EFFECTS OF AGE ON PAIN SENSATION AND ITS TREATMENT IN RATS

By

JEREMIAH DAVID MITZELFELT

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To my wife, who is probably the only person who could survive living with me while I wrote this

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LIST OF ABBREVIATIONS

µg	microgram
µl	microliter
µl/hr	microliter per hour
µm	micrometer
5-HT	serotonin
ALS	anterolateral system
ANOVA	analysis of variance
BDNF	brain-derived neurotrophic factor
C	Celsius
cDNA	complementary deoxyribonucleic acid
cm	centimeter
CNS	central nervous system
DLF	dorsolateral funiculus
DNA	deoxyribonucleic acid
DRG	dorsal root ganglion
e.g.	*exempli gratia*
F344 x BN	Fischer 344 x Brown Norway
g	grams
g/day	grams per day
GABA	γ-aminobutyric acid
HCl	hydrochloric acid
IACUC	Institutional Animal Care and Use Committee
kg	kilograms

L/min	liters per minute
L4	lumbar region 4
LTP	long-term potentiation
m/s	meters per second
mg/kg/day	milligrams drug per kilogram body weight per day
min	minute
mRNA	messenger ribonucleic acid
mtDNA	mitochondrial deoxyribonucleic acid
n	number of subjects
NaOH	sodium hydroxide
NMDA	N-methyl-D-aspartate
O_2	oxygen
OIH	opioid-induced hyperalgesia
PAG	periaqueductal gray
PCR	polymerase chain reaction
PNS	peripheral nervous system
qRT-PCR	quantitative real-time polymerase chain reaction
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
RVM	rostral ventromedial medulla
s	seconds
S1	sacral region 1
SEM	standard error of the means
SGN	spiral ganglion neurons

Tbp	TATA box binding protein
TD-NMR	time domain-nuclear magnetic resonance
TRP	transient receptor potential
TRPA1	transient receptor potential ankyrin type 1
TRPM8	transient receptor potential melastatin type 8
TRPV1	transient receptor potential vanilloid type 1
TRPV2	transient receptor potential vanilloid type 2
TRPV3	transient receptor potential vanilloid type 3
TRPV4	transient receptor potential vanilloid type 4
VPL	ventral posterolateral nucleus of the thalamus

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By

Jeremiah David Mitzelfelt

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Chronic pain is a common medical complaint in aged individuals, and as the relative age of the population in the United States continues increase, the number of older persons suffering from chronic pain is going to increase. At the same time, there has been an increased acceptance of the use of opioid drugs for the alleviation of chronic pain. This has resulted in a dramatic increase in the number of older individuals taking opioids for pain relief for long periods of time. Unfortunately, how age affects both chronic pain and the effectiveness of long-term opioid use is not fully understood. This study investigated the effects of aging on the analgesic and secondary effects of fentanyl as well as the effects of age on the development and intensity of chronic pain using a relatively new animal model of chronic pain.

With increased age, the opioid fentanyl produced decreased levels of antinociception, but increased weight loss. These results suggest that fentanyl is a less than ideal drug for treatment of chronic pain in older individuals as the decrease in antinociception cannot be overcome by increasing the dose due to increased weight loss.

To assess the effects of aging on the development of chronic pain, acidic saline was injected into the left gastrocnemius muscle of adult and aged rats. Mechanical and thermal pain was tested using von Frey filaments and the thermal preference procedure respectively. Repeated acidic saline injection produced decreased withdrawal thresholds to mechanical stimulation for both ages, although thresholds decreased more slowly in older animals. Acidic saline injection produced no change in the thermal sensitivity for either age, although acidic saline injection did increase expression of heat sensitive receptors in the younger animals. While more research is warranted, the repeated acidic saline injection model of chronic pain is a useful model for studying the development of chronic pain due to its distinct acute, transitional, and chronic pain periods.

Overall, these studies demonstrated age-related differences in opioid action and chronic pain development. These results highlight the importance of research across ages allowing for development of effective treatments for patients at all ages.

CHAPTER 1
INTRODUCTION

The Problem of Aging

As the “baby boomer” generation begins to reach the age of 65, there is an increased focus on aging research (Kalapatapu and Sullivan, 2010). This research is important as it is expected that within the United States, the number of individuals that is 50 years and older will top 112 million persons (Colliver et al., 2006), and the number of individuals 65 and over to account for 20% of the population in 2030 (Kalapatapu and Sullivan, 2010). With current estimates of the US population at around 300 million, individuals over the age of 65 could account for approximately one-third of the population.

What is Aging?

While aged individuals continue to account for a greater portion of the population, and research focused specifically at aging increases, the questions of what aging is, why is occurs, and why “aged” individuals should be studied separately from “adult” individuals arise (Gavrilov and Gavrilova, 2006). An exact definition of what aging is has yet to be agreed upon, although aging can generally be thought of as the time-dependent loss of function that begins after an organism has reached reproductive competence. While this is a very general and broad definition, it is necessary, because aging produces a complex phenotype with high levels of variability, not only across species, but also between individuals within a specific species. Therefore, a precise definition of aging, which can encompass these high levels of variability, is difficult to produce.

Theories of Aging

As the rich diversity of the aging process makes it difficult to define what exactly aging is, it also makes it difficult to explain exactly why we age. An attempt has been made at identifying a unifying theory for aging, however, this review of the literature was unsuccessful (Medvedev, 1990). Below are several theories that have been developed to describe the process of aging at all levels of analysis from the population level down to the molecular level.

Evolutionary theory

At the population level, the evolutionary theory of aging addresses the issue that aging may be a result of natural selection pressure on a species based on two distinct, but not exclusive theories (Gavrilov and Gavrilova, 2006). The first theory is the mutation accumulation theory (Charlesworth, 2001; Gavrilov and Gavrilova, 2006). This theory suggests that mortality is a natural process due to a decline in pressure from natural selection after reproductive age has been reached. Any mutation in a gene that leads to death in old age will be passed on because the effects of the mutation are not realized until after reproduction. Therefore, there is no pressure from natural selection to select against these mutations. This lack of selective pressure is in contrast to genetic mutations that lead to mortality prior to reaching a reproductive age. These mutations are selected against, because carriers of these mutations die before they reach an age to pass on the mutations. Because mutations that lead to death in late life are not selected against by natural selection, multiple mutations can accumulate within a population over time, which can lead to limits on life-span and aging effects within a population.

The second theory of aging that functions at the population level is the antagonistic pleiotropy theory (Gavrilov and Gavrilova, 2006; Williams, 1957). This theory suggests that genes that are harmful late in life may actually be actively selected for in a population, if these genes are beneficial early in life. While this theory may sound the same as the mutation accumulation theory, there is a major difference between the two theories. In the mutation accumulation theory, genes that lead to mortality late in life are passively selected for due to their deleterious effects occurring after reproductive age has been reached. This is in contrast to the antagonistic pleitoropy theory where the genes are actively selected for, due to their importance in assisting an organism in reaching reproductive age. While these theories are different, it is important to stress that they are not mutually exclusive and work through similar mechanisms.

Cellular level

While the evolutionary theories are useful to describe aging and limits of life span at the population level, they do not explain the mechanisms through which genetic mutation can lead to aging and/or death. At the cellular level, there are numerous theories on how changes in cellular processes can lead to aging.

All of the theories center on the idea of the inability of the cell to fully repair and maintain itself as time progresses, which leads to aging symptoms. The free radical (Harman, 1956) and mitochondrial (Miquel et al., 1980) theories of aging focus on the role of reactive oxygen species (ROS) in the aging process. During metabolism, reactive oxygen species are formed. ROS can then attack the DNA leading to degradation or mutation and a decreased functioning of the cell. While the free radical theory suggests that damage to cellular DNA by ROS is the cause of aging, the

mitochondrial theory speculates that ROS damage to mtDNA leads to loss of function in mitochondria and thus the symptoms of aging.

Other theories of aging, such as the lysosomal, apoptosis, and protein aggregation theories of aging, suggest that a decline in the systems that remove damaged cellular structures and molecules lead to either increased ROS or a decline in function of the cell itself and thus aging symptoms. The inflammation theory of aging rests on the idea that increased ROS can increase inflammatory molecules, which in turn increase ROS production (Chung et al., 2009).

Molecular level

At the molecular level, many theories address the decline in DNA replication and protein synthesis with increased age. The codon restriction theory (Strehler et al., 1971) suggests that protein synthesis declines with aging due to the decrease in mRNA translation due to difficulties decoding mRNA codons. Abnormal protein production is also implicated in the error catastrophe theory, which suggests that proper gene expression declines with age, resulting in increased abnormal protein production (Gershon and Gershon, 1976; Goel and Ycas, 1975, 1976). While these theories focus on the decrease in the accuracy of RNA or DNA expression with age, the telomere theory of aging (Harley, 1991; Olovnikov, 1973) suggests that aging is due to the progressive shortening of telomeres during mitotic cell division. This shortening suggests there is a limit to number of replications a cell can go through, and thus a mitotic limit on lifespan as a loss in ability to undergo mitosis would limit the repair functions for many cells.

Sensory Decline in Aging

At the systems level, all these cellular and molecular changes can combine to produce the physiological and behavioral symptoms associated with aging. Even within the nervous system, which could be spared some of the aging effects described above due to its limited capacity for cell division and repair, there is a loss of function with increased age. With aging, there are decreases within each sensory modality, which can result in loss of function and independence in older individuals.

Presbycusis, or age-related hearing loss, affects almost half the population over the age of 75 years old (Gates and Mills, 2005). Presbycusis can be caused by either damage to the hair cells, or due to loss of spiral ganglion neurons (SGN), which relay information from the hair cells to the central nervous system (CNS) (Bao and Ohlemiller, 2010). While SGN loss is often secondary degeneration due to hair cell loss, primary degeneration of SGNs can occur (Ohlemiller and Frisina, 2008; Schacht and Hawkins, 2005). This primary degeneration is suggested to be caused by decreases in ROS mitigation (Bai et al., 1997; Keithley et al., 2005; McFadden et al., 1999; Niu et al., 2007; Pickles, 2004; Yamasoba et al., 2007). This loss of auditory ability can make interacting with others difficult leading to increased social isolation in the elderly.

Within the visual system, there are many age-related declines that occur, but not all of them are due to nervous system deficits (Jackson and Owsley, 2003; Spear, 1993). However, much of the loss of acuity in the visual system can be attributed to neural deterioration (Weale, 1975, 1987). Other age-related deficits in the visual system that are due to neural problems can include impairment of contrast sensitivity in low light (Ginsburg, 1984; Haegerstrom-Portnoy et al., 1997; Pelli et al., 1988), which may be due to age-related loss of rods (Curcio et al., 1993; Jackson et al., 1999). While cone

levels are maintained through old age, older individuals can develop difficulties in color discrimination that may be neural in origin (Jackson and Owsley, 2003). Temporal resolution can also be affected (Coppinger, 1955; Misiak, 1947). All of these deficits can make activities like driving unrealistic in aged individuals, resulting in loss of functional independence.

While taste and smell are two distinct senses, both are chemosensory in nature (Purves et al., 2008), and the age-related declines in these systems are very similar (Rolls, 1999). As both of these senses are involved in the direct detection of specific groups of chemicals, this detection ability is what is lost with aging. Within taste, age-related declines are often associated with either loss in salt (Murphy and Withee, 1986) or sweet sensitivity (De Jong et al., 1996), although these declines may be relatively minor in most adults. Similar declines are observed in olfaction, although to a greater magnitude (Doty et al., 1984; Tepper and Genillard-Stoerr, 1991), with deficits occurring in odor detection (Schiffman et al., 1976), and odor identification ability (Schiffman, 1977). Declines in taste and smell can alter the flavor of food, and may contribute to decreases in quantity and quality of food intake, resulting in health problems such as hypertension, or excessive weight loss.

Somatosensory Decline in Aging

The final sensory system in the body is the somatosensory system. The somatosensory system is possibly the most complex sensory system (Purves et al., 2008), responding to numerous stimulus modalities from mechanical stimulation (touch, pressure, vibration) to temperature to chemical detection to limb position to pain, the somatosensory system is almost a catch-all sensory system that covers all sensory input not covered by the eyes, ears, nose, or mouth (Bear et al., 2001). However, even

with all the complexity within the somatosensory system, age-related declines are observed (Shaffer and Harrison, 2007; Sturnieks et al., 2008; Wang and Albers, 2009; Wickremaratchi and Llewelyn, 2006). For mild mechanical stimuli, decreases in sensitivity have been demonstrated in the elderly in response to tactile (Bruce, 1980; Kenshalo, 1986; Thornbury and Mistretta, 1981) and vibratory stimulation (Kenshalo, 1986), along with decreases in spatial acuity on the skin (Bolton et al., 1966; Gescheider et al., 1994; Schimrigk and Ruttinger, 1980; Stevens and Patterson, 1995). Age-related declines are also observed in proprioception (Shaffer and Harrison, 2007; Sturnieks et al., 2008), with changes to muscle spindle number and function thought to be responsible for proprioceptive declines in aging (Kararizou et al., 2005; Liu et al., 2005; Miwa et al., 1995; Swash and Fox, 1972). Combined with deficits in other sensory systems, this loss of proprioception can interfere with mobility leading to functional impairments in older individuals (Sturnieks et al., 2008).

The Problem of Pain

One aspect of the somatosensory system that was not discussed above is pain. Pain is a complex sensory experience, and how pain perception and thresholds change as age increases is still not fully understood. However, as pain can interfere with daily functioning, it is important to understand how pain changes in the aged population, especially since they are susceptible to sensory decline in other systems that can lead to functional impairment.

What is Pain?

To characterize how aging affects pain, one must first understand what pain is, and why we feel pain. Pain is often described as a sensory signal that indicates either current or potential tissue damage (Dickinson et al., 2010). While this definition is useful

in explaining why we feel pain, as a protective mechanism to bodily harm, it is unable to explain chronic pain, situations where tissue damage is lacking, but pain is still experienced. Chronic pain states highlight the importance in drawing the distinction between nociception and pain. Nociception is the signaling of tissue damage by the somatosensory system, while pain is the perception of unpleasant sensations in the body (Bear et al., 2001). While these two concepts are closely related, and activation of the nociceptive system often leads to the perception of pain, they do not always occur together. Nociceptive neurons can signal tissue damage without the perception of pain, and pain can be perceived without any activation of the nociceptors (Bear et al., 2001). To fully understand how nociception and pain can occur jointly and separately, one must understand the organization and functioning the nociceptive system.

Neurobiology of Nociception and Pain

Peripheral nervous system

Unlike other somatosensory modalities, which use specialized nerve endings to transduce environmental stimuli to neuronal signals, the nociceptive system relies on free nerve endings for transduction (Purves et al., 2008). Nociceptors respond to a variety of noxious stimuli, from mechanical to thermal to chemical, allowing for numerous types of stimuli to elicit a pain response. Noxious signals are transmitted to the central nervous system through two different types of neurons, classified by axon diameter, presence of myelin, and conduction speed.

Initial nociceptive signals are received in the central nervous system by Aδ fibers. These neurons are the larger of the two nociceptive neurons (1-5 μm diameter), although they are smaller than the Aβ fibers responsible for non-noxious touch (Purves et al., 2008). These fibers are lightly myelinated, and have conduction speeds between

5-30 m/s and respond to either mechanical stimulation or mechanical and thermal stimulation (Purves et al., 2008). Conversely, mechanical, thermal, and chemical stimuli can activate C-fibers, which are smaller (0.2-1.5 μm), unmyelinated, and have slower conduction speeds (0.5-2 m/s). Typically, the initial sharp pain perceived immediately after an injury is a result of Aδ fiber signaling, while the prolonged dull pain perceived after the initial pain is due to C-fiber signaling. While these two fiber types transmit different information, they both enter into the spinal cord through the dorsal horn, where they synapse onto second order neurons within the CNS. Aδ fibers synapse onto neurons in either in laminae 1 and 5 of the dorsal horn, while C-fibers synapse onto neurons in laminae 1 and 2 of the dorsal horn.

Central nervous system

The second order neurons that receive input from Aδ or C-fibers travel medially, and cross the midline through the anterior white commissure (Haines, 2008). These fibers then travel towards the brain in the anterolateral system (ALS). The majority of these fibers travel rostrally in the spinal cord, through the brainstem, to the thalamus where they synapse in the ventral posterolateral nucleus of the thalamus (VPL) (Haines, 2008). The third order neurons then travel up to the somatosensory cortex. While most of the second order neurons synapse within the VPL, there are braches of the ALS that synapse along other areas throughout the brainstem and midbrain. Within the brainstem, branches of the ALS are sent out to synapse onto neurons within the reticular formation, while in the midbrain, branches innervate the periaqueductal gray (PAG) and the intralaminar nucleus of the thalamus.

Descending modulation of nociception

While the preceding neural pathway describes how nociceptive signals are transmitted from the periphery to the brain, there are also mechanisms by which the brain can modulate the intensity of this signal. These signals originate in the somatosensory cortex of the brain and pass to the PAG, either directly or through the amygdala or hypothalamus. Neurons from the PAG then descend to various nuclei in the brainstem. These nuclei then project neurons down to the dorsal horn where they can modulate the incoming nociceptive signal.

One of the most studied of these nuclei is the rostral ventral medulla (RVM), which contains the raphe nuclei, responsible for much of the serotonergic input to the rest of the body. The RVM has is directly involved in the modulation of pain through two sub-sets of neurons. These neurons are termed either “off” cells or “on” cells dependent on their activity level during noxious stimulation (Fields et al., 1991). Traditionally, these cells have been regarded as neurons that are not serotonin (5-HT) containing (Gao and Mason, 2000), although recent research may suggest that a population of “on” cells are serotonergic in nature (Bee and Dickenson, 2008). This distinction is important, as in the classic model of descending inhibition of pain, neurons from the PAG synapse onto 5-HT containing neurons within the raphe nuclei. These 5-HT neurons then project onto interneurons within the dorsal horn of the spinal cord. Activation of these interneurons presynaptically inhibits output from C-fiber neurons, which dampens the incoming nociceptive signal (Purves et al., 2008). While this descending serotonergic input can inhibit the transmission of pain through the spinal cord, other serotonergic input from the RVM can have a facilitatory effect on noxious mechanical input from the periphery (Bee and Dickenson, 2008). This facilitatory system is important due to its apparent role in

the maintenance of persistent pain after nerve injury, as well as a potential target for pharmacological treatment and intervention (Bee and Dickenson, 2008).

The interplay between the ascending nociceptive signal from the PNS through the ALS and the descending antinociceptive pathways from the PAG demonstrate how nociception can be separated from pain. Even though the nociceptors in the PNS are activated, with enough input from the PAG, the nociceptive signal may not be strong enough to activate second order neurons in the spinal cord, and therefore preventing the nociceptive signal from reaching the brain and being perceived as pain. This ability of the brain to block nociceptive signals has an important biological function in survival as it allows one to not perceive the pain from an injury in order to escape a potentially life threatening situation. In contrast, the converse condition where pain is perceived without nociception, is maladaptive and can interfere with normal functioning (Dickinson et al., 2010).

Acute versus Chronic Pain

While the above distinction between nociception and pain is important to point out, clinically another distinction should be made between the concepts of acute pain and chronic pain. Acute pain is associated with tissue damage, subsides with the healing process, and is thought to predominantly involve activation of Aδ fibers (Markenson, 1996). Using the above definitions of nociception and pain, the clinical condition of acute pain would be a condition where nociception and pain occur jointly, and work to protect the body from impending or continuing damage. However, chronic pain is pain that persists past the healing process (Merskey and Bogduk, 1994), or occurs during 3 of the past 6 months (Apkarian et al., 2009; Neville et al., 2008). Chronic pain is

therefore pain perception that exists without nociception, and is believed to be primarily signaled by C-fibers (Markenson, 1996).

Mechanisms of Chronic Pain

As chronic pain often exists without nociception, there have to be mechanisms for how the brain can perceive pain without a nociceptive signal. These mechanisms occur throughout the nervous system, although why these changes occur is still not fully understood.

Peripheral sensitization

Much of the research into the mechanisms of chronic pain focuses on sensitization. This may be in the PNS or the CNS either spinally or supra-spinally. Sensitization in the periphery develops primarily due to a sensitization of the nociceptors (Gold and Gebhart, 2010; Julius and Basbaum, 2001). These receptors can either lower their threshold of activation, as with transient receptor potential vanilloid type 1 (TRPV1) channels which activate at room temperature when in an acidic environment (Montell, 2005; Tominaga et al., 1998), or increase the magnitude of their response, which results in greater depolarization of nociceptive neurons, and potentially increased pain signaling (Gold and Gebhart, 2010; Petersen and LaMotte, 1993). However, TRPV1 channel levels have been shown to decrease with age in mice (Wang and Albers, 2009), and preliminary studies in our lab have shown similar decreases in the transient receptor potential vanilloid type 4 (TRPV4) channel in aged rats (Figure 4-11). Wang and Albers (2009) also showed a decrease in TRPV1 activation in aged mice. These findings suggest that older individuals may have decreased mechanisms for sensitization of peripheral pain receptors. While repeated stimulation can desensitize some receptors, such as the TRPV1 channels (Mandadi et al., 2004; Mandadi and

Roufogalis, 2008), repeated stimulation of the transient receptor potential vanilloid type 3 (TRPV3) channels located in keratinocytes can lead to greater response, and thus sensitization within the periphery (Mandadi and Roufogalis, 2008).

Sensitization of peripheral sensory receptors such as the TRP channels occurs primarily due to environmental stimulation, such as thermal or chemical exposure, but sensitization in the periphery can also occur due to peripheral nerve damage, leading to prolonged pain (Gold and Gebhart, 2010). Peripheral nerve injury can lead to PNS sensitization by changing the location and number of nociceptors (or their inhibitors) in damaged nerves (Gold and Gebhart, 2010; Howe et al., 1977; Kohno et al., 2005; Michaelis et al., 1999; Porreca et al., 1999; Tsuzuki et al., 2001), how these receptors activate (Gold and Gebhart, 2010), or even change some receptors from inhibitory to excitatory (Birder and Perl, 1999). These receptors can be mechanical or thermal sensory receptors (Howe et al., 1977; Michaelis et al., 1999), voltage gated ion channels (Porreca et al., 1999), signaling molecule receptors (Tsuzuki et al., 2001), or nociception inhibiting receptors such as opioid receptors (Kohno et al., 2005). In aged individuals, the mechanisms for peripheral nervous system repair are depressed (Lamoureux et al., 2010; Verdú et al., 2000), and therefore, this population may be more inclined to develop neuropathic pain.

Central sensitization of the spinal cord

Much of the sensitization in the spinal cord occurs at the synapses between the peripheral nociceptive neurons and the secondary afferent neurons in the CNS that relay peripheral sensory information to the brain (Rygh et al., 2005). Peripheral nerve injury can release a host of signaling chemicals into the spinal cord that can lead to central sensitization (Basbaum et al., 2009). The release of adenosine-5’-triphosphate

and brain-derived neurotrophic factor (BDNF) into the dorsal horn has been shown to activate microglia in the outer lamina. These activated microglia migrate towards C-fiber terminals and release numerous cytokines and chemokines that lead to increased pain signaling. BDNF activation of the TrkB receptor on lamina I neurons has been shown to alter the Cl^- gradient, resulting in γ-aminobutyric acid (GABA) input to become depolarizing as opposed to hyperpolarizing, resulting in this previously inhibitory GABAergic input to become excitatory to these neurons (Coull et al., 2005), and thus increase pain signaling as opposed to attenuating it. Inhibition of the microglial response has been shown to block the development of persistent pain in nerve-ligated rats (Lin et al., 2007a). Studies have shown that natural nociceptive stimulation in the periphery can induce long-term potentiation (LTP) of C-fibers that terminate in lamina II of the spinal cord (Sandkühler and Liu, 1998). This LTP is dependent on multiple neurotransmitter systems and requires activation of glutamate receptors, such as the *N*-methyl-D-aspartate (NMDA) and metabotropic glutamate receptors, or substance P receptors (Liu and Sandkühler, 1997; Liu and Sandkühler, 1995; Randić et al., 1993; Rudebeck et al., 2008) such as the Neurokinin-1 receptor.

All of these changes can lead to greater transmission of pain signals from the periphery through the spinal cord to the brain or lead to previously non-painful stimulation now being perceived as painful (i.e. allodynia). LTP and other mechanisms of synaptic sensitization allow previously sub-threshold inputs to become supra-threshold, and thus increase transmission of pain signals. However, the mechanisms involved in LTP and other forms of synaptic plasticity have been shown to be depressed with aging throughout the nervous system (Clayton and Browning, 2001; Corsini et al.,

1999; Komatsu, 1994; Korzick et al., 2001). This decreased level of potential plasticity should lead to less spinal sensitization, and therefore less chronic pain in older individuals. This decreased plasticity may also be involved in decreased opioid tolerance development in older individuals (Mao and Mayer, 2001; Wang et al., 2005).

Supra-spinal sensitization

The brain is able to modulate pain signals through descending inputs to the spinal cord. However, numerous studies have shown that these areas in the brain are susceptible to alteration during peripheral pain input, and thus contribute to the chronic pain state. As noted above, the RVM is involved in chronic pain through descending fibers that can either increase or decrease the pain signal. Within the RVM there are "on" cells that facilitate pain signals in the dorsal horn, and "off" cells that inhibit this transmission (Fields et al., 1991; Wang et al., 2009). The RVM has been shown to be an area of action for opioid antinociception (Pan et al., 1990) presumably by inhibiting "on" cells as targeted ablation of neurons containing μ-opioid receptors has been shown to block or reverse neuropathic pain (Bee and Dickenson, 2008; Porreca et al., 2001). Similar effects have been shown through direct application of lidocaine to the RVM as well as the PAG, another brain region involved in descending control of pain (Pertovaara et al., 1996). The neurons that facilitate pain signals are possibly activated endogenously by cholecystokinin (Kovelowski et al., 2000) and travel through the dorsolateral funiculus (DLF), as lesions to the DLF attenuates neuropathic pain in rats (Ossipov et al., 2000).

Pain and Aging

Epidemiological studies suggest that chronic pain rates are higher in older age groups (Bruckenthal et al., 2009; McCarthy et al., 2009; Neville et al., 2008; Rustøen et

al., 2005; Walid and Zaytseva, 2009). One population based study of chronic pain across ages showed that the majority of responders with chronic pain were over the age of 50 (Neville et al., 2008). Another study looking at chronic pain in different age groups showed the highest reports of chronic pain in the 60-81 year old group, in which 31.2% of respondents reported chronic pain (Rustøen et al., 2005). Pain incidence continue to rise throughout aging as one study found pain rates to be 35% in people 60-69 years old, and as high as 56% for patients over the age of 90 (Bruckenthal et al., 2009), with a majority of these patients being female (McCarthy et al., 2009). Compounding the problem is that many of these patients also present with psychiatric disorders such as anxiety or depression (Walid and Zaytseva, 2009), and it is unclear if older individuals experience more pain, or if they report pain more readily as pain can have a greater impact on quality of life for the elderly (Thomas et al., 2004).

Nociception in older humans

Overall, nociception is the process of relaying noxious environmental stimuli from nociceptive receptors, through the peripheral nervous system and spinal cord to the brain. Sensitization of responses can occur at the peripheral receptors, or at synapses within the spinal cord and brain, increasing the nociceptive signal, which can lead to chronic, persistent pain. However, studies suggest that most of the mechanisms involved in sensitization are depressed in aged individuals. Therefore, one would expect rates of chronic pain to be lower in older individuals.

This concept is consistent with laboratory experiment in humans which show that across many forms of stimulation, pain thresholds are either increased or show no change with age (for review see Gibson and Farrell, 2004). These studies covered multiple forms of stimulation, (e.g. mechanical, thermal), but stimulation was applied in

isolated applications, which would measure the nervous systems ability to transmit nociceptive signals. This increase in threshold suggests a decrease in sensitivity to painful stimulation, which is consistent with anatomical studies that have shown deficits in nociceptive nerve function (Adler and Nacimiento, 1988; Drac et al., 1991; Kakigi, 1987; Namer, 2010; Verdú et al., 2000) as well as nociceptive neuron number (Drac et al., 1991; Namer, 2010; Verdú et al., 2000). Substance P, a neurotransmitter associated with nociception, has also been shown to be decreased in the skin of aged humans (Gibson and Farrell, 2004), suggesting decreased nociceptive signaling in the periphery.

Nociception in older animals

Nociceptive signaling and aging has also been investigated experimentally using animals. Pain assessment in animals is difficult due to a lack of verbal communication, and as a result, animal models of pain assessment rely on changes in behavior. Traditionally, these models used noxious stimuli (e.g. thermal, electrical, mechanical, or chemical) to elicit a reflexive behavior (e.g. tail withdrawal, tail flick, paw licking and guarding). Changes in pain sensitivity are measured by changes in the latency, duration, or magnitude of the reflexive response. These measures are useful for measuring changes in thresholds and nociception, but do not require processing above the spinal cord reflex circuit, as shown by studies of reflexive withdrawals to noxious stimuli in animals that have do not have an intact spinal cord (Jensen and Smith, 1982; Silva et al., 1997). Due to this, these methods do not necessarily measure changes in pain perception.

Numerous studies have investigated changes in reflexive pain response during aging. While the results are mixed across studies (for review see Gagliese and Melzack, 2000), most studies of reflexive pain from a thermal stimulus show either no effect of

age in the tail-flick test (Crisp et al., 1994; Ghirardi et al., 1994; Girardot and Holloway, 1985; Goettl et al., 2000; Goicoechea et al., 1997; Hamm and Knisely, 1986a, b; Hamm et al., 1986; Knisely and Hamm, 1989), or an increase in latency as age increases for tail-flick (Akunne and Soliman, 1994; Goettl et al., 2000; Hess et al., 1981; Onaivi et al., 1994) or hot plate tests (Akunne and Soliman, 1994; Goettl et al., 2000; Hess et al., 1981). These reflexive tests assess changes in spinal reflexes, which activate the PNS and the spinal cord, suggesting a decrease in peripheral nociception with age.

Pain perception in older humans

While nociceptive signaling may be decreased in the periphery in older adults, pain is a complex phenomenon that can involve mechanisms beyond simple transmission of sensory signals from the periphery through the spinal cord to the brain. These mechanisms can involve the integration of multiple signals in the spinal cord, processing of incoming nociceptive signals in the brain, and descending modulation of nociceptive signals in the spinal cord. Although older adults report no change or increased thresholds of pain in response to single exposure to noxious stimulation, other studies have investigated changes in pain tolerance (as opposed to thresholds) during aging. These studies used trains of repeated stimulation, which has been shown to increase the intensity of pain due to integrative mechanisms in the spinal cord (Li et al., 1999; Mendell, 1966; Price et al., 1977; Vierck et al., 1997) in response to C-fiber signaling (Mendell, 1966; Price et al., 1977). In these studies, older participants reported greater pain intensity and unpleasantness than younger participants (Edwards and Fillingim, 2001; Farrell and Gibson, 2007; Harkins et al., 1986; Woodrow et al., 1972). This suggests that the central mechanisms involved in pain processing are actually increased in older adults. Another study assessing age differences in endogenous

mediation of pain showed that a paradigm that induced inhibition of pain in younger adults actually produced a facilitation of pain in older adults (Edwards et al., 2003). These studies suggest that there may be mechanisms of nociception or pain that are increased in older humans, although the exact mechanisms are not fully known.

Pain perception in older animals

To better understand the mechanisms involved in these changes in pain perception, there is a need for increased research in pain using animal models. Importantly more research is needed looking across ages as most preclinical research is focused on young, male animals. However, the previously reported studies of pain and aging in animals do not show the increases in pain perception observed in the human studies.

These differences may be explained by differences in signaling mechanisms between acute and chronic pain. Traditional reflexive tests predominately activate Aδ-nociceptive fibers (Yeomans et al., 1996; Yeomans and Proudfit, 1996), while the human studies showing increases in pain perception used assessments that more selectively activate C-fibers. Therefore, to better study age-related changes in pain perception in animals, pain assessment methodologies that selectively activate C-fibers need to be developed and/or utilized (Vierck et al., 2008b). Recently, operant-based thermal pain assessments have been developed that can assess changes in sensitivity to non-noxious thermal stimulation (Marcinkiewcz et al., 2009; Morgan et al., 2008; Vierck et al., 2005; Yezierski et al., 2010). The thermal escape procedure measures change in thermal sensitivity by allowing the animal to choose between a dark room with a metal floor that can be heated or cooled and a brightly lit room with a floor at room temperature. With both floors at room temperature, the bright light is aversive to

the rats, and rats will exhibit preference for the dark room (Morgan et al., 2008; Vierck et al., 2008a). However, if the floor in the dark room is heated or cooled, the rat must decide which is least aversive, the temperature of the floor or the bright room. Increased age leads to increased escape times from both cold and hot plates. A conceptually related procedure uses two chambers with floors that can be independently heated or cooled. This allows for direct comparisons of non-noxious stimuli, as well as changes in hot versus cold temperature sensitivity. These studies have shown age differences in thermal sensitivity, with older animals showing greater sensitivity to both heat and cold with increasing age (Yezierski et al., 2010). These procedures often times show age-related differences that are the opposite from the data using reflexive thermal tests. While those tests showed either no age difference or a decrease in thermal sensitivity with increased age, the operant-based tests demonstrate greater thermal sensitivity with increased age, highlighting the importance of using the proper behavioral test for pain assessment based on the experimental question and/or human clinical situation that is of interest.

Chronic pain in older animals

While the above studies are important for showing baseline differences in thermal sensitivity across ages, they focused on acute pain by testing animals that were not already in a pain state. To this end, numerous models of chronic pain have been developed, in an effort to reflect the wide variety of chronic pain conditions in the human population (for review see Blackburn-Munro, 2004). Animal models exist to study inflammatory ('t Hart and Amor, 2003; Calvino et al., 1987; Colpaert, 1987), joint (Kehl et al., 2000; Radhakrishnan et al., 2003), neuropathic (Vos et al., 1994; Wang and Wang, 2003; Xu et al., 1992), visceral (Boucher et al., 2000; Laird et al., 2001),

musculoskeletal (Sluka et al., 2001), and other types of pain (Kehl et al., 2003; Shimoyama et al., 2002; Wang and Wang, 2003).

As with basic studies in pain perception as a function of age, multiple studies have looked at age effects on pain responses in many of these models of chronic pain. In inflammatory models, aged animals have shown less hyperalgesia to mechanical stimulation (Fathalla et al., 2004; Gagliese and Melzack, 1999; Kitagawa et al., 2005), but greater hyperalgesia to thermal stimulation (Zhang et al., 2004). Across neuropathic models, aged animals show either an increase (Crisp et al., 2003; Kim et al., 1995; Ramer and Bisby, 1998), a decrease (Chung et al., 1995), or no change in mechanical thresholds (Crisp et al., 2003) depending on the type of neuropathic pain model used and ages of animals. Similar results are observed with thermal thresholds with increases (Ramer and Bisby, 1998), decreases (Chung et al., 1995; Crisp et al., 2003), or no differences observed (Kim et al., 1995; Novak et al., 1999). While these animal models are useful for testing new treatments, and understanding the mechanisms involved in various types of chronic pain, most do not allow for the study of the transition from acute to chronic pain. Being able to isolate this transition experimentally can allow for the identification of new treatment targets that can prevent further development of chronic pain. Inflammatory models inject inflammatory agents directly into the animal, instantly creating a pain state. Similar conditions exist with the neuropathic pain models where nerves are damaged inducing a chronic pain state. In all of these models, there is no way to induce an acute pain state with which to compare underlying cellular and molecular mechanisms that may be different between an acute and chronic pain state.

Therefore, these models do not help in the development of treatments to prevent the development of chronic pain.

The Difficulties of Opioids

Chronic pain is becoming a more common medical diagnosis, as opposed to simply a symptom, and is especially common in older individuals (Bruckenthal et al., 2009; Colliver et al., 2006; McCarthy et al., 2009; Neville et al., 2008; Rustøen et al., 2005; Walid and Zaytseva, 2009). Unfortunately, across the spectrum of chronic pain, including lower back pain (Apkarian et al., 2009; Chenot et al., 2007), chronic post-surgical pain (Akkaya and Ozkan, 2009), neuropathic pain (Cavenagh et al., 2006; Colombo et al., 2006; Jensen et al., 2009), and chronic pelvic pain (Dalpiaz et al., 2008), there is a lack of effective treatments. While antiepileptic and antidepressant drugs are most commonly used for in the treatment of chronic pain (Colombo et al., 2006; Jensen et al., 2009), opioids are often prescribed for the treatment of acute pain and their use is increasing for chronic pain (Atluri et al., 2003; Bell et al., 2009; Benyamin et al., 2008; Brixner et al., 2006; Garcia del Pozo et al., 2008; Pergolizzi et al., 2008; Trescot et al., 2008b).

How Do Opioids Work?

With the increase in opioid prescriptions for the treatment of chronic pain, it is important to understand how opioids work, as well as some of the drawbacks to their long-term use for chronic pain. Opioids bind to opioid receptors that are located throughout the PNS and CNS. Within the CNS, opioid receptors are located in many of the areas involved in nociception and pain (Julien, 2005). Opioids have an inhibitory effect when they bind to neurons in the dorsal horn of the spinal cord, which limits the nociceptive signal. In the brain, opioids have an excitatory effect on neurons in the PAG

and other mid-brain and brain stem nuclei (Julien, 2005). This excitation can increase the descending mediation of nociceptive signals thus limiting the intensity of nociception that reaches the brain for perception as pain.

Why are Opioids Bad?

While opioids are useful for the blocking of nociception, they do possess limitations and drawbacks to their use. While opioid receptors are located in many of the brain regions to help mediate pain and nociception, they are also unfortunately located in areas that control respiration, and thus large doses of opioids can be fatal due to a suppression of respiration (Julien, 2005). One serious concern with opioids, especially in regards to long term use, is that their effectiveness in pain relief decreases due primarily to changes in opioid receptor levels and function (Collett, 1998) resulting in the development of tolerance (Benyamin et al., 2008), although there is some evidence that tolerance develops more slowly in older individuals (Buntin-Mushock et al., 2005). A more recently described, but related phenomenon is opioid-induced hyperalgesia (OIH) (Bekhit, 2010). In OIH, opioid administration becomes pronociceptive as opposed to antinociceptive. OIH has been demonstrated in preclinical studies in response to both acute (Kayan et al., 1971) and chronic (Celerier et al., 2001; Laulin et al., 1999) opioid administration, although conclusive evidence of OIH in a clinical setting has yet to be shown (Fishbain et al., 2009; Tompkins and Campbell, In Press). Patients of prolonged opioid use can also develop physical dependence, and experience withdrawal sickness in the absence of opioids (Benyamin et al., 2008; Cavalieri, 2005; Johnson et al., 1989).

Opioids and Aging

Aging can presumably have effects on both the pharmacokinetics and pharmacodynamics of opioid action. Previous studies have shown that aged individuals

have higher opioid plasma levels than younger individuals for weight-matched doses (Björkman et al., 1998; Pesonen et al., 2008; Scott and Stanski, 1987; Singleton et al., 1988). This increase in plasma level could be attributed to a decrease in clearance from lower renal function in aged individuals (ElDesoky, 2007). Both μ- (Smith and Gray, 2001) and κ-opioid receptor agonists (Smith and French, 2002) have been shown to be more potent in aged rats. In clinical situations, aged individuals require a lower dose of fentanyl than younger individuals to achieve the same analgesic effect (Scott and Stanski, 1987). Conversely, studies looking at changes in μ-opioid receptor number have shown a decrease in number with aging (Amenta et al., 1991; Gerald et al., 2008; Hess et al., 1981; Messing et al., 1980) without changes in binding affinity at these receptors (Hess et al., 1981). In terms of changes in functioning of opioid receptors with age, little research has been completed. However, in a study on the functioning of G protein-coupled receptors (GPCR) in general showed that GPCR function decreases with age (Alemany et al., 2007). As opioid receptors are a type of GPCR it is expected that the functioning of opioid receptors also will decrease with age, although to what extent is still unknown. Increased understanding of how opioid receptor number, binding affinity, and function are important to maintain adequate treatment of pain throughout the lifetime. In studies of chronic administration of opioids and aged individuals, aged individuals are less likely to develop tolerance to opioids than younger individuals (Mercadante and Arcuri, 2007), and animal studies have shown this is not due to changes in pharmacokinetics, but could be due to a decrease in molecular plasticity required for the development of tolerance (Wang et al., 2005).

Rationale for the Current Study

With the above information in mind, these studies were designed based on three primary issues:

- The relative age of the population in the United States is increasing
- Chronic pain is becoming a more common medical diagnosis, especially among older individuals
- Opioids are being prescribed for the treatment of chronic pain at increasing rates.

These three conditions could set the stage for conditions where older individuals are being prescribed opioids for extended periods to treat chronic pain. However, elderly patients are often undertreated when it comes to addressing chronic pain (Auret and Schug, 2005; Bernabei et al., 1998; Chodosh et al., 2004; Gianni et al., 2010; McNeill et al., 2004). This is partially due to a lack of understanding on what effects aging can have on the long-term ability of opioids to treat pain. Therefore, this study aims to increase understanding of the relationship between aging, chronic opioid administration, and chronic pain.

Rationale for Animal Models

Long-term studies of aging in the human population can be difficult and costly. While many of the questions addressed in this dissertation could be asked in humans, the use of animal models provides several important advantages. Animal studies allow for greater knowledge and control of the subject's history and experiences, as well as greater control over the experimental environment to limit confounding variables, which allows for a greater ability to interpret data. In addition, in order to develop more effective treatments for chronic pain the underlying mechanisms of chronic pain must be better understood. To facilitate this, it is necessary to collect tissue for cellular and

molecular studies. However deciding to use animal models is not a simple decision, as there are numerous species and strains that can be used for research, as well as numerous behavioral and experimental models to assess pain, nociception, and the effects of opioid administration.

The following studies all use Fischer 344 x Brown Norway (F344 x BN) rats. Rats were chosen as the experimental animal because our lab was experienced in the care and handling of rats, many of the experimental models used were designed for rats, and the need for tissue collection required the use of an animal large enough to collect tissue from. Within biomedical research, many different strains of rats are used. The F344 x BN strain was selected because it is often used as a model of non-pathological, "normal" aging (Carter et al., 2004; Carter et al., 2002; Spangler et al., 1994). While the F344 strain was originally used as the rat model for aging, its high rates of nephropathy with aging has led many to start using the F344 x BN for aging studies (Weindruch and Masoro, 1991).

Justification of the Use of Fentanyl

While there are numerous opioids that are used for the treatment of pain, fentanyl is increasingly used for a wide range of conditions including epidural anesthesia, chronic back pain, and cancer pain; (Bell et al., 2009; Bhambhani et al., 2010; de Leon-Casasola, 2008; Hong et al., 2010; Manchikanti and Singh, 2008; Pergolizzi et al., 2008; Rauck et al., 2009), and the development of novel formulations and delivery systems, such as the transdermal patch, makes fentanyl easy to administer chronically in outpatient situations (Grape et al., 2010). Furthermore, we were interested in studying relatively long-term administration of an opioid (i.e. one month in a rat). To avoid repeated bouts of anesthesia and surgery, we utilized osmotic minipumps that could

deliver drug over a 28-day period – to maintain constant levels of drug delivery these pumps required a relatively potent drug such as fentanyl to be used (e.g. effective doses of morphine could not be delivered in this manner).

Justification of the Use of Thermal Preference/Sensitivity Procedure

As noted above, chronic pain is often signaled by C-fibers (Cooper et al., 1986; Raja et al., 1988; Schmelz, 2009; Staud et al., 2008) and while chronic pain is often studied in animals; traditional measures of pain in animals focused more on reflexive nociception that is signaled by Aδ fibers (Yeomans et al., 1996; Yeomans and Proudfit, 1996). With this in mind, these studies used the thermal preference procedure to measure changes in thermal sensitivity and pain. This procedure uses slower deliveries of non-noxious heat that can more selectively activate C-fibers. In addition, because the animals are actively deciding which plate to stand on, the test requires neural processing above the spinal level, and therefore may detect any changes in the supra-spinal areas involved in pain processing.

Overall, it is important to better understand how aging, chronic pain, and opioid administration all interrelate. However, in order to understand how three variables interrelate, one must first understand how the variables interact in pairs. To this end, these studies examined at two separate conditions:

- The relationship between aging and chronic fentanyl administration
- The relationship between aging and chronic pain development and severity

The experiment in Chapter Two investigates the effects of age and chronic fentanyl administration on thermal sensitivity using the thermal preference procedure. As a follow up, Chapter Three investigates the influence of age on the secondary,

adverse effects of chronic fentanyl administration using a battery of physiological and physical performance tests. In Chapter Four, the effects of age on the development of chronic pain are assessed using a relatively new animal model of chronic pain, with pain measured both reflexively using mechanical stimulation via von Frey filaments and through operant measures using the thermal preference procedure.

CHAPTER 2
EFFECTS OF CHRONIC FENTANYL ADMINISTRATION ON THERMAL SENSITIVITY ACROSS AGES USING AN OPERANT-BASED THERMAL PREFERENCE PROCEDURE

Opioids have been used to treat essentially every type of pain in humans, including acute and chronic pain, for pre-, peri-, and postsurgical situations, for both cancer and non-malignant conditions, and their use is increasing and becoming more widely accepted with the rates of prescriptions for all opioids increasing steadily in the past few years, with some increasing greater than 500% since 1997 (Atluri et al., 2003; Bell et al., 2009; Benyamin et al., 2008; Brixner et al., 2006; Garcia del Pozo et al., 2008; Manchikanti and Singh, 2008; Pergolizzi et al., 2008; Trescot et al., 2008a). This is especially relevant to the aged population where chronic pain due to diffuse conditions (Delgado-Guay and Bruera, 2008; Fine, 2001, 2004; Pergolizzi et al., 2008), such as neuromuscular pain (Helme and Gibson, 2001; Thomas et al., 2004) and arthritis (Donald and Foy, 2004), and to disease-related conditions such as cancer (Potter and Higginson, 2004; Rao and Cohen, 2004), is more prevalent, with estimates of as many as 40% of aged individuals in the community (e.g. > 50 years of age) and 80% in nursing homes (mean age of 75 years) reporting pain that interferes with daily functioning (Fox et al., 1999; Gagliese, 2009; Rustøen et al., 2005; Scudds and Robertson, 2000; Thomas et al., 2004; Zarit et al., 2004). With the number of people in the United States over the age of 65 expected to more than double by 2050 (Kalapatapu and Sullivan, 2010), it is expected that an increased number of aged individuals will be prescribed opioids for longer periods of time. However, how increased age influences the various effects of opioids is not fully understood (Chan and Lai, 1982; Crisp et al., 1994; Hoskins et al., 1986; Islam et al., 1993; Kavaliers et al., 1983;

Kramer and Bodnar, 1986; Saunders et al., 1974; Smith and Gray, 2001; Webster et al., 1976).

This study investigated the influence of age on the antinociceptive effectiveness of opioids during chronic administration. As chronic opioid administration is most often in response to a chronic pain condition, an operant based pain assessment was used to better isolate the nociceptive processes involved in chronic pain. In the current study, fentanyl was continuously administered at a dose of 1.0 mg/kg/day for 28 days via osmotic minipumps, as this dosing protocol has previously been shown to produce antinociception (i.e. pain relief) in rats across a wide age range (Morgan et al., 2008; unpublished data). Based on previous studies suggesting that aged individuals require lower doses of opioids to achieve an analgesic effect (Scott and Stanski, 1987), it is hypothesized that older animals will show a greater magnitude of antinociception due to opioid administration, and will develop tolerance more slowly than younger animals.

Materials and Methods

Animals, Treatments, and Experimental Design

For this study 27 male Fischer 344 x Brown Norway rats (F344 X BN), obtained from the National Institute of Aging colony at Harlan Industries (Indianapolis, IN), at three ages (16, 20, and 24 months of age at time of pump implant) was used. All animals were individually housed in a climate (temperature and humidity) controlled colony with a 12-hr light/dark cycle with food and water available *ad libitum*. Animals were cared for in accordance with the regulations of the IACUC and in accordance with the “Guide” (ILAR, 1996). In addition, animals were assessed on a weekly basis for signs of overt health problems by using a standardized form; measures included, but

were not limited to, checking for sudden decline in body weight, redness around the eyes and nostrils, ruffled coat, open sores on the tail, and haunched posture.

Upon arrival in the colony, the animals were given 2 weeks to acclimate before thermal preference testing began. Animals were then given four days of training on the thermal preference device. Each day the plates were set to different temperatures. Throughout all phases of testing, animals were pseudo-randomly assigned to start on the “hot” or “cold” plate and was counterbalanced within each group; however, no animal could start on the same side more than two days in a row to prevent the development of side biases. After this initial training, hot and cold aversion thresholds were determined. Each day one plate was set to 27.5°C (neutral) and the other plate was set to a temperature ranging from 10-45°C in 3°C increments. Temperatures for each day were pseudo-randomly determined, with each temperature condition tested once, and a temperature warmer or cooler than 27.5°C could not be tested more than two days in a row, to prevent development of side biases.

After threshold testing, baseline testing for thermal preference testing was started. Animals were tested on three conditions, “cold versus neutral” (15 and 27.5°C), “hot versus neutral” (45 and 27.5°C), and “hot versus cold” (plate temperatures were 45 and 15°C). These conditions allowed for the independent testing of sensitivity to hot, sensitivity to cold, as well as preference for hot or cold. Animals were tested six days a week, with each condition tested twice a week.

Animals within each age group were then randomized to receive osmotic minipumps containing either saline (n=14) or fentanyl (n=13). Animals were given 24 hours to recover then thermal preference testing resumed. Animals were tested similarly

to baseline testing: 3 conditions (hot versus cold, hot versus neutral, cold versus neutral), six days a week, with each condition twice a week for 4 weeks. After four weeks, the pumps were removed, and animals tested six days a week for another 4 weeks. The exact order of temperature presentation during testing is presented in Table 2-1.

Surgery and Drug Delivery

Osmotic minipumps (Alzet, Durect Corp., Cupertino, CA) filled with either fentanyl or saline were implanted subcutaneously in the right hindquarter during isoflurane anesthesia (1.5% at 1.0 L/min O_2). Minipumps delivered fluid at a rate of 2.58 µl/hr for 28 days. Fentanyl was delivered at a dose of 1 mg/kg/day. Four weeks after pump implantation, animals were lightly anesthetized and the pumps removed.

Thermal Preference

Apparatus

The thermal preference chambers were custom-made and consist of the following components. Two aluminum plates (11.5” x 7”; Smalls Design and Manufacturing, Portland, TN) that contain channels throughout connected to an input and output valve. These valves are connected to recirculating water baths (Model RTE-7; Thermo Scientific) which can maintain water at particular temperatures ranging from -25 to 150°C via insulated Tygon® tubing. The plates are surrounded by a Plexiglas chamber with two compartments separated by a removable partition with a cutout (3.5” x 3.5”) allowing the animal to traverse between the two compartments (Magnum Wood LLC, Gainesville, FL). Each chamber is opaque, 17” tall, and has no cover. This allows an overhead camera connected to an Ethovision tracking system (Noldus Information Technology, Wageningen, Netherlands) to record behavior.

Testing procedure

The general thermal preference procedure that used is as follows. The rat is placed into the thermal preference apparatus for a 15-minute session, and is given access to both components and presumably escapes from aversive stimulation to the other compartment. The number of “switches” from one compartment to the other, the time of each switch, cumulative duration on each side, and total distance travelled is recorded. The Ethovision tracking system (Noldus Information Technology, Wageningen, Netherlands) both collects and analyzes these data and makes a permanent record (both the digitized track and a video copy) of each session. Animals that never changed plates from the starting plate were assumed to have not sampled the other temperature and their data from that session were excluded from analysis.

Body Weight

Body weight was measured for each animal prior to each thermal preference test. Average body weights were calculated for each animal weekly throughout testing. Osmotic minipump weight was determined by measuring animals prior to, and after pump implant, and pump weight was subtracted from recorded body weights during drug administration session.

Locomotor Activity

General locomotor activity was measured by recording the total distance traveled during each thermal preference test using the Ethovision tracking system. Locomotor activity was analyzed separately based on the temperature settings for each trial. As each temperature test condition was presented twice in a week, the average distance traveled for each subject was calculated, and the group means for each week are presented.

Data Analysis and Statistics

For the baseline measure of body weight a one-way ANOVA with age as a factor was performed. For body weight data collected during and after drug administration, separate two-way ANOVAs were performed for each age group with test session and drug as the main factors. A subsequent two-way ANOVA was performed on fentanyl-treated animals with age and test session as factors.

The relative preference was calculated by taking the amount of time in seconds spent on the hotter side during the trial, and subtracting 450 (50% of trial time in seconds). This number was then divided by 450, and multiplied by 100. Using this calculation, animals that spend equal time on each plate have a relative preference of zero, whereas preference for the hotter plate falls above the zero line, and preference for the colder plate falls below the zero line.

For both locomotor and thermal preference data at baseline, a two-way repeated-measures ANOVA, with age and temperature setting as the factors was performed, as well as one-way ANOVAs across ages for each temperature setting. For locomotor and thermal preference data collected during and after drug administration, data were separated by temperature setting. Within each temperature setting, separate two-way repeated measure ANOVA were performed for each age with administered drug and test session as main factors. A separate two-way repeated-measures ANOVA was performed on the fentanyl-treated animals with age and test session as factors. For thermal preference testing, data were analyzed both as actual time spent on each plate, and as time spent on plate as a percent of baseline. Locomotor data were analyzed as a percent of baseline.

For all statistical tests, differences were considered statistically significant with a *p*-value of less than 0.05. Student-Newman-Keuls post-hoc tests were performed when appropriate. All statistical testing was performed using SigmaStat version 3.11 (Systat Software, Inc, San Jose, CA).

Results

Baseline Measures

Threshold testing

A range of temperatures from 10° to 45°C were compared to a neutral temperature (27.5°C) to assess where the threshold for hot and cold aversion occurred for each age (Figure 2-1). Temperatures were separated into temperatures cooler than neutral and warmer than neutral (Figure 2-1A, B respectively). Two-way repeated-measures ANOVAs were performed and in each case there were statistically significant main effects of temperature (cooler: $F_{5, 117} = 7.776$, $p < 0.001$; warmer: $F_{5, 116} = 7.881$, $p < 0.001$), and no differences between ages. Based on these results, 16° and 45°C were selected as the temperatures for the cold and hot plates during the thermal preference testing. These temperatures were selected because animals spent approximately the same amount of time on these temperatures so they could be considered equally "aversive." However, these temperatures were not so aversive that animals spent no time on these plates, and thus any increase in aversion to these temperatures could be observed.

Body weight

At baseline, body weight differed across ages ($F_{2, 24} = 10.431$; $p < 0.001$), with the 24-month old animals weight significantly more than the 16- and 20-month old animals (Figure 2-2A).

Locomotor activity

Locomotor activity during baseline testing is shown for each temperature setting across ages in Figure 2-2B. Locomotor activity was significantly different for each temperature setting, with the greatest levels of locomotor activity occurring during the hot versus cold temperature comparison, and the least amount of activity during the cold versus neutral comparison (main effect of temperature: $F_{2, 48} = 40.757$, $p < 0.001$). The 24-month old animals also showed significantly greater locomotor activity than the other two age groups (main effect of age: $F_{2, 24} = 4.367$, $p = 0.024$). Within each temperature comparison, one-way ANOVAs showed there were significant differences between ages for the cold versus neutral ($F_{2, 24} = 4.279$, $p = 0.026$) and hot versus neutral comparisons $F_{2, 24} = 4.601$, $p = 0.020$). Post-hoc analysis showed that 24-month old animals had significantly greater levels of activity than 16-month old animals during the cold versus neutral comparison, and greater activity than both 16- and 20-month old animals during the hot versus neutral comparison (Figure 2-2B).

Thermal preference

The relative preference for each temperature comparison across ages is shown in Figure 2-2C. In the cold versus neutral comparison and hot versus neutral comparison, all animals showed a strong preference for the neutral plate. However, during the hot versus cold comparison, 16- and 20-month old animals showed a slight preference for the cold plate, while 24-month old animals showed a slight preference for the hot plate. Primary statistical analysis showed a main effect of temperature ($F_{2, 46} = 127.616$, $p < 0.001$), with each comparison significantly different from the others. One-way ANOVAs were performed across ages within each temperature, but there were no significant differences across ages during this baseline assessment.

Body Weight

Changes in body weight during drug administration and withdrawal are shown in Figure 2-3. Fentanyl administration decreased body weight for all three ages, which was maintained through 4 weeks of drug administration, and all three ages showed weight recovery during withdrawal. For all three ages, there was a significant drug x session interaction (16-month: $F_{7, 56} = 16.648$, $p < 0.001$; 20-month: $F_{7, 42} = 11.272$, $p < 0.001$; 24-month: $F_{7, 49} = 12.550$, $p < 0.001$). By week two of drug administration, fentanyl-treated animals had significantly lower body weight than saline-treated animals for both the 20- and 24-month old animals. This significant decrease in body weight continued through the second week of withdrawal for both ages. When fentanyl-treated animals were compared across ages, there was a significant age x session interaction ($F_{14, 70} = 4.057$, $p < 0.001$), and 24-month old animals had significantly lower body weight than 16-month old animals starting in the fourth week of drug administration and continuing through the fourth week of withdrawal.

Cold versus Neutral

Locomotor activity

Locomotor activity during cold versus neutral sessions, for each age, is shown in Figure 2-4. In general, fentanyl administration increased locomotor activity during the later weeks of drug administration, although this increase did not persist through drug withdrawal. There was a significant drug x session interaction for 16- and 20-month old animals (16-month: $F_{7, 55} = 2.231$, $p = 0.045$; 20-month: $F_{7, 42} = 3.746$, $p = 0.003$) with fentanyl-treated animals showing significantly increased locomotor activity during drug administration weeks 2 through 4 compared to saline-treated animals. Although fentanyl administration increased locomotor activity in 24-month old animals (Figure 2-4C), there

was no statistically significant difference across drug, session, or drug x session interaction. When fentanyl-treated animals were compared across ages (Figure 2-4D), there was a significant main effect of session ($F_{7, 69} = 5.940$, $p < 0.001$), with weeks 3 and 4 of drug administration having significantly greater activity than drug week 1 and all four weeks of withdrawal.

Thermal preference

The average time spent on the cold plate for each age is shown in Figure 2-5. Overall, fentanyl administration showed little effect on sensitivity to cold. All three ages showed greater time spent on the cold plate during the first test after the start of drug administration, however this effect was not maintained through the rest of drug administration. Statistical analysis showed a main effect of session for the 20-month old animals ($F_{16, 85} = 2.479$, $p = 0.004$). Post-hoc analysis showed that animals spent greater time on the cold plate during first drug session than the fourth drug session, and all withdrawal sessions with the exception of the first withdrawal session (Figure 2-5B). There was a significant interaction between drug and session for the 24-month old animals ($F_{16, 103} = 2.340$, $p = 0.005$), with fentanyl-treated animals spending more time on the cold plate than saline-treated animals during the first, sixth, and eighth drug session, as well as the first withdrawal session (Figure 2-5C). When fentanyl-treated animals were compared across ages, there was a significant main effect of session ($F_{16, 146} = 4.841$, $p < 0.001$), with animals spending more time on the cold plate during the first drug session than all other sessions (Figure 2-5D).

The time spent on the cold plate was also analyzed as a percent of time spent on cold plate during baseline testing. Similar to the raw data, there was little change from baseline in the time spent on the cold plate (Figure 2-6), with the exception being the

first drug session. Statistical analysis showed a main effect of session for the 16-month old animals ($F_{15, 108} = 2.007$, $p = 0.021$). There was a main effect of both drug and session for the 20-month old animals (drug: $F_{1, 6} = 8.148$, $p = 0.029$; session: $F_{15, 80} = 2.487$, $p = 0.005$). Overall, fentanyl-treated animals spent more time on the cold plate than saline-treated animals, and animals spent more time on the cold plate during the first drug session than all the withdrawal sessions with the exception of the first (Figure 2-6B). As with the raw data, there was a significant drug x session interaction for the 24-month old animals ($F_{16, 96} = 2.223$, $p = 0.010$), with fentanyl-treated animals spending more time on the cold plate than saline-treated animals during the first, sixth, and eighth drug session as well as the first withdrawal session (Figure 2-6C). There was no statistically significant difference in the effects of fentanyl across age groups, although fentanyl-treated animals did spend more time on the cold plate during the first drug session compared to all other sessions (session main effect: $F_{15, 136} = 6.493$, $p < 0.001$).

Hot versus Neutral

Locomotor activity

As with during cold versus neutral sessions, fentanyl administration increased locomotor activity for all ages during hot versus neutral sessions (Figure 2-7). However, unlike the cold versus neutral sessions, there was a significant drug x session interaction for all three ages (16-month: $F_{7, 56} = 5.463$, $p < 0.001$; 20-month: $F_{7, 41} = 3.505$, $p = 0.005$; 24-month: $F_{7, 49} = 7.221$, $p < 0.001$). Post-hoc analysis showed that fentanyl-treated animals had significantly higher activity than saline-treated animals during weeks 2 through 4 of drug administration for the 16- and 20-month old animals (Figure 2-7A, B), but only during weeks 3 and 4 for the 24-month old animals (Figure 2-7C). Compared across ages, fentanyl-treated animals showed significantly greater

activity during drug weeks 2 through 4 compared to drug week 1, and all four withdrawal weeks (main effect of session: $F_{7, 69} = 11.968$, $p < 0.001$; Figure 2-7D).

Thermal preference

Fentanyl administration increased the amount of time spent on the hot plate during hot versus neutral sessions (Figures 2-8, 2-9). Throughout drug administration, fentanyl-treated animals spent more time on the hot plate than on the neutral plate, a reversal from behavior during baseline assessment. However, this change in preference did not extend through the withdrawal period. Interestingly, 24-month old saline-treated animals also showed increased time spent on the hot plate, although this increase was not as great or as consistent as the fentanyl-treated animals (Figure 2-8C).

Within each age, there was significant drug x session interaction (16-month: $F_{16, 127} = 6.107$, $p < 0.001$; 20-month: $F_{16, 89} = 2.363$, $p = 0.006$; 24-month: $F_{16, 110} = 2.017$, $p = 0.018$). Fentanyl-treated animals spent significantly more time on the hot plate than saline-treated animals during drug sessions 2 through 8 for both 16- and 20-month old animals, and this increase persisted through the first withdrawal session for the 16-month old animals. Fentanyl administered animals spent more time on the hot plate than saline animals, but only during drug sessions 4 and 8 for the 24-month old animals. Compared to baseline, 16-month old fentanyl-treated animals spent significantly more time on the hot plate during drug sessions 2 through 8 and during the first withdrawal session. For 20-month old animals, there was a significant increase in time spent on the hot plate for fentanyl administered animals during drug sessions 4 through 6, and fentanyl administered 24-month old animals never spent a significantly greater amount of time on the hot plate compared to baseline. There was a main effect of session when fentanyl-treated animals were compared across ages ($F_{16, 149} = 14.307$, $p < 0.001$), with

animals spending more time on the hot plate during drug sessions 2-8 and the first withdrawal session compared to baseline (Figure 2-8D).

When data were analyzed as a percent of baseline, similar results to the raw data were observed with a significant interaction between drug and session for all ages (16-month: $F_{15, 119} = 5.231$, $p < 0.001$; 20-month: $F_{15, 83} = 2.524$, $p = 0.004$; 24-month: $F_{15, 103} = 1.925$, $p = 0.029$), with 16- and 20-month old fentanyl-treated animals spending significantly more time on the hot plate compared to saline-treated animals during drug sessions 2 through 8 (Figures 2-9A and 2-9B). This increase persisted through the first withdrawal session for 16-month old animals. When fentanyl-treated animals were compared, there was a main effect of session ($F_{15, 139} = 13.024$, $p < 0.001$). Post-hoc analysis showed that animals spent significantly more time on the hot plate during drug sessions 2 through 8, compared to all withdrawal sessions with the exception of the first session. Animals also spent significantly more time on the hot plate during drug sessions 4 and 5 compared to drug session 1 (Figure 2-9D).

Hot versus Cold

Locomotor activity

During hot versus cold sessions, changes in locomotor activity were similar to those observed during hot versus neutral session (Figure 2-10). There was a significant drug x session interaction for all three ages (16-month: $F_{7, 56} = 2.925$, $p = 0.011$; 20-month: $F_{7, 42} = 3.779$, $p = 0.003$; 24-month: $F_{7, 47} = 3.374$, $p = 0.005$), with 16-month old fentanyl-treated animals having greater activity than saline-treated animals during drug weeks 2 through 4 (Figure 2-10A). Fentanyl-treated animals had greater activity than saline-treated animals in the 20- and 24-month old age groups, but during drug weeks 3 and 4 (Figure 2-10B, C). When fentanyl-treated animals were compared across ages,

results were similar to hot versus neutral sessions (Figure 2-10D). There was a significant main effect of session ($F_{7, 69} = 6.745$, $p < 0.001$), with drug weeks 2 through 4 having significantly greater activity than drug week 1 and all four weeks of withdrawal.

Thermal preference

Fentanyl administration increased the time spent on the hot plate during hot versus cold tests for all ages (Figures 2-11 and 2-12), although 24-month old, saline-treated animals also showed increased preference for the hot plate during hot versus cold sessions. For 16-month old animals, there was a significant drug x session interaction when raw data were analyzed ($F_{16, 127} = 1.838$, $p = 0.033$), with fentanyl animals spending more time on the hot plate during all drug sessions except session 2, and withdrawal sessions 2, 6, 7, and 8 (Figure 2-11A). Compared to baseline, fentanyl administered animals spent a significantly greater time on the hot plate during drug session 5. Analysis of raw data for 20-month old animals showed main effects for both drug and session (drug: $F_{1, 6} = 14.406$, $p = 0.009$; session: $F_{16, 95} = 2.302$, $p = 0.007$), with fentanyl-treated animals spending a greater amount of time on the hot plate compared to saline-treated animals (Figure 2-11B). There was a main effect of session when raw data for the 24-month old animals was analyzed ($F_{16, 106} = 3.502$, $p < 0.001$), with animals spending a greater amount of time on the hot plate during drug sessions 3 through 8 and withdrawal sessions 1, 3, 5, 6, and 7, when compared to baseline (Figure 2-11C). When compared across ages, there was a main effect of session for fentanyl-treated animals($F_{16, 155} = 6.709$, $p < 0.001$), which spent more time on the hot plate for all sessions compared to baseline except for the last withdrawal session (Figure 2-11D).

When hot versus cold data for the 16-month old animals were analyzed as a percent of baseline, there was a main effect of drug ($F_{1, 119} = 12.032$, $p = 0.008$), with

fentanyl-treated animals spending more time on the hot plate than saline-treated animals (Figure 2-12A). Main effects of both drug and session were obtained with 20-month old animals (drug: $F_{1, 6} = 27.093$, $p = 0.002$; session: $F_{15, 89} = 2.170$, $p = 0.013$) with fentanyl-treated animals spending more time on the hot plate than saline-treated animals (Figure 2-12B). Analysis of 24-month old animals showed a significant drug x session interaction ($F_{15, 99} = 1.865$, $p = 0.036$) with fentanyl-treated animals spending significantly more time on the hot plate than saline-treated animals during drug session 4, and withdrawal sessions 1 and 3 (Figure 2-12C). When fentanyl-treated animals were directly compared (Figure 2-12D), 20-month old animals have a significantly greater increase in time on the hot plate compared to the other ages (age main effect: $F_{2, 10} = 8.225$, $p = 0.008$). In addition, all fentanyl-treated animals spend more time on the hot plate during drug sessions 2 through 8 and withdrawal session 1, compared to the last withdrawal session (session main effect: $F_{15, 145} = 3.880$, $p < 0.001$).

Discussion

A potential medical crisis is approaching with increases in the age of the population (Colliver et al., 2006; Kalapatapu and Sullivan, 2010), the rates of chronic pain (Bruckenthal et al., 2009; Colliver et al., 2006; McCarthy et al., 2009; Neville et al., 2008; Rustøen et al., 2005; Walid and Zaytseva, 2009), and the use of opioids to treat chronic pain (Caudill-Slosberg et al., 2004; Ghodse, 2003; Kalso et al., 2003; Rowbotham et al., 2003) resulting in greater numbers of aged individuals taking opioids for longer periods of time. Unfortunately, how older individuals respond to chronic opioid administration compared younger individuals is not fully understood. The purpose of this study was to assess the relationship between increased age and the antinociceptive properties of chronic fentanyl administration using an operant based thermal preference

procedure. Overall, this study showed that chronic fentanyl had a decreased antinociceptive effect as age increased.

Baseline Differences

At baseline, older animals spent more time on the warmer plate than younger animals. Although these differences were not statistically significant, that may be due to the relatively small age difference between the groups. Increased preference for the warmer plate is consistent with previous studies (Yezierski et al., 2010), and could be explained by other studies which have shown that tolerance for cold temperatures decreases with age (Scarpace, 1997). Older animals also showed greater locomotor activity than younger animals. This could also be a symptom of less tolerance for extreme temperatures, which forced the older animals to change plates more often than younger animals. The oldest animals also had the highest body weight at the start of the experiment, which was expected as animals generally add body weight with age.

Body Weight

During fentanyl administration animals of all ages lost weight and this effect increased with age, with older animals losing weight more quickly, and losing a greater percent of their body weight during drug administration. While these results were not unexpected, as chronic opioid agonist administration has previously been shown to decrease body weight (Binsack et al., 2006; Levine et al., 1988; Li et al., 2010), they do suggest that fentanyl has a greater effect on older individuals than younger individuals do, which should be a concern in a clinical setting.

Locomotor Activity

One of the most common side effects of opioid administration is sedation (Manchikanti and Singh, 2008), therefore, the locomotor activity of animals during the

thermal preference procedure was recorded. After one week of fentanyl administration, locomotor activity increased for all fentanyl administered animals. These results suggest that sedation may not be a major concern with chronic fentanyl administration, and similar results have been observed in clinical setting with human patients (Agarwal et al., 2007). However, increases in locomotor activity were lowest in the oldest animals, suggesting that opioids have a diminished effect with increasing age, which is contradictory to the results observed with body weight.

Thermal Preference

Overall, chronic fentanyl administration had a greater effect on sensitivity to heat as opposed to cold. Animals showed an increase in time spent on the cold plate initially, but quickly became tolerant to these effects (i.e. within one week). Interestingly, animals still displayed increased locomotor activity during cold versus neutral trials, even though their thermal preference did not change. This suggests that the increase in locomotor activity is not due to the animals changing plates more often, and that the effects of opioids on locomotor activity and thermal preference are dissociable and mediated through different neurobiological systems.

Chronic fentanyl administration did alter tolerance for heat, as all animals increased their time on the hot plate during fentanyl administration. As with locomotor activity, there was a decreased effect of chronic fentanyl administration with increasing age, suggesting that older individuals are less sensitive to the antinociceptive effects of fentanyl. It is difficult however, to assess how diminished the antinociceptive effects of fentanyl are in the oldest animals tested as 24-month old, saline-treated animals also showed a decrease in sensitivity to heat. These animals did not show a decrease in body weight or an increase in locomotor activity, so they were not mistakenly

administered fentanyl, although an explanation for this decrease in heat sensitivity is not clear. One possible explanation is that the decrease in heat sensitivity observed in 24-month old animals is an age-related change, and thus observed in both saline and fentanyl administered animals. If this explanation were correct, it would suggest that chronic fentanyl administration had either no effect on thermal preference in older animals, or any effects were occluded by the age related shift in thermal preference. The findings that sensitivity to heat returned following removal of the minipumps argues against this interpretation however. It is clear that additional research is necessary to fully understand the effects of chronic fentanyl administration on thermal sensitivity in older animals.

The overall results with fentanyl are similar to previous studies in our lab assessing the effects of acute morphine on thermal preference across ages. Both that study and the current study showed that opioid administration, either acute or chronic, decreased aversion to hot temperatures, yet had little effect on cold aversion. Of particular interest is that the antinociceptive effects of fentanyl were maintained throughout 4 weeks of drug administration, suggesting that thermal preference is resistant to the development of opioid tolerance. This findings are in stark contrast to previous studies that assessed the effects of chronic fentanyl administration on reflexive tail withdrawal from hot water (Morgan et al., 2008). This study showed a rapid development of tolerance to the effects of fentanyl during chronic administration using similar experimental parameters (e.g. various ages, same sex and rat strain, similar drug administration protocols). Together, these data highlight the suggestion that there are distinct physiological systems mediating nociception assessed using these two

behavioral procedures. Furthermore, as in the human clinical situation, the development of tolerance is not a necessary outcome of chronic drug treatment suggesting that there may be situations in which long-term opioid use for chronic pain conditions is appropriate.

Implications and Future Directions

Older individuals are being prescribed opioids in greater numbers to alleviate pain, and taking them for longer periods of time. However, how increased age affects the antinociceptive properties of opioids is not fully understood. The results of this study suggest that opioid efficacy decreases with advancing age, at least in respects to antinociception. However, these results are far from definitive. The age range for this study was limited and even the youngest age tested could be considered an aged rat. This was done to assess animals across the range of middle aged to pre-senescent, similar to the current age range of the “baby boomer” generation. However, to understand the relationship between aging and opioids, a greater age range will need to be tested.

This study measured changes in thermal sensitivity in males. It is important to extend these studies to females as well for two reasons. One, females generally live longer than males, and therefore will make up a greater portion of the aged population. Second, some chronic pain conditions are more common in females (McCarthy et al., 2009), and therefore animal studies should be conducted in females in order to better model the conditions that are observed in clinical settings. Previous studies using the thermal preference procedure have shown sex differences in thermal preference (Vierck et al., 2008a), and these differences between males and females need to be assessed following opioid administration as well.

This study used a new operant based procedure for pain assessment in animals. While the justification for the use of this procedure is explained in Chapter 1, it is important to note that opioid effects with this procedure have not been fully characterized in animals, regardless of sex or age. Therefore, it is important to continue to test not a range of doses of fentanyl on this procedure, but also other opioid and non-opioid analgesic drugs need to be tested on this procedure. Two important drugs to consider are duloxetine and pregabalin, non-opioid drugs that are commonly prescribed for the treatment of chronic pain. With the thermal preference procedure designed to selectively activate the processes involved in chronic pain, it is important to assess the effects of drugs commonly used in the treatment of chronic pain.

Table 2-1. Temperature presentation for thermal preference testing

Session	Week	Day	Temperatures	
Threshold	1	1	45	27.5
		2	27.5	16
		3	33	27.5
		4	27.5	10
		5	27.5	22
		6	39	27.5
	2	1	30	27.5
		2	27.5	25
		3	27.5	13
		4	42	27.5
		5	36	27.5
		6	27.5	19
Baseline	1	1	45	27.5
		2	45	16
		3	27.5	16
		4	45	16
		5	45	27.5
		6	27.5	16
Drug	1	1	27.5	16
		2	45	27.5
		3	45	16
		4	45	27.5
		5	27.5	16
		6	45	16
	2	1	27.5	16
		2	45	16
		3	45	27.5
		4	27.5	16
		5	45	27.5
		6	45	16
	3	1	27.5	16
		2	45	16
		3	45	27.5
		4	45	16
		5	27.5	16
		6	45	27.5
	4	1	45	16
		2	45	27.5
		3	27.5	16
		4	45	27.5
		5	45	16
		6	27.5	16
Withdrawal	1	1	45	16
		2	27.5	45

Table 2-1. Continued

Session	Week	Day	Temperatures	
Withdrawal	1	3	16	27.5
		4	45	16
		5	27.5	16
		6	45	27.5
	2	1	27.5	16
		2	45	16
		3	45	27.5
		4	27.5	16
		5	45	27.5
		6	45	16
	3	1	45	27.5
		2	45	16
		3	27.5	16
		4	45	27.5
		5	45	16
		6	27.5	16
	4	1	45	16
		2	45	27.5
		3	27.5	16
		4	45	27.5
		5	45	16
		6	27.5	16

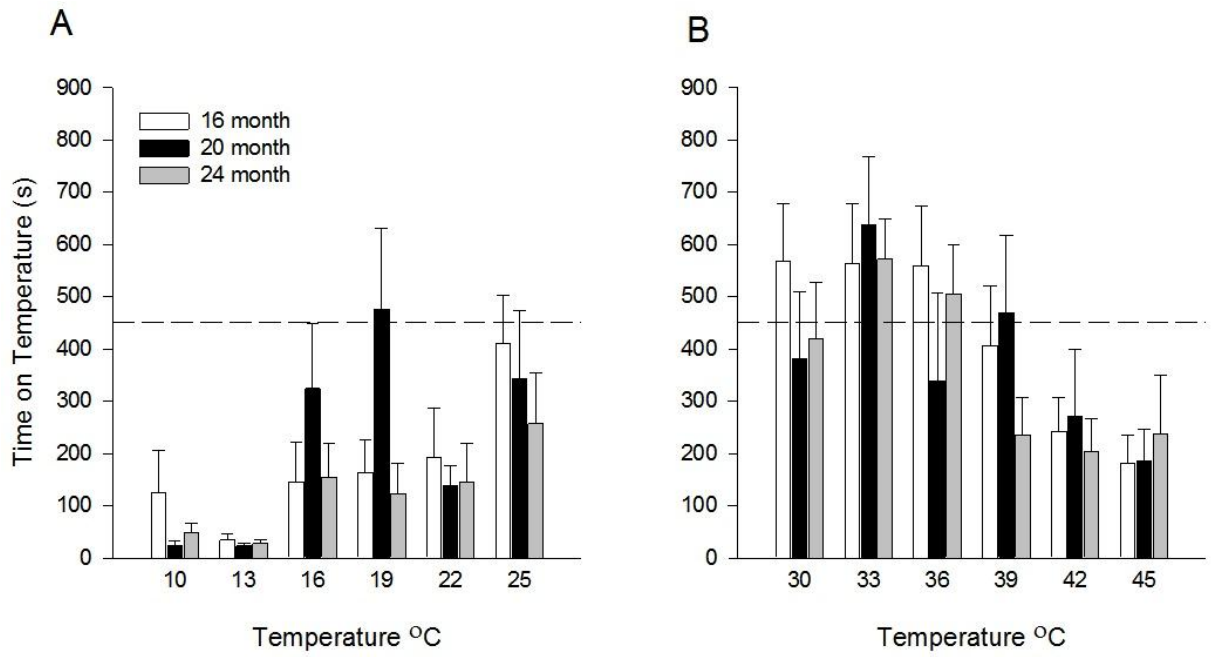

Figure 2-1. Temperature thresholds. A) Sensitivity to cooler temperatures. B) Sensitivity to warmer temperatures. The time spent on each temperature when compared to neutral (27.5°C) is shown for each age (mean ± SEM). Dashed line represents 450 seconds, or equal time on each plate. Predictably the closer the temperature was to neutral, the less aversive the temperature was, although there were no statistically significant differences between ages.

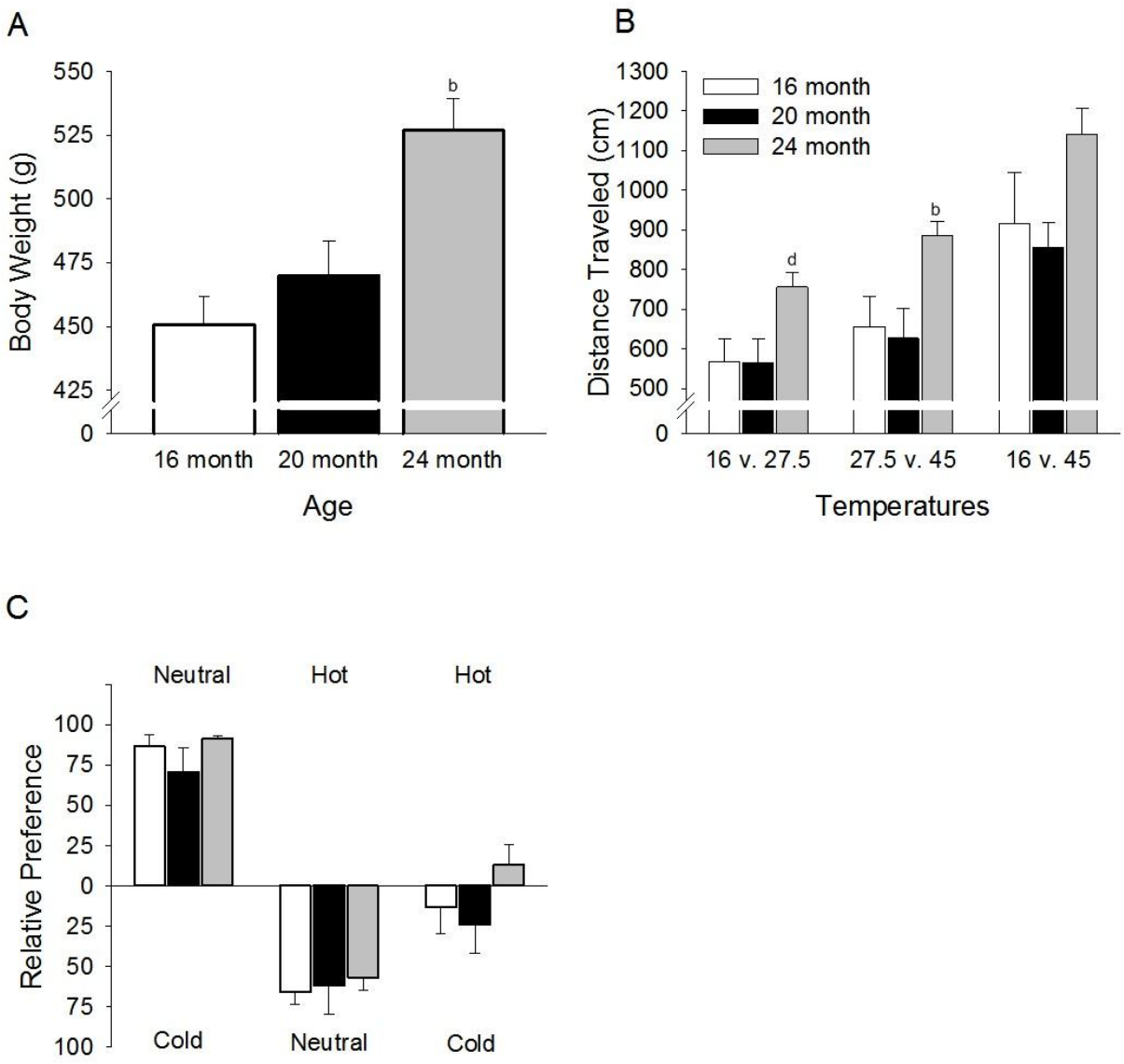

Figure 2-2. Baseline measures. A) Body weight. B) Locomotor activity. C) Thermal preference. Assessments made before mini-pump implantation. A) Body weight increased as a function of age. B) The oldest animals showed greater locomotor activity during all three temperature comparisons. C) Animals showed a preference for the neutral plate when compared to cold or hot plates. When hot and cold plates were compared, the oldest animals showed a slight preference for the hot plate, while the other ages showed a slight preference for the cold plate, although these differences are not statistically significant. $^{b}p < 0.05$ compared to all other ages, $^{d}p < 0.05$ compared to 16-month old animals.

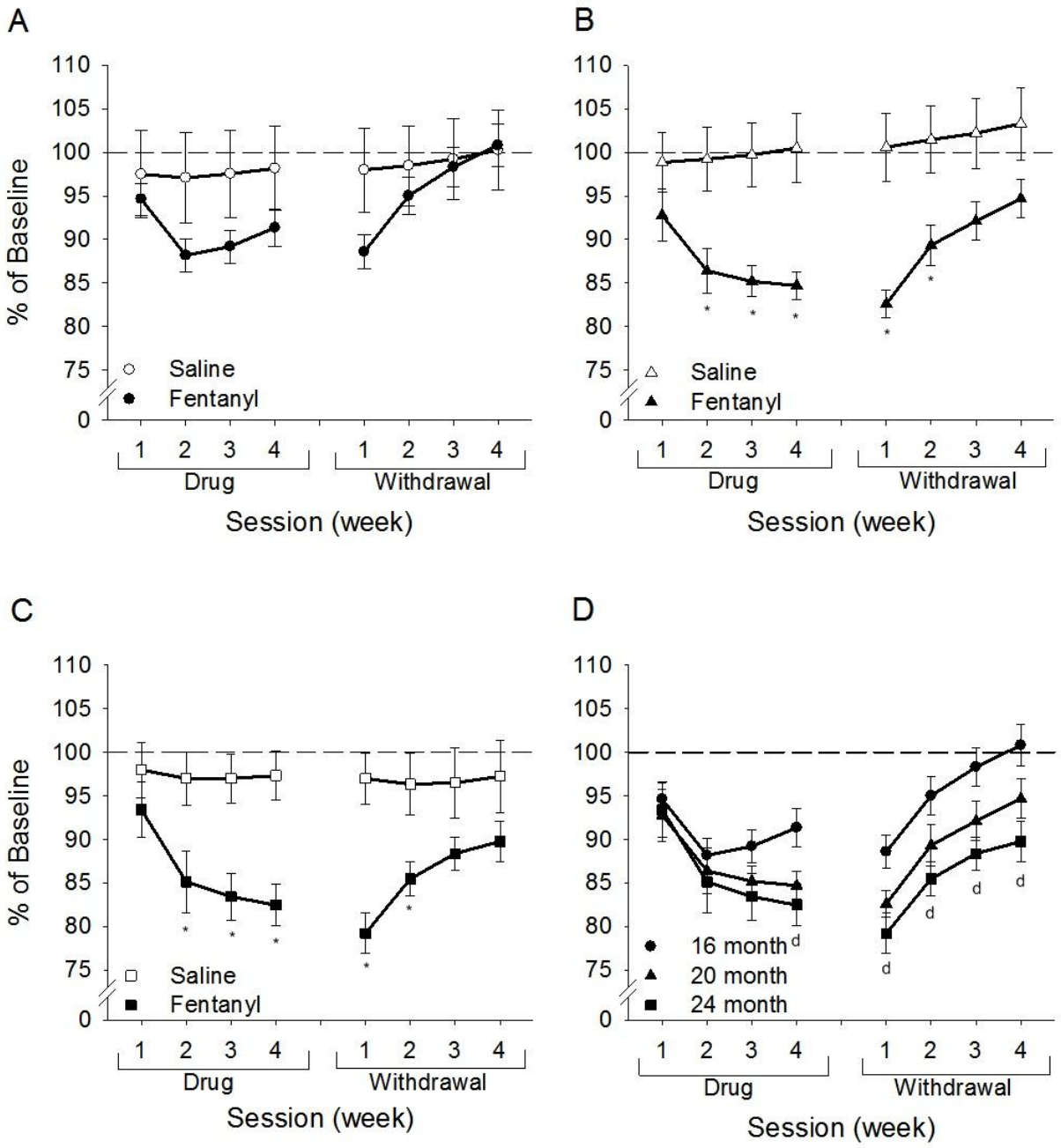

Figure 2-3. Body weights during drug administration and withdrawal. A) 16-month old animals. B) 20-month old animals. C) 24-month old animals. D) Comparison of fentanyl administered animals across ages (mean ± SEM). Fentanyl administration decreased body weight for all ages after one week of drug administration. All ages showed a recovery of body weight during withdrawal, although only 16-month old animals showed a full recovery of body weight. Animals showed increased weight loss in an age dependent manner during drug administration, as well as slower recovery of body weight during withdrawal. *$p < 0.05$ compared to saline animals, $^{d}p < 0.05$ compared to 16-month old animals.

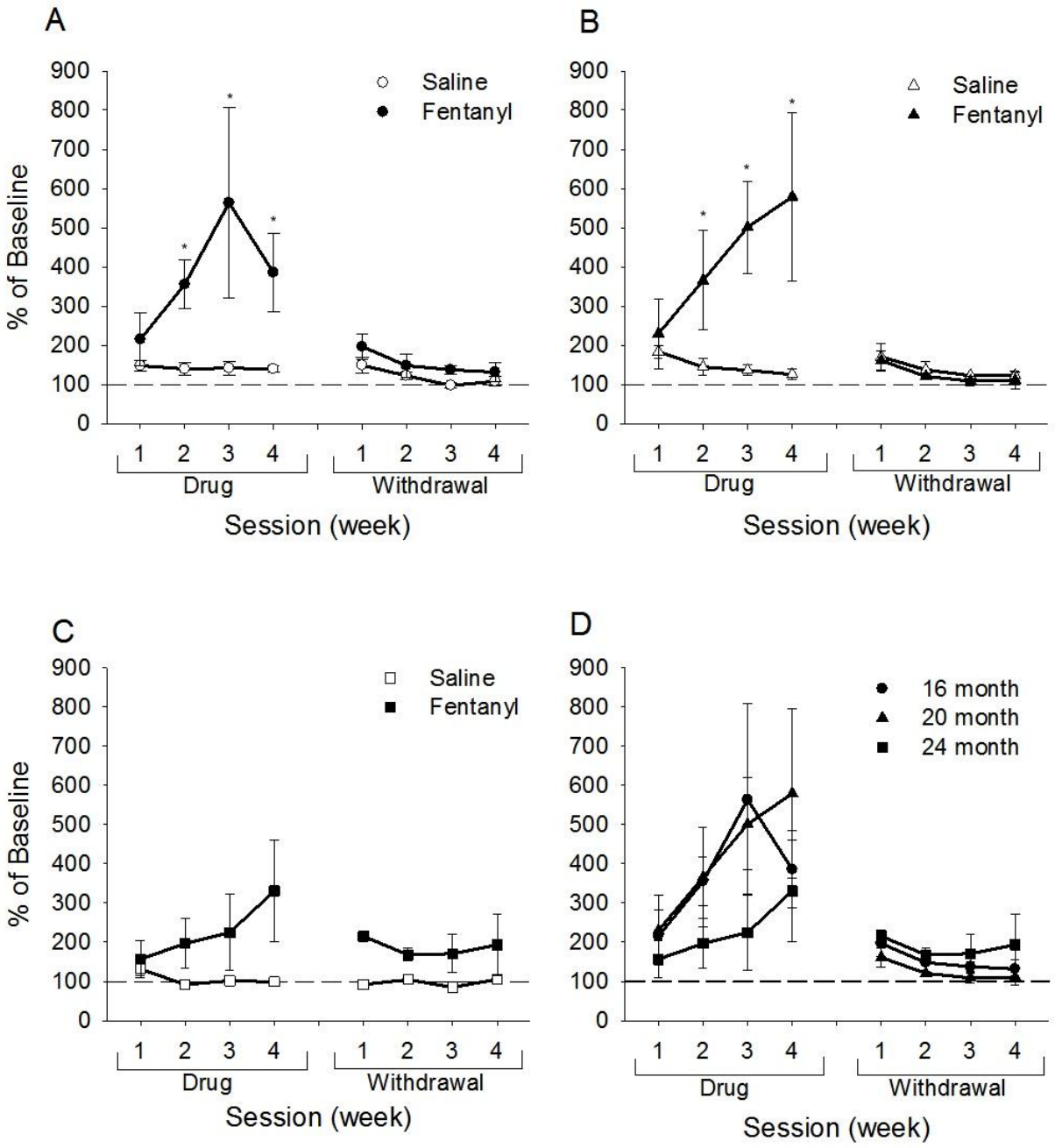

Figure 2-4. Change in locomotor activity during cold versus neutral temperature comparison. A) 16-month old animals. B) 20-month old animals. C) 24-month old animals. D) Comparison of fentanyl administered animals across ages. Weekly averages of locomotor activity as a percent of baseline are shown (mean ± SEM). Fentanyl administration increased activity after the first week of administration in all ages, although this increase did not persist through withdrawal. There was no statistically significant effect of age on changes in locomotor activity in fentanyl-treated animals. *$p < 0.05$ compared to saline administered animals.

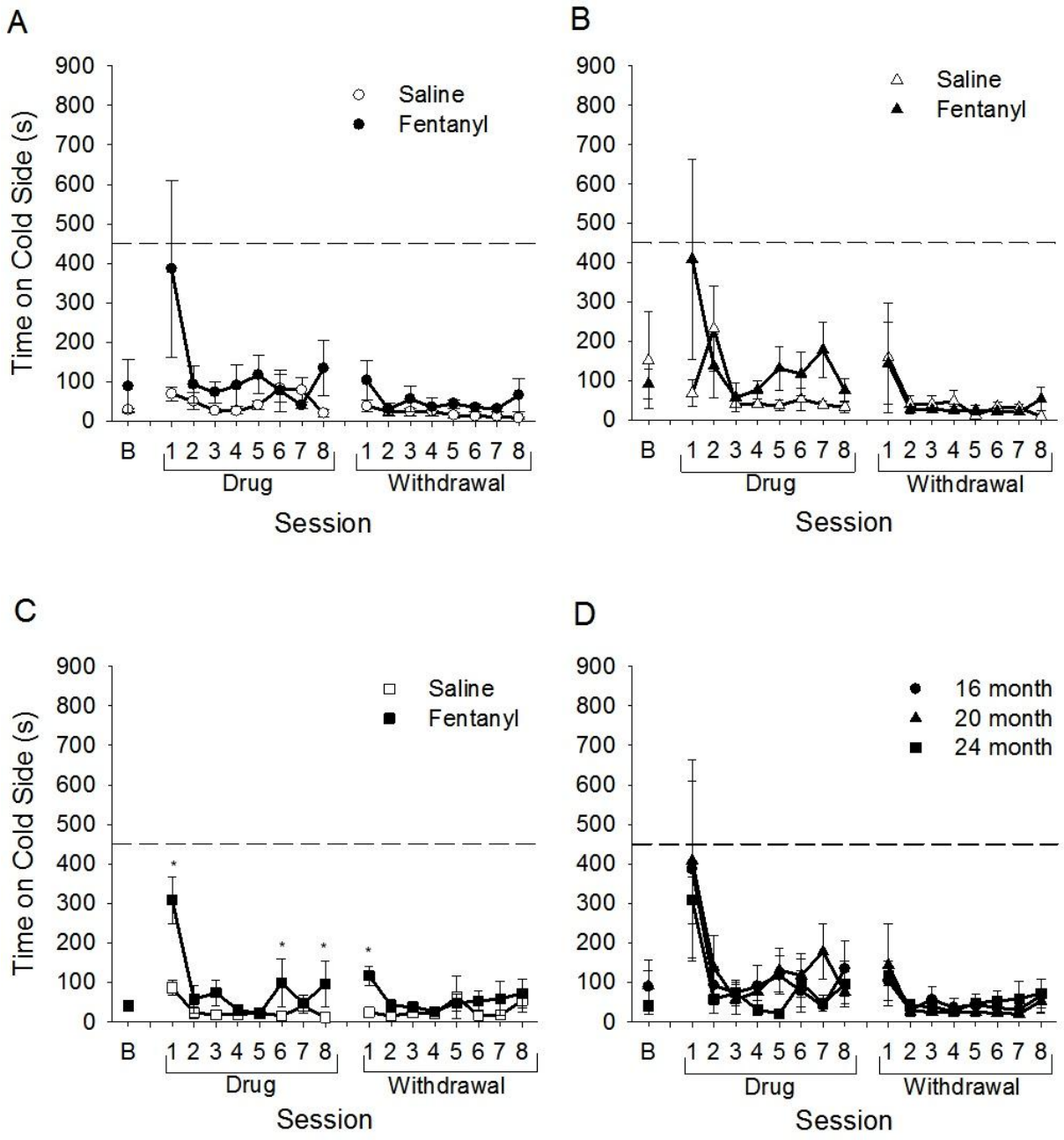

Figure 2-5. Sensitivity to cold during drug administration and withdrawal. A) 16-month old animals. B) 20-month old animals. C) 24-month old animals. D) Comparison of fentanyl administered animals across ages. Time spent on cold plate during cold versus neutral sessions (mean ± SEM). Overall animals spent more time on the neutral plate than the cold plate. Fentanyl decreased sensitivity to cold during the first session, and animals quickly developed tolerance to these effects. Fentanyl administration increased the amount of time spent on the cold plate during the first drug session compared to all other sessions. *$p < 0.05$ compared to saline administered animals.

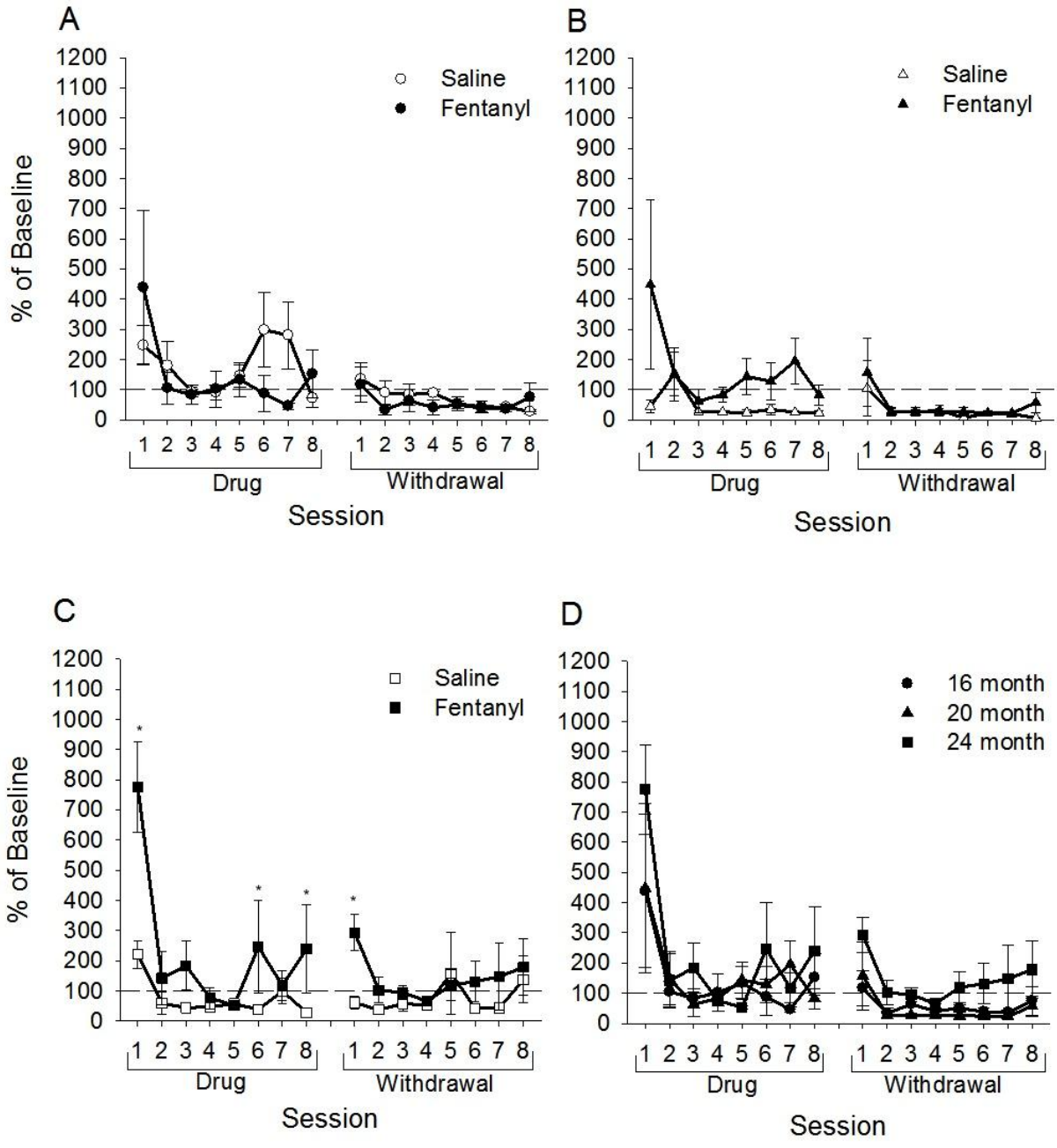

Figure 2-6. Change in cold sensitivity during drug administration and withdrawal. A) 16-month old animals. B) 20-month old animals. C) 24-month old animals. D) Comparison of fentanyl administered animals across ages. Time spent on cold plate as a percent of baseline (mean ± SEM). *$p < 0.05$ compared to saline administered animals.

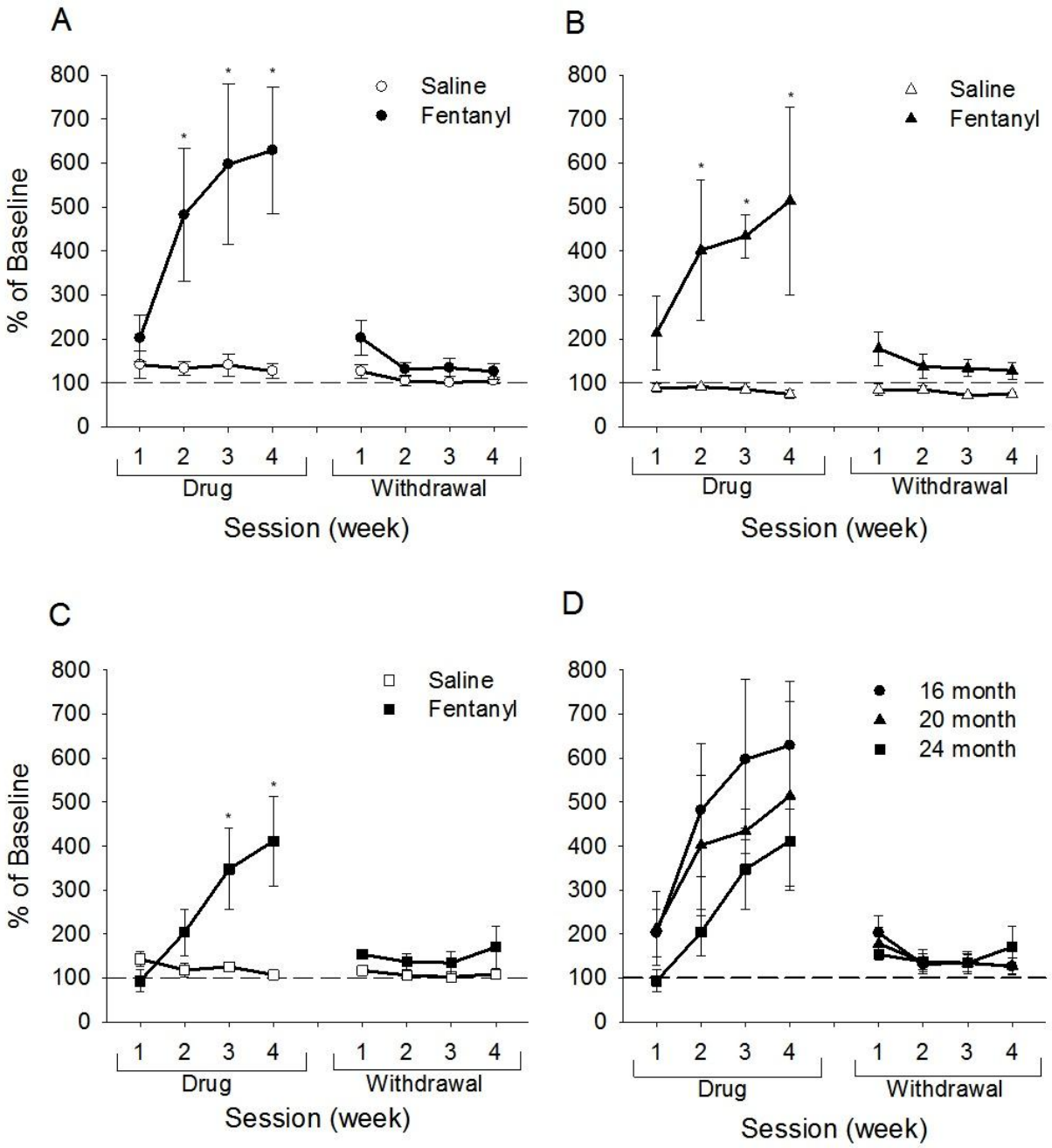

Figure 2-7. Change in locomotor activity during hot versus neutral temperature comparison. A) 16-month old animals. B) 20-month old animals. C) 24-month old animals. D) Comparison of fentanyl administered animals across ages. All other details as in Figure 2-4. Fentanyl administration significantly increased locomotor activity across all ages, although this increase took longer to develop in 24-month old animals. *$p < 0.05$ compared to saline administered animals.

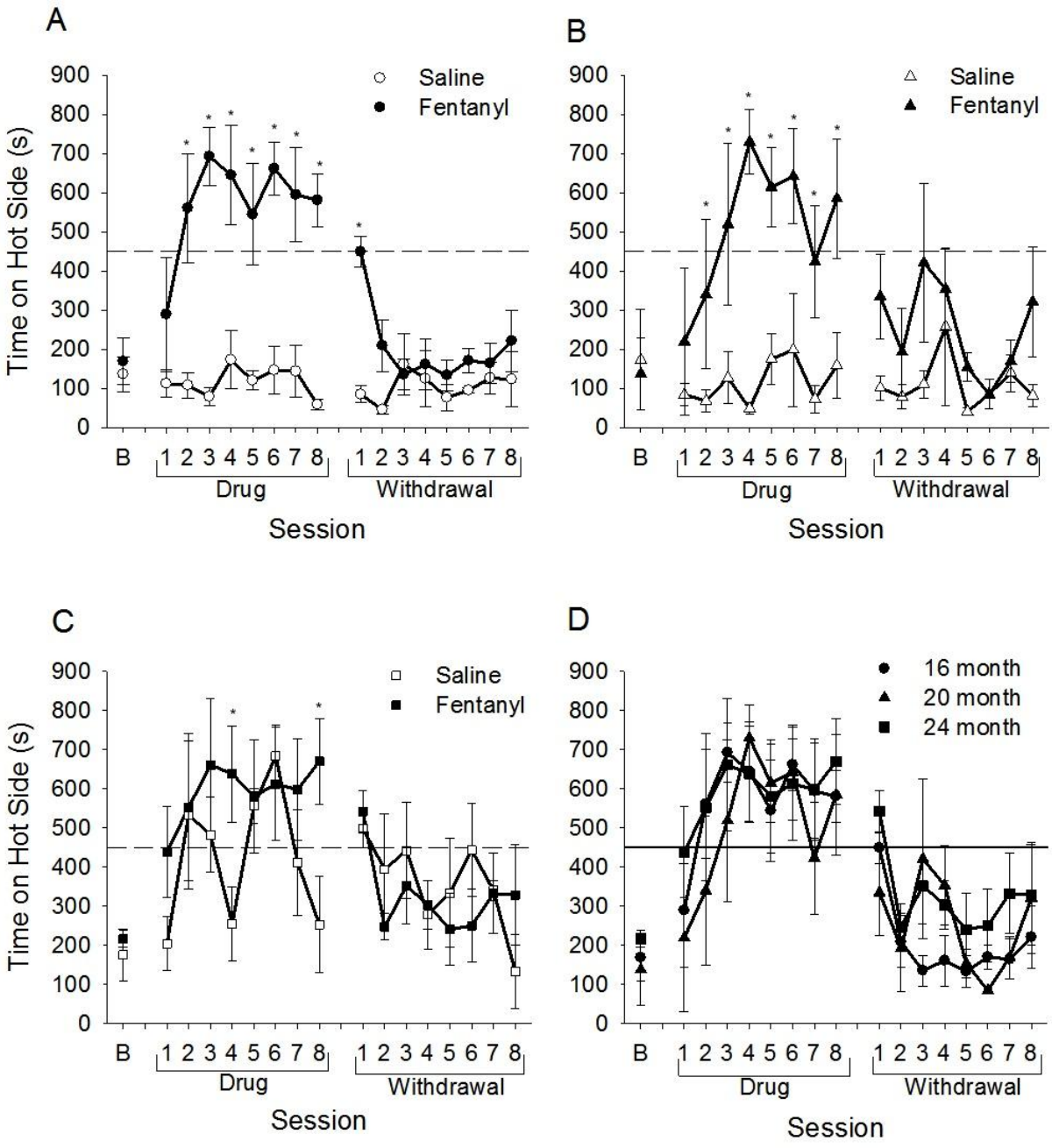

Figure 2-8. Sensitivity to hot during drug administration and withdrawal. A) 16-month old animals. B) 20-month old animals. C) 24-month old animals. D) Comparison of fentanyl administered animals across ages. Time spent on hot plate (mean ± SEM), all other details as in Figure 2-5. Fentanyl administration increased time spent on hot plate for all ages, however, 24-month old saline administered animals also increased time spent on hot plate. Decrease in hot sensitivity did not persist through withdrawal. *$p < 0.05$ compared to saline administered animals.

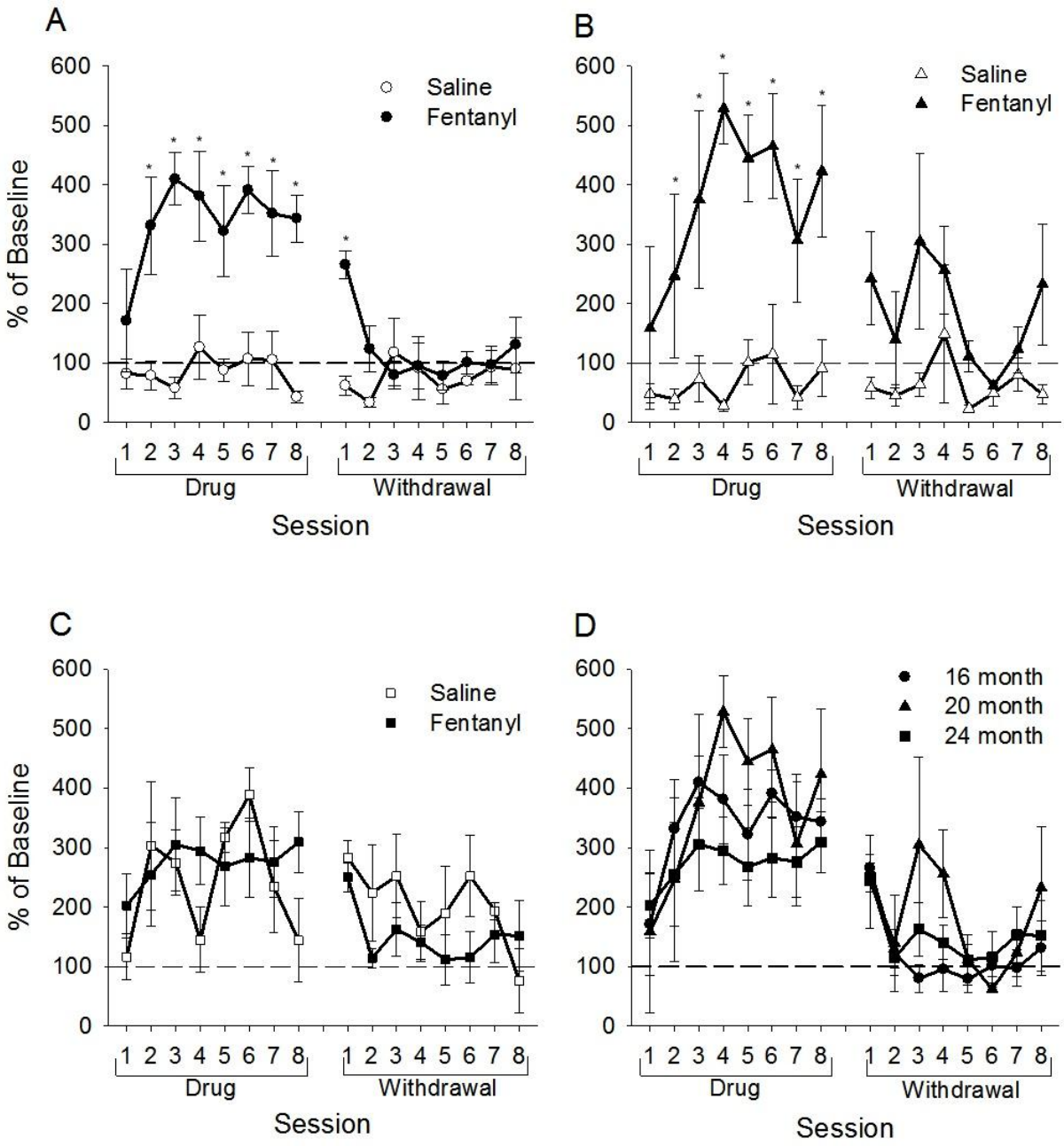

Figure 2-9. Change in sensitivity to hot during drug administration and withdrawal. A) 16-month old animals. B) 20-month old animals. C) 24-month old animals. D) Comparison of fentanyl administered animals across ages. Other details similar to those in Figure 2-6. Fentanyl administration decreased sensitivity to hot for 16-month and 20-month old animals. Both fentanyl- and saline-treated 24-month old animals had increase time on the hot plate during drug sessions. *$p < 0.05$ compared to saline administered animals.

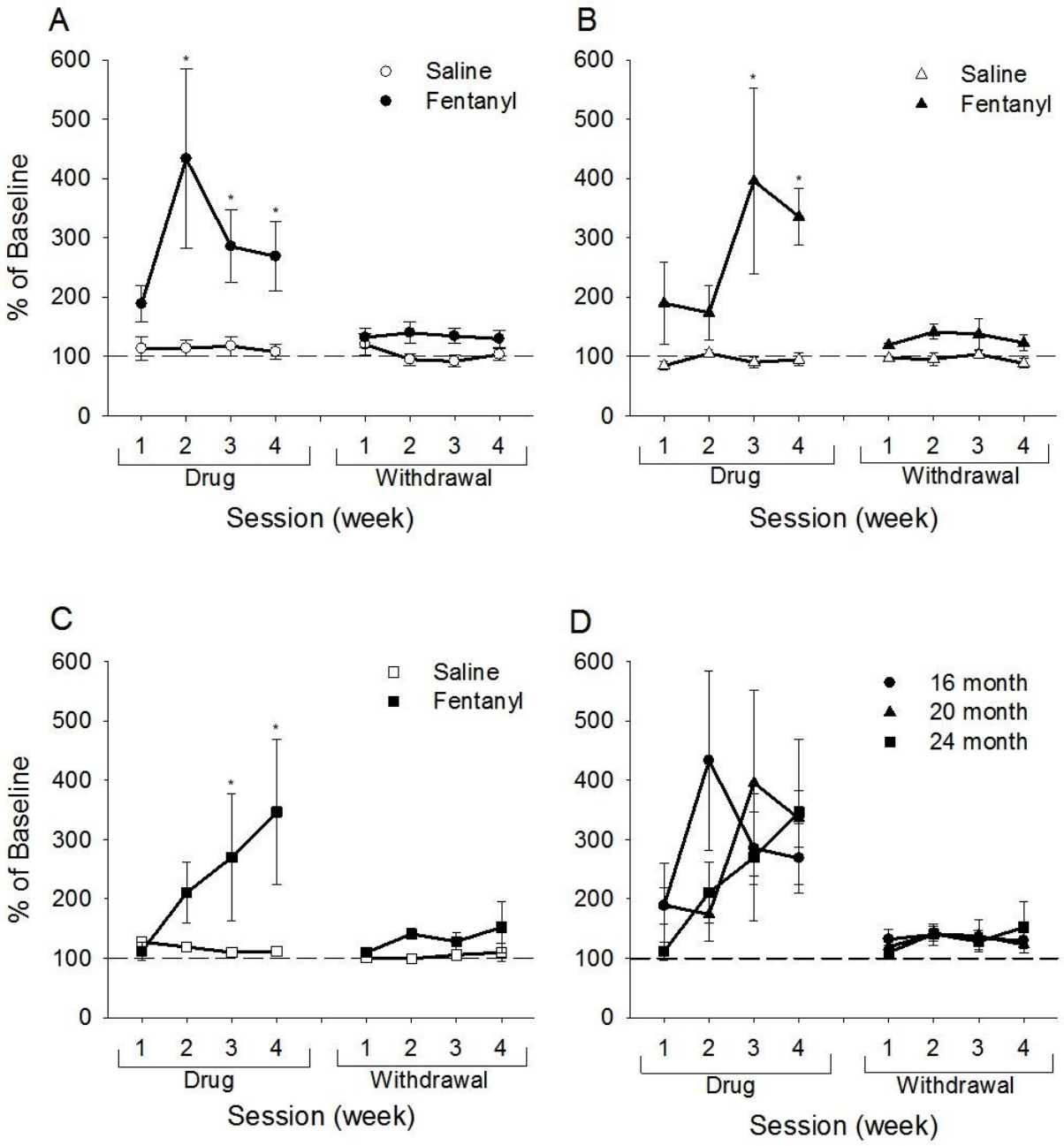

Figure 2-10. Change in locomotor activity during hot versus cold temperature comparison. A) 16-month old animals. B) 20-month old animals. C) 24-month old animals. D) Comparison of fentanyl administered animals across ages. Other details similar to those in Figure 2-4. Fentanyl administration increased locomotor activity in all ages, although this effect took longer to develop in older animals. Increases in locomotor activity did not persist into withdrawal sessions. *$p < 0.05$ compared to saline administered animals.

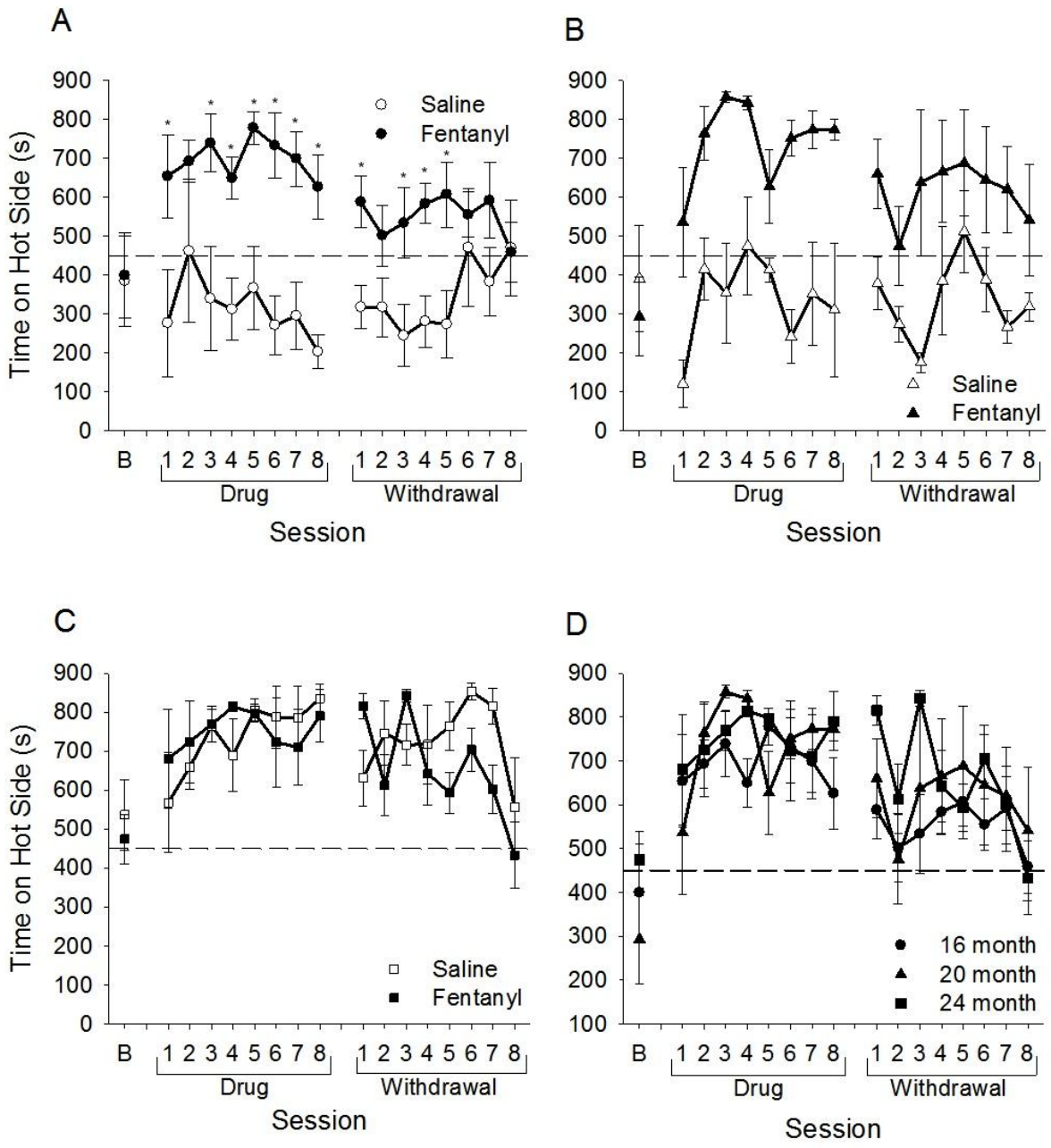

Figure 2-11. Thermal preference during drug administration and withdrawal. A) 16-month old animals. B) 20-month old animals. C) 24-month old animals. D) Comparison of fentanyl administered animals across ages. Other details similar to Figure 2-8. Fentanyl administration increased time spent on hot plate for all ages. This increase persisted through much of the withdrawal period. 24-month old saline-treated animals also increased time spent on hot plate during drug and withdrawal sessions. *$p < 0.05$ compared to saline administered animals.

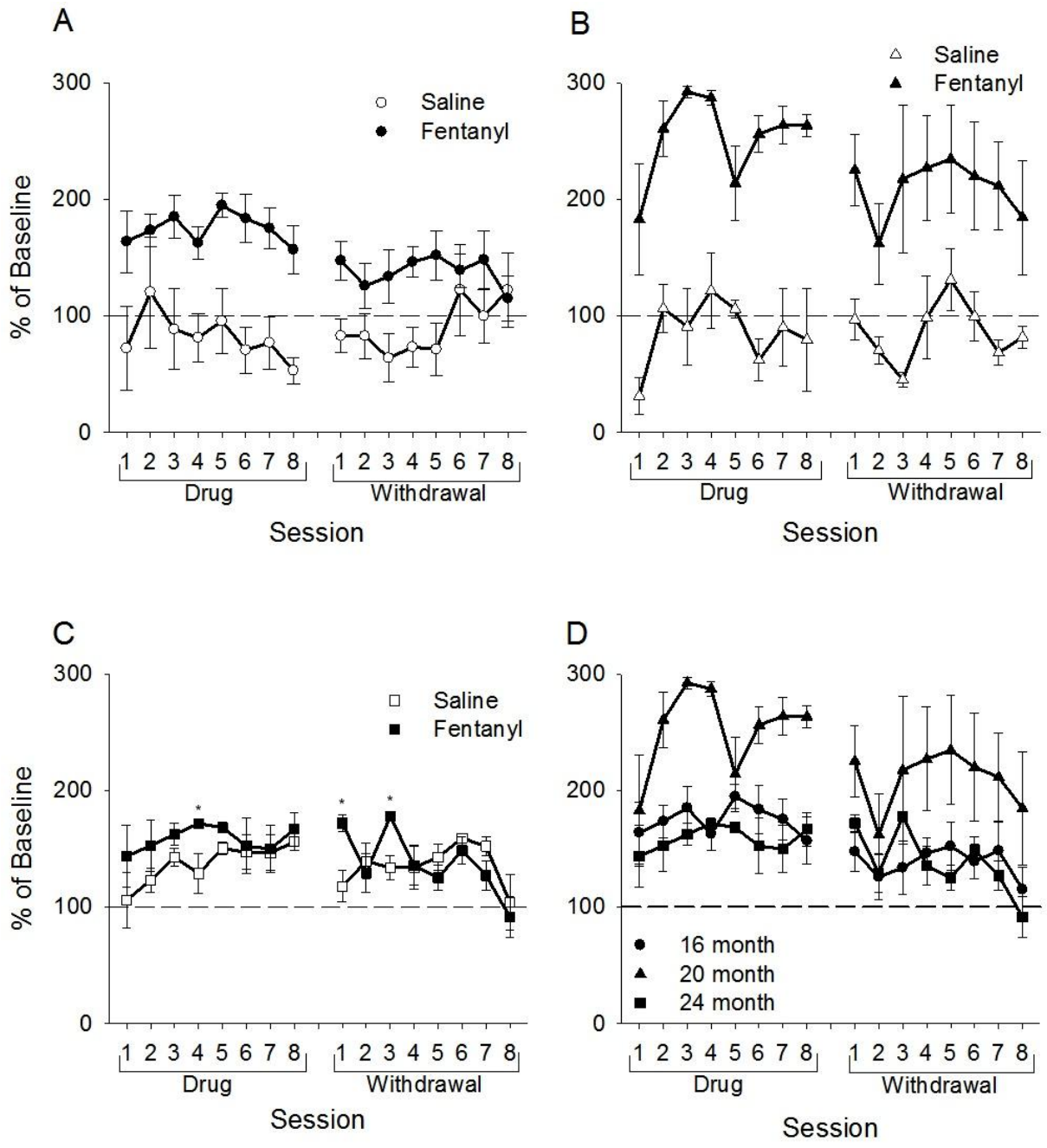

Figure 2-12. Changes in thermal preference during drug administration and withdrawal. A) 16-month old animals. B) 20-month old animals. C) 24-month old animals. D) Comparison of fentanyl administered animals across ages. Other details similar to Figure 2-6. Fentanyl administration increased time spent on hot plate for all ages, with the greatest increase occurring in 20-month old animals. *$p < 0.05$ compared to saline administered animals.

CHAPTER 3
DIFFERENTIAL AGE EFFECTS IN PHYSIOLOGICAL AND BEHAVIORAL MEASURES AFTER CHRONIC FENTANYL ADMINISTRATION

Based on the preceding study, the results suggested that older individuals may require greater doses of opioids to treat chronic pain[1]. Unfortunately, this pain is often undertreated in aged individuals (Auret and Schug, 2005; Bernabei et al., 1998; Chodosh et al., 2004; Gianni et al., 2010; McNeill et al., 2004). This problem is due in part to the fact that physicians are often reluctant to prescribe opioids to the elderly, given that the full spectrum of adverse side effects (e.g. nausea, constipation, and sedation) (Benyamin et al., 2008; Byas-Smith et al., 2005; Herndon et al., 2002; Swegle and Logemann, 2006) in this particular population is not known (Hutchinson et al., 2007; Lin et al., 2007b; Thomason et al., 1998; Wilder-Smith, 2005).

Preclinical studies have demonstrated that various behavioral and physiological measures in animals can function as correlates to human measures of declining physical function (Carter et al., 2002; Moser, 2000). These tests evaluate not only basic health metrics such as body weight and composition (fat mass versus muscle mass), but also physical performance measures related to muscle strength, agility, and overall activity. The purpose of the present study was to apply these evaluation tools to study the effects of chronic opioid administration and withdrawal on physical function in rats of various ages. Increased understanding of how age influences the effects of opioids can result in minimizing adverse outcomes, and consequently lead to more effective pain management in the elderly. While the previous study focused on ages that represent the

[1] The contents of this chapter have been previously published: Mitzelfelt, J. D., Dupree, J. P., Seo, D. O., Carter, C. S., Morgan, D., 2011. Effects of chronic fentanyl administration on physical performance of aged rats. Exp Gerontol 46, 65-72.

current age range of the “baby boomer” generation, this study assessed the effects of fentanyl across a greater range from adulthood through senescence to assess age-related changes throughout life, as opposed to only changes that occur in later life.

Materials and Methods

Animals, Treatment Conditions, and Experimental Design

Male F344 X BN rats, obtained from the National Institute of Aging colony at Harlan Industries (Indianapolis, IN) across four age groups (12, 18, 24, 30 months of age during baseline testing) were used in the present study. This range of ages represents adulthood, middle-age, pre-senescent and senescent portions of the lifespan. Animals were individually-housed in a temperature- and humidity-controlled colony room with a 12-hr light/dark cycle (lights on at 6 AM) with food and water available *ad libitum*. All surgery and testing was performed during the light cycle. Animals were cared for in accordance with the regulations of the IACUC and with the “Guide for the Care and Use of Laboratory Animals” (ILAR, 1996). In addition, animals were assessed on a weekly basis for signs of overt health problems as in the previous study.

The experimental timeline is shown in the Table 3-1. In brief, upon arrival in the colony, the animals were given 2 weeks to acclimate before baseline testing began for body composition, grip strength, and open field with 2-3 days between each test. Animals within each age group were then randomized to receive osmotic minipumps containing either fentanyl (n = 32) or saline (n = 36). After four weeks of drug administration pumps were removed. In the end, 27 fentanyl- and 35 saline-treated animals completed the entire study (12 month: 5 fentanyl, 9 saline; 18 month: 9 fentanyl, 9 saline; 24 month: 7 fentanyl, 9 saline; 30 month 6 fentanyl, 8 saline). The

other six animals did not complete the experiment either due to adverse interactions between fentanyl and isoflurane or loss of pump during the experiment. Of primary interest were the behavioral and physiological effects of fentanyl at approximately one week and one month of chronic fentanyl administration. These time points were chosen to be analogous to either short-term treatment regimens related to surgery or outpatient clinical situations, or longer-term disease conditions associated with chronic pain. For ease of description, these time points are referred to as “early” and “late” periods of chronic drug administration or withdrawal. Behavioral tests were conducted on different days, and the order of animal testing was counterbalanced across ages. In general, the duration of the testing was less than 3 hours, and was conducted in the middle of the light/inactive phase.

Surgery and Drug Delivery

Osmotic minipumps (Model # 2ML4, Alzet, Durect Corp., Cupertino, CA) containing fentanyl or saline were implanted subcutaneously in the right hindquarter of animals while maintained on isoflurane anesthesia (1.5% at 1.0 L/min O_2). Minipumps delivered fluid at a rate of 2.58 µl/hr for 28 days. Fentanyl was delivered at a dose of 1 mg/kg/day. Four weeks after pump implantation, animals were anesthetized and the pumps removed.

Behavioral and Physiological Testing

Food consumption

Twenty-four hour food consumption data were collected at 7 and 28 days after pump-implantation.

Body weight and composition

Body weight was measured weekly for all animals. Determination of body composition was assessed by time domain-nuclear magnetic resonance (TD-NMR) using a Minispec analyzer (Bruker Optics, The Woodlands, TX). TD-NMR testing allows for rapid (approximately 1 min) assessment of body composition in awake, restrained animals. Absolute values for fat, lean, and fluid mass were recorded. At each time point, each animal was tested twice and the average of those results is reported.

Open-field activity

Animals were placed into Plexiglas open-field testing chambers (690 cm x 555 cm) for 5 min and movement was tracked using an overhead camera and computer software (EthoVision, Noldus Information Technology, Wageningen, Netherlands). General activity levels were determined by assessing the total distance traveled. The amount of time spent along the margin of the open field as opposed to the center was taken as a measure of anxiety. The margin was defined as a 3-cm wide strip around the outside of the box.

Grip strength

Forelimb grip strength was measured using a Chatillon force gauge (Ametek, Largo, FL). Animals were placed so their forepaws were on a wire grid connected to the force gauge. Animals were then pulled away from the wire grid, while the force meter recorded the maximum force exerted on the wire grid. Animals were given three consecutive trials, and the maximum force was taken as a measure of grip strength.

Rotarod

Agility and balance were tested using a Rotamex® rotarod device (Columbus Instruments, Columbus, OH). Animals were given two days of training, in which they

were placed on the 3.5-inch rotarod turning at a rate of 4 rpm. Animals falling in less than 30 s were placed immediately back on the drum for another trial with a maximum of three trials and a time limit of 60 s. Two days after the second training session, baseline performance was assessed. Animals falling prior to 60 s were immediately given a second trial, and trials were a maximum of 300 s in duration.

Statistics

For baseline assessments, one-way ANOVA with age as a factor was used. In cases of unequal variance and non-normal data, a Kruskal-Wallis one-way analysis of variance on ranks was performed. For drug administration and withdrawal data, primary statistical analysis consisted of separate two-way repeated measures ANOVA comparing age and treatment phase within each drug group. Subsequent statistical analyses consisted of two-way ANOVA comparing age and drug group for each phase of testing (i.e. early drug, late drug, early withdrawal, and late withdrawal). Student-Newman-Keuls post-hoc tests were performed where appropriate. Differences were considered statistically significant when *p*-values were less than 0.05. All statistical tests were performed using SigmaStat version 3.11 (Systat Software, Inc, San Jose, CA).

Results

Baseline Characteristics

Age differences in physiology and physical performance prior to drug administration are shown in Figure 3-1. Food consumption at baseline differed across ages (H = 18.11, 3 d.f., p <0.001) with 12-month old animals consuming less than 24- and 30-month old animals. There were also significant age-related differences in body weight ($F_{3, 77}$ = 27.94, p <0.001), such that 12-month old animals weighed less and the 24-month old rats weighed more than all other ages. Regarding body composition, 12-

month old animals had significantly less fat mass than all other ages ($F_{3, 76}$ = 5.64, p = 0.002) and significantly more lean mass than 24- and 30-month old animals (H = 10.87, 3 d.f., p = 0.012). During the initial assessment of physical performance measures, 12-month old animals had greater general activity than all others ($F_{3, 75}$ = 3.04, p = 0.034), but there were no age differences in time spent in the margin of the activity chamber (a commonly used measure of anxiety), grip strength, or rotarod time.

Food Consumption

Food consumption was altered in fentanyl-treated animals (Figure 3-2; $F_{4, 93}$ = 85.01, p = <0.001). During early chronic drug administration, food consumption was significantly decreased relative to baseline, and tolerance appeared to develop to this effect as food consumption returned to baseline levels over the 4 weeks of drug administration. During both early and late withdrawal periods, food consumption was above baseline in all age groups. This change in food consumption was also seen when the fentanyl-treated rats were directly compared to saline-treated rats during early drug and withdrawal, and following four weeks of withdrawal.

Body Weight

Figure 3-3 shows a significant interaction of age and phase for the fentanyl-treated animals ($F_{12, 102}$ = 4.14, p <0.001). Fentanyl administration decreased body weights significantly during early and late drug administration and during early withdrawal, and this effect was greatest in the older animals. During late withdrawal, body weight for 12-month old animals had returned to baseline, but other ages had not fully recovered, with the body weight for 30-month old animals being significantly less than 12-month old animals. Across all phases, direct comparison of fentanyl versus saline treatment revealed lower body weights in fentanyl-treated animals.

Body Composition

Using TD-NMR decreases in both fat mass and lean mass during drug administration and withdrawal were observed (Figure 3-4). Similar to body weight, there was an interaction of age and phase for both fat and lean mass in fentanyl-treated animals (fat: $F_{12, 104} = 2.18$, $p = 0.018$; lean: $F_{12, 104} = 2.50$, $p = 0.006$). Fentanyl-treated animals lost both fat and lean mass during early and late drug administration as well as during early withdrawal. During late withdrawal, fat and lean mass had returned to baseline levels for 12-month old animals, but older animals were slower to recover, with 30-month old animals showing significantly lower fat and lean mass levels relative to 12-month old animals. During all phases, fentanyl-treated animals had significantly lower levels of fat and lean mass than saline-treated animals. In a direct comparison of tissue type (fat versus lean) and across ages, fentanyl had a greater effect on fat mass than lean mass (all p values < 0.05).

General Activity

In saline-treated animals, there was a habituation-like process with a decrease in activity across phases (Figure 3-5A; $F_{3, 94} = 23.81$, $p = <0.001$). In fentanyl-treated animals, activity during the late withdrawal period was significantly decreased relative to baseline ($F_{3, 81} = 8.88$, $p = <0.001$). Comparison of fentanyl versus saline showed that saline-treated animals had significantly lower levels of activity during early drug, late drug, and late withdrawal phases. The amount of time spent along the edges of the open field device is often taken as a measure of anxiety. There were little differences in this measure across ages or phases with the exception of a long-lasting increase (i.e. during late withdrawal) in the fentanyl-treated animals (Figure 3-5B; $F_{1, 54} = 4.46$, $p = 0.039$).

Grip Strength

During acute drug administration (Figure 3-6A), age had a significant impact on maximum grip strength similar to previous studies (Carter et al., 2004; Forster and Lal, 1999) with grip strength decreasing with age ($F_{3, 59} = 4.19$, $p = 0.009$). Fentanyl administration decreased grip strength across ages, but this effect was resolved during late withdrawal (early drug: $F_{1, 59} = 5.28$, $p = 0.025$; late drug: $F_{1, 56} = 7.907$, $p = 0.007$).

Rotarod

During all treatment phases (Figure 3-6B), there was a prominent age effect with older animals having impaired rotarod performance relative to younger animals (early drug: $F_{3, 55} = 5.20$, $p = 0.003$; late drug: $F_{3, 52} = 9.11$, $p = <0.001$; late withdrawal: $F_{3, 50} = 7.11$, $p = <0.001$). However, there was no effect of fentanyl administration on rotarod performance during any phase.

Discussion

There is growing concern over the increasing use of opioids to treat chronic pain in the elderly primarily because of the potential increased sensitivity to the adverse side effects (Benyamin et al., 2008; Hutchinson et al., 2007; Wilder-Smith, 2005). Unfortunately, an increased risk to these effects is not well documented (Bernabei et al., 1998; Fine, 2004; Pergolizzi et al., 2008). The purpose of this study was to assess differential effects of chronic fentanyl administration across ages on various measures of physiology and behavior. Overall, the most profound effects of fentanyl administration were on food consumption, body weight, and body composition. Fentanyl administration resulted in decreases in food consumption and a long-term decrease in body weight, primarily due to decreases in fat mass. Even after a month of withdrawal from fentanyl administration, only the youngest rats had returned to baseline body weight. Given the

literature demonstrating the difficulty of regaining unexpected or unintentional body weight loss and the detrimental functional consequences of such loss in older persons (e.g. (Lee et al., 2005; Locher et al., 2007; Miller and Wolfe, 2008; Ritchie et al., 2008), the current findings suggest that body weight should be monitored not only during chronic drug administration but for extended periods following cessation of drug treatment.

Physiological consequences of chronic opioid administration

There is a large literature documenting the effects of opioids on food consumption (e.g. (Bodnar, 2004), and in general it is known that acute administration of opioid agonists increases food consumption in rats (Sanger and McCarthy, 1981) while opioid antagonists decrease food consumption (Glass et al., 1999; McLaughlin and Baile, 1983; Yuan et al., 2009). However, studies looking at chronic administration of morphine show decreases in food intake and body weight (Binsack et al., 2006; Levine et al., 1988; Li et al., 2010), while chronic opioid antagonist administration increases food consumption and weight gain (Chen et al., 2004). As hypothesized, chronic fentanyl administration resulted in a decreased food consumption within the first week, and animals became tolerant to this effect as food consumption had returned to baseline after 4 weeks of fentanyl administration. During withdrawal, fentanyl-treated animals showed an increase in food consumption. Clinically, these data suggest that at the beginning of chronic opioid administration, it is important to make sure that patients are receiving adequate food intake even though these effects are not persistent through the duration of opioid administration or withdrawal.

One predictable consequence of decreases in food consumption is a change in body weight. Of importance is the fact that while obesity leads to major health risks in

younger populations, studies have shown that in individuals 60 years old and older, being underweight has a greater risk of mortality and disability than being overweight or even obese (Flegal et al., 2005; Marcell, 2003; Miller and Wolfe, 2008; Paddon-Jones et al., 2008). In the current study, fentanyl administration resulted in a decrease in body weight for all ages throughout the 28 days of drug administration. Using TD-NMR, body composition changes were followed through drug administration and withdrawal, and the overall pattern of decreases in fat and lean (muscle) mass followed a similar course as the changes in body weight. Although food consumption returned to baseline levels by the end of drug administration and increased through 1week of withdrawal, body weight (and both fat and lean mass components) remained decreased through withdrawal. In general, the effect of fentanyl administration on fat mass relative to lean mass was of a greater magnitude and longer lasting. The fact that only the youngest animals fully regain their body weight at the end of withdrawal even though there are no age differences in food consumption suggests that the decrease in food consumption is not entirely responsible for the decrease in body weight. However, although older individuals may take longer to regain body weight after ending chronic fentanyl administration, lean mass is recovered more quickly than fat mass, which is an important factor in the health of aged individuals (Marcell, 2003).

Behavioral consequences of chronic opioid administration

Given the increasing use of fentanyl, the high prevalence of chronic pain, and the aging of the population, it is critical to identify some of the functional consequences of chronic fentanyl administration across a range of ages. This was especially important as we identified profound changes in body weight and composition that were longer-lasting

in the oldest animals and could lead to decreased strength and functional independence, and eventually increased frailty, disability, and mortality.

Similar to previous studies, there were age-related differences in overall activity levels assessed in an open field at baseline, and during the initial drug administration phase declines were observed in rotarod and grip strength (Carter et al., 2004; Forster and Lal, 1999). Fentanyl appeared to have effects on both activity levels (increases) and grip strength (decreases) at the late drug time point, and these effects were not maintained into the late withdrawal phase. These findings are consistent with the literature showing that repeated administration of mu opioid agonists (including fentanyl and morphine) can result in long-lasting increases in locomotor activity levels in rats (e.g. Khallouk-Bousselmame and Costentin, 1994; Powell and Holtzman, 2001; Rauhala et al., 1995; Trujillo et al., 2004). Clinically, extended fentanyl administration increases activity, although it is unclear if this is a direct effect of fentanyl or a by-product of mitigation of chronic pain in these patients (Agarwal et al., 2007). Age-related decreases in rotarod performance were maintained throughout all phases of drug/saline administration, however fentanyl did not influence performance. These findings suggest that while chronic fentanyl administration has lasting effects on physiological measures, this does not necessarily translate into decrements observed in these behavioral measures. Importantly, these data provide information suggesting that strong opioids such as fentanyl do not necessarily result in more dramatic and potentially dangerous adverse behavioral side effects with increases in age.

Implications

The relative age of the population is increasing, there is a high prevalence of age-related chronic pain in this population, and opioids are being used more commonly for a

greater number of clinical conditions. The potential adverse physiological and behavioral outcomes of chronic opioid administration in aged populations are relatively unknown. The present study characterized some differential consequences of chronic fentanyl administration to rats of various ages. Chronic opioid administration had effects on both physiological measures (body weight, body composition, food consumption) and behavioral tests (grip strength and open field). Lasting effects were only observed in these physiological measures. Fentanyl administration decreased body weight (both fat mass and lean, muscle mass) across all ages, but older animals took longer to recover body weight following cessation of fentanyl administration. This slow recovery in body weight occurs even in the face of apparent tolerance to the anorectic effects as food consumption levels returned to then surpassed baseline levels of intake. On the positive side, these dramatic changes in body weight and consumption did not necessarily translate into long lasting functional deficits assessed using a number of behavioral measures. Taken together, these data suggest that chronic opioid administration to aged individuals can have dramatic and long-lasting effects that should be routinely assessed even following prolonged periods of withdrawal from the drug.

Table 3-1. Timeline of experimental events

Phase	Day	Experimental event
Baseline	0	Arrival in lab
	14-42	Open field, grip strength, TD-NMR, Rotorod, food consumption, and body weight measures
Drug / saline administration	0	Implant pump
	7	Body weight, food consumption, Rotorod
	8	TD-NMR
	10	Open field, grip strength, TD-NMR, Rotorod, food consumption, and body weight measures
	11	Grip strength
	21	Rotarod
	22	TD-NMR
	24	Open field
	25	Grip strength
	28	Food consumption, body weight
Withdrawal	0	Remove pump
	6	Body weight
	7	Food consumption, TD-NMR
	21	Rotarod
	22	TD-NMR
	24	Open field
	25	Grip strength
	28	Food consumption, body weight

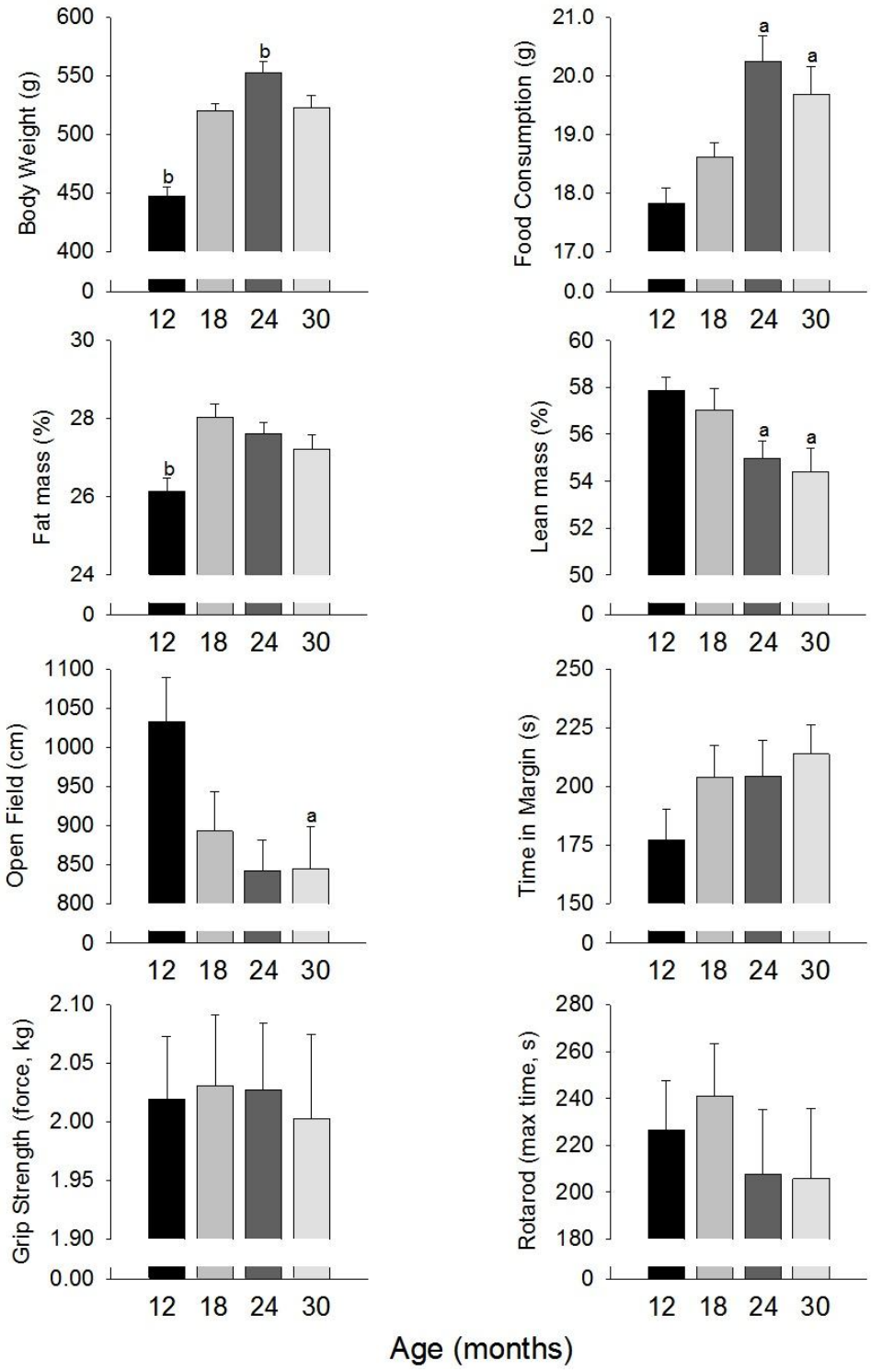

Figure 3-1. Baseline measures. Assessments made before implantation of osmotic pumps across the four age groups. Data are presented as means ± SEM. [a]$p < 0.05$ compared with 12-month old animals, [b]$p < 0.05$ compared with all other ages.

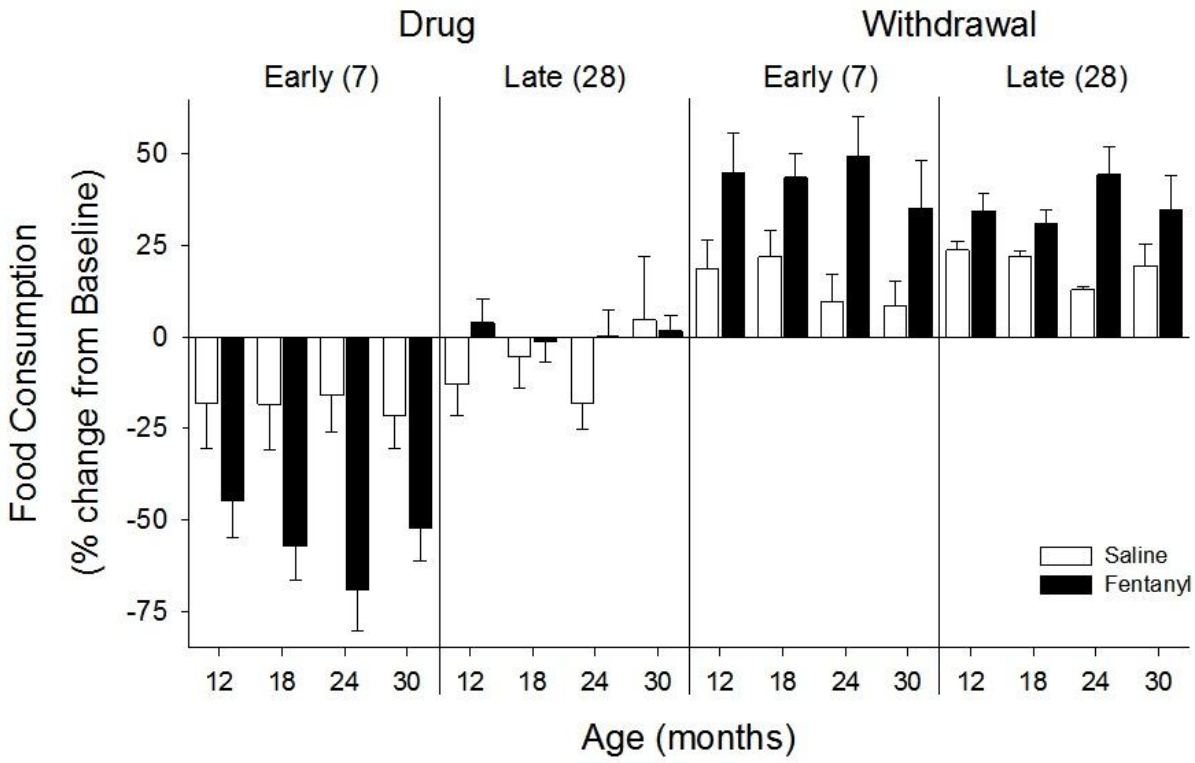

Figure 3-2. Food consumption. Changes in food consumption during fentanyl/saline administration ("Drug" and "Withdrawal"). Each phase is divided into an "Early" and "Late" stage, and the number in parentheses represents the number of days following the mini-pump implantation or removal that the measure was taken. Data are presented as percent change from baseline (mean ± SEM) for each age group. Note that fentanyl decreased food consumption across ages during early assessments, and increased food consumption throughout withdrawal.

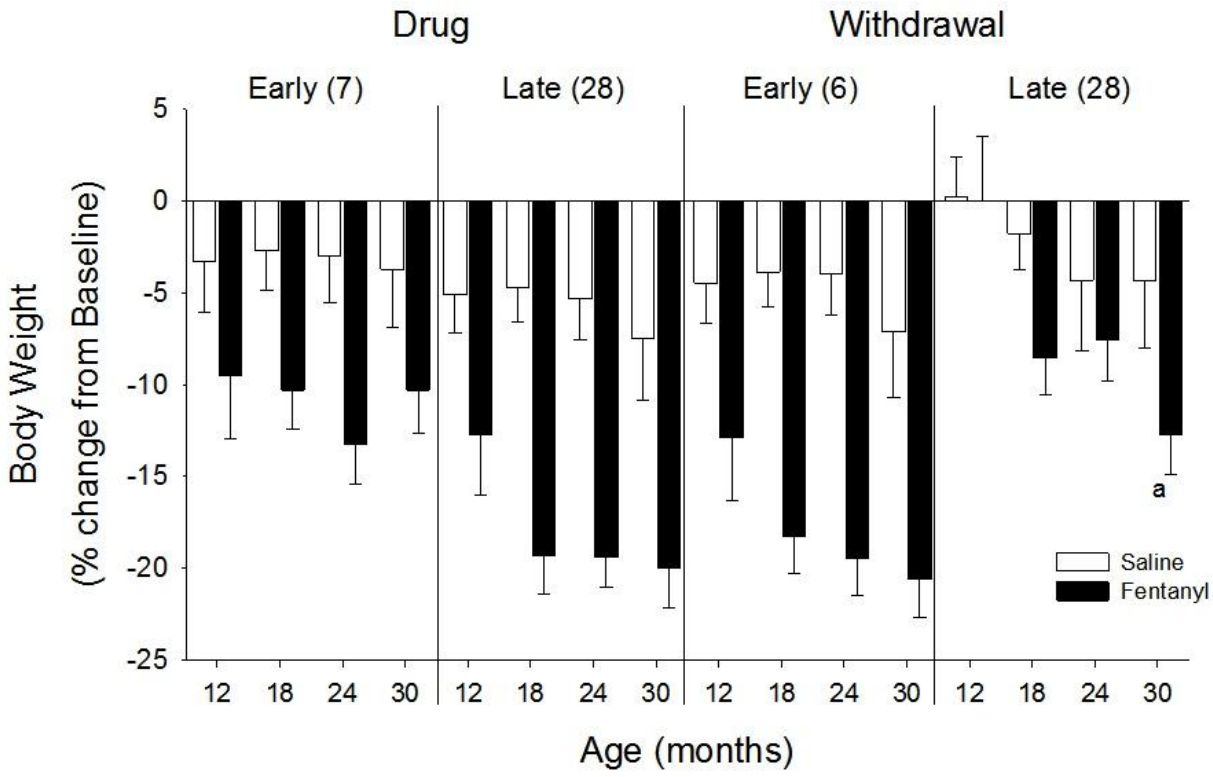

Figure 3-3. Body weight. Changes in body weight during fentanyl/saline administration and withdrawal. Other details as in Figure 3-2. Note that fentanyl resulted in decreased body weight across ages during the Early and Late drug administration, and during Early withdrawal. By 28 days of withdrawal, the youngest animals returned to baseline levels. [a]$p < 0.05$ compared with 12-month old animals.

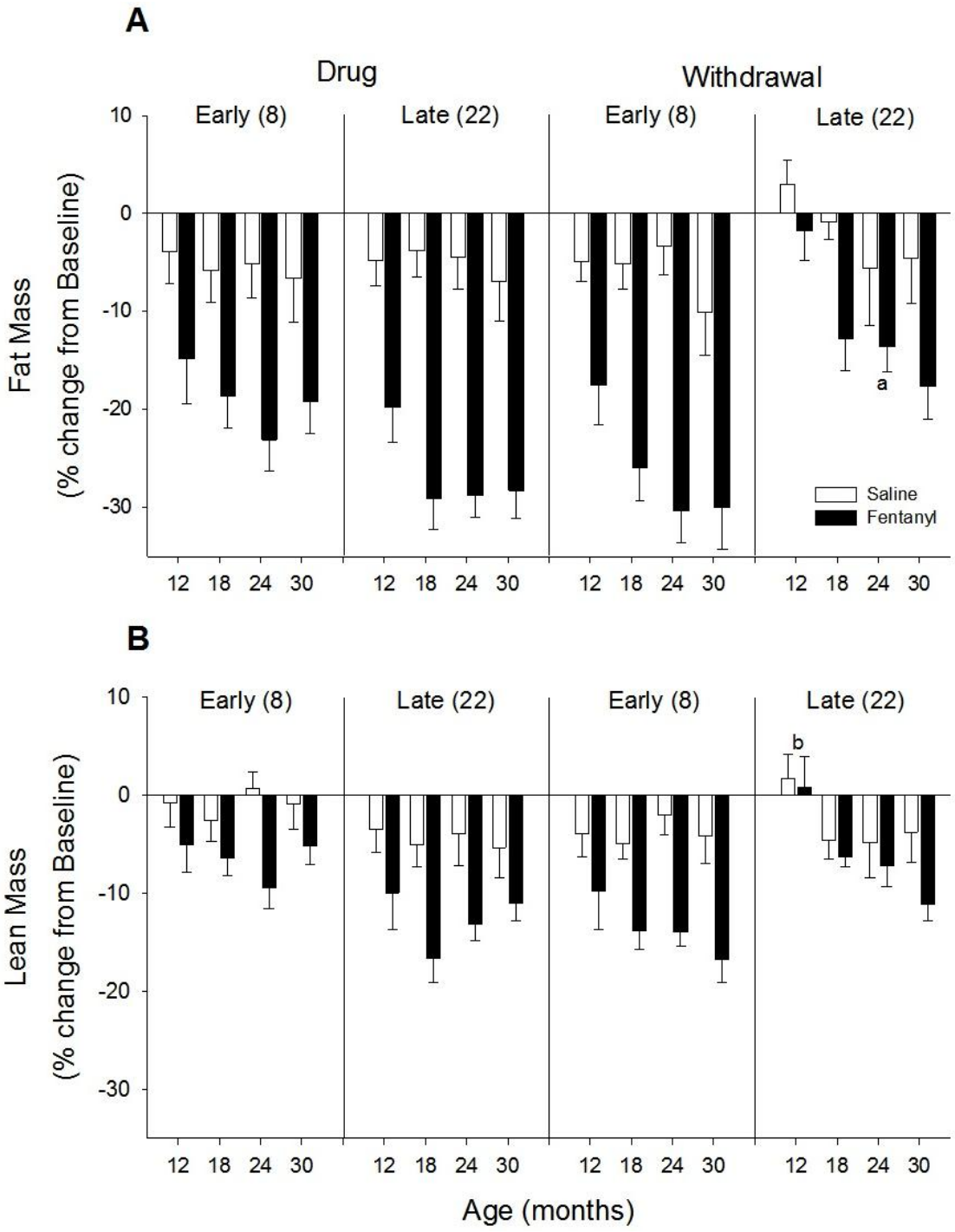

Figure 3-4. Body composition. A) Fat mass. B) Lean/muscle mass. Changes during fentanyl/saline administration and withdrawal. Other details as in Figure 3-2. Note that fentanyl resulted in decreased fat and muscle mass across ages during Early and Late drug administration, and during Early withdrawal. By 22 days of withdrawal, the youngest animals returned to baseline levels. [a]$p < 0.05$ compared with 12-month old animals, [b]$p < 0.05$ compared with all other ages.

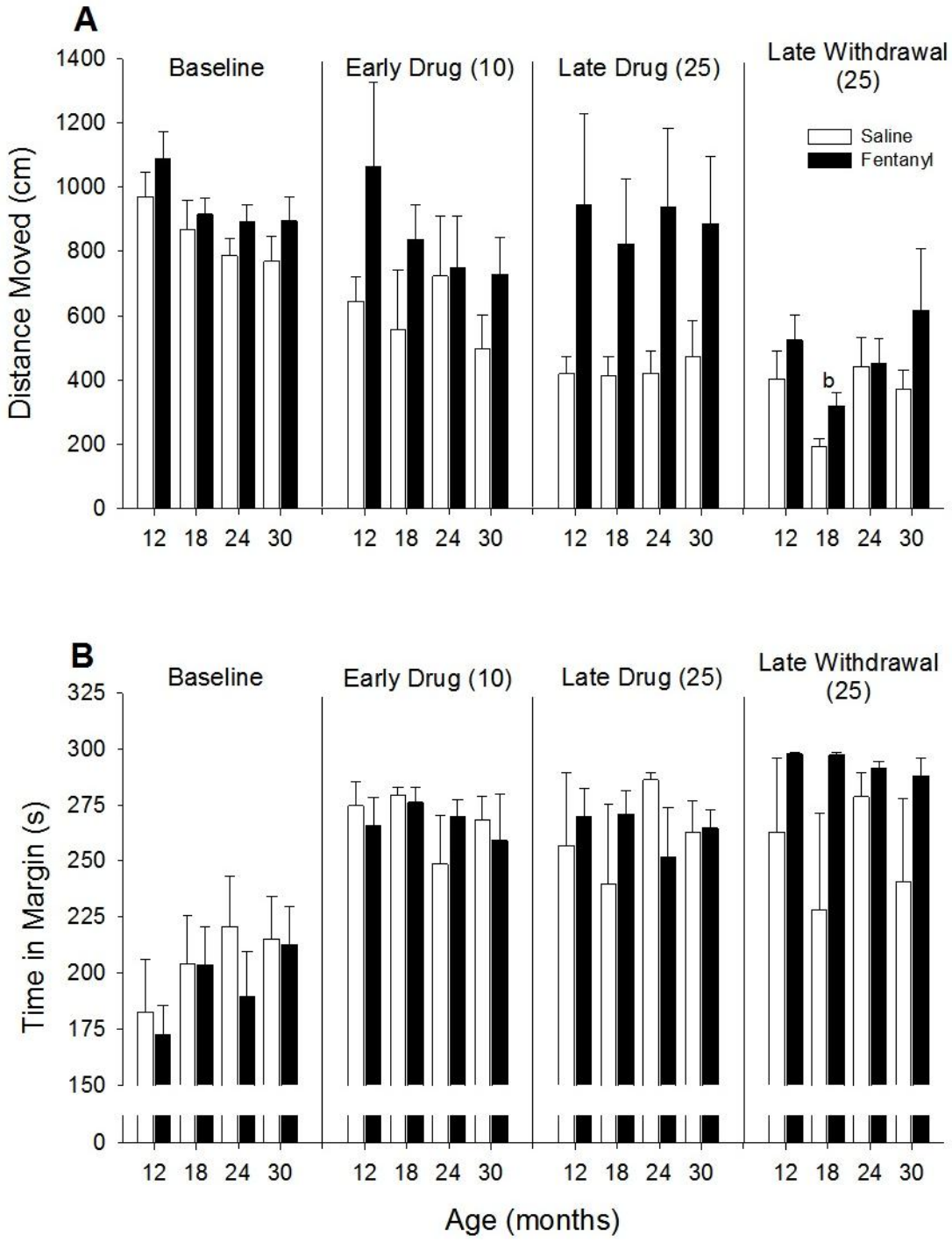

Figure 3-5. Open field activity. A) Total distance traveled. B) Time spent in the margin. Other details as in Figure 3-2. Note that saline-treated animals showed decreased activity over repeated testing (i.e. habituation), whereas fentanyl resulted in heightened levels of activity throughout drug administration. Following 24 days of drug withdrawal, fentanyl-treated animals spend essentially the entire session in the margin, a finding consistent with increased levels of anxiety. $^{b}p < 0.05$ compared with all other ages.

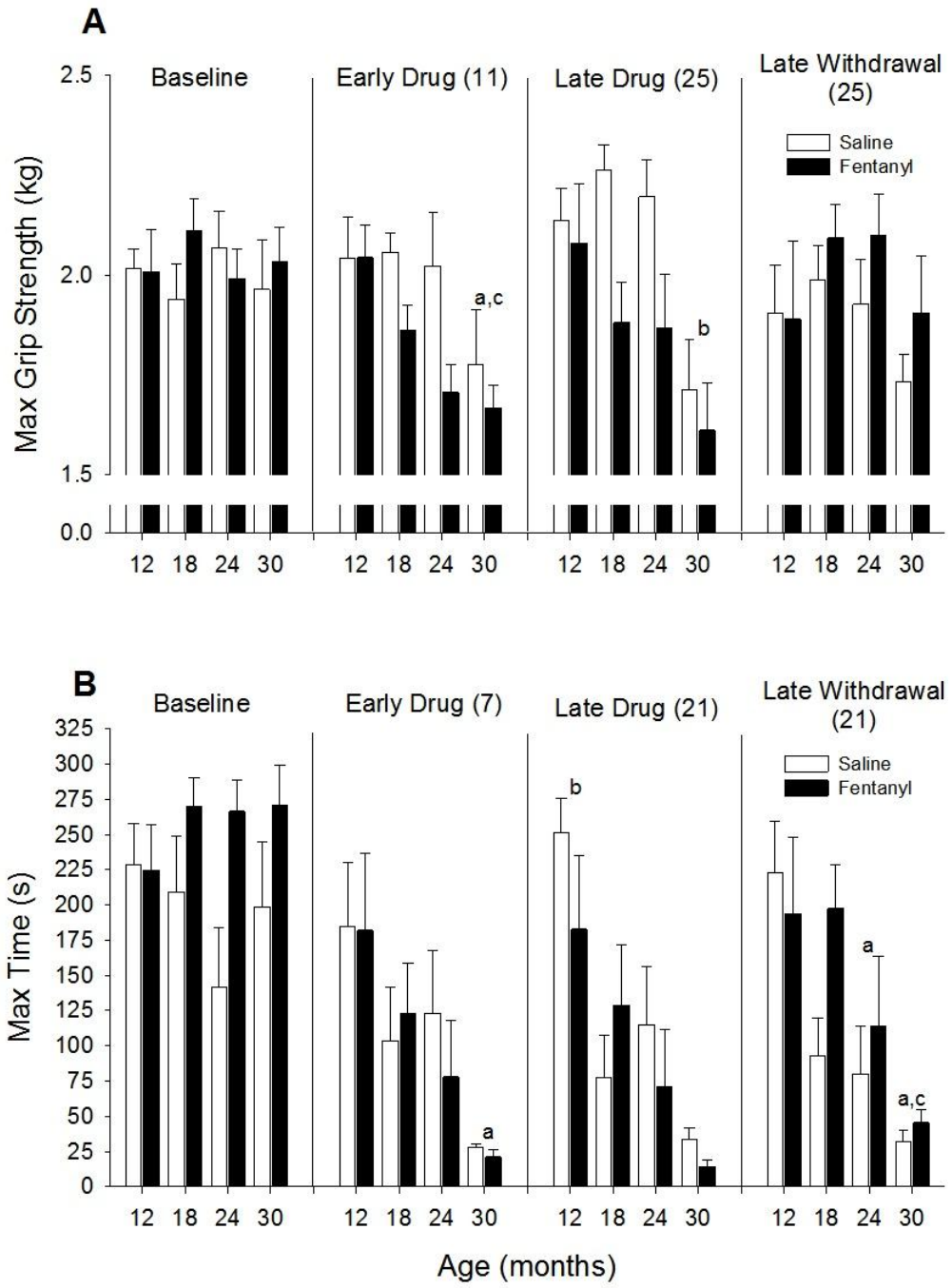

Figure 3-6. Physical performance measures. A) Maximum grip strength B) Time spent on rotarod. Other details as in Figure 3-2. Note decreases in grip strength during fentanyl administration although this decrease does not persist after 25 days of withdrawal. [a]$p < 0.05$ compared with 12-month old animals, [b]$p < 0.05$ compared with all other ages, [c]$p < 0.05$ compared with 18-month old animals. For rotarod performance, there were decreases in time with increasing age regardless of drug treatment. [a]$p < 0.05$ compared with 12-month old animals, [b]$p < 0.05$ compared with all other ages, [c]$p < 0.05$ compared with 18-month old animals.

CHAPTER 4
AGE DIFFERENCES IN MECHANICAL AND THERMAL SENSITIVITY AFTER REPEATED INTRAMUSCULAR ACIDIC SALINE INJECTIONS

While the previous two experiments focused on the relationship between aging and opioids, it is important to note that the animals were not in a chronic pain state. In order to understand how aging, opioids, and chronic pain all interact, we must first better understand the effects of aging on the development and intensity of chronic pain.

Although there are numerous animal models for chronic pain (for review see (Blackburn-Munro, 2004) which have been useful for testing new treatments and understanding the mechanisms involved in various types of chronic pain, most do not allow for the study of the transition from acute to chronic pain. Being able to isolate this transition experimentally can allow for the identification of new treatment targets that can prevent further development of chronic pain. Inflammatory models inject inflammatory agents directly into the animal, instantly creating a persistent pain state, and similar conditions exist with the neuropathic pain models where nerves are damaged inducing a chronic pain state.

The recently developed repeated intramuscular injection of acidic saline model may be uniquely suited to fill this role (Sluka et al., 2001). In this model, acidified saline is injected into the same gastrocnemius muscle (i.e. unilateral injections) 2-5 days apart. After the second injection, animals show bilateral mechanical hypersensitivity, but little change in thermal sensitivity assessed with standard reflexive measurements for up to 4 weeks. Interestingly, after the first injection, animals show a temporary change in mechanical sensitivity. This lack of persistent pain after one injection allows for the comparison of animals that have induced acute and chronic pain, which may serve useful for understanding the transition from acute to chronic pain. This model for chronic

pain was also selected because it did not require an invasive surgery, which can produce complications in older animals. Lastly, this model fails to affect locomotor activity (Bement and Sluka, 2005) which is necessary for relevant to behavior assessed in the thermal preference procedure.

Initial studies using the repeated acidic saline injection model have shown the development of sensitization to mechanical stimulation, but not thermal stimulation (Sluka et al., 2001). However, this study used a reflexive measure for the thermal stimulation, which may have not activated the C-fiber neurons that are involved in chronic pain transmission. Within the PNS, there are ion channels that respond to thermal stimulation. These channels are members of the transient receptor potential (TRP) superfamily of cation channels that are found throughout the body. Within the somatosensory neurons, there are TRP channels that respond to selective ranges of temperatures from cold (TRPA1: < 17°C), to extreme heat (TRPV2: > 52°C) (Venkatachalam and Montell, 2007). These TRP channels allow calcium to enter the neurons, which could lead to changes in other receptor levels, and thus sensitization. Therefore, these TRP channels may be involved in the generation of chronic pain due to their activation by a range of temperatures and other stimuli, which could lead to sensitization of C-fibers. However, as previous studies looked at thermal sensitivity in response to extreme thermal stimulation, they may have not activated TRP channels that respond to non-noxious temperatures, but are involved in sensitization.

Therefore, this study investigated age-related differences in the development and magnitude of chronic pain using a repeated intramuscular acidic saline injection model of chronic pain (Sluka et al., 2001). Sensitization to mechanical stimulation was

assessed as a replication of previous studies using the acidic saline injections, as well as to assess any age differences in the development of mechanical sensitivity. Sensitivity to thermal stimulation was tested using the thermal preference procedure. The thermal preference procedure was used because it allows for the assessment of changes in sensitivity to non-noxious temperatures as well as involving higher cortical processing that may be involved in chronic pain states. Changes in expression of mRNA levels for thermo-sensitive TRP channels in the DRG of the hind limbs were also assessed as a potential mechanism for any changes in thermal sensitivity. It is hypothesized that older animals will be less sensitive to acidic saline injections due to decreased neuroplasticity (Wang et al., 2005). It is hypothesized that an increase in temperature specific TRP channels will be correlated with the development of thermal hyperalgesia and/or allodynia in acidic saline injected animals. Consistent with behavioral results, younger animals will have greater increase of TRP channels compared to older animals.

Materials and Methods

Animals, Treatment Conditions, and Experimental Design

For this study, female F344 x BN rats were obtained from the National Institute of Aging colony at Harlan Industries (Indianapolis, IN). Rats were either 8- (n = 12) or 28-months old (n = 10) at time of first saline injection (see below). Animals were housed under the same conditions as previous studies, with all experiments performed during the light cycle.

After arrival in the colony, animals were allowed to acclimate for 2 weeks before testing began. Animals were trained on the thermal preference procedure 5 days a week for 4 weeks on the temperatures described below. After training, baseline thermal

preference data was collected with two trials at each temperature condition. The next day, baseline data for mechanical withdrawal thresholds were collected. The following day, animals were injected with either acidic (pH = 4.0 ± 0.1, n = 12) or neutral (pH = 7.0 ± 0.1, n = 10) saline and mechanical withdrawal and thermal preference data were collected 4, 24, and 48 hours after injection. The next day a second saline injection was given. Mechanical withdrawal and thermal preference were tested 4 hours later, and then 5-7 days a week for the next month. After the end of pain testing, animals were sacrificed and the dorsal root ganglions (DRGs) for the hind limbs (L4-S1) were extracted for quantitative real-time polymerase chain reaction (qRT-PCR) experiments.

Acidic Saline Injections

Induction of a chronic pain state was achieved by using the repeated intramuscular injection of acidic saline initially described in Sluka et al. (2001). Briefly, physiological saline was pH adjusted using hydrochloric acid (HCl) or sodium hydroxide (NaOH) to produce saline that was either acidic (pH = 4.0 ± 0.1) or neutral (pH = 7.0 ± 0.1). Either acidic or neutral saline (100 µl) was injected into the left gastrocnemius muscle of each animal while maintained on isoflurane anesthesia (1.5% at 1.0 L/ min O_2). Two acidic saline injections administered 2-5 days apart to the same muscle are sufficient to induce chronic mechanical hyperalgesia in rats (Sluka et al., 2001). For this study, the injections were administered three days apart so that each thermal preference condition could be tested after the first injection.

Pain Assessment

Thermal preference

Thermal preference testing was performed using the same equipment as in Chapter 2, and testing procedures were similar; trials were 15 min in length although the temperature conditions tested were slightly different. The three temperature conditions tested were: sensitivity to hot, sensitivity to cold, and hot versus cold, with the temperatures of the floors were 30° and 45°C, 30° and 15°C, and 15° and 45°C respectively for each condition.

Mechanical withdrawal

Mechanical withdrawal testing was performed in plastic cages (13 x 8 x 11 inches) with a floor of wire mesh (0.25-inch openings). The cage was placed on a metal wire rack allowing access to the plantar surface of the hind paw while the rat is unrestrained and resting comfortably. Each day the rats were given a few minutes to acclimate to the chamber prior to testing.

After acclimation, von Frey filaments (EB Instruments, Pinelas Park, FL) were applied to the plantar surface of each hind paw. The 50% withdrawal threshold, i.e. the amount of force that would result in paw withdrawal 50% of the time, was determined using the up-down method adopted from Chaplan et al. (1994). Testing started with a sub-threshold filament, which was applied to the plantar surface of the hind paw until it bent. If the animal did not withdrawal its paw within ~ one second of the filament bending, the test was scored as a “miss” and the next largest filament was tested. Filament size was increased until the animal withdrew its paw, which was scored as a “hit.” After this test, the filament size was decreased. Four more tests were performed after the first “hit” on that paw for that day, with filament size being decreased or

increased depending on if the animal withdrew its paw or not. The pattern of “hits” and “misses” were recorded for the last six tests, and used determine the 50% withdrawal threshold (Chaplan et al., 1994). Testing was performed on both hind paws, ipsilateral and contralateral to the site of injection. On days where both mechanical withdrawal and thermal preference testing was performed, animals were returned to their home cage for 15 min after mechanical withdrawal testing prior to the start of thermal preference testing.

Thermo-Sensitive Transient Receptor Potential Channel Expression Quantification

Dorsal root ganglion extraction

At the end of the pain assessment study, animals were deeply anesthetized with isoflorane (4% at 2.0 L/min O_2), then rapidly decapitated. The spine was exposed by making an incision along the top of the spine from the base of the tail to the top of the rib cage. Muscle was disconnected from the spine by make incisions alongside the spine from the base of the tail to the base of the rib cage. The spinal cord was the exposed by removing the dorsal portion of the vertebra using rongeurs. The spinal cord was then carefully removed leaving the dorsal root ganglion on the ventral portion of the spine. The ipsilateral and contralateral DRG were collected separately for the hind limb region from vertebrae lumbar 4 (L4) to sacral 1 (S1). The DRG from each side were placed in cryovials with 500 µl of RNA*later*® solution (Ambion Inc., Austin, TX) and snap frozen in liquid nitrogen.

Ribonucleic acid extraction and complementary deoxyribonucleic acid synthesis

Extracted DRGs were removed from liquid nitrogen and weighed. They were then placed in 500 µl of TRI reagent® (Sigma, St. Louis, MO). The tissue was then

homogenized using a polytron homogenizer (Model: Power Gen 500, Fisher Scientific, Pittsburgh, PA). RNA was then isolated following the protocol for TRI reagent®. RNA concentrations were determined using a spectrophotometer (Model: NanoDrop 1000, Thermo Scientific, Waltham, MA). One µg of RNA was then converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA).

Ribonucleic acid expression quantification

The primers for each gene tested are listed in Table 4-1. RNA expression for target genes was then quantified using Power SYBR® Green PCR master mix (Applied Biosystems, Carlsbad, CA) in a real time PCR machine (Model: 7300 Real Time PCR system, Applied Biosystems, Carlsbad, CA). For this study, the thermo-sensitive transient receptor potential (TRP) channels that are found in the DRG (TRPV1, TRPV3, TRPV4, TRPM8, and TRPA1) were studied. These channels were selected because they respond to temperatures within the range tested in the thermal preference procedure. All TRP channel expression levels were normalized using the TATA box binding protein (Tbp) gene as a standard, and expression levels were quantified using the delta-delta Ct method (Livak and Schmittgen, 2001), with the 8-month old, neutral saline group serving as the control group.

Statistical Analysis

Statistical analysis consisted of t-tests, one-way and two-way repeated measures ANOVAs as appropriate. Student-Newman-Keuls post-hoc tests were performed where appropriate. Differences were considered statistically significant when *p*-values were less than 0.05. All statistical tests were performed using SigmaStat version 3.11 (Systat Software, Inc, San Jose, CA).

Results

Baseline Measures

Prior to first saline injection, baseline measures for body weight, thermal preference, locomotor activity, and mechanical withdrawal threshold were recorded (Figure 4-1). When body weight was measured at baseline, 28-month old animals weighed significantly more than 8-month old animals, $t(19) = 17.950$, $p < 0.001$ (Figure 4-1A).

The relative preference during each temperature comparison was calculated as in Chapter 2 (Figure 4-1B). Animals of both ages avoided the cold plate when it was paired with either a neutral or a hot plate. Animals showed no preference for either plate during the hot versus neutral comparison. Comparison of relative preference across temperatures and ages showed a main effect of temperature ($F_{2, 35} = 43.788$, $p < 0.001$), with each temperature comparison significantly different from the other.

During these baseline thermal preference tests, locomotor activity was also recorded. Generally, animals traveled a greater distance during the hot versus neutral and hot versus cold temperature comparisons. Statistically, there was a main effect of temperature ($F_{2, 38} = 6.843$, $p = 0.003$), with locomotor activity during the cold versus neutral sessions significantly less than the other two temperature comparisons (Figure 4-1C).

Prior to saline injection, baseline withdrawal thresholds to mechanical stimulation were also measured (Figure 4-1D). Comparison was made across both ages and foot (relative to side of future saline injections), with there being no statistically significant difference for age, side, or age x side interaction (age: $F_{1, 19} = 0.061$, $p = 0.807$; side: $F_{1, 19} < 0.001$, $p = 0.995$; age x side interaction: $F_{1, 19} = 0.199$, $p = 0.660$).

Body Weight

Changes in body weight after saline injection as a percentage of baseline body weight are shown in Figure 4-2. The 8-month old animals show an increase in body weight over time (Figure 4-2A), and comparison across session and pH of saline injection showed a main effect of session ($F_{20, 200} = 20.089$, $p < 0.001$). The 28-month old animals showed a decrease in body weight from baseline after saline injection (session main effect: $F_{20, 140} = 1.809$, $p = 0.025$). As there was no statistically significant difference in body weight for either age based on pH of saline injection, changes in body weight were compared across ages, regardless of pH group (Figure 4-2C). While there was a consistent decrease in body weight for the 28-month old animals after the first saline injection, the 8-month old animals showed an initial decrease in body weight, which had recovered by 9 days after the second saline injection. Statistical analysis showed a significant interaction between age and session ($F_{20, 380} = 5.832$, $p < 0.001$).

Mechanical Stimulation Withdrawal Threshold

The 50% withdrawal threshold to mechanical stimulation across session is shown in Figure 4-3 and Figure 4-4 using a semi-log plot with the y-axis on a logarithmic scale and the x-axis on a linear scale. The semi-log plot was used based on the Weber-Fechner law, and the common use of logarithmic functions for perceptual responses, including perceptions of weight and force.

Data for withdrawal thresholds of the foot ipsilateral to saline injections are shown in Figure 4-3. After the second saline injection, animals that received acidic saline injections showed a significant decrease in withdrawal threshold, thus they were more sensitive to mechanical stimulation. Interestingly, as time progressed, animals injected with neutral saline also showed decreased withdrawal thresholds through day 24 after

the second saline injection. It was speculated that this increased sensitivity was due to overtesting. Animals were subsequently not tested for one week then retested, and withdrawal thresholds for the neutral saline injected animals increased to near baseline levels. For 8-month old animals (Figure 4-3A), there was a significant pH x session interaction ($F_{25, 250} = 5.260$, $p < 0.001$) with acidic saline injected animals showing a significantly lower withdrawal threshold than neutral saline injected animals during all sessions after the second saline injection except for the session 1, 3, 22, 23 and 24 days post injection. Acidic and neutral saline injected animals showed no statistically significant differences during session 1, 3, 23, and 24 days after the second injection, and neutral saline injected animals had a significantly lower threshold during the session 22 days after the second saline injection. Analysis of 28-month old animals (Figure 4-3B), showed significant interaction between pH and session ($F_{25, 175} = 2.952$, $p < 0.001$). Post-hoc analysis showed that acidic saline injected animals had a significantly lower withdrawal threshold for almost all sessions starting 3 days after the second saline injection with the exception of sessions 4, 16, 20, 23, and 24 days after the second injection. Acidic saline injected animals were statistically compared across ages, with a significant age x session interaction ($F_{25, 225} = 1.937$, $p = 0.006$). Post-hoc analysis showed that 8-month old animals injected with acidic saline had significantly lower withdrawal thresholds than 28-month old acidic saline injected animals on sessions 16 and 31 days after the second injection.

Withdrawal thresholds for the foot contralateral to the saline injections are shown in Figure 4-4. As with the ipsilateral foot, withdrawal thresholds decreased after acidic saline injection, and remained decreased throughout testing for animals injected with

acidic saline. For animals injected with neutral saline, these animals also showed decreased thresholds from baseline, with thresholds similar between neutral and acidic injected animals during sessions more than 21 days after the second saline injection. Withdrawal thresholds for 8-month old animals are shown in Figure 4-4A. Statistical analysis showed a significant pH x session interaction for these animals ($F_{25, 250}$ = 4.622, $p < 0.001$), with acidic saline injected animals showing lower withdrawal thresholds than neutral saline injected animals for all sessions between 5 and 20 days after the second saline injection. Similar results were observed for 28-month old animals (Figure 4-4B), with a significant pH x session interaction observed ($F_{25, 175}$ = 2.068, $p <$ 0.001). Post-hoc analysis showed that acidic saline injected animals had lower withdrawal thresholds than neutral saline injected animals for session 2, 5, and 9-22 days after the second saline injection. Withdrawal thresholds for acidic saline injected animals were statistically compared across age groups, with a significant main effect of session ($F_{25, 225}$ = 27.214, $p < 0.001$). Post-hoc analysis showed that withdrawal thresholds were significantly decreased from baseline for all sessions starting with the session one day after the second saline injection.

Cold versus Neutral

Locomotor activity

Changes in locomotor activity after saline injection during cold versus neutral temperature comparisons are shown in Figure 4-5. With repeated testing, locomotor activity decreased from baseline all groups. For the 8-month old animals, there were significant changes in locomotor activity across sessions (session main effect: $F_{4, 40}$ = 3.499, p = 0.015), with the session three days after the second saline injection having the least amount of activity (Figure 4-5A). Locomotor activity also decreased for 28-

month old animals (session main effect: $F_{4, 25} = 6.776$, $p < 0.001$), with the sessions 3, 9, and 13 days after the second saline injection showing significantly less activity that the session 4 hrs after the first saline injection (Figure 4-5B). As there were no differences in locomotor activity based on the pH of saline injection, locomotor activity was compared across ages regardless of saline injection group (Figure 4-5C). There were no differences across ages, but a main effect of session ($F_{4, 73} = 8.707$, $p < 0.001$).

Thermal preference

Changes in time spent on the neutral plate, as a percent of baseline, after intramuscular injections during cold versus neutral comparisons are shown in Figure 4-6. Generally, animals spent an increased time on the neutral plate after saline injections. For 8-month old animals (Figure 4-6A), there was a significant difference between saline injection groups, with neutral saline injected animals showing greater increase in time spent on neutral plate (main effect of pH: $F_{1, 10} = 5.902$, $p = 0.035$). However, there were no statistically significant differences in time sent on the neutral plate for 28-month old animals (Figure 4-6B). When animals that were injected with acidic saline were compared across ages (Figure 4-6C), there were also no statistically significant differences.

Hot versus Neutral

Locomotor activity

Changes in locomotor activity after saline injections for the hot versus neutral thermal preference sessions are shown in Figure 4-7. Data are presented for 8-month (Figure 4-7A), 28-month (Figure 4-7B), and across ages (Figure 4-7C). For all three, animals showed a general decrease in activity after saline injection. Statistical analysis

showed a significant main effect of session for all three (8-month: $F_{10, 100} = 4.416$, $p < 0.001$; 28-month: $F_{10, 70} = 2.145$, $p = 0.032$; across ages: $F_{10, 190} = 5.830$).

Thermal preference

Changes in time spent on the hot plate after intramuscular saline injections for the hot versus neutral comparisons are shown in Figure 4-8. Data are presented for 8-month (Figure 4-8A), 28-month (Figure 4-8B), and between ages of acidic saline injected animals (Figure 4-8C). Two-way repeated measure ANOVAs were performed across session and pH for both 8-month and 28-month old animals, although there were no statistically significant differences. A two-way repeated measure ANOVA was also performed for acidic saline injected animals (age and session main effects), although there were no statistically significant differences in this comparison.

Hot versus Cold

Locomotor activity

Changes in locomotor activity after saline injection during hot versus cold thermal preference sessions are shown in Figure 4-9. Data for 8-month old animals are shown in Figure 4-9A. Animals injected with acidic saline show a slight decrease in activity compared to animals injected with neutral saline, although these differences are not statistically significant. Saline injection significantly decreased locomotor activity for 28-month old animals (session main effect: $F_{4, 27} = 6.085$, $p = 0.001$), with the sessions after the second saline injection showing significantly less activity than the session after the first saline injection (Figure 4-9B). When animals were compared across ages, regardless of saline injection, there was a significant age X session interaction ($F_{4, 75} = 2.956$, $p = 0.025$).

Thermal preference

Changes in time spent on the hot plate after saline injection for the hot versus cold comparison are shown in Figure 4-10. For 8-month old animals (Figure 4-10A), there were no statistically significant changes in time spent on the hot plate after saline injection. For 28-month old animals (Figure 4-10B), animals injected with neutral saline showed a significant increase in the amount of time spent on the hot plate compared to animals injected with acidic saline (main effect of pH: $F_{1, 7} = 13.952$, $p = 0.007$). When acidic saline injected animals were compared across ages (Figure 4-10C), there were no statistically significant differences in time spent on hot plate, either between ages ($F_{1, 9} = 0.007$, $p = 0.936$) or across sessions ($F_{4, 36} = 1.130$, $p = 0.358$). There was also no statistically significant interaction between age and session ($F_{4, 36} = 2.508$, $p = 0.059$).

Relative Expression of Thermo-Sensitive Transient Receptor Potential Channels

After the conclusion of behavioral testing, changes in expression of RNA of thermo-sensitive TRP channels was assessed the DRGs for sensory neurons of the hind limbs. TRP channels were separated into those that respond to heat (TRPV1, TRPV3, and TRPV4) and cold (TRPA1, TRPM8). Relative RNA expression was then quantified for DRGs ipsilateral and contralateral to the side of saline injection. Relative expression was quantified separately for each TRP channel and side.

Heat sensitive transient receptor potential channels

The relative expressions for the heat sensitive TRP channel RNA ipsilateral to saline injection are shown in Figure 4-10A. Relative expression was greatly increased for all TRP channels in the 8-month old acidic saline injected animals. Although there are large standard errors due to the small sample size stemming from difficulties

processing the tissue. Two-way ANOVAs comparing age and pH were performed independently for each channel, with a significant main effect of pH for the TRPV3 channel ($F_{1, 11}$ = 7.964, p = 0.017) with acidic saline injected animals showing greater expression than neutral saline injected animals. On the contralateral side, there was a large increase in expression of the TRPV4 channel RNA for the 8-month old acidic saline injected animals, and little change for all other groups. Unfortunately, due to difficulties processing the tissue, the relative expression of the TRPV1 channel RNA could not be quantified for the 8-month old acidic saline injected animals (Figure 4-11C). Statistical analysis showed a main effect of age for the TRPV4 channel on the contralateral side ($F_{1, 9}$ = 5.482, p = 0.044), with the 8-month old animals showing greater expression of the TRPV4 channel RNA than 28-month old animals.

Cold sensitive transient receptor potential channels

The relative expression of RNA for cold sensitive TRP channels is shown in Figures 4-11 for the ipsilateral (panel B) and contralateral (panel D) sides. On the ipsilateral side, all groups show a slight increase in expression of both TRPA1 and TRPM8 RNA compared to the 8-month old neutral saline injected animals, although these increases are not statistically significant. On the contralateral side, 28-month old animals show an increase in expression of TRPA1 RNA compared to 8-month old animals, although this increase is not statistically significant. For TRPM8 RNA, the 28-month neutral saline injected animals show an increase in expression relative to all other groups, although again this increase in not statistically significant.

Discussion

Studies suggest that chronic pain rates are higher in older individuals (Bruckenthal et al., 2009; Colliver et al., 2006; McCarthy et al., 2009; Neville et al., 2008; Rustøen et

al., 2005; Walid and Zaytseva, 2009). However, based on the known mechanisms of chronic pain, one would predict that chronic pain rates would be lower in older individuals, since many of the mechanisms of chronic pain, for example LTP, are decreased in older individuals. These mechanisms are thought to be responsible for the maintenance of chronic pain, and not for the transition of acute pain to chronic pain. Unfortunately, the biological mechanisms underlying the transitions to chronic pain states are not currently understood, as most animal models of chronic pain induce chronic pain states from the start and do not allow for the study of this transition. The purpose of this study was to assess the role of aging on the development and maintenance of chronic pain following repeated intramuscular injections of acidic saline.

As with initial studies examining this model (Sluka et al., 2001), acidic saline injections decreased withdrawal thresholds to mechanical stimulation bilaterally. However, there was no change in thermal sensitivity when measured by the thermal preference procedure even though qRT-PCR analysis showed a change in thermo-sensitive TRP channels in acidic saline injected animals.

Baseline Differences

As with the previous studies, there was an increase in body weight with increased age, and as expected, these female animals weighed less than males as similar ages. In fact, 12-month old male animals from the second study weighed more at baseline than the 28-month old female animals in this study. While there were no changes in body weight after acidic saline injection, suggesting this is not a concern with this procedure, the stark differences in body weight between males and females highlight the importance of conducting research on both sexes, as these differences can have effects on treatment outcomes.

At baseline, there was no difference between the ages for thermal preference, and animals showed a strong aversion to the cold plate, regardless of which temperature was the alternative, even though the younger males in the first study showed a preference for the cold plate when paired with the hot plate. These results are consistent with previous studies (Vierck et al., 2008a) in which females had a greater sensitivity to cold than males, and further highlight the need to study both sexes. Also at baseline, there were no differences in locomotor activity or mechanical withdrawal thresholds between the two ages, so any differences in thermal preference, locomotor activity, or mechanical withdrawal thresholds after acidic saline injection are most likely an age-related difference in the response to these injections, and not a result of baseline differences.

Mechanical Sensitivity

Repeated acidic saline injections decreased mechanical withdrawal thresholds for both 8- and 28-month old animals. This decrease was observed for hind paws both ipsilateral and contralateral to the acidic saline injections, which suggests sensitization in the CNS. On the ipsilateral side, 8-month old animals showed a decrease in withdrawal thresholds as quickly as 4 hours after the second injection. However, 28-month old animals did not show a decrease in withdrawal thresholds until 2 days after the second injection. This suggests that the mechanisms for the development of chronic pain are depressed in older animals, even though older individuals have higher rates of chronic pain. On the contralateral side, decreases in withdrawal threshold occurred similarly between the two ages, suggesting that the mechanisms for central sensitization of mechanical sensitivity are separate from those responsible for developing sensitivity to mechanical stimulation, and are not affected by aging.

For animals injected with neutral saline, there was also a decrease in withdrawal thresholds, although these decreases developed later in testing. These decreases could be a combination of not obtaining a stable baseline prior to saline injection, as animals were tested once before the first saline injections, and a by-product of testing animals daily for withdrawal thresholds. Daily testing could affect withdrawal thresholds by either sensitizing the paws of animals due to repeated stimulation, or lifting of the foot in response to stimulation could have become a learned behavior by the animals to shorten time spent in the testing chamber. There is some evidence of decreases in withdrawal thresholds in neutral saline animals being a product of repeated testing, as a slight increase in withdrawal threshold is observed whenever there is more than one day between test sessions, and thresholds returned to near baseline levels during the last test session, which was performed one week after the previous session.

Thermal Sensitivity

Unlike mechanical withdrawal testing, there were no changes in thermal preference due to acidic saline injection for either age, during any of the three temperature comparisons. These findings are consistent with previous studies that showed no change in thermal sensitivity after acidic saline injection (Sluka et al., 2001). Although the thermal sensitivity testing performed in the previous study potentially activated a different group of nociceptive neurons than the thermal preference procedure, the current results suggest that acidic saline injections could have no effect on thermal sensitivity.

Transient Receptor Potential Channels

Even though there were no behavioral changes in thermal preference observed, DRGs for the dermatomes responsible for the hind limbs were collected and expression

of mRNA for thermo-sensitive TRP channels assessed. Due to difficulties processing the small amount of tissue collected, the number of samples for each TRP channel decreased to a maximum of three, which made statistical analysis of TRP channel expression difficult. However, increases in relative expression for the acidic saline injected 8-month old animals was observed for heat sensitive TRP channels on both the ipsilateral and contralateral side, with the exception of contralateral TRPV1 which could not be quantified for this group. For TRPV4, which responds to temperatures just above room temperature, there was a decrease on the ipsilateral side for both neutral and acidic saline injected 28-month old animals. Previous studies have suggested that the TRPV4 channel is responsible for the degree of sensitivity to heat (Todaka et al., 2004), and the decreases in this TRP channel in aged animals may be a potential mechanism for the shift in thermal preference to warmer temperatures observed in older animals (Yezierski et al., 2010). An increase in expression of cold sensitivity TRP channels was also observed for 28-month old animals, which could also contribute to the shift towards warmer thermal preferences.

Implications and Future Directions

Overall, this study investigated the effects of increased age on the development and maintenance of chronic pain after repeated acidic saline injection. While the results of this study were not definitive, the results do suggest that older animals may develop chronic pain at slower rates than younger animals in this model of chronic pain. While these results contradict clinical data that suggest older individuals are more likely to suffer chronic pain, this could result from the chronic pain observed in these animals is an experimentally induced form of chronic pain. Therefore, the mechanisms for the development of chronic pain may be decreased in older animals (e.g. LTP, protein

synthesis), and therefore they develop induced chronic pain at slower rates. In the clinical setting, chronic pain may develop slowly over one's lifetime, and therefore these decreases in cellular mechanisms may not affect the development of chronic pain in older adults. In fact, increased age may lead to greater rates of chronic pain simply because they have had a longer period in which chronic pain could develop. It is important to note however, that the change in mechanical sensitivity was similar between young and old animals, suggesting that old animals experience a similar degree or intensity of chronic pain compared to younger animals.

There were no changes in thermal sensitivity for either age after acidic saline injection, even though qRT-PCR results showed increases in heat sensitive TRP channels in younger animals after acidic saline injection. Numerous explanations may explain the difference between the molecular and behavioral tests. Increases in TRP channel expression were observed in the ipsilateral side, suggesting that these changes occur locally due to exposure to acidic saline. Due to this, increased thermal sensitivity in one limb may not be enough to overcome the baseline thresholds and/or sensitivity of the other three unaffected limbs, and thus there is no change in behavior. In future experiments, it may be necessary to inject both one hind limb and one forelimb, or possibly inject all four limbs to observe changes in thermal sensitivity. It is important to also note that an increase in mRNA expression does not always lead to an increase in receptor number or function; therefore, it is important to also perform studies quantifying changes in protein levels for these receptors. One other possibility is that we failed to test the most appropriate temperatures during the thermal preference procedure. The greatest change in mRNA levels was observed for the TRPV3 receptor, although a

temperature that would maximally activate this receptor was not tested. Future experiments should assess a gradient of temperatures versus a neutral temperature to investigate any changes in thermal sensitivity across the entire range of innocuous temperatures.

Overall, this study suggests that there are important changes in the sensory systems involved in thermal detection, and these changes are age dependent. Therefore, it is important to continue to use the acidic saline injection model of chronic pain in conjunction with the thermal preference procedure to assess exactly what role thermo-sensitive TRP channels may play in chronic pain. Better understanding of the role of these channels may lead to the discovery of novel therapeutic targets for the treatment and/or prevention of chronic pain.

Table 4-1. Timeline of experimental testing

Phase	Day	Experimental event
Baseline	0	Arrive in lab
	14-42	Thermal preference training
	43-49	Thermal preference baseline measures
	50	Mechanical withdrawal baseline measures
First saline injection	0	Inject saline
	4 hr	Mechanical withdrawal; cold versus neutral
	1	Mechanical withdrawal; hot versus cold
	2	Mechanical withdrawal; hot versus neutral
Second saline injection	0	Inject saline
	4 hr	Mechanical withdrawal; cold versus neutral
	1	Mechanical withdrawal; hot versus cold
	2	Mechanical withdrawal; hot versus neutral
	3	Mechanical withdrawal; cold versus neutral
	4	Mechanical withdrawal; hot versus cold
	5	Mechanical withdrawal; hot versus neutral
	6	Mechanical withdrawal; hot versus cold
	9	Mechanical withdrawal; cold versus neutral
	10	Mechanical withdrawal; hot versus neutral
	11	Mechanical withdrawal; hot versus cold
	12	Mechanical withdrawal; hot versus neutral
	13	Mechanical withdrawal; cold versus neutral
	16-21	Mechanical withdrawal; hot versus cold
	22-24	Mechanical withdrawal
	31	Mechanical withdrawal
	32-33	Animals sacrificed

Table 4-2. Primer list for genes tested using quantitative real-time polymerase chain reaction

Gene	Accession Number	Base Pairs	Primer Sequence
Tbp	NM_001004198	60	5’ TCCCTCCTCTGCACTGAGATC
			3’ GCAGCACAGAGCAAGCAACT
TRPV1	NM_031982	60	5’ TTGAACGGCGGAACATGA
			3’ CCTGGACATCTGCTCCATTCTC
TRPV3	NM_001025757	59	5’ CAAGACCTCTCCCCCAATCTT
			3’ AGAGGCACTGCCGGATGTT
TRPV4	NM_023970	59	5’ CAGCCGCACATCGTCAACTA
			3’ CGCCTCATATCGGCTTTCTT
TRPM8	NM_134371	63	5’ TGGCCTCGTATCGTTTAGGAA
			3’ ACGTAGTACCAGAGCAGCTTCTTG
TRPA1	NM_207608	61	5’ GCTGAGATCGACGGGAGTGT
			3’ GACGTAAAAGCTGAGGCCAAA

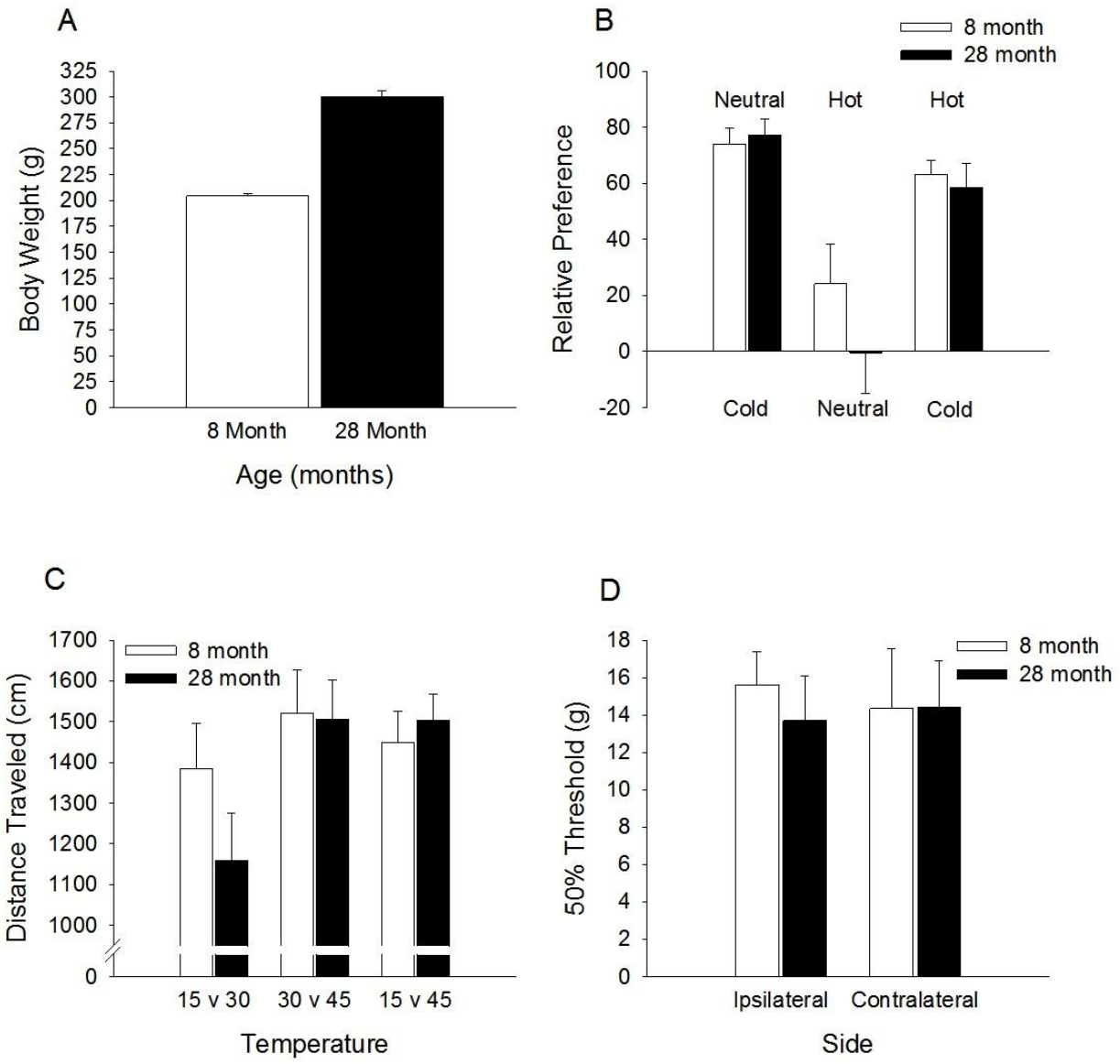

Figure 4-1. Baseline measures. A) Body weight. B) Thermal preference. C) Locomotor activity. D) Mechanical withdrawal. Comparisons across ages prior to saline injection (mean ± SEM). A) As expected, older animals had greater body weight than younger animals. B) Thermal preference by temperature comparison, there were no statistically significant differences between ages in relative preference, but preference was significantly different across temperature comparisons. C) Locomotor activity showed no statistically significant differences across ages, but activity was significantly less during the cold versus neutral comparison. D) Mechanical withdrawal showed no differences between ages or side of body prior to saline injection.

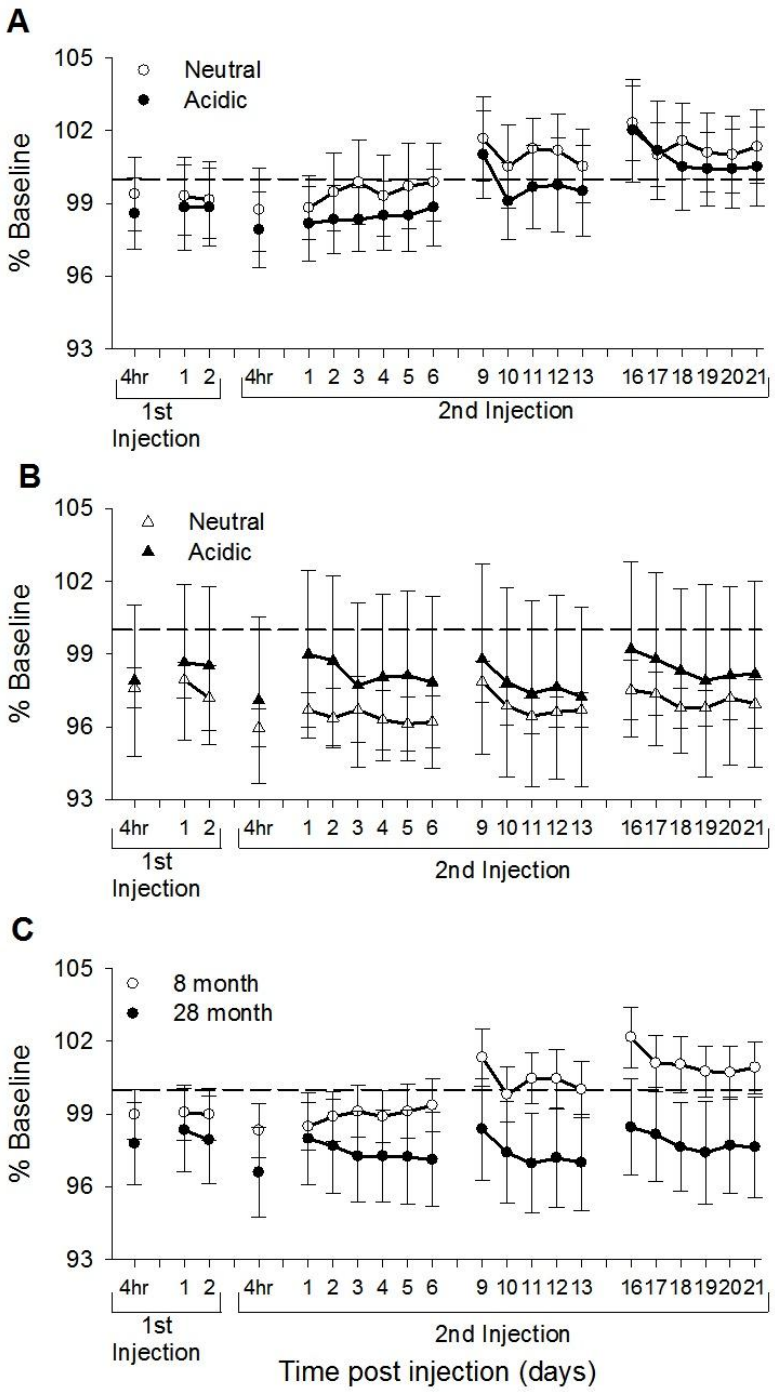

Figure 4-2. Changes in body weight after repeated intramuscular saline injection. A) 8-month old animals. B) 28-month old animals. C) Across ages. Data are presented as percent of baseline body weight (mean ± SEM). Note the slow increase in body weight for 8-month old animals. C) Due to lack of difference between injection groups, pH groups were combined within each age and compared across ages. Both age groups showed decreased weight immediately after saline injections, but only 8-month old animals recovered this weight over time.

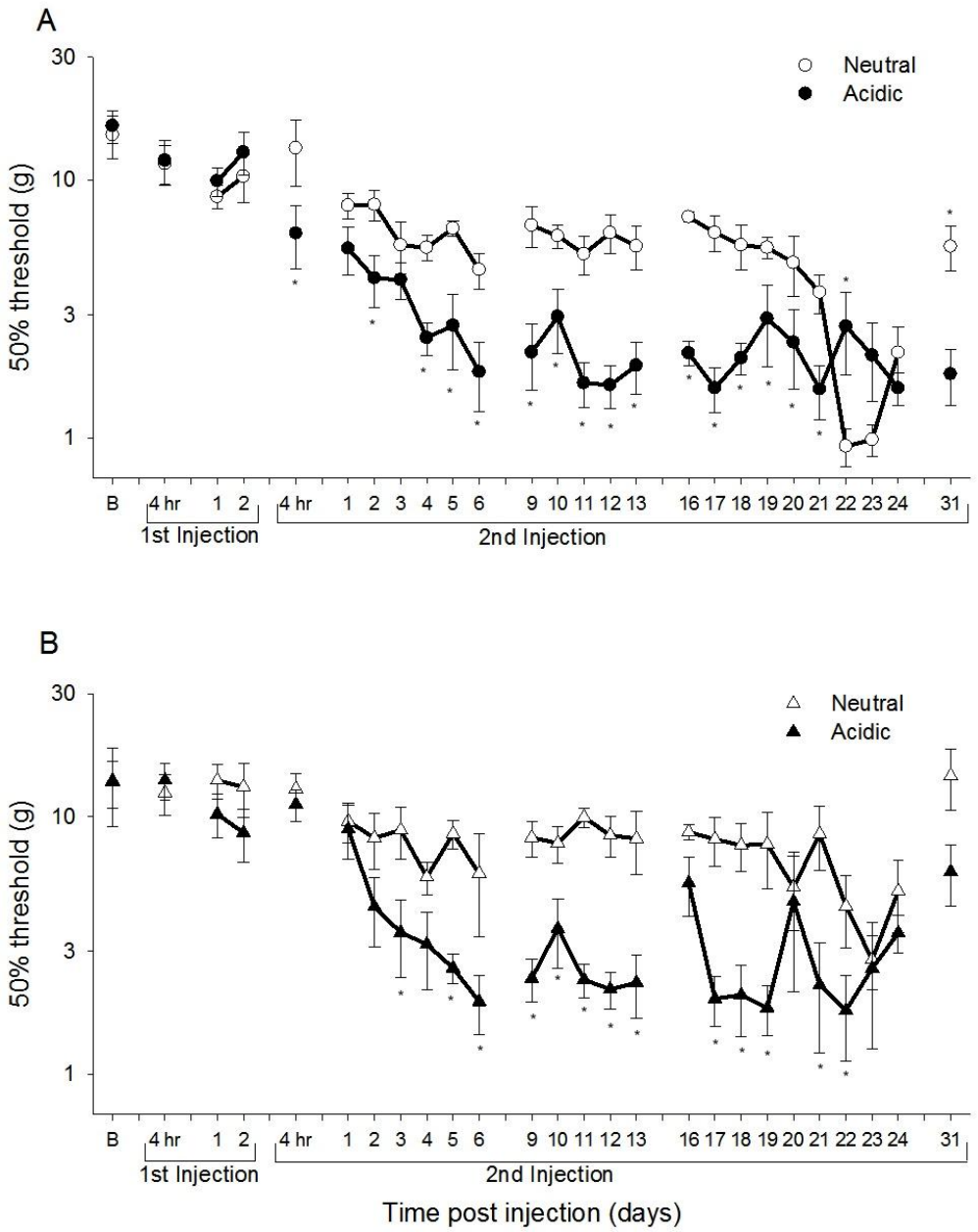

Figure 4-3. Mechanical stimulation withdrawal threshold of ipsilateral foot to intramuscular saline injection. A) 8-month old animals. B) 28-month old animals. A) Acidic saline injection significantly lowered withdrawal thresholds in 8-month old animals after the second injection. Withdrawal thresholds decreased within 4 hrs of second injection and remained decreased for three weeks after the second injection. B) Withdrawal thresholds were significantly lower for acidic saline injected animals than neutral saline injected animals in the 28-month age group. Notice though that withdrawal thresholds decreased more slowly compared to 8-month old animals, suggesting slower development of chronic pain in older animals. *$p < 0.05$ compared to neutral saline injected animals.

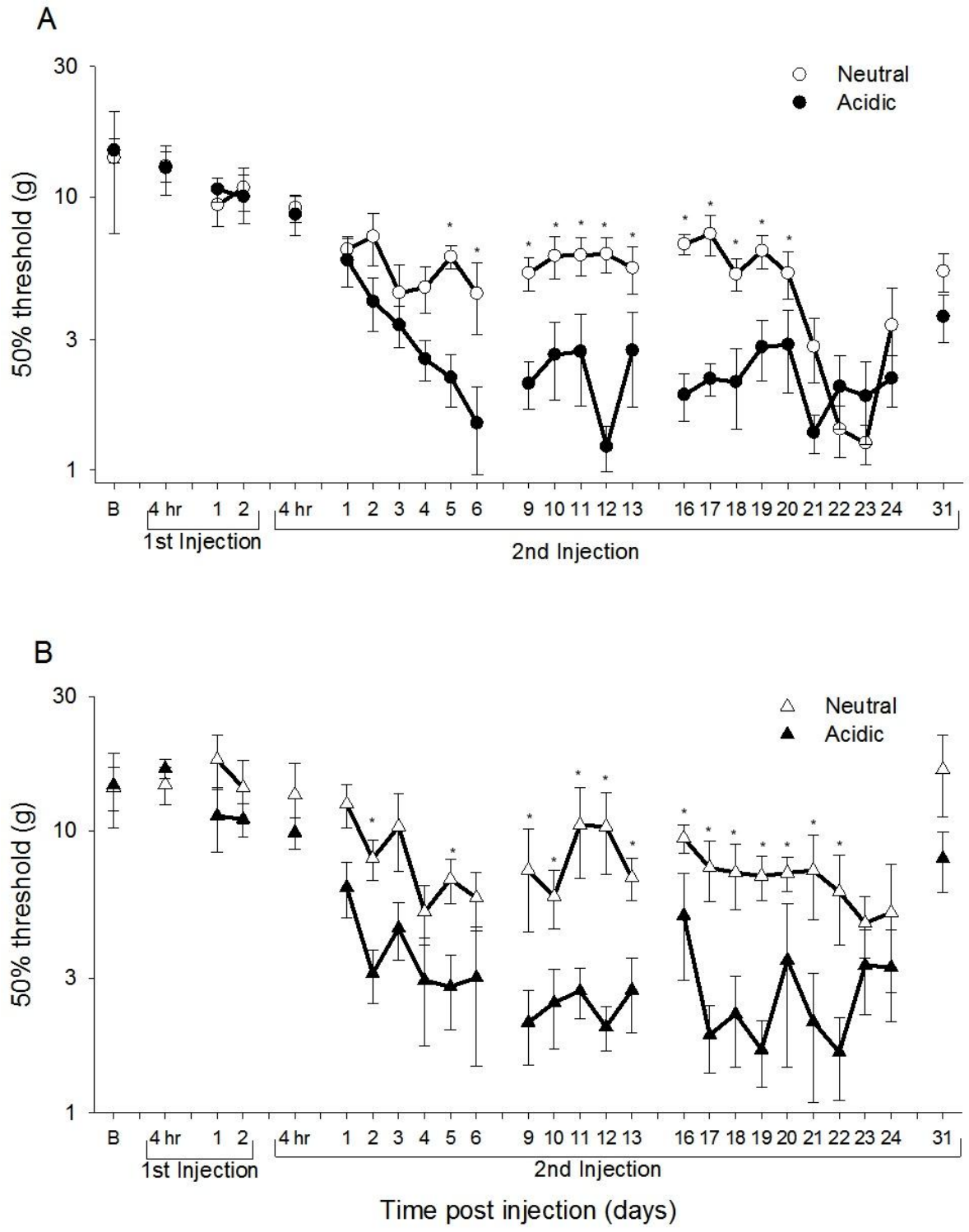

Figure 4-4. Mechanical stimulation withdrawal thresholds for foot contralateral to intramuscular saline injection. A) 8-month old animals. B) 28-month old animals. Similar to the ipsilateral foot, acidic saline injection significantly decreased withdrawal thresholds compared to neutral saline injected animals. *$p < 0.05$ compared to neutral saline injected animals.

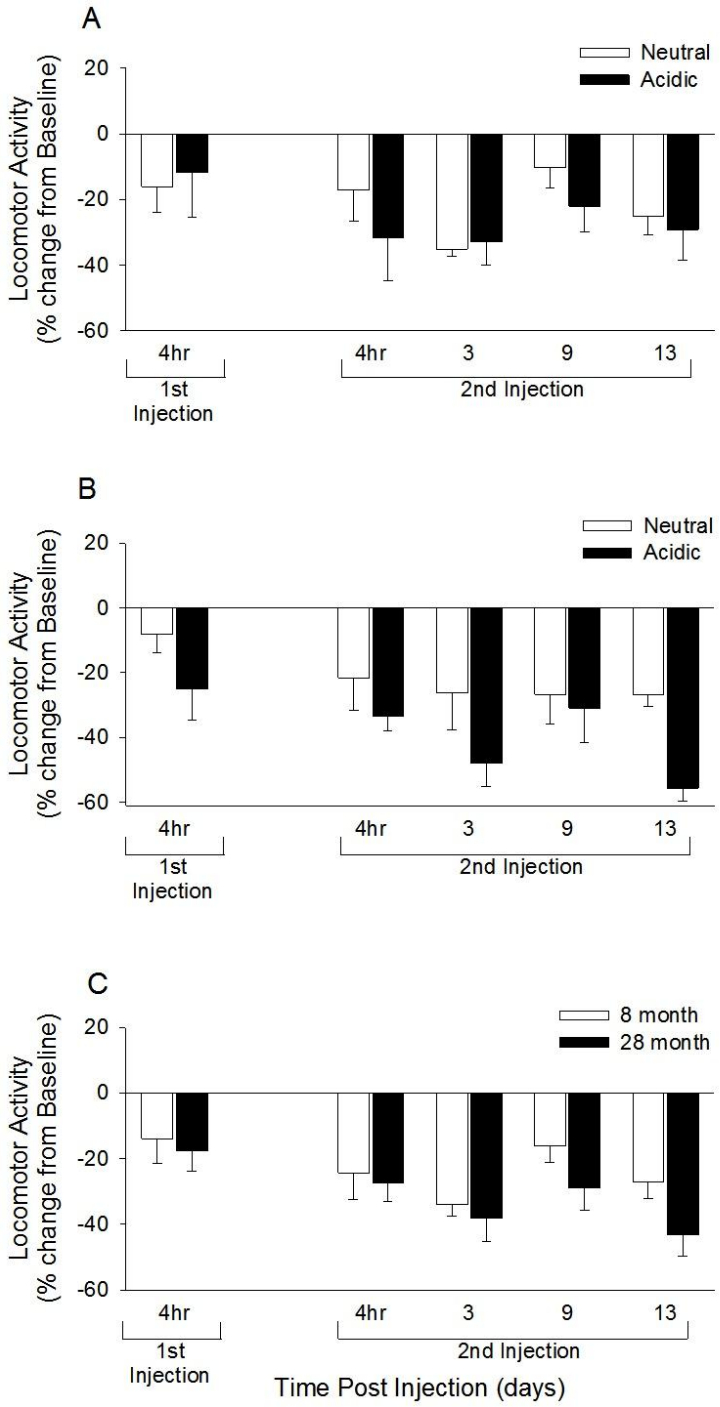

Figure 4-5. Change in locomotor activity after repeated intramuscular saline injection during cold versus neutral sessions. A) 8-month old animals. B) 28-month old animals. C) Across ages. Other details as in Figure 4-2. There were no differences in locomotor activity due to pH of saline injection, although activity did decrease for all groups over time. C) There were no statistically significant differences in locomotor activity between ages when animals were compared regardless of pH group.

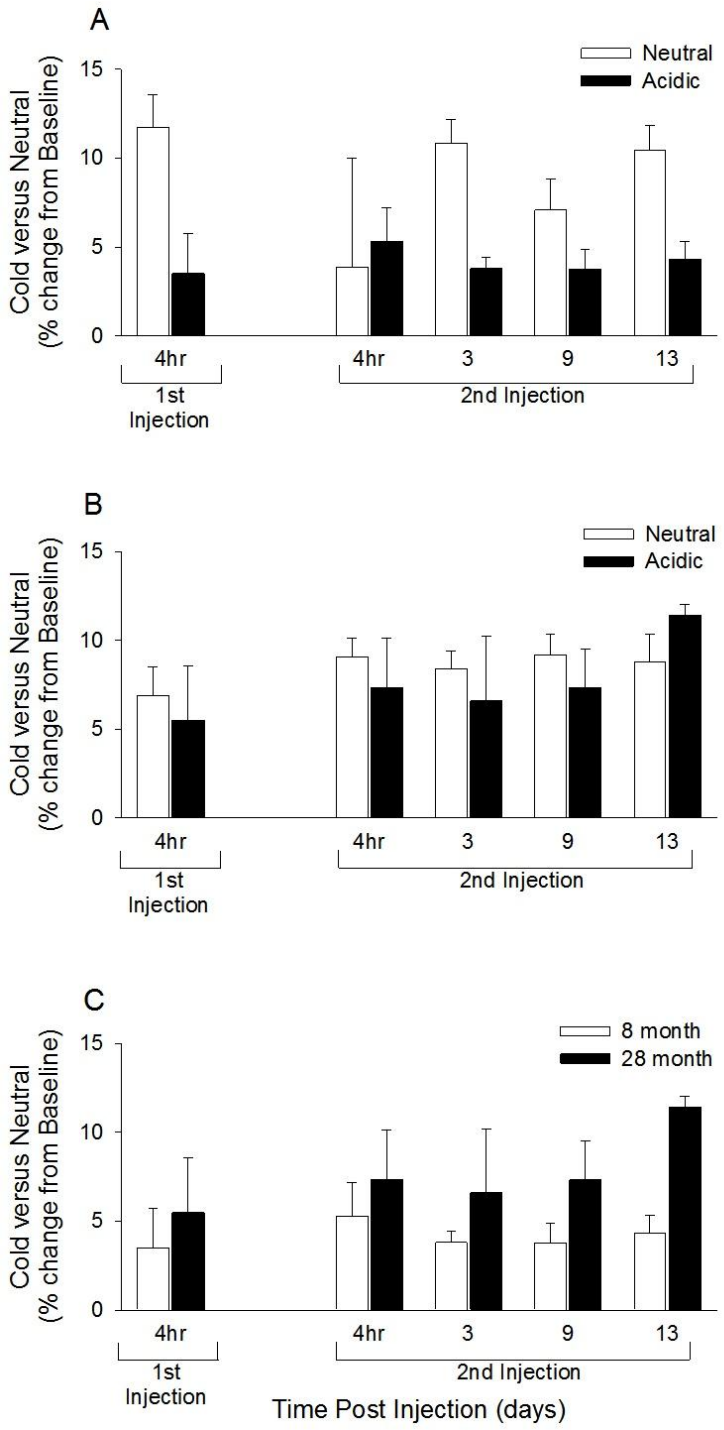

Figure 4-6. Change in time on neutral plate during cold versus neutral sessions after intramuscular saline injections. A) 8-month old animals. B) 28-month old animals. C) Acidic saline injected animals. Data are presented as mean ± SEM. A) Neutral saline injection increased the amount of time spent on neutral plate while acidic saline had no effect in 8-month old animals. B) Saline injection had no effect on time spent on neutral plate for either group in 28-month old animals. C) No statistically significant differences being shown.

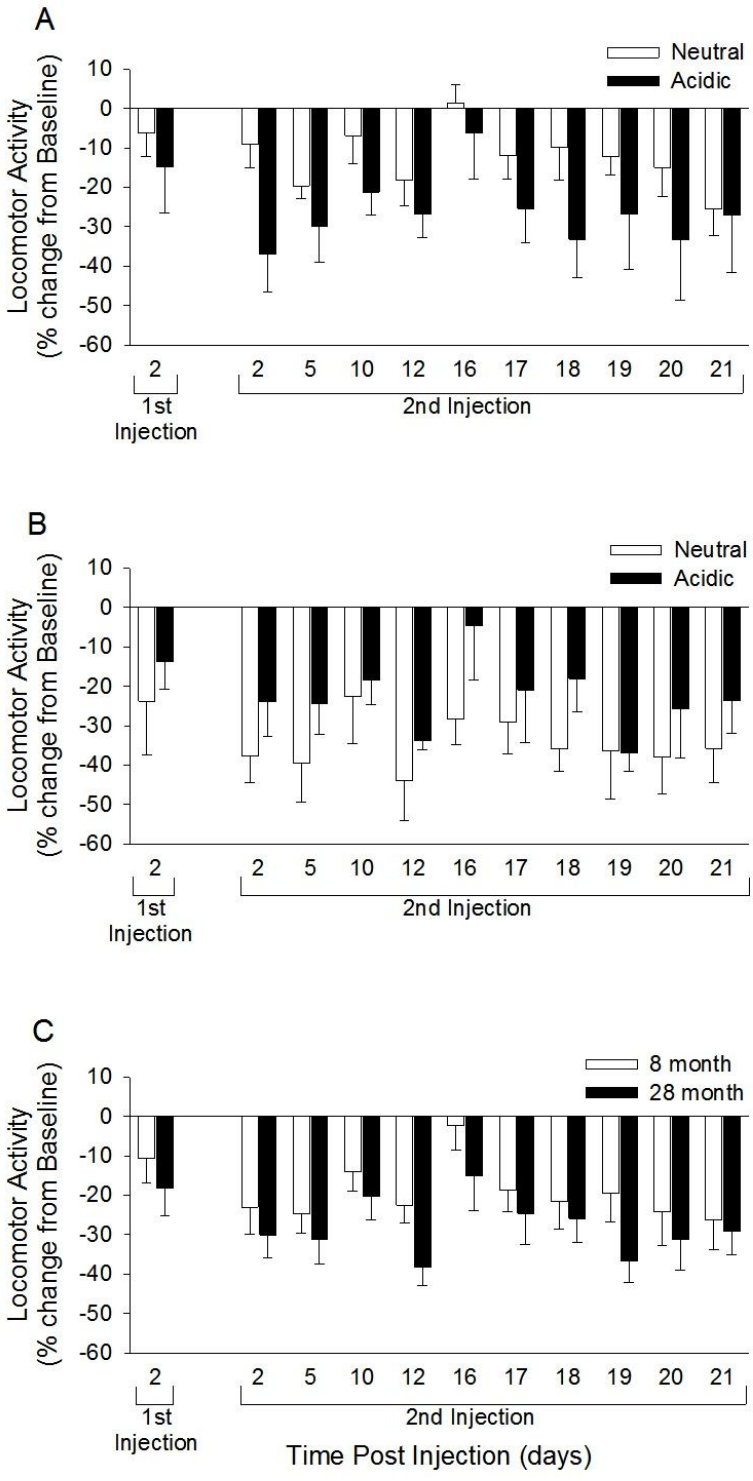

Figure 4-7. Changes in locomotor activity after intramuscular saline injections during hot versus neutral sessions. A) 8-month old animals. B) 28-month old animals. C) Across ages. Other details as in Figure 4-5. Generally, saline injection decreased locomotor activity across all groups with no statistically significant differences between pH of injection or ages.

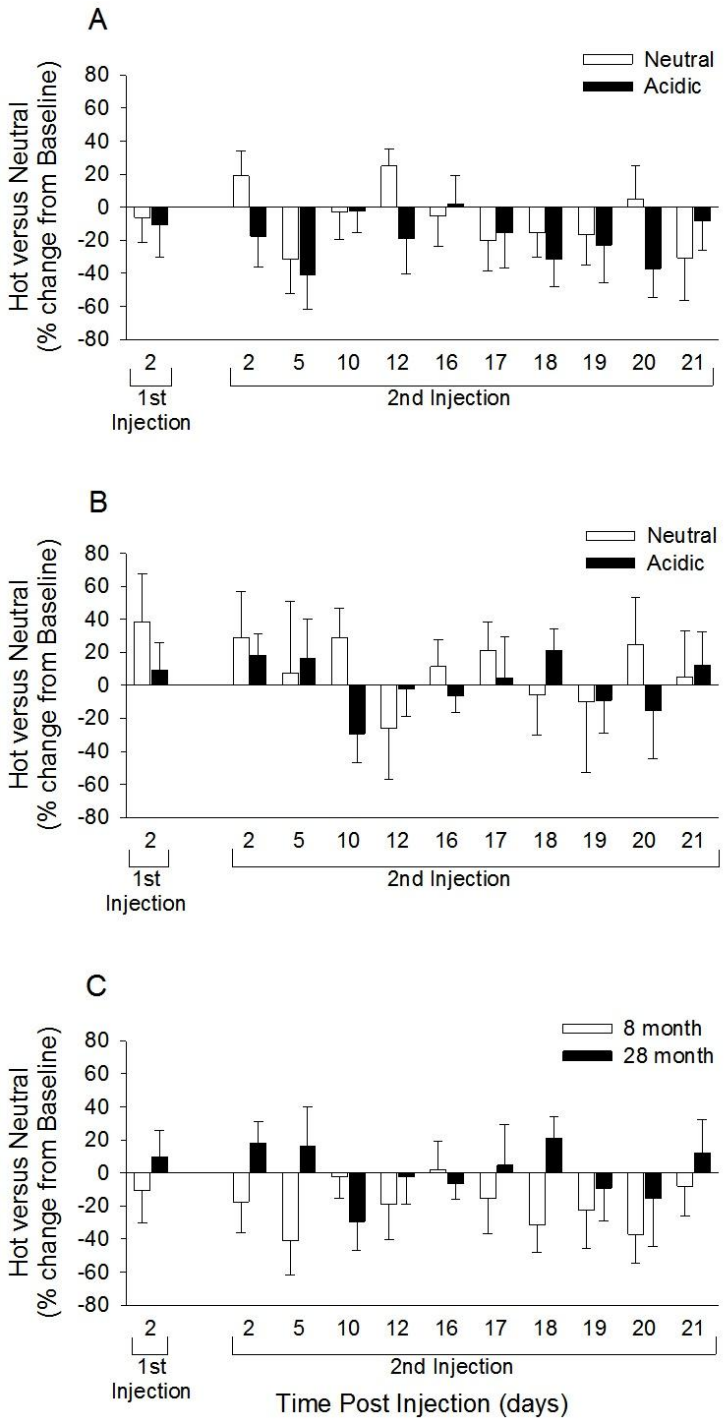

Figure 4-8. Change in time spent on hot plate during hot versus neutral sessions after intramuscular saline injections. A) 8-month old animals. B) 28-month old animals. C) Acidic saline injected animals. Other details as in Figure 4-6. Changes in time spent on hot plate were variable across sessions for each pH group and age with no statistically significant differences across any comparison. Overall, acidic saline injected animals did spend less time on the hot plate than neutral saline injected animals.

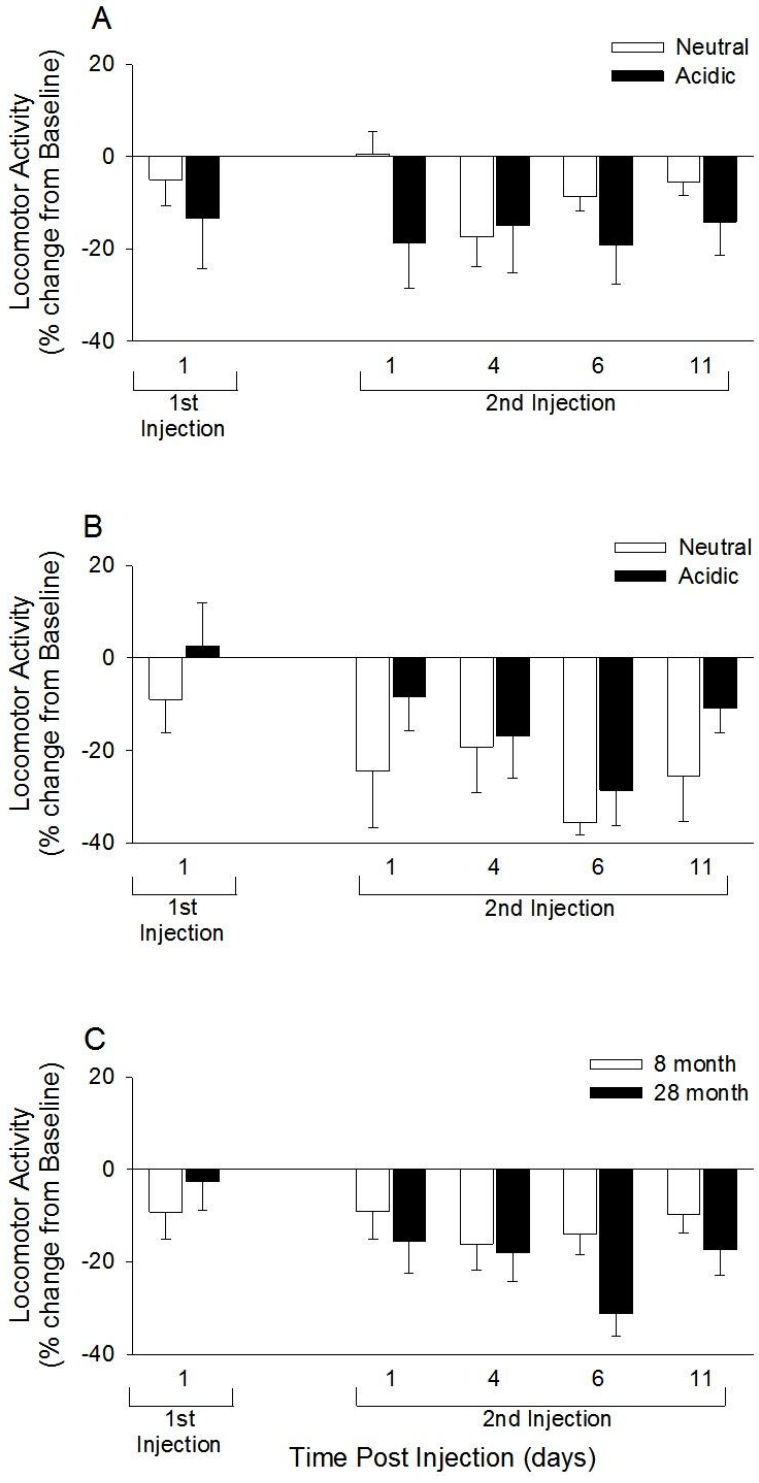

Figure 4-9. Changes in locomotor activity after intramuscular saline injections during hot versus cold comparisons. A) 8-month old animals. B) 28-month old animals. C) Across ages. Other details as in Figure 4-5. Generally, saline injection decreased locomotor activity across all groups with no statistically significant differences between pH of injection or ages.

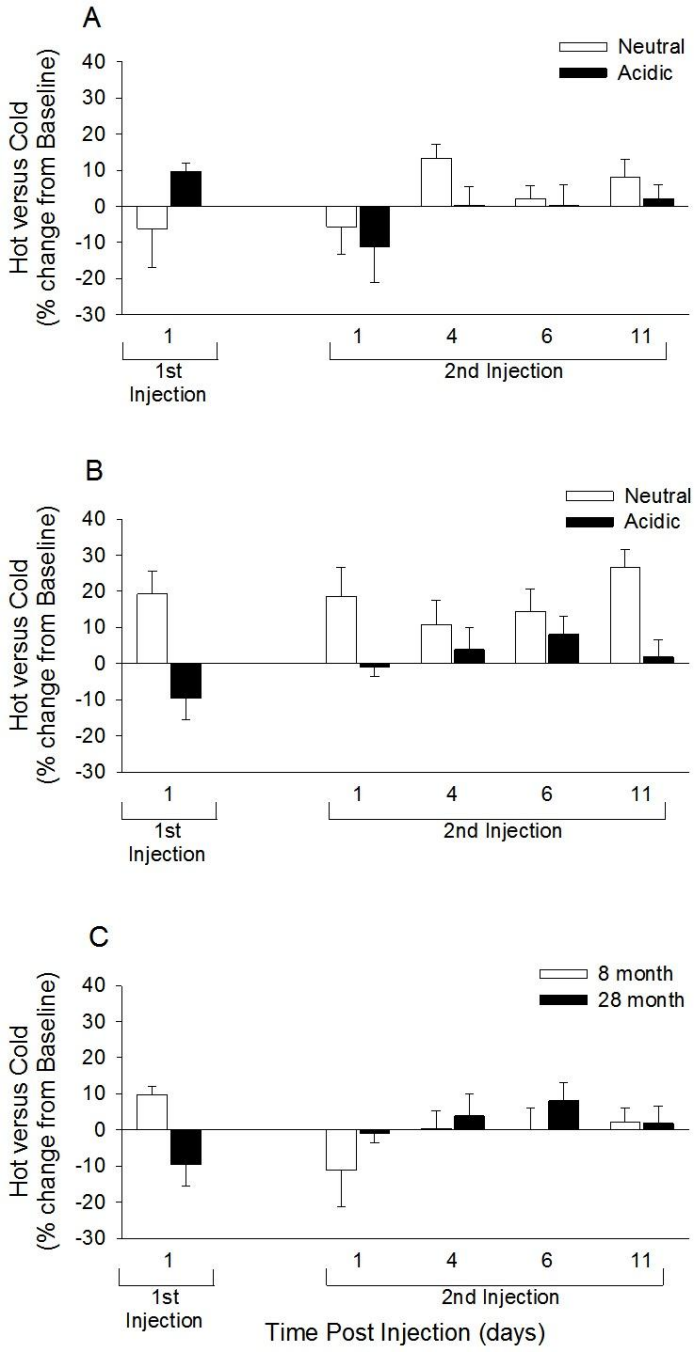

Figure 4-10. Change in time spent on hot plate after intramuscular saline injections during hot versus cold comparisons. A) 8-month old animals. B) 28-month old animals. C) Acidic saline injected animals. Other details as in Figure 4-6. A) Acidic saline injection had no effect on thermal preference in 8-month old animals. B) Acidic saline injection had no effect on thermal preference in 28-month old animals, although neutral saline injected animals did show a significant increase in the amount of time spent on the hot plate. C) There was no difference in time spent on hot plate between the two ages of acidic saline injected animals.

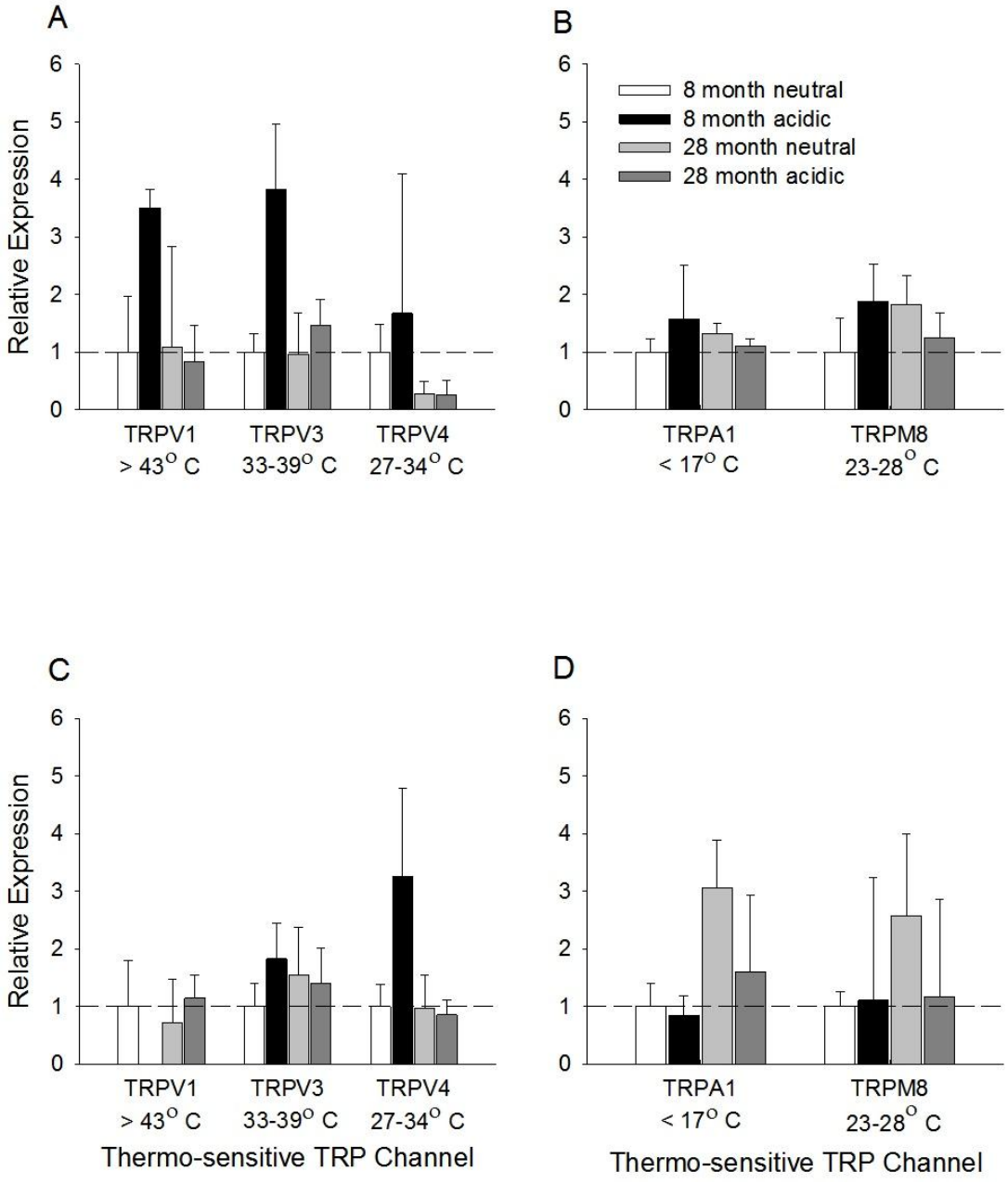

Figure 4-11. Relative expression of thermo-sensitive transient receptor potential channels after repeated saline injection. A) Ipsilateral heat sensitive channels. B) Ipsilateral cold sensitive channels. C) Contralateral heat sensitive channels. D) Contralateral cold sensitive channels. Expression levels are normalized to the 8-month old neutral saline group for each TRP channel on each side (ipsilateral and contralateral). Acidic saline injection had a greater effect on the heat sensitive TRP channels than the cold sensitive channels, with the greatest effects observed in the 8-month old acidic saline animals. The large levels of variability are due to small sample sizes.

CHAPTER 5
CONCLUSIONS AND FUTURE DIRECTIONS

The population in the United States is growing older and unfortunately, chronic pain is a common condition in older individuals (Bruckenthal et al., 2009; Colliver et al., 2006; McCarthy et al., 2009; Neville et al., 2008; Rustøen et al., 2005; Walid and Zaytseva, 2009). In conjunction with this, opioids are being prescribed more often for the treatment of chronic pain (Atluri et al., 2003; Bell et al., 2009; Benyamin et al., 2008; Brixner et al., 2006; Garcia del Pozo et al., 2008; Pergolizzi et al., 2008; Trescot et al., 2008b). This creates a situation where older individuals will be taking opioids in greater quantities for longer periods of time. Unfortunately, lack of a complete understanding of how aging affects both the effectiveness of opioids and chronic pain leads to under treatment of many aged individuals (Hutchinson et al., 2007; Lin et al., 2007b; Thomason et al., 1998; Wilder-Smith, 2005). The purpose of this study was to use animal models of both aging and chronic pain to assess the influence of aging on the development of chronic pain and the antinociceptive and secondary adverse effects of long-term opioid administration.

Previous studies in our lab had begun to assess the effects of increased age on opioid modulation of thermal sensitivity. The initial study assessed the effects of chronic fentanyl administration on reflexive tail withdrawal, and showed rapid development of tolerance to antinociceptive effects of fentanyl (Morgan et al., 2008). A second study assessed acute morphine antinociceptive effects using operant procedures. This study showed a decrease in heat sensitivity, but no effect on cold temperatures. Both of these studies compared adult (7- or 12-month old) to senescent (30- or 28-month old) animals. The studies within this project were designed to extend these findings by

assessing the age-related differences in the antinociceptive effects of chronic opioids using an operant procedure, assess chronic opioid effects across a greater set of ages, and to begin assessing age-related differences in chronic pain, so that future studies could assess the relationship between age, chronic pain, and opioids.

Overall, there were age differences within each of the studies in this project. The first two studies suggest that fentanyl is not an ideal analgesic drug for chronic administration in older individuals as older rats showed less antinociceptive response to chronic fentanyl administration, but greater weight loss. In the third study, older animals showed a slower development of mechanical sensitivity and no increase in mRNA levels of thermo-sensitive TRP channels compared to younger animals. While these results are not definitive on the relationship between age, opioids, and chronic pain, they do demonstrate the importance of research involving older animals and patients, as there are apparent differences between adult and aged individuals.

Drawbacks to Current Project

Although this project has highlighted some of the effects of aging on the development of chronic pain and responsiveness to long-term opioid administration, some drawbacks limit this project. The biggest drawback to this project is the lack of consistency in subjects across the studies. For each study, a different range of ages or different sex was used. Our initial studies examining responsiveness to acute administration of morphine used animals that were ~12- or 27 months of age. The first study described here focused on ages of animals that resemble the current age range of the “baby boomers”, while the second study sampled ages that covered adulthood through senescence as age-related changes may occur earlier in adulthood, and age-

related changes may continue to occur throughout late life. The third study compared the extremes by examining young adult and aged animals. Although it was useful to sample many different ages in these studies to assess where differences may occur, these differences make it difficult to compare results across the studies.

While the use of female animals in the final study was important as data suggest that chronic pain affects a greater portion of females than males (McCarthy et al., 2009), this difference made it difficult to compare the thermal preference results between the first and last study, and relate it to previous studies in the lab. However, females were used due to greater chronic pain rates in females (McCarthy et al, 2009), and the fact that the repeated acidic saline injection model of chronic pain had never been assessed in female rats. Using males in the final study would have allowed two more age groups to be assessed in the thermal preference procedure increasing the understanding of how age affects thermal sensitivity. However, by including both males and females in the project, differences between the sexes in thermal sensitivity were shown, demonstrating the need to investigate both sexes in future research.

Between the first and last study, different temperatures are also used for the thermal preference procedure. For the first study, the neutral temperature used was the temperature in the middle of the range tested, and the hot and cold temperatures were selected experimentally from the threshold testing as two temperatures that were equally aversive compared to the neutral temperature. However, the third study used temperatures for cold, neutral, and hot that was more in line with other thermal preference studies performed in the lab. These tests used a higher neutral temperature which is closer to the core body temperature of the rats (Gordon, 2008) and has shown

to be a preferred temperature in other studies in our lab. While these experimental differences make direct comparisons between the two studies difficult, the temperatures used are similar enough (27.5 vs. 30°C) that different results would not be expected if the same temperatures had been used between the two studies. This is further supported by other studies in the lab that assessed the effects of acute morphine on thermal preference using the temperature settings used in the third study. These studies showed that acute morphine also decreased sensitivity to the hot plate, but had no effect on preference for the cold plate. This study also showed that older male rats had a preference for warmer temperatures than younger rats, and these older animals had greater locomotor activity than younger rats. These results are all similar to results from first study suggesting that the temperature difference between the first and third study are not significant enough to change behavior. The results from this experiment were similar to those of the first study suggesting that these minor temperature differences are not enough to affect thermal preference in rats.

Although the studies within this project all investigate the effects of aging, they all used cross-sectional designs. While cross-sectional designs are useful to assess difference between ages, they have the potential to misrepresent aging effects due to group differences, since age is a between-subject variable in cross-sectional studies. While longitudinal studies may have a stronger experimental design, they require much longer periods to acquire data, and therefore are not always feasible. Furthermore, longitudinal studies investigating the types of questions examined here would necessarily include repeated drug administration (known to influence later drug sensitivity) and longer-term/repeated pain testing (which could influence subsequent

assessments). In terms of the thermal preference data, a recent longitudinal study of thermal sensitivity in female F344 x BN rats has shown similar age differences in thermal preference as this study has shown that older animals have a preference for warmer temperatures than younger animals do. These studies suggest that while a cross-sectional design may not be the most robust way to study aging effects, in terms of the thermal preference procedure, cross-sectional and longitudinal studies yield similar results.

Improvements to Current Project

Long-term research projects are often dynamic and rarely finish in the same manner as they were originally planned. While this fluidity is often a result of hypotheses being incorrect, or unexpected data opening new avenues for research, one can always retrospectively improve a project once it is completed. Although the numerous different ages that were assessed across the studies were useful to obtain a wider picture of aging, the lack of consistency across studies made comparisons difficult. This is especially apparent for the thermal preference data in the first and third studies. If this project were to be redone, it would be useful to match the ages for these two studies to allow for some comparison of thermal sensitivity in control animals across the sexes. Using three ages, such as 8-, 18-, and 28-months old, would allow for assessment of adult, middle-aged, and old animals without increasing subject numbers substantially. With these two studies using subjects of the same age, it would be logical to use the same ages for the second study. While this would be a reduction in age groups for this study, it would most likely not affect the results as the youngest age group would be even younger, there would still be representation in the middle of the age groups, and

the oldest age group would be two months younger, so the same age effects should be observed.

The other changes that should be made to this project if it were to be redone involve the third study. For this study, there were no changes in thermal sensitivity, even though there was an increase in thermo-sensitive TRP channel mRNA in younger animals. While this could be a result of there being no relationship between thermo-sensitive TRP channels and thermal sensitivity, it could also be due to the fact that changes in mRNA levels were only induced in one of four limbs in the rats. Therefore, changes in sensitivity within this one limb may not be enough to change overall behavior. If the study were to be repeated it would be useful to inject acidic saline into both a hind limb and a forelimb to see if this would be enough to alter behavior.

It would also be useful to have a more direct approach in testing changes in thermal sensitivity. Instead of retesting the three temperature comparisons used in this project, it could be more effective to select temperatures that have been shown to maximally activate each respective thermo-sensitive TRP channel and test those temperatures against a neutral temperature. Testing in this manner would be a more focused assessment of how changes in TRP channel expression may contribute to thermal sensitivity. It might also allow for the testing of a more noxious hot temperature that would activate the TRPV2 channel. This channel responds to noxious heat above 52°C (Venkatachalam and Montell, 2007), and was not discussed previously in this project, because the experiment was limited to a maximum temperature of 45°C. However, all thermo-sensitive TRP channels located within the DRG could then be tested for changes in sensitivity to acidic saline injection by testing a wider range of

temperatures. It should be noted however that temperatures greater than 45°C elicit a variety of unconditioned behaviors (paw licking and guarding) that were intentionally avoided in the present studies in order to optimize the possibility of selectively activating physiological systems thought to be involved to chronic pain states.

Future Directions

Short of redoing the current project to make the improvements mentioned above, there are other studies that could be conducted that build off the current project. While it is mentioned above that injecting both hind and forelimbs with acidic saline to possibly induce changes in sensitivity, it would also be interesting to assess if acidic saline into the hind limb can produce mechanical hyperalgesia in the forelimb. It is accepted that central sensitization occurs to produce bi-lateral hyperalgesia after acidic saline, although no one has assessed if this sensitization travels along the spinal cord to produce hyperalgesia at the forelimbs. This study would be easy to conduct, and may show age-related differences in the susceptibility to sensitization within the spinal cord, as measured by the degree of hyperalgesia observed in the forelimb. It is also important to assess changes in thermo-sensitive TRP channels at the protein level as opposed to the expression level. The current study used qRT-PCR to assess changes in mRNA levels, but these changes do not always correlate with increases in channel numbers. Assessing protein levels of thermo-sensitive TRP channels after acidic saline injection with Western blots could confirm changes in channel numbers.

Summary

Aging influences pain processing. Previous and current studies in the laboratory suggest that there are differences in sensitivity to thermal stimulation dependent on the

age of the animal. The effects of opioids depend on the age of the animal, and we have shown this following acute morphine and chronic fentanyl administration. The thermal sensitivity procedure used here is sensitive to age differences, and when compared to standard reflex-based assessments of pain sensitivity (e.g. tail withdrawal) shows a different profile of sensitivity to opioid administration (e.g. tolerance develops to the antinociceptive tail withdrawal response, however no tolerance develops to the “thermal sensitivity” response). Lastly, new models of chronic pain induction (i.e. the acidic saline intramuscular injection procedure) results in profound changes in mechanical sensitivity without dramatically altering thermal sensitivity, and results in an interesting transition from acute to chronic pain that may be useful for the development of novel pharmacological targets for the treatment of pain, especially in older subjects.

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BIOGRAPHICAL SKETCH

Jeremiah David Mitzelfelt was born in Lexington, Nebraska. As the middle of three children, he grew up mostly in Gypsum, Colorado, and graduated high school from Eagle Valley High School in 1998. He attended one year of college at the University of Puget Sound in Tacoma, Washington in 1998-1999 before taking time off to grow up and gain some direction. He enrolled in Regis University in 2002 and graduated with a Bachelor of Science. in honors in neuroscience from Regis University in 2005.

Upon earning his bachelor's degree, Jeremiah enrolled in the University of Florida Interdisciplinary Program in Biomedical Sciences Ph.D. program in August of 2005. During his studies, Jeremiah met his wife, Megan Greenlee, whom he married in October of 2010. After completing his Ph.D., Jeremiah started a post-doctoral research position at Emory University in Atlanta, Georgia under the mentorship of Dr. Shawn Hochman.