Short Communication

Blockade of Cav2.1-mediated NMDA receptor signaling disrupts conditioned fear extinction

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HIGHLIGHTS

- We studied physiological role of Cav2.1 in consolidation of extinction.
- Mice received α-agatoxin IVA showed impaired extinction and increased Arc expression.
- Lower dose of intra-mPFC injections of MK-801 blocked extinction in Cav2.1 mutant. Cav2.1-mediated glutamatergic pathway plays role in mPFC-dependent fear extinction. Combined pharmacological and genetic approach is useful to study neuronal circuits.

ARTICLE INFO

Article history:
Received 11 September 2013
Received in revised form 17 October 2013
Accepted 20 October 2013
Available online 28 October 2013

Keywords:
Cav2.1
Fear extinction
Neuronal circuits
α-Agatoxin IVA
Rolling Nagoya mice

ABSTRACT

Although fear extinction requires N-methyl-D-aspartate (NMDA) receptor signaling, Cav2.1-regulated synaptic function in extinction remains unknown. This study examined whether Cav2.1-mediated signaling plays role in consolidation of extinction. Wild-type mice received intracerebroventricular injection of Cav2.1 blocker (α-agatoxin IVA, 4.0 pg/site) showed impaired extinction behavior and increased expression of CREB-dependent gene Arc in medial prefrontal cortex (mPFC). Intra-mPFC injections of NMDA receptor antagonist (MK-801, 0.5 μg/midline), which was ineffective in wild-type controls, blocked extinction in heterozygous rolling Nagoya (rol+/-) mice carrying Cav2.1c1 gene mutation rol +/- mice. These results indicate that Cav2.1-mediated NMDA receptor signaling is functional pathway in mPFC-dependent fear extinction. Our results also indicate that the combination of pharmacological and genetic approaches can be used to study functional signaling pathways in neuronal circuits.

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In recent years, there has been a growing interest in the neural mechanisms of fear extinction [1], as extinction may be a potential target for the treatment of neuropsychiatric diseases. Animals learn to associate a previously neutral or conditioned stimulus (CS) with an aversive or unconditioned stimulus (US) during fear conditioning. Subsequent re-exposure to the CS alone triggers two competing processes. Brief re-exposure to the CS initiates a reconsolidation process that serves to stabilize or maintain the original CS-US memory [2]. In contrast, more prolonged re-exposure to the CS leads to the formation of an inhibitory extinction (CS-no US) memory [3]. Hence, extinction procedures do not erase the original fear memory, but yield a new safety memory that inhibits fear under certain conditions. Previous study reported that fear extinction requires a distributed neural circuit in the brain, especially the medial prefrontal cortex (mPFC) [4]. Glutamatergic synaptic transmission is implicated in extinction learning. Injections of N-methyl-D-aspartate (NMDA) receptor antagonists impair extinction memory [4]. Blocking protein synthesis in the mPFC prevents the formation of extinction memory, and activation of cAMP-responsive element-binding protein (CREB)-mediated transcription is induced in the mPFC in the consolidation phase of extinction [5]. Cav2.1 (P/Q-type) channels mediate the presynaptic machinery for glutamate release [6]. NMDA receptor and Cav2.1 express broadly in the central nervous system [7,8], suggesting that Cav2.1-regulated NMDA receptor signaling regulates the CREB cascade involved in extinction memory in the mPFC. Thus, administration of a Cav2.1 blocker

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http://dx.doi.org/10.1016/j.bbr.2013.10.033
should result in dysfunctional fear extinction. However, the exact physiological role of Cav2.1-regulated extinction has not yet been determined.

The Cav2.1 is a molecular complex comprising several subunits: α1, α2-δ, β, and γ [9]. The α1 subunit is essential for channel function and determines fundamental channel properties [9]. Rolling Nagoya mice carrying a mutation in the αδ subunit of Cav2.1 channel (Cav2.1αδ) exhibit severe ataxia after about two weeks of age, whereas two-month-old heterozygous Rolling Nagoya (roll+) mice have no apparent deficits [10]. Thus, the combination of lower dose administration of an NMDA receptor antagonist and roll/+ mice should result in dysfunctional fear extinction compared with wild-type (+/+) mice, because precise regulation of neurotransmitter release through Cav2.1 plays an important role in neuronal circuits.

In this study, to examine the relationship between Cav2.1-mediated signaling in the mPFC and extinction memory formation, we investigated the extinction of conditioned fear using the wild-type mice received intracerebroventricular injection of Cav2.1 blocker (ω-agatoxin IVA) and the rol/+ mice that received intramPFC injections of NMDA receptor antagonist (MK-801). We used immunohistochemical analysis to characterize the relationship between Cav2.1-mediated signaling and the expression of the CREB-dependent gene activity-regulated cytoskeleton-associated protein (Arc) after prolonged re-exposure. The studies presented here demonstrate the importance of mPFC-dependent Cav2.1-mediated NMDA receptor signaling in the consolidation of fear extinction.

All animal procedures were approved by the Animal Experiments Committee of RIKEN. The Rolling Nagoya mouse strain was provided by the RIKEN BioResource Center (Ibaraki, Japan) and backcrossed to C57BL/6j mice (Charles River Japan, Kanagawa, Japan) for 12 generations. Male +/+ and rol/+ F1 progeny were genotyped by polymerase chain reaction (PCR) [10] and were 2 months of age when tested. The mice were given free access to water and food pellets (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and were housed under a 12/12-h light/dark cycle (lights on from 08:00 to 20:00) at 23 ± 1 °C and 55 ± 5% humidity. Testing was performed during the light phase of the cycle. All experiments were conducted blind to the treatment condition of the mouse.

Mice were trained and tested in conditioning chambers (17.5 × 17.5 × 15 cm) that had a stainless steel rod floor through which footshocks could be delivered (Med Associates, Inc., St. Albans, VT, USA). Training consisted of placing a mouse in the chamber and delivering a series of unsignaled three footshocks at 1-min intervals (first footshock at 148 s after placement in the chamber). Mice were returned to their home cages 30 s after the final footshock. Twenty four hours after training, mice were re-exposed to the conditioning context for 30 min. One day following the extinction session, the mice were tested (5 min in context). Freezing behavior was automatically measured (Winroo version 5.5 software; Mitani Corporation, Tokyo, Japan).

The following two drugs were used: Cav2.1 blocker, ω-agatoxin IVA (Peptide Institute, Osaka, Japan) and NMDA receptor antagonist, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydromaleate (MK-801, Sigma-Aldrich, Tokyo, Japan). For the infusion studies, ω-agatoxin IVA (10, 40, or 80 pg/µL) and MK-801 (1, 5, or 10 µg/µL) were dissolved in saline (vehicle). Under anesthesia and using standard stereotaxic procedures, stainless-steel guide cannulae (22-gauge) were implanted into the lateral ventricle (posterior to bregma, −0.34 mm; lateral to midline, ±0.9 mm; ventral from the dura, −2.3 mm) or mPFC (anterior to bregma, +1.9 mm; lateral to midline, ±0.0 mm; ventral from the dura, −2.3 mm). Mice were allowed to recover for at least 1 week following surgery. The mice were briefly anesthetized with isoflurane to facilitate insertion of the injection cannula (26-gauge). Infusions into the lateral ventricle (0.1 µL/side) or mPFC (0.1 µL/midline) including prelimbic cortex (PLC) and infralimbic cortex (ILC) are accomplished at a rate of 0.05 µL/min immediately after the re-exposure session. The injection cannula was left in place for 2 min following the infusion. The drug doses were determined according to previous report [10,11]. The doses used in the present study did not produce non-specific motor or sedating effects [10,11]. Mice that were not treated with drugs received an equivalent volume of vehicle.

Mice that received intracerebroventricular injection immediately after the re-exposure session were anesthetized with sodium pentobarbicone 90 min after the re-exposure session and were perfused with phosphate-buffered saline (PBS) 0.1 mM sodium fluoride (NaF) containing 4% paraformaldehyde. The brains were then removed, fixed overnight, and transferred into 30% sucrose. Coronal sections (30 µm) were cut on a cryostat. Consecutive sections were incubated overnight with an anti-Arc rabbit polyclonal primary antibody (1:1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in the blocking solution. The sections were incubated with biotinylated goat anti-rabbit IgG (SAB-PO kit; Nichirei Biosciences, Tokyo, Japan), followed by incubation at room temperature in streptavidin-biotin-peroxidase complex (SAB-PO kit). Quantification of Arc-positive cells in sections (bregma between 2.00 and 1.88 mm) of PLC and ILC was determined with a computerized image analysis system (Winroof version 5.5 software). Immunoreactive neurons were counted bilaterally with a fixed sample window across (200 × 200 μm) at least three sections by an experimenter blind to the treatment condition.

Data are presented as means ± standard error of the mean (SEM). Statistical analyses for the behavioral and immunocytochemical studies were conducted using Excel Statistics 2006 (SSRI, Tokyo, Japan). Data were analyzed using repeated measures analysis of variance (ANOVA) followed by Tukey’s post-hoc tests.

We examined intracerebroventricular injection effects of ω-agatoxin IVA on the consolidation of extinction. Our experimental design is shown in Fig. 1A. We used four groups of mice (n = 10 each) given systemic injections of 0 (vehicle), 1, 4, or 8 pg/side ω-agatoxin IVA. There were no significant differences among groups in the extinction training session [F(15,216) = 0.816, P > 0.05] (Fig. 1B). However, the groups significantly differed in the percentage of freezing during the test [F(3,36) = 161.680, P < 0.01] (Fig. 1C). The mice given 4 pg/side ω-agatoxin IVA showed greater freezing behavior than the mice given vehicle. These results show that blockade of Cav2.1-mediated signaling impairs consolidation of extinction. Correct placement of the guide cannula was verified by ink injection after experiments (data not shown).

The intracerebroventricular injection study is difficult to evaluate the site of action of the cascade in the brain. Because previous studies reported that fear extinction requires CREB expression in the mPFC [5], we examined Arc expression patterns using mice (n = 8 each) given the injections of vehicle or 4 pg/side ω-agatoxin IVA immediately after re-exposure session. The experimental design is shown in Fig. 2A. All groups showed decreasing levels of freezing over the 30-min re-exposure session [F(5,84) = 0.732, P > 0.05]. After the 90-min re-exposure session, significantly higher Arc expression was detected in mPFC regions, including the prelimbic cortex (PLC) and the infralimbic cortex (ILC), in the vehicle-injected mice compared with the ω-agatoxin IVA-injected mice [PLC: F(1,14) = 86.562, P < 0.01, ILC: F(1,14) = 19.523, P < 0.01] (Fig. 2B). These results indicate that Cav2.1-mediated signaling in the mPFC is necessary for consolidation of extinction.

We examined whether rol/+ mice showed normal acquisition and extinction of contextual fear conditioning. Freezing times in the first trial block (0–5 min) of the re-exposure session were 60.9 ± 2.71% and 59.7 ± 1.11% for rol/+ and +/+ mice, respectively (n = 10 each) [F(1,18) = 1.332, P > 0.05]. During the re-exposure session (i.e., extinction training session), no significant difference was
detected between rol/+ and +/+ mice \(F(5,108) = 0.483, P > 0.05\). Freezing behavior decreased over time within the 30-min re-exposure session in both rol/+ and +/+ mice. Measurements obtained 24 h after the re-exposure session confirmed that the extinction of conditioned freezing did not significantly differ between rol/+ and +/+ mice \(F(1,18) = 0.437, P > 0.05\). We also examined freezing behavior without the 30-min re-exposure session in both rol/+ and +/+ mice \((n = 10)\). Measurements obtained 48 h after acquisition confirmed that the freezing did not significantly differ between rol/+ and +/+ mice \(F(1,18) = 0.287, P > 0.05\), suggesting that these conditions were specific for the extinction process. These results showed that the rol/+ mice exhibited normal sensitivity to the footshock and normal acquisition and extinction of conditioned fear.

To examine whether mPFC-dependent Cav2.1-mediated NMDA receptor signaling plays a role in the consolidation of fear extinction, we used microinfusion analysis in the rol/+ mice. The experimental design is shown in Fig. 3A. We examined eight groups of mice \((n = 10)\), including four groups each of +/+ and rol/+ mice that were given intra-mPFC injections of 0 (vehicle), 0.1, 0.5 or 1.0 μg/midline MK-801. Although there were no significant differences among the groups during the extinction training session and all groups showed decreasing levels of freezing over the 30-min re-exposure session \(F(15,432) = 0.368, P > 0.05\) (Fig. 3B), the groups differed significantly in freezing percentage on the test [genotype effect: \(F(1,72) = 24.778, P < 0.01\); dose effect: \(F(3,72) = 135.448, P < 0.01\); genotype × dose interaction: \(F(3,72) = 36.245, P < 0.01\)] (Fig. 3C). The rol/+ mice given 0.5 μg/midline MK-801 showed higher freezing behavior than the dose-matched +/+ mice. The +/+ mice given 1.0 μg/midline MK-801 showed more freezing behavior than the rol/+ mice given vehicle. The rol/+ mice given 0.5 or 1.0 μg/midline MK-801 had greater freezing behavior than the rol/+ mice given vehicle. Fig. 3D shows the infusion cannula placement.
in the mPFC, including the PLC and ILC. Thus, mPFC-dependent NMDA receptor signaling is necessary for consolidation of extinction.

Previous studies have demonstrated that NMDA receptors are not necessary during extinction training session (extinction acquisition), but are necessary for long-term retention of extinction (extinction consolidation) [12]. Therefore, we examined whether the Cav2.1-mediated NMDA receptor signaling is important for consolidation of extinction (i.e., after extinction training). The o-agonaxin IVA inhibits presynaptic Cav2.1 function [9] and reduces glutamate release [13]. We showed that o-agonaxin IVA (4 pg/side) blocked extinction memory, suggesting that the signal cascade activated by Cav2.1-regulated glutamate binding to the NMDA receptor plays a role in the consolidation of extinction. Because Cav2.1 and NMDA receptor are present at a variety of synapses [7,8], intracerebroventricular injection study does not identify the specific brain regions using Cav2.1-mediated signaling that are engaged the formation of extinction memory. A major goal in extinction research is to understand the molecular and circuit mechanisms underlying each of the phases of the extinction experience. It is clear that a network of brain regions is involved in encoding extinction learning as well as storage of the extinction memory necessary for its later retrieval under the appropriate environmental conditions. In this regard, studies of expression and microinfusion in the specific neural region would be useful for elucidating the relevant neuronal mechanisms.

Activation of CREB-mediated transcription is induced in the mPFC during the consolidation of extinction phase [5]. The mPFC is engaged after extinction training occurs [14]. Our results showed that intracerebroventricular injection of o-agonaxin IVA immediately after the re-exposure session blocks Arc expression in the mPFC of wild-type mice. The results indicate that the mPFC is an important region in extinction memory formation, particularly in consolidation of the extinction phase, and that information within the mPFC is likely processed by Cav2.1-regulated glutamatergic transmission.

Because the Ca²⁺ current amplitude exhibited a 40% reduction in homozygous rolling Nagoya mice compared with wild-type controls [15], the Cav2.1 mutation in rol/+ mice may result in a decreased Ca²⁺ influx and lower the threshold for glutamate release, thereby playing a role in impairment of extinction memory formation. This possibility was confirmed using intra-hippocampal injections of a combination of sub-threshold doses of MK-801 in the Y-maze test, which trigger short-term memory deficits in the rol/+ mice, but not wild-type controls [16]. In this study, intra-mPFC injections of MK-801 (0.5 μg/midline) were ineffective in wild-type controls, but impaired extinction behavior in rol/+ mice. These results demonstrate that Cav2.1-regulated glutamatergic signaling

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Fig. 3. Intra-mPFC injection effects of MK-801 on freezing percentage. The experimental design shown (A) was used to collect the data presented below. Effect of MK-801 on the freezing percentage is shown during the extinction training session (B) and on the CS re-exposure test (C). This coronal drawing shows the location of the MK-801 injection aimed at the mPFC, including the prelimbic cortex (PLC) and infralimbic cortex (ILC) (D). *P < 0.01 compared with the appropriate control (Tukey's test).
in the mPFC is crucial for the consolidation of extinction and that using combinations of subthreshold doses of different genes and drugs is a suitable approach for identifying molecular and circuit mechanisms.

NMDA receptor-dependent bursting in the mPFC is required for the initial 2 h of post-training processing of extinction [4]. The bursting would be generated by instructive Cav2.1-regulated input signals from other regions. Indeed, our study showed that blocking Cav2.1-regulated glutamatergic signaling in the mPFC induces dysfunctional fear extinction. These results indicate that Cav2.1-mediated NMDA receptor signaling in the mPFC plays an important role after extinction training in the acquisition of a vital aspect of the extinction experience, by putting together relationships between different input source(s) and the mPFC. This, in turn, serves to shift behavioral strategies to rapidly inhibit the previously learned fear response during extinction retrieval.

Within the mPFC, PLC and ILC have been implicated in the expression of fear [17] and the consolidation of extinction [5]. The PLC projects mainly to excitatory neurons involved in fear expression [18], whereas the ILC projects mainly to inhibitory neurons involved in suppressing fear after extinction [19]. Both glutamatergic [20] and GABAergic synaptic transmission within the mPFC contribute to extinction learning. Although direct evidence of the neural circuit mechanisms of extinction learning and recall within the mPFC and between the mPFC and other regions have yet to be examined, this information would aid in understanding the nature and cause of extinction impairments that contribute to psychopathology.

In conclusion, we showed that Cav2.1-mediated NMDA receptor signaling is functional pathway in mPFC-dependent consolidation of extinction behavior of conditioned fear. Our results also suggest that rol/+/ mice represent a useful model for delineating changes in extinction memory associated with Cav2.1-mediated glutamatergic signal transduction and that different combinations of subthreshold doses of pharmacological agents and genetic origins in local infusion studies are useful for inducing the effects of phenotypes in silent mutants and for identifying functional signaling pathways in specific neuronal circuits.

Acknowledgments

This work was supported by China 973 project (2010CB529604) to WL, and Grants-in-Aid for Scientific Research KAKENHI (22500396) to ET.

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