Genes, Brain and Behavior
Official publication of the International Behavioural and Neural Genetics Society

Genes, Brain and Behavior (2012) **11:** 1020–1031 doi: 10.1111/j.1601-183X.2012.00842.x

Loss of EphA4 impairs short-term spatial recognition memory performance and locomotor habituation

R. Willi[†,‡,], C. Winter[§], F. Wieske[§], A. Kempf[†,‡], B. K. Yee[¶,††], M. E. Schwab[†,‡,*] and P. Singer[¶,††,*]**

[†]Brain Research Institute, University of Zurich, [‡]Department of Health Sciences and Technology, ETH Zurich, Zurich, Switzerland, [§]Department of Psychiatry and Psychotherapy, University Hospital Carl Gustav Carus at the Technical University Dresden, Dresden, Germany, [¶]Laboratory of Behavioral Neurobiology, Department of Biology, ETH Zurich, Schwerzenbach, Switzerland, [**]Present address: CNS Discovery, F. Hoffmann-La Roche Ltd, Basel, Switzerland, and [††]Present address: Robert Stone Dow Neurobiology Laboratories, Legacy Research Institute, Portland, OR, USA
**Corresponding authors: P. Singer, Robert Stone Dow Neurobiology Laboratories, Legacy Research Institute, 1225 NE 2nd Ave, Portland, OR 97232, USA. E-mail: psinger@downeurobiology.org; and*
M. E. Schwab, Brain Research Institute, University of Zurich, Zurich, Switzerland. E-mail: schwab@hifo.uzh.ch

EphA4 receptor (EphA4) tyrosine kinase is an important regulator of central nervous system development and synaptic plasticity in the mature brain, but its relevance to the control of normal behavior remains largely unexplored. This study is the first attempt to obtain a behavioral profile of constitutive homozygous and heterozygous EphA4 knockout mice. A deficit in locomotor habituation in the open field, impairment in spatial recognition in the Y-maze and reduced probability of spatial spontaneous alternation in the T-maze were identified in homozygous *EphA4*$^{-/-}$ mice, while heterozygous *EphA4*$^{+/-}$ mice appeared normal on these tests in comparison with wild-type (WT) controls. The multiple phenotypes observed in *EphA4*$^{-/-}$ mice might stem from an underlying deficit in habituation learning, reflecting an elementary form of nonassociative learning that is in contrast to Pavlovian associative learning, which appeared unaffected by EphA4 disruption. A deficit in motor coordination on the accelerating rotarod was also demonstrated only in *EphA4*$^{-/-}$ mice – a finding in keeping with the presence of abnormal gait in *EphA4*$^{-/-}$ mice – although they were able to improve performance over training. There was no evidence for substantial changes in major neurochemical markers in various brain regions rich in EphA4 as shown by post-mortem analysis. This excludes the possibility of major neurochemical compensation in the brain of *EphA4*$^{-/-}$ mice. In summary, we have demonstrated for the first time the behavioral significance of EphA4 disruption, supporting further investigation of EphA4 as a possible target for behavioral interventions where habituation deficits are prominent.

Keywords: Anxiety, cognition, EphA4, hippocampus, learning, memory, plasticity

Received 19 April 2012, revised 29 July 2012 and 22 August 2012, accepted for publication 25 August 2012

The Eph receptor A4 (EphA4) is a member of the Eph family of receptor tyrosine kinases (Ephs). Ephs and their cell surface-associated ephrin ligands are important regulators of central nervous system (CNS) development; they are involved in the guidance of axonal growth and establishment of neural connectivity (Egea & Klein 2007; Pasquale 2008). In the mature brain, Ephs regulate neuron–glia communication and play a role in synaptic plasticity (Klein 2009; Murai & Pasquale 2011). Among the subtypes, EphA4 shows widespread expression throughout the CNS starting from early development and persisting in the mature brain (Greferath et *al*. 2002). In the adult hippocampus, EphA4 is expressed on dendritic spines of pyramidal neurons and axon terminals (Bouvier *et al*. 2008; Carmona *et al*. 2009; Filosa *et al*. 2009; Murai *et al*. 2003; Tremblay *et al*. 2007), where it interacts with ephrin-A3 to shape dendritic spine morphology and influences synaptic plasticity (Carmona *et al*. 2009; Filosa *et al*. 2009; Murai *et al*. 2003). Eph dysfunction might be relevant to neuropsychiatric and neurodegenerative diseases (Inoue *et al*. 2009; Yamaguchi & Pasquale 2004) characterized by abnormal dendritic spines in the hippocampus and cortex (Glantz & Lewis 2000; Kaufmann & Moser 2000). Specifically, a genetic mouse model of Alzheimer's disease overexpressing human amyloid-β protein precursor was accompanied by reduced hippocampal EphA4 expression (Simón *et al*. 2009), suggesting that aberrant EphA4-mediated signaling might contribute in part to the memory deficits in this model.

However, the functional significance of EphA4 in normal behavior and cognition remains largely unexplored. This study is the first attempt to characterize the impact of constitutive genetic deletion of EphA4 on behavior and cognition in adulthood. Five behavioral tests were performed, including a test of motor coordination using the accelerating rotarod, open field locomotor activity, anxiety-related behavior in the elevated plus maze, spontaneous alternation in the T-maze, spatial recognition memory in the Y-maze and conditioned context freezing. Performance in the last four tests is sensitive, albeit not exclusively so, to hippocampal damage. They were selected because EphA4 is not only highly expressed in the hippocampus but also

Table 1: Sequence of behavioral testing and sample size of each experimental group per test

Cohort	Experiments	Duration (days)	Number of mice (WT/HT/KO)
Cohort I	Accelerating rotarod	3	10/9/9
Cohort II	Open field	2	11/12/9
	Y-maze	2	11/12/9
	T-maze	1	11/12/9
Cohort III*	Elevated plus maze	1	12/12/12
	Contextual fear conditioning	4	12/12/11†

The numbers illustrated represent the animals used in each of the experiments, after taking into account any unexpected loss of data or exclusion criterion. WT, *EphA4*$^{+/+}$; HT, *EphA4*$^{+/-}$; KO, *EphA4*$^{-/-}$.
*Brains of some of the animals from cohort III were dissected 4 weeks after completion of the last behavioral test and subsequently used for HPLC analyses.
†Data from one KO animal were excluded from analysis because it fell sick.

involved in hippocampal synaptic plasticity, such as long-term potentiation (Grunwald *et al*. 2004); and therefore *EphA4* may assume a role in hippocampus-dependent behavior. Both homozygous (*EphA4*$^{-/-}$) and heterozygous (*EphA4*$^{+/-}$) EphA4 knockout (KO) mice were tested and compared with WT littermate controls (*EphA4*$^{+/+}$).

We demonstrate here for the first time that *EphA4*$^{-/-}$ mice are associated with reduced locomotor habituation and impaired spatial novelty detection. In addition, we report a deficit in motor coordination and balance on the accelerating rotarod, which is most likely related to altered neuronal connections in the spinal cord and impaired hind limb gaits previously demonstrated in these animals (Akay *et al*. 2006; Dottori *et al*. 1998; Kullander *et al*. 2001). We also found no indication of potential developmental alterations in major neurochemical markers in the brain, although the contribution of other developmental factors cannot be excluded. This study highlights a critical role of EphA4 in short-term habituation processes and novelty assessment.

Materials and methods

Animals

Subjects were all adult male mice with a pure C57BL/6J genetic background. They comprised *EphA4*$^{-/-}$ (KO), *EphA4*$^{+/-}$ (HT) and *EphA4*$^{+/+}$ (WT) mice derived from *EphA4*$^{+/-}$ × *EphA4*$^{+/-}$ breeding. The generation of the mutant mice has been fully described elsewhere (Dottori *et al*. 1998; Kullander *et al*. 2001). Briefly, the line was originally created by electroporation of the target vector construct into W9.5 embryonic cells and two positive clones carrying a single targeted mutation of the *EphA4 gene* were injected into (C57BL/6 × C57Bl/10) F2 blastocysts. Chimeric mice were then mated to C57BL/6J mice. The resulting heterozygous EphA4 KO mice were backcrossed with pure C57BL/6J mice for more than 10 generations. The genotype of each animal was determined by polymerase chain reaction of tail DNA using specific probes for the *EphA4 gene* as described previously (Dottori *et al*. 1998; Kullander *et al*. 2001).

The animals were housed on a reversed 12/12-h light/dark cycle (lights on at 2000 h) under temperature- (21°C) and humidity-controlled (55%) conditions. Littermates were caged in groups of maximally six and maintained on *ad libitum* food and water. Testing was performed in 2- to 4-month-old mice and took place during the dark phase of the light/dark cycle. Three different cohorts of animals were used to accomplish all behavioral and cognitive tests of interest. The use of different cohorts served to minimize potential confounding factors associated with prolonged behavioral testing and aging. All necessary information regarding the design of the behavioral experiments is provided in Table 1, including the sequence and duration of the behavioral tests and the number of animals included in the final analyses. In general, the least stressful test was conducted first and the most stressful test last in order to minimize stress-related carry-over effects across tests.

We observed a reduction of body weight in *EphA4*$^{-/-}$ mice relative to *EphA4*$^{+/-}$ and *EphA4*$^{+/+}$ mice. The body weight was measured in 10-week-old animals (mean ± SEM): *EphA4*$^{-/-}$: 23.1 ± 0.6 g (n = 12), *EphA4*$^{+/-}$: 28.8 ± 0.6 g (n = 12) and *EphA4*$^{+/+}$: 28.8 ± 0.3 g (n = 12). It significantly differed between groups ($F_{2,33} = 38.01$, $P < 0.001$) and *post hoc* analysis confirmed that *EphA4*$^{-/-}$ mice were significantly lighter than *EphA4*$^{+/-}$ and *EphA4*$^{+/+}$ mice ($P < 0.001$).

All procedures described in this study had been approved by the Cantonal Veterinary Office in Zurich and are in agreement with the Principles of Laboratory Animal Care (National Institutes of Health publication no. 86–23, revised 1985). All efforts were made to minimize the animals' suffering or discomfort.

Western blotting

Two adult naive mice of each genotype were killed by cervical dislocation and brains were quickly dissected. For each animal, one complete hemisphere and the hippocampus from the other hemisphere were separately homogenized in CHAPS lysis buffer [60 mM CHAPS, 20 mM Tris (pH 8.0), 1 mM ethylenediaminetetraacetic acid (EDTA), Complete Mini EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland) and 1% phenylmethylsulfonyl fluoride in ethanol] using a EUROSTAR power basic stirrer (IKA, Staufen, Germany). The samples were then incubated for 30 min on ice and centrifuged for 15 min at 4°C and 15 000 ***g***. Total protein concentrations were determined using the Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA, USA). Equal amounts of protein (20 μg) were resolved by 4–12% NuPAGE gels (Invitrogen, Carlsbad, CA, USA) and transferred onto polyvinylidene fluoride membranes (GE Healthcare Osmonics, Bucks, UK). After blocking, membranes were incubated with primary antibodies (mouse anti-EphA4 receptor, Invitrogen, 1:200; mouse anti-GAPDH, Abcam, Cambridge, UK, 1:20 000) overnight at 4°C, washed and incubated with horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse IgG, 1:10 000) for 1 h at room temperature. Protein bands were detected with a chemiluminescent substrate system (SuperSignal West Pico; Pierce Biotechnology, Rockford, IL, USA). Images for Western blots were scanned and quantified with a chemiluminescent imaging system (Stella 3200; Raytest, Straubenhardt, Germany).

Motor coordination on the accelerating rotarod

Motor coordination was assessed using the rotarod. The device (Model 7650, Ugo Basile; Biological Research Apparatus, Comerio, Italy) and procedures have been fully described before (Willi *et al*. 2009). Five mice were tested concurrently. They were placed on the rotating drum at a baseline speed of 4 r.p.m., and the rotation rate

of the drum was then linearly increased from 4 to 40 r.p.m. over a period of 5 min. A trial ended when the animal fell off the rotating drum, or when a maximum of 5 min had elapsed. The latency to fall was recorded to index performance. Each mouse was given four trials per day with an intertrial interval of 1 h over three consecutive days.

Spontaneous locomotor activity in the open field

Spontaneous locomotor activity was evaluated using a standard open field exploration test. The apparatus consisted of four identical white open field arenas (40 × 40 × 35 cm) as described elsewhere (Meyer *et al*. 2005). Animals were allowed to freely explore the open field for 1 h on each of two consecutive days. Locomotor activity was indexed by the distance traveled (in centimeter) in the entire open field arena, which was expressed as a function of 5-min bins. As an additional measure, the number of rearings was counted. Finally, the time spent in the central region of the open field (13.33 × 13.33 cm) on the first testing day served as a measure to index phobia toward exposed places (Prut & Belzung 2003). All dependent measures were calculated by the EthoVision tracking system (Noldus Information Technology, Wageningen, the Netherlands).

Spatial recognition memory in the Y-maze

Short-term spatial recognition memory was examined by a spatial novelty preference task in the Y-maze as previously described (Vuillermot *et al*. 2011). This test is based on the natural tendency of rodents to preferentially explore novel over familiar spatial environments (Dellu *et al*. 1992). The Y-maze was made of transparent Plexiglas and comprised three identical arms (50 × 9 × 10 cm), radiating from a central triangle (8 cm on each side) and spaced 120° apart from each other. The test consisted of two phases separated by a variable time interval (delay). Each animal was assigned two arms (start arm and familiar arm) to which they were exposed during the sample phase. The remaining third arm constituted the novel arm to be used in the second phase (test phase). Allocation of arms (start, familiar and novel) was counterbalanced within each experimental group. Access to the novel arm was blocked during the sample phase. To start a trial, the animal was released from the end of the start arm facing the center of the maze. After entering the central triangular area, the animal was allowed to freely explore both the start and familiar arms for 5 min. In the test phase, the animal was returned to the maze for another 5-min exploration period with all arms accessible. Two delay intervals (2 and 15 min) between sample and test phases were tested in the same animals (short delay first). For each delay, the Y-maze was set up in a different room with a distinct set of extra-maze cues. During the delay, the animal was kept in a waiting cage in the testing room. To avoid olfactory cues, the entire maze floor was covered with fresh saw-dust prior to each visit to the maze. The time spent in each of the three arms and the distance traveled in the entire maze was recorded for each trial by the EthoVision v.3.2 tracking software (Noldus Technology). To index spatial novelty preference, the relative time spent in the novel arm during the choice phase was calculated by the following formula: [(time spent in the novel arm/time spent in all arms) × 100%].

Spontaneous alternation in the T-maze

Spontaneous alternation in the T-maze is considered a test of spatial working memory and it also makes use of the natural preference of rodents to explore a novel environment (Deacon & Rawlins 2006). The apparatus consisted of a modified elevated plus maze made of clear Plexiglas as fully described before (Muhia *et al*. 2010). One of the open arms was permanently closed off to create a T-maze (one open arm and two closed arms). Animals received two successive non-rewarded trials. Briefly, the animal was first allowed to enter one of the two equally unfamiliar choice arms (i.e. trial 1). The criterion of choosing an arm was defined as a complete body turn into an arm. After being confined to the chosen arm for 30 second, the animal was removed from the maze and then placed back on the start arm (i.e. trial 2), and once again allowed to choose freely between the same two arms that now differed in terms of familiarity – namely, a visited vs. an unvisited arm. Wild-type mice are expected to show a preference for the novel arm in the second trial, which is scored as a spontaneous alternation. The emphasis on the two-trial protocol – with only one opportunity to assess alternation on the second trial – was intended to be a more stringent and specific procedure than the multi-trial version as fully explained by Lalonde (2002) and Deacon and Rawlins (2006).

Anxiety-related behavior in the elevated plus maze

Unconditioned fear and anxiety-like behavior were assessed in the elevated plus maze test. The apparatus was an opaque Plexiglas plus maze, formed by two open (30 × 5 cm) and two enclosed (30 × 5 × 15 cm) arms radiating from a central platform (5 × 5 cm) as described before (Willi *et al*. 2009, 2010). The test began by placing the animal on the central platform with its head facing one of the open arms. The mouse was then allowed to freely explore the maze for 5 min. Anxiety-related behavior was measured by percent time spent in the open arms [time in open arms/(time in open + closed arms) × 100%] and percent entries made into the open arms [entries into open arms/(entries into open + closed arms) × 100%]. In addition, the total distance traveled in the entire maze (in centimeter) was recorded as a measure of locomotor activity. The raw data were calculated by the EthoVision tracking software (Noldus Technology).

Contextual fear conditioning

Contextual fear conditioning was assessed by the conditioned freezing paradigm using a procedure adapted from Pietropaolo *et al*. (2007). Two sets of four chambers were used to provide two distinct contexts (identified as context A and context B). Context A comprised four metal operant boxes (30 × 25 × 29 cm, model E10-10; Coulbourn Instruments, Allentown, PA, USA), each installed in a ventilated and sound-insulated Coulbourn Instruments chest. The animal was confined to a rectangular Plexiglas enclosure (17.5 × 13 cm) in the center of the operant box. The grid floor was made of stainless steel rods (4 mm in diameter) spaced at 10-mm intervals center to center, through which scrambled electric shocks (unconditioned stimulus, 0.25 mA) could be delivered (model E13-14; Coulbourn Instruments). Constant illumination was provided by a 2.8-W incandescent house light in each chamber. Context B comprised four semicircular Plexiglas enclosures (19 cm in diameter) resting on a smooth plastic floor. Each chamber was installed in a ventilated and sound-insulated wooden cabinet. Illumination was similar to that described for context A. In addition, a digital camera was mounted 30 cm directly above the area of interest in each chamber, and images were captured at a rate of 1 Hz. Freezing behavior was evaluated by comparing successive frames as described by Richmond *et al*. (1998). In summary, the automated image analysis algorithm compares and calculates the number of pixels differing between two images (192 × 144 = 27 648 pixels, at 8-bit grey scale) taken 1 second apart, with a criterion of freezing set at 0.05% – i.e., fewer than 14 pixels being different between the two images. The validity of this algorithm had been directly affirmed with blind hand-scoring.

The test procedures consisted of two phases: conditioning and tests of context freezing. On day 1, conditioning was conducted in context A and comprised three successive 1-second foot shock deliveries. Each shock was preceded and followed by a 180-second inter-shock interval (ISI). Context tests took place on days 2–4. Subjects were returned to context A on days 2 and 4 for a period of 300 seconds in the absence of any discrete stimulus. On day 3, freezing behavior was measured in a neutral context (context B) for a period of 300 seconds. Freezing responses in contexts A and B were compared with each other to dissociate conditioned freezing from general nonspecific freezing behavior. The percent time freezing across successive 180-seconds ISIs during conditioning and for each context session was calculated.

High-performance liquid chromatography

To evaluate whether there were basal neurotransmitter alterations in the brains of EphA4 KO mice, post-mortem neurochemical analysis

was performed using high-performance liquid chromatography (HPLC) as previously described (Enard *et al*. 2009; Winter *et al*. 2009). Mice were killed by cervical dislocation, their brains quickly dissected, frozen on dry ice and stored at −80°C until further processing. Tissue samples from both hemispheres were processed from the medial prefrontal cortex, caudate putamen, nucleus accumbens, hippocampus, amygdala and cerebellum via micropunches of 1-mm diameter. Samples were homogenized by ultrasonication in 0.1 N perchloric acid at 4°C immediately after collection by micropunches. Homogenates (100 μl) were then added to equal volumes of 1 N sodium hydroxide for protein content measurement. The remaining homogenates were centrifuged at 17 000 ***g*** and 4°C for 10 min. Aliquots of the supernatants were added to equal volumes (20 μl) of 0.5 M borate buffer and stored at −80°C for subsequent analyses of amino acids. The remaining supernatants were used for immediate measurement of monoamines and their metabolites. The levels of monoamines (dopamine and serotonin) and their metabolites (dihydroxyphenylacetic acid and 5-hydroxyindoleacetic acid) were measured by HPLC with electrochemical detection as fully described before (Felice *et al*. 1978; Sperk 1982; Sperk *et al*. 1981). Thus, the perchloric acid extracts were separated through a column (Prontosil 120-3-C18-SH; Bischoff Analysentechnik und -geräte GmbH, Leonberg, Germany) at a flow rate of 0.55 ml/min. The mobile phase consisted of 80 mM sodium dihydrogen phosphate, 0.85 mM octane-1-sulfonic acid sodium salt, 0.5 mM EDTA disodium salt, 0.92 mM phosphoric acid and 4% 2-propanol (all chemicals were purchased from Merck KGaA, Darmstadt, Germany). Monoamines were detected using an electrochemical detector (Model 41000; Chromsystems Instruments & Chemicals GmbH, Munich, Germany) at an electrode potential of 0.8 V. Sample analysis was performed based on peak areas using a computer-based chromatography data system (CSW 1.7; DataApex Ltd, Prague, Czech Republic) in relation to the mean of applied calibration solutions. Glutamate, γ-aminobutyric acid, glutamine and taurine were determined using methods described previously (Piepponen & Skujins 2001). Briefly, amino acids underwent pre-column derivatization with *o*-phthalaldehyde-2-mercaptoethanol using a refrigerated autoinjector and were then separated through an HPLC column (ProntoSil C18 ace-EPS) at a flow rate of 0.6 ml/min and a column temperature of 40°C. The mobile phase was 50 mM sodium acetate (pH 5.7) in a linear gradient from 5% to 21% acetonitrile. Derivatized amino acids were detected by their fluorescence at 450 nm after excitation at 330 nm.

Statistical analysis

Behavioral data (except spontaneous alternation data) were subjected to parametric analysis of variance (ANOVA), followed by Fisher's least significant difference (LSD) *post hoc* comparisons or restricted ANOVAs whenever appropriate. Data sets that failed to comply with the homoscedasticity assumption of parametric ANOVA according to the Levene's test of equal error variances were either subjected to appropriate data transformation or analysis by nonparametric tests. Accordingly, the open field data were logarithmically (base *e*) transformed prior to ANOVA; and the two anxiety indexes (percent time in, or entries into, open arms) were analyzed by the Kruskal–Wallis test. The neurochemical (HPLC) data were analyzed by multivariate analysis of variance (MANOVA). Finally, the numbers of alternating vs. non-alternating animals obtained in the spontaneous T-maze alternation task were analyzed using Fisher's exact test. All statistical analyses were carried out by PASW Statistics (version 18).

Results

Brain EphA4 protein expression

Western blot analysis of whole brain and hippocampal lysates of *EphA4*$^{-/-}$, *EphA4*$^{+/-}$ and *EphA4*$^{+/+}$ animals showed the following pattern of results: EphA4 levels in heterozygous EphA4 KOs were reduced by about 40–50%, whereas EphA4 was completely absent in homozygous EphA4 KOs (Fig. 1).

Motor coordination on the accelerating rotarod

The latency to fall was analyzed by a 3 × 3 × 4 (genotype × days × trials) repeated measures ANOVA, which confirmed the presence of a general improvement in motor performance over days (days: $F_{2,50} = 45.37$, $P < 0.001$) and showed a significant main effect of genotype ($F_{2,25} = 10.02$, $P < 0.001$) that was accompanied by a marginally significant genotype × days interaction ($F_{4,50} = 2.35$, $P = 0.067$). Subsequent *post hoc* analyses showed that *EphA4*$^{-/-}$ mice fell significantly earlier (i.e. shorter latency) than *EphA4*$^{+/+}$ ($P < 0.01$) or *EphA4*$^{+/-}$ animals ($P < 0.001$) (Fig. 2).

Spontaneous locomotor activity in the open field

Locomotor activity indexed by horizontal distance traveled in the open field appeared higher in the *EphA4*$^{-/-}$ mice on both test days, and this effect seemed to be accompanied by an increase in variability (Fig. 3). Levene's test of equality of variances confirmed that the *EphA4*$^{-/-}$ group exhibited significantly higher variance in the average distance traveled across the 2 days relative to the other two groups ($F_{2,29} = 11.99$, $P < 0.001$), as illustrated by the estimated standard deviations: WT = 238.41, HT = 213.08 and KO = 638.60 cm per 5-min bin. To meet the homoscedasticity assumption of parametric ANOVA, the activity data were subjected to a logarithmic transformation to minimize the impact of between-group difference on variance (Levene's test: $P = 0.20$).

The 3 × 2 × 12 (genotype × days × 5-min bins) repeated-measures ANOVA of the log-transformed activity data yielded a significant main effect of genotype ($F_{2,29} = 8.59$, $P < 0.001$), indicating that locomotor activity was enhanced in the *EphA4*$^{-/-}$ mice (Fig. 4). Subsequent *post hoc* analyses confirmed that the overall distance traveled was significantly increased on both days in *EphA4*$^{-/-}$ mice relative to *EphA4*$^{+/+}$ mice (day 1: $P < 0.001$, day 2: $P < 0.01$) and *EphA4*$^{+/-}$ mice (day 1: $P < 0.001$, day 2: $P < 0.01$). Hyperlocomotion in *EphA4*$^{-/-}$ mice mainly resulted from a failure of within-session habituation which was evident on both days. This gave rise to a significant main effect of 5-min bins ($F_{11,319} = 24.13$, $P < 0.001$) and a genotype × bins interaction ($F_{22,319} = 1.86$, $P < 0.05$). Irrespective of genotype, locomotor activity was reduced on the second day compared to the first day, yielding a main effect of days ($F_{1,29} = 16.43$, $P < 0.001$). The genotype × days interaction did not achieve statistical significance. Parallel analysis of the number of rearings recorded in the open field did not yield any significant effects (data not shown).

In addition, the time spent in and entries made into the central region of the open field on the first day were calculated (Fig. 5) and subjected to separate one-way ANOVAs, which yielded a significant genotype effect (time: $F_{2,29} = 9.13$, $P < 0.01$; entries: $F_{2,29} = 16.65$, $P < 0.001$). Subsequent Fisher's LSD comparisons showed that *EphA4*$^{-/-}$ mice spent significantly more time in and made significantly more entries into the central region than the other two groups ($P < 0.01$), indicating that *EphA4*$^{-/-}$ mice were less anxious in exploring open and exposed spaces.

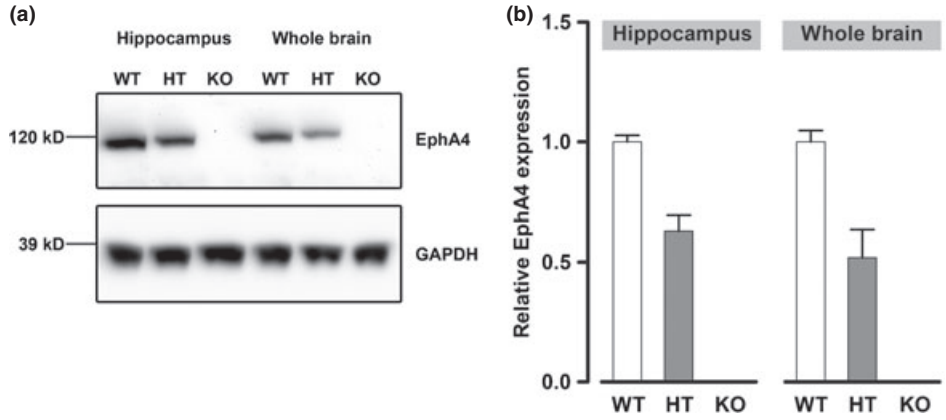

Figure 1: Endogenous EphA4 protein levels in *EphA4*$^{-/-}$ mice. (a) Immunoblotting with an anti-EphA4 antibody and an anti-GAPDH antibody as internal standard. Total brain and hippocampal lysates from *EphA4*$^{+/+}$ (WT), *EphA4*$^{+/-}$ (HT) and *EphA4*$^{-/-}$ (KO) mice were loaded in the respective lanes. Molecular weight of the markers is indicated on the left. (b) Densitometry of immunoblots for relative EphA4 levels. EphA4 expression was reduced by about 40–50% in HT and completely absent in KO mice. All values are mean ± SE. $n = 2$ per genotype.

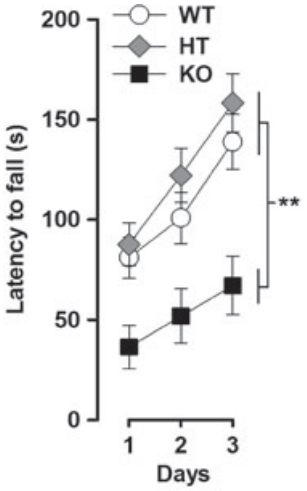

Figure 2: Decreased motor coordination on the accelerating rotarod in *EphA4*$^{-/-}$ mice. Latency to fall is expressed as a function of days. *EphA4*$^{-/-}$ (KO) mice displayed significantly reduced motor coordination compared to *EphA4*$^{+/+}$ (WT) and *EphA4*$^{+/-}$ (HT) mice. $^{**}P < 0.01$ based on Fisher's LSD *post hoc* comparison. All values are mean ± SE. WT, $n = 10$; HT, $n = 9$; KO, $n = 9$.

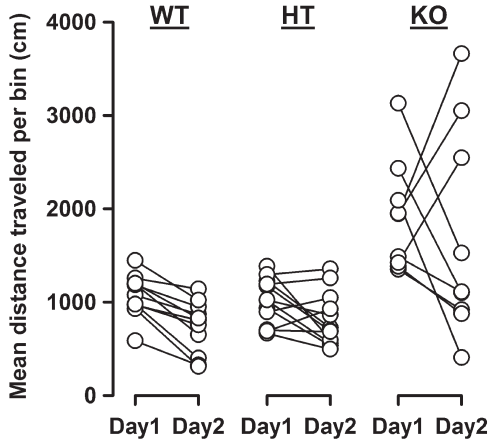

Figure 3: Individual differences in open field activity. This illustrates that the *EphA4*$^{-/-}$ (KO) group exhibited higher variability compared with the *EphA4*$^{+/+}$ (WT) and *EphA4*$^{+/-}$ (HT) groups, which was confirmed by Levene's test of equality of variances (see *Results*). The genotype difference appeared more pronounced on day 2 (estimated group standard deviation: WT = 281.27, HT = 285.09, KO = 1122.84 cm) than on day 1 (estimated group standard deviation: WT = 224.32, HT = 245.26, KO = 592.10 cm). The activity data were therefore log transformed prior to parametric ANOVA as illustrated in Fig. 4. All values are mean ± SE. WT, $n = 11$; HT, $n = 12$; KO, $n = 9$.

Spatial recognition memory in the Y-maze

Spatial recognition memory in the Y-maze was indexed by the relative time spent in the novel arm during the choice phase of this test. Analysis of percent time in the novel arm by a 3 × 2 (genotype × delays) repeated measures ANOVA yielded only a significant main effect of genotype ($F_{2,29} = 3.98$, $P < 0.05$). Subsequent *post hoc* analyses confirmed a significant decrease in spatial novelty preference in *EphA4*$^{-/-}$ mice relative to *EphA4*$^{+/+}$ ($P < 0.05$) and *EphA4*$^{+/-}$ mice ($P < 0.05$) (Fig. 6a). Likewise, the absolute time spent in the novel arm was significantly reduced in the *EphA4*$^{-/-}$ mice compared with the other two groups across the entire 5-min test period ($F_{2,29} = 4.06$, $P < 0.05$; Fig. 6b). In line with this impression, a 3 × 2 × 5 (genotype × delays × 1-min bins) ANOVA showed only a significant main effect of genotype ($F_{2,29} = 3.98$, $P < 0.05$). In addition, locomotor activity was analyzed by a 3 × 2 × 5 (genotype × delays × 1-min bins) repeated

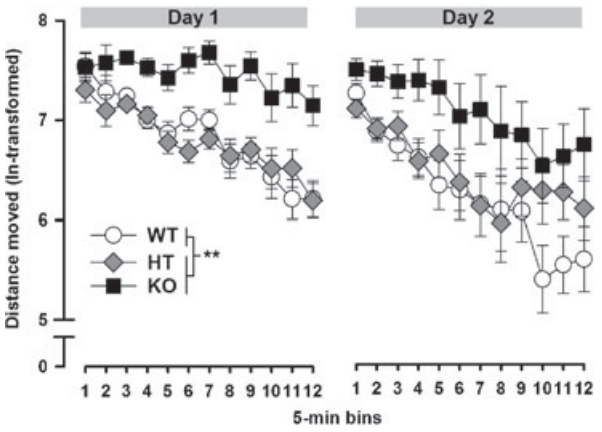

Figure 4: Increased spontaneous locomotor activity in *EphA4*$^{-/-}$ mice. Distance moved (cm) is expressed as a function of 5-min bins (*ln* transformed). A clear habituation effect is lacking in *EphA4*$^{-/-}$ (KO) mice on both testing days, as evidenced by a significant genotype × 5-min bins interaction ($P < 0.05$). Furthermore, *EphA4*$^{-/-}$ mice displayed a significant overall increase in distance moved compared to *EphA4*$^{+/+}$ (WT) and *EphA4*$^{+/-}$ (HT) mice. $^{**}P < 0.01$ based on Fisher's LSD *post hoc* comparison. All values are mean ± SE. WT, $n = 11$; HT, $n = 12$; KO, $n = 9$.

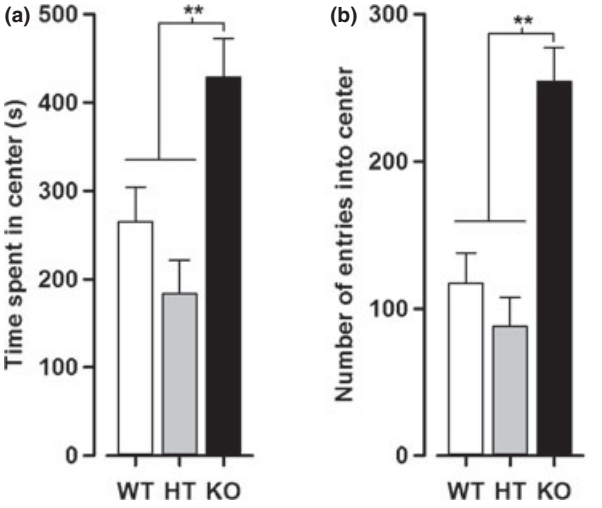

Figure 5: Reduced anxiety levels in *EphA4*$^{-/-}$ mice. Time spent in (a) and number of entries made into (b) the central part of the open field arena were increased in the *EphA4*$^{-/-}$ (KO) mice relative to *EphA4*$^{+/+}$ (WT) and *EphA4*$^{+/-}$ (HT) mice. $^{**}P < 0.01$ based on Fisher's LSD *post hoc* comparison. All values are mean ± SE. WT, $n = 11$; HT, $n = 12$; KO, $n = 9$.

measures ANOVA of distance moved (Fig. 6c). Consistent with the activity data obtained in the open field experiment, *EphA4*$^{-/-}$ mice were hyperactive ($F_{2,29} = 3.80$, $P < 0.05$), which was associated with weaker locomotor habituation as confirmed by the presence of a significant genotype × bins interaction ($F_{8,116} = 3.10$, $P < 0.05$). To exclude a potential confounding effect of hyperlocomotor activity on the Y-maze novelty preference of the *EphA4*$^{-/-}$ mice, we performed a restricted 3 × 2 × 3 (genotype × delay × 1-min bins) ANOVA of percent time spent in the novel arm over the first 3 min of the test when locomotor activity was comparable between groups. The main effect of genotype remained highly significant ($F_{2,29} = 6.20$, $P < 0.01$), and additional Fisher's LSD *post hoc* comparisons again indicated that *EphA4*$^{-/-}$ mice spent significantly less time in the novel arm compared with both *EphA4*$^{+/+}$ and *EphA4*$^{+/-}$ mice ($P < 0.05$). One-sample *t*-tests further verified that *EphA4*$^{+/+}$ and *EphA4*$^{+/-}$ mice, but not *EphA4*$^{-/-}$ mice, showed a significant preference for the novel arm above chance ($P < 0.05$). It is therefore valid to conclude that the Y-maze phenotype was independent of the concomitant hyperactivity phenotype.

Spontaneous alternation in the T-maze

The tendency to select the unvisited arm on the second trial (i.e. alternate) was markedly reduced in *EphA4*$^{-/-}$ compared with *EphA4*$^{+/+}$ and *EphA4*$^{+/-}$ mice. 10 out of 11 *EphA4*$^{+/+}$ and 10 out of 12 *EphA4*$^{+/-}$ mice alternated their response, whereas only 3 out of 9 EphA4A$^{-/-}$ mice alternated. Fisher's exact test yielded a significant effect ($P < 0.05$) for the observed pattern of alternating vs. non-alternating animals.

Anxiety-related behavior in the elevated plus maze

Anxiety-related behavior in the elevated plus maze test was indexed by percent time spent in the open arms and percent entries made into the open arms. Because the ANOVA assumption of equal variances was violated due to higher variability in the *EphA4*$^{-/-}$ group, the data were analyzed by the nonparametric Kruskal–Wallis test, which failed to show a significant group difference ($P > 0.2$). Respective box plots are shown in Fig. 7. In addition, we analyzed the absolute time spent in the central part of the plus maze, which showed no significant group difference ($F_{2,33} = 0.79$, $P = 0.46$) based on one-way ANOVA (equality of variance assumption was met). The mean center time per group was: *EphA4*$^{+/+}$ = 41.10 seconds, *EphA4*$^{-/+}$ = 40.35 seconds, *EphA4*$^{-/-}$ = 33.30 seconds (standard error of difference between sample means = 4.85 seconds). Furthermore, the absolute time spent in the open arms as well as locomotor activity as indexed by the distance traveled in the entire maze did not differ between groups (data not shown).

Contextual fear conditioning

The development of conditioned freezing was analyzed by a 3 × 4 (genotype × ISIs) repeated-measures ANOVA of percent time freezing. Acquisition of conditioned freezing was apparent in all three groups by the increase in freezing levels across successive ISIs. This led to a significant main effect of ISIs ($F_{3,96} = 110.95$, $P < 0.001$). Notably, ANOVA showed no significant main effect of genotype or interaction between genotype and ISIs, suggesting that KO of EphA4 did not compromise the development of conditioned freezing to the context (Fig. 8a).

Context freezing in the conditioned context A (retrieval test sessions on days 2 and 4 of the experiment) was

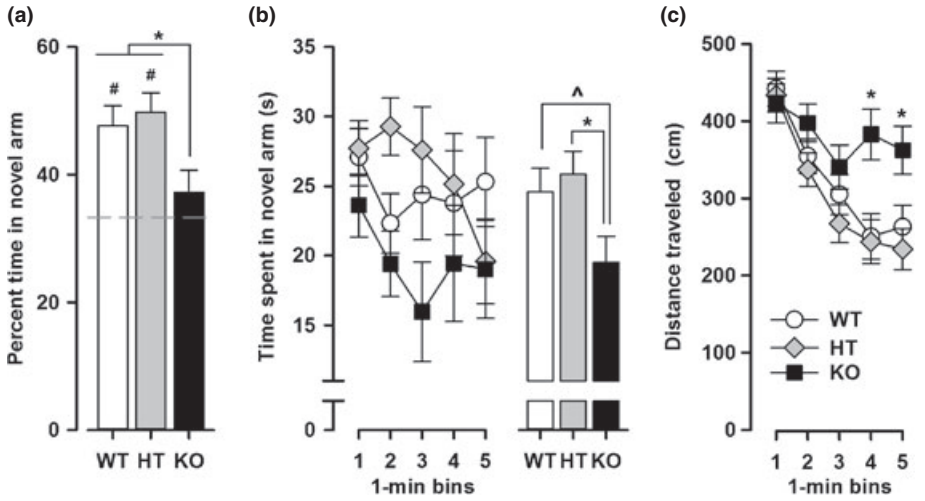

Figure 6: Impaired spatial recognition memory in the Y-maze in *EphA4*$^{-/-}$ mice. (a) Percent time spent in the novel (previously unexplored) arm during the test phases. *EphA4*$^{-/-}$ (KO) mice performed close to chance level (as represented by the dashed line) in both tests and they showed significantly lower spatial novelty preference compared to *EphA4*$^{+/+}$ (WT) and *EphA4*$^{+/-}$ (HT) mice. (b) The absolute time spent in the novel arm was also reduced in the KO animals relative to the other two groups. (c) Locomotor activity during the test phase showed a habituation deficit in the KO group as indicated by a significant two-way (genotype × bins) interaction. *$P < 0.05$, ^$P = 0.06$ based on Fisher's LSD *post hoc* comparison. # indicates that performance was significantly above chance level ($P < 0.05$). All data were averaged across the two delay conditions. All values are mean ± SE. WT, $n = 11$; HT, $n = 12$; KO, $n = 9$.

analyzed by a 3 × 2 (genotype × test sessions) repeated measures ANOVA of percent freezing (Fig. 8b). The freezing response did not differ between groups but was generally lower on the second relative to the first context re-exposure session as indicated by a significant effect of test sessions ($F_{1,32} = 24.60$, $P < 0.001$). Hence, successive re-exposure to the conditioned context from one test session to the next was sufficient to induce a significant reduction of the conditioned freezing response. This reduction corresponds to the operational definition of experimental extinction (Pavlov 1927), although we cannot exclude the possibility that memory reconsolidation processes [triggered by the first conditioned stimulus (CS) re-exposure] might also be implicated in the determination of freezing levels observed on the second re-exposure to the conditioned context (Suzuki *et al*. 2004; Yamada *et al*. 2009). Irrespective of whether extinction learning or reconsolidation might predominate here, our freezing data clearly indicate that neither acquisition nor expression of conditioned context freezing was altered by EphA4 deletion. Furthermore, freezing behavior in the neutral context B (day 3 of the experiment) also did not differ between genotypes ($F < 1$). The duration of freezing recorded in the neutral context B remained low, and it was substantially below that observed during re-exposure to context A (the conditioned context) on day 2 ($F_{1,32} = 178.03$, $P < 0.001$) and day 4 ($F_{1,32} = 178.03$, $P < 0.001$) of the experiment.

Brain neurotransmitter levels

Standard HPLC analyses were performed in order to identify putative effects of EphA4 deletion at the neurotransmitter level. Basal levels of the major neurotransmitters and their metabolites in selected brain regions of each genotype are depicted in Table 2. Analysis of neurochemical concentrations by a one-way MANOVA failed to show a significant effect of genotype using Wilks' Lambda test [$\Lambda = 0.02$, $F_{df} = 0.48$, $P = 0.85$]. In fact, the overall effect of genotype was far from statistical significance, indicating that the pattern of measured neurochemical markers did not substantially differ between groups.

Discussion

This study showed that constitutive homozygous deletion of EphA4 yielded multiple phenotypes on (1) the open field test of spontaneous motor activity and motor habituation, (2) the Y-maze test of short-term familiarity judgment underlying spatial recognition and (3) spontaneous alternation on the T-maze , without affecting Pavlovian contextual fear conditioning. Furthermore, a deficit in motor function was detected in *EphA4*$^{-/-}$ mice, which is most likely related to the hind limb coordination problems previously identified in these animals (Akay *et al*. 2006; Dottori *et al*. 1998; Kullander *et al*. 2001).

Anatomical studies of *EphA4*$^{-/-}$ mice have shown that genetic deletion of EphA4 led to dysfunctional central pattern generators in the spinal cord responsible for the coordination of limb alternation underlying normal walking (Kiehn & Butt 2003; Kullander *et al*. 2001, 2003), as indicated by the 'hopping gait' in *EphA4*$^{-/-}$ mice (Dottori *et al*. 1998; Kullander *et al*. 2001) possibly attributable to an

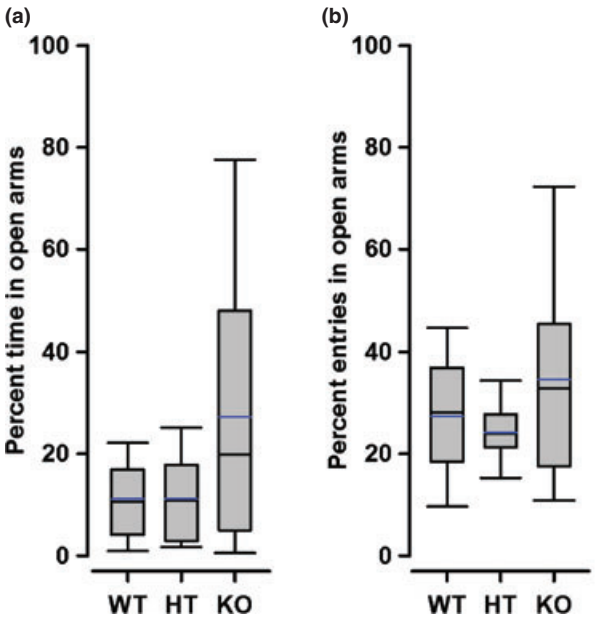

Figure 7: Unaltered anxiety-related behavior in the elevated plus maze in *EphA4*$^{-/-}$ mice. Percent time spent in the open arms (a) and percent entries made into the open arms (b) are shown as standard box plots. The blue line denotes group mean, and the ends of the whiskers refer to the 5th and 95th percentiles, respectively. WT, $n = 12$; HT, $n = 12$; KO, $n = 12$.

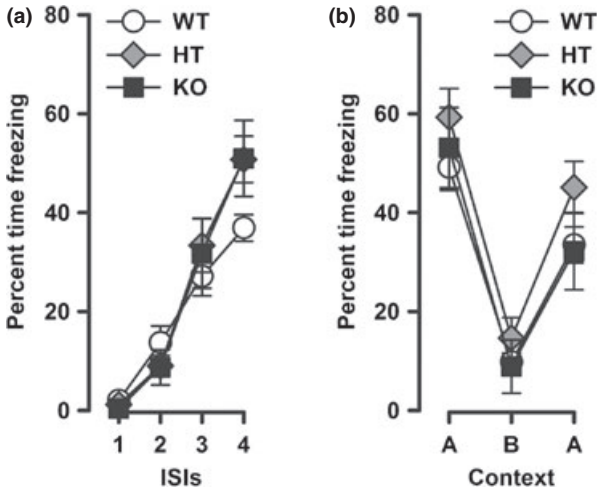

Figure 8: Unaltered fear conditioning in the context freezing paradigm in *EphA4*$^{-/-}$ mice. (a) Expression of freezing behavior in the context across successive ISIs during conditioning. Freezing is expressed as the percent time freezing per ISI. *EphA4*$^{-/-}$ (KO), *EphA4*$^{+/-}$ (HT) and *EphA4*$^{+/+}$ (WT) mice exhibited similar freezing levels. (b) Freezing to the context 24, 48 and 72 h after conditioning. This is expressed as the percent time spent freezing to context A and context B, respectively. Again, all groups exhibited similar freezing levels. All values are mean ± SE. WT, $n = 12$; HT, $n = 12$; KO, $n = 11$.

excitation/inhibition imbalance in the spinal cord (Restrepo *et al*. 2011). The severe deficit in rotarod performance in the *EphA4*$^{-/-}$ mice reported here is also novel, but not surprising. Despite this deficit, *EphA4*$^{-/-}$ mice nonetheless were able to demonstrate an improvement over training, suggesting that EphA4 deletion did not completely disrupt motor skill learning, which apparently requires the cerebellum (Lalonde & Strazielle 2007; Llinas & Welsh 1993). Hence, even though EphA4 is heavily expressed in the cerebellum from neonatal to adult life (Greferath *et al*. 2002; Karam *et al*. 2000; Liebl *et al*. 2003; Martone *et al*. 1997; Xiao *et al*. 2006) and is implicated in cerebellar wiring (Cesa *et al*. 2011; Karam *et al*. 2000), its deletion did not completely prevent cerebellum-dependent motor skill learning.

A previous study has reported that *EphA4*$^{-/-}$ mice were less active in the open field than *EphA4*$^{+/-}$ mice (Dottori *et al*. 1998), but examination of locomotor activity in that study was limited to 5 min, and the authors did not provide any data on WT controls for comparison. This reported phenotype is seemingly opposite to our current finding of hyperlocomotor activity in *EphA4*$^{-/-}$ mice. However, the hyperlocomotor activity phenotype did not emerge until habituation became evident in the *EphA4*$^{+/-}$ and *EphA4*$^{+/+}$ mice, which emerged after the first 5 min and persisted until the end of the 1-h test. This observation was replicated on the second test session 24 h later. In contrast to this deficit in within-session motor habituation, habituation across test sessions as evidenced by the overall reduction of activity levels from day 1 to day 2 was not significantly altered in *EphA4*$^{-/-}$ mice. Thus, the open field locomotor activity phenotype suggests an underlying deficit in short-term, but not in long-term, locomotor habituation.

At the same time, we also observed that the *EphA4*$^{-/-}$ mice spent significantly more time in the central zone of the open field, which might be interpreted as anxiolysis (Prut & Belzung 2003); but this interpretation should be cautioned because the behavior of *EphA4*$^{-/-}$ mice in the elevated plus maze test of anxiety was not significantly altered. Additional tests of anxiety would be warranted to ascertain whether the null results in the elevated plus maze might represent a type-II error attributable to the higher level of individual variability amongst the *EphA4*$^{-/-}$ mice.

Short-term habituation is a critical factor that influences spontaneous alternation behavior in the T-maze and spatial recognition memory in the Y-maze (Honey & Good 2000; Sanderson *et al*. 2010). Both tests are based on the innate motivation of mice to explore novel environments, whereby the preferential exploration of a novel over a familiar environment is commonly taken as an index of spatial novelty detection (Dellu *et al*. 1992; Dember & Fowler 1958). Wagner's sometimes opponent process (SOP) model (Wagner 1981) describes short-term habituation as a shift from the center to the periphery of attention, such that stimuli in the periphery of attention are less able to elicit a response. It follows that the novel arm in the Y-maze and the T-maze commands more attention and therefore elicits more intense exploration than the familiarized, visited arm, which would be in the periphery of attention according to Wagner's SOP model. Short-term habituation is defined as decrement in

Table 2: Basal brain neurotransmitter levels in WT vs. EphA4-deficient mice

Region	Group	Dopamine	DOPAC	Serotonin	5-HIAA	Glutamate	Glutamine	Taurine	GABA
mPFC	WT	15.23 ± 5.67	5.97 ± 1.92	14.53 ± 2.91	2.40 ± 0.71	99.90 ± 6.04	36.63 ± 1.53	94.16 ± 4.57	19.16 ± 1.90
	HT	19.74 ± 8.47	6.27 ± 1.42	14.50 ± 1.56	2.04 ± 0.28	97.17 ± 5.85	38.63 ± 1.93	90.45 ± 3.41	22.64 ± 4.01
	KO	15.05 ± 4.91	5.95 ± 1.84	15.52 ± 1.23	2.99 ± 0.33	98.38 ± 4.33	36.19 ± 2.57	92.22 ± 3.74	17.80 ± 2.35
CPu	WT	745.51 ± 38.56	57.81 ± 9.81	10.79 ± 1.56	2.41 ± 0.64	78.94 ± 4.19	41.13 ± 1.93	113.61 ± 6.51	14.57 ± 0.59
	HT	830.04 ± 31.04	48.49 ± 3.21	11.27 ± 1.78	2.59 ± 0.54	76.30 ± 2.88	40.34 ± 1.81	106.66 ± 4.69	13.99 ± 0.54
	KO	800.78 ± 34.27	50.93 ± 2.24	8.90 ± 0.61	2.63 ± 0.26	75.79 ± 2.90	40.46 ± 1.14	110.45 ± 4.26	14.00 ± 0.42
NAc	WT	259.35 ± 30.81	31.03 ± 3.24	22.42 ± 2.07	5.37 ± 1.06	70.31 ± 2.67	46.23 ± 2.48	74.66 ± 3.47	44.09 ± 5.27
	HT	396.73 ± 21.67	37.10 ± 1.99	25.44 ± 2.23	5.00 ± 0.58	75.97 ± 4.22	46.08 ± 2.85	76.80 ± 3.92	44.33 ± 6.51
	KO	248.26 ± 47.81	25.94 ± 4.67	22.77 ± 4.45	4.42 ± 0.77	73.71 ± 3.62	42.11 ± 3.22	80.84 ± 3.79	28.12 ± 5.69
HPC	WT	6.03 ± 0.77	1.72 ± 0.12	14.15 ± 1.18	5.88 ± 0.93	84.40 ± 3.68	37.12 ± 1.52	81.94 ± 3.49	23.37 ± 0.73
	HT	7.13 ± 0.88	1.97 ± 0.25	17.34 ± 1.28	7.71 ± 0.92	84.30 ± 2.95	37.56 ± 1.71	81.01 ± 3.67	23.34 ± 0.89
	KO	6.42 ± 0.85	1.72 ± 0.19	17.80 ± 0.73	6.63 ± 0.85	84.37 ± 4.56	38.58 ± 2.05	88.34 ± 4.42	23.66 ± 1.46
AM	WT	27.06 ± 6.57	4.18 ± 0.71	29.93 ± 4.42	5.77 ± 0.82	84.77 ± 3.73	43.03 ± 1.53	72.99 ± 3.23	25.26 ± 1.41
	HT	21.44 ± 1.97	3.20 ± 0.35	30.38 ± 3.31	5.95 ± 0.64	94.28 ± 6.06	46.89 ± 2.17	80.62 ± 5.18	24.78 ± 1.40
	KO	34.70 ± 5.02	5.26 ± 0.33	31.18 ± 1.63	6.06 ± 0.44	85.35 ± 4.70	43.64 ± 2.21	77.02 ± 4.23	24.36 ± 1.62
CB	WT	5.32 ± 0.52	1.65 ± 0.21	8.28 ± 0.93	2.46 ± 0.55	57.38 ± 1.56	27.73 ± 1.01	29.85 ± 1.10	16.84 ± 0.46
	HT	4.84 ± 0.61	2.12 ± 0.38	12.88 ± 2.04	3.04 ± 0.53	58.05 ± 2.10	28.56 ± 1.63	31.28 ± 3.20	16.62 ± 1.16
	KO	5.43 ± 0.47	2.24 ± 0.41	9.00 ± 0.62	2.34 ± 0.26	56.96 ± 2.17	28.59 ± 1.16	29.73 ± 1.15	16.82 ± 0.64

Neurotransmitter levels are expressed as mean concentrations in nmol/mg protein (± SE); $n = 7–11$.
5-HIAA, 5-hydroxyindoleacetic acid; AM, amygdala; CB, cerebellum; CPu, caudate putamen; DOPAC, dihydroxyphenylacetic acid; GABA, γ-aminobutyric acid; HPC, hippocampus; mPFC, medial prefrontal cortex; NAc, nucleus accumbens.

behavioral response when a stimulus is presented repeatedly and is considered an elementary form of nonassociative learning (Thompson 2009; Thompson & Spencer 1966). In this sense, locomotor hyperactivity, disruption of alternation behavior in the T-maze and impaired spatial novelty detection observed in the Y-maze in the *EphA4*$^{-/-}$ mice may reflect a form of cognitive deficit specific to nonassociative learning. By contrast, associative learning appears to be unaffected in these animals as evidenced by intact Pavlovian fear conditioning. There is indeed evidence that performance in the T-maze and Y-maze tests primarily relies on short-term habituation but is largely independent of associative learning (Sanderson *et al*. 2010). Hence, a specific deficit in short-term habituation may provide a parsimonious account of the three phenotypes identified in the *EphA4*$^{-/-}$ mice in the open field as well as the Y- and T-mazes.

Although the use of a constitutive KO model does not allow one to dissect region-specific mechanisms, speculations on the involvement of relevant brain regions can guide further investigation. One plausible candidate region is the hippocampus where EphA4 is critical for the regulation of synaptic plasticity including long-term potentiation (Grunwald *et al*. 2004). Hippocampal lesions not only induce hyperactivity and retard habituation (Poucet 1989) but also robustly impair spontaneous alternation in the T-maze and spatial recognition memory in the Y-maze (Bannerman *et al*. 2004; O'Keefe & Nadel 1978). Studies of neuronal activity also suggest a specific role of the hippocampus in the detection of spatial novelty (Rinaldi et *al*. 2010; Zhu *et al*. 1997). Furthermore, several theories have linked the hippocampus to the ability to compare the present state of the world with what is expected based on retrieved memories to guide appropriate behavioral response (Gray 1982; McNaughton 2006; Vinogradova 2001). According to this concept, Gray (1982) suggested that an important function of the hippocampus is to increase arousal and attentional processing following detection of a novel stimulus and thus to enhance exploration (McNaughton 2006; Vinogradova 2001). However, the involvement of hippocampal EphA4 and in particular the contribution of hippocampal dysfunction to the behavioral phenotypes identified in *EphA4*$^{-/-}$ mice should not be overestimated, because hippocampus-dependent contextual fear conditioning remained intact in *EphA4*$^{-/-}$ mice. Instead, contextual fear conditioning seems to be regulated by another member of the EphA receptor family, namely the EphA5 subtype (Gerlai *et al*. 1999). Nevertheless, the role of EphA4 as a potential regulator of hippocampal functions certainly deserves further investigation by using region-specific KO models in order to examine whether disruption of EphA4 in the hippocampus alone would be sufficient to reproduce the behavioral phenotypes seen in constitutive KOs here. Another candidate brain region that warrants consideration is the striatum, which is rich in EphA4, and where EphA4 regulates the development of the spatial patterning of the striatum during maturation (Passante *et al*. 2008). The striatum is implicated in the regulation of locomotion and novelty-evoked behavioral activation (Berns *et al*. 1997; Hooks and Kalivas 1995; Rinaldi et *al*. 2010). Although there is evidence for a participation of the striatum in spatial novelty detection and spontaneous alternation behavior, its precise role is in need of further clarification (Cigrang *et al*. 1986; Hagan *et al*. 1983; Lalonde 2002; Roullet *et al*. 2001; Taghzouti *et al*. 1985; Thullier *et al*. 1996; Usiello *et al*. 1998). The striatum is also known for its key role in procedural and habit learning (Knowlton et al. 1996; Mishkin & Petri 1984; Yin et al. 2004), which has not been explicitly tested in this study. Nonetheless, a contribution of altered striatal function to the behavioral phenotypes of the *EphA4*$^{-/-}$ mice should be considered, although not necessarily in exclusion of a parallel hippocampal contribution in our constitutive KO model.

Here, none of the behavioral phenotypes identified in the homozygous EphA4 KOs was evident in the heterozygous KOs, suggesting that a single copy of the *EphA4 gene* with about 50% of normal protein expression was insufficient to significantly alter behavior in the present experiments. Furthermore, no changes in various neurochemical markers were detected across diverse brain regions rich in EphA4 protein expression. These negative findings support the suggestion that the observed behavioral effects resulted indeed from the genetic disruption of EphA4 rather than potential developmental compensation in other neurotransmitters – although the contribution of additional developmental factors cannot be excluded, which should be best addressed by temporally controlled gene deletion or local pharmacological blockade in adult mice.

In conclusion, our study shows that constitutive homozygous deletion of EphA4 leads to a distinct set of behavioral phenotypes including impaired short-term habituation and novelty detection. Targeting EphA4 might open novel avenues for manipulating such cognitive processes, which are critical for normal memory functions and selective attention (Schmajuk 1997).

References

Akay, T., Acharya, H.J., Fouad, K. & Pearson, K.G. (2006) Behavioral and electromyographic characterization of mice lacking EphA4 receptors. *J Neurophysiol* **96**, 642–651.

Bannerman, D.M., Rawlins, J.N., McHugh, S.B., Deacon, R.M., Yee, B.K., Bast, T., Zhang, W.N., Pothuizen, H.H. & Feldon, J. (2004) Regional dissociations within the hippocampus–memory and anxiety. *Neurosci Biobehav Rev* **28**, 273–283.

Berns, G.S., Cohen, J.D. & Mintun, M.A. (1997) Brain regions responsive to novelty in the absence of awareness. *Science* **276**, 1272–1275.

Bouvier, D., Corera, A.T., Tremblay, M.E., Riad, M., Chagnon, M., Murai, K.K., Pasquale, E.B., Fon, E.A. & Doucet, G. (2008) Presynaptic and post-synaptic localization of EphA4 and EphB2 in adult mouse forebrain. *J Neurochem* **106**, 682–695.

Carmona, M.A., Murai, K.K., Wang, L., Roberts, A.J. & Pasquale, E.B. (2009) Glial ephrin-A3 regulates hippocampal dendritic spine morphology and glutamate transport. *Proc Natl Acad Sci U S A* **106**, 12524–12529.

Cesa, R., Premoselli, F., Renna, A., Ethell, I.M., Pasquale, E.B. & Strata, P. (2011) Eph receptors are involved in the activity-dependent synaptic wiring in the mouse cerebellar cortex. *PLoS One* **6**, e19160.

Cigrang, M., Vogel, E. & Misslin, R. (1986) Reduction of neophobia in mice following lesions of the caudate-putamen. *Physiol Behav* **36**, 25–28.

Deacon, R.M. & Rawlins, J.N. (2006) T-maze alternation in the rodent. *Nat Protoc* **1**, 7–12.

Dellu, F., Mayo, W., Cherkaoui, J., Le Moal, M. & Simon, H. (1992) A two-trial memory task with automated recording: study in young and aged rats. *Brain Res* **588**, 132–139.

Dember, W.N. & Fowler, H. (1958) Spontaneous alternation behavior. *Psychol Bull* **55**, 412–428.

Dottori, M., Hartley, L., Galea, M., Paxinos, G., Polizzotto, M., Kilpatrick, T., Bartlett, P.F., Murphy, M., Kontgen, F. & Boyd, A.W. (1998) EphA4 (Sek1) receptor tyrosine kinase is required for the development of the corticospinal tract. *Proc Natl Acad Sci U S A* **95**, 13248–13253.

Egea, J. & Klein, R. (2007) Bidirectional Eph-ephrin signaling during axon guidance. *Trends Cell Biol* **17**, 230–238.

Enard, W., Gehre, S., Hammerschmidt, K. *et al*. (2009) A humanized version of Foxp2 affects cortico-basal ganglia circuits in mice. *Cell* **137**, 961–971.

Felice, L.J., Felicem, J.D. & Kissinger, P.T. (1978) Determination of catecholamines in rat brain parts by reverse-phase ion-pair liquid chromatography. *J Neurochem* **31**, 1461–1465.

Filosa, A., Paixao, S., Honsek, S.D., Carmona, M.A., Becker, L., Feddersen, B., Gaitanos, L., Rudhard, Y., Schoepfer, R., Klopstock, T., Kullander, K., Rose, C.R., Pasquale, E.B. & Klein, R. (2009) Neuron-glia communication via EphA4/ephrin-A3 modulates LTP through glial glutamate transport. *Nat Neurosci* **12**, 1285–1292.

Gerlai, R., Shinsky, N., Shih, A., Williams, P., Winer, J., Armanini, M., Cairns, B., Winslow, J., Gao, W. & Phillips, H.S. (1999) Regulation of learning by EphA receptors: a protein targeting study. *J Neurosci* **19**, 9538–9549.

Glantz, L.A. & Lewis, D.A. (2000) Decreased dendritic spine density on prefrontal cortical pyramidal neurons in schizophrenia. *Arch Gen Psychiatry* **57**, 65–73.

Gray, J.A. (1982). *The Neuropsychology of Anxiety*. Oxford University Press, London.

Greferath, U., Canty, A.J., Messenger, J. & Murphy, M. (2002) Developmental expression of EphA4-tyrosine kinase receptor in the mouse brain and spinal cord. *Mech Dev* **119** (Suppl. 1), S231–238.

Grunwald, I.C., Korte, M., Adelmann, G., Plueck, A., Kullander, K., Adams, R.H., Frotscherm, M., Bonhoefferm, T. & Klein, R. (2004) Hippocampal plasticity requires postsynaptic ephrin-Bs. *Nat Neurosci* **7**, 33–40.

Hagan, J.J., Alpert, J.E., Morris, R.M.G. & Iversen, S.D. (1983) The effects of central catecholaminergic depletions on spatial learning in rats. *Behav Brain Res* **9**, 83–104.

Honey, R.C. & Good, M. (2000) Associative components of recognition memory. *Curr Opin Neurobiol* **10**, 200–204.

Hooks, M.S. & Kalivas, P.W. (1995) The role of mesoaccumbens--pallidal circuitry in novelty-induced behavioral activation. *Neuroscience* **64**, 587–597.

Inoue, E., Deguchi-Tawarada, M., Togawa, A., Matsui, C., Arita, K., Katahira-Tayama, S., Sato, T., Yamauchi, E., Oda, Y. & Takai, Y. (2009) Synaptic activity prompts gamma-secretase-mediated cleavage of EphA4 and dendritic spine formation. *J Cell Biol* **185**, 551–564.

Karam, S.D., Burrows, R.C., Logan, C., Koblar, S., Pasquale, E.B. & Bothwell, M. (2000) Eph receptors and ephrins in the developing chick cerebellum: relationship to sagittal patterning and granule cell migration. *J Neurosci* **20**, 6488–6500.

Kaufmann, W.E. & Moser, H.W. (2000) Dendritic anomalies in disorders associated with mental retardation. *Cereb Cortex* **10**, 981–991.

Kiehn, O. & Butt, S.J. (2003) Physiological, anatomical and genetic identification of CPG neurons in the developing mammalian spinal cord. *Prog Neurobiol* **70**, 347–361.

Klein, R. (2009) Bidirectional modulation of synaptic functions by Eph/ephrin signaling. *Nat Neurosci* **12**, 15–20.

Knowlton, B.J., Mangels, J.A. & Squire, L.R. (1996) A neostriatal habit learning system in humans. *Science* **273**, 1399–1402.

Kullander, K., Mather, N.K., Diella, F., Dottori, M., Boyd, A.W. & Klein, R. (2001) Kinase-dependent and kinase-independent functions of EphA4 receptors in major axon tract formation in vivo. *Neuron* **29**, 73–84.

Kullander, K., Butt, S.J., Lebret, J.M., Lundfald, L., Restrepo, C.E., Rydstrom, A., Klein, R. & Kiehn, O. (2003) Role of EphA4 and EphrinB3 in local neuronal circuits that control walking. *Science* **299**, 1889–1892.

Lalonde, R. (2002) The neurobiological basis of spontaneous alternation. *Neurosci Biobehav Rev* **26**, 91–104.

Lalonde, R. & Strazielle, C. (2007) Brain regions and genes affecting postural control. *Prog Neurobiol* **81**, 45–60.

Liebl, D.J., Morris, C.J., Henkemeyer, M. & Parada, L.F. (2003) mRNA expression of ephrins and Eph receptor tyrosine kinases in

the neonatal and adult mouse central nervous system. *J Neurosci Res* **71**, 7–22.
Llinas, R. & Welsh, J.P. (1993) On the cerebellum and motor learning. *Curr Opin Neurobiol* **3**, 958–965.
Martone, M.E., Holash, J.A., Bayardo, A., Pasquale, E.B. & Ellisman, M.H. (1997) Immunolocalization of the receptor tyrosine kinase EphA4 in the adult rat central nervous system. *Brain Res* **771**, 238–250.
McNaughton, N. (2006) The role of the subiculum within the behavioural inhibition system. *Behav Brain Res* **174**, 232–250.
Meyer, U., Feldon, J., Schedlowski, M. & Yee, B.K. (2005) Towards an immuno-precipitated neurodevelopmental animal model of schizophrenia. *Neurosci Biobehav Rev* **29**, 913–947.
Mishkin, M. & Petri, H.L. (1984) Memories and habits: some implications for the analysis of learning and retention. In Squire, L.R. & Butters, N. (eds), *Neuropsychology of Memory*. Guilford, New York, NY, pp. 287–296.
Muhia, M., Yee, B.K., Feldon, J., Markopoulos, F. & Knuesel, I. (2010) Disruption of hippocampus-regulated behavioural and cognitive processes by heterozygous constitutive deletion of SynGAP. *Eur J Neurosci* **31**, 529–543.
Murai, K.K. & Pasquale, E.B. (2011) Eph receptors and ephrins in neuron-astrocyte communication at synapses. *Glia* **59**, 1567–1578.
Murai, K.K., Nguyen, L.N., Irie, F., Yamaguchi, Y. & Pasquale, E.B. (2003) Control of hippocampal dendritic spine morphology through ephrin-A3/EphA4 signaling. *Nat Neurosci* **6**, 153–160.
O'Keefe, J. & Nadel, L. (1978). *The Hippocampus as a Cognitive Map*. Oxford University Press, Oxford.
Pasquale, E.B. (2008) Eph-ephrin bidirectional signaling in physiology and disease. *Cell* **133**, 38–52.
Passante, L., Gaspard, N., Degraeve, M., Frisén, J., Kullander, K., De Maertelaer, V. & Vanderhaeghen, P. (2008) Temporal regulation of ephrin/Eph signalling is required for the spatial patterning of the mammalian striatum. *Development* **135**, 3281–3290.
Pavlov, I.P. (1927). *Conditioned Reflexes*. Oxford University Press, London.
Piepponen, T.P. & Skujins, A. (2001) Rapid and sensitive step gradient assays of glutamate, glycine, taurine and gamma-aminobutyric acid by high-performance liquid chromatography-fluorescence detection with o-phthalaldehyde-mercaptoethanol derivatization with an emphasis on microdialysis samples. *J Chromatogr B Biomed Sci Appl* **757**, 277–283.
Pietropaolo, S., Mintz, M., Feldon, J. & Yee, B.K. (2007) The behavioral sequela following the prevention of home-cage grid-climbing activity in C57BL/6 mice. *Behav Neurosci* **121**, 345–355.
Poucet, B. (1989) Object exploration, habituation and response to a spatial change in rats following septal or medial frontal cortical damage. *Behav Neurosci* **103**, 1009–1016.
Prut, L. & Belzung, C. (2003) The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. *Eur J Pharmacol* **463**, 3–33.
Restrepo, C.E., Margaryan, G., Borgius, L., Lundfald, L., Sargsyan, D. & Kiehn, O. (2011) Change in the balance of excitatory and inhibitory midline fiber crossing as an explanation for the hopping phenotype in EphA4 knockout mice. *Eur J Neurosci* **34**, 1102–1112.
Richmond, M.A., Murphy, C.A., Pouzet, B., Schmid, P., Rawlins, J.N. & Feldon, J. (1998) A computer controlled analysis of freezing behaviour. *J Neurosci Methods* **86**, 91–99.
Rinaldi, A., Romeo, S., Agustín-Pavón, C., Oliverio, A. & Mele, A. (2010) Distinct patterns of Fos immunoreactivity in striatum and hippocampus induced by different kinds of novelty in mice. *Neurobiol Learn Mem* **94**, 373–381.
Roullet, P., Sargolini, F., Oliverio, A. & Mele, A. (2001) NMDA and AMPA antagonist infusions into the ventral striatum impair different steps of spatial information processing in a nonassociative task in mice. *J Neurosci* **21**, 2143–2149.
Sanderson, D.J., McHugh, S.B., Good, M.A., Sprengel, R., Seeburg, P.H., Rawlins, J.N. & Bannerman, D.M. (2010) Spatial working memory deficits in GluA1 AMPA receptor subunit knockout mice reflect impaired short-term habituation: evidence for Wagner's dual-process memory model. *Neuropsychologia* **48**, 2303–2315.
Schmajuk, N.A. (1997). *Animal Learning and Cognition: A Neural Network Approach*. Cambridge University Press, Cambridge.
Simón, A.M., de Maturana, R.L., Ricobaraza, A., Escribano, L., Schiapparelli, L., Cuadrado-Tejedor, M., Perez-Mediavilla, A., Avila, J., Del Rio, J. & Frechilla, D. (2009) Early changes in hippocampal Eph receptors precede the onset of memory decline in mouse models of Alzheimer's disease. *J Alzheimers Dis* **17**, 773–786.
Sperk, G. (1982) Simultaneous determination of serotonin, 5-hydroxyindoleacetic acid, 3,4-dihydroxyphenylacetic acid and homovanillic acid by high performance liquid chromatography with electrochemical detection. *J Neurochem* **38**, 840–843.
Sperk, G., Berger, M., Hortnagl, H. & Hornykiewicz, O. (1981) Kainic acid-induced changes of serotonin and dopamine metabolism in the striatum and substantia nigra of the rat. *Eur J Pharmacol* **74**, 279–286.
Suzuki, A., Josselyn, S.A., Frankland, P.W., Masushige, S., Silva, A.J. & Kida, S. (2004) Memory reconsolidation and extinction have distinct temporal and biochemical signatures. *J Neurosci* **24**, 4787–4795.
Taghzouti, K., Louilot, A., Herman, J.P., Le Moal, M. & Simon, H. (1985) Alternation behavior, spatial discrimination, and reversal disturbances following 6-hydroxydopamine lesions in the nucleus accumbens of the rat. *Behav Neural Biol* **44**, 354–363.
Thompson, R.F. (2009) Habituation: a history. *Neurobiol Learn Mem* **92**, 127–134.
Thompson, R.F. & Spencer, W.A. (1966) Habituation: a model phenomenon for the study of neuronal substrates of behavior. *Psychol Rev* **73**, 16–43.
Thullier, F., Lalonde, R., Mahler, P., Joyal, C.C. & Lestienne, F. (1996) Dorsal striatal lesions in rats. 1: effects on exploration and motor coordination. *Arch Physiol Biochem* **104**, 300–306.
Tremblay, M.E., Riad, M., Bouvier, D., Murai, K.K., Pasquale, E.B., Descarries, L. & Doucet, G. (2007) Localization of EphA4 in axon terminals and dendritic spines of adult rat hippocampus. *J Comp Neurol* **501**, 691–702.
Usiello, A., Sargolini, F., Roullet, P., Ammassari-Teule, M., Passinom, E., Oliverio, A. & Mele, A. (1998) N-methyl-D-aspartate receptors in the nucleus accumbens are involved in detection of spatial novelty in mice. *Psychopharmacology* **137**, 175–183.
Vinogradova, O.S. (2001) Hippocampus as comparator: role of the two input and two output systems of the hippocampus in selection and registration of information. *Hippocampus* **11**, 578–598.
Vuillermot, S., Joodmardi, E., Perlmann, T., Ogren, S.O., Feldon, J. & Meyer, U. (2011) Schizophrenia-relevant behaviors in a genetic mouse model of constitutive Nurr1 deficiency. *Genes Brain Behav* **10**, 589–603.
Wagner, A.R. (1981) SOP: a model of automatic memory processing in animal behavior. In Spear, N.E. & Miller, R.R. (eds), *Information Processing in Animals: Memory Mechanisms*. Erlbaum, Hillsdale, NJ, pp. 5–47.
Willi, R., Aloy, E.M., Yee, B.K., Feldon, J. & Schwab, M.E. (2009) Behavioral characterization of mice lacking the neurite outgrowth inhibitor Nogo-A. *Genes Brain Behav* **8**, 181–192.
Willi, R., Weinmann, O., Winter, C., Klein, J., Sohr, R., Schnell, L., Yee, B.K., Feldon, J. & Schwab, M.E. (2010) Constitutive genetic deletion of the growth regulator Nogo-A induces schizophrenia-related endophenotypes. *J Neurosci* **30**, 556–567.
Winter, C., Djodari-Irani, A., Sohr, R., Morgenstern, R., Feldon, J., Juckel, G. & Meyer, U. (2009) Prenatal immune activation leads to multiple changes in basal neurotransmitter levels in the adult brain: implications for brain disorders of neurodevelopmental origin such as schizophrenia. *Int J Neuropsychopharmacol* **12**, 513–524.
Xiao, D., Miller, G.M., Jassen, A., Westmoreland, S.V., Pauley, D. & Madras, B.K. (2006) Ephrin/Eph receptor expression in brain of adult nonhuman primates: implications for neuroadaptation. *Brain Res* **1067**, 67–77.
Yamada, D., Zushida, K., Wada, K. & Sekiguchi, M. (2009) Pharmacological discrimination of extinction and reconsolidation

of contextual fear memory by a potentiator of AMPA receptors. *Neuropsychopharmacology* **34**, 2574–2584.
Yamaguchi, Y. & Pasquale, E.B. (2004) Eph receptors in the adult brain. *Curr Opin Neurobiol* **14**, 288–296.
Yin, H.H., Knowlton, B.J. & Balleine, B.W. (2004) Lesions of dorsolateral striatum preserve outcome expectancy but disrupt habit formation in instrumental learning. *Eur J Neurosci* **19**, 181–189.
Zhu, X.O., McCabe, B.J., Aggleton, J.P. & Brown, M.W. (1997) Differential activation of the rat hippocampus and perirhinal cortex by novel visual stimuli and a novel environment. *Neurosci Lett* **229**, 141–143.

Acknowledgments

We thank Rüdiger Klein for kindly providing the *EphA4* mouse line and Joram Feldon for providing access to the animal keeping and behavioral testing facilities necessary for the reported experiments. This work was supported by grants of the Swiss National Science Foundation and the National Centre for Competence in Research 'Neural Plasticity & Repair' of the Swiss National Science Foundation. All authors declare that they have no conflicts of interest to disclose.