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Neuromodulatory Effect of Kappa Opioid Receptor Activation on Spinal Network Activity

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Neuromodulatory Effect of Kappa Opioid Receptor Activation on Spinal Network Activity

by

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Abstract

Dynorphin is a potent anti-nociceptive neuropeptide ubiquitously expressed throughout the peripheral and central nervous system. Previous studies have shown that dynorphin is co-packaged and co-released with orexin, a pro-locomotory neuropeptide known to be involved in goal-directed movement. Why then is an inhibitory neuropeptide co-released with an excitatory pro-locomotory neuropeptide orexin, and what is its role?

In this thesis the expression of kappa opioid receptors (KOR) on motoneurons was demonstrated using RNAscope Assay. Consistent with the observation of other studies, my results show that KOR activation significantly reduced spontaneous network activity, increased motoneuron excitability, and increased presynaptic inhibition.

These results suggest that dynorphin modulates the spinal motor network rather than inhibits it. This also explains why the co-release of orexin and dynorphin does not have an antagonistic effect. My work suggests that perinatally dynorphin can have potent effects on spinal cord networks. This raises the possibility that dynorphin release may contribute to the development and correct function of spinal cord motor networks.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
aCSF	Artificial cerebrospinal fluid
AP-5	Amino-5-phosphonovaleric acid
Ca^{2+}	Calcium ion
CPG	Central pattern generator
DNQX	6,7-dinitroquinoxaline-2,3-dione
DYN	Dynorphin
GABA	Gamma amino butyric acid
GPCR	G-protein coupled receptor
G-protein	Guanine nucleotide-binding protein
IR^{+}	Immunoreactive
KOR	Kappa opioid receptor
NMDA	N-methyl-D-aspartate
nor-BNI	Norbinaltophimine
SCI	Spinal cord injury

Chapter One

Chapter One: Introduction

1.1. General Introduction

Locomotion is arguably one of the essential characteristics of living organisms necessary for adaptation and survival. The elegance of unimpeded locomotion is often not sufficiently appreciated until impaired or lost.

The importance of neuromodulators in sculpting spinal network activity and locomotor output cannot be overemphasized. This is evidenced by the several ongoing research studies aimed at elucidating their role in shaping spinal locomotor activities both at the cellular and network level (Schwartz *et al.,* 2005; Dunbar *et al.*, 2010; Humphreys & Whelan, 2012; Sharples *et al.*, 2015). Dynorphin is a potent neuromodulator whose role in modulating spinal network activity has thus far been poorly investigated. Although work from the 1980's suggested a dynamic effect of dynorphin on muscle activity (Faden & Jacobs, 1983; Stevens *et al.,* 1987; Bakshi *et al.*, 1992a), it was not until recently that the role of dynorphin in modulating locomotor output was considered, revealing that dynorphin was co-localized with a pro-locomotory neuropeptide, orexin (Hagan *et al.*, 1999; Ida *et al.*, 1999; Nakamura *et al.*, 2000; Duxon *et al.*, 2001; Matsuzaki *et al.*, 2002; Stone *et al.*, 2005; Arendt *et al.,* 2013; Inutsuka *et al.,* 2014; Li *et al.*, 2014). Orexin, a neuropeptide expressed and released by a class of neurons exclusively present in the lateral hypothalamus has been shown to promote goal-directed locomotion. Since about 94% of orexinergic neurons also co-release the neuropeptide dynorphin (Chou *et al.,* 2001), the question then is: does dynorphin itself modulate spinal motor network activity?

Several lines of evidence suggest that dynorphin has a potent antinociceptive role within the spinal cord. Dynorphin is known to bind to Kappa opioid receptors, which are present both pre- and post-

synaptically and are G-protein coupled (Bruijnzeel, 2009; Jüngling *et al.*, 2016). Kappa opioid receptors are of the $G_{i/o}$ class of GPCRs and are known to be inhibitory upon activation (Attali *et al.*, 1989; Konkoy & Childers, 1989; Prather *et al.*, 1995; Bruijnzeel, 2009; Al-Hasani & Bruchas, 2011). Since kappa opioid receptors are present both presynaptically and postsynaptically, their dynorphin-mediated activation results in the reduction in neurotransmission across synapses (Galligan & Akbarali, 2014). Dynorphin, therefore, mediates its well-known analgesic effect by reducing neurotransmission onto the spinothalamic pathway, a pathway necessary for conducting nociceptive neural inputs (Willis, 1991; Martelli *et al.*, 2010). Studies into the role of dynorphin in modulating motor output started in the 1980's (Faden & Jacobs, 1983; Stevens *et al.*, 1987; Podvin *et al.*, 2016). These studies involved the intrathecal application of dynorphin followed by electromyographical recordings of changes in muscle activity and accompanying motor dysfunction defined as long-lasting paresis. Until my thesis study, there have been no prior investigations yielding mechanistic insight into the network and cellular effects of dynorphin in modulating spinal motor function.

To address this knowledge gap, I will address three main questions: i). Do motoneurons express kappa opioid receptors? ii). What is the effect of activating KOR on spinal spontaneous activity? iii). Does KOR exert direct effects on the intrinsic membrane properties of motoneurons? My data suggests that kappa opioid receptors are present on motoneurons and extracellular recordings on isolated *en bloc* spinal cord preparations indicates that dynorphin inhibits spontaneous activity. Dorsal root potential (DRP) recordings, which give an index of presynaptic inhibition, shows that there is an increase in presynaptic inhibition. Conversely, whole-cell patch clamp data indicates that dynorphin modulates the intrinsic membrane properties of motoneurons to increase their overall excitability.

1.2. Background

Generally, locomotion is an output of the coordinated activities of spinal neural circuits that act as "central pattern generators" (CPGs) (Marder & Bucher, 2001; Goulding, 2009; Dunbar *et al.*, 2010). CPGs can intrinsically generate a rhythmic output to drive flexor and extensor muscles to control limb movement without phasic supraspinal or sensory inputs (Brown, 1911; Grillner, 1973; Forssberg *et al.*, 1980; Grillner & Zangger, 1984). Propriospinal inputs mostly act to initiate and modulate locomotor activities within the spinal cord. Descending control of locomotion from brain areas such as the motor cortex, brainstem, and lateral hypothalamus is mediated by the release of neurotransmitters such as monoamines (dopamine, serotonin), glutamate, and neuropeptides such as orexin (Kim *et al.*, 2017; Noga *et al.*, 2017; Jean-Xavier & Perreault, 2018; Sharma *et al.*, 2018). The Whelan lab studies the descending control of locomotion from several brain regions including the A11, A13, Mesencephalic Locomotor Region (MLR), and the Medullary Reticular Formation (MRF) (Koblinger *et al.*, 2014; Sharples *et al.*, 2014, 2015; Sharples & Whelan, 2017). In addition to elucidating the connectivity between these regions and their axonal projections to the spinal cord, the Whelan lab also studies the neuromodulatory effects of neurotransmitters and neuropeptides such as dopamine and orexin, on the control of spinal locomotor output. Proximal to ventral tegmental area (VTA) and zona incerta are a unique class of neurons located in the lateral hypothalamus (LHA) (Date *et al.*, 1999; Yoshida *et al.*, 2006; Konadhode *et al.*, 2014; Tsujino & Sakurai, 2009, 2013). These orexinergic neurons are known to send axonal projections to most regions of the central and peripheral nervous system. The Whelan lab and a host of other groups have explored the modulatory effect of orexin on locomotion both at the spinal and supraspinal regions (Nakamura *et al.*, 2000; Matsuzaki *et al.*, 2002; Li *et al.*, 2014).

My interest in the neuromodulatory effect of dynorphin on spinal locomotor output was triggered by the work of Chou *et al.* (2001) which suggested that about 94% of orexinergic neurons within the LHA co-express dynorphin. Furthermore, this group went on to show, using immunogold labeling, that dynorphin is co-packaged within the same dense core vesicles. By implication, this means that each time there is a release of orexin there is also a co-release of dynorphin.

As for the pro-locomotory effects of orexin, studies suggest that orexin plays a role in goal-directed behaviors (motivation activation) including arousal and sleep/wake transitions, appetite, and locomotion (Mahler *et al.*, 2014). Intracerebroventricular injection of orexin in mice have been shown to increase locomotion (Mang *et al.*, 2012). Work in the Whelan lab has also examined the effects of orexin on fictive locomotion and intrinsic membrane properties that are indicative of locomotory activities (Biswabharati *et al.*, - Unpublished). Bath application of orexin resulted in depolarization of motoneurons and an increase in firing activity of ventral root neurograms obtained from *en bloc* recordings. Orexin increased the frequency of fictive locomotion bouts studied using en bloc isolated spinal cord preparations from neonatal mice. Since the foregoing studies validate the pro-locomotory effect of orexin on spinal locomotor activity, the next logical line of inquiry is to investigate the contribution of dynorphin in modulating spinal locomotor activities.

1.3. Dynorphin and Neuropathic pain

Dynorphin was first isolated from the porcine pituitary and described by Goldstein and colleagues in 1979 (Goldstein *et al.*, 1979), this tridecapeptide was named dynorphin (meaning: dynamis = “power”; Orphin = Opioid) due to its extraordinary potency upon interaction with KORs. Most studies on dynorphin have considered its role in neuropathic pain, chronic pain, addiction and the

modulation of various affective states. Dynorphin is a KOR agonist whose analgesic and antinociceptive effects have been well studied. Several post-translational proteolytic processing of the prodynorphin precursor results in variants including dynorphin A, dynorphin B, α-neoendorphin and 'Big' dynorphin, an intermediate precursor of dynorphin A and B containing amino acid sequence for both (Schwarzer, 2009).

Several studies have shown that upon binding with KORs, dynorphin mediates effects including the inhibition of cAMP synthesis (Sharma *et al.*, 1977), calcium channels (Rusin *et al.*, 1997), activation of potassium channels (North *et al.*, 1987), increased intracellular calcium levels (Jin *et al.*, 1992) and modulation of NMDA receptor-mediated synaptic currents (Caudle *et al.*, 1994). The antinociceptive effects of dynorphin are achieved by its presynaptic inhibition of N-type calcium channels (Gross & Macdonald, 1987; Tallent *et al.*, 1994). The release of neurotransmitters from the presynaptic terminal is calcium-dependent; therefore, the inhibitory action of KOR activation on N-type Calcium channels results in the reduction in neurotransmitter release from primary sensory afferents. Postsynaptically, KOR activation also results in the activation of inwardly rectifying potassium channels resulting in hyperpolarization (Monod *et al.*, 2014). These have a summative effect of reducing the transmission of nociceptive inputs in neurons of the spinothalamic pathway involved in the conduction of nociceptive stimuli from the periphery to the central nervous system (CNS). Studies suggest that under pathological conditions an increased expression of dynorphin can mediate its neurodegenerative effects via promiscuous interaction with other receptors such as bradykinin receptors and NMDA receptors (Schwarzer, 2009). Work by Lai *et al.* (2006) suggests that the low basal expression level of dynorphin mediates antinociception under physiological conditions by binding to KOR. On the other hand, in pathological and chronic pain conditions, there is an upregulation of dynorphin expression which

then allows dynorphin to interact with bradykinin receptors (Kajander *et al.*, n.d.; Iadarola *et al.*, 1988; Draisci *et al.*, 1991; Malan *et al.*, 2000; Rosén *et al.*, 2000; Wang *et al.*, 2001a; Lai *et al.*, 2009). This leads to the activation of P/Q and L-type calcium channels resulting in a pronociceptive effect (Luo *et al.*, 2008). Dynorphin's interaction with bradykinin and NMDA receptors results in excitotoxicity, due to the excessive influx of calcium ions, and can trigger downstream apoptotic cascades. Intrathecal injection of dynorphin induces pain hypersensitivities such as allodynia and hyperalgesia that are remedied by administration of anti-dynorphin antibodies (Malan *et al.*, 2000; Gardell *et al.*, 2004). The body of evidence suggests that while dynorphin can mediate analgesic effects under normal physiological conditions, it can also result in neuropathologies when present in supraphysiological levels such as during spinal cord injuries, and inflammation (Long *et al.*, 1986, 1994; Bakshi *et al.*, 1992a; Tachibana *et al.*, 1998; Hauser *et al.*, 1999; Vanderah *et al.*, 2000; Wang *et al.*, 2001; Gupta & Hubscher, 2012).

1.4. Effect of Dynorphin on Motor Function

Several behavioral and electromyographical studies have focused on the effects of dynorphin on motor function (Faden & Jacobs, 1983; Stevens *et al.*, 1987; Bakshi *et al.*, 1992b). Dynorphin has been shown to mediate its effects on motor output via opioid and non-opioid (i.e., naloxone-insensitive) mechanisms. In 1983 Faden and Jacobs studied the effect of intrathecal administration of dynorphin in rats and observed that it induced dose-dependent hind limb paresis. The observed dynorphin-induced paraplegia could not be replicated by μ- or δ-selective enkephalins and beta-endorphin (Faden & Jacobs, 1983). They furthered their investigation by showing that the observed effects of dynorphin and dynorphin-related peptides (including dynorphin (1-17), DYN-(1-13), DYN-(1-8) and α-neo-endorphin) acted via non-opioid mechanisms since they could not be

blocked by naloxone, a kappa-opioid receptor (KOR) blocker (Faden & Jacobs, 1984). Moreover, they showed that whereas kappa-selective agonists such as bremazocine, MRZ and U-50488H did not cause motor dysfunction, des-Tyr-dynorphin (DYN-(2-17)), a non-opiate truncated dynorphin, caused paralysis. Studies have also shown that DYN-(2-17) has a high affinity for NMDA receptors (Schwarzer, 2009).

Years later, a subsequent study by Faden and his colleagues suggested that the effects of dynorphin on motor functions is mediated by both kappa opioid receptors and NMDA receptors (Bakshi *et al.*, 1992b). This phenomenon has been corroborated by several studies that investigated dynorphin-induced motor impairments which can be restored to varying degrees depending on the application of either KOR antagonists or NMDA receptor blockers (Wollemann & Benyhe, 2004; Chen *et al.*, 2012; Bannister *et al.*, 2014).

1.5. Distribution of Dynorphin in the Brain

There are three distinct opioid prohormones: proopiomelanocortin (POMC), proenkephalin, and prodynorphin (Bakshi *et al.*, 1992a). Enzymatic cleavage of these prohormones results in various splice variants: POMC gives rise to melanocortin and endorphins (α, β, and γ), proenkephalin generates Leu- and Met-enkephalin (α, β, and γ), while prodynorphin produces 17-amino acid dynorphin A (1-17), 13-amino acid dynorphin B (1-13), 32-amino acid big dynorphin (1-32), alpha- and beta-neoendorphin. Other shorter and potent splice variants of dynorphin have also been isolated and purified from different brain regions.

Several studies suggest a strong overlap in the expression profile of dynorphin A with other prodynorphin splice variants in the brain (Schwarzer, 2009; Chavkin, 2013). Studies also show that many prodynorphin splice variants are often found co-expressed in the same neurons (Watson

et al., 1983). Studies indicate that there might be more widespread expression pattern of dynorphin B compared to dynorphin A (Khachaturian *et al.*, n.d.; Weber *et al.*, 1982; Weber & Barchas, 1983).

Brain Region	Dynorphin A	Brain Region	Dynorphin B
Medial Forebrain Bundle	High	Ventral Pallidum	High
Lateral Hypothalamic Area (LHA)	High	Internal Capsule	High
Substantia Nigra	High	Substantia Nigra	High
Hippocampus	Low	Hippocampal Formation	High
Nucleus Accumbens (NAc)	Low	Nucleus Accumbens (NAc)	High
Caudate Putamen	Low	Caudate Putamen	Low
Paraventricular Hypothalamic Nucleus (PVN)	Low	Paraventricular Hypothalamic Nucleus (PVN)	Low
Supraoptic Nucleus	Low	Supraoptic Nucleus	Low
Nucleus of the Solitary Tract	Low	Bed Nucleus of the Stria Terminalis (BNST)	Low
	Low	Central Nucleus of the Amygdala (CeA)	Low

Table 1.1: Brain regions showing dynorphin A and B expression.

1.6. Kappa Opioid Receptors

Several pharmacological studies have revealed the existence of four main types of opioid receptors: mu (μ)-opioid receptors, kappa (κ)-opioid receptors, and delta (δ)-opioid receptors, opioid receptor-like receptor (ORL).

Opioid Receptor	**Known Subtypes**	**Primary Endogenous Ligand**
Mu (μ)-opioid receptors (MOR)	μ_1, μ_2, μ_3	Enkephalin
Kappa (κ)-opioid receptors (KOR)	κ_1, κ_2, κ_3	Dynorphin
Delta (δ)-opioid receptors (DOR)	δ_1, δ_2	Enkephalin, β-endorphin
Opioid receptor-like receptor (ORL)	ORL_1	Nociceptin

Table 1.2: Summary of opioid receptors, known subtypes and endogenous ligands.

Dynorphin is the endogenous ligand for KOR. However, studies have shown that MORs have a low affinity for dynorphin (Zhang *et al.*, 1998). There are three KOR subtypes: κ_1, κ_2, κ_3. Dynorphin is known to mediate its effects via interactions with the various subtypes of KORs. The functional implication of dynorphin interaction with KORs differs depending on the receptor subtype. For example, dynorphin has been reported to interact with KOR2 on CA3 pyramidal cells in the hippocampus results in inhibition of NMDA-mediated synaptic currents (Caudle *et al.*, 1994);

whereas, other work reports that interaction of dynorphin with KOR1 on isolated spinal neurons results in increased NMDA currents and calcium transients (Hauser *et al.*, 1999). Dynorphin can also interact with NMDA receptors and bradykinin receptors. The KOR agonist, U69, 593, that I've used in my thesis, is a highly selective agonist specific for κ_1 KOR subtype. Unlike endogenous dynorphin, studies have shown that U69593 does not interact with NMDA receptors or alter their activity (Hjelmstad & Fields, 2001). Studies have also shown that KORs can interact with and form heterodimers with delta opioid receptors, forming a receptor complex having high affinity for dynorphin A and decreased affinity for U69593. Kappa opioid receptors, like most opioid receptors, acts by binding with Gi/Go G-protein coupled receptors. $G_{i/o}$ – GPCR signaling results in inhibition of adenylate cyclase activity, lowering of CAMP levels and PLC-mediated increase in intracellular Ca^{2+} levels. Norbinaltorphimine (norBNI) is a highly selective non-competitive antagonist that specifically blocks the activity of κ_1 KOR subtype.

1.7. Hypothesis

The general hypothesis of my work is as follows: **dynorphin will not just inhibit but modulate spinal motor network function**.

This hypothesis was investigated via the following aims:

1. Investigate the expression of KOR on motoneurons (Using RNAscope assay)
2. Characterization of the effects of dynorphin on spinal network activity
3. Characterization of the effects of dynorphin on the intrinsic membrane properties of motoneurons.

Chapter Two

Methods

Chapter Two: Method

All experiments were performed on neonatal C57BL/6 mice (Charles River Laboratories, Wilmington, MA), postnatal day 0 – 3 (weight 1.3 - 2.8 g; n = 56). The animals were anesthetized by hypothermia following procedures approved by the University of Calgary Health Sciences Animal Care Committee, (AC12-0152) which follows the guidelines published by the Canadian Council on Animal Care.

2.1. Tissue Preparation

2.1.1. Isolated Spinal Cord Preparation

Anesthetization by hypothermia was used and is an approved method since neonatal mice aged P0 – P3 cannot fully regulate their body temperature (Lagerspetz, 1966). Following decapitation and evisceration, the remaining tissue was placed in a dissection chamber. The tissue was superfused with carbogenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (aCSF; containing in mM: 4 KCl, 1.5 $CaCl_2$, 128 NaCl, 0.5 Na_2HPO_4, 1 $MgSO_4$, 21 $NaHCO_3$, and 30 D-glucose). A ventral laminectomy was performed to expose the spinal cord, and the ventral and central dorsal roots were then carefully cut to release the cord from the vertebral column. The spinal cord was transversely transected at the T1 – T3 segment and isolated from the vertebral column to the level of the cauda equina. The isolated spinal cord was then transferred to the recording chamber filled with superfused aCSF heated to 27^{o}C. The roots were then attached with tight-fitting suction electrodes, and the preparation was allowed to recover for one hour.

2.1.2. Slice Preparation for Whole-cell Patch Clamping

For acute spinal cord slice preparations, the isolated spinal cord was transversely transected at T13 and then mounted on an agar block stabilized in an upright position in the slicing chamber using 20% gelatin. The slicing chamber was quickly filled with ice-cold carbogenated sucrose-aCSF solution (in nM: 25 NaCl, 188 sucrose, 1.9 KCl, 10 $MgSO_4$, 1.2 Na_2HPO_4, 26 $NaHCO_3$, and 25 D-glucose) continuously bubbled and kept chilled using ice pellets. Using a vibratome (VT1000S; Leica, Bussloch, Germany) 250 µm slices were cut and transferred to a recovery chamber containing aCSF held at about 36°C. The slices were allowed to recover for one hour before being transferred to the recording chamber.

2.2. Pharmacology

2.2.1. Pharmacology for Spontaneous Activity Recording

In experiments studying the effects of the KOR agonist on spontaneous activity the specific KOR agonist U69,593 (Sigma-Aldrich; CAS: 96744-75-1) was used. Bath application of 1 µM U69,593 was used to activate KOR. KOR activity was blocked using the specific non-competitive antagonist, nor-BNI (Tocris; 100 nM dissolved in water). The dose-response experiments were performed using the following concentrations of U69,593: 30 nM, 100 nM, 300 nM and 1 µM. Vehicle experiments were performed using 50% ethanol (EtOH) while sham experiments, studying changes in activity over time, were conducted without bath application of any drug. The drug was bath-applied in 200 mL aCSF that was carbogenated and recirculated. However, due to the well-published high binding-affinity of U69,593 to KOR, the washout experiments were performed using aCSF without U69,593.

2.2.2. Pharmacology for Whole-cell Patch-Clamp Recording

Fast neurotransmitter blockers were used to study the effect of KOR activation on intrinsic properties of motoneurons. The following blockers were used: picrotoxin (Sigma-Aldrich; $GABA_A$ Receptor blocker; [50 µM]), APV (Tocris; NMDA receptor antagonist; [50 µM]), DNQX (Tocris; AMPA and kainate receptor antagonist; [20 µM]), and strychnine hydrochloride (Sigma-Aldrich; glycine receptor antagonist; [10 µM]). U69,593 (Sigma-Aldrich; [1 µM]) was used as the specific KOR agonist. The internal pipette solution contained (in mM) 130 K-gluconate, 7 NaCl, 0.1 EGTA, 10 HEPES, 0.3 $MgCl_2$, 2 ATP, and 0.5 GTP, adjusted to pH 7.3 using KOH. The osmolarity of the internal pipette solution was within the 270 - 280 mOsm range.

2.3. Electrophysiology

2.3.1. Spontaneous Activity Recording on Isolated Spinal Cord Preparation

Neurograms (recorded neural activities) were obtained using tight-fitting suction electrodes hooked up to the right and left ventral roots of the second lumbar segment (RL2 and LL2) and the right and left ventral roots of the fifth lumbar segment (RL5 and LL5) of the isolated spinal cord. The suction electrode was filled with the bath aCSF. The recorded signals were band-pass filtered (AC: 0.1 – 1 kHz), sampled at 20 kHz, and digitized (Digidata 1440, Molecular Devices, Sunnyvale, CA), acquired using Clampex 10.6 software (Molecular Devices). Obtained data were analyzed using Clampfit 10.6 (Molecular Devices) and Axograph X (Version 1.7.0; John Clements).

2.3.2. Dorsal Root Potential (DRP) and Monosynaptic Response (MSR) Recording

The effect of KOR activation on DRP and MSR was tested on isolated spinal cord preparation obtained as aforementioned. Tight-suction electrodes were attached onto the L5 ventral root, ipsilateral L5 dorsal root, and ipsilateral L4 dorsal root. The L5 dorsal root was stimulated using a stimulus isolator (A360 World Precision Instruments, Sarasota, FL; AMPI Master 8 pulse generator, Jerusalem, Israel) and electrotonically propagated potentials were recorded from the ipsilateral L4 dorsal root. The elicited monosynaptic (reflex) reflex was recorded from the ipsilateral L5 ventral root. The acquired neurograms were amplified (1000 – 20,000 times), low-pass [direct current (DC) 1 kHz] filtered and digitized at 2 – 5 kHz for future analysis.

2.3.3. Whole-cell Patch-Clamp Recording

Patch-clamping experiments were done on putative motoneurons identified by their low input resistance and high capacitance which correlates with their size when visualized using infrared light transmission and differential interference contrast optics (BX51; Olympus Optical, Center Valley, PA). The lab has validated this approach for identifying motoneurons (Nakanishi & Whelan, 2010). Patch electrodes were pulled using a P87 Flaming/Brown puller (Sutter Instruments, Novato, CA) with resistances in the range of 2 – 4 MΩ. The liquid junction potential was calculated and corrected using the pClamp 10.6 software to be 16.1 mV. Data were recorded using a low pass filter 10 kHz and digitized with a sampling rate of 20 kHz (Digidata 1440, Molecular Devices). The computer-controlled microelectrode current and voltage clamp amplifier, Multiclamp Commander 700B (Molecular Devices) was used.

2.4. Data Collection and Analysis

2.4.1. Effect of Kappa Opioid Receptor Agonist (U69593) on Spontaneous Activity

The isolated spinal cord preparation was allowed to recover for 1 hour in the recording chamber. Baseline activity was then recorded for 15 minutes after which KOR agonist (U69,593) was bath applied for 30 minutes. The drug was washed out using fresh aCSF for 30 minutes. For antagonist experiments, the preparation was pre-treated with nor-BNI for 30 minutes before the application of U69,593. Only the last 15 minutes of each treatment condition (i.e. baseline, antagonist, agonist, and washout conditions) were considered for data analysis. This is because preliminary dose-response experiment data show that the effect of U69,593 sets approximately 6 minutes after bath application and peaks after 15 minutes.

The neurograms were first filtered in Clampfit 10.6 (Molecular Devices) and then exported to Axograph X, where the signals were then rectified and analyzed to measure parameters such as the number of spikes (known as events), amplitude, and frequency of the recorded neural activity.

2.4.2. Effect of U69593 on Dorsal Root Potential and Monosynaptic Response

To study the effect of U69,593 on DRPs and MSRs the stimulation threshold (i.e., the smallest current intensity to elicit a response) was first determined. Preliminary studies tested the effect of the drug at 3, 5 and ten times the threshold and found the highest effect at 5 times the threshold (5T). Since it is important to have a stable baseline DRP/MSR before studying the impact of any drug, the DRP/MSR every spinal cord preparation was observed in real time. This was done by stimulating the cord at 5T every 30 seconds and plotting out the DRP/MSR using Microsoft Excel.

It typically takes about 2 hours for the preparations to achieve a stable DRP/MSR baseline. The baseline DRP/MSR was then taken for 15 minutes after which U69,593 was bath applied for 30 minutes. U69,593 was washed out using fresh aCSF for 30 minutes. The KOR antagonist, nor-BNI, was pre-applied before U69,593 treatment for antagonist experiments. The preparation was constantly stimulated at 5T for the entire duration of the experiment.

Clampfit 10.6 (Molecular Devices) was used to perform offline analysis of the results. The amplitude of the last ten responses was considered for baseline, nor-BNI, U69593 and washout conditions.

2.4.3. Effect of U69,593 on Intrinsic Properties of Motoneurons

After recovering the slices for 1 hour in the recovery chamber, the slices were then transferred into the recording chamber and held down using a harp. The patch electrode was then robotically guided (using manipulators) to create a giga-seal (with resistance > 1000 Ω). By applying gentle negative pressure, the patch is broken into to allow recording in a whole-cell mode. The cell was left in the whole-cell conformation for 5 minutes to allow for dialysis of the cytoplasm with the patch electrode internal solution.

Fast synaptic blockers (picrotoxin, DNQX, APV, and strychnine) were then bath-applied for 10 minutes followed by current clamp recordings of the baseline intrinsic membrane properties such as input resistance and rheobase. U69,593 was then applied for 10 minutes, followed by a 10-minute washout using fresh aCSF containing the same concentration of fast synaptic blockers. Data were only analysed from experiments for cells that maintained access resistance with changes not greater than 15%. The data were analysed off-line using Clampfit 10.6.

2.5. Statistical Analysis

All statistical analyses were performed using SigmaPlot 13.0 (Systat Software Inc., San Jose, CA, USA). The effect of the drug treatment was compared to the baseline and washout using a non-parametric repeated measure ANOVA test followed by a Holm-Sidak post hoc analysis. Mann-Whitney test rank sum tests were performed when the assumption of normality was not met using a Shapiro-Wilk test. Test for significance was considered at P value < 0.05.

2.6. RNA In Situ Hybridization (RNAscope Assay)

2.6.1. Introduction

This is an RNA in situ hybridization assay kit (Advanced Cell Diagnostics, Newark, CA) that is a preferred method of showing the expression of G-protein Coupled Receptors (GPCRs) (Cabañero *et al.*, 2016; Samineni *et al.*, 2017). From personal communication with experts in the field such as Dr. Michael Bruchas, Dr. Jose Moron-Concepcion, and Dr. Charles Chavkin, I was informed that immunostaining for KORs using available antibodies shows low-specificity for labeling GPCRs and there are currently no validated antibodies for immunolabeling of KORs. I was introduced to RNAscope assay technology by Dr. Bruchas and traveled to his lab at the Washington University, St. Louis Missouri, under the Eyes High Learn-from-the-best Award. Conversely, RNAscope assays are highly specific for labeling mRNA. This is because RNAscope assay utilizes two independent double-Z probes that hybridize in tandem to the target RNA sequence. The tandem hybridization of the double-Z probes is necessary for the binding of pre-amplifiers, amplifiers and labeled probes (figure 2.1).

Using this tool, we ran a multiplex fluorescence assay showing the expression of ChAT (for motoneurons) and KORs.

2.6.2. RNAscope Assay Method

There are two types of RNAscope assay methods: freshly frozen tissue assay, and fixed tissue assay. The fresh frozen tissue (FFT) assay method was used for this project. For this method, the spinal cord was first isolated as described. The isolated spinal cord was then transected at the T12 -T13 segment level and flash frozen. To flash freeze, the spinal cord was put in a mold containing Optimal Cutting Temperature compound (OCT - VWR International, Radnor, PA, USA) and then dipped in a pre-chilled (using dry ice) solution of isobutane (2-methylpropane) for 30 seconds to 1 minute. The flash frozen tissue was stored in a -80 ^{o}C freezer. When needed, the tissues were recovered from the -80 ^{o}C freezer and equilibrated for one hour at -20 ^{o}C. The tissue was then sectioned using a cryostat (Leica CM1850 UV, Leica Biosystems, Ontario, Canada) and mounted onto superfrost plus glass slides (VWR International).

The mounted tissue was first fixed by immersion in freshly-made 4% paraformaldehyde (PFA) for 15 minutes. This was followed by dehydration steps which involved immersing the tissue for 5 minutes in 50%, 70% and 100% ethanol (in that order). The slides were then air-dried for 5 minutes, and a hydrophobic barrier was created around the tissue using a hydrophobic pen. The tissue was then treated with Protease IV reagent for 30 minutes at room temperature; this step exposes the antigen to the probe and is also a critical control step that can be modified for RNAscope-Immunohistochemistry assay. The probe was then applied and the tissue was incubated at 40 ^{o}C using the humidity control unit (Hybez oven – Advanced Cell Diagnostics, Newark, CA). The tissue was then treated with a series of amplifiers thus: Amplifier 1 (Amp 1 - incubation time:

30 minutes), Amp 2 (15 minutes), Amp 3 (30 minutes), and finally, Amp A, B or C, which contains the fluorophore (15 minutes). All amplification steps were performed in the Hybez oven with the temperature maintained at 40 ^{o}C. There was always a wash step (using wash buffer provided in a kit) between the probe and amplification steps. DAPI was then added for 30 seconds, followed by addition of a mounting medium. Coverslips were mounted and sealed using nail polish.

2.6.3. Image Visualization

Imaging was performed using confocal microscopes available at the HBI-AMP facility at the University of Calgary, Foothills Campus. Images were collected using the Nikon A1R Confocal Laser Scanning Microscope (Nikon Canada Inc., Ontario, Canada) and Leica TCS SP8 inverted laser confocal microscope (Leica Microsystems Inc., Concord, Ontario, Canada).

For Nikon A1R microscopy, the following objectives were used: Plan Apo 10x/0.45 NA air, and Plan Fluor 20x/0.75 NA multi-immersion. Lasers used for imaging include 405 nm diode laser (DAPI), 488 nm Ar-Ion (Alexa 488 - ChAT), and 561 nm diode laser (Alexa 546 - KOR). The images were further processed offline using the proprietary software, NIS-Elements. For imaging on Leica SP8 confocal microscope, the following objectives were used: 10x / 0.40 NA (HC PL APO CS2), and 25x / 0.95 NA water (HC FLUOTAR, 2.4mm WD, 0.17 VISIR). Imaging was performed using the following lasers: 405 nm diode laser (DAPI), 488 nm diode laser (Alexa488 - ChAT), and 552 nm diode laser (Alexa 546 - KOR). Offline image processing was done using LAS X proprietary software.

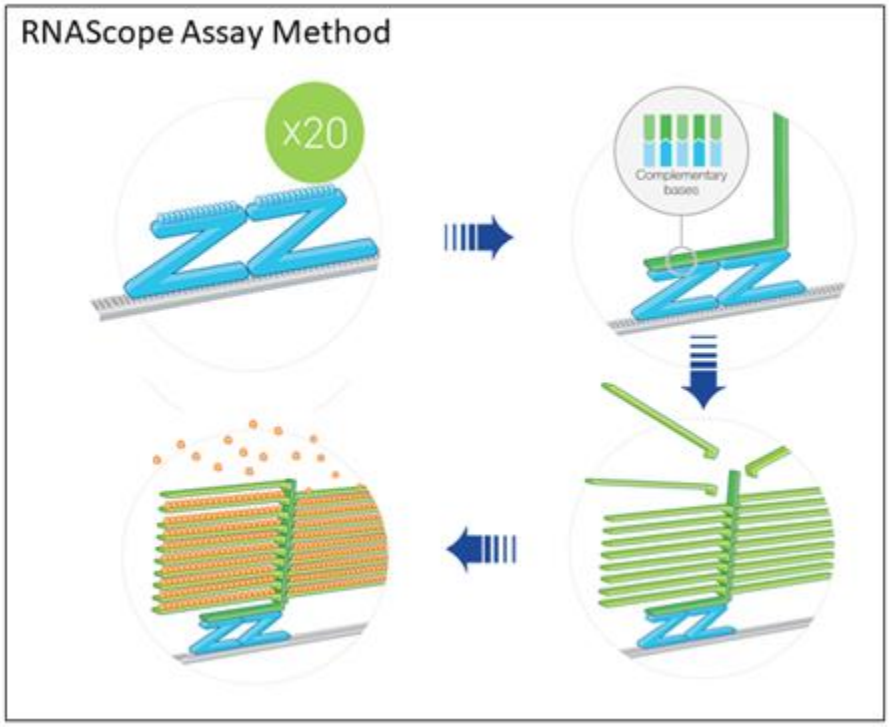


Figure 2.1: Image showing procedure for RNAscope assay. Each of the double-Z probe recognizes one-half of the target sequence. The L-shaped pre-amplifiers only bind to adjacently bound Z probes to improve sensitivity and specificity of the assay. The amplifiers are then added (Amp 1 - 3) and finally, Amp 4, containing the fluorophore is then applied to give fluorescence.

(Image obtained from ACD Bio Website: https://acdbio.com/science/how-it-works)

Chapter Three

Results

Chapter Three: Results

3.1. Introduction

A high degree of correlation has been observed between KOR (κ-1 subtype) mRNA-expression and binding in several regions of the brain such as caudate putamen, dorsomedial and ventromedial hypothalamus, amygdala, and nucleus accumbens (Mansour *et al.*, 1995).

KORs have been shown to have analgesic effects by modulating the ascending and descending spinothalamic pathway. Different populations of dorsal root ganglia (DRG) cells have been shown to express KOR which is involved in the presynaptic processing of sensory transmission of nociceptive inputs (Vanderah, 2010). Within the DRG, KOR has been shown to be mostly localized in small and medium diameter neurons, which is consistent with their role in nociception. KORs (as well as μ- and δ-opioid receptors) have also been shown to have higher expression on the superficial layers of the spinal cord (lamina I and II) and lower expression in the remaining dorsal and ventral horn (Besse *et al.*, 1992; Arvidsson *et al.*, 1995; Minami *et al.*, 1995). Furthermore, consistent with established spinal KOR expression pattern, in his investigation of the laminar pattern of preprodynorphin mRNA expression in a model of peripheral inflammation and hyperalgesia, the work of Ruda *et al.* (1988) showed that 66% of preprodynorphin immunoreactive (IR^{+ve}) cells were found in lamina I-II, followed by 29% in lamina V-VI. Images of KOR and prodynorphin expression from the Allen Brain Atlas further corroborate the dorsal-dominant expression pattern (figure 3.1A). The work of Wang *et al.* (2009) gave insight to the subcellular distribution of KOR in the dorsal horn neuron of rat spinal cord. Using electron microscopy, they showed that while KOR was detected in axon terminals, axons, dendrites, and soma, the dendrites contained more KOR than other neuronal compartments. These studies suggest

that there is a correlation between spinal lamina distribution pattern of KOR and dynorphin expression.

3.2. Results

3.2.1. Kappa Opioid Receptor Expression on Motoneurons

Most studies showing the expression of KORs and/or dynorphin (or preprodynorphin mRNA) within the spinal cord have focused on pain modulation and pruritus (Tominaga *et al.*, 2007; Martelli *et al.*, 2010; Kardon *et al.*, 2014). None have considered the expression of KORs on motoneurons. This is of key importance to my work because if motoneurons are the final neurons in the motor pathway, then KOR is capable of modulating the amplitude of motor output and sculpting the final locomotor pattern.

We adopted the a RNAscope assay, a kit-based *in situ* hybridization technique, used to reliably show mRNA expression (Cabañero *et al.*, 2016; Samineni *et al.*, 2017) since antibodies against KOR are not reliable.

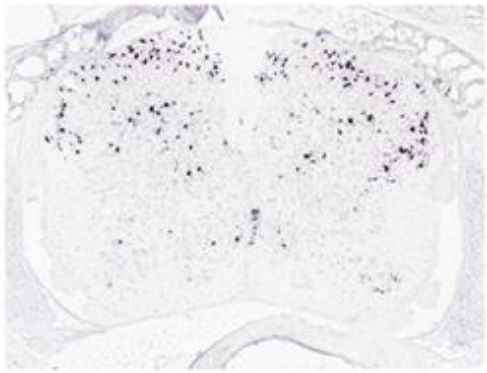
Using the RNAscope assay, the expression of KOR relative to ChAT-positive cells were studied in C57BL/6 neonate mice, aged P0 – P3 (figure 3.1 B and C). Choline acetyltransferase (ChAT), is a transferase enzyme involved in the synthesis of the neurotransmitter acetylcholine. It is a specific marker for cholinergic cells. The ChAT probe is in the green channel (Alexa 488: λ_{em} 540 ± 10 nm) while the KOR probe was in orange channel (Atto 550: λ_{em} 574 ± 10 nm). A pseudocolor (Red) was applied to the KOR channel to improve visualization and distinction between the KOR IR^{+ve} and merged cells. The merged channel indicates that there were motoneurons that co-label for KOR IR^{+ve} and ChAT IR^{+ve}. DAPI, in the blue channel (λ_{em} 461 nm), stains for cell nuclei.

These results suggest that motoneurons also express KOR and the effect of KOR activation on spinal spontaneous activity or locomotor output might be mediated via the direct effect dynorphin on motoneurons.

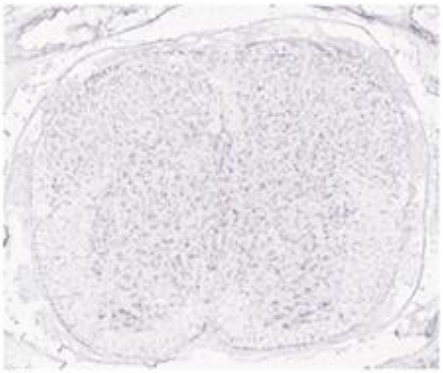
Two sets of controls were performed for RNAscope assay. The first is the positive and negative control kit probes that come with the assay kit. The positive control from the kit labels some housekeeping genes that are important for cell cycle in mammalian species. The negative control labels a bacterial gene. The purpose of this control provided in the kit is to ensure that the genes observed were mammalian. The second control used was a tissue from brain sections known to express the target gene. The ventral tegmental area (VTA) is known to express KORs and ChAT (Mansour *et al.*, 1994; Polter *et al.*, 2017). Data obtained were comparable to those obtained from Bruchas Lab.

A

Transverse section of lumbar spinal cord segment showing the expression of prodynorphin mRNA (i) and kappa opioid receptors (ii)



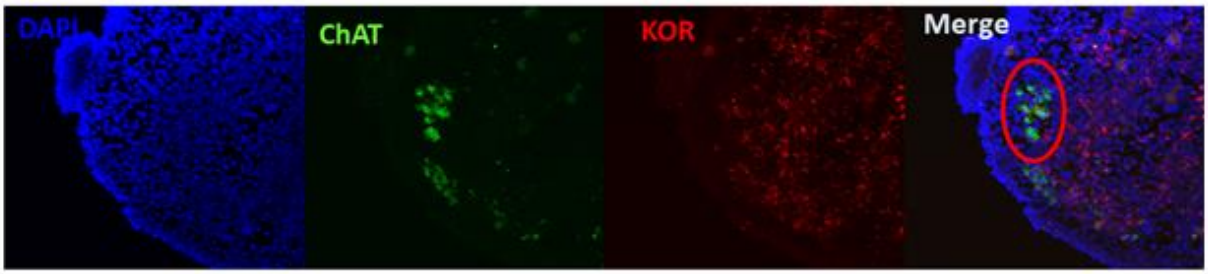
(i) Prodyn



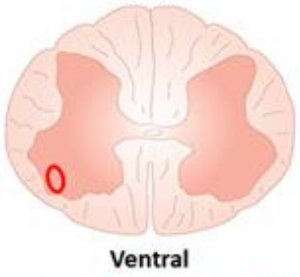
(ii) Oprk1

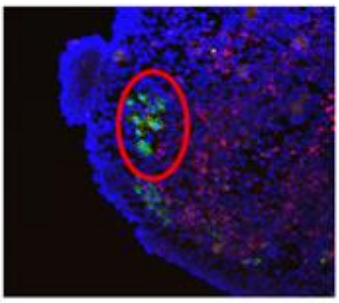
B

Co-immunolabeling of ChAT and KOR



C





Ventrolateral section of the Lumbar spinal cord showing ChAT/KOR co-immunolabeling

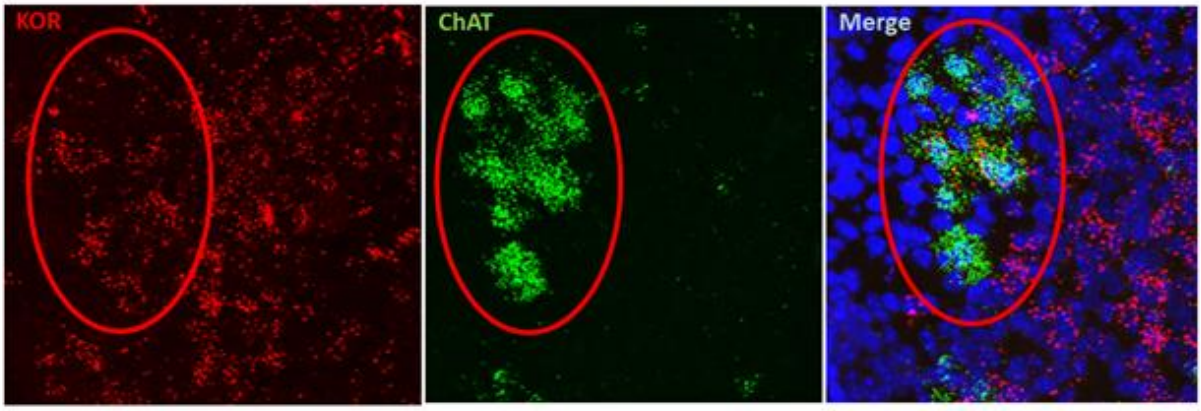


Figure 3.1: Expression of KORs in lumbar segment of neonate mice spinal cord. A) In situ hybridization images from Allen brain atlas – Mouse Spinal Cord (http://mousespinal.brain-map.org/) showing the expression of prodynorphin mRNA (i) and KORs (κ_1 subtype) (ii) in postnatal day 4 (P4) mice. B) Transverse section of lumbar spinal cord segment from P3 mouse showing co-labeling of KOR (Red channel) and ChAT (Green channel) using RNAscope assay. Representative images are shown. C) Zoomed in image of the ventrolateral section of the lumbar spinal cord showing expression of KOR, ChAT and the merge channel. Images were obtained using the Leica SP8 Confocal microscope and processed offline using LAS X, proprietary software for Leica SP8.

Tissue Sections	ChAT IR^{+ve}	ChAT/KOR IR^{+ve}
1	23	4
2	13	3
3	29	8
4	6	2
Total	71	17

Table 2.1: Table showing the quantification of Chat IR^{ve} cells and ChAT/KOR IR^{+ve} co-labelled cells.

3.3. Discussion

The results of the RNAscope assay in figure 3 suggests that there is an expression of KOR on motoneurons. Manual counting of cells from 4 lumbar spinal cord slices show that of the 71 Chat IR^{+ve} cells, 17 of them were ChAT/KOR IR^{+ve} co-labelled cells (Table 2.1). This suggests that the neuromodulatory effects of KOR activation might be mediated by its direct action on motoneurons. The RNAscope assay is a very sensitive and specific technique used to show mRNA expression. This assay employs the use of a double-Z probe: each of the Z-probe has two sides with one side recognizing one-half of the target sequence and the other half having half of the binding sequence for the L-shaped pre-amplifier (figure 2.1). The pre-amplifier will bind only when there are two Z-probes bound adjacent to each other the create the complete binding sequence of the pre-amplifier. This way the RNAscope assay overcomes the problem of off-target interaction which plagues most antibodies used to show expression of GPCRs such as KOR.

3.3.1. Caveats for RNAscope Assay

The primary challenge with using RNAscope assay to show expression of KOR is the difficulty in quantifying the results. This is often the case with targets having low basal mRNA expression level such as KOR. While DAPI may assist in the qualitative recognition of a cell, it will be very challenging to use software such as Image J and Imaris for quantification. One way to resolve the issue of low KOR expression might be to check for co-labeling in a mouse model of peripheral inflammation or chronic pain. This is because studies have shown that KOR expression increases following inflammation, spinal cord injury, or chronic pain (Pol *et al.*, 2003; Pol & Puig, 2004; Xu *et al.*, 2004; Hughes *et al.*, 2014). This will have increased KOR expression and improve the signal to enhance quantification using the software.

The second limitation of using mRNA expression to infer protein expression pattern is the possibility of post-translational protein trafficking. This means that the site of mRNA expression might be different from where the protein (i.e. KOR receptor) is eventually expressed. One way to resolve this might be to use KOR-knockout mice to test for the effect of KOR activation on synaptically isolated motoneurons.

3.4. Effect of Kappa Opioid Receptor Activation on Spinal Network Activity

3.4.1. Introduction

The spinal cord serves as an integration center for neural signals that generate and modulate locomotor activity (McCrea, 2001; Rybak *et al.*, 2002; Yang & Gorassini, 2006; Hultborn & Nielsen, 2007; Markin *et al.*, 2010; Guertin, 2012). A complex network of neurons within the spinal cord, known as the central pattern generator (CPG), can generate locomotor patterns independent of any sensory or supraspinal inputs. For a drug to impact spinal locomotor output it can act on one or both levels of the CPG: the rhythm generator and the pattern formation layers (McCrea, 2001; Markin *et al.*, 2010; Guertin, 2012). However, for a neuropeptide such as dynorphin, whose modulatory effects on spinal neural activities is largely unknown, we chose to examine its effect using an in vitro isolated spinal cord neonatal preparation ideally suited to examining effects on network function.

One classic feature of developing neural networks is spontaneous activity which occurs in diverse areas of the brain such as hippocampus, spinal cord, brainstem, and retina. Spontaneous activity represents the episodic discharge of neural networks at a frequency between 0.1 – 2 Hz with each episode lasting about 5 – 90 seconds (O'Donovan *et al.*, 1998; Tabak *et al.*, 2000). These episodes of neural activity are punctuated by bouts of quiescence. Although the exact mechanisms of spinal spontaneous activity generation are still poorly understood, studies suggest that it is a result of a complex interplay between the excitatory and inhibitory components of developing synaptic connections onto network neurons. While the highly excitable neuronal components of the network drive the bouts of episodic bursting activities, the inhibitory components mediate the moments of depression and quiescence.

To study spontaneous activity in isolated spinal cord preparations using extracellular recording techniques, suction electrodes were used to record neurograms from the ventral roots (right and

left L2 and L5 segments). Since spontaneous activity is best observed in the developing network, C57BL/6 mice aged P0 – P3 were used for these experiments.

3.4.2. Results

3.4.2.1. Results of Vehicle, Dose-Response and Sham Experiments

The KOR agonist used, U69,593, was dissolved in 50% ethanol, prepared by mixing equal volumes of water and absolute ethanol (Hjelmstad & Fields, 2001; Margolis *et al.*, 2003, 2005; Poole *et al.*, 2007; Xia *et al.*, 2008). A 10 mM stock aliquot of U69593 (Sigma Aldrich; Mwt: 356.50 g/mol) was first prepared by dissolving the 5 mg solid U69593 in 1.4025 L (or 1,403 µL) 50% ethanol. The stock was aliquoted into 20 µL vials and stored in -20 ^{o}C freezer. To achieve a working concentration of 1 µM, 20 µL of 10 mM U69,593 stock was added to 200 mL aCSF.

Several publications show that 1 µM was the most popular concentration of U69,593 used to activate KORs (Connor & Christie, 1998; Emmerson & Miller, 1999; Hjelmstad & Fields, 2001; Margolis *et al.*, 2008; Kang-Park *et al.*, 2013; Zhang & van den Pol, 2013). Vehicle experiments were performed, and spontaneous activity was recorded from isolated spinal cord preparations using 200 mL of aCSF into which 20 µL of 50% ethanol was added instead of U69,593. The vehicle had no significant effect on the number of events (n = 6, Baseline = 1758.8 ± 253.3, Drug = 2192.2 ± 927.2, Washout = 2196.3 ± 1023.6; P = 0.956) or amplitude (n = 6, Baseline = 43.9 ± 10.7, Drug = 44.3 ± 10.6, Washout = 45.3 ± 10.5; F(2,10) = 1.239, P = 0.331) of recorded neural activity (Figures 3.2. A, B and C).

Since there are was no published work showing the effect of U69,593 on spontaneous activity recorded from isolated spinal cord preparations, I performed a dose-response experiment to obtain

the concentration of the drug that will completely abolish spontaneous activity. Four concentrations were used to test for dose-response: 30 nM **Events:** (n = 3, Baseline = 2199.7 ± 284.3, Drug = 1576.7 ± 671.9, Washout = 436.7 ± 325.5; P = 0.003) ; **Amplitude**: (n = 3, Baseline = 46.0 ± 9., Drug = 45.2 ± 9.4, Washout = 45.5 ± 16.6; P = 0.975), 100 nM **Events:** (n = 4, Baseline = 1942 ± 779.5, Drug = 900.3 ± 768.9, Washout = 1324.5 ± 1517.8; P = 0.149); **Amplitude**: (n = 4, in μV: Baseline = 39.9 ± 7.4, Drug = 42.1 ± 9.2, Washout = 37.9 ± 11.3; P = 0.525), 300 nM **Events:** (n = 7, Baseline = 766 ± 927.2, Drug = 226.4 ± 328, Washout = 219 ± 293; P = 0.016); **Amplitude**: (n = 7, in μV: Baseline = 40.3 ± 13.7, Drug = 36.5 ± 10.5, Washout = 32.2 ± 10.9; P = 0.276); and 1 μM **Events:** (n = 7, Baseline = 1674 ± 861.1, Drug = 144.7 ± 216.7, Washout = 500 ± 615.9; P = 0.002); **Amplitude**: (n = 7, in μV: Baseline = 36.3 ± 12.5, Drug = 30.4 ± 8.8, Washout = 31.4 ± 9.5; P = 0.029); (figure 3.3. A). Statistical analysis of the data using a Mann-Whitney ANOVA with repeated measures and Holm-Sidak method for post hoc analysis, indicated that the 1 μM concentration had the most significant effect in decreasing number of spontaneous activity events and amplitude. The IC_{50} was calculated to be 83.68 nM (Log IC50 = 1.92, SEM = 0.37) (figure 3.3. B).

The longest experiments using nor-BNI as an antagonist to block KOR activity (lasting 105 minutes), it was necessary to make sure that there were no time-dependent effects observed. To do this, spontaneous activity was recorded for 105 minutes using only aCSF. The results of these sham experiments showed that there was no time-dependent effect on the number of events (n = 4, 15 mins = 2755.8 ± 1507.7, 45 mins = 2542.8 ± 1393, 75 mins = 2141.8 ± 937.4, 105 mins = 3049.3 ± 1776.1; P = 0.149) or amplitude (n = 4, in μV: 15 mins = 42.7 ± 9.9, 45 mins = 44.0 ±

10.5, 75 mins = 43.9 ± 10.6, 105 mins = 45.3 ± 12.2; P = 0.079) of the recorded neural activities (Figures 3.2. D and E).

3.4.2.2. Effect of KOR Activation

Bath application of 1 μM U69,593 was observed to block spontaneous activity in the isolated spinal cord preparation. Axograph X was used to analyze the number of events (neural activity above a set threshold) and amplitude of each event. Comparing between baseline and U69,593 revealed that there was a statistically significant decrease in the number of events (n = 7, Baseline = 1674 ± 861.1, Drug = 144.7 ± 216.7, Washout = 500 ± 615.9; $F(2,12) = 12.24$, P = 0.002) and their amplitude (n = 7, Baseline = 36.3 ± 12.5, Drug = 30.4 ± 8.8, Washout = 31.4 ± 9.5; $F(2,11) = 4.95$, P = 0.029); post hoc analysis using Holm-Sidak method) (figures 3.4. A, B, C, and G).

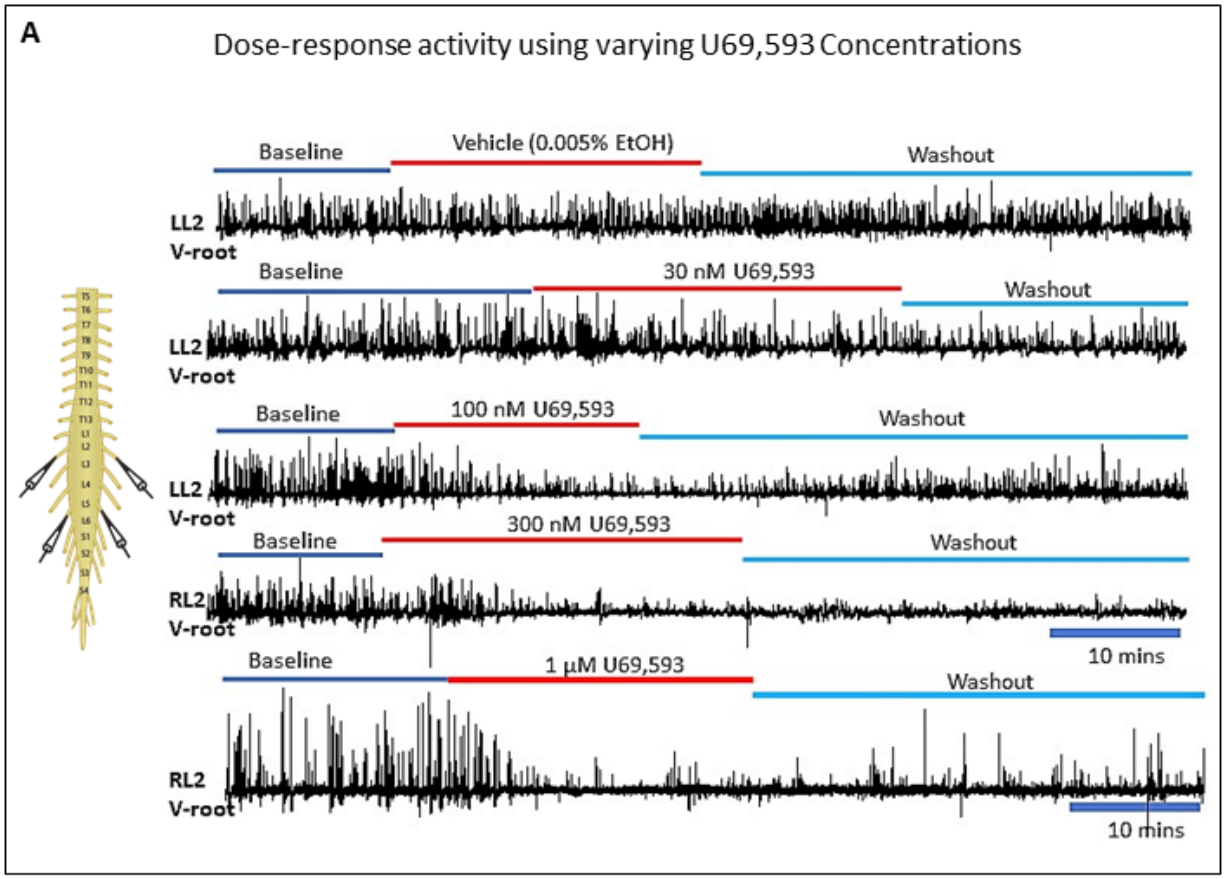
To verify the involvement of KOR in mediating the observed changes in spontaneous activity, the highly specific and selective KOR antagonist, nor-BNI was pre-applied before U69,593 addition. Blocking KOR activation using nor-BNI prevented the KOR-mediated reduction in spontaneous activity from baseline level, **Events:** (n = 8, Baseline = 1061 ± 727.4, NorBNI = 2296.1 ± 1469.1, KOR = 2282.3 ± 1395.7, Washout 3166.8 ± 2096; P = 0.980); **Amplitude**: (n = 8, in μV: Baseline = 32.9 ± 4.7, NorBNI = 34.5 ± 5, KOR = 39.9 ± 12.1, Washout 36.2 ± 6.4; P = 0.768) (figures 3.4. D, E and F).

The data show that washout of the drug using aCSF does not return the activity to baseline levels. This is either due to U69,593 not being easily washed off, or U69,593 is inducing some plastic changes that are not reversible by washing off. To test whether it is due to the high-affinity nature of U69593 to KOR further experiments using nor-BNI will need to be performed. If nor-BNI reverses the effect of U69,593 then the observed non-reversal of activity might be due to

ineffective washout; if it does not restore the activity, then it might be due to U69,593-mediated plasticity that altered the network activity (Schmid *et al.*, 2013).

3.4.3. Limitations

Data analysis comparing baseline and washout activity for experiment using nor-BNI to block KOR activation showed a statistically significant increase in the number of events. A possible explanation for this might be the activity of constitutively active KOR (Polter *et al.*, 2017). The application of nor-BNI might have blocked their constitutive activity resulting in the increase in spontaneous activity. To address this, the use of a KOR knockout mouse model would be appropriate.


A
Dose-response activity using varying U69,593 Concentrations
Baseline
Vehicle (0.005% EtOH)
Washout
LL2
V-root
Baseline
30 nM U69,593
Washout
LL2
V-root
Baseline
100 nM U69,593
Washout
LL2
V-root
Baseline
300 nM U69,593
Washout
RL2
V-root
10 mins
Baseline
1 µM U69,593
Washout
RL2
V-root
10 mins

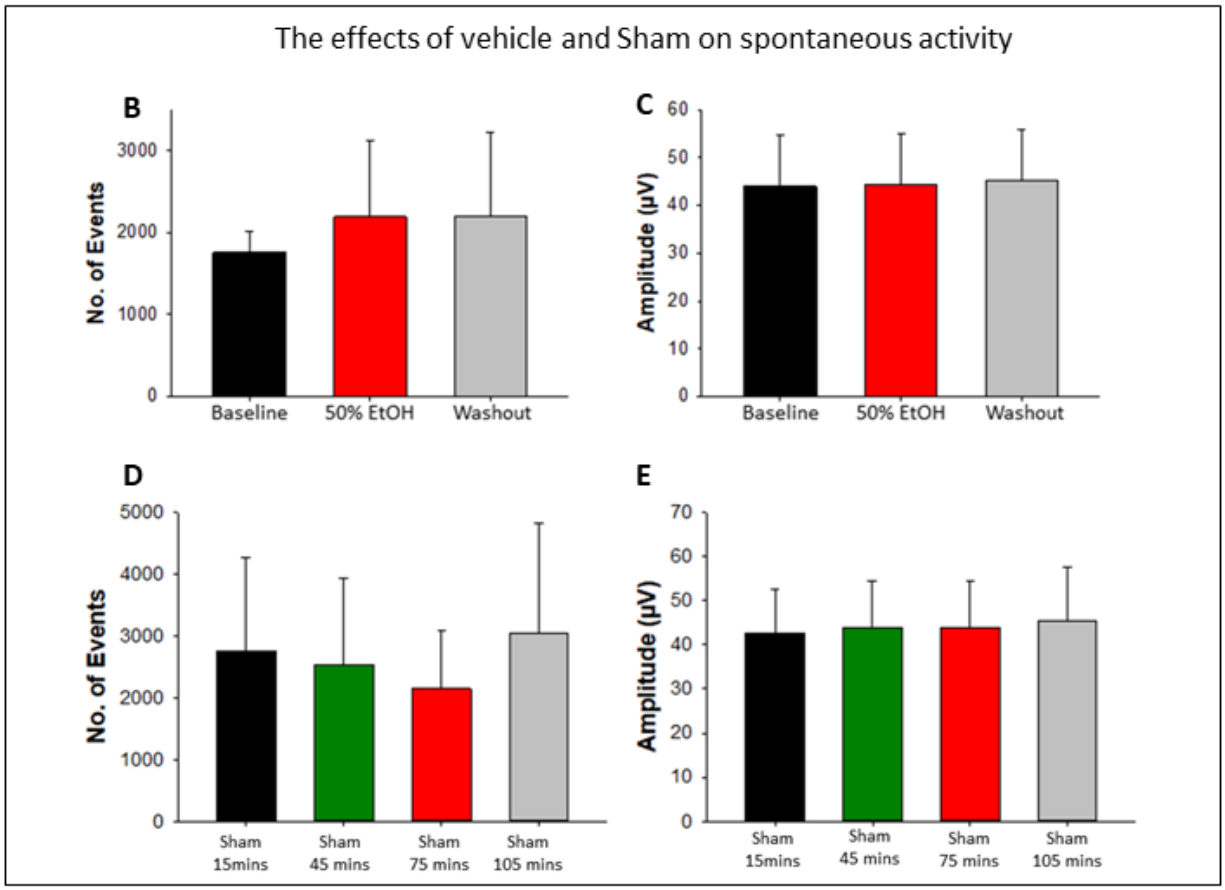
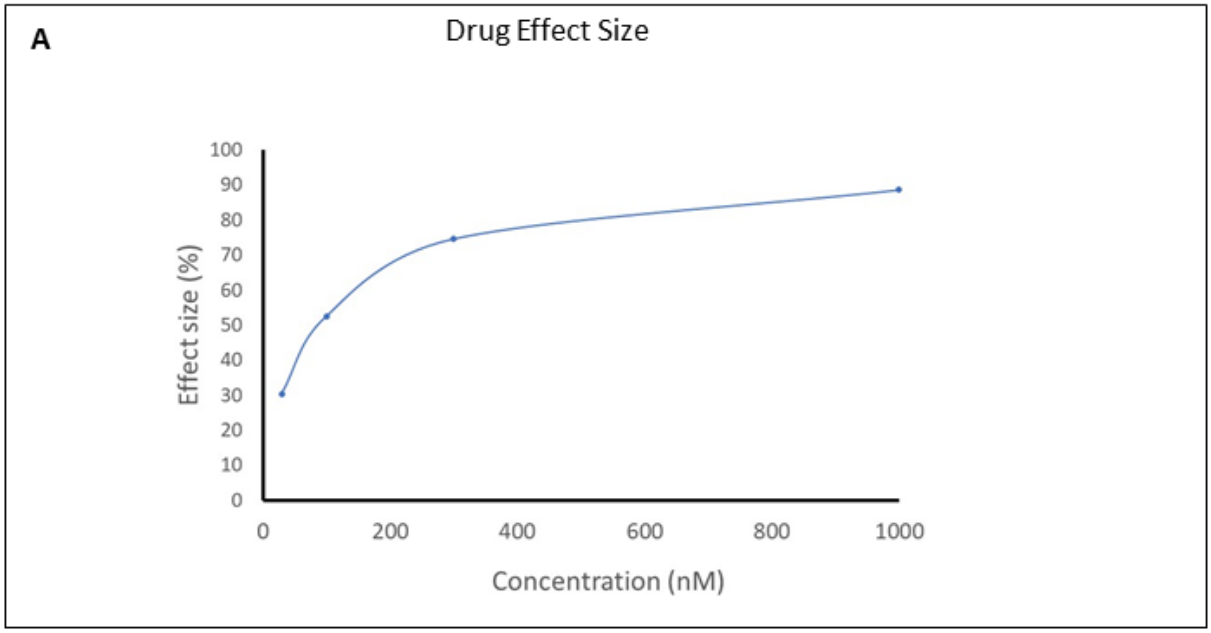

The effects of vehicle and Sham on spontaneous activity
B
No. of Events
3000
2000
1000
0
Baseline
50% EtOH
Washout
C
Amplitude (µV)
60
50
40
30
20
10
0
Baseline
50% EtOH
Washout
D
No. of Events
5000
4000
3000
2000
1000
0
Sham 15mins
Sham 45 mins
Sham 75 mins
Sham 105 mins
E
Amplitude (µV)
70
60
50
40
30
20
10
0
Sham 15mins
Sham 45 mins
Sham 75 mins
Sham 105 mins

Figure 3.2: KOR antagonist, U69593 suppressed spontaneous activity in a dose-dependent manner. Recorded traces and graphs showing dose-response and results of vehicle and sham experiments. A) Neurograms showing the effect of vehicle application, 30 nM, 100 nM, 300 nM, and 1 μM U69593. Neurograms were recorded from ventral roots of neonate mouse spinal cord (P0 – P3). Only representative traces shown. B) and C) Graph of data for vehicle experiments (50% ethanol) which shows that the vehicle had no significant effect on the number of events ($n = 6$; $P = 0.956$) or amplitude ($n = 6$; $P = 0.331$) of recorded neural activity. D) and E) Graph of result for sham experiments showing that there was no time-dependent effect on the number of events ($n = 4$; $P = 0.149$) or amplitude ($P = 0.079$) of the recorded neural activities for a duration of 105 minutes ($n = 4$; $P = 0.149$).


A
Drug Effect Size
Effect size (%)
100
90
80
70
60
50
40
30
20
10
0
0
200
400
600
800
1000
Concentration (nM)

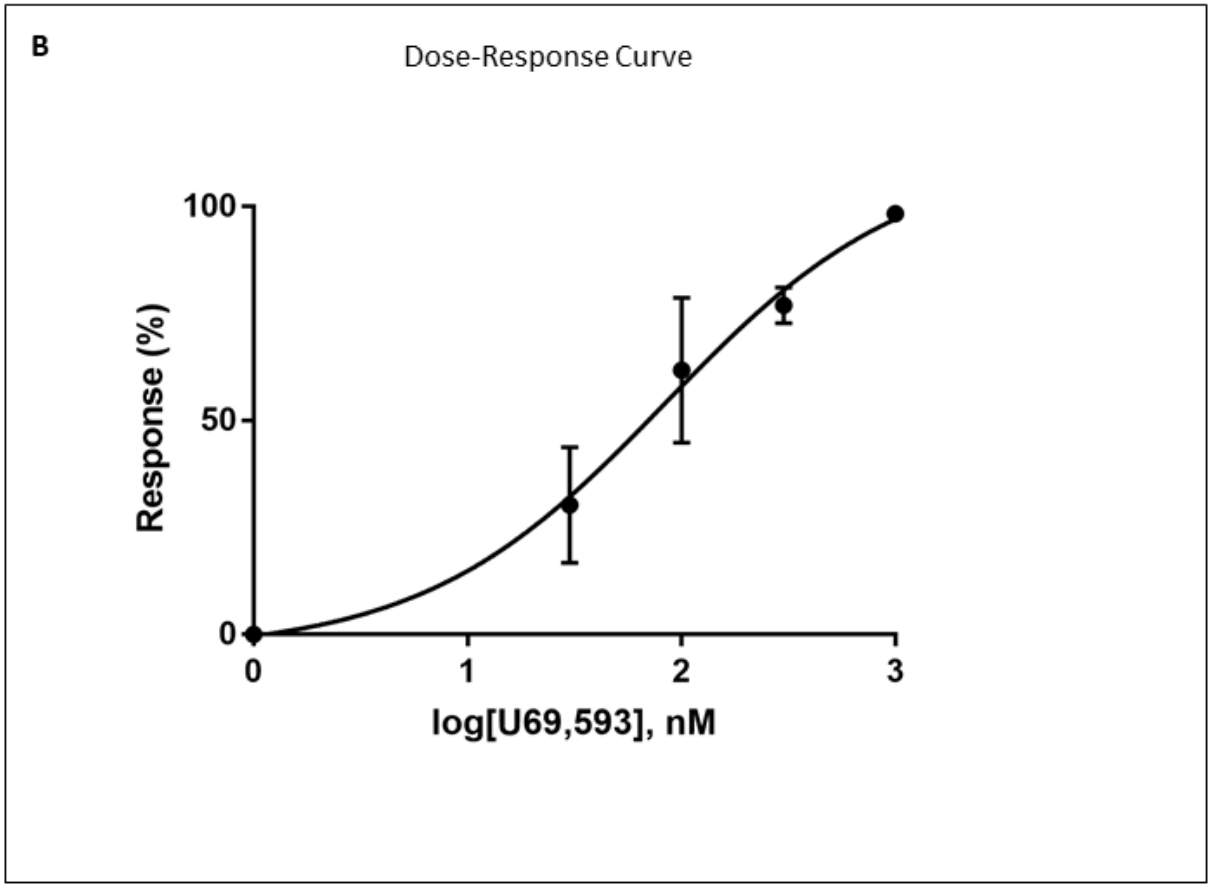
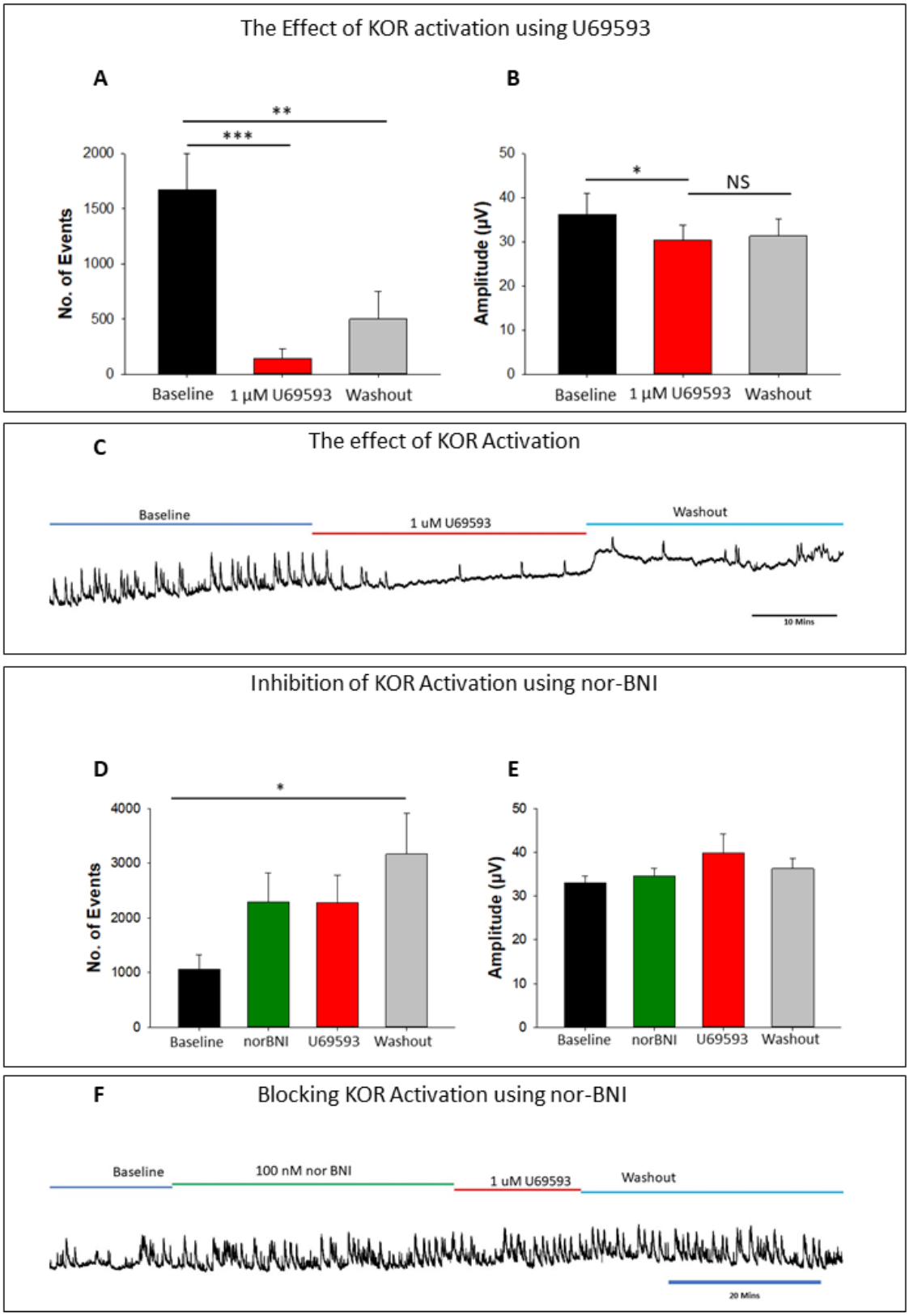

B
Dose-Response Curve
Response (%)
100
50
0
0
1
2
3
log[U69,593], nM

Figure 3.3: Dose response to KOR activation. A) Effect size of varying U69,593 concentrations: 30 nM, 100 nM, 100 nM, and 1 µM. B) Dose-response curve showing maximum KOR activation at 1 µM U69593 concentration. The IC_{50} was calculated from the fitted-line (4 parameter logistic equation) to be 83.68 nM (Log IC50 = 1.923, SEM = 0.372) . This was done using the GraphPad Prism Software (Version 7.04)


The Effect of KOR activation using U69593
A
B
**
***
No. of Events
2000
1500
1000
500
0
Baseline
1 µM U69593
Washout
Amplitude (µV)
50
40
30
20
10
0
*
NS
Baseline
1 µM U69593
Washout
C
The effect of KOR Activation
Baseline
1 uM U69593
Washout
10 Mins
Inhibition of KOR Activation using nor-BNI
D
E
*
No. of Events
4000
3000
2000
1000
0
Baseline
norBNI
U69593
Washout
Amplitude (µV)
50
40
30
20
10
0
Baseline
norBNI
U69593
Washout
F
Blocking KOR Activation using nor-BNI
Baseline
100 nM nor BNI
1 uM U69593
Washout
20 Mins

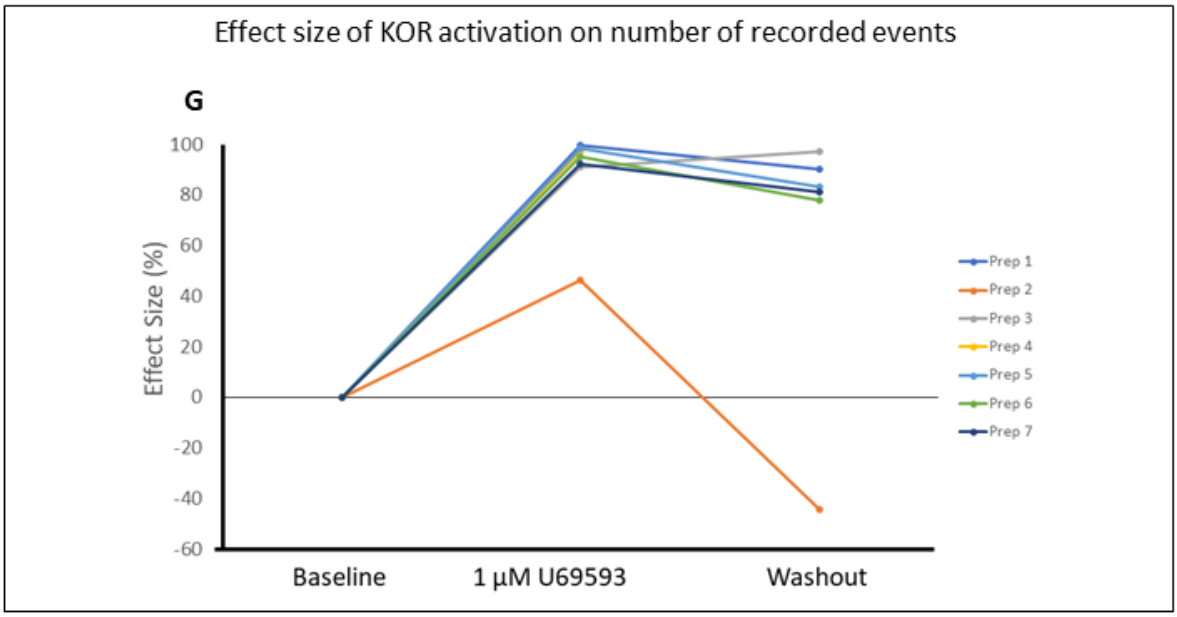


Figure 3.4: Results showing the effect of KOR activation using U69,593. A) and B) Charts showing the effect of KOR activation using 1 µM U69,593 on the number of events (n = 7; P = 0.002) and amplitude (n = 7; P = 0.029). C) A representative trace showing the inhibitory effect of KOR activation on spinal spontaneous activity using 1 µM U69,593. D) and E) Charts showing the blocking of KOR activation using 1 µM nor-BNI. Comparing baseline activity and activity after application of U6959 there is no significant decrease in the number of events (n = 7; P = 0.980) and amplitude (P = 0.768) of recorded activities. F). Representative traces showing the blocking of KOR activation using 1 µM nor-BNI. The sustenance of the baseline level of spontaneous activity can be qualitatively appreciated. G) Graph of effect size shows the percentage difference between baseline, U69593 application, and washout conditions. Since there are differences in baseline spontaneous activity levels between isolated spinal cord preparations, this graph shows the percentage difference all normalized to the baseline.

3.5. Effect of Kappa Opioid Receptor Activation on Presynaptic Inhibition

3.5.1. Introduction

The spinal cord in mammalian systems serves as the first integration and processing center for sensory signals entering the central nervous system (CNS). One mechanism employed by the spinal cord to filter the barrage of sensory stimuli it receives is known as presynaptic inhibition (PSI) (Hochman *et al.*, 2010; Shreckengost *et al.*, 2010). Sensory information carried by action potentials across a neuron can only be transferred to other neurons in that sensory pathway if the generated action potential is sufficient to cause depolarization of the axon terminal. This depolarization activates N-type Ca^{2+} channels causing an influx of Ca^{2+} which is necessary for the release of neurotransmitters into the synaptic cleft. PSI is accomplished by depolarization of the primary afferent terminals (primary afferent depolarization – PAD), therefore, leading to a reduction in Ca^{2+} influx at the terminals and consequently a decrease in neurotransmitter release. PSI is mainly mediated by the activity of a bi-synaptic or tri-synaptic network of GABAergic inhibitory interneurons within the dorsal horn.

Early in development, there is a predominant expression of the NKCC1 cation-chloride cotransporter. NKCC1 maintains a high intracellular Cl^- concentration such that the activation of $GABA_A$ receptor (chloride channel) becomes depolarizing (Kaila, 1994; Owens & Kriegstein, 2002; Vanhatalo *et al.*, 2005; Blaesse *et al.*, 2009; Hyde *et al.*, 2011). Therefore, activation of chloride channels (such as $GABA_A$ receptors) results in a depolarization of the cell. The antidromic electrotonically propagated depolarizing potential is known as dorsal root potential (DRP). PSI is achieved when action potentials reaching the sensory terminals results in neurotransmission that activates GABAergic interneurons. The release of GABA activates $GABA_A$ receptors expressed

on the presynaptic afferents causing the release of Cl^- from the neuron, resulting in depolarizing potentials that can be recording extracellularly at the dorsal roots as DRPs (figure 3.5. A).

Several studies have shown a dorsal-dominant expression pattern of KOR in the spinal cord. Studies in brain regions such as central amygdala, accumbens, and substantia gelatinosa neurons, and in the spinal cord, have revealed presynaptic activity of KOR activation (Besse *et al.*, 1990; Randić *et al.*, 1995; Kohno *et al.*, 1999; Kang-Park *et al.*, 2013; Tejeda *et al.*, 2017). The presynaptic presence of KOR on sensory afferents and its high expression in spinal dorsal horn prompted the question of whether KOR modulates DRP which might give insight into its mechanism of action in modulating spinal network activity. The work of Cabaj *et al.* (2017) suggests that neurotransmitters (such as serotonin) can modulate afferent inputs by acting on DRGs or neurons in the dorsal horn. Several other like studies have demonstrated the afferent modulation of spinal network or locomotor output (García-Ramírez *et al.*, 2014; Fidelin *et al.*, 2015). This section of my work, therefore, investigated the effect of KOR activation on generated DRPs, as it might give insight into the mechanism of dynorphin's neuromodulatory actions.

3.5.2. Results

3.5.2.1. Effect of KOR Activation on DRP

Bath application of 1 µM U69,593 was observed to significantly decrease the amplitude of the elicited DRPs (n = 5, Baseline = 828 ± 185.9, KOR = 760.4 ± 175.1, Washout 685.4 ± 259.6; p = 0.039) (figure 3.5. B). The result of antagonist data reveal that the observed effect was blocked using nor-BNI, a selective KOR agonist (n = 3, Baseline = 1099.8 ± 716.3, NorBNI = 1067.6 ± 676.7, KOR = 1056.8 ± 663.6, Washout 1089.9 ± 656.3; p = 0.396) (figure 3.5. C). DRPs took a

while to stabilize (120 minutes) before recording could commence. Differences in the baseline amplitude between isolated spinal cord preparations necessitated the normalization to baseline and calculation of the effect size (%) (figures 3.5. D). The monosynaptic reflex (MSR) was also recorded from the ipsilateral ventral root on the same segment as the stimulating electrode. KOR activation did not have any significant effect on amplitude of elicited MSR (n = 5; P = 0.093) (figures 3.6. A - C).

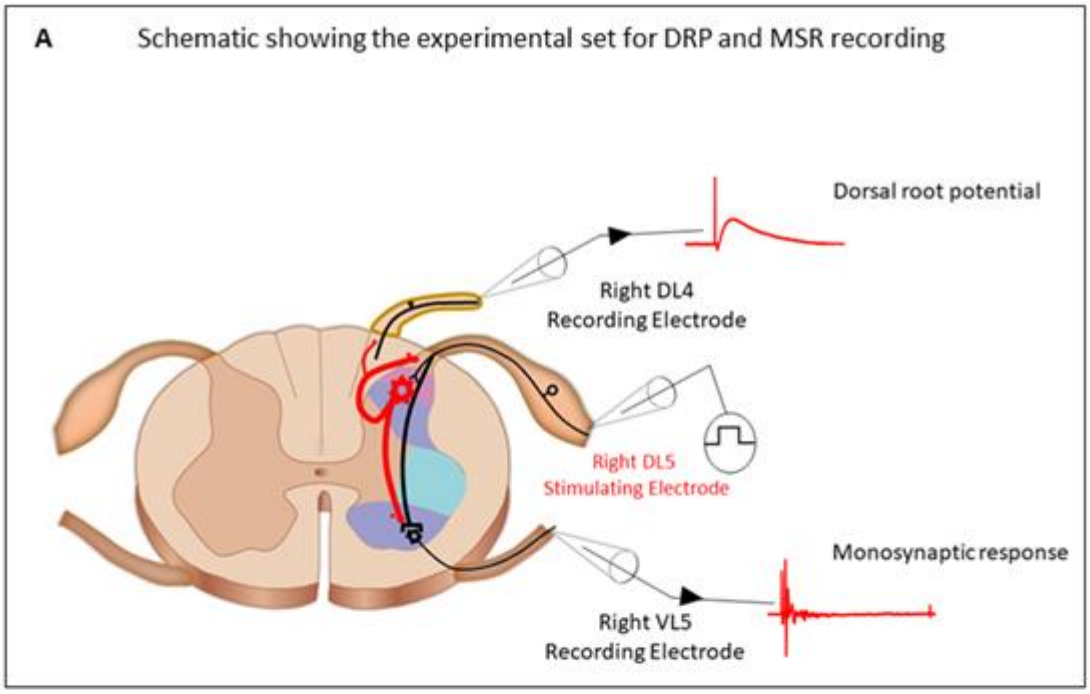
3.5.3. Discussion

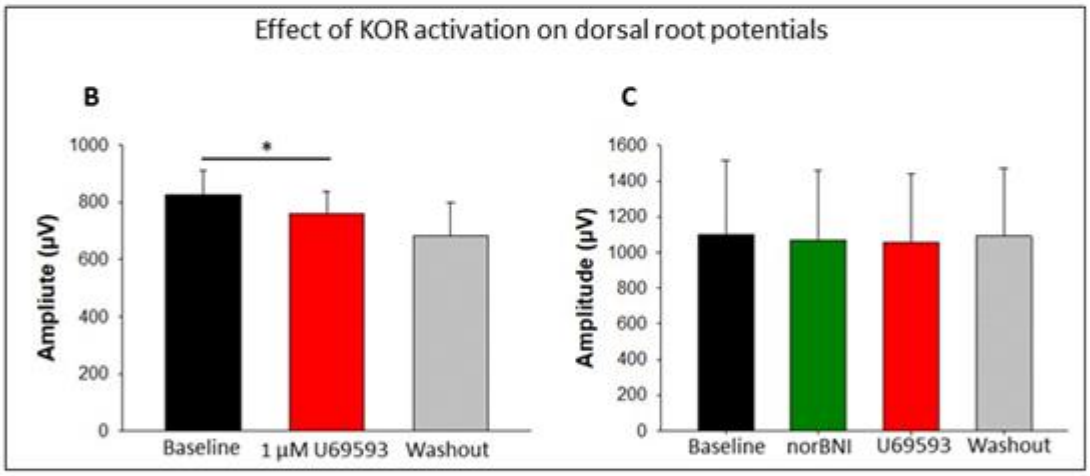
The observed decrease in DRP was consistent with the known activity of KOR. If U69593 activated KOR present in presynaptic afferents, this will result in the inhibition of N-type calcium current (Tallent *et al.*, 1994), leading to less activation of GABAergic interneurons, less GABA release and less $GABA_A$ receptor-mediated depolarization of the presynaptic afferent.

Further studies can confirm the involvement of GABAergic interneurons by blocking GABA receptors using picrotoxin and gabazine. It will also be interesting to study the expression of GABA (i.e. *vgat* – Slc32a1) in relation to KOR expression within the spinal cord. It was very challenging to obtain a stable MSR recording. This could be addressed using mephenesin which generally blocks polysynaptic activities. Although from the work of Shreckengost *et al.* (2010), mephenesin will cause an overall reduction in the MSR amplitude, it is still the best tool to reduce network synaptic inputs onto motoneurons in the MSR pathway. This will allow for more precise MSR recording and study of the effect of KOR activation on MSR.

What is the implication of PSI in movement? Studies have shown that presynaptic inhibition of spinal sensory neural feedback is necessary for the execution of smooth movement (Rudomin & Schmidt, 1999). The presynaptic inhibitory control of spinal GABAergic interneurons over

sensorimotor transmission is required for performance of precise limb movement (Fink *et al.*, 2014). Studies in patients with spasticity have shown that co-contraction of antagonist muscles in these patients is due to abnormal regulation of presynaptic inhibition and disynaptic reciprocal inhibition (Morita *et al.*, 2001).


A
Schematic showing the experimental set for DRP and MSR recording
Dorsal root potential
Right DL4
Recording Electrode
Right DL5
Stimulating Electrode
Monosynaptic response
Right VL5
Recording Electrode


Effect of KOR activation on dorsal root potentials
B
Ampliute (μV)
1000
800
600
400
200
0
*
Baseline
1 μM U69593
Washout
C
Amplitude (μV)
1600
1400
1200
1000
800
600
400
200
0
Baseline
norBNI
U69593
Washout

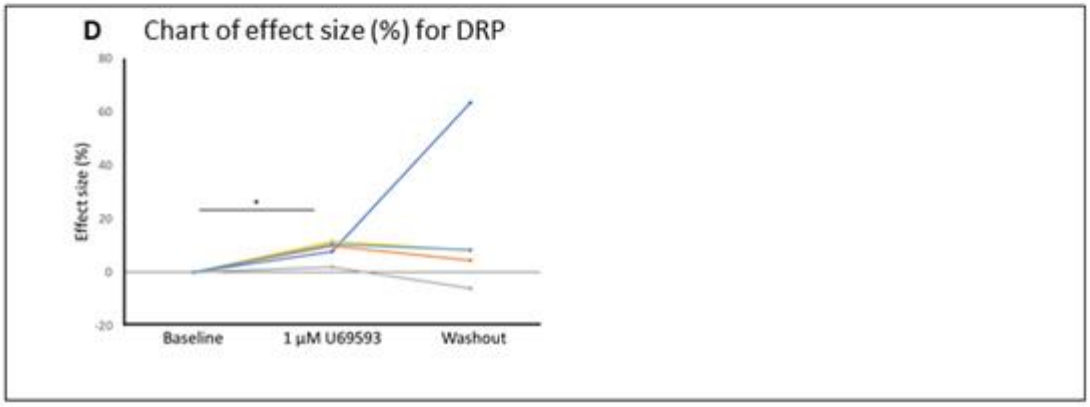

D
Chart of effect size (%) for DRP
Effect size (%)
80
60
40
20
0
-20
*
Baseline
1 μM U69593
Washout

Figure 3.5: Setup and results of DRP experiments: A) Diagram showing the setup for DRP and MSR recording. All recordings are made on the ipsilateral side of the spinal cord (Right side, on the diagram). The stimulating electrode is placed on the dorsal root L5 segment (DL5), DRP recording electrode placed on the adjacent dorsal root L4 segment (DL4), and MSR is recorded from the ventral root L5 segment (VL5). B) Chart showing significant decrease in DRP amplitude following KOR activation using 1 μM U69593 ($n = 5$; $P = 0.039$). C) Chart showing inhibition of KOR activity using 100 nM nor-BNI. D). Graph of the effect size (%) with values normalized to baseline.

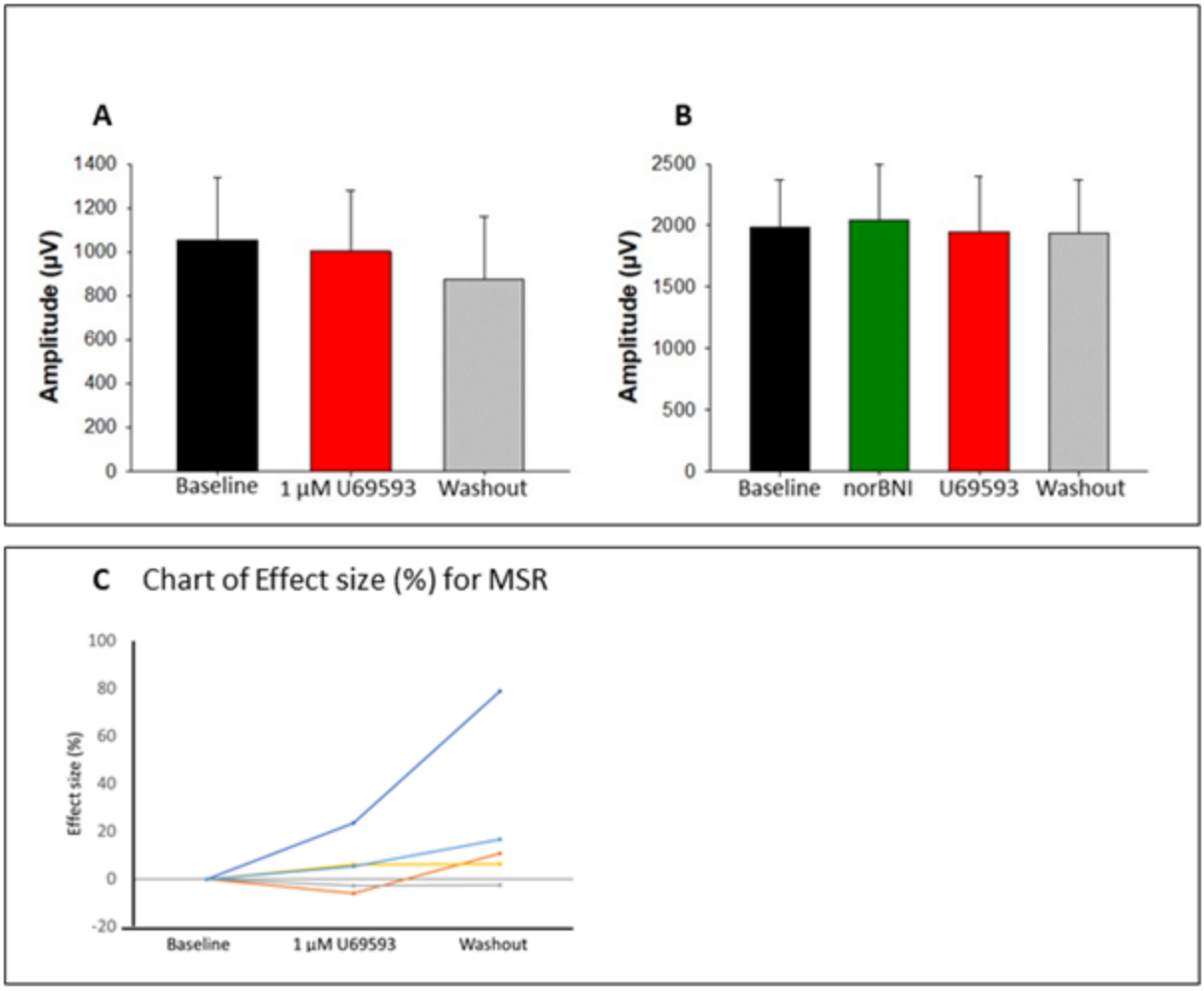


Figure 3.6: Results of MSR experiment: A). and B) Chart showing no significant effect of KOR activation on MSR amplitude (n = 5; P = 0.093). C) Graph is showing no significant effect in magnitude of MSR following KOR activation. However, the power of the performed test was 0.329, which was below the desired power of 0.800.

3.6. Effect of KOR Activation on Motoneurons

3.6.1. Introduction

Having studied the expression pattern of KOR and its modulatory effects on spinal network activity, my research then focused on looking at the impact of KOR activation on the intrinsic properties of motoneurons. Since the results of *in situ* hybridization assay (RNAscope assay) suggested that KORs are expressed on motoneurons, it is logical to expect that KOR activation should have some effects on the motoneurons that will, in turn, sculpt the activity of the entire network.

To study this, whole-cell patch clamp experiments were performed on fast-synaptically isolated motoneurons (figure 3.7. B).

The patch clamp technique allows for the study of certain key intrinsic membrane properties: resting membrane potential, membrane capacitance, input resistance, rheobase, afterhyperpolarization (AHP), persistent inward currents (PIC), post-inhibitory rebound (PIR), and spike frequency adaptation. Changes in these intrinsic properties might portend an effect on locomotor network by the treatment condition (figure 3.7. A).

3.6.2. Results

Changes in intrinsic motoneuron properties were studied in synaptically isolated motoneurons to ensure that there were no network activities synaptically modulating the properties of the cells or the activity of KORs.

3.6.2.1. Effect of KOR Activation on Input Resistance

Activation of KOR using 1 μM U69,593 resulted in a significant increase in the input resistance of motoneurons (n = 19; P = 0.002; post hoc analysis using Holm-Sidak method) (figure 3.7. D). An increase in input resistance is consistent with the inhibitory properties of KOR activation known to decrease the conductance of voltage-gated sodium and calcium channels (Chieng & Bekkers, 2001; Su *et al.*, 2002a, 2002b, 2009; Leffler *et al.*, 2012). Further studies are needed to identify the receptor(s) on motoneurons affected by KOR activation.

3.6.2.2. Effect of KOR Activation on Rheobase

Rheobase is the smallest current needed to initiate an action potential. Action potentials are usually generated when depolarization of neuron reaches the action potential threshold (about -55 mV) for motoneurons. KOR activation using 1 μM U69,593 resulted in a significant decrease in the rheobase (n = 19; P = 0.038) (figure 3.7. C). This is consistent with the effect on input resistance; an increase in the rheobase suggests that KOR activation altered certain conductance in the neuron to increase its excitability.

3.6.3. Caveats

Some further experiments are required to confirm the results. The data are still considered to have been obtained from putative motoneurons. This is because motoneurons were identified using parameters such as their high capacitance (88.73 ± 5.31 pF – which indicates cell size), input resistance (99.47 ± 6.45 MΩ), and ventrolateral positioning of the neurons in the lumbar spinal cord (Nakanishi & Whelan, 2010) (Figure 3.7. A). The gold standard for identifying motoneurons would be to fill the recorded cells with biocytin and perform a post hoc immunostaining for ChAT

and biocytin. Co-immunolabeling of these will confirm the identity of the patched cell as motoneuron. However, work from our lab and others suggests that using my approach alone identifies the motoneurons with a high degree of certainty (Nakanishi & Whelan, 2010). The second limiting factor to this study is that the experiments blocking KOR activation (using nor-BNI) have not been done, however, my data show that nor-BNI effectively blocks U69,593's actions.

3.6.4. Future Directions

It will be of interest to investigate whether KOR activation and modulation of motoneuron activity also happens indirectly by its action on postsynaptic neurons in the network. This can be studied by recording mini-inhibitory/excitatory postsynaptic currents (mini-IPSCs/mini-EPSCs) in synaptically intact motoneurons. The pervasive expression pattern of KOR in the spinal cord suggests that it might also mediate its neuromodulatory effect on spinal network/locomotor activity by other indirect mechanisms.

A

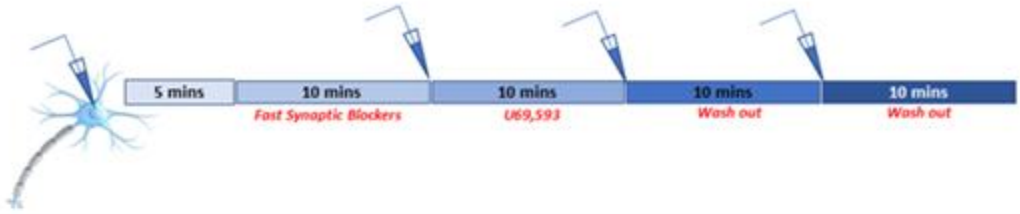
Summary of Intrinsic membrane properties of motoneurons

(Nakanishi & Whelan, 2010)

Property	Average	Std Dev.	P0-P3 Mean	P0-P3 SE	P8-P11 Mean	P8-P11 SE
Cm (pF)	113.74	35.41				
Ra (Mohms)	13.00	5.38				
Vm (mV)	-80.57	6.13				
Rin (Mohms)	107.38	54.76	99.47	6.45	87.27	10.23
AP Amplitude (mV)	73.59	7.86	74.06	1.16	74.90	2.47
AP Time-to-peak (ms)	0.88	0.23	0.98	0.04	0.81	0.06
AP Threshold (mV)	-54.05	3.70	-51.86	0.77	-56.00	2.12
Rheobase Current (pA)	210.00	70.19	157.60	8.41	287.70	40.71
First Spike Freq (Hz/pA)	0.16	0.12				
Average Freq (Hz/pA)	0.08	0.03				
Steady state Freq (Hz/pA)	0.07	0.03				
Number of Mice (n)	n=14		n=72		n=36	

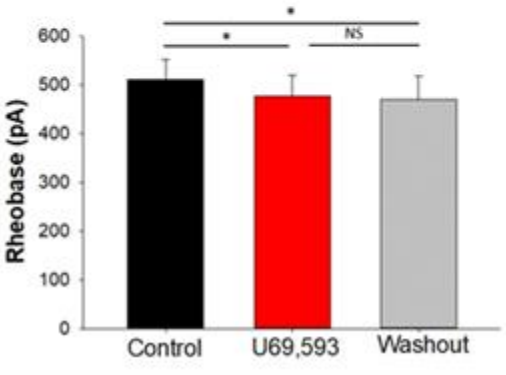
B

Procedure for motoneuron patch clamp experiment

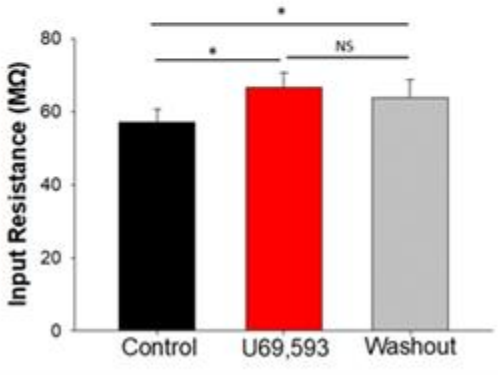


Effect of KOR activation on intrinsic properties of motoneurons

C



D



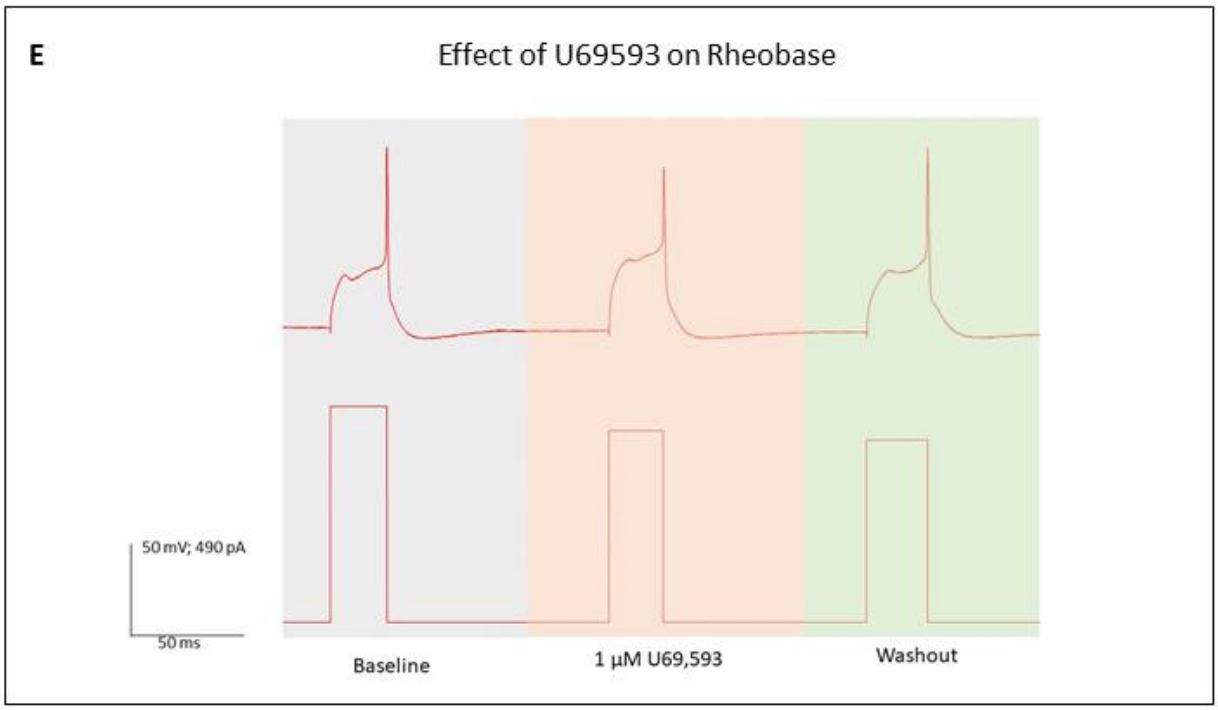


Figure 3.7: Effect of KOR on intrinsic membrane properties of motoneurons: A) Intrinsic membrane properties of motoneurons without addition of any drugs. This is compared to data published earlier by the Whelan lab (Nakanishi & Whelan, 2010). B) Flow-chart for patch clamping experiment. C) Effect of KOR activation on input resistance (n = 19; P = 0.002). D). Effect of KOR activation on rheobase (n = 19; P = 0.038). E). Representative trace showing the effect of U69,593 on rheobase: Baseline = 800 pA; 1 µM U69,593 = 790 pA; Washout = 750 pA.

3.7. General Discussion

While most studies on dynorphin focus on its role in addiction and anti-nociception, the few studies on its effect on motor function have only been behavioral. My work adds to the literature by providing data showing, at the network and cellular level, the modulatory effects of dynorphin and KOR activation on spinal motor network activity.

The only study of dynorphin's modulatory role using electrophysiological measurements considered its effects on muscle activity. Two main limitations affect the interpretation and application of data obtained from this study: 1) Recording muscle activity (i.e., population electromyographical activity) is not sufficient to explain the effect of dynorphin on the cells enervating the muscles, that is, motoneurons. 2) It can be argued that the concentrations of dynorphin inducing observed paresis in the work of pioneers like Yaksh are not physiological (Stevens *et al.*, 1987). Different concentrations of the same neuromodulator can mediate different effects. For instance, studies in the Whelan lab have shown that varying concentrations of dopamine can result in different patterns of rhythmic motor activity (Sharples & Whelan, 2017).

My work is the first to study the modulatory effects of dynorphin/KOR activation on spinal motor networks using extracellular recording techniques. Spontaneous activity, though poorly understood, provides an index into neural activities that occur in maturing motor circuits and networks. The use of neonates (P0 – P3) in this study to elucidate the effects of dynorphin on developing motor networks is important because it provides an understanding of the role of peptides at birth, and the activities of developing network model those observed during regeneration and recovery following spinal cord injury.

The primary purpose of my work was to elucidate the effect of dynorphin on spinal network activity. Studies have shown that dynorphin and orexin are co-expressed and co-released from the

same vesicles. Electrophysiological recordings in the Whelan lab have demonstrated that orexin increases the excitability of motoneurons and increases the frequency of fictive locomotion in isolated mouse spinal cord preparations (Biswabharati *et al.*, - Submitted). The co-expression and co-release of dynorphin with a pro-locomotory neuropeptide, orexin, further hinted to the possible neuromodulatory properties of dynorphin on spinal motor output. I, therefore, hypothesized that dynorphin or KOR activation would not inhibit, but modulate spinal motor function and network activity.

3.7.1. KOR Activation Inhibits Spontaneous Activity

Results from my work studying the effect of KOR activation using U69,593 on neonate mice isolated spinal cord demonstrated an inhibitory effect. This is consistent with the mode of action of KORs, which are $G_{i/o}$-type GPCRs, known for their inhibitory functions. Several studies showing the expression pattern of KOR and prodynorphin in the spinal cord have revealed that they are ubiquitously expressed throughout the spinal cord.

From my thesis, I have demonstrated that bath application of KOR agonist results in a significant decrease in the number and amplitude of spontaneous neural activity in the lumbar spinal cord. KOR involvement was investigated by blocking the observed effect using the KOR-selective antagonist, nor-BNI. The effects observed were not time-dependent as no reduction in activity was seen in the sham experiments. Data from vehicle experiments ensured that the solvent for KOR agonist (U69,593) was not affecting spontaneous activity in any way.

Spontaneous activity is a general feature of the developing nervous system (O'Donovan, 1999). Studies have shown that spontaneous activity also plays an important role in axon guidance and pathfinding type. Knowing that dynorphin and KOR are ubiquitously expressed in the brain, it is

possible that they also play an important role in modulation spontaneous activity in the developing brain.

3.7.2. Effect of KOR Activation on Presynaptic Inhibition

My work also investigated the effect of KOR activation on PSI, a phenomenon that is necessary for the execution of smooth, precise movement. Results from my work show that activation of KOR results in increased PSI (i.e. decreased DRPs Amplitude). This was also verified by using nor-BNI to block KOR activation and consequently, prevent the decrease in DRPs. The observed effects were not due to spontaneous changes in the network resulting in fluctuations in DRPs. This is because the DRPs (before drug application) were recorded and plotted out in real time to observe the attainment of a stable baseline.

PSI is a control mechanism used to filter sensory neural inputs at the spinal cord. Fink *et al.* (2014) have shown that skilled movements require sensory feedbacks onto a network of GABAergic interneurons that exert their control via PSI. Genetic ablation of this class of *Gad 2*-expressing GABAergic interneurons was shown to impair Goal-directed movements. Previous studies have shown that KOR has a dorsal-dominant expression, especially in laminar I and II of the dorsal horn. These results suggest that KOR activation might be important for the execution of precise, skilled movements. For instance, presynaptic inhibition might be necessary to control the gain of reflexes while sensory afferents are actively firing during locomotion.

3.7.3. Effect of KOR activation on Intrinsic Membrane Properties of Motoneurons

Using whole-cell patch clamp techniques the effect of U69,593 on motoneurons was tested on fast-synaptically isolated motoneurons. My results show that KOR activation increases motoneuron

excitability by increasing input resistance and decreasing the rheobase. These data are still preliminary as it lacks nor-BNI antagonist experiments to further verify the involvement of KOR, but it is likely since nor-BNI effectively blocked actions of U69,593. This result is further supported by RNAscope assay result showing the expression of KOR on motoneurons. This implies that the effect of KOR activation on spinal motor networks is also mediated by direct activation of KOR present on motoneurons, the last neurons in the motor pathway.

3.7.4. Limitations

One limitation of this study was that KOR activation was done using a KOR agonist (U69,593) instead of dynorphin. While U69,593 is known to be highly selective for KOR-1 subtype, dynorphin is known to interact via all three known subtypes of KORs. Studies have also shown that dynorphin can mediate different effects depending on the receptor subtype it interacts with (Staley *et al.*, 1997)**.** On the other hand, using dynorphin will make interpretation of the data impossible as it will be difficult to tell which receptor subtype is mediating the observed effects. Moreover, dynorphin peptides will be susceptible to proteolytic degradation and will make it difficult to tell what concentration of the peptide is reaching the receptors and mediating the effects. The solution to this problem will be to perform these experiments using dynorphin on knockout mouse expressing one subtype of KOR.

Another limitation is with the patch clamp experiments. While fast neurotransmission involving AMPAR, NMDAR, Glycine and GABA receptors were blocked, cholinergic neurotransmission was left intact. Therefore, the contribution of spinal cholinergic interneurons such as the $V0_c$ is not accounted for. Cholinergic neurotransmission has been recently shown to be important for the control of motoneuron excitability and spinal locomotor output (Zagoraiou *et al.*, 2009; Bertuzzi

& Ampatzis, 2018). This is even more important as studies have shown that $V0_c$ interneurons make direct synapse onto motoneurons (Frank, 2009). This confound can be resolved using the nicotinic acetylcholine blocker, mecamylamine.

3.7.5. Future Directions

To further validate the involvement of dynorphin and KOR activation in mediating the observed neuromodulatory effects, the expression of KOR relative to GABAergic interneurons within the spinal cord will need to be done. Several studies have shown that dynorphin can act pre-synaptically and post-synaptically. Therefore, it will be important to know whether dynorphin also directly modulates the activity of GABAergic interneurons.

The analgesic property of dynorphin has been well studied. However, it will be interesting to see how dynorphin modulates locomotion and pain, together. This can be accomplished using the leg-attached preparation. I obtained some preliminary data showing the effect of dynorphin on locomotion. Fictive locomotion was initiated using the following drugs: N-methyl-D(L)-aspartic acid (NMDA, 5 µM), dopamine (DA, 50 µM) and serotonin (5-HT, 10 µM). My preliminary data show that application of 1 – 3 µM U69,593 did not stop fictive locomotion. Instead, these concentrations of dynorphin altered the duration and phase of the locomotor bursts. These data suggest that dynorphin may not just inhibit movement as the data from spontaneous activity experiment might indicate. Rather, dynorphin will modulate the network activity to improve locomotor output. Going back to the original motivation for this work, it will also be important to test the effect of dynorphin and orexin (co-applied) on fictive locomotion. This will yield insight as to why there are a co-expression and co-release of dynorphin and orexin.

3.7.6. Conclusions

The result of my work has demonstrated that dynorphin/KOR activation does not just inhibit but modulates spinal network activity. While KOR activation acts on the entire network to decrease spontaneous activity, it increases presynaptic inhibition in the dorsal horn, a phenomenon necessary for filtering and integrating sensory feedback to improve motor output. The result of my research has contributed to filling the knowledge gap necessary for understanding the role of dynorphin in modulating spinal network activity and locomotor output. Furthermore, two most important symptoms of spinal cord injuries (SCIs) are neuropathic pain and motor impairment. The well-established antinociceptive property of dynorphin in conjunction with its neuromodulatory effects on spinal network output makes it a very important target in the treatment of spinal cord injuries and other movement-related disorders.

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