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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;