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# Learning and memory impairment in Eph receptor A6 knockout mice

Katerina V. Savelieva, Indrani Rajan, Kevin B. Baker, Peter Vogel, Wendy Jarman, Margaret Allen, Thomas H. Lanthorn*

*Neuroscience Research, Lexicon Pharmaceuticals Incorporated, 8800 Technology Forest Place, The Woodlands, TX 77381-1160, USA*

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ABSTRACT

Genetic inhibition of the ephrin receptor (EphA6) in mice produced behavioral deficits specifically in tests of learning and memory. Using a fear conditioning training paradigm, mice deficient in EphA6 did not acquire the task as strongly as did wild type (WT) mice. When tested in the same context 24 h later, knockout (KO) mice did not freeze as much as WT mice indicating reduced memory of the consequences of the training context. The KO mice also displayed less freezing when presented with the conditioning stimulus (CS) in a separate context. In the hidden platform phase of the Morris water maze (MWM) task, KO mice did not reach the same level of proficiency as did WT mice. KO mice also exhibited less preference for the target quadrant during a probe trial and were significantly impaired on an initial reversal of the platform. These findings suggest that EphA6, in line with a number of other Eph receptors and their ephrin ligands, is involved in neural circuits underlying aspects of learning and memory.



Ephrin receptors (Eph) are receptor tyrosine kinases whose activity can be modulated by interaction with ligands, known as ephrins. The Eph family of receptors is subdivided into two classes, EphA and EphB (e.g. Ref. [17]). The EphA receptors interact with ephrin-A ligands, glycosylphosphatidylinositol (GPI)-anchored proteins. Members of the Eph receptors have been reported to be involved in the development of neural projection pathways (e.g. [17]). EphA6 may be particularly important for vomeronasal projections [12].

The functions of a number of Eph receptors and ephrins have been investigated. Several studies have focused on their activity in adult brain and overall in the hippocampus, in particular on learning and memory processes, using knockouts (KOs), transgenics, and various pharmacological manipulations (reviewed in Refs. [17,19,22]). EphB2, EphB3, EphA4, and EphA5 have been reported to have effects on processes and receptors involved in learning and memory. An elegant immunoadhesion-mediated inhibition or enhancement of EphA5 function in the adult hippocampus was shown to modulate two different hippocampal-dependent behaviors [8]. Inhibiting EphA5 exclusively in the adult hippocampus resulted in a significant deficit in context dependent fear conditioning, but not in cued fear conditioning. An EphB2 null mouse line generated by homologous recombination was evaluated in Morris water maze (MWM) [9]. However, since the KO's showed impaired learning in the visible phase of the test interpretation of the results was difficult.

The phenotype resulting from genetic inhibition of EphA6 has not been reported. EphA6 may be particularly interesting to examine as it has been reported to be more strongly expressed in brain relative to other Eph receptors [11]. Expression of EphA6 is high in the hippocampus, various regions of cortex, and the retina [13,15]. This localization suggests that EphA6 could also have effects on processes involved in learning and memory. As part of a collaborative effort between Genentech and Lexicon Pharmaceuticals to analyze the function of about 500 secreted and transmembrane proteins, we have investigated the behavior, including fear conditioning and MWM, of mice in which EphA6 has been genetically inhibited.

Standard homologous recombination strategy was used to delete exon 1 of EphA6 (NM_007938) ([1], Supplemental Methods and Fig. 1) in 129Sv/Ev$^{Brd}$ (LEX2) embryonic stem (ES) cells. Targeted ES cell clones were injected into C57BL/6J-*Tyr*$^{c\text{-}Brd}$ (albino) blastocysts and the resulting chimeras were mated to C57BL/6J-*Tyr*$^{c\text{-}Brd}$ (albino) females. Hets thus generated were used to produce F2 wild type (WT), Het, and KO littermates used in all assays.

All work was performed in accordance with Public Health Service Policies, the Animal Welfare Act, and the Lexicon Pharmaceuticals Incorporated Policy on the humane care and use of vertebrate animals. All experiments were approved by the Institutional Animal Care and Use Committee of Lexicon Pharmaceuticals, Inc. Animals used for all behavioral studies were male and female KO and WT littermates bred on a mixed (albino) C57BL/6J-*Tyr*$^{c\text{-}Brd}$ × 129S5/SvEv$^{Brd}$ genetic background [23]. All mice were maintained at Lexicon and were 11–12 weeks old and weighing 25–30 g at the time of testing. They were housed in groups of five in 30 cm × 20 cm × 20 cm acrylic cages with food and water

* Corresponding author. Tel.: +1 281 863 3120; fax: +1 281 863 8094.
*E-mail address:* tlanthorn@lexpharma.com (T.H. Lanthorn).

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freely available under a standard 12 h light/dark cycle (lights on at 7 a.m.).

Mice were tested in a standardized behavioral phenotyping battery. The data acquisition and analysis were performed by experimenters blind to genotype for all assays. A comprehensive phenotypic analysis (including a subset of behavioral tests derived from the Irwin screen) revealed no notable abnormalities across a wide range of behaviors as well as assays for cardiac, immune system, endocrine, and ophthalmic function (as an example, the phenotypic screen of VGLUT1 mice is accessible at http://www.informatics.jax.org/external/ko/lexicon/2383.html).

The assays in which no significant differences were detected between genotypes are described in Supplementary methods.

Fear conditioning was carried out using eight conditioning chambers (Coulbourn Instruments, Allentown, USA). For details regarding procedures on each day (training, context test and cued test—each separated by 24 h) see Supplemental methods. Freezing was recorded using video camera and Actimetrics FreezeFrame software.

For the Morris water maze the setup consisted of a circular pool 2 m in diameter and 40 cm in depth (Accuscan Instruments, Inc., Columbus, OH) and a WaterMaze Video Tracking System (Actimetrics, Inc., Wilmette, IL). The pool was filled to a depth of 30 cm with water maintained at 24–26 °C. In order to hide the visibility of the escape platform, the water was made opaque by the addition of non-toxic water-based paint. The escape platform (circular, 20 cm in diameter) was positioned 0.5 cm below the water surface in the middle of one of the quadrants (N, S, E, or W), designated as the test quadrant. Mice were held in the holding cage under the heat lamp between trials. There were three learning phases and two probe trials. The first phase was a pre-training (visible platform)-phase in which 19 WT mice (12 males and 7 females) and 18 KO mice (11 males and 7 females) were trained. During this phase the platform was made visible with a local clue (conical tube in a cylinder), which was put on the platform. The maze was surrounded with a curtain in order to hide all extra-maze clues. The mouse was released into the pool facing the wall of one of the quadrants (except the quadrant where the platform was located). The trial ended as soon as the mouse climbed onto the platform and remained on it for 10 s. Mice that failed to find the platform within 90 s were guided to it by the experimenter and had to stay on it for 10 s before being removed and placed back into the holding cage. This phase had two trials per day for 4 days, with inter-trial interval of 15 min. The next phase was the hidden training phase. Only those mice that learned to find visible platform during pre-training were included into hidden platform training (18 WT mice (11 males and 7 females) and 17 KO mice (10 males and 7 females)). This phase had two trials per day for 7 days. The releasing point differed at each trial, and different sequences of releasing points were used from day to day. The probe trials occurred prior to trial #11 (training on day 6), and 24 h after the last hidden training trial. During the probe trial, the platform was removed from the pool, and the mouse was placed into the pool facing the wall in the quadrant opposite from the training quadrant. The percentage of time spent in each quadrant during 60 s trial was recorded. In order to assess working memory of the mice the hidden phase was followed by 2 days of reversal phase. The reversal phase constitutes changing the location of the hidden platform on each of the 2 days to a quadrant that differs from training quadrant. Four training trials were run with each reversal. In order to have similar baseline performance, only those mice that performed above chance in the last probe trial (indicating that they learned the location of the hidden platform) were included in the reversal phase training (14 WT mice (8 males and 6 females) and 10 KO mice (6 males and 4 females)). Latency to reach the platform, path length and velocity were recorded for each trial. All trials were recorded by video camera and WaterMaze software (Actimetrics, Inc.). More details regarding MWM procedure can be found in Supplemental methods.

The Statistica 7.0 software package (StatSoft, Inc.) was used to determine significant differences between groups. The data from different tests was analyzed using unpaired two-tailed *t*-tests or RM ANOVA with genotype and sex as main effects, and trial as a repeated measure. Where no significant effect of sex or sex × genotype interaction was determined the data from both sexes was analyzed together. The data is presented as Mean ± S.E.M. The lines on the dot-plot graphs represent group means.

EphA6 KO mice appeared normal in terms of weight, length and in a battery of physiological and metabolic assays (data not shown) routinely run on all the mice generated with genetic deficiencies at Lexicon Pharmaceuticals [1,3]. General activity, anxiety, motor coordination, acoustic startle, sensorimotor gating, depressive-like behaviors and acute pain sensitivity did not differ from WT controls as assessed in the open field, platform (modification of the light:dark test as described by Pogorelov et al. [20]), marble burying, inverted screen, pre-pulse inhibition, tail suspension, and hot plate tests (Supplemental Table 1). No structural changes, particularly no neuropathology, were evident by histological examination (Supplemental Fig. 2).

In fear conditioning, the time spent freezing during training increased across trials in the WT mice (freezing after shock, freezing during the trace period between the tone and the shock [freezing to tone], and freezing preceding each tone). Although freezing also increased in the KO mice, there was a significant difference between genotypes. Specifically, freezing to tone, a possible specific index of learning the cued conditioning, was lower in the KO mice (Fig. 1A). The RM ANOVA revealed significant effects of geno-

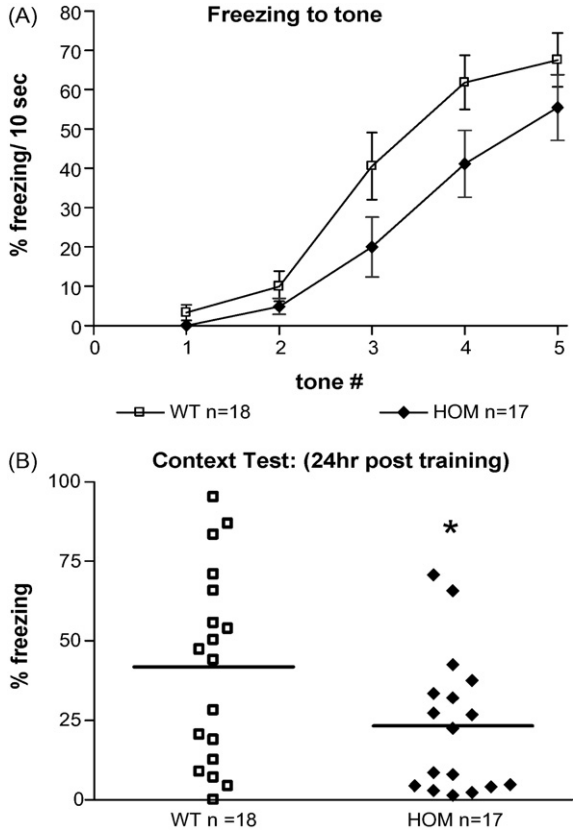


**Fig. 1.** (A) Freezing in response to tone during the 10 s trace interval after each tone termination during acquisition in trace conditioning assay. $p < 0.05$, RM ANOVA. (B) Freezing behavior during context test.* $p < 0.05$ (unpaired *t*-test).