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Neuronal correlates of sustained fear in the anterolateral part of the bed nucleus of stria terminalis

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ABSTRACT

As part of the extended amygdala network, the bed nucleus of the stria terminalis (BNST) was shown to be critically involved in processing sustained fear responses to diffuse and unpredictable threats. However, neuronal activity patterns in relation to sustained components of the fear response remain elusive, so far. We used a fear training paradigm with unpredictable pairing of conditioned and unconditioned stimuli allowing distinction between phasic and sustained components of conditioned fear, and recorded single units in the anterolateral part of the BNST (BNSTal) in freely behaving mice. An objective, non-biased cluster-analysis was performed for each identified single unit on specific waveform-, activity-, stimulus-dependent and LFP-related parameters. The analysis revealed three distinct neuronal subpopulations of *biphasic*-, *sustained fear on*- and *fear off*-neurons. Results show that activities of *biphasic*- and *sustained fear on*-neurons temporally coincide with the shift from phasic to sustained components of the fear response. Presentation of non-conditioned auditory stimuli resulted in a variety of neuronal responses in BNSTal with no indication of biphasic response profiles. It is suggested that fear conditioning sharpens neuronal response profiles in BNSTal with *biphasic*-cells signaling phasic and sustained fear. These results confirm the pivotal role of BNST in processing sustained fear on the neuronal level, thereby complementing pharmacological experimental animal and human imaging data.

1. Introduction

Fear and anxiety constitute important mechanisms to cope with harmful situations. Despite many similarities, fear and anxiety possess conspicuous differences concerning behavioral expressions and underlying neuronal mechanisms (Davis, Walker, Miles, & Grillon, 2010; Tovote, Fadok, & Luthi, 2015; Walker, Toufexis, & Davis, 2003). As parts of the extended amygdala network, the central amygdala (CeA) and the bed nucleus of stria terminalis (BNST) are considered crucial elements for mediating these distinct components of fear responses (Fox, Oler, Tromp, Fudge, & Kalin, 2015; Walker & Davis, 2008). Previous studies on fear and anxiety demonstrated selective, yet related functions for CeA and BNST in response to threatening stimuli. Cue-conditioning studies in rodents revealed that CeA is critically involved in mediating fear responses to short discrete cues, while BNST is not (Campeau & Davis, 1995; Walker & Davis, 1997). By contrast, BNST lesions were shown to alter conditioned fear to contexts or long and unpredictable cues, but not short, discrete cues (Davis et al., 2010; Sullivan et al., 2004). Furthermore, BNST lesions selectively decreased light-enhanced startle, as well as startle enhancing effects of the anxiogenic peptide corticotropin releasing factor (CRF), while both effects were disrupted by administration of anxiolytic agents in respective paradigms conducted on rodents (Davis, Walker, & Lee, 1997a; Walker & Davis, 1997), suggesting unconditioned fear responses to be also based on BNST activity. Thus, available data suggest the existence of two related but dissociable threat-response systems with CeA mediating rapid stimulus-specific responses to imminent threats and BNST generating long-lasting responses to distal threat (Walker et al., 2003). Therefore, it is assumed that CeA is mainly involved in acute fear processes, while BNST seems to be specifically related to prolonged states of apprehension resembling anxiety (Davis et al., 2010). However, studies using pharmacological (Burghardt & Bauer, 2013; Ravinder, Burghardt, Brodsky, Bauer, & Chattarji, 2013) and viral manipulations (Sink, Walker, et al., 2013) or lesions (Duvarci, Bauer, & Pare, 2009) in BNST were shown to affect cued fear mechanisms. Other studies demonstrated CeA microcircuits

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to be also involved in the generation of anxiety-like behavior (Ciocchi et al., 2010; Haubensak et al., 2010; Tye et al., 2011).

While fear and anxiety thus seem to involve neuronal circuits which share similar input and ultimate output stations (Davis, Walker, & Lee, 1997b; Davis et al., 1997a), the neurophysiological basis of the two dissociable response systems remains elusive, so far. One study aiming at the neuronal correlates underlying these distinct systems used auditory and contextual fear conditioning in combination with electrophysiological recordings in the anterolateral (BNSTal) and anteromedial BNST (BNSTam) (Haufler, Nagy, & Pare, 2013). After auditory fear conditioning, mainly inhibitory conditioned stimulus (CS) responses were found in a subpopulation of BNSTal, while positive CS responses were found in cells of BNSTam. However, such opposing effects were found in rather small neuronal populations (~20%). Activity changes of BNSTal and BNSTam during retrieval of contextual fear paralleled results from cue-conditioning. Thus, these findings suggest regional differences in relation to learned fear in distinct subnuclei of BNST which is also shown for anxiety. Two recent studies demonstrated the existence of functional subregions in BNST, which modulate different dimensions of anxiety via efferents to lateral hypothalamus (LH), parabrachial nucleus (PB) and ventral tegmental area (VTA) (Jennings et al., 2013; Kim et al., 2013). For instance, the anteroventral BNST (BNSTav)-VTA-pathway has been shown to bidirectionally regulate anxious states and respective changes in reward-seeking behavior (Jennings et al., 2013). Additionally, two distinct BNST subregions have been shown to exert contrasting effects on anxious states. While activation of the oval nucleus (BNSTov) promoted anxiety, recruitment of cells in the anterodorsal group of BNST (BNSTad), reduced anxiety via excitatory projections from basolateral amygdala (BLA) (Kim et al., 2013).

Conditioning paradigms using short, discrete cues fail to evoke anxiety-like states. On the other hand, tests for innate or learned anxiety provide poor control over onset and termination of applied stimuli, and therefore might fail to detect immediate, acute fear-like responses to distal threats, as well as potential transition phases between acute fear and anxiety-like states. To reveal distinct neural circuits of fear and anxiety and their potential interactions in one paradigm and to improve control over stimulus applications, Walker and Davis (2008) developed the sustained fear paradigm which constitutes a modulated form of context-conditioning in rats. Acoustic clicker stimuli of variable duration are paired with footshocks in a pseudo-randomized fashion, to impose unpredictability on upcoming aversive events and thereby creating a threatening context during prolonged CS presentation (8 min) in subsequent testing sessions. During testing, animals showed highest startle responses during the first minute of CS-presentation, probably reflecting a strong initial acute fear-like component. Davis et al. termed this initial component "phasic fear". Phasic fear was followed by a later phase of long-lasting anxious apprehension which they called "sustained fear" (Davis et al., 2010). Subsequent studies have shown that phasic and sustained components of fear are pharmacologically dissociable (Miles, Davis, & Walker, 2011) and involve CeA and BNST as parts of the two interrelated response-systems within the extended amygdala (Walker, Miles, & Davis, 2009; Walker, Yang, et al., 2009; Walker et al., 2003). In particular, the interplay between the lateral division of the CeA (CeAl) and its CRF-containing projections to the BNSTal has been proposed to be a critical element for sustained fear responses (Walker & Davis, 2008). As the sustained fear paradigm resembles respective protocols from human studies (Grillon, Baas, Lissek, Smith, & Milstein, 2004) it constitutes a unique approach for translational research on fear and anxiety.

To better assess underlying genetic, molecular and neuronal mechanisms of fear and anxiety in genetically modified animals and to make use of the immense potential for developing specific intervention strategies, we recently adapted the sustained fear paradigm for application in freely moving mice (Daldrup et al., 2015; Seidenbecher, Remmes, Daldrup, Lesting, & Pape, 2016). Here, we used a training paradigm with unpredictable CS–US pairings, and a 6 min CS-presentation with superimposed startle inducing bursts during retrieval and combined this sustained fear paradigm with extracellular local field potential (LFP) and single unit recordings in the BNSTal in freely behaving mice to identify the neuronal correlates of phasic and sustained fear behavior.

2. Material and methods

2.1. Animals

All experiments were performed in accordance to the European Communities Council directive (86/609/EEC), with the regulations of German law and as approved by the local animal care committee of LANUV NRW (AZ 84-02.04.2012.A206). Animals were kept in a 12 h light/dark cycle provided with food and water ad libitum. Experiments were conducted with 9–12 weeks old male C57BL/6J mice (n = 22, M&B Taconic, Berlin, Germany).

2.2. Electrode implantation

Micro-wire arrays (MWA, 1 array, 8 electrodes and one reference/array per brain region; Stablohm 650; California Fine Wire) were implanted under stereotaxic control (David Kopf Instruments). The tip of each wire was gold-plated by passing a cathodal current of 1 mA while wires were submerged in a gold solution to reduce the impedance to a range of 150–300 kΩ. Under deep pentobarbital anesthesia (50 mg/kg i.p.), supplemented by subcutaneous injection of Carprofen (Rimadyl; 5 mg/kg), electrodes were implanted in the BNSTal, following the parcellation scheme by Ju and Swanson (Ju & Swanson, 1989), of the left hemisphere at the following stereotactical coordinates (Franklin & Paxinos, 1997): +0.15 mm AP; 0.9 mm ML; 3.8 mm DV from bregma. Electrodes were fixed with dental cement. Experiments involved a ground electrode, positioned close to the midline over the cerebellar region (5.8/0.5 mm from bregma) of the right hemisphere. At the end of the experiments animals were killed by an overdose of pentobarbital (100 mg/kg, i.p.), location of recording sites were marked by small electrolytic lesions (1 mA anodal current for 10 s), and brains were rapidly removed and fixed in 4% phosphate-buffered formaldehyde (pH 7.4). Electrode positions were identified in 50 μM cresyl violet counterstained frontal brain sections (Fig. 1A).

2.3. Behavioral paradigm

After 7–10 days of surgical recovery, one group of animals (n = 17) underwent a fear conditioning paradigm (Fig. 1B) (Daldrup et al., 2015). In brief, 36 startle-eliciting white noise bursts (50 ms duration, inter-burst interval: 30 s) were presented to mice in an adaptation session (day 1) in context A. Fear conditioning (day 2) was performed in a standardized fear conditioning chamber (Fear Conditioning System, TSE, Bad Homburg, Germany, context B). Mice were presented four 10 kHz tones (conditioned stimulus CS; 75 dB, pseudo-randomized presentation with variable duration of 29, 9, 19 and 14 s, ISI 30 s) each co-terminated with a 1 s-footshock (unconditioned stimulus US; scrambled, 0.4 mA). Conditioning was repeated in a second session 6 h later. 24 h after fear conditioning (day 3), animals were tested for fear memory retrieval. Under Forene anesthesia (isofluran, 1-chloro-2,2,2 tri fluoroethyldifluoromethylether) animals were connected to a swivel commutator of the recording device. Experiments in context A