DOPAMINERGIC MODULATION OF THE AUTONOMIC NERVOUS SYSTEM: *IN VITRO* AND *IN VIVO* EVIDENCE FROM THE MOUSE

by

Tracy L. Johnson

Director of Thesis: Stefan Clemens, Ph.D.

Home Department: Department of Physiology

Central nervous system (CNS) function depends on both the connections between the underlying neurons and neural circuits and their activity. Neuronal activity in turn can be classified as neurotransmission and neuromodulation, where neuromodulation serves as a means to adapt neurotransmission to the different behavioral needs of the animal. Here, using the well-described monosynaptic stretch reflex (MSR) spinal circuit as a tool, we compared in the *in vitro* mouse spinal cord preparation the modulatory actions of DA in spinal lumbar segments that mediate somatosensory input and locomotor output with segments that additionally house the final common output of the autonomic nervous system (ANS). As the ANS contributes to the activation of the cardiac system and as a dysfunction of the DA system and the DA D3 receptor is associated with an increased prevalence of hypertension, we next addressed the potential role of DA modulation on the ANS *in vivo*, and in particular its role in hypertension and with age.

In the first part of this thesis, we provide evidence that DA exerts opposing modulatory effects in ANS-containing segments when compared to segments void of ANS innervation and that this switch in DA modulation in the thoracic spinal cord is reversed by gap junction blockers.

In the second part of the study, we show that aging-related increases in blood pressure and cardiac function in control wild-type (WT) animals were accompanied by bradycardia in the oldest animals. Interestingly, young D3 receptor knockout (D3KO) mice displayed blood pressure and heart rate values that were significantly increased over their age-matched WT controls but similar to those of the old WT group. Ultrasound echocardiography revealed aging-related increases in heart ventricle size in WT animals, but no similar changes in D3KO. In contrast, functional analyses revealed that ejection fraction and fractional shortening were compromised in old WT animals and similar to young D3KO mice. Subsequent histological assays demonstrated an aging-related interstitial fibrosis that peaked in old WT and that was similar to old WT in young D3KO mice.

Taken together, our data suggest that DA–mediated neuromodulatory actions of spinal cord circuits are dependent on the underlying spinal circuitry, and they suggest that a dysfunction of the D3 receptor pathway is sufficient to mimic the increased hypertension and cardiac remodeling observed in the aging heart.

DOPAMINERGIC MODULATION OF THE AUTONOMIC NERVOUS SYSTEM: *IN VITRO* AND *IN VIVO* EVIDENCE FROM THE MOUSE

A Thesis

Presented to the Faculty of the Department of Physiology

Brody School of Medicine at East Carolina University

In Partial Fulfillment of the Requirements for the Degree

Master of Science in Biomedical Science

by

Tracy L. Johnson

June 2012

© Tracy L. Johnson, 2012

DOPAMINERGIC MODULATION OF THE AUTONOMIC NERVOUS SYSTEM:

IN VITRO AND *IN VIVO* EVIDENCE FROM THE MOUSE

by

Tracy L. Johnson

APPROVED BY:

DIRECTOR OF THESIS	Stefan Clemens, Ph.D.
COMMITTEE MEMBER	Alexander Murashov, M.D., Ph.D.
COMMITTEE MEMBER	Kori Brewer, Ph.D.
COMMITTEE MEMBER	Richard Ray, Ph.D.
CHAIR OF THE DEPARTMENT OF PHYSIOLOGY	Robert Lust, Ph.D.
DIRECTOR OF THE BIOMEDICAL SCIENCE MASTERS PROGRAM	Richard Franklin, Ph.D.
DEAN OF THE GRADUATE SCHOOL	Paul J. Gemperline, Ph.D.

DEDICATION

I dedicate my work in graduate school to my late father, Cecil Edward Denison, Jr. I was a Daddy's girl from the start and to lose you was the hardest thing I've ever had to endure. 14 years later and I still think about you every day. I hope you know how much I still love and miss you.

ACKNOWLEDGMENTS

First and foremost, I would like to thank my mentor, Dr. Stefan Clemens, for allowing me to work in his lab for the past 2.5 years. Easy, uncomplicated, and painless are not words I would use to describe my years in graduate school. However, Dr. Clemens not only welcomed me into his lab, but he made me feel wanted and even though things were mostly hard, complicated, and painful (all from my end), he never gave up on me.

I thank my committee, Dr. Stefan Clemens, Dr. Kori Brewer, Dr. Richard Ray, and Dr. Alexander Murashov for giving me their time and guidance in this process.

I thank Dr. Benjamin Keeler for his support and advice, without which I would have never been able to finish my degree. I couldn't have asked for a better person to share the lab with during graduate school.

I thank my friend, Fatiha Moukdar, who encouraged me every single day of graduate school to keep going. I am leaving North Carolina with two things I didn't have when I came here: a Master's Degree and the best friend I'll ever have.

I thank the faculty, graduate students, and staff of the Physiology Department, particularly Dr. Robert Lust, who greatly facilitated my transfer from the PhD program to the Master's Program.

I thank Dr. Richard Franklin and the Biomedical Sciences Masters Degree Program for allowing me to complete my degree.

I thank Jim and Sheryl York and Cecil E. Denison, Jr., for being the best parents I could have asked for in my life.

Last, but not least, I thank my family: my husband, Matt and children, Nathan and Abigail for their love and support during the past 3 years.

TABLE OF CONTENTS

LIST OF TABLES……….……………………………………….…….……………….............

LIST OF FIGURES.……………………………………….…………………..…………

LIST OF ABBREVIATIONS………………………………..………….……........................

CHAPTER 1: GENERAL INTRODUCTION………………………….........................1

Definitions……….………………………………………………………………..……

Specific Aims………………………………..……………………………………

CHAPTER 2: METHODS………..……………………………………………….………..10

CHAPTER 3: SEGMENT-SPECIFIC CHANGES IN SPINAL CORD CIRCUITRY

LEADING TO DIFFERENTIAL DOPAMINERGIC MODULATION OF REFLEXES……22

Summary.……………………………………………………………….…………

Introduction.………………………………………………………………………….…

Results.………………………………………………………………………..……

Discussion.………………….……………………………………………………..

CHAPTER 4: THE DOPAMINE D3 RECEPTOR KNOCKOUT MOUSE MODELS

AGING-RELATED CHANGES IN AUTONOMIC FUNCTION………………..……......73

Summary.…………………………………………………………………………..…

Introduction.…………………………………………………………….……………

Results.…………………………………………………………………..…………

Discussion…………………………………………………………………………

CHAPTER 5: CONCLUSION…….…………………………………..……………….....99

REFERENCES.…………………………………………………………………….....103

APPENDIX….……….…………………………...……………………….…………….…117

Animal user protocols..………………………………………………………………..

LIST OF TABLES

CHAPTER 3: SEGMENT-SPECIFIC CHANGES IN SPINAL CORD CIRCUITRY LEADING TO DIFFERENTIAL DOPAMINERGIC MODULATION OF REFLEXES

Table 3.1 Summary of differential MSR DA responses

CHAPTER 4: THE DOPAMINE D3 RECEPTOR KNOCKOUT MOUSE MODELS AGING-RELATED CHANGES IN AUTONOMIC FUNCTION

Table 4.1: Animal weights

Table 4.2: Echocardiogram analysis

Table 4.3: Morphometric analysis

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

Figure 1.1: Model of dose-dependent modulation of DA

CHAPTER 2: METHODS

Figure 2.1: Electrophysiology experimental setup

Figure 2.2: Typical example of an ultrasound echocardiogram

CHAPTER 3: SEGMENT-SPECIFIC DIFFERENCES IN SPINAL CORD CIRCUITRY LEADING TO A REVERSAL OF DOPAMINERGIC REFLEX MODULATION

Figure 3.1: Stimulation of the lumbar, thoracic, and sacral dorsal roots evoked short-latency monosynaptic and longer latency reflex

Figure 3.2: Presence of MSR and LLR in thoracic, lumbar, and sacral segments

Figure 3.3: DA and DA receptor agonist and antagonist modulation of MSR in lumbar and IML-containing segments

Figure 3.4: DA and DA receptor agonist and antagonist modulation of LLR in lumbar and IML-containing segments

Figure 3.5: 5-HT and NA modulation of MSR in lumbar and IML-containing segments

Figure 3.6: 5-HT and NA modulation of LLR in lumbar and IML-containing segments

Figure 3.7: MSR response to gap junction blockers in combination with DA and DA receptor agonists and antagonists in IML-containing segments

Figure 3.8: Potential model for Specific Aim #1

CHAPTER 4: THE DOPAMINE D3 RECEPTOR KNOCKOUT MOUSE MODELS AGING-RELATED CHANGES IN AUTONOMIC FUNCTION

Figure 4.1: Blood pressure, heart rate, and rate-pressure product of awake WT and D3KO animals

Figure 4.2: Echocardiogram traces from 2-month-old, 1-year-old, and 2-year-old WT and 2-month-old D3KO animals

Figure 4.3: Interstitial fibrosis in WT (2 months to 2 years) and young (2 months) D3KO animals

LIST OF ABBREVIATIONS

5-HT	Serotonin (5-hydroxy-tryptamine)
ACSF	Artificial cerebrospinal fluid
ANS	Autonomic Nervous System
cAMP	Cyclic adenosine monophosphate
CNS	Central Nervous System
CPG	Central pattern generator
D3KO	D3 receptor knockout
DA	Dopamine
GI	Gastrointestinal
IML	Intermediolateral cell column
LLR	Long-latency reflex
MCSA	Myocyte cross-sectional area
MMP	matrix metalloproteinase
MSR	Monosynaptic reflex
NA	Noradrenaline
NIA	National Institutes of Aging
PNS	Parasympathetic Nervous System
RLS	Restless Legs Syndrome
RPP	Rate pressure product
SEM	Standard error
SNS	Sympathetic Nervous System
SPN	Sympathetic preganglionic neuron

SRA	Spinal reflex amplitude
WT	Wild type

CHAPTER 1: GENERAL INTRODUCTION

Spinal Cord and the Autonomic Nervous System

The central nervous system (CNS) is comprised of the brain and spinal cord and has two divisions: somatic and autonomic, both of which are present in the spinal cord. The somatic nervous system innervates skeletal muscle and controls voluntary movement, while the autonomic nervous system (ANS) innervates smooth and cardiac muscle as well as glands, and controls involuntary functions (adapted from www.ndrf.org). The ANS is further subdivided into the sympathetic nervous system (SNS) and the parasympathetic nervous system division (PNS). While the components of the somatic nervous system are located in each segment of the spinal cord, the ANS is represented only in some segments: in humans, the SNS is located in the spinal segments T1-L2, while the PNS is located in the brainstem and spinal segments S2-S4 (Charles Watson, 2008). These spinal cord segments contain the final common output of the autonomic nervous system – the sympathetic preganglionic neurons (SPNs). The specific neuronal circuitry found in each segmental division in the spinal cord is at the center of attention for numerous research laboratories, but despite their efforts, spinal cord circuitry is still not fully understood. Identification of the anatomical and functional circuitry of the spinal cord has major implications for understanding both neurological diseases and injury states.

Nervous System Circuitry for Spinal Reflex

One of the simplest neural networks is the monosynaptic reflex (MSR) circuit. The coordination of the input and output aspects of this circuit contribute to the

contraction and relaxation of a muscle *in vivo* (Chen et al., 2003). This careful control of the state of the muscle is important during proprioception and helps determine the final motor output of spinal motor circuits (Rossi-Durand, 2006). A reduced spinal cord preparation is typically used to study the MSR and other types of reflex activity such as long-latency reflex (LLR) *in vitro* (Clarac et al., 2000, Chen et al., 2003, Rossi-Durand, 2006). As demonstrated first by Sherrington, the circuits for spinal reflex can be manipulated by descending systems to produce complex voluntary movements (Sherrington, 1906, 1910). However, reflex systems do not simply act in the background to perform the task of local regulation of voluntary movements that are more or less directly executed by descending systems; reflexes can be functional outside of the influence of the brain, and the spinal cord is by itself capable of producing not only reflexes, but also the coordinated, rhythmic movements of locomotion (McCrea, 1986). Importantly, spinal reflexes can be modulated by different pharmacological substances such as serotonin, noradrenaline, dopamine, glutamate, GABA, etc. (Carp and Anderson, 1982, Hounsgaard and Kiehn, 1989, Tanabe et al., 1990, Crick and Wallis, 1991, Kiehn et al., 1999, Levant and McCarson, 2001, Machacek et al., 2001, Clemens and Hochman, 2004, Clemens et al., 2006, Juvin et al., 2007, Lapointe et al., 2009, Clemens et al., 2012). As such, reflex modulation is an indication of the changes that can be imposed onto the hard-wired circuits in the spinal cord and that can effectively shape different behavioral outcomes to meet the needs of the animal (Marder and Calabrese, 1996, Clemens, 1998, 2011, Marder, 2011).

Dopamine

Dopamine (DA) is a monoaminergic neurotransmitter discovered over 50 years ago by Avid Carlsson, which has been shown to have wide-reaching effects in many areas of the body, including the brain, spinal cord, and organ systems outside of the CNS, such as the heart and kidney (Carlsson and Waldeck, 1958, Adam, 1980, Lokhandwala and Amenta, 1991, Jaber et al., 1996, Ozono et al., 1996, Ricci et al., 1998, Hussain and Lokhandwala, 2003, Banday and Lokhandwala, 2008). The actions of DA are widely studied in the CNS, primarily in the brain, where it plays a major role in reward-driven learning, motor control, and cognition, along with many other functions (Jaber et al., 1996); less studied are the effects of DA in the spinal cord. Evidence suggest that all DA neurons are located in the brain, but DA fibers also descend from the brain into the spinal cord exclusively from a small DA cluster in the A11 region of the dorsal hypothalamus (~150 neurons in the mouse, 300 in rat). They project extensively through the entire cord, including the deep dorsal horn, responsible for pain perception, the intermediolateral nucleus (IML), the final common output of the sympathetic nervous system, and the motoneuron pools, which control leg locomotor output (Bjorklund and Skagerberg, 1979, Skagerberg et al., 1982, Lindvall et al., 1983, Skagerberg and Lindvall, 1985, Holstege et al., 1996). Despite this well-established DA projection pattern, few studies have examined the modulatory actions of DA in the spinal cord. These studies have shown that DA can have effects on unidentified neurons in the IML that are slow and long lasting, and they can be inhibitory, excitatory or mixed, pointing to the modulatory potential of DA (Gladwell and Coote, 1999b, Gladwell and Coote, 1999a). The studies also revealed that the DA-induced inhibitory actions are controlled

by a direct membrane activation, whereas the excitatory actions are synaptically-mediated (Gladwell and Coote, 1999a). The first complete map of DA receptor expression and distribution in the rodent spinal cord was completed recently (Zhu et al., 2007) and it was reported that, in the lumbar spinal cord, all DA receptors are present throughout the spinal gray matter, and that at least a subset of motoneurons contains both D1 and D2 receptors alike. DA acts through two different receptor subfamilies, D1-like (D1, D5) and D2-like (D2, D3, D4) and has a lower affinity for the D1-like receptors compared with the D2-like receptors (Fig 2.1). There is mounting evidence that spinal neurons may be able to react to DA in a dose-dependent manner due to the differing affinities of DA receptors towards DA (Clemens et al., 2012).

Model of Dose-Dependent Modulation of DA

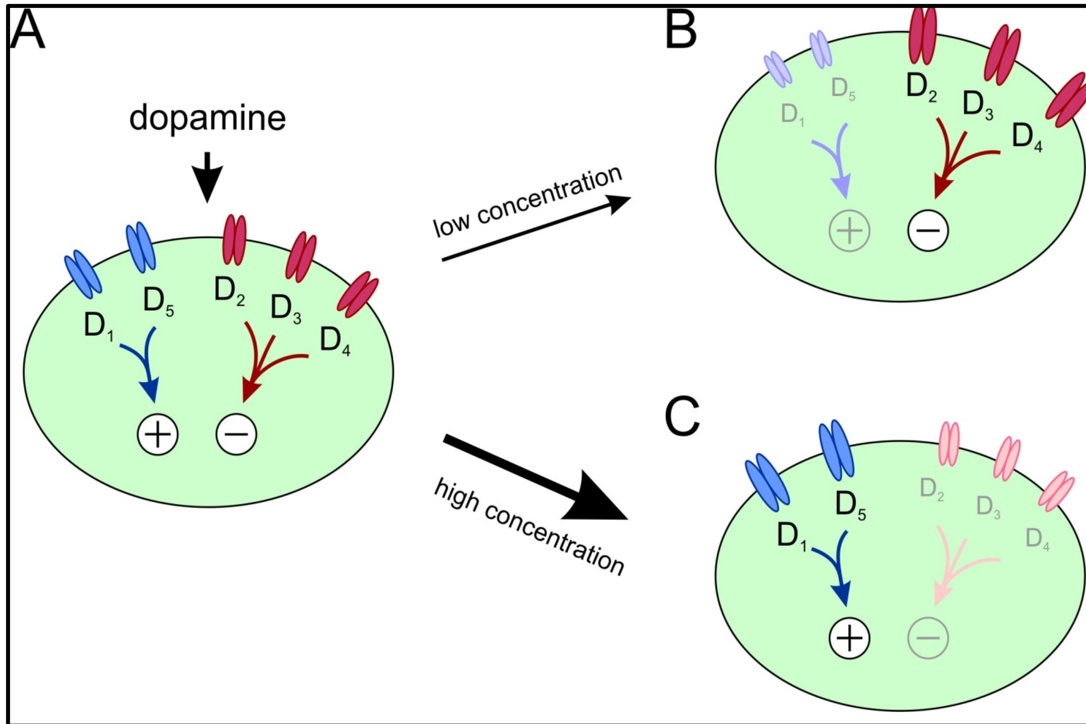


Figure 1.1

Figure 1.1 Legend

The affinity of the different receptor subtypes towards DA is D2-like >> D1-like. Thus under low DA conditions, DA will bind first to D2-like receptors, which tend to mediate overall inhibitory actions. At higher levels of DA, additional D1-like receptors will be activated, which generally mediate excitatory effects.

Studies on DA-mediated effects on spinal cord function have focused almost exclusively on the lumbar segments and the role DA may play in the recruitment of the central pattern generator (CPG) for locomotion. Recruitment of the CPG appears to occur at higher of DA concentrations (Cazalets et al., 1995, Han et al., 2007), presumably via D1-like receptor pathways (Han and Whelan, 2009). DA has also been shown to directly modulate spinal reflex in the lumbar segments of the spinal cord (L2-L5) (Clemens and Hochman, 2004). Together these data suggest that individual motoneurons in the spinal cord can adjust their output as a function of ambient DA release.

Specific Aim #1:

Test the hypothesis that dopamine modulates autonomic and somatic circuits differently *in vitro*.

Problem: Most studies focused on DA action in the spinal cord have focused on dopaminergic changes in spinal reflex only in the lumbar segments of the cord, and until this point it has been unknown if segments containing autonomic innervation have a similar change in reflex amplitude with the addition of DA.

Hypothesis: Changes in reflex amplitude in different segments of the spinal cord mediated by dopamine are dependent on the presence or absence of the ANS.

Approach: In the first part of this study, we studied the role of DA in modulating spinal reflex. Experimentally, monosynaptic and long-latency reflexes (MSR and LLR) are evoked in an *in vitro* rodent spinal cord preparation and dopamine is bath applied to observe a drug-dependent change in reflex (MSR, LLR, or both). Our study sought to

uncover if there might be segment-specific differential dopaminergic modulation of spinal cord reflex in thoracic, lumbar, and sacral segments of the mouse.

Aging-related Changes in Autonomic Output

In Specific Aim #1, we wanted to determine if DA would have different effects on spinal reflex in segments of the spinal cord with and without autonomic innervation using an *in vitro* approach. We next asked the question if a DA effect on the autonomic nervous system would also be relevant *in vivo*. Along with a clear role in the CNS and in modulating spinal cord reflexes, DA is also known to be involved in the development of hypertension and DA receptor populations are present in both the heart and the kidney (Adam, 1980, Lokhandwala and Amenta, 1991, Jose et al., 1992, Ozono et al., 1996, Ricci et al., 1998, Cavallotti et al., 2010). In particular, a role for the D3 receptor has been suggested in the increased blood pressure (Jaszlits et al., 1985, Jose et al., 2002, 2003, Polakowski et al., 2004, Yang et al., 2004, Zeng et al., 2004a, Staudacher et al., 2007). Interestingly, a genetically modified knockout animal for this receptor (D3KO) exhibits both hyperactivity and hypertension (Asico et al., 1998). The D3KO mouse has been introduced as a potential animal model for the sensory-motor and sleep disorder Restless Legs Syndrome (RLS)(Clemens et al., 2006, Paulus et al., 2007). Patients suffering from this disorder often also express hypertension (Winkelman, 1999, Hening et al., 2007, Winkelman et al., 2008, Walters and Rye, 2010) and RLS pathology and associated symptoms worsen with age (Ferri et al., 2008). In humans, treatment with D3-receptor agonists can remediate both RLS symptoms and the associated hypertension (Manconi et al., 2010). These findings then underline the

possibility that the D3 receptor system may play a pivotal role in the emergence of aging-related hypertension. While a recent study suggested a role of intrarenal DA deficiency on hypertension and decreased longevity (Zhang et al., 2011), in that effort the role of the D3 receptor was not addressed.

Specific Aim #2:

Test the hypothesis that in vivo ANS function is under DA modulatory influence.

Problem: Aging animals and humans exhibit increased blood pressure over the course of a lifetime. If the D3KO mouse shows an early increase in blood pressure, this animal could be used as a new model for advanced aging.

Hypothesis: The D3 receptor plays a key role in regulating global autonomic tone and the loss of this receptor will result in early-onset hypertension.

Approach: To study the role of DA on changes in cardiovascular components of the ANS, *in vivo* measurements of blood pressure, heart rate, functional echocardiography, and post-mortem fibrosis measurements are done on the aging WT mouse vs. the D3KO mouse. The results will show how a system-wide knockout of the functioning D3 receptor will affect the animal in terms of regulation of the ANS.

CHAPTER 2: METHODS

All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of East Carolina University according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (US NIH, Pub. No. 85-23, rev. 1996). Copies of the approval notices are submitted in the Appendix.

In vitro Electrophysiology Experiments

Animals

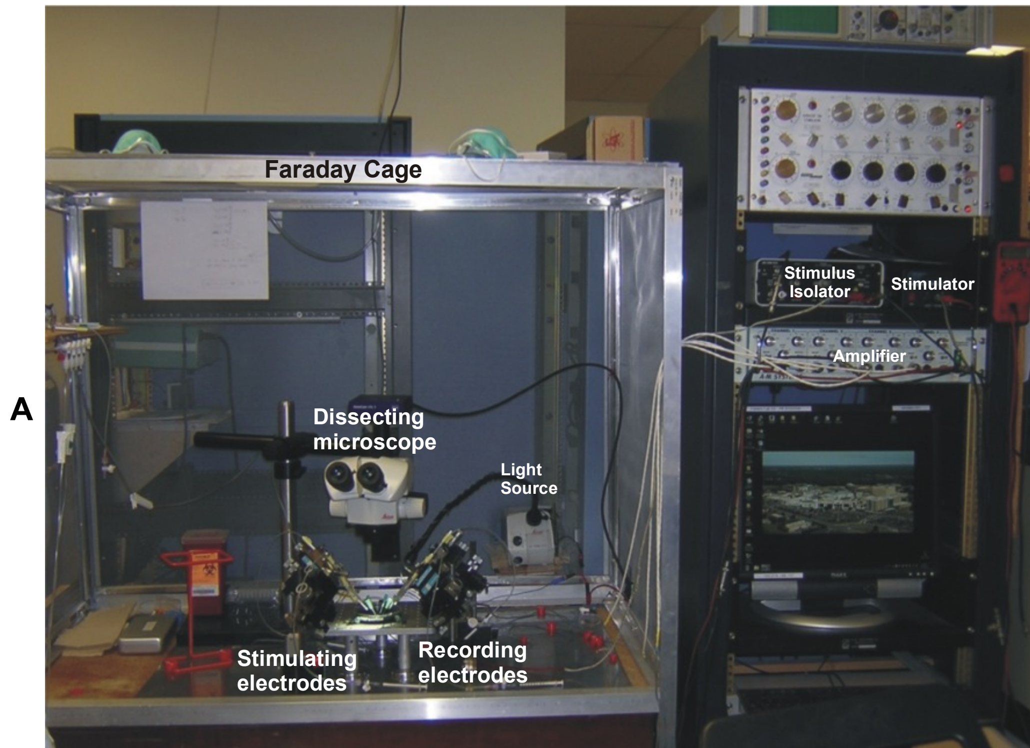
A total of 80 wild type mice (C57BL/6) of either sex (postnatal day 5 to 14) were anesthetized with an i.p. injection of a ketamine/xylazine mix (18mg/ml and 2mg/ml at 0.05 ml/10g body weight). The spinal cord was quickly dissected and placed in a Sylgard-lined (Dow Corning, Midland, MI) Petri dish in ice-cold sucrose artificial cerebrospinal fluid (ACSF) containing: sucrose 87g/L, D-glucose 4.5g/L, $NaHCO_3$ 2.2 g/L, KCl 0.19g/L, NaH_2PO_4 0.17g/L, $MgCl_2$ 0.15g/L, $CaCl_2$ 0.04g/L at a pH of 7.4, aerated with 95% O_2/5% CO_2. After the dura mater was opened (usually within 10 minutes of decapitation), the high sucrose-ACSF was washed out against regular ACSF containing: 7.5g/L NaCl, 0.14g/L KCl, 0.35g/L $CaCl_2$, 0.32g/L $MgCl_2$, 1.8g/L glucose, 0.16g/L $KHPO_4$, and 2.2g/L $NaHCO_3$ at a pH of 7.4, and oxygenated with 95% O_2/5% CO_2.

To record reflexes and to avoid interference from the cross-extension reflex circuitry, spinal cord preparations were hemisected mid-sagittally, which also served to better oxygenate the tissue and allow for better access of ACSF. Dorsal and ventral roots were identified and pinned out as needed with small Minutien pins (Fine Science

Tools, Foster City, CA). Glass suction electrodes were placed on the dorsal and ventral roots of thoracic segments (T6-T13), lumbar segments (L1-L6), or sacral segments (S1-S2). Stimulations and recordings were either done with two sets of recordings in parallel (e.g. one dorsal stimulus/ventral recording on a thoracic segment and one dorsal stimulus/ventral recording on a lumbar segment), or with one segment (e.g. one dorsal stimulus/ventral recording on a sacral segment)(see Fig 2.1). Dorsal roots were stimulated with constant current pulses of 50–500 μA, and a duration of 50–500 μs, at interstimulus intervals of 30–60 s. Reflex responses were recorded from the corresponding ventral roots and amplified with a four-channel extracellular AC amplifier (AM-Systems, Model 1700, Sequim, WA), and digitized with a Digidata 1322A, using pClamp 10.2 software (Axon Instruments, Union City, CA). To allow comparisons, reflex responses were rectified, and the calculated integrals of these responses were measured and compared between epochs of identical duration before and after drug application. Reflex amplitudes were normalized to the mean of the control values and are reported here as percentage changes from the control (pre-drug) conditions. Comparisons were made between the averaged amplitudes of the last 10 consecutive reflex responses measured before drug application and the averaged amplitudes of the last 10 consecutive reflex responses during the drug application.

Electrophysiology Experimental Setup

A



B

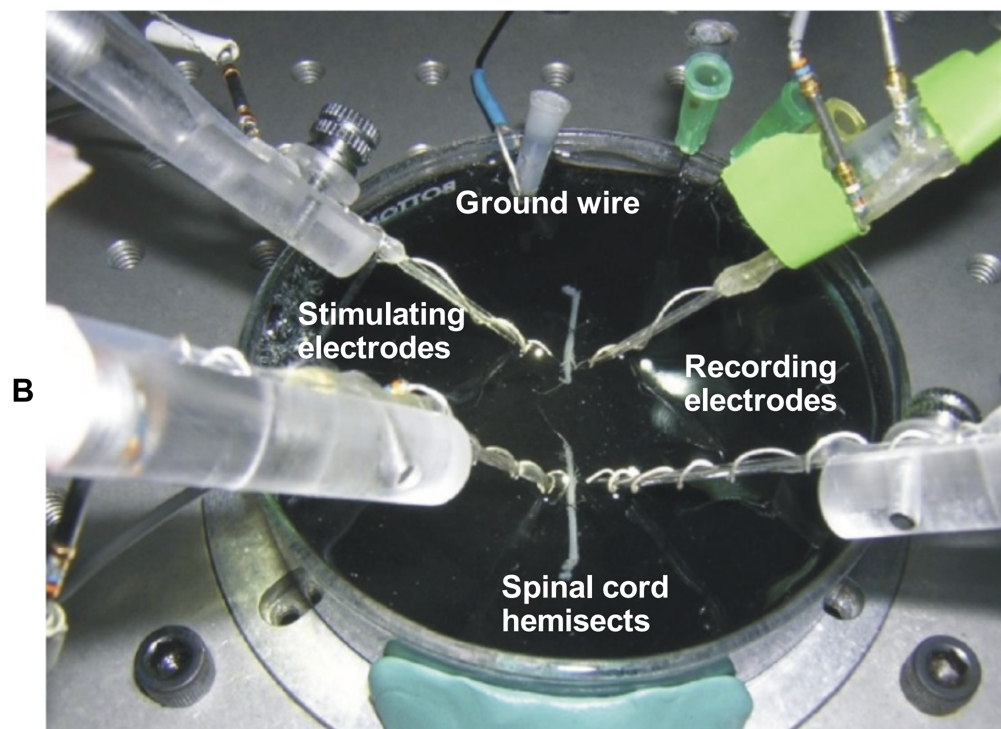


Figure 2.1

Figure 2.1 Legend

A. This photograph shows the Faraday cage setup for an *in vitro* electrophysiology experiment. The Faraday cage contains the dissecting microscope and the petri dish with the spinal cord hemisects in ACSF. Two pair of electrodes (two stimulating and two recording) are attached to matching dorsal and ventral roots of different segments of the spinal cord in order to record reflex activity.

B. This photograph shows the typical setup for an *in vitro* electrophysiology experiment. Shown are two hemisects of the same spinal cord, with the dorsal roots positioned on the left and the ventral roots positioned on the right. Two pairs of stimulating electrodes are attached to the dorsal roots on the left and two pairs of recording electrodes are attached to the ventral roots on the right. With this preparation, we can easily record from different segments in the same experiment.

Pharmacology

After a stable response in the ventral root recordings was established, typically 30–60 min after onset of the stimulation protocol, drugs (drug-dependently dissolved in distilled water or ethanol) were bath applied for durations of 30-60 min. The following chemicals were used in this part of the study: Dopamine (1 μM, together with 9 mM sodium meta-bisulfite, used as an antioxidant, both from Sigma-Aldrich). The DA receptor agonists applied included: quinpirole (D2 receptor agonist, 1 μM; Tocris Bioscience), raclopride (D2 receptor antagonist, 10 μM; Sigma-Aldrich), and SKF38393 (D1 receptor agonist, 10 μM; Tocris Bioscience). We also tested the effects of the other monoamines, noradrenaline (10 μM; Sigma Aldrich) and serotonin (5-HT, 10 μM; Tocris Bioscience). In addition to the monoamines, we also tested the effects of the gap junction blockers carbenoxolone (100 μM; Aldrich Chemical) and quinine (10 μM; Sigma Aldrich). Tests of the effects of the individual drug carrier substances or antioxidants (ethanol or $Na_2S_2O_5$) at their final concentrations used in the experiments did not lead to any of the effects on reflex modulation that were observed with the additional presence of the respective drug (data not shown).

Several drugs were often compared in the same animal. In these cases, following the drug application, we interrupted the stimulation protocol and washed the preparation (three to four times the bath volume over a 3–5 min period) with room temperature ACSF and subsequently let the preparation recover from the drug application for an additional 30-60 min. During this time, we repeated the stimulation protocol to assure the recovery of the reflex responses. In general, reflex responses returned to pre-drug amplitudes, and these “recovered” reflex amplitudes in turn

became the pre-drug control of the subsequent drug application. With this protocol we were able to test several drugs per experiment.

Statistical Analysis

All values are given as mean ±SEM. We used SigmaPlot 11.0 (SPSS Science, Chicago, IL) to analyze the data and test for significant differences in the course of an experiment using parametric or nonparametric tests where appropriate. Differences were considered significant if $p<0.05$.

In vivo Autonomic Evaluation and Histology

Animals

A total of 42 animals were used across all the *in vivo* experiments addressing the role of DA on autonomic function and with aging. Wild type (WT) mice (C57BL/6) were divided into three separate age groups: 2-3 month-old (n=10), 1-year-old (12-14 months, n=12), and 2-year-old mice (24-27 months, n=10). 2-month- and 1-year-old WT animals had been bred and kept on site, 2-year-old mice were obtained from the National Institutes of Aging (NIA). D3 receptor knockout (D3KO) animals on a C57BL/6 background (B6.129S4-*Drd3*^{tm1Dac}/J) were obtained from Jackson Laboratory, Bar Harbor, ME, and maintained as a breeding colony at ECU. Animals were housed in the Brody School of Medicine Animal Facility with a 12:12-h light-dark cycle and free access to standard mouse chow and water.

Blood Pressure Measurements

Systolic and diastolic blood pressure and heart rate in awake mice were measured using a noninvasive tail cuff measurement system (Model SC1000 Blood Pressure Analysis System, Hatteras Instruments, Cary, NC). Animals were placed on a warming platform with a temperature of 37-38° C for comfort, and a magnetic metal holder was used to restrict movement that could potentially affect readings. A traditional tail-cuff occluder was placed proximal on the mouse's tail, which was then immobilized with tape in a V-shaped block between a light source above and a photoresistor below with a LED assembly to detect the pulse. The tail was carefully taped at the tip to prevent the mice from pulling out of the cuff. Data were displayed, recorded, and stored for analysis on a PC (SC1000 Comm Windows, v2.50) and were exported and saved to SigmaPlot (v11; Systat Software, Inc., Chicago, IL) for analysis. The minimum pulse amplitude was set to 20%, the systolic threshold to 5%, and the maximum pressure to occlude blood flow in the tail to 200 mmHg.

Measurements took approximately 8 minutes to complete and consisted of 5 preliminary cycles and 10 measurement cycles. The preliminary cycles served to acclimate the mice, while the subsequent cycles provided the measurements for systolic and diastolic blood pressure and heart rate. Each cycle included two phases: pulse detection and blood pressure acquisition. Pulse detection took ~10 seconds once animals were properly acclimated and systolic/diastolic pressure readings took ~20 seconds. The warming platform was cleaned after each session to remove any odors and minimize stress for subsequent animals. Measurements were performed on sequential days and at similar times to maintain consistency and to avoid circadian

perturbations. Only animals of the same age group or strain were measured on a given day, and recording sessions were repeated daily, generally for 4-6 days. Further, to avoid potential problems stemming from the acclimation phase during the first sessions, we only analyzed data from the last 3 sessions per animal. These readings were then averaged to produce a single data point for each session, for systolic and diastolic blood pressure, and heart rate, respectively.

Functional Echocardiography

Left ventricle echocardiograms of the 3 differently aged WT and the young D3KO animals were taken with the Vevo 2100 Imaging system (VisualSonics, Toronto, ON, CA) and the data analyzed off-line. Animals were anesthetized by exposure to 2% isoflurane and were placed on a platform under a fumehood, where the anesthesia was maintained via a nose cone. The chests were depilated with a chemical hair remover to minimize ultrasound attenuation, and a contact gel (Aquasonic, Parker Laboratories, Fairfield, NJ) was applied to the thorax surface to optimize visibility of the heart and the left ventricle. Parasternal long- and short-axis views were acquired in M-mode traces made from B-mode images. In order to obtain and calculate the measurements of the different parameters, the LV area tool was used to trace the anterior and posterior walls of the left ventricle. After the tracings were completed, the imaging software calculated the following parameters: Heart Rate (BPM), Volume (systolic, µl), Volume (diastolic, µl), Stroke Volume (µl), Ejection Fraction (%), Fractional Shortening (%), Cardiac Output (ml/min), Diameter (systolic, mm), Diameter (diastolic, mm), LV mass (mg), LV mass / body weight (mg)(See Fig 2.2).

Typical Example of an Ultrasound Echocardiogram

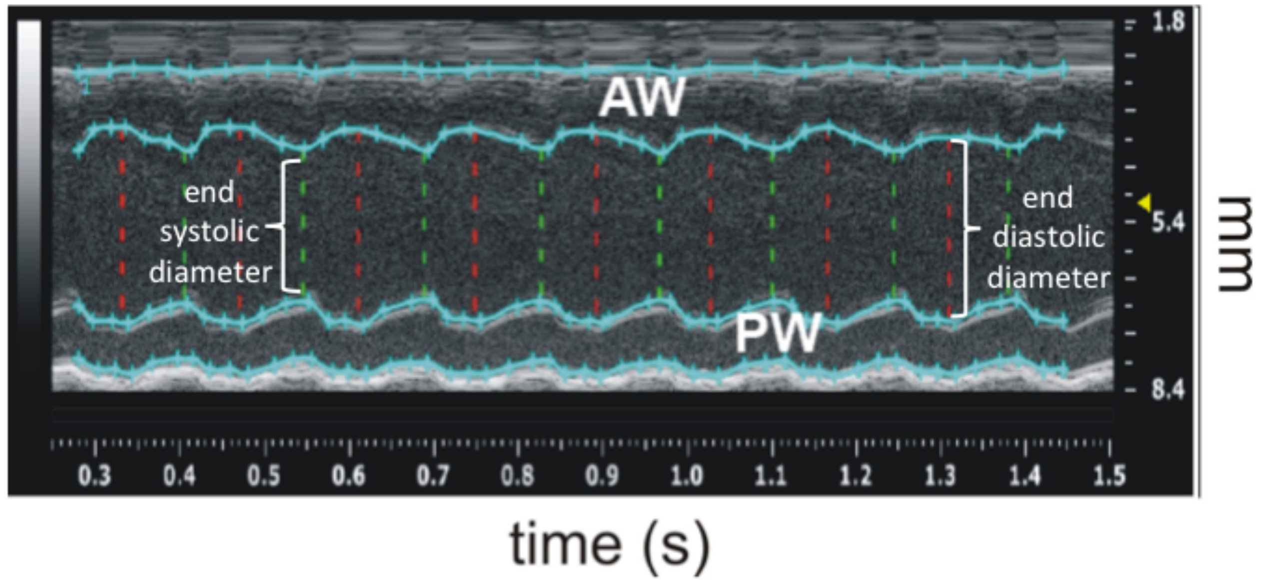


Figure 2.2

Figure 2.2 Legend

The LV area tool (blue line) was used to trace the anterior and posterior walls of the left ventricle. The anterior wall is labeled AW and the posterior wall is labeled PW in this example. The green lines show the internal dimension of the systolic diameter and the red lines show the internal dimension of the diastolic diameter as calculated by the Vevo 2100 system.

Tissue Harvesting and Histology

After the *in vivo* data were acquired, animals were deeply-anesthetized by exposure to urethane and decapitated, the hearts quickly dissected out, fixed in zinc-based fixative (n=5 per group and strain), processed, and embedded according to routine procedures for histological analyses (Dries et al., 2011).

Slides stained with hematoxylin and eosin were used for morphometric analysis of left ventricular area, chamber area, and myocyte cross-sectional area (MCSA). Briefly, for ventricular and chamber areas, each of the 4 sections per heart were imaged at 20x, the measures were averaged per animal, and each of those averages were used to calculate the average for all 5 hearts in each group. For MCSA, 3 images in both the epicardial and endocardial regions of 2 sections per hearts were taken. In each image, the area of 3-8 myocytes with centrally located nuclei was measured and recorded. The average of these numbers were calculated for each animal and then those numbers were averaged for n=5 per group (Virag et al., 2010).

Fibrosis Staining

Picrosirius Red Protocol was performed according to routine procedures. Briefly, slides were de-paraffinized and rehydrated, immersed in a 0.1% picrosirius red/fast green solution (Sigma-Aldrich, 365548, F7258, P6744) for 30 min, cleared and coverslipped (Virag et al., 2007). Tissue collagen was determined by taking 4 images at 400x in 2 sections per heart and using Adobe Photoshop to count the number of red and green pixels. The percent collagen was calculated as the number of red pixels/red +

green pixels x 100%, the numbers were averaged for each animal, and the data presented are the average of 5 animals per group.

Statistical Analysis

All data are presented as means ± standard error (SEM). SigmaPlot 11 (Systat Software, Inc., Chicago, IL) was used to analyze data and to test for significant differences in the course of an experiment. ANOVA was performed on data at a minimum $p<0.05$ threshold, and appropriate post-hoc tests were applied to evaluate differences between treatment groups.

CHAPTER 3: SEGMENT-SPECIFIC DIFFERENCES IN SPINAL CORD CIRCUITRY LEADING TO A REVERSAL OF DOPAMINERGIC REFLEX MODULATION

SUMMARY

Dopamine (DA) innervation is thought to be present in the entire segmentally organized spinal cord, where DA's actions are mediated by D1- and D2-like receptors alike. However, the modulatory actions of DA in the different regions of the spinal cord are poorly understood and research has focused on lumbar segments L3-L5 and their role in locomotion or reflex modulation. In contrast, the action of DA in those spinal cord segments that house the autonomic nervous system (ANS, T1-L2 and, in mouse, L6-S1) have not been addressed. Using electrophysiological and pharmacological approaches of the isolated neonatal spinal cord *in vitro*, we provide evidence that DA modulation of the spinal reflex circuitry exerts opposing modulatory effects in the mouse spinal cord, and that these differences coincide with the presence or absence of the ANS. In particular, bath-application of low DA levels and D2-receptor agonists decreased spinal reflex responses in the L3-L5 segments, but significantly facilitated them in the thoracic and sacral segments studies. Conversely, D1-receptor activation facilitated reflex responses in L3-L5, but decreased them in the ANS-containing segments.

Further, the region-specific modulatory effects were specific for dopaminergics. While bath-application of other monoamines (serotonin and noradrenaline) was able to modulate spinal reflex amplitudes, we did not find any region-specific differences as was found with the dopaminergics.

As gap junctions play an important role in the interneuronal communication in both the ANS and between motoneurons, we decided to test if they might also be involved in the location-specific modulation under DA. Application of both carbenoxolone and quinine reversed the reflex DA-modulation of responses in ANS-containing spinal segments, but had no additional effect in L3-L5 segments.

Thus our data suggest that DA modulates the spinal reflex circuitry in a region-specific manner, dependent on the presence or absence of the ANS, and that these actions might be mediated via gap junctions.

INTRODUCTION

Understanding the circuitry of the spinal cord is essential for understanding the global function of the spinal cord not only under normal circumstances, but also following changes that occur in the injury or disease state. In this study, we address monoaminergic-mediated changes in activity between sensory, motor, and autonomic neuronal cell populations in the spinal cord.

The study of spinal reflex is one tool that can be used to understand the circuitry of the spinal cord (Pinco and Lev-Tov, 1993, Chen et al., 2003). Two broad divisions can be made when assessing reflex activity: The monosynaptic reflex (MSR) is two neuron pathway: a large-diameter sensory Aα neuron originating from a muscle spindle receptor directly synapses upon α motoneurons in the spinal cord, which project homosynaptically and monosynaptically to the muscle containing the Aα neuron. The simplicity of connection between these two cells makes it the most well studied spinal reflex (Frank and Mendelson, 1990, Davidoff, 1992, Pierrot-Deseilligny and Mazevet, 2000, Voerman et al., 2005, Rossi-Durand, 2006).

In contrast, polysynaptic, or long-latency reflexes (LLR) likely follow a different path and they are mediated by more than two neurons. LLR can be produced by several possible mechanisms: activation of small-diameter afferent fibers (Aδ and C) evoked by high intensity stimuli, repetitive firing of motoneuron pools, or activation of interneuronal pathways can all contribute to the generation of the LLR (Clemens and Hochman, 2004). The circuitry of both types of reflex activity can be studied in detail with an *in vitro* isolated neonatal rodent spinal cord preparation; in this model MSR and

LLR amplitude, once stabilized, can be modulated by the addition of drugs to the preparation (Fulton, 1986, Jiang et al., 1999). See Fig 3.1.

Stimulation of the lumbar, thoracic, and sacral dorsal roots evoked short-latency monosynaptic and longer latency reflex.

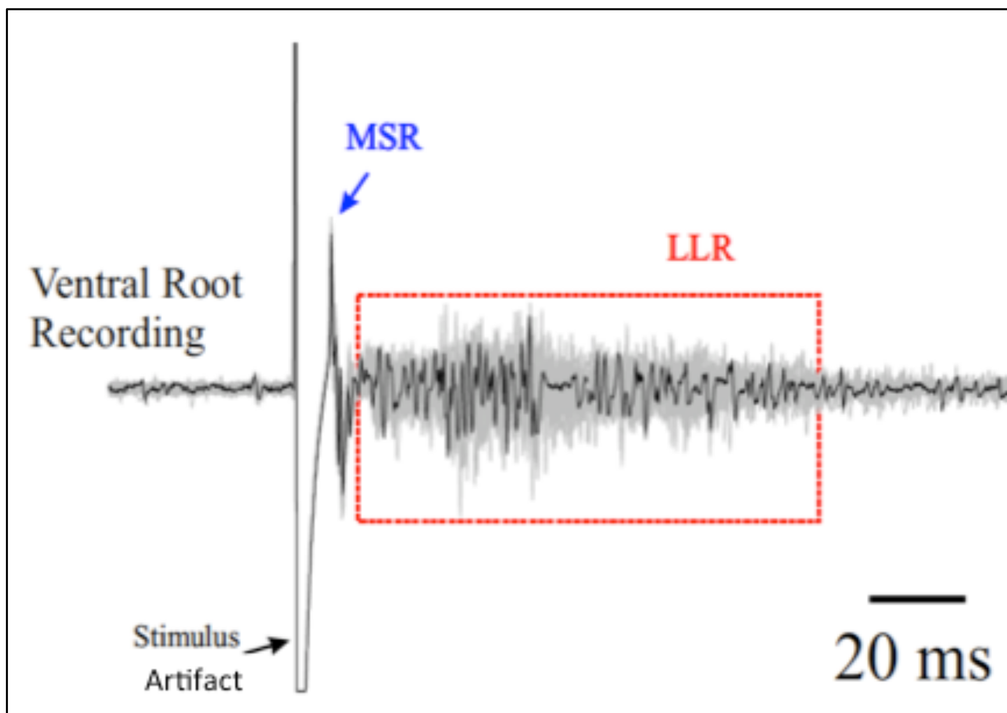


Figure 3.1

Figure 3.1 Legend

The monosynaptic reflex (MSR) was measured at 3-5 ms from the end of the stimulus artifact. A baseline of amplitudes of the MSR was established as a control before drugs were applied to the bath. Longer latency reflexes (LLR) were measured from the end of the MSR and following a typical 2-5 ms period of inactivity until approximately 65 ms following the onset of reflex activity.

Monoamines are one class of neurotransmitters known to modulate spinal cord function, including MSR and LLR (Kitazawa et al., 1985, Lew et al., 1992, Tanaka et al., 1997, Kiehn et al., 1999). Serotonin (5-HT) and noradrenaline (NA) have been studied in detail in this context, but the role of spinal dopamine (DA) is still not as well characterized as the other monoamines (Garraway and Hochman, 2001, Clemens and Hochman, 2004). The only source of spinal DA is the A11 cell group in the dorsal posterior hypothalamus, which sends projections to most locations in the spinal cord (Skagerberg et al., 1982, Lindvall et al., 1983, Skagerberg and Lindvall, 1985, Holstege et al., 1996). A recent study in a non-human primate (the macaque monkey) suggests that DA itself is not projected from the A11 cell group, but instead the precursor to DA, L-DOPA, travels from the brain to the spinal cord, where L-DOPA can be converted to DA (Barraud et al., 2010). All DA receptors (D1-D5) have been found in all lamina of the spinal cord and the activation of these specific DA receptors mediate downstream effects on spinal cord reflex (van Dijken et al., 1996, Gladwell and Coote, 1999b, Gladwell et al., 1999, Levant and McCarson, 2001, Zhu et al., 2007). There are two families of DA receptors: D1-like receptors (D1, D5), which tend to induce excitatory actions (increasing intracellular levels of cAMP), and D2-like receptors (D2, D3, D4), which generally activate inhibitory second messenger pathways (Carp and Anderson, 1982, Gajendiran et al., 1996). The DA system is present at birth, allowing the neonatal rodent spinal cord preparation to be useful in studying dopaminergic reflex modulation (Kudo and Yamada, 1985, Reisert et al., 1990). Both the presence of DA and DA receptors throughout the spinal cord indicate that dopamine can impact the functional output of this system.

Studies on the role of DA in modulating spinal reflex have primarily focused on its action in the lumbar segments of the spinal cord (Clemens and Hochman, 2004, Juvin et al., 2007). This information is useful in examining the circuitry involved in locomotion; however, there are other segments with different populations of neurons that could be influenced by dopaminergic reflex modulation. For example, in humans, the spinal cord has autonomic innervation from segments T1-L2 (sympathetic) and from S2-S4 (parasympathetic). In mice, the segmental organization of this innervation differs slightly with respect to parasympathetic innervation, with autonomic cell bodies located from L6-S1. The presence of these cell types opens up the possibility for reflex circuit modulation that is not seen in the segments that lack autonomic input (in mice, L3-L5).

In search for a possible mechanism for the interaction of spinal somatic and autonomic motoneurons, there is evidence that neurons exiting the spinal cord and originating in the IML (sympathetic preganglionic neurons – SPNs) are connected to each other electrically via gap junctions. The same study found populations of motoneurons are also electrically coupled (Marina et al., 2008). "A homeodomain transcription factor, Hb9, is expressed by embryonic motoneurons and functions during development to consolidate motoneuron identity" (Arber et al., 1999, Thaler et al., 1999, Wilson et al., 2005). Cells expressing Hb9 include ventral motoneurons and sympathetic preganglionic neurons found in the intermediolateral cell column and the central autonomic area, suggesting these cells stem from the same pool during development (Wilson et al., 2005). This information, added to the fact that the individual populations of somatic and autonomic motoneurons are coupled electrically, led us to

ask if it is possible for the population of motoneurons and sympathetic/parasympathetic preganglionic neurons might also be connected to each other via gap junctions.

The goal of this study is to test the potential role of the ANS in modulating spinal reflexes. More specifically, we are interested in how DA can modulate spinal reflex in segments of the spinal cord containing ANS innervation, a novel idea that has not yet been explored in other laboratories studying spinal cord reflex circuitry.

RESULTS

Reflexes were analyzed from a total of 80 hemisect spinal cords. Electrical current stimulation of the lumbar, thoracic, and sacral dorsal roots evoked both a short-latency monosynaptic reflex (MSR) and a longer-latency reflex (LLR). The MSR was identified and measured at 3-5 ms after the end of the stimulus artifact (Clemens and Hochman, 2004), Fig. 3.2. A baseline of spinal reflex amplitudes (SRAs) was established as a control before drugs were applied to the bath. LLRs emerged generally 10-15 ms after the end of the stimulus artifact and lasted for approximately 50-65 ms. LLRs were inherently more variable than MSRs and could not be recruited in all experiments, thus providing a smaller dataset than the MSRs.

Presence of MSR and LLR in Thoracic, Lumbar, and Sacral Segments

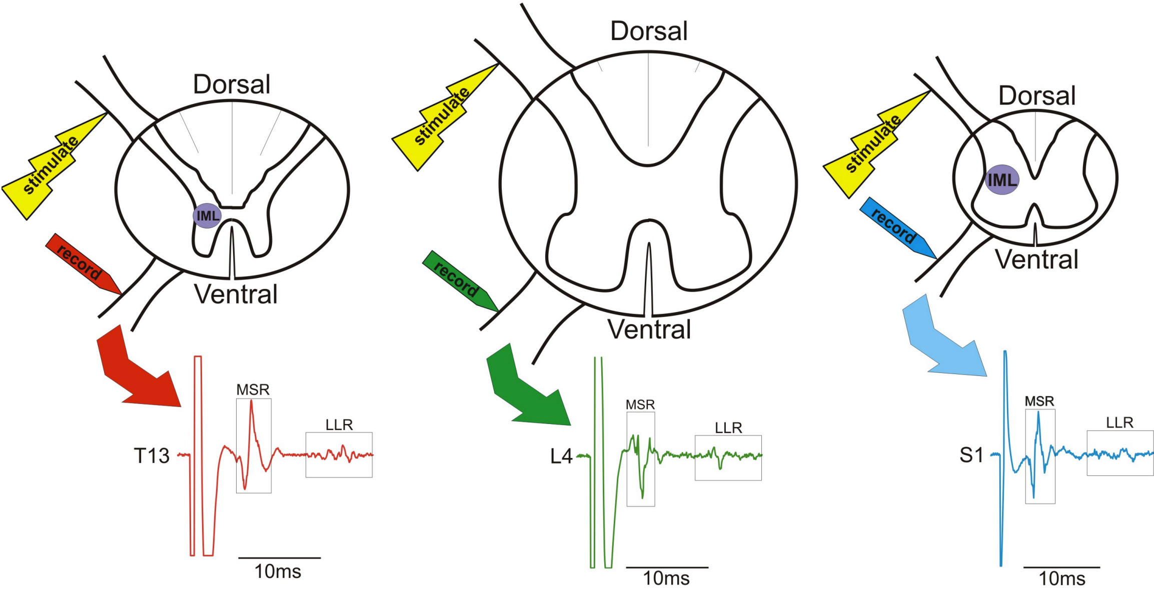


Figure 3.2

Figure 3.2 Legend

Both MSR and LLR could be evoked in thoracic, lumbar, and sacral segments. Traces shown are from raw data generated in each of the respective segments shown (T13, L4, and S1). Roots are stimulated on the dorsal side and recorded on the ventral side. The MSR is visible within 10ms of the stimulation artifact and last from 3-5ms in duration. The LLR follows a 2-5ms period of quiet activity and can last for hundreds of milliseconds.

Effect of Dopaminergics on the MSR

In order to study the effects of DA and DA-receptor ligands on the MSR amplitude between segments of the spinal cord with and without the IML, we bath-applied the following compounds: 1 μM DA (considered a low concentration), 1 μM quinpirole (D2 agonist), 10 μM raclopride in combination with 1 μM DA (D2 antagonist), and 10 μM SKF 38393 (D1 agonist). See Fig 3.3.

DA and DA Receptor Agonist and Antagonist Modulation of MSR in Lumbar and IML-containing Segments

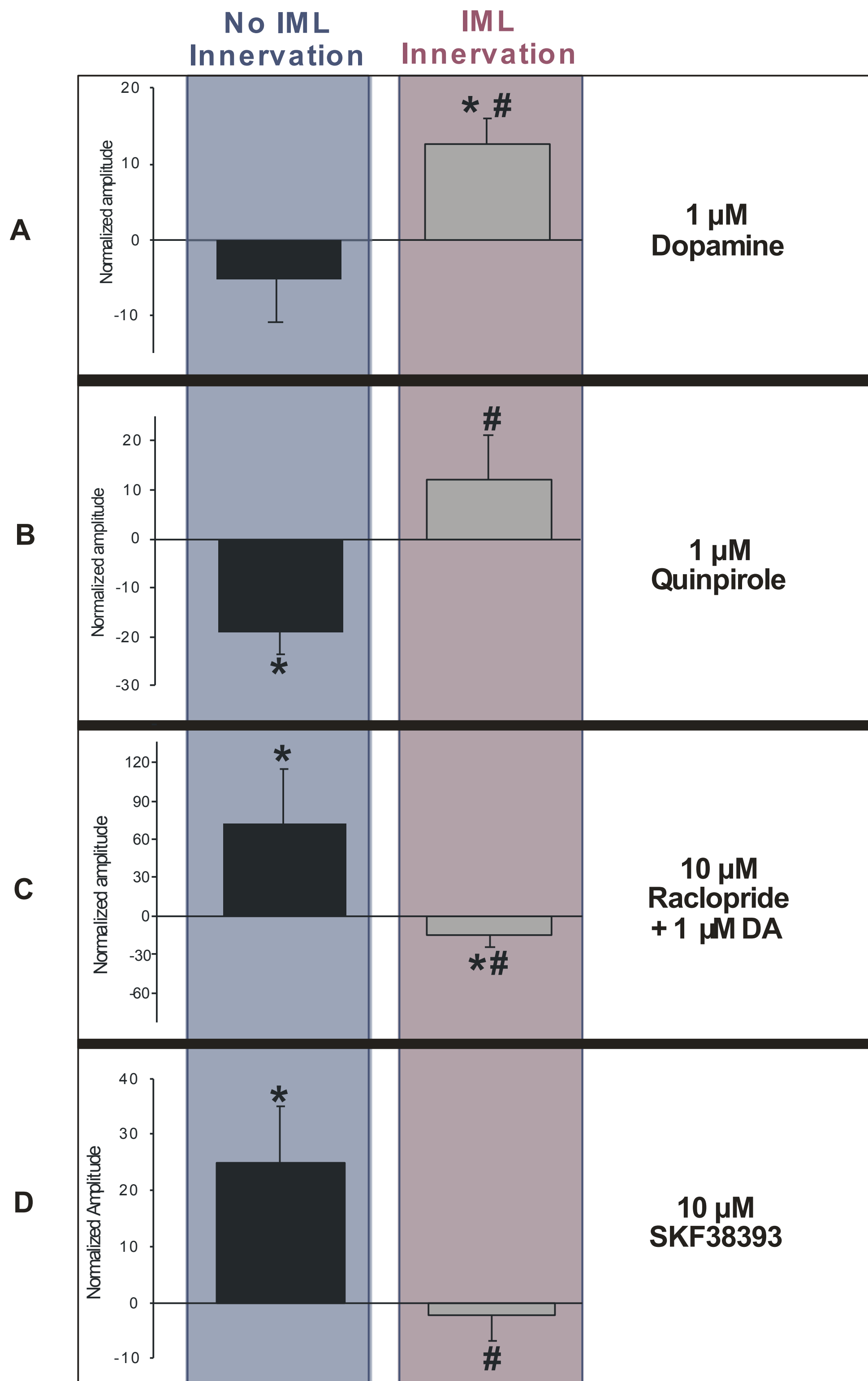


Figure 3.3

Figure 3.3 Legend

A. Application of 1 μM DA to the bath led to a decrease from baseline that was not significance in the lumbar segments (L3-L5) of the spinal cord (left bar, n=8). Application of 1 μM DA to the bath led to an increase in MSR amplitude from baseline measurement in the segments of the spinal cord containing IML (T1-L2, L6-S1) leading to an overall increase in amplitude from baseline (right bar, n=26). The difference in MSR amplitude response to 1 μM DA application between the lumbar segments of the spinal cord with no IML compared to all segments with IML was significant.

B. In lumbar segments (L3-L5) of WT mice, with the application of 1 μM quinpirole, the reflex amplitude was decreased from baseline in all experiments (left bar, n=8). In IML-containing segments (T1-L2, L6-S1), however, had an overall change from baseline that was not significant (right bar, n=7). The difference in MSR amplitude response to 1 μM quinpirole application between the lumbar segments (L3-L5) of the spinal cord with no IML compared to all segments with IML was significant.

C. The application of 10 μM raclopride and 1 μM DA to lumbar segments (L3-L5) of WT mice produced an overall increase significant in reflex amplitude from baseline (left bar, n=12). In contrast, IML-containing segments (T1-L2, L6-S1) had an overall decrease in reflex amplitude in response to this drug combination (right bar, n=12). The difference in MSR amplitude response to 10 μM raclopride and 1 μM DA application between the lumbar segments of the spinal cord with no IML compared to all segments with IML was significant.

D. The application of 10 μM SKF 38393 to lumbar segments (L3-L5) caused an overall increase from baseline in the lumbar segments (left bar, n=14). Conversely, the

application of 10 µM SKF 38393 to thoracic and sacral segments of the spinal cord produced no significant change from baseline (right bar, n=6). The difference in MSR amplitude response to 10 µM SKF 38393 application between the lumbar segments of the spinal cord compared to thoracic/sacral segments of the cord was significant.

* = different from baseline

= different from effects on lumbar segments

Effect of low dopamine

Application of 1 μM dopamine (DA) in the lumbar segments (L3-L5) caused in 6 of 8 applications a depression of the MSR amplitude (-10±5% of control) and in 2 of 8 applications an increase in the reflex amplitude (10±1% of control). The overall effect of the application of 1 μM DA was no significant change from baseline (-6±5% of control, $p<0.052$)(Fig 3.3A, first bar).

In contrast, in IML-containing spinal segments (T1-L2, L6-S1), application of 1 μM DA to the bath led to an increase in MSR amplitude from baseline measurements in 20 of 26 applications (18±4% of control). In 6 of 26 applications, the MSR showed a decrease from baseline (-6±1% of control)(Fig 3.3A, right bar), leading to an overall significant increase in amplitude from baseline (13±3% of control, $p<0.001$).

The difference in MSR amplitude response to 1 μM DA application between the lumbar segments of the spinal cord with no IML compared to all segments with IML was significant ($p<0.01$).

Effect of the D2 receptor agonist quinpirole

In order to further explore the differential dopaminergic modulation of MSR between non-IML and IML-containing segments, we tested the effects of a D2-preferring agonist quinpirole on spinal reflex amplitude (Fig 3.3B). D2-like receptors (D2, D3, and D4) have a higher affinity to DA than D1-like receptors (D1, and D5), and quinpirole has been reported to have a 30 times higher affinity to D2 over D3 receptors when cloned from human tissue (Sokoloff et al., 1990, Freedman et al., 1994, Sautel et al., 1995) and

a 10 times higher affinity to D2 over D3 receptors when cloned from rat tissue (Sokoloff et al., 1990, Sautel et al., 1995, Clemens and Hochman, 2004).

We found that, in lumbar segments (L3-L5), application of 1 μM quinpirole mimicked the effect of 1 μM DA and decreased the MSR amplitude in 8 of 8 experiments (-17±4% of control, $p<0.001$)(Fig 3.3B, left bar).

In contrast, in IML-containing segments (T1-L2, L6-S1), quinpirole caused in 5 out of 6 applications an increase in reflex amplitude (21±9% of control), and in only 1 application a decrease from baseline (-18±6% of control). The overall change from control was an increase by 16±9% ($p<0.11$)(Fig 3B, right bar).

The difference in MSR amplitude response to the application of quinpirole application between the lumbar segments (L3-L5) and IML-containing segments with IML was significant ($p<0.02$).

Effect of the D2 receptor antagonist raclopride on the MSR

Next, we asked if blocking the D2 receptor could reverse the effect see with both 1 μM DA and the D2 agonist quinpirole. The D2 receptor antagonist, raclopride (10 μM), applied in combination with 1 μM DA to better reveal the consequences of D2 receptor inactivation, was added to non-IML and IML-containing segments (Fig. 3.3C)(Lidow et al., 1989, Clemens et al., 2012).

The combined application of raclopride and DA to lumbar segments (L3-L5) of WT mice produced a significant increase in MSR amplitude over baseline control (70 ±39% of control, $p<0.003$), with 2 of 12 applications showing a decrease from baseline

(-7±3% of control) and 10 of 12 applications showing an increase in reflex amplitude from baseline (85±45% of control)(Fig 3.3C, left bar).

In contrast, IML-containing segments (T1-L2, L6-S1) showed a consistent and significant decrease in reflex amplitude in response to this drug combination (-17±6% of control, $p<0.003$)(Fig 3.3C, right bar). In 10 of 12 applications the MSR amplitude decreased (-22±6% of control), and was not altered in 2 experiments (8±8% of control).

As with the application of the D2-agonist quinpirole, application of raclopride caused significantly different responses in MSR amplitude modulation between L3-L5 and IML-containing spinal segments ($p<0.001$).

Effect of D1 receptor agonist SKF 38393

We have seen that differences between the dopaminergic modulation of spinal reflex exist between IML and non-IML containing segments. Next, we tested if this was only mediated via the D2 receptor, or if the other class of DA receptors would have the same effect. SKF 38393 is a D1 receptor agonist and has been shown to stimulate cAMP production (Setler et al., 1978).

Application of SKF 38393 (10 µM) to the lumbar segments L3-L5 significantly increased the MSR amplitude from baseline in 12 out of 14 applications (by 31±10% of control). In 2 of 14 applications, the reflex amplitude was decreased (by -13±0.08% from control). In these segments, the overall increase was significant (25±10% of control, $p<0.001$)(Fig 3.3D, left bar).

Conversely, application of SKF 38393 to thoracic and sacral segments of the spinal cord produced no significant change from baseline ($p<1$)(Fig 3.3D, right bar).

Specifically, in 3 of 6 applications, the reflex amplitude was increased (5±0.5% of control) and in 3 of 6 applications the reflex amplitude was decreased (-9±7% of control).

The difference in MSR amplitude response to the application of SKF 38393 between lumbar segments L3-L5 and IML-containing thoracic/sacral segments was significant ($p<0.04$).

See Table 3.1 for a summary of the DA-mediated effects on the MSR between segments with and without autonomic innervation.

Table 3.1 Summary of Differential MSR DA Responses

	Spinal level	Dopamine (DA)	D2 agonist	D2 antagonist	D1 agonist
Sympathetic IML T1-L2	T1-L2	↑	↑	↓	↓
No IML L3-L5	No IML L3-L5	↓	↓	↑	↑
Parasympathetic IML L6-S1	L6-S1	↑	↑	↓	↓

Effect of Dopaminergics on the LLR

In order to study the effects of DA and DA-receptor ligands on the LLR amplitude between segments of the spinal cord with and without the IML, we bath-applied the following compounds: 1 μM DA (considered a low concentration), 1 μM quinpirole (D2 agonist), 10 μM raclopride in combination with 1 μM DA (D2 antagonist), and 10 μM SKF 38393 (D1 agonist). See Fig 3.4.

DA and DA Receptor Agonist and Antagonist Modulation of LLR in Lumbar and IML-containing Segments

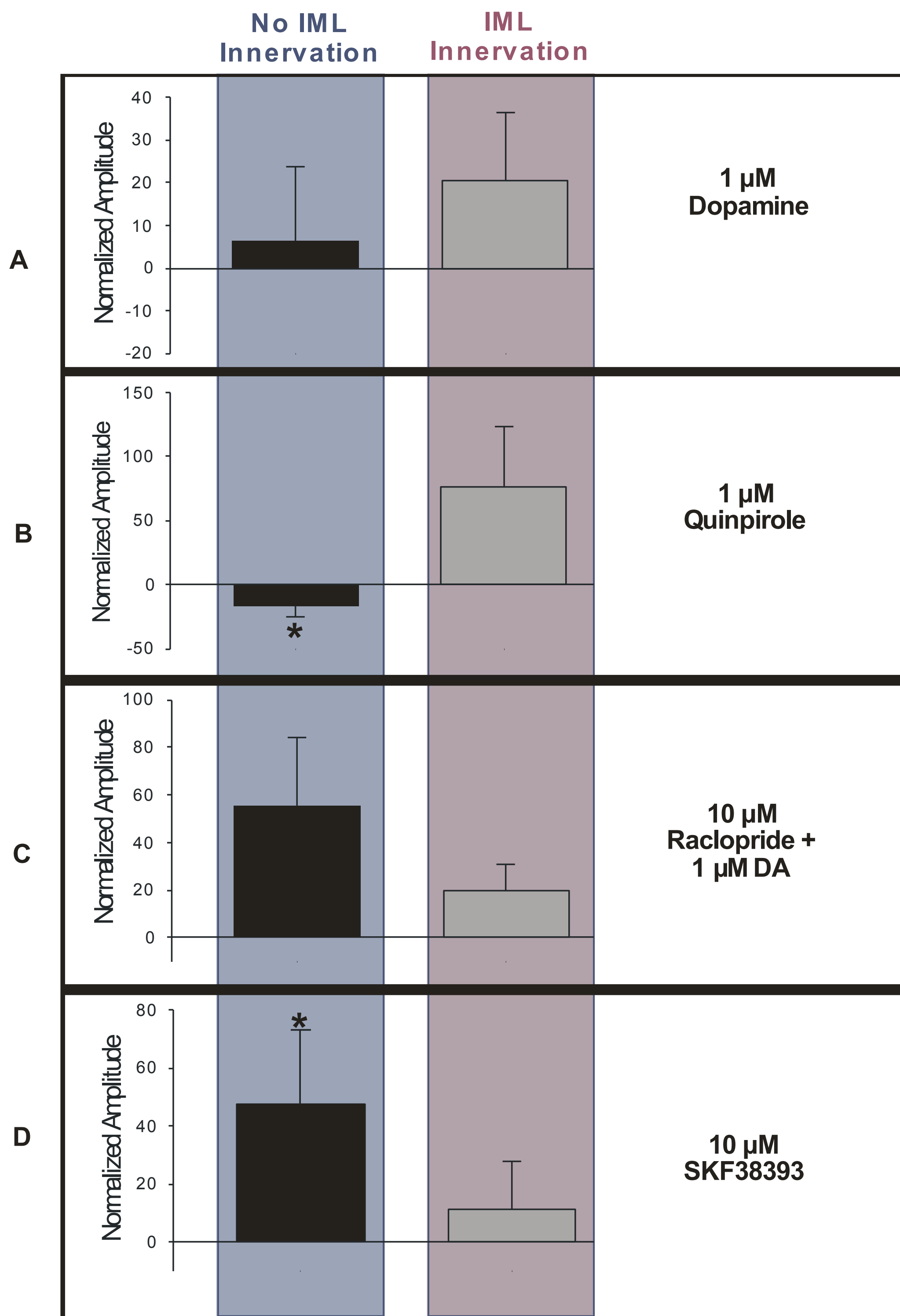


Figure 3.4

Figure 3.4 Legend

A. Application of 1 μM DA to the bath generally led no significant change from baseline in the lumbar segments (L3-L5) of the spinal cord (left bar, n=8). Application of 1 μM DA to the bath generally led no significant change in LLR from baseline in the segments of the spinal cord containing IML (right bar, n=14). The difference in MSR amplitude response to 1 μM DA application between the lumbar segments of the spinal cord with no IML compared to all segments with IML was not significant.

B. In lumbar segments (L3-L5) of WT mice, with the application of 1 μM quinpirole, the reflex amplitude was significantly reduced from baseline (left bar, n=7). In IML-containing segments (T1-L2, L6-S1), however, 4 out of 5 applications increased in reflex amplitude (right bar, n=5). The difference in MSR amplitude response to 1 μM quinpirole application between the lumbar segments (L3-L5) of the spinal cord with no IML compared to all segments with IML was not significant.

C. The application of 10 μM raclopride and 1 μM DA to lumbar segments (L3-L5) of WT mice led to no overall change in reflex amplitude from (left bar, n=8). IML-containing segments (T1-L2, L6-S1) had no overall change in reflex amplitude in response to this drug combination (right bar, n=5). The difference in LLR amplitude response to 10 μM raclopride and 1 μM DA application between the lumbar segments of the spinal cord (L3-L5) and segments with IML was not significant.

D. The application of 10 μM SKF 38393 to lumbar segments (L3-L5) significantly increased the reflex amplitude from baseline in the lumbar segments (left bar, n=10). Conversely, the application of 10 μM SKF 38393 to thoracic and sacral segments of the

spinal cord produced no significant change from baseline (right bar, n=6). The difference in LLR amplitude response to 10 μM SKF 38393 applications between the lumbar segments of the spinal cord compared to thoracic/sacral segments of the cord approached significance.

* = different than baseline

= different than lumbar

Effect of low dopamine

Application of 1 μM DA to the bath generally had no significant effect on the modulation of LLR amplitudes in the lumbar segments (L3-L5) of the spinal cord (Fig 3.4A, left bar). Specifically, in 5 of 8 applications, DA depressed the reflex amplitude (-23±11% of control) and in 3 of 8 applications it increased the reflex amplitude (56±22% of control), leading to no overall to a change from baseline (6 ±17% of control, $p<0.44$).

Similarly, 1 μM DA had no significant effect on LLR amplitudes in the segments of the spinal cord containing IML (T1-L2, L6-S1). Specifically, in 9 of 14 applications, the LLR amplitude was increased from baseline (40±22% of control). In 5 of 14 applications, the LLR showed a decrease from baseline (-15±6% of control)(Fig 3.4A, right bar), leading to a change in overall slight increase in LLR amplitudes (to 20±16% of control ($p<0.21$).

Thus, unlike the MSR, we did not find a significant difference in LLR amplitude response to 1μM DA application between the lumbar segments L3-L5 and IML-containing segments ($p<0.43$).

Effect of the D2 receptor agonist quinpirole

In lumbar segments L3-L5, application of 1 μM quinpirole significantly decreased the reflex amplitudes in 6 of 7 experiments by 25±4% of control (Fig 3.4B, left bar), and increased it in 1 experiment. Overall, the applications of 1 μM quinpirole lead to a significant decrease in LLR amplitudes in L3-L5 lumbar segments (-16±9% of control, $p<0.02$).

In IML-containing segments however, the effects of the D2-receptor agonist were mixed. Quinpirole increased the LLR amplitude in 4 of 5 applications (109±44% of control) and decreased it in 1 experiment, thus yielding to an overall increase to 76±47% of control ($p<0.15$)(Fig 3.4B, right bar).

While the quinpirole-induced decrease in LLR amplitude in L3-L5 segments was significant, the difference in LLR amplitude response between the lumbar segments and non-IML-containing segments was not significant ($p<0.11$).

Effect of the D2 receptor antagonist raclopride

In contrast to the MSR findings, application of raclopride and DA to L3-L5 segments had no effect on LLR amplitudes, with 3 of 8 applications showing a decrease from baseline (-8±5% of control) and 5 of 8 applications showing an increase in reflex amplitude from baseline (94±36% of control). The overall change from baseline was 56±28% of control, $p<0.36$. (Fig 3.4C, left bar)

Similarly, the raclopride/DA mix did not significantly alter LLR amplitudes in the IML-containing segments (overall: 20±11% of control, $p<0.11$, Fig 3.4C right bar). In one experiment, we observed a slight decrease in reflex amplitude from baseline (-14±4% of control) and in 3 applications we found trend towards an increase in reflex amplitude (28±9% of control).

Consequently, we did not observe any significant difference between L3-L5 and IML-containing spinal segments ($p<0.12$).

Effect of the D1 receptor agonist SKF 38393

Similar to the effects observed on the modulation of the MSR amplitude, application of SKF 38393 to the segments L3-L5 overall significantly increased the reflex amplitude from baseline (48±25% of control, p<0.02)(Fig. 3.4D, left bar). In 8 of 10 applications we observed a strong increase (63±29% of control), and in 2 of 10 applications, a slight decrease in LLR amplitude (-14±8% of control).

Conversely, application of the D1-receptor agonist to thoracic and sacral segments produced no significant change from baseline (p<0.07)(Fig. 3.4D, right bar). Specifically, in 5 of 6 applications, the reflex amplitude was increased (127±7% of control) and in 1 of 6 applications the reflex amplitude was decreased (-77±4% of control).

Overall, the LLR responses to SKF 38393 in L3-L5 and IML-containing segments showed a strong differential trend and approached significance (p<0.056).

Effect of Other Monoamines on the Spinal Reflex

To test if the differential effects of DA modulation on IML-containing spinal segments and lumbar segments (L3-L5) were specific to DA or a result of general monoamine modulation, we tested other monoamines in the same segments. Serotonin (5-HT) and noradrenaline (NA) have well-characterized responses in the lumbar segments (L3-L5), but similarly to DA, have not been studied in detail with respect to reflex modulation in the IML-containing segments.

Effect of Other Monoamines on MSR

In addition to DA and DA-receptor ligands, we applied the other monoamines 5-HT and NA to the in vitro spinal cord preparation to observe the change in amplitude of the MSR in segments of the spinal cord with and without the IML. See Fig 3.5.

5-HT and NA Modulation of MSR in Lumbar and IML-containing Segments

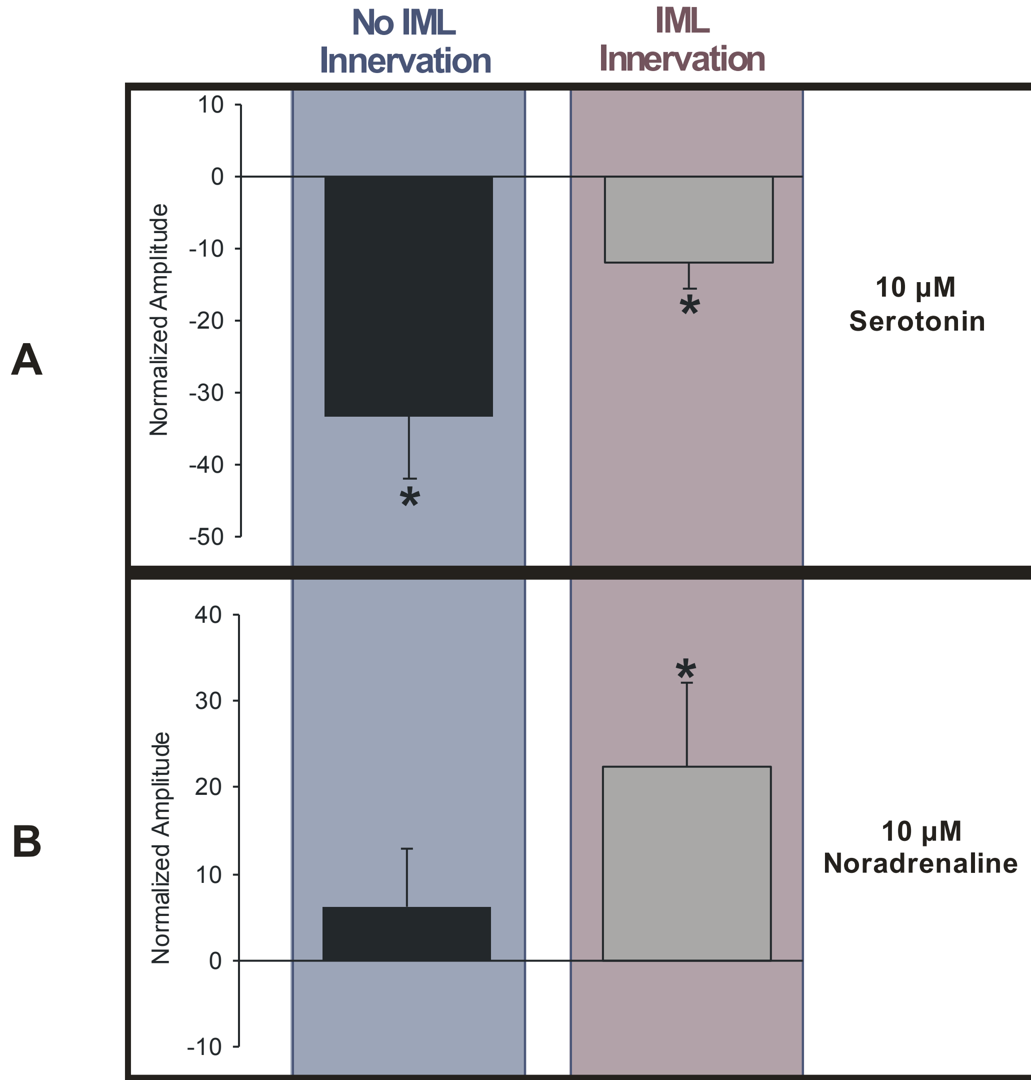


Figure 3.5

Figure 3.5 Legend

A. The application of 10 μM 5-HT to the lumbar segments (L3-L5) led to an overall decrease from baseline in all applications (left bar, n=9). Similarly, the application of 5-HT to IML-containing segments produced an overall decrease from baseline in all applications (right bar, n=7).

B. The application of 10 μM NA to the lumbar segments (L3-L5) led to a slight albeit insignificant increase from baseline (left bar, n=11), and increased SRA in IML-containing segments significantly (right bar, n=13). We did not observe any significant differences in the effects of either 5-HT or NA modulation between L3-L5 and IML-containing segments.

* = different than baseline

= different than lumbar

Effect of 5-HT

Application of 5-HT (10 μM) to the lumbar segments (L3-L5) led to a consistent and significant decrease from baseline in all 9 experiments (by -36±10% of control, $p<0.001$)(Fig 3.5A, left bar). Similarly, the application of 5-HT to IML-containing segments produced an overall decrease from baseline in 7 of 7 applications (by -18±3% of control, $p<0.001$)(Fig 3.5A, right bar). We did not find any significant difference between the lumbar (L3-L5) and IML-containing segments with the application of 5-HT ($p<0.1$).

Effect of NA

In contrast to 5-HT, application of NA (10 μM) to the lumbar L3-L5 segments led to no overall change from baseline (6±7% of control, $p<0.5$)(Fig 3.5B, left bar). Specifically, in 6 of 10 applications, MSR amplitudes increased from baseline (15±10% of control), while in 4 of 10 applications, MSR amplitudes were slightly decreased (-6±4% of control). In contrast, application of NA to IML-containing segments led to an overall increase over the control (by 24±9%, $p<0.02$)(Fig 3.5B, right bar). Specifically, in 10 of 13 applications, the reflex amplitude was increased from baseline (33±4% of control). In 3 of 13 applications, the reflex amplitude was decreased (-6±1% of control). Despite the significant increase in MSR amplitude there was no significant difference between L3-L5 and IML-containing segments with the application of NA ($p<0.2$).

Effect of Other Monoamines on the LLR

In addition to DA and DA-receptor ligands, we applied the other monoamines 5-HT and NA to the in vitro spinal cord preparation to observe the change in amplitude of the LLR in segments of the spinal cord with and without the IML. See Fig 3.6.

5-HT and NA Modulation of LLR in Lumbar and IML-containing Segments

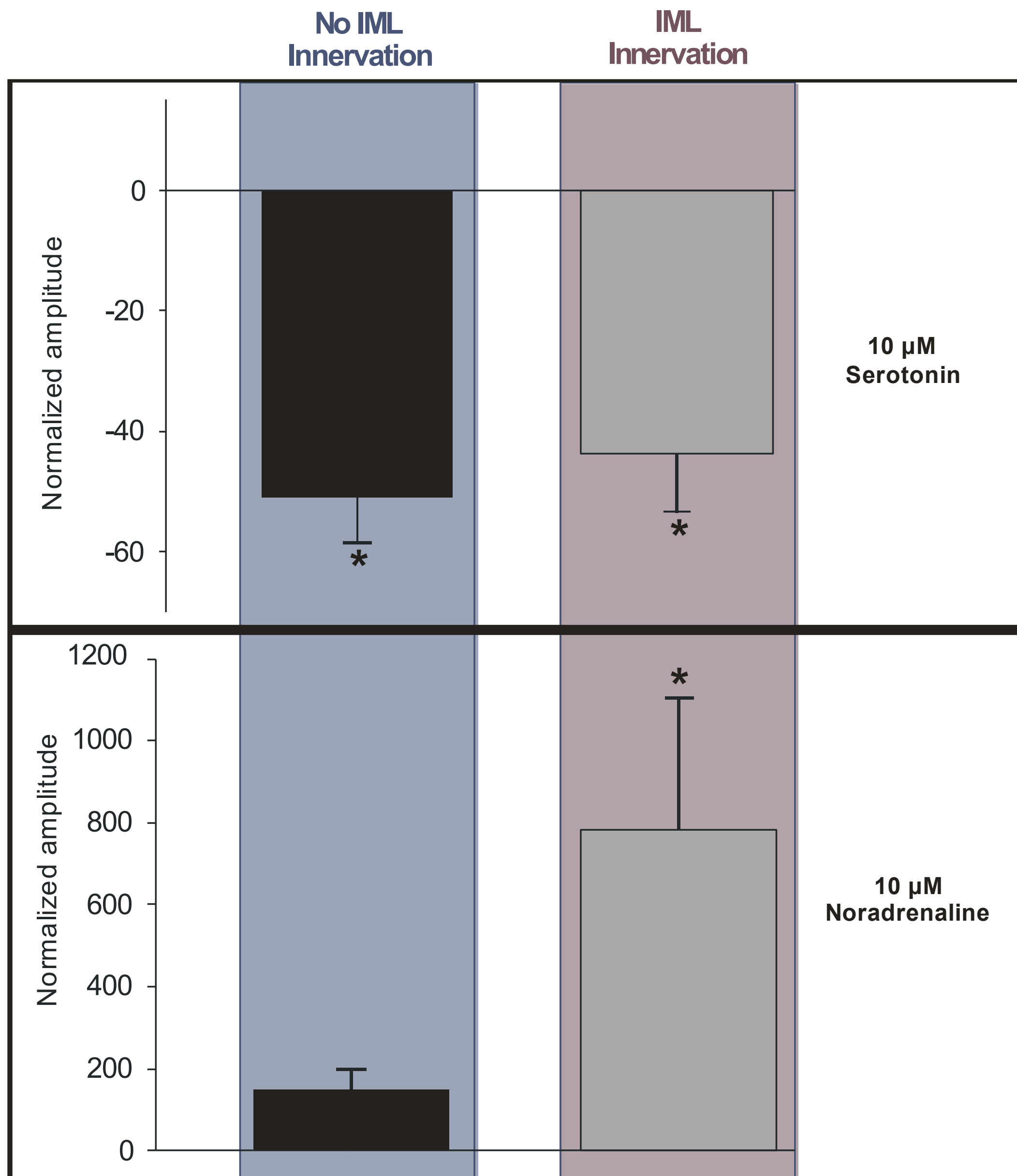


Figure 3.6

Figure 3.6 Legend

A. The application of 10 μM 5-HT to the lumbar segments (L3-L5) led to an overall decrease from baseline (left bar, n=4). Similarly, the application of 5-HT to IML-containing segments produced no overall change from baseline (right bar, n=2). There was no significant difference between the lumbar (L3-L5) and IML-containing segments (T1-L2, L6-S1) with the application of 5-HT.

B. The application of 10 μM NA to the lumbar segments (L3-L5) led to no overall change from baseline (left bar, n=5). The application of 10 μM NA to IML-containing segments (T1-L2, L6-S1) led to an overall increase from baseline in all experiments (right bar, n=4). There was no significant difference between lumbar (L3-L5) and IML-containing segments (T1-L2, L6-S1) with the application of 10 μM NA.

* = different than baseline

= different than lumbar

Effect of 5-HT

Application of 5-HT to the L3-L5 segments led to a consistent significant decrease from baseline in 4 of 4 applications (-51±7% of control, $p<0.03$)(Fig 3.6A, left bar). The application of 5-HT to IML-containing segments produced no overall change from baseline in 2 of 2 applications (-44±10% of control, $p<0.33$)(Fig 3.6A, right bar). Importantly, with the application of 5-HT, we did not find any significant difference between L3-L5 and IML-containing segments (T1-L2, L6-S1)($p<0.6$).

Effect of NA

Application of NA to L3-L5 segments led to no significant change from baseline (50 ±47% of control, $p<0.31$)(Fig 3.6B, first bar). Specifically, in 2 of 5 applications, the reflex amplitude was increased from baseline (163±15% of control), while in 3 of 5 applications, the reflex amplitude was decreased (-25±14% of control). In IML-containing segments, application of NA to IML-containing segments led to an overall increase from baseline in 4 of 4 experiments (783±321% of control, $p<0.03$)(Fig 3.6B, second bar). There was a trend for significance between lumbar (L3-L5) and IML-containing segments (T1-L2, L6-S1) with the application of NA ($p<0.06$). Previous studies have noted a larger increase from baseline with the application of this concentration of NA and there is no opposite effect seen between lumbar (L3-L5) and IML-containing segments; more experiments are necessary to rule out an actual difference between these segments, but for the purposes of this study, we will assume that if there is a difference with NA, it is not of the same magnitude as what is seen with DA.

Together, these data suggest that the switch in modulation seen between lumbar (L3-L5) and IML-containing segments is DA-specific and does not extend to include all monoamines.

Effect of Dopaminergic Drugs in Combination with Gap Junction Blockers

We applied gap junction blockers (quinine and carbenoxolone), in combination with dopaminergic drugs, to the IML-containing segments of the spinal cord to identify a possible route of communication between somatic and autonomic motor neurons. These drugs, when applied with the dopaminergics, reversed the effects previously seen with DA and DA receptor ligands applied alone in those segments. The application of the gap junction blocker alone had no effect on either the lumbar (L3-L5) or IML-containing segments (T1-L2, L6-S1)(data not shown).

The application of 10 μM quinine and 1 μM DA to IML-containing segments reversed the effect seen previously with 1 μM DA to that area. Specifically, in 3 of 5 applications, the reflex amplitude was increased relative to baseline (8±3% of control). In 5 of 8 applications, the reflex amplitude was decreased when compared to baseline (-4±1% of control). The overall effect of this drug combination in the IML segments was no change from baseline (0±3% of control, Fig. 3.7A). The change from IML-containing segments with only 1μM DA approached significance ($p<0.054$).

The application of 10μM quinine and 1μM quinpirole (n=2) and 100μM carbenoxolone and 1μM quinpirole (n=1) to the IML-containing segments also reversed the effect seen previously with 1 μM quinpirole to that area. In all three applications, the

amplitude was reduced to -45±13% of control (Fig. 3.7B). The change from IML-containing segments with only 1μM quinpirole was significant ($p<0.001$).

Further, a single application of 100μM carbenoxolone, 10μM raclopride, and 1μM DA in the IML-containing region reversed the effect seen previously with the application of 10 μM raclopride and 1 μM DA to that area (28% of control, Fig. 3.7C).

Taken together, the application of gap junction blockers quinine and carbenoxolone reversed the DA-mediated effect seen on the MSR in the IML-containing segments.

LLR was present with the application of dopaminergic drugs and gap junction blockers (and generally followed the same pattern of change in amplitude as the MSR), however, there were too few experiments available to present a complete analysis.

MSR Response to Gap Junction Blockers in Combination with DA and DA Receptor Agonists and Antagonists in IML-containing Segments

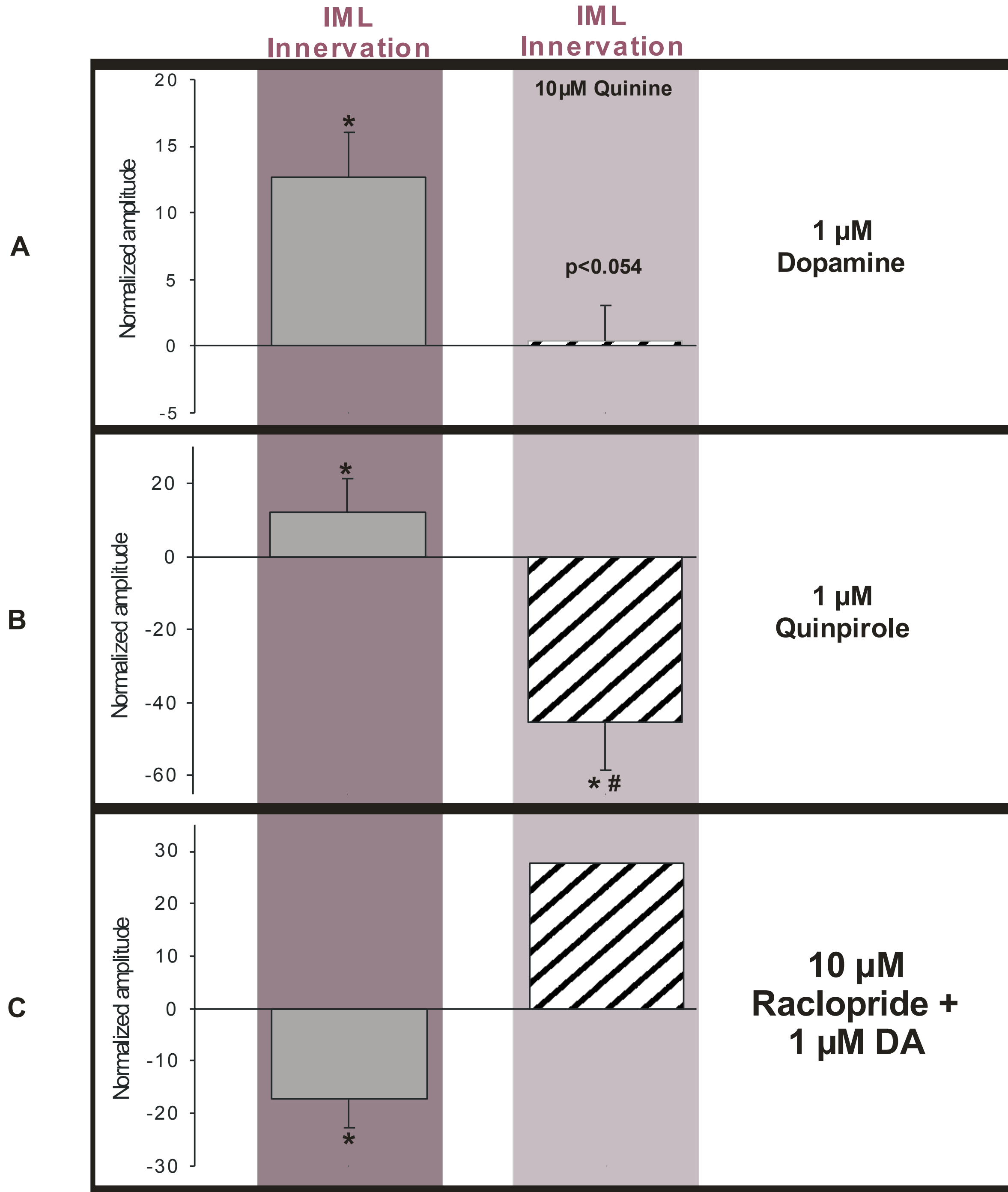


Figure 3.7

Figure 3.7 Legend

A. The application of 10 μM quinine and 1 μM DA to IML-containing segments reversed the effect seen previously with 1 μM DA to that area. The overall effect of this drug combination in the IML segments was no change from baseline (right bar, n=8). The change from IML-containing segments with only 1μM DA was near significance.

B. The application of 10 μM quinine or 100 μM carbenoxlone with 1 μM quinpirole to the IML-containing segments reversed the effect seen previously with 1 μM quinpirole to that area. In all three applications, the amplitude was significantly reduced from baseline (right bar, n=3). This change from IML-containing segments with only 1μM quinpirole was significant.

C. One application of 100μM carbenoxolone, 10 μM raclopride, and 1 μM DA in the IML-containing region caused the MSR amplitude to reversed the effect seen previously with the application of 10 μM raclopride and 1 μM DA to that area (right bar, n=1).

* = different than baseline

= different than lumbar

DISCUSSION

In this study we present the first evidence that the modulation of spinal cord reflex circuits by descending monoamines is associated with the presence of the absence of the spinal autonomic nervous system (ANS). In particular, we show that in the isolated spinal cord *in vitro*, dopaminergics modulate the monosynaptic reflex circuitry in a region-specific opposing manner. Application of low DA or a D2-receptor agonist induced depression of spinal reflexes in spinal segments void of ANS innervation (L3-L5) that was mirrored by a corresponding reflex facilitation in spinal segments containing an intermediolateral nucleus (IML). In contrast, a D1-receptor agonist-mediated facilitation of spinal reflexes in L3-L5 was paralleled by a decrease in reflex amplitude in IML-containing segments. Intriguingly, these differential modulatory effects were DA-specific, as the monoamines 5-HT or NA were unable to evoke similar region-specific neuromodulatory actions. Further, bath-application of the gap junction blocker quinine was able to prevent the differential DA-modulatory effects in the IML-containing segments. Quinine not only interferes with gap junctions, but also has been shown to modulate K+ channels in addition to gap junctions. This suggests that the region-specific DA modulation of spinal reflex could be mediated through gap junctions, IML-affiliated K+ channels, or both (Findlay, et al, 1985, Srinivas et al., 2001).

Effect of Dopaminergics on the MSR

Modulatory effects of DA

We report that a low concentration of DA (1 μM) can modulate spinal reflex in an opposing manner in different segments of the spinal cord. As seen in previous studies,

this concentration of DA tends to have an inhibitory effect on the MSR in WT mice (Clemens and Hochman, 2004). However, when the same concentration of the drug is applied to segments of the spinal cord containing autonomic innervation, spinal reflex is instead facilitated. This is a novel finding, which has not been published to date. In a 2012 study, a tadpole spinal cord preparation was used to study these opposing modulatory effects of DA on a central pattern generator during fictive swimming. Under low DA, the bursts of tail-based swimming movement was decreased, while under high DA, it was increased. With the use of DA ligands, the reduction in locomotor activity seen with low DA is mimicked with a D2 agonist and the increase in locomotor activity with high DA is mimicked with the D1 agonist (Clemens et al., 2012). Thus, the application of low DA (1 μM) would be expected to preferentially activate the D2-like receptors over the D1-like receptors, therefore causing a decrease in cellular excitability. This is generally what we found in the lumbar (L3-L5) segments. However, we observed a facilitation of reflex strength in the segments with autonomic innervation, suggesting the segments containing the IML have cells that respond differently to DA than what has previously been reported in the lumbar segments.

Modulatory effects of DA receptor agonists and antagonists

The application of D2 and D1-specific receptor agonists and antagonists further revealed the differential response between lumbar (L3-L5) and IML-containing (T1-L2, L6-S1).

The D2 specific agonist quinpirole mimicked in the lumbar segments the MSR amplitude change seen with 1 μM DA. This outcome has been seen in previous studies

using this technique (Clemens and Hochman, 2004, Clemens et al., 2012). Furthermore, the same application of this drug in the IML-containing segments facilitated the MSR amplitude, again similar to what was observed with the application of low DA in those segments. This suggests that the low application of DA used in these experiments was preferentially targeting the D2-like receptors over the D1-like receptors. We conclude from this data that the D2-like receptors mediate the inhibition of reflex strength in the lumbar (L3-L5) segments, and likewise, can also bring about the facilitation of reflex strength in the IML-containing segments.

The D2 specific antagonist raclopride was used to verify the effect seen with the D2 agonist; it opposed the response seen with 1 μM DA in the lumbar segments and mimicked the actions of 1 μM DA in the IML-containing segments. The effect of raclopride in the L3-L5 segments agree with what has been reported previously (Clemens et al., 2012), however the effect of raclopride in the IML-containing segments has not previously been reported.

We next tested if the differential response seen between segments with and without the IML was exclusively mediated by the D2 receptor. The D1 specific agonist SKF 38393 increased the MSR amplitude in the L3-L5 segments, however in the IML-containing segments, the MSR amplitude was not different than baseline and the difference between these two regions was significant. The effects of this drug in this specific preparation have not been previously reported. From this data we conclude that the reversal of reflex strength seen between lumbar (L3-L5) and IML-containing segments can occur through both D2-like and D1-like receptors.

Effect of Dopaminergics on the LLR

Modulatory effects of DA

After we observed the differential MSR response between segments of the spinal cord with and without autonomic innervation, we investigated if this effect would be similar in the LLR circuit as well. Previous studies have shown that the MSR and LLR can be modulated differentially by DA, albeit at a different concentration than what we used (we used 1 μM DA, while a previous study used 1 μM-100 μM DA and the differential modulation of LLR was seen at only the 10 μM application)(Clemens and Hochman, 2004). In our hands, 1 μM DA did not have a conclusive effect on LLR; possibly because we did not have enough experiments with LLR present when only DA was added to the bath (L3-L5: n=8; T1-L2, L6-S1: n=14) and/or because of the large amount of variation across experiments.

Modulatory effects of DA receptor agonists and antagonists

As with DA, the LLR response to D2 and D1-specific receptor agonists and antagonists showed a similar trend to the MSR response in the case of the D2 agonist quinpirole in the L3-L5 and IML-containing segments. In the case of the D2 antagonist raclopride, the LLR change was not significant for either the L3-L5 or the IML-containing segments. The D1 agonist SKF 38393 in the L3-L5 segments did show an increase from baseline similar to what was seen with the MSR and the IML-containing segments were also similar to the MSR in that the change was not significantly different from

baseline. From this data we conclude that the switch in DA modulation of spinal cord reflex is circuit specific to the MSR and does not apply to the LLR circuitry.

Modulatory Effects of Other Monoamines on the MSR and LLR

In the lumbar spinal cord, neuromodulatory actions of the monoamines, 5-HT and NA, have been studied in detail (Lew et al., 1992, Tanaka et al., 1997, Kiehn et al., 1999, Garraway and Hochman, 2001, Clemens and Hochman, 2004). However, their actions have not been studied in the context of modulating spinal reflex in the spinal cord segments that contain the IML. Past studies have indicated that 5-HT tends to depress spinal reflex activity in the rat neonatal spinal cord (Machacek et al., 2001). We found a similar depression of reflex in the L3-L5 segments (both MSR and LLR) to what has been found in previous studies (Crick and Wallis, 1991, Machacek et al., 2001). Importantly, we did not see any difference in 5-HT modulation of spinal reflex between the lumbar segments of the spinal cord without IML when compared to the segments with IML. This was a first indication that the region-specific differential modulation of spinal reflex occurs was DA-specific.

Early studies on the precursor to DA and NA (L-DOPA) showed a facilitation of reflex activity (MSR and LLR) in the L4-L5 segments in the rat spinal cord (Tanabe et al., 1990). This was thought to occur through spinal α_1 and α_2-adrenoreceptors, pointing to a role for NA in modulating these reflexes. A more recent study involving the noradrenergic modulation of activity in the lumbar dorsolateral nucleus (located in the L6 segment) of the rat showed an increase in excitability of these motoneurons (Yashiro et al., 2010). We observed a small increase in spinal reflex amplitudes from baseline in

the L3-L5 segments, and a substantial increase from baseline in the IML-containing segments. However, there was no significant change between the two areas of the cord under NA, thus supporting the hypothesis of a DA-specific differential modulation.

Modulatory Effects of Gap Junction Blockers on Spinal Reflex

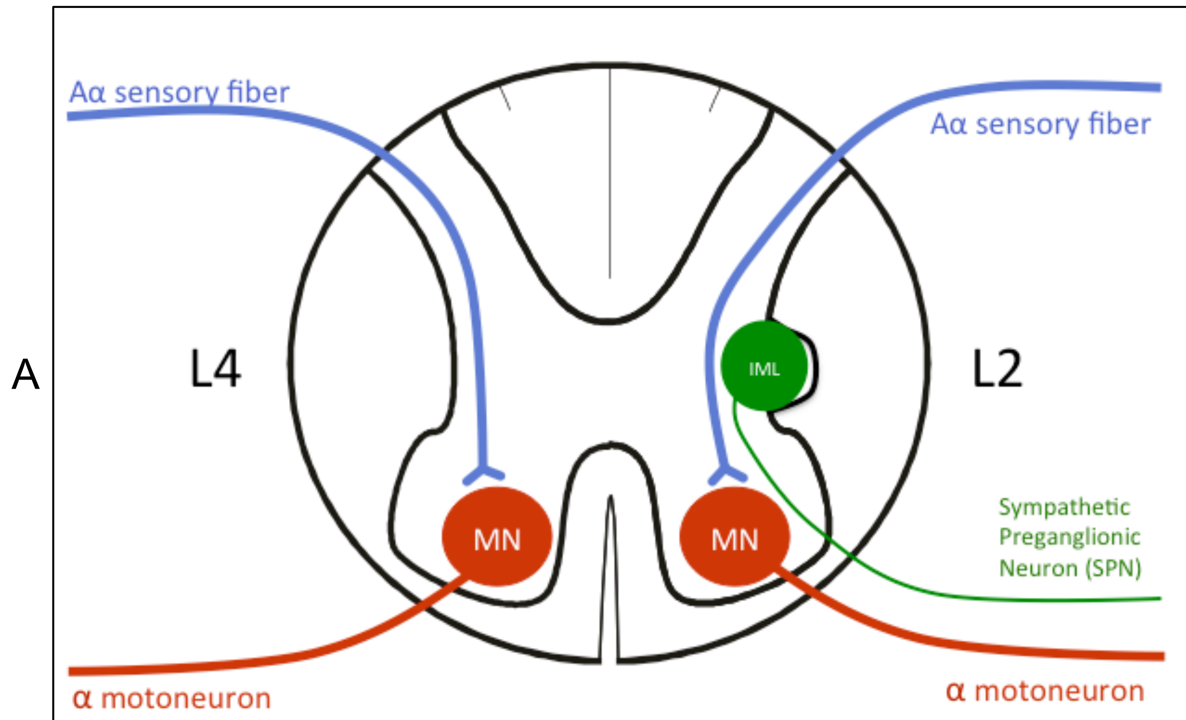
Motoneurons are coupled to each other via gap junctions, as are sympathetic preganglionic neurons (SPNs)(Marina et al., 2008), however it remains unknown if such gap junctions also exist between motoneurons and SPNs. We therefore sought to explore the potential role of gap junctions in the differential DA-drive modulatory actions observed. Gap junctions formed by connexin36 (Cx36) are known to be specific to neural tissue (Hormuzdi et al., 2001, Christie et al., 2005). Quinine is a specific blocker of Cx36 gap junction channels, while carbenoxolone targets Cx43, another neural gap junction channel (Srinivas et al., 2001, Russo et al., 2008). Both gap junction blockers, when applied in combination with dopaminergics, were able to reverse the effects of the dopaminergic drugs in the IML without the gap junction blocker. However, both quinine and carbenoxolone also exert non-specific actions. For example, in addition to blocking gap junctions, quinine is known block G-protein coupled inwardly rectifying K+ (GIRK) channels in many different tissues, including pancreas, heart, nervous system, and liver (Lebrun et al., 1982, Cherubini et al., 1984, Jung and Brierley, 1984, Cook and Haylett, 1985). GIRK channels are activated by G-proteins, either through direct interaction of the G-protein and the ion channel, or through second messenger pathways (Brown, 1990, Breitwieser, 1996, Dascal, 1997). "The wide distribution of GIRK subunits in channels are modulated by G proteins, most via second the brain and the potential for

regulation of neurotransmitters suggest important physiological roles for GIRKs in the central nervous system" (Dascal, 1997). DA is known to regulate the activity of the GIRK channel, through both D1 and D2 receptors, in neural tissue (Dascal, 1997, Kuzhikandathil and Oxford, 2002, Marowsky et al., 2005, Sahlholm et al., 2008). D1 receptors in paracapsular intercalated cells in the amygdala have been shown to inhibit the activity of these cells on a systemic level, while at the same time causing excitation of interneurons in the basolateral amygdala (Marowsky et al., 2005). The inhibition of activity occurring through the D1 receptors is thought to occur not through the classical cAMP-mediated pathway, but instead the D1 receptor is thought to be mediating a decrease in excitability of the system through the opening of GIRK channels (Marowsky et al., 2005). Another paper presented evidence that a D1 specific antagonist has the ability to close GIRK channels in the Chinese hamster ovary cell (Kuzhikandathil and Oxford, 2002). Based on these observations, we believe a possible mechanism for the differential modulation of reflex through the actions of DA in the cells of the IML could involve D1 receptor-mediated opening of the GIRK channel in those cells. The GIRK channel is notably present on tonically active cells (such as SPNs), and serves the role of regulating the excitability of these cells (Dascal, 1997, Takigawa and Alzheimer, 2002). Figure 3.8 illustrates the model we put forth to explain the DA-specific differential modulation of spinal cord reflex. In Figure 3.8A, the circuitry of two segments, L2 and L4, are presented, showing only the monosynaptic reflex circuit and the presence of the IML and SPN on only the L2 segment. In Figure 3.8B, these circuits are examined in more detail in the L2 segment, showing the presence of the D1 and D2 receptors on the motoneuron and on the IML. The IML has the added presence of the

GIRK channel, which would be either opened or closed upon activation by the D1 or D2 receptor. If the activation of the D1 receptor opens the GIRK channel in the IML, as it does in the paracapsular intercalated cells of the amygdala, the excitability of the IML could be decreased through the D1 receptor (Marowsky et al., 2005). Gap junction connections between SPNs and motoneurons would provide a pathway for the excitability of the SPN to impact the excitability of the motoneuron. Similarly, carbenoxolone can also have nonspecific effects on ion channels (Rekling et al., 2000, Rouach et al., 2003, Vessey et al., 2004), perhaps including GIRK channels. Thus, it is possible that the gap junction blockers used in our experiments were acting on ion channels in the IML region. A dual effect of quinine or carbenoxolone could also explain both the mechanism of action on the DA receptor side and the SPN to motoneuron side.

Potential Model for Specific Aim #1

A



B

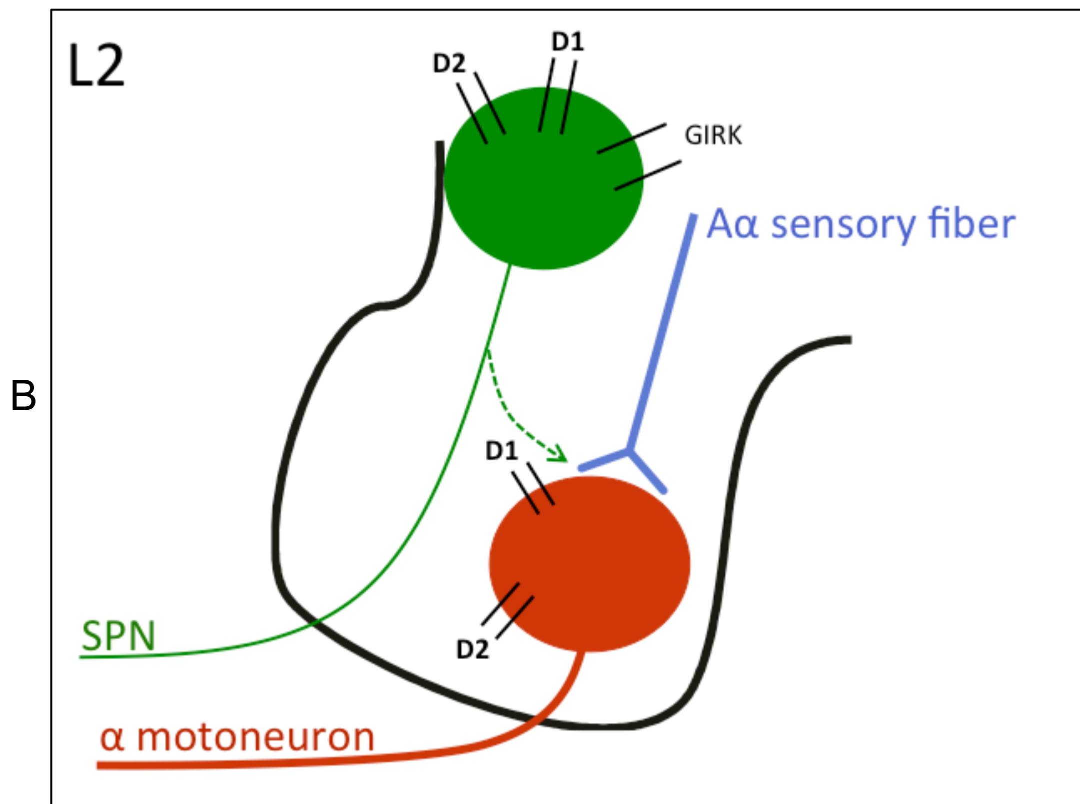


Figure 3.8

Figure 3.8 Legend

A. Monosynaptic circuits are shown in both the L4 segment (no presence of IML) and the L2 segment (IML is present).

B. Focusing on the L2 segment, D1-like and D2-like receptors are present on the motoneuron cells and the IML cells. As shown in previous publications, the D1 receptor has the ability to open GIRK channels, therefore decreasing the excitability of the SPN. The D2 receptor could do the opposite, closing the GIRK channels and increasing the excitability of the SPN. In this model, the SPN sends a presynaptic projection to the motoneuron and gap junctions provide a pathway for the excitability of the SPN to impact the excitability of the motoneuron.

The results of this study indicate that dopaminergic modulation of spinal reflex (MSR and LLR) occur in segment-specific manner. The lumbar segments (L3-L5) of the spinal cord without autonomic innervation respond in an opposite manner to those segments that do contain either sympathetic or parasympathetic preganglionic cell bodies. While there is some evidence that gap junctions play a role in the purported communication between these preganglionic autonomic motoneurons and somatic motoneurons, the mechanism of action could possibly also be dependent on an altered modulation of ion channels in the IML, in particular K+.

There is strong *in vitro* evidence from this study that the presence of DA can have an impact on the ANS; we next asked if this monoamine could impact the ANS *in vivo* as well.

CHAPTER 4: THE DOPAMINE D3 RECEPTOR KNOCKOUT MOUSE MODELS AGING-RELATED CHANGES IN AUTONOMIC FUNCTION

SUMMARY

The increased prevalence of hypertension with aging is well established, as is a role for the dopamine D3 receptor in the general pathophysiology of hypertension. To determine the specific effects of D3 dysfunction on autonomic output and cardiac involvement in aging associated hypertension, non-invasive blood pressure, pulse measurements, and echocardiography were used to compare cardiac structure and function in differently aged wild-type (WT) animals (2 months, 1 year, 2 years) and D3 receptor knockout mice (D3KO). In WT animals, aging-related increases in blood pressure and cardiac function, accompanied by bradycardia in the oldest animals were observed. Interestingly, 2-month-old D3KO mice displayed blood pressure and heart rate values that were significantly increased over their age-matched WT controls, and that were comparable to those of the 2-year-old WT group. Echocardiography revealed aging-related changes in WT animals, but we did not find significant differences between age matched young WT and D3KO mice. Histology demonstrated the appearance of interstitial fibrosis in the young D3KO mice that was comparable to the age-related fibrosis seen in 2 year-old WT mice. Our data suggest that the D3KO animal may serve as a model to better understand the role of the dopamine D3 receptor in the aging heart, and in particular its role in the pathophysiology of cardiovascular fibrosis.

INTRODUCTION

The role of dopamine (DA) in the modulation of spinal reflex has been well characterized in several previous studies (Carp and Anderson, 1982, Lindvall et al., 1983, Garraway and Hochman, 2001, Levant and McCarson, 2001, Clemens and Hochman, 2004, Clemens et al., 2012). DA plays a role in shaping somatic motor output from the spinal cord (CPG) (Harris-Warrick et al., 1995, Lapointe et al., 2009, Clemens et al., 2012), but may also have an influence on neural circuits in other segments of the spinal cord that do not contribute to locomotion (cf. Chapter 3). Therefore, we wanted to know how the loss of a functional DA receptor would impact global autonomic motor output and cardiovascular performance at the level of the whole animal.

Measurement of both blood pressure and heart rate variability are noninvasive ways to assess the autonomic tone of the cardiovascular system (de Champlain et al., 1980, Stein et al., 1994, Ino-Oka et al., 2011, Davis et al., 2012). Blood pressure is ultimately determined by three factors: cardiac output, fluid volume, and peripheral resistance. Neural reflexes involving both the SNS and PNS mediate the short-term control of blood pressure and together they exert control over all of these factors. The SNS is the main neural regulator of peripheral resistance, as the autonomic innervation of the vasculature is primarily sympathetic. A change in sympathetic tone will increase or decrease the diameter of blood vessels, subsequently regulating peripheral resistance. Heart rate and stroke volume (cardiac output = HR x SV) are controlled by the PNS and SNS; therefore both divisions of the ANS have an impact on the cardiac output side of the equation. The SNS also exerts an influence on changes in

hemodynamics and tubule transport in the kidney, and is the primary contributor to neural control of the long-term regulation of blood pressure (Boron and Boulpaep, 2002).

The rise in blood pressure with age is an indicator of an increase in sympathetic tone that occurs during the lifespan of an animal (de Champlain et al., 1980, Esler et al., 2002, Thijssen et al., 2006, Malpas, 2010). A role for DA in the pathophysiology of hypertension has been well established (Planz et al., 1976, Goldberg, 1984, Andrejak and Hary, 1986, Kuchel, 1990, Lundberg et al., 1990, Kuchel and Kuchel, 1991), and there is evidence for a role of both D1 and D3 receptors in the pathophysiology of hypertension (Asico et al., 1998, Felder et al., 2000, Sato et al., 2000, Zeng et al., 2004a, Zeng et al., 2005, Yu et al., 2006, Zeng et al., 2006, Zeng et al., 2007, Banday and Lokhandwala, 2008, Zeng et al., 2008, Yu et al., 2009, Tayebati et al., 2011). As the highest affinity DA receptor is the D3 type (Clemens et al., 2006, Pivonello et al., 2007), we wanted to assess its contribution to the emergence of hypertension. The D3 receptor is expressed in the heart (Gomez Mde et al., 2002), and several mechanisms have been reported by which a failure of the D3 receptor system in the periphery might account for an increase in blood pressure (Asico et al., 1998, Muhlbauer et al., 2000, Jose et al., 2002), including an interaction with the D1 receptor subtype (Jose et al., 1999, Zeng et al., 2004a, Zeng et al., 2004b), with angiotensin II (Zeng et al., 2003), endothelin binding receptors (Yu et al., 2009), and the G protein-coupled receptor kinase 4 in the kidney (Kim et al., 2005, Villar et al., 2009, Jose et al., 2010). Since increases in autonomic output and corresponding cardiac function observed with age are well established, and since D3 receptor knockout animals have been found to

express hypertension (Asico et al., 1998), we addressed the question if a dysfunction of the D3 receptor system can mimic aging-related changes in the murine animal model.

Using non-invasive blood pressure measurements, functional echocardiography, and histology on the hearts of differently-aged WT animals (2-3 months, 1 year, 2 years), we found an aging-related increase in blood pressure and cardiac dysfunction, which was accompanied by bradycardia in the oldest animals. Intriguingly, the 2-3 month old D3KO cohort displayed blood pressure and heart rate values that were significantly increased over their age-matched WT controls but similar to those of the 2 year-old WT group. While the echocardiography did reveal morphological differences between young and old WT, we did not observe any such differences between the young and age-matched WT and D3KOs. However, functional data derived from the echocardiography indicate that fractional shortening and ejection fraction showed an aging-related decline in WT, and that these parameters were similar between old WT and young D3KO.

Histological analyses revealed an aging-related increase in interstitial fibrosis in the myocardium of WT animals, with fibrosis in the 2 year-old WT animal group equivalent to that observed in the 2 month-old D3KO animals.

Taken together, our data suggest that aging-related changes in hypertension and cardiac fibrosis of WT animals are partially mimicked in young D3KOs. Thus, D3KOs may provide a model to understand the role of the dopamine D3 receptor in aging heart function, and in particular its role in the associated fibrotic remodeling.

RESULTS

Weight of Animals

One of the common reasons for a chronic change in blood pressure is related to a change of the fluid volume in the body, which is associated to the overall body weight. Table 4.1 lists body weights of the four animal cohorts. We observed significant increases in body weight between 2-month-old WTs (26.83±0.47g) and 1-year-old WTs (42.49±2.2 g), between 2-month-old WT and 2 years-old WTs (35.15±1.13g), and between 1-year-old WTs and D3KOs (28.96±0.63g, $p<0.001$). However, we did not detect any differences between young WTs and young D3KOs, suggesting that any potential differences in blood pressure or heart rate did not arise from differences in body mass or fluid volume between the age-matched animals.

Animal Weights

	2 mo WT	1 yr WT	2 yrs WT	2 mo D3KO
Body weight (g)	26.83 ± 1.48	42.49 ± 6.98 ♯	35.15 ± 3.57 §	28.98 ± 1.99 ♪

Table 4.1

Average values ± S.E.
♯ = significant difference between 2 mo WT and 1 yr WT
§ = significant difference between 2 mo WT and 2 yrs WT
♪ = significant difference between 1 yr WT and 2 mo D3KO

Autonomic Evaluation of WT and D3KO Animals

Non-invasive blood pressure and heart rate recordings were obtained from a total of 30 WT of different ages and 6 young D3KO animals. WT animals were grouped in cohorts of young (2-3 months, n=10), 1-year-old animals (n=12), and 2-year-old animals (n=8). On average, animals in each group were subjected to 5.7±0.15 sessions, with the first 3 sessions used to acclimate the animals and the last 3 sessions used for data analysis. On average, 7.6±0.13 readings/parameter/session were made, and a total of 5305 systolic and diastolic blood pressure and pulse measurements were used for data analysis.

In WT animals, aging was accompanied by a gradual increase in systolic and diastolic blood pressures (Fig. 4.1). Systolic values were similar between 2-month- and 1-year-old animals (94.17±1.94 mmHg and 96.57±1.95 mmHg, respectively) but significantly increased by year 2 to 143.76±3.57 mmHg ($p<0.001$, Fig. 4.1A). In contrast, diastolic values showed a continuous and significant rise from 42.09±0.65 mmHg at 2 months over 56.59±1.67 at 1 year, to 83.97±3.3 at 2 years of age ($p<0.001$, Fig. 4.1B). Interestingly, systolic blood pressure in 2 month-old D3KOs was similar to the 2-year-old WT cohort (141.85±2.72 mmHg), but was significantly higher than both the 2-month- and 1-year-old WTs (Fig. 4.1A). Similarly, diastolic values of the young D3KOs (69.71±1.1 mmHg) were significantly higher than the 2-month- and 1-year-old WTs (Fig. 4.1B, $p<0.001$).

The increase in blood pressure readings in the aging WT mice was accompanied by a significant decrease in heart rate with age from 714.88±6.96 beats-per-minute (bpm) at 2 months and 735.59±8.7 bpm at 1 year to 660.85±9.3 bpm (Fig. 4.1C,

$p<0.001$). The bradycardia in the 2-year-old WT was again mimicked in the young D3KOs (632±15.32 bpm, Fig. 4.1C). To assess the oxygen requirements and work load of the hearts, the rate pressure product (RPP) was calculated as a product of measured systolic blood pressure x heart rate (Fig. 4.1D). The RPP in WT rose by 41% from 67302±1430 at 2 months and 70936±1151 at 1 year, to 94974±2530 in the 2-year-old animals ($p<0.001$), and was increased by 33% in the 2-months-old D3KOs over their age-matched WT controls (89565±2176, n.s. from 2-year-old WT) ($p<0.001$, Fig. 4.1D). Together, these data provide evidence that aging-related changes observed in WT can already be found in young D3KOs.

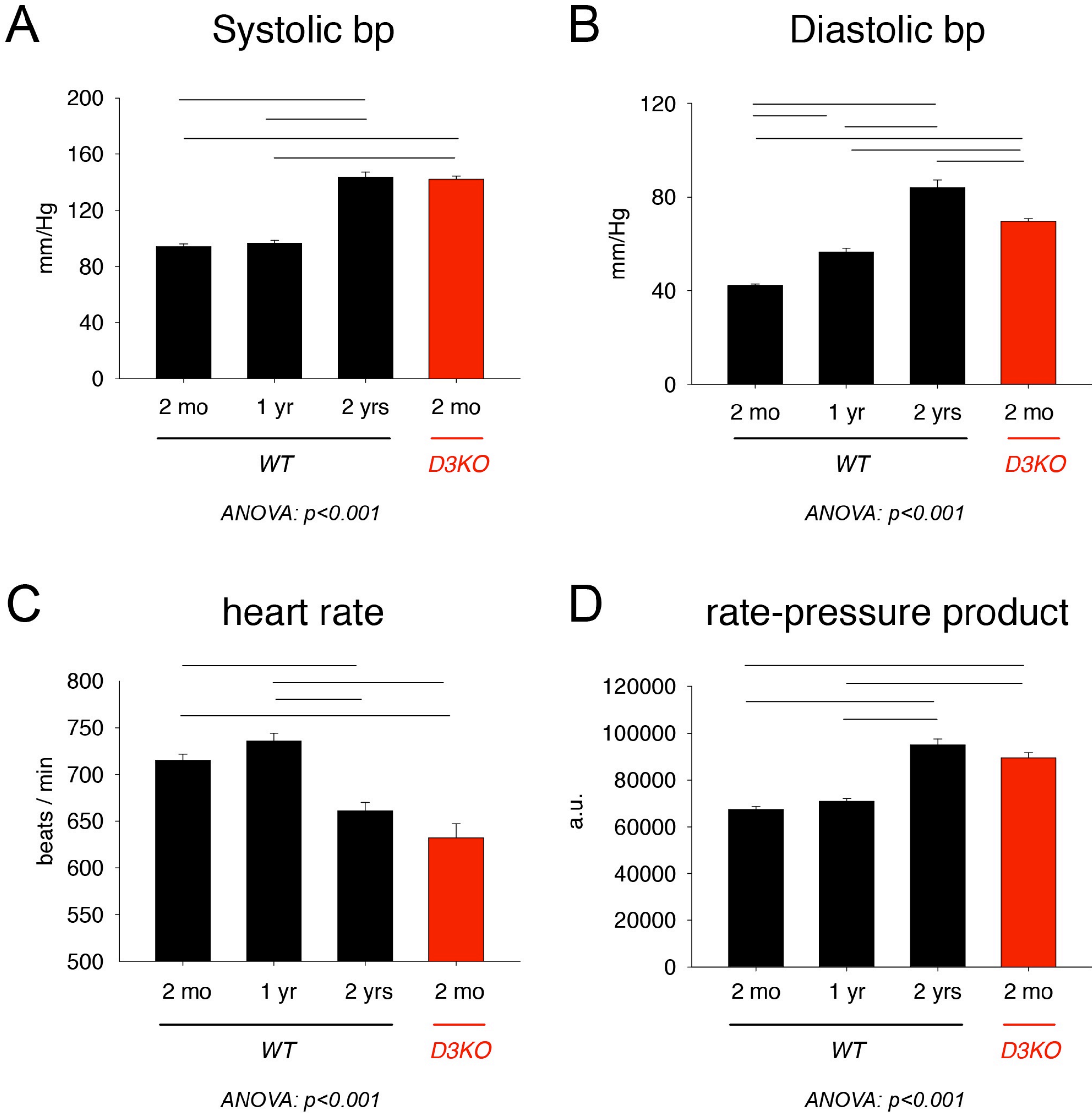


Figure 4.1

Figure 4.1 Legend

Horizontal lines indicate significant differences between groups.

A. Systolic blood pressure is stable in WT from 2 months to 1 year of age (94.17 +/- 1.94 mmHg and 96.57 +/- 1.95 mmHg, respectively), but significantly increases from year 1 to year 2 (96.57 +/- 1.95 mmHg to 143.76 +/- 3.57 mmHg, respectively) ($p<0.001$, ANOVA). Systolic blood pressure in 2 month-old D3KOs was similar to the 2 year-old WT cohort (141.85 +/- 2.72 mmHg), and was significantly higher than both the 2 month- and 1 year-old WTs (Fig. 4.1A).

B. Diastolic blood pressure increases gradually with age in WT and reaches its peak at 2 years of age (42.09 +/- 0.65 mmHg at 2 months over 56.59 +/- 1.67 at 1 year, to 83.97 +/- 3.3 at 2 years of age, $p<0.001$, Fig. 1B). Young D3KO show levels that are not as high as 2 years old WT, but significantly higher than 2 month- or 1 year-old WTs (69.71 +/- 1.1 mmHg, $p<0.001$, ANOVA, Fig. 4.1B).

C. Heart rate reaches a peak in 1-year-old WT and drops significantly in 2 year-old WT (714.88 +/- 6.96 beats-per-minute (bpm) at 2 months and 735.59 +/- 8.7 bpm at 1 year to 660.85 +/- 9.3 bpm, Fig. 1C, $p<0.001$). Values are similar between 2 year-old WT and 2 month-old D3KO (632 +/-15.32 bpm, Fig. 4.1C).

D. The RPP in WT rose by 41% from 67302 +/- 1430 at 2 months and 70936 +/-1151 at 1 year, to 94974 +/-2530 in the 2 year-old animals ($p<0.001$), and was increased by 33 % in the 2 months-old D3KOs over their age-matched WT controls (89565 +/-2176, n.s. from 2 year-old WT) ($p < 0.001$, Fig. 4.1D).

Echocardiography

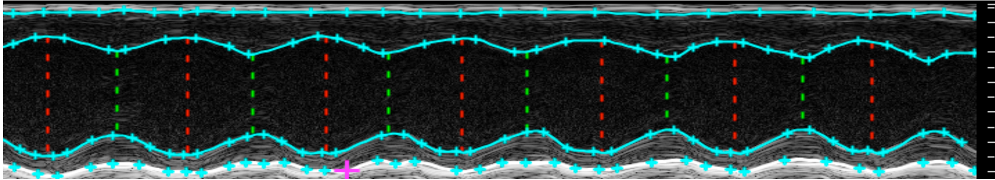
To determine if aging-related changes in blood pressure and heart rate in WT and D3KOs were related to cardiac function, we performed ultrasound echocardiograms in anesthetized animals. Echograms were obtained and analyzed from 2-month-old WTs (n=9), 1-year-old WTs (n=7), 2-year-old WTs (n=6), and 2-month-old D3KOs (n=5), and data are reported in Table 4.2. Overall, ten morphological and functional parameters were measured or calculated and compared between all groups. Comparing 1-year-old WT with 2-month-old WT mice, we found significant increases in systolic diameter ($p<0.024$), lateral ventricle (LV) mass ($p<0.003$), systolic volume ($p<0.019$), diastolic volume ($p<0.028$), and cardiac output ($p<0.001$). At 2 years of age, WT animals exhibited further increases in systolic diameter ($p<0.024$) and systolic volume ($p<0.019$), but significantly reduced ejection fraction ($p<0.033$) and fractional shortening ($p<0.017$) when compared to the young group. Interestingly, when adjusted for body weight, LV mass was no longer significantly different across all WT age groups.

In contrast, when comparing the 2-year-old WTs with the 2-month-old D3KOs, we found significant reductions in systolic and diastolic diameters ($p<0.024$ and $p<0.027$, respectively), total ($p<0.003$) and body weight-corrected ($p<0.006$) LV mass, diastolic volume, stroke volume, and cardiac output ($p<0.028$, $p<0.045$, and $p<0.001$, respectively). LV mass, LV mass corrected for bodyweight, systolic and diastolic volume, stroke volume, and cardiac output were also significantly different between 2-month-old D3KOs and 1-year-old WTs. Importantly, both ejection fraction and fractional shortening were significantly decreased in D3KO over their age-matched WT controls ($p<0.012$ and

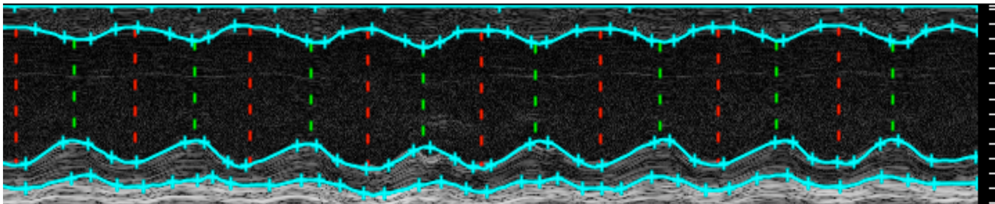
$p<0.01$, respectively), suggesting that cardiac dynamic function is compromised in young D3KOs.

Echocardiogram Traces from 2-month-old, 1-year-old, and
2-year-old WT and 2-month-old D3KO Animals

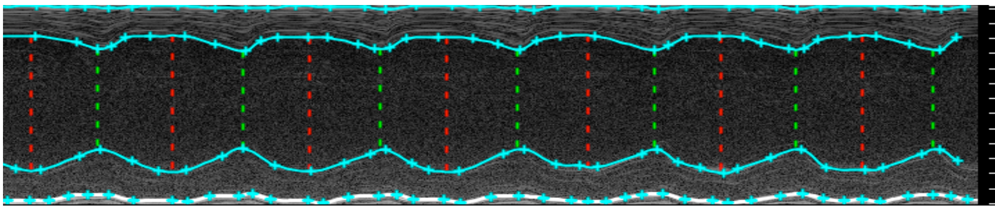
2 month WT



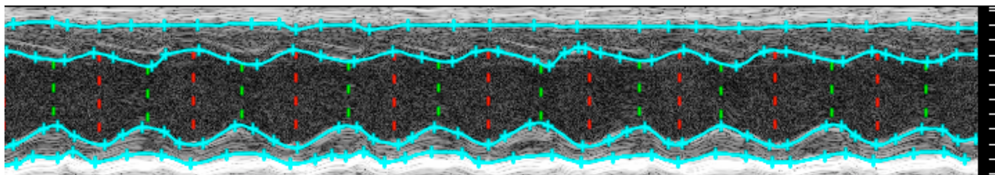
1 year WT



2 year WT



2 month D3KO



5 mm

250 ms

Figure 4.2

Figure 4.3 Legend

The LV area tool (blue line) was used to trace the anterior and posterior walls of the left ventricle. An aging-related increase in anatomical parameters (systolic and diastolic diameters) was revealed after analysis of the echocardiograms, but no difference between age-matched WT and D3KO. In contrast, fractional shortening and ejection fraction (parameters of cardiac function) decrease in WT with age, and are in young D3KO similar to those of the 2-year old WT.

Green: systolic diameter

Red: diastolic diameter

Echocardiogram Analysis

	2 mo WT	1 yr WT	2 yrs WT	2 mo D3KO
Systolic diameter (mm)	2.22 ± 0.56	2.73 ± 0.45 ♯	2.83 ± 0.26 §	2.21 ± 0.25 ♪
Diastolic diameter (mm)	3.51 ± 0.52	4.05 ± 0.57	3.9 ± 0.38	3.26 ± 0.25 ♪
Ejection fraction (%)	68 ± 3.2	61.65 ± 1.6	53.18 ± 3.9 §	55 ± 2.9 €
Fractional shortening (%)	37.76 ± 2.5	32.84 ± 1	27.39 ± 2.6 §	28 ± 2.9 €
LV mass (mg)	94.66 ± 13.88	138.52 ± 32.56 ♯	133.64 ± 17.38 §	72.29 ± 10.67 ♪, +
LV mass / body weight (%)	0.35 ± 0.05	0.33 ± 0.08	0.38 ± 0.05	0.25 ± 0.04 €, ♪, +
Systolic volume (µl)	18.6 ± 10.57	29.21 ± 10.71 ♯	31.11 ± 6.57 §	18.05 ± 3.05 +
Diastolic volume (µl)	52.99 ± 18.5	74.28 ± 22.62 ♯	67.17 ± 15.85	43.81 ± 8.31 ♪, +
Stroke volume (µl)	34.39 ± 8.2	45.07 ± 12.41	36.06 ± 14.31	25.76 ± 7.96 ♪
Cardiac output (ml/min)	12.55 ± 2.8	21.14 ± 5.52 ♯	14.59 ± 5.23	9.32 ± 3.14 ♪, +

Table 4.2:

Average values ± S.E.
♯ = significant difference between 2 mo WT and 1 yr WT
§ = significant difference between 2 mo WT and 2 yrs WT
€ = significant difference between 2 mo WT and 2 mo D3KO
♪ = significant difference between 1 yr WT and 2 mo D3KO
+ = significant difference between 2 yrs WT and 2 mo D3KO

Morphometric Analyses

We next assessed the morphometric features of the hearts, and measured LV, LV chamber area, and myocyte cross-sectional areas (MCSAs) of endo- and epicardium in WT and D3KO animals (Table 4.3). We found an aging-related trend in WT animals for an increase in LV and LV chamber area, a significant increase in both myocyte cross-sectional area (MCSA) endo- and epicardium area in 1-year-old animals ($p<0.001$), and a concomitant bi-phasic change in MCSA endocardium area with a peak in the 1-year-old animals ($p<0.001$). In contrast, MCSA areas were not significantly different between age-matched WT and D3KOs. Thus the increase in blood pressure and the bradycardia observed in young D3KOs appears to not stem from changes in LV or MCSA areas.

Fibrosis Measurements

Cardiac fibrosis is a hallmark of heart disease and hypertension (Biernacka and Frangogiannis, 2011). We therefore assessed with histological approaches the levels, expression pattern, and qualitative characteristics of fibrosis-related collagen in the heart (Fig. 4.3). Fig. 4.3A shows images from the myocardium of the left ventricle of each of the different age groups of WT and D3KO. There is an increase in collagen staining (represented by red staining) in the group of aging WT mice from 2 months to 2 years of age and the amount of collagen in the 2-month-old D3KO is more similar to the 2-year-old WT. After normalization to the 2 months-old WT, collagen expression in the myocardium increased in the 2 year-old cohorts by 24.8 +/- 6.1 %, and by 34.8 +/- 8.5% in the 2 months-old D3KOs (ANOVA, $p=0.002$)(Fig. 4.3B). This suggests that interstitial

fibrosis might play a critical role in the emergence of hypertension and bradycardia in the young D3KOs.

Morphometric Analysis

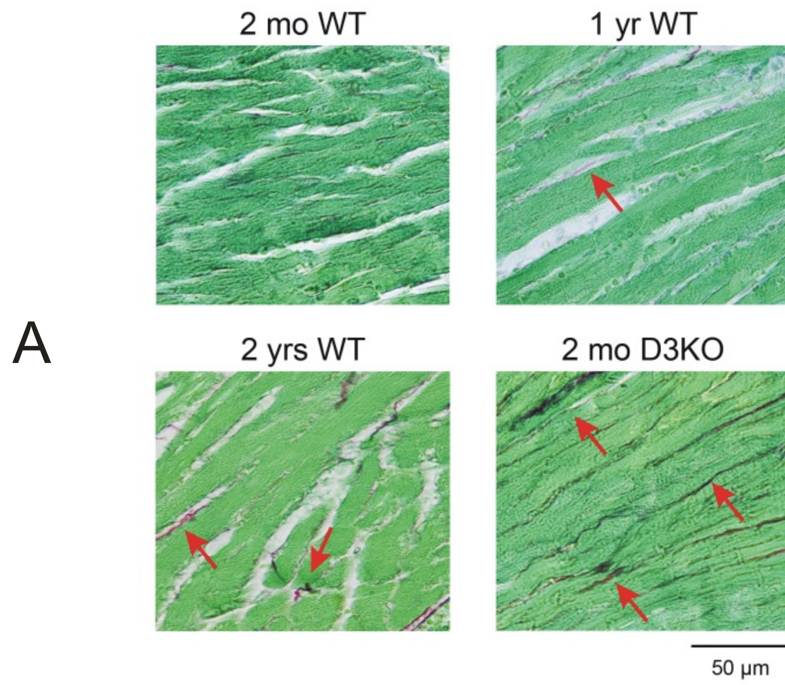
	2 mo WT	1 yr WT	2 yrs WT	2 mo D3KO
LV area (mm^2)	15.79 ± 1.62	20.38 ± 0.87	20.2 ± 1.3	15.02 ± 0.76 ♪, **+**
LV chamber area (mm^2)	2.11 ± 0.05	2.24 ± 0.22	2.58 ± 0.21	1.51 ± 0.09 **+**
MCSA epicardium (mm^2)	230.2 ± 13.58	323.96 ±16.54 ♯	267.8 ±12.67	230.93 ±13.52 ♪
MCSA endocardium (mm^2)	284.3 ± 13.98	340.93 ± 12.57 ♯	254.72 ± 9.66 ¶	265.37 ± 8.26 ♪

Table 4.3:

Average values ± S.E.
♯ = significant difference between 2 mo WT and 1 yr WT
§ = significant difference between 2 mo WT and 2 yrs WT
♪ = significant difference between 1 yr WT and 2 mo D3KO
¶ = significant difference between 1 yr WT and 2 yrs WT

Interstitial Fibrosis in WT (2 months to 2 years) and Young (2 months) D3KO Animals

A



B

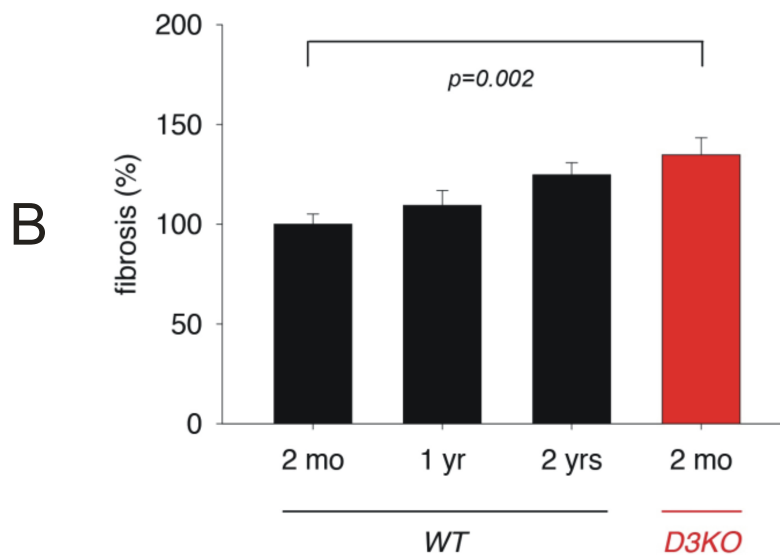


Figure 4.3

Figure 4.3 Legend

A. Representative images of the myocardium of the left ventricle of 2 month-, 1 year- and 2-year-old WT, and 2 month-old D3KO mice. Images show an increased amount and thickness of fibrillar collagen in WT with age, and the levels of collagen in 2 year-old WT are comparable to the levels in the young D3KOs.

B. A quantification of the fibrosis reveals a significant increase in fibrosis staining in young D3KOs over the age-matched WT control, even surpassing the fibrosis levels of the 2-year-old cohort.

DISCUSSION

We present evidence that aging is associated with an increase in autonomic function and interstitial fibrosis, and that the dysfunction of the dopamine D3 receptor can be associated with the emergence of aging-related changes in cardiac function and cytoarchitecture in a young D3KO animal. Hypertension is defined as an abnormal rise in blood pressure, which, regardless of origin, results in either an increase in systemic vascular resistance or an increase in cardiac output, or both. Thus, our data are consistent with the idea that D3KOs may be a new animal model to understand the role of the dopamine D3 receptor in aging heart function, and in particular its role in cardiac remodeling associated with hypertension. The rise in blood pressure during the aging process in humans is common and has been well-established in the literature for many years (Amery et al., 1978). This seems to translate well with our group of aging WT mice, which showed an overall steady increase in blood pressure with age. Specifically, the systolic measurement stayed level from 2 months to 1 year, but then was significantly increased from 1 year to 2 years, while the diastolic measurement increased significantly over each time point. In our aging WT mice, we are likely seeing the normal progression of hypertension during the aging process from an early stage (borderline) to a later stage of the fully developed disease with no clear, identifiable cause (primary/essential)(Julius, 1988). The literature on the why borderline hypertension develops and how it shifts from early to late stage is confusing and full of differing opinions about exactly which system(s) is/are responsible. Among others, defects are cited in the renal and renin/angiotensin system (Loney et al., 2002, Failor and Capell, 2003, Hartman et al., 2003, Freel and Connell, 2004), the SNS (Julius et al.,

1971, Julius, 1988, DeQuattro and Feng, 2002), and others think there are abnormalities during cardiovascular development which lead to the development of primary hypertension (Safar and Boudier, 2005). In cases of primary hypertension, there is evidence that during the early stages of the disease there is first an increase in cardiac output, which subsequently leads to an increased vascular resistance (Julius et al., 1971, Julius, 1988, Palatini and Julius, 2009). In long-term hypertensive patients, the blood volume returns to normal levels, but the vascular resistance remains (Conway, 1963, Harburg et al., 1964, Sivertsson, 1970, Julius, 1988). This division between the stages of hypertension is important in determining the mechanism behind the rise in blood pressure. During the borderline stage of hypertension, the mechanism seems to be primarily neurogenic in origin and a pharmacological intervention to block the autonomic drive tends to reduce cardiac output and reduce blood pressure (Julius et al., 1971, Julius, 1988, Palatini and Julius, 2009). In the established phase of hypertension, however, cardiac output normalizes and "patients show a high total peripheral resistance, their plasma norepinephrine values are not elevated, and there is good evidence of a structural, non-neurogenic component in the elevation of their vascular resistance" (Conway, 1963, Harburg et al., 1964, Sivertsson, 1970, Julius, 1988). In contrast to the aging WT, our young D3KO (2-months-old) mice showed systolic blood pressure nearly equal to that of 2-year-old WT animals and diastolic blood pressure that was significantly increased over 2-month-old and 1-year-old WT, but lower than 2-year-old WT.

We considered the changes in weight and cardiac function as potential explanations for increased blood pressure in in the young D3KO mice. Hypertension

can arise from changes in three parameters: cardiac output, fluid volume, and/or peripheral resistance. The weight of our WT animals increased from 2 months of age to 1 year of age, but did not continue to increase (and may even begin to decrease) from 1 year of age to 2 years of age. The systolic and diastolic blood pressure, however, did increase from their first to second year. This indicates that in the WT animals the blood volume was increasing from 2 months to 1 year, but leveled off or began to drop by 2 years of age, while at the same time the blood pressure of the older animals still significantly increased during that time. Added to the data on the weight of the mice is the fact that the heart rate drops at the same time point that the weight of the animals drops, providing evidence that the cardiac output (heart rate x stroke volume) is reduced with age. Finally, the functional echocardiography confirms that the cardiac output of the 1-year-old WT does increase significantly from 2-months-old, but the 2-month-old is no different from the 2-year-old animals for this parameter. The D3KO animals showed an increased blood pressure at an early age, but showed no differences in weight when compared to the age-matched WT, indicating blood volume was similar between the young D3KO and young WT. Also, the heart rate of these animals was lower than any of the WT age groups and echocardiography measurements indicate a normal cardiac output. By considering the change in these parameters of weight/fluid volume (young D3KO and young WT weights are not different), heart rate (young D3KO have even lower heart rate than young WT), and functional echocardiography (cardiac output is the same in young D3KO and young WT), the driving force behind this increase in blood pressure seen in young D3KO mice seems to be a change in vascular resistance over a change in cardiac output. If the mechanism behind the development of early-onset

hypertension in the young D3KO animals were working through a change in vascular resistance through a remodeling of the vasculature, this would be more similar to what is seen in the older WT animals and older humans during later stages of the disease. This indicates that the young D3KO could be a useful model of autonomic dysfunction with advanced aging and that the physiology and vascular structure is more similar to what is found in the aged animal.

Adding to the idea that the lack of the D3 receptor could be driving a change in the structure of the vasculature is data collected from the use of dopaminergic drugs in the treatment of CNS disorders. Dopamine D2 receptor agonists (specifically ergot derivatives), such as cabergoline or pergolide, have been used in the treatment of Parkinson's disease for many years. As early as 1995, patients treated with these drugs have been noted to develop fibrosis in conjunction with the delivery of the drug (Jimenez-Jimenez et al., 1995). More recent studies from "population studies of patients with Parkinson's disease compared with non-Parkinsonian controls have reported that pergolide and cabergoline have a similar risk of inducing fibrotic changes in cardiac valve leaflets. The fibrotic changes cause thickening, retraction, and stiffening of valves, which result in incomplete leaflet coaptation and clinically significant regurgitation, and have necessitated surgical valve replacement in some patients" (Antonini and Poewe, 2007). Others have since provided more evidence that these D2-specific agonists are indeed involved in producing drug-induced fibrotic lesions in the heart (Andersohn and Garbe, 2009, Antonini et al., 2009, Apostolakis et al., 2009, Bhattacharyya et al., 2009, Izgi et al., 2010, Perez-Lloret and Rascol, 2010), however the mechanism for this D2 receptor-driven increase in cardiac fibrosis has not yet been

fully determined. Although the focus in all of these studies was on D2-specific receptor agonists, it is interesting to reiterate that we have shown evidence that the young D3KO from our study shows an increased amount and thickness of fibrillar collagen in WT with age, and the levels of collagen in 2 year-old WT are comparable to the levels in the young D3KO.

Layered on top of the dysfunction of the cardiovascular system involving either fluid balance, peripheral resistance, or both, is the fact that ultimately both of these hemodynamic factors can be disturbed by an increase in sympathetic activity (Lund-Johansen, 1994, Grassi, 2010). The D3KO animal lacks a key inhibitory dopamine receptor, which is found throughout the spinal cord, including the sites of autonomic control (i.e. IML) that are involved in the regulation of blood pressure. In the aging WT mouse, we can see a normal progression in the development of blood pressure: increase in weight (fluid volume), increase in cardiac output, and increase in blood pressure from 2 months of age to 1 year of age. From the first to second year, however, the cardiac output and weight drops back to what was seen in the 2-month-old animal, but the blood pressure remains high. This has been previously noted as the switch from a dysfunction in cardiac output to a dysfunction in vascular resistance from early stage hypertension (neurogenic in origin) to late stage hypertension (non-neurogenic in origin). However, in our D3KO animals, we see the components of a dysfunction in vascular resistance when compared to the 2-month-old WT animals, without the changes in cardiac output (i.e. they are at the late-stage of hypertension). We cannot, however, rule out a neurogenic role for the D3KO animals in the development of high blood pressure (even if they are physiologically at the late stage) because of the fact the

spinal cord of these animals lack the D3 receptor and therefore could also have a partially unchecked SNS drive from that component of blood pressure regulation in addition to the development of fibrosis.

We have shown an aging related increase in blood pressure in WT mice from 2 months to 2 years of age. The blood pressure measurements of 2–month-old D3KO animals, however, were most similar to the oldest WT animals (2-years-old). We believe the young D3KO animals provide a new model of aging, which exhibit not only a similar increase in blood pressure, but similar changes to the vasculature present in older WT animals.

CHAPTER 5: CONCLUSION

The overall hypothesis of this study is that DA plays a role in regulating the autonomic nervous system with both central and peripheral effects. We can see evidence of this from both the perspective of the underlying spinal cord circuitry and from the *in vivo* physiological changes in global autonomic output.

In the first aim, we asked if DA modulation of spinal reflex would be similar in direction and magnitude in all segments. Almost all previous studies on DA-mediated effects on spinal cord function have focused exclusively on the lumbar segments and the role DA may play in the recruitment of the central pattern generator (CPG) for locomotion (Cazalets et al., 1995, Han et al., 2007). In addition to its role in modulating CPG activity, DA has also been shown to directly modulate spinal reflex in the lumbar segments of the spinal cord (L2-L5)(Clemens and Hochman, 2004). In our study, we were interested DA modulation of reflex in segments of the spinal cord outside the lumbar region involved in locomotion, specifically, those segments that contain autonomic innervation. *In vitro* data from mouse spinal cord show that areas containing autonomic innervation (SNS and PNS) respond differentially to DA and DA receptor agonists and antagonists than segments without the ANS. Furthermore, this result seems to be DA-specific, as noradrenaline and serotonin had similar effects in both the lumbar and ANS-containing segments. We conclude that DA mediates *in vitro* autonomic and somatic circuits in the spinal cord differently and this effect is circuit specific for MSR. We have also shown that a gap junction blocker can reverse the DA effect, pointing also to a role for either gap junctions or ion channels in what we have observed. Therefore we conclude that the switch in modulation between segments in

the spinal cord with and without autonomic innervation could be mediated by GIRK channel modulation by DA in the IML and this channel activity is regulated only by DA and not by other monoamines. In this scenario, GIRK channels are present on the tonically active cell bodies of the IML, but are not present on motoneurons. The ability of DA to regulate the opening and closing of the GIRK channel would ultimately regulate the activity of the SPN and gap junction connections between SPNs and motoneurons would provide a pathway for the excitability of the SPN to impact the excitability of the motoneuron.

After our *in vitro* experiments pointed to a clear role for DA in modulating the ANS, we asked what impact DA would have on the ANS *in vivo.* We used a knockout mouse model lacking a key inhibitory DA receptor and compare differences in autonomic output in these animals to aging WT mice. The D3KO mouse is has been noted as hypertensive and hyperactive, with known defects in the renal system (Asico et al., 1998). Additionally, the D3 receptor has the highest affinity for DA and any functional problems associated with DA are likely to be linked to this receptor (Clemens et al., 2006, Pivonello et al., 2007). From these studies, we can see that DA likely has a role in the control of autonomic function *in vivo* in the D3KO animal. It is also known that compromised autonomic function is commonly seen in the aging population (e.g. hypertension, cardiac disease)(de Champlain et al., 1980, Esler et al., 2002, Thijssen et al., 2006, Malpas, 2010). Therefore in the second aim of our study, we used the well-established background for aging-associated compromise in autonomic function to test how the cohort of aging WT animals compared to the young D3KO animal in terms of *in vivo* blood pressure and functional echocardiography, along with histological fibrosis

data. These experiments provided further evidence that DA does have an effect in the intact animal and is relevant to regulation of the ANS in not only the *in vitro* setting, but also *in vivo*. The blood pressure and heart rate changes over time in the WT animals show an increase in autonomic tone associated with aging. We have provided evidence in this study that animals lacking the D3 receptor show signs of advanced aging not only from the standpoint of an increase in blood pressure, but because these animals show an increase in fibrosis not typically seen until the WT animals reach 2 years of age. We conclude that the D3 receptor is plays a major role in the regulation of cardiac measures of autonomic function and that the increase in global autonomic tone in the D3KO mice mimics what is seen in aging WT animals. Whether the hypertension is caused directly by changes in the SNS due to the lack of the D3 receptor in the CNS or from peripheral effects, the autonomic changes observed in the young D3KO and old WT ultimately lead to high blood pressure. This almost has to cause (if it's not being itself being cause by it already) autonomic dysfunction because the blood pressure in the animals is so high, the regulatory system (mainly SNS) is going to be trying to bring it down. In either case, the autonomic nervous system is dysfunctional. This study is important because it provides the first evidence that the D3KO mouse could be considered as a new model of advanced aging.

Future studies to address the unanswered questions that remain include: intracellular/patch clamp recording for cell-specific experiments, as opposed to our current method, which is recording from a population of cells. We would expect that if gap junctions exist between motoneurons and SPNs, dye-coupling experiments would show movement of dye between these cells. Another way to help define the role of gap

junctions in a possible communication between motoneurons and SPNs would be to use a gap junction blocker during an intracellular recording. In this type of experiment, we would be able to visualize the response of a single motoneuron when the gap junction connection from the motoneuron to the SPN is blocked. If in the presence of the gap junction blocker, the motoneuron responds similarly to what has been seen in lumbar segments of the spinal cord without autonomic innervation, we would have further evidence that the SPN is influencing the excitability of the motoneuron through gap junctions. Also within the *in vitro* setting, using either the suction electrode or intracellular approach, use of more specific ion channel blockers would more clearly define a mechanism for the differential DA-mediated MSR effect seen between segments of the spinal cord with and without autonomic innervation. We could use K+ channel blockers specific for the GIRK channel to remove the influence of that single channel on the activity of the IML and record the subsequent response of both the SPN and the motoneuron. Additionally, studies to determine the mechanism of a D3-mediated increase in fibrillar collagen in the D3KO animal, such as western blots to analyze the levels of different types of collagen that are found in WT and D3KO animals. Levels of matrix metalloproteinases (MMPs), which are responsible for the breakdown of collagen, could be used to determine if they could provide a mechanism for the accumulation of collagen in the D3KO animals. If the levels or activity of MMPs in the young D3KO resemble what is seen in the older WT, this could give us further evidence that an increase in collagen and subsequent increase in stiffening of the tissue of the heart or vasculature could be the key factor in early-onset hypertension seen in the young D3KO animals.

REFERENCES

Adam WR (1980) Aldosterone and dopamine receptors in the kidney: sites for pharmacologic manipulation of renal function. Kidney international 18:623-635.

Amery A, Wasir H, Bulpitt C, Conway J, Fagard R, Lijnen P, Reybrouck T (1978) Aging and the cardiovascular system. Acta cardiologica 33:443-467.

Andersohn F, Garbe E (2009) Cardiac and noncardiac fibrotic reactions caused by ergot-and nonergot-derived dopamine agonists. Movement disorders : official journal of the Movement Disorder Society 24:129-133.

Andrejak M, Hary L (1986) Enhanced dopamine renal responsiveness in patients with hypertension. Clinical pharmacology and therapeutics 40:610-614.

Antonini A, Poewe W (2007) Fibrotic heart-valve reactions to dopamine-agonist treatment in Parkinson's disease. Lancet neurology 6:826-829.

Antonini A, Tolosa E, Mizuno Y, Yamamoto M, Poewe WH (2009) A reassessment of risks and benefits of dopamine agonists in Parkinson's disease. Lancet neurology 8:929-937.

Apostolakis EE, Baikoussis NG, Tselikos D, Koniari I, Prokakis C, Fokaeas E, Karanikolas M (2009) Cardiac surgery in a patient with retroperitoneal fibrosis and heart valvulopathy, both due to pergolide medication for Parkinson's disease. Journal of cardiothoracic surgery 4:65.

Arber S, Han B, Mendelsohn M, Smith M, Jessell TM, Sockanathan S (1999) Requirement for the homeobox gene Hb9 in the consolidation of motor neuron identity. Neuron 23:659-674.

Asico LD, Ladines C, Fuchs S, Accili D, Carey RM, Semeraro C, Pocchiari F, Felder RA, Eisner GM, Jose PA (1998) Disruption of the dopamine D3 receptor gene produces renin-dependent hypertension. J Clin Invest 102:493-498.

Banday AA, Lokhandwala MF (2008) Dopamine receptors and hypertension. Curr Hypertens Rep 10:268-275.

Barraud Q, Obeid I, Aubert I, Barriere G, Contamin H, McGuire S, Ravenscroft P, Porras G, Tison F, Bezard E, Ghorayeb I (2010) Neuroanatomical study of the A11 diencephalospinal pathway in the non-human primate. PloS one 5:e1330Bhattacharyya S, Schapira AH, Mikhailidis DP, Davar J (2009) Drug-induced fibrotic valvular heart disease. Lancet 374:577-585.

Biernacka A, Frangogiannis NG (2011) Aging and Cardiac Fibrosis. Aging and disease 2:158-173.

Bjorklund A, Skagerberg G (1979) Evidence for a major spinal cord projection from the diencephalic A11 dopamine cell group in the rat using transmitter-specific fluorescent retrograde tracing. Brain Res 177:170-175.

Boron W, Boulpaep E (eds.) (2002) Textbook of Medical Physiology: Saunders.

Breitwieser GE (1996) Mechanisms of K+ channel regulation. The Journal of membrane biology 152:1-11.

Brown DA (1990) G-proteins and potassium currents in neurons. Annual review of physiology 52:215-242.

Carlsson A, Waldeck B (1958) A fluorimetric method for the determination of dopamine (3-hydroxytyramine). Acta physiologica Scandinavica 44:293-298.

Carp JS, Anderson RJ (1982) Dopamine receptor-mediated depression of spinal monosynaptic transmission. Brain research 242:247-254.

Cavallotti C, Mancone M, Bruzzone P, Sabbatini M, Mignini F (2010) Dopamine receptor subtypes in the native human heart. Heart and vessels 25:432-437.

Cazalets JR, Borde M, Clarac F (1995) Localization and organization of the central pattern generator for hindlimb locomotion in newborn rat. J Neurosci 15:4943-4951.

Charles Watson GP, Gulgun Kayalioglu (ed.) (2008) The Spinal Cord A Christopher and Dana Reeve Foundation Text and Atlas: Elsevier.

Chen HH, Hippenmeyer S, Arber S, Frank E (2003) Development of the monosynaptic stretch reflex circuit. Current opinion in neurobiology 13:96-102.

Cherubini E, North RA, Surprenant A (1984) Quinine blocks a calcium-activated potassium conductance in mammalian enteric neurones. British journal of pharmacology 83:3-5.

Christie JM, Bark C, Hormuzdi SG, Helbig I, Monyer H, Westbrook GL (2005) Connexin36 mediates spike synchrony in olfactory bulb glomeruli. Neuron 46:761-772.

Clarac F, Cattaert D, Le Ray D (2000) Central control components of a 'simple' stretch reflex. Trends in neurosciences 23:199-208.

Clemens S (1998) Expression, modulation dynamique et interactions de deux réseaux neuronaux stomatogastriques des Crustacés: Etudes électrophysiologiques in vivo. In: Thesis: Ecole Doctorale des Sciences Biologiques et Médicales; Specialité: Neurosciences et pharmacologie, pp 1-105 Bordeaux: Bordeaux 1.

Clemens S (2011) La neuromodulation dynamique des circuits neuronaux: Flexibiltes et restrictions imposees au finctionnement physiologique. In: Habilitation a Diriger des rRecherches - Ecole Doctorale Sciences de la Vie - Sciences de la Sante, pp 1-258 Bordeaux: Bordeaux Victor Segalen.

Clemens S, Belin-Rauscent A, Simmers J, Combes D (2012) Opposing modulatory effects of D1- and D2-like receptor activation on a spinal central pattern generator. Journal of neurophysiology 107:2250-2259.

Clemens S, Hochman S (2004) Conversion of the modulatory actions of dopamine on spinal reflexes from depression to facilitation in D3 receptor knock-out mice. The Journal of neuroscience : the official journal of the Society for Neuroscience 24:11337-11345.

Clemens S, Rye D, Hochman S (2006) Restless legs syndrome: revisiting the dopamine hypothesis from the spinal cord perspective. Neurology 67:125-130.

Conway J (1963) A vascular abnormality in hypertension. A study of blood flow in the forearm. Circulation 27:520-529.

Cook NS, Haylett DG (1985) Effects of apamin, quinine and neuromuscular blockers on calcium-activated potassium channels in guinea-pig hepatocytes. The Journal of physiology 358:373-394.

Crick H, Wallis DI (1991) Inhibition of reflex responses of neonate rat lumbar spinal cord by 5-hydroxytryptamine. British journal of pharmacology 103:1769-1775.

Dascal N (1997) Signalling via the G protein-activated K+ channels. Cellular signalling 9:551-573.

Davidoff RA (1992) Skeletal muscle tone and the misunderstood stretch reflex. Neurology 42:951-963.

Davis JT, Rao F, Naqshbandi D, Fung MM, Zhang K, Schork AJ, Nievergelt CM, Ziegler MG, O'Connor DT (2012) Autonomic and hemodynamic origins of pre-hypertension: central role of heredity. Journal of the American College of Cardiology 59:2206-2216.

de Champlain J, Cousineau D, Lapointe L (1980) Evidences supporting an increased sympathetic tone and reactivity in a subgroup of patients with essential hypertension. Clinical and experimental hypertension 2:359-377.

DeQuattro V, Feng M (2002) The sympathetic nervous system: the muse of primary hypertension. Journal of human hypertension 16 Suppl 1:S64-69.

Dries JL, Kent SD, Virag JA (2011) Intramyocardial administration of chimeric ephrinA1-Fc promotes tissue salvage following myocardial infarction in mice. J Physiol 589:1725-1740.

Esler M, Hastings J, Lambert G, Kaye D, Jennings G, Seals DR (2002) The influence of aging on the human sympathetic nervous system and brain norepinephrine turnover. American journal of physiology Regulatory, integrative and comparative physiology 282:R909-916.

Failor RA, Capell PT (2003) Hyperaldosteronism and pheochromocytoma: new tricks and tests. Primary care 30:801-820, viii.

Felder RA, Eisner GM, Jose PA (2000) D1 dopamine receptor signalling defect in spontaneous hypertension. Acta Physiol Scand 168:245-250.

Ferri R, Manconi M, Lanuzza B, Cosentino FI, Bruni O, Ferini-Strambi L, Zucconi M (2008) Age-related changes in periodic leg movements during sleep in patients with restless legs syndrome. Sleep Med 9:790-798.

Findlay I, Dunne MJ, Ullrich S, Wollheim CB, Petersen OH (1985) Quinine inhibits Ca2+-independent K+ channels whereas tetraethylammonium inhibits Ca2+-activated K+ channels in insulin-secreting cells. FEBS Lett 185(1):4-8.

Frank E, Mendelson B (1990) Specification of synaptic connections mediating the simple stretch reflex. The Journal of experimental biology 153:71-84.

Freedman SB, Patel S, Marwood R, Emms F, Seabrook GR, Knowles MR, McAllister G (1994) Expression and pharmacological characterization of the human D3 dopamine receptor. The Journal of pharmacology and experimental therapeutics 268:417-426.

Freel EM, Connell JM (2004) Mechanisms of hypertension: the expanding role of aldosterone. Journal of the American Society of Nephrology : JASN 15:1993-2001.

Fulton BP (1986) Motoneurone activity in an isolated spinal cord preparation from the adult mouse. Neuroscience letters 71:175-180.

Gajendiran M, Seth P, Ganguly DK (1996) Involvement of the presynaptic dopamine D2 receptor in the depression of spinal reflex by apomorphine. Neuroreport 7:513-516.

Garraway SM, Hochman S (2001) Modulatory actions of serotonin, norepinephrine, dopamine, and acetylcholine in spinal cord deep dorsal horn neurons. Journal of neurophysiology 86:2183-2194.

Gladwell SJ, Coote JH (1999a) Fast excitatory post synaptic potentials and their response to catecholaminergic antagonists in rat sympathetic preganglionic neurones in vitro. Neurosci Lett 268:89-92.

Gladwell SJ, Coote JH (1999b) Inhibitory and indirect excitatory effects of dopamine on sympathetic preganglionic neurones in the neonatal rat spinal cord in vitro. Brain research 818:397-407.

Gladwell SJ, Pyner S, Barnes NM, Coote JH (1999) D(1)-like dopamine receptors on retrogradely labelled sympathoadrenal neurones in the thoracic spinal cord of the rat. Experimental brain research Experimentelle Hirnforschung Experimentation cerebrale 128:377-382.

Goldberg LI (1984) Dopamine receptors and hypertension. Physiologic and pharmacologic implications. The American journal of medicine 77:37-44.

Gomez Mde J, Rousseau G, Nadeau R, Berra R, Flores G, Suarez J (2002) Functional and autoradiographic characterization of dopamine D2-like receptors in the guinea pig heart. Can J Physiol Pharmacol 80:578-587.

Grassi G (2010) Sympathetic neural activity in hypertension and related diseases. American journal of hypertension 23:1052-1060.

Han P, Nakanishi ST, Tran MA, Whelan PJ (2007) Dopaminergic modulation of spinal neuronal excitability. J Neurosci 27:13192-13204.

Han P, Whelan PJ (2009) Modulation of AMPA currents by D(1)-like but not D(2)-like receptors in spinal motoneurons. Neuroscience 158:1699-1707.

Harburg E, Julius S, McGinn NF, McLeod J, Hoobler SW (1964) Personality Traits and Behavioral Patterns Associated with Systolic Blood Pressure Levels in College Males. Journal of chronic diseases 17:405-414.

Harris-Warrick RM, Coniglio LM, Barazangi N, Guckenheimer J, Gueron S (1995) Dopamine modulation of transient potassium current evokes phase shifts in a central pattern generator network. The Journal of neuroscience : the official journal of the Society for Neuroscience 15:342-358.

Hartman RP, Kawashima A, King BF, Jr. (2003) Evaluation of renal causes of hypertension. Radiologic clinics of North America 41:909-929.

Hening WA, Allen RP, Chaudhuri KR, Hornyak M, Lee HB, Winkelman J, Yoakum R (2007) Clinical significance of RLS. Mov Disord 22 Suppl 18:S395-400.

Holstege JC, Dijken Hv, Buijs RM, Goedkengt H, Gosens T, Bongers CM (1996) Distribution of dopamine immunoreactivity in the rat, cat, and monkey spinal cord. J Comp Neurol 376:631-652.

Hormuzdi SG, Pais I, LeBeau FE, Towers SK, Rozov A, Buhl EH, Whittington MA, Monyer H (2001) Impaired electrical signaling disrupts gamma frequency oscillations in connexin 36-deficient mice. Neuron 31:487-495.

Hounsgaard J, Kiehn O (1989) Serotonin-induced bistability of turtle motoneurones caused by a nifedipine-sensitive calcium plateau potential. The Journal of physiology 414:265-282.

Hussain T, Lokhandwala MF (2003) Renal dopamine receptors and hypertension. Experimental biology and medicine 228:134-142.

Ino-Oka E, Urae J, Sekino M, Satoh M, Takeuchi K, Sekino H, Ohtaki Y, Inooka H (2011) Blood pressure and sympathetic nerve tone relation during hemodialysis may reflect cardiovascular dysfunction. Internal medicine 50:2741-2748.

Izgi C, Feray H, Cevik C, Saltan Y, Mansuroglu D, Nugent K (2010) Severe tricuspid regurgitation in a patient receiving low-dose cabergoline for the treatment of acromegaly. The Journal of heart valve disease 19:797-800.

Jaber M, Robinson SW, Missale C, Caron MG (1996) Dopamine receptors and brain function. Neuropharmacology 35:1503-1519.

Jaszlits L, Tardos L, Borsy J (1985) The role of central dopamine (DA) receptors in the regulation of blood pressure. Polish journal of pharmacology and pharmacy 37:405-410.

Jiang Z, Carlin KP, Brownstone RM (1999) An in vitro functionally mature mouse spinal cord preparation for the study of spinal motor networks. Brain research 816:493-499.

Jimenez-Jimenez FJ, Lopez-Alvarez J, Sanchez-Chapado M, Montero E, Miquel J, Sierra A, Gutierrez F (1995) Retroperitoneal fibrosis in a patient with Parkinson's disease treated with pergolide. Clinical neuropharmacology 18:277-279.

Jose PA, Eisner GM, Felder RA (1999) Role of dopamine in the pathogenesis of hypertension. Clinical and experimental pharmacology & physiology 26:S10-13.

Jose PA, Eisner GM, Felder RA (2002) Role of dopamine receptors in the kidney in the regulation of blood pressure. Current opinion in nephrology and hypertension 11:87-92.

Jose PA, Eisner GM, Felder RA (2003) Regulation of blood pressure by dopamine receptors. Nephron Physiology 95:p19-27.

Jose PA, Raymond JR, Bates MD, Aperia A, Felder RA, Carey RM (1992) The renal dopamine receptors. Journal of the American Society of Nephrology : JASN 2:1265-1278.

Jose PA, Soares-da-Silva P, Eisner GM, Felder RA (2010) Dopamine and G protein-coupled receptor kinase 4 in the kidney: role in blood pressure regulation. Biochimica et biophysica acta 1802:1259-1267.

Julius S (1988) Transition from high cardiac output to elevated vascular resistance in hypertension. American heart journal 116:600-606.

Julius S, Pascual AV, London R (1971) Role of parasympathetic inhibition in the hyperkinetic type of borderline hypertension. Circulation 44:413-418.

Jung DW, Brierley GP (1984) The permeability of uncoupled heart mitochondria to potassium ion. The Journal of biological chemistry 259:6904-6911.

Juvin L, Simmers J, Morin D (2007) Locomotor rhythmogenesis in the isolated rat spinal cord: a phase-coupled set of symmetrical flexion extension oscillators. The Journal of physiology 583:115-128.

Kiehn O, Sillar KT, Kjaerulff O, McDearmid JR (1999) Effects of noradrenaline on locomotor rhythm-generating networks in the isolated neonatal rat spinal cord. Journal of neurophysiology 82:741-746.

Kim KM, Gainetdinov RR, Laporte SA, Caron MG, Barak LS (2005) G protein-coupled receptor kinase regulates dopamine D3 receptor signaling by modulating the stability of a receptor-filamin-beta-arrestin complex. A case of autoreceptor regulation. J Biol Chem 280:12774-12780.

Kitazawa T, Saito K, Ohga A (1985) Effects of catecholamines on spinal motoneurones and spinal reflex discharges in the isolated spinal cord of the newborn rat. Brain research 351:31-36.

Kuchel O (1990) Peripheral dopamine in essential hypertension. An early defense against hypertension failing during its progression? Am J Hypertens 3:104S-107S.

Kuchel OG, Kuchel GA (1991) Peripheral dopamine in pathophysiology of hypertension. Interaction with aging and lifestyle. Hypertension 18:709-721.

Kudo N, Yamada T (1985) Development of the monosynaptic stretch reflex in the rat: an in vitro study. The Journal of physiology 369:127-144.

Kuzhikandathil EV, Oxford GS (2002) Classic D1 dopamine receptor antagonist R-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride (SCH23390) directly inhibits G protein-coupled inwardly rectifying potassium channels. Molecular pharmacology 62:119-126.

Lapointe NP, Rouleau P, Ung RV, Guertin PA (2009) Specific role of dopamine D1 receptors in spinal network activation and rhythmic movement induction in vertebrates. The Journal of physiology 587:1499-1511.

Lebrun P, Malaisse WJ, Herchuelz A (1982) Paradoxical activation by glucose of quinine-sensitive potassium channels in the pancreatic B-cell. Biochemical and biophysical research communications 107:350-356.

Levant B, McCarson KE (2001) D(3) dopamine receptors in rat spinal cord: implications for sensory and motor function. Neuroscience letters 303:9-12.

Lew H, Jordan C, Jerger J, Jankovic J (1992) Acoustic reflex abnormalities in cranial-cervical dystonia. Neurology 42:594-597.

Lidow MS, Goldman-Rakic PS, Rakic P, Innis RB (1989) Dopamine D2 receptors in the cerebral cortex: distribution and pharmacological characterization with [3H]raclopride. Proceedings of the National Academy of Sciences of the United States of America 86:6412-6416.

Lindvall O, Bjorklund A, Skagerberg G (1983) Dopamine-containing neurons in the spinal cord: anatomy and some functional aspects. Ann Neurol 14:255-260.

Lokhandwala MF, Amenta F (1991) Anatomical distribution and function of dopamine receptors in the kidney. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 5:3023-3030.

Loney EL, Farrant J, Watkinson A (2002) Renovascular hypertension. Radiographic methods. The quarterly journal of nuclear medicine : official publication of the Italian Association of Nuclear Medicine 46:283-294.

Lund-Johansen P (1994) Newer thinking on the hemodynamics of hypertension. Current opinion in cardiology 9:505-511.

Lundberg J, Lundberg D, Norgren L, Werner O (1990) Dopamine counteracts hypertension during general anesthesia and hypotension during combined thoracic epidural anesthesia for abdominal aortic surgery. Journal of cardiothoracic anesthesia 4:348-353.

Machacek DW, Garraway SM, Shay BL, Hochman S (2001) Serotonin 5-HT(2) receptor activation induces a long-lasting amplification of spinal reflex actions in the rat. The Journal of physiology 537:201-207.

Malpas SC (2010) Sympathetic nervous system overactivity and its role in the development of cardiovascular disease. Physiological reviews 90:513-557.

Manconi M, Ferri R, Zucconi M, Clemens S, Rundo F, Oldani A, Ferini-Strambi L (2010) Effects of acute dopamine-agonist treatment in restless legs syndrome on heart rate variability during sleep. Sleep Med.

Marder E (2011) Variability, compensation, and modulation in neurons and circuits. Proc Natl Acad Sci U S A 108 Suppl 3:15542-15548.

Marder E, Calabrese RL (1996) Principles of rhythmic motor pattern generation. Physiol Rev 76:687-717.

Marina N, Becker DL, Gilbey MP (2008) Immunohistochemical detection of connexin36 in sympathetic preganglionic and somatic motoneurons in the adult rat. Autonomic neuroscience : basic & clinical 139:15-23.

Marowsky A, Yanagawa Y, Obata K, Vogt KE (2005) A specialized subclass of interneurons mediates dopaminergic facilitation of amygdala function. Neuron 48:1025-1037.

McCrea DA (1986) Spinal cord circuitry and motor reflexes. Exercise and sport sciences reviews 14:105-141.

Muhlbauer B, Kuster E, Luippold G (2000) Dopamine D(3) receptors in the rat kidney: role in physiology and pathophysiology. Acta Physiol Scand 168:219-223.

Ozono R, O'Connell DP, Vaughan C, Botkin SJ, Walk SF, Felder RA, Carey RM (1996) Expression of the subtype 1A dopamine receptor in the rat heart. Hypertension 27:693-703.

Palatini P, Julius S (2009) The role of cardiac autonomic function in hypertension and cardiovascular disease. Current hypertension reports 11:199-205.

Paulus W, Dowling P, Rijsman R, Stiasny-Kolster K, Trenkwalder C, de Weerd A (2007) Pathophysiological concepts of restless legs syndrome. Mov Disord 22:1451-1456.

Perez-Lloret S, Rascol O (2010) Dopamine receptor agonists for the treatment of early or advanced Parkinson's disease. CNS drugs 24:941-968.

Pierrot-Deseilligny E, Mazevet D (2000) The monosynaptic reflex: a tool to investigate motor control in humans. Interest and limits. Neurophysiologie clinique = Clinical neurophysiology 30:67-80.

Pinco M, Lev-Tov A (1993) Modulation of monosynaptic excitation in the neonatal rat spinal cord. Journal of neurophysiology 70:1151-1158.

Pivonello R, Ferone D, Lombardi G, Colao A, Lamberts SW, Hofland LJ (2007) Novel insights in dopamine receptor physiology. European journal of endocrinology / European Federation of Endocrine Societies 156 Suppl 1:S13-21.

Planz G, Gierlichs HW, Hawlina A, Planz R, Stephany W, Rahn KH (1976) A comparison of catecholamine concentrations and dopamine-beta-hydroxylase activities in plasma from normotensive subjects and from patients with essential hypertension at rest and during exercise. Klinische Wochenschrift 54:561-569.

Polakowski JS, Segreti JA, Cox BF, Hsieh GC, Kolasa T, Moreland RB, Brioni JD (2004) Effects of selective dopamine receptor subtype agonists on cardiac contractility and regional haemodynamics in rats. Clinical and experimental pharmacology & physiology 31:837-841.

Reisert I, Schuster R, Zienecker R, Pilgrim C (1990) Prenatal development of mesencephalic and diencephalic dopaminergic systems in the male and female rat. Brain research Developmental brain research 53:222-229.

Rekling JC, Funk GD, Bayliss DA, Dong XW, Feldman JL (2000) Synaptic control of motoneuronal excitability. Physiological reviews 80:767-852.

Ricci A, Bronzetti E, Fedele F, Ferrante F, Zaccheo D, Amenta F (1998) Pharmacological characterization and autoradiographic localization of a putative dopamine D4 receptor in the heart. Journal of autonomic pharmacology 18:115-121.

Rossi-Durand C (2006) Proprioception and myoclonus. Neurophysiologie clinique = Clinical neurophysiology 36:299-308.

Rouach N, Segal M, Koulakoff A, Giaume C, Avignone E (2003) Carbenoxolone blockade of neuronal network activity in culture is not mediated by an action on gap junctions. The Journal of physiology 553:729-745.

Russo RE, Reali C, Radmilovich M, Fernandez A, Trujillo-Cenoz O (2008) Connexin 43 delimits functional domains of neurogenic precursors in the spinal cord. The Journal of neuroscience : the official journal of the Society for Neuroscience 28:3298-3309.

Safar ME, Boudier HS (2005) Vascular development, pulse pressure, and the mechanisms of hypertension. Hypertension 46:205-209.

Sahlholm K, Nilsson J, Marcellino D, Fuxe K, Arhem P (2008) Voltage-dependence of the human dopamine D2 receptor. Synapse 62:476-480.

Sato M, Soma M, Nakayama T, Kanmatsuse K (2000) Dopamine D1 receptor gene polymorphism is associated with essential hypertension. Hypertension 36:183-186.

Sautel F, Griffon N, Levesque D, Pilon C, Schwartz JC, Sokoloff P (1995) A functional test identifies dopamine agonists selective for D3 versus D2 receptors. Neuroreport 6:329-332.

Setler PE, Sarau HM, Zirkle CL, Saunders HL (1978) The central effects of a novel dopamine agonist. European journal of pharmacology 50:419-430.

Sherrington CS (1906) Observations on the scratch-reflex in the spinal dog. The Journal of physiology 34:1-50.

Sherrington CS (1910) Flexion-reflex of the limb, crossed extension-reflex, and reflex stepping and standing. The Journal of physiology 40:28-121.

Sivertsson R (1970) The hemodynamic importance of structural vascular changes in essential hypertension. Acta physiologica Scandinavica Supplementum 343:1-56.

Skagerberg G, Bjorklund A, Lindvall O, Schmidt RH (1982) Origin and termination of the diencephalo-spinal dopamine system in the rat. Brain research bulletin 9:237-244.

Skagerberg G, Lindvall O (1985) Organization of diencephalic dopamine neurones projecting to the spinal cord in the rat. Brain Res 342:340-351.

Sokoloff P, Giros B, Martres MP, Bouthenet ML, Schwartz JC (1990) Molecular cloning and characterization of a novel dopamine receptor (D3) as a target for neuroleptics. Nature 347:146-151.

Srinivas M, Hopperstad MG, Spray DC (2001) Quinine blocks specific gap junction channel subtypes. Proceedings of the National Academy of Sciences of the United States of America 98:10942-10947.

Staudacher T, Pech B, Tappe M, Gross G, Muhlbauer B, Luippold G (2007) Arterial blood pressure and renal sodium excretion in dopamine D3 receptor knockout mice. Hypertension research : official journal of the Japanese Society of Hypertension 30:93-101.

Stein PK, Bosner MS, Kleiger RE, Conger BM (1994) Heart rate variability: a measure of cardiac autonomic tone. American heart journal 127:1376-1381.

Takigawa T and Alzheimer C (2002) Phasic and tonic attenuation of EPSPs by inward rectifier K+ channels in rat hippocampal pyramidal cells. J Physiol 539:67-75.

Tanabe M, Ono H, Fukuda H (1990) Spinal alpha 1- and alpha 2-adrenoceptors mediate facilitation and inhibition of spinal motor transmission, respectively. Japanese journal of pharmacology 54:69-77.

Tanaka Y, Nakayama K, Shigenobu K (1997) Inhibitory actions of ONO-3708 on the stretch-induced contraction potentiated by hemolysate/oxyhemoglobin studied in dog cerebral artery. Research communications in molecular pathology and pharmacology 98:303-311.

Tayebati SK, Lokhandwala MF, Amenta F (2011) Dopamine and vascular dynamics control: present status and future perspectives. Current neurovascular research 8:246-257.

Thaler J, Harrison K, Sharma K, Lettieri K, Kehrl J, Pfaff SL (1999) Active suppression of interneuron programs within developing motor neurons revealed by analysis of homeodomain factor HB9. Neuron 23:675-687.

Thijssen DH, de Groot P, Kooijman M, Smits P, Hopman MT (2006) Sympathetic nervous system contributes to the age-related impairment of flow-mediated

dilation of the superficial femoral artery. American journal of physiology Heart and circulatory physiology 291:H3122-3129.

van Dijken H, Dijk J, Voom P, Holstege JC (1996) Localization of dopamine D2 receptor in rat spinal cord identified with immunocytochemistry and in situ hybridization. The European journal of neuroscience 8:621-628.

Vessey JP, Lalonde MR, Mizan HA, Welch NC, Kelly ME, Barnes S (2004) Carbenoxolone inhibition of voltage-gated Ca channels and synaptic transmission in the retina. Journal of neurophysiology 92:1252-1256.

Villar VA, Jones JE, Armando I, Palmes-Saloma C, Yu P, Pascua AM, Keever L, Arnaldo FB, Wang Z, Luo Y, Felder RA, Jose PA (2009) G protein-coupled receptor kinase 4 (GRK4) regulates the phosphorylation and function of the dopamine D3 receptor. J Biol Chem 284:21425-21434.

Virag JA, Dries JL, Easton PR, Friesland AM, DeAntonio JH, Chintalgattu V, Cozzi E, Lehmann BD, Ding JM, Lust RM (2010) Attenuation of myocardial injury in mice with functional deletion of the circadian rhythm gene mPer2. Am J Physiol Heart Circ Physiol 298:H1088-1095.

Virag JA, Rolle ML, Reece J, Hardouin S, Feigl EO, Murry CE (2007) Fibroblast growth factor-2 regulates myocardial infarct repair: effects on cell proliferation, scar contraction, and ventricular function. Am J Pathol 171:1431-1440.

Voerman GE, Gregoric M, Hermens HJ (2005) Neurophysiological methods for the assessment of spasticity: the Hoffmann reflex, the tendon reflex, and the stretch reflex. Disability and rehabilitation 27:33-68.

Walters AS, Rye DB (2010) Evidence continues to mount on the relationship of restless legs syndrome/ periodic limb movements in sleep to hypertension, cardiovascular disease, and stroke. Sleep 33:287.

Wilson JM, Hartley R, Maxwell DJ, Todd AJ, Lieberam I, Kaltschmidt JA, Yoshida Y, Jessell TM, Brownstone RM (2005) Conditional rhythmicity of ventral spinal interneurons defined by expression of the Hb9 homeodomain protein. The Journal of neuroscience : the official journal of the Society for Neuroscience 25:5710-5719.

Winkelman JW (1999) The evoked heart rate response to periodic leg movements of sleep. Sleep 22:575-580.

Winkelman JW, Shahar E, Sharief I, Gottlieb DJ (2008) Association of restless legs syndrome and cardiovascular disease in the Sleep Heart Health Study. Neurology 70:35-42.

Yang Z, Sibley DR, Jose PA (2004) D5 dopamine receptor knockout mice and hypertension. Journal of receptor and signal transduction research 24:149-164.

Yashiro K, Thor KB, Burgard EC (2010) Properties of urethral rhabdosphincter motoneurons and their regulation by noradrenaline. The Journal of physiology 588:4951-4967.

Yu C, Yang Z, Ren H, Zhang Y, Han Y, He D, Lu Q, Wang X, Yang C, Asico LD, Hopfer U, Eisner GM, Jose PA, Zeng C (2009) D3 dopamine receptor regulation of ETB receptors in renal proximal tubule cells from WKY and SHRs. Am J Hypertens 22:877-883.

Yu P, Asico LD, Luo Y, Andrews P, Eisner GM, Hopfer U, Felder RA, Jose PA (2006) D1 dopamine receptor hyperphosphorylation in renal proximal tubules in hypertension. Kidney international 70:1072-1079.

Zeng C, Armando I, Luo Y, Eisner GM, Felder RA, Jose PA (2008) Dysregulation of dopamine-dependent mechanisms as a determinant of hypertension: studies in dopamine receptor knockout mice. American journal of physiology 294:H551-569.

Zeng C, Asico LD, Wang X, Hopfer U, Eisner GM, Felder RA, Jose PA (2003) Angiotensin II regulation of AT1 and D3 dopamine receptors in renal proximal tubule cells of SHR. Hypertension 41:724-729.

Zeng C, Felder RA, Jose PA (2006) A new approach for treatment of hypertension: modifying D1 dopamine receptor function. Cardiovascular & hematological agents in medicinal chemistry 4:369-377.

Zeng C, Wang D, Asico LD, Welch WJ, Wilcox CS, Hopfer U, Eisner GM, Felder RA, Jose PA (2004a) Aberrant D1 and D3 dopamine receptor transregulation in hypertension. Hypertension 43:654-660.

Zeng C, Wang D, Yang Z, Wang Z, Asico LD, Wilcox CS, Eisner GM, Welch WJ, Felder RA, Jose PA (2004b) Dopamine D1 receptor augmentation of D3 receptor action in rat aortic or mesenteric vascular smooth muscles. Hypertension 43:673-679.

Zeng C, Zhang M, Asico LD, Eisner GM, Jose PA (2007) The dopaminergic system in hypertension. Clin Sci (Lond) 112:583-597.

Zeng CY, Yang ZW, Wu LJ, Asico LD, Felder RA, Jose PA (2005) [Hypertension in D3 dopamine receptor deficient mice]. Zhonghua xin xue guan bing za zhi 33:1132-1136.

Zhang MZ, Yao B, Wang S, Fan X, Wu G, Yang H, Yin H, Yang S, Harris RC (2011) Intrarenal dopamine deficiency leads to hypertension and decreased longevity in mice. J Clin Invest 121:2845-2854.

Zhu H, Clemens S, Sawchuk M, Hochman S (2007) Expression and distribution of all dopamine receptor subtypes (D1 - D5) in the mouse lumbar spinal cord: A real-time polymerase chain reaction and non-autoradiographic in situ hybridization study. Neuroscience 149:885-897.

APPENDIX

Animal User Protocols

Animal Care and Use Committee
East Carolina University
212 Ed Warren Life Sciences Building
Greenville, NC 27834
252-744-2436 office • 252-744-2355 fax

May 29, 2009

Stefan Clemens, Ph.D.
Department of Physiology
Brody 6N-98
ECU Brody School of Medicine

Dear Dr. Clemens:

Your Animal Use Protocol entitled, "Dopamine Modulation of Spinal Cord Pathways in Young and Old Mice," (AUP #Q273) was reviewed by this institution's Animal Care and Use Committee on 5/29/09. The following action was taken by the Committee:

"Approved as submitted"

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure

East Carolina University is a constituent institution of the University of North Carolina. An Equal Opportunity/Affirmative Action Employer.

Animal Care and
Use Commitee
212 Ed Warren Life
Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

March 16, 2012

Stefan Clemens, Ph.D.
Department of Physiology
Brody 6N-98
ECU Brody School of Medicine

Dear Dr. Clemens:

Your Animal Use Protocol entitled, "Role of Dopamine Modulation in the Spinal Cord" (AUP #Q273a) was reviewed by this institution's Animal Care and Use Committee on 3/16/12. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Scott E. Gordon, Ph.D.
Chairman, Animal Care and Use Committee

SEG/jd

enclosure

East Carolina University is a constituent institution of the University of North Carolina. An equal opportunity university.