SOCIAL TRANSFER OF PAIN IN THE MOUSE

By

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GENERAL DISCUSSION

LIST OF ABBREVIATIONS

AAV8 – Adeno-associated virus, serotype 8; Viral vector for designer receptors transfection

CFA – Complete Freunds Adjuvant; Induces long lasting local inflammation in the hindpaw

CNO – Clozapine-N-oxide; Selectively binds to and activated designer receptors

Co-Housed – Groups that are individually housed within their own cages, but housed and tested in the same room as another experimental group

Diaz – Diazepam; Prototypical anxiolytic of the benzodiazepine family

DREADD – Designer receptors exclusively activated by designer drugs; Synthetic G-protein couple receptors exclusively activated by the synthetic ligand CNO

EtOH – Ethanol; Alcohol solution for drinking (3-10% v/v)

H_2O – Tap water for drinking; represents a “control” group

hM4Di –Designer receptor activating the Gi inhibitory pathway

Mor - Morphine

NLX - Naloxone

NoWD – No withdrawal, or continuous drinking

Olf – Olfactory; Mice that were housed in a room in which they received exposure to bedding from other mice

Veh – Vehicle; Vehicle treatment were “control” mice that were either housed in the same room as another experimental group or in a separate room

VF – Von Frey; Mechanical testing technique

WD – Withdrawal; For EtOH, WD sessions were 24 h, for Mor, WD was tested 48 h post injection

EXPERIMENTAL GROUP DESCRIPTIONS

References to group names are also described in the methods of each chapter. (alphabetical order)

CFA/Co-Housed – Complete Freunds Adjuvant (CFA); Mice that received a hindpaw injection that leads to long term local inflammation (CFA) and that were housed and tested in the same room as vehicle (PBS) treated bystander mice.

EtOH/Co-Housed/WD – Ethanol (EtOH), Withdrawal (WD); Mice that were given 24 h access to alcohol and water bottles with weekly 24 h sessions of forced abstinence. These mice were housed and tested in the same room as bystander mice drinking only water throughout.

EtOH/Co-Housed/NoWD - No Withdrawal (NoWD); Mice that were given continuous access to alcohol and water bottles without any sessions of abstinence. These mice were housed and tested in the same room as bystander mice drinking only water throughout.

EtOH/Co-Housed/WD-Veh – Vehicle (Veh); Mice in the “diazepam” experiment that were drinking alcohol and then treated with a vehicle injection during the second session of forced abstinence. These mice were housed in the same room as a group of bystander mice.

EtOH/Co-Housed/WD-Diaz – Diazepam (diaz); Mice in the "diazepam" experiment that were drinking alcohol and then treated with an injection of diaz during the second session of forced abstinence. These mice were housed in the same room as a group of bystander mice.

EtOH/Separate/WD – Mice that were given 24 h access to alcohol and water bottles with weekly 24 h sessions of forced abstinence, but were housed in a separate room without any other treatment groups.

H_2O/Co-Housed – Water drinking (*H_2O)*; "Bystander" mice with access to water only, but housed and tested in the same room as mice drinking alcohol and experiencing sessions of WD.

H_2O/Co-Housed/NoWD – "Bystander" mice with access to water only, but housed and tested in the same room as mice continuously drinking alcohol with no WD sessions.

H_2O/Olfactory-CTRL – Mice housed in a separate room and exposed to bedding from naïve colony animals.

H_2O/Olfactory-WD – Mice housed in a separate room and exposed to bedding from primary and bystander mice housed in an adjacent room.

H_2O/Co-Housed/Diaz – Bystander mice given a single injection of diazepam prior to the 2nd test session

H_2O/Co-Housed/Veh – Control bystander mice in the diazepam experiment given a single vehicle injection

H_2O/Separate – Control mice housed and tested in a separate, yet adjacent room as Co-Housed (primary and bystander) mice.

Mor/Co-Housed/WD – Primary mice that received 2 injections of morphine, and were tested during 2 sessions of withdrawal from morphine

Veh/Separate - Vehicle; A comparison group of mice that received vehicle treatments and were housed in a "separate" room to control for testing/experimental/housing procedures

Veh/Co-Housed - Vehicle; "Bystander" mice which received vehicle treatments, but were housed in the same room (Co-Housed) as mice that were subjected to noxious stimuli

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"It will feel better when it quits hurting." – Kyle Lusk

I think this quote is a perfect description of pain- in that, there is no clear explanation for when or why something hurts, no obvious reason for how much something hurts, and sometimes there is just no clear end in sight. You just have to keep going until it is over, you persist until you reach the end- which is a place you might not recognize until you are there. Sometimes grad school felt that way, especially while writing my dissertation.

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CERTIFICATION

School of Medicine

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CERTIFICATE OF APPROVAL

This is to certify that the PhD dissertation of
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ABSTRACT

Pain is a multifaceted process, with sensory, behavioral, and emotional components that can be dramatically influenced by psychosocial and environmental factors. One way to explore the complicated interactions among the psychological and social determinants of pain is through examination of pain communication. Pain communication has coevolved with affective circuits to guide actions to enhance fitness in social animals. Recognition of another's pain can lead to the avoidance of harm, or trigger empathy and caregiving behavior. The spectrum of pain behavior ranges from basic alarm cues to empathy, involving multiple sensory modalities.

The aim of these studies was to explore the social communication of pain and characterize the "social transfer of pain," a phenomenon in which the presence of "primary" animals experiencing hyperalgesia leads to congruent pain behavior in "bystander" animals that are housed and tested in the same room.

In chapter one of this dissertation I demonstrate the social transfer of hyperalgesia, when primary animals are subjected to persistent inflammation or withdrawal from opioids or alcohol. This socially transferred hyperalgesia is demonstrated in mechanical, thermal or chemical nociceptive tests. I also show that the transfer is mediated by olfactory cues, does not involve visually dependent emotional contagion, and cannot be explained as stress-induced hyperalgesia. In chapter two, I explore some of the neural mechanisms responsible for the expression of hyperalgesia in bystander mice using the alcohol withdrawal paradigm. Bystander mice demonstrate enhanced Fos expression within the anterior cingulate (ACC) and anterior insula (AI),

whereas the primary mice show a marked increase in Fos in the dorsal medial hypothalamus (DMH). Inactivation of the ACC via inhibitory Gi-coupled designer receptors exclusively activated by designers drugs (DREADDs), but not the somatosensory cortex reverses the expression of hyperalgesia in both primary and bystander mice.

Together, these studies further our understanding of the social modulation of pain, and demonstrate that hyperalgesia can be induced solely by social factors, in the absence of tissue damage. In addition, my data show that an abnormal pain state can result from voluntary alcohol drinking in the mouse. Finally, these studies suggest that the common practice of housing control animals in the same room with experimental animals may not be appropriate because of the possibility of social transfer of physiological states.

INTRODUCTION

Understanding Pain

"[Pain finds itself] halfway between the world of emotions and the realm of sensations." (Moscoso, 2012)

The word 'pain' can be used to describe a wide variety of experiences and processes, including everything from the description of the physiological response to injury, to an emotional reaction to psychological distress. The inherently complex nature of the experience and description of pain has created long-standing difficulties regarding the investigation of pain and its mechanisms. Pain has long been described as an essential and inevitable part of the human condition, and the consideration and investigation of pain can be dated back to some of the earliest philosophers. For example, Galen (130 - circa 200 AD) theorized that pain was strictly a physical sensation involving "violent irritation of nerves" (Siegel, 1970), and Descartes (1596-1650 AD) proposed that pain is a "disturbance [caused by injury that] passes along the nerve until it reaches the brain" (Descartes & Hall, 1972). This thinking reflected dualist approaches that conceptualized the mind and body as functioning independently, and many of the methods for studying pain have focused solely on the physical aspects ("body") of the experience. By contrast, Aristotle (384–322 BC) considered pain as an emotion rooted in the heart (Gross, 1995), and it has even been argued that, "'Pain' is how we name and perceive an event, a feeling, a sensation. Pain is not the event, feeling, or sensation itself" (Bourke, 2014). This latter description implies that pain is not a physiological event, but our perception and communication of the experience. Despite

centuries of research, the true nature of pain has not been precisely conceptualized, and such polarized views leave our understanding of the mechanisms of pain lacking.

Historically, the formulation of the gate control theory of pain in the mid 20th century (Melzack, 1965) encouraged the view that pain is a psychological phenomenon that can be modulated by peripheral and central nociceptive systems, and should be treated with biomedical *and* psychosocial interventions. In fact, the contemporary definition of pain used by the International Association for the Study of Pain (IASP) is based upon the multidimensional definition proposed by Melzack & Casey (1968) which includes sensory-discriminative, affective-motivational and cognitive-evaluative dimensions. Specifically, the IASP defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Merskey & Bogduk, 1994). This definition highlights the importance of both the sensory and emotional components of pain, while also including the concept that tissue damage is not necessary for the experience of pain. Perhaps this definition is imperfect, but importantly it posits that the experience of pain is much more than just a biophysical response to injury. It is now a commonly accepted that pain is a multifaceted process having sensory, behavioral, cognitive, and emotional components that are dramatically influenced by psychosocial and environmental factors. Research exploring environmental, social, and psychological factors involved in the pain experience in humans and animal models is on the rise, though attention to biological factors governing pain has previously dominated research, especially in animal models.

Nociceptive Processing

"Pain and suffering are always inevitable." (Dostoevsky, 1866)

"Nociception" refers to the processing of afferent information related to tissue damage, and "nociceptive neurons" are neurons that respond to tissue damage . Nociception is distinguished from "pain," which is a sensory experience that may or may not be linked to tissue damage. Three major processes must be taken into account when considering nociception: *transduction*, *transmission*, and *modulation*.
Primary afferent nociceptors have cell bodies in the dorsal root ganglion (DRG) adjacent to the spinal cord, and the first order nociceptive neurons. Primary afferents are described as "pseudo-unipolar," with one process extending peripherally to innervate the tissue (skin, viscera or deep tissues), and the other running centrally to synapse onto second order neurons in the dorsal horn of the spinal cord. Primary afferent nociceptors fall into the so-called "A-delta" and C-fiber classes. Aδ fibers are lighty myelinated and have a role in rapid pain sensations that can be described as "sharp." C fibers are smaller in diameter, unmyelinated, and contribute to slower, "dull" or "burning" sensations.

Transduction refers to the activation of the peripheral terminal by damaging or potentially damaging (noxious) stimuli. This information is then *transmitted* from the periphery into the central nervous system. Second order nociceptive neurons in the dorsal horn decussate and transmit information into the brain via ascending pathways, such as the spinothalamic and spinoreticular tracts. The spinothalamic tract is thought to be the major ascending pathway. It is somatotopically organized, arising from

neurons in laminae I, IV and V of the dorsal horn. The thalamus then projects to the cortical areas such as the somatosensory cortex and the anterior cingulate cortex, areas which are thought to deal with the sensory discriminative and affective aspects of the pain experience, respectively (Applebaum, Leonard, Kenshalo, Martin, & Willis, 1979; Kenshalo & Isensee, 1981; Kenshalo & Perkins, 1984; Rainville, 2002).

Transmission of nociceptive information through the spinal cord is subject to bidirectional *modulation* via descending controls exerted by brainstem structures that project to the dorsal horn. One structure important in pain modulation is the periaqueductal grey (PAG), which received input from cortical areas (prefrontal cortex, anterior cingulate cortex). Neurons in PAG express opioid receptors and play an important role in opioid analgesia. The PAG influences the dorsal horn through a relay in the rostroventromedial medulla (RVM), which is capable of facilitating or inhibiting spinal nociceptive processing via specialized cell populations (Heinricher et. al., 2009). This system exerts descending control, leading to analgesia or hyperalgesia depending upon competing behavioral priorities and homeostatic demands. For example, the PAG is responsible for opioid-mediated inhibition of nociceptive inputs, and thus acts within an endogenous pain inhibitory system, particularly during extreme stress (Bolles & Fanselow, 1980). On the other hand, during inflammation and chronic opioid exposure descending facilitation of spinal nociception via this PAG-RVM system leads to hyperalgesia (for review, *see*: Heinricher et. al., 2009)

The brain is an active receiver of nociceptive signals, and is capable of modulating the perception of pain. Certain pain experiences are dependent upon the

transduction and transmission of nociceptive signals to the brain, where a dynamic interplay between pain inhibition and facilitation occurs and leads to perception of acute and chronic pain. This modulation occurs as dictated by behavioral, environmental and physiological priorities. Therefore, it is possible for nociception to occur within the periphery and/or at the level of the brain, *without* the presence of pain due to descending inhibition. Conversely, pain often occurs *without* the activation of nociceptors, for example, neuropathic pain arises from within the nerve itself, rather than from direct stimulation of nociceptors. Additionally, many patients report persistent pain in the absence of any obvious injury, including hypersensitivity to normally innocuous stimuli (e.g., fibromyalgia, chronic idiopathic pain; Phillips & Clauw, 2011).

Pain and Affective systems

"The lower animals, like man, manifestly feel pleasure and pain, happiness and misery." (Darwin, 2003)

Pain can act as a warning of actual or potential injury, and is beneficial in motivating escape and defensive behavior. From an evolutionary perspective, it can be argued that pain acts as a mechanism to increase the probability of survival (Melzack, 1973). For example, humans with a congenital insensitivity to pain have decreased life expectancies (Nagasako, Oaklander, & Dworkin, 2003) suggesting that the inability to experience pain has a selective disadvantage. It has been hypothesized that pain is adaptive because there is increased fitness for those who experience and suitably respond to tissue damage or modify behavior to facilitate healing or avoid further

damage (Wall, 2000). However, adaptive value depends on the physical and social context. Moreover, not all pain is adaptive. Chronic pain, for example, rarely has a protective function.

The ability to engage in reflexive withdrawal from noxious stimuli is phylogenetically continuous from invertebrates to humans. For example, basic avoidance of noxious stimuli is seen in crustaceans (Barr & Elwood, 2011), signaling the capacity for anticipatory behavior and fear learning in this species. Behaviors motivated by emotional arousal evolved earlier than those driven by complex cognitive capacities, allowing animals to rapidly evaluate threatening and aversive stimuli and respond in the most adaptive manner. Accordingly, pain has developed in conjunction with affective circuits to guide actions according to the organism's priorities (Barkow, Cosmides, & Tooby, 1995). Arguably, the most debilitating component of pain is the aspect of unpleasantness and/or suffering, and this emotional state demands the attention of the sufferer and can act as a strong motivator to guide behavior.

In sum, the relationships between self-preservation, pain, and affect function through a series of nested evolutionary processes, which are subject to social and environmental contingencies.

Biopsychosocial Model of Pain

"When a person screams in pain, the actual pain is only half the noise they make. The other half is the terror at being forced to accept that they exist." (Cicero, 2006)

George Engel (1977) is credited as one of the first to emphasize the importance of simultaneously addressing biological, psychological and social dimensions of illness. This model has come to be known as the “biopsychosocial model” of pain, and is now the most widely accepted philosophical approach for the investigation and treatment of pain. This theoretical framework considers the perception of pain as a subjective interpretation of nociceptive processing based upon an array of factors, including an individual’s genetic composition, psychological status, cognitive functioning and social environment. From this perspective, pain is not a secondary symptom of tissue pathology nor is the amount of pain experienced proportional to tissue damage (Wall, 1979). This conceptual model highlights the importance of assessing the sensory-discriminative, cognitive-evaluative and motivational features of pain. Disentangling these three components can be difficult, as they are interdependent (for review, *see*: Gatchel, et. al., 2007). In sum, the biopsychosocial approach has been recognized as essential to fully understand and treat pain, though most neuroscientific investigation has focused on the biological aspects of the pain experience.

Social Communication of Pain

“Pain always has a specific language, whether it is a cry, a sob, or a tensing of the features, and it is a language in itself as well.” (Rey, 1995)

It has been postulated that in order to properly evaluate pain, one must also consider the emotional *response*, and the elicited behavior used to *communicate* the pain, distress, and suffering (Loeser 1982). Accordingly, it has recently been posited

that the biopsychosocial model may be better understood when investigated in regard to pain communication (Hadjistavropoulos, et. al, 2011). Recognition of another's pain can lead to the avoidance of harm, or trigger empathy and caregiving behavior. Pain behaviors have evolved to act as a social signal, which can benefit not only the object of the pain, but the social group as a whole. The ability to communicate the presence of pain and respond to another's pain could have a genetic component. Pain communication can occur both automatically and at levels requiring purposeful cognitive processing (K. D. Craig, 2015; Hadjistavropoulos & Craig, 2002) and ranges from basic alarm cues to empathy. In fact, pain communication has been defined as "inclusive of actions that may or not be intentionally sent, as well as the intentional and unintentional reactions of receivers to the cues or signs" (Hadjistavropoulos et al., 2011) and responses depend upon social and environmental context. To simplify the investigation of such processes, the social communication of pain can be broken down into component parts, including the expression of pain by an individual and the perception and interpretation of this information by an observer.

Like the experience of pain, expression of pain has emerged throughout the course of animal evolution and is conserved in humans. Though there are many uniquely human adaptations in the communication of pain, including verbal communication, much can be learned from other social mammals. The same selective pressures supporting communication that brought about the development of language (Pinker, 1997) would have existed to communicate information by nonverbal means, including facial expression (Fridlund, 1991) and body movement (e.g., guarding). It has

been suggested that these two types of nonverbal pain behaviors can be categorized as communicative and protective, respectively (Sullivan, 2008). Any expression of pain aimed at conveying information to an observer can be considered communicative pain behavior, whereas protective pain behaviors are aimed at reducing further injury or supporting healing. In fact, protective pain behaviors may be functionally distinct from communicative pain behaviors, but can have a communicative influence if they are received by others. Thus, the way that pain is expressed may serve different functions for the individual, but still act as a social cue depending upon the circumstances.

The social communication of pain goes beyond just the expression of pain by an individual, and must include some sort of interaction with an observer. To best understand something as multifaceted as the social communication of pain, theories of communication must be considered. Three types of communication exist (Duck & McMahan, 2011) that may give insight into pain behaviors (Hadjistavropoulos et al., 2011): *Communication as action*, in which a message is sent or received, *communication as interaction* where messages are sent, received and interpreted, and finally, *communication as transaction*, in which messages are exchanged. These types of communications are not restricted to humans, but occur in other species, although the mode of communication may differ in its modality or level of complexity. Such communicative actions or transactions may occur automatically/reflexively or in an intentional/controlled manner for both the subject expressing the pain and/or the observer reacting to the signal. Reflexive actions have been studied in rodents (A. D. Craig, 2009; K. D. Craig & Prkachin, 1983; Prkachin & Craig, 1979), and humans

(Hadjistavropoulos & Craig, 2002) and have been shown to serve as a form of pain communication, leading to reactions in observers.

The expression of pain is also perceived by observers, representing more than just communication as action. Thus, the communication of pain must be elicited via some signaling system (Plesker & Mayer, 2008), emitted from the sender and interpreted by the receiver, and may employ multiple sensory modalities. For example, both auditory and visual cues have been shown to mediate the social transfer of fear, in which an “observer” rodent views a “demonstrator” receive a painful electric shock paired with a conditioned stimulus (CS; e.g., tone), and later demonstrates freezing behavior to the CS (E. J. Kim, Kim, Covey, & Kim, 2010) or to the learning context (Q. Chen, Panksepp, & Lahvis, 2009; Jeon et al., 2010). Specifically, Chen et. al (2009) found that observers immediately orient to the demonstrator as the shock was being applied (indicative of the importance of visual cues), and orient to playbacks of conspecific distress vocalizations that occur in response to shock (indicative of the importance of auditory cues). These studies demonstrate communication as interaction, in that, visual and auditory cues about a painful experience are sent by demonstrators and received and interpreted by observers. In these studies, pain is communicated via an auditory signal, and leads to observational learning. The fact that rodents are able to detect and respond to the affective state of their social partners is viewed as a form of empathy (Panksepp & Lahvis, 2011), defined as “the generation of an affective state more appropriate to the situation of another compared to one's own” (Hoffman, 1975).

Social communication of pain has also been explored in the form of “emotional contagion,” an endophenotype of empathy, which can be defined as a reflexive behavioral process where the perception of a behavioral change in an individual appears to automatically activate the same process in another individual (Panksepp & Lahvis, 2011). In a foundational study, Langford and colleagues (2006) demonstrated communication of pain as a transaction, and showed that when pairs of mice are given identical noxious stimuli and tested together, they display increased pain behaviors compared to being tested alone or with another mouse that has not received the noxious stimulus. This “social modulation of pain” was dependent upon visual cues and the familiarity of the dyads. These findings have been extended with the recent observation that mice housed for several weeks in the same cage as conspecifics subjected to peripheral nerve injury exhibit enhanced responding in the acetic acid-induced writhing test (Baptista-de-Souza et al., 2015). This behavior appeared to represent a form of stress-induced hyperalgesia (Jennings, Okine, Roche, & Finn, 2014a), but the sensory channel mediating this social communication of pain was not investigated. These studies indicate that the presence of a conspecific in pain can have a physiological and behavioral effect through social cues.

Visual cues have thus been demonstrated to communicate pain in mice. However, other sensory modalities are likely to play a role as well. For example, it has been demonstrated that olfactory cues can act as the channel of social communication. In mice, chemical signaling contained in excretory products can convey individuality, age and disease status (Beauchamp & Yamazaki, 2003), and facilitate the formation

and maintenance of social relationships (Hurst, 1990). For example, exposure to chemical cues from tumor-bearing mice leads to behavioral and neuroimmune changes in cagemates (Alves, Ribeiro, & Palermo-Neto, 2012). In humans, fear-related chemosignals can influence associative learning (D. Chen, Katdare, & Lucas, 2006; Prehn, Ohrt, Sojka, Ferstl, & Pause, 2006). Thus, the social modulation of behavioral and physiological states through chemical communication is well demonstrated. Olfactory communication of pain has not been studied extensively. However, rats will display analgesia following exposure to olfactory chemosignals from a conspecific that had received an electric shock (Fanselow, 1985a), indicating the activation of endogenous pain control mechanisms following a social-olfactory cue. Neuropathic pain behavior can also be altered simply by co-housing with rats exhibiting high levels of neuropathic pain behavior following nerve injury (Raber & Devor, 2002). These observations demonstrate olfactory cues, like visual cues, communicate information capable of altering nociceptive responding.

To summarize, pain can serve as a social cue and may be communicated via visual, olfactory, or chemical channels. These cues may or may not be intentionally sent, and may lead to intentional and unintentional reactions of receivers. The method of communication likely depends upon the species, context and level of pain.

Neural Mechanisms of Social Communication of Pain

"Not even one's own pain weighs so heavy as the pain one feels with someone, for someone." (Kundera, 2004)

A large body of research has documented the reliable activation of a distributed brain network (often labeled the “pain matrix”) that relays nociceptive information from the spinal cord to the cerebral cortex through cooperating ascending and descending pathways (Price, 2000; Rainville, 2002). As discussed above, nociceptive signals are received by subcortical regions, such as the thalamus, amygdala and nucleus accumbens, as well as cortical regions including the somatosensory cortices, insula, anterior cingulate and prefrontal areas (Dubé et al., 2009; Price, 2000; Rainville, 2002; Staud, Craggs, Robinson, Perlstein, & Price, 2007). These areas project down to the periaqueductal grey, brainstem, and spinal cord to form descending pathways. As previously mentioned, pain is much more than just the perception of nociceptive inputs and neurophysiological activation during pain may therefore represent sensory discriminative, cognitive, emotional, or social aspects of the experience. In fact, it has been shown that attention (Rainville, Carrier, Hofbauer, Bushnell, & Duncan, 1999a; Rainville, Hofbauer, Paus, Duncan, Bushnell, & Price, 1999b), emotion (Apkarian, Bushnell, Treede, & Zubieta, 2005), and expectation (Koyama, McHaffie, Laurienti, & Coghill, 2005) can even alter the magnitude of brain activation during an acute noxious stimulus.

Based upon brain imaging, anatomical and electrophysiological studies, multiple pain-related regions have been proposed as important for different aspects of the pain experience. For example, one set of brain regions is consistently linked to the discriminative aspects of pain “sensation,” whereas a divergent, yet [possibly] overlapping neural circuit is linked to the “emotional” aspects of the pain experience

(Bingel et al., 2004; Peyron et al., 1999; Rainville, Duncan, Price, Carrier, & Bushnell, 1997). It has been suggested information about sensory information related to stimulus location and intensity is processed via a spinothalamic pathway projecting to the somatosensory cortices, as these areas (or cells within these areas) have been demonstrated to be active during noxious stimulation in humans and monkeys (S1/S2; (M. C. Bushnell, Ceko, & Low, 2013; Chudler, Anton, Dubner, & Kenshalo, 1990; Kenshalo & Isensee, 1981; Kenshalo & Perkins, 1984; Kenshalo & Isensee, 1983). On the other hand, the anterior cingulate (ACC) is thought be a part of the neural circuitry that regulates the emotional aspects of pain, as it is a part of the classic limbic system and has been consistently been shown to be activated during pain (Hutchison, Davis, Lozano, Tasker, & Dostrovsky, 1999; Lenz et al., 1998). More specifically, it has been suggested that the ACC is critical to assessing the salience and affective quality of pain (Downar, Crawley, Mikulis, & Davis, 2002; Downar, Mikulis, & Davis, 2003) whereas the insula may be important for perception of and attention to a variety of aversive cues (Mériau et al., 2009; A. Simmons, Matthews, Stein, & Paulus, 2004). Furthermore, lesions of S1/S2 in humans have been shown to produce to deficits in pain localization and sensation (Ploner, Freund, & Schnitzler, 1999a), whereas lesions of the ACC lead to reductions in the emotional components response to pain or reported “unpleasantness” of pain (Foltz & White, 1962; Hebben, Corkin, Eichenbaum, & Shedlack, 1985). Despite the apparent localization of different aspects of pain, the sensory and affective components of pain are generally highly correlated and it is difficult to disentangle distinct neural mechanisms.

The process of pain communication begins with the individual's perception of the pain, activation of pain neural circuitry and expression of a pain signal that is communicated to observers. According to some investigators, similar neural circuits are activated during a behavior and during observation of that same behavior in another (Jackson & Decety, 2004; Jackson, Meltzoff, & Decety, 2005; Preston & de Waal, 2002). Observation-related activation can be conceptualized as "priming" the regions involved in the actual behavior to elicit rapid response and facilitate learning. Accordingly, the receipt of a pain signal from another individual leads to a neurophysiological reaction in the observer. In fact, observation of another's pain is sufficient to activate portions of the pain matrix such as the ACC and anterior insula (AI; (Botvinick et al., 2005; Jackson, Brunet, Meltzoff, & Decety, 2006; Saarela et al., 2007; Singer et al., 2006; 2004), depending upon the environment and context of the interaction. For example, one study demonstrated that perception of another's pain led to activation of the ACC, and the amount of activation positively correlated with the intensity of the pain observed, as rated by the observer (Jackson et al., 2005). Additionally, the ACC and AI have been shown to be activated in humans when a participant receives an electric shock ("self") *and* when they see a cue indicating that someone else ("other") is receiving shock (Singer et al., 2004; 2006). The AI may play an important role in the perception of, and attention to, the aversive aspects of pain communication, as it has been shown to be activated by disgusting odors and facial expressions of disgust/pain (Krolak-Salmon et al., 2003; Phan, Wager, Taylor, & Liberzon, 2002; Phillips et al., 1997; Wicker et al., 2003). Although the perception of

pain in self and other share neural commonalities, these experiences likely require distinct subregions that do not perfectly overlap (Morrison & Downing, 2007).

Dissertation Studies

"Pain is the root of knowledge." (McCullough, 2014)

To sum up the literature and theories discussed above, environmental, social, and psychological (reflective, higher level cognitive) factors are recognized to be integral to the pain experience in humans and non-human animals. Pain and affective systems are closely interrelated and it is probable that they developed to promote safety and survival. One way in which these systems work in concert is through social communication: in mammals, the expression of pain behavior can act as a social cue when observed by others in the social environment, leading to behavioral reactions that vary from avoidance to helping. Evidence also suggests that the expression and observation of pain may lead to activation of similar, but potentially divergent neural circuitry. However, the direct study of the social communication of pain has largely been restricted to humans and/or has been conducted and interpreted in terms of understanding endophenotypes of empathy.

The current studies were designed to explore the social communication of pain. The first chapter of this thesis tests whether the presence of "primary" animals experiencing hyperalgesia alters nociceptive responding of "bystander" animals that are housed and tested in the same room, but not subjected to any initial noxious stimulus. I observed that bystanders display hyperalgesia congruent with that experienced by

primary animals subjected to persistent inflammation or withdrawal from opioids or alcohol, as tested by mechanical, thermal or chemical modalities. I then found that the transfer of this hyperalgesia is communicated by olfactory cues, does not involve visually mediated emotional contagion, and cannot be explained as stress-induced hyperalgesia. Chapter Two of this thesis investigated the potential neural mechanisms involved in the social transfer of hyperalgesia. Fos immunoreactivity indicated differential activation of the ACC, AI and the dorsal medial hypothalamus in primary and bystander animals. I then investigated whether the ACC was required for the expression of socially transferred hyperalgesia via chemogenetic inactivation.

CHAPTER 1: Characterization of Social Transfer of Pain in the Mouse

"As the pain sweeps through / Makes no sense for you" David Bowie (1986)

(This chapter has been reformatted for inclusion in this dissertation from: Smith, M.L., Hostetler C.M., Heinricher, M.M. & Ryabinin, A.E. (2016; *in revision*) Social Transfer of Pain in Mice. *Science Advances*; Contributions: Caroline Hostetler analyzed corticosterone samples and Alfredo Zuniga collected behavioral data in handling induced convulsion experiment)

Abstract

A complex relationship exists between the psychosocial environment and the perception and experience of pain, and the mechanisms of the social communication of pain have yet to be elucidated. The present study examined the social communication of pain, and demonstrates that “bystander” mice housed and tested in the same room as mice subjected to inflammatory pain or withdrawal from morphine or alcohol develop corresponding hyperalgesia. Olfactory cues mediate the transfer of hyperalgesia to the bystander mice, which can be measured using mechanical, thermal and chemical tests. Hyperalgesia in bystanders does not co-occur with anxiety or sensory hyper-reactivity, and cannot be explained by visually dependent emotional contagion or stress induced hyperalgesia. These experiments reveal the multifaceted relationship between the social environment and pain behavior, and support the use of mice as a model system for investigating such factors. Additionally, these experiments highlight the need for proper consideration of how experimental animals are housed and tested.

Pain is both a sensory and emotional experience, and is dramatically influenced by psychosocial and environmental factors (Hadjistavropoulos et al., 2011; Krahé, Springer, Weinman, & Fotopoulou, 2013; Loggia, Mogil, & Bushnell, 2008). Clinically significant chronic pain often manifests in the absence of tissue damage, yet most investigations of the neural mechanisms governing these disorders rely upon activation of nociceptive pathways with a noxious stimulus, and are only beginning to consider social influences. Like humans, rodents are capable of complex social behaviors, and increasing evidence suggests that social and environmental variables also impact pain responsiveness in these species (Fanselow, 1985b; Raber & Devor, 2002; Sorge et al., 2014).

Pain is an adaptive process that can serve as a warning of actual or potential injury, enhancing the survival of the individual and its social group. As a social cue, recognition of another's pain can lead to the avoidance of harm, or trigger empathy and caregiving behavior. The communication of pain is a complex process, and the spectrum of this behavior ranges from basic alarm cues to empathy, involving multiple sensory modalities. The social communication of pain has been explored in the form of emotional contagion, and previous studies have demonstrated the importance of visual and auditory cues in certain contexts. For example, these foundational studies have demonstrated that the presence of a familiar conspecific responding to an acute noxious stimulus or in an ongoing pain state can modulate the response of a test animal responding to the same noxious input, with enhanced (Langford et al., 2006; Z. Li et al., 2014; Raber & Devor, 2002) or diminished (Gioiosa, Chiarotti, Alleva, & Laviola, 2009)

pain behaviors, depending on the experimental paradigm. Visual cues are thought to play a primary role in mediating this transfer, with paired animals displaying synchronous pain behaviors described as “emotional contagion” (Langford et al., 2006). These findings have been extended with the recent observation that mice housed for several weeks in the same cage as conspecifics subjected to peripheral nerve injury exhibit enhanced responding in the acetic acid-induced writhing test (Baptista-de-Souza et al., 2015). This behavior appeared to represent a form of stress-induced hyperalgesia (Jennings, Okine, Roche, & Finn, 2014b), since the cagemates of the nerve-injured animals demonstrated changes in behavior on the elevated plus maze and in the open-field test, that are thought to represent anxiety-like behavior.

The current studies were designed to further explore the social communication of pain and test whether the presence of “primary” animals experiencing hyperalgesia affects “bystander” animals that are housed and tested in the same room, but not subjected to any initial noxious stimulus. We observed that bystanders display hyperalgesia congruent with primary animals subjected to persistent inflammation or withdrawal from opioids or alcohol as tested by mechanical, thermal or chemical modalities. The transfer of this hyperalgesia is mediated by olfactory cues, does not involve visually dependent emotional contagion, and cannot be explained as stress-induced hyperalgesia

Results

Hyperalgesia in “bystander” mice housed in the same room as mice subjected to persistent inflammation or undergoing opiate withdrawal

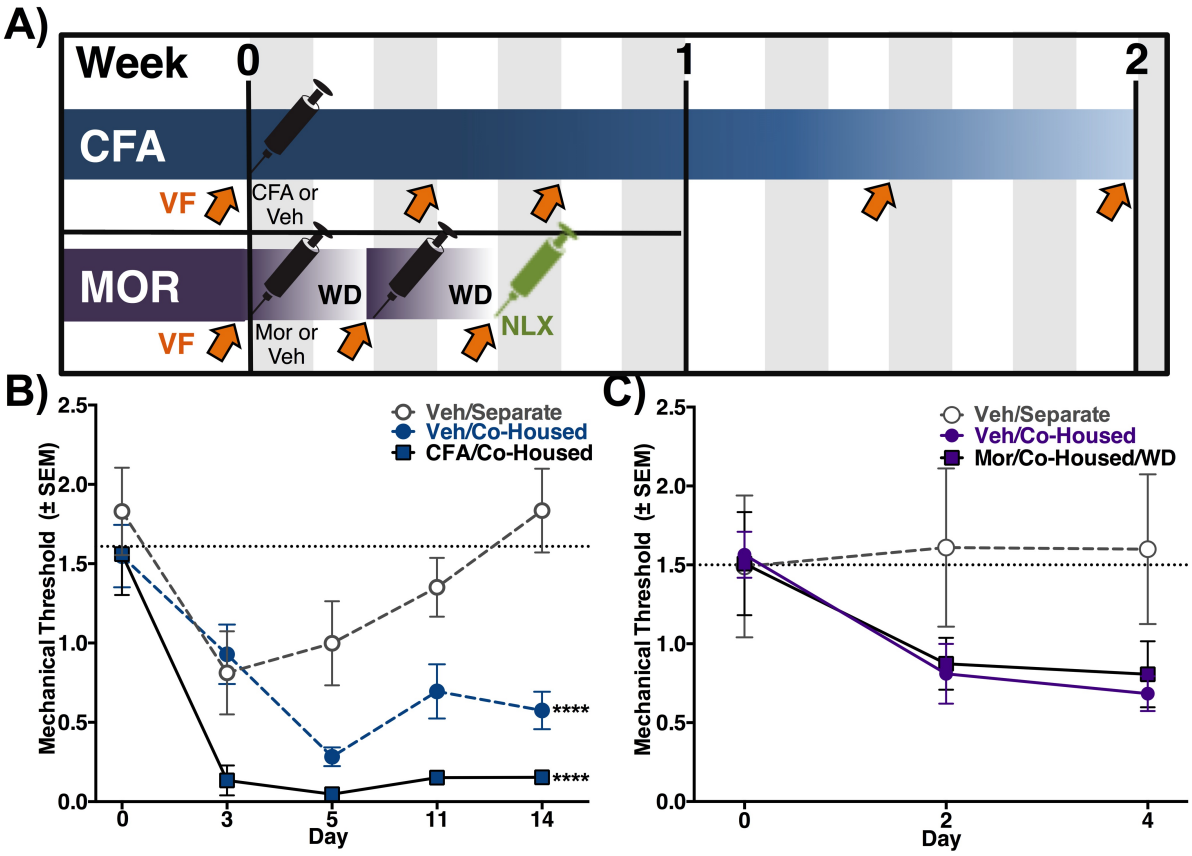
To investigate the effect of the social environment on nociceptive behavior, we conducted experiments in which mice were either: housed and tested in the same room as mice that received a persistent noxious stimulus (Co-Housed) or housed and tested in a separate room (Separate). All mice were individually housed in cages with wire cagetops and assessed at several timepoints for mechanical responsiveness using calibrated von Frey filaments applied to the plantar surface of the left hindpaw. In this first experiment, following testing for basal mechanical thresholds, PBS (vehicle; Veh), or Complete Freund's Adjuvant (CFA) was injected into the plantar surface of the tested paw (Fig 1.1A). CFA is well known to induce long-lasting, localized inflammation and hyperalgesia (Hylden, Nahin, Traub, & Dubner, 1989; Iadarola, Brady, Draisci, & Dubner, 1988). Injection of Veh led to modest hypersensitivity that resolved by the third test session in mice housed in their own separate room (Veh/Separate; Fig 1.1B). As expected, CFA-treated animals demonstrated a robust and persistent mechanical hypersensitivity for the entire two-week timecourse (CFA/Co-Housed; Fig 1.1B). However, mice injected with PBS but housed in the same room as the CFA-injected mice (Veh/Co-Housed) *also displayed pronounced hypersensitivity* that was evident for the two weeks (Fig 1.1B). This experiment indicates that "bystander" mice housed in the same room as mice experiencing CFA-induced hypersensitivity exhibit congruent hypersensitivity.

To determine the generalizability of this acquired hypersensitivity in bystander mice, we examined the potential for the transfer of alternate hyperalgesic states. Hyperalgesia is known to occur during opiate withdrawal, and therefore we investigated

the ability of bystanders to acquire hypersensitivity when housed and tested in the same room as primary mice experiencing morphine withdrawal-induced hypersensitivity. Mechanical sensitivity was assessed during two sessions of spontaneous withdrawal from morphine (48 h after injection; *see* Fig 1.1A). Accordingly, immediately after the baseline test, a subcutaneous injection of morphine base (300 mg/kg; Mor/Co-Housed/WD) or vehicle in a sustained-release emulsion (Veh/Co-Housed/WD) was given. The first mechanical test occurred 48 h later, immediately followed by the second injection of Mor or Veh. This treatment regime has been demonstrated to induce profound physical dependence in mice (Bagley, Chieng, Christie, & Connor, 2005; Bellchambers, Chieng, Keay, & Christie, 1998; Chieng & Christie, 1996). Two days after each injection, withdrawal from morphine led to evident hypersensitivity compared to basal mechanical thresholds or to vehicle-treated mice housed in a separate room (Veh/Separate; Fig 1.1C). As in the previous experiment, vehicle-treated mice that were housed in the same room as mice experiencing hyperalgesia also demonstrated significant mechanical hypersensitivity (Veh/Co-Housed/WD; Fig 1.1C). To confirm that the morphine-treated mice developed dependence, naloxone (NLX; 10.0 mg/kg i.p.) was given 24 h after the final test session. This dose of NLX precipitated withdrawal in morphine-treated mice, leading to jumping, wet dog shakes and paw tremors (Fig S1.1), confirming that this dose of morphine is sufficient to induce physical dependence. This experiment indicates that the transfer of hyperalgesia from primary experimental mice to vehicle-treated bystanders housed in the same room is not specific to inflammatory stimuli, but can also be demonstrated during morphine WD-induced hyperalgesia.

Figure 1.1: Social transfer of CFA and morphine withdrawal-induced pain

A) Experimental timeline of experiments presented in panels B and C. B) Mice subjected to intraplantar CFA injection showed a robust and persistent decrease in mechanical sensitivity for all test sessions (CFA/Co-Housed; n = 8) compared to Veh injected mice housed in a separate room (Veh/Separate; n = 8). Veh-injected mice housed in the same room as CFA-injected mice (Veh/Co-Housed; n = 8) demonstrated significantly decreased mechanical thresholds compared to Veh/Separate mice during the last 3 test sessions. This resulted in significant differences between groups ($F_{2,21}$ = 30.0, p<0.0001) across time ($F_{4,84}$ = 27.6, p<0.0001), and a significant interaction between these variables ($F_{8,84}$ = 9.1, p = 0.003) according to repeated measures ANOVA. C) Co-housed mice injected with either a slow release morphine emulsion (Mor/Co-Housed/WD; n = 7) or vehicle emulsion (Veh/Co-Housed; n = 8) every other day demonstrated significant decreases in mechanical thresholds on the two test sessions compared to vehicle-injected mice housed in a separate room (Veh/Separate; n = 7). Repeated measures ANOVA showed a significant effect of treatment ($F_{2,19}$ = 7.4, p = 0.004), and a significant effect of time ($F_{2,38}$ = 5.7, p = 0.006). Following a significant interaction, Bonferroni posthoc analyses were conducted, and differences compared to control according to are represented by (*).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Veh/Separate
Veh/Co-Housed
Mor/Co-Housed/WD
Mechanical Threshold (± SEM)
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1.0
0.5
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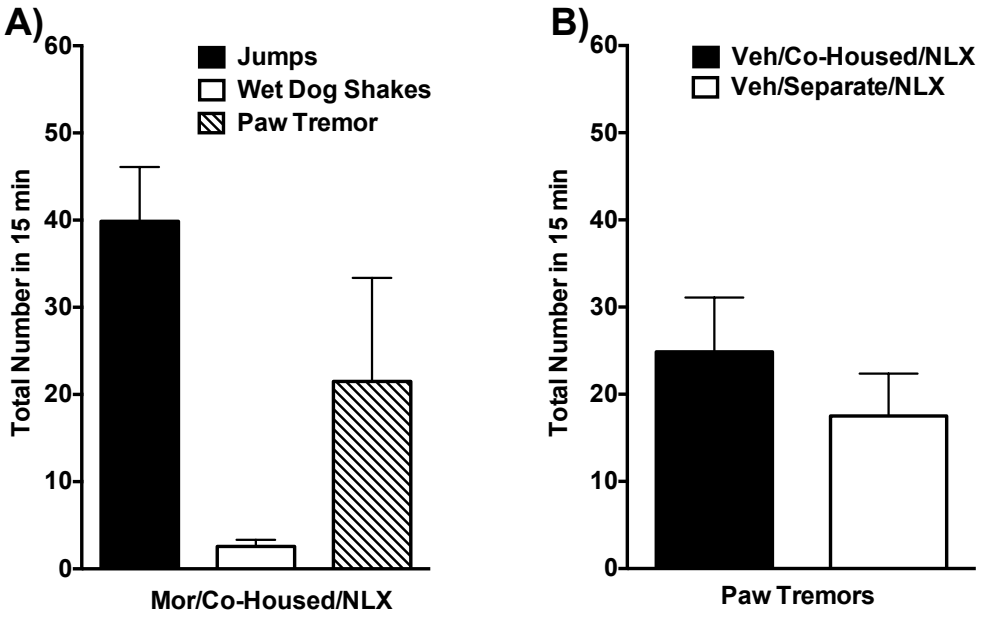
0
2
4
Day

Supplementary Figure S1.1: Naloxone precipitates withdrawal behaviors in morphine treated mice

Opiate withdrawal induced behaviors, such as A) Jumping, wet dog shakes and paw tremor in morphine treated mice immediately following the final test session and injection of naloxone (NLX; 10.0mg/kg, i.p.). B) Paw tremors were also evident in vehicle treated mice.



Hyperalgesia in "bystander" mice housed in the same room as mice undergoing alcohol withdrawal

If transfer is a general phenomenon that occurs with any hyperalgesic state, it should be seen in conditions in which the treatment does not specifically target pain-transmission (CFA) or pain-modulation (morphine) systems. We therefore tested animals undergoing alcohol withdrawal, since hyperalgesia and spontaneous pain are well documented during alcohol withdrawal in humans, although understudied in rodents (Apkarian et al., 2013; Egli, Koob, & Edwards, 2012). Thus, we used a standard voluntary drinking protocol to test whether alcohol withdrawal would lead to hypersensitivity in alcohol-withdrawn and control (water-drinking) mice housed in the same room. We exposed mice to a 24 h-access 2-bottle choice drinking procedure (Giardino, Cocking, Kaur, Cunningham, & Ryabinin, 2011; Smith, Li, & Ryabinin, 2014). In the initial experiment, mice were individually housed in cages with wire tops containing water and introduced to increasing concentrations of ethanol (EtOH, 3-10% v/v) with weekly 24 h sessions of imposed abstinence from ethanol (Withdrawal; WD; Fig 1.2A). Mice given ethanol (EtOH/Co-Housed/WD) voluntarily drank 9.4 ± 0.9 g/kg/day (mean ± SEM; Table S1.1), and were housed and tested in a room with ethanol-naïve control mice drinking only water (H_2O/Co-Housed). Additional ethanol and water-drinking control groups were individually housed and tested in separate rooms (EtOH/Separate/WD and H_2O/Separate groups, respectively). Basal nociceptive thresholds were determined at the beginning of the protocol and each group was tested weekly thereafter (Fig 1.2A).

At the end of the first session of abstinence, mice in the EtOH/Co-Housed/WD group exhibited significant mechanical sensitivity relative to baseline (Fig 1.2B). This hypersensitivity was maintained in subsequent withdrawal sessions, and mechanical thresholds were decreased by 68 ± 2% relative to baseline (mean ± SEM) at the third withdrawal session. Notably, H_2O/Co-Housed mice demonstrated equivalent hypersensitivity by the second week of testing, and demonstrated an overall 62 ± 2% decrease at the third and final test session (Fig 1.2B). Animals drinking ethanol but housed in a separate room without a water-drinking group (EtOH/Separate/WD) also displayed significant hypersensitivity during withdrawal (Fig 1.2B). However, control mice that drank only water and were housed without an ethanol group in the same room (H_2O/Separate) did *not* develop hypersensitivity at any point (Fig 1.2B).

We repeated this experiment in female mice, and found that similar to males, females developed significant hypersensitivity during alcohol withdrawal (EtOH/Co-Housed/WD; Fig 1.2C). Again, congruent mechanical sensitivity was also observed in water-drinking control mice housed in the same room (H_2O/Co-Housed/WD), and in this case, mechanical thresholds exhibited by the bystanders were significantly lower than those displayed by the primary mice experiencing alcohol withdrawal. As with males, female mice drinking water but housed in a separate room maintained stable mechanical thresholds for the 3 weeks of testing (H_2O/Separate; Fig 1.2C). These data demonstrate that, following voluntary drinking in both male and female mice, episodes of acute withdrawal lead to reduced mechanical thresholds in both alcohol-withdrawn mice and in water-consuming control mice housed in the same room.

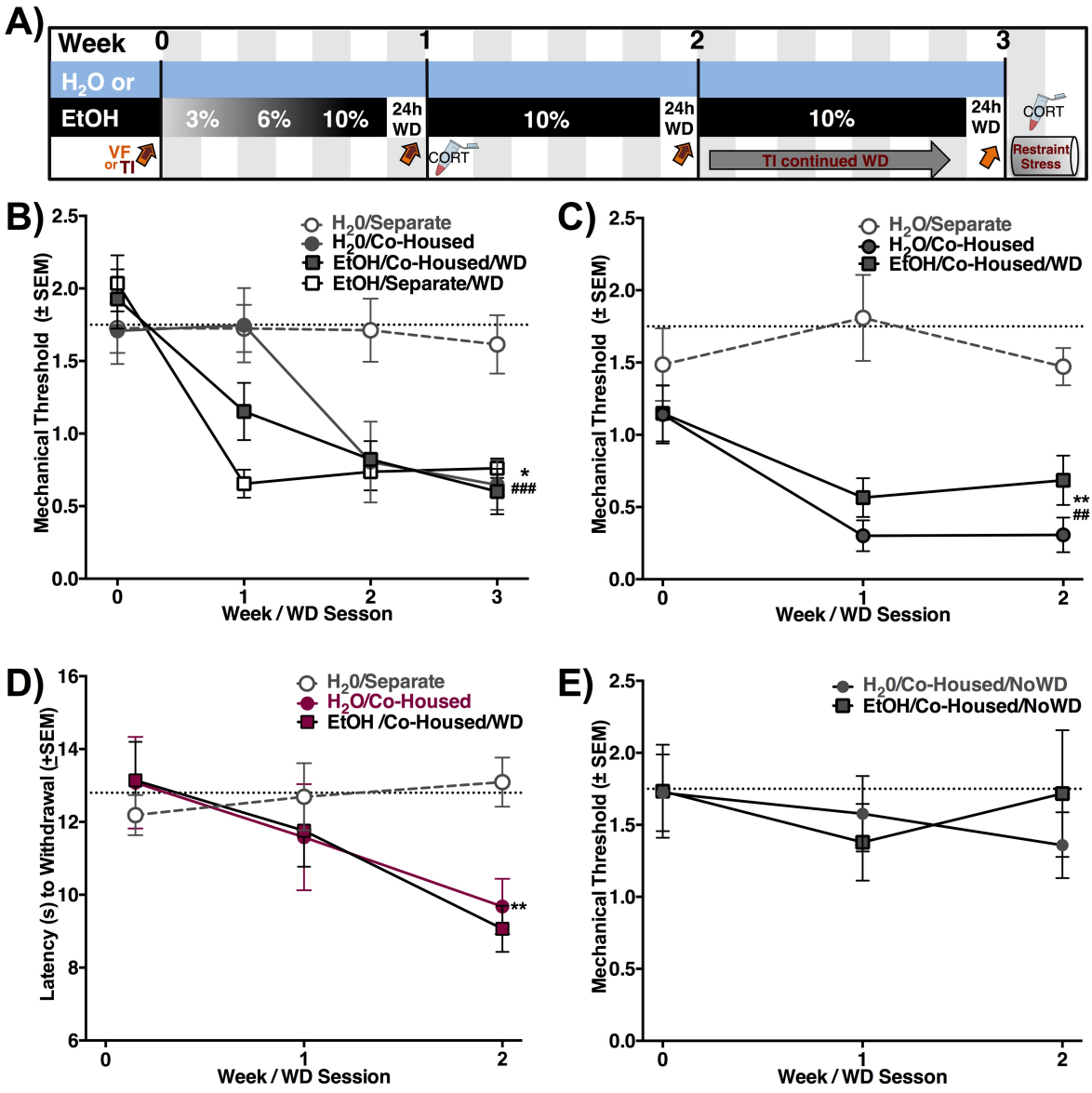
To further investigate nociceptive responsiveness in this paradigm, we assessed thermal sensitivity in an additional set of male mice by immersing the tips of their tails into a 46 °C water bath. As with mechanical thresholds, both the EtOH/Co-Housed and H_2O/Co-Housed groups demonstrated significantly decreased withdrawal latencies compared to H_2O/Separate mice by the second 24 h withdrawal session (Fig 1.2D). Thus, alcohol-withdrawn and bystander mice display abnormal responses to non-noxious mechanical *and* thermal stimuli.

Additional experiments were conducted to further characterize hyperalgesia in both the primary (alcohol-exposed) and bystander (water-drinking) mice. First, we verified that the mechanical hypersensitivity in the Co-Housed groups was related specifically to *withdrawal* from ethanol, and not merely the consumption of ethanol, or presence of ethanol-related olfactory and/or behavioral cues. In this experiment, we gave an independent set of mice constant ethanol access (EtOH/Co-Housed/NoWD). Neither this group, nor water-drinking mice housed in the same room (H_2O/Co-Housed/NoWD), displayed changes in mechanical sensitivity at any point (Fig 1.2E). The lack of changes in nociceptive thresholds indicate that alcohol-drinking mice are not primarily demonstrating alcohol-related neuropathy (Chopra & Tiwari, 2012) at these time points, as the displayed hypersensitivity is contingent upon withdrawal. These data further indicate that the hypersensitivity displayed by water-drinking mice cannot be attributed to the odor of alcohol, presence of alcohol metabolites, or the cues related to behavioral intoxication in the alcohol-drinking mice. Thus, the behavior in both groups is specific to the hypersensitivity experienced during alcohol withdrawal.

Figure 1.2: Social transfer of alcohol withdrawal (WD)-induced mechanical sensitivity to nearby water drinking controls

A) Experimental timeline of experiments presented in panels B-E. Von Frey (VF); Ethanol (EtOH, 3-10% v/v). B) Ethanol drinking (EtOH/Co-Housed/WD; n = 14 males/group) mice demonstrate a significant decrease in mechanical thresholds following 1 WD session that is matched by water drinking control mice housed in the same room (H_2O/Co-Housed; n = 10 males) by week/WD session 2. Ethanol drinking control mice housed in an adjacent room (EtOH/Separate/WD; n = 12 males) also demonstrate enhanced mechanical sensitivity between 1-3 Weeks/WD sessions. Water-drinking mice in an adjacent room (H_2O/Separate; n = 14 males) display stable mechanical thresholds across the timecourse. Repeated measures ANOVA comparing mechanical sensitivity of male mice over time revealed significant main effects of week ($F_{3,138} = 26.16$, $p<0.0001$), treatment ($F_{3,46} = 6.69$, $p = 0.0008$, and a significant interaction $F_{9,138} = 4.97$, $p<0.0001$. Bonferroni posthoc analysis revealed significant differences between H_2O/Separate and: H_2O/Co-Housed, EtOH/Co-Housed/WD and EtOH/Separate/WD. C) In a separate experiment utilizing female mice (n = 7-8/group), H_2O/Separate (n = 8) mice never significantly deviated from baseline. Interestingly, both Co-Housed groups demonstrated decreased mechanical thresholds during the first and second WD sessions, with the Bystander group (H_2O/Co-Housed; n = 7) reaching the lowest level. Repeated measures ANOVA demonstrated significant main effects of treatment ($F_{2,19} = 13.0$, $p = 0.0003$), week ($F_{2,38} = 7.1$, $p<0.002$), as well as a significant interaction ($F_{4,38} = 4.4$, $p<0.005$). Bonferroni posthoc analysis revealed significant

differences between H_2O/Separate and: H_2O/Co-Housed and EtOH/Co-Housed/WD. D) When tested for thermal sensitivity by immersing the tail into a hot water bath, Co-Housed EtOH (n = 8) and H_2O (n = 8) mice demonstrate significantly shorter withdrawal latencies on the second WD session compared to H_2O/Separate mice according to one-way ANOVA on the second WD session ($F_{2,21} = 9.8$, $p = 0.001$). E) Ethanol mice with continuous access/no withdrawal sessions (EtOH/Co-Housed/NoWD; n = 7), and H_2O mice housed in the same room (H_2O/Co-Housed/NoWD; n = 7) did not demonstrate any alterations in mechanical sensitivity following 2 weeks of ethanol exposure. There were no significant differences between groups according to repeated measures ANOVA ($p>0.05$). Significant changes ($p<0.05$) from baseline according to Bonferroni posthoc analyses are represented by (#). Mean basal responses of all groups represented by dotted line (---). Significant differences compared to control ($p<0.05$) are represented by (*).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Latency (s) to Withdrawal (±SEM)
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H20/Co-Housed/NoWD
EtOH/Co-Housed/NoWD
Mechanical Threshold (± SEM)
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Supplementary Table S1: Average mechanical thresholds and alcohol intake

Overall average decreases in mechanical thresholds of all alcohol-withdrawn mice and mean alcohol intakes (v/v; ±SEM) by concentration.

Experiment	Length	Room	Average Decrease in Mechanical Threshold (%)	Mean Alcohol Intake (g/kg) by Concentration			Mean Alcohol Preference by Concentration		
				3%	6%	10%	3%	6%	10%
1	3 weeks	Co-Housed	58.04 ± 7.3%	2.63 ± 0.37	5.69 ± 0.61	9.44 ± 0.89	0.48 ± 0.06	0.51 ± 0.06	0.6 ± 0.2
		Separate	-	2.98 ± 0.65	4.05 ±0.88	7.85 ± 0.55	0.5 ± 0.08	0.53 ± 0.02	0.75 ± 0.09
2-4	2 weeks	Co-Housed	79.1 ± 7.0%	2.53 ± .321	4.35 ± 0.53	8.51 ± 1.02	0.679 ± 0.09	0.72 ± 0.64	0.71 ± 0.08

Hyperalgesia is communicated to bystanders via olfactory cues

The lowered nociceptive threshold exhibited by the bystander mice suggests that these mice acquired hypersensitivity due to cues within the social environment. To determine the sensory channel mediating this communication, we utilized the alcohol withdrawal paradigm, and assessed the ability of olfactory cues to provoke hyperalgesia. Accordingly, a group of naïve animals housed in a separate room were exposed to bedding from the primary and bystander (Co-Housed) mice. That is, following a single session of withdrawal, and daily for the next week (during drinking and the second withdrawal session; Fig 1.3A), small amounts of bedding from EtOH/Co-Housed/WD and H_2O/Co-Housed mice, which both displayed hypersensitivity, were placed in empty cages without cagetops in a separate room containing control mice (H_2O/Olfactory-WD). Exposure to bedding from the hypersensitive Co-Housed mice induced significant mechanical hypersensitivity in the otherwise treatment-naïve mice within 24 h (H_2O/Olfactory-WD; Fig 1.3B). This hypersensitivity cannot be attributed merely to cues associated with novel mouse bedding, as exposure to bedding from unfamiliar, but experimentally naïve mice had no effect on the behavior of a separate group of water-drinking mice housed in an adjacent room (H_2O/Olf-CTRL; Fig 1.3B). This finding demonstrates that olfactory cues released into the social environment by mice experiencing hyperalgesia are sufficient to rapidly provoke congruent hypersensitivity in nearby mice.

Alcohol withdrawn and bystander mice demonstrate non-synchronous hyperalgesia during the formalin test

To further confirm that the abnormal nociceptive responsiveness in alcohol-withdrawn and bystander mice represents hyperalgesia, we administered a noxious chemical stimulus to mice that had previously demonstrated mechanical hypersensitivity. Therefore, at the completion of the mechanical testing, subsets of mice from previous experiments (n = 6-8; Fig 1.2D & 1.3B) were subjected to the formalin test (Dubuisson & Dennis, 1977; Tjølsen, Berge, Hunskaar, Rosland, & Hole, 1992). Briefly, formalin was injected into the plantar surface of the hindpaw, and nocifensive paw-licking behavior was quantified during the two phases of the formalin test. A low concentration of formalin (1.5%) was used to avoid ceiling effects. We found that all groups that previously displayed mechanical hypersensitivity (EtOH/Co-Housed/WD, H_2O/Co-Housed, and H_2O/Olf-WD) also exhibited enhanced nocifensive responding in the second phase of the formalin test compared to controls, which had exhibited normal mechanical thresholds (EtOH/Co-Housed/NoWD and H_2O/Co-Housed/NoWD). The latter groups had been directly or indirectly exposed to ethanol, but never experienced withdrawal or been housed with animals undergoing withdrawal (Fig 1.3C). Socially transferred hyperalgesia is thus observed across three distinct modalities of nociception (chemical, thermal, and mechanical).

In order to test whether the mice exhibited visually-dependent emotional contagion during the formalin test, we examined the synchrony of nocifensive behaviors of mice tested within the same sessions (Langford et al., 2006). We estimated whether

licking behavior was correlated across time within groups of 6-8 mice tested within proximity of each other (Fig S1.2A). Licking behavior among animals tested together was not synchronized, and the between-subject variance was comparable to that of randomly grouped mice (Fig S1.2B). This analysis indicated that these mice do not exhibit synchronized behavior during testing.

Hyperalgesia in alcohol withdrawn and bystander mice does not occur in conjunction with a state of ongoing anxiety or altered corticosterone levels

To determine whether the hypersensitivity exhibited by the H_2O/Co-Housed or EtOH/Co-Housed/WD mice was dependent upon a state of generalized anxiety, or could be described as stress-induced hyperalgesia (Imbe, Iwai-Liao, & Senba, 2006; Jennings, Okine, Roche, & Finn, 2014b), we conducted several independent experiments. First, we examined behavior on the elevated plus maze (EPM), one of the most widely utilized measures of anxiety-like behavior (Rodgers & Dalvi, 1997). The EPM consisted of two white open arms (anxiety-producing) and two black opaque high-walled arms, and the amount of time spent in each of these areas was recorded, as reported by our lab previously (Weitemier & Ryabinin, 2005). During the second WD session, no differences were observed between the groups in any measure on the EPM (Fig 1.3D, Fig S1.3). The lack of differences suggests that the hypersensitivity displayed by both groups at this timepoint does not occur in conjunction with a state of ongoing anxiety.

In the next experiment, we treated groups of H_2O/Co-Housed and EtOH/Co-Housed/WD mice with a prototypical anxiolytic (diazepam, Diaz; 1.0 mg/kg) or vehicle

(Veh) prior to the second mechanical test session. Diazepam had no effect on mechanical threshold in any group (Fig 1.3E), although this dose of diazepam was sufficient to reverse another phenotype (handling-induced convulsions) triggered by acute ethanol withdrawal in a separate group of mice (Fig S1.4), and has previously been shown to reverse anxiety-like behavior on the EPM in C57BL/6J mice (Paterson, Iwunze, Davis, Malekiani, & Hanania, 2010). The inability of diazepam to alter the hypersensitivity exhibited in either the primary mice undergoing alcohol withdrawal or the bystander mice further argues that the presence of anxiety is not necessary for the presentation of hyperalgesia in either group.

To determine whether the hypothalamic-pituitary (HPA) axis was activated during alcohol-induced or socially transferred hyperalgesia, we examined plasma corticosterone levels (CORT; Table 1.1) at several timepoints. Blood was taken immediately after sacrifice following the final mechanical test session, and there were no differences in plasma CORT levels between groups of mice sacrificed at the end of the 1st or 3rd withdrawal sessions, or following 8 days of extended withdrawal (Table 1.1). The lack of altered plasma CORT indicates that activation of the HPA axis is not the primary underlying mechanism for the abnormal pain behavior exhibited by either mice experiencing alcohol withdrawal or socially influenced bystanders. Because acute measurement of CORT does not assess stress-responsivity in these mice, we tested the CORT response to 30 min of restraint stress following 8 days of extended withdrawal (Fig 1.2A). As expected, all groups displayed an enhancement in CORT in response to restraint stress, but there were no differences between Co-Housed and the

H_2O/Separate groups in the CORT response (Fig 1.3F).

Taken together, these experiments indicate that although Co-Housed mice demonstrate mechanical, thermal and chemical hyperalgesia, it is not dependent upon a state of concurrent anxiety or simultaneous activation of the HPA axis, and does not lead to long-term adaptations in the stress response.

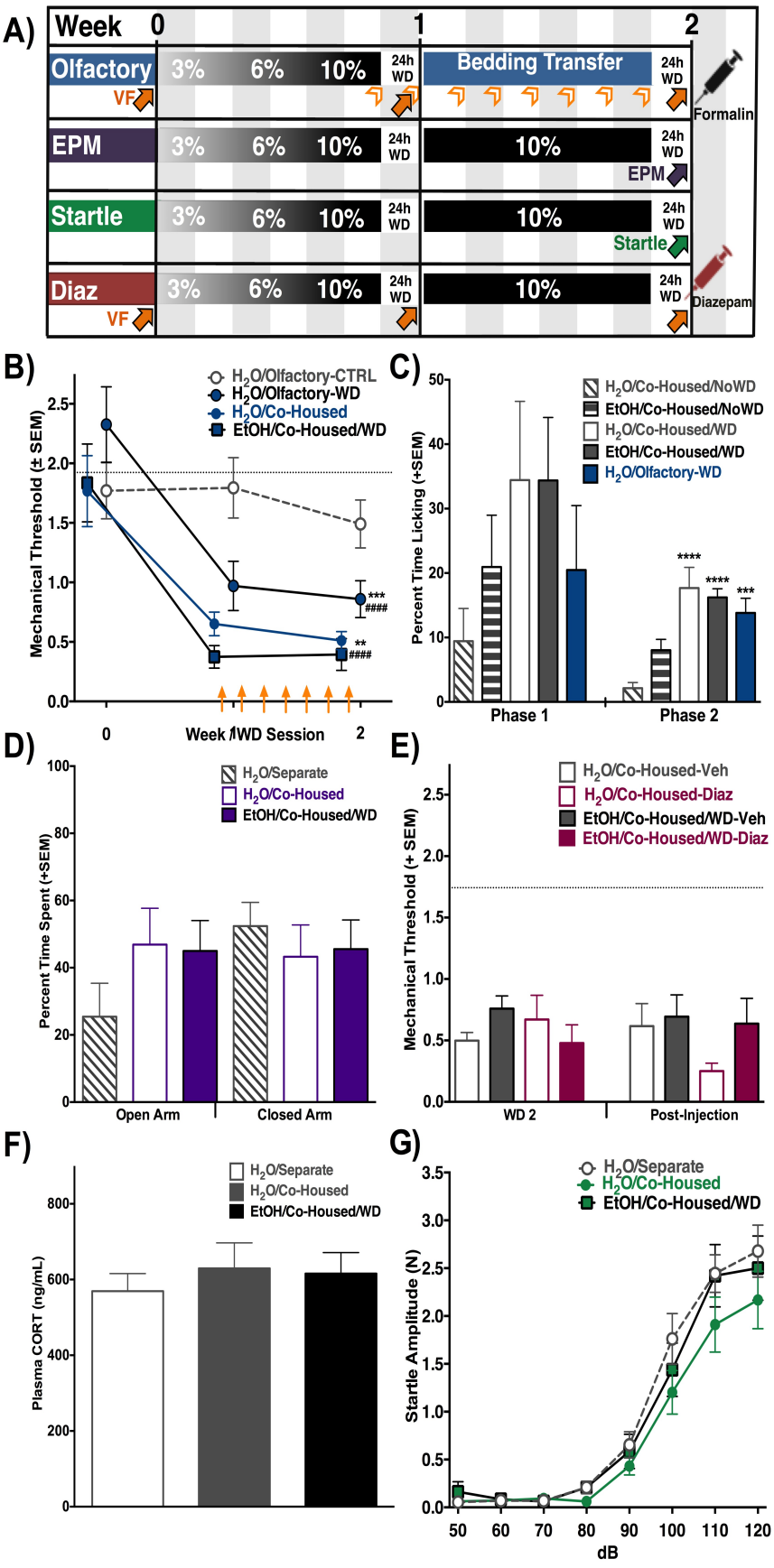
Alcohol withdrawn and bystander mice demonstrate normal responses to acoustic startle

Finally, it could be theorized that EtOH/Co-Housed/WD and H_2O/Co-Housed/WD display hyper-reactivity to novel stimuli across multiple sensory systems (e.g., auditory). To investigate this possibility, we examined acoustic startle responses as a measure of hyperacusis (Hayes, Radziwon, Stolzberg, & Salvi, 2014) and sensory hyper-reactivity. The acoustic startle procedure consisted of exposure to 18 trials of 60-120 dB tones in 10 dB increments in random order, with variable inter-trial intervals. There were no differences between any of the groups in acoustic startle responses (Fig 1.3G), indicating that EtOH-withdrawn and bystander mice do not demonstrate hyperacusis, or an exaggerated response to a novel, startling stimulus. This finding reveals the specificity of this phenotype to pain-related systems and argues against an overall sensory hyper-reactivity.

Figure 1.3: Social transfer occurs via alcohol-withdrawal-specific olfactory cues and this state leads to chemical and thermal hyperalgesia

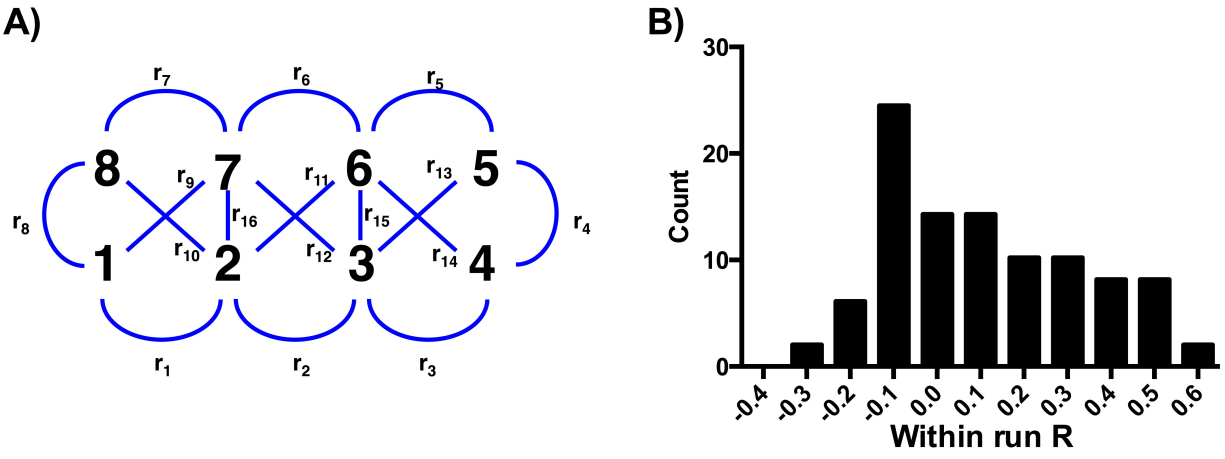
A) Experimental timeline for panels B-G. B) When a group of mice housed in a separate room (H_2O/Olfactory-WD; n = 8) was exposed to bedding from the cages of H_2O/Co-Housed (n = 9) and EtOH/Co-Housed/WD (n = 8), they demonstrated significant decreases in mechanical thresholds within 24 h. Mice exposed to bedding from naïve water drinking mice maintained baseline levels of sensitivity (H_2O/Olfactory-CTRL; n = 16). H_2O/Co-Housed and EtOH/Co-Housed/WD mice began the experiment 1 day prior to H_2O/Olfactory-WD mice, and transfer of bedding is represented by orange arrows. Repeated measures ANOVA revealed a significant effect of treatment ($F_{3,37} = 7.3$, $p = 0.0006$) and test session ($F_{2,74} = 26.7$, $p < 0.0001$), as well as a significant interaction ($F_{6,74} = 3.3$, $p = 0.0068$). C) The mechanical hypersensitivity in groups of mice from the Olfactory experiment (H_2O/Co-Housed, EtOH/Co-Housed and H_2O/Olfactory-WD) and the NoWD experiment (Fig 1D) manifests as hyperalgesia following a low concentration (1.5%) formalin, in a pattern that was significant during the second phase of the formalin test according to one-way ANOVA ($F_{4,30} = 10.19$, $p < .0001$). D) There were no significant differences in the percent of time spent on closed or open arms for any group (H_2O/Separate, n = 9; EtOH/Co-Housed, n = 9; and H_2O/Co-Housed, n = 9) according to ANOVA ($p > 0.05$). E) H_2O/Co-Housed (n = 14) and EtOH/Co-Housed/WD (n = 14) were treated with diazepam (Diaz; 1.0mg/kg; n = 7) or vehicle (Veh; n = 7) 20 min prior to the second von Frey test. Diazepam had no effect on mechanical thresholds in any group, according to ANOVA ($p > 0.05$). F) According to a two-way ANOVA comparing treatment

groups, there were no changes (p>0.05) between groups (n = 8/group) following 30 min of restraint stress on the 8th day after recovery from hyperalgesia (behavioral data in Fig 2D). G) Acoustic startle responses did not differ between Co-Housed (n = 8/group) and Separate (n = 8) mice according to repeated measures ANOVA (p>0.05). Significant changes (p<0.05) from baseline according to Bonferroni posthoc analyses are represented by (#). Significant differences compared to control (p<0.05) are represented by (*). Mean basal responses of all groups represented by dotted line (---).


A)
Week 0
1
2
Olfactory
3%
6%
10%
24h WD
Bedding Transfer
VF
Formalin
EPM
10%
EPM
Startle
Startle
Diaz
Diazepam
B)
H2O/Olfactory-CTRL
H2O/Olfactory-WD
H2O/Co-Housed
EtOH/Co-Housed/WD
Mechanical Threshold (± SEM)
Week 1WD Session
C)
H2O/Co-Housed/NoWD
EtOH/Co-Housed/NoWD
H2O/Co-Housed/WD
EtOH/Co-Housed/WD
H2O/Olfactory-WD
Percent Time Licking (+SEM)
Phase 1
Phase 2
D)
H2O/Separate
H2O/Co-Housed
EtOH/Co-Housed/WD
Percent Time Spent (+SEM)
Open Arm
Closed Arm
E)
H2O/Co-Housed-Veh
H2O/Co-Housed-Diaz
EtOH/Co-Housed/WD-Veh
EtOH/Co-Housed/WD-Diaz
Mechanical Threshold (+ SEM)
WD 2
Post-Injection
F)
H2O/Separate
H2O/Co-Housed
EtOH/Co-Housed/WD
Plasma CORT (ng/mL)
G)
H2O/Separate
H2O/Co-Housed
EtOH/Co-Housed/WD
Startle Amplitude (N)
dB

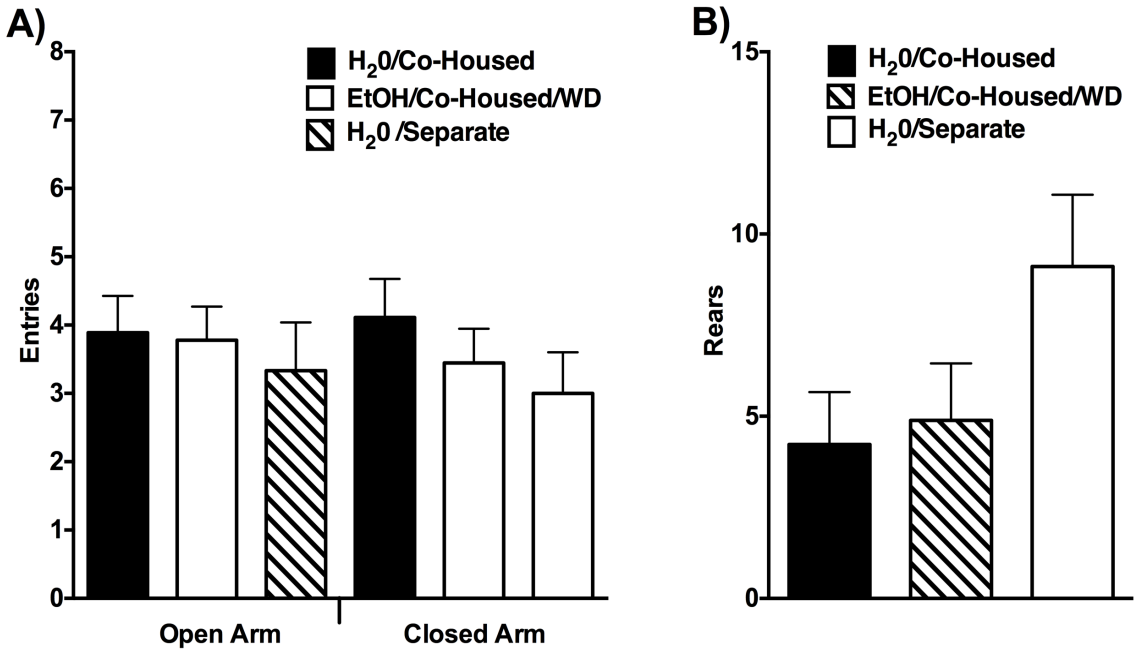
Supplementary Figure S1.2: Non-synchrony of nocifensive behavior in primary and bystander mice

To determine the synchrony of nocifensive behavior during the formalin test, correlations were conducted on the 5-8 mice that were given formalin and tested next to each other at the same time. Correlations were run on mice with visual access to each other during the text (resulting in 5-19 correlations per run). A) Representative example of 16 correlations that were conducted for 8 mice given formalin in the same run. B) A histogram of correlative values of nocifensive behavior calculated between mice during all runs. These correlations were used to calculate a grand average correlation (R = 0.107), which was no different from that of the randomly paired mice (permuted data; R = 0.108).



Supplementary Figure 1.3: No differences in behavior on elevated plus maze

There were no differences between groups (n = 9/group) in A) open or closed arm entries or B) rearing according to ANOVA ($p > 0.05$).



Supplementary Figure 1.4: Diazepam attenuates handling induced convulsions (HIC) following acute EtOH withdrawal

Six hours after an injection of 4 g/kg EtOH (i.p.), an injection of 1.0 mg/kg (i.p.) attenuated HICs. According to a repeated measures ANOVA there were significant differences between groups ($F_{1,7} = 46.06$, $p = 0.0003$), across time ($F_{12,84} = 33.09$, $p < 0.0001$), and a significant interaction ($F_{12,84} = 20.17$, $p < 0.0001$). Bonferroni posthoc revealed significant differences between groups ($p < 0.05$) following Diazepam injection, as represented by (*).

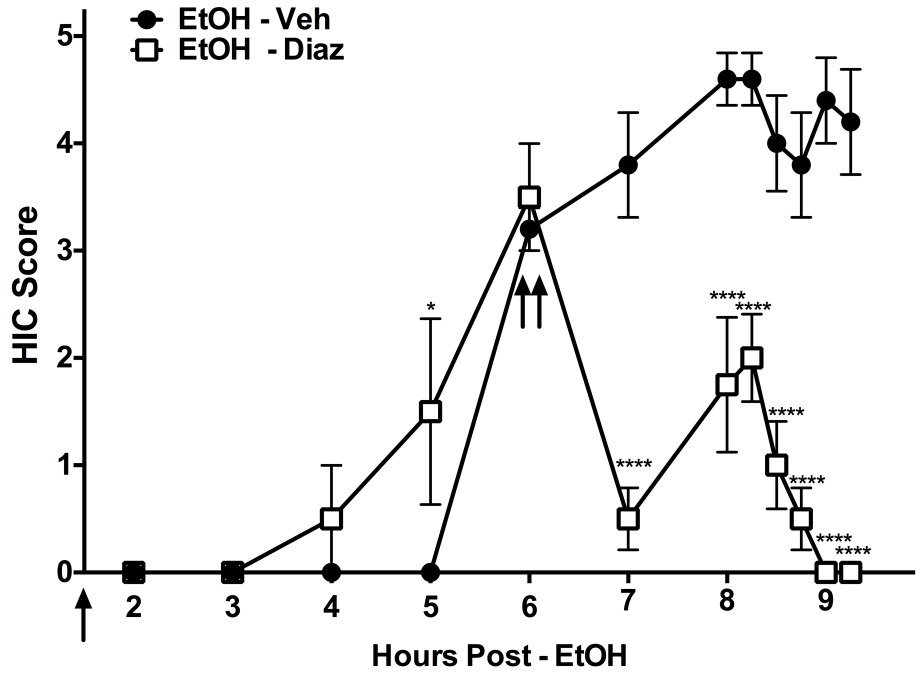


Table 1.1: No changes between groups in plasma corticosterone levels

When examining plasma CORT (taken immediately post-mortem) in separate groups of mice, there were no changes in the mean (±SEM) plasma CORT levels ($p > 0.05$) between groups (n = 5-12) following: 1 week of drinking and 1 WD session (WD 1), 3 weeks of drinking and 3 WD sessions (WD 3) or 4 weeks of drinking and 4 WD sessions followed by 7 days of extended WD (xtend).

Time of Sacrifice	H20/Co-Housed	EtOH/Co-Housed/WD	EtOH/Separate	H20/Separate
WD 1	-	204.4±	-	192.9±
WD 3	279.3±44.57	310.7±63.68	337.4±37.2	381.3±44.33
7 days xtend	288.4±22.4	319.8±45.5	271.5±30.39	314.8±41.06

Discussion

Our findings reveal that exposure to olfactory cues from “primary” mice experiencing hyperalgesia can trigger hyperalgesia in mice housed and tested in the same environment (bystanders). These “bystander” mice demonstrate hypersensitivity that does not require injury or noxious stimulation, but which is acquired following exposure to olfactory cues in the social environment. Under the current experimental conditions, this phenomenon reliably occurs during multiple pain states, including local inflammation (CFA) and hypersensitivity during drug withdrawal (morphine- or alcohol-induced). This socially transferred hyperalgesia can be measured by standard mechanical, thermal and chemical pain tests. Furthermore, we demonstrate that the phenomenon of social transfer can occur via an olfactory mechanism, as 24 h of exposure to bedding from hyperalgesic mice was sufficient to induce hyperalgesia in otherwise naïve mice. However, we cannot eliminate the possibility that other sensory modalities could also play a role. These findings highlight the importance of environmental and social variables in conducting and interpreting preclinical pain research. At the same time, they help elucidate the relationship between alcohol abuse and pain.

It is well known that social and environmental factors influence pain in humans, and these variables have also been shown to modulate pain behaviors in preclinical models, leading to analgesia (Gioiosa et al., 2009) or hyperalgesia (Langford et al., 2006; Z. Li et al., 2014; Raber & Devor, 2002) depending on the paradigm. Studies identifying these factors represent the foundation of our understanding of empathy in the

form of emotional contagion and social modulation of pain in rodents. However, the current results differ from previous findings (Langford et al., 2006; Z. Li et al., 2014), in that the hypersensitivity exhibited by bystander animals is not associated with emotional contagion acquired via visual cues (Langford et al., 2006), nor does it represent modification of an existing pain state (Gioiosa et al., 2009; Langford et al., 2006; Raber & Devor, 2002). Specifically, previous studies have relied upon a nociceptive trigger and contemporaneous visual cues or explicitly stressful stimuli, whereas the current results demonstrate a socially induced pain state that occurs in the absence of: tissue damage, visually dependent emotional contagion/synchronous behavior, concurrent anxiety, or simultaneous activation of the HPA axis. The hyperalgesia demonstrated by bystanders is nearly identical to that seen in animals subjected to withdrawal (from either an opioid or alcohol), but is not as severe as that seen in mice subjected to persistent localized inflammation induced with CFA. This indicates that differences among groups can be maintained in some paradigms, and may be related to the magnitude of hyperalgesia in the “primary” animals. The magnitude of socially transferred hypersensitivity was greater in female compared to male bystanders. This is intriguing since females demonstrate higher levels of empathy than males (O'Brien, Konrath, Grühn, & Hagen, 2013), and thus, social transfer may play a role in the overrepresentation of females in many chronic pain conditions like migraine and fibromyalgia (Fillingim, King, Ribeiro-Dasilva, Rahim-Williams, & Riley, 2009). However, the current studies exclusively examined reflexive responses and did not investigate whether the pain experience (which includes emotional components) is identical in these groups of mice, and

therefore it will be important to compare the affective states of bystander mice in future studies.

Olfactory cues in the social environment have been shown to induce physiological and behavioral changes that are not accompanied by measurable changes in CORT or a concurrent state of anxiety (as assessed by standard measures such as EPM (Alves et al., 2012). Although others have reported a social influence on pain as a form of stress-induced hyperalgesia (Baptista-de-Souza et al., 2015), the hyperalgesia observed in bystanders in the present studies was not contingent upon a simultaneous state of anxiety, nor was it associated with contiguous activation of the HPA axis or long-term changes in stress-induced activation of the HPA axis. This follows from the absence of altered CORT levels, the inability of diazepam to attenuate the expression of mechanical hypersensitivity, the lack of changes in the elevated plus maze and acoustic startle behavior, and the normal response to restraint stress. However, we cannot rule out the possibility that the HPA axis is activated in the bystander animals at some point during the acquisition of hyperalgesia. For example, the hyperalgesia displayed by bystanders could be triggered by stress, leading to neuroadaptations that maintain hyperalgesia in the absence of ongoing HPA axis activation (Rainville et al., 1997; Wiech & Tracey, 2009).

The lack of changes in the response to restraint stress in the present experiments is in agreement with lack of evidence for increased anxiety during withdrawal from voluntary alcohol self-administration in mice (Cox et al., 2013). In fact, drinking in the standard two-bottle choice procedure in mice has been argued to be a

poor model of alcoholism in part because of the lack of overt signs of pathological effects after prolonged history of drinking (Dole, Ho, & Gentry, 1985). The observation of hyperalgesia displayed during abstinence from voluntary drinking in the present study provides a potentially translational sign of withdrawal following that developed within a single week of alcohol drinking in the two-bottle choice procedure. Previously, hyperalgesia during alcohol withdrawal has only been demonstrated in the rodent after prolonged self-administration (Fu et al., 2015), forced alcohol exposure (Egli et al., 2012; Gatch, 2006; 2009; Gatch & Lal, 1999), or dependence-inducing escalated drinking procedures (Kliethermes, Cronise, & Crabbe, 2004; Perez & De Biasi, 2015; Wallis, Rezazadeh, & Lal, 1995). Thus, it is tempting to speculate that previous studies did not detect hypersensitivity during withdrawal from standard 2-bottle choice drinking because it was communicated via olfactory cues to nearby water-drinking mice, the typical control group. This social transfer could obscure any between-group differences. Regardless, the current observations illuminate the relationship between alcohol abuse and pain disorders, which has been amply demonstrated in humans (“Chronic Pain: Lifetime Psychiatric Diagnoses,” 1985), but understudied in animal models, despite apparent similarities in neuroanatomical substrates (Egli et al., 2012). Finally, the short time course utilized in the current studies, as well as the lack of changes in nociceptive responding in the absence of withdrawal, indicate that the hyperalgesia seen in alcohol drinking mice does not represent alcohol induced neuropathy.

The current findings also have broader methodological implications for rodent studies. It is common for experimental groups to be housed and tested with or near their

respective comparison groups, in order to control for environmental confounds. The present findings demonstrate that a physiologically relevant behavioral state can be transmitted between rodents housed throughout a room via olfactory cues. Although the experimental conditions employed here may have maximized the potential for social transfer via an olfactory channel (cages had wire tops with no filter lids to permit access to drinking bottles, and the mice were tested in the room in which they were housed), the manner in which experimental animals are housed and tested should be considered as a factor in experimental design. Our findings expand the concern raised by a recent study which has suggested that mice undergoing neuropathic pain can induce hypernociception in cagemates (Baptista-de-Souza et al., 2015).

The current studies elucidate the complex relationship between social-environmental cues and pain behavior while supporting the use of rodents as models for understanding the multidimensional aspects of chronic pain and alcoholism. Finally, further investigation of the social transfer of pain may prove to be relevant to chronic pain disorders in human patients that have no obvious noxious cause and are highly influenced by social and environmental factors

Materials and Methods

Animals

A total of 251 adult C57BL/6J mice were used in all experiments (n = 7-16/group) with the exception of 1 experiment examining handling induced convulsions, in which male DBA/2J mice (DBA; n = 12) were used. Male mice were used in all experiments, with

the exception of the experiment represented in Fig 1.1C which females were used. Mice were delivered from The Jackson Laboratory (Sacramento, CA) at 7-8 weeks of age, housed 3-5 per cage, and spent at least 1 week acclimating to our colony room (12:12 schedule; lights on 06:00 hours) before being individually housed and transferred to the experimental room (12:12 schedule; lights off between 09:30-10:30 hours) for an additional 7-day acclimation period prior to the initiation of the experiment. For all experiments, mice were housed in a temperature (20-22^{O} C)- and humidity-controlled environment with *ad libitum* access to food (LabDiet 5001; LabDiet, Richmond, IN) and tap water. All protocols were approved by the Oregon Health & Science University animal care and use committee and performed within the National Institutes for Health Guidelines for the Care and Use of Laboratory Animals, as well as the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research.

Experimental Rooms

Four separate experimental rooms (80-100 sq. ft.) were used in the current studies. These rooms exist within an isolated 750 sq. ft. suite and are connected via a common hallway containing a sink and supplies. Each room has an adjustable light cycle, and is separated from the common hallway by a door. For each experiment, treatment groups were rotated among physical rooms in the suite to prevent room-specific environmental factors from confounding the results. For all sets of experiments, one room contained "Co-Housed" experimental and control "Bystander" (vehicle treated or water drinking) groups, and adjacent rooms contained "Separate" control groups of mice tested concurrently. Overall there was no effect of any single housing room on behavior, as the

behaviors were predictable according to the treatment/social condition and were unaffected by the physical room the experiment took place in.

Cage Details

Mice were individually housed in standard polycarbonate “shoebox” cages (7.25″W x 11.5″D x 5″H) with wire cagetops and no filter lids. Bedding was fresh at the beginning of the experiment, was not exchanged during the course of the experiment. Within each room, individual cages were placed 2-6 inches apart on metal housing racks. A range of 8-64 mice were housed in a single room during a given experiment, although in the majority of cases, only 8-24 mice were in a single room. The number of mice in a room did not lead to any obvious changes in the measured behaviors.

Drugs

Complete Freunds Adjuvant (CFA) is 1 mg *Mycobacterium tuberculosis* (H37Ra, ATCC 25177)/ml of emulsion in 85% paraffin oil and 15% mannide manooleate. The vehicle in this experiment was the same volume of phosphate-buffered saline (PBS). Morphine base (300 mg/kg) was delivered s.c. in an emulsion that consisted of 50 mg of morphine base suspended in 0.1 ml of Arlacel A (mannide monooleate), 0.4 ml of light liquid paraffin and 0.5 ml of 0.9% w/v NaCl, and the vehicle for these experiments was the suspension lacking morphine. Naloxone (10.0 mg/kg; Sigma) was dissolved in saline and injected i.p. EtOH solutions for drinking (v/v) were prepared from 95% ethyl alcohol in tap water for drinking, and in saline for injection (20% v/v). For acute ethanol withdrawal, a 4 g/kg (i.p.) dose was injected. Diazepam (Sigma) was injected at a dose of 1.0 mg/kg (i.p.). Diazepam was dissolved in Tween 20 until it produced a clear

solution, and then diluted with saline. The final concentration of Tween 20 in the solution was 1%. The vehicle used in the diazepam experiment contained 0.9% saline with 1% Tween. Formalin was made from paraformaldehyde (PFA; Sigma) and diluted into PBS for a final concentration of 1.5% formalin, or 0.56% PFA.

Noxious Stimuli

CFA-Induced Inflammatory Pain: To examine the social transfer of chronic inflammatory pain, mice were housed in two adjacent rooms, tested for basal mechanical thresholds to von Frey stimulation and then lightly restrained and immediately injected with either PBS (PBS/Co-Housed or PBS/Separate) or 10 μl CFA (CFA/Co-Housed) into the intraplantar surface of the left hindpaw, which is known to reliably induce long lasting pain (Ren & Dubner, 1999). Mice were then tested on days 3, 5, 11 and 14 post-injection.

Morphine Withdrawal (WD): To determine whether WD from a drug of abuse would lead to the social transfer of pain, mice were individually housed and tested in two neighboring rooms (Co-Housed or Separate). Mice were tested for basal mechanical sensitivity to von Frey stimulation of the hindpaw. Immediately following the baseline test, mice were injected with either a slow release morphine base (300 mg/kg, Mor/Co-Housed/WD; n = 8) or vehicle suspension lacking morphine (Veh/Co-Housed/WD; n = 8 or Veh/Separate n = 8). Forty-eight and 96 h post injection, mice were tested again and then injected with their assigned treatment. Immediately following the final test on day 5, all mice were injected with 10 mg/kg naloxone and rated for morphine-withdrawal related behavior such as jumping, wet dog shakes, paw tremor and diarrhea by an

experimenter that was blind to treatment assignments during scoring.

Forced Abstinence/Alcohol Withdrawal (WD): To examine whether WD from alcohol resulted in increased pain sensitivity and its social transfer, mice were given continuous access to two bottles: one containing water and the other - a solution of EtOH. Once weekly (2 h into the dark cycle) EtOH bottles were removed and replaced with bottles containing water for 24 h. Thus, for the first week of drinking all mice received each 3% and 6% EtOH (v/v) for 2 days and 10% EtOH (v/v) for one day followed by 24 h of withdrawal. On each following week (in relevant experiments) the mice were allowed access to 10% EtOH for 6 days followed by 24 h of withdrawal.

Pain Tests

Mechanical Sensitivity: Responses to mechanical stimulation by von Frey hairs (0.01 to 2g plastic fibers) were determined in the plantar surface of the left hindpaw. Normal response was considered as: withdrawal, shaking or licking the paw. Mechanical thresholds were tested using the Up-Down technique (Chaplan, Bach, Pogrel, Chung, & Yaksh, 1994). This method uses stimulus oscillation around the response threshold to determine the median 50% threshold of response. Mice were allowed to acclimate to the plexiglass enclosure on top of a wire testing rack for 40 min on 2 days prior to the start of the experiment and for 10-20 min before each test session. The testing rack was located within each testing room near the housing rack and illuminated with a dim red lamp. Mechanical sensitivity was assessed prior to treatment exposure (baseline), and mice were then assigned to treatment group based upon basal mechanical thresholds. Testing then occurred each week following 24 h of withdrawal unless stated otherwise.

All behavioral testing was conducted by a single experimenter, with the exception of the handling induced convulsions experiment, which was conducted by a different lab member. During testing, the experimenter was blind to the individual treatment assignments within each room.

Thermal Sensitivity: Mice were tested for thermal nociceptive sensitivity at baseline and during 2 weekly withdrawal sessions using the heat-evoked tail withdrawal reflex. Two days prior to the first test session, mice were habituated to handling (light restraint in a soft cloth), and the tip of their tail (5 cm from the end) was immersed into room temperature water. On the test days, mice were lightly restrained and the tail was submerged into 46° C water to detect the response (flicking the tail out of water), which give baselines of approximately 15 s. Two tail withdrawal measurements were taken 10 min apart and averaged for a single data point for each animal. A stopwatch was used to determine the latency to flick the tail (Pradhan, Smith, Zyuzin, & Charles, 2014). Experimenter was blind to treatment group assignment during testing. All mice from this experiment were also used in the restraint stress experiment (described below).

Chemical Sensitivity: A subset of mice from: 1) EtOH/Co-Housed/WD and H_2O/Co-Housed, 2) H_2O/Olfactory-WD and 3) H_2O/Co-Housed/NoWD and EtOH/Co-Housed/NoWD groups (see Experimental Procedures) received a formalin test following the 2nd 24 h withdrawal session. Immediately following the final mechanical test mice were injected with 1.5% formalin (Sigma) into the plantar surface of the left hindpaw. A low dose of formalin was chosen in order to avoid a potential ceiling effect. Following injection, the mice were placed into individual plexiglass chambers on the testing rack

and digitally videotaped for 60 min for later analysis. Since no nocifensive behaviors were demonstrated between 46-60 min, these timepoints were excluded from analysis. Using a stopwatch, an experimenter blind to group assignment sampled video files for 5 s at 1-min intervals for pain behavior. Nocifensive behavior was defined as licking/biting the injected paw. These data were analyzed as percent time spent licking during every 5s interval. The first phase was defined as 0-5 min post injection and the second phase as 11-45 min post-injection. To determine synchrony of licking behavior (as described elsewhere (Langford et al., 2006), we calculated all possible correlations between mice tested during the same session that were in visual range of each other. This led to 3-5 correlations per mouse, depending on testing conditions, as 6-8 mice were tested during each experimental run. We then took the average of those correlations (*R;* Fig S1.2) and then calculated the grand average of $R \pm$ SD across the 3 experimental runs ($R =$ 0.107±0.22). The data was then permuted 100 times, creating random pairings of mice, and allowing for calculations of the grand mean and standard deviation for this data. We found that the actual standard deviation of the mice run together was not significantly different than that of randomly grouped mice (permuted data; $R = 0.108 \pm 0.007$, $p =$ 0.97), suggesting a lack of synchrony in licking behavior.

Experimental Procedures

Ethanol Intake Procedures: During the 7-day acclimation period, mice received 24 h access to two bottles with metal sipper tubes (containing water) on either side of the cage, with food evenly distributed along the wire cage top. Following acclimation and/or baseline testing, mice either received access to 2 bottles of water only (H_2O mice) or 1

bottle of water and 1 bottle of alcohol (EtOH mice). *24 h Access Two Bottle Choice:* EtOH mice received 24 h access to two bottles: one containing tap water and once containing increasing concentrations of EtOH (3-10%) dissolved in tap water. Both 3% and 6% were available for 2 days, after which the animals had access to 10% EtOH for the remainder of each experiment. Fluid levels from each of the two bottles were recorded on a daily basis during the second hour of the dark cycle. The locations of the bottles on the cages (left vs. right) were alternated every other day to avoid the potential confound of an inherent side preference. Further, when multiple treatment groups were housed in a single room, the treatment-assignment was randomly assigned across the cage locations, to avoid any confound related to the treatment of neighboring cages.

No Withdrawal (no WD): To examine whether the mere presence of: 1) alcohol cues in the room or 2) cues related to the behavior of intoxicated neighbors was enough to elicit mechanical hypersensitivity in the water-drinking mice, we co-housed water drinking mice with an EtOH drinking group that did not experience any forced abstinence (Fig 2B; H_2O/Co-Housed/NoWD; EtOH/Co-Housed/NoWD). Mechanical testing occurred on the 7^{th} and 14^{th} days, 2h into the dark cycle. This experiment was conducted at the same time as the olfactory experiment (Fig 1.3B, described below) and these mice were subjected to the formalin test (described below) immediately following their final mechanical test.

Olfactory Stimuli: To examine the sensory method of social transfer, 3 neighboring rooms were utilized. One room contained EtOH/Co-Housed/WD and H_2O/Co-Housed mice, (Fig 2A,D). In 2 adjacent rooms mice were given access to water only

(H_2O/Olfactory). The Co-Housed mice (which received either ethanol and water or water only), began their schedule one day prior to H_2O/Olfactory mice, and thus had experienced 24 h of abstinence from alcohol when the first bedding was collected. This experiment followed the same timeline as all other 2 week experiments, with the exception that on the 7th day dirty bedding was removed from cages (~5g/ea. cage) of *all* mice in the Co-Housed room, e.g., EtOH/Co-Housed/WD *and* H_2O/Co-Housed/WD mice (n = 32/group; both groups displayed hypersensitivity at this time), or from cages of water drinking mice in the animal colony (n = 45). Bedding from each set of mice (Co-Housed or colony; ~40-50g total/day) was mixed and placed into 3 empty cages with wire cagetops. The 3 cages containing bedding from Co-Housed mice were set (evenly spaced) on the housing rack of the one of the rooms containing water-drinking mice (H_2O/Olfactory-WD). As a control for novel mouse bedding cues, the 3 cages containing dirty bedding from mice in the animal colony were placed on the housing rack of water drinking mice in the final room (H_2O/Olfactory-CTRL). Bedding from both sets of mice (Co-Housed and colony) was continually removed, combined and placed into these cages each day for 1 week. This was done to match the experience of continuous exposure to olfactory cues experienced in the Co-Housed room. H_2O/Olfactory-WD and H_2O/Olfactory-CTRL mice were tested for mechanical sensitivity 24 h after the first bedding exposure, and one week later. The Co-Housed/Olfactory-CTRL experiment was run twice in 2 separate rooms to ensure the reliability of this effect. There were no statistical differences between the groups in the first and second experiment, thus these were combined to create single groups of 16 mice.

Elevated Plus Maze (EPM): To explore the possibility that anxiety was present in Co-Housed mice, we examined EPM activity in groups of Co-Housed mice, (H_2O- and EtOH-drinking) mice as well as H_2O/Separate mice following the second 24 h withdrawal session. Testing occurred in the experimental/housing rooms. The EPM apparatus (Med Associates, Inc., St. Albans, VT, USA) consisted of two black opaque high-walled arms and two white open arms (51-cm long x 8-cm wide) elevated 60 cm off the ground. Small lamps were placed over the open arms, and the closed arms remained un-lit, resulting in respective lux values of 95 and 2. Mice were placed in the center platform facing a closed arm, and the following variables were scored live by an experimenter blind to treatment group assignment during a 5 min test: entries and time spent in open arms, closed arms, and rearing behavior, grooming, urination, and fecal boli. Between each session, the EPM was cleaned with water and a sponge, and thoroughly dried with paper towels. Data is presented as percent time spent, or number of occurrences (+SEM).

Diazepam Treatment: In a separate group of Co-Housed (H_2O- and EtOH-drinking) mice, following baseline testing, subjects were counterbalanced into four groups: H_2O mice that received vehicle (H_2O/Co-Housed-Veh) or diazepam (H_2O/Co-Housed-Diaz) and EtOH mice that received vehicle (EtOH/Co-Housed/WD-Veh) or diazepam (EtOH/Co-Housed/WD-Diaz). For habituation, saline injections were given immediately prior to the first test session (in 24 h WD). Following the 2nd 24 h session of WD, mice were weighed, injected and placed on the testing rack. The mechanical test took place 20 min later (Fig 3A,E).

Handling Induced Convulsions (HICs): To verify that a 1.0 mg/kg dose of diazepam would successfully reverse another commonly used alcohol withdrawal phenotype (Goldstein & Pal, 1971) we examined the ability of diazepam to attenuate HICs in DBA/2J mice, which reliably display this behavior (Crabbe, 1992) (Supplementary Fig 4). We used this strain of mice because C57BL/6J mice (used in all other experiments) do not reliably display this behavior (HICs, (Crabbe, Keith, Kosobud, & Stack, 1983), yet display similar anxiolysis as DBA/2J in both the EPM and light dark box following a 1.0 mg/kg dose of diazepam (Griebel, Belzung, Perrault, & Sanger, 2000). Additionally, the same dose of diazepam actually leads to lower brain concentrations in DBA/2J mice compared to C57BL/6J mice, suggesting that C57BL/6J mice should be more sensitive to the same dose of diazepam (Crabbe, Gallaher, Cross, & Belknap, 1998). Following a 4.0 g/kg i.p. EtOH injection, DBA/2J mice were scored for HICs, as reported in detail elsewhere(Metten & Crabbe, 1994). Individual baselines are subtracted from HIC scores, and data are shown as mean (±SEM) group response across time (h).

Acoustic Startle: To test for auditory hypersensitivity we conducted a separate experiment in which acoustic startle responses were investigated on the second WD session. The same drinking/WD protocol was used as described for all other alcohol-drinking experiments, with the following exceptions: On the second WD session (24 h after removal of EtOH bottles), mice were removed from home cages and placed into the acoustic startle chambers (Kinder Scientific, San Diego) present in the housing/testing room. For the first 5 min, mice were not subjected to any tone (habituation). All tones were separated by random inter-trial intervals (ITI; 15-30s).

Following habituation, the session began [and ended] with 3 three no tone trials. Following the three no tone trials, 60-120 dB tones were played (10 dB increments) in a randomized order for a total of 24 trials. Data is plotted as group mean ± SEM acoustic startle response to increasing intensity tones.

Restraint Stress: Mice from the tail immersion (TI) experiment were allowed one week of recovery in their experimental/housing room. On the 8th day after the last TI test, mice were removed from homecages and placed in standard plexiglass restrainer tubes on a table in their respective housing rooms for 30 min. Immediately following removal from the restraint devices, mice were sacrificed via CO_2 inhalation and trunk blood was taken for CORT analysis.

Corticosterone Analysis: Immediately after the final mechanical test, or following 30 min restraint stress, mice were sacrificed by CO_2 inhalation, and trunk blood was collected for corticosterone (CORT) analysis (Table 1.1). Samples were kept on ice, and then centrifuged and plasma was removed and stored at -20^{o} until analyzed. CORT was assayed using a commercially available radioimmunoassay kit (MP Biomedicals, Solon, OH) with plasma samples diluted 1:200 and run in duplicate. The intra-assay coefficient of variation was 4.67%; the inter-assay coefficient of variation was 5.5%.

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Author Contributions:

M.L.S, M.M.H and A.E.R conceived of the studies, designed experiments and wrote the paper. M.L.S. performed all experiments, with the exception of the corticosterone analysis, which was conducted by C.M.H., who also provided editorial comments.

CHAPTER 2: Neural Mechanisms of the Social Transfer of Pain

“Pain ... has a structure. It has a floor plan. It has designs more intricate than a chambered nautilus, features more baroque than the most buttressed Gothic cathedral.”

Dan Simmons (Simmons, 2011)

(This chapter has been modified for inclusion in this dissertation from: Smith, M.L., Heinricher, M.M. & Ryabinin, A.E. (Ready for Submission), All authors contributed to the design of experiments and the writing of the manuscript, Smith, M.L. conducted all experiments)

Abstract

Pain is a multifaceted process, with sensory, behavioral, cognitive and emotional components that can be dramatically influenced by psychosocial factors. Expression of pain by an individual can be socially communicated to nearby conspecifics via visual or olfactory cues. The perception of another's pain can lead to physiological and behavioral changes in an observer. For example, it has recently been demonstrated as the "social transfer of hyperalgesia," whereby "primary" mice exposed to a noxious stimulus induce a congruent state of hyperalgesia in "bystander" mice housed and tested in the same room. The current studies were designed to investigate the neural mechanisms responsible for the social transfer of hyperalgesia and found enhanced Fos-ir in the anterior cingulate (ACC) and anterior insula (AI) of bystander mice, and in the dorsal medial hypothalamus (DMH) of primary mice. Chemogenetic inactivation of the ACC but not the primary somatosensory cortex reversed the expression of hyperalgesia in both primary and bystander mice.

Pain is a multifaceted sensory, emotional, cognitive, and social experience that is processed via multiple neural pathways often labeled as the “pain matrix” (Hadjistavropoulos et al., 2011). It has been posited that one such pathway encodes the “sensory discriminative” aspects of pain, whereas a divergent, yet [possibly] overlapping neural circuit is linked to the “emotional” aspects of the pain experience (Bingel et al., 2004; Peyron et al., 1999; Rainville et al., 1997; Rainville, Carrier, Hofbauer, Bushnell, & Duncan, 1999a). In fact, some studies suggest that the primary somatosensory cortices (S1/S2) encode sensory information related to pain location and intensity (Apkarian et al., 2005; Chudler et al., 1990; Kenshalo & Isensee, 1981; Kenshalo & Perkins, 1984; Kenshalo & Isensee, 1983), while the anterior cingulate (ACC) and anterior insula (AI) are involved in processing the affective and motivational aspects of the pain experience (Apkarian et al., 2005; Rainville, 2002; Rainville et al., 1997). It has been suggested that the ACC is involved in assessing the salience and affective quality of pain (Downar et al., 2002; 2003), whereas the AI may be important for the aversive aspects of pain communication, as it is activated during disgust and while watching others in pain (Krolak-Salmon et al., 2003; Phan et al., 2002; Phillips et al., 1997; Wicker et al., 2003).

It has been difficult to elucidate the neural circuitry that underlies the pain experience, as cortical regions activated during pain receive afferent inputs from multiple routes and lead to multiple descending modulatory circuits (Bushnell et al., 2013). Additionally, pain is highly influenced by psychosocial and environmental factors, creating a complex array of feedback loops between emotion, cognition and pain (M. C. Bushnell et al., 2013). Some have theorized that these neural circuits may converge

within the ACC, as this region is implicated in both physical (Apkarian et al., 2005; Peyron et al., 1999) and social pain (rejection, exclusion, heartbreak; (Rotge et al., 2015), as well as cognitive tasks related to goal-directed responding (Botvinick, Braver, Barch, Carter, & Cohen, 2001; van Veen, Cohen, Botvinick, Stenger, & Carter, 2001) and conflict detection (Derbyshire, Vogt, & Jones, 1998).

Understanding pain is further complicated by the fact that noxious stimulation is not necessarily required for the experience of pain or activation of the pain matrix (Wall, 1979; Lamm, Decety, & Singer, 2011). A number of human studies have demonstrated activation of brain areas within the pain matrix in the absence of a noxious insult, including activation of the ACC during empathy for another's pain (Yesudas & Lee, 2015). Additionally, chronic pain often manifests in the absence of tissue damage (van Wilgen & Keizer, 2012), and chronic pain patients display abnormal activity throughout the pain matrix (Sprenger & Borsook, 2012; Staud & Rodriguez, 2006). Far fewer animal studies have explored such "top-down" mechanism of pain, and the studies that have been done utilize explicitly stressful stimuli or focus upon behavioral output rather than neural mechanisms.

Chapter One of this thesis demonstrates that exposure to the olfactory cues of conspecifics experiencing hyperalgesia leads to congruent pain behavior (social transfer of hyperalgesia). This hyperalgesia may require different neural mechanisms from that which is engaged via noxious insult and activation of peripheral afferent pathways or from that which is explicitly initiated by stress. The aim of the current study was to elucidate the neural circuitry responsible for the expression of socially transferred

hyperalgesia, using Fos immunohistochemistry and inactivation of the ACC and S1 via chemogenetics.

Results

Enhanced Fos Activation in Bystander and Primary Mice

We used a paradigm in which mice were allowed voluntary access to alcohol for one week followed by 24-h session of withdrawal (*see* Chapter 1). These "primary" mice demonstrate significant hypersensitivity during withdrawal that is socially transferred to "bystander" control mice that are housed and tested in the same room but are drinking only water. This socially transferred hyperalgesia is communicated via olfactory cues (Chapter 1). To explore the neural circuits involved in the expression of hyperalgesia in primary (EtOH/Co-Housed/WD) and bystander (H_2O/Co-Housed/WD) mice, we compared brain Fos immunoreactivity in targeted brain regions in these groups compared to their control mice housed in a separate room (H_2O/Separate). We examined Fos as a measure of neural activation *during the third session of abstinence of alcohol* (withdrawal; WD), as brains were taken immediately following the completion of the 3rd mechanical test (Fig 2.1A). Between-group differences were seen in 3 of 21 brain regions analyzed (Table 2.1). First, when compared to the H_2O/Separate control group, bystander mice (H_2O/Co-Housed) demonstrated enhanced Fos immunoreactivity (Fos-*ir)* in the anterior insula (AI; Fig 2.1B,E) and anterior cingulate (ACC; Fig 2.1C,F). By contract, increased Fos-*ir* was seen in the dorsal medial hypothalamus (DMH) in primary mice (EtOH/Co-Housed/WD) compared to bystanders (H_2O/Co-Housed; Fig

2.1D,G).

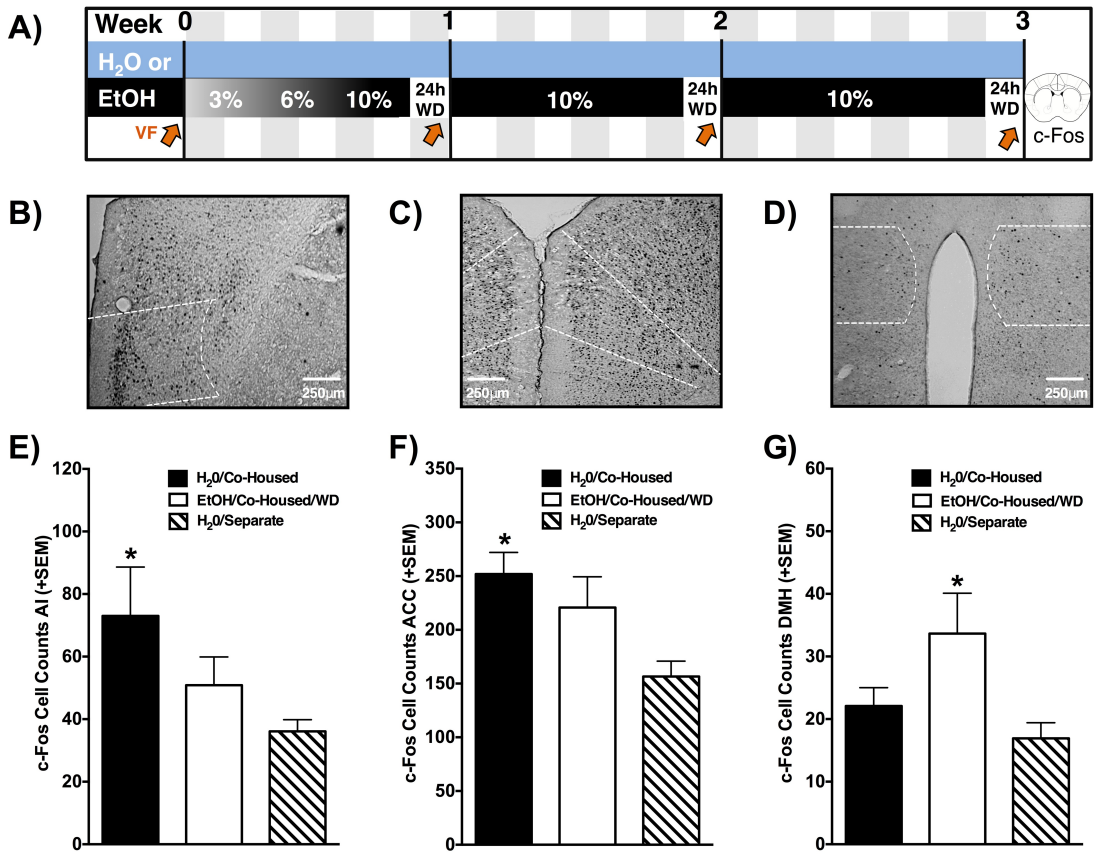
Table 2.1: Fos immunoreactivity in primary and bystander mice

Mean (±SEM) c-Fos positive cell counts for experimental each group per brain area examined. ANOVA values are presented in the right column, with significant values represented in **bold** and significant Bonferroni post hoc comparison to control (H_2O/Separate) represented by an **(*)** ($p < 0.05$).

Brain area	H20/Co-Housed	EtOH/Co-Housed/WD	H20/Separate	ANOVA
Anterior Cingulate (CG1)	251.9 ± 20.18*	220.8 ± 28.66	156.6 ± 14.28	**F(2,14) = 4.77**
Anterior Cingulate (CG2)	77.33 ± 14.12	75.14 ± 20.05	73.56 ± 5.65	F(2,15) = 0.017
Insula (GI)	24.46 ± 6.201	23.89 ± 2.695	19.08 ± 2.752	F(2,16) = 0.547
Insula (AI)	73.02 ± 15.59*	50.86 ± 9.032	36.1 ± 3.73	**F(2,15) = 3.779**
Somatosensory	449.6 ± 76.52	659.8 ± 168.7	542.1 ± 44.79	F(2,16) = 1.147
Dorsal Lateral Septum	6.5 ± 1.351	6.958 ± 2.021	6.893 ± 1.542	F (2, 17) = 0.023
Intermediate Lateral Septum	19.44 ± 2.637	23.71 ± 1.429	23.82 ± 2.326	F (2, 17) = 1.250
Ventral Lateral Septum	12.27 ± 2.743	14.36 ± 3.342	13.65 ± 0.991	F (2, 17) = 0.183
Nucleus Accumbens	23.02 ± 3.265	25.76 ± 5.959	21.65 ± 6.323	F (2, 16) = 0.155
Bed Nucleus of the Stria Terminalis (anterior)	39.14 ± 5.47	48 ± 11.1	30.89 ± 2.2	F (2, 17) = 1.561
Bed Nucleus of the Stria Terminalis (posterior	23.33 ± 4.889	25.45 ± 2.91	20.53 ± 8.444	F (2, 15) = 1.472
Dentate Gyrus	31.27 ± 6.609	39.14 ± 6.744	36.19 ± 5.337	F (2, 17) = 0.4011
Posterolateral Cortical Amygdaloid	16.6 ± 1.027	17.3 ± 3.412	24.69 ± 2.328	F (2, 16) = 2.845
Posteromedial Cortical Amygdaloid	8.722 ± 0.604	7.571 ± 1.242	9.571 ± 1.852	F (2, 17) = 0.5452
Basolateral Amygdala	17.78 ± 4.901	12.13 ± 2.592	12.61 ± 12.61	F (2, 15) = 0.7956
Central Nucleus of the Amygdala	10.02 ± 2.409	5.833 ± 1.153	9.063 ± 1.194	F (2, 15) = 1.687
Paraventricular Nucleus	12.13 ± 3.29	11.17 ± 3.55	3.4 ± 0.75	F(2,12) = 2.584
Dorsal Medial Hypothalamus	22.08 ± 2.939	33.67 ± 6.438*	16.93 ± 2.48	**F (2, 16) = 4.236**
Centrally Projecting Edinger Westphal	11.38 ± 1.799	12.16 ± 3.073	7.988 ± 1.276	F (2, 17) = 1.150
Periaqueductal Gray	63.9 ± 8.827	79.96 ± 19.14	44.98 ± 7.049	F (2, 17) = 2.073
Substantia Nigra	6.107 ± 1.603	7.917 ± 2.546	4.679 ± 2.298	F (2, 17) = 0.5495
Ventral Tegmental Area	9.43 ± 4.66	4.88 ± 2.54	2.93 ± 1.02	F(2,15) = 1.115

Figure 2.1: Differentially enhanced Fos in primary and bystander mice

A) Timeline of data collection (behavioral data in Fig 1.2B), Blue bar represents Bystander mice, black bar represents alcohol drinking mice, with corresponding EtOH % (v/v); "VF" and orange arrows represent von Frey testing; "WD" represents withdrawal from alcohol; Representative photomicrographs of Fos-ir in the B) anterior insula (AI), C) anterior cingulate (ACC) and D) dorsal medial hypothalamus (DMH). As evidenced by one way ANOVA, H_2O/Co-Housed/WD mice displayed an increase in c-Fos cell counts in the: E) AI ($F_{2,15} = 3.8$, $p = 0.046$) and the F) Anterior cingulate (ACC; $F_{2,14} = 4.8$, $p = 0.026$). Whereas EtOH withdrawal (EtOH/Co-Housed/WD; n = 14) induced enhanced c-Fos ir in the F) DMH ($F_{2,16} = 4.2$, $p = 0.033$). Significant differences compared to control (H_2O/Separate; $p < 0.05$) according to Bonferroni posthoc analyses are represented by (*).



Inhibition of the Anterior Cingulate, but not Primary Somatosensory Cortex Reverses Hyperalgesia in Primary and Bystander Mice

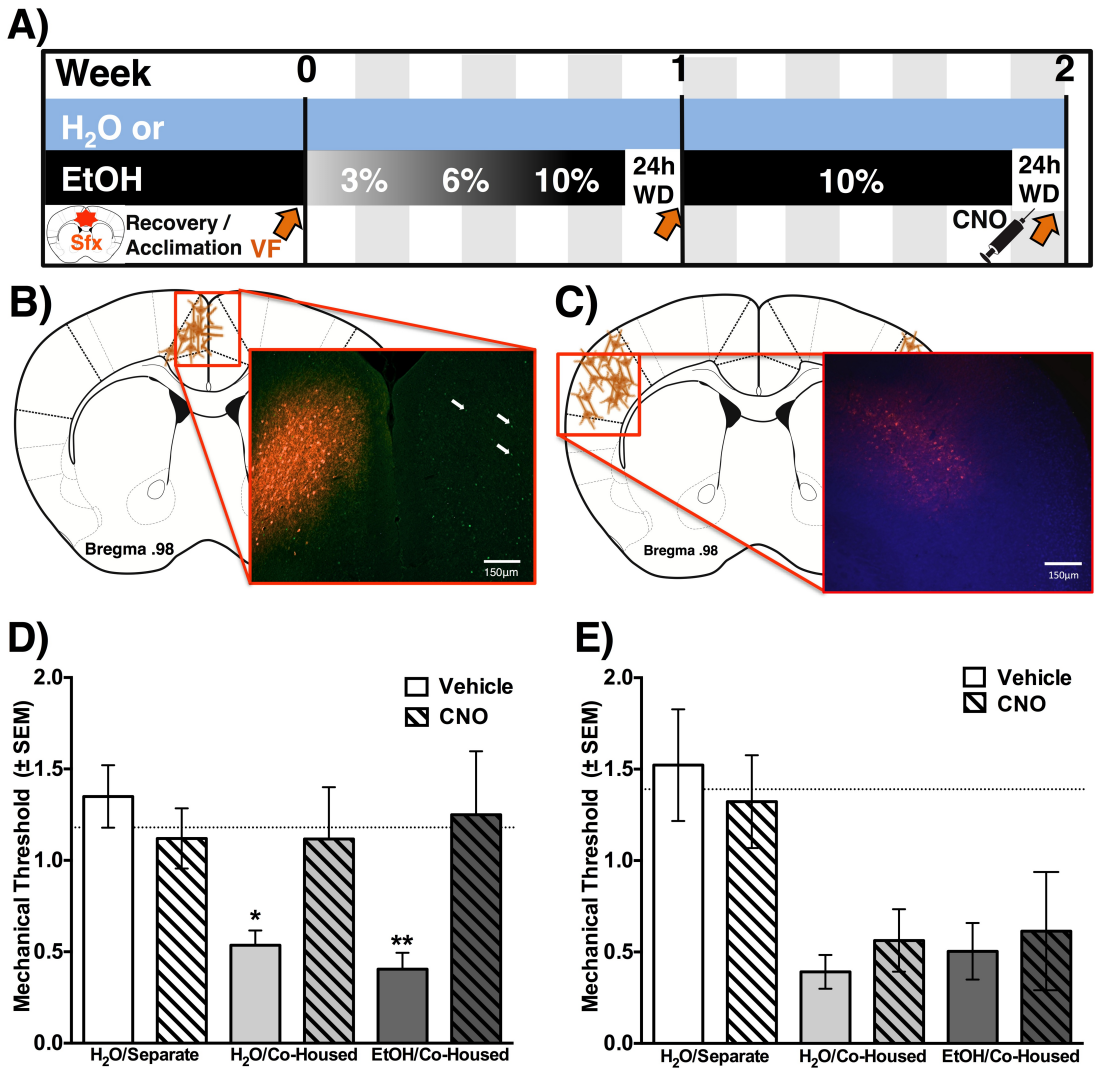
To determine whether the ACC is required for the expression of mechanical hypersensitivity in these mice, we utilized chemogenetic technology, or Designer Receptors Exclusively Activated by Designer Drugs (DREADDs; (Armbruster, Li, Pausch, Herlitze, & Roth, 2007; Rogan & Roth, 2011), which are synthetic G-protein coupled receptors that display selective sensitivity to the pharmacologically inert drug clozapine-N-oxide (CNO). At least one week prior to the start of the experiment (Fig 2.2A) mice were transfected with an AAV virus carrying a Gi-coupled inhibitory DREADD (hM4Di) microinjected unilaterally into the ACC (Fig 2.2B). Prior to the 2nd WD session, mice were injected with either CNO or vehicle (Veh). Both the H_2O/Co-Housed and the EtOH/Co-Housed mice demonstrated significant decreases in mechanical thresholds on the 2nd WD session that were reversed by inactivation of the ACC (Fig 2.2D) via injection with 1.0 mg/kg CNO (CNO; n = 5/group) compared to injection with vehicle (Veh; n = 6/group). Mice transfected with the DREADD virus that were housed and tested in a separate room (H_2O/Separate) did not demonstrate any changes in mechanical threshold regardless of CNO or Veh treatment (Fig 2.2D). When the same viral DREADD construct was transfected into the somatosensory cortex (Fig 2.2C) there was no effect of CNO injection (inhibition of the somatosensory cortex) in primary (EtOH/Co-Housed/WD) or bystander (H_2O/Co-Housed/WD) mice, (Fig 2.2 E; $F_{3,21}$ = 1.233, p = 0.323) when comparing these to mice which received vehicle injection on the second test session. Again, there were no changes in mechanical thresholds

H_2O/Separate mice transfected with the DREADD virus in the somatosensory cortex and treated with CNO or Veh (Fig 2.2E). In combination with the Fos data, these results suggest that the ACC, but not the somatosensory cortex, is required for the expression of socially transferred- and alcohol withdrawal induced-hyperalgesia.

Figure 2.2: Inhibition of ACC, but not somatosensory cortex reverses hyperalgesia in primary and bystander mice

A) Timeline of data collection and experimental manipulation: “Sfx” refers to surgery, which took place 7-14 days prior to beginning of experiments; Blue bar represents bystander mice, black bar represents alcohol drinking mice, with corresponding EtOH % (v/v); “VF” and orange arrows represent von Frey testing; “WD” represents withdrawal from alcohol; Black syringe represents CNO injection at the beginning of the 2nd WD session. A) Representative photomicrograph of unilateral hM4Di viral expression within the ACC (orange) and c-For labeling (green, arrows). C) Representative photomicrograph of hM4Di viral expression within the somatosensory cortex (orange) and DAPI staining in blue. D) Both H_2O/Co-Housed (n = 5-6/group) and EtOH/Co-Housed (n = 5-6/group) mice transfected with the hM4Di DREADD virus demonstrated significant decreases in mechanical thresholds on the 2nd WD session compared to Separately housed controls. According to ANOVA this led to a significant difference between groups ($F_{1,30}$ = 4.79, p = 0.037), as well as a significant interaction ($F_{2,30}$ = 3.37, p = 0.048). This hypersensitivity was reversed by inactivation of the ACC via injection with 1.0 mg/kg CNO (n = 6), compared to injection with vehicle (Veh; n = 5), as CNO groups were no longer significantly different from separately housed controls according to Fishers LSD. E) H_2O/Co-Housed (n = 5-7/group) and EtOH/Co-Housed (n = 6/group) mice bilaterally transfected with the hM4Di DREADD virus into the somatosensory cortex demonstrated significant decreases in mechanical thresholds on the 2nd WD session compared to separately housed controls (n = 5-6), leading to

significant differences between groups according to ANOVA ($F_{2,29}$ = 14.88, p<0.0001), but no significant effects of treatment or an interaction, indicating that inactivation of the somatosensory cortex had no effect on hypersensitivity. Mean basal responses of all groups represented by dotted line (---). Significant differences compared to control (p<0.05) are represented by (*).



Discussion

These results confirm our previous finding that alcohol-withdrawal induced hyperalgesia is socially transferred from these “primary” mice to “bystander” mice housed in the same room, and begin to elucidate the neural mechanisms involved in the expression of these behaviors.

Examination of Fos-*ir* revealed enhanced neural activity in the ACC and AI of bystander mice when compared to separately housed water-drinking controls. Chemogenetic inactivation of the ACC verified that this area is required for the expression of mechanical hypersensitivity in bystander mice.

Alcohol-withdrawn mice also demonstrated an enhancement in Fos within the ACC that was comparable to that of bystanders, but this increase was not statistically different from H_2O/Separate mice. Nevertheless, unilateral inhibition of the ACC also reversed hyperalgesia in primary mice during the 2^{nd} test session, indicating that this region is necessary for expression of this alcohol withdrawal-induced hyperalgesia at this timepoint. Additionally, there was a enhancement in Fos within the DMH of the primary mice, suggesting that this area may be distinctly important for alcohol-withdrawal, and not socially transferred hyperalgesia.

Bilateral inhibition of the somatosensory cortex did not alter hypersensitivity in any of the mice, indicating the relative importance of the ACC in this behavior. This finding is in accordance with the Fos data, as there was enhanced activity within the ACC of the primary and bystander mice, but not within the somatosensory cortex of any group.

It is usually thought that the somatosensory cortices are responsible for determining the sensory qualities of pain such as intensity and location of nociceptive stimuli (Chudler et al., 1990; Kenshalo & Isensee, 1981; Kenshalo & Perkins, 1984; Ploner, Schmitz, Freund, & Schnitzler, 1999b), whereas the ACC is believed to play a role in the affective/emotional components of the pain experience (Apkarian et al., 2005). The current findings may fit into this framework, as this is the second session in which the mice are experiencing [presumably] diffuse hyperalgesia induced by obfuscated stimuli (alcohol withdrawal or olfactory cues). Indeed we showed previously that hypersensitivity as measured by a range of stimulus modalities, including thermal and chemical, as well as mechanical, which was used in the current study (Chapter 1). It is tempting to speculate that during the second experience with these diffuse alcohol WD (primary mice) and social-olfactory (bystander mice) cues, the hyperalgesia is represented in higher-level cognitive areas within the cortex, leading to a “top-down” activation of pain circuitry.

However, the ACC is not a pain-specific region, and is involved in general affect, attention and motor preparation (Devinsky, Morrell, & Vogt, 1995). Thus, the involvement of this brain area may not be related specifically to the hyperalgesia in these mice, but some other aspect of the experience. Additionally, specific neuronal populations within the ACC have distinct roles in pain and nociception. For example, Kang and colleagues (2015) recently demonstrated that *inhibition* of ACC pyramidal neurons reverses CFA-induced hypersensitivity, an effect that is also seen through *activation* of parvalbumin (PV)-containing neurons. By contrast, activation of

somatostasin (SOM)-containing neurons had no effect on nociception. Considering the multiple, functionally distinct cell populations within the ACC, it is possible that our inhibition studies targeted multiple unique cell populations with potentially varied involvement in socially transferred pain. However, we were able to see a reversal of hypersensitivity, indicating the overall importance of the ACC to this behavior. In future studies, it will be essential to determine if there are unique microcircuits (defined by protein or activity-specific markers) within the ACC governing both alcohol withdrawal-induced and socially transferred hyperalgesia.

Alcohol withdrawn mice show a robust enhancement of Fos in the DMH, suggesting that slightly different circuitry may underlie the state in the primary compared to bystander mice. This is interesting considering the role of the DMH in stress induced hyperalgesia (Wagner et. al, 2013). However, this activation could be related to another component of the withdrawal process, and may be unrelated to hyperalgesia. Notably, the state of hyperalgesia in the bystander mice does not appear to activate the DMH.

In summary, these studies replicate the presence of social transfer of hypersensitivity from mice experiencing alcohol withdrawal to control mice housed and tested in the same room. This model allows for the investigation of hyperalgesia triggered by a social signal rather than inflammation or injury. The current studies demonstrate that the ACC is necessary in this pain model, and indicate that potentially divergent neural circuits govern the expression of socially transferred hyperalgesia compared to hyperalgesia expressed during alcohol withdrawal.

Materials and Methods

A total of 103 adult male C57BL/6 mice from the Jackson Laboratory (Sacramento, CA) were used in these experiments. The Fos analysis was conducted on the brains of 27 mice for which the behavioral data are presented in Chapter 1 of this thesis (Fig 1.2B). All mice were delivered at 7-8 weeks of age. Upon arrival, the mice were housed 3-5 per cage and spent at least 1 week acclimating to our colony room (12:12 schedule; lights on 06:00 hours) before being subjected to stereotaxic surgery. For all experiments, mice were housed in a temperature (20-22^{O} C)- and humidity-controlled environment with *ad libitum* access to food (LabDiet 5001; LabDiet, Richmond, IN) and tap water. All protocols were approved by the Oregon Health & Science University animal care and use committee and performed within the National Institutes for Health Guidelines for the Care and Use of Laboratory Animals, as well as the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research.

Viruses

Adeno-associated (serotype 8) inhibitory (hM4Di) DREADD (AAV8-hSyn-hM3D/hM4D-Gi)-mCherry) virus (UNC Vector Core, North Carolina), were used. The description of these types of viruses have been well documented elsewhere (Armbruster, et. al., 2007; Roth, 2016; Rogan & Roth, 2011). Briefly, DREADDs are synthetically engineered muscarinic acetylcholine [G-protein coupled] receptors that are minimally activated by acetylcholine, but *display potent activation to the pharmacologically inert drug* clozapine-N-oxide (CNO) and couple to Gq (hM3Dq) or Gi (hM4Di) signaling pathways,

creating neuronal activation or inhibition, respectively. This allows for direct control of specific neuronal populations via systemic injection of CNO, and in the absence of this ligand, DREADDs have no known physiologic activity.

Drugs

Clozapine-N-Oxide (1.0 mg/kg, i.p., Sigma) was dissolved in 0.5-1.0% dimethylsulfide (DMSO). Vehicle consisted of saline with a matching percentage of DMSO. All injections were delivered intraperitoneally (i.p.).

Surgical procedures

One to two weeks prior to the start of each experiment, mice were transported to a suite for stereotactic surgery. Mice were anesthetized via 5% isoflurane delivered in oxygen via a precision vaporizer (DatexOhmeda, WI). Following induction, mice were maintained under 1-2% isoflurane anesthesia and secured in a stereotaxic frame (Kopf, 900 series). A glass injector attached to a Hamilton syringe (1.0 µL) via plastic tubing was used to inject 150nL-300nL (unilateral and bilateral, respectively) of virus into the following brain areas: Anterior Cingulate (ACC, defined as CG1 in Paxinos and Franklin, 2009; unilateral, 40^{o} angle, A/P 1.1mm from Bregma, M/L 0.629mm, D/V 0.979mm) and Primary Somatosensory cortex (bilateral, no angle, A/P .98mm from Bregma, M/L 3.1mm, D/V 1.375mm). Unilateral injections were carried out within the ACC, due to the known connectivity between the right and left hemispheres, and due to pilot studies, which displayed that, unilateral inhibition was sufficient for an effect. A non-cre dependent DREADD was injected into CG1 or somatosensory cortex, as defined by Paxinos & Franklin (2001). All injections occurred over the course of 5 min and injectors

were left in place for 10 min and extracted over the course of 5 min. Following recovery from anesthesia, mice were individually housed and transported back to the animal colony for 7-14 days to allow for transfection of the virus and recovery from surgery. Following recovery, all mice were exposed to the experimental procedures described above for alcohol drinking and mechanical testing.

DREADD Activation: For all experiments, CNO (i.p.) administration occurred immediately prior to placement on the testing apparatus on the final test session. Following 20-30 min acclimation to the test rack (and to allow for CNO distribution) mice were tested for mechanical sensitivity as described previously. Immediately following the mechanical test, mice were sacrificed via CO_2 inhalation and brains were extracted for placement analysis.

Alcohol Intake Procedures

Following surgery (during the 7-day acclimation period), mice received 24 h access to two bottles with metal sipper tubes (containing water) on either side of the cage, with food evenly distributed along the wire cage top. No filter tops were used. Following acclimation and baseline testing, mice either received access to 2 bottles of water only (H_2O mice) or 1 bottle each water and alcohol (EtOH mice).

24 h Access Two Bottle Choice: EtOH mice received 24 h access to two bottles: one containing tap water and once containing increasing concentrations of EtOH (3-10%) dissolved in tap water. Fluid levels from each of the two bottles were recorded on a daily basis 2 h into the dark cycle. The locations of the bottles on the cages (left vs. right) were alternated every other day to avoid the potential confound of an inherent side

preference. Further, when multiple treatment groups were housed in a single room, the treatment-assignment was randomly assigned across the cage locations, to avoid any confound related to the treatment of neighboring cages.

Alcohol Withdrawal (WD): Once weekly (2 h into the dark cycle) EtOH bottles were removed and replaced with bottles containing water for 24 h. Thus, for the first week of drinking all mice received each 3% and 6% EtOH for 2 days and 10% EtOH for one day followed by 24 h of withdrawal. On each following week the mice were allowed access to 10% EtOH for 6 days followed by 24 h of withdrawal.

Mechanical Sensitivity

Responses to mechanical stimulation by von Frey hairs (0.01 to 2 g plastic fibers) were determined in the plantar surface of the left hindpaw. Normal response was considered as: withdrawal, shaking or licking the paw. Mechanical thresholds were tested using the Up-Down technique (Chaplan, 2008). This method uses stimulus oscillation around the response threshold to determine the median 50% threshold of response. Mice were allowed to acclimate to the plexiglass enclosure on top of a wire testing rack for 40 min on 2 days prior to the start of the experiment and for 10-20 min before each test session. The testing rack was located within each testing room near the housing rack and illuminated with a dim red lamp. Mechanical sensitivity was assessed prior to treatment exposure (baseline), and mice were then assigned to treatment group based upon basal mechanical thresholds. Testing then occurred each week following 24 h of withdrawal. All behavioral testing was conducted by a single experimenter. During testing, the experimenter was blind to the individual treatment assignments within each

room.

Tissue Processing and Immunohistochemistry

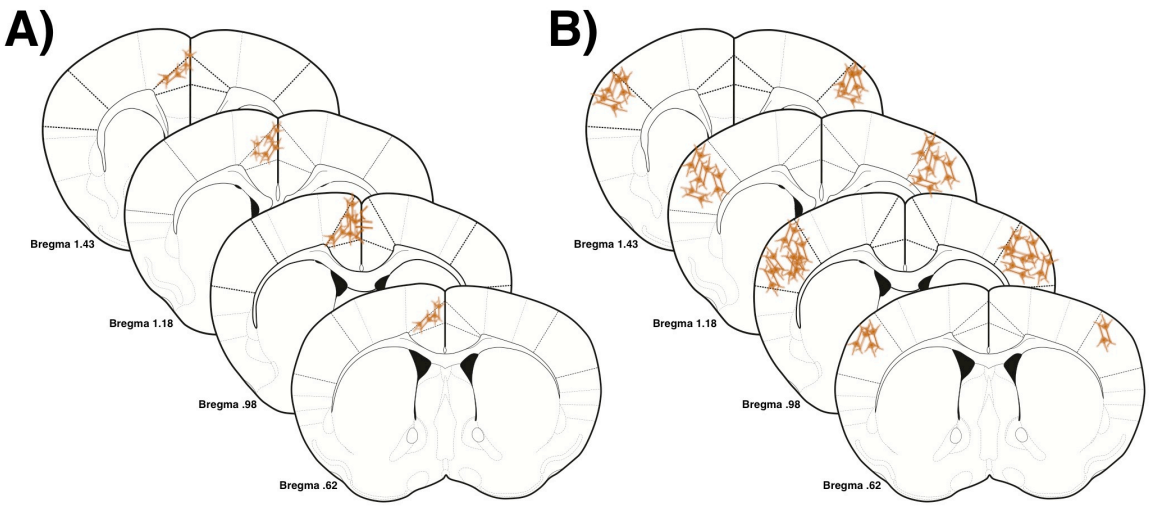
Fos: Brains were taken immediately after the third and final test session for a subset of mice, so that Fos ir would correspond to the neural activation related to the state immediately prior to testing (60-90 min prior to collection). Mice were sacrificed by CO_2 inhalation; brains were extracted, post-fixed for 24 h in 2% paraformaldehyde/PBS and cryopreserved in 20% and then 30% sucrose/PBS. Brains were sliced at 30 µm across the entire brain. Slices containing 20 brain regions of interest were selected for analysis. Brain regions were defined by the Franklin and Paxinos (2001) Mouse Brain Atlas parameters. Slices containing ACC for one animal in the H_2O/Co-Housed group were damaged and removed from analysis. The tissue was processed for Fos immunohistochemistry using standard avidin-biotin-DAB protocols(Bachtell, Tsivkovskaia, & Ryabinin, 2002; Ryabinin et al., 2000). Immunopositive cells were counted manually when there were relatively low numbers of cells, allowing for reliable counts. For cortical regions automatic cell counting was done using ImageJ Software due to the high number of cells present in these regions. All analyses were conducted by an experimenter that was blind to treatment condition. The immunohistochemical reaction was run twice, and the counts were averaged between the 2 batches containing 2-4 slices for each region, for each mouse per batch (average of 4-8 slices per mouse). This average served as a single data point for statistical analysis. There were no interactions of batch and factors of interest when batch was included in an ANOVA as a factor.

DREADD Tissue Processing: Following extraction, brains were post-fixed for 24 h in 2% paraformaldehyde/phosphate-buffered saline (PBS) and cryopreserved in 20-30% sucrose/PBS. Then brains were sliced at 30 µm and processed for mCherry and [in some cases] Fos immunohistochemistry. Unless noted otherwise, all steps were performed in 0.3% Triton-X/Tris-buffered saline (TBS) and preceded by three washes in TBS. The sections were rinsed for 30 min in 1% sodium borohydride in TBS, and blocked in 5% normal donkey serum (Jackson Laboratories) for 45 min. The tissue was then incubated with 1:1000 goat polyclonal Fos antibody (Santa Cruz) and 1:2500 rabbit polyclonal DS-Red (Clontech). This was followed by 1 h incubations with AlexaFluor 555-labeled and AlexaFluor 488-labeled secondary antibodies (raised in donkey) (Invitrogen). Finally, slices were washed with PBS, mounted on gelatinized slide and coverslipped with Prolong Gold (Invitrogen). Co-localization of immunoreactivity was quantified manually using a Leica DM4000 microscope. Viral infusions were considered a "hit" when neuronal expression of the virus was limited to the boundaries of the chosen brain region (as defined by Franklin and Paxinos, 1997). When spread of the virus was beyond this, it was considered a "miss".

This procedure led to the following exclusions: 13 were excluded of 46 total CG1 surgeries (final n= 33), and 3 exclusions of 38 of Somatosensory cortex surgeries (final n = 35). Expression was seen from ~Bregma 1.43 to .62 (Fig 2.3). Any misses were excluded from all analyses.

Figure 2.3: Average viral location and spread of hM4Di virus

Mice with average expression levels were used as “representative” in terms of transfection location and spread. Expression was visually qualitatively assessed, and each slice was assigned a score of +, ++ or +++, using these scores, slices from the mice representing the mean level of expression were used to create the illustrations below. These values are represented by the amount of orange “expression.” When examining all individuals, the average spread occurred from Bregma 1.43 to Bregma .62. Viral expression for ACC and somatosensory transfections were strongest around Bregma .98, and virus was typically not present beyond the displayed representations A) Viral spread for unilateral ACC transfection and B) bilateral somatosensory cortex transfection.



GENERAL DISCUSSION

“When you've suffered a great deal in life, each additional pain is both unbearable and trifling.” (Martel, 2001)

Pain is now conceptualized as a “biopsychsocial” phenomenon that includes sensory, cognitive, emotional and social components. The majority of pain research has focused on the biological mechanisms of pain, and less attention has been given to social factors related to the pain experience. This thesis characterizes a model of the social communication of pain that can be used to examine of biological, psychological and social factors related to the experience of pain. In addition, these studies being to characterize a novel measure of alcohol withdrawal within the mouse.

Summary of Findings

Chapter One characterized a novel phenomenon in which the presence of “primary” animals experiencing hyperalgesia leads to a congruent state of hyperalgesia in “bystander” animals that are housed and tested in the same room. Hyperalgesia is transferred from primary mice to bystanders following diverse noxious stimuli, including local inflammation (intraplantar CFA) or withdrawal from drugs of abuse (morphine or alcohol) and can be measured by mechanical, thermal and chemical nociceptive tests. Bystander mice are housed in their own individual cages with wire cagetops, and nociceptive testing occurred at the same time as primary mice, allowing for the communication of various social cues (olfactory, visual, auditory) during this paradigm. I

established that this pain communication likely occurs via olfactory cues, as experimentally naïve mice demonstrate significant mechanical hypersensitivity following just 24 h of exposure to soiled bedding from primary/bystander mice. Primary and bystander mice do not demonstrate synchronized behavior during nociceptive testing, indicating that visually dependent emotional contagion cannot be used to explain the current findings. Finally, bystander mice do not demonstrate any changes in anxiety-like behavior or alterations in corticosterone levels, suggesting that stress and/or anxiety are not required for the expression of hyperalgesia. In sum, these studies indicate that an abnormal pain state can be transferred between nearby conspecifics via olfactory cues, and that this does not appear to depend on mechanisms related to activation of the HPA axis and can not be explained by stress induced hyperalgesia.

In Chapter Two, I examined the potential neural mechanisms underlying hyperalgesia in both primary and bystander mice. Utilizing the alcohol withdrawal paradigm, I examined Fos-*ir* (as a measure of neural activation) across the brain to identify potential regions of interest. Fos was quantified across 21 brain regions and the results indicated that the anterior cingulate (ACC), anterior insula (AI) and dorsal medial hypothalamus (DMH) displayed differential levels of Fos-*ir* when comparing primary and/or bystander to control mice. These findings pointed to brain regions of interest within the “pain matrix,” that are thought to be important to the affective aspects of pain (the ACC and AI). Considering the Fos data and previous research regarding the pain matrix, I chose to investigate whether the ACC and/or primary somatosensory cortex (S1) were required for the expression of hyperalgesia in the primary and bystander

mice. I therefore used chemogenetic technology (designer receptors exclusively activated by designer drugs; DREADDs) and found that inactivation of the ACC, but not the S1 is required for the expression of hyperalgesia in both primary and bystander mice.

Alcohol Use Disorders and Pain

These studies help illuminate the relationship between alcohol abuse and pain disorders, which has been amply demonstrated in humans (Katon, et. al., 1985), but understudied in animal models, despite similar neuroanatomical substrates (Egli, et. al., 2012). For some individuals, alcohol abuse precedes the development of chronic pain, whereas in others, alcohol consumption occurs as a mechanism for coping with chronic pain. Moreover, chronic drinking can lead to severe pain during and following the withdrawal process (Gatch, 1999; Jochum et. al, 2012). Although pain is often reported as a symptom of withdrawal in humans, it has never been reported following voluntary drinking in the C57BL/6J (B6) mouse. In fact, drinking in the two-bottle choice procedure in B6 mice has been argued to be a poor model of alcoholism for reasons including lack of overt signs of pathological effects after prolonged history of drinking (Dole, et. al., 1985). The studies described in Chapter One and Chapter Two demonstrate reliable hyperalgesia in the B6 mouse during abstinence from voluntary drinking, and potentially demonstrates that a translational sign of withdrawal develops following short term two-bottle choice drinking. However, further exploration of this abstinence-induced hyperalgesia needs to be conducted in order to determine whether this can be used as

a model withdrawal in the mouse. Important studies will include: comparison to mouse strains that reliably display other symptoms of withdrawal such as handling induced convulsions (HIC's; e.g.: DBA/2J mice), and investigation of the timecourse of development. Studies in Chapter One already demonstrate that hyperalgesia is not dependent upon a state of anxiety, or activation of the HPA axis, due to the lack of changes in elevated plus maze behavior and corticosterone levels, respectively. Additionally, the mechanisms underlying WD-induced hyperalgesia appear to differ from those required for the expression of handling induced convulsions, as evidenced by the fact that diazepam pre-treatment is sufficient to reverse HIC's, but not hyperalgesia (Chapter 1). These studies differentiate the current state of hyperalgesia from other potential indicators of physical withdrawal, at least in the B6 mouse.

The neural substrates underlying alcohol dependence are known to be critical to the transmission and perception of pain (Egli et. al., 2012; Apkarian et. al., 2012), though the exact relationship between alcohol use disorders and pain conditions has yet to be elucidated. The studies in Chapter Two of this dissertation demonstrate that the anterior cingulate (ACC) is required for the expression of both alcohol withdrawal-induced and socially transferred pain. It has been previously demonstrated that this area is important for the affective components of the pain, as evidenced by human and animal literature (Zhuo, et. al., 2006; Rainville, et. al., 1997). Interestingly, inhibition of another area known to be involved activated during pain (somatosensory cortex) did not have an effect on mechanical thresholds in alcohol withdrawn or bystander mice. These studies provide further evidence that symptoms related to alcohol abstinence rely upon

neural circuitry that is fundamental to the experience of pain. It will be interesting to determine whether there are divergent circuits or specialized cell populations activated during alcohol withdrawal induced hyperalgesia compared to other abnormal pain states.

Central Sensitization and Non-Nociceptive Pain

As mentioned in the Introduction of this dissertation, in the event of acute, "nociceptive" pain, nociceptors are activated by noxious stimulation that is either sufficient to produce or threaten tissue damage. The extent of nociception depends upon the nature and degree of damage, and this process is terminated following healing. This is a presumably protective process, which immediately elicits both reflexive withdrawal and eventually encourages complex behavioral strategies geared toward termination of damage and avoidance of such stimuli. Considering this, acute pain has a clear purpose: to avoid injury and promote healing. One way in which this system functions is through "sensitization," or the process by which the threshold for nociceptive responding is decreased and responses to subsequent stimuli are enhanced (Woolf & Walters, 1991). In most cases, this state of sensitivity is terminated in the absence of ongoing injury. However, it is possible for sensitization to persist, leading to a lasting state of heightened sensory experiences. This state of "central sensitization" can occur following functional, chemical, and structural changes within the central nervous system (Latremoliere & Woolf, 2010).

Pain often has no clear relation to tissue damage, nor does it correlate with

healing (Wall, 1979), and persistent pain does not have a clear protective purpose, and may involve sensitization in the periphery and/or centrally. This pain occurs spontaneously, and can be provoked by normally innocuous stimuli (allodynia), lead to enhanced responses to noxious stimuli (hyperalgesia) and spread beyond the site of injury (secondary hyperalgesia). Central sensitization provides a mechanistic explanation for some types of persistent or chronic pain conditions, and reveals that central nervous system changes can result in pain in the absence of noxious stimuli, injury, or peripheral pathology. Central sensitization can be triggered by repeated noxious stimulation, inflammation, or nerve injury, and leads to plasticity within the dorsal horn of the spinal cord, and even plasticity within the cortex (Latremoliere & Woolf, 2010).

Psychological and behavioral factors may also important to the maintenance of sensitization in certain cases (Gracely, et. al., 2004). Furthermore, social and environmental factors such as early life trauma and emotional stress have been implicated in the development of central sensitivity-related pain syndromes like fibromyalgia (McLean & Clauw 2004). These findings are examples of central, or "top-down" types of alterations in nociceptive sensitivity and pain. Perhaps these findings are unsurprising considering the biopsychosocial model of pain. Based on the current dissertation studies, it appears that under certain conditions, social cues are capable of inducing an abnormal pain state. In this case, exposure to olfactory cues (related to hyperalgesia) induces a similar state in otherwise naïve mice. Since this "socially transferred" hyperalgesia is an abnormal, non-noxious pain induced by

social/environmental cues, it is tempting to speculate that this model could be representative of a “top-down” induction of pain, and could be used to model syndromes that lack a clear cause (e.g., fibromyalgia). However, additional studies need to be conducted to further explore the mechanisms underlying socially transferred hyperalgesia, for example, it will be important to determine whether this state is indicative of central sensitization, and if these mice are actually in pain (and not just displaying enhanced nociception).

Modeling the Social Communication of Pain

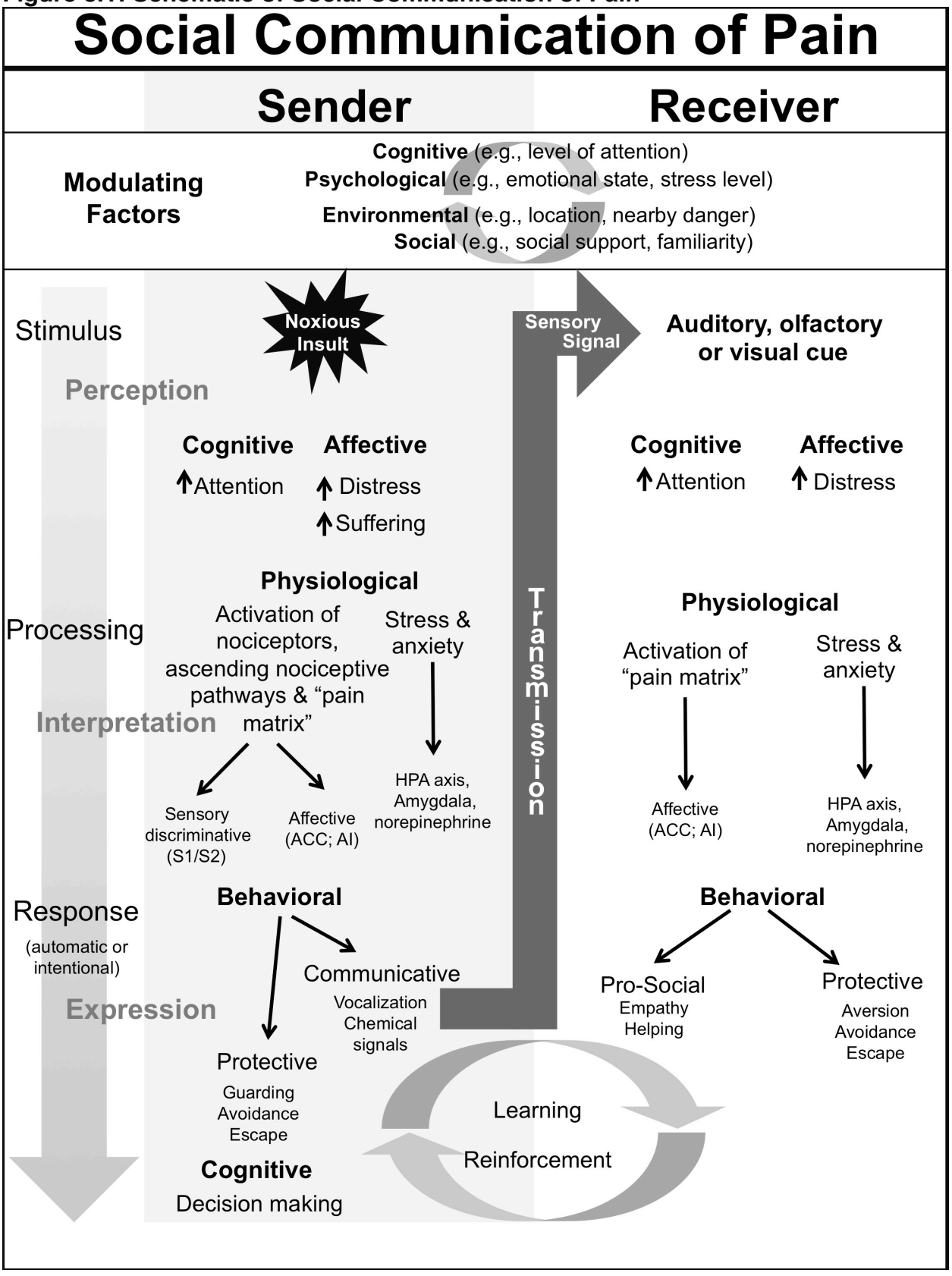
Figure 3.1 illustrates a schematic framework to conceptualize the social communication of pain. As briefly described in the introduction, this process requires the perception, interpretation, and expression of pain on the part of a “sender,” which is automatically or intentionally communicated to a “receiver” via a sensory signal. This signal is then perceived and interpreted by the receiver leading to a response.

Modulating Factors

As represented in the diagram, this entire process is modulated by cognitive, psychological, environmental and social factors, supporting the biopsychosocial model of pain and pain communication. The state of the individual when the stimulus is received (either a noxious stimulus or sensory signal) can determine how the stimulus is interpreted and the direction of nociceptive response (analgesia or hyperalgesia; Lumley et. al., 2011). For example, an aversive emotional state with low arousal enhances pain, whereas negatively valenced emotions with high arousal (such as fear) reduce pain

(Rhudy & Meagher, 2000). Factors like the level of available attention and cognitive load can also reduce subjective pain ratings (Wiech et al., 2005). Examples of modulating factors are represented in the diagram, but are not meant to be all-inclusive.

Figure 3.1: Schematic of Social Communication of Pain



Stimulus Perception:

The process begins with noxious stimulation in the sender, which activates ascending nociceptive pathways and/or areas of the pain matrix responsible for sensory discrimination and affective responses, typically enhancing attention and leading to distress. Sensory discrimination consists of determining aspects of the experience such as the quality, location and intensity of the painful stimulus, and it is thought that the somatosensory cortex plays a large role in this processing (Lamm et al., 2011; Ploner, Schmitz, Freund, & Schnitzler, 1999b). This process also includes emotional responses to pain, like suffering, anger, fear and defeat. These affective states are thought to depend upon the ACC (Lamm et al., 2011; Rainville et al., 1997; Zhuo, 2006). In fact, lesion of the somatosensory or cingulate cortices leads to deficits in sensory discrimination and the affective aspect of pain, respectively (Foltz & White, 1962; Ploner, Freund, & Schnitzler, 1999a).

During the social communication of pain, the sympathetic nervous system may be activated leading to activation of the HPA axis. However, it is important to note that activation of the HPA axis and/or stress response is a *possible parallel process* and pain perception and control do not *require* HPA axis activation. If the stress response occurs during pain, it can be categorized into two stages: immediate *defensive arousal,* followed by *recovery* (Chapman, Tuckett, & Song, 2008). Defensive arousal occurs to enable adaptive behaviors (e.g., escape), and leads to activation of the locus coeruleus noradrenergic system, the hypothalamo-pituitary-adrenocortical (HPA) axis based in the hypothalamic periventricular nucleus (PVN), and the sympathoadrenomedullary (SAM)

axis (Padgett & Glaser, 2003). Under these circumstances, appropriate sensory stimulation can generate an allostatic response that involves an ensemble of interdependent nervous system, endocrine and immune processes. This may or may not include processes such as central sensitization with regard to nociceptive processing. Stressors (including social stressors) can compound the allostatic load of sensory stimulation, or act alone to dysregulate the system.

The recovery stage represents a slower process of behavioral adaptation and return to normalcy that begins prior to the completion of the defensive arousal stage. Integrated activation of nociceptive and stress systems represents a dynamic process, and as represented by the currents studies, certain indicators of stress and/or anxiety may not be measureable during the presence of an abnormal pain state. In terms of the current studies, it is possible that the defensive arousal stage was terminated prior to the point we chose to take CORT measurements, and the mice were in a state of recovery, thus leading to a negative result. However, the lack of anxiety-like behavior or acoustic-hyper-reactivity in these mice suggests that it is unlikely that they were experiencing a heavy allostatic load at this timepoint. Additionally, the restraint stress experiment suggests that there were not long term changes in the stress response in these mice. Again, the timecourse of these behaviors should be further explored before making definitive conclusions regarding the involvements of stress systems in this behavior. However, when considering just the current evidence, it is possible that either the stress response was not required for the development of hyperalgesia in this paradigm, or the timecourse or chosen measurements did not detect changes that were

present.

Response and Expression:

Physiological and affective processes then lead to responses in the form of protective and/or communicative behaviors (Sullivan, 2008). These responses are "inclusive of actions that may or not be intentionally sent, as well as the intentional and unintentional reactions of receivers to the cues or signs" (Hadjistavropoulos et al., 2011). Any expression of pain aimed at conveying information to an observer can be considered communicative pain behavior, whereas protective pain behaviors are aimed at reducing further injury or promoting recovery. For example, reflexive withdrawal of a limb is an automatic, protective behavior, whereas facial expression or vocalization can be a communicative behavior that is automatic or intentional. However, it is not always this straightforward, as pain communication is a dynamic process. The behaviors involved in pain communication may be interrelated and determined by physiological, social, and environmental factors. Facial displays and vocalizations represent two types of communicative behaviors have been extensively studied. It has been suggested that facial displays might be important when others are in close proximity, whereas vocalizations could be utilized when others are not in view (Grunau & Craig, 1987; Zeskind, Sale, Maio, Huntington, & Weiseman, 1985). However, as evidenced by the current studies, olfactory communication is also an important channel for social communication. However, this has been studied far less extensively.

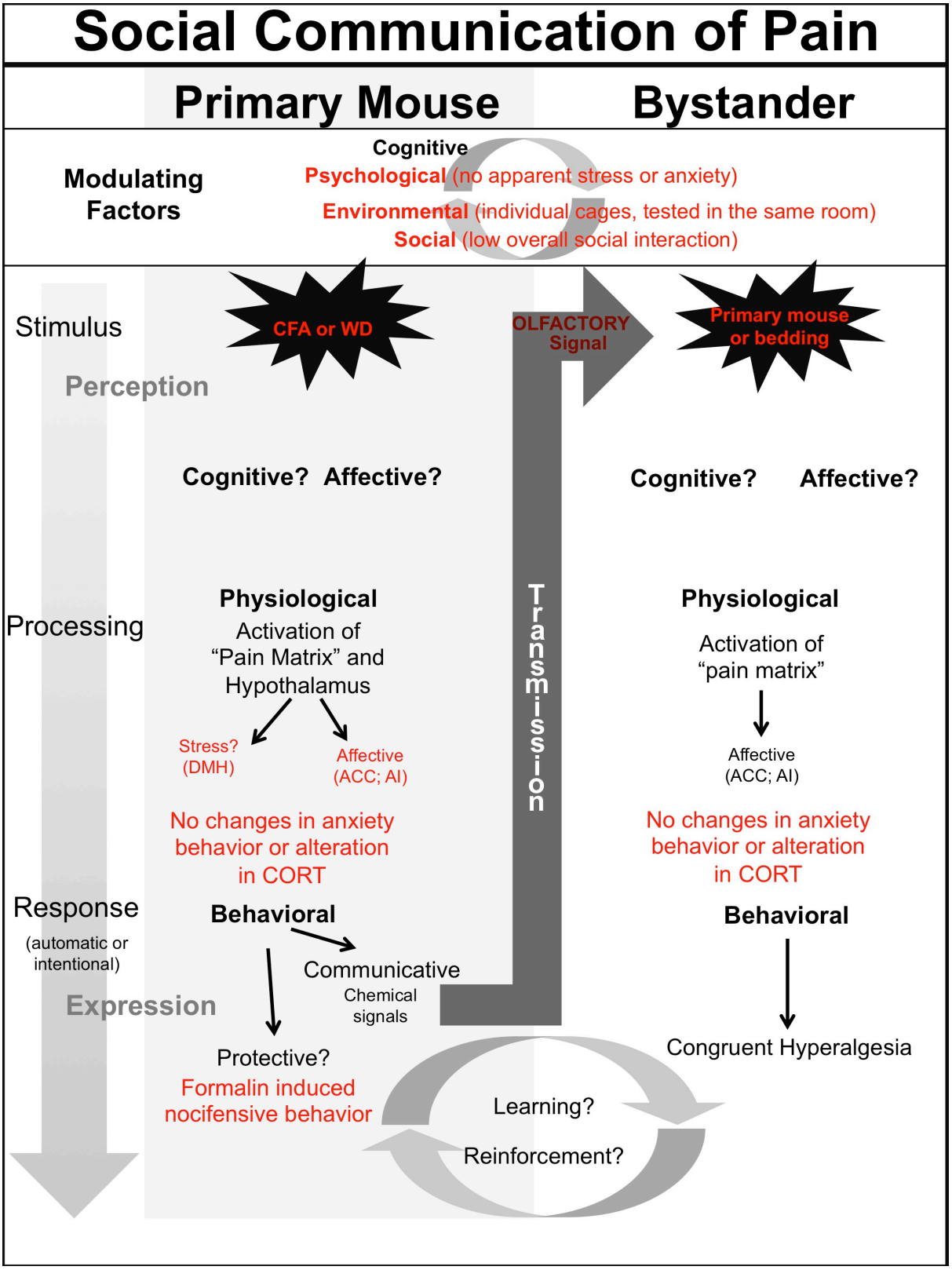
Transmission:

Once a behavior and/or sensory signal is expressed by the sender, this cue can

be transmitted to a receiver. Then the signal acts as a stimulus to engage a similar process in the receiver as experienced by the sender. For example, an auditory cue (such as a cry) perceived by the receiver can engage attention, and/or elicit distress. The interpretation of this cue will have physiological effects, and lead to a behavioral response. This response could range from protective behavior (avoidance of the sender) to pro-social behavior (approach and helping), depending upon a complex array of factors.

Using the schematic outlined in Fig 3.1 it becomes clear that there are several levels to examine social communication, and a variety of parallel processing and modulating factors that have yet to be elucidated in terms of the sender and the receiver. Fig 3.2 shows how the current studies have probed the social communication of pain, and how the current model of “social transfer of pain” can be used to further explore this process. As demonstrated in this schematic, the cognitive and affective states of the primary and bystander mice are unknown, and only certain aspects of each stage of social communication have been examined. Therefore, this model leaves room for future experiments to further investigate the social communication of pain at several levels.

Figure 3.2: Schematic Including Current Dissertation Studies



Final Comments

The results of this dissertation provide novel data for the fields of pain, alcohol and social behavior research: For the pain field, I demonstrate that an abnormal pain state can be induced solely by social factors without any prior tissue damage. For the alcohol field, I demonstrate that hyperalgesia occurs following short-term voluntary alcohol drinking, which may represent a measure of alcohol dependence in this preclinical model. Finally, for *all* studies using animal models, I show that the common practice of housing control animals in the same room (or even in the same cage) with experimental animals may not be appropriate because of the possibility of social transfer of physiological states.

As mentioned above, each of these areas need to be further investigated in ways that are beyond the scope of this dissertation. For example, determining whether bystander mice are in a state of pain (including some type of affective component, such as conditioned avoidance) will be important for the development of this pain model. Additionally, detailed examination of the timecourse of alcohol-withdrawal hyperalgesia and comparison of this behavior to other withdrawal symptoms will further inform whether or not these mice are, in fact, demonstrating signs of dependence. It will be important to determine the various conditions in which social transfer of pain (or other physiological states) occurs. For example, it will be important to determine whether filter tops, or cage filtration will reduce the effects of olfactory cues and, in turn, inhibit the development of this behavior. Furthermore, it will be interesting to explore whether familiarity of the animals, or their innate sociability modulates the acquisition or

expression of socially transferred hyperalgesia. Studies such as these might inform the question of whether this behavior is related to “empathy.” These are just a few examples of the possible future directions for this project. Nevertheless, these studies have laid the foundation for a novel animal model that is important to the fields of both pain and alcohol research and describe a novel way to explore the social communication of pain.

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