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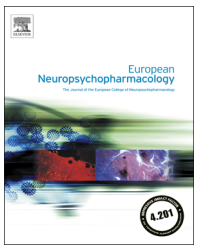

Sonic hedgehog signaling regulates amygdalar neurogenesis and extinction of fear memory

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Abstract
It is now recognized that neurogenesis occurs throughout life predominantly in the subgranular zone (SGZ) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricle. In the present study, we investigated the relationship between neurogenesis in the amygdala and extinction of fear memory. Mice received 15 tone-footshock pairings. Twenty-four hours after training, the mice were given 15 tone-alone trials (extinction training) once per day for 7 days. Two hours before extinction training, the mice were injected intraperitoneally with 5-bromo-3-deoxyuridine (BrdU). BrdU-positive and NeuN-positive cells were analyzed 52 days after the training. A group of mice that received tone-footshock pairings but no extinction training served as controls (FC+No-Ext). The number of $BrdU^+/NeuN^+$ cells was significantly higher in the extinction (FC+Ext) than in the FC+No-Ext mice. Proliferation inhibitor methylazoxymethanol acetate (MAM) or DNA synthesis inhibitor cytosine arabinoside (Ara-C) reduced neurogenesis and retarded extinction. Silencing *Sonic hedgehog* (*Shh*) gene with short hairpin interfering RNA (shRNA) by means of a retrovirus expression system to knockdown *Shh* specifically in the mitotic neurons reduced neurogenesis and retarded extinction. By contrast, over-expression of *Shh* increased neurogenesis and facilitated extinction. These results suggest that amygdala neurogenesis and Shh signaling are involved in the extinction of fear memory.

1. Introduction

Repeated presentations of non-threatening cues (conditioned stimulus, CS) to the subject without pairing with aversive stimuli (unconditioned stimulus, US) result in a gradual decrease in the conditioned response (CR) (Falls et al., 1992; Myers and Davis, 2007). This procedure, termed extinction training, has been applied in psychological treatments to extinguish inappropriate and exaggerated fear such as anxiety and phobias. The precise mechanisms of exposure therapy have received extensive attention but its whole picture is still not clear. Proposed mechanisms include extinction-induced silencing of amygdala fear neurons (Hobin et al., 2003; Herry et al., 2008; Amano et al.,

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2010; Livneh and Paz, 2012) likely through increasing inhibitory plasticity (Ehrlich et al., 2009; Makkar et al., 2010; Kullmann et al., 2012), ubiquitin- and proteasome-dependent protein degradation (Lee et al., 2008), induction of striatal-enriched protein tyrosine phosphatase (STEP) expression and subsequently resulting in AMPARs endocytosis (Mao et al., 2006) and induction of spine formation on the same dendritic branches where fear conditioning caused spine elimination (Lai et al., 2012). Intriguingly, Trouche et al. (2013) using a *c-fos*-based transgenic mouse have shown that extinction training induced target-specific remodeling of amygdala perisomatic inhibitory synapses originating from parvalbumin and cholecystokinin-positive interneurons (Trouche et al., 2013).

Sonic hedgehog (Shh) is a member of the Hedgehog (Hh) family of secreted signaling proteins functions as a chemical signal in transmitting information to embryonic cells required for normal development. Shh pathway is important for developing nervous system, including midbrain and ventral forebrain neuronal differentiation and neuronal precursor proliferation (Ericson et al., 1995; Hynes et al., 1995; Chiang et al., 1996; Wechsler-Reya and Scott, 1999; Britto et al., 2002; Ruiz i Altaba et al., 2002). Activation of Shh signaling occurs through binding to a receptor complex including Patched (Ptc-1) and Smoothened, a G-protein coupled receptor (Sims et al., 2009). Patched is an integral membrane protein with 12 transmembrane domains that acts as an inhibitor of Smoothened activation. The pathway downstream of the Smoothened receptor has remained somewhat unclear, but involves the Gli zinc-finger transcriptional factors (Traiffort et al., 2010).

It is now established that neurogenesis occurs throughout life predominantly in the subgranular zone (SGZ) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricle (Ming and Song, 2005; Balu and Lucki, 2009). Neurogenesis in the adult hippocampus is required for trace eyeblink conditioning (Shors et al., 2001). Consistent with this report, ablated neurogenesis impaired contextual fear conditioning, but did not impair cued fear conditioning, which is independent of the hippocampus (Saxe et al., 2006; Imayoshi et al., 2008). Contradictorily, it was also reported that ablation of hippocampal neurogenesis had no effect on learning, even in hippocampus-dependent memory tasks such as the contextual fear memory task and the Morris water maze task (Shors et al., 2002; Snyder et al., 2005; Zhang et al., 2008). Recently, it has been shown that block of neurogenesis via cranial ray irradiation robustly inhibited social avoidance, highlighting a unique functional role for newly born neurons in an antidepressant-like effect (Lagace et al., 2010).

Under normal conditions, adult neurogenesis was not detected in the amygdala. However, under the conditions of pentylenetetrazole-induced epilepsy (Park et al., 2006), olfactory bulbectomy and enriched environment (Keilhoff et al., 2006; Okuda et al., 2009) increase in BrdU-positive cells was found in the amygdala. In the present study, we investigated the relationship between neurogenesis in the amygdala and fear extinction (Okuda et al., 2009). We found that inhibition of neurogenesis with proliferation inhibitor methylazoxymethanol acetate or with DNA synthesis inhibitor cytosine arabinoside impaired fear extinction. Silencing *Shh* gene with short hairpin interfering RNA reduced neurogenesis and retarded extinction. Conversely, overexpression of *Shh* gene increased neurogenesis and facilitated extinction suggesting the involvement of amygdala neurogenesis and Shh signaling in the extinction of fear memory.

2. Experimental procedures

2.1. Animals

Four-five mice were housed in a room with controlled temperature (21 ± 2 °C) and humidity (55 ± 5%) under a 12/12 h light/dark cycle. Both food and water were available ad libitum. The experiment is processed according to the Institutional Animal Care and Use Committee of the National Cheng-Kung University.

2.2. Pavlovian fear conditioning and extinction models

Adult male C57BL/6J mice (2 months old) were used in this study. We use chamber A in room A and chamber B in room B for each auditory fear conditioning training and extinction session (Hung et al., 2014). Training of auditory fear conditioning was started with a 120 s acclimation period in room A. Mice were presented with a tone (20 s, 80 dB, 5 kHz) that co-terminated with foot shock (1 s, 0.7 mA). This tone-footshock pairing procedure was repeated 15 times with an inter-trial interval (ITI) of 2 min. Ethanol (75%) was used for cleaning in training sessions. In extinction session, mice are placed back into room B for 120 s acclimation period, and then received 15 tone-alone trials (20 s, 80 dB, 5 kHz) without foot-shock with inter-trial intervals (ITI) of 2 min. Acetic acid (1%) was used for cleaning between each test session. Freezing responses were recorded by camera and analyzed by Freeze Scan software (Med Associates, St. Albons, VT).

2.3. Surgery and drugs application

Mice were anesthetized with chloral hydrate (400 mg/kg, i.p.) and mounted on a stereotaxic apparatus (Kopf). The mice were implanted cannulas (26 gauge stainless steel tubing) into the amygdala (anterior $= -1.6$ mm; lateral $= \pm 3.5$ mm and depth $= -4.7$ mm). After 7 days recovery, cytosine arabinoside (Ara-C, 1 mM/1 μl/per side) (Colon-Cesario et al., 2006) dissolved in saline was bilaterally infused into the amygdala in a volume of 1.0 μl at rate of 0.1 μl/min. The infusion cannulas were left in place for 2 min before being withdrawn. Mice received intraperitoneal injection of methylazoxymethanol acetate (MAM, 7 mg/kg, Sigma Aldrich, St. Louis, USA) (Dupret et al., 2005) once per day for 7 consecutive days before extinction learning.

2.4. BrdU labeling

Bromodeoxyuridine (BrdU, 300 mg/kg; Sigma Aldrich, St. Louis, USA) was injected intraperitoneally into the mouse 2 h before extinction training. The mice were sacrificed at 52 day after fear conditioning for the detection of newborn cells (Bischofberger, 2007).

2.5. Immunofluorescent staining

The fixation and immunofluorescent staining processes were adopted from Kitamura et al. (2009). Mice were deeply anesthetized and perfused intracardially with saline followed by 4% paraformaldehyde in phosphate buffered saline (PBS). Brains were removed, and cryoprotected the tissues by immersion in PBS containing 30% sucrose. The brain was cut in 20 μm coronal sections on a cryostat, thaw-mounted onto gelatin slides and stored at -20 °C until use. Sections were pretreated with saline-sodium citrate (SSC) buffer at 85 °C for 15 min, and then incubated in 2 N