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The roles of Eph receptors in contextual fear conditioning memory formation

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ABSTRACT

Eph receptors regulate glutamate receptors functions, neuronal morphology and synaptic plasticity, cellular events believed to be involved in memory formation. In this study we aim to explore the roles of Eph receptors in learning and memory. Toward that end, we examined the roles of EphB2 and EphA4 receptors, key regulators of synaptic functions, in fear conditioning memory formation. We show that mice lacking EphB2 (EphB2$^{-/-}$) are impaired in short- and long-term contextual fear conditioning memory. Mice that express a carboxy-terminally truncated form of EphB2 that lacks forward signaling, instead of the full EphB2, are impaired in long-term, but not short-term, contextual fear conditioning memory. Long-term contextual fear conditioning memory is attenuated in CaMKII-cre;EphA4$^{lx/-}$ mice where EphA4 is removed from all pyramidal neurons of the forebrain. Mutant mice with targeted kinase-dead EphA4 (EphA4^{KD}) exhibit intact long-term contextual fear conditioning memory showing that EphA4 kinase-mediated forward signaling is not needed for contextual fear memory formation. The ability to form long-term conditioned taste aversion (CTA) memory is not impaired in the EphB2$^{-/-}$ and CaMKII-cre;EphA4$^{lx/-}$ mice. We conclude that EphB2 forward signaling is required for long-term contextual fear conditioning memory formation. In contrast, EphB2 mediates short-term contextual fear conditioning memory formation in a forward signaling-independent manner. EphA4 mediates long-term contextual fear conditioning memory formation in a kinase-independent manner.

1. Introduction

EphB2 and EphA4 receptors are intimately involved in regulating molecular and cellular events believed to be needed for memory formation such as regulation of NMDA and AMPA receptors functions and neuronal morphogenesis (Klein, 2009; Lamprecht & LeDoux, 2004; Sheffler-Collins & Dalva, 2012). EphB2 directly interacts and cooperates with NMDA receptors (NMDARs) in synapse formation and plasticity and promotes tyrosine phosphorylation of NMDAR subunit by Src family kinases in dissociated neurons and in hippocampal slices (Dalva et al., 2000; Grunwald et al., 2001; Henderson et al., 2001; Takasu, Dalva, Zigmond, & Greenberg, 2002). EphB2 activates AMPAR function to induce mossy fiber LTP and postsynaptic intracellular signaling, resulting in EphB2 clustering, possibly by interaction with the AMPAR-binding protein GRIP (Contractor et al., 2002). In addition, EphB2 is involved in spine morphogenesis mostly through controlling actin regulatory proteins. For example, the Rac1 guanine exchange factor (GEF) Tiam1 is involved in regulating spine development in an EphB2-dependent manner (Tolias et al., 2005). EphB2 is also involved in synaptic plasticity. At the CA3-CA1 synapse, EphB2 mediates long-lasting long-term potentiation (LTP), a physiological model of memory, in a kinase-independent manner (Grunwald et al., 2001). EphA4 binds ephrin-A ligands but can also bind ephrin-B ligands. EphA4 regulates the level of GluR1 subunit of AMPA receptor in synapse in hippocampus (Fu et al., 2011). EphA4 is involved in regulating spine morphology through the actin cytoskeleton (e.g. Murai, Nguyen, Irie, Yamaguchi, & Pasquale, 2003; Zhou et al., 2007). EphA4 is also involved in synaptic plasticity. For example, EphA4/ephrin-A3 signaling regulates LTP through glial glutamate transport downstream to ephrin-A3 (Filosa et al., 2009), EphA4 is required for the early stages of LTP at the CA3-CA1 synapse and similar to EphB2 acts in a kinase-independent fashion (Grunwald et al., 2004) and EphA4 is needed for lateral amygdala LTP formation (Deininger et al., 2008).

In the present study we are interested to elucidate whether EphB2 and EphA4 are needed for fear memory formation. Toward that end, we used the contextual fear conditioning paradigm. In this paradigm an association is formed between the context (conditioned stimulus (CS)) and an aversive mild footshock

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(unconditioned stimulus (US)) (Davis & Whalen, 2001; Fanselow & LeDoux, 1999; LeDoux, 2000; Maren, 2005; Sah, Faber, Lopez De Armentia, & Power, 2003). The putative site of contextual fear conditioning memory, the hippocampus, has been identified (Kim & Fanselow, 1992; Phillips & Ledoux, 1992). EphB2 and EphA4 are expressed in the hippocampus and are involved in hippocampal synaptic plasticity (e.g. Filosa et al., 2009; Grunwald et al., 2001, 2004). In the present study we have used genetically modified mice to explore the roles of EphB2 and EphA4 receptors in contextual fear conditioning memory formation.

2. Material and methods

2.1. Mice

The generation of EphB2$^{-/-}$ and EphB2$^{lacZ/lacZ}$ mice has been described previously (Henkemeyer et al., 1996) and the distribution and level of EphB2 in these mice were characterized (Grunwald et al., 2001). Mutant mice were maintained in a heterozygous state on a 129XC57BI/6 background. The generation of Kinase-Dead EphA4 (EphA4^{KD}) mice was described previously (Kullander et al., 2001). EphA4^{flox} (Herrmann et al., 2010) mice were crossed with CaMKII-Cre mice (Minichiello et al., 1999) to obtain CaMKII-cre;EphA4$^{lx/-}$ mice. Colonies founder mice were obtained from Prof. Ruediger Klein (Max Planck Institute of Neurobiology, Martinsried, Germany) and were bred at University of Haifa. Mice were housed at 22 ± 2 °C in a 12 h light/dark cycle, with free access to food and water. No significant differences are observed in the weight of the mice between the wild type and the genetic modified littermate mice (in grams): EphB2$^{-/-}$: wt-25.75, EphB2$^{-/-}$-25.0 ($p > 0.8$); EphB2$^{lacZ/lacZ}$: wt-21.66, EphB2$^{lacZ/lacZ}$-21.5 ($p > 0.7$); EphA4^{KD}: wt-27.4, EphA4^{KD}-25.03 ($p > 0.1$); CaMKII-cre;EphA4$^{lx/-}$: wt-23.5, CaMKII-cre;EphA4$^{lx/-}$-23.33 ($p > 0.9$). Behavioral experiments were approved by the University of Haifa Institutional Committee for animal experiments in accordance with National Institutes of Health guidelines.

2.2. Mice genotyping

DNA was extracted from mice tail using Kapa express extraction kit (KAPA biosystems) and subjected to PCR (Dreamtaq polymerase, Thermo Scientific) using the following primers and conditions: *EphB2 KO*-Primers ACG ATT GCC TAG GCT CTT GGA GTA G; GGG TAC ATC TCA GTG GTA GAA TG; GTC AGT TTC ATA GCC TGA AGA ACG. PCR Program: 94 °C 2 min, (94 °C 15 s; 61 °C 40 s; 72 °C 1 min and 30 s)X35, 72 °C 10 min. DNA was run on 1% agarose gels (wt band ~600 bp; mutant band ~300 bp). *EphB2-LacZ*-CAC AAG TCA TTT TTG CCA CTC TAG; TAA AAC GAC GGG ATC ATC GCG AGC C; GGA CAG AGC TAG GCT ATA GAC CCA G. PCR Program: 94 °C 2 min, (94 °C 15 s; 61 °C 40 s; 72 °C 1 min and 30 s)X35, 72 °C 10 min. DNA was run on 1% agarose gels (Wt band ~700 bp; Mut band ~400 bp). *EphA4-KD*-Primers: EphA4 mutant: 5′GAC TCT AGA GGA TCC ACT AGT GTC GA; TTT TCT CCC TCT TTA AGC AAG GAT CAA GC. EphA4 wt: CAA TCC GCT GGA TCT AAG TGC CTG TTA GC: ACC GTT CGA AAT CTA GCC CAG T. PCR Program: 94 °C 3 min, (94 °C 30 s, 65 °C 30 s, 72 °C 1 min)X35, 72 °C 10 min (Wt band ~450 bp; Mut band ~200 bp). CaMKII-cre;EphA4$^{lx/}$-EphA4^{lx}-GCA CAC TTA GCA ATT CAG TGT GGG; CAG TTA ATT AGT GGT GGG TTC CT PCR program: (94 °C 4 min (94 °C 30 s, 72 °C 1 min)X 39, 72 °C 7 min, keep 4 °C (EphA4/lx ~300 bp). CaMKII-Cre-GCC TGC ATT ACC GGT CGA TGC AAC GA; GTG GCA GAT GGC GCG GCAACA CCA TT: PCR program: 94 °C 3 min (94 °C 1 min; 67 °C 1 min, 72 °C 1 min) X35, 72 °C 5 min, keep at 4 °C (~800 bp). EphA4-: GAC TCT AGA GGA TCC ACT AGT GTC GA; TTT TCT CCC TCT TTA AGC AAG GAT CAA GC, PCR program: 94 °C 3 min (94 °C 1 min; 65 °C 1 min, 72 °C 1 min) X35, 72 °C 5 min, keep at 4 °C (~200 bp).

2.3. Fear conditioning

Fear conditioning took place in a Plexiglas rodent conditioning chamber with a metal grid floor (Coulbourn Instruments). Mice were allowed to acclimate to the conditioning chambers for 2 min followed by 3 pairings (EphB2 mice) or 1 pairing (EphA4 mice) of a tone (CS; 30 s, 2800 Hz, 85 dB) that was co-terminated with a foot shock (US; 2s, 0.75 mA). The intertrial interval (ITI) was 120 s in average. Mice were tested 1 h after training for contextual fear conditioning short-term memory (STM). Different groups of animals were tested for contextual fear conditioning long-term memory (LTM) 24 h after training. Mice were tested for contextual fear conditioning in the same conditioning chamber. Freezing of mice was evaluated for 5 min. Behavior was recorded and the video images were transferred to a computer equipped with an analysis program. The percentage of changed pixels between two adjacent 0.5 s images was used as a measure of activity.

2.4. Conditioned taste aversion

Saccharin (0.5% (gr/l), sodium salt) was used as the unfamiliar taste in training (i.e., the conditioned stimulus (CS)) and injection of LiCl (0.14 M, 2% of body weight, i.p.) as the malaise-inducing agent (unconditioned stimulus (US)). At day 1 the mice were weighted and the water was taken from them. The mice were trained for 3 days to drink water once a day for 30 min from two pipettes. On the conditioning day (day four), they were allowed to drink the saccharin solution instead of water from similar pipettes for 30 min, and 50 min later they were injected with LiCl. In next day the mice were allowed to recover with 2 pipettes of water for 30 min. The next day mice were subjected to a multiple choice test (2 pipettes with water and 2 pipettes with saccharin). The behavioral data are presented in terms of aversion index, defined as [ml water/(ml water + ml saccharin)] × 100 consumed in the test. The higher the aversion index, the more the mice prefer water to the conditioned taste (saccharin) and the stronger the memory to the CS.

2.5. β-Galactosidase assay

Wild type mice and EphB2$^{lacZ/lacZ}$ mice were scarified by decapitation. Brains were excised and immediately frozen in liquid nitrogen. After freezing, brains were covered with aluminum foil and kept at −80 °C until sectioning. Fifty μm brain sections were sliced with a cooled cryostat (Leica, CM1900) and mounted on Super Frost-coated slides. The slides with the slices were washed twice for 10 min with 0.01 M PBS, and then were fixated for 10 min at room temperature (RT) with the fixation solution (2% formaldehyde and 0.2% glutaraldehyde in PBS). After fixation the slides were washed again with PBS and incubated with the X-Gal staining solution (SigmaAldrich) at 37 °C for at least 2 h. The tissue was removed from the staining solution and the slides were washed for 30 min with PBS. After washing, Slow Fade antifade medium (Invitrogen) was added to each dry slice. Slides were kept in the dark at 4 °C until image acquisition.

2.6. Immunohistochemistry

Wild type and CaMKII-Cre;EphA4$^{lx/-}$ mice were anesthetized by injection of Ketamin/Xylazine and transcardially perfused with 30 ml of cold 0.01 M PBS solution, followed by 30 ml of 4%