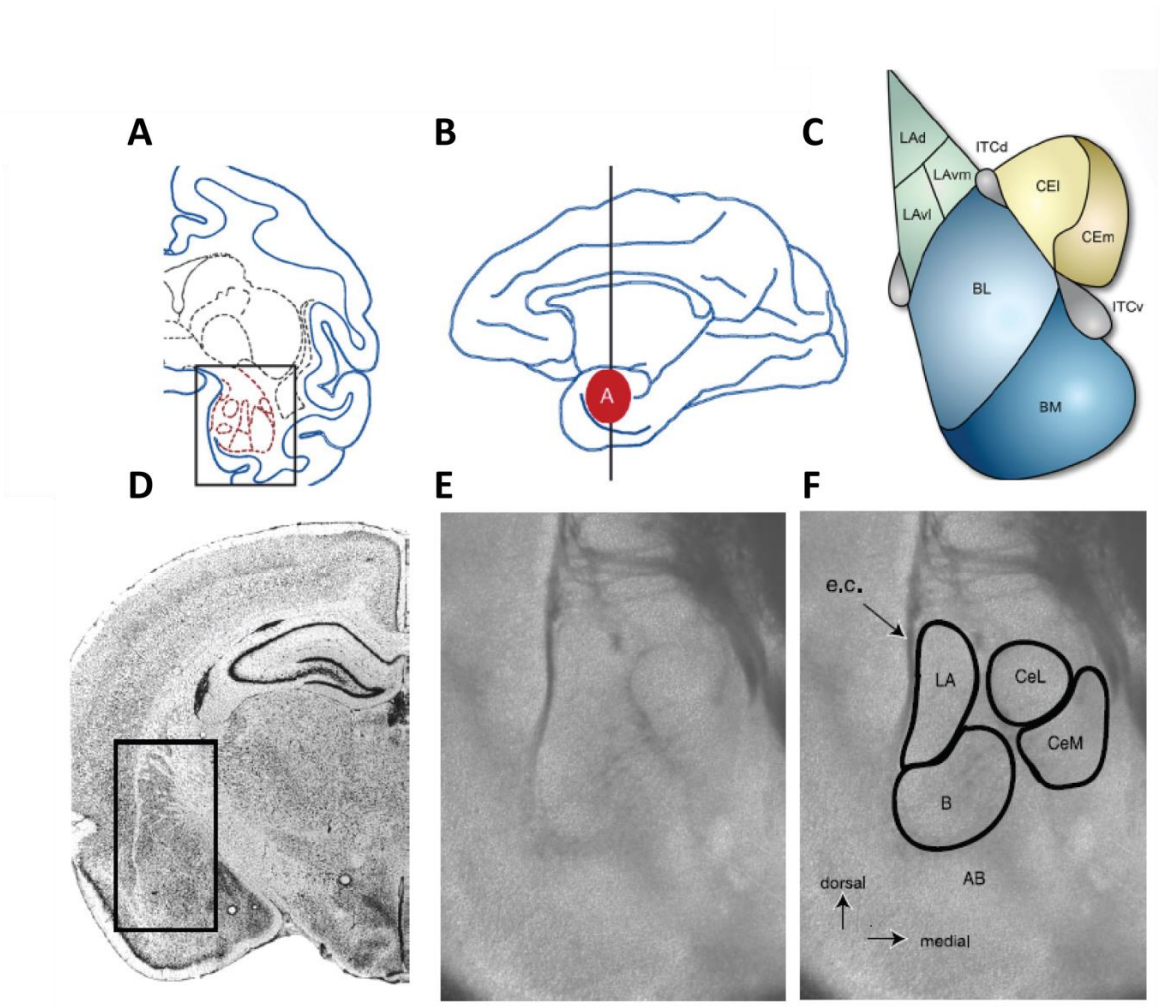


Figure 5. Amygdala anatomy **(A)** Coronal section of the rhesus monkey brain, showing amygdaloid nuclei in the black rectangular box, depicted with red dashed lines; **(B)** Medial aspect of the right hemisphere of the brain, line indicates position of coronal section in **(A)**. **Taken from** (Murray 2007); **(C)** Simplified structure of the amygdala cortex like structures are depicted in blue, while striatum-like subnuclei in yellow. **Taken form** (Lee et al. 2013); **(D)** Coronal section of the rodent brain with Nissl-staining, position of amygdala is indicated in the rectangular box; **(E) and (F)** Magnification of **(D)** with assigned amygdaloid subnuclei. **Taken from** (Sah et al. 2003); **Abbreviations: (C)** LAd, LAvl, LAvm,- dorsal, ventrolateral, ventromedial lateral amygdala, respectively; BL, basolateral nucleus; BM, basomedial nucleus; CEl, lateral part of the central amygdala; CEm, medial part of the central amygdala; lITC, lateral paracapsular intercalated cell mass; ITCd, dorsal part of the intercalated cell mass; ITCv, ventral part of the intercalated cell mass; **(F)** LA, lateral amygdala; B, basal nucleus; AB, accessory basal nucleus; CeL, lateral part of the central nucleus; CeM, medial part of the central nucleus.

The amygdala is a very heterogeneous structure consisting of around 12 nuclei, where some parts have properties closely related to the cortical organization while others are more similar to the striatum (Figure 5C). Very important nuclei, especially in terms of fear and anxiety, are found in the basolateral complex (BLA), consisting of the lateral (LA), the basolateral (BL), and the basomedial (BM) nuclei, the latter also being known as the accessory basal (AB) nucleus. Additional parts are the central nucleus (CeA) which is divided in a lateral (CeL) and a medial (CeM) region and the intercalated cell masses (ICMs) (Duvarci & Pare 2014).

In general, the LA is believed to be the main entry point for sensory information to the amygdala, whereas the CeM is the main output structure which sends projections to a variety of effector regions (Pape & Pare 2010).

1.3.1 Anatomical Organization

BLA

The cellular architecture of the BLA is often related to that of the cerebral cortex, because it consists of mainly (~80%) spiny glutamatergic neurons (principal neurons) with mostly regular spiking phenotype. The second class of neurons in the BLA is a minority (~20%) of sparsely spiny, local-circuit GABAergic interneurons (Pape & Pare 2010). While the majority of GABAergic interneurons in the BLA are local-circuit cells, just recently a small subset of neurons, projecting to the basal forebrain have been found (McDonald et al. 2012). Pharmacologically it is important to mention that BLA principal cells exhibit spike frequency adaption via differential expression of voltage- and CA2+-dependent K+ conductance (Sah et al. 2003). The adaptation is strongly reduced by corticosterone and norepinephrine, thereby increasing the excitability of these cells to emotional conditions (Duvarci & Paré 2007). In the rodent BLA there are at least five classes of GABAergic interneurons, the two main types of which express parvalbumin (PV+) or somatostatin (SOM+) (McDonald & Betette 2001; McDonald & Mascagni 2002).

CeA

The central nucleus, divided into the CeL and CeM, mainly contains GABAergic neurons (Pare & Smith 1993; Saha et al. 2002) and resembles the constitution of the striatum. In the

CeA, principle cells are connected by GABAergic synapses and it was shown that local application of glutamate in the CeL evoked inhibitory post synaptic potentials in CeL neurons (Lopez de Armentia & Sah 2004). It is an interesting fact that CeL neurons extensively project to the CeM, but there are merely weak projections from the CeM to the CeL (Jolkkonen & Pitkänen 1998). It has just recently been found that populations of neurons in the CeL project to CeM cells which contact different brainstem sites. CeL neurons which express the oxytocin receptor (OR^+) form contacts to CeM neurons that innervate the periaqueductal gray (PAG). But CeM cells which project to the dorsal vagal complex (DVC) get input from OR^- CeL neurons (Viviani et al. 2011). Further distinction can be made because numerous OR^+ cells also express subunit δ of protein kinases C (PKC-δ), whereas the OR^- cells express somatostatin (SOM) and corticotropin releasing hormone (CRH) (Haubensak et al. 2010; Li et al. 2013; Duvarci & Pare 2014).

1.3.2 THE AMYGDALA AND FEAR LEARNING

Fear learning was probably most intensively studied in the fear conditioning paradigm where tone-footshock pairings are applied for associative fear learning. As mentioned earlier, a sound (CS) is paired with a footshock (US) which induces associative learning. The physiological and behavioral responses will fall under the control of the CS. After a few pairings the tone alone will lead to defensive responses (CR).

The lateral amygdala seems to be critical for the acquisition and retention of fear conditioning, as lesions of the LA have shown to prevent fear conditioning and expression (LeDoux et al. 1990; Amorapanth et al. 2000; Gale et al. 2004). The central nucleus appears to be the main output region of the amygdala, with connections to the hypothalamus and brainstem (Kapp et al. 1979; Cain & LeDoux 2008).

The auditory thalamus and cortex both project to the lateral nucleus of the amygdala (LA), where inputs converge even on a single cell (Romanski & LeDoux 1991; Romanski et al. 1993). LeDoux and colleagues revealed that projections from the thalamus provide the amygdala with fast, but imprecise auditory signals (LeDoux et al. 1991). More elaborate representations of cortical inputs from auditory and other sensory systems arise from association areas rather than from primary cortical regions, but these are slower because several connections are involved.

Within the lateral amygdala CS and US information can converge onto single neurons (Romanski et al. 1993). The current hypothesis is that CS input to LA is relatively weak in the beginning, so the CS is unable to elicit fear responses. However, when the CS is presented in association with a significant event (e.g. the shock (US)), the specific CS synapses get potentiated and as a result can now interact with fear effector regions like the CeM to elicit a fear response. However, the LA is not connected directly to the CeM (Pitkänen et al. 1997), instead the information reaches the central medial nucleus indirectly, via glutamatergic projections from the basal amygdala (BA) or inhibitory GABAergic connections from the CeL (Duvarci & Pare 2014). Different studies have already shown that BA neurons enhance activity due to fear conditioning and in addition, BA inactivation leads to reduced conditioned fear responses (Amano et al. 2011).

Also the CeL projections to the CeM have now been investigated in more detail (Ciocchi et al. 2010). Selective inactivation of the CeL impaired fear acquisition, while inactivation of CeM impaired fear expression. However, how the inhibitory input from the CeL to CeM could lead to an increased neuronal activity in the CeM remained unclear. In the same study

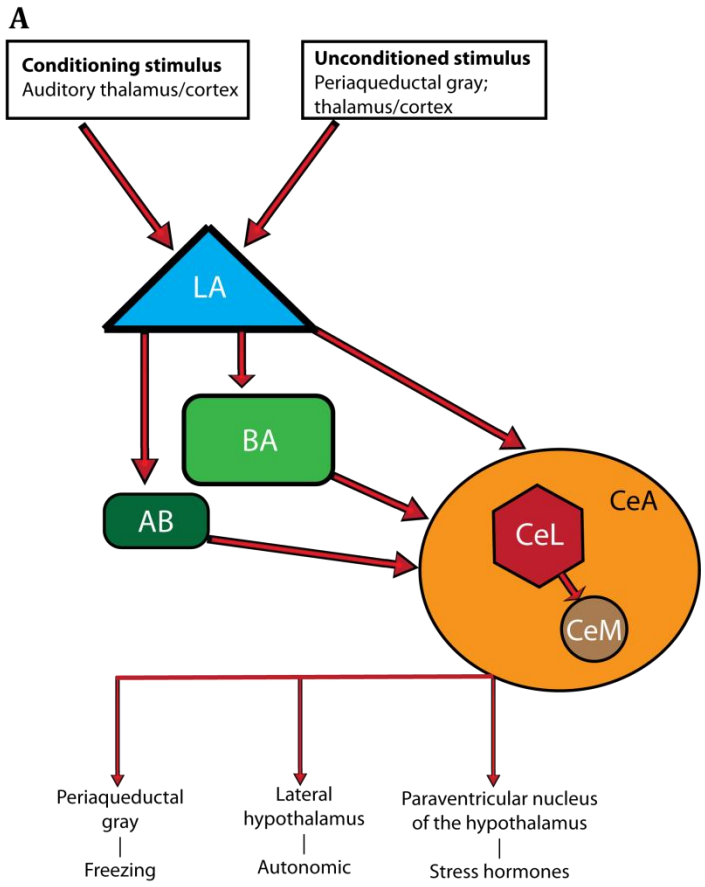


Figure 6. Amygdala fear conditioning circuits The convergence of the conditioned stimulus (CS) and the unconditioned stimulus (US) in the amygdala is essential for fear conditioning. On the level of the lateral amygdala (LA) the sound (CS) and the shock (US) converge, leading to synaptic plasticity in the LA. The LA is connected directly to the central nucleus, or indirectly via projections to the basal nucleus and accessory basal nucleus. Finally, the medial nucleus of the central amygdala, as the main output, sends information to downstream regions that control expression of conditioned fear. **Adapted from** (Johansen et al. 2011).
Abbreviations: LA, lateral amygdala; BA, basal amygdala; AB, accessory basal amygdala; CeA, central amygdala; CeL, lateral part of the central amygdala; CeM, medial part of the central amygdala.

it was shown that in the CeL there are two populations of neurons, one with an inhibitory (CeL-Off) and the other with an excitatory (CeL-On) response to the CS after fear conditioning (Ciocchi et al. 2010). It was shown that CeL-Off neurons correspond to the PKC-δ^+ neurons that also express the oxytocin receptor mentioned earlier (Ciocchi et al. 2010; Haubensak et al. 2010). These two populations reciprocally inhibit each other and, importantly, gate amygdala output.

1.3.3 THE AMYGDALA AND VALENCE

It has been a well known fact for a long time, that the amygdala is involved in learning and expression of negative experiences and fear responses, respectively (see section 1.3.2). However, over the last decades more and more studies also report amygdaloid activity to positive experiences and reinforcers. In one of these studies Paton et al. looked at activity in the amygdala regarding positive or negative reinforcement in monkeys (Paton et al. 2006). In their study, visual stimuli acquired positive or negative valence through Pavlovian conditioning. After animals had learned the task, they inverted the stimuli-valence association and checked for cellular activity in amygdaloid nuclei. Paton et al. observed a change in neuronal activity over trials, reflecting the reinforcer reassignment. In addition, the animals' responses to the stimuli correlated closely with the neuronal activity. Interestingly, the changes in neural firing were mostly seen in the BLA. Therefore it seems that the amygdala with its various subnuclei could play an important role in integrating and processing valence information and finally communicating it to different output structures. The ability to change neural activity in concert with a shift in valence could account for a hub function of the amygdala in adapting emotional relevance depending on the situation encountered.

1.4 SCIENTIFIC QUESTION

Numerous studies have used Pavlovian conditioning as proxy to explore emotional behaviors (see section 1.3.2). Although these studies provided us with a valuable conceptual and neuronal framework for understanding basic mechanisms of fear expression and learning, we are still lacking information of how the brain computes more naturally threatening situations.

Pavlovian fear conditioning takes advantage of associating sensory like sounds or odors with a shock. These simple one dimensional stimuli only partially mimic realistic situations of naturally occurring threat. Typically, threats which can be found in almost every natural environment and have a profound role in animal reaction are: predators, attacks from conspecifics, and dangerous features of the environment (Endler 1986) – all of which are complex objects in space (e.g. the predator). For successful reduction of damage, defensive responses have to be finely tuned to the threat (Blanchard & Blanchard 2008). For example it was shown that, though the same threat is presented, there is a switch from flight to freezing behavior when an escape route is blocked (Blanchard 1997). This behavior can then be further dissected into underlying features: Freezing will be more advantageous if the prey was not already detected (Eilam 2005). An escape response is further influenced by the distance and the speed of a predator (Cooper 2006). These studies underscore the complexity of threat evaluation and response and emphasize the importance of spatial features within such situations.

To understand fear in more realistic environments, we have to include these features. Therefore, we aimed to develop a paradigm to analyze behavioral responses to 3D objects in a spatially defined arena. We asked the question: How does the brain control behavioral towards such objects? We reasoned that this might be implemented in a straightforward manner by interaction of neuronal systems for valence and spatial information. But what are the underlying circuits that influence reactions to valenced objects and how would these reactions change, if we perturb those circuits? A good starting point to look for spatial representations in the brain was the entorhinal cortex (EC) contributing spatial and object information (Moser et al. 2008; Tsao et al. 2013) and the amygdala assigning valenced behavioral output.

1.5 THE ENTORHINAL CORTEX

1.5.1 ANATOMICAL ORGANIZATION

The entorhinal cortex (EC) (Brodman area 28) is a brain area located in the temporal lobe and is partially enclosed by the rhinal (olfactory) sulcus, wherefrom it also derived its name. It was first described in the 20th century by Ramon y Cajal as a peculiar part of the posterior

temporal cortex, which is strongly connected to the hippocampus (Cajal 1902). Today the EC is conceived as the nodal point between the hippocampal formation and a variety of multimodal association areas such as parietal and prefrontal cortex (Canto et al. 2008). In addition, studies revealed connections of the EC with a variety of subcortical areas (Pitkänen et al. 2000). Although the entorhinal cortex harbors a high diversity of cellular subtypes, combinations of different methods like electrophysiological and connectivity experiments

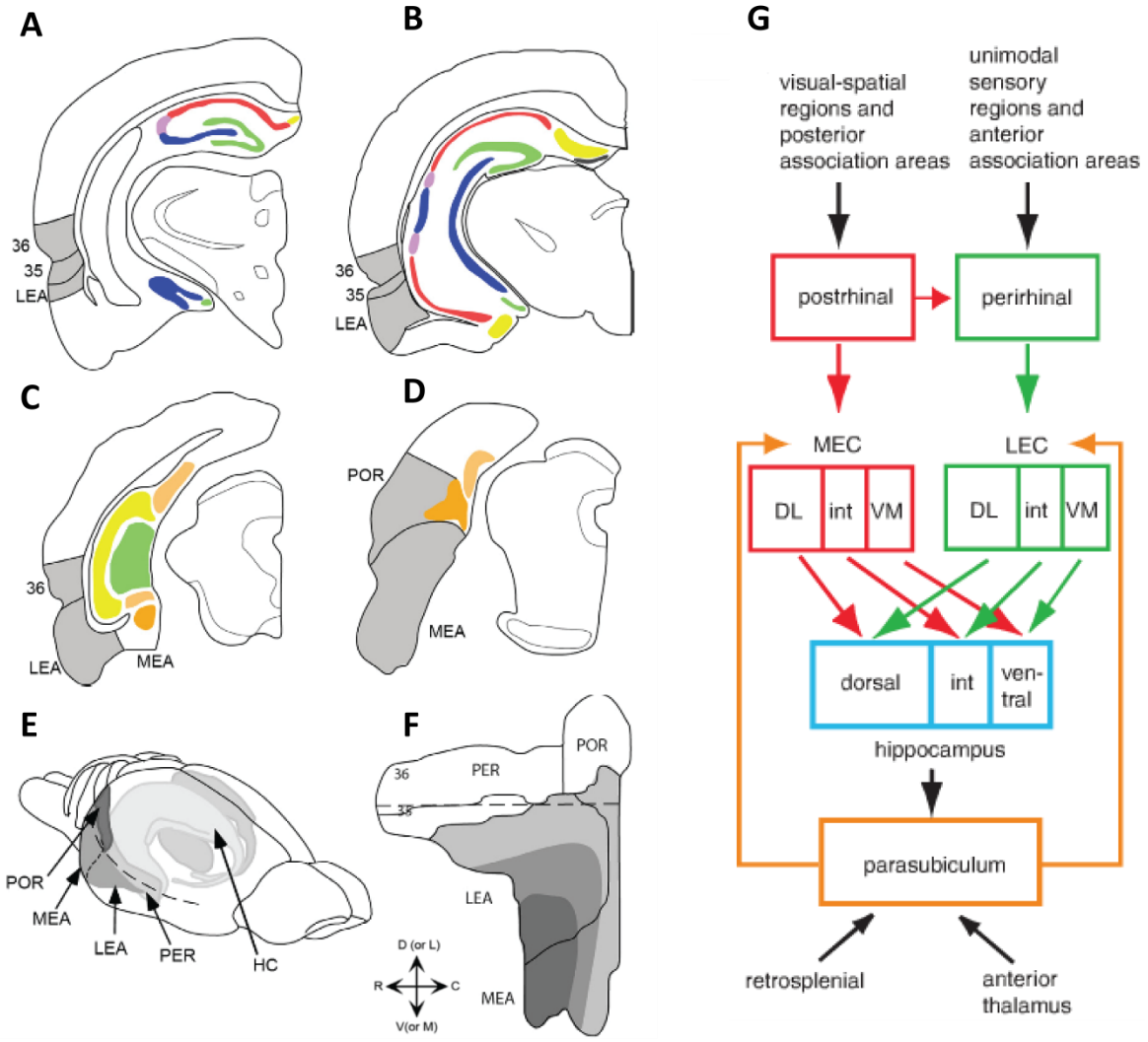


Figure 7. Basic anatomy of entorhinal cortex **(A to D)** Coronal brain sections of a rodent brain with area 35 and 36 of perirhinal cortex as well as LEA, MEA and POR depicted in gray. Hippocampal areas are shown in colors: dentate gyrus (green), CA-3 (blue), CA-2 (purple), CA-1 (red) and subiculum (yellow); **(E)** Position of parahippocampal structures and the hippocampus on the surface of the rat brain; **(F)** 2-dimensional unfolded map, indicating medial band in dark gray, intermediate band in medium gray and lateral band of EC in light gray. Compass depicts topographical position on the unfolded map. **Taken from** (Agster & Burwell 2013); **(G)** Main pathways to the hippocampus. Dissociation of different bands of the EC to the hippocampus are indicated. **Taken from** (Hargreaves et al. 2005); **Abbreviations: (A to F)** LEA, lateral part of the entorhinal cortex; MEA, medial part of the entorhinal cortex; POR, postrhinal cortex; PER, perirhinal cortex; HC, hippocampus; **(G)** MEC, medial part of the entorhinal cortex; LEC, lateral part of the entorhinal cortex; DL, int, VM,- dorsolateral, intermediate, ventromedial band of the EC, respectively.

suggest a main division into a medial entorhinal cortex (MEC) and lateral entorhinal cortex (LEC) (Hargreaves et al. 2005; Fyhn et al. 2004; Canto et al. 2008). In general, the LEC occupies a more rostrolateral position, whereas a more caudomedial position is seen for the medial entorhinal area (Figure 7). The lamination of these regions can be seen as a transition between the three layered allocortex and the six layered neocortex (Canto et al. 2008). As already stated, the entorhinal cortex is extensively connected to the hippocampus. In fact, all parts of the EC project to all regions of the hippocampus. Although the common view that layer I-III of the EC are the strongest input structures to and the deep layers the main recipients of the hippocampus, recent data argues against this simple dissection (Sewards & Sewards 2003). The topographical organization of entorhinal-hippocampal connectivity has to be taken into account, because the connections organize into a dorsolateral, an intermediate and a medial band (Figure 7F). It was confirmed that the dorsal band of the EC projects to the dorsal hippocampus and areas situated more in the ventral axis, especially in the LEC, are more strongly connected to the ventral hippocampus (Canto et al. 2008). The same seems to be true for reciprocal connections (Kerr et al. 2007). For the broad view it can be said that there is evidence that the MEC is more associated with the dorsal hippocampal system and the LEC more with the ventral hippocampal structures (Agster & Burwell 2013). This organization was also shown to be of behavioral importance, because lesions in the dorsal hippocampal and entorhinal portion lead to severe deficits in spatial learning and recall, an effect not seen in ventral lesions. However, the latter had a profound effect on fear related behavior, that was not seen for dorsal lesions (Steffenach et al. 2005; Kjelstrup et al. 2002).

1.5.2 CONNECTIONS

CORTICAL-ENTORHINAL CONNECTIVITY

According to Kerr et al. 30% of total afferents to the LEC arrive from cortical areas (Figure 8A), with strongest inputs from the piriform cortex, followed by insular regions and then the frontal areas, the LEC also projects back to these regions (Kerr et al. 2007). The MEC by contrast, receives much stronger input from occipital, parietal, and cingulate regions.

Very strong connections from cortical areas to the entorhinal system arrive also from the postrhinal (parahippocampal) and perirhinal cortices (Burwell 2000). According to connectivity, the LEC receives its major input from the perirhinal cortex and gives input to

the hippocampus via the lateral perforant pathway, whereas the postrhinal (parahippocampal) structure is the predominant input to the MEC and provides information to the hippocampus through the medial perforant path (Hargreaves et al. 2005).

SUBCORTICAL-ENTORHINAL CONNECTIVITY

The entorhinal cortex has several subcortical connections (Figure 8B), for example to the basal forebrain, claustrum, basal ganglia, thalamus, hypothalamus, and brainstem (Burwell & Witter 2002). It also receives inputs from the ventral tegmental area (VTA) (Beckstead 1978).

Subcortical structures strongly innervate the lateral entorhinal cortex, with strongest afferents from the olfactory area. Also the MEC receives subcortical inputs, e.g. from claustrum and dorsal thalamus (Kerr et al. 2007). Very detailed information is present for the entorhinal connections to the amygdala (McDonald & Mascagni 1997).

ENTORHINAL CORTEX-AMYGDALA CONNECTIVITY

Several studies looked at the connectivity pattern of the entorhinal cortex with the amygdala. Studies in different animals have shown that the EC projects to the amygdala and that particularly the lateral portion of the entorhinal cortex (LEC) is strongly connected to several

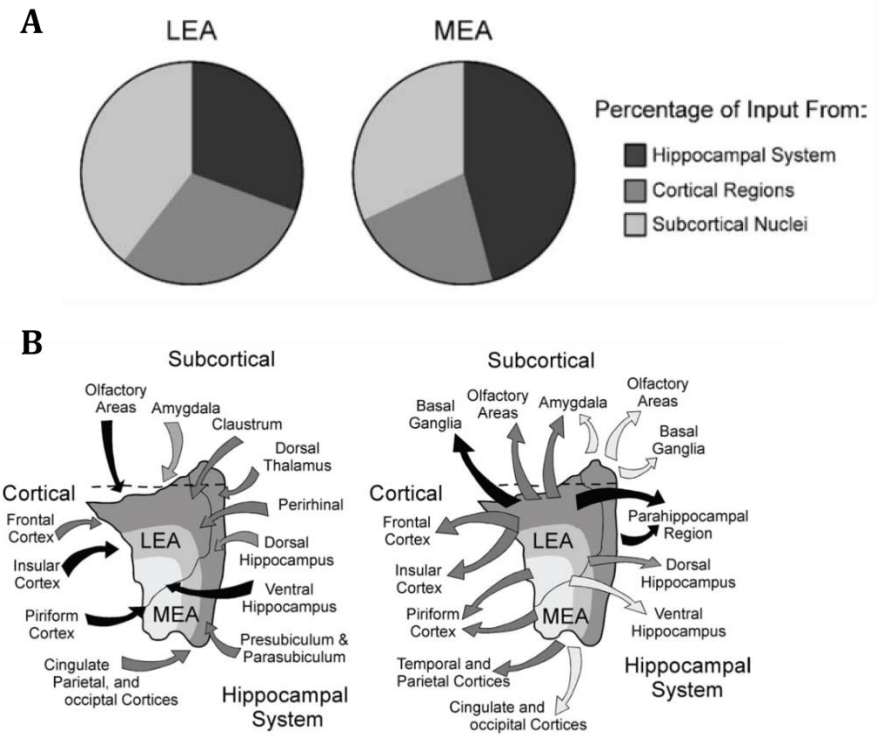


Figure 8. Connectivity of the entorhinal cortex to different brain areas **(A)** Percentage of inputs to LEA (=LEC) and MEA (=MEC); **(B)** Detailed afferent and efferent connections from and to subcortical areas. White, light gray and dark gray area indicates the dorsolateral, intermediate and medial band, respectively. Arrow color indicates connection strength with white being weakly, gray being medium and black being strongly connected. **Taken from** (Kerr et al. 2007); **Abbreviations:** LEA, lateral part of entorhinal cortex; MEA, medial part of entorhinal cortex.

amygdaloid subnuclei. In fact, the amygdala is one of the main output targets of the LEC. This connectivity is also topographically organized and the projections originate mainly in the deep layers of the LEC (McDonald 1998). Strongest projections to the amygdala arrive from the dorsolateral (dlLEC) and the ventrolateral (vILEC) bands of the LEC. Main targets for the dlLEC are the lateral portion of the basal nucleus as well as the accessory basal nucleus. The same projections are seen for the ventrolateral LEC, but basal projections are shifted medially. The vlLEC has profound connections to the lateral nucleus which are quite weak from the dlLEC. In addition, the most anterior parts of dlLEC and vlLEC, near to where they merge with the amygdalopiriform transition area, project to the lateral nucleus of the central amygdala (CeL) (McDonald & Mascagni 1997). The heaviest projections from the amygdala derive from the lateral, basal, accessory basal, medial, and posterior cortical nuclei. They terminate mainly in the layers 3 or 5 of the EC. Most connections seem to be reciprocal. One exception is the central amygdala, which receives inputs from the EC but does not project back to it (Pitkänen et al. 2000).

1.5.3 The Entorhinal Cortex and Function

Medial Entorhinal Cortex

The medial entorhinal cortex received major interest after it was shown that cells projecting to the hippocampus from layers II and III exhibit sharply tuned spatial firing, much like place cells, but with the difference that each cell had multiple firing fields (Fyhn et al. 2004). The firing fields of each neuron form a periodic triangular grid that covers the entire environment (Hafting et al. 2005). Because of the grid related activity pattern, these cells were named grid cells. The grid cells provide the animal with information about its changing position within the environment and also seem important for the activity of hippocampal place cells (Moser et al. 2008). Together with head direction cells, which show activity when a rat moves its head in a specific direction (Sargolini et al. 2006) and border cells, which fire in the vicinity of walls or edges (Solstad et al. 2008), the medial entorhinal cortex obviously plays a fundamental role in spatial orientation (Figure 9A to C).

LATERAL ENTORHINAL CORTEX

No spatial selectivity-neither in a simple, nor in a cue-rich environment was found in the LEC (Hargreaves et al. 2005; Yoganarasimha et al. 2011).

As already mentioned, the lateral entorhinal cortex receives major input from the perirhinal cortex. The perirhinal cortex is associated with several unimodal sensory areas and appears to be involved in object configuration, object-recognition and familiarity (Norman & Eacott 2005; Aggleton & Brown 1999; Murray et al. 2007). Therefore, it had been proposed that the LEC provides information about local and non-spatial environmental cues, different from the MEC, which processes global and spatial frame information. Deshmukh & Knierim were the first to show this double dissociation of MEC and LEC (Deshmukh & Knierim 2011). When they put discrete objects within an arena, cells in the lateral entorhinal cortex of rats, foraging for food, fired in the vicinity of objects and others at a distance to the objects.

Interestingly, different cells with a variety of activity patterns to discrete objects were found. Some cells fired in response to the presentation of multiple objects, in multiple sessions. These could encode object location or generalized attention to external landmarks (Deshmukh & Knierim 2011). Further understanding of the nature of object-dependent firing of cells in the LEC derived from electrophysiological investigations of rats in a very similar setting as described above. Rats were allowed to freely explore an open field, where objects were presented. Together with object-cells described before, Tsao et al. also reported on cells which showed activity when the rat was in the vicinity of the location where an object had been experienced in the trial before (Figure 9E) (Tsao et al. 2013). They called these cells object-trace cells, as it seemed that the cells showed the trace of the position were an object had been. This trace firing field was stable over several days. When the object was moved to different locations, the cells activity followed the object position. When the object subsequently was removed from the chamber, the trace field firing reappeared at the position, where the rat had been trained to meet the object.

But this activity was not confined to visual objects, because upon activation of the medial forebrain bundle (MFB), a structure associated with reward sensation, within a specific region of the arena, cells in the LEC responded. On the next day, cells were again active at the same spot in the arena although the MFB was not activated again (Tsao et al. 2013). The group was able to show that, indeed, cells in the LEC showed activity in the vicinity of an

object and that additionally, there was a population of neurons that was active where objects had been before. This activity was not restricted to physical objects, but seemed to imply a broader field of sensory inputs. The idea that the LEC is not just involved in physical object cognition, but uses a variety of external sensory stimuli was further supported by Lovett-Barron et al. (Lovett-Barron et al. 2014). Probing the neuronal activity to different stimuli in LEC, they observed highest responses to negative associated air puffs, but also firing upon visual stimulation and weakly to tone presentation. Together these studies provide extensive evidence that the LEC indeed plays an important role in object and, in a broader view, external sensory processing.

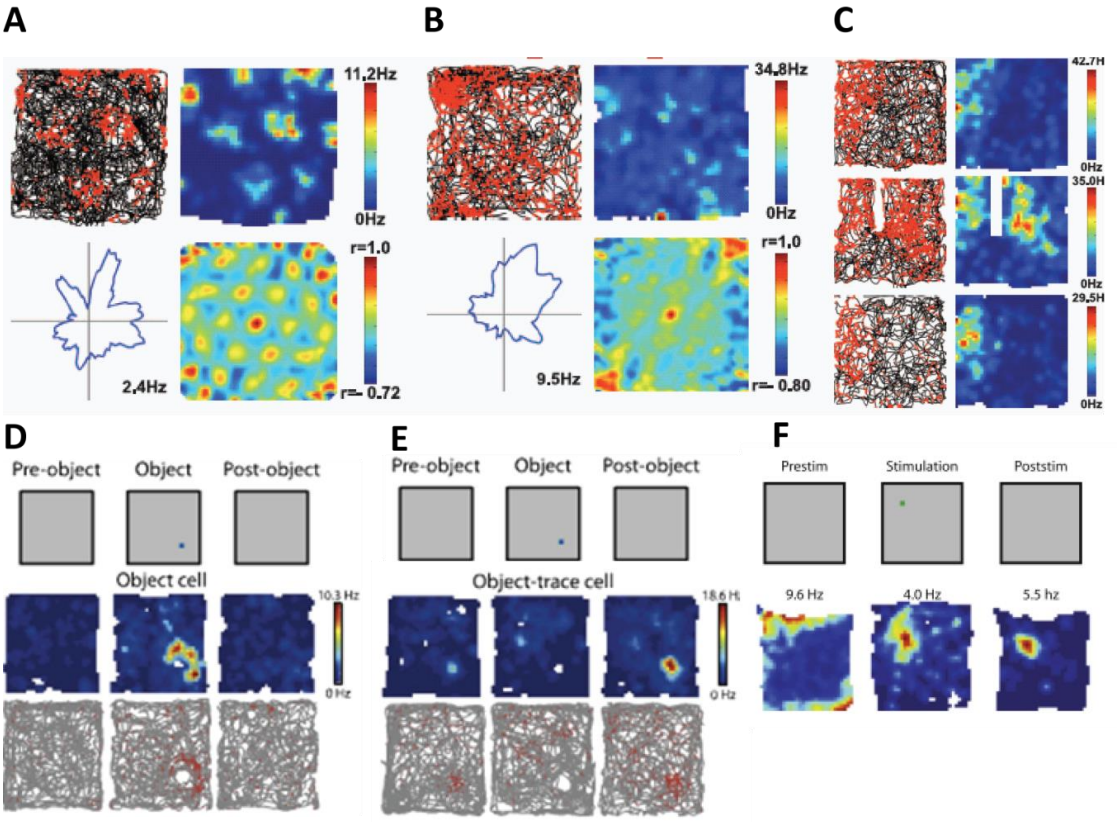


Figure 9. MEC and LEC cellular activity patterns **(A, B, C)** Recordings from MEC; **(D, E, F)** Recordings from LEC; **(A)** Firing pattern of grid cells. **(Upper left):** Trajectory with superimposed firing fields in red. **(Upper right):** Color coded rate map and scale bar with minimum and maximum firing rates. **(Lower left):** Polar plot that shows firing rate as a function of head direction and peak rate. **(Lower right):** Color-coded auto-correlation map with scale bar indicating minimum and maximum correlation values. Scale of autocorrelograms is twice the scale of the rate map; (**B)** Head direction cell, raster same as **(A)**; **(C)** Border cell, in the first trial the rats were free to explore an empty arena, in the subsequent trial a wall was placed in the box. On the third trial, the wall was removed again and rats explored the empty box. Raster same as **(A** and **B) (Upper left** and **right). Taken from** (Zhang et al. 2013); **(D)** Recording from object cell in LEC. **(Top):** Cartoon of three trial experiment, in the pre-object trial no object was in the box, in the object trial an object was placed in the box, the post-object trial is equal to pre-object trial. **(Middle):** Example cell with object response, rate map is shown with color coded scale bar indicating minimum and maximum rates. **(Bottom):** Path plots with corresponding activity spikes overlaid; **(E)** Activity pattern of object-trace cell, same raster as **(D)**; **(F)** Stimulus activated cell recording. **(Top):** Cartoon shows MFB activation in a confined location of the arena. **(Middle):** Example cell, showing trace field corresponding to stimulation location. **Taken from** (Tsao et al. 2013).

The representation in LEC of "What is out there" and "Where is it" nicely fits a model wherein the lateral entorhinal cortex provides the hippocampus with the content of an experience, based on a local frame of reference, that could be derived from external sensory inputs (Knierim et al. 2014).

1.6 SPACE AND THE MODEL OF VALENCE

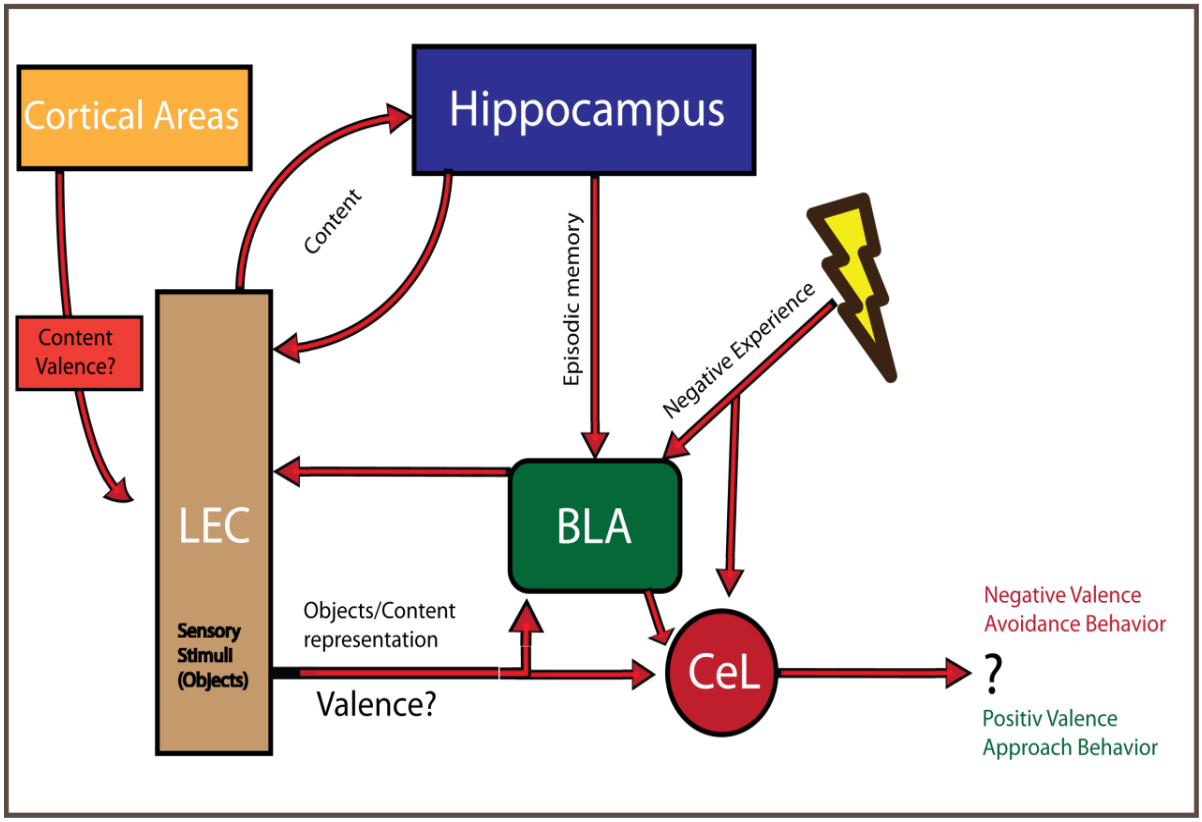
The eminent role of the entorhinal cortex in processing and representing objects of an experience (see section 1.5.3), raises the question of how this might contribute to emotional behavioral responses. One possibility of translating positive and negative emotions into a more spatial environment is the concept of approach and withdrawal (Davidson & Irwin 1999). The approach system facilitates appetitive behavior which will lead to positive affect. The behavior is directed towards a goal, so a positive experience associated with a place, object or other stimulus would facilitate this behavior and lead to a longer stay in the place or at the object. This also means that the distance to the given stimulus should be reduced, to reach the goal. The opposite holds true for the withdrawal system. This system strengthens the withdrawal from a source of aversive stimulation. Therefore a negative affect will be generated and an individual will try to increase the distance to the source of aversive stimulation (see (Davidson & Irwin 1999)). Somewhere in the brain, this switching of spatial behavior has to be processed and there has to be interplay of context, distance and object information to induce a fine tuned response. The entorhinal cortex, with its strong connections, on the one hand to the hippocampus and on the other hand to a variety of cortical and subcortical structures, would be in a favorable position to be involved in this function. Firstly, it processes and represents context and content information of an experience. Secondly, EC is connected to different brain structures also associated with valence representation and thirdly, it is also strongly connected to the amygdala, a center point for emotional functions (see section 1.3 and 1.5.2). Indeed, the amygdala also seems to be involved in processing distance related aspects of emotion. Kennedy et al. investigated the phenomenon of personal space (Kennedy et al. 2009). It is known that people normally hold a specific distance when interacting. Close distances especially, e.g. during discussion, feel rather uncomfortable. Kennedy et al. tested the concept of personal space with S.M., a woman with complete bilateral amygdala damage. When comparing her ratings of comfortable

distance to an unknown person with healthy controls, she repeatedly demonstrated a notable lack of discomfort at close distances. In addition, Kennedy et al. observed higher amygdala response when participants knew that an experimenter maintained a close distance to them during a functional magnetic resonance imaging (fMRI) experiment.

1.7 AIM AND EXPERIMENTAL APPROACH

The entorhinal cortex plays a crucial role in the representation of context/space ("Where am I") and content/objects ("What is out there") (Eichenbaum et al. 2007). Several studies already showed a dissociation of context representation in MEC and content processing in LEC. However, content representation in the LEC seems to span a wide range of external sensory information. Although MFB activation within a certain area in an environment was already shown to induce activity in specific cells in the LEC (Tsao et al. 2013), to our knowledge no one ever investigated behavior towards real 3D objects coupled with an emotional value in mice with similar object-shock association tasks that we have established. It is still completely unclear which brain structures could drive an approach or avoidance behavior when an object is associated with a valence. Although fear has already been dissected in great detail, it is not understood what makes an animal keep a distance and avoid a threatening stimulus. We expect the LEC to be a hub between cortical and subcortical structures, especially important for the integration of sensory stimuli, which also implies the integration of positive or negative associations to the encountered stimulus. Although the amygdala is also involved in the processing of spatial components of an emotional experience (Kennedy et al. 2009), it seems rather unlikely that the spatial portion itself is encoded in this structure. Rather more likely is the integration of spatial information from the entorhinal cortex to the amygdala. As already discussed before (see section 1.5.2), the EC receives input from different association areas. Together with the knowledge, that sensory stimuli e.g. 3D objects are represented within the LEC, it seems possible that already highly processed valenced stimuli are represented therein. The extensive connections from the LEC to the amygdala could form a microcircuit for then evaluating the object-experience association and subsequently triggering of the proper response. It is also possible, that specific LEC neuronal populations project to certain amygdaloid subnuclei and therefore induce physiological changes, important for approach or avoidance behavior.

A



B

	Tone	Context	Object
Amygdala	X	X	X
Hippocampus		X	
Entorhinaler cortex			X?

Figure 10. Hypothesis model for LEC-Amygdala microcircuit function **(A)** LEC receives innervations from different cortical areas and generates object/content representation. It is strongly connected with the hippocampus and sends content information to that structure. Together with context information, the hippocampus produces episodic memories of an experience. There are also direct connections from the LEC to the amygdala (here BLA and CeL shown). The LEC to CeL connection could sever as a fast pathway for important object information, essential for immediate behavioral responses. The output of that pathway should trigger an avoidance or an approach response. BLA is connected to several cortical structures. Maybe content information from the LEC is evaluated with already experienced episodic memories coming from the hippocampus, which then initiate the proper behavioral response to the situation and would therefore represent the highly processed and memorized pathway of object information. If the valence associated to the object is already intrinsically processed in the LEC or comes in at the level of the amygdala has to be tested; **(B)** Table of brain structures involved in fear conditioning to different sensory stimuli. The amygdala is needed for tone-fear conditioning. The hippocampus and the amygdala are essential for the context-fear conditioning. We expect the amygdala and the entorhinal cortex (i.e. the LEC) to be important for the association of objects with negative experiences.

We investigated the LEC connection to the BLA and the CeL of the amygdala for several reasons: It is suggested that the primary information flow during affective responding involves sensory input to the BLA (Rosenkranz & Grace 1999). Several studies displayed an important role for EC-BLA connections in different tasks e.g. context fear conditioning, latent inhibition or inhibitory avoidance (i.e. affective memory) (Barak & Weiner 2010; Sparta et al. 2014; Rosenkranz & Johnston 2007).

The CeA, comprising the CeL and CeM was long considered a simple output station of the amygdala to downstream targets, important for the expression of fear (LeDoux 2000). However, over the last years different laboratories discovered very dynamic activity of the CeL dependent on the task and situation (Pape 2010).

The BLA drives both CeL and CeM, which receive glutamatergic input from the BLA (Figure 10). The CeL, in turn, gates CeM output and as a result higher or lower freezing upon fear conditioning (Ciocchi et al. 2010; Haubensak et al. 2010).

Here, we explored the interaction of space/object systems in LEC and valence/behavioral output system in BLA and CE in mediating object related behaviors. We first planned to inject mice with retrograde tracers in order to investigate the pattern and strength of projections from the LEC to the BLA and the CeL. Next, we mapped neuronal activity in the LEC-amygdala network to neutral and negatively valenced objects by immediate early gene expression, to test if components of this system discriminate between neutral and negatively valenced objects. We then aimed to perform NMDA mediated excitotoxic lesions of the anterior dorsolateral LEC (adlLEC), the area with strongest projections to the CeL amygdala (see (McDonald & Mascagni 1997)). To test if the LEC functionally contributes to object related emotional processing, we introduced an object-fear conditioning task with which we were able to associate a 3D object with a negative experience and contrasted those to fear responses in a Pavlovian trace fear conditioning task. Projection specific optogenetic manipulations should then pinpoint if LEC to amygdala projections selectively modulate avoidance responses to 3D objects but not tones.

Collectively, these experiments should provide us, on the one hand, with LECs role in processing negative associated stimuli, which can be objects as well as sounds; and on the other hand, answer the question, if direct LEC-amygdala connections are selectively modulating emotional responses to objects.

2. MATERIAL AND METHODS

2.1 MATERIALS

2.1.1 ANIMALS

Inbreed male C57BL/6J mice aged 2-3 months were used for surgery. All subjects were maintained at 21ºC under a 14h/10h light/dark circadian circle, independent of daylight-saving time. Mice were supplied with free access to food and water. After surgery there was a two week recovery time before experiments started. Analgesics and antibiotics were provided via drinking water for 10-14 days after surgery.

2.1.2 CHEMICALS AND REAGENTS

SOLUTIONS

Sterile water as well as Phosphate buffered saline (PBS) (10x and 1x) were provided by the In-House IMP media kitchen.

Bovine Serum Albumin (BSA) was bought as solid powder from Sigma Aldrich. For the blocking solution, used in immunochemistry 1% BSA in PBST (Phosphate buffered Saline + TritonX-100 (Sigma-Aldrich)) (m/V) was prepared.

Heparin Sodium Salt (≥ 180 USP units/mg, porcine intestinal mucosa, Sigma Aldrich Handels-GmbH) was diluted to achieve 10U/ml Heparin/PBS concentration.

Paraformaldehyde (PFA) was obtained from MORPHISTO® Evolutionsforschung und Anwendung GmbH as Paraformaldehyde (PFA) 4%, in PBS ph 7.4.

Sucrose Crystals from Fluka Biochemika were used to prepare 15% Sucrose/PBS (m/V)

NMDA: ≥98% (TLC) was obtained from Sigma Aldrich (CAS Number 6384-92-5) and diluted in PBS to achieve a final concentration of 50mg/ml.

MOUNTING

Fluorescence Mounting medium was obtained from Pathology Products Dako Oesterreich GmbH.

It was used for mounting of fluorescently labeled tissue and slides.

The Embedding medium Tissue Tek from Sakura Finitech B.V. was used for embedding and for cryoprotection of fixed tissue before further processing.

ADHESIVES

Dental adhesive resin cement obtained from Superbond C&B, Sun Medical Co., Ltd and acrylic resin cement (JetSet 4 Liquid, Lang Dental Manufacturing Co., Inc.) were used to mount the implanted cannulas on the mouse skull.

CHOLERA TOXIN B SUBUNIT

The cholera toxin, secreted by the bacterium Vibrio Cholerae, is a protein complex consisting of an A and B subunit (Faruque 2008). The A subunit is an ADP-ribosyltransferase, which interferes with G-protein signaling and leads to its prolonged activity. The B subunit is essential for binding to the nerve cell membrane. Mechanistically it binds to the GM1 ganglioside which facilitates entry into the cell. Especially in the nervous system there is a high density of gangliosides, which are concentrated on synaptic membranes (Conte et al. 2009). A recombinant and non-toxic Cholera toxin B subunit (CTB) has been developed, which can be conjugated to fluorescent molecules. This provides a potent tool for retrograde tracing, as the CTB travels from the synapse to the cell soma (Luppi et al. 1990).

CTB-conjugates were obtained from Invitrogen as CTB-Alexa Fluor-488 (C-22841), CTB-Alexa Fluor-555 (C-22843) or CTB-Alexa Fluor-647 (C-34778) as dry powder (Life Technologies®). For injection, the powder was dissolved in 8.7% glycerol/PBS to yield a final concentration of 1g/µl.

2.1.3 VIRUSES AND CONSTRUCTS

AAV

Adeno-associated virus (AAV) was first discovered as a contaminant of adenovirus stocks in the 1960s, and although AAVs have been studied for the past 50 years, and approximately 80% of the population are seropositive for anti-AAV antibodies, little is known about the natural infection process (Weitzman & Linden 2011). AAVs are members of the Dependovirus genus of the parvoviruses that can replicate under a diverse set of conditions (Berns & Giraud 1996). AAVs are helper-dependent viruses, meaning that they require the replication machinery of other viruses. Adenovirus (Ad) and herpes simplex virus (HSV) can serve this function. In the absence of these assistants, the AAV-genome will be stably maintained in the host cell as episomes or be integrated into the genome (Shin et al. 2012).

The combination of very low toxicity in the central nervous system and its efficient delivery of genetic material together with long-lasting gene expression in the infected cells, made AAVs to a widely used and powerful tool for manipulations of post-mitotic neuronal cells (Betley & Sternson 2011).

2.1.4 CRYOSTAT

The fixed and frozen brains were sliced with MB DynaSharp Microtome Blades (Thermo Scientific) into 20µm thick coronal sections on a Microm HM560 cryostat. The slices were put on to microscopy slides (Thermo Scientific).

2.1.5 MICROSCOPY

To examine slides Axio Imager Z2 Stativ epifluorescence microscope was used.
For image acquisition a laser scanning confocal microscope (LSM 700 Axiolmager) with 20x/0.8 LD LCI plan-apochromat, multidimension objective was used. Laser Diode 405nm 25mW, Argon 458/488/514 30mW, DPSS 651nm 15mW, HeNe 633nm 5mW were used.

2.1.6 ANESTHETICS AND ANTIBIOTICS

Ketamine was purchased from OGRIS Pharma Vertriebs-GmbH as Ketasol (100mg/ml). For application it was mixed in PBS to achieve a concentration of 10mg/ml. Injection were done intraperitoneally (ip.) at a volume of 10µl/g of body weight. Surgical procedure started after plantar reflexes were absent.

Xylazine was purchased as Xylasol (20mg/ml) from OGRIS Pharma Vertriebs-GmbH. To get a final concentration of 1mg/ml, Xylazine was diluted in PBS.

Medetomidine as Domitor (Medetomidine Hydrochloride (1mg/ml)), was obtained from Orion Corporation. 0.8ml Domitor were mixed with 1ml of ketamine and brought to a final volume of 10ml with PBS. 0.3-0.7ml of the final solution was injected ip. for deep anesthesia before perfusion started. Perfusion procedure was delayed until plantar reflexes were absent.

Isoflurane was obtained from Abbot Laboratories as Isoflo (100% w/w). For surgery, mice were kept anaesthetized during the whole procedure. For further details see (*Stereotaxic Surgery*)

Carprofen was obtained as Rimadyl (50mg/ml) from Pfizer Corporation Austria.
Rimadyl was diluted in PBS to achieve 4µg/g of body weight and a volume of 200ul. This was used for postoperative ip. injection. After surgery carprofen was supplied in the drinking water for 10 days at a concentration of 250mg/l.

Enrofloxacine, as Baitryl (100mg/ml), was from KVP Pharma + Veterinaer Product GmbH. It was provided via drinking water for 10 days post surgery at a concentration of 400mg/ml.

Lidocaine obtained as Xylanaest (1%) from Gebro Pharma. 100µl Xylanaest were injected under the skin of the animal's head before cutting the skin and starting surgery.

Ophthalmic Ointment: Animal eyes were lubricated with Refobacin from Merck GmbH, before starting the surgery, after anesthesia. The gel contained the broad-spectrum antibiotic gentamicin (3mg/g).

2.1.7 STEREOTAXIC SURGERY EQUIPMENT

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

Further Instruments for Stereotaxic Surgery

Non rupture earbars model 1922
Electrode Holder model 1970
Micro Manipulator model 1940
Alignment Indicator model 1905
Stereotaxic drill model 1911
Centering Scope 40X model 1915

All Models from Kopf

FOR INJECTION

Mirco4 Micro Syringe Pump Controller (World Precision Instruments)

Nanoliter 2000 injector (World Precision Instruments)

Micropipette Puller: Model P-97, Sutter Instruments

35in Glass Capillaries (World Precision Instruments) were used for stereotaxic solution delivery.

2.1.8 OPTOGENETICS TOOLS

Laser Power supply: DPSSL-Driver Power supplies (2-4A)

Lasers: 473nm, 50mW: MBL-III-50mW; 12127218; Doric lenses

447nm, 50mW; doric lenses

FC/FC mono fiber patch cord cable (multimode optical fiber) 200µm core diameter, NA=0.22

FC mono fiber to dual fiber ferrules: Doric lenses

Zirconia sleeves as receptacles for ferrule to cannula: Doric lenses

2.1.9 CANNULAS

Cannulas for implantation and chronic light delivery via laser into the brain were provided by Thorlabs, Inc. (CFMLC22L05 - Fiber Optic Cannula, Ø1.25 mm Ceramic Ferrule, Ø200 µm Core, 0.22 NA, L=5 mm)

2.1.10 FIBER PATCH CABLES

Diameter: 200µm, varnished black or white

Thorlabs/Doric lenses

2.1.11 ANTIBODIES

PRIMARY

type	species		supplier	Product number
Anti-c-Fos (polyclonal IgG)	Rabbit		Abcam®	ab7963
Anti-WFS-1 (IgG)	Rabbit		Proteintech ™	11558-1-AP
Anti-PKC-δ (IgG 2b)	Mouse		BD Biosciences	610398
Anti-NeuN (monoclonal IgG 1)	Mouse		Merck Millipore	MAB377
Anti-NeuN-fox3 (polyclonal IgY)	Chicken		Abcam®	ab131624

SECONDARY:

type	species	color	supplier	Product number
Anti-Rabbit(IgG (H+L))	Goat	Alexa-Fluor ® 633	Life technologies ®	A-21071
Anti-Rabbit (IgG (H+L))	Goat	Alexa-Fluor ® 488	Life technologies ®	A-11008
Anti-Mouse (IgG (H+L))	Donkey	Alexa-Fluor ® 488	Life technologies ®	A-21202
Anti-Mouse (IgG (H+L))	Goat	Alexa-Fluor ® 633	Life technologies ®	A-21052
Anti-Chicken (IgG (H+L))	Goat	Alexa-Fluor ® 633	Life technologies ®	A-21103
Anti-Chicken (IgG (H+L))	Goat	Alexa-Fluor ® 488	Life technologies ®	A-11039
Anti-Chicken (IgG (H+L))	Goat	Alexa-Fluor ® 568	Life technologies ®	A-11041
DAPI (4'6-Diamidino-2-Phenylindole, Dilactate)		Blue~460nm	Invitrogen	D3571

2.1.12 ANIMAL SHOCKER

Precision regulated animal shocker H13-15 (Coulbourn Instruments)

2.2 METHODS

2.2.1 OPTOGENETICS

Optogenetics involves the introduction of genes encoding light-sensitive transmembrane ion channels (microbial opsins) which allow for the excitation or inhibition of expressing cells with high specificity and temporal precision (Aston-Jones & Deisseroth 2013). As electrically excitable cells react on a timescale as short as milliseconds, high temporal accuracy is needed (Zhang et al. 2006). 40 years ago already, scientists knew that there are microorganisms that produce proteins which are gated by visible light, thereby regulating the flow of ions across the plasma membrane (Deisseroth 2011). In 2005 it was possible for the first time to efficiently introduce a microbial opsin gene without any additional components, which led to the expression of the channel protein and resulted in light responsive neurons (Boyden et al. 2005). These opsins can be introduced into neurons by using transgenic animals and via viral vectors which contain the genes for the expression of these molecules (Zhang et al. 2006). Several types and derivatives of opsins have already been used, but a small subset of proteins has shown to be especially useful due to their physical and chemical properties. One of these is Channelrhodopsin-2 (ChR2), a light gated cation channel, derived from the green alga *Chlamydomonas reinhardtii* (Figure 11) (Zhang et al. 2006). ChR2 is a seven-transmembrane protein with the light sensitive all-*trans* retinal (ATR) bound in the center of the channel. When 470nm blue light is directed at the protein, ATR isomerizes. That leads to a conformational change of the channel and allows an inward cation current, to be evoked within 50µs of illumination (Nagel et al. 2003). This was the first time that neurons could be activated at the temporal precision of single action potentials and also over multiple action potential trains (Boyden et al. 2005). Therefore optogenetics provides us with a tool for minimally invasive, genetically targeted and temporally precise manipulation of neural

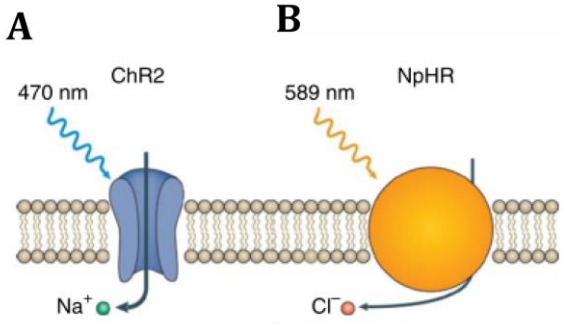


Figure 11. Example of microbial opsins used in optogenetics **(A)** Channelrhodposin-2 (ChR2) from *Chlamydomonas reinhardtii*. Upon blue light stimulation the channel opens and cations can enter the cell, leading to depolarization; **(B)** Halorhodopsin (NpHR) from *Natronomonas pharaonis* is activated upon yellow light delivery. It acts as a chloride pump and leads to hyperpolarization of a cell. **Taken from** (Zhang et al. 2010).

circuits in freely behaving animals (Deisseroth 2011; Tsai et al. 2009).

2.2.2 VIRUS CONSTRUCTS

For AAV-mediated delivery of channelrhodopsin-2 (ChR2) we utilized AAV constructs provided by Addgene.

VIRUS DELIVERY

Viruses directly from http://www.med.upenn.edu/gtp/vectorcore/

Plasmids from https://www.addgene.org/

CHR2-CONSTRUCTED

pAAV-EF1a-DIO-hChR2(H134R)-mCherry-WPRE-HGHpA (Addgene 20297)

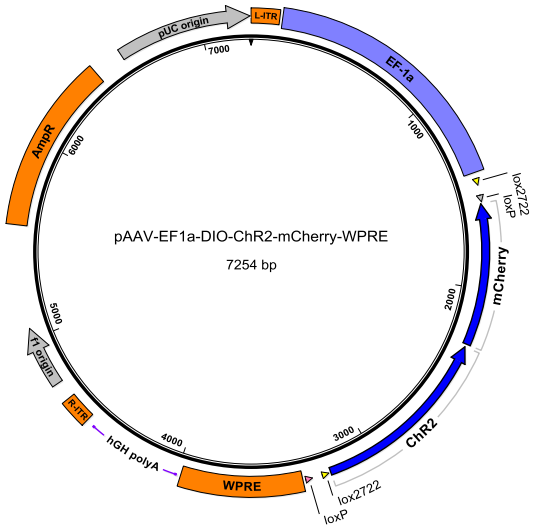


Figure 12. DIO-hChR2(H134R)-mCherry ChR2-mcherry is expressed in cells which express Cre-recombinase. When Cre-recombinase is present in the same cell, ChR2(H124R)-mCherry fragment is irreversibly shwitched and ChR2-mCherry is expressed. **Abbrivations**: L-ITR/R-ITR, Inverted Terminal Repeats; EF1a, elongation factor 1a promotor; ChR2, Channelrhodopsin 2 (H134R); WPRE, Woodchuck Hepatitis Post-Transcriptional Regulatory Element; AmpR, Ampicillin-resistance gene; dfox, double-floxed; DIO, Double-floxed Inverted Open reading frame; loxP/lox2722 – independent recognition sites for Cre-recombinase.

2.2.3 THE CRE-RECOMBINASE SYSTEM

The Cre recombinase-system is a very powerful method to restrict gene expression to specific brain regions and/or other cell types (Tsien et al. 1996). Nowadays it is a widely used tool to precisely manipulate from whole tissues to neuronal subpopulations in several ways, for example express genes in a Cre-dependent manner.

The system was identified in the bacterial phage P1 and efficiently applied to mammalian tissue (Sauer & Henderson 1988). It requires the Cre-recombinase protein and its recognition site, called loxP-sequence. Two of these 34 bp loxP recognition sequences can be inserted

into the genome, so that they flank a single exon to a whole gene (a “floxed gene”) (Tsien et al. 1996). As the Cre protein is a site-specific recombinase, the orientation of the loxP sites is important for the outcome of the recombination reaction (Tronche et al. 2002). The combination of different orientations of the loxP sites can result in an excision or insertion, as well as an inversion of the flanked gene region (Van Duyne 2001). It is therefore possible to precisely turn gene expression on or off dependent on which loxP combination is used. Because the recombination process is dependent on Cre-recombinase, recombination will only occur in tissue/cells expressing this protein (Schnütgen et al. 2003).

2.2.4 STEREOTAXIC SURGERY

Male mice 2-3 months old were deeply anaesthetized with isoflurane (100%) and placed in a stereotaxic frame (Kopf). The tongue was carefully pulled out of the mouth to prevent blockage of the airways and the head was positioned within the anesthesia nosepiece. To stabilize the temperature around 35ºC a heating pad was used. The head of the animal was fixed with non-rupturing ear bars. Before opening the skin, 0.1ml of Lidocaine were injected under the scalp. The skull was exposed by opening the scalp with a scalpel (longitudinal incision along the saggital suture to expose bregma and lambda). A stereotaxic alignment indicator was used to adjust lateral and anterior-posterior axis. Bregma was used as the standardized landmark and position of 0. Stereotaxic coordinates were taken from (Paxinos & J 2007). The skull was perforated with a stereotaxic mounted drill at the desired coordinates. Drills of different size where used, depending on the requirements (0.8mm, 0.6mm, 0.3mm). Before withdrawing injection solution, pulled glass needles were filled with mineral oil without creating air bubbles. A micro 4 syringe pump controller was used to regulate collection and injection volumes. To precisely inject into the region of interest, the glass capillary position was set to zero at the anterior-posterior position of the injection site above the saggital suture of the skull. Afterwards the needle was moved above the center of the drilled hole and slowly lowered until the ventral position was reached. Injection rate was set to 20nl/min. After the injection was finished the glass needle was left in place for another 5 min to guarantee complete injection and good diffusion of the solution.

To insert cannulas, a metal holder was used which could be fixed on the stereotaxic precision arm. To set the zero position, the same procedure as for the injection needle was used. After

inserting the cannula, dental cement was applied. After the cement was completely dried, the holder was lifted.

For postoperative care, drinking water was supplied with Carprofen and Enrofloxacine for 10-14 days.

The coordinates for injections were (anteriorposterior, mediolateral, dorsoventral, respectively in mm relative to bregma):

Region	**Coordinates (mm)**		
	bregma	**lateral**	**ventral**
dlLEC	-2.70	4.58	-4.60
BLA	-1.58	3.30	-5.10
CeL	-1.35	2.90	-4.85

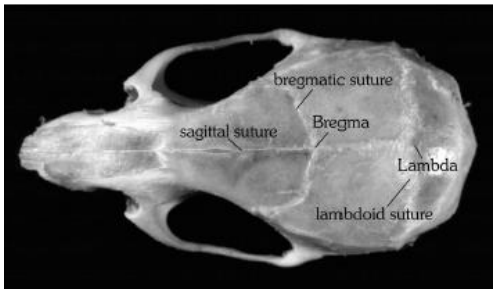


Figure 13. Top view of the mouse skull Indicated are reference points important for stereotaxic surgery. **Taken from** (Paxinos & J 2007).

2.2.5 CTB-INJECTION INTO AMYGDALA

CTB retrograde tracers, conjugated to a fluorophore were injected into BLA or CeL. 30µl was injected into BLA and 20µl into CeL. To distinguish between cell populations connected to the different amygdaloid subnuclei, different fluorophores were used. Seven days after injection, the mice were sacrificed and coronal cryostections were taken for immunohistochemistry. Sections of CeL were stained for PKC-δ (marker of a subpopulation of CeL) to confirm accuracy of injection site. LEC sections were grouped into anterior, medial and posterior portions (see representative pictures: Figure 17C and D). Regions of

interest were assigned by hand and CTB-positive cells were counted with an automated program.

2.2.6 VIRAL INJECTIONS

Viral particles were delivered by stereotaxic injection with a Micro 4 controller equipped with a Microject system (Nanoliter 200 injector) (World Precision Instruments).

2.2.7 IMMEDIATE EARLY GENE (IEG)-EXPRESSION AS NEURONAL ACTIVITY MARKER

The activity of the lateral entorhinal cortex was measured by the expression of the immediate early gene c-fos. The counting of c-fos positive cells is a standard procedure for assessing neuronal activity in different brain regions. Three experimental groups were investigated: to show baseline activity, one group of mice (n=5) was left in their homecages (group: homecage) and no experiments were done before perfusion. The second group (n=5) underwent a Novel Object recognition task (Figure 14), divided into two consecutive days. 90min after the Novel Object session ended for a given mouse, it was perfused and the brain was cut for staining with anti c-fos antibody. As we were not just interested in object novelty, but also in the involvement of the LEC in valence to objects, a different group of mice performed an Object-Shock Conditioning task. Therefore not a single cohort of mice was used, but the control group from the NMDA-experiments (see section 3.3.1) was placed back in the arena and reexposed to the objects, where one of these objects was associated with a shock in previous trials (Object reexposure group). Same procedure for perfusion, cutting and immunohistochemistry for c-fos staining was used. LEC, BLA and CeL regions were marked by hand and c-fos positive cells were counted with an automated program. Additionally, c-fos was measured in the auditory cortex (AuX) for all groups to make sure that IEG patterns in LEC, BLA and CeL do not lack specificity.

2.2.8 NMDA-LESION

N-methyl-D-aspartete receptors (NMDARs) are glutamate gated ion channels that are widely expressed in the central nervous system and are found in a variety of different subtype compositions. They derived their name from the high affinity to the synthetic chemical

NMDA. NMDARs play a key role in excitatory synaptic transmission and are best known for their involvement in learning and memory formation. These properties are closely connected to the high permeability for calcium ions upon activation of the channel. The flow of calcium ions through the plasma membrane into the cell is also a fundamental process involved in neuronal plasticity and excitotoxic damage (Paoletti & Neyton 2007). It has been shown that high concentrations of glutamate or application of NMDA causes cell damage which can ultimately cause neuronal death. Several detrimental effects are triggered via production of nitric oxide compounds and free radicals by interaction of Ca2+ with mitochondria upon high levels of intracellular calcium (Lau & Tymianski 2010). This effective destruction of neuronal tissue can also be used as a tool in neuroscience. Application of NMDA in a brain region of interest will lead to cell death, depending on the concentration and volume used. So it is a method to precisely and reliably destroy brain tissue, this is called a lesion. For example see (Yoon & Otto 2007).

Histological examination of LEC lesion was done by Cresyl-Violet and NeuN staining after the mice were sacrificed. For lesion experiments 85nl solution were injected bilaterally in the region of interest.

2.2.9 PERFUSION

Before perfusion mice were deeply anesthetized with a Metedomidine/Ketamine mixture. When plantar reflexes were completely absent, the paws were fixed to the surface of a grid and the thorax was opened with surgical scissors. For solution delivery, a 21G butterfly was inserted 3mm into the left ventricle of the heart. Before opening the perfusion valve, the right atrial auricle was incised. First 10U/ml Heparin/PBS was applied. After blood was washed out, 4% PFA/PBS was injected in a volume of 30-60ml depending on the perfusion performance. For extracting the fixed brain, 3 cuts, from the posterior of the skull to the auditory canals and along the saggital suture were done. For better fixation, the brain was incubated in a 15ml falcon tube filled with 4% PFA for 2h. Afterwards the solution was exchanged with 15% sucrose/PBS for dehydration and stored in 4ºC overnight. To prepare the brain for cutting on the cryostat, it was embedded with a cryoprotective embedding medium in a plastic cryoblock and deeply frozen in dry ice, immersed in 96% ethanol for several minutes. For longer storage, the embedded brains were put to -80ºC.

2.2.10 IMMUNOHISTOCHEMISTRY

For immunofluorescent labelling, mice were transcardially perfused, first with heparin(0.013%) in PBS, second with 4% paraformaldehyde in PBS.

Brains were removed and cryoprotected for 16h at 4ºC in 15% sucrose. Cryo-sections 20–30 µm thick were dried in air for 30 min and then rehydrated in PBST (PBS plus 0.1% Triton X-100). Non-specific binding was blocked with 1% BSA in PBST for 30min. Primary antibodies, diluted 1:300–1:1,000 in blocking solution, were incubated for overnight at 4ºC in a humidified chamber. The slides were washed three times in PBST for 10min. Standard secondary antibodies together with 4' 6-diamidino-2-phenylindole (both 1:1000 dilution) in blocking solution were applied on the slides. After incubation for 2 h at room temperature the unbound primary and secondary antibodies were washed out by incubating three times in PBST for 10min. Sections were mounted in Fluorescence Mounting Medium (Dako; S302380) and covered with cover-slips for further analysis.

2.2.11 OPTOGENETIC STIMULATION

Laser light was delivered by a 447nm or 473nm laser (50mW) connected to a multimode optical fiber. This was connected to a dual ferrule (ZF1.25) optical cable which could be plugged on the cannulas implanted into the mouse brain. Stimulation was performed with laser pulses of 20Hz and 20ms in length. Intensity was 5mW per cannula.

2.2.12 BEHAVIOR

For object related experiments a 60x60 or 40x40 open field arena surrounded by walls of different texture were used. A thin metal plate covered the floor to allow for current flow.

NOVEL OBJECT TASK

For the novel object task a 60x60cm plastic open-field arena was used which was surrounded by (20cm high) plastic walls. On the first day (Habituation) and the second day (Novel Object), white plastic walls were used. A 10cm plastic dinosaur placed in the center of the arena was used as the object.

For habituation, control and treatment mice (both groups n=5) were put in the arena for 10min and afterwards placed back into their homecages. On the second day (Novel Object) the

control group was placed again in the empty open field for 10min. The treatment group was allowed to freely explore the open field with the object placed in the arena for 10min (Figure 14). A third group was left in their homecage for the whole experiment.

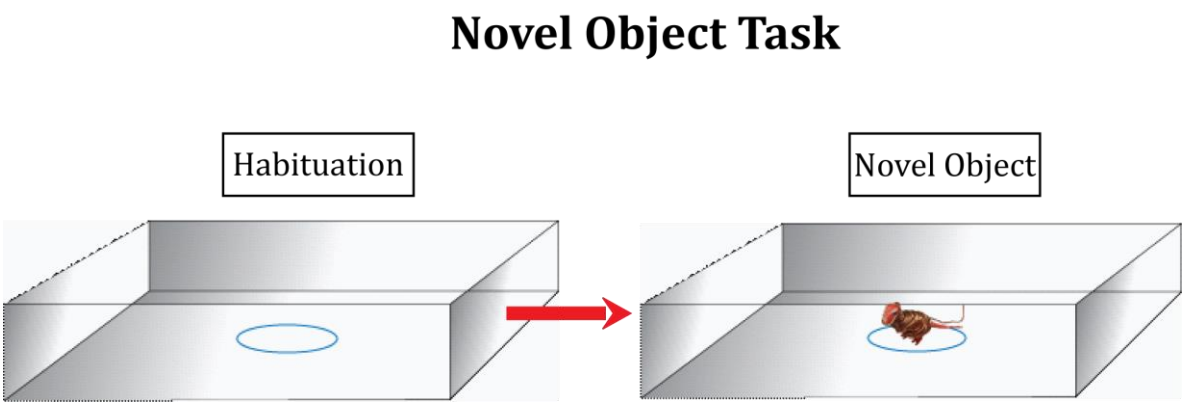


Figure 14. Novel Object paradigm Novel Object Task cartoon shows the consecutive trials. First mice were placed in an empty chamber **(Habituation)**, on the next day an object was placed in the center of the arena before mice were put inside **(Novel Object)**. For analysis see Appendix 1.

90min after the test mice were sacrificed and perfused for immunohistochemical analysis.

Establishment of an Object-Fear Conditioning Paradigm

A 40x40 open field arena, surrounded by white walls, covered with black doted paper sheets was used. A 10cm high plastic dinosaur, wrapped in orange wire, served as the object. It was randomly positioned in one of the arena corners for each trial. Position was assigned randomly for each mouse in each trial. The object position was never allowed to be the same in the next trial. To provide voltage to the object an animal shocker (Coulbourn Instruments) was used. One pole was connected to the metal floor, the other to the object which was insulated to the floor. Therefore if the animal touched the object, current flow was possible and a shock was applied. During habituation, mice were allowed to freely explore the arena with the object inside. Next day (Conditioning), the shock group received a shock automatically when a mouse touched the dinosaur. For the control group no voltage was applied to the object. On the third day (testing), memory was tested with the object in the arena, but without voltage applied. Every trial lasted 20min. (n=4 both groups) (Figure 16).

OBJECT-FEAR CONDITIONING

A 40x40 open field arena surrounded by white walls was used as the standard context for the experiment (context A). To provide a different context, walls with black dotted paper sheets were used (context B).

A 10cm high plastic dinosaur wrapped in orange wire and a polished metal toy car were used as objects to guarantee clear discrimination (Figure 21). For each mouse just one of the objects was energized (from now on referred to as "shock object"). The other object was also connected to a similar wire, but without voltage supply (from now on referred to as "neutral object").

On the first day (Habituation) mice were allowed to freely explore the open field and the objects. On the second day (Conditioning) one of the objects was connected to the animal shocker to provide voltage and mice were put in the arena. For the testing session, context B was used to ensure object-context dissociation. All trials lasted 20min. Objects were placed in one of the four corners in a randomized order. Additionally, no object was allowed to be placed at the same position it had been in the trial before. The energized object (dinosaur or car) was chosen randomly for each mouse.

Object-Fear Conditioning with Optogenetic Stimulation

The same 40x40 open field arena as well as the same two objects were used for this behavioral experiment. Each trial had a duration of 10min. Object position as well as selection for the shock and neutral object were randomly chosen.

White walls covered by black dotted sheets or wooden texture represented different contexts. The order of different context presentation was also interchanged for half of the mice.

For habituation mice were allowed to freely explore context A and B (each 10min). Afterwards the animals were put back in the context they first encountered and laser light was applied always when the mouse entered a pre-defined circular "object area" closely surrounding the "shock object" selected beforehand. During this trial no shock was provided to the "shock object". The neutral object received no manipulation (Figure 24).

On the second day, mice were tested in the context A without any optogenetic manipulation for 10min (Appendix 6). Thereafter the shock object was energized, so that a given mouse would receive a shock when touching it (Conditioning). Additionally laser light was

automatically applied whenever an animal enters the "shock object area" AND the "neutral object area", a similar pre-defined region closely surrounding the neutral object.

On the next day (Testing), the mice were tested in the context B without shock or laser stimulation (Appendix 6). After 10min the laser was periodically turned ON and OFF for 2.5min respectively (Figure 24).

FEAR CONDITIONING BOXES

Pavlovian fear conditioning to sounds was performed in Skinner boxes, lined with sound absorbing foamed material. Different plastic boxes (squares or triangles) were used as different contexts. To strengthen context dissociation either sole ethanol 70% or ethanol 70% mixed with lemon odor were used for cleaning of the different environments.

The animals were recorded by a camera placed above the boxes fixed on the roof.

Shocks could be delivered via a metal grid, inserted as the floor of the boxes and controlled by an animal shocker (Coulbourn Instruments).

TRACE FEAR CONDITIONING

Pavlovian trace fear conditioning represents a special form of associative conditioning, where a CS (e.g. sound) is followed by a US (e.g. footshock), after a stimulus free interval, called the trace. As a result, trace conditioning needs more cognitive resources than simple delay conditioning, where CS and US are contiguous (Esclassan et al. 2009). Thus, trace fear conditioning is thought to involve associative learning, because events are casually related, but discontinuous in time (Bangasser et al. 2006). It was already shown that several brain regions are required to perform this task (Clark et al. 2002; Han et al. 2003).

Two brain regions especially important for this task are the hippocampus (HPC) and the entorhinal cortex (EC).

Several studies have shown that structures in the hippocampal formation are needed to bridge the temporal gap between CS and US (Wallenstein et al. 1998; Bangasser et al. 2006). It is also suggested, that processing of the trace could already occur upstream of the hippocampus in the entorhinal cortex (Ryou et al. 2001).

A paper by Esclassan et al. showed that EC lesion leads to a disruption in conditioned fear response to a tone in a trace fear conditioning paradigm, but not in delayed fear conditioning

(Esclassan et al. 2009). Interestingly, these studies preferentially targeted the MEC. As we were interested in the processing and representation of sensory information within the LEC, we decided to use a similar paradigm, to see if LEC lesion does not just change behavior to visual 3D objects, but also influences the reaction to sounds. In addition we chose the trace fear conditioning protocol because of its higher complexity compared to delay fear conditioning. Therefore it should be more sensitive for perturbations within circuits that process fear memory systems.

Protocol

Trace fear conditioning protocol was adapted from Han et al. (Han et al. 2003).

On the first day (Habituation) animals were placed in context A for 10min.

Immediately afterwards trace conditioning was performed (Conditioning).

For this, a sound (70dB, 3kHz) was played for 10sec, followed by a trace of 20sec and a footshock (0.4mA) of 2sec. This was repeated 10 times. An inter-trial interval (ITI) of 188sec was introduced between each trial.

On the second day mice were placed again in context A for 10min to test fear to the context. Two hours later the animals explored context B for 10min. Then 10sec of sound followed by 20sec trace but without an ITI were repeated 10x for testing the fear conditioning (Testing) (Figure 15).

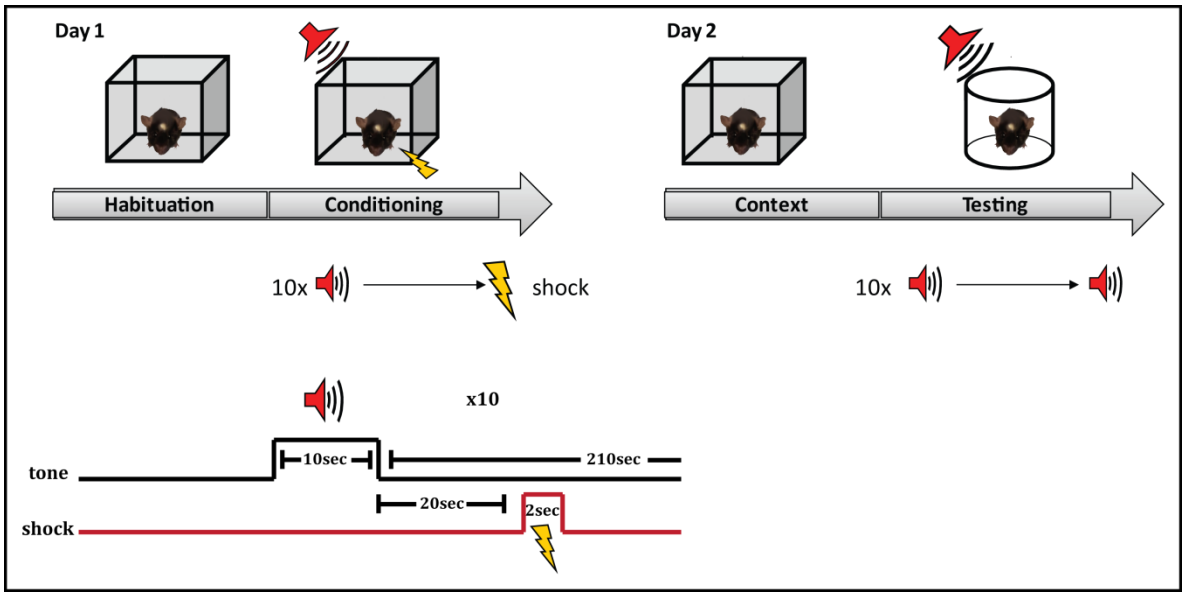


Figure 15. Trace-Fear Conditioning protocol (Top): Cartoon of the trace-fear conditioning protocol with a Habituation and Conditioning session on the first day and a context fear test as well as the tone fear test on the second day. **(Bottom):** Schematic of the fear-conditioning protocol. A sound was played for 10sec, a 20sec trace was introduced before a 2sec shock was applied. Intertrial interval (ITI) was 188sec. This procedure was repeated 10 times. In the testing session, the sound was played, after a 20sec gap the sound was played again, this was repeated 10 times and freezing was measured during sound presentation.

Trace Fear Conditioning Protocol with Optogenetic Stimulation

The duration and also the number of repetitions of the tone, trace and shock remained the same as in the simple trace fear conditioning experiment, with the only difference of shock strength (0.3mA instead of 0.4mA) to prevent behavioral ceiling effects.

On the first day (Habituation) mice were placed in context A for 5min.

Immediately afterwards, the trace conditioning was done, where blue laser light was activated during the whole period of tone, trace and shock.

On the second day mice re-explored context A for 5min to measure context fear memory and then performed the testing session. During the testing session the sound was played for 10sec followed by a 20sec trace period. This was repeated 10 times with no laser activation during the whole session (Figure 25).

Video Recording and Behavior Analysis

Videos were recorded with cameras placed above the behavior chambers and the readouts were quantified with video-tracking software Any-maze (Stoelting Co.) or EthoVisionXT (Noldus Information technology).

As parameters of interest we analyzed the animals total distance traveled and total time freezing during a given trial. In object conditioning tasks we also examined the time the animals spent within a virtual circular area, closely surrounding the objects. These areas were defined within the Any-maze software protocol, and had the same size for the shock object area and the neutral object area. We also measured relative preference for the objects. Therefore we introduced a discrimination index comparing the time an animal spent close to the shock object with the time the animal was close to the neutral object.

Discrimination index: (Time shock object – Time neutral object)/(Time shock object + Time neutral object). As a result, a value close to 1 would mean a longer stay close to the shock object in relation to the neutral object and -1 the opposite.

2.2.13 STATISTICAL ANALYSIS

For statistical analysis GraphPad PRISM (GraphPad Software, Inc.) was used. For two groups and only one parameter of interest, unpaired t-test with parametric distribution (*significance if $P<0.05$) was used. Three or more groups were tested on one parameter with One-way ANOVA and Multiple Comparisons Holm-Sidak test. Three or more groups were tested on more than one parameter with Two-Way ANOVA and Multiple Comparisons Holm-Sidak Test. Different sessions (e.g. Habituation, Conditioning, and Testing) were analyzed separately.

3. RESULTS

3.1 ESTABLISHING AN OBJECT-FEAR CONDITIONING TASK

We first transformed classical Pavlovian tone foot shock conditioning into a behavioral paradigm that allows to study conditioned behavioral responses to 3D objects. We used a plastic open field arena with a metal plate covering the floor delivering electric foot shock as US, and wire wrapped plastic objects as CSs (see section 2.2.12). The behavioral paradigm was termed object-fear conditioning. With this setting we were able to associate a 3D object deliberately with a shock. One trial was sufficient to establish a strong and long lasting avoidance behavior to the object in mice (Figure 16).

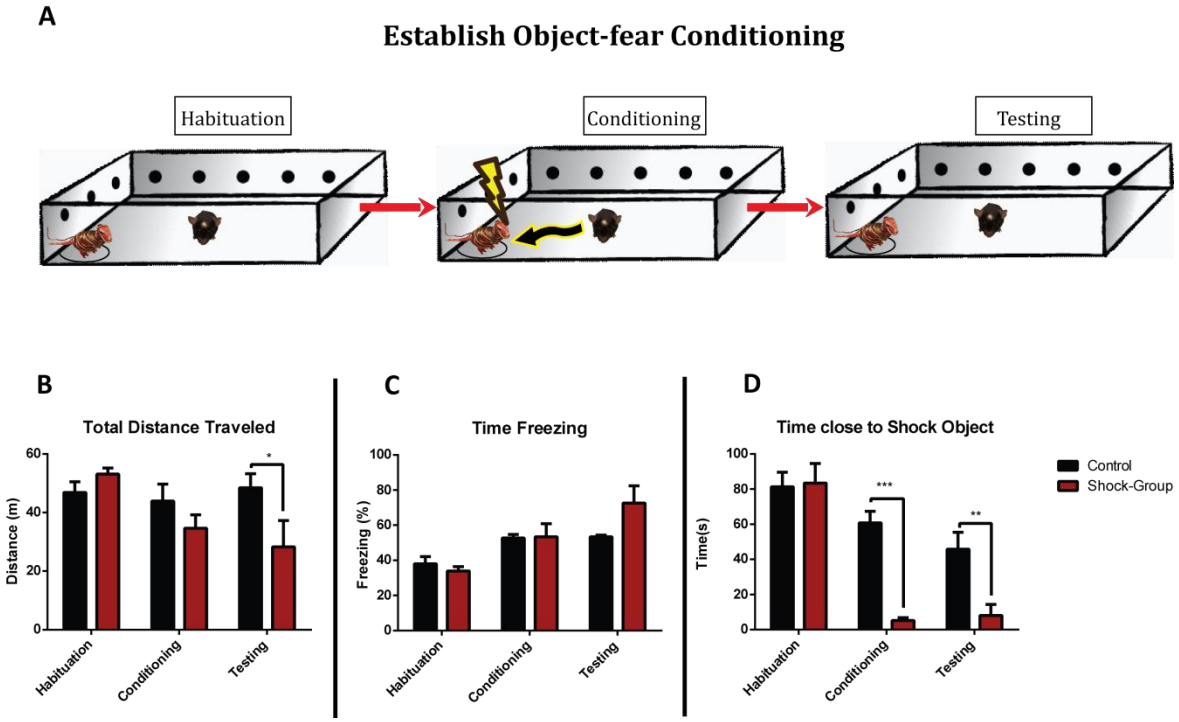


Figure 16. Establish and object-fear conditioning task **(A)** For establishing the Object-Fear conditioning task, a simple paradigm was introduced. On the first day mice were placed in the arena with an object in one of the corners **(Habituation)**, on the subsequent day, mice received a shock when they touched the object **(Conditioning)**, on the third day, animals were placed again in the arena with the object, but no shock was applied **(Testing)**; **(B)** In the Testing session, Shock-Group mice traveled significantly less compared to the Control group; **(C)** No difference was seen for the time freezing during all sessions; **(D)** During habituation, both groups explored the object and therefore spent time close to the object. After voltage was applied to the object in the conditioning session, the Shock-Group spent significantly less time in the vicinity of the object to prevent further pain by the shock. The object-shock association was memorized very well, what can be seen on the testing day, were the treatment group still prevented the object. (Control n=4; Shock-Group n=4). *Statistical significance was tested with Two-Way ANOVA and Holm-Sidak Multiple Comparisons test. Data plotted as mean ± SEM.*

3.2 Architecture and Activity of the Entorhinal-Amygdala Network in Object-Fear Conditioning

3.2.1 Retrograde Tracing from Amygdala to the Lateral Entorhinal Cortex

The EC is known to have profound connections to the amygdala. We were particularly interested in the projection of the LEC - the area with high density of object responsive cells (Tsao et al. 2013) – to BLA and CeL, the major amygdala nuclei. To differentiate between projections to these amygdaloid subnuclei, we used dual-color CTB: by injecting these CTB conjugated to two different fluorophores into two different amygdala nuclei, we were able to dissociate projections patterns from LEC to BLA and CeL by counting CTB positive cells in the LEC. Percentage CTB-positive cells are illustrated in Figure 18 and show a difference in their distribution for BLA and CeL as well as in the anterior to posterior positions. A higher percentage of CTB-positive cells can be seen in the anterior and medial portion of LEC projecting to CeL. Only around 0.5% of CeL projecting cells are CTB-positive in the posterior part of LEC. The opposite was true for retrogradely labeled cells from BLA. A lower portion of positive cells was seen in the anterior and medial part but apparently higher percentage of labeled cells in the posterior LEC. Comparing CeL and BLA innervating LEC cells, the connection from the lateral entorhinal cortex seems stronger to BLA than to CeL, as even the highest percentage of positive cells in CeL was lower than BLA projection populations in every layer. However, the anterior and medial portion of the dlLEC seems to project quite equally to the CeL and the BLA. Importantly, CTB-back labeling of the CeL and the BLA confirmed profound innervations of the dlLEC to amygdaloid subnuclei, an area of the EC where also object cells are found. Although it is not possible to identify overlap of object cells and CTB-positive cells with this method, the existence of amygdala projecting cells was a first step toward that possibility.

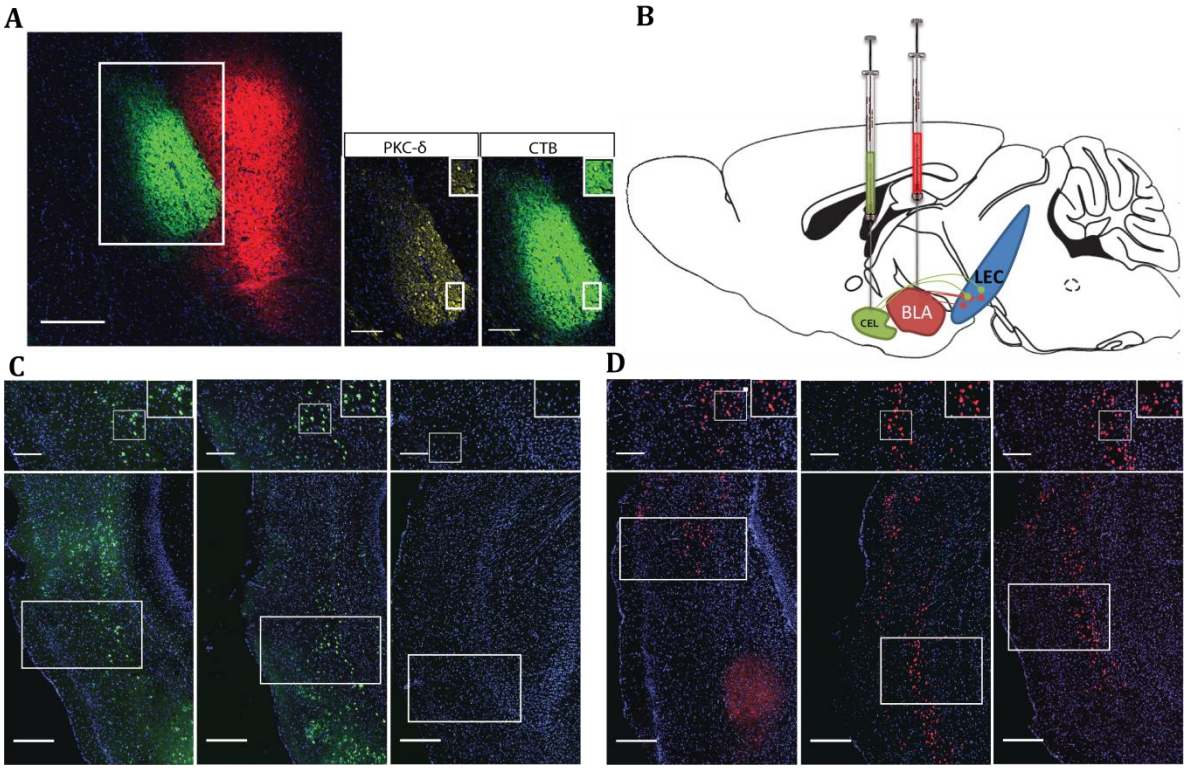


Figure 17. CTB- Injection into CeL and BLA **(A)** Injection side shows CTB-488 **(**green**)** injection into CeL and CTB-555 (red) injection into BLA. Magnification of large white rectangular box of CeL shows PKC- δ staining **(Middle)** and CTB over PKC- δ staining **(Right).** Small white rectangular boxes indicate cellular magnification; **(B)** Schematic of CTB injection shown on a saggital view of the rodent brain, with CTB-Injection into CeL or BLA and retrograde tracing into the LEC; **(C and D)** LEC coronal sections, examples from anterior **(Left)**, medial **(Middle)** and posterior **(Right)** parts of the LEC for **(C)** CeL and **(D)** BLA injections. Area indicated by the large rectangular box is always shown as magnification on the top of the picture. Small rectangular box show cellular magnification. *Scale bar for low magnification 200µm and for high magnification 100µm*.

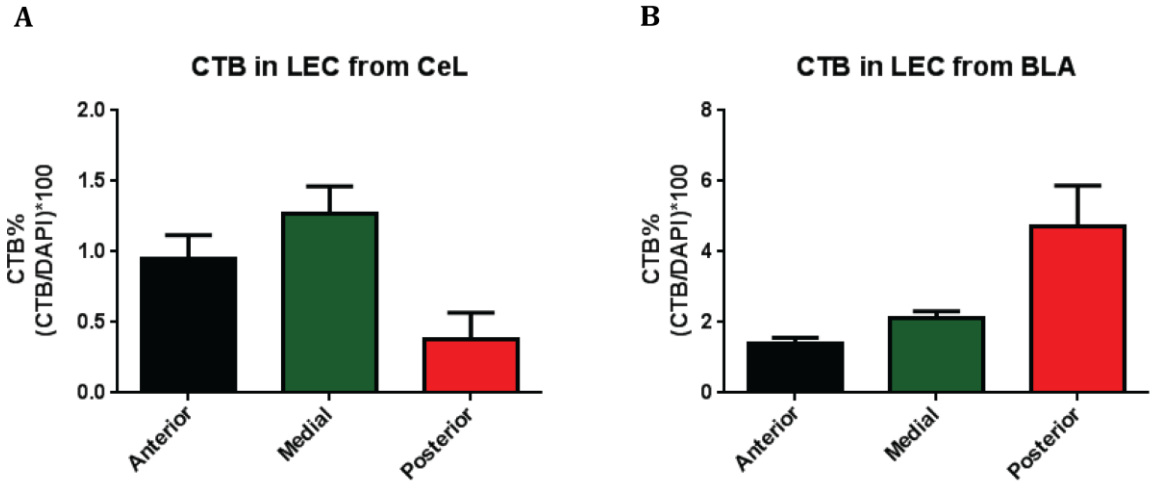


Figure 18. Quantification of CTB backlabeling of amygdaloid injections CTB-positive cells in LEC were counted from brain sections where CTB was injected into **(A)** CeL (n=8) or **(B)** BLA (n=3). Brain sections were grouped into anterior, medial and posterior portions on the basis of the Allen brain atlas by observation of the scientist. *CTB% as relative values compared to DAPI positive cells. At least 3 sections per region were counted*.

3.2.2 ACTIVATION OF LEC AND CEL AFTER SHOCK-OBJECT REEXPOSURE

The previous tracing experiments established an anatomical network between LEC, BLA and CeL. To investigate if this network is functionally linked to object-related behaviors, we wanted to test if these regions are activated in object-fear conditioning tasks. As explained in section 2.2.7 the immediate early gene (IEG) c-fos was used as a genetic marker for neuronal activity. We tested the activation of the LEC, BLA and CeL components of this network by novel objects (see section 2.2.12) in one cohort, while another cohort of mice was re-exposed to an object which had already been associated with a shock previously. The activity within the brain regions of interest should change dependent on the experience with the object; that is, the negatively valenced more relevant object should induce higher levels of activity in this network, that in turn should correlate with stronger behavioral reponses. C-fos was also counted in the auditory cortex (AuX) as an outgroup for unspecific activation. Figure 19B shows a significantly higher activity for LEC and the CeL in the Shock-Object reexposure group compared to the Homecage animals (Interaction: $F(4, 32) = 4.695$; $P = 0.0043$) with (Holm-Sidak $P<0.05$; $P<0.001$, respectively). More c-fos were also counted in CeL comparing Novel Object and Shock-Object reexposure group ($P<0.0001$). The c-fos data clearly showed that the LEC, as well as the CeL were profoundly activated when mice were confronted with a previously negatively associated object. Although there is a trend for higher activation of the BLA during the Shock-Object reexposure, we found no significant difference compared to the control group. Therefore it seems that novel and negatively valenced objects are discriminated in LEC and CeL. To confirm that c-fos activation was not due to possible shocks when touching the object during the reexposure session, the time spent close to the object was correlated to the c-fos expression in the LEC, CeL, BLA and auditory cortex (AuX). Interestingly, a negative correlation was seen between time and c-fos expression for the LEC (Pearson r=-0.8899, R square=0.7919, P(two tailed)=0.0431) (Figure 19C). One could argue that the higher activity in LEC reflects stronger association of the shock with the object and therefore mice avoided the object more strongly. The within-group correlation was not seen for the CeL and the BLA. However, the trend is evident across groups, that is, novel object groups are higher than the Shock-Object reexposure groups. Note that the AuX does not follow these within and across group trends. (CeL: Pearson r=0.2323,

R squared=0.05394, P(two-tailed)=0.5799; BLA: r= -0.02419, R=0.00058, P=0.9547; AuX: r=-0.7653, R= 0.5857, P=0.2347) (Figure 19C).

Taken together, c-fos mapping revealed a strong activation of the LEC and the CeL to the exposure to a negatively associated object, indicating that these structures should have important functions related to that experience and its behavioral response.

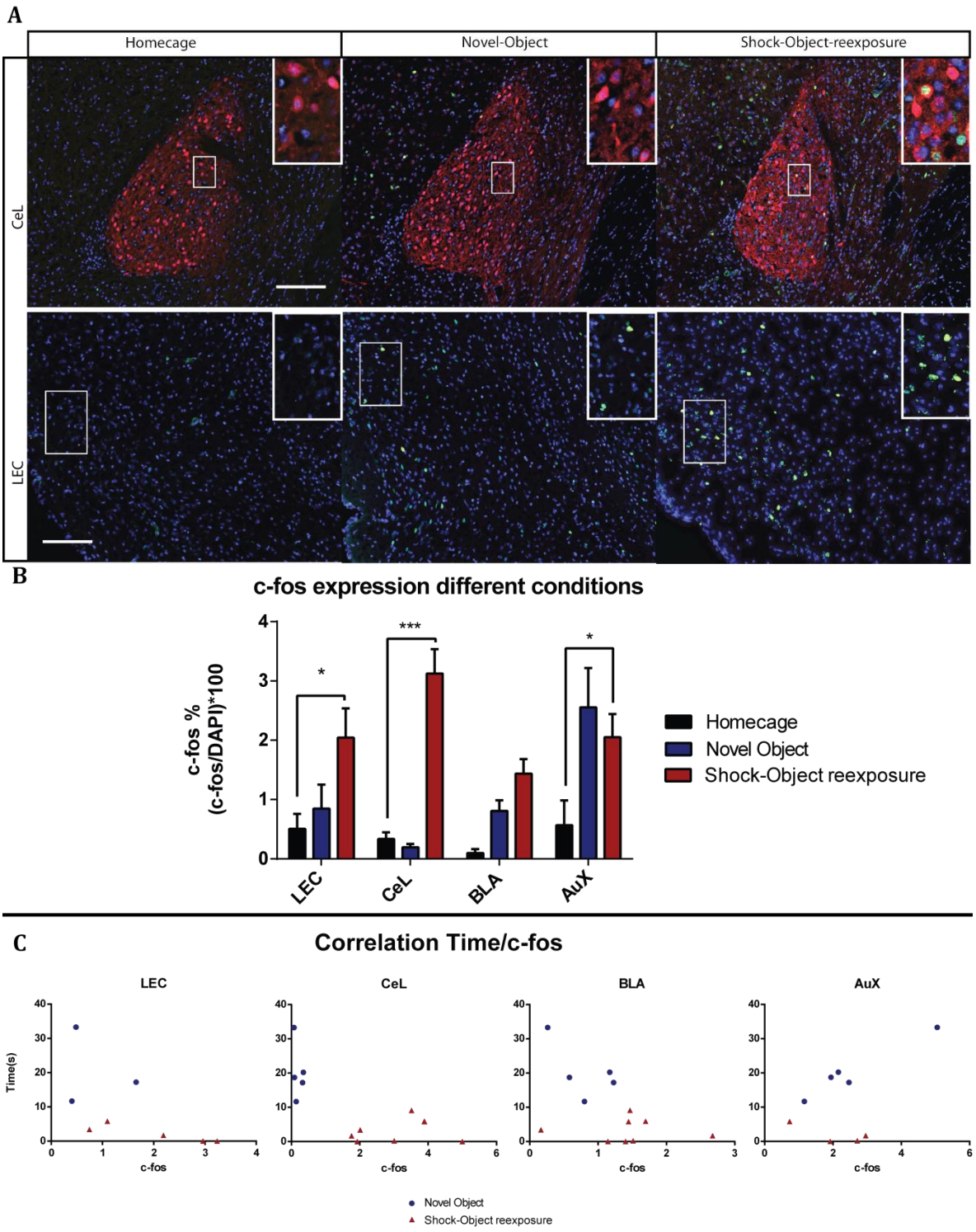


Figure 19. Shock-object reexposure induced c-fos expression in LEC and CeL **(A)** Example pictures from CeL and LEC under different conditions. CeL was co-stained for PKC- δ **(red)**; **(B)** Quantification of c-fos expression counted in 3 different areas. *c-fos% as relative values compared to DAPI positive cells. At least 3 sections per region counted*; **(C)** Correlation between time spent close to the object area against c-fos expression (%). No correlation between the c-fos expression in CeL, BLA or AuX to the time that animals spent close to the object were seen for the Shock-Object reexposure group, in contrast to the LEC. (Novel Object group (n=3-5), Shock Object-reexposure group (n=5-8)). *With *p<0.05, **p<0.01, ***p<0,001, ****p<0.0001. Statistical significance was tested with Two-Way ANOVA and Holm-Sidak Multiple Comparisons test. Data plotted as mean ± SEM. Scale bar: 100µm.*

3.3 A ROLE FOR LEC IN OBJECT-FEAR CONDITIONING

3.3.1 NMDA-LESIONS

The previous evidence suggests that the LEC is involved in processing negative object associations or memory to negative valenced objects. We wanted to corroborate this by establishing a causal role for this structure in controlling spatial valenced object behavior. To address this, NMDA injections into the LEC were performed and assessed post behavioral testing by neuron-specific immunohistochemistry. The staining confirmed profound cell loss in the injected region (Figure 20B). Apart from one animal, all NMDA lesioned mice (n=10) had bilateral lesions with different extension and size. Sham lesioned mice which were injected with the same amount of PBS, showed no or marginal cell death (n=9) (Figure 20B). For some NMDA-injected animals the lesions expanded to the surrounding regions of the LEC, especially the perirhinal cortex (PRh) and a ventral portion of the CA-1 region of the hippocampus (Figure 20A). The strongest neuronal loss was confined to the LEC in most of the cases.

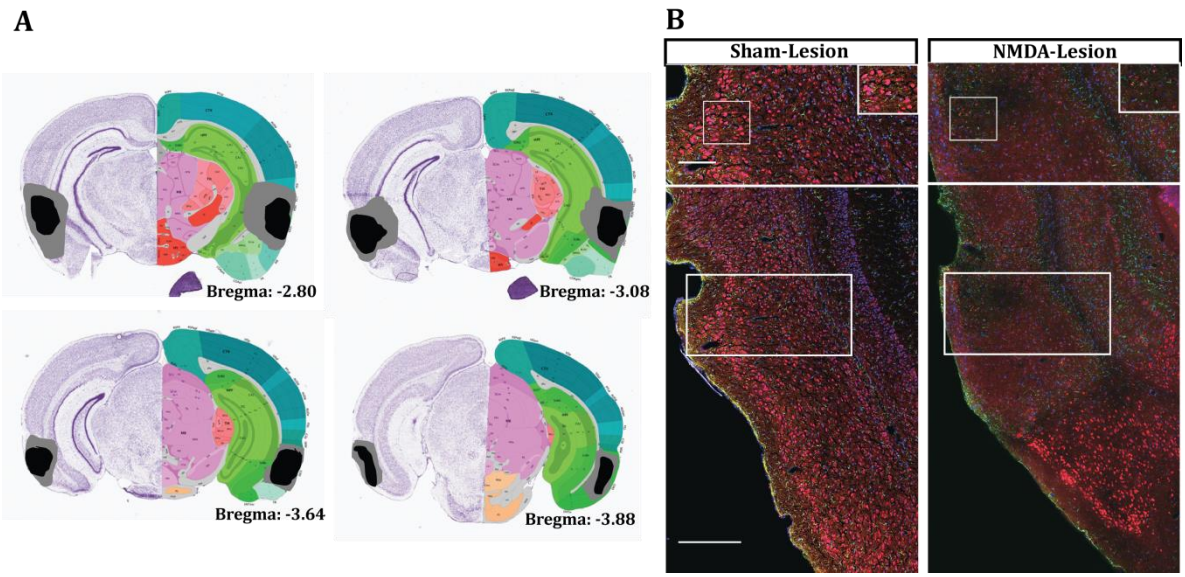


Figure 20. NMDA-Lesion **(A)** NMDA-Lesion size for different anterior-posterior positions. Position is indicated in mm relative to Bregma. Approximate damage by NMDA-lesion with minimum (black) and maximum (gray) lesion size indicated. **Coronal sections taken from (http://mouse.brain-map.org)**; **(B)** Example pictures from Sham-Lesion and NMDA-Lesion of LEC. Magnification of white rectangular box on the top of the pictures. Coronal slices were stained for DAPI **(blue)** and NeuN **(red)**. *Scale bar: low magnification 200µm, high magnification 100µm*.

NMDA-Lesions modulate Object-Fear Conditioning

First we wanted to test the NMDA-lesioned mice on our object-fear conditioning task, where one object was associated with a negative experience, here a shock, and the other, clearly dissociable object, remained neutral. We introduced the second object to assess effects on discriminatory performance vs. generalization.

Figure 21A shows a schematic of the object-fear conditioning paradigm. No difference was seen for both groups in the habituation and conditioning session (Figure 21B). Interestingly, NMDA-lesioned mice spent significantly less time in the vicinity of the shock object compared to the control animals in the testing phase (unpaired t-test: P=0.0447). This suggests a role of LEC in processing the memory of the negatively valenced object. Importantly, as the total distance traveled and the total time freezing were not significantly different comparing the two groups (Appendix 2), we can exclude an overall increase in general anxiety and it depicts a rather specific reaction to the association of the object with a shock. Nevertheless, there was a trend towards a shorter time period the NMDA-lesioned mice spent also near the neutral object (Appendix 2). This trend was not reflected in the discrimination index, which was calculated to depict preference for one of the objects (Figure 21C). There we saw significantly higher discrimination ability for the NMDA-lesioned mice than the controls (unpaired t-test: P=0.0449).

Although histological examination confirmed extensive destruction of the dlLEC tissue by NMDA lesion, mice displayed no functional deficits in learning or remembering the association of the shock with one of the objects. There was also no impairment in discriminating the shock object from the neutral object. In contrast, we saw an increased avoidance of the shock object during the testing trial as well as stronger discrimination between the objects for the NMDA-lesioned group. Thus dlLEC lesion does not impair, but amplifies shock-object avoidance.

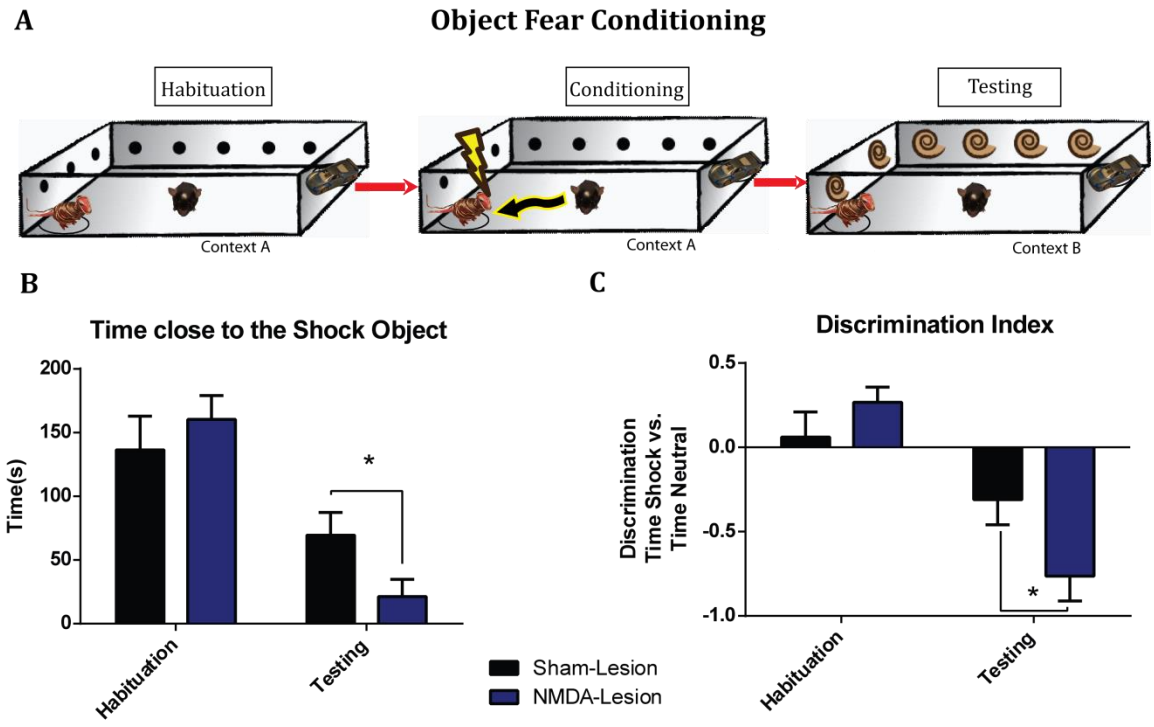


Figure 21. NMDA-lesioned mice show increased fear response to a negatively conditioned object **(A)** Cartoon of object fear conditioning behavioral protocol for NMDA-lesioned mice. In the **Habituation** session mice were placed in the arena (context A) and allowed to freely explore the two objects. In the next trial, mice were placed in the same arena, but one of the objects was energized, so the mouse received a shock upon touching the object **(Conditioning)**. The third trial was a **Testing** session in a novel context B; **(B)** Shown is the time animals spent close to the shock-associated object in the habituation and testing sessions. In the testing session, mice with lesions of the LEC spent significantly less time in the vicinity of the shock object compared to the Sham-lesioned control; **(C)** Discrimination index indicates relative preference for one of the objects. A higher discrimination is seen for the NMDA-lesioned group in the testing session. *Sham-lesion group n=9, NMDA-lesion group n=10. Statistical significance was tested with unpaired t-test. Data plotted as mean ± SEM.*

TRACE FEAR CONDITIONING

To see if the effect of increased fear, as seen towards the shock-object by NMDA destruction of the lateral entorhinal cortex, is also present for other modalities, we used a paradigm were we associated a sound with a shock. To increase the sensitivity of the assays, and to probe for entorhinal associated memory, the animals were tested in a so called trace-fear conditioning paradigm. Trace-fear conditioning contains a gap, without any stimulus between the tone (CS) and the shock (US) presentation, called the trace (section 2.2.12).

Conditioning was not influenced by the lesion as seen in Figure 22A. When we checked for the fear to the context the mice were conditioned in, we saw a significantly higher percentage of freezing for the NMDA-lesioned group (unpaired t-test: P=0,019). However, similar effects were seen in a novel context where the mice were tested ($P<0.05$), as well as to the reexposure to the tone (CS) after training ($P<0.05$). These results indicate a general involvement of LEC in processing and memory of negative (not only object-) associated

stimuli. Although a generalization effect was seen for the trace-fear conditioning experiment, where LEC-lesioned animals developed higher freezing to the same and to a novel context as well as the tone presentation after conditioning, we saw a quite specific behavior for the object shock conditioning. LEC-lesioned animals showed a persistent avoidance of the shock object, which was decreased in the control group within a novel context. The simplest explanation for the behavioral data so far, is that LEC does not store object and trace memory itself, but rather gates fear responses to threats by signaling safety.

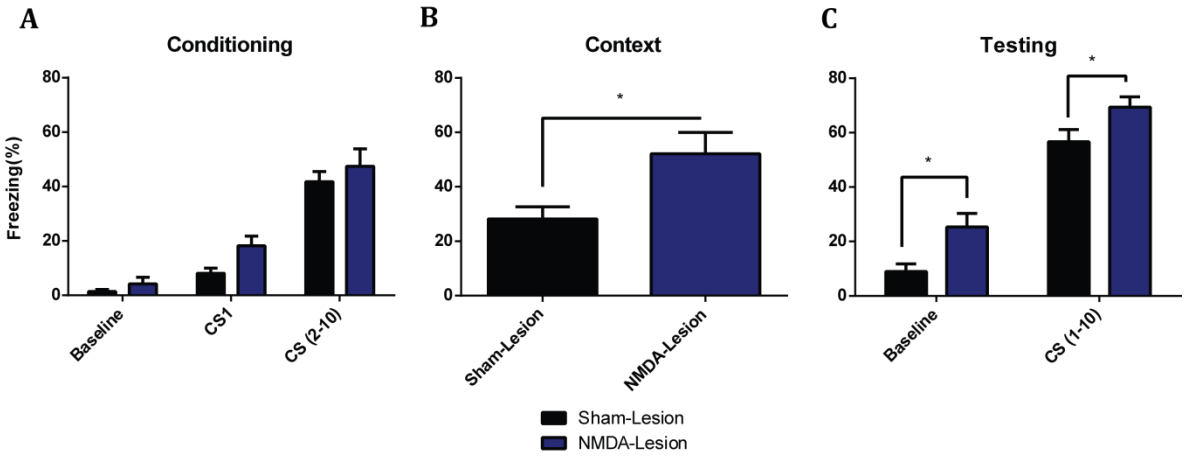


Figure 22. Higher freezing to different stimuli in TFC paradigm in the NMDA-lesioned group **(A)** Percentage of freezing during the Conditioning session, both groups were able to learn the association; **(B)** Reexposure to the conditioning context induced higher freezing in NMDA-lesioned mice; **(C)** In the testing session, NMDA-lesioned animals showed significantly higher freezing during a 2min baseline session as well as to the sound presentations. *Statistical significance was tested with unpaired t-test and with One-Way or Two-Way ANOVA and Holm-Sidak Multiple Comparisons test. Data plotted as mean ± SEM.*

3.4 Optogenetic Dissection of LEC-Amygdala Microcircuits in Object and Tone Fear

3.4.1 Optogenetic Targeting of LEC-Amygdala Projections

The observation that impairment of the lateral entorhinal cortex influenced behavior towards objects and context in fear conditioning paradigms raised the question of which circuits are involved in the processing of these functions. The amygdala as one of the essential brain structures for emotional processing (see section 1.2.4) and given that it is connected to and activated together with LEC by negatively valenced objects, could play a crucial role in

regulating behavioral responses to threatening objects within the brain. To explore this possibility, we performed optogenetic manipulations to selectively activate the connection from the LEC to the BLA or to the CeL. To gain projection specific optogenetic control, we expressed ChR2 in LEC (by stereotactic injections of 1:10 Cre expressing and Cre-dependent ChR2-expressing AAVs (section 2.2.3)). This resulted in high expression levels within the region of interest (Figure 23A). During the same surgery, cannulas for laser light delivery during optogenetic stimulation were implanted above BLA (n=5) or CeL (n=5). Therefore only synapses projecting from LEC to the specific subnucleus would get activated upon light stimulation. Control mice (n=10) were injected with Cre-expressing and Cre-dependent GFP expressing AAV and implanted with cannulas above BLA (n=5) or CeL (n=5). As no significant differences were seen for the control groups they were pooled and tested as a single group (n=10).

ChR2-expression levels were examined after sacrifice and sectioned with cryostat. The extent of infections were correlated with behavioral data (Appendix 4).

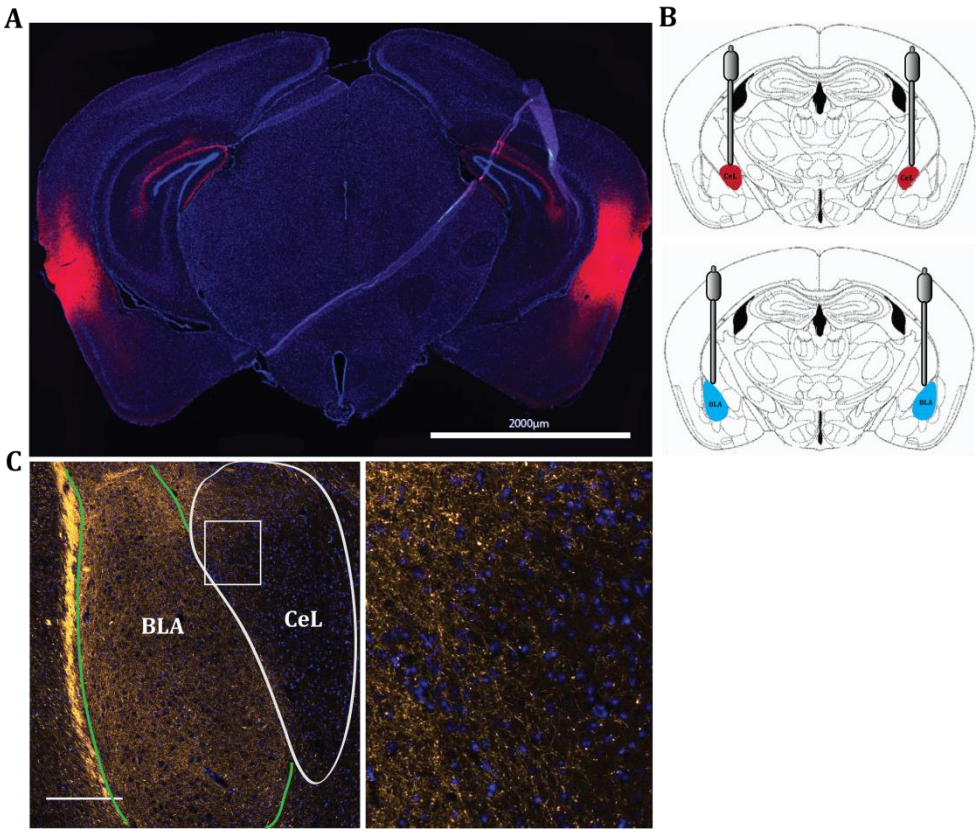


Figure 23. ChR2 expression in LEC **(A)** Representative picture of ChR2 expression in LEC; **(B)** Cartoon showing cannulation sites for optogenetic fiber activation of **(Top)** CeL or **(Bottom)** BLA; **(C) (Left):** ChR2 positive fibers from LEC to BLA and CeL. Strong fiber innervations are seen for BLA. CeL shows strongest innervations at the anterior pole. **(Right):** Magnification of white rectangular box. *Scale bar 200µm.*

To test the hypothesis that direct LEC-amygdala projections are important for object valence communication within the brain we first probed mice on an object-fear conditioning task (Figure 24A), similar to the experiment conduced on the NMDA-lesioned mice. Several laser activation patterns were used to address for specific behavioral questions (section 2.2.12). For example, during conditioning the laser was active when the animal was close to the shock object AND when it was close to the neutral object. We used this experimental setup to exclude possible association of the laser and the shock alone, which would give us a drop in time spent next to the neutral object during laser on phases as well.

OBJECT-FEAR IS GATED THROUGH LEC-CEL PROJECTIONS

During the habituation phase, where the laser was activated whenever an animal was close to the predefined shock object, BLA as well as CeL activated mice spent significantly more time around the object, compared to the control group (Interaction $F(2, 17)=14.53$, $P=0.0002$), (Holm-Sidak $P<0.01$ CeL and BLA), (Figure 24B), that is, both projections increased the intrinsically positively valenced behavioral reactions to the object. The conditioning phase revealed no significant differences. During the testing phase, animals with light guides above CeL spent significantly more time in the very proximity of the shock object (Interaction $F(2, 17)$ $P=0.0153$) with (Holm-Sidak $P<0.01$), which was not observed for the control and the BLA group. Thus, the testing session revealed a dissociation between BLA and CeL mice: the activation of BLA had no effect on the aversion to the shock associated object (while increasing positively valenced reactions during habituation), whereas the CeL activation decreased negatively valenced responses to the shock object. As a consequence the animals stayed for longer time periods close to the object (Figure 24C). The most likely interpretation is that activating LEC projections to CeL, gated the aversive response to the shock object, whereas activation of BLA projection amplified the intrinsic valence response to the object. Interestingly, all manipulations left no memory trace as no

difference in object valence was seen in an independent trial looking for memory effects (Appendix 6).

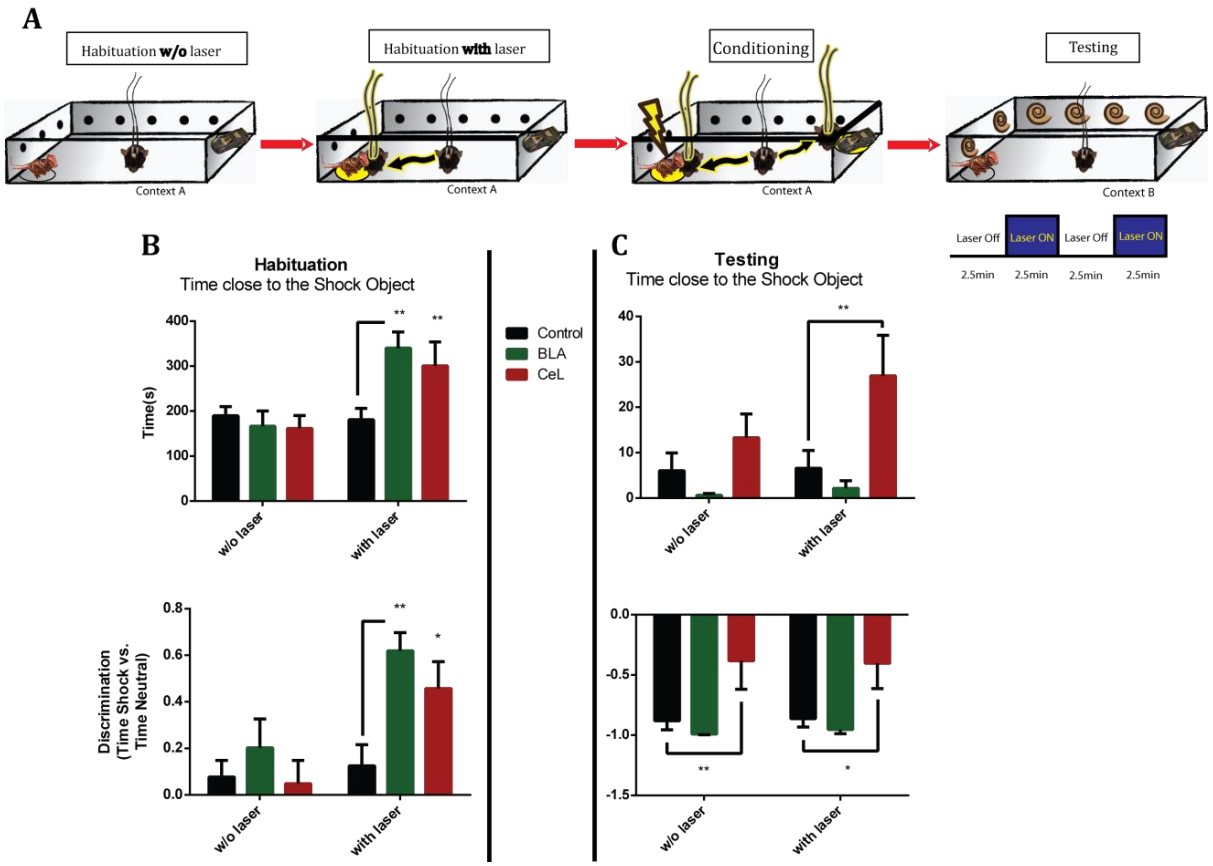


Figure 24. Optogenetic activation led to valence stability or shift **(A)** Cartoon of the Object-Fear Conditioning task with optogenetic stimulation. First a habituation session in context A was performed **(Habituation w/o laser)**. Subsequently the laser got activated when the mouse was close to the shock object (here the dinosaur) **(Habituation with laser)**. In the next trial the laser was activated when the animal was in the vicinity of the shock object but also when it was close to the neutral object. In addition, mice received a shock when they touched the shock object **(Conditioning).** The **Testing** session was performed in context B. No shock was applied, but the laser was activated consecutively for 2.5min and intermitted for the same time period (see schematic under the testing cartoon); **(B)** Comparing the habituation sessions w/o laser and with laser. When looking at time, the animals spent close to the shock object, laser activity lead to a significant increase in time;
(C) In the testing session, periods without laser and periods with laser activation were summed up and time close to the shock object was compared. During periods where the laser was activated, mice with cannulas on the CeL spent more time in the vicinity of the shock-object. Animals with activation of the LEC-BLA connection did not show this effect. *Statistical significance was tested with Two-Way ANOVA and Holm-Sidak Multiple Comparisons test. Data plotted as mean ± SEM.*

3.4.2 LEC-Amygala Projections have only a minor Role in Trace-Fear Conditioning

To test for a dedicated role for LEC-Amygdala system in mediating object- but not general fear-responses, we next conducted TFC in the same animals. The laser was activated during the whole period of tone, trace and shock presentations in the conditioning trial, but was never turned on in the testing session (Figure 25A). Percentage of freezing revealed a

decrease in the conditioning phase for the BLA group during CS presentation 2-10 (Interaction F(4, 34) P=0.0434) with (Holm-Sidak P<0.001) (Figure 25B). However, no significant differences were detected for all groups comparing the freezing parameters in the testing session. These results suggest only a minor role for LEC-BLA or CeL projections in mediating responses to auditory cues, as opposed to their strong participation in object responses.

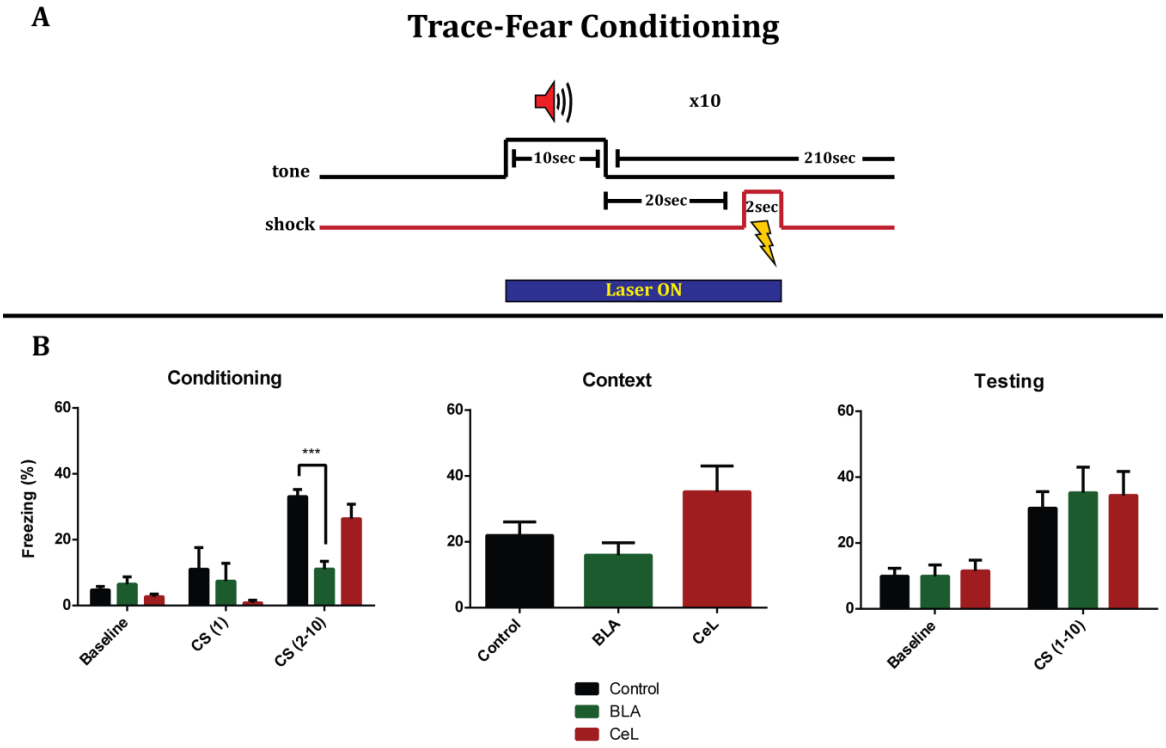


Figure 25. Optogenetic stimulation does not change behavior in trace fear conditioning task **(A)** Schematic of trace fear conditioning and the time period the laser was turned on indicated by the blue bar under the protocol; **(B)** Freezing values during **(Left):** Conditioning, **(Middle):** Context reexposure and **(Right):** Testing session of the trace-fear conditioning task. Significantly lower freezing was seen for the BLA group during the conditioning phase, this difference is not seen any more during the testing session. *Statistical significance was tested with One-Way or Two-Way ANOVA and Holm-Sidak Multiple Comparisons test. Data plotted as mean ± SEM.*

3.4.3 Delay Fear Conditioning

Given that BLA and CeL projections from the lateral entorhinal cortex clearly influence behavior towards objects, but had no consistent effect in TFC, we asked if maybe delay fear conditioning behavior is influenced upon activation of these connections. We used a simple DFC protocol (see section 2.2.12) and activated the laser in the conditioning phase during tone presentation and shock delivery (Appendix 8). In the testing session no perturbations

were applied. No differences were found in freezing parameters for the three groups tested (Appendix 8 and Appendix 9). This result further indicates an involvement of the direct connection form LEC to BLA and CeL in tasks associated with physical objects, but just a small contribution in the realm of sounds, if any at all.

4. DISCUSSION

4.1 THE LEC-AMYGDALA NETWORK IN PROCESSING VALENCED OBJECTS

It is extremely important for an organism to associate objects with an appropriate significance (discussed in section 1.2). Irrelevant stimuli should be ignored whereas important experiences should trigger proper reactions. Therefore a negative encounter with an object must lead to defensive or avoidance behavior, whereas positive experience should increase time spent at a given opportunity. The entorhinal cortex has already for a long time been implicated in several aspects of processing spatial and temporal information. Entorhinal dissociation into a medial and a lateral entorhinal cortex (MEC and LEC, respectively) already suggested decades ago, has proven methodologically helpful for understanding the structural framework in the brain which assembles consistent and uniform experiences and finally the formation of episodic memories (Knierim et al. 2014). The partitioning into apparently two information streams within the entorhinal cortex carried out by these subdivisions built the basis for many studies which showed that the medial entorhinal cortex with its grid cell, border cell and head direction cell systems (Boccara et al. 2010) provides a global reference frame, primarily using internally generated, self-motion cues (Neunuebel et al. 2013) and that the LEC with its object responsive cells and activity to content within an environment provides a local reference frame (Knierim et al. 2014).

We proposed that this is in part being mediated by the LEC-Amygdala interactions. These interactions (i) play an important role for the LEC in object processing, providing other brain areas with the information of the content of an object related experience; (ii) they assign emotional relevance (valence) to an object and/or (iii) drive approach or avoidance responses towards such objects (see section 1.6).

Our anatomical mapping identified the dlLEC to amygdaloid subnuclei as major input to the basolateral amygdala (BLA) and the lateral part of the central amygdala (CeL) (Figure 17). The fact that the dlLEC has recently been identified to have a high density of object responding cells points towards a role for this connection in processing object related information. To assess the function of this network in object guided behavior in mice we

designed an object-fear conditioning task which allowed us to explicitly study the behavioral effects to neutral and negatively valenced objects (Figure 21).

When we investigated the c-fos expression pattern, as proxy for neuronal activity, evoked by a novel and a negatively associated object, LEC and CeL, but not BLA, displayed high activity specifically to the reexposure to the negatively associated object and a positive correlation of dlLEC activation with behavioral avoidance responses. This strongly suggests, that particularly the dlLEC-CeL projections relay valence related object-information.

4.1.1 LEC GATES OBJECT AND CONTEXTUAL FEAR

Based on these anatomical studies, NMDA-lesions were focused on the dorsolateral portion anterior part of the LEC, which were found to have strongest projections to the CeL and proposed to derive from that area. Further support for this notion comes from the fact that LEC lesions had profound effects on object-related behaviors. The additional role for LEC in gating also contextual fear responses can in part be explained by impairment LEC functions mediated through connections to other, extra-amygdala brain regions.

The increased avoidance behavior we observed during shock object-reexposure, but not during habituation (Figure 21), though unexpected at first, can be embedded in a list of studies, pointing towards a role of the entorhinal cortex in active inhibition of negative episodic memory formation (Baldi & Bucherelli 2014). This broader impairment is also in line with higher freezing values in LEC lesions but not selective optogenetic LEC-amygdala manipulations (see below), observed in the trace fear conditioning task, where fear was increased to the conditioning context, novel context and the CS presentation (Figure 22).

4.1.2 LEC-CEL PROJECTION GATES OBJECT-FEAR

c-fos activity mapping and functional experiments suggest a role for adLEC projections, in in object-fear processing. To address this function directly, we activated the direct inputs from the adlLEC to BLA or CeL nuclei of the amygdala. To this end, we expressed ChR2 in the LEC, but activated just the axons that project to a specific region by light delivery via implanted cannulas what enabled high specificity in microcircuit manipulations (Packer et al. 2013).

In our object-fear conditioning task, we found increased object preference upon activation of the LEC to BLA and the LEC to CeL projections (Figure 24). Therefore we propose that the direct pathway from the LEC to the amygdala attributes positive valence to objects via activation of amygdaloid nuclei and drives an approach behavior towards the object. This hypothesis is also in line with neuronal activity seen in the LEC, after MFB activation (Tsao et al. 2013).

Interestingly, the picture changed considerably after we had associated an object with a negative valence (i.e. shock). LEC-CeL activation connection again increased approach behavior, while in LEC-BLA activation group, the positive valence effect reversed. The shift in BLA response upon object-shock association is in line with its role as a top down controller in the amygdala (Duvarci & Pare 2014). It also matches the results Redondo et al. received from experiments where he optogenetically manipulated the dentate gyrus (DG) or the BLA neurons in valence shifting tasks (Redondo et al. 2014). He was able to show that stable DG neuronal populations maintained the capability of changing valence from positive to negative and vice versa. In contrast, BLA neuronal populations, once associated with positive or negative valence, remained with that determination.

Thus, the LEC-CeL pathway seems to encode a **stable positive object valence**, which manifested in a strong approach behavior to a neutral object and a diminished avoidance behavior towards a negative associated object. Surprisingly at first, the valence encoded within the LEC-CeL microcircuit was positive. As a consequence, immediate avoidance responses have to be transmitted somewhere else.

In contrast, the LEC-BLA connection encodes **variable object valence** that was reprogrammed for avoidance behavior (or at least had no positive valence effect anymore). We think that the object-shock association induced a change within the BLA, probably controlled also by inputs from the hippocampus. These changes led to a shift of the BLA output activity, maybe by inhibition of CeL activity and/or by sending negative object associations to other descending structures to elicit an avoidance response. A neutral object does not induce BLA-valence association and therefore will not initiate the formation of a neuronal population engram. Thus the information flow can run consecutively from the LEC to the BLA and to the CeL and induce, while activated, the observed approach response.

After the negative association is encoded, the BLA inhibit or alter CeL activity, in addition probably other areas get activated (Figure 26).

Importantly, these effects were solely seen in the object conditioning task, with no effects on conditioning to sounds (Figure 25). The idea, that the connections investigated in this study are activated during an object specific experience is further supported by experiments done by Pinelopi Pliota in our lab. She was able to show that activation of the LEC to CeL connection on an Elevated Plus Maze (EPM) as well as activation of this connection in one compartment in a Place-Preference assay (PP) had no influence on the behavior of the mice (see Appendix 10 and Appendix 11). These results provide strong evidence that the LEC-CeL pathway activation does not lead to a general release of fear, which would have been seen in more time spent on the open arm of the EPM. As there was also no preference inducible for the chamber the laser was activated in during the PP, the increased time animals spent during laser activation close to an object seems also to be a very object specific effect. Hence it seems that the projections we were looking at provide mainly valence information about visual objects and the relevance connected to them. Why the pure activation of that circuit led to a positive valence towards an object remains a very interesting question. At this point, maybe our NMDA-lesion results can help to resolve the puzzle. It does not seem that no activity in the adlLEC to amygdala circuit leads to a simple negative representation, as NMDA lesioned mice showed no avoidance of the objects per se. But after a negative experience was associated with an object, our results provide evidence that mice impaired within this microcircuit cannot “recalibrate” the system. Fear stayed high and avoidance behavior remained over long time periods (Figure 21). Here we have to stress, that NMDA-lesions were strongest in areas where most LEC-CeL connections had been found. We hypothesize, that the LEC-CeL microcircuit is involved not just in representing but also in shifting the object valence to facilitate proper responses. The NMDA-lesion data as well as our optogenetic results indicate that the LEC to CeL microcircuit gates object related negative valence and avoidance responses and therefore prevents detrimental persistence of negative experiences with objects. This effect is also very interesting regarding several psychological pathologies, as different phobias show an

inordinately high fear response to specific objects. Moreover, these results also suggest that the default reaction to objects is to avoid, and that approach will have to be actively encoded by gating avoidance through the LEC-CeL network.

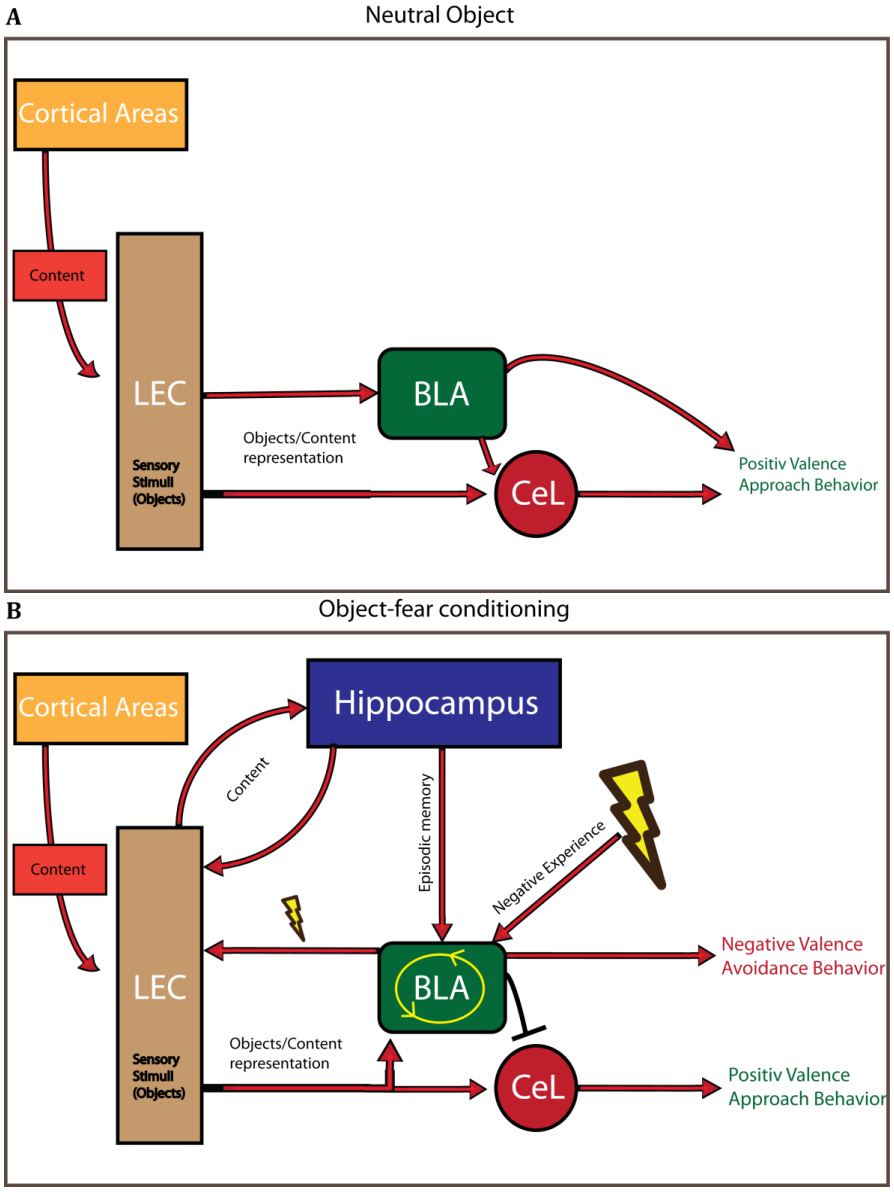


Figure 26. LEC-amygdala microcircuit induces experience dependent valence shift **(A)** Microcircuit when exposed to a neutral object. Content information arises from several cortical areas and facilitates an object representation in the LEC. Upon LEC-amygdala activation in the vicinity of the object, the animals resided more time close to the object. This indicates that a positive valence is experienced during the microcircuit was active. LEC to BLA as well as to CeL showed the same positive, approach directed response. It is not clear, if the BLA just passively transmits neutral object representation to the CeL, or actively targets structures which induce an approach related behavior; **(B)** Microcircuit after the object was associated with a negative valence (i.e. shock). The association will be processed in several brain regions and also lead to the formation of episodic memory in the hippocampus. Additionally neuronal activity will be adjusted in the amygdala to induce fast avoidance behavior when the object is encountered the next time. LEC-CeL connection seems not to be influenced by that modification, as activation led to increased approach behavior. Therefore this microcircuit probably encodes a hardwired positive valence signal to objects, independent of the experience made. In contrast, the LEC-BLA activation was not able to induce approach responses. The valence shift could have been a consequence of a switch in BLA neuronal engram activity due to the shock association. It is possible that BLA inhibit the CeL output and/or project to other structures important for the avoidance response.

4.2 FUTURE DIRECTION AND OUTLOOK

We have shown by optogenetic manipulation that the direct axonal activation from the adlLEC to the BLA or the CeL of the amygdala lead to a prolonged stay close to the object, when the laser was activated in the vicinity of that object. After the object was associated with a shock, only activation of the CeL increased approach behavior maybe via a shift in valence and so a decreased fear to the negative associated object. This effect was not seen for the BLA activation.

With preliminary data that BLA and CeL projecting neurons from LEC are in large parts separate populations, and evidence from other labs that neurons which respond to objects are present in the LEC, it would be very interesting to see if there is an overlap of one or both of these populations with object cells. If no overlap of these populations is seen, but still object specific activity of BLA and/or CeL innervating cells from LEC could still be observed, it would raise the possibility that there are not just object representing cells, but also 'object-valence' cells which provide the emotional significance connected to an object to form an entire episodic memory trace. This information could be further processed in the amygdala and combined with information from other brain areas to induce approach or avoidance behavior. Additionally, there seems to be a hierarchical order within the amygdala, depending on the subnuclei, the LEC is projecting to. As LEC-CeL activation was able to reverse or at least weaken the withdrawal behavior, but LEC-BLA did not. The shift from positive to negative association could probably take place within the BLA or via integration from different inputs. Therefore detailed dissection of the connection on a cellular level has to be performed to understand the exact information content these neuronal pathways send to other structures and to decipher the role these communication plays in formation of episodic memory and emotion.

A very helpful tool in these regards could be the genetically encoded calcium indicator GCaMP combined with an endoscopic microscope. With this system it would be possible to selectively express GCaMP in the LEC and watch the neuronal activity while the animal is performing the object fear conditioning task. This method could also be applied to the amygdala and provide real time information about the cellular activity during the experiment. Together with projection specific markers it would be possible to identify the activity pattern and the specificity of the neuronal populations involved in the task.

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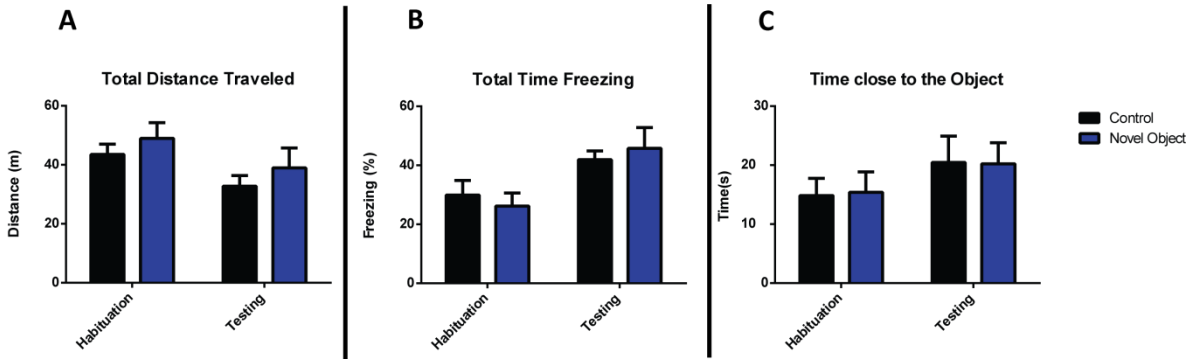
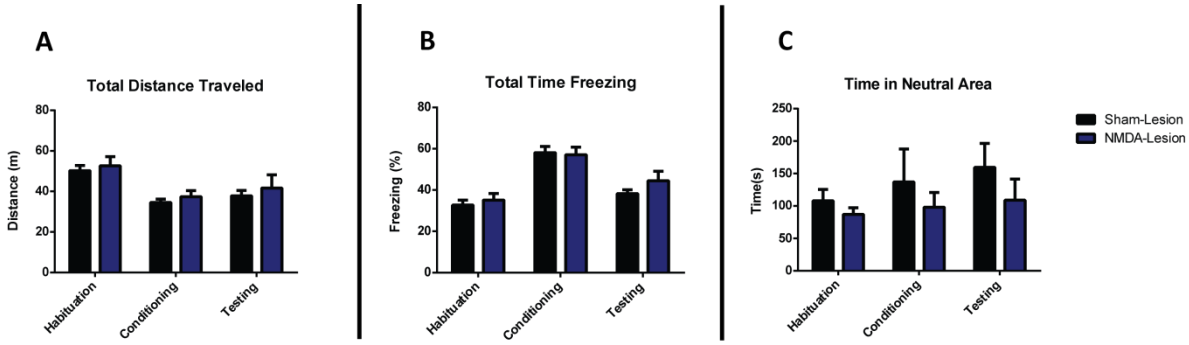
6. APPENDIX

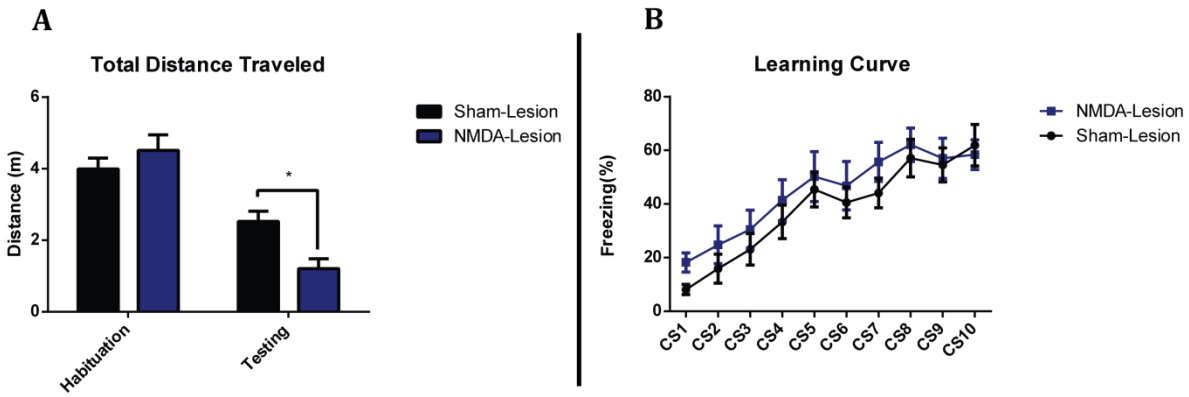
6.1 FIGURES



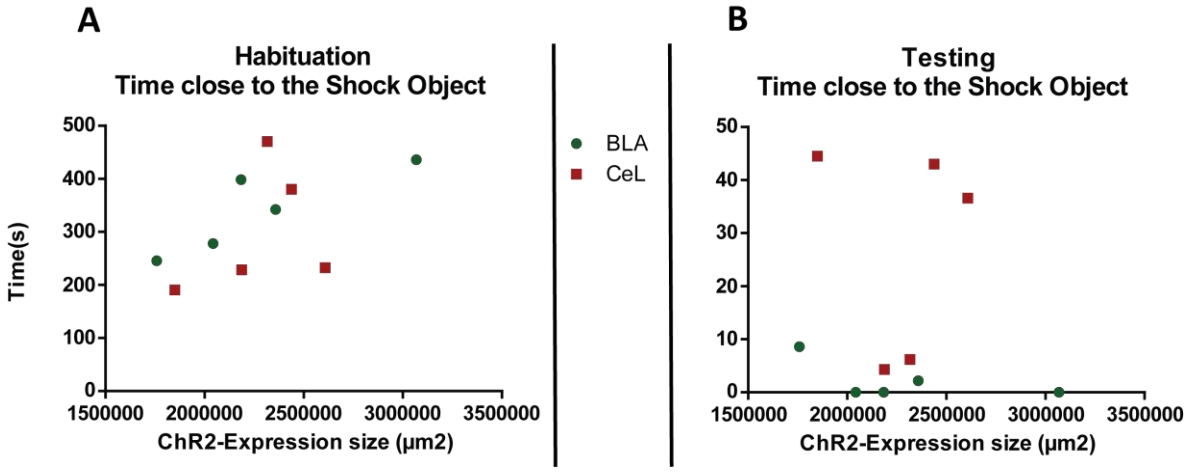
Appendix 1. Novel Object task On the first day (**Habituation**), mice were placed in an open field arena to explore the environment. On the second day (Testing), the control group was put back in the arena. For the Novel Object group, an object was placed in the center of the open field and mice were allowed to explore the object. 90min after the testing session, mice were perfused for further analysis (**Figure 14**); **(A)** Total distance traveled revealed no differences for the groups. The same was true for **(B)**; **(C)** Interestingly, there was also no difference in the time that animals spent close to the object, although no object was placed in the arena for the control group. (Control n=5; Novel Object n=5). *Statistical significance was tested with unpaired t-test and Two-Way ANOVA and Holm-Sidak Multiple Comparisons test. Data plotted as mean ± SEM.*



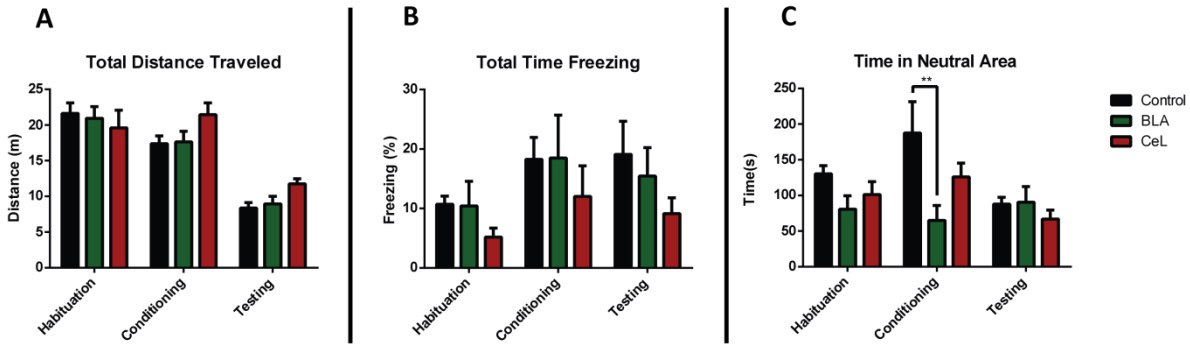
Appendix 2. Object-Fear Conditioning parameters from NMDA-lesion group For explanation of behavioral paradigm see **Figure 21**; No differences were seen in **(A)** Total distance traveled and **(B)** Total time freezing between groups; **(C)** Although not significant, there is a trend that NMDA-lesioned mice spent less time close to the neutral object during Conditioning and Testing. (Sham-Lesion n=9, NMDA-Lesion n=10). *Statistical significance was tested with unpaired t-test and Two-Way ANOVA and Holm-Sidak Multiple Comparisons test. Data plotted as mean ± SEM.*



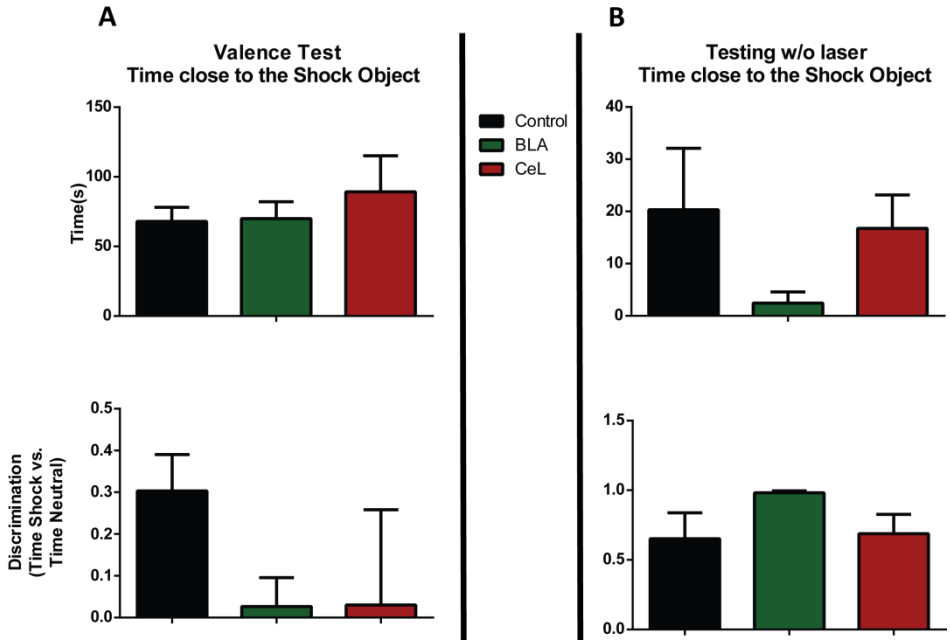
Appendix 3. Trace-Fear Conditioning from NDMA-lesion group For explanation of behavioral paradigm see **Figure 15**; **(A)** During the testing session, NMDA-lesion group traveled less distance compared to the control-group, which is a sign for higher fear; **(B)** The association of the sound with the shock was not impaired in the treatment group, as freezing to the CS (sound) was not different between groups. *Statistical significance was tested with Two-Way ANOVA and Holm-Sidak Multiple Comparisons test. Data plotted as mean ± SEM*



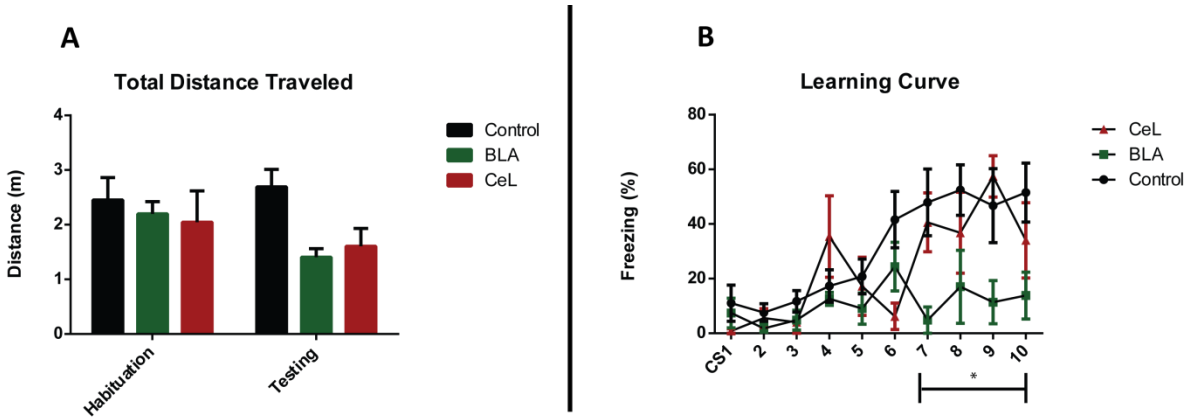
Appendix 4. Correlation of the expression size of ChR2 in the LEC and the time the animals spent close to the shock-object Single animals were plotted; **(A)** During the habituation session, were the laser was activated when animals were in the vicinity of the object (Habituation with laser, see fig.21), there is a trend for the BLA-group indicating that higher ChR2 expression leads to more time close to the object (Pearson r=0.8572, R square= 0.7347, P=0.0634); **(B)** In the testing session no correlation for expression size and time was seen. (BLA n=5, CeL n=5).



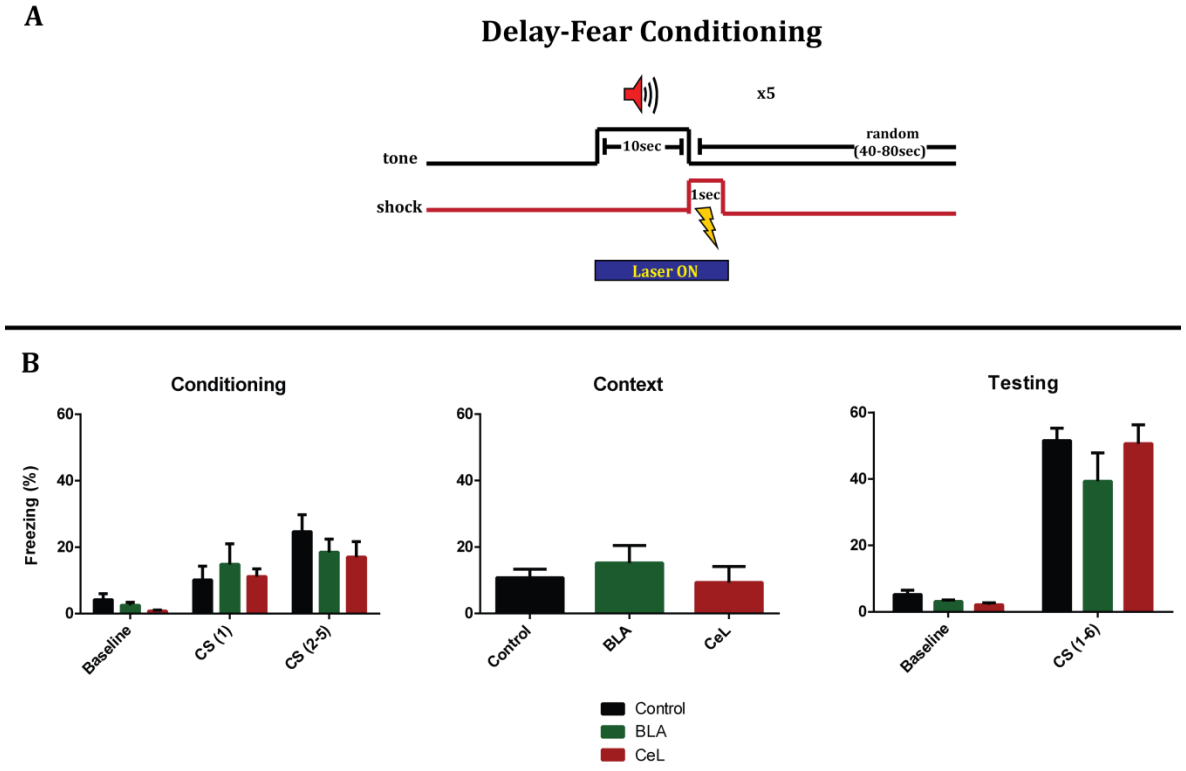
Appendix 5. Object-Fear Conditioning parameters with optogenetic manipulations For explanation see **Figure 24**; **(A)** No differences between groups was seen in the total distance traveled for all groups during all sessions; **(B)** A trend for lower freezing levels is seen for the CeL-group during all sessions; **(C)** During the conditioning session, mice from the BLA-group spent significantly less time close to the neutral object compared to the other groups. *Statistical significance was tested with Two-Way ANOVA and Holm-Sidak Multiple Comparisons test. Data plotted as mean ± SEM.*



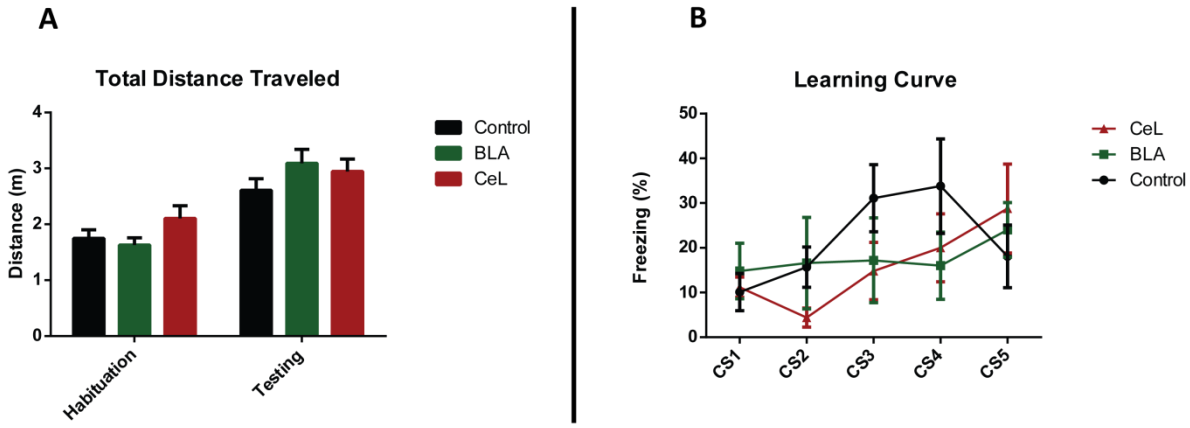
Appendix 6. No differences in the valence test and the testing session without laser indicating expression effect of laser activation For explanation see section 3.4.1; **(A)** The time animals spent close to the shock object was measured in the valence test. This session was performed after the habituation session with laser activation to look for memory effects of LEC-BLA or LEC-CeL projections upon laser-object association. No significant differences were seen; **(B)** After conditioning, the first testing session was without laser activation. Analysis of the time, the animals spent close to the shock object revealed no significant differences for all groups. *Statistical significance was tested with One-Way ANOVA and Holm-Sidak Multiple Comparisons test. Data plotted as mean ± SEM.*



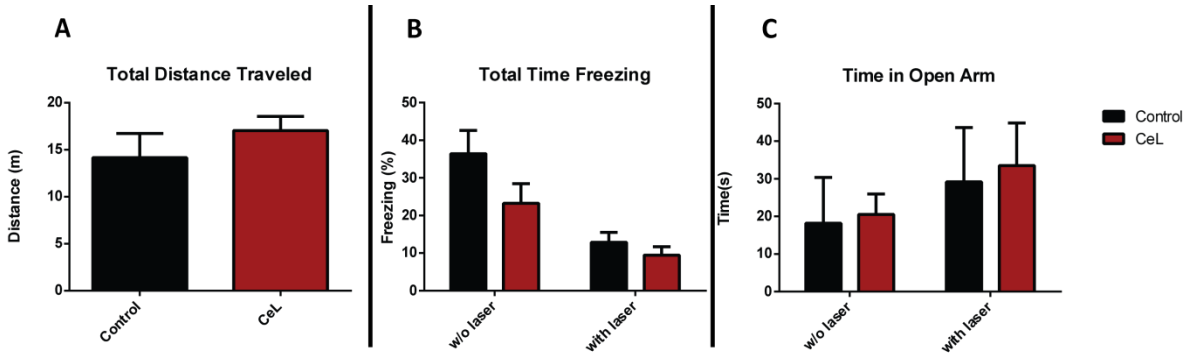
Appendix 7. Trace-Fear Conditioning with optogenetic manipulation For explanation see **Figure 24**; **(A)** Total distance traveled showed no significant differences; **(B)** When looking at the learning curve of the CS (sound) with the US (shock), we saw a significant lower amount of freezing for the BLA group, starting at CS-7. Therefore BLA-group showed hardly any freezing response to the CS presentations during the conditioning phase. During the conditioning session, the laser was activated during the CS presentation, the trace and the US presentation. No effect is seen during the testing session anymore (**Figure 24**). So the effect seems to be immediate and not on memory. *Statistical significance was tested with Two-Way ANOVA and Holm-Sidak Multiple Comparisons test. Data plotted as mean ± SEM.*



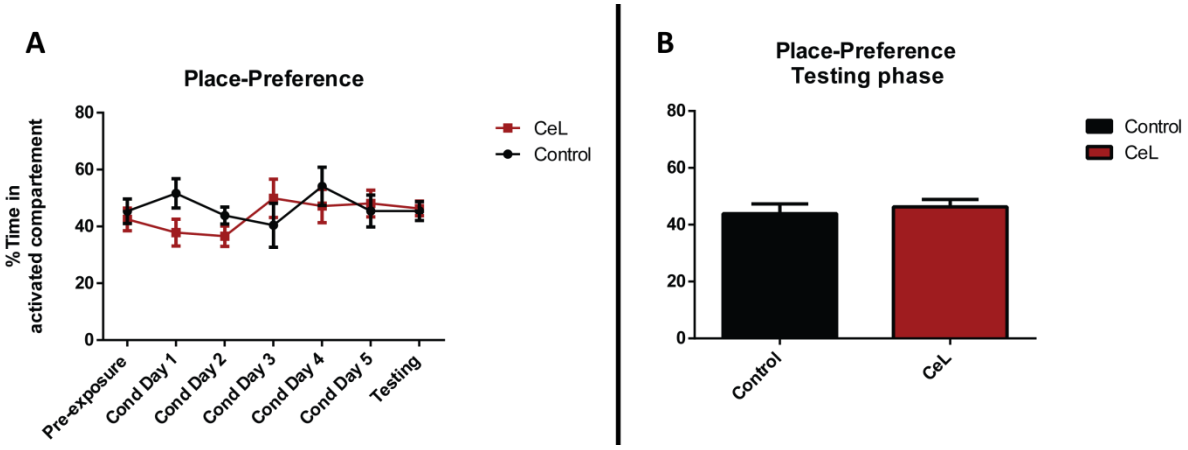
Appendix 8. Delay- fear conditioning with optogenetic activation **(A)** Delay-fear conditioning protocol, no trace separates the tone and shock presentations. Blue bar indicates laser activation during tone and shock presentation during conditioning; **(B)** Freezing values during **(Left):** Conditioning, **(Middle):** Context reexposure and **(Right):** Testing session of the trace-fear conditioning task. No significant differences are seen. *Statistical significance was tested with One-Way or Two-Way ANOVA and Holm-Sidak Multiple Comparisons test. Data plotted as mean ± SEM.*



Appendix 9. Delay-Fear Conditioning with optogenetic manipulation is not changed For explanation see **Appendix 8**; No differences seen for **(A)** Total distance traveled or **(B)** During learning in the conditioning session. *Statistical significance was tested with Two-Way ANOVA and Holm-Sidak Multiple Comparisons test. Data plotted as mean ± SEM.*



Appendix 10. Optogenetic activation of LEC-CeL connection does not change basic anxiety levels Animal performance on the EPM is a well established paradigm for investigating general anxiety. A plus shaped apparatus with two closed and two open arms is used. Additionally the apparatus is elevated around 50cm from the floor. Animals will prevent the open arms and hide within the closed compartments. This behavior would increase when anxiety level is high, and decrease when it's low. During this specific experiment, the connection from LEC to CeL was repetitively activated for 2.5min. Plotted are the sums of laser Off and laser On phases; **(A and B)** No differences are seen for the total distance traveled and the total time freezing during the session, respectively; **(C)** Also the time the mice spent in the open arms is not different for both groups. Experiments performed by Pinelopi Pliota. (Control n=7, CeL n=6). *Statistical significance was tested with One-Way or Two-Way ANOVA and Holm-Sidak Multiple Comparisons test. Data plotted as mean ± SEM.*



Appendix 11. LEC-CeL activation does not influence general preference for one compartment For the Place-Preference assay, an apparatus with two dissociable compartments is used. The compartments are connected through a corridor in the center of the apparatus. LEC to CeL connection was always activated when the animals were in one of the compartments. A preference for that compartment would indicate a positive and/or rewarding association; **(A)** Percentage of time, animals spent in the compartment where the laser was activated is shown over all days. In the Pre-exposure session, mice were placed in the apparatus without laser activation. During Cond Day1-5, the laser was activated in one compartment. During the Testing phase, no laser was applied and the animals were allowed to explore both compartments. No significant difference is seen during all sessions; **(B)** Testing session alone. Similar values for the time animals spent in the compartment with laser activation for both groups are observed. Experiments performed by Pinelopi Pliota. (Control n=7, CeL n=6). *Statistical significance was tested with unpaired t-test and Two-Way ANOVA and Holm-Sidak Multiple Comparisons test. Data plotted as mean ± SEM.*

6.2 ABSTRACT

Emotions are the main drivers for reacting to, and recall of, significant events. They are crucially needed to promote survival and prevent death. Fear is probably the most basic emotion and, in turn, can be addressed easily in experimentally tractable models. Most studies used Pavlovian fear conditioning as a model. However, although these findings established a profound knowledge of brain mechanisms and structures important for emotional processing, they could not address questions about spatial components of fear reactions: normally the first response to a threat is to run away and avoid it. Therefore systems in the brain are needed to trigger this avoidance reaction and to encode their spatial components. As a consequence the brain must represent the environment, the stimulus (i.e. an object) and the spatial relationship of the stimulus with oneself (i.e. the distance) to create an appropriate response.

One brain area which is probably involved in these tasks is the entorhinal cortex (EC). The (EC) is located in the temporal lobe and is partially enclosed by the rhinal (olfactory) sulcus. It is strongly connected with cortical as well as subcortical parts of the brain. Because of its pronounced connections to the hippocampus, the EC represents a nodal point for the communication of cortical areas with the hippocampus. Through the combination of different methods, the EC was divided into a medial entorhinal cortex (MEC) and a lateral entorhinal cortex (LEC). In the early 2000s, the EC received major interest after it was shown that cells projecting to the hippocampus exhibit sharply tuned spatial firing. The firing fields of each neuron formed a hexagonal grid, which covered the entire environment. Therefore these cells were named grid cells. Later on, Deshmukh & Knierim observed neuronal activity in the LEC, when discrete objects were present. Some cells displayed firing activity in the vicinity of these objects and others on a specific distance to them. Together with these so called object cells, in a constitutive study Tsao et al. found neurons in the LEC which were active in an area, where an object had been the trial before, named object-trace cells. These object-trace cells therefore displayed the memory of an object position. Importantly, the EC has strong connections to the amygdala - a brain structure embedded in the limbic system and central hub for fear processing.

Based on these findings, we hypothesized that interactions between LEC and amygdala might encode spatial avoidance responses to emotionally relevant objects. To explore this

possibility, we focused on the direct projections from the LEC to the basolateral amygdala (BLA) because of its role as a main entry point for sensory information and the lateral part of the central amygdala (CeL) as one of the main output structures under direct control of the BLA. First, we used neuronal tracers to assess the connectivity pattern and strength from the LEC to the BLA and the CeL and confirmed the anterior part of the dorsolateral LEC (adlLEC) as the major lateral entorhinal input to BLA/CeL. We then established a new object-fear conditioning task, where a 3D object could be associated with a shock to induce fear and avoidance from the object. We compared neuronal activity in the LEC and the amygdaloid subnuclei after mice performed a novel object task or after they were reexposed to the negatively valenced (i.e. shocked) object. The experiment revealed significantly stronger activation of the LEC and the CeL on the reexposure to the threatening object. To further dissect the functional role of the LEC-BLA and LEC-CeL connections, we used a combination of excitotoxic lesions and projection specific optogenetic manipulations. When we activated those connections in the vicinity of a neutral object, we observed a preference for that object. After the very same object was associated with a shock, LEC-BLA activation had no influence on the reaction of the animal. The LEC-CeL activation resulted in a strong attenuation of the avoidance response, while LEC lesions induced the converse. Importantly, the LEC-amygdala network is specific for object-shock association, as these manipulations had no effect on tone/foot shock conditioning. Taken together, this study provides first evidence, that LEC-amygdala microcircuits are involved in the processes that link objects to emotions, predominately through LEC-CeL connections. Moreover, we find that this connection operates through gating a default avoidance response that is expressed upon exposure to a negatively valenced object.

6.3 ZUSAMMENFASSUNG

Emotionen sind essentielle Faktoren um auf wichtige Ereignisse reagieren zu können und sich diese auch einzuprägen. Sie fördern das Überleben und verhindern den Tod. Furcht ist wahrscheinlich eine der ältesten und grundsätzlichsten Emotionen und ermöglicht daher auch die Erforschung an Modellorganismen. Die meisten Studien verwendeten Pawlow‘sche Furcht Konditionierung um die Grundmechanismen des Furcht-Lernens im Gehirn zu verstehen. Obwohl diese Experimente eine Vielzahl an wichtigen Erkenntnissen über die Prozesse im Hirn hervorbrachten, war es nie möglich räumliche Komponenten der Furchtantwort zu behandeln. Befindet man sich in einer gefährlichen Situation, ist die erste Reaktion typischerweise Flucht. Dies bedeutet, dass gewisse Systeme im Gehirn diese Reaktion einleiten müssen. Dies erfordert eine Repräsentation von der Umgebung, dem Objekt von dem Gefahr ausgeht und die räumliche Beziehung in der man mit dem Objekt steht. Die zuletzt genannte kann auch als die Distanz zum Objekt gesehen werden.

Eine Hirnstruktur die wahrscheinlich eine wichtige Rolle für die Bewältigung dieser Aufgaben spielt ist der Entorhinale Kortex (EC). Diese Struktur befindet sich im Temporallappen und ist teilweise von der rhinalen Furche umgeben. Der EC ist sowohl mit dem Kortex, als auch mit subkortikalen Regionen stark verbunden. Durch seine ebenfalls intensiven Verbindungen mit dem Hippocampus wird der EC als ein Kontenpunkt für die Kommunikation zwischen kortikalen und hippocampalen Strukturen gesehen. Durch eine Vielzahl an Experimenten wurde eine funktionelle Teilung des EC in einen medialen entorhinalen Kortex (MEC) und einen lateralen entorhinalen Kortex (LEC) vorgeschlagen.

Großes Interesse erlangte der EC in den frühen 2000er Jahren, als Nervenzellen gefunden wurden, die genau definirte räumliche Aktivität aufwiesen. Das Aktivitätsmuster jeder dieser Neuronen formte ein hexagonales Gitter welches den gesamten vorhanden Raum ausfüllte. Aus diesem Grund wurden die Zellen auch Gitter Zellen (Grid cells) genannt. In einer späteren Studie konnten Deshmukh & Knierim neuronale Aktivität im LEC feststellen, die jedoch an das Vorhandensein von Objekten im Raum gebunden war. Einige Nervenzellen waren nur in der unmittelbaren Nähe eines Objekts aktiv, andere nur in einer bestimmten Distanz dazu. Diese Zellen wurden als Objekt Zellen (Object cells) bezeichnet. Tsao et al. fand wiederum eine zusätzliche Population an Zellen im LEC. Diese Zellen waren jedoch nur an einem Ort aktiv, wo ein Objekt in einem vorangegangenen Versuch positioniert war.

Diese Neuronen bekamen den Namen: Objekt-Spur Zellen (Object-trace cells), da sie die Erinnerungsspur eines Objekts darstellten. Ein weiteres wichtiges Merkmal des EC ist außerdem seine starke Verbindung zur Amygdala, eine Hirnstruktur die sich im limbischen System befindet und sehr wichtig für die Verarbeitung von Angst/Furcht Prozessen ist.
Aufgrund dieser Erkenntnisse vermuten wir, dass die Kommunikation zwischen dem LEC und der Amygdala eine wichtige Rolle in der Verarbeitung von Flucht Reaktionen in Verbindung mit emotionell relevanten Objekten spielt. Um diese Vermutung zu untermauern haben wir uns auf die direkten Verbindungen des LEC mit der basolateralen Amygdala (BLA) und dem lateralen Teil der zentralen Amygdala (CeL) fokussiert. Die BLA wurde aufgrund ihrer wichtigen Funktion als primäre Eintrittsstelle für viele sensorische Informationen ausgewählt. Die CeL wiederum ist eine zentrale Stelle für die Weitergabe der Information zu anderen Hirnstrukturen und wird direkt von der BLA kontrolliert. Als erstes nutzten wir neuronale Tracer um das Verbindungsmuster und die Verbindungsstärke des LEC zur CeL und BLA zu erkennen. Dabei konnten wir bestätigen, dass der anteriore Anteil des LEC (adlLEC) die vorrangige LEC Verbindung zur BLA/CeL darstellt. Als nächstes etablierten wir eine neue Methode um ein plastisches (3D) Objekt mit einem Schock zu assoziieren. Dies führte zu Furcht vor dem Objekt und einer Vermeidungsreaktion. Die Methode wurde Objekt-Furcht Konditionierung genannt. Mit bestimmten Markern konnten wir nun die neuronale Aktivität im LEC und in den amygdaloiden Untereinheiten testen. Wir verglichen die Aktivität nachdem Mäuse ein neues Objekt erkunden durften, oder sie zum wiederholten Male mit einem negativ valenzierten (also geschockten) Objekt konfrontiert wurden. Dieses Experiment zeigte eine signifikante Erhöhung der neuronalen Aktivität, nachdem die Tiere wieder mit dem negativ assoziierten Objekt konfrontiert wurden, in den Strukturen des LEC und CeL. Um noch mehr über die Funktion der LEC-BLA und LEC-CeL Verbindung zu erfahren nutzten wir eine Kombination aus exzitotoxischen Läsionen und optogenetischer Manipulation. Durch die direkte optogenetische Aktivierung der Verbindungen in der Nähe eines neutralen Objektes, induzierten wir eine Vorliebe für dieses Objekt. Nachdem dieses Objekt jedoch mit einem Schock assoziiert wurde, hatte die LEC-BLA Aktivierung keinen Einfluss mehr auf das Verhalten der Mäuse. Im Gegensatz dazu, führte die Aktivierung der LEC-CeL Verbindung jedoch zu einer starken Abnahme des Vermeidungsverhaltens dieser Tiere, während die Läsionen des LEC genau das Gegenteil

induzierte. Hierbei muss noch erwähnt werden, dass diese Effekte sehr Objekt spezifisch zu sein scheinen, da die Effekte in Ton/Fußschock Konditionierungen nicht auftraten.

Zusammengefasst zeigt unsere Studie, dass der LEC-Amygdala Schaltkreis in den Prozessen die Objekte mit Emotionen verbinden involviert zu sein scheint. Dies dürfte vor allem über die LEC-CeL Verbindung passieren. Weiteres konnten wir herausarbeiten, dass diese Verbindung über die Steuerung einer vorgegebenen Vermeidungs-Reaktion läuft, die aktiviert wird wenn man einem negativ valenzierten Objekt ausgesetzt ist.

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