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Behavioral analysis of NR2C knockout mouse reveals deficit in acquisition of conditioned fear and working memory

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ABSTRACT

N-methyl-*D*-aspartate (NMDA) receptors play an important role in excitatory neurotransmission and mediate synaptic plasticity associated with learning and memory. NMDA receptors are composed of two NR1 and two NR2 subunits and the identity of the NR2 subunit confers unique electrophysiologic and pharmacologic properties to the receptor. The precise role of NR2C-containing receptors *in vivo* is poorly understood. We have performed a battery of behavioral tests on NR2C knockout/*nβ*-galactosidase knock-in mice and found no difference in spontaneous activity, basal anxiety, forced-swim immobility, novel object recognition, pain sensitivity and reference memory in comparison to wildtype counterparts. However, NR2C knockout mice were found to exhibit deficits in fear acquisition and working memory compared to wildtype mice. Deficit in fear acquisition correlated with lack of fear conditioning-induced plasticity at the thalamo-amygdala synapse. These findings suggest a unique role of NR2C-containing receptors in associative and executive learning representing a novel therapeutic target for deficits in cognition.

1. Introduction

Glutamate mediates majority of the excitatory neurotransmission in the mammalian central nervous system. There are three major classes of ionotropic glutamate receptors classified on the basis of sequence similarity and pharmacology (Erreger, Chen, Wyllie, & Traynelis, 2004; Traynelis et al., 2010). One such class of ionotropic glutamate receptors is the *N*-methyl-*D*-aspartate (NMDA) receptor, which is involved in key physiologic events such as synaptic plasticity and neural development. The NMDA receptor is a tetrameric receptor composed of two NR1 and two NR2 subunits (Monyer et al., 1992). The identity of the NR2 subunit (NR2A–D) confers unique electrophysiologic and pharmacologic properties to the NMDA receptor. NR2A- and NR2B-containing receptors are expressed widely throughout the central nervous system and are essential for integration of environmental stimuli and synaptic processes of learning and memory (Gao et al., 2010; Tang et al., 1999; Zhao et al., 2005). In contrast, the role of NR2C- and NR2D-containing receptors is poorly understood. The NR2C subunit is abundantly expressed in the cerebellar granule neurons first appearing at postnatal day 10 in rodents (Cathala, Misra, & Cull-Candy, 2000; Farrant, Feldmeyer, Takahashi, & Cull-Candy, 1994; Karavanova, Vasudevan, Cheng, & Buonanno, 2007; Monyer, Burnashev, Laurie, Sakmann, & Seeburg, 1994; Wenzel, Fritschy, Mohler, & Benke, 1997). NR2C mRNA is also expressed in the olfactory bulb, thalamus, retrosplenial cortex, pontine and vestibular nuclei (Karavanova et al., 2007; Wenzel et al., 1997) and certain interneurons in the cerebral cortex and hippocampus (Monyer et al., 1994).

Activation of the NMDA receptor is both ligand- and voltage-dependent. NMDA receptors are inactive under resting membrane potential due to Mg^{2+}-block (Mayer, Westbrook, & Guthrie, 1984). However, NR2C-containing receptors exhibit relatively low sensitivity to Mg^{2+}-block compared to NR2A- and NR2B-containing receptors (Cull-Candy, Brickley, & Farrant, 2001; Monyer et al., 1994). This property allows the NR2C-containing receptors to be activated by ambient glutamate without the requirement for prior depolarization as seen in layer 4 spiny stellate cells in barrel cortex and reticular thalamic nuclei (Binshtok, Fleidervish, Sprengel, & Gutnick, 2006; Zhang, Llinas, & Lisman, 2009). Deletion of NR2C gene leads to higher charge transfer in cerebellar granule cells in agreement with lower open probability of NR2C-containing receptors (Dravid, Prakash, & Traynelis, 2008; Ebralidze, Rossi, Tonegawa, & Slater, 1996; Lu et al., 2006) but does not affect general

Abbreviations: NMDA, *N*-methyl-*D*-aspartate; NR1, NMDA receptor subunit 1; NR2, NMDA receptor subunit 2.

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motor coordination or motor learning (Ikeda et al., 1995; Kadotani et al., 1996).

A number of pharmacological studies have identified a role of NMDA receptors in a variety of learning and memory functions including spatial learning (Handelman, Contreras, & O'Donohue 1987; Heale & Harley, 1990; Morris, Anderson, Lynch, & Baudry 1986) and working memory (Honey et al., 2003). Moreover, pharmacological and receptor knockout studies have identified a crucial role of NMDA receptors in fear conditioning and fear extinction (Gao et al., 2010; Sakimura et al., 1995; von Engelhardt et al., 2008; Zhang et al., 2008; Zhao & Constantine-Paton, 2007). The acquisition of fear primarily occurs through integration of thalamic and cortical excitatory glutamatergic inputs to the lateral amygdala (LA) (Goosens & Maren, 2004; LeDoux, 2000; LeDoux, Iwata, Cicchetti, & Reis, 1988). During auditory fear conditioning LA receives auditory inputs from posterior intralaminar nucleus (PIN) and medial sector of medial geniculate nucleus (MGM) (Campeau & Davis, 1995; LeDoux, Farb, & Ruggiero, 1990). Auditory inputs to PIN and MGM originate in the inferior colliculus (LeDoux, Ruggiero, Forest, Stornetta, & Reis, 1987). It has also been shown that MGM may play a role in regulation of fear response by receiving both CS and US inputs and relaying the convergent inputs to LA (Antunes & Moita, 2010; Weinberger, 2010). LA may also receive auditory inputs indirectly from the auditory cortex which receives projections from thalamic nuclei. The basal amygdala (BA) and LA is interconnected with the central nucleus of amygdala (CeA) which sends out projections to centers involved in mediating specific fear responses. Basolateral amygdala (BLA) is also considered to be the site for fear memory storage and fear conditioning leads to long-lasting strengthening of afferents from the thalamus to the amygdala (LeDoux, 2000; Maren, 2001; Radley et al., 2007; Rumpel, LeDoux, Zanod, & Malinow, 2005). Intra-amygdala infusion of NMDA receptor antagonists block the acquisition of conditioned fear (Fendt, 2001; Gewirtz & Davis, 1997; Miserendino, Sananes, Melia, & Davis, 1990) and NMDA-dependent long-term potentiation has been reported in the amygdala (Huang & Kandel, 1998; Maren & Fanselow, 1995). Further studies suggest that NR1/NR2B receptors play a predominant role in the acquisition of fear whereas NR1/NR2A receptors have a generalized effect on synaptic plasticity (Rodrigues, Schafe, & LeDoux, 2001; Walker & Davis, 2008). The role of NR2C-containing receptors in fear conditioning and other memory processes is poorly understood.

Due to the predominant expression of NR2C-containing receptors in mature cerebellar granule cells, previous behavioral studies using NR2C knockout mice have primarily focused on behaviors related to cerebellar function (Ikeda et al., 1995; Kadotani et al., 1996). However, given the unique biophysical properties (Cull-Candy et al., 2001; Monyer et al., 1994) and forebrain expression of NR2C subunit (Karavanova et al., 2007; Wenzel et al., 1997), a detailed behavioral analysis examining the role of NR2C-containing receptors in non-cerebellar functions is warranted. Here we report that mice lacking the NR2C subunit (Karavanova et al., 2007) exhibit a deficit in fear acquisition and working memory. These specific behavioral deficits suggest that NR2C-containing receptors may regulate unique neuronal circuits in the brain.

2. Methods

2.1. General behavior protocol

We used a NR2C knock-out/$n\beta$-galactosidase knock-in mouse C57BL/6 strain, where $n\beta$-galactosidase is inserted after the translation initiation site of the NR2C gene (Karavanova et al., 2007). Experimental procedures were performed on male NR2C +/+ and NR2C −/− littermate mice at 1–2 months of age. Animals were group housed on a 12:12 light–dark cycle with *ad libitum* access to food and water. Prior to all behavioral procedures, animals were handled for 3 days at the approximate time of the day the procedures were to occur. All procedures took place in the light phase of the light–dark cycle unless indicated otherwise. Unless indicated otherwise, all experimental surfaces were thoroughly cleaned with 70% ethanol between trials. Behavior procedures were video-recorded and scored by an individual blind to the genotype of the animal via a random coding system of the video files. All behavioral procedures were approved by the Creighton University Institutional Animal Care and Use Committee and conformed to the 1996 NIH *Guide for the Care and Use of Laboratory Animals*.

2.2. Fear conditioning/extinction/testing apparatus (tests 1–5)

For fear conditioning, mice were placed in a Plexiglas rodent conditioning chamber (chamber A; model 2325-0241 San Diego Instruments, San Diego, CA) with a metal grid floor that was enclosed in a sound-attenuating chamber. The chamber was illuminated with either red or white light depending on the type of conditioned stimulus (CS, tone or light) associated with the unconditioned stimulus (US, foot-shock), indicated below. Chamber A was cleaned with a 19.5% ethanol, 1% vanilla solution to give the chamber a distinct scent. For extinction training and CS testing; mice were placed in a novel Plexiglas chamber (chamber B; model 2325-0241 San Diego Instruments, San Diego, CA) with different visual cues and a solid Plexiglas floor to minimize generalization to the conditioning chamber. Chamber B was cleaned with a 70% ethanol solution, scented with linen-scented air freshener and illuminated with a 40 watt white light unless indicated otherwise. White noise was provided in each isolation cabinet with a fan. A web-camera (Logitech QuickCam) was mounted at the top of each isolation chamber to videotape all sessions.

2.2.1. Tone-cue conditioning (test 1)

Prior to conditioning (day 0) animals were acclimated to chamber A for 30 min. On the day of conditioning (day 1) mice were placed in chamber A for 3 min followed by three CS–US pairings. The CS was an 85 dB, 3 kHz tone delivered for 30 s with a 1 min inter-trial interval (ITI). The US was a 0.8 mA foot-shock delivered for 2 s that co-terminated with the CS. Mice were removed from chamber A 1 min after the final CS–US pairing. On testing day (day 2), the mice were placed in chamber B and after a 2 min delay exposed to the CS for 2 min and removed from the chamber 2 min later. The procedure for long-term memory testing (day 7) was the same as that on day 2 testing. Tests 2–5 were variations of the fear conditioning procedure used in test 1.

2.2.2. Context conditioning (test 2)

To determine contextual influence on fear conditioning in NR2C −/− mice, context conditioning and testing were performed. Animals were not pre-exposed to the conditioning chamber (chamber A) prior to conditioning. Conditioning proceeded as described in test 1. On testing day (day 2) instead of being placed into chamber B the mice were placed in chamber A for 4 min, freezing behavior was scored during the entire duration of context exposure.

2.2.3. Light-cue conditioning (test 3)

The CS was changed from a tone to light cue to assure that the observed deficit in fear conditioning was independent of cue perception. Acclimation, conditioning and testing procedures were the same as described test 1, except that instead of a tone, the CS for conditioning and testing was a white light. Also, the houselight in chambers A and B were provided by a 25 watt red light.