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Research report

EphB2 reverse signaling regulates learned opiate tolerance *via* hippocampal function

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HIGHLIGHTS

- Identifies mechanistic role for EphB2 reverse signaling in opiate tolerance.
- Effect of EphB2 opposes that previously described for EphB1 signaling.
- EphB2 accelerates opiate tolerance *via* hippocampal-dependent mechanism.
- Provides mechanistic basis for prior work on associative cues and opiate tolerance.

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ABSTRACT

Despite significant progress, many uncertainties remain regarding molecular and cellular mechanisms governing opiate tolerance. We report that loss of EphB2 receptor reverse signaling results in a marked acceleration of morphine tolerance *in vivo*. EphB2 null mice exhibited no significant difference in brain or blood morphine metabolism, mu opiate receptor affinity or binding capacity. Motor and sensory performance for EphB2 null mice was also comparable to controls for both morphine naïve or tolerized states. Regional distributions of mu opioid receptor, CGRP and substance P were also unaltered in EphB2 null mice. However EphB2 null mice, but not animals homozygous for kinase dead version of EphB2, exhibited significant modification of context-dependent anti-nociceptive responses following chronic morphine treatment. To verify the changes seen in EphB2 null mice arise from impairment of hippocampal learning, discreet bilateral lesions of the dorsal hippocampus were produced in wildtype mice demonstrating striking similarities to that seen in EphB2 null mice for opiate-dependent behavior. The results demonstrate that EphB2 reverse signaling plays a unique and requisite role in inhibiting the development of opiate-dependent tolerance *in vivo*.

1. Introduction

Mu opioid receptor signaling has traditionally been viewed in the context of signalling events arising within receptor containing cells [1–4]. However a substantial body of evidence demonstrates that opiate response can be strongly influenced by associative learning [5–10]. This is evidenced by hyperalgesia seen in morphine-tolerant animals subjected to novel environmental cues, the extinction of morphine tolerance in animals presented with environmental cues previously associated with morphine but subsequently associated with placebo, and the impedance of tolerance acquisition during interspersion of placebo sessions between morphine treatments (partial reinforcement) [6–8]. Such findings suggest that a form of Pavlovian conditioning operates with respect to morphine exposure and contextual cues, and that such associations are critical in opiate tolerance. However aspects of the molecular mechanisms underlying this effect remain unclear. We have examined the role of EphB2 in the development of opiate tolerance and observed that it plays a key role in regulating Pavlovian features of morphine-dependence.

Erythropoietin-producing hepatocellular carcinoma (Eph) receptors represent the largest family of receptor tyrosine kinases

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and play critical roles in cellular navigation and tissue patterning as well as topographic organization [11,12]. Eph receptors are classified into two major sub-groups, EphA and EphB depending upon ligand binding preferences [13]. Ephrin (Eph receptor interacting) ligand A's are bound to the cell's outer surface *via* GPI linkage while ephrin B's are transmembrane proteins exhibiting their own intracellular signaling capabilities [14,15]. EphB-family receptors are therefore unusual in that both receptor and ligand are capable of directing intracellular signaling. Termed bidirectional signaling, such propagation may occur through either a receptor-mediated (forward) or ephrin mediated (reverse signaling) mechanisms [16,17]. In order to distinguish which form of signaling may govern a particular set of cellular effects, a series of kinase-dead or ephrin-modified mutants have been created for various Eph family members. We and others have previously demonstrated that loss of EphB2 attenuates hippocampal LTP [18,19], and that combinatorial loss of EphB1, EphB2 and EphB3 demonstrate their roles in proper development of hippocampal dendritic spines [20]. Postnatally, we and others have demonstrated that EphB2 is largely confined to regions of the hippocampus and cortex undergoing synaptic modification [18,19,21]. To examine the role of EphB2 in regulating morphine tolerance *in vivo*, we utilized kinase dead and null mutants of EphB2, demonstrating that loss of EphB2 reverse signaling strongly potentiates acquisition of morphine tolerance. Mu opioid receptor binding capacity, affinity, spinal receptor levels, as well as rates of morphine metabolism and measures of sensory/motor performances were unaltered between EphB2 null mice and controls. By contrast, EphB2 null mice differed strikingly compared to controls in their perceptual responses to morphine. Interestingly the effects seen in EphB2 null mice oppose those previously described for inhibition of EphB1 forward signaling [22]. The potentiation of morphine tolerance in conjunction with impaired hippocampal learning in EphB2 mice suggests competition between hippocampal and extra-hippocampal learning in response to morphine. Induction of bilateral lesions to the dorsal hippocampi of control mice results in development of altered morphine responsiveness similar to that seen in EphB2 nulls. This study provides the first evidence that EphB2 reverse signaling plays a novel role in attenuating the rate of morphine tolerance through effects on hippocampal associative learning *in vivo*.

2. Materials and methods

2.1. Animals and agents utilized

Wildtype, heterozygotes, and EphB2 targeted knock-out mice were generated as littermates from crosses of heterozygous EphB2 lineages as described previously [18]. EphB2 targeted lines in which the intracellular kinase domain was replaced through in-frame fusion with beta-galactosidase (kinase dead, designated as EphB2-N2) with wild type, heterozygous and homozygous mice of this series generated through heterozygous intercrosses. Mice used for analyses were 3–5 month age-matched siblings weighing between 25 and 35 g. Oprm1 null mice were obtained from Jackson Laboratory. For all experiments, wild type littermates served as controls for EphB2 null mutants and heterozygotes, with all genotypes evaluated simultaneously for a given assay. Outbred CD1 wild type mice as above were also evaluated for the assays indicated, served as an external genetic control, and were utilized for hippocampal lesion experiments. All procedures and protocols were in accordance with the Canadian Council on Animal Care (CCAC) and the University of Toronto Faculty of Medicine and Pharmacy Animal Care Committee. All efforts were made to minimize animal suffering, with mice euthanized by Avertin overdose and cervical dislocation. Morphine sulphate was obtained from Professional Compounding Centers of America (PCCA, Houston, Texas) and freshly prepared at 1.5 mg/mL in 0.9% saline and administered at a dose of 10 mg/kg i.p. Naltrexone was purchased from Tocris Cookson (Ballwin, MO) and prepared fresh in water. Morphine-3-glucuronide (M3G) was kindly provided by the laboratory of Dr. Sandy Pang (National Institutes on Drug Abuse). For tests of morphine tolerance, mice were injected with 10 mg/kg morphine sulphate i.p. twice per day (morning and afternoon) at 8 h intervals over a period of six days. Sensory tests were performed 15 (tail pinch) or 30 (tail flick) minutes following morning morphine injection on days 1, 3 and 6. For experiments performed on day 7, mice were split into two groups, remaining either in their home environment or alternatively transported to a novel environment prior to receiving timed injection of morphine with behavioral assessment.

2.1.1. Preparation LC/MS/MS standards

Stock solutions of morphine and M3G were prepared in sterile 0.9% saline. All subsequent working solutions for LC/MS/MS were prepared from serial dilutions of the standard in acetonitrile and stored at −20 °C until used. Whole blood and brain homogenates for standards were obtained from non-injected morphine naive mice. Standard solutions consisted of blank blood and blank brain homogenates spiked with known concentrations of the working standard creating a calibration curve in the desired concentration range. Caffeine was used as the internal standard and prepared in water at a stock concentration of 3 mg/mL.

2.1.2. Brain and blood sample collection, LC/MS/MS

EphB2 wildtype and null animals were given a bolus injection of 10 mg/kg morphine sulphate. Blood was then collected terminally by heart puncture at 30, 60, or 90 min after injection. Blood was frozen immediately at −80 °C until analyzed. Brain samples were homogenized in 0.1 N perchloric acid (Sigma–Aldrich), to a final concentration of 0.33 g tissue/mL homogenate and stored at −80 °C until analyzed. Upon thawing, brain homogenates were sonicated for 10 min in ice water. Samples were then spun at 15,000 rpm for 10 min, and the supernatant collected and neutralized with 2 M NaOH. If not used immediately samples were stored at −80 °C until analyzed.

2.2. LC/MS/MS sample purification

A 10 μL aliquot of the caffeine (3 μg/mL) internal standard was added to each 100 μL of blood or brain homogenate. Samples were mixed with equal volume of methanol and acetonitrile and the proteins precipitated. Following vortexing for 60 s, samples were centrifuged at 13,000 × *g* for 10 min and the supernatant transferred to Sep-Pak Vac C18 3cc cartridges (200 mg; Waters, Milford, MA, USA). Each cartridge was pre-conditioned with 2 × 1 mL acetonitrile followed by 2 × 1 ml Millipore water. After loading, samples were eluted with 2 × 1 mL of acetonitrile and eluents pooled and dried under a stream of nitrogen at room temperature.

2.2.1. LC–MS/MS analysis

Blood and brain samples were reconstituted with 200 μL and 100 μL, respectively of the mobile phase (70% of water with 0.1%v/v formic acid and 30% acetonitrile with 0.1%v/v formic acid). The reconstituted sample was injected (1 uL blood sample or 35 uL brain sample) into the LC/MS/MS system for analysis. Samples were analyzed using a 6410 Triple Quad LC/MS/MS instrument (Agilent Technologies) with ESI source in positive ion mode. Samples were separated on a C18 column (XTerra MS 3.5 μm, 4.6 × 150 mm) at flow rate of 1 mL/min. Mobile phase consisted of HPLC grade water (A) and acetonitrile (B) both containing 0.1% formic acid. The following gradient was run: 0–1 min, 4% (B); 4–5 min, 4% (B); 5–9 min 4–100% (B); 9–10 min, 100% (B); 10–11 min 100–4% B;

11–16 min, 4% (B). MS parameters were as follows: gas temperature 350 °C, nebulizer pressure 50 psi, drying gas (nitrogen) 11 L/min and VCap 3500 V. Using MRM monitoring the following transitions were observed: morphine-3-glucoronide (m/z 462 → 286, RT 6.7 min), morphine (m/z 286.1 → 165, RT 7.8 min) and caffeine (m/z 195 → 138, RT 11.4 min). Fragmentor voltage (Frag) and collision energy (CE) settings for each compound follows: morphine – Frag 155 V, CE 42 V, morphine-3-glucuronide – Frag 160 V, CE 32 V, caffeine – Frag 85 V, CE 20 V.

2.2.2. Novel object recognition

Mice from each genotype were placed in a clean empty test cage in the presence of visual cues for 5 min, followed by habituation with the initial (3) test objects (Fig. S3) for an additional 15 min. Object 2 was then displaced to the novel location and object interactions assessed for a period of 5 min. A new novel object was then introduced in position 1 of the test cage and interactions examined for an additional 5 min period. Animal interaction with test objects was scored using an automated 16 quadrant IR beam recorder.

2.2.3. Immunohistochemistry/histochemistry

Brain and spinal sections from wild type, heterozygous, EphB2 null mice, or mu opioid receptor (MOR, *Oprm*) knockout mice and controls were prepared following intracardial perfusion of saline followed by 4% paraformaldehyde in 0.9% NaCl,0.1 M phosphate buffered saline pH 7.4 (PBS). Following 2 h of post-fixation, tissues were then dissected and processed for paraffin embedding. Seven micron wax sections were then prepared for immunohistochemistry. To verify *in situ* the specificity and fidelity of MOR antisera utilized, MOR null and wildtype littermate tissues were used as negative and positive controls respectively. Following dewaxing and peroxidase treatment (3% H_2O_2 for 30 min), slides were washed (3 × 5 min in PBS) and antigen retrieval performed by incubating in 10 mM sodium citrate, pH 6.0 at 100 °C for 5 min in a pressure cooker. Following cooling, sections were incubated at 1:1000 with anti-MOR antisera (Immunostar) diluted in blocking solution (5% goat serum, 0.25% Tween-20 in PBS) overnight at 4 °C. Following washing, slides were incubated at 1:200 with biotinylated goat anti-rabbit secondary antisera (Vector Labs) for two hours at room temperature. Slides were subsequently washed and incubated with avidin-horseradish peroxidase as per manufacturer's instructions (Vector Labs) for 45 min and visualized using 3,3-diaminobenzidine. Additional antisera (minus antigen retrieval) were utilized as follows: GFAP (1:400, Dako), beta-galactosidase (1:200, MP Biomedical), beta III tubulin (TUJ1 1:500, Cedarlane). For fluorescent assays, Alexa Fluor™ 488 goat anti-rabbit or Alexa Fluor™ 594 goat anti-mouse antisera (1:400 from Invitrogen) were utilized as indicated. For fluorescent sections Hoechst 33258 (Sigma) was utilized as a nuclear marker. Images were collected on a Nikon Model E1000R is a motorized fluorescence microscope equipped with DAPI, FITC, TRITC, Texas Red and Cy5/DiD filter cubes, with images collected on a cooled Hamamatsu ORCA 285CCD camera. Western analyses were performed as described previously [23] and analyzed using an Alpha-Innotech imager with SuperSignal West Pico ECL chemi-luminescent detection substrate (Fisher). Histochemical detection of beta-galactosidase activity was performed as described previously [24].

2.2.4. Radioligand binding

Radioligand binding studies were performed to determine the intrinsic affinity and total mu opiate binding capacity of wild type, heterozygous and EphB2 null littermates. Assays were performed by varying concentrations of [^{3}H]-naloxone (61.1Ci/mmol, PerkinElmer,) against a constant concentration of the non-specific opioid antagonist naltrexone. Samples were obtained from the dorsal spinal cord and superior colliculus (due to its high relative expression of EphB2 and MOR with limited expression of alternate opiate receptors). Samples were collected at the time of sacrifice and frozen at −80 °C until used. Samples were subsequently thawed, homogenized *via* mechanical disruption, centrifuged at 12,000 × *g* for 30 min and re-suspended in binding buffer. Protein concentrations were determined using BCA assay (Fisher) as per the manufacturer's instructions. 230 μg of total protein homogenate was then incubated for 2.5 h at room temperature with 10 μL of [^{3}H]-naloxone (concentrations: 1.78×10^{-8}, 1.33×10^{-8}, 1×10^{-8}, 5.62×10^{-9}, 4.22×10^{-9}, 3.16×10^{-9}, 1.78×10^{-9}, 1×10^{-9}, or 5.62×10^{-10}) and 10 μL of naltrexone (100 μM) or vehicle in binding buffer (50 mM Tris, 3 mM $MgCl_2$, 1 mg/mL BSA, 1 mM EDTA, pH 7.4). Binding reactions were then terminated by filtration in a Harvester apparatus with proteins collected onto Whatman glass microfiber filters (GE Healthcare). Filters were then washed three times with wash buffer (50 mM Tris, 3 mM $MgCl_2$, pH 8.0), dried and immersed in scintillate (Ultima Gold, PerkinElmer) overnight prior to counting. For each experimental series, wildtype, EphB2 heterozygote, and EphB2 null samples were examined in parallel.

2.2.5. Passive avoidance assay

Passive avoidance apparatus consisted of a light and dark chamber containing identical stainless steel gridded floors. Mice were confined to the lightened chamber for a period of 30 s, followed by a period of free access to the alternative (darkened) module. Time of initial entry into the darkened chamber was recorded as Transfer Latency Time (acquisition TLT). Following 10 s in the darkened chamber, mice were subjected to a single foot shock of 0.5 mA for 5 s (0.7 mA for outbred CD1 animals). Following a period of 30 s in the dark chamber, animal were allowed to access light chamber for two minutes and returned to their home cage. Twenty-four hours following acquisition mice were re-introduced into the light chamber and the time for dark chamber entry recorded. Tests were terminated at 300 s. Light chamber luminance was maintained at 80 lux.

2.2.6. Activity monitor

EphB2 null mice, heterozygotes and littermate controls were examined in a 25 cm × 42 cm open field pen for a period of 1–2 h. Spontaneous motor activity was recorded using an automated movement detection system (AM1053 activity monitors; Linton Instrumentation, UK), consisting of 24 infrared beams forming a two level grid network. Displacement of the animal resulting in beam interruption was used to record motor activity. Studies were repeated in a similar manner with lesioned and sham operated controls. For monitoring of morphine-induced hyperactivity, EphB2 wild-type and nulls were tested for 1 h to determine drug naïve basal performance. Twenty-four hours later, animals were injected with a single dose of 10 mg/kg morphine or vehicle and their activity analyzed for the subsequent 90 min period. This was repeated with lesioned and sham operated control animals. All animals were examined during the same one hour period each day. Chamber luminance was maintained at 80 lux.

2.2.7. Opiate dependent learned context control

Following six days of twice daily morphine exposure treatment groups were divided on day 7. While one group was retained to their home environment, the other was transferred to a novel environmental setting. Visual cues were altered in the novel environment whereas cage size, ambient light, and noise levels were held constant between the two groups which were retained in adjoining rooms of the animal colony. Animals were allowed to accommodate to their relative environment for a period of 15 min prior to receiving scheduled morphine doses.

2.2.8. Tail flick assay

Anti-nociceptive responses were determined through measurement of response latency to warm water tail-immersion [25,26]. Response latencies were plotted as the time to response following immersion of the tail in a given bath. Responses of separate test groups to tail immersion were monitored at 55 °C. Animals were placed in a Plexiglas mouse retainer allowing free tail movement with 2 cm of the tail tip immersed in water at time = 0. The time upon removal was then determined with a maximum allowable immersion of 15 s to prevent tissue injury. Tail flick assays were performed 30 min following morphine administration.

2.2.9. Tail pinch assay

Tail pinch was performed as previously reported [27] using surface flat forceps of dimension 0.5 cm with persistent closing force of 180 g. Pressure was applied at the proximal third of the animal's tail. Nociceptive responses were determined as a function of the latency required for response. To avoid tissue injury, a maximum response time of 10 s was set. Tail pinch assays were performed at 15, 30, 45, 60, 90 and 120 min following morphine administration.

2.2.10. Von Frey fiber test

Mechanoceptive function was assessed *via* foot withdrawal response using a graded series of Von Frey filaments (displacement forces, 0.008–10 g, tip diameter 100 μm) upon application of each probe to the plantar surface of the hindpaw. Each animal was assessed on alternating hindpaws on two separate occasions for a given ascending force for each of the filaments indicated. Interstimulus interval was approximately 20 s. Withdrawal threshold was defined as the force which evoked a minimum detectable withdrawal reflex in >50% of cases for a given bending force. Results were plotted directly as raw data for each individual without internal normalization, plotted as a function of the gram force required to induce plantar withdrawal of the hindlimb.

2.2.11. Hippocampal lesions

Adult male CD1 mice 2–3 months of age, weighing between 35 and 42 g were surgically anesthetized using 2.5% Avertin (0.2 mL/kg body weight) and a 1 cm incision made along the dorsal scalp over the sagittal suture. At the midpoint between lambda and bregmal sutures, two 0.2 mm burr holes were drilled on either side of the sagittal midline at a displacement of 1.8 mm. A 0.1 mm diameter platinum-iridium electrode with an exposed tip of 0.5 mm was then placed to a depth of 1.5 mm at each site. Electrolytic lesions were produced within the dorsal hippocampus using a single constant direct current discharge of 3 s at 3 mA. Following surgery, the scalp incision was closed and animals allowed to recover for 72 h. Sham operated controls underwent the same procedure as experimental animals minus the electrode insertion. After completion of all behavioral testing, mice in which electrolytic lesions had been performed were perfused with 4% paraformaldehyde as above and their brains removed for retrospective verification of lesion placement. In these animals, serial 7 micron paraffin sections were collected at intervals of 200 microns through the full extent of the lesion for thionin staining.

2.2.12. Gait analysis

Gait dynamics were recorded using a customized motor driven transparent treadmill with ventral plane videography (*L*: 156 cm, *W*: 5 cm). A digital video camera was mounted below the transparent treadmill belt to capture stride features. A Plexiglas chamber (*H*: 9 cm, *L*: 20 cm, *W*: 5 cm) housed the treadmill belt allowing the animal to freely move within the viewing chamber. Gradations along the bottom of the chamber coordinated distance measurements. Video images were collected at rate of 60 frames per second. Each animal was weighed (mean weight 30 ± 5 g) and acclimatized to the chamber for a period of 2 min. Gait was analyzed at a treadmill speed of 10.9 cm/s with analysis performed on $N \geq 20$ individual steps.

2.2.13. Grip strength

A commercial grip strength dynamometer was employed to monitor grip strength. Forelimb and hindlimb measurement were performed. Scores were determined for the best 4 out of 7 grips and the averages response calculated.

2.2.14. Edge performance test

Motor coordination and balance was evaluated by the ability of mice to traverse a 3 mm wide beam toward an enclosed safety platform. Beam was 30 cm long and 28 cm in height. A score of 1 was assigned to animals traversing easily without undue effort, could reverse on the 3 mm edge and reach platform easily from a distance of 15 cm. A score of 2 was given to animals reaching the platform with substantial difficulty, and only access platform from distances <7 cm. A score of 3 was assigned to animals who crossed with extreme difficulty (tremor) in edge navigation and could make progress on beam, but are only able to maintain grip for 8–10 s. A score of 4 was assigned to animals unable to traverse beam when placed on edge and cannot maintain grip.

2.2.15. Platform performance at 90 degree incline

Animals were timed for their climbing speed up a 90° inclined wire mesh (2 cm) platform of height 28 cm, width 14 cm.

2.2.16. Hindlimb extension reflex

Mice were suspended by the tail at a distance of 10 cm above a supportive surface and the extent of hindlimb extension was observed in 5 independent lifts. A score of (0) corresponded to absence of hindlimb extension or clutching of limbs close to the body; (1) to extension but without reflex upon whisker-surface contact; (2) to extension reflex in hindlimbs including splaying of toes upon whisker contact; (3) to hindlimb extension immediately prior to whisker contact; (4) to early vigorous hindlimb extension reflex.

2.2.17. Statistics

Latency and concentration differences among groups over time were tested using two-way ANOVA with repeated measurement (time) followed by Bonferroni post hoc tests using GraphPad Prism software v5.0 (GraphPad Software Inc., La Jolla, CA, USA). Three-way ANOVA was performed to measure latency differences among groups over time and days using SPSS 16 (SPSS Inc., Chicago, IL, USA). Student's *t*-test was used to compare differences between each test group and its corresponding control using Microsoft Excel. All data are presented as mean ±SEM. Statistical results are considered significant if $P \leq 0.05$.

3. Results

3.1. Loss of EphB2 significantly accelerates the rate of morphine tolerance

To determine the effect of EphB2 ablation on baseline behavioural function, several sensory and behavioural analyses were performed on EphB2 null mice, wild-type and EphB2 heterozygous littermates. Examination of baseline mechanoceptive and thermoceptive responses using tail pinch and tail flick assays respectively (Fig. 1A–D, time 0; Fig. S1A), as well as Von Frey plantar fibre response and static and dynamic measures of motor performance (Fig. S1B–G) revealed no intrinsic sensory or motor differences. To ascertain the distribution of EphB2 within relevant sensory loci within the adult CNS, histochemical staining for

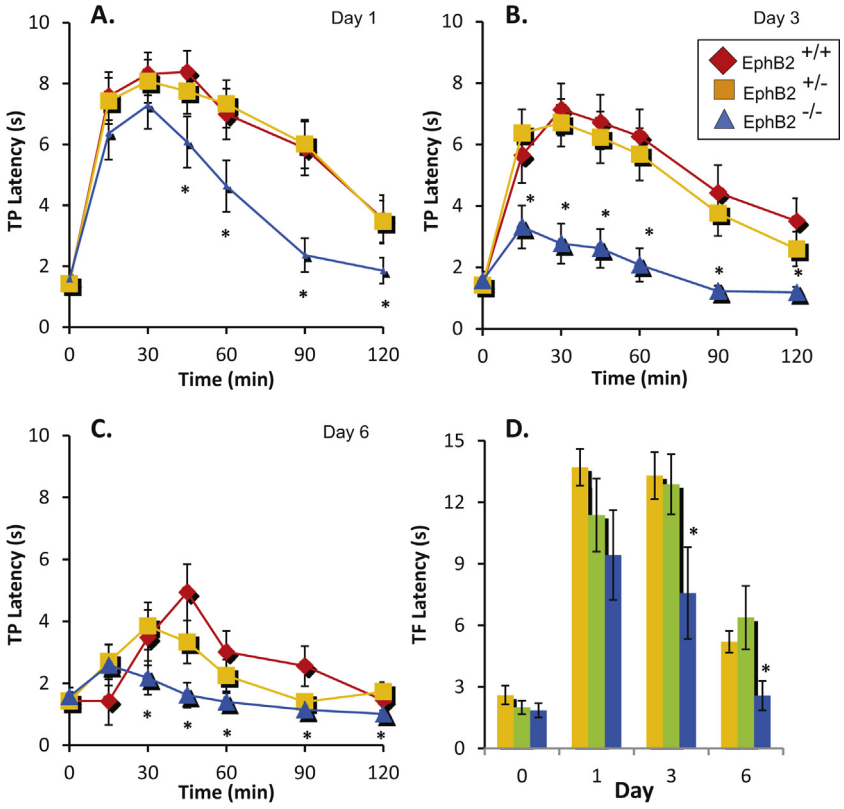

Fig. 1. Analysis of antinociceptive responses to tail pinch following morphine administration in wild type (diamond), heterozygous (square) and EphB2 (triangle) null mice ($N > 22$ animals/group). (A) Anti-nociceptive responses following initial exposure to morphine on day 1. EphB2 null mice initially exhibit tail pinch latencies similar to wild type littermates. By 45 min following morphine treatment however, EphB2 null mice demonstrate significantly lower TP latencies compared to controls. (B) Tail pinch latencies on day 3; (C) Tail pinch latencies on day 6. (D) Tail flick responses 30 min following morphine treatment on days 1, 3 and 6 compared to pre-treatment controls (0). Similar to that seen in tail pinch analyses, EphB2 null mice (blue) display significantly lower levels of anti-nociception compared to heterozygous (yellow) or EphB2 N2/N2 mice (green) on days 3 and 6 ($N > 8$ animals/group, error bars ±SEM), *comparison of EphB2 null mice to wild type and heterozygous littermates at $p < 0.05$. (TP = tail pinch, TF = tail flick). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

beta-galactosidase was performed using mice heterozygous for the EphB2 N2 allele (EphB2 $^{N2/+}$) in which the region from the intracellular juxtamembrane to the C-terminal region have been replaced through in-frame fusion with beta-galactosidase as previously described [18,21]. Due the level of homology present among various EphB family members and EphA4, and lack of EphB2-specific antisera, identification using beta-galactosidase provides an unambiguous means of determining the cellular and subcellular localization of EphB2 [18,21]. As shown in Fig. S1(H–K), EphB2 is persistently expressed within the adult nervous system at a variety of CNS loci involved in sensory perception. Given that loss of EphB2 did not result in detectable changes in baseline sensory function we examined the response of EphB2 null mutants to anti-nociceptive sensory adaptation, using the well-characterized model of opiate-dependent tolerance.

Upon initial exposure to morphine EphB2 null mice show similar anti-nociceptive properties during the first 30 minutes to that seen in wild type littermates in response to applied tail pinch (Fig. 1A). However EphB2 null mice subsequently exhibited significantly faster rates of decay in morphine-dependent analgesia compared to both heterozygous and wild type littermates. Analysis by two-way ANOVA revealed a significant interaction [$F(6,240) = 2.91$, $p < 0.05$] (genotype × time – repeated measure). As shown in Fig. 1B the trend toward accelerated tolerance in EphB2 null mice continues following repeated exposure to morphine, with two-way ANOVA again revealing a significant interaction [$F(6,240) = 6.79$, $p < 0.05$] (genotype × time – repeated measure). Following six days of treatment wild-type, EphB2 heterozygotes and EphB2 null mice all exhibit a significant degree of tolerization toward the analgesic effects of morphine (Fig. 1C). Analysis of thermoceptive (tail flick) response in EphB2 null mutants versus controls following morphine treatment (Fig. 1D), demonstrates findings similar to those seen in tail pinch assays. Thus loss EphB2 does not alter baseline mechanoceptive or thermoceptive sensory function, but rather accelerates the development of morphine tolerance. Analysis of mice homozygous for a targeted deletion of the EphB2 kinase domain (EphB2$^{N2/N2}$, Fig. S2A–C) further demonstrates that the accelerated pattern of morphine tolerance observed in EphB2 null mice is independent of receptor kinase function, arising instead from as a result of reverse signaling through cognate ephrin ligands.

3.2. *EphB2 null mice do not exhibit context dependent reversal of morphine tolerance*

Previously we have demonstrated that loss of EphB2 reduces steady state levels of postsynaptic NMDA receptors, with resulting attenuation of glutamatergic signaling affecting features such as LTP [18,28]. Given that NMDA antagonists such as MK-801 have been shown to attenuate the development of morphine induced tolerance, we sought to investigate the mechanism whereby loss of EphB2 acts to potentiate such signaling. Previous work by Siegel et al. [6,7,9] has demonstrated that chronic morphine exposure represents a cue-associative form of Pavlovian conditioning which can alter systemic drug response. Under conditions of chronic morphine treatment in the presence of persistent contextual cues, wild-type animals undergo a reproducible association between the local environment and receipt of morphine. As a result ani-

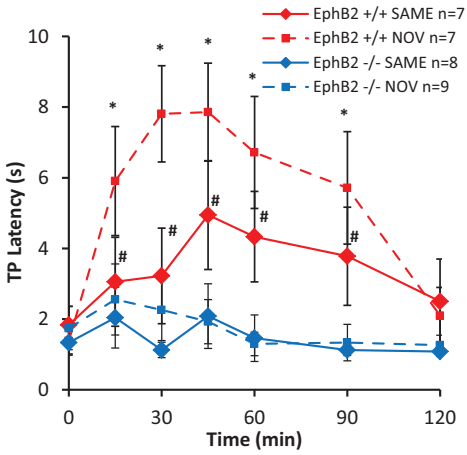

Fig. 2. Anti-nociceptive responses of EphB2 null mice and controls upon switching to a novel environment following 7 days of morphine treatment. Consistent with previous reports [5], tolerized wild-type animals exhibit a significant increase in anti-nociceptive response following transfer to a novel (NOV) environment. By contrast EphB2 null mice exhibit no such enhancement following transfer to novel environment.
Legend: EphB2 wild-type, same environment (diamond, solid); EphB2 wild-type, novel environment (square, dotted); EphB2 null, same environment (diamond, solid), EphB2 null, novel environment (square, dotted). *Wild-type versus EphB2 null mice for novel environment at $p < 0.05$; #EphB2 wild-type, same versus novel environment at $p < 0.05$. TP = tail pinch, error bars are ±SEM.

mals learn to respond to available contextual cues, displaying a homeostatically opposed hyperalgesic response to counter the next (anticipated) dose of morphine induced analgesia. Robbed of this learned response upon switching to a novel environment, a full analgesic response is again seen upon subsequent morphine exposure. In the event that loss of EphB2 signaling altered such a response, it would be predicted that such animals would be unable to compensate for the loss of such contextual learning. In order to investigate this, EphB2 null mice and control litter mates were treated twice per day with morphine over a period of 6 days. On day 7, EphB2 null mice and controls were retained in either their normal environment, or removed to a novel environment. At the appropriate time animals received their daily injection of morphine and anti-nociceptive responses were assessed 15 min following drug exposure. As shown in Fig. 2, EphB2 control littermates placed in a novel environment exhibited significant enhancement in morphine dependent anti-nociceptive response compared to those retained in their normal contextual environment. By contrast, EphB2 null mice exhibited no apparent enhancement in morphine dependent analgesic response following removal to a novel environment. Analysis of this context-dependent learning using three way ANOVA found significant interactions between genotype (EphB2 null or control), environment (novel or control) and time (0, 15, 30, 45, 60, 90 and 120 min) [$F(6,162) = 5.99$, $p < 0.05$].

3.3. Loss of EphB2 does not alter MOR expression, distribution or opiate binding affinity

Similar to other EphB-family members, EphB2 is extensively distributed within primary and secondary sensory loci of the adult nervous system (Fig. 1S). To determine whether loss of EphB2 results in an altered expression or distribution of the mu opioid receptor, MOR distribution was examined within the spinal cord and other CNS sites. As shown in Fig. 3A EphB2 null mutants and control littermates exhibit similar laminar distributions of MOR within the dorsal spinal cord. As indicated in Fig. 3B, EphB2 wildtypes, heterozygous and null mutants exhibit similar patterns of MOR distribution within regions such as patch components of the striatum. Western analysis of total MOR levels within the dorsal spinal cord similarly demonstrated no significant differences between EphB2 null mutants and wildtype (Fig. S2D). Furthermore competitive radioligand ligand binding analyses using [^{3}H]-naloxone tritiated versus naltrexone demonstrated similar Kd and Bmax *in vivo* [EphB2$^{+/+}$: 2.74×10^{-7}, 2012 pM/mg protein; EphB2$^{-/-}$: 2.54×10^{-7}, 1835 pM/mg protein]. To determine whether loss of EphB2 resulted in a disturbance of primary sensory architecture, markers of both peptidergic (calcitonin gene related peptide, CGRP terminating largely in LI and the outer two-thirds of lamina II) and non-peptidergic (isolectin B4, IB4 centered in lamina II) nociceptive afferents were examined in the lumbar spinal cord (L2). As shown in Figure 3C, both EphB2 null and wildtype morphine-treated (day 6) animals exhibit similar laminar distributions of CGRP and IB4, which remained comparable to that seen in morphine naïve controls (EphB2$^{N2/+}$ shown for comparison). Analysis of morphine-treated EphB2 wildtype, null, and morphine naïve EphB2$^{N2/+}$ mice similarly demonstrated no significant difference in distribution of either IB4 or CGRP positive sensory neurons within L4–L5 dorsal root ganglia.

3.4. Loss of EphB2 does not alter morphine metabolism

Inhibition of EphB2 resulted in a significant reduction in temporal extent of morphine-induced analgesia. To determine whether loss of EphB2 might somehow alter the tissue availability of morphine or otherwise modify its metabolism, we examined blood and brain levels of morphine and its main metabolite morphine-3-glucuronide (M3G) *via* LC/MS/MS analysis. Fig. 4A and B indicate the observed concentration of morphine and M3G respectively within the brain following initial drug exposure. As these data indicate, no significant difference in concentration between EphB2 null and wildtype littermates is observed for any time point measured. EphB2 null and wildtype littermates displayed similar trends in morphine and M3G decline over time. Similar trends are seen in Fig. 4C and D demonstrating morphine and M3G kinetics respectively within whole blood following initial drug exposure. Similarly analysis of the brain (Fig. 4E) and blood (Fig. 4F) in wildtype and EphB2 null mice following repeated morphine exposure (day 7) demonstrated no significant difference for either morphine or M3G in either group. Taken together, loss of EphB2 does not appear to significantly alter morphine kinetics or metabolism.

3.5. EphB2 null mice exhibit deficiencies in contextual learning

Previous studies have demonstrated that in addition to direct modification in mu opiate receptor signaling, the development of morphine tolerance represents a form of learned conditioning [29–31]. As indicated above EphB2 null mutants do not exhibit an enhancement in morphine dependent analgesia following removal to a novel environment. In order to determine whether these features are morphine-specific or the result of a more general deficit in learning and memory, EphB2 null mutants and controls were examined in several paradigms of conditioned learning in the absence of morphine. Response to single-trial passive avoidance conditioning was examined for both groups as shown in Fig. 5A. In this paradigm, animals freely explored light and dark compartments of the chamber prior to a single round of training with delivery of a mild foot shock in one compartment. Enhanced latency to enter the environment in which the aversive stimulus was previously delivered is taken as a measure of associative learning and memory. As shown in the figure, EphB2 null mice exhibited significantly lower retention times compared to controls despite similar acquisition times between both groups.

A feature of morphine exposure in naïve rodents is the induction of hyperactivity [32–34]. To examine this property in EphB2 null

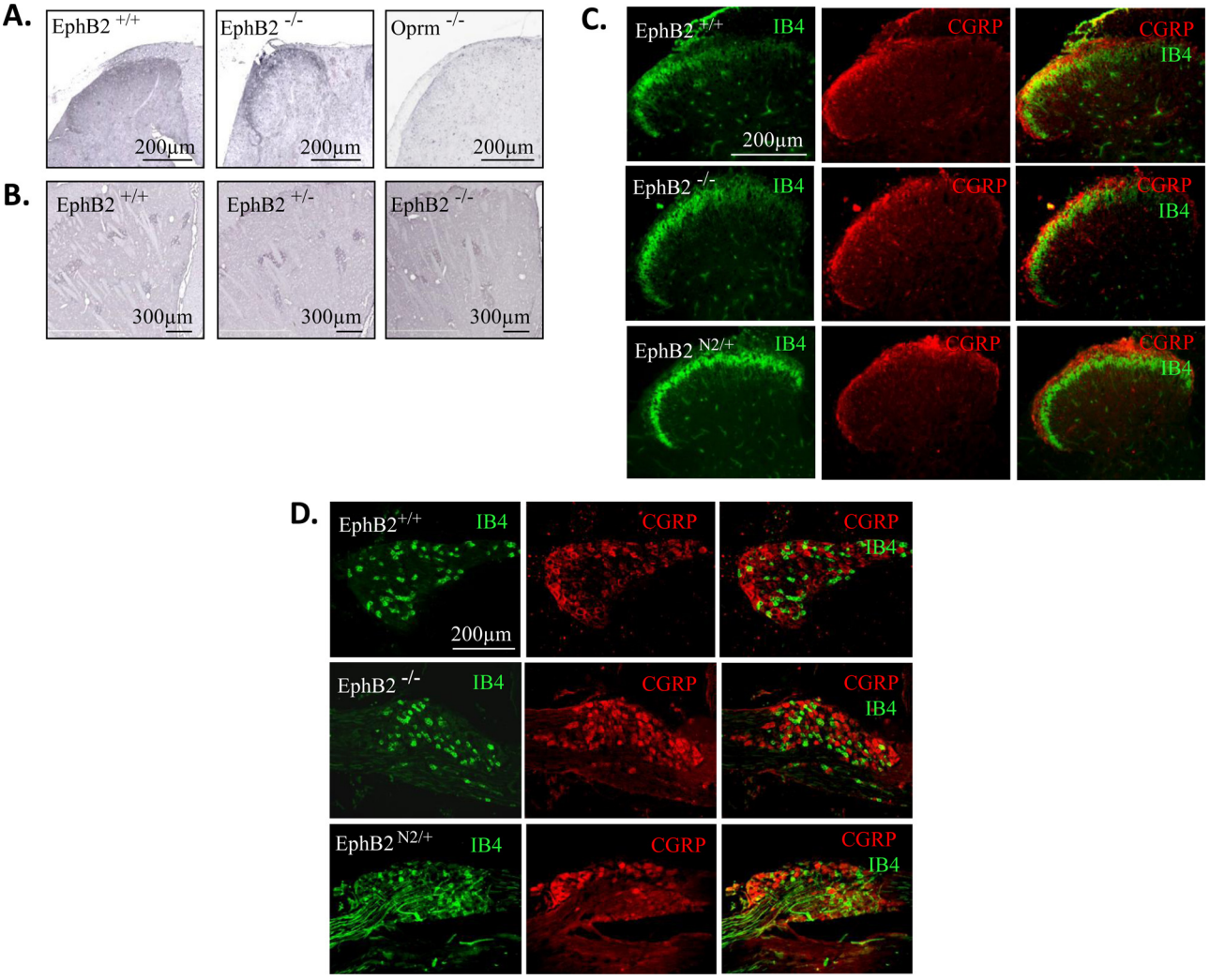

Fig. 3. Distribution mu opioid receptor and nociceptive markers in EphB2 null mice and control littermates. Loss of EphB2 does not alter cellular distribution of MOR in (A) spinal cord or (B) striatum. (A) Distribution of mu opioid receptor in lumbar spinal cord of EphB2 wild type, null and Oprm kockouts respectively. (B) Distribution of mu opioid receptor in striatum of EphB2 wild type, heterozygous, and null mice. (C) Immunohistochemistry of the peptidergic and non-peptidergic markers CGRP and IB4 respectively within in dorsal laminae of the lumbar spinal cord. Comparative sections of morphine treated EphB2 wild type and EphB2 null mice are shown, together with those of morphine naïve EphB2 N2/+ mice. No disruption of the cellular organization of the indicated markers is seen in EphB2 null mice prior to or following morphine treatment compared to controls. (D) CGRP/IB4 immunohistochemistry in dorsal root ganglia (L4–L5). No significant difference in staining intensity or organization of CGRP and IB4 is observed between morphine-treated EphB2 wild type, null or EphB2 N2/+ morphine-naïve animals.

mice, mutants and controls were each allowed to freely explore a novel environment for a period of one hour. Compared to controls, mice lacking EphB2 exhibit persistently higher levels of exploratory activity (Fig. 5B). Such elevations in open field activity have traditionally been interpreted as a result of enhanced motor output, anxiety, or a reduction in habituation and/or learning [35]. Examination of EphB2 null mice versus controls demonstrated no significant difference in intrinsic locomotor ability (Fig. S1C–G), or anxiety as suggested by initial latency to dark chamber entry (Fig. 5A). The elevation in exploratory activity seen in EphB2 mice may therefore arise from impairment in spatial cue recognition. Notably however, the effect seen in EphB2 null mutants is not an all-or-none event. Despite significant impairment, EphB2 null mice do experience a degree of habituation in both the presence and absence of morphine; suggesting that some forms of learning are intact in these animals. Following one hour of exploration, morphine naive animals received an injection of morphine and their open field activity recorded for an additional hour. In wildtype littermates and EphB2 heterozygotes, such treatment produced the expected elevation in activity as shown in Fig. 6A. Similar to controls, naïve EphB2 null mice also responded initially to morphine exposure with a period of enhanced activity, suggesting that these animals show no baseline difference in their initial response to morphine. However in EphB2 null mice this elevated activity was not sustained compared to control littermates. Interestingly we have previously observed a similar phenomenon in EphB2 null mice with respect to stability of their NMDA dependent long-term potentiation [18]. However it should be noted that EphB2 null mice are not deficient with respect to all aspects of place recognition, as demonstrated by their equivalent performance compared to age-matched littermate controls in both novel object recognition and object displacement challenge (Fig. S3), suggesting a specific role in contextual opiate learning.

3.6. *Bilateral lesions of the dorsal hippocampus mimic the behavioural responses seen in EphB2 null mice*

A number of prior studies have demonstrated the significance of associative and contextual learning in the regulation of morphine-dependent behaviors such as tolerance [6–8]. To determine the

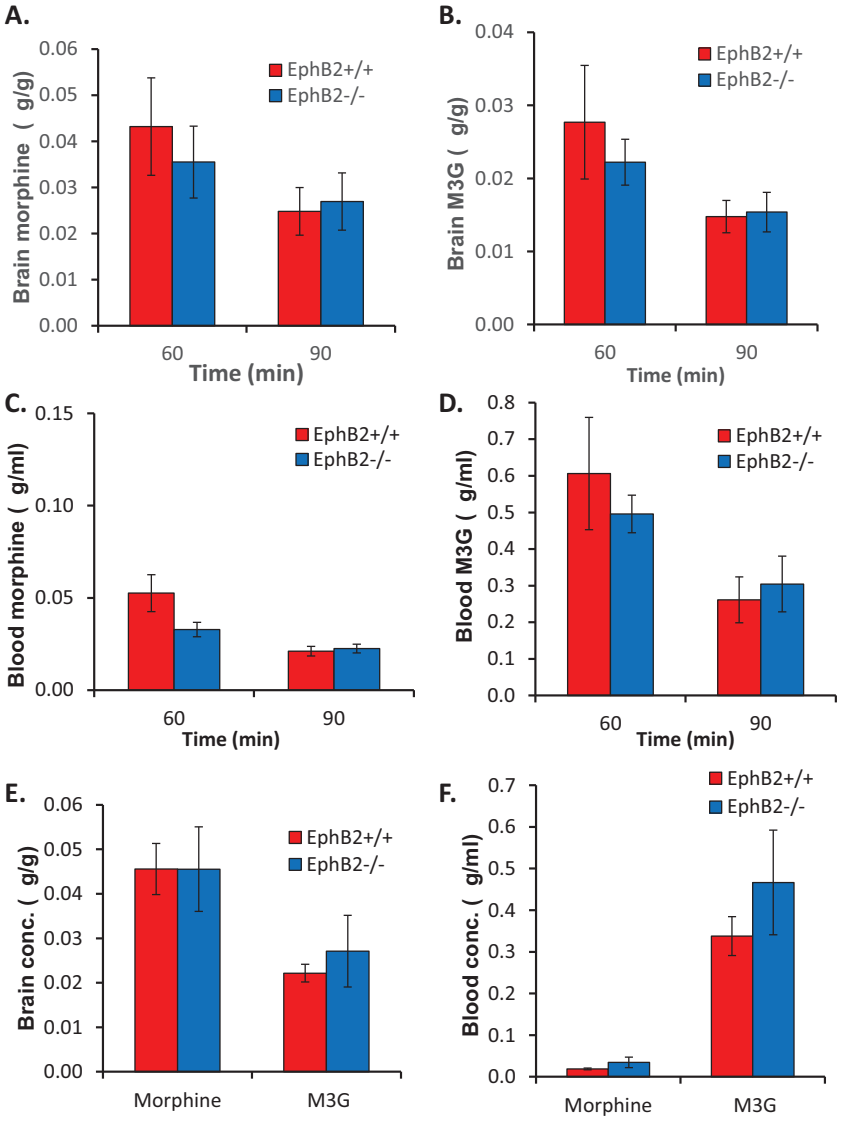

Fig. 4. LC/MS/MS Analysis of brain and blood morphine metabolism in EphB2 null mice and controls ($N > 6$ animals/group/time point). Brain morphine (A) and M3G (B) levels in EphB2 null and wild type littermates upon initial exposure to morphine. (C and D) Kinetic analysis of blood morphine and M3G levels respectively in EphB2 mice. (E and F) Analysis of brain 60 min following morphine treatment. (E) and blood (F) morphine and M3G levels in wild type and EphB2 null mice following repeated morphine exposure (day 7, $N > 4$ animals/group). *Wild-type versus EphB2 null mice at $p < 0.05$. Error bars are ±SEM.

significance of spatial contextual cues in regulating the effect seen in EphB2 null mice toward morphine tolerance and define the neuroanatomic locus of these effects, bilateral electrolytic lesions were performed in the dorsal hippocampus of male adult wildtype animals. As shown in Fig. 5C, bilaterally lesioned animals do indeed exhibit a substantial impairment in single-trial passive avoidance conditioning compared to sham-operated controls. Baseline acquisition times between the two groups were not significantly altered. Analysis of such animals with respect to spontaneous exploratory activity (Fig. 5D), demonstrates a similar trend to that seen in EphB2 null mice compared to sham operated controls. Bilateral disruption of dorsal hippocampal circuitry thus results in impedance in contextual learning similar to that seen in EphB2 mutants. With respect to morphine-induced hyperactivity, EphB2 null and lesioned animals both show significant differences compared to sham-operated controls and EphB2 wildtypes in their maintenance of morphine-mediated spontaneous activity (Fig. 6A and B). Similarly, following acquisition of morphine tolerance on day 7, lesioned animals did not show enhancement of morphine-dependent analgesia following transfer to novel environment (Fig. 6C); similar to responses seen in EphB2 null mice (Fig. 2). By contrast, sham-operated animals (Fig. 6D) exhibited a significant enhancement of morphine-dependent analgesic response upon transfer to a novel environment similar to EphB2 wild-types. Such findings suggest that changes in morphine-mediated behavior seen in EphB2 null mutants are mediated through alteration of hippocampal contextual learning.

4. Discussion

In the present study we examined the role which EphB2 plays in regulating morphine tolerance. We observe that loss of EphB2 significantly potentiates the development of morphine-dependent tolerance compared to wildtype and heterozygous littermates. EphB2 null mice also showed changes in context-dependent response to morphine, as shown by the absence of enhanced anti-nociception following introduction to a novel environment compared to control littermates. By contrast, mice homozygous for a targeted deletion of the EphB2 kinase domain demonstrated patterns of morphine tolerance indistinguishable from that seen in

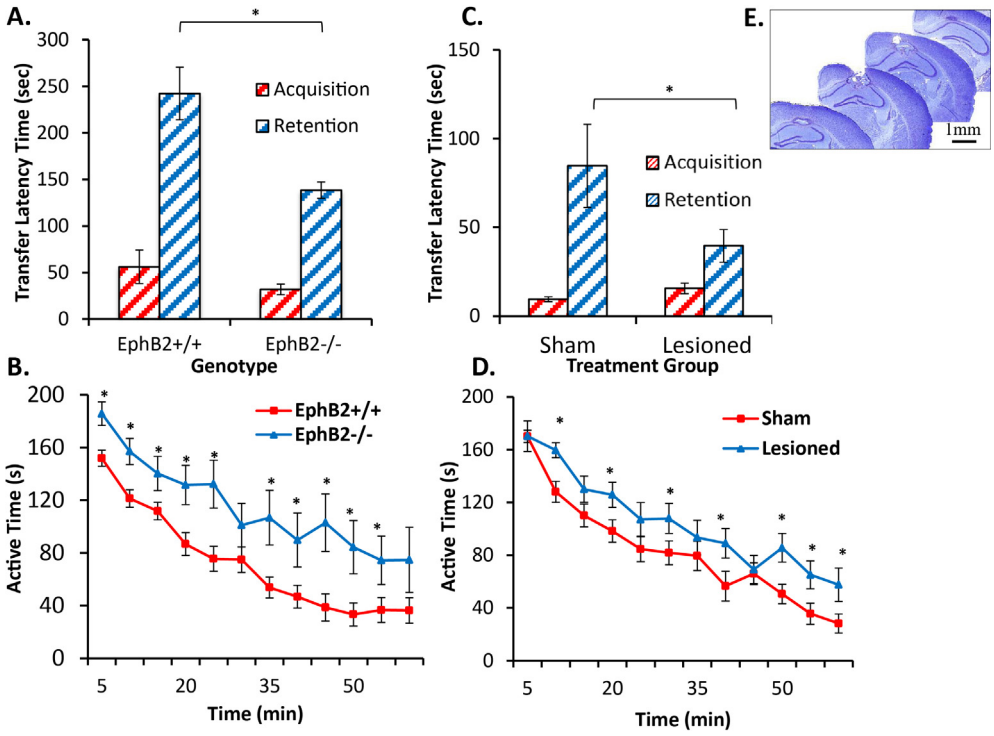

Fig. 5. Behavioral responses of EphB2 null mice and hippocampal-lesioned wild type animals. (A) Acquisition and retention times following single pass passive avoidance training for EphB2 null and wild type littermates ($N = 10$ and 14, respectively). (B) EphB2 null mice exhibit elevated exploratory activity compared to wild-types littermates ($N = 14$ and 18, respectively). (C) Passive avoidance acquisition and retention times in lesioned and sham operated wild type animals ($N = 19$ and 14, respectively). Similar to EphB2 null mice, lesioned animals show significantly lower levels of retention in passive avoidance assays compared to controls. (D) Lesioned animals exhibit a similar increase in exploratory activity compared to sham operated controls ($N = 19$ and 16, respectively). (E) Example of electrolytic lesion affecting dorsal hippocampus. Error bars are shown ±SEM, *comparison of wild type and EphB2 null mice at $p < 0.05$.

wild-types. The accelerated tolerance seen following loss of EphB2 thus appears to arise as a result of the loss of EphB2 reverse signaling in these mutants.

Prior electrophysiologic studies have demonstrated that EphB2 enhances NMDA-dependent signaling within the hippocampus through the promotion of receptor stability [18,28]. Loss of EphB2 *in vivo* has been shown to reduce both the magnitude and stability of NMDA-dependent LTP at CA1 and dentate gyrus synapses. Consistent with this, our analysis of context-dependent associative learning in EphB2 null mice demonstrate an impairment in hippocampal-dependent function. Yet if loss of EphB2 inhibits hippocampal learning, why does it result in a potentiation of morphine-dependent tolerance; a form of learning? The key may lie in the relative influence of EphB2 on hippocampal versus extra-hippocampal forms of learning. Studies in several systems in mammals have demonstrated that disruption of hippocampal processing can actually enhance stimulus response and discrimination learning [36–39]. This effect is thought to arise as a result of a reduction in proactive competitive interference between the hippocampal and extra-hippocampal sites of learning; thereby enhancing configural learning [36]. Impedance of hippocampal activity thus facilitates single cue learning at sites such as the striatum and amygdala [39], suggesting that these systems normally compete with one another in the intact animal. As a result, hippocampal inactivation enhances conditioned taste aversion mediated by sites such as the amygdala, and analyses of bidirectional competition between the striatum and hippocampus during learning reveals enhanced performance in win-stay strategy in radial arm maze tasks following the induction of hippocampal lesions [37,39]. Thus, the reduced efficiency of EphB2 null mice to process hippocampal-dependent contextual information may underlie the enhanced ability of these animals to respond to morphine-dependent single cue learning.

Previous studies have examined the role of NMDA-dependent signaling in the development of morphine tolerance utilizing the non-competitive NMDA antagonist MK-801 [40–42]. In these studies, pharmacologic treatment with MK-801 induces an *attenuation* of morphine tolerance, similar to results seen following spinal inhibition of EphB1. Mice heterozygous for EphB1 have also been reported exhibit an attenuation of morphine tolerance [22]. By contrast loss of EphB2 signaling results in a change in learning and memory similar in many respects to that seen following our bilateral ablation of dorsal hippocampal signaling. Examination of both native and morphine-induced exploratory activity in these animals, as well as performance passive avoidance task demonstrate an altered pattern of contextual perception similar to that observed in EphB2 null mice. Mechanistically, previous studies have demonstrated a rapid context-dependent effect of morphine sensitization upon AMPA receptor distribution within the hippocampus, notably a decrease in synaptic levels of GluA2 and elevation of synaptic GluA1 as a result of enhanced receptor insertion [43]. These effects are correlated with both enhanced S845 phosphorylation on GluA1 and promotion of stargazin association with the receptor. In addition, impairment of hippocampal LTP following morphine exposure has been shown to influence NMDA receptor signaling [44,45]. This is intriguing given that we have previously observed loss of EphB2 signaling significantly reduces both the stability of hippocampal LTP and levels of synaptic NMDA receptors due to the role of EphB2 in localizing AMPA and NMDA receptors within the synaptic bouton [18,46]. Taken together, such findings suggest the existence of a requisite molecular network regulating contextual opiate associations, connecting EphB2 reverse signaling to morphine-dependent reductions in hippocampal LTP through

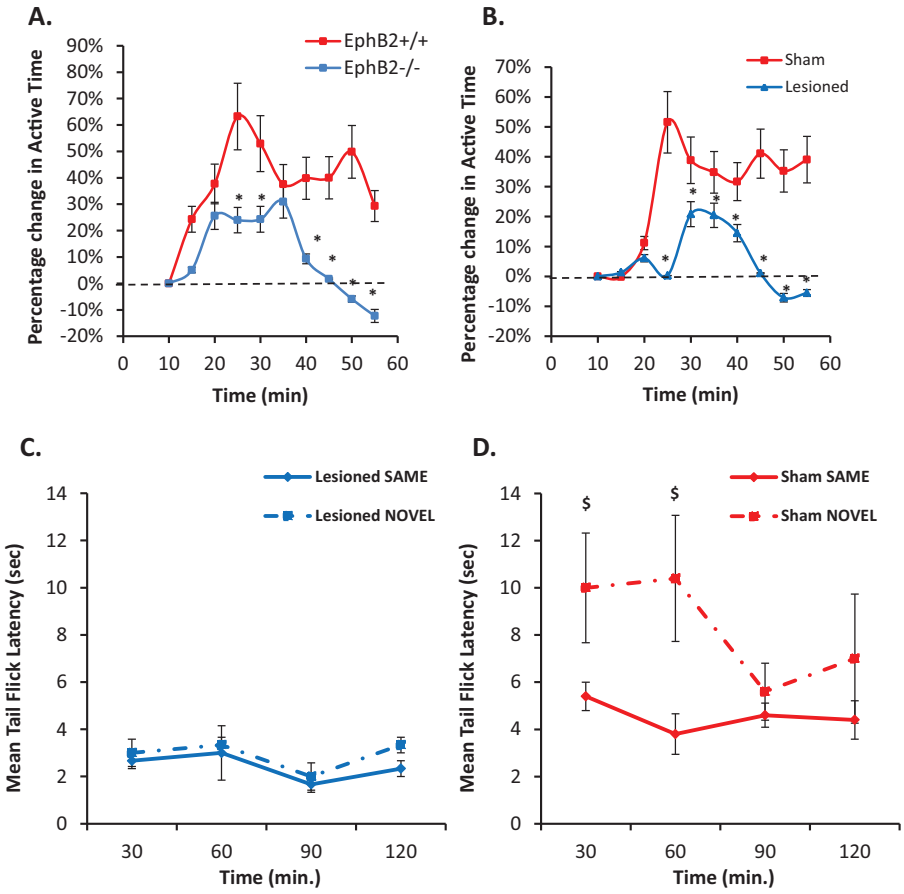

Fig. 6. Behavioral responses of EphB2 null mice and hippocampal-lesioned wild type animals. (A and B) Exploratory activity following morphine treatment. Both wild type and EphB2 null mice ($N \geq 8$ animals/group) exhibit morphine induced hyperactivity. However morphine induced activation is quickly diminished in EphB2 null mice similar to lesioned animals. $^{*}p < 0.05$ for time indicated. (C and D) Anti-nociceptive responses of sham-operated and lesioned animals upon transfer to a novel environment following 7 days of morphine treatment ($N \geq 6$ animals/group).
Legend: (C) (diamond solid) sham-operated, home environment; (square dashed) sham operated, novel environment; (D) (diamond solid) lesioned animals, home environment; (square dashed) lesioned animals, novel environment. $^{\$}$sham lesioned animals, novel versus home environment at $p < 0.05$. Error bars are ±SEM.

modification of NMDA/AMPA signaling. Indeed the effects seen in EphB2 null mice fit well with the notion that EphB2 reverse signaling represents a requisite molecular link in opiate signaling, connecting morphine-dependent alterations in hippocampal LTP to behavioral adaptations such as tolerance through modulation of NMDA /AMPA receptor signaling.

Initial exposure to morphine induces a period of hyperactivity in EphB2 null mice similar to that seen in wildtype littermates. However the *period* of this effect is significantly reduced in EphB2 null mice compared to controls, consistent with that seen in wildtype mice receiving bilateral hippocampal lesions. The lack of sustained morphine induced hyperactivity in EphB2 null mice is notable given its similarity to the reduced stability of LTP previously described [18]. Loss of EphB2-mediated signaling does not appear to modify baseline motor or sensory function, or alter initial responses to morphine exposure compared to heterozygous or wildtype littermate controls.

Nor does loss of EphB2 appear to alter the expression, distribution or avidity of mu opioid receptors at those CNS sites examined. In addition the pharmacokinetics of morphine metabolism appears unaltered in EphB2 null mice compared to controls. Analysis of associative contextual learning in EphB2 null mice demonstrates deficits in learning consistent with our previous description of impairments in hippocampal LTP for these animals [18]. In addition, EphB2 null mice do not exhibit extinction of morphine tolerance upon presentation of novel environmental cues, in contrast to results seen for EphB2 littermate controls and described previously in wildtype animals [5,10,47]. The nature of these effects suggests a modification in associative pathways involved in integrating learned responses to morphine rather than direct modification of morphine dependent signal transduction. These effects highlight several important differences with respect to how different members of the EphB family regulate morphine-dependent signaling. The effects seen in EphB2 null mice are propagated *via* reverse signaling as demonstrated by the wildtype pattern of morphine tolerance seen in kinase dead EphB2 $^{N2/N2}$ mice. By contrast, morphine dependent effects regulated by EphB1 appear to be mediated through forward receptor signaling, the ablation of which retards the development of morphine tolerance [22]. In addition we show that animals heterozygous for the null allele of EphB2 (EphB2$^{+/-}$) exhibit wildtype patterns of morphine responsiveness, whereas EphB1 heterozygotes are reported to exhibit responses similar to those seen for EphB1 knockouts [48]. A further distinction involves the pattern with which EphB2 and EphB1 are expressed within the central and peripheral nervous systems. Though EphB2 is persistently expressed in neurons at several central and peripheral loci involved in mediating morphine responsiveness, we observe that expression of EphB2 is not significantly altered at these sites in the presence or absence of following morphine treatment, sciatic nerve transection or chronic constriction injury (Fig. S1L–M); in contrast to EphB1 [49,50]. Thus it appears both mechanistically and functionally these EphB family receptors operate at different levels in

the hierarchy of sensate control to exert opposing influences on development of morphine tolerance.

Building upon the work of Battaglia et al. [51], several studies have examined the role of Eph/ephrin B-family signaling in the development of thermal hyperalgesia and mechanical allodynia following neuropathic injury [22,49,52], as well as the development of morphine tolerance and withdrawal using these models [22]. In these models of induced neuropathic pain, inhibition of Eph/ephrin B signaling through either genetic lesion of EphB1 or pharmacologic application of soluble EphB1 or B2 Fc, inhibited the development of neural hyperalgesia. By contrast pharmacologic application soluble dimeric ephrin B1 or B2 Fc promotes hyperalgesia induction. These effects have been shown to relate to the direct spinal influences of Eph/ephrin B signaling, with inhibition suppressing hyperexcitability in DRG sensory neurons, thereby preventing sensitization of central nociceptive neurons in the dorsal horn [48,52]. Thus in contrast to our findings with EphB2, ablation of EphB1 signaling appears to directly modify spinal excitability with respect to morphine exposure. The NMDA dependence of these phenomena has been demonstrated through use of the non-competitive antagonist MK-801, which both prevents development of hyperalgesia and attenuates morphine tolerance and withdrawal in the above paradigms.

As noted previously, the multivariate nature of Eph receptors and their ligands provide an opportunity to create complex patterns of biological response within disparate neural loci [14,16,21]. An example of this is seen in the distribution and response of Eph receptors within the hippocampus and spinal cord. Within the hippocampus, the varied distribution of EphB2 versus EphB1 at different loci within this circuit allows imposition of distinct patterns of regulatory activity on effects such as NMDA signaling [18,20,53]. By contrast in dorsal lamina of the spinal cord, both EphB1 and B2 are co-expressed but EphB2 does not go undergo upregulation following morphine exposure or neuropathic injury in contrast to EphB1 [49,52]. Thus under conditions of chronic morphine exposure, the EphB1 signaling influence may be expected to predominate at such sites under these circumstances. Such multilayered systems of signaling may explain the varied time dependent response of drugs such as morphine in which aggregate signaling response is modified over time; ultimately resulting in a varied behavioral response following chronic treatment. Thus in the present study we have described a novel aspect of EphB2 receptor function underlying a modification of learning and memory relevant to the development of opiate tolerance. Examination of EphB2 reverse signaling reveals that EphB2 regulates the competing associative features of morphine-dependent learning (phm.utoronto.ca/~jeffh/Huroy_sup_mat.pdf).

Author contributions

All authors contributed to the work presented in this paper. S.H. designed and performed experiments, analysed data and assisted in the production of the manuscript; A.K. designed and performed experiments and analysed data; L.M. assisted in the preparation and analysis of samples for mass spectrometry; C.L.C. designed and analyzed data for mass spectrometry experiments; S.R.G. designed, implemented and analyzed data related to radio-ligand binding experiments; D.v.d.K. assisted in all elements of experimental design, analysis of data and editing of the manuscript; J.T.H. designed and performed experiments, analyzed data and wrote the manuscript.

Disclosure of potential conflicts of interest

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbr.2015.09.023.

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