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Prefrontal NMDA receptors expressed in excitatory neurons control fear discrimination and fear extinction

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ABSTRACT

N-methyl-D-aspartate receptors (NMDARs) are critically involved in various learning mechanisms including modulation of fear memory, brain development and brain disorders. While NMDARs mediate opposite effects on medial prefrontal cortex (mPFC) interneurons and excitatory neurons, NMDAR antagonists trigger profound cortical activation. The objectives of the present study were to determine the involvement of NMDARs expressed specifically in excitatory neurons in mPFC-dependent adaptive behaviors such as fear discrimination and fear extinction. To achieve this, we tested mice with locally deleted *Grin1* gene encoding the obligatory NR1 subunit of the NMDAR from prefrontal CamKIIα positive neurons for their ability to distinguish frequency modulated (FM) tones in fear discrimination test. We demonstrated that NMDAR-dependent signaling in the mPFC is critical for effective fear discrimination following initial generalization of conditioned fear. While mice with deficient NMDARs in prefrontal excitatory neurons maintain normal responses to a dangerous fear-conditioned stimulus, they exhibit abnormal generalization decrement. These studies provide evidence that NMDAR-dependent neural signaling in the mPFC is a component of neural mechanism for disambiguating the meaning of fear signals and supports discriminative fear learning by retaining proper gating information, *viz.* both dangerous and harmless cues. We also found that selective deletion of NMDAR from excitatory neurons in the mPFC leads to a deficit in fear extinction of auditory conditioned stimulus. These studies suggest that prefrontal NMDARs expressed in excitatory neurons are involved in adaptive behavior.

1. Introduction

Normal brain functioning relies critically on the ability to keep fear memories distinct and resistant to confusion. Fear behavior is controlled by adaptive processes including discrimination, generalization and extinction, which are likely regulated by separate neural mechanisms. While fear memory accuracy is critical for survival and balanced fear generalization allows avoidance of dangerous situations, circuit and molecular level mechanisms for fear discrimination remain unclear. Multiple memory systems theory postulates that different types of memory are consolidated via hardwired pathways (Squire, 1992). In tone fear conditioning, tone [conditional stimulus (CS)]-foot shock [unconditional stimulus (US)] associations are directly encoded through synaptic plasticity in the amygdala, which receives direct auditory inputs (Medina, Repa, Mauk, & LeDoux, 2002). During contextual fear conditioning, the contextual stimulus (CS) is encoded by the dorsal hippocampus whose outputs are subsequently associated with the US through synaptic plasticity in the amygdala (Kim & Fanselow, 1992; Maren & Fanselow, 1995), and later consolidated by the hippocampal–prefrontal circuitry (Frankland & Bontempi, 2005; Frankland, Bontempi, Talton, Kaczmarek, & Silva, 2004; Quinn, Ma, Tinsley, Koch, & Fanselow, 2008; Tse et al., 2011; Zelikowsky et al., 2013). In fact, the medial prefrontal cortex (mPFC) can compensate for absence of dorsal hippocampus in contextual fear learning (Zelikowsky et al., 2013). In addition, fear behavior is differentially regulated by infralimbic (IL) and prelimbic (PL) subregions of the

Abbreviations: ANOVA, analysis of variance; BLA, basolateral amygdala; CREB, cAMP response element binding protein; HAT, histone acetyltransferase; IL, infralimbic cortex; mPFC, medial prefrontal cortex; NMDAR, N-methyl-D-aspartate receptors; PL, prelimbic cortex; repeated measures-ANOVA, RM-ANOVA.

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mPFC (Courtin, Bienvenu, Einarsson, & Herry, 2013; Quirk & Mueller, 2008; Sierra-Mercado, Padilla-Coreano, & Quirk, 2010; Sotres-Bayon, Cain, & LeDoux, 2006) via fear excitation and inhibition, respectively (Sierra-Mercado et al., 2010; Sotres-Bayon & Quirk, 2010), which may be due to differential connectivity with the amygdala (Gabbott, Warner, Jays, Salway, & Busby, 2005; Vertes, 2004). For example, differential conditioning increases unit and field responses within the amygdala to the conditioned stimulus, paired with US (CS+), whereas responses to the second stimulus that was never paired with US (CS−) decreased (Collins & Pare, 2000).

Studies show that mPFC lesions enhance generalization. In absence of IL mPFC, rats become more fearful of a novel environment after fear conditioning (Zelikowsky et al., 2013). In addition, lesions of mPFC disrupts discrimination of more discrete multiple odor stimuli (DeVito, Lykken, Kanter, & Eichenbaum, 2010). Furthermore, inactivation of pathways (in either direction) between mPFC and nucleus reuniens of thalamus (NR) enhances fear memory generalization (Xu & Sudhof, 2013; Xu et al., 2012). We have recently demonstrated that prefrontal hypofunction of transcription regulators implicated in the mechanism underlying long-term memory consolidation results in abnormal generalization decrement during contextual and auditory fear discrimination learning in mice (Vieira et al., 2014). These data indicate that the prefrontal circuit might be involved in fear discrimination between the conditioned stimulus CS+ (reinforced with a foot shock) and CS− (nonreinforced).

There is a strong evidence for prefrontal N-methyl-D-aspartate receptors (NMDARs) in mechanism underlying extinction of conditioned fear (Burgos-Robles, Vidal-Gonzalez, Santini, & Quirk, 2007; Santini, Muller, & Quirk, 2001). While fear extinction is widely considered as a new learning event rather then forgetting (Maren & Quirk, 2004), it is postulated that fear extinction involves inhibition of an existing response (Bouton & Nelson, 1994). In agreement with the data showing that lesions in the mPFC produce deficit in extinction of conditioned fear (Gewirtz, Falls, & Davis, 1997; Morgan, Romanski, & LeDoux, 1993; Orsini, Kim, Knapska, & Maren, 2011; Orsini & Maren, 2012; Quirk, Russo, Barron, & Lebron, 2000), consolidation of fear extinction memory recruits mechanisms controlled by NMDARs, mitogen-activated protein kinase and protein synthesis (Quirk & Mueller, 2008; Sotres-Bayon, Diaz-Mataix, Bush, & LeDoux, 2009). Involvement of NMDAR in mPFC-dependent learning mechanism is supported by the studies showing that NMDAR receptors are effective mediators of synaptic plasticity in prefrontal excitatory neurons [e.g. (Hirsch & Crepel, 1991)]. However, NMDARs in the mPFC mediate opposite effects on interneurons and excitatory neurons (Homayoun & Moghaddam, 2007). Pharmacological blockers of NMDAR trigger profound cortical activation in behaving rodents (Jackson, Homayoun, & Moghaddam, 2004) and human volunteers (Breier et al., 1997; Lahti, Holcomb, Medoff, & Tamminga, 1995; Suzuki, Jodo, Takeuchi, Niwa, & Kayama, 2002; Vollenweider, Leenders, Oye, Hell, & Angst, 1997) suggesting that the effect of NMDAR antagonists in pharmacological studies is predominately targeted to inhibitory neurons producing disinhibition of excitatory network. The objectives of the present study were to determine involvement of NMDARs expressed specifically in CamKIIα positive excitatory neurons in mPFC-dependent adaptive behaviors such as in fear discrimination and fear extinction.

Based on the studies discussed above, discrimination between dangerous, fear-conditioned CS+ and nonreinfoced CS− auditory cues likely involves mPFC functional interactions. Still unknown are the neural mechanisms underlying the attainment of fear memory accuracy for appropriate discriminative responses to CS+ and CS− stimuli. To explore the potential impact of prefrontal NMDARs on fear discrimination, we generated mutant mice with locally deleted obligatory subunit of the NMDAR in prefrontal excitatory CamKIIα positive neurons and examined their capability to distinguish between dangerous, fear-conditioned stimulus and nonreinforced stimulus in fear discrimination procedure. For behavioral evaluations, we used an auditory fear discrimination task that depends on the ability to distinguish discrete auditory cues constructed of frequency modulated (FM) upward or downward tone sweeps. This auditory fear discrimination task indicated that NMDAR-dependent neural signaling within mPFC circuitry is an important component of the mechanism for disambiguating the meaning of fear signals. We have also demonstrated that NMDAR inactivation in the prefrontal excitatory neurons impairs fear extinction.

2. Materials and methods

2.1. Subjects

The UC Riverside Institutional Animal Care and Use Committee approved all procedures in accordance with the NIH guidelines for the care and use of laboratory animals. We used C57BL/6J mice for all experiments. Mice were weaned at postnatal day 21, housed 4 animals to a cage with same sex littermates with ad libitum access to food and water and maintained on a 12 h light/dark cycle. Old bedding was exchanged for fresh autoclaved bedding every week.

2.2. Surgery

We used the same rescue surgery protocol as described previously (Vieira et al., 2014) Briefly, 2–3-month-old mice were separated into individual cages prior to surgery. Anesthesia was induced by placing individual mice in chamber filled with isoflurane. After induction, anesthesia was maintained by mounting the mouse in a heated stereotaxic apparatus and supplying a constant flow of isoflurane/oxygen mix. After adjusting the ear bars, bite bar, and nose clamp, the scalp was shaved, sanitized, and incised along the midline. A dental drill was used to thin the skull over the injection sites. The thinned bone was then removed with a needle tip. A 5-μl calibrated glass micropipette [8 mm taper, 8 μm internal tip diameter] was fitted with a plastic tube connected to a 10-ml syringe and lowered onto a square of Parafilm containing a 4-μl drop of virus. After filling the micropipette, it was lowered to the proper stereotaxic coordinates and pressure was applied to the syringe to inject 1 μl of solution at a rate of 50 nl/min. After completing the bilateral injection and removing the micropipette, the skin was sutured and antibiotic was applied to the scalp. The mouse was kept warm by placing its cage on a heated plate and injected with buprenorphine [0.05 mg/kg] for pain relief. The water bottle in the cage was mixed with meloxicam [1 mg/kg] to relieve pain during subsequent recovery days. Animals were monitored for any signs of distress or inflammation for 3 days after surgery. Behavioral experiments were initiated 3 days after surgery. The mPFC was targeted at the following stereotaxic coordinates: Bregma; AP 1.8, ML ± 0.4, DV 1.4.

2.3. Viruses

Surgical procedures were standardized to minimize the variability of HSV virus injections, using the same stereotaxic coordinates for the mPFC and the same amount of HSV injected into the mPFC for all mice. CRE and/or mCherry under control of CamKIIα Promoter were cloned into the HSV amplicon and packaged using a replication-defective helper virus as previously described (Lim & Neve, 2001; Neve & Lim, 2001). The viruses were prepared by Dr. Rachael Neve (MIT, Viral Core Facility). The average titer of the

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