THE ROLE OF EPHB2 RECEPTORS IN THE DEVELOPMENT OF MORPHINE TOLERANCE

By

Ashlin Katherine Kanawaty

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Pharmaceutical Sciences
Faculty of Pharmacy, University of Toronto

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The role of EphB2 receptors in the development of morphine tolerance

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Ashlin Kanawaty

Department of Pharmaceutical Sciences, University of Toronto

ABSTRACT

Recently we have begun to investigate a novel role of EphB receptors in opiate-dependant analgesia. EphB2-β-galactosidase knockins demonstrate that EphB2 is persistently expressed within a number of neural pathways involved in MOR-mediated nociception *in vivo* and that EphB2 colocalizes with markers of the MOR at the cellular level in the spinal cord and dorsal root ganglia. Despite demonstrating wild-type levels of sensory and motor activity, EphB2 null mice exhibit a significantly altered analgesic response to repeated (but not naive) opiate exposure compared to controls. Investigation of EphB2 null mice and wild type animals revealed no differences in MOR protein levels or affinity. Analysis of this opiate-mediated tolerance suggests that associative phenomena play a substantial role in mediating the analgesic effects observed, possibly due to deficiencies in CA1-mediated learning. Therefore, loss of EphB2 may diminish context-dependent learning and that such learning plays a substantial role in regulating morphine-dependent tolerance.
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siRNA-mediated suppression of EphB2 in the dorsal hippocampus
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<tr>
<td>AMPA</td>
<td>2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CXC4R</td>
<td>Chemokine receptor 4</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’-diaminobenzidine</td>
</tr>
<tr>
<td>DAMGO</td>
<td>[D-Ala2, N-MePhe4, Gly-ol]-enkephalin</td>
</tr>
<tr>
<td>DOR</td>
<td>Delta opioid receptor</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced luminol-based chemiluminescence</td>
</tr>
<tr>
<td>Eph receptor</td>
<td>Erythropoietin producing hepatocellular receptor</td>
</tr>
<tr>
<td>Ephrin</td>
<td>Eph family receptor interacting protein</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-Aminobutyric acid</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GIRK channel</td>
<td>G protein-coupled inwardly-rectifying potassium channel</td>
</tr>
<tr>
<td>GLU</td>
<td>Glutamate</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein coupled receptor</td>
</tr>
<tr>
<td>Grb4</td>
<td>Growth factor receptor-bound protein 4</td>
</tr>
<tr>
<td>GRIP1</td>
<td>Glutamate receptor-interacting protein 1</td>
</tr>
<tr>
<td>GRK2</td>
<td>G protein-coupled receptor kinase 2</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>JMR</td>
<td>Juxtamembrane region</td>
</tr>
<tr>
<td>KD</td>
<td>Kinase domain</td>
</tr>
<tr>
<td>KIF5</td>
<td>Kinesin superfamily protein 5</td>
</tr>
<tr>
<td>KOR</td>
<td>Kappa opioid receptor</td>
</tr>
<tr>
<td>LBD</td>
<td>Ligand binding domain</td>
</tr>
<tr>
<td>LMW-PTP</td>
<td>Low molecular weight protein tyrosine phosphatase</td>
</tr>
<tr>
<td>LTP</td>
<td>Long term potentiation</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MOR</td>
<td>Mu opioid receptor</td>
</tr>
<tr>
<td>N-WASP</td>
<td>Neural Wiskott-Aldrich syndrome protein</td>
</tr>
<tr>
<td>N1</td>
<td>Nuk 1 (EphB2) null mutant</td>
</tr>
<tr>
<td>N2</td>
<td>Nuk 2 (EphB2) kinase dead mutant</td>
</tr>
<tr>
<td>Nck</td>
<td>Nck adaptor protein</td>
</tr>
<tr>
<td>NIK</td>
<td>NF-κB-inducing kinase</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>PAG</td>
<td>Periaqueductal gray matter</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDZ</td>
<td>An acronym combining the first three letters of three different proteins: postsynaptic density protein (PSD95),</td>
</tr>
</tbody>
</table>
Drosophila disc large tumour suppressor (Dlga), zonula
occludens-1 protein (zo-1)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>PDZ-RGS3</td>
<td>PDZ-Regulator of G protein signaling 3</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKCγ</td>
<td>Protein kinase C gamma</td>
</tr>
<tr>
<td>PLL</td>
<td>Poly-L-lysine</td>
</tr>
<tr>
<td>PSD95</td>
<td>Postsynaptic density protein 95</td>
</tr>
<tr>
<td>RGC</td>
<td>Retinal ganglion cell</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>RVM</td>
<td>Rostral ventral medulla</td>
</tr>
<tr>
<td>SAM</td>
<td>Sterile α motif</td>
</tr>
<tr>
<td>SFK</td>
<td>Src family kinase</td>
</tr>
<tr>
<td>SH2</td>
<td>Src-homology 2 domain</td>
</tr>
<tr>
<td>SHEP1</td>
<td>SH2-domain-containing Eph receptor-binding protein 1</td>
</tr>
<tr>
<td>TLT</td>
<td>Transfer latency time</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION
1.1 Eph receptors

“Eph” or erythropoietin producing hepatocellular receptors, were originally discovered in 1987 in a hepatoma cell line ETL-1[1]. Eph receptor kinases were first discovered during attempts to identify receptor tyrosine kinases which might mediate cancer aggression. As a result of these studies, Eph receptors were first classified as orphan receptors with no known ligands or functions for several years before it was discovered they act as key regulators of cell sorting and axon guidance during development. Today, the Eph family of receptor tyrosine kinases represents the largest family of all known axon guidance molecules (netrins, semaphorins, etc.).

Following sequencing of the mammalian genome, known mammalian Eph receptors can be categorized into two major subfamilies termed A and B (see Figure 1). For each of these, their cognate ligands (ephrins) can be similarly divided into A and B subclasses. EphrinA ligands are attached to the plasma membrane by a glycosylphosphatidylinositol (GPI) linkage, whereas ephrinB ligands contain both transmembrane and intracellular components. Thus Eph receptor signaling is unique in that its membrane-bound ligands can exhibit both “reverse” (ligand) as well as “forward” (receptor-mediated) signaling. Though EphA and EphB receptors typically bind ephrinA or ephrinB-class ligands respectively, there are several known instances of interclass promiscuity. Significantly, the EphA4 receptor (EphA-family receptor bearing the strongest structural similarity to EphB2) exhibits a relatively high affinity for ephrinB2, while EphB2 exhibits affinity for ephrins B1, B2, A5 and B3. These interactions are summarized in Table 1.
**Figure 1. Structure of Eph receptor and ligands.** Within the extracellular domain, the Eph receptor is comprised of a ligand binding domain (N-terminus) followed by a cysteine-rich region and two fibronectin type three repeats. The receptor juxtamembrane region contains several tyrosine residues important in subsequent receptor trans-autophosphorylation and activation. Proximal to this is the core kinase domain followed by the sterile alpha motif (SAM) domain and C-terminal PDZ-binding motif. With respect to Eph ligands, ephrinA and B families differ with respect to the form of their membrane attachment. EphrinA ligands are connected to the plasma membrane by glycosylphosphatidyl inositol (GPI) linkages, while ephrinB ligands possess transmembrane and intracellular motifs capable of signaling. Because of this intracellular domain, signaling conducted downstream of the Eph receptor is referred to as forward signaling, while signaling through the ligand is referred to as reverse signaling.
Table 1. Affinities of different ephrin-Fc molecules for the EphB2 receptor ligand binding domain. Values were determined from pull down assays using isolated ectodomains of the receptor and ligands. Kᵢ is given in nmol/L.
<table>
<thead>
<tr>
<th>Ephrin-Fc ligand</th>
<th>$K_i$(nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EphrinB2</td>
<td>0.24</td>
</tr>
<tr>
<td>EphrinB1</td>
<td>1.2</td>
</tr>
<tr>
<td>EphrinA5</td>
<td>28</td>
</tr>
<tr>
<td>EphrinA2</td>
<td>240</td>
</tr>
<tr>
<td>EphrinA4</td>
<td>540</td>
</tr>
<tr>
<td>EphrinA1</td>
<td>740</td>
</tr>
<tr>
<td>EphrinB3</td>
<td>1000</td>
</tr>
<tr>
<td>EphrinA3</td>
<td>2000</td>
</tr>
</tbody>
</table>

Since their discovery Eph receptors have been shown to regulate a wide array of cellular interactions. Among these, Eph receptors have been shown to play a prominent role in several forms of cell-cell communication including cell sorting and axon guidance. Specific neuronal examples include guidance of retinal[2, 3], cortical[4] and spinal[5] axons during development; while prominent cell sorting phenomena include neural crest cells[6], angiogenesis[7] and development of intestinal villia[8]. Because of their critical role in proper cell-cell sorting, derangements of Eph/ephrin expression have been implicated in pathologic responses such as cancer[9], neurodegeneration[10] and drug dependence[11].

1.2 Eph structure and signaling

Like most receptor tyrosine kinases, the Eph receptor fundamentally consists of an ectodomain, transmembrane region and intracellular signaling unit (Figure 1). Within the ectodomain there exists the N-terminal ligand binding core followed by two fibronectin type III repeats and a cysteine-rich region. The ligand binding domain (Figure 2) is organized around a central hydrophobic core formed by aromatic side chains. The G-H loops of the ephrin inserts into a hydrophobic channel created by D, E and J strands of the receptor. The core of the ephrin-binding interface consists of critical contacts with the G-H loop upon ligand binding, and it is this region which confers much of the ligand specificity exhibited by individual Eph receptors[12, 13]. The critical subunit recognition loop, the H-I loop, of EphA receptors is 13 amino acids in length, while those of the B subclasses is 17 residues. Consistent with its similar ligand binding specificity to Eph B-class receptors, the EphA4 receptor exhibits a valine at position 54 similar to EphB
receptors. By contrast, EphA receptors typically incorporate isoleucine or methionine at this position[14].

The intracellular domain contains several critical signaling domains: the juxtamembrane region (JMR), the kinase domain, a sterile alpha motif (SAM) domain, as well a C-terminal PDZ-binding motif. The JMR is a 47 amino acid region occupying residues 573-620 of the EphB2 receptor. This region contains two critical tyrosine residues which are phosphorylated following full activation of the kinase domain upon ligand binding. The kinase domain consists of an N-terminal and C-terminal lobe. The N terminal lobe is involved in coordinating binding of ATP, while the C terminal lobe is involved in coordinating the binding of magnesium and subsequent catalysis of ATP. In the unbound (inactive, autoinhibited) state, the N and C lobes interact to form the nucleotide binding site and catalytic cleft. In this state, the unphosphorylated JMR interacts with the C terminal component of the kinase domain, causing movement of Tyr 750 towards the C terminal lobe resulting in steric hindrance. In the activated state, phosphorylation of JMR residues Tyr 604 and 610 results in a conformational change and charge repulsion from the kinase domain, allowing binding of this region to SH2 domain-containing proteins β strands 1 and 2 of the N terminal lobe, in combination with the G loop, form a flexible flap that interacts with the adenine base, ribose sugar and non-hydrolizable phosphate groups of the ATP molecule. Of the C-terminal lobe, β strands 7 and 8 contribute to the cleavage and transfer of the γ-phosphate group to the substrate. These phosphate groups can be transferred to the JMR or KD (in EphA4 these sites are Tyr 779 (activation site), Tyr798 and Tyr841) of adjacent Eph receptors in a phenomenon
Figure 2. Structure of the EphB2 ligand binding domain and juxtamembrane region/kinase domain. A. Crystal structure of the EphB2 LBD. This domain assumes a compact jellyroll beta-sandwich consisting of 11 antiparallel β-strands. B. JMR (red) and KD N-terminal (green) and C-terminal (blue) lobes.
known as “trans-autophosphorylation”, where these phosphate groups serve to activate the receptor or act as docking sites for intracellular signaling molecules[15, 16].

The SAM domain represents a relatively low affinity motif (ranging from 500 uM to 5 mM) which is thought to play a role in time-dependent homotypic oligomerization. However this has only been demonstrated in isolated SAM domains proteins and not complete Eph receptors. The PDZ motif consists of a C-terminal valine, but also contains upstream elements which impart structural specificity (Ile-Gln-Ser-Val-Glu-Val in the case of EphB2). The PDZ motif acts as a docking site for a wide variety of intracellular signaling molecules and phosphatases which act to inactivate Eph signaling activity[17].

As indicated above, upon binding clustered cognate ligand, Eph receptors can initiate a variety of intracellular signaling events. Though typically affiliated with an enhancement of cellular repulsion, the specific cellular responses elicited by ligand binding (typically attraction or repulsion of cells or axons) depend in large part upon the nature of the down-stream signaling proteins present in any given cell. At present the most well characterized Eph signal transduction pathway is that regulating by Rho family GTPases such as Rho, Rac and Cdc42. In the case of axon guidance, transient activation of Rho by phosphorylated Eph receptors results in a temporary inhibition of Cdc42 and Rac activity, ultimately resulting in enhanced depolymerization of actin cytofilaments within targets such as nascent growth cones. This is thought to be the principal mechanism whereby activation of Eph receptors results in growth cone collapse[18].

Alternatively, during synaptogenesis, EphB2 can inhibit RhoA and activate Rac and Cdc42, promoting the proliferation of dendritic spines and synapse formation (Figure 3; discussed in detail below). In addition, Eph receptor activation has also been shown to
regulate integrin activity, altering integrin interaction with the extracellular matrix. Specifically, the recruitment of Nck, PI3K and/or LMW-PTP can result in an inhibition FAK leading to integrate activation, promoting adhesion. Alternatively activation of molecules such as SHEP1 leads to reductions in integrin activation and thus diminished adhesion (Figure 4)[19, 20].

1.3 Ephrin structure and signaling

Ephrin ligands contain a structurally characterized ligand binding motif (LBD) at the extracellular N-terminus, a transmembrane domain and non-enzymatic cytoplasmic tail terminated in a PDZ binding motif (Figure 1). Unlike Eph receptors, the class specificity of ephrin ligands is designated by the G-H loop loops of the ligand binding domain which interface with the hydrophobic channel previously described for Eph receptors[13, 21-25]. Upon binding to Eph receptors, several tyrosine residues located within the intracellular tail of ephrin receptors undergo phosphorylation through the actions of cytoplasmically localized kinases. Such binding results in a destabilization of the local hairpin structure within the intracellular domain, promoting the attachment of a variety of signaling molecules. These signaling events have been termed "reverse signaling" in order to differentiate them from signaling events induced directly via Eph phosphorylation directly. Like Eph receptors, activated ephrins can induce a number of different signal transduction pathways. One of the more well characterized these involves activation of ephrins by Src family kinases. Phosphorylation of ephrin tyrosine residues within the intracellular domain induces binding of SH2-containing proteins such as Grb4. Grb4 can subsequently activate molecules such as Rac, Abi-1 and FAK, which ultimately
Figure 3. Eph receptor signaling through Rho-family GTPases. Generally, Eph receptor activation is known to elicit several events which influence cytoskeletal remodeling, principally on regulators such as RhoA, Rac, Cdc42 and Abi, as well as ERK/MAPK pathways.
Activation of EphB2 will enhance spine formation through the inhibition of RhoA and the activation of Rac and Cdc42.
Figure 4. Eph receptors and integrins. Eph receptors can both activate and inactivate \( \alpha_6\beta_3 \) integrins via Nck/LMW-PTP signaling or Ras/FAK signaling, respectively.
Figure 5. Ephrin signaling and integrins. Ephrin ligands themselves can elicit both immediate and delayed signaling cascades. During fast signaling, ephrins phosphorylate SFKs enhancing its activity, which subsequently increases integrin and Grb4 activity. Stimulation of Grb4 leads to subsequent activation of Axin, Abi-1 and CAP. In the slow component, PTP-BL inactivates both the SFKs and ephrin.
**Figure 6. Ephrin signaling and GPCRs.** Ephrins may bind PDZ-RGS3 via their PDZ domain. PDZ-GRS3 acts to couple G proteins back to the CXCR4 GPCR, which is crucial in the guidance of granule cell neurons within the cerebellum during development.
influence the dynamics of cytoskeletal organization within the cell. PDZ binding proteins such as PTP-BL can interact with activated ephrins to promote dephosphorylation, thus countering levels of ephrin activation (Figure 5)[19]. Ephrins are also known to promote the re-coupling of G proteins with their G protein coupled receptors (GPCRs), as in the case of the CXC4R receptor, which ultimately regulates granule cell migration in the cerebellum[26] (Figure 6).

1.4 Nature of the Eph-ephrin oligomer

The precise organization and dynamics of activated Eph-ephrin receptor complexes remains somewhat ambiguous. Co-crystals and biochemical analyses of ligand binding domains from specific Eph receptors / ephrin complexes have suggested the formation of two ephrin binding domains in conjunction with two Eph ectodomains. However it has also been theorized that high affinity interaction between single Eph and ephrin binding domain may initially create a heterodimer (Figure 7A). According to this model, sufficient concentrations of these heterodimers promote the development of higher-order complexes consisting of two heterodimer pairs to form a so-called heterotetramer (sequential step model; Figure 7B). Like all receptor tyrosine kinases, the close apposition of Eph receptors along the cell membrane induced as a result of complex formation is thought to promote autophosphorylation of adjacent kinase domains; resulting in receptor activation. Furthermore it has been postulated that as a consequence of additional lower affinity interactions through motifs such as the SAM domain, there may exist a time-dependent aggregation of heterotetrameric complexes following ligand binding which may be physiologically relevant to the regulation of Eph-induced activity.
(Figure 7C). At present only the EphB2-ephrinB2 complex has been definitively isolated as a tetramer[12].

As indicated above formation of Eph complexes typically results in axonal or cellular repulsion[20]. However truncated forms of Eph receptors have been identified which promote adhesion rather than repulsion[20, 27]. An example of this is seen with the EphA7 receptor, in which three variants have been identified: one full length isoform and two truncated isoforms which lack the kinase domain. These truncated forms of the EphA7 receptor are co-expressed with the full-length isoform in regions such as the cranial neural folds of E8.5 mice. Upon binding to ephrinA5, found in the dorsal edge of the cranial neural folds, the presence of truncated isoforms of EphA7 act to inhibit levels of natural phosphorylation seen in the full length receptor, ultimately resulting in a shift of ligand binding from repulsion to adhesion[27].

With respect to EphB2, three unique mRNAs have been described. Of these transcripts – 4, 5 and 11 kb in length – only the 4 kb (EphB2v) mRNA has been examined. EphB2v encodes a full length EphB2 receptor, but varies from the original in several aspects. EphB2v lacks one arginine residue immediately adjacent to the C-terminal side of the transmembrane domain and bears two conservative amino acid changes from leucine (EphB2) to valine (EphB2v). In addition, there is also a 93 nucleotide deletion at the 3’ end of the coding region which makes the C-terminus of EphB2v 70 amino acids longer than EphB2, and results in a frameshift, translocating the stop codon almost 300 nucleotides downstream of its location in the full length EphB2 receptor. EphB2v contains an additional polyadenosine stretch following the polyadenylation tail at the 3’ end, which is absent in the original full length EphB2
**Figure 7. Oligomerization states of EphB2 with ephrinB2.** A. EphB2 (blue) will form heterodimers with ephrinB2 (red) on adjacent cells (one Eph receptor, one ephrin). B. Heterodimers will further aggregate to form heterotetramer (two Eph receptors, two ephrins). This complex is believed to be sufficient for trans-autophosphorylation and activation of Eph receptors to occur. C. The formation of higher order oligomers is theorized to occur between tetrameric units at newly formed interfaces that only exist in the assembled tetramer. However, such structures have not yet been crystalized.

414(6866): p. 933-8
receptor. No distinct physiologic differences in these isoforms have yet been described[28].

1.5 Expression pattern of EphB2

Using a targeted knockin of the EphB2 receptor in conjunction with β-galactosidase (Figure 8), we and others have thoroughly characterized both the embryonic and adult expression pattern of EphB2. In specific regions these findings have been verified by anti-sense in situ hybridization, as well as Western blotting of sub-dissected tissues and immunohistochemistry[29-32]. The presence of EphB2 targeted gene deletions (i.e. knockouts) in conjunction with the described fusion has allowed the fidelity of each of the above such methods to be verified. The utilization of such reagents has demonstrated that published immunohistochemical reports regarding EphB2 must be interpreted with caution, as there presently exists no truly EphB2 specific antisera (regardless of claims). Typically such reagents exhibit a degree of cross-reactivity to other closely related Eph receptors due to the high degree of amino acid conservation present between related members. An overview of EphB2 expression during development and in adult stages relevant to the neuroanatomic features described in thesis is provided in Table 2.

The earliest appearance of EphB2 during development occurs at E8.5-9.0 within rhombomeres r3 and r5 and in the developing mesencephalon. Consistent with its role in axon guidance, EphB2 expression continues to spread as development proceeds, appearing within the retina and preoptic areas, otic vesicle, ventral midbrain and hypothalamus, trigeminal and vestibular ganglia, as well as cranial and spinal nerves by E10.5[31]. EphB2 begins to be expressed in a dorsal-ventral gradient within retinal
ganglion cells around E13. EphB2 expression continues during this period within pioneer axons of many of the above sites until such point as they reach their targets of innervation[29]. EphB2 is also present in some sensory epithelia adjacent to the sites, such as the semicircular canals, utricle and stria vascularis of the cochlea at E14.5[30]. During this period EphB2 expression also becomes elevated along the ventral aspect of the forebrain, a site for which proper guidance of the posterior track of the anterior commissure is crucial. Indeed, the first described neuroanatomic defect seen in mice lacking EphB2 is the aberrant guidance of axons within the pars anterior of the anterior commissure[31]. In such EphB2 knockout animals, these axons mis-project toward the floor of the forebrain due to the lack of EphB2-dependent repulsive cues. Though expressed primarily within the central nervous system during development, EphB2 is also known to be expressed at several extra neural sites such as the heart and gut.

During the latter phase of embryonic development (~E18.5), EphB2 expression within the brain is largely shut down, with the exception of several sites such as the superior colliculus and ventral forebrain. In the period between E18.5 and postnatal day 7 (P7) EphB2 throughout the CNS becomes substantially reduced, largely shutting down save for low expression within sites known to undergo significant synaptic modification such as the entorhinal cortex, cingulate and hippocampus (principally the CA3 and dentate gyrus). An exception to this pattern is seen in spinal motor and sensory nerves, which continue axonal expression of EphB2 until the early postnatal period. Beginning around P7, expression of EphB2 once again increases however only at distinct sites and with a substantially altered pattern of cellular expression. Whereas the prenatal expression of EphB2 is largely focused on expression within developing neural axons,
**Figure 8. Generation of EphB2 mutants.** A. The Nuk1 (N1) mutation was created by a 1.4 kb deletion of EphB2 (amino acids 29-50) and replacement with a neomycin resistance cassette. B. Nuk2 (N2) mutants were developed by insertion of an in-frame bacterial lacZ sequence in place of amino acids 622-707, which constitute the JMR, KD and other C terminal motifs.
Table 2. Timeframe of EphB2 expression in the developing mouse. The relative age of onset of EphB2 is given for key regions of the mouse. Though EphB2 receptors tend to be expressed strongly in specific CNS regions during periods of neural conductivity, EphB2 expression is maintained in a few select CNS regions during early postnatal and adult stages of life.
<table>
<thead>
<tr>
<th>AGE AT ONSET OF EPHB2 EXPRESSION</th>
<th>REGION OF EPHB2 EXPRESSION</th>
</tr>
</thead>
<tbody>
<tr>
<td>E8.5</td>
<td>Head folds (neuroectodermal cells and heart, rhombomeres r3 and r5, forebrain, midbrain)</td>
</tr>
<tr>
<td>E9.25</td>
<td>Hypothalamus, tegmentum of midbrain[31]</td>
</tr>
<tr>
<td>E10.5</td>
<td>Preoptic area, ventral midbrain and hindbrain, posterior neural tube, eye, trigeminal ganglia, vestibular ganglia, otic vesicle, trigeminal nerves, oculomotor nerves, spinal motor nerves</td>
</tr>
<tr>
<td>E11.5</td>
<td>Pioneer axons (vestibulo-acoustic VIII ganglion sensory neurons), endolymphatic duct</td>
</tr>
<tr>
<td>E12.5</td>
<td>Inner ear efferent axons, facial motor neurons, floor plate marking ventral midline</td>
</tr>
<tr>
<td>E13.5</td>
<td>Retinal ganglion cells, outer retina</td>
</tr>
<tr>
<td>E14.5</td>
<td>Epithelial cells of semicircular canals, utricle and stria vascularis of the collea, hippocampus, optic chiasm, trigeminal ganglion and tongue</td>
</tr>
<tr>
<td>E16</td>
<td>EphB2 expression shuts off in the brain (excluding superior colliculus and ventral forebrain)</td>
</tr>
<tr>
<td>P7</td>
<td>Medial entorhinal cortex, cingulate cortex and hippocampus (CA3/DG)</td>
</tr>
<tr>
<td>P10</td>
<td>Neocortex (layers IV and VI), Purkinje dendrites of the cerebellum, amygdala, geniculate, thalamic and hypothalamic nuclei, dorsal horn of the spinal cord and dorsal root ganglia</td>
</tr>
</tbody>
</table>

[29] |[30] |[31] |[32]
postnatal expression of EphB2 proceeding beyond P7 is largely characterized by expression within neural dendrites and synaptic densities. Consistent with this by postnatal day 10 EphB2 is strongly expressed in layers of the cingulate, neo- and entorhinal cortices, the hippocampus, amygdala, geniculate, and thalamic centers, as well as Purkinje cells of the cerebellum[32]. Interestingly, although expression of EphB2 is extinguished in spinal motor neurons during early period of postnatal development, EphB2 becomes strongly upregulated in dorsal lamina (I-III) of the spinal cord and in dorsal root ganglia (DRG) during subsequent postnatal development. For each the above sites, expression of EphB2 persists through adulthood.

1.6 EphB2 mutant mice

Over the past two decades, genetic nulls of almost every Eph receptor and ephrin ligands have been generated in mice[31, 33-37]. My thesis research focuses on physiologic properties relevant to the EphB-family receptor tyrosine kinases. With this in mind I have principally focused on two distinct targeted knockins of the EphB2 gene: a standard null allele of EphB2 which we have termed “N1”; and the “N2 line”, in which the cytoplasmic kinase domain EphB2 has in frame two β-galactosidase (Figure 8). As a result, homozygous mice do not exhibit EphB2 dependent kinase activity, nor do they express functional Eph SAM or PDZ-binding motifs[31]. Heterozygous EphB2 N2 / + mice have been utilized previously to examine the cellular and intracellular pattern of EphB2 expression both developmentally and following specific stimuli, while homozygous EphB2 N2 mice are useful in determining the role of EphB2-dependent kinase signaling in a given physiologic response. In addition, given the relationship of EphB2 to other
closely related (and potentially functionally homologous) EphB-type receptors (most notably EphA4 and EphB3), combinatorial null mutants of EphB2/B3, EphB2/A4 and EphB1/B2 have been previously generated by our laboratory. To further probe the role of EphB-family receptors combinatorial EphB1/B2/B3 triple null mutants have also been developed[38]. Such examinations have revealed both unique and complex functional redundancies which exist among EphB-family receptors in several regions of the CNS[38, 39]. For example in our own laboratory we previously determined that interactions of several EphB-family receptors are all that are required to regulate each of the principle axon guidance steps required to correctly specify the primary interhemispheric connections within the mouse anterior forebrain[39]. Similarly studies by ourselves[32] and others[40] in the adult have demonstrated the unique and redundant roles which EphB-family receptors play in regulating synaptic signaling within the hippocampus.

1.7 Eph receptors and genetic background, examples from the inner ear

In addition to the anterior commissure and hippocampus, EphB2 null mice have been suggested to exhibit abnormalities with respect to innervation of the inner ear[30, 34]. Initial studies of EphB2 null mutants indicated that a subgroup of these animals exhibited circling behavior and other phenotypic changes consistent with abnormal vestibular function. These characteristics were reported to be observed with 100% penetrance in EphB2B3 double homozygotes. Analysis of these mutants revealed an abnormality in axon guidance in inner ear efferent (IEE) axons. Similar axon guidance defects were reported in EphB2 null and N2/N2 homozygous mice, however, these errors were
corrected by E14.5 and did not result in an abnormal phenotype in the adult[30]. Further investigation of colchear function revealed that there is no difference in distortion product otoacoustic emissions (DPOAE), an indicator of frequency modulation in the inner ear, between EphB2 null mutants and wild type controls. This suggests that these animals likely experience no deficits in hearing[34]. Cowan and colleagues also determined that EphB2, acting in conjunction with ephrinB2, is responsible for regulating the organization of epithelial cells within the semicircular canal. Consistent with this, EphB2 null mutants were reported to exhibit significant reduction in the diameter of the semicircular canals. The authors also provide evidence that EphB2 interacts with aquaporin via the PDZ domain-containing protein Pick-1 in the semicircular epithelial cells, proposing that disruption of the signaling results in impaired fluid production. With respect to these findings however, it is important to note that a number of investigators have commented upon the fact that the described “EphB2-dependent” circling behavior appears only in the specific genetic background utilized for the above study, and is not seen for EphB2 null mutants on a variety of other genetic backgrounds. This suggests that the above defects may be strongly or entirely dependent upon other genetic factors present within the specific background examined[30]. As a result of such features, the findings of my thesis studies are based upon results obtained for EphB2 N1 or N2 mutants which have been derived upon a minimum of two distinct genetic backgrounds.
1.8 Eph/ephrin-B hippocampal interactions

Analysis of hippocampal function in EphB-family mutants reveal these receptors are found both pre- and postsynaptically in several regions of the hippocampus[32, 38, 41-52]. It was subsequently discovered that both Eph receptors and ephrins, could effect change on hippocampal synaptic function through their actions on several specific signaling pathways as illustrated in Figure 9. The role of this receptor family in regulating spine morphogenesis and LTP has now been thoroughly examined[32, 38, 41-52]. In addition to the original finding of a requirement for EphB2 in regulating synaptic spine formation and LTP[32], several additional receptors and ligands within this group have subsequently been shown to regulate this process; notably EphA4[43, 50], ephrinB3[43, 47, 49, 50, 52] and ephrinB2[38, 43, 51]. With respect to EphB2, we and others have demonstrated that this receptor plays a role in stabilizing synaptic levels of NMDA receptor. As a result, loss of EphB2 results in a destabilization of postsynaptic activity dependent signaling, ultimately manifesting as reduction in the efficacy and stability of long-term potentiation (LTP) at sites such as Schaffer collaterals. The presence of wild type levels of LTP in homozygous EphB2 in N2/N2 indicates that this phenomenon is mediated by either EphB2/ephrin adhesion, or ephrin-mediated reverse signaling[32].

1.9 EphB2 and the synapse – current molecular model

With respect to the molecular mechanism by which EphB2 regulates spine morphogenesis, four key mediators are known to be responsible for this process: GRIP-1, RhoA, Rac1 and Cdc42 (Figure 9). Activation of these molecules by EphB2 has been
reported to result in both the formation and reduction of dendritic spines, depending on
the cellular cascade elicited. Work from several groups has demonstrated that GRIP1 is
an important mediator this effect. GRIP1 is thought to mediate these effects through
interaction with the PDZ binding motif of EphB2 to anchor EphB2 to additional signaling
proteins within dendrites to maintain surface expression of EphB2, reduce the number of
primary dendrites and preserve mossy fiber LTP (Figure 9A)[41, 45]. EphB2 dependent
activation of RhoA has been suggested to be a key regulator of spine morphogenesis.
Moeller and colleagues found that association of EphB2 with FAK, paxillin, Grb2 and
Src results in phosphorylation of Src, which then phosphorylates FAK and paxillin. FAK
activation leads to activation of RhoA, which linearizes actin filaments and ultimately
results in shortening of dendritic filopodia (Figure 9B)[46]. Penzes et al. demonstrated
that Rac1 could also be activated by RacGEF kalirin-7, which subsequently influences
activity levels of PAK1, which then modulates actin rearrangements and ultimately spine
morphogenesis (Figure 9C)[42]. Lastly, Cdc42 is known to be activated by EphB2,
intersectin-1 and N-WASP (plays a synergistic role, not required) to properly regulate
spine morphology. Dominant negative constructs of Cdc42, intersectin-1 and N-WASP
transfected into hippocampal neurons all result in the formation of longer and thinner
spines (Figure 9D)[53].

Subsequent functional and morphologic studies have highlighted the functionally
redundant character which exists among various EphB-family members with respect to
spine morphogenesis. Loss of EphB2 or EphA4 alone results in relatively mild
perturbations in hippocampal spine formation. In contrast, combinatorial knockouts of
these EphB-family members results in a significantly greater perturbation in spine
formation and morphogenesis. EphB triple knockouts (EphB1-/-B2-/-B3-/-) have also been shown to result in the development of long thin and immature spines which lack proper localization NMDA and AMPA receptors and PSD-95[38].

With respect to EphB2-dependent ligands, ephrinB2 has been shown to interact with GRIP via its PDZ domain. Using two different ephrinB2 knock-in mice, one in which four of the five tyrosine phosphorylation sites (Y307, Y314, Y319, Y334) in the cytoplasmic domain were replaced with phenylalanine (Y33 was replaced with leucine to conserve interaction between ephrinB2 and GRIP-1) and one where the valine of the PDZ domain is deleted (disabling interaction with GRIP-1), it was shown that phosphorylation of ephrinB2, coupled with interaction with GRIP, is essential for ephrinB2 to regulate spine density and size. Inhibition of ephrinB2 phosphorylation or interaction with GRIP was found to reduce surface expression of ephrinB2, diminish LTP and result in deregulation of NR2A and Src phosphorylation. However, only interaction with GRIP, not ephrinB2 phosphorylation, was necessary for proper LTD and depotentiation[51].

1.10 Hippocampal ephrin interactions

With respect to ephrin ligands, ephrinB3 is posed to be a major binding partner for the observed EphB-mediated regulation of spine morphogenesis in the hippocampus[43, 47, 49, 50, 52]. Using ephrinB3 null mice, it has been demonstrated that this ephrin ligand is required for normal levels of LTP, as null mutants show reduced CA3-CA1 LTP[52]. Interestingly, application of forskolin largely ameliorates observed reductions in LTP ephrinB3<sub>lacZ/lacZ</sub> mutants in hippocampal slices, suggesting that this effect could be mediated in part through the actions of adenylyl cyclase[47]. Mice lacking ephrinB3
**Figure 9. EphB2 receptor signaling in hippocampal neurons during adulthood.** A. GRIP1, through interaction with EphB2 via its PDZ domain, forms a complex with EphB2, GluR2 subunits of AMPA receptors and KIF5 in dendritic spines. This unit promotes circulation of EphB2 from the Golgi apparatus to the plasma membrane and mossy fiber LTP while reducing the number of primary dendrites on postsynaptic cells. B. Upon activation, EphB2 can associate with FAK, paxillin, Grb2 and Src, which results in phosphorylation of Src and subsequent phosphorylation of FAK and paxillin. FAK activation leads to activation of RhoA, which linearizes actin filaments and results in shortening of dendritic filopodia. C. EphB2 can also activate Rac1 via activation of its RacGEF Karilin-7. Rac1 then activates PAK, which leads to actin rearrangements acting in favour of spine morphogenesis. D. Activation of Cdc42 by EphB2, intersectin-1 and N-WASP regulates proper morphological formation of spines.
D.

PROPER MORPHOLOGICAL DEVELOPMENT OF DENDRITIC SPINES
express higher levels of synapsin and synaptobrevin, while exhibiting decreased NMDA receptor expression; consistent with findings seen in EphB2 null mice. EphrinB3 mice exhibited in decreased mEPSC amplitudes at the Schaffer collaterals, reduced AMPA current and increased phosphorylation of the glutamate receptor NR2B subunit[49].

Though finding such as those given above have suggested that ephrinB3 is a major ligand mediator of spine morphogenesis and LTP in the hippocampus, recent publications have also proposed a role for ephrinA3[50] and ephrinB2[38, 43, 51] in these processes. EphrinA3 is known to be present on hippocampal astrocytes, where it acts to reduce glutamate transport into the cells by decreasing the expression of glutamate transporters GLT-1 and GLAST. Lack of ephrinA3 results in increased spine length and spine neck length and abnormal spine morphology (more mushroom-shaped spines as opposed to stubby-shaped spines), as well as poor performance in context-dependent learning tasks. Astrocytic ephrinA3 is believed to activate EphA4 on postsynaptic cells, which induces dendritic spine retraction[50].

1.11 Long-range Eph-dependent hippocampal interactions. In addition to spine morphogenesis, EphB2, and to a lesser extent EphB3, have been shown to regulate distal guidance of medial hippocampal axons to targets in the dorsal lateral septum. It also appears that axon fascicles innervating the lateral septum fail to defasciculate after reaching the target. Loss of EphB2 or its kinase domain results in failure of hippocampal axons to defasciculate through ephrinB3 expressing regions surrounding the septum. Axon targeting effects were observed in kinase dead EphB2 homozygous mutants suggesting that this process is dependent upon kinase mediated reverse signaling[44].
EphB1 has also been shown to be necessary for the proper migration of neural progenitor cells (NPCs) from the subgranular zone. EphB1 knockout mice display fewer NPCs, which tend to mislocalize beyond the subgranular zone into the granular and molecular layers. This reduction in NPCs was observed to be even more severe in the EphB1/B2 double knockouts. It was found that ephrinB3 in the hilus and inner molecular layer is required for proper localization and dendritic branching of these NPCs[54].

1.12 Opiates and man

The benzylisoquinoline alkaloid morphine was originally derived from the opium poppy, *Papaver somniferum*. *Papaver* is the Greek word for ‘poppy’, while *somniferum* translates from the Latin ‘sleep inducing’. Writings describing the opium poppy are seen in ancient Egyptian and Sumerian texts dated from ~4000 BCE, and opium is believed to have been harvested in Europe for at least 4000 years. Presently opium is grown legally in countries such as India and Turkey, in addition to being raised illegally in nations like Iran. Although morphine is considered to be the most prominent alkaloid produced by *Papaver somniferum* (10-17%), several additional compounds which contribute to the observed euphoric and sedative effects associated with extracts of the opium poppy include: codeine (0.7-4%), thebaine (0.5-2%) and benzylisoquinolines such as papaverine (0.5-1%). As a relatively potent mu opioid receptor agonist (see below), morphine and its analogues are primarily used clinically for the treatment of pain, and morphine is still widely regarded as a gold standard with respect to analgesics. Though highly effective at reducing the perception of painful stimuli, morphine also exhibits a well-characterized
propensity for abuse, tolerance and addiction, and as such is tightly regulated in most countries[55].

1.13 Opiates and their receptors

Opiates represent a large class of medically (morphine, heroin, codeine, hydromorphone, oxycodone, fentanyl and methadone to name a few) and physiologically (endorphins, enkephalins, dynorphins, endomorphins) important molecules which bind to one or more classes of opioid receptors: mu (MOR), delta (DOR) and kappa (KOR)[55]. Both endogenous and synthetic opioids target these receptors with different pharmacologic profiles[56, 57]. A fourth related receptor termed ORL1 (opiate receptor like-1) is not generally considered part of the formal opioid receptor family[58]. Molecular cloning and pharmacologic studies of these receptors sub-types has over the past 25 years has demonstrated that each of these receptors exhibits unique pharmacologic and signaling properties with respect to the opiates listed above[56, 57, 59]. Key properties of these are summarized in Table 3. As my thesis studies focuses principally upon pharmacologic action centered around the role of the mu opioid receptor, I will concentrate my comments here.

1.14 Opioid receptor structure

Like many membrane proteins, the crystal structure of the opioid receptors remains to be definitively elucidated. Broadly, opioid receptors belong to the superfamily of G protein-coupled receptors (GPCRs). Within this family, they have been categorized as class A (Rhodopsin receptor family), subgroup γ. Based upon structural homology, sequence
Table 3. Overview of opioid receptor expression and function. For each subclass of opioid receptor, the expression pattern and physiological functions are indicated.
<table>
<thead>
<tr>
<th>OPIOID RECEPTOR</th>
<th>EXPRESSION WITHIN THE CNS</th>
<th>PHYSIOLOGICAL EFFECT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>μ</strong></td>
<td>Cortex (laminae III and IV) Thalamus Hypothalamus Striatum (striosomes) Periaqueductal gray matter Median raphe nucleus Hippocampus Superior/inferior colliculus Olfactory bulbs Substantia gelatinosa Lamina I and II\textsubscript{outer} of the dorsal spinal cord Dorsal root ganglia</td>
<td>Analgesia Physical dependence Euphoria Respiratory depression Miosis Reduced GI motility</td>
</tr>
<tr>
<td><strong>δ</strong></td>
<td>Pontine nuclei Amygdala Olfactory bulbs Deep cortex Hippocampus Nucleus accumbens Striatum Laminae II\textsubscript{inner} through V of the dorsal spinal cord</td>
<td>Weak analgesia Anti-depressant Physical dependence</td>
</tr>
<tr>
<td><strong>κ</strong></td>
<td>Hypothalamus Periaqueductal gray matter Claustrum Striatum Cortex (laminae V-VI) Substantia gelatinosa</td>
<td>Analgesia Physical dependence Sedation Miosis Inhibition of ADH release Dysphoria</td>
</tr>
<tr>
<td><strong>ORL\textsubscript{1}</strong></td>
<td>Cortex Amygdala Hippocampus Septal nuclei Habenula Hypothalamus</td>
<td>Anxiety Depression Appetite Development of tolerance to mu agonists</td>
</tr>
</tbody>
</table>
comparisons and a variety of biochemical investigations, is believed that opioid receptors are comprised of seven membrane spanning helices arranged in an anti-clockwise orientation (Figure 10). Class A receptors generally have relatively short extracellular N-termini, and possess an intracellular C-terminal domain which can be phosphorylated by several targets to serve as docking sites for additional intracellular signaling molecules[56]. The binding pocket for endogenous and synthetic opioid ligands is believed to lie within a defined element of the seven transmembrane domain core, where components of the N-terminus together with extracellular loops 1 and 3 form a binding motif (Figure 11). The precise structure of this interface among the different opiate receptor subtypes is believed to be the primary component imparting selectivity among the described opioid ligands[57].

There is evidence to suggest that opioid receptor subtypes may form higher-order oligomers within the plasma membrane. Biochemical analysis of receptor subtypes, pharmacologic profiling, and molecular characterizations such as Western blotting, radioligand binding, confocal microscopy and BRET analysis have supported the presence of dimeric and higher order forms of mu, delta and kappa opioid receptors in both the rodent and human CNS[58-61]. However at present, the prevailing pharmacologic model of opiate receptors focuses upon the dimeric form of homo-oligomers (see below). Like other GPCR's, it is thought that hydrophobic forces present between select transmembrane domains and coiled-coil interactions mediate the primary driving forces to promote these higher-order states[59]. Biochemical evidence from several groups suggest that in addition to homo-oligomers (e.g. MOR-MOR dimers), opiate receptors may be capable of forming hetero-oligomers structures (e.g. MOR-DOR)
Figure 10. Structural schematic of the human mu opioid receptor. The MOR consists of an extracellular domain, seven transmembrane domains and a C-terminal intracellular domain. Red amino acids indicate those which are conserved among the various opioid subclasses. N-linked glycosylation sites are denoted by square brackets in the N terminus, while palmitoylation sites of cysteine residues are indicated with zigzag lines in the C-terminus.
Figure 11. Mu opioid receptor binding pocket. Depicted in the schematic is β-funaltrexamine (β-FNA), a mu antagonist, within the MOR binding pocket. The ‘message’ interaction occurs between the amine group and Asp III:08 and the phenol moiety and His VI:17 (solid dark blue circles). This is presupposed for all opioid binding interactions in all three receptor subclasses. The ‘address’, though it has not been determined definitively, is believed to be attributed to attractions between Lys V:05 and some portion of the affinity label of β-FNA (solid red circle). Tyr VI:19 and Cys VII:06 are believed to be important in binding morphine (dashed pink circles).

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under certain conditions[58]. The properties of MOR-DOR dimers have been characterized in several cell lines using epitope tagged constructs[58, 62]. Such complexes demonstrate a reduced affinity for several synthetic opioid agonists (DPDPE, DAMGO) but show higher affinities for endogenous ligands such as endomorphin-1 and Leu-enkephalin. Interestingly these MOR-DOR complexes exhibit little apparent desensitization in contrast to MOR or DOR homodimers, and show altered internalization kinetics. In addition, MOR-DOR heterodimers are insensitive to pertussis toxin, which acts to catalyze the ADP-ribosylation of G_{ia} subunits, suggesting that such heterooligomers may act through alternative signaling cascades compared to their parent homodimers[63]. Despite these findings, evidence for the presence of bona fide MOR-DOR heterodimers in vivo remains controversial. Evidence supporting the presence of MOR-DOR heterodimers has shown that mice lacking MOR appear not to be susceptible to deltorphin II dependence[64] and display diminished DOR-mediated activities[65-67], while DOR-1 deficient mice fail to exhibit tolerance to morphine in both hot plate and tail flick tests over an eight day morphine (5 mg/kg, s.c.) treatment[68]. Contrastingly, a study published by Scherrer et al. in 2009 examining the pattern of mu and delta receptor expression via targeted knockin of epitope tags of the DOR using homologous recombination found that mu and delta receptors are not coincidently expressed in populations of sensory neurons, arguing against a physiological role for MOR-DOR heterodimers[69].

At present there is no evidence suggesting associations between mu and kappa receptors[59]. Byren and colleagues investigated the possibility of MOR-KOR heterodimers in vitro and found that while myc-tagged KORs immunoprecipitated with
FLAG-tagged DORs, they did not do so with FLAG-tagged MORs[70]. This is consistent with the observation that MORs and KORs elicit opposing physiological responses, possibly due to their unique placements on neurons in a variety of regions of the nervous system[71].

1.15 Binding specificity of opioid receptors

In the absence of a definitive crystal structure, pharmacophore models of the opioid receptors have been developed based on point mutations and domain swapping among receptor classes. Additional structural insights have been gained from comparison of these receptors to related receptor such as the β-adrenergic receptor. Some opiates are clearly selective for a specific opioid subclass while others are more nonspecific, leading to the development of the “message-address” concept[57]. Conserved elements common to most opiate receptors are defined as message, while structural entities unique to a specific opiate comprise the address. In this context, the tyramine moiety of opioids comprises message, with the amine and phenol moiety binding to Asp8 of transmembrane domain III (Asp III:08) and His17 of transmembrane domain VI (His VI:17), respectively. By contrast, substituents along the C-ring comprise address components (Figure 11). It has been determined that these components bind to highly variable regions of the receptor which extend from the top of transmembrane domain VI to its C-terminus[57]. Specifically, positions TM VI:23 and TMVII:03 are responsible for binding of the address regions of opiates. Selective binding is achieved by mutual attraction (pairing of complimentary residues between ligand and receptor) and combined with steric exclusion (conformational interference of receptor-ligand interaction). For
example, the tyramine moiety (the “message”) of kappa agonist gNTI forms salt bridges with Asp III:08 and His VI:17, while its guanidinyl group (the “address”) interacts with Glu VI:23 via another salt bridge.

Interestingly, nonopiates such as fentanyl do not abide by the message-address theory. They instead have unique epitopes which minimally coincide with traditional opiates that bind to the pharmacophore. The specific biochemical interactions that occur are described in the sections below. Relative avidities of ligands and agonists for the different opioid receptors are displayed in Table 4.

1.16 Endogenous opioid ligands

The endogenous ligands of opioid receptors are typically small peptides. The core sequence, or message, of all endogenous opioids is Tyr-Gly-Gly-Phe-Met/Leu. Three families of endogenous opioids have been identified to date: endorphins, enkephalins and dynorphins. β-endorphin, the lone constituent of the endorphin family, has the greatest affinity for mu opioid receptors, demonstrating slightly lower affinity toward DORs and little to no affinity toward KORs. Transcripts for the β-endorphin precursor are made mainly in the anterior pituitary, by a small subset of cells that produce ACTH, which is derived from the same precursor molecule proopiomelanocortin. In non-human mammals, this precursor is also produced in the intermediate lobe of the pituitary. β-endorphin is also produced in smaller amounts by the arcuate nucleus of the medial basal hypothalamus, the nucleus of the solitary tract and nucleus commissuralis[72].

The enkephalin family consists of Met-enkephalin, Leu-enkephalin, Met-enkephalin-Arg^6-Phe^7, Met-enkephalin-Arg^6-Gly^7-Leu^8 and peptide E. These molecules
Table 4. Binding affinities of small molecules to opioid receptors. Binding affinities (nmol/L) of opioids to the three main subclasses of opioid receptors are indicated, as measured in guinea pig brain homogenates. Values denoted in red indicate antagonistic activity at the receptor site.
<table>
<thead>
<tr>
<th>Drug</th>
<th>$\delta$</th>
<th>$\kappa$</th>
<th>$\mu$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>90</td>
<td>317</td>
<td>1.8</td>
</tr>
<tr>
<td>DAMGO</td>
<td>-</td>
<td>-</td>
<td>0.6</td>
</tr>
<tr>
<td>Normorphine</td>
<td>310</td>
<td>149</td>
<td>4.0</td>
</tr>
<tr>
<td>Levorphanol</td>
<td>5.6</td>
<td>9.6</td>
<td>0.6</td>
</tr>
<tr>
<td>Codeine</td>
<td>&gt;1000</td>
<td>-</td>
<td>2700</td>
</tr>
<tr>
<td>Methadone</td>
<td>15.1</td>
<td>1628</td>
<td>4.2</td>
</tr>
<tr>
<td>Fentanyl</td>
<td>151</td>
<td>470</td>
<td>7.0</td>
</tr>
<tr>
<td>Alfentanil</td>
<td>21.200</td>
<td>&gt;10.000</td>
<td>30</td>
</tr>
<tr>
<td>Sufentanil</td>
<td>23</td>
<td>124</td>
<td>1.6</td>
</tr>
<tr>
<td>Lofentanil</td>
<td>0.24</td>
<td>0.6</td>
<td>0.023</td>
</tr>
<tr>
<td>Carfentanil</td>
<td>3.3</td>
<td>43</td>
<td>0.024</td>
</tr>
<tr>
<td>Pethidine</td>
<td>4345</td>
<td>5140</td>
<td>385</td>
</tr>
<tr>
<td>Pentazocine</td>
<td>106</td>
<td>22.2</td>
<td>7.0</td>
</tr>
<tr>
<td>Butorphanol</td>
<td>13</td>
<td>7.4</td>
<td>1.7</td>
</tr>
<tr>
<td>Nalbuphine</td>
<td>163</td>
<td>66</td>
<td>6.3</td>
</tr>
<tr>
<td>(±)Pentazocine</td>
<td>467</td>
<td>8.7</td>
<td>39</td>
</tr>
<tr>
<td>Buprenorphine</td>
<td>1.3</td>
<td>2.0</td>
<td>0.6</td>
</tr>
<tr>
<td>Substance</td>
<td>IC50</td>
<td>IC25</td>
<td>IC10</td>
</tr>
<tr>
<td>----------------------------</td>
<td>-------</td>
<td>-------</td>
<td>------</td>
</tr>
<tr>
<td>Naloxone</td>
<td>27</td>
<td>17.2</td>
<td>1.8</td>
</tr>
<tr>
<td>Naltrexone</td>
<td>9.4</td>
<td>6.5</td>
<td>0.46</td>
</tr>
<tr>
<td>Cyprodime</td>
<td>356</td>
<td>176</td>
<td>0.4</td>
</tr>
<tr>
<td>(-)Bremazocine</td>
<td>0.78</td>
<td>0.075</td>
<td>0.62</td>
</tr>
<tr>
<td>(+)Tifluadom</td>
<td>290</td>
<td>4.1</td>
<td>22</td>
</tr>
<tr>
<td>(+)U50,488H</td>
<td>9200</td>
<td>0.69</td>
<td>435</td>
</tr>
<tr>
<td>(-)Ethylketazocine</td>
<td>5.2</td>
<td>2.2</td>
<td>2.3</td>
</tr>
<tr>
<td>Leu-enkephalin</td>
<td>1.8</td>
<td>&gt;10,000</td>
<td>150</td>
</tr>
</tbody>
</table>

exhibit high affinity for DORs, with little affinity for the other subclasses. The exception is Met-enkephalin-Arg\textsuperscript{6}-Gly\textsuperscript{7}-Leu\textsuperscript{8}, which exhibits equal affinity for delta and mu opioid receptors. Proenkelphalin expression is more robust, exhibiting its presence at endocrine and CNS sites such as the adrenal medulla, gastrointestinal tract and throughout the brain and spinal cord[72].

The dynorphin family is composed of α-neo-endorphin, β-neo-endorphin, dynorphin A\textsubscript{L} (long), dynorphin A\textsubscript{S} (short) and dynorphin B. This family has high affinity for KORs and, except for the long and short variants of dynorphin A, little affinity for mu and delta receptors. Dynorphin A\textsubscript{S} has a mild affinity for DORs while dynorphin A\textsubscript{L} shows moderate affinity for DORs and MORs. The precursor molecule for dynorphin is known to be expressed in the gut, posterior pituitary, hypothalamus, brainstem and other areas of the brain[72].

Opioid peptides can act as autocrine, paracrine and endocrine regulators. Within the CNS, they are commonly delivered to their targets through local release or long fiber projections to distant targets. Thus, endogenous opioids, such as β-endorphin or Met-enkephalin, can reach mu opioid receptors within the brain and spinal cord, as well as peripheral sites, via fiber tracts or the blood. These peptides and their affinities are indicated in Table 5.
Table 5. Binding affinities of endogenous opioid peptides for opioid receptors.

Binding affinities (nM) of opioids to the three main subclasses of opioids are indicated, as measured in membrane preparations from mouse brains.
<table>
<thead>
<tr>
<th>Ligand</th>
<th>δ</th>
<th>κ</th>
<th>μ</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-endorphin</td>
<td>4.0</td>
<td>15.6</td>
<td>1.0</td>
</tr>
<tr>
<td>Met-enkephalin</td>
<td>5.8</td>
<td>1566.0</td>
<td>22.3</td>
</tr>
<tr>
<td>Leu-enkephalin</td>
<td>9.4</td>
<td>3697.0</td>
<td>68.2</td>
</tr>
<tr>
<td>Met-enkephalin-Arg&lt;sup&gt;6&lt;/sup&gt;-Phe&lt;sup&gt;7&lt;/sup&gt;</td>
<td>3.9</td>
<td>19.2</td>
<td>6.9</td>
</tr>
<tr>
<td>Met-enkephalin-Arg&lt;sup&gt;6&lt;/sup&gt;-Gly&lt;sup&gt;7&lt;/sup&gt;-Leu&lt;sup&gt;8&lt;/sup&gt;</td>
<td>9.0</td>
<td>9.3</td>
<td>23.7</td>
</tr>
<tr>
<td>Dynorphin A (1-8)</td>
<td>11.5</td>
<td>2.3</td>
<td>17.7</td>
</tr>
<tr>
<td>Dynorphin A (1-17)</td>
<td>6.3</td>
<td>0.3</td>
<td>2.6</td>
</tr>
<tr>
<td>Dynorphin B (1-13)</td>
<td>20.5</td>
<td>1.4</td>
<td>12.6</td>
</tr>
<tr>
<td>α-Neo-Endorphin</td>
<td>2.6</td>
<td>3.6</td>
<td>4.0</td>
</tr>
<tr>
<td>β-Neo-Endorphin</td>
<td>9.8</td>
<td>5.0</td>
<td>15.6</td>
</tr>
</tbody>
</table>

1.17 Exogenous opioid ligands

The term opiates broadly describes a group of narcotic alkaloids found naturally in the opium poppy and semisynthetic derivatives thereof. These exogenous opioid agonists/antagonists tend to display promiscuity among all opioid receptors classes, but may exhibit preference for a particular subclass. Mu-specific agonists are perhaps the most well known of all synthetic and naturally derived opiates. Mu agonists may be divided into opiate (morphine, codeine, heroin) and nonopiate (fentanyl) ligands (Figure 12).

Morphine as indicated above is a phenanthrene alkaloid with additional ring modifications[73]. Morphine has a half-life of roughly two hours in humans, and 50 minutes in mice[74]. The C3 phenolic, C6 secondary alcoholic and tertiary amine groups confer the activity of morphine[73, 75, 76]. Though it has been suggested that the 6 α-hydroxyl group of the C ring (so-called boat conformation) may impart specificity, there is as yet no direct experimental evidence to support this claim[57].

While morphine has the highest affinity for the mu receptor, it does have small, but non-negligible affinities for delta and kappa receptors (Table 4). Although morphine primarily acts through MORs, it has been known to induce diminished analgesia through activation of DORs and induce KOR-mediated antinociceptive and respiratory effects in MOR knockout mice[66]. Therefore, it is important to remember that though the MOR is the chief mediator of the morphine response, DORs can contribute despite their significantly lower affinity.

Upon administration morphine is taken up by the liver and metabolized to two main products: morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G)
Figure 12. Structures of MOR ligands. The structures of several MOR agonists, antagonists and partial agonists are depicted
through the actions of UGT 1A3 and UGT 2B7 respectively (Figure 13). Roughly 60% of total morphine is converted to M3G, while only 10% is converted to M6G, with the remaining 30% distributed among other metabolites such as normorphine, 3-acetylmorphine, morphine-6-sulfate, 6-acetylmorphine, morphine-N-oxide, morphine-3-sulfate and pseudomorphine (Figure 13)[73, 75, 76]. Although glucuronidation products are the main metabolites of morphine, Frolich and colleagues investigated the affinities and potencies of the other minor metabolites. It was found through radioligand binding studies and FRET analysis that some of these metabolites possessed affinities and potencies (measured as a function of Gi activation and recruitment of beat-arrestin to the MOR) comparable with morphine, while others exhibited lower affinities and levels of Gi activation and β-arrestin recruitment by the MOR (Table 6)[75].

M3G has little to no affinity for MOR, or any other opioid receptors, and very low potency (Table 6)[73, 75, 76]. It has been postulated that M3G may antagonize the analgesic and respiratory effects of morphine and M6G, thereby contributing to tolerance. Rats which received M3G intrathecally developed hyperaesthesia and allodynia[77]. Intracerebroventricular administration of M3G has been reported to induce some symptoms of withdrawal, such as wet dog shakes and salivation, as well as increased exploration, excessive grooming[78], EEG spiking and epileptiform discharges[79]. This excitatory activity is exacerbated, rather than diminished, by opioid antagonists naloxone and naltrexone, suggesting that M3G is mediating its effects through non-opioid sites. While some have suggested that M3G may act through NMDA receptors to mediate its excitatory effects, this is unlikely as M3G has a very low affinity for NMDA receptor binding sites[78].
Figure 13. Metabolism of morphine. Morphine is metabolized in the liver into two main products: morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G). Also shown are the metabolisms of codeine and heroin, two morphine precursors. Heroin is deacetylated once it crosses the blood brain barrier into morphine, while codeine is converted to morphine and several other products. Compounds highlighted in red display a significant affinity for the MOR.
Table 6. Affinity and potency profiles of morphine and its metabolites for the MOR.

Affinity values were obtained by competition displacement experiments with $[^3\text{H}]$-naloxone in 293T cells. Potencies were determined by FRET analysis of the MOR with $\beta$-arrestin and $G_{\alpha_i}$ protein. EC$_{50}$ values represent the concentrations at which half of the maximal response was observed.
M6G has been demonstrated to have an affinity for MORs comparable to that of morphine[75], while exhibiting low affinities for delta and kappa opioid receptors (Table 6). Although M6G has been reported to be up to 50 times more potent than the parent compound by some[57, 73], one group has shown that M6G has a similar potency to morphine[75]. Interestingly, this particular glucuronidation product exhibits a longer elimination half life (roughly 3.2 hours, compared to roughly 2 hours, in humans)[80] and longer duration of effect compared to morphine. Following subcutaneous administration, morphine exhibits peak effects at 20 minutes, while M6G does not reach peak until one hour. By oral administration, morphine and M6G are observed to reach their peak effect after 45 minutes and two hours, respectively[81]. M6G is known to induce many of the same effects as morphine (sometimes with greater potency), namely analgesia, respiratory depression, tolerance, nausea and vomiting and increased urinary retention[73].

In the conversion of morphine to dihydromorphinone (a minor metabolite) by morphine 6-dehydrogenase, a toxic intermediate known as morphinone is produced. Morphinone is then usually converted into dihydromorphinone by interaction with glutathione[82, 83]. While morphinone is known to cause respiratory depression[84], this metabolite has been proposed by a number of scientists to react with sulfhydryl groups of opiate receptors, thereby irreversibly inactivating and blocking analgesia[82, 85, 86]. Interestingly, pretreatment of mice with alkylated forms of morphinone have been reported to induce cross-tolerance to morphine[85], suggesting that endogenous modification of this molecule may interfere with morphine-induced antinociception.
Two common precursors of morphine are heroin (diacetylmorphine) and codeine (Figure 13). Neither parent compound alone possesses a strong affinity for the MOR, but instead are converted to morphine and its metabolites, capable of binding to the MOR[87, 88]. The diacetylation of morphine present in heroin increases its lipophilicity, allowing it to readily cross the blood brain barrier. Once in the CNS, heroin is converted to 6-monoacetylmorphine which is then deacetylated to morphine. M3G and M6G are the main metabolic products[88]. The metabolism of codeine is slightly more complex. Codeine can be converted to morphine to yield M3G and M6G. However, it may also be metabolized into norcodeine and codeine-6-glucuronide[87]. Codeine itself exhibits a relatively low affinity for the MOR, as do its metabolites compared to morphine - its primary active metabolite. Fentanyl is a nonopiate agonist known to be potent analgesics[55, 57]. The structural features conferring this activity include an N-phenyl-N-piperidinyl propionamide moiety and a phenethyl group[57]. Fentanyl is roughly 100-300 more potent than morphine, but like morphine induces low levels of receptor internalization per receptor. However, because fentanyl binds to fewer MORs to generate the same level of analgesia, relative to morphine, it incurs less PKC phosphorylation overall and thus less tolerance[89]. It widely utilized in clinical practice for the treatment of pain. At present it is unclear what the precise pharmacophore of fentanyl and its analogues are.

D-Ala2-MePhe4-Gly-ol5-enkephalin (DAMGO) is a highly specific mu agonist and is considered the gold standard MOR agonist in terms of specificity. DAMGO is extremely selective for MORs, having far lower affinities for DORs and KORs (Table 4), making it a useful tool in isolating mu-specific responses and effects in the laboratory. He


*et al.* have shown that co-administration of DAMGO with morphine facilitates endocytosis of the MOR, reduces superactivation of cAMP normally observed following chronic morphine treatment and diminishes morphine tolerance[90].

### 1.18 Exogenous inhibition of opioid receptors

In addition to agonists, opiate receptors also have a well-characterized repertoire of antagonists and partial agonists. Naloxone and naltrexone are opioid antagonists which both display a preference for mu sites, but still exhibit significant avidity for both DORs and KORs[55, 57]. Both compounds possess a 3’ hydroxyl group, consistent with the theory that this functional group is important for binding to MORs. In the clinic, naloxone and naltrexone are used to treat opioid overdose and opioid addiction, respectively[91]. The partial agonist buprenorphine is a unique opioid in that its pharmacological classification depends on its site of action. While buprenorphine acts as a partial agonist for the MOR, it is a full agonist for ORL1 and DORs, and a competitive antagonist at the KOR. It displays high affinity for mu and kappa sites and lower avidity for delta and ORL1[55].

### 1.19 Physiologic actions of morphine

Through binding to the mu opioid receptor, the presence of morphine results in a variety of physiological effects. Principally morphine is known for its ability to induce analgesia and the feeling of euphoria[55]. In contrast sensations associated with euphoria in the presence of morphine are thought to be governed by modification dopamine releasing pathways arising within the ventral tegmental area (VTA) and terminating in the nucleus
accumbens (NA)[92]. Morphine stimulates postsynaptic MORs in the NA, which leads to diminished release of GABA and dynorphin (which acts on postsynaptic KORs in the VTA) from NA axon terminals onto neurons in VTA. These VTA neurons, which project back to the NA, release greater amounts of dopamine into the NA, thereby inducing feelings of reward and thus a state of dependence[93]. As indicated above, morphine does exhibit a lower but measurable affinity toward DORs and KORs which is able to generate low levels of analgesia (but not euphoria), and may play a role in addiction[55, 94].

Although addiction is a major consequence of persistent MOR activation, my project does not focus on this specific MOR action. Interestingly, recent work has demonstrated the potential for EphB receptors to regulate this pharmacologic phenomenon[11]. In addition, the results of this project pose the possibility of a cognitive or conditioned component of addiction which may involve the modulation of NMDA receptors by EphB2.

High doses of morphine can result in acute adverse effects. Respiratory depression can occur via MOR activation in the raphe magnus[95] and possibly due to activation of MORs in the alveolar walls of bronchioles[96]. Another well characterized effect of morphine is that it reduces gastrointestinal mobility within the myenteric plexus. Penagini and colleagues have shown that morphine reduces the transient rate of the lower esophageal sphincter and decreases volumes in the proximal stomach[97]. Mechanistically, morphine elicits the release of nitric oxide via the mu3 receptor in the stomach, small intestine and colon which reduces GI motility[98]. The inhibition of peristalsis in the intestines, combined with reduced secretion and increased fluid absorption in the gut, often leads to constipation[99].
In addition to GI relaxation, mu agonists (e.g. morphine and fentanyl) are known to induce nausea through activation of mu_1 receptors in the area postrema. Ligand binding at this site does not require penetration through the blood brain barrier. In contrast morphine can also activate mu_2 receptors, in sites such as the in the nucleus tractus solitarius, opposing the emetic effects within the area postrema. It has therefore been postulated that the lipid solubility of a given opiate ligand may regulate the degree to which such agents act as emetics or anti-emetics. For example fentanyl which exhibits relatively high lipid solubility, easily diffuses through the blood brain barrier; counteracting its own emetic effects. In comparison, morphine is comparatively less lipophilic, therefore retaining a larger percentage outside of the CNS proper with a resulting higher propensity to induce vomiting for a given dose[100]. A third well-characterized effect of morphine intoxication is miosis, following activation of mu_3 receptors which induces nitric oxide release with subsequent constriction of the pupils[101].

1.20 Opioid analysis of MOR function using knockout mice
Generally, the opioid receptor gene is comprised of three codons, the first encoding the extracellular domain and transmembrane domain I, the second coding for transmembrane domains II-IV, and the third encompassing transmembrane domains V-VII together with the cytoplasmic tail. MOR gene structure differs from the other opioid receptors with respect to the 3’ coding region where the last 12 codons are located on a fourth exon, as opposed to the entire gene being condensed into three exons, as seen in the DOR and KOR[94]. The MOR localizes to murine chromosome 10[102], while DORs and KORs
are found on chromosomes 4[103] and 1[102], respectively, in the mouse. In humans, MORs, DORs and KORs can be found on chromosomes 6[104], 1[105] and 8[106], respectively.

The MOR gene is known to have two promoters: exon 1 promoter and exon 11 promoter[107]. Exon 1 promoter is located 1.5 kb upstream of exon 1[108-112] and is proposed to be a dual promoter, where the dominant proximal promoter is 500 bp apart from the distal promoter[113-115]. This promoter contains several tumor specific promoter (tsp) sites[107] and multiple GC-rich cis-acting elements Sp1[116, 117] and AP-2[107]. It is known to regulate transcription of 16 transcript variants[107]. The more recently discovered exon 11 promoter is located in the 5’ region flanking exon 11 and is more evident in neural cells. Exon 11 promoter contains a TATA box within its core and lacks the multiple Sp1 and AP-2 sites seen in exon 1 promoter[118]. In addition, it only has one tsp site, compared to the several found in the exon 1 promoter. Exon 11 promoter drives transcription of the remaining 9 variants. Based on sequence differences and proteins found to bind to each promoter, exon 11 promoter is thought to be a typical eukaryotic promoter that associates with RNA polymerase II, while exon 1 promoter favours a “housekeeping” gene mode[107].

Four major forms of the mu opioid receptor are proposed to exist: mu_1, mu_2, mu_3 and the morphine-6β-gluruconide binding form. However, up to 25 different splice variants of the MOR-1 gene have been detected in the mouse (8 in the rat, 11 in humans)[107]. Even more interesting, all variants of the MOR are currently attributed to one genetic locus. These 25 variants of the MOR differ mainly with respect to the C-terminal splice variants encoded by downstream exons[118, 119]. Using RT-PCR[119]
and antibodies raised against exon-specific peptides[120-123], it was revealed that there are distinct expression patterns, with small areas of overlap, among variants. Analysis of variant MORs has shown that while the differential forms of the MOR exhibit similar affinities for exogenous MOR ligands[118, 119, 124-128], there were some subtle differences in binding to endogenous peptides dynorphin A[127] and ß-endorphin[119] among some receptor forms. Previously, Rossi et al. observed that antisense oligonucleotides targeting exons 1-4 abolished morphine-induced analgesia, but not M6G-induced analgesia[129-131]. Consistent with this, studies in mice lacking either MOR-1 exon 1 or MOR-1 exon 2 showed that while exon 1-deficient mice experienced M6G-induced analgesia but not morphine-induced analgesia, exon 2-deficient mice experienced neither[132]. In addition, marked differences were observed in potency and efficacy of agonist-induced G protein activation[124, 128, 133] and forskolin-stimulated cAMP accumulation[124] between the variants. There was little correlation between affinity, potency and percentage of maximal stimulation among the agonists. Receptor internalization was also observed to differ between variants[123, 134]. These observations are in keeping with the logic that MOR variants have identical N-terminal extracellular domains (which determines affinity) and transmembrane domains, but altered C-terminal domains (the site of phosphorylation and G protein interaction).

Null mutations for the three primary opioid receptors (mu, delta and kappa) have been produced at present in mice using homologous recombination and have been extensively studied[94]. In addition, knockouts of a number of opioid peptides have also been produced[135-139]. Presently, five distinct MOR gene modifications have been produced in mice[132, 140-143]. Three of these have focused upon replacing exon 1 with
a neomycin resistance cassette[132, 142, 143], while the other two have replaced or inserted a neomycin cassette into exon 2[140, 141]. In each case the resulting modified allele should result in a completely nonfunctional protein. Radioligand binding studies have confirmed the absence of mu binding sites in these MOR knockout animals using a variety of mu-dependent ligands (i.e.. Morphine, DAMGO, endomorphins)[132, 140-142, 144-146]. In addition, several groups have shown the lack of agonist-induced G protein activation using [$^{35}$S]guanosine 5’ -3-O-(thio)triphosphate (GTP$_y$S) binding assays[66, 146-149]. Such studies have also demonstrated that the absence of mu receptors does not affect the interaction of G proteins with DORs or KORs[65, 66, 148].

Behavioural/sensory analyses of these animals have examined the role of MORs influencing a number of functions including locomotion, nociception, and emotional response. Intrinsic locomotion studies have reported no or a slight reduction in motor activity[141-143]. Diminished motor activity would be consistent with the observation that wild type animals exposed to morphine exhibit hyperactivity, as opposed to MOR knockouts which do not. Upon administration of a variety of mu agonists (morphine, heroin, methadone, DAMGO, etc.), mu-specific behaviours such as morphine-induced analgesia[132, 140-142, 150, 151], reward[141, 152] and withdrawal[141, 152] are abolished in MOR-deficient animals. These results are reproducible and widely accepted by most scientists. Interestingly, some studies have reported M6G and heroin-induced analgesia in mice lacking exon 1 of the MOR-1 gene[132]. Schuller and colleagues found that the receptor which binds to M6G requires exon 2, but not exon 1, of the MOR-1 gene upon examination of mice which lack exon 2 of MOR-1. This was further validated with [$^{3}$H]-M6G binding in the brain and antisense probe targeting of exon 2. Sensory studies
in MOR null mice demonstrate that these animals exhibit increased thermal
sensitivity[66, 141, 142, 153], reduced sensitivity to chemically-induced noxious
stimuli[151], and unchanged sensitivity to mechanoreceptive pain[150]. Such findings
suggest that MORs mediate some, but not all forms of pain sensation. In models of
chronic pain, MOR null mice actually exhibit faster recovery from induced hyperalgesia
following administration of complete Freund’s adjuvant (CFA). Interestingly, recovery
was blocked by delta-specific antagonist naltrindole. It was then found that the MOR
knockout mice were more sensitive to delta agonist-induced analgesia, suggesting that
these mice have heightened DOR activity in circumstances of chronic pain[153]. Stress-
induced analgesia is also known to contain an MOR-dependent component which is
partially reversed upon administration of mu opioid antagonists[154]. In this paradigm,
MOR knockouts exhibited a reduction in late, but not early stage stress-induced analgesia
compared to wild type controls[155]. Analyses of anxiety/depression using forced swim
tests in MOR knockouts have likewise demonstrated a role for mu opioid receptors in
regulating these behaviors in mice[156].

In the hippocampus, MOR null mice display decreased levels of LTP in the
dentate gyrus but not the CA1[157]. No functional assays have followed up these
findings. Highest concentrations of MOR are typically found in inhibitory basket cells of
the hippocampus which are known to modulate the activity of excitatory pyramidal
neurons[158]. Therefore agents such as morphine could potentially regulate hippocampal
LTP by blocking inhibition of hippocampal pyramidal excitation by GABAergic basket
cells in addition to more indirect means.
Outside the CNS, MORs play a role in hematopoiesis and proper lymphocyte development. Animals lacking MORs exhibit greater numbers of femoral bone marrow granulocytes/macrophages, erythroid, and multipotential progenitor cells in vivo[143] and reduced basal proliferation of thymic and splenic T cells compared to wild type controls[159]. Deletion of the MOR also results in a modest increase in breathing frequency, suggesting that MORs influence tonal control of respiration[160].

1.21 MOR signaling: Analgesia

The acute effects of MOR activation have been characterized in recent years. Presynaptically localized MORs inhibit calcium influx and cAMP production via adenylate cyclase resulting in reduced neurotransmitter release[161, 162]. Postsynaptically, MOR activation enhances outward potassium currents via G protein-regulated inwardly rectifying potassium (GIRK) channels[161, 163], and inhibit the activity of NMDA and AMPA receptors (Figure 14)[161, 162]. The actions MORs are most thoroughly investigated in the rostral ventral medulla (RVM) of the spinothalamic pathway with respect to pain response. Within the RVM there exist two primary cell types whose actions can be segregated based upon the firing patterns observed prior to the induction of painful stimuli as shown in Figure 15: 1) ON cells, which fire at a fast rate prior to the induction of a withdrawal reflex from painful stimuli and 2) OFF cells, which are silent prior to the induction of a withdrawal reflex from painful stimuli. MORs are postsynaptically localized on “ON cells”, where they directly inhibit the excitatory actions of NMDA and AMPA receptors through enhancement of the activity of outward potassium currents via GIRK channels and inhibiting receptors. For OFF cells,
presynaptically localized MORs on GABAergic neurons indirectly promote OFF cell activity by inhibiting presynaptic GABA release[161]. By reducing “on cell” activity and increasing “off cell” activity, MORs act to reduce the extent of withdrawal reflex.

1.22 Morphine tolerance

For many years it has been appreciated that recurrent and prolonged exposure to morphine can result in the development of tolerance against the analgesic effects of the morphine, enhancing the potential for addiction. While a general molecular mechanism for morphine tolerance has been proposed for the immediate effects of morphine, there are still a number of factors which are not addressed by the current model. The binding of morphine, as well as activation of other GPCRs (e.g. CGRP receptor, group I metabotropic glutamate receptors, NK-1 receptor), induces G protein mediated activation of the phospholipase C pathway, resulting in the activation of protein kinase C. PKC subsequently phosphorylates the C-terminus of the MOR at specific sites, resulting in desensitization of the receptor[164-167]. Interestingly this is not observed with other MOR agonists such as DAMGO. When DAMGO binds to the MOR, the receptor is phosphorylated by G protein-coupled receptor kinase (GRK) instead of PKC, resulting in an internalization of the phosphorylated receptor with resultant dephosphorylation and recycling of the receptor back to the cell membrane, a process known as re-sensitization[89, 94]. Compared to other agonists, morphine causes relatively high desensitization and low resensitization compared to DAMGO, meaning that it induces a higher degree of
Figure 14. Presynaptic and postsynaptic signaling of the MOR. Presynaptically localized MORs can inhibit local adenylate cyclase activity causing calcium channels to close; ultimately reducing the amount of neurotransmitter released. Postsynaptically, MORs can alter signaling by inducing hyperpolarization through activation of GIRK channels, thereby inhibiting NMDA and AMPA receptors signaling.
Figure 15. Neurologic actions of the MOR depend on their synaptic localization. Postsynaptically localized MORs can induce hyperpolarization of postsynaptic on-cells, thereby decreasing the firing rate of these excitatory cells just prior to the withdrawal reflex and resulting in the induction of analgesic effects. Presynaptic MORs act to reduce GABA release of off-cells, increasing their activity prior to the withdrawal reflex and thus generating analgesia.
ANALGESIA

ON cell

Hyperpolarization

MOR agonist

GLU

GABA

OFF cell

Inhibition of GABA release

Presynaptic cell

Postsynaptic cell

Presynaptic cell

Postsynaptic cell

↑ = withdrawal reflex upon painful stimulation
tolerance[89]. Once in the desensitized state, MORs are uncoupled from their G proteins, resulting in adaptive cellular changes and AC superactivation over prolonged exposure[168-170]. AC superactivation leads to increased basal release of CGRP, a neuropeptide involved in the transmission of pain, via cAMP/PKA activation of neurotransmitter release[171]. Therefore, morphine tolerance develops as a result of high levels of MOR desensitization and increase release of nociceptive neuropeptides over time. (Figure 16)

Since different ligands induce different degrees of tolerance, the RAVE hypothesis was developed in order to rate the unique index of tolerance for each ligand. The RAVE value is known as the relative activity (RA) versus endocytosis.

\[
\text{Tolerance} = \frac{RA}{VE} = \frac{\text{relative activity}}{\text{endocytosis}}
\]

Specifically, relative activity is measured as a function of GIRK channel activation, while endocytosis is defined as the number of receptors internalized. The RAVE hypothesis is useful in comparing different opioid ligands. Considering the example above, DAMGO (low degree tolerance) has a low RAVE value, while morphine (high degree of tolerance) has a relatively higher RAVE value. An example other opioids which exhibit of low, moderate and high RAVE values are enkephalin, methadone and fentanyl. It is important to note that the RAVE value is not correlated with the affinity or potency of the ligand[89].
1.23 Thesis rationale and hypotheses

**EphB2 receptors and LTP:** Studies from our laboratory have demonstrated that EphB2 knockout animals have impaired LTP in CA1 neurons of the hippocampus. This was found to be due to reduced levels of NMDA receptor expression on the cell surface in EphB2 -/- mice; it was proposed that EphB2 acts to stabilize the NMDA receptor on the plasma membrane[32]. Thus, the involvement of EphB2 in hippocampal LTP has the potential to affect opiate-dependent learning.

**EphB receptors and sensory function:** Previously intrathecal injection of EphB2-Fc, a molecule which potentially interferes with EphB/ephrinB signaling, reduces morphine-dependent withdrawal. EphB receptors were also found to be up-regulated during morphine treatment and withdrawal in the dorsal laminae of the spinal cord. In addition, it was reported that intrathecal application of EphB2-Fc attenuates a number of molecular events associated with morphine withdrawal, such as elevated levels of c-Fos, increases in CGRP during treatment and increases in phosphorylated forms of ERK, CREB and the NR2B subunit of NMDA receptors[11]. Although this sheds light on the involvement of Eph receptors in opiate withdrawal, the role of these receptors in opiate tolerance still remains unclear. Therefore, one of the major aims of my thesis will focus on the role of EphB2 in morphine tolerance.

Several Eph receptors/ligands are known to map close to schizoaffective loci in man. Studies using RNA in situ hybridization and histochemical analysis have demonstrated the presence of a number of Eph at neural loci associated with dependence throughout development and adulthood. Eph receptors and ephrins are present in regions associated with schizophrenia and drug dependence, such as the
nucleus accumbens (NAc), caudate-putamen (CPu), ventral tegmental area (VTA) and substantia nigra (SN)[31, 172-175]. These receptors are also reported in the periaqueductal gray (PAG) matter, a region involved in the processing of pain[175].

In addition, some Eph receptors and ephrin ligands are reported to exhibit increased levels of expression in these areas following exposure to cocaine or amphetamine[172, 176, 177].

_Hypotheses:_

1) EphB2-mediated signaling in the hippocampus regulates the conditioned response to morphine.

2) EphB2 in the spinal cord and dorsal root ganglia regulates the pharmacologic actions of MOR signaling to modulate morphine tolerance
**Figure 16. Proposed mechanism of morphine tolerance.** Different MOR agonists have been shown to induce different degrees of tolerance. This is believed to be the result of differential phosphorylation of the MOR receptor by distinct groups of kinases, which ultimately culture the amount of receptor internalized and recycled. A. DAMGO, which induces low levels tolerance, induces activation of GRK. GRK-mediated phosphorylation of the MOR induces high levels of receptor internalization and recycling. B. In contrast, morphine induces PKCγ-mediated phosphorylation of the MOR. This results in significantly lower levels receptor internalization, leaving desensitized MORs on the cell surface. As a result greater numbers of MOR receptors are unable to re-couple with their G proteins, inducing higher degree of tolerance.
A. 

DAMGO → MOR 

ARVE value 
MILD TOLERANCE 

Adenylate cyclase 

GRK2 → INTERNALIZATION → DEPHOSPHORYLATION 

RESENSITIZATION
B.

Morphine

↑ RAVE value
SEVERE TOLERANCE

Adenylate cyclase

ATP  ↑ cAMP

↑ Release of substance P and CGRP

↑ PAIN

INTERNALIZATION

DESENSITIZATION
CHAPTER 2: MATERIALS AND METHODS
2.1 Animals

Two types of genetically modified mice were used in this study: the N1 and N2 lines (Figure 8). The N1 line consists of a deletion at the 5’ segment of the EphB2 locus, encompassing amino acids 29-50 and replacement with a neomycin resistance cassette. These animals do not express the EphB2 receptor. The N2 line contains a 1 kilobase deletion of the EphB2 locus that includes the kinase domain and all subsequent C-terminal regions (amino acids 622-707) and replacement with an in-frame bacterial sequence for the enzyme beta-galactosidase. These animals express the ectodomain, transmembrane domain and part of the juxtamembrane region of the EphB2 receptor, however, they are kinase dead[31]. All animals were raised on a 129S1/SVImj x CD1 background.

2.2 Pharmaceuticals

Morphine sulphate was obtained from the laboratory of Derek van der Kooy. Morphine was dissolved at a concentration of 1.5 mg/mL in 0.9% saline and administered at a dose of 10 mg/kg intraperitoneally. Baclofen (RBI), a GABA\(_B\) agonist, was dissolved in 0.9% saline at a concentration of 2 mg/mL.

2.3 Assessment of motor and sensory modalities

*Open field activity:* Previous work by Stephanie Ho in our laboratory has demonstrated that the EphB2 null mice do not exhibit significant motor deficits with respect to an array of motor tasks. In order to assess higher cognitive functions in EphB2 null mice, EphB2 nulls and control littermates were examined in open field activity assays. Animals were
tested in a standard 24 x 42 cm field for a period of one hour. Spontaneous activity was measured using an infrared Linton AM1053 monitoring system; with results interpreted using Microsoft Excel 2000.

*Von frey fiber test:* To assess basic mechanoceptive function, EphB2 null animals and controls were examined using a graded series of Von frey filaments. Each filament is rated against known bending force, and the plantar surface of the hind footpad of each animal was assessed unilaterally for two separate exposures for each of the indicated fiber calibers. Results were graphed as a function of the force in grams required for reproducible recognition of mechanoceptive pressure.

*Tail pinch assay:* To assess cognitive awareness of mechanoceptive/nociceptive function, standard tail pinch assay was employed. The assay utilized a constant closing force of 29 Newtons, as this pressure was determined to be minimally sufficient to consistently evoke a response in untreated wild type animals of each of the genetic backgrounds employed. Prior to and following morphine injection, tail pinch assay was performed for the proximal third of the tail at 15, 30, 45, 60, 90 and 120 minutes following morphine introduction. In untreated wild-type animals, based-time latencies were 1-2 seconds. Assay response times were limited to 10 seconds.

*Tail flick assay:* To assess thermoceptive function, the tail flick assay was employed. To perform these tests at the analysis time points and before morphine treatment, the distal third of the tail was introduced to water held at a temperature of 55°C. In untreated wild-type animals baseline latencies were 2-3 seconds. Assay response times were limited to 15 seconds to avoid injury.
2.4 Morphine dosing and behavioral response

Animals were injected with 10 mg/kg morphine sulphate twice per day in the morning and afternoon at intervals of 8 hours for a period of six days (Figure 17). To assess higher cortical influences on morphine analgesia, on day 7 groups of mice either remain in their home environment, or alternatively were placed in a novel environment prior to receiving their daily injection of morphine sulfate prior to behavioral assessment.

2.5 Histochemistry/immunohistochemistry/Western blotting

Primary antisera used included antibodies to CGRP (polyclonal, chicken, 1:100, Neuromics), substance P (polyclonal, rabbit, 1:5000, Chemicon), p75 (polyclonal, rabbit, 1:1000, Reichart lab), beta-galactosidase (polyclonal, rabbit, 1:100, MP Biomedical; or monoclonal, mouse, 1:50, Promega) and the MOR (rabbit, polyclonal, 1:1000, Immunostar), as well as the FITC-conjugated isolectin IB4 (10ug/mL, Sigma). Secondary antibodies used included Alexa Fluor 488 goat anti-rabbit (1:200, Invitrogen) for anti-MOR, Dylight 488 goat anti-chicken (1:200, Jackson Immunoresearch) for anti-CGRP, Alexa Fluor 546 goat anti-rabbit (1:200, Invitrogen) for anti-substance P, anti-p75 and anti-beta-galactosidase (Rb) and Alexa Fluor 488 goat anti-mouse (1:200, Invitrogen) for anti-beta-galactosidase (Ms).

Localization of EphB2 was routinely determined using animals heterozygous for the EphB2 N2 mutation (EphB2 N2/+) expressing beta-galactosidase, C-terminal transmembrane domain and juxtamembrane tyrosine residues (see Figure 8). Given the EphB2-dependent expression of this protein and its fusion to the EphB2 coding sequence, analysis of β-galactosidase expression in these animals can be used as a definitive tag for
Figure 17. Morphine treatment regimen. Mice were treated with morphine (10 mg/kg, i.p.) for a period of six days. Animals were injected twice per day, once in the morning and once in the evening, 8 hours apart. Tail pinch (TP) and/or tail flick (TF) tests were performed before morphine exposure (day -1) and on days 1, 3 and 6 of treatment after the morning injection.
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the positional expression of EphB2. These animals were therefore utilized to examine the distribution and expression of EphB2 as indicated in the studies described. For the indicated studies, morphine-treated or naïve animals were lethally anesthetized, perfused with phosphate buffered saline and were immersed in fixative (5mM EGTA, 2mM MgCl$_2$, 0.2% gluteraldehyde dissolved in 0.1M sodium phosphate, pH 7.3) for 30 minutes. Following three 10 minute washes with LacZ wash buffer (2mM MgCl$_2$, 0.01% deoxycholate, 0.02% NP-40 dissolved in 0.1M sodium phosphate, pH 7.3), requisite tissue samples were then isolated and incubated in X-gal solution (1mg/mL X-gal, 0.2mg/mL potassium ferrocyanide, 1.6mg/mL potassium ferricyanide dissolved in LacZ wash buffer) at 37°C until sufficient staining had appeared (approximately two hours).

To determine the cellular localization of EphB2 with respect to MOR expression, EphB2 N2/+ animals were perfused with 4% paraformaldehyde (PFA) dissolved in 0.1M phosphate buffered saline (PBS). Spinal cord (T13-L1) and dorsal root ganglia (DRG; L2-L5) samples were subsequently taken and processed for frozen or wax sectioning as indicated below.

*Paraffin wax sectioning:* Tissues were embedded in paraffin wax according to standard procedures and 7µm sections obtained and mounted onto poly-L-lysine (PLL) coated slides. Slides were incubated for $\geq$4 hours at 55°C to allow adherence. Wax sections to be probed with MOR antibody underwent antigen retrieval (10mM sodium citrate, pH 6.0, heated in a pressure cooker for 10 min to bring the water to a boil, followed by an additional two minutes at a boil. Sections were then incubated with anti-MOR (1:1000, Immunostar) diluted in blocking solution (5% goat serum, 0.25% Tween-20 dissolved in PBS) overnight at 4°C, followed by washing (3x5 minutes in blocking buffer) and
incubated in biotinylated goat anti-rabbit secondary antibody (1:200, Vector Labs) for two hours at room temperature. Tertiary reagent (avidin-horseradish peroxidase, Vector Labs) was then added for 45 minutes, followed by development using diaminobenzidine as previously described.

*Frozen sectioning:* Cerebral, spinal and DRG tissue were either obtained from either PFA-perfused animals or taken and flash frozen directly. Flash frozen DRGs were embedded directly in freezing media (OCT, Fisher). Spinal tissues were postfixed overnight followed by nutation in 30% sucrose in 0.1M PBS overnight. Tissues were then embedded in OCT and cryostat sections obtained at 14 µm. Sections were thaw-mounted onto PLL coated slides and incubated overnight at 4°C to promote adherence. Sections were then incubated with primary antisera (see below) overnight at 4°C. Following washing, fluorescently tagged secondary antibody was then applied at room temperature for 2 hours. Where needed, cell nuclei were visualized using Hoechst 33258 (1:500, Sigma) dissolved in 0.1M PBS applied for 10 minutes.

To verify the fidelity of antisera used in determining MOR expression, *Oprm* (MOR) null mice and controls were examined using several commercial MOR antisera by each of the methods described above. These results demonstrated that only the Immunostar antiserum for the MOR when used in conjunction with antigen retrieval on paraffin sections resulted in the expected pattern of MOR expression (localizes staining in the dorsal lamina of the spinal cord) in the wild type with no staining observed in comparable knockout animals (please see Figure 27).

*Western blotting:* For Western analyses, tissues were immediately dissected out following sacrifice and homogenized in a lysis buffer (50 mM Tris, 10mM EDTA, 1.5% NP-40 and
1mM PMSF). Tissues were nutated overnight at 4°C in lysis buffer and centrifuged for 10 min in a micro-centrifuge at 13,000 rpm in order to extract the cell supernatants. Fifty micrograms of lysate were then mixed with 8 uL of 2x SDS sample buffer and heated at 37°C for 15 minutes. Samples were again centrifuged at 13,000 rpm and loaded onto a 12% acrylamide gel[178]. Samples were separated by SDS-PAGE electrophoresis for one hour and fifteen minutes at 25mA and 500V. Following separation proteins were transferred to a nitrocellulose membranes over two hours at 230mA, 90V. Nitrocellulose membranes were then incubated with rabbit polyclonal anti-MOR antibody (Immunostar, 1:5000) dissolved in 5% milk powder in TBS-T (45mM NaCl, 3mM Tris, 0.05% Tween-20) overnight for 4°C. Following washing membranes were incubated with HRP-conjugated secondary antibody (1:3000, Bio-Rad Laboratories), then visualized using enhanced chemiluminescence (ECL, Thermo Scientific). For purposes of quantitation, levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:10 000, Sigma) in tissue isolates were used as a measure of a loading control.

2.6 Radioligand binding

Radioligand saturation binding studies to determine the affinity and total binding capacity of the MOR in EphB2 +/+ , +/- and -/- littermates were determined in the presence of varying concentrations of [3H]-naloxone against a constant concentration of the nonspecific opioid antagonist naltrexone[179]. Because of their low levels of potentially interfering delta and kappa opioid receptors, tissues of the superior colliculus were chosen for these assays. This site were chosen for analysis because EphB2 is expressed here in the presence of relatively high concentrations of MOR with no or limited
expression of other opiate receptors. Tissues samples were collected at the time of sacrifice and frozen at -80°C until used. Prior to radioligand binding tissues were thawed at room temperature and protein concentration determine using BCA assay according to standard procedures[178]. Roughly 230 µg of protein was incubated at room temperature for two hours and thirty minutes with a given concentration of [3H]-naloxone ((1.78 x 10^{-8}, 1.33 x 10^{-8}, 1 x 10^{-8}, 5.62 x 10^{-9}, 4.22 x 10^{-9}, 3.16 x 10^{-9}, 1.78 x 10^{-9}, 1 x 10^{-9}, or 5.62 x 10^{-10}) additions adjusted to a volume of 10 µL) and 10 µL of naltrexone (100 µM) or binding buffer (50 mM Tris, 3 mM MgCl₂, 1 mg/mL BSA). The samples were then run through a Harvester apparatus with naloxone-bound proteins collected onto a Whatman glass microfiber filter (GE Healthcare). Filters were washed three times with wash buffer (50 mM Tris, 3 mM MgCl₂) following filtration, then immersed in scintillate (Ultima Gold, Perkin Elmer) overnight prior to counting. The following day results were counted and interpreted using Graphpad Prism 5.0 and software developed by Dr. Jim Wells[180].

2.7 Cognitive tasks

Passive avoidance. As shown in Figure 18, the testing apparatus consists of 25 x 25 cm light and dark chambers. Acquisition. Mice are initially confined to the light chamber for a period of 30 seconds, at which time the divide separating the two chambers is opened with animals allowed to freely explore both spaces. Transfer latency time (TLT) is defined as the period of time it takes animals to cross from the light into the dark chamber (the normally preferred chamber for mice) following opening of the divide door. Once inside the darkened chamber, the divide door is closed for a period of 10 seconds and animals experience a one-time provisional shock through the stainless steel floor grid
(0.5mA for 5 seconds). Thirty seconds following shock, the chamber divide is once again opened and mice are allowed to freely return to the light chamber. The time required for mice to return to light chamber is subsequently recorded. Retention. Twenty-four hours following acquisition mice are re-introduced into the light chamber with the divide open. The time required to move into the dark chamber is recorded. Cut off time for the assay was set at 300 seconds (Figure 18).

Object recognition. The response of EphB2 null mice, heterozygous and control littermates were assessed with respect to novel object recognition. In this paradigm, animals are placed in a quiet room in a clear box and exposed sequentially to four different conditions (Figure 19): (1) primary habituation - exposure to empty test cage for 5 minutes; (2) secondary habituation - exposure to three identical objects placed at each of three corners of the test cage for a period of 15 minutes; (3) displaced object - object 2 is moved to the empty corner of the box and responses assessed over a period of 5 minutes; (4) novel object - the displaced object is removed from the box, with one of the remaining objects replaced with a new novel object. Responses were assessed over a period of 5 minutes. A three-minute break is employed between each of the four phases given above, during which time in the animal is returned to its home cage.
Figure 18. Passive avoidance paradigm. A. Acquisition phase: mice were introduced to the light chamber and allowed 30 seconds to explore the environment. After this, the door was opened and mice were permitted to enter the dark chamber of their own accord and the transfer latency time (TLT) was recorded. Once inside the dark chamber, mice received an electric shock of 0.5 mA for 5 seconds. The total time in the dark chamber was 30 seconds, after which the door was opened and mice were permitted to leave. B. Retention phase: Mice were placed back into the light chamber with the door open 24 hours later and the transfer time latency was recorded. Cut off time was 300 seconds.
**Figure 19. Object recognition paradigm.** Mice were first habituated to the environment for five minutes, followed by a second habituation phase to the objects for 15 minutes. In the third phase, the object 2 was moved (displaced object), and in the final phase the displaced object was removed and object 1 was exchanged for a novel object. Each phase was separated by a three minute break in the home cage.
**HABITUATION 1**
(5 min)

**HABITUATION 2**
(15 min)

**DISPLACED OBJECT**
(5 min)

**NOVEL OBJECT**
(5 min)

---

Observer
CHAPTER 3: RESULTS
3.1 Assessment of basic motor and sensory response in EphB2 null mice

*Basic motor function:* A previous graduate student in the lab evaluated the performance of EphB2 null mice in a number of motor tasks, including the hindlimb extension, edge performance, hindlimb grip response, grip strength, wire platform locomotion and 90° incline climb (Stephanie Ho, Thesis). It was observed that EphB2 knockouts also showed significantly poorer performance in the wire platform locomotion test relative to EphB2 +/− mice. Otherwise, these animals exhibited wild type-like performance in all other motor tasks described above. Therefore, EphB2 knockout mice possess mostly normal basic motor functions, exhibiting deficiencies in only specific tasks involving motor coordination and balance.

*Von frey filament test:* Sensory response to a series of filaments of graded bending force have long been utilized to assess features up mechanosensitive response in rodent lineages[181-183]. In this assay, the animal’s response following contact with a graded filament of specific force displacement is placed along the plantar surface of the murine hindlimb is utilized to determine their sensitivity to a given sensory stimulus. I therefore examined the response of EphB2 null mice with respect to this measure. As shown in Figure 17, analysis of wild type and EphB2 +/−, −/− and N2/N2 littermates indicates that there is no significant intrinsic difference in levels of mechanosensitivity among any group compared to wild type controls within the limits of assay sensitivity (Figure 20).

*Tail pinch assay:* Physical responses to a given pressure applied to the proximal third of the rodent tail represents another widely utilized measure of
nociceptive/mechanoeptive function[184-186]. As indicated in Figure 21A, neither EphB2 null or kinase dead mice exhibit any intrinsic difference in tail pain sensitivity compared to littermate controls. As a result these analyses, a tail pinch force of 29 Newtons was selected to be the standard force utilized in subsequent tail pinch analyses.

Tail flick assay: In order to determine the intrinsic thermoceptive response of EphB2 null mice, the response of immersion of the distal third of the tail into a water bath of 55° C was assessed. The tail flick assay represents one of the most widely utilized measure of thermoceptive/nociceptive function in rodents[187-189]. As indicated in Figure 21B, no significant difference in intrinsic thermoceptive sensitivity was observed following immersion at 55°C. Further studies were therefore performed at a temperature of 55° C as this represents the most widely utilized standard reference temperature for tail flick measurements in rodents.

Taken together the above results suggest that EphB2 null or EphB2 N2/N2 mice do not exhibit significant perturbations in the basic features of their primary sensory and/or motor responses.

Exploratory (open field) activity: EphB2 null mice and controls were individually introduced into a novel environment and their spontaneous motor activity was monitored over a one hour period as a measure of exploratory activity. Open field motor performance was therefore utilized as a measure of exploratory activity for EphB2 null mice and control littermates. As shown in Figure 22, examination of spontaneous motor activity in EphB2 null mice following introduction into a novel
Figure 20. Examination of mechanoeception in EphB2 mice. EphB2 null mice and controls were examined with respect to stimulus response against von Frey fibers of graded potential. With respect to intrinsic mechanoeception, no statistically significant differences were observed between EphB2 null or kinase dead mice and controls. Error bars represent standard error of the mean.
Figure 21. Examination of basal mechanical and thermal nociception in EphB2 mice. A. Mechanical nociception was measured as a function of tail pinch response, in which the proximal third of the tail was pinched and the amount of time for the mouse to respond was measured. There was no significant difference in response time observed between wild type and both N1 and N2 mutants. B. Thermal nociception was assessed using the tail flick assay at 55°C. There was no significant difference in the response time observed between wild type and both N1 and N2 mutants. Error bars represent standard error of the mean.
A.

Baseline tail pinch response

Tail pinch latency (sec)

- EphB2 +/+ n=13
- EphB2 +/- n=14
- EphB2 -/- n=16
- EphB2 N2/ N2 n=7
Baseline tail flick response (55°C)

- EphB2 +/+ n=8
- EphB2 +/- n=10
- EphB2 +/- n=7
- EphB2 N2/ N2 n=8

Tail flick latency (sec)
environment indicates that mice lacking EphB2 initially exhibit elevated levels of motor activity which is subsequently reduced throughout exposure to the environment. A similar trend is seen in EphB2 heterozygous and wildtype littermates. However analysis of motor activity (activity per 10 minute time period) indicate that at almost every point of the analysis EphB2 heterozygote and null mice exhibit significantly elevated levels of spontaneous motor activity compared to the wild type controls. As indicated in the figure, analysis of the total motor activity during the one-hour exposure period indicates that EphB2 heterozygote and null mice showed 1.6 and 2.2-fold elevations in total activity compared to wild type littermates. Interestingly EphB2 heterozygotes exhibited activity levels which were intermediate to those seen for EphB2 null mice and wildtype littermates. EphB2 N2/N2 mice lacking the EphB2 tyrosine kinase, SAM, and PDZ domains exhibit no significant difference in motor activity compared to wild type controls. As EphB2 N2/N2 mice generally show no differences from controls with respect to their physiology or behaviour. Investigations of these mice in our lab have revealed wild type levels of surface NMDA receptors and normal hippocampal LTP[32].

In conjunction with the above experiments, this suggests that the elevation in motor activity seen for naïve EphB2 null mice upon exposure to a novel environment are the results of a change in the integrated response of EphB2 null mice to the environment rather than the result of a change in primary motor or sensory responsiveness. The observed difference in activity responses seen in
Figure 22. Open field behavior in EphB2 mice. Spontaneous motor activity was monitored in EphB2 null mice and controls for a period of 60 minutes following introduction into a novel environment. A. Activity is displayed as a function of distance traveled per five minute period. B. Average total distance traveled each genetic group. Compared to wild type animals, EphB2 null mice exhibit somewhat elevated levels of spontaneous movement over the majority of exploration period. Error bars represent standard error of the mean, asterisk indicates statistical difference that p<0.05, EphB2 +/+ vs. EphB2 +/- or -/-.
B.

![Bar graph showing total distance (m) for different genotypes.]

- EphB2 +/+ n=10
- EphB2 N2/ N2 n=10
- EphB2 +/- n=18
- EphB2 -/- n=10
EphB2 null mice versus EphB2 N2/N2 mice suggest that these effects are mediated via some action of Eph/ephrin reverse signaling.

3.2 Morphine related responses of EphB2 null mice

Compared to controls, EphB2 null mice exhibit no intrinsic differences in sensory responsiveness for any assay utilized. Given that EphB2 continues to be expressed during the adult period at a number of sites known to be involved in regulating the central perception of pain, I examined the analgesic response of EphB2 null mice following administration of morphine. As shown in Figure 23, the response of EphB2 null mice to morphine during the first 30 minutes following exposure is not significantly different to that seen in EphB2 heterozygous wildtype littermates.

However subsequently, EphB2 null mice exhibit a trend toward reduced analgesic response compared to heterozygous and wildtype littermates during this initial exposure of morphine. For initial morphine exposure, this trend achieves statistical significance at 45 minutes. Repeated exposure to morphine along the dosing schedule shown in Figure 17 can result in reduced analgesic response over time (induction of morphine tolerance)[190, 191]. By day 3 of morphine dosing, tail pinch analysis of EphB2 null mice clearly demonstrates a substantial statistically significant reduction in morphine-induced analgesia responses compared to that seen in EphB2 heterozygotes and wildtype littermates (Figure 24). Analysis of morphine-dependent response following 6 days of dosing demonstrates the expected outcome in wild type animals (Figure 25), showing a significant reduction
in tail pinch latencies compared to that seen in these animals on day 1 (i.e. induction of morphine tolerance in wild type animals). Consistent with the responses seen on day 3, EphB2 null mice exhibit an almost complete abrogation of morphine-dependent analgesia. This altered responsiveness (i.e. reduction in tail pinch latency) is observed in both the tail pinch and tail flick assay (Figure 26). Interestingly kinase dead EphB2 N2/N2 mice show responses similar to that seen for control littermates in both tail pinch and tail flick assays, suggesting that presence of the EphB2 ectodomain is sufficient to allow propagation of the morphine-dependent effects seen in wild type mice. Thus though EphB2 null mice do not exhibit intrinsic differences with respect to sensory responsiveness compared to control littermates, the time-dependent analgesic response observed with morphine is significantly altered.

3.3 Analysis of mu opioid receptor levels in the dorsal spinal cord

To rule out that the effects observed in EphB2 null mice might reflect potential intrinsic differences in mu opioid receptors in these animals, MOR levels were investigated at several key CNS sites via Western analysis of sub-dissected CNS loci. Analysis of the performance of several published commercial MOR antisera indicated that these antisera were not reliable with respect to detection of the protein of interest. Analysis of various MOR antisera via immunohistochemistry of paraffinized spinal cord sections from wildtype and MOR null mice (Jackson Laboratories) eventually resulted in the identification of a commercial MOR
Figure 23. Tail pinch latency following day one of morphine treatment in EphB2 null and kinase dead mice. Nociceptive/mechanosensitive response was measured by tail pinch using an artery clamp before morphine treatment or every 15-30 minutes following morphine administration on day 1. EphB2 knockout mice display a similar response to morphine in the first 30 minutes following morphine injection. However, knockout mice exhibit significantly lower latency times for the remainder of the time examined compared to wild type controls. EphB2 N2/N2 mice behave similarly to wild type animals. Error bars represent standard error of the mean. (*) p<0.05 for EphB2 +/- vs. EphB2 -/- mice.
Figure 24. Tail pinch latency following day three of morphine treatment in EphB2 null and kinase dead mice. As previously described, nociceptive/mechanooceptive response was measured by tail pinch using an artery clamp before morphine treatment or every 15-30 minutes following morphine administration on day 3. EphB2 -/- animals showed significantly lower latency times compared to wild type animals, while EphB2 N2/N2 mice behaved like EphB2 +/- . Error bars represent standard error of the mean. (*) p<0.05 for EphB2 +/- vs. EphB2 -/- mice.
Day 3

Tail pinch latency (sec)

Time after morphine injection (min)

- EphB2 +/+  
  n=22

- EphB2 +/-  
  n=26

- EphB2 -/-  
  n=24

- EphB2 N2/ 
  N2 n=7
Figure 25. Tail pinch latency following day six of morphine treatment in EphB2 null and kinase dead mice. As previously described, nociceptive/mechanoceptive response was measured by tail pinch using an artery clamp before morphine treatment or every 15-30 minutes following morphine administration on day 6. EphB2 -/- animals showed significantly lower latency times compared to wild type animals, while EphB2 N2/N2 mice behaved like EphB2 +/+. Error bars represent standard error of the mean. (*) p<0.05 for EphB2 +/+ vs. EphB2 -/- mice.
Day 6

[Graph showing tail pinch latency over time after morphine injection for different genotypes: EphB2 +/+ (n=22), EphB2 +/- (n=26), EphB2 /- (n=24), EphB2 N2/N2 (n=7).]
Figure 26. Tail flick latency following morphine treatment in EphB2 null and kinase dead mice. For testing purposes the terminal 2 cm of the subject’s tail was placed in contact with 55°C water before or 30 minutes following morphine administration on day 0, 1, 3 and 6. Despite similar intrinsic latencies, EphB2 null mice exhibit significantly lower morphine induced latencies at days 3 and 6 of treatment compared to controls. Error bars represent standard error of the mean. (*) p<0.05, EphB2 +/- vs. EphB2 -/-.
The bar chart shows the tail flick latency (sec) on different days of morphine treatment for different genotypes:

- EphB2 +/-(n=10)
- EphB2 N2/(N2 n=8)
- EphB2 -/- (n=8)

The x-axis represents the day of morphine treatment, with two time points highlighted:
- Prior to morphine treatment
- 30 minutes following morphine treatment
antiserum which reliably detected a protein exhibiting a staining pattern identical to that established for the MOR in the spinal cord (Figure 27). This antiserum (Immunostar) was subsequently utilized to determine levels of both monomeric and dimeric MOR species (apparent MW 55 and 105 kDa respectively) in various tissue extracts. It should be noted that this antiserum also recognized an unrelated protein product of ~43 kD. Initial analysis of protein extracts derived from the dorsal spinal cord (a site known to be enriched for MOR) suggested no significant difference in levels of MOR expression between EphB2 null and heterozygous mice (Figure 28). Animals from separate litters of EphB2 null mice were paired with heterozygous and wildtype counterparts, confirming an absence of significant differences in total MOR protein levels among these groups.

3.4 Functional analysis of mu opioid receptors in EphB2 null mice using [³H]-naloxone

In order to verify that the functional characteristics of mu opioid receptors in EphB2 null mice were comparable to that seen in littermate controls, tritiated naloxone binding (followed by cold chase with naltrexone) was performed[179]. For these assays, analyses were performed on material derived from the superior colliculus. The superior colliculus was chosen due to its expression of MOR in the absence of other contaminating (kappa, delta) opioid receptors. Although MOR desensitization, thought to be a crucial process in morphine tolerance, has been shown to occur within specific areas of the brain and in the spinal cord[89], there have been no reports to date which examine desensitization in the superior colliculus.
Figure 27. Verification of specificity of MOR antibody utilized. Several MOR antisera were utilized in attempts to characterized patterns of MOR staining in EphB2 null mice and controls. Antisera were tested against both wild type (left) and MOR knockout (right) C57Bl6 mice under several different fixation conditions. For the result shown, paraffin sections taken from spinal cord level T10 underwent antigen retrieval prior to MOR staining. Shown in the figure, observed staining was confined to the outer dorsal laminae in adult wildtype specimens, while no such staining was observed in knockout tissue. Scale bars represent a distance of 100 μm.
Figure 28. Analysis of MOR levels in the spinal cords of EphB2 null mice. Tissue levels of monomeric (55 kD) and dimeric (105 kD) MOR were examine by Western blot shown in EphB2 null mice (n=3) and controls (n=2). No significant change in total MOR levels were observed between control and EphB2 null mice. Error bars represent standard error of the mean.
Analysis of three independent replicates of tissue samples derived from three
dependent animals indicated that the $K_d$ for collicular extracts was similar for
EphB2 null and wildtype mice (Table 6), and consistent with one report which
examined tritiated naloxone binding in amphibian brain extracts[192]. Likely due to
the compositional complexity of the primary material used, the affinity constant
directly derived using whole brain extracts are an order of magnitude greater than
that seen for similar studies using genetically engineered purified monolayer cell
cultures[55, 179, 193]. Further work is being done to determine if the receptor
density differs between wild types and knockouts.

3.5 Distribution of EphB2 in the adult murine CNS

Using EphB2 N2/+ knockin mice, the expression pattern of EphB2 was definitively
determine through β-galactosidase histochemistry. Fusion of EphB2 to β-
galactosidase allows for the unequivocal identification of cells expressing this
protein. Like the endogenous protein, the EphB2/β-galactosidase fusion exhibits a
similar membranous localization and is detected in growing axons during
development[30, 31]. Thus loss of the tyrosine kinase, SAM or PDZ domains within
the EphB2/beta-galactosidase fusion does not appear to significantly alter patterns
of intracellular localization. However, it is presently unclear whether this EphB2/β-
galactosidase fusion exhibits similar protein turnover kinetics to that seen for
endogenous EphB2.

As shown in Figure 29, EphB2 was observed to be present in adult animals at
a variety of CNS loci known to be involved in the perception and processing of
mechanoceptive, nociceptive thermoceptive stimuli. Regions of prominent EphB2 expression in this regard include the dorsal laminae of the spinal cord (Figure 29A), dorsal root ganglia (Figure 29B), thalamus (Figure 29C), hypothalamus (Figure 29C), anterior cingulate and somatosensory cortices (Figure 29E), amygdala (Figure 29C) and periaqueductal grey (Figure 29D). In contrast EphB2 was not strongly expressed in adult mice in regions associated with dependence (nucleus accumbens, striatum, ventral tegmental area and substantia nigra) with the exception of the lateral septal nuclei. β-galactosidase histochemistry for detection of EphB2 was performed in both naïve and morphine-treated (6 days) mice. For none of the above sites was a significant difference in the nature and/or extent of β-galactosidase staining observed between naïve and morphine treated animals.

3.6 Distribution of sensory neuronal sub-classes within the dorsal root ganglia and pattern of innervation

In order to determine whether developmental loss of EphB2 may alter the composition of sensory neuronal classes within the dorsal root ganglia, an assessment was made of the two major sensory subclasses related to pain in EphB2 null mice and controls, similar to work previously performed in the laboratory on ShcB and ShcC mutants[194]. Specifically, we examined the spatial patterning of peptidergic and non-peptidergic sensory neurons. Sensory subclasses were identified immunohistochemically based on their expression of the markers CGRP (peptidergic C fibers) or IB4 (non-peptidergic C fibers)[69, 195]. No major
Table 7. Binding affinity of $[^3H]$-naloxone to the superior colliculus. No
significant difference was found in the binding affinity between EphB2 +/+ (10.5
nM) and +/- (7.8 nM) mice in superior colliculus samples.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>$K_d$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EphB2 +/+ (n=4)</td>
<td>10.5</td>
</tr>
<tr>
<td>EphB2 -/- (n=3)</td>
<td>7.8</td>
</tr>
</tbody>
</table>
Figure 29. Histochemical analysis of EphB2 expression in the sensory nervous system. Observed patterns of beta-galactosidase staining were similar in naïve and morphine-treated adult EphB2 N2/+ mice. Prominent sites of EphB2 expression associated with sensory function are A. the dorsal root ganglia (DRG; L2-L5), B. dorsal laminate of the spinal cord (laminae I-III), C. the thalamus (TH), hypothalamus (HYP), amgdala (AMYG), D. periaqueductal gray (PAG), and E. anterior cingulate cortex (ACC). EphB2 is also present in the hippocampus (HIP).
differences in the staining intensity or distribution of CGRP or IB4 staining were observed in DRGs taken from L2-L5 spinal levels for EphB2 null mice versus controls (Figure 30). In order to verify that these populations remain similar between EphB2 null mice and control littermates controls following morphine treatment, the above assessment was repeated in animals following the completion of morphine treatment (6 days).

The natural distribution of EphB2 in the above DRG populations was assessed using EphB2 N2/+ mice, which demonstrated that EphB2 is broadly expressed across these populations of both peptidergic and non-peptidergic sensory neurons. While EphB2 showed colocalization with peptidergic (CGRP, substance P, p75 and the MOR) and non-peptidergic (IB4) markers, the relative level of EphB2 expression was not uniform in all sensory neurons (Figure 31). However this variance does not appear to be correlated to a particular DRG neuronal subclass as described above and was not further investigated.

3.7 Organization of spinal sensory inputs in EphB2 null mice and controls

In order to determine whether loss of EphB2 may alter the pattern of sensory innervation into ascending spinal tracts, the laminar pattern of spinal sensory inputs in EphB2 null mice and littermate controls was assessed. Similarly as described above, we examined markers of peptidergic (CGRP) and non-peptidergic C (IB4) fibers. EphB2 null mice exhibited a wild type-like staining pattern of CGRP+ (peptidergic pain fibers, laminae I and IIouter) and IB4+ (non-peptidergic pain fibers, lamina IIinner) axons (Figure 32).
Using EphB2 N2/+ mice as indicated above, the pattern of endogenous EphB2 expression was assessed in dorsal spinal lamina. EphB2 expression was observed within laminae I – III of the dorsal horn of the spinal cord and showed coincident expression with both peptidergic (CGRP, substance P and p75) and non-peptidergic (IB4) markers (Figures 33). In addition, we observed similar staining intensity and distribution of the MOR between wild type and EphB2 knockout paraffin spinal cord sections (T13; Figure 33E). Thus with respect to structural organization, EphB2 null mice appear to exhibit no significant perturbations with respect to either DRG sensory neuron class, or the pattern of sensory input to a sending spinal tracts within the dorsal horn of the spinal cord compared to control littermates.

3.8 Influence of higher learning on morphine induced tolerance

Given the absence of significant differences in intrinsic sensory responsiveness between EphB2 null mice and controls, the similar structural organization of their fundamental (DRG and spinal) sensory inputs, and their comparative responsiveness to morphine in the naïve state, we sought to determine whether the difference seen in EphB2 null mice versus controls following continued exposure to morphine was perhaps a function of some higher integrative aspect of sensory control. As this was unlikely to be simply a result of altered higher order sensory processing (in CNS loci such as the thalamus) due to their similar response to morphine in the naïve state, we began to examine (based on suggestions from Dr.
Figure 30. Immunohistochemical staining of CGRP and IB4 in the dorsal root ganglia (L2-L5). No significant difference in staining extent or intensity was observed between naïve and morphine-treated animals. For the figures shown: A. represents sections from a morphine-treated EphB2 +/+ mouse; B. morphine-treated EphB2 -/- mouse; and C. EphB2 N2/+ morphine-naïve animals.
Figure 31. Distribution of EphB2 in DRG neurons as a function of neuronal marker. A. EphB2 shares cellular localization with CGRP+ cells, B. substance P+ cells, C. IB4+ cells, and D. p75+ cells. E. EphB2 is highly expressed in some MOR+ cells, but displays lower levels of expression in others. Therefore the expression of EphB2 overlaps with markers of both peptidergic (CGRP, substance P, p75 and MOR) and non-peptidergic (IB4) primary afferent neurons. Scale bars represent 100 µm.
Figure 32. Immunohistochemical localization of CGRP and IB4 within the dorsal laminae of the spinal cord (T13-L1). CGRP was utilized as a marker of peptidergic primary afferent neurons, while and IB4 was utilized as a marker of non-peptidergic primary afferent neurons. No significant difference in staining extent or intensity was observed between naïve or opiate-treated morphine-treated EphB2 mice. For the figures shown: A. represents sections taken from a morphine-treated EphB2 +/+ mouse; B. morphine-treated EphB2 +/- mouse; and C. EphB2 N2/+ morphine-naïve animals
Figure 33. Analysis of EphB2 staining as a function of spinal cord laminar markers. As shown in panel A, in the adult spinal cord, EphB2 expression is observed in laminae I, II and III, overlapping regions of both A. IB4 (lamina II_{inner} marker), B. CGRP (laminae I and II_{outer} marker), and C. substance P (laminae I and II_{outer} marker), as well as D. p75 (laminae I and II_{outer}). E. MOR staining reveals similar pattern in wild type (left) and EphB2 -/- (right) mice. Scale bars represent a distance of 100 μm.
Derek van der Kooy) whether EphB2 null mice might not have an altered learned response to morphine. As indicated in the introduction, there is some prior experimental basis to suggest that at least a component of morphine-induced tolerance may be regulated through learning. In addition, prior investigations in the laboratory have demonstrated that EphB2 null mice exhibit a reduction in hippocampal LTP[32]. We therefore examined the potential of alterations in learning to influence the morphine induced responses seen in EphB2 null mice. As a first step in this process I verified for myself the experimental results previously described, in which morphine tolerated wild type animals exhibited an increase in morphine dependent analgesic response upon transfer to a novel environment (so-called anticipatory analgesia). As shown in Figure 34, consistent with previous results, tolerized wild type and heterozygous EphB2 mice do indeed show a significant increase in their morphine dependent analgesic response when removed to a novel environment. In accordance with prior studies, this is taken as evidence to indicate that wild type and heterozygous EphB2 mice are capable of constructing a form of associative connection between morphine and surrounding environmental cues which impinge upon nociceptive perception. In contrast, also shown in Figure 34, EphB2 null mice do not show such an anticipatory reversal of morphine dependent responsiveness when transferred to the same novel environment; suggesting that the above associations are impaired following loss of the EphB2 receptor tyrosine kinase.
3.9 Functional analysis of hippocampal learning – Passive avoidance

In order to assess the intrinsic functional aspects of hippocampal learning in EphB2 null mice and controls, several well-characterized assays examining different aspects of hippocampal dependent behavior were examine. In the first of these (passive avoidance) animals underwent single-pass training to an aversive stimulus with their response to this training assessed 24 hours later. Transfer latency time, the time it takes animals to freely transfer from the light to the dark chamber (see Figure 19), was assessed for EphB2 null mice and controls prior to and following single-pass training. As shown in Figure 35, wild type animals exhibited average transfer latency times of 56 ± 18 seconds in the acquisition phase. Similarly in the acquisition phase, EphB2 heterozygous and null animals exhibited transfer latency times of 39 ± 11 and 32 ± 6 seconds, respectively (see Figure 35). Thus no significant intrinsic differences were observed in the transfer latency times of mice for each of these groups into the dark chamber initially. Following single pass training, wildtype animals exhibited the expected increase in transfer latency times to 242 ± 28 seconds, while EphB2 heterozygote mice exhibited a time of 190 ± 30 seconds. In contrast, EphB2 null mice exhibit significantly lower transfer latency times into the dark chamber following single-pass training relative to EphB2 +/+ mice (138 ± 9 seconds). Thus in contrast to wild type and EphB2 heterozygous mice, EphB2 nulls appear deficient in their ability to learn this integrating task of contextual environmental hippocampal learning.
Figure 34. Context-dependent morphine response in EphB2 null mice.

Following morphine treatment as per the indicated schedule for 6 days, environmental context used for morphine injection was altered for half of the sample population on day 7. On that day, mice were injected and tested in either the same (SAME) or a novel (NOV) environmental setting. Similar to previous reports, EphB2 controls who received treatment in a novel experimental environment exhibited elevated levels of morphine induced analgesia (light blue) compared to those which underwent testing in the normal experimental environment (dark blue). In contrast, EphB2 null mice exhibit significantly lower levels of tolerance to morphine in both the novel and home environments (yellow and green, lines respectively). Error bars represent standard error of the mean. (*) p<0.05, novel env., EphB2 +/+ vs. EphB2 -/-; (#)p<0.05, same env., EphB2 +/+ vs. EphB2 -/-; ($)p<0.05, novel env., EphB2 +/+ vs. EphB2 +/-mice.
Day 7

![Graph showing tail pinch latency over time after morphine injection for different genotypes. The graph includes lines for EphB2 +/- SAME (n=7), EphB2 +/- NOV (n=7), EphB2 +/- SAME (n=8), EphB2 +/- NOV (n=8), EphB2 +/+] SAME (n=8), EphB2 +/+] NOV (n=9). Significant differences are indicated by symbols: $ for p < 0.05, # for p < 0.01.]
3.10 Specifying functional deficits in hippocampal learning - Object recognition

In order to further delineate the functional deficits present EphB2 null mice with respect to hippocampal-dependent tasks, the response of EphB2 null mice and controls were assessed in the object recognition task as described (see Methods and Figure 18). Interestingly, EphB2 null mice responded similar to control littermates, showing comparable levels of attendance to displaced and introduced novel objects (Figures 36B and 36C respectively). Similarly, no differences were observed with respect to the percentage of time attending objects in the second habituation phase (Figure 36A). These data suggest that EphB2 null mice have intact hippocampal function.
Figure 35. Passive avoidance in EphB2 +/+ , +/- and -/- mice. EphB2 +/+ and +/- animals exhibited shorter transfer latency times (TLTs) during acquisition, while scoring slower TLTs during the retention phase. EphB2 -/- mice also demonstrated fast transfer latency times during acquisition, but showed significantly faster transfer latency times during retention compared to controls. Error bars represent standard error of the mean. (*) p<0.05, EphB2 +/+ vs. EphB2 -/-.
Figure 36. **Object recognition in EphB2 +/+, +/- and -/- mice.** A. During habituation phase 2, mice spend approximately equal amounts of time attending to the three identical objects. B. In the displaced object phase, EphB2 null animals demonstrate greater attention to object 2 (displaced object), similar to wild type controls. C. Similar to the previous phase, EphB2 null mice spend more time attending to the novel object like controls. Error bars represent standard error of the mean.
B.

Displaced object

% of time attending to object

Object

EphB2 +/+ n=9
EphB2 +/- n=15
EphB2 -/- n=10
C.

**Novel object**

![Bar graph showing time spent attending to novel objects under different conditions.](image)

- EphB2 +/+ n=3
- EphB2 +/- n=13
- EphB2 -/- n=8

% of time attending to object

Object

1
2
3
CHAPTER 4: DISCUSSION
4.1 EphB2 null mice do not significantly differ from wildtype littermates with respect to a number of sensory and motor features

Prior to performing sensory investigations of morphine-treated EphB2 null mice and controls, it was necessary to first ascertain the intrinsic sensory and motor behaviour of these mice to determine if there was any underlying defect in basal function. With this in mind, we examined the performance of EphB2 mice in a variety of sensory and motor tasks. Based upon the aggregate results of these sensory (Von frey filament, tail pinch and tail flick tests) and motor (rotorod, hindlimb extension, edge performance, hindlimb grip response, grip strength, wire platform locomotion and 90° incline climb) tasks, it appears that EphB2 null mice exhibit little or no significant defects with respect to basic motor performance, and no difference in sensory behaviour, compared to control littermates. Similar findings are seen for EphB2 N2/N2 mice. Taken together these results indicate that EphB2 is not required either developmentally or in the adult state for proper execution of basic sensory and most motor functions. Previously, the laboratory has undertaken an extensive neuro-anatomic investigation of the role which EphB2 plays in regulating the target specific innervation of spinal motor neurons (Stephanie Ho, thesis). The results of these investigations demonstrate that while EphB2 does regulate some aspects of target-specific of motor innervation, the perturbations induced in EphB2 null mice do not lead to a significant modification of motor function (likely as a result of developmental adaptation).

In our current investigations, however, one aspect of motor behavior was observed to be altered in EphB2 null mice. Analysis of spontaneous open field
behavior demonstrated that EphB2 null mice exhibit an approximately 50% increase in total activity (distance traveled) during the one-hour test period. In contrast, EphB2 N2/N2 mice showed total activity measurements which did not significantly differ from that seen in EphB2 heterozygous or wildtype littermates. The absence of differences in baseline motor behavior in EphB2 null mice compared to controls suggests that this increase in motor activity may be a result of differences in higher-order cortical processing affecting motor performance. In this regard, spontaneous motor activity (in the absence of differences in baseline motor performance) has been suggested to represent a measure of exploratory behavior[196-198] or anxiety[199-201]. Thus a possible explanation for the heightened motor activity seen in EphB2 knockouts is that they may be more anxious, or exhibit greater exploratory behavior due to greater novelty seeking or an inability to habituate to their new environment. In this regard it should be noted that though EphB2 null mice consistently exhibit greater activity levels than control littermates, they do show the same general trend as these groups in that the initially high levels of exploratory activity upon introduction to a novel environment become reduced as time progresses. Given that EphB2 has previously been shown to regulate levels of active NMDA receptors within Schaffer collateral/CA1 connections in the hippocampus[32, 40], and that loss of EphB2 at this site results in impaired LTP strength and stability, it is possible that the observed effects may be tied to alterations in learning. Mechanistically, loss of EphB2 reduces surface expression and activity half-life of NMDA receptors, inducing a state of hypoactivity. Interestingly, genetic or pharmacologically induction of NMDA hypoactivity at
cortical and hippocampal sites has previously been associated with increased motor activity in several rodent models of schizophrenia[202]. In such cases the inability of higher cortical centers to properly “filter” important sensory information is thought to represent an important component underlying the observed elevations in motor activity.

Another potential cause of motor perturbation would be defects in axon guidance affecting dopaminergic tracts of the substantia nigra, pars compacta and ventral tegmental regions terminating in the striatum. Alterations in dopamine release within the striatum have been shown to induce hyperactivity in mice, for example over-expression of dopamine D2 receptors in the striatum[203]. Since EphB2 is developmentally expressed within dopaminergic neurons during formation of these tracks[31], this might at first glance appear to be a plausible option.

As no prior investigation of sensory function had been performed in EphB2 null mice, I proceeded to determine the baseline performance of these animals in response to an array of different sensory stimuli (nociceptive, mecanoceptive, thermoceptive). As indicated in the results, EphB2 null mice show no detectable alterations compared to control for any of the sensory assays examined. Consistent with this, anatomical examination of the major nociceptive classes of sensory neurons within dorsal root ganglia (ie. peptidergic and non-peptidergic C neurons/fibers) indicates no significant loss or displacement of any of these classes, and examination of the pattern of sensory innervation into ascending sensory tracts along the dorsal spinal laminations also indicate no anatomical perturbation.
Though it is not yet been thoroughly investigated, it remains possible (as observed in the motor system) that loss of EphB2 could developmentally alter fine aspects of sensory axon guidance at a level which does not result in functionally detectable perturbations in sensory performance in the adult state. Additionally, as previously described in many other regions of the nervous system, loss of EphB2 may be functionally compensated for by an array of functionally homologous family members expressed in these targets[38]. Irrespective of which of these ultimately proves to be the case, EphB2 null use appear to exhibit functionally normal responses with respect to the baseline sensory modalities examined.

4.2 EphB2 null mice exhibit an enhanced reduction of morphine-induced analgesia following time-dependent exposure to morphine

During twice-daily exposure to 10 mg/kg morphine over a period of six days (a protocol previously recognized to induce morphine tolerance), EphB2 null mice exhibit an accelerated pattern of morphine tolerance. Despite initially identical responses to morphine in naïve EphB2 null mice and controls, EphB2 knockouts exhibited significantly lower latency times with respect to tail pinch at latter time points (45 minutes) even upon first exposure to morphine. This pattern was exacerbated, being significantly different from controls when tested on injection days 3 and 6. EphB2 wild type, heterozygotes, and EphB2 N2/N2 mice showed the expected degree of tolerance development with continued exposure to morphine, however no significant differences were observed among these groups when compared to one another on test days 1, 3, or 6. Thus: (1) In both the tail flick and
tail pinch assays EphB2 heterozygotes behavior cannot be distinguished from that of wild type animals and; (2) The observed behavior does not require the endodomain of EphB2 as responses of EphB2 N2/N2 mice were not statistically different from that seen in wild type controls. Thus the altered responsiveness seen in EphB2 null mice must be a function of altered interactions between EphB2 and its cognate ligand via the ephrin reverse signaling. In attempting to clarify the mechanism by which EphB2 influences morphine tolerance, it is interesting to note that despite the day of treatment (1, 3 or 6), the intrinsic baseline sensory responses of EphB2 null mice prior to injection of morphine is equivalent to that seen in wild types controls. Such findings support the notion that loss of EphB2 acts in a rather acute manner to alter mu opioid-based analgesia rather than modifying some long-term aspect of the system. Indeed if the complete developmental loss of EphB2 induced a persistent long-term inhibition of mu opioid receptor function, then the effects observed would be expected to be more akin to that seen in Oprm null mice[141]. Instead intrinsic baseline sensory responsiveness appears unaltered in EphB2 null animals, with only the induced response to morphine affected, suggesting an alteration in the higher-order processing of morphine-related responsiveness (be it changes in mu opioid receptor signaling, or the integrated responses of to these inputs). To examine the validity of this concept further, studies were required which examine both the expression pattern and neuroanatomic nature of sensory components in EphB2 null mice (see below).

Though no one has previously examined the relationship between EphB2 and morphine tolerance, one prior study has investigated the role of EphB receptors in
opiate withdrawal. In a study performed by Liu et al., mice received seven escalating
doses of morphine (20, 40, 60, 80, 100, 100 mg/kg) i.p. every 8 hours, followed by
naloxone following the final dose in order to induce withdrawal. Though the
molecular mechanisms governing opiate withdrawal versus tolerance are thought to
be distinct, it is interesting to note that the investigators observed that intrathecal
infusion of EphB2-Fc, an ephrinB antagonist, significantly attenuated symptoms of
morphine withdrawal following of the administration of naloxone. The results
suggest that inhibiting productive association of one or more EphB2 receptors with
ephrinB ligands diminishes the extent of signaling involved in mediating symptoms
of opiate withdrawal. Specifically, intrathecal injection of EphB2-Fc was shown to
attenuate rises in CGRP, CREB, NR2B, c-Fos and ERK observed during chronic
morphine exposure[11]. In order to determine the identity of the appropriate
ephrin ligands involved the investigators examined the response of EphB1 knockout
mice. EphB1 has been shown to become elevated following chronic exposure to
morphine in the spinal cord. This group also claims that EphB1 knockout mice
exhibit diminished signs of naloxone-precipitated withdrawal (unpublished)[11].

With respect to this latter point, it is important to note as indicated above
that EphB-family receptors are highly homologous and advertised "EphB-family
specific" antiserum have a propensity to cross-react with closely related family
members. For example a number of prior claims have been made regarding
elevations of specific EphB receptors following certain forms of pain
stimulation[204, 205], none of which were found to be true during subsequent
follow-ups using with more precise reagents including EphB2 N2/+ knockin mice.
With respect to the pharmacologic use of Eph-Fc’s, is also important to note that due to ligand promiscuity, injection of a particular EphBx-Fc does not necessarily indicate that this entity represents the physiologic ligands regulating the phenomenon under study. For example, intrathecal pharmacologic application of EphB2-Fc would disrupt a number of Eph/ephrin B-family interactions far beyond those physiologically regulated by EphB2.

EphB receptors have also previously been investigated with respect to neuropathic pain. One study using intrathecal administration of EphB1-Fc, demonstrated that inhibition of ephrinB-family signaling inhibited Src-mediated phosphorylation of NR2B and prevented hyperalgesia and alldynia in the carrageenan induced model of nociceptive inflammation[206]. Similarly Kobyashi and colleagues showed through immunohistochemical means that ephrinB2 is up-regulated in the DRG following crush injury and that administration of an ephrinB2-specific siRNA reduces behavioral and mechanical alldynia in this model[205]. In addition, with respect to nociceptive signaling following chronic constriction of the sciatic nerve, the effects of EphB1-Fc and EphB2-Fc antagonist have been examined electrophysiologically with respect to neural excitability of small caliber DRG neurons (which project C fibers directly involved in mediating sensation of painful stimuli). These studies suggest that broad inhibition of EphB-family signaling reduced the induction of hyperexcitability (sensitization) of small DRG neurons, as well as reducing levels of long-term potentiation induced at synapses between C fibers and postsynaptic dorsal horn neurons in the spinal cord. Consistent with this, studies have found that the administration of EphB1-Fc prior to chronic constriction
of the sciatic nerve prevents the induction long-term potentiation at the above synapse, suggesting that in some manner EphB-family signaling plays a role in regulating chronic responses to painful stimuli. Similar to the other injury models described above, chronic constriction of the sciatic nerve was reported to induce a rise in ephrinB1 and EphB1 following injury as determined by Western blotting[204].

Taken together, the above studies indicate that EphB-family signaling plays a role in mediating several aspects of nociceptive sensory perception. In the majority of cases the specific ligand/receptor interactions which physiologically regulate these events have not yet been determined. With respect to downstream signaling, at present several interesting correlations to specific signaling pathways have been observed. However the role of these in regulating EphB-mediated nociceptive signaling have yet to be definitively established in the majority of cases. In our studies of mu opioid receptor signaling, loss of EphB2 does not appear to alter the intrinsic state of mu opioid receptor responsiveness, as shown by similar baseline responses in morphine naïve animals compared controls. However the loss of EphB2 appears to interfere with some aspect of the temporal changes induced following chronic exposure morphine. To better understand the mechanism of this effect it was therefore first necessary to determine several basic aspects of mu opioid receptor function in EphB2 null mice.
4.3 Mu opioid receptor levels *in vivo* do not significantly differ between EphB2 mutants and control littermate at several critical CNS sites

Western analyses were performed using sub-dissected CNS loci and tissues from EphB2 null mutants and controls. Based upon the conditions utilized to perform these analyses, and the intrinsic stability of the mu opioid receptor complex itself, both the biologically active (dimeric) and associated (monomeric) forms of the opioid receptors were observed, consistent with previous observations described below. The similarity in the levels of *total* MOR observed in the spinal cord between EphB2 mutants and controls indicates that loss of EphB2 *per se* does not alter expression or stability of MOR within the mammalian central nervous system. However it should be noted that due to the nature by which these assays were performed (analysis of whole tissue extracts), they do not address the relative level of MOR receptors at the cell membrane versus interior cellular compartments.

Further analyses in the above tissues examining membrane versus cytoplasmic preparations would be required to shed light upon such issues. As both internalized (endosome) and surface (plasma membrane) MORs would be present within membrane preparations, Western analyses of a membrane preparation would not be reflective of the true ratio of internalized to surface MORs. Instead, a biotinylation assay of cells transfected with both MOR and EphB2 constructs would allow us to properly determine this ratio. I have performed some preliminary investigations examining the amounts of functional MORs, which are currently underway. As such, the Western analyses only examine levels of total MOR protein, do not address issues such as relative functional activity MORs in different murine
lines (see functional activity below) or specific patterns of protein phosphorylation (see future studies).

With respect to molecular analysis of MORs, historically there has been significant confusion with respect to proper identification of the monomer species in vivo. While several groups have reported monomer being in the range of 66-79 kD[207-210], others stated have stated molecular weights as low as 44 kD[126] or 51 kD[211]; significantly below the predicted molecular weight of 53 kD for the Oprm gene product[112]. Garzon and colleagues, have described bands from tissue extracts molecular weights of 43, 51 and 58 kD, which they ascribe to different mu splice variants[212]. While at least 25 different mRNA splice variants have been identified in mouse and 11 MOR splice variants in humans[107], the molecular weights of these different variants has not been determined as these different mRNA splice forms have been discovered primarily through RNA technologies. To speculate, one might expect differences in molecular weights, but not at the magnitude of discrepancies given in the literature. In our hands, using several different commercial MOR antisera did indeed result in the identification of protein products in the range of those described above. Thus descriptions of the alternative molecular weight species described above should be viewed with caution, and may represent closely related structural species. Indeed, even the MOR antisera which we ultimately chose to utilize for the present studies exhibited a band of ~43 kDa in a variety of tissue extracts examined. Without a standard in the field to follow, we chose to focus our attention on bands consistent with the predicted size of the monomer protein[112] and with one study which observed an MOR band at 102
kD[213], which they believed to be a dimer. Based upon the results obtained, while it remains possible that MORs in EphB2 null mice may exhibit an altered subcellular localization compared to that seen in control littermates, total levels of MOR protein in EphB2 null mutants appear unaltered compared to their cognate wildtypes.

4.4 In the adult nervous system EphB2 exhibits cellular co-localization with the mu opioid receptor at several CNS sites involved in processing sensory input

Our laboratory and others have demonstrated the identify and presence of EphB2 at a number of CNS sites also known to express the MOR (Figure 37). EphB2 N2/+ mice were used as a tool to specifically and unequivocally probe for the presence of EphB2 protein (the close structural homology of EphB2 to other Eph family receptors precludes the use of any currently available antisera - despite the fact that several "EphB2-specific" antisera are commercially advertised). EphB2 expression was examined at these sites which included the dorsal root ganglion, dorsal horn of the spinal cord, periaqueductal gray, thalamus, hypothalamus, somatosensory cortex and anterior cingulate cortex in naïve and morphine treated animals (Figure 29). Despite the fact that EphB2 is known to be expressed in the adult state at such sites for more than a decade, no detailed analysis nor specific function for EphB2 at these sites has been previously ascribed.

Examination of EphB2 in the dorsal root ganglia and spinal cord, two sites which have been consistently implicated in Eph-mediated control of nociceptive stimuli, demonstrated that EphB2 was present in laminae I through III of the dorsal
Figure 37. Expressions of EphB2 and the MOR in the ascending and descending nociceptive pathways. Pathways in purple are part of the ascending sensory pathways and spinothalamic tract. Inhibitory (green), excitatory (red) and serotonergic (yellow) descending pathways are also shown. We have observed that EphB2 is expressed in many of the same areas as the MOR in regions such as the dorsal root ganglia (DRG), dorsal horn of the spinal cord, periaqueductal gray (PAG) matter, thalamus, hypothalamus and somatosensory and anterior cingulate cortices.
horn of the spinal cord was robustly expressed in a number of primary afferent neurons in the dorsal root ganglia. Within the spinal cord, analysis of several well-characterized laminal markers of dorsal horn layers (CGRP, substance P and p75 mark laminae I and II\textsubscript{outer}; IB4 marks lamina II\textsubscript{inner}) indicated no disruption or modification of these boundaries in EphB2 null mice compared to wildtype controls. For dorsal root ganglia from lumbar segments, expression of EphB2 was also seen across each of the two major recognized subclasses of nociceptive neurons at this locus (peptidergic and non-peptidergic). Consistent with findings observed in the spinal cord, no anatomic perturbation of neuronal subclasses was observed in EphB2 null mice compared controls, with similar neural numbers and expression levels seen for each of the markers utilized (CGRP, substance P, p75 and the MOR mark peptidergic nociceptors; IB4 marks non-peptidergic nociceptors). Thus within the dorsal root ganglia and spinal cord, EphB2 appears to be broadly and persistently expressed in the adult state; both within those neurons which express MOR and those which do not.

The cellular co-expression of EphB2 and MOR suggests the possibility that EphB2 may directly modulate MOR signaling complexes in order to regulate time-dependent aspects of morphine exposure. Alternatively, this interaction might be an indirect one, mediated through either additional protein-protein interactions or through modulation at a completely disparate CNS site. In cases where the EphB2 receptor has been shown to modulate the action of other CNS receptors[19, 20, 32, 40] indirectly through other protein mediators. As explained in the Introduction (“Eph structure and signaling”) and illustrated in Figure 4, Eph receptors can both
activate or inactivate integrins via several independent signaling pathways. Activation of integrins can occur through Eph-mediated activation of Nck/NIK or LMW-PTP, while inactivation can occur via Eph-mediated activation of SHEP-1/R-Ras/R-Raf1 or FAK[19, 20]. In another example of receptor modulation, EphB2 is known to regulate surface expression of NMDA receptors in the hippocampus[32, 40]. In our current studies this also appears to be the case in that (based upon observations obtained using EphB2 N2/N2 mice) these interactions occur through ephrinB mediated mechanisms. In this regard ephrinBs have been reported to regulate other CNS receptors in vivo[19, 26]. As described in the introduction, ephrinBs promote the recoupling of G proteins to the CXC4R receptor in the guidance of cerebellar granular axons (Figure 6)[26]. This illustrates that ephrinBs have the potential to modulate GPCRs, such as the MOR. Similar to Eph receptors, ephrins can lead to activation of integrins through activation of SFKs, ultimately affecting cellular adhesion or repulsion (Figure 5)[19].

In order to determine the intrinsic functional capacity mu opioid receptors in EphB2 null mice, I next examined several functional aspects of these receptors in vivo.

### 4.5 MOR functional activity is not altered in EphB2 null mutants

As a first step towards understanding the functional capabilities of new opiate receptors in EphB2 null mice I examined properties of [3H]-naloxone binding in tissue extracts taken from EphB2 null mice and littermate controls. Analysis of superior colliculus lysates incubated with different concentrations of tritiated
naloxone and a given concentration of cold naltrexone revealed that the affinity constant of naloxone for the MOR is similar between EphB2 knockouts and wild types. These results demonstrate that the innate capacity of mu opioid receptors to bind ligand is not impaired or enhanced in EphB2 null mice; at least for the tissue investigated. The determined Kd in tissue extracts from EphB2 null mice and controls was consistent with values previously reported for primary brain preparations[192]. It should be noted that the values determined were almost an order of magnitude greater than that observed for purified MOR preparations isolated from engineered cell cultures, likely due to the lower relative levels of MOR higher nonspecific binding seen in such preparations[55, 179, 193]. Despite comparable results in spinal cord preparations, the superior colliculus was chosen as a major target of in vivo analysis due to its enrichment in both MOR[214] and EphB2. Additional [3H]-naloxone binding assays are being performed using purified membrane preparations. Taken together these findings are consistent with those observed from in the functional assays, in which EphB2 null mice exhibit no intrinsic difference in baseline performance compared to littermate controls regarding MOR function. Rather only the time-dependent aspects of MOR signaling appear to be altered in these animals. To further understand the functional mechanisms of this MOR response I chose to examine a known element of EphB2 signaling which might impinge upon the state-dependent behavior of MOR signaling: context dependent learning. This approach was investigated rather than pursuing targeted inhibition of potential signal transduction pathways at individual neural loci in vivo (which likely would first require definitive identification of the
ephrinB target(s) of interest) in order to gain insight into the general manner by which EphB2 might influence MOR time-dependent MOR function.

4.6 EphB2 null mice do not exhibit an enhancement in analgesia following morphine exposure in a novel environment

As indicated above, while direct biochemical interactions between EphB2/ephrins and the mu opioid receptor may underlie the observed accelerated morphine tolerance, there is also evidence to suggest that such behavior may be influenced by context dependent learning directed from higher-order brain centers. The idea that the extent of morphine-dependent nociceptive analgesia can be modulated through learning pathways was first suggested almost 40 years ago by Shep Siegel[215, 216]. Siegel and colleagues initially demonstrated this phenomenon by pairing by the stimulus (morphine) with specific contextual cues; ultimately demonstrating that this resulted in an anticipatory modification of sensory response. That the altered sensory behavior (manifest by the extent of sensory response to a nociceptive stimulus following morphine injection) was indeed dependent to some degree upon contextual cues was demonstrated by performing the identical experimental in two slightly different contexts. Animals were treated with morphine (5 mg/kg) for a series of four injections separated by 48 hour intervals and were testing using the hot-plate assay, whereby animals are placed on a hot plate (54°C) and the latency to lick their paws is used as a measure of analgesia. Four groups of animals were used in this experiment: 1) M-HP: animals that received morphine for all four injections in the same environment and were tested on the hot plate following each injection;
2) M-CP: animals that received morphine for all four injections in the same environment but for which the hot plate was only turned on following the fourth injection (to prevent the animal from being accustomed to paw licking, the experimental endpoint); 3) M-CAGE: animals that were injected in a different environment on session 4 than on the previous three trials, but were only tested on the hot-plate during the final session; 4) S: animals which received saline in the same environment for all four injections and were tested on the hot plate following each injection. Siegel observed that while M-HP and M-CP groups exhibited a tolerated response to morphine in the fourth and final session, the M-CAGE group showed significantly higher paw lick latency times, exhibiting a more naïve-like response to the morphine[147]. The results obtained (which in subsequent years have been replicated) demonstrated that when placed in a novel environment the extent of apparent analgesic response was enhanced, indicating that there exists a contextual component to the regulation of morphine dependent analgesia. With this in mind we sought to examine whether EphB2 null mice, which exhibit a reduced capacity for hippocampal long-term potentiation, might exhibit a modified response with respect to morphine dependent contextual learning. Initial analyses aimed at replicating the basic observation made by Siegel and colleagues using wild type mice were successful; and these were followed by analysis of EphB2 null mice and controls. As discussed in the results we observed that EphB2 null mice, unlike their wild type littermates, are unable to perform this context-dependent modification of morphine-dependent sensory analgesia. Given that prior electrophysiologic investigations which established impairment in EphB2 null mutants in NMDA-
dependent LTP response at Schaffer collateral/CA1 synapses, we sought to examine the functional features of hippocampal-dependent contextual memory in these animals.

4.7 EphB2 null mutants exhibit deficits in hippocampal-dependent learning

To assess contextual memory in EphB2 null mice, animals were examined into well-characterized (hippocampal-dependent) tasks: passive avoidance and novel object recognition. The passive avoidance task tests an animals’ ability to pair a specific contextual cue (in this case a darkened chamber) with an aversive stimulus (foot shock). As expected EphB2 control littermates performed well in this task, demonstrating significantly longer transfer latency times into the darkened chamber following training compared to that seen during the pre-training session. In contrast, EphB2 null mutants did not exhibit as great of an increase in post-training transfer latency times as seen for control littermates. Thus it appears that EphB2 null mutants are impaired with respect to this form of hippocampal-dependent contextual memory task. However EphB2 null mutants do not appear to be impaired with respect to all forms of hippocampal learning. Mice lacking EphB2 exhibited responses similar to that seen for littermate controls in the object recognition task. Though on its face the findings observed for these two hippocampal-dependent tasks appear somewhat contradictory, prior lesion studies targeting specific regions of the hippocampus have suggested that successful performance of the passive avoidance task requires coordinated signaling from CA1/CA3 circuits[217], while the novel object recognition task is more dependent upon CA3/dentate gyrus signaling[218]. The functional findings obtained are
therefore not inconsistent with prior electrophysiologic studies performed in hippocampal slices of EphB2 mutants which demonstrated diminished CA1 LTP[32]. Taken together, the above findings suggest that (1) a temporal component of morphine-dependent analgesia is tied to contextual learning, (2) that EphB2 null mutants lack this ability in response to morphine, and (3) that EphB2 mutants are also deficient in independent tests of context-dependent hippocampal memory, which are reliant upon CA1 signaling. CA1 hippocampal circuitry in EphB2 null mice has previously been shown through electrophysiologic investigations to exhibit deficiencies with respect to NMDA-dependent LTP. Finally, EphB2 null mutants, which exhibit no intrinsic motor deficiencies compared to control littermates, exhibit significantly elevated levels exploratory activity when placed in a novel open field environment. Numerous studies have previously identified the hippocampus as one of the key integrating sites for association of sensory and spatial information[219, 220]. The most parsimonious explanation for the results obtained is that a hippocampal-dependent disruption in the association between specific sensory stimuli and other contextual cues underlies the observed deficit morphine-dependent behavior seen in EphB2 null mutants. While such a finding is consistent with results obtained, the evidence as presented is by no means definitive. Other potential explanations still exist to explain the aberrant morphine-dependent behavior seen in EphB2 null mutants. These include, direct ephrin-mediated modification of MOR receptor desensitization and/or downstream signaling, the induction of altered morphine metabolism in EphB2 null mutants following chronic morphine treatment, and non-hippocampal alterations in contextual memory. With
respect to the above, experiments can be designed to address the validity of each of these alternatives (see future studies below). Clearly one means by which the “EphB2-mediated disruption of CA1 hippocampal signaling altering morphine tolerance” idea could be tested would be to specifically impair EphB2 signaling within the hippocampus of adult wildtype mice. Prior to undertaking the challenging experimental task of bilaterally inhibiting EphB2 function within the hippocampus, however, it would first be advisable to firmly establish that this neural locus was indeed the site principally responsible for the observed modification of morphine signaling seen in EphB2 null mice (as other possibilities could potentially exist). With this in mind, I attempted to perform this task by permanently inhibiting hippocampal function bilaterally through the use of kainic acid (please refer to the Appendix). Students in the laboratory have previously utilized this method to perform unilateral chemical lesions of hippocampal CA1-3 pyramidal neurons (Anish Kanungo, Henderson laboratory, Ph.D. thesis 2009). However attempts to replicate this feat bilaterally were met with a number of difficulties due to more extensive nature of the network affected. Subsequent efforts to reduce levels of neural excitation using both GABA_B (and GABA_A) agonists were made, however neither ultimately proved acceptable with respect to reducing mortality. Experiments performed subsequently in the laboratory have focused on inhibiting hippocampal function through bilateral electrolytic lesions and through pharmacologic inhibition of neural excitation. A summary of my procedures performed in this regard is given in the Appendix Table 8. It is hoped that experiments currently being performed in the laboratory will soon clarify whether
the hippocampus is indeed centrally involved in the EphB2-dependent events involved in regulating the observed modification of morphine dependent signaling.

4.8 Concluding remarks/future studies

Analysis of morphine metabolism in EphB2 null mice: Current studies have demonstrated that the intrinsic responsiveness of EphB2 null mice versus littermate controls is similar. However it is possible that continued exposure to morphine may induce changes in morphine metabolism. As some glucuronidated morphine metabolites (M6G) are themselves biologically active, such a change in P450 (CYP) mediated metabolism could potentially mediate some of the changes seen in EphB2 null mice. Arguing against this is the fact that primary sites of morphine metabolism such as the liver never express EphB2 during either developmental or adulthood. Nevertheless some degree of morphine metabolism occurs locally within the CNS, and in either event, potential differences in morphine metabolism in EphB2 null versus controls mice should be definitively determined. Therefore the investigation of morphine and its major metabolites as a function of time, both systemically (i.e. as measured in blood plasma) and locally (i.e. CNS as measured in brain parenchyma and cerebrospinal fluid), would be useful in this regard.

Anatomic localization of morphine dependent learned tolerance effects: The data presented in the current thesis demonstrates for the first time that EphB2 null mice exhibit a morphine specific defect with respect to mechanisms of tolerance, and provides evidence to suggest that a significant component of this effect may be influenced by the learned behaviors of the nociceptive response. However, the
information as presented does not definitively identify a specific locus for these EphB2 mediated effects (though suggests it may be acting centrally). As a first step toward establishing the locus of such effects, neural inhibition of anatomical sites of interest such as the hippocampus could be undertaken. During the course of my thesis, initial steps to perform such bilateral inhibitions of the CA1 hippocampal network chemically using kainic acid were undertaken (see Appendix). However this route of inhibition was later abandoned for technical reasons. Current efforts in the laboratory have focused upon alternative methods to reversibly and irreversibly inhibit hippocampal signaling.

*Definitive established locus of EphB2 morphine dependent effect:* Data presented in the current thesis establish that EphB2 is causally involved in influencing aspects of morphine dependent tolerance. However the specific neural network(s) which EphB2 influences have not presently been identified. Once the neural loci regulating the described morphine-dependent tolerance effects have been identified (likely through local nonspecific inhibition of neural signaling), inhibition of EphB2 (through siRNA or ephrinB1-Fc) at this site could definitively establish the role of this receptor tyrosine kinase in mediating the effects described in the current study.

*Pharmacologic modulation of the MOR by EphB2/ephrinBs:* To further clarify the nature of EphB2 – MOR interactions, immunoprecipitation approaches could be employed using either EphB2, or more likely cognate EphB2 ligands to ascertain whether the MOR can be pulled down in tissue lysates. Beyond this, identifying the pattern of MOR receptor desensitization and internalization in EphB2 null mice versus controls may also be useful in understanding mechanistic interactions.
between EphB2 and the MOR. It would also be of interest to examine the specific
nature of MOR phosphorylation status the presence and absence of EphB2, and the
kinases involved. As PKC is known to induce greater desensitization of the MOR and
reduce receptor recycling relative to β-arrestin (Johnson 2006), differential
phosphorylation of the MOR could account for differences in the internalization
kinetics.

The present studies describe for the first time a novel role for the EphB2
receptor in regulating morphine tolerance. While further studies will be required in
order to more definitively localize the CNS features principally responsible for
meeting these EphB2 dependent effects, it is clear that the receptor tyrosine kinase
EphB2 plays a unique and substantial role in morphine tolerance. Indeed it is
remarkable that given the considerable functional overlap which exists among
various Eph-family receptors that a single such member should exert such powerful
influence. Though regulation of morphine responsiveness is a well-studied aspect of
both molecular pharmacology and neuroscience, the present findings highlight how
incomplete our understanding opiate mediated signaling remains. Much still
remains to be learned. Further understanding of the molecular mechanisms by
which EphB2 regulates morphine tolerance may one day lead to the development of
new therapies to modify the tolerizing aspects of this important opiate, and lead to a
greater understanding of the role which contextual learning may play in the
modification of nociceptive response.
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KAINIC ACID-INDUCED ABLATION OF THE DORSAL HIPPOCAMPUS

Hippocampal surgery

In an attempt to determine the nature of the cognitive defects seen in EphB2 null mice, microinjection of the excitotoxin kainic acid into the dorsal hippocampus was performed based upon stereotactic coordinates define from the three-dimensional MRI atlas previously developed in the laboratory[221]. Mice were anesthetized with fresh 2.5% Avertin (0.2 ml/kg), placed into stereotactic frame and cannulae introduced bilaterally at a point midway between lamda and bregma suture intersections, 1.7 mm lateral of the sagittal suture at a depth of 1.15 mm below the dura.

Three hundred nanoliters of 20 mM kainic acid (Tocris Biosciences) was then delivered over three minutes prior to removal of the cannula (6 nmoles per hippocampi). Mice were sutured and allowed to cover for 48 hours. To prevent seizurogenic activity, mice were given baclofen (20 mg/kg total) at different time points before and after surgery. Details are explained in the Appendix and summarized in Table 8.

These studies consisted of three basic experimental groups, wildtype animals, sham operated controls and kainic acid treated mice. Mice were allowed to recover 48 hours prior to analysis of hippocampal function using the passive avoidance assay. Mice were then perfused transcardially with 4% PFA, and 7 µm brain sections prepared throughout the region of injury in order to retroactively determine the position and extent of the lesion induced.
Bilateral inhibition of hippocampal function – chemical destruction of CA1 hippocampal neurons using kainic acid

Using standard procedures for unilateral hippocampal lesions previously established in our lab, adult mice were given 200-400 nL of kainic acid (20 mM) per hippocampi. Analysis of these results demonstrated that under these conditions a dose of 300 nL kainic acid infused over a period of 3 minutes lead to the most reproducible lesions within CA1 neurons. However due to elevated levels of postoperative deaths (which normally never occurs in our CNS procedures), this protocol was modified such that kainic acid was given in conjunction with of GABA$_B$ agonist baclofen (20 mg/kg) given intraperitoneally just prior to surgery or both before and after surgery to limit the global extent of induced excitatory stimulation (such pharmacologic manipulations do not locally impede the rate or level of CA1-3 neuronal degeneration). Despite these efforts, residual self excitatory effects manifesting in lethality due to interruption respiratory or cardiac rhythm led to an abandonment of this procedure as a means to bilaterally inhibit hippocampal function. Table 8 summarizes the modifications made in our attempt to induce cell death in the dorsal hippocampus and the outcomes. Subsequent efforts in laboratory have devised new methods in which to pursue these investigations.
Table 8. Summary of approaches taken to induce hippocampal ablation using kainic acid (KA). Several strategies were employed to induce local interruption of hippocampal circuits within the dorsal hippocampus. Initial attempts using 200 nL/hippocampus of 20 mM KA did not induce extensive enough cell death within the dorsal hippocampus and thus higher doses were employed. However, successful bilateral lesions of the dorsal hippocampus were found to be associated with a significant increase in mortality. Due to the epileptogenic potential of kainic acid-induced lesions, attempts were made to reduce excitatory discharges using GABAB agonist baclofen. Baclofen showed limited protection, but did not inhibit lesion formation if administered in two i.p. 10 mg/kg injections (one prior to surgery and the other one hour post-op).
<table>
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<th>Amount of KA/hippocampus</th>
<th>Baclofen dosage</th>
<th>Mortality</th>
<th>Mice exhibiting full bilateral lesion</th>
<th>Condition of surviving mice</th>
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<tr>
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<td>200 nL</td>
<td>-</td>
<td>2/2</td>
<td>-</td>
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<tr>
<td>3</td>
<td>400 nL</td>
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<td>1/3</td>
<td>Poor</td>
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<tr>
<td>4</td>
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<td>20 mg/kg i.p. one hour post-surgery</td>
<td>5/5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>300 nL</td>
<td>One dose, 20 mg/kg i.p., prior to surgery OR Two doses, 10 mg/kg i.p. prior to surgery and one hour post-surgery</td>
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<td>0/1</td>
<td>Good</td>
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<tr>
<td>6</td>
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siRNA-MEDIATED SUPPRESSION OF EPHB2 IN THE DORSAL HIPPOCAMPUS

Hippocampal surgeries

Surgeries were performed by the same protocol described above except using EphB2-specific siRNA in place of kainic acid. Four different siRNAs targeting EphB2 receptor mRNA were tested in EphB2 N2/+ animals (siRNA-B2-1: GGCAAGAUGUACUUCCAAA; siRNA-B2-2: CGGACAAGCUACAACACUA; siRNA-B2-3: GUUCGUCAUUGCCAUCGU; siRNA-B2-4: GCAGUACACCUCUGAGAUC). The siRNA (ThermoFisher Scientific), mixed with Dharmafect transfection reagent, (300 nL total) 30 minutes prior to the surgery, was injected at a concentration of 183.5 μM into the right hippocampus, such that the effective concentration within the hippocampus following dispersion of the siRNA would be 5 μM. The left hippocampus remained untreated as an internal control. Mice were allowed four days to recover from surgery, at which point they were perfused with 4% PFA and brain tissue was extracted. Frozen sections (30 um) were incubated with polyclonal rabbit LacZ antibody (1:100, MP Biomedical) to determine extent of inhibition of EphB2 expression. A DAPI stain was also performed to determine ensure any reduction in EphB2 was due to decreased expression instead of cell death.

siRNA inhibition of EphB2 expression in the dorsal hippocampus

To discern if the absence of EphB2 in the hippocampus was responsible for the disruption in learned tolerance, we attempted to suppress EphB2 expression with
siRNA in the hippocampus of wild type CD1 mice. Inibition of EphB2 expression was attempted *in vivo* in the hippocampus using microinjection of four separate siRNAs. We determined that injection of either of the four alone did not cause any major reduction in EphB2 expression in the dorsal hippocampus, as determined by *lacZ* immunohistochemistry (FIG). Further parameterization of the procedure will be required in order to determine if EphB2 specifically in the hippocampus is critical for morphine-dependent learning.
Figure 37. siRNA suppression of EphB2 in the dorsal hippocampus. Four different EphB2-specific siRNA oligonucleotides were injected into the right dorsal hippocampus of EphB2 N2/+ mice (A-D). The left hippocampus served as a reference control. Three days after surgery, mice were sacrificed and brains removed prior to staining for beta-galactosidase to determine the relative extent of EphB2 protein suppression. For each set of figures, the control hippocampus is shown to the left while the EphB2 siRNA treatment is shown on the right. Within the limits of detection, no significant differences in EphB2 protein expression levels were observed using these treatments within the hippocampus.
B. Control  EphB2-specific siRNA

LacZ

DAPI

Merged
C.  

Control  EphB2-specific siRNA

LacZ

DAPI

Merged