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Isoform-Specific Roles of Extracellular Signal-Regulated Kinases in Pain

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by

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ABSTRACT OF THE DISSERTATION

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Professor Robert Gereau, Chairperson

The extracellular signal-regulated kinase (ERK) isoforms, ERK1 and ERK2, are believed to be key signaling molecules in nociception and nociceptive sensitization. Studies utilizing inhibitors targeting the shared ERK1/2 upstream activator, mitogen-activated protein kinase kinase (MEK), and transgenic mice expressing a dominant negative form of MEK have established the importance of ERK1/2 signaling. However, these techniques do not discriminate between ERK1 and ERK2. To dissect the function of each isoform in pain, mice with a targeted genetic deletion of ERK1 (*ERK1*$^{-/-}$) and mice with a conditional deletion of ERK2 in nociceptors (*ERK2*$^{f/f}$*;Na*$_V$*1.8-Cre*) were used. Although both isoforms are activated (phosphorylated) following inflammation, deletion of ERK1 had no effect in several models of chemical nociception, inflammatory pain, and neuropathic pain. In contrast, conditional deletion of ERK2 in nociceptors attenuates nociceptive spontaneous behavior in the second phase of the formalin test, reduces inflammatory mechanical hypersensitivity, and eliminates heat hypersensitivity due to the inflammatory mediator, nerve growth factor (NGF). Nociceptive sensitization was not reduced in all models tested, since *ERK2*$^{f/f}$*;Na*$_V$*1.8-Cre* mice developed robust heat hypersensitivity to other inflammatory and chemical insults. Biochemical analysis of both lines revealed that eliminating one ERK isoform led to elevated phosphorylation of the remaining isoform at baseline, which could explain the lack of a phenotype in *ERK1*$^{-/-}$ mice. However, this is probably not the case since the elevation in spinal

cord ERK2 phosphorylation above baseline following noxious stimulation is not affected by the deletion of ERK1. It is also possible that other intracellular signaling cascades may compensate for the loss of ERK1. This seems less probable since systemic MEK inhibition attenuates formalin-induced spontaneous nociceptive behaviors similarly in *ERK1*$^{-/-}$ and WT littermate controls. These experiments demonstrate an isoform-specific role for ERK2 on the behavioral level. On the cellular level, ERK2 is also partially required for innervation of the epidermis by peptidergic nociceptive afferents that express the NGF receptor, TrkA. The partial reduction in epidermal innervation is not accompanied by cell loss, suggesting a defect in axon growth or maintenance. Fiber loss is unlikely to account for all behavioral phenotypes observed in *ERK2*$^{f/f}$*;Na*$_V$*1.8-Cre* mice since remaining epidermal fibers have previously been shown to play important roles on the behavioral level. Additionally, *ERK1*$^{-/-}$ mice have exaggerated NGF-induced heat hypersensitivity but do not have altered epidermal innervation. Overall, these data highlight the complicated interplay between ERK1 and ERK2 *in vivo* and suggest that ERK2 is the critical isoform for nociceptor sensitization.

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This work is dedicated to my father, whose success in science and in life I can only hope to emulate,

and to my best friend and true love, C.A.W.

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Chapter 1: Introduction

The advantages of nociception

The detection of noxious, potentially damaging stimuli is an important function of the sensory nervous system. This process, termed nociception (Loeser and Treede, 2008), and its modulation represent the culmination of evolutionary adaptation. The ability to sense and avoid potentially lethal threats provides an obvious advantage for survival (Scholz and Woolf, 2002). Equally important is the ability to recover from injury. In mammals, tissue damage is associated with an immune response, inflammation, and eventual wound repair. Although physiologic wound healing is relatively robust, the process would be severely hampered by repeated injury. In this light, enhanced nociception seems to provide some benefit. If the injured tissues become more sensitive to noxious stimuli, the organism may guard against reinjury. A similar phenomenon occurs with innocuous stimuli, which become exquisitely noxious following an injury. Enhanced nociception may not always be adaptive, however. In life-threatening situations, it may be more advantageous to silence nociceptive information in order to escape or resist a threat. This function of the nociceptive system has been observed in many organisms and can be appreciated in our own personal accounts of sports injuries or heroic actions in extreme situations. Thus, the nociceptive system not only faithfully detects noxious stimuli but can also enhance or repress nociception in a context-appropriate fashion. The overall benefit of the nociceptive system is highlighted by patients with severe inherited deficits in the nociceptive sensory system due to loss-of-function of $Na_V1.7$ or TrkA (Nagasako et al., 2003; Drenth and Waxman, 2007). With congenital insensitivity to pain, patients acquire self-inflicted facial and oral injuries, burn injuries, undetected fractures and subsequent orthopedic complications. In many ways, the nociceptive system is largely beneficial.

Chronic pain

Pain is distinct from nociception (Loeser and Treede, 2008). The International Association for the Study of Pain defines pain as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage.” Nociception is a sensory process that may or may not be associated with the percept of pain. For example, modulation of nociception may prevent the percept of pain during stress. Chronic pain can be defined as persistent or intractable pain that may or may not be associated with injury. One broad class of

chronic pain is pain due to injury of the nervous system, or neuropathic pain (Costigan et al., 2009). Common examples of neuropathic pain include painful peripheral neuropathies, such as carpal tunnel syndrome or postherpetic neuralgia, pain resulting from central nervous system damage, such as spinal cord injury or stroke, and pain associated with limb amputation, such as phantom limb pain. In neuropathic pain, the damaged nervous system signals as if noxious stimuli were occurring, even when they are not. Moreover, plastic changes in the undamaged nervous system amplify these inappropriate nociceptive inputs and contribute to the perception of pain. This dual effect results in persistent pain that is unrelated to external stimuli. In contrast, inflammatory pain arises from a normally functioning nociceptive system. As mentioned above, inflammatory pain can be thought of as beneficial in order to allow wound healing. Although biological phenomena often make sense in the light of evolutionary adaptation, this teleological argument does little for patients suffering from pain following traumatic injury or surgery. Another example of inflammatory pain includes rheumatoid arthritis, in which inflammatory mediators persist within joints and enhance nociception. Injury and arthritis are often referred to as nociceptive pain, since the nociceptive system is not damaged.

Inflammatory and neuropathic pain share important features. Both can persist for long periods of time, compromising patients' daily activities and reducing their quality of life. Both forms of chronic pain involve enhanced or sensitized nociception. In inflammatory pain, nociceptive afferents respond more vigorously to noxious stimuli and become sensitive to previously innocuous stimuli. This results from a process known as peripheral sensitization, in which inflammatory mediators enhance nociceptor excitability, reduce sensory thresholds, and initiate transcriptional changes thought to underlie long-term sensitization (Woolf and Ma, 2007). Certain types of peripheral sensitization also occur due to nerve injury. Although nerve injury may lead to areas of denervation and insensitivity, intact axons that comingle with injured axons can acquire new properties which contribute to enhanced sensitivity to noxious and innocuous stimuli (Costigan et al., 2009). Inflammatory and neuropathic pain both involve central sensitization (Costigan et al., 2009; Sandkuhler, 2009). Second order neurons found in the superficial dorsal horn of the spinal cord receive input from nociceptors, transmitting this information to higher brain centers. After injury or inflammation, the incoming nociceptive information is amplified (Ikeda et al., 2006; Sandkuhler, 2009). For example, the same nociceptive input that produced low frequency firing in dorsal

horn neurons will evoke increased firing frequencies as a result of central sensitization. Similar plasticity can occur in other higher order structures in the context of inflammatory or neuropathic pain. Although the pathophysiological mechanisms of inflammatory and neuropathic pain are quite different, they share basic themes including plasticity at the level of the nociceptor and at the level of the spinal cord, termed peripheral and central sensitization respectively.

Despite significant progress in our understanding of chronic pain, treatments for chronic pain conditions are limited. The mainstays of pain control are non-steroidal anti-inflammatory drugs (NSAIDs) and opiates. These drugs are analgesic in nociceptive pain, but are often not completely effective in inflammatory pain and have almost no efficacy in many neuropathic conditions (Kissin, 2010). Moreover, these analgesics are limited by their side-effect profiles. NSAIDs are associated with gastric and renal toxicity, and opiate dosage is restricted by sedating effects and constipation. In a concerted effort to find novel pain medications, researchers have uncovered several promising pharmacological targets. In the 1990's, TRPV1 was identified as a molecular sensor of noxious heat and protons, and it has enjoyed great interest by pharmaceutical companies since then (Szallasi et al., 2007). Even with a body of evidence suggesting that its expression is restricted to nociceptive sensory neurons, functional evidence has suggested that TRPV1 modulates synaptic plasticity in hippocampus and plays an important role in thermoregulation (Szallasi et al., 2007; Alter and Gereau, 2008; Gibson et al., 2008; Khairatkar-Joshi and Szallasi, 2009). Both functions represent potential side-effect pitfalls when considering TRPV1 antagonists for the treatment of pain. Instead of targeting sensory transduction machinery like TRPV1, it may be more fruitful to target molecules that sensitize sensory transduction. The detection of noxious stimuli is not the main clinical problem in chronic pain. Sensitization processes, occurring in the central and peripheral nervous systems, should be the main focus for developing new chronic pain therapies. One promising area of investigation involves indentifying intracellular signaling molecules that play an important role in nociceptive sensitization.

The ERK1/2 toolbox

Extracellular signal-regulated kinases (ERK) 1 and 2 are mitogen-activated protein kinases (MAPK) that have become a focus of pain research. ERK1/2 are part of a three-tiered intracellular signaling module of serially arranged protein kinases. Activated Raf phosphorylates and activates

the MAP kinase kinase MEK1/2, which goes onto phosphorylate and activate ERK1/2 (Fig. 1, English et al., 1999; Pearson et al., 2001). Much information about the function of this signaling cascade has been gleaned using protein kinase inhibitors and phospho-specific antibodies. Since many protein kinases share a high degree of homology, selective inhibitors are rare (Davies et al., 2000). Fortunately, several relatively selective inhibitors of MEK1/2 have been discovered, perhaps as a result of their mode of action. PD98059 (Dudley et al., 1995) and U0126 (Favata et al., 1998) do not bind at MEK1/2 active site, but instead inhibit MEK1/2 activity through a non-competitive mechanism probably involving allosteric modulation of MEK (Fischmann et al., 2009). Since the only known substrate of MEK1/2 is ERK1/2, inhibition of MEK1/2 is a way to selectively block ERK1/2 activity. It is important to point out that at each level of this three-tiered intracellular signaling system, there are several different isoforms with relatively high sequence homology. PD98059 and U0126 have similar potency at both MEK isoforms, MEK1 and MEK2 (Servant et al., 1996; Favata et al., 1998). Since *in vitro* assays have shown that both MEK1 and MEK2 can phosphorylate both ERK1 and ERK2 (Pearson et al., 2001) and since MEK1/2 inhibitors decrease the phosphorylation status of both ERK1 and ERK2 in cells or *in vivo* (see Chapters 2 and 3, Servant et al., 1996; Karim

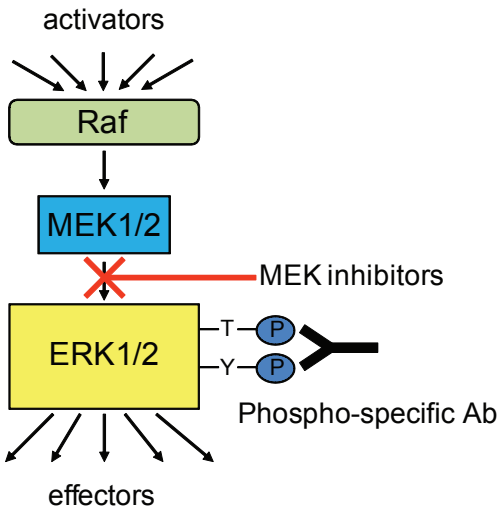

Figure 1: MAP Kinase Module Raf, MEK1/2, and ERK1/2 are serially arranged protein kinases that integrate signals from cell surface receptors and second messenger systems (activators). Upon dual phosphorylation of ERK1/2 at threonine and tyrosine residues within the phosphorylation loop, ERK1/2 kinase activity increases dramatically, acting as a molecular switch. Activated ERK1/2 phosphorylates many downstream effector molecules, such as ion channels, cytoskeletal proteins, or transcription factors. To study this pathway, MEK inhibitors can be used to block ERK1/2 activation. Phospho-specific antibodies specifically recognize dually phosphorylated ERK1/2, allowing for the detection of ERK1/2 activation.

et al., 2001; Carrasquillo and Gereau, 2007), MEK1/2 inhibitors are likely to prevent signaling between every combination of MEK and ERK isoform. Therefore, MEK1/2 inhibitors provide no information about which specific MEK1/2 or ERK1/2 isoform is important. Phospho-specific antibodies to ERK1/2 allow for the measurement of ERK1/2 activation (phosphorylation). Since the ERK domain phosphorylated by MEK, known as the phosphorylation loop, is highly conserved between ERK1 and ERK2 (Fig. 2), phospho-specific antibodies bind both phosphorylated ERK1 (pERK1) and pERK2. Therefore, immunostaining experiments cannot be used to distinguish isoform specific activity. Western blotting does allow for this sort of analysis since ERK1 and ERK2 are different sizes. However, this method is limited since tissue homogenates used in Western blotting represent a heterogeneous mixture of cells. Nevertheless, using these tools has produced a wealth of information about the role of ERK1/2 in nociception and nociceptive sensitization.

ERK1/2 and nociception

Studies using rodent models have demonstrated that ERK1/2 activation occurs rapidly in nociceptive afferents following noxious stimulation. In anesthetized rats, pERK1/2 immunostaining can be observed in cell bodies of afferents, found in dorsal root ganglia (DRG), by stimulating the paw for 2 minutes using a heated water bath (Dai et al., 2002). At 42°C, a small but significant increase in the number of pERK1/2-positive DRG neurons is detected when compared with untreated control rats. Increasing the temperature of the stimulus to noxious temperatures (46°C-60°C) linearly increases pERK1/2-positive cell bodies. Heat-induced ERK1/2 activation is associated with the noxious heat sensor, TRPV1. Roughly 80% of pERK1/2-positive cell profiles colocalize with TRPV1, and TRPV1 antagonists reduce pERK1/2 immunoreactivity. Activation of TRPV1 by hindpaw injection of capsaicin, the pungent component of chili peppers, also induces pERK1/2 immunoreactivity in the cell bodies of DRG neurons. Overall these data suggest that acute noxious heat leads to ERK1/2 activation via TRPV1 within minutes. However, the cell body of nociceptive afferents is quite distant from the initial site of sensory transduction, raising the question of how heat stimuli could lead to somatic ERK1/2 phosphorylation so quickly. In a critical experiment, Dai et al. applied topical lidocaine to the sciatic nerve, which contains the axons of nociceptors innervating the hindpaw. Following this nerve conduction block, capsaicin-induced pERK1/2 immunoreactivity was completely abrogated, indicating that electrical activity was necessary for somatic ERK1/2 activation. Voltage-gated calcium channels likely connect membrane depolarization with ERK1/2

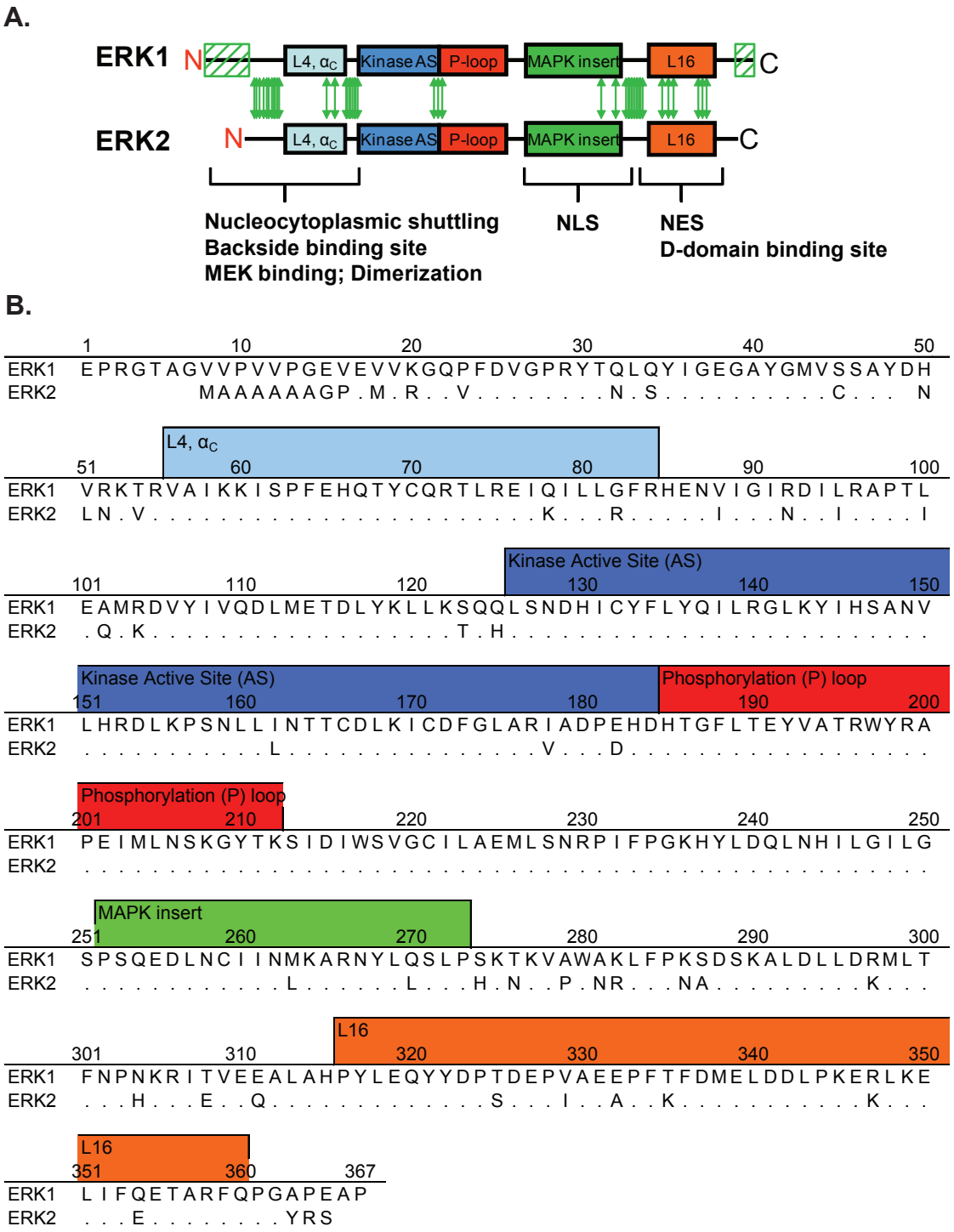

Figure 2: Comparison of ERK1 and ERK2 structure A. Diagram of ERK1 and ERK2 structure annotated with putative cellular functions and important domains. Information compiled from Cobb and Goldsmith, 2000, English et al., 1999, Pearson et al., 2001, Kinoshita et al., 2008, and Marchi

et al., 2008. Isoforms share approximately 90% sequence homology; however differences (denoted by green arrows or hatched boxes) occur throughout the proteins, especially at the termini. **B.** Primary amino acid sequence comparison of ERK1 and ERK2 (Boulton et al., 1991). Amino acid numbering is based on ERK1 sequence. ERK2 sequence appears below ERK1 sequence with shared amino acids denoted by periods (.) and nonhomologous sequence listed.

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activation via calcium sensitive kinases such as CaMK or certain PKC isoforms (Fields et al., 1997). Somatic ERK1/2 phosphorylation is poised to initiate changes in transcription by nuclear translocation and phosphorylation of transcription factors such as CREB. It is unclear whether noxious stimulation as described above would initiate these processes, which are better understood in persistent inflammatory pain models (see discussion below). Nevertheless, it is clear that ERK1/2 becomes phosphorylated in the soma of DRG following noxious heat.

Although the nociceptor soma represents the physical site of transcriptional modulation, post-translational modifications can also affect the function of sensory neurons. Functionally significant post-translation modifications can occur throughout the subcellular extent of the nociceptor. This has been most studied in the terminals of nociceptors that innervate target tissues, such as the skin, and contain sensory transduction machinery. One potential mechanism for modulating nociceptor sensitivity is to modulate sensitivity of this transduction machinery. For example, the phosphorylation of TRPV1 by PKC, PKA, and Src enhance TRPV1 function by increasing channel open probability, reversing desensitization of the channel, and increasing exocytosis of the channel, respectively (Huang et al., 2006). It is therefore quite interesting that subcutaneous injection of capsaicin into the plantar surface of the hindpaw (intraplantar) results in rapid increases in pERK1/2 immunoreactivity in epidermal free nerve endings (Dai et al., 2002). Moreover, this activation of ERK1/2 is behaviorally relevant. The injection of capsaicin in humans causes a burning pain that rapidly resolves within 15 minutes but is accompanied by hypersensitivity to heat and mechanical stimuli lasting about one hour (Torebjork et al., 1992). This is recapitulated in rodents, producing hypersensitivity to heat with a similar timecourse (Dai et al., 2002). Interestingly, intraplantar injection of a MEK inhibitor immediately before capsaicin reduces heat hypersensitivity and concomitantly attenuates pERK1/2 immunoreactivity in fine nerve endings of the skin. Future experiments should directly test whether noxious heat stimulation induces ERK1/2 activation in peripheral terminals, as one might expect. However, based on the above results, it appears that ERK1/2 is activated following noxious heat

and that ERK1/2 activation in afferent fibers is required for behavioral sensitization.

ERK1/2 phosphorylation in nociceptive afferents has been observed after other types of noxious stimuli. These include noxious cold (Mizushima et al., 2006). At temperatures between 28°C and 20°C, pERK1/2-positive cells are rare, ~1% of the total number of DRG neurons, and similar to the frequency observed in naïve animals. Exposure of the paw to a 16°C water bath for 2 min (10 s in bath, 10 s out of bath, repeated 6 times) yields significantly more pERK1/2-positive cells, and their frequency progressively increases with colder temperatures down to 4°C, at which ~10% of all neurons label for pERK1/2. As an interesting parallel to the discussion above, cold-induced pERK1/2 immunostaining co-occurs with in situ hybridization signal for sensory transduction channels that are activated by cold, TRPM8 and TRPA1. Approximately 80% of the pERK1/2-positive cell profiles colocalize with TRPM8 mRNA, while ~10% of pERK1/2-positive cells also express TRPA1 mRNA. In addition to noxious heat and noxious cold, noxious mechanical stimulation increases pERK1/2 staining in the soma of DRG (Dai et al., 2002). A similar stimulation paradigm (10 s of pinch followed by 10 s without pinch, repeated 6 times) yields significant increases pERK1/2-positive DRG neurons. Additionally, electrical stimulation of the sciatic nerve that evokes Aδ- and C-fiber compound action potentials is sufficient to induce pERK1/2 immunostaining in DRG neurons (Dai et al., 2002). Clearly, noxious stimulation in several modalities (heat, cold, mechanical) leads to ERK1/2 activation in DRG neurons.

Given these observations, there are several important considerations regarding the function of ERK1/2 phosphorylation in DRG neurons. It appears that innocuous stimuli do not lead to ERK1/2 activation. Paw stimulation with temperatures between 20°C and 38°C have no effect on pERK1/2 immunostaining above the background levels observed in naïve rats (Dai et al., 2002; Mizushima et al., 2006). Additionally, electrical stimulation at current amplitudes that evoke Aβ compound action potentials is not sufficient to increase pERK1/2 in DRG neurons. Since changes in pERK1/2 above baseline values are not affected by innocuous stimulation, it seems plausible that ERK1/2 activation is related to nociception and may be involved in nociceptive sensitization. Another related consideration is the effect of MEK inhibitors in naïve animals. Inhibition of ERK1/2 in unstimulated rats by intraplantar injection of MEK1/2 inhibitors has no effect on their sensitivity to short applications of heat or mechanical stimuli (Hao et al., 2008). These experiments suggest that low levels of basal ERK1/2 phosphorylation do not modulate the sensation of acute noxious stimuli.

Therefore, it seems that innocuous stimuli do not increase pERK1/2, and basal levels of pERK1/2 do not actively modulate acute nociception.

In the preceding discussion, a tacit distinction has been made about acute nociception and noxious stimulation. This is a helpful distinction, but in regards to ERK1/2 signaling, its boundaries are only roughly known. Noxious stimulation with heat, cold, pinch, or pulses of electrical current all increase pERK1/2. All paradigms used involve repeated or prolonged stimulation – 2 minutes of continuous exposure to heat, for example. This is a dramatically different stimulus than briefly applied heat stimuli used to assess acute nociception. In a well characterized behavioral assay measuring acute heat sensation known as the Hargreaves test (Hargreaves et al., 1988; Mogil et al., 2001), the mouse or rat is placed on a glass platform within a small enclosure, and a radiant heat source is then applied to the plantar surface of the paw. The amount of time between the onset of the heat source and withdrawal of the paw is measured. Heat intensity is calibrated to yield paw withdrawal latencies of ~10 seconds, and a cutoff time is also used, typically 20 seconds. It is unlikely that these short heat applications are sufficient to induce pERK1/2 in nociceptors, although this has not been directly tested. Additionally, as part of the test, the interval between applications of radiant heat is between 10 and 15 minutes. With this protocol, sensitization to repeated application of these brief stimuli has not been observed (Hargreaves et al., 1988). Therefore, on the behavioral level, acute nociception and noxious stimulation are very different.

Given these results, it is interesting to speculate that the magnitude and/or duration of ERK1/2 activation may determine whether behavioral sensitization occurs. Evidence directly addressing this question is limited. For instance, the behavioral relevance of ERK1/2 activation following two-minute heat stimulation has not been addressed, since the experiments were carried out in anesthetized animals and MEK inhibitors were not used. However, in a burn injury model, a first-degree burn with associated behavioral hypersensitivity occurs after exposing the paw to a 52°C metal surface for 45 seconds (Sorkin et al., 2009). ERK1/2 phosphorylation occurs at temperatures below 52°C, but at lower levels than those observed after capsaicin injection (Dai et al., 2002). Interestingly, pERK1/2 immunostaining occurs in more cells at temperatures higher than 52°C. Therefore, there may be a threshold of ERK1/2 activation which if crossed initiates peripheral and behavioral sensitization to heat. A greater magnitude of ERK1/2 activity above this threshold may produce more exaggerated or longer lasting hypersensitivity. Although the amount

of ERK1/2 activity is likely important, the duration of ERK1/2 phosphorylation may also be relevant. In other cell types, the timecourse of ERK1/2 activation determines whether cells continue to divide or begin to differentiate (Murphy et al., 2002; Murphy and Blenis, 2006). It will be interesting to determine the timecourse of ERK1/2 after noxious stimulation. Comparison with the timecourse of capsaicin-evoked pERK1/2 (Dai et al., 2002) will allow a better understanding of the relationship between ERK1/2 activation and behavioral sensitization.

Given that noxious stimulation and capsaicin injection result in robust activation of ERK1/2 in primary afferents, it is not too surprising that similar stimuli result in phosphorylation of ERK1/2 in spinal cord dorsal horn neurons. Noxious heat, noxious cold, noxious mechanical stimuli, and C-fiber level electrical stimulation, applied for several minutes with protocols similar to those described above, all lead to increases in pERK1/2 immunoreactivity in the dorsal horn (Ji et al., 1999; Kominato et al., 2003; Polgar et al., 2007; Honda et al., 2008). ERK1/2 activation appears to occur primarily in the superficial lamina of the dorsal horn (lamina I and II), which receive inputs from nociceptive afferents (Ji et al., 1999). The staining pattern includes cell profiles which colocalize with the neuronal marker NeuN (Fukui et al., 2007). A subset of pERK1/2-positive cells also stains for the neurokinin 1 (NK1) receptor, which labels spinal cord neurons with axons that project to higher brain centers (Polgar et al., 2007). Besides staining within clearly defined cell bodies, images also reveal staining in the neuropil, which may include staining from primary afferent terminals projecting from the DRG and staining from the dendritic arbor of dorsal horn neurons (Ji et al., 1999). As with pERK1/2 staining in the DRG, innocuous stimuli do not activate ERK1/2. Light touch stimulation of the hindpaw for 2 min does not induced ERK1/2 phosphorylation in the spinal cord dorsal horn (Ji et al., 1999). Additionally, in a slice preparation consisting of spinal cord sections with attached dorsal nerve roots, stimulation of Aβ fibers which generally transmit innocuous light touch is not sufficient to induce pERK1/2 immunostaining. In accordance with *in vivo* data, C-fiber strength electrical stimulation and bath application of capsaicin both produce robust pERK1/2 immunostaining. Therefore, as with ERK1/2 activation in primary sensory neurons, it appears that only noxious stimulation results in pERK1/2 immunoreactivity. Although specific for nociceptive activity, the behavioral relevance of pERK1/2 immunoreactivity following noxious stimulation is unclear, since application of MEK inhibitors to the spinal cord by intrathecal injection has no effect on baseline responses to acute nociception (Ji et al., 1999; Ji et al., 2002; Obata et al., 2003; Hu

et al., 2006; Karim et al., 2006) (see discussion above regarding the distinction between noxious stimulation and acute nociception). To better understand the functional importance of pERK1/2 activation in nociceptors and spinal cord for behavioral sensitization, other models have been used.

ERK1/2 in chemical nociception and sensitization

The injection of noxious chemicals into the hindpaw, such as capsaicin or formalin, produces robust behavioral sensitization (Tjolsen et al., 1992; Torebjork et al., 1992). As mentioned above, capsaicin injection not only evokes minutes of burning pain it also triggers subsequent hypersensitivity to heat and mechanical stimuli. To determine the behavioral relevance of capsaicin-induced ERK1/2 activation in nociceptors, MEK inhibitors were injected into the paw before capsaicin injection. MEK inhibition attenuates heat hypersensitivity but has no effect on mechanical hypersensitivity (Dai et al., 2002), suggesting that afferent ERK1/2 activation is necessary for capsaicin-induced heat hypersensitivity. Capsaicin injection also increases pERK1/2 immunostaining in the spinal cord dorsal horn with a peak in the number of pERK1/2 cell bodies at 2 min which is sustained for 30 minutes – a similar timecourse to that observed in DRG (Ji et al., 1999; Kawasaki et al., 2004). The staining pattern of capsaicin-evoked pERK1/2 immunoreactivity is similar to that observed after noxious stimulation, occurring robustly in neuronal cell bodies (Kawasaki et al., 2006). The presence of pERK1/2 immunostaining is not too surprising, given that noxious heat stimulation and C-fiber stimulation both induced pERK1/2 (Ji et al., 1999). In this case, however, capsaicin injection allowed for a critical test of the behavioral relevance of ERK1/2 activation in the spinal cord. Indeed, intrathecal pretreatment of a MEK inhibitor attenuated capsaicin-induced mechanical hypersensitivity (Kawasaki et al., 2004). These data indicate that capsaicin induces ERK1/2 activation at the level of the DRG and spinal cord dorsal horn and that ERK1/2 activation is necessary for behavioral sensitization that accompanies capsaicin injection.

In experimental animals, it is difficult to assess spontaneous pain, and many pain models rely on hypersensitivity to stimuli (Mogil et al., 2001). However, there are often stereotyped spontaneous responses expressed by animals that occur after injection of noxious chemicals, and these behaviors may more closely parallel spontaneous pain in humans than evoked withdrawal reflexes. Immediately following intraplantar capsaicin injection, mice lick the injected paw (Sakurada et al., 1992). However, this only occurs for 5 minutes and has completely subsided by 10 minutes,

potentially limiting its experimental utility. The effect of MEK inhibition on capsaicin-induced spontaneous behavior has not yet been reported. Another model of spontaneous chemical pain is the formalin test. Formalin is a dilute solution of paraformaldehyde, the injection of which elicits robust spontaneous behaviors including licking, lifting and flinching of the injected paw which can last for 1 hour (Tjolsen et al., 1992). Formalin injection results in two distinct phases of spontaneous behaviors. Immediately following injection, the time spent performing spontaneous behaviors is quite high. By 5-10 minutes after injection, spontaneous behaviors subside but then resume over the following 30-40 minutes, forming a second peak. This biphasic behavioral response correlates with distinct mechanisms. The first phase is attributed to direct stimulation of nociceptive afferents, while the second phase is thought to result from continued nociceptor activity and peripheral sensitization as well as central sensitization within the spinal cord dorsal horn. Evidence supporting a role for nociceptive afferents comes from *in vivo* electrophysiological recordings indicating activity among Aβ-, Aδ-, and C-fibers following injection (Tjolsen et al., 1992; Puig and Sorkin, 1996). Although Aβ activity subsides with 5-10 minutes, firing frequencies of Aδ- and C-fibers persist well into the 2nd phase. Systemic administration of NSAIDs decreases the 2nd phase of the formalin test (Shibata et al., 1989; Tjolsen et al., 1992). Inhibition of a variety of inflammatory mediators impacts only the 2nd phase as well, suggesting that peripheral, inflammatory processes are important in the 2nd phase. Active processes in the spinal cord dorsal horn also appear to be important (Tjolsen et al., 1992). After 1st phase activity, local anesthesia of the injected paw with lidocaine reduces but does not eliminate spontaneous behaviors (Coderre et al., 1990), indicating that peripheral activity is not sufficient for the full expression of formalin-induced behaviors. More recent studies have indentified activity-dependent synaptic strengthening in the spinal cord dorsal horn following formalin injection, which is likely to contribute to the behavioral response (Ikeda et al., 2006; Sandkuhler, 2009). Therefore, the formalin test provides an interesting model to assess both acute chemical nociception as well as sensitization in both nociceptors and spinal cord dorsal horn.

Formalin injection induces ERK1/2 activation, and this activation is required for spontaneous behaviors observed in the formalin test (Ji et al., 2009). The original study investigating a potential role for ERK1/2 in pain revealed that formalin injection rapidly increased pERK1/2-positive cell profiles in rat spinal cord dorsal horn (Ji et al., 1999). Shortly thereafter, a similar observation was made in mice (Karim et al., 2001). The timecourse of ERK1/2 activation is fairly rapid, peaking at

3 minutes after injection and persisting for at least 30 minutes (Ji et al., 1999; Karim et al., 2001). Quantitative Western blot analysis also shows a significant increase in ERK1/2 activation (Karim et al., 2001). Since ERK1 and ERK2 are slightly different sizes (44 and 42 kDa, respectively), Western blotting provides a direct assessment of ERK1 and ERK2 activity. Although pERK1 and pERK2 both increased after formalin, the amount of change was greater for pERK2. Additionally, the pERK2 band was consistently darker than the pERK1 band. Although enticing, these observations do not directly address the relative roles of ERK1 and ERK2, since antibody binding affinity may be different between isoforms and since phosphorylation does not necessarily imply functional relevance. To address functional relevance without specifically interrogating each isoform, MEK inhibitors were applied to spinal cord by intrathecal injection (Ji et al., 1999; Karim et al., 2001; Tsuda et al., 2007). Intrathecal pretreatment with PD98059 dose-dependently reduced the 2nd phase of the formalin test, without affecting the 1st phase (Ji et al., 1999; Karim et al., 2001). Moreover, this was accompanied by a significant decrease in pERK1/2 immunostaining (Karim et al., 2001). Additionally, intrathecal PD98059 after the 1st phase was also sufficient to reduce 2nd phase behaviors (Ji et al., 1999), suggesting that ERK1/2 actively contributes to central sensitization downstream of afferent activity in the 1st phase. Less evidence exists about the role of afferent ERK1/2 following formalin injection. One study has reported that pERK1/2 immunoreactivity occurs in the soma of DRG neurons following formalin injection (Tsuda et al., 2007). Although Tsuda et al. reported an attenuated 2nd phase following intrathecal MEK inhibition with U0126, they did not investigate the effects of U0126 on formalin-induced DRG pERK1/2, leaving the question of the behavioral relevance of DRG ERK1/2 activity unanswered. Additionally, intrathecal MEK inhibition would affect both DRG and spinal cord ERK1/2 activity (see discussion below), making it difficult to conclude about the relative importance of ERK1/2 in either location. In addition to pharmacological studies, transgenic expression of a dominant negative MEK1 construct in all neurons also decreases formalin-induced spontaneous behaviors (Karim et al., 2006). Experiments with pharmacologic and genetic targeting of MEK1/2-ERK1/2 signaling all indicate that ERK1/2 activation is necessary for formalin-induced spontaneous behaviors.

ERK1/2 in models of inflammatory pain

Formalin- and capsaicin-induced spontaneous behaviors are ultimately short-lived compared to the pain complaints of patients suffering from chronic pain. Much work in the pain field

has involved the development of additional rodent models that more closely recapitulate human disease. Inflammatory pain states, such as rheumatoid arthritis, have been modeled by injecting complete Freund's adjuvant (CFA), which is a suspension of heat-killed *Mycobacterium tuberculosis* (Neugebauer et al., 2007). Subcutaneous injection of CFA into the plantar surface of the hindpaw produces local inflammation as well as robust hypersensitivity to heat and mechanical stimuli which persists for at least one week (Bennett, 2001). CFA injection into the hindpaw induces pERK1/2 immunostaining in DRG (Obata et al., 2003; Tamura et al., 2005). The number of pERK1/2-positive cell profiles increases as early as 6 hours, peaks 1 day after injection, and subsides 3 days after the injection. pERK1/2 immunoreactivity occurs primarily in small-diameter neurons, which are mostly unmyelinated C-fiber afferents (Lawson and Waddell, 1991). The effect of MEK inhibitors applied to the hindpaw has not yet been reported. However, in a related inflammatory model involving CFA injection into the knee joint, passive joint movement induced pERK1/2 activation in fibers innervating the synovial lining of the joint (Seino et al., 2006). More importantly, injection of U0126 before passive movement dose-dependently reduced already established behavioral hypersensitivity. This indicates that long-term inflammation due to CFA induces ERK1/2 phosphorylation in peripheral terminals, which contributes to behavioral sensitization. Another study applied MEK inhibitors intrathecally and claimed that diminished sensitization following hindpaw injection of CFA was due to inhibition of ERK1/2 in DRG neurons (Obata et al., 2003). Although DRG pERK1/2 immunostaining also decreased following MEK inhibition, intrathecal drug delivery almost certainly also affects processes in the spinal cord (see below). Therefore, it is unclear whether ERK1/2 activation in the DRG soma is behaviorally relevant. Finally, it is interesting to note that Western blot analysis indicated that both ERK1 and ERK2 phosphorylation increased 1 d after CFA injection.

CFA-induced inflammation of the paw also leads to ERK1/2 activation in the spinal cord. In both rats and mice, CFA injection induces a significant increase in pERK1/2 immunoreactivity in superficial lamina of the spinal cord dorsal horn (Ji et al., 2002; Adwanikar et al., 2004). The number of pERK1/2-positive cell profiles peaks at the earliest time point reported, 10 min, and then slowly diminishes over the course of several days (Ji et al., 2002). By Western blot, significantly elevated ERK1/2 activation was observed at 7 days (Adwanikar et al., 2004). Western blot analysis also indicated that both ERK1 and ERK2 phosphorylation was robustly elevated 30 min and 7 days following CFA injection (Ji et al., 2002; Adwanikar et al., 2004). Clearly, CFA-induced paw

inflammation produces dramatic and sustained increases in ERK1/2 activity in the spinal cord dorsal horn. Unlike other shorter term models discussed above, ERK1/2 protein expression may also be affected by long-term inflammation. Three days after CFA injection, ERK1 and ERK2 expression is slightly but significantly increased in superficial dorsal horn (Xu et al., 2008). A similar phenomenon was observed at extended time points (12 h) after formalin injection (Li et al., 2004), although this would have no bearing on the initial spontaneous behaviors and may or may not be behaviorally relevant. Given these changes in expression, it is tempting to speculate that ERK1/2 may be part of a positive feedback loop which would amplify signal transduction within dorsal horn neurons. It is unlikely that increased expression accounts for the observed increases in ERK1/2 phosphorylation since, in most studies, phospho-specific bands are normalized to total protein bands.

To address the behavioral importance of ERK1/2 activation in the spinal cord, researchers have applied the MEK inhibitor, U0126, intrathecally and measured hypersensitivity to mechanical and heat stimulation following CFA injection (Ji et al., 2002). Using osmotic pumps, U0126 was delivered before CFA injection and then throughout behavioral testing. Animals treated with U0126 had significantly less CFA-induced mechanical and heat hypersensitivity, although hypersensitivity was not completely abolished. Additionally, post-treatment with U0126 after behavioral sensitization had been established also significantly attenuated mechanical and heat hypersensitivity. This indicates that continued ERK1/2 activity is important to maintain CFA-induced hypersensitivity. A different group reported similar results using intrathecal delivery of U0126 and found attenuation of CFA-induced hypersensitivity (Obata et al., 2003). However, they also reported a significant decrease in CFA-induced ERK1/2 activity within DRG as a result of intrathecal MEK inhibition. Taken together, these studies demonstrate that intrathecal drug delivery can exert effects in both spinal cord and DRG, complicating the interpretation of the above results. Despite this uncertainty, it can still be concluded that ERK1/2 activation is important for sensitization. Future experiments to determine the anatomic locus of ERK1/2 activity may aid in drug design. If peripheral ERK1/2 is required for sensitization, then peripherally restricted antagonists may be therapeutic but avoid CNS-related side effects.

The CFA model described above in detail is just one example among many others demonstrating an involvement for ERK1/2 in inflammatory pain. ERK1/2 activity has also been implicated in several other models that utilize different inflammatory agents (carrageenan, bee

venom; Galan et al., 2002; Yu and Chen, 2005; Hu et al., 2006; Hao et al., 2008) injected into different peripheral tissues (temperomandibular joint, knee joint; Seino et al., 2006; Suzuki et al., 2007). Inflammatory mediators which are required for CFA-induced hypersensitivity also feed into ERK1/2 signaling. Analyzing the effects of each mediator on ERK1/2 activity provides more detailed mechanistic information about signaling pathways upstream of ERK1/2. For example, nerve growth factor (NGF) is required for CFA-induced hypersensitivity since function neutralizing antibodies attenuate CFA-induced hypersensitivity (Woolf et al., 1994; Pezet and McMahon, 2006). Exogenous NGF alone is also sufficient to induce heat and mechanical hypersensitivity (Pezet and McMahon, 2006). In relation to ERK1/2, intraplantar injection of NGF induces ERK1/2 phosphorylation in the soma of DRG neurons (Averill et al., 2001). Pretreatment with intraplantar MEK inhibitors attenuates NGF-induced heat and mechanical hypersensitivity (Zhuang et al., 2004; Malik-Hall et al., 2005). This indicates that ERK1/2 activation is required for NGF-induced thermal hypersensitivity. Other chemical messengers implicated in inflammation have also been shown to induce ERK1/2 activation in sensory afferents, including bradykinin (Rashid et al., 2004; Korkmaz et al., 2008), nitric oxide (Freeman et al., 2008), and epinephrine (Aley et al., 2001). Together, these data suggest that ERK1/2 in nociceptive afferents may integrate signals from several distinct inflammatory mediators. When acting in concert, as is the case after CFA injection, ERK1/2 is required for behavioral sensitization.

ERK1/2 in models of neuropathic pain

Neuropathic pain is a general term that applies to chronic pain conditions which involve damage to the nervous system (Costigan et al., 2009). It can involve damage to the central nervous system or peripheral nervous system, termed central and peripheral neuropathic pain respectively, and can be caused by multiple different insults. Although in most cases of peripheral nerve damage sensory function is actually lost, in some cases pain does develop (Bennett, 2001; Costigan et al., 2009). In this light, neuropathic pain represents a gain of positive neurological symptoms including spontaneous pain in the absence of injury, hypersensitivity to light touch or heat, and parasthesias (Costigan et al., 2009). To study the active processes that underlie neuropathic pain, several animals models have been developed (Bennett, 2001). Peripheral neuropathic pain models involve transection or partial transection of peripheral nerves, nerve entrapment accompanied with inflammation within the nerve sheath, transection of nerve roots which contribute to large peripheral

nerves, as well as other variations.

ERK1/2 phosphorylation has been observed in both the spinal cord and dorsal root ganglion in several of these models. In dorsal root ganglion, transection of the sciatic nerve induces pERK1/2 immunoreactivity in medium- to large-diameter myelinated neurons expressing NF200 as well as glial satellite cells that surround neuronal soma within the DRG (Obata et al., 2003). Additionally, pERK1/2 immunostaining colocalizes with ATF3, which labels axotomized DRG neurons (Tsujino et al., 2000). ERK1/2 activation within axotomized DRG neurons was confirmed with a different model involving transection of the L5 nerve root. The sciatic nerve is composed of afferents from L3-L5, so the L5-spinal nerve ligation (L5-SNL) model allows for the neat segregation of injured (L5) and uninjured (L3-L4) afferents. Virtually all L5 DRG neurons express ATF3, indicating axotomy. Within the injured L5 ganglion, pERK1/2 immunoreactivity occurs primarily in medium- to large-diameter DRG neurons, although small diameter neurons expressing pERK1/2 were also observed (Obata et al., 2004b). Interestingly, pERK1/2 immunostaining was not significantly elevated in the uninjured L4 DRG. In a third model in which the sciatic nerve is loosely ligated with chromic gut sutures and inflammation of perineural tissues leads to constriction (chronic constriction injury, CCI; Bennett, 2001), a similar observation is made in which pERK1/2 activation occurs primarily in larger diameter, injured neurons (Obata et al., 2004a). Taken together, nerve injury induces ERK1/2 activation only in axotomized afferents, most of which are medium to large diameter neurons. This is in contrast with the cellular distribution of pERK1/2 immunostaining following inflammation, in which ERK1/2 activation is primarily observed in small-diameter DRG neurons.

This also suggests that ERK1/2 may play distinct roles behaviorally and mechanistically in inflammatory and neuropathic pain. To test this, researchers have again used the pharmacological approach. Intrathecal MEK inhibitors delivered before sciatic nerve transection and then continuously throughout testing reduce autotomy behavior, which is the self-induced damage of the denervated hindpaw (Obata et al., 2003). Additionally, intrathecal MEK inhibition attenuates hypersensitivity to mechanical stimuli following CCI or L5-SNL, with little or no effect on thermal hypersensitivity (Obata et al., 2004a; Obata et al., 2004b). However, as mentioned above, intrathecal application of inhibitors affects spinal cord processing as well, making conclusions about the specific role of peripheral ERK1/2 activity in neuropathic pain somewhat tenuous.

This is especially true given that ERK1/2 activation has been observed in spinal cord dorsal horn following nerve injury. L5-SNL induces ERK1/2 phosphorylation in spinal cord dorsal horn with an interesting timecourse and cellular localization (Zhuang et al., 2005). Immediately following spinal nerve transection, pERK1/2 immunoreactivity increases in the superficial dorsal horn. In neurons, this increase occurs rapidly (within 10 min) and appears to subside over 6 hours. However, Western blot analysis indicates a sustained increase in both pERK1 and pERK2 from 1 day to 21 days after the injury. Apparently, this is due to increases in ERK1/2 phosphorylation in non-neuronal cells. At 2 days post-injury, OX42-positive microglia account for most of the pERK1/2 immunostaining, while at 21 days post-injury most of the pERK1/2 immunostaining occurs within GFAP-positive astrocytes. In the CCI model, persistent pERK1/2 increases were also observed, although the specific cell type was not addressed (Song et al., 2005). To test whether spinal ERK1/2 activation was necessary for behavioral sensitization in these models, researchers used intrathecal delivery of MEK inhibitors. In the studies mentioned above focusing on DRG ERK1/2 activation, continuous intrathecal administration of MEK inhibitors reduced mechanical hypersensitivity without significant effect on thermal hypersensitivity (Obata et al., 2004a; Obata et al., 2004b). Similar results were obtained in the CCI model with nonselective antisense oligonucleotides to both ERK1 and ERK2 (Song et al., 2005). However, in this study CCI-induced heat hypersensitivity was also affected. This may be due to differences in administration, i.e. multiple intrathecal injections versus osmotic pump delivery or subtle differences in the CCI surgery. Regardless, it seems that ERK1/2 activity is important for the behavioral effects of CCI. In the L5-SNL model, MEK inhibition also decreases behavioral hypersensitivity (Zhuang et al., 2005). Interestingly, both the amplitude and duration of analgesia following intrathecal MEK inhibition increased at later time points after L5-SNL, perhaps suggesting that persistent ERK1/2 activity in microglia or astrocytes is necessary for the long-term maintenance of hypersensitivity. Of course, this would also imply that neuronal ERK1/2 activity is not as important for the maintenance of nerve-injury induced sensitization. Overall, it is clear that sciatic nerve injury initiates ERK1/2 activity in DRG and spinal cord. Intrathecal administration of MEK inhibitors or ERK1/2 antisense attenuates behavioral sensitization following injury, indicating that ERK1/2 activity is required for injury-induced hypersensitivity in models of neuropathic pain.

ERK1/2 in the nociceptive brain

Thus far, the discussion has focused on the activation and behavioral importance of

ERK1/2 in primary afferents and spinal cord. However, recent studies are revealing critical roles of higher brain structures in chronic pain (Tracey, 2005). Certainly, the percept of pain involves the brain, with the somatosensory cortex being involved in sensory-discriminative aspects of the sensation of pain, such as the location, quality, and duration of a noxious stimulus. However, other structures are playing previously unappreciated roles in the affective component of pain, such as attaching negative emotional valence to the noxious stimulation. Moreover, recent data suggests that ERK1/2 activation in these structures may contribute to rodent correlates of affective pain. Noxious stimulation results in ERK1/2 phosphorylation in limbic structures including the anterior cingulate cortex (Wei and Zhuo, 2008; Cao et al., 2009) and the amygdala (Carrasquillo and Gereau, 2007). In the anterior cingulate cortex, ERK1/2 is activated following formalin injection to the paw (Cao et al., 2009). MEK inhibition in the ACC has no effect on formalin-induced spontaneous behaviors or CCI-induced mechanical or thermal hypersensitivity. However, MEK inhibition prevents the association between formalin injection and contextual cues in a conditioned place aversion task (formalin-conditioned place aversion, F-CPA). Interestingly, if rats underwent F-CPA, re-exposure to the formalin-associated context actually resulted in increases ERK1/2 phosphorylation, mimicking ERK1/2 activation seen immediately following formalin injection. This molecular correlate of memory retrieval is functionally relevant because MEK inhibitors delivered after F-CPA erase aversion for the formalin-associated context. Importantly, MEK inhibition in the ACC has no effect on spatial learning and no effect on conditioned place aversion to shock, which unlike formalin does not sensitize nociceptive spinal dorsal horn neurons. Cao et al. also observed increases in the phosphorylation of ERK1 and ERK2 in the ACC using Western blot, indicating that both isoforms are activated. Overall, this study demonstrated that ERK1/2 activity may be involved in memories associated with painful stimuli, which may relate to the affective component of pain in humans.

ERK1/2 activity is also important in other areas of the brain that are involved in chronic pain. Formalin induces ERK1/2 activation in the amygdala (Carrasquillo and Gereau, 2007). As opposed to the ACC, ERK1/2 activity in the amygdala contributes to stimulus-evoked hypersensitivity. Formalin-induced mechanical hypersensitivity, which lasts hours after the injection of formalin, is diminished by infusion of MEK inhibitors into the amygdala (Carrasquillo and Gereau, 2007). Interestingly, activation of ERK1/2 using agonists or activators of upstream pathways is sufficient

to induce hypersensitivity to mechanical stimuli without any injury or noxious stimulus (Carrasquillo and Gereau, 2007; Kolber et al., 2010). These studies indicate that ERK1/2 activity in the amygdala is both necessary and sufficient for mechanical hypersensitivity. Other areas of the brain involved in nociception have also been examined. ERK1/2 activation has been observed in the brainstem (Imbe et al., 2005; Imbe et al., 2009), which is the site of several important nuclei that are part of descending modulatory circuits that participate in the processing of nociceptive input in the spinal cord. CFA induces pERK1/2 in the nucleus raphe magnus (NFM), and application of MEK inhibitors to the rostral ventromedial medulla attenuates CFA-induced heat hypersensitivity (Imbe et al., 2005; Imbe et al., 2008). Since cells within the NRM can either facilitate or inhibit nociception (Mason, 2005), the MEK inhibitor study suggests that the net effect of ERK1/2 activation in the NRM is facilitatory and thereby increases heat hypersensitivity. ERK1/2 activation has also been reported in the locus coeruleus (Imbe et al., 2009), but it is unclear what behavioral effect this might have since inhibitor studies have not yet been reported.

ERK1/2 – Upstream Activators and Downstream Targets

Although sustained noxious stimulation, inflammatory pain, and neuropathic pain are distinct in many ways, they share some common mechanisms (Costigan et al., 2009). Similar sensitization processes occurring peripherally and centrally seem to be present in many different models of pain. An excellent example of this is the importance of ERK1/2 signaling at the level of the primary afferent and the spinal cord in multiple models of inflammatory and neuropathic pain. Although expression and behavioral experiments have been outlined in detail above, the precise activators and downstream targets have not yet been discussed. Much recent work has demonstrated that similar mechanisms of sensitization involving ERK1/2 can be found in the context of many different pain models, suggesting that ERK1/2 may be an attractive target for the treatment of pain.

ERK1/2 has been implicated in peripheral sensitization and is thought to integrate signals from a variety of channels and receptors. Activation of sensory transduction channels, such as TRPV1, lead to ERK1/2 activation (Dai et al., 2002; Zhuang et al., 2004). The observation that capsaicin induces ERK1/2 phosphorylation *in vitro* (Zhuang et al., 2004) argues for cell autonomous activation of ERK1/2, which could result from TRPV1-mediated Ca^{2+} influx and activation of Ca^{2+}-

sensitive kinases such as PKC or CaMK (Rosen et al., 1994; Agell et al., 2002). It is tempting to speculate that Ca^{2+} influx from other TRP channels, such as TRPA1, may activate ERK1/2 through a similar mechanism, which would explain the observed increase of pERK1/2 *in vivo* following noxious cold (Mizushima et al., 2006). Besides sensory transduction channels, receptor tyrosine kinases and G-protein coupled receptors feed into ERK1/2 activation in DRG neurons. The best example of receptor tyrosine kinase coupling to ERK1/2 is the receptor for NGF, TrkA (Averill et al., 2001; Zhuang et al., 2004; Malik-Hall et al., 2005). *In vivo* and *in vitro* evidence suggests that ERK1/2 phosphorylation occurs directly downstream of TrkA, since pERK1/2 occurs within TrkA-positive neurons and short applications (10 minutes) of NGF activate ERK1/2 in cultured cells (Averill et al., 2001; Zhuang et al., 2004). TrkA is likely to signal through adaptor proteins (Shc/Grb2/SoS) in order to recruit Ras and ultimately activate the Raf-MEK1/2-ERK1/2 cascade (Malik-Hall et al., 2005). Parallel signaling cascades downstream of TrkA include the PLC-PKC and PI3K-Akt cascades. Although these three cascades can be distinct and functionally isolated in other cell types, it appears that there is some cross-talk in DRG neurons. *In vivo* and *in vitro* studies have indicated that PI3K inhibitors attenuate NGF-induced ERK1/2 activation (Zhuang et al., 2004; Malik-Hall et al., 2005). PI3K-Ras interaction has been observed before in DRG neurons (Jones et al., 2003), supporting the idea that NGF-TrkA intracellular signaling likely involves the intersection of PI3K and ERK1/2. Several G-protein coupled receptors also activate ERK1/2 in primary afferents. Epinephrine activates ERK1/2 in DRG neurons via β2-adrenergic receptors (Aley et al., 2001). Interestingly, this is independent of PKC and PKA signaling but instead involves $G_{i/o}$ and Ras. Bradykinin has also been shown to activate ERK1/2 *in vivo* and *in vitro* (Rashid et al., 2004; Tang et al., 2006), probably through B2 receptors. These are just a few examples of important signaling molecules that activate ERK1/2 cell autonomously.

After integrating signaling from a variety of upstream receptors and channels, ERK1/2 phosphorylates downstream effector proteins which go on to sensitize nociceptors. Conceptually, this could occur rapidly via direct phosphorylation of effector proteins, such as sensory transduction channels or voltage-gated ion channels, or via phosphorylation of intermediate kinases and transcription factors which would lead to transcriptional changes. Recent studies have identified both mechanisms at play in the context of peripheral sensitization. In nociceptors, ERK1/2 activity has been linked to sensitization of TRPV1 (Zhuang et al., 2004). Specifically, TrkA binding to NGF

leads to activation of PI3K and then ERK1/2 in cultured DRG neurons. Patch clamp recordings of capsaicin-evoked currents were then used to assess NGF-induced sensitization, which was found to depend on both PI3K and ERK1/2 activity. Several other protein kinases regulate TRPV1 function, including but not limited to PKA, PKC, Src and CDK5 (Bhave and Gereau, 2004; Huang et al., 2006; Stucky et al., 2009). One plausible mechanism for ERK1/2-dependent TRPV1 modulation could involve the placement of ERK1/2 upstream of one of the above kinases. Indeed, interplay among these pathways has been reported previously in other systems (Della Rocca et al., 1997; English et al., 1999; Parekh et al., 1999; Maudsley et al., 2000; Kalia et al., 2004; Utreras et al., 2009). It is also possible that ERK1/2 may directly phosphorylate TRPV1, since predicted ERK1/2 phosphorylation sites do occur in cytoplasmic TRPV1 domains (NetPhos 2.0 search, unpublished). One putative ERK1/2 site can be phosphorylated by CDK5, which has also been implicated in heat hypersensitivity *in vivo* (Pareek et al., 2007). This supports a model in which direct phosphorylation of TRPV1 by ERK1/2 may enhance TRPV1 function and, additionally, posits interaction between ERK1/2 and CDK5 at this phosphorylation site. Of course, this must be tested directly by mutagenesis studies. The use of MEK inhibitors has established a role for ERK1/2 in sensitizing TRPV1 and elucidating the precise molecular machinery involved will be an interesting area of investigation.

In addition to ERK1/2 modulation of TRPV1, ERK1/2 may sensitize nociceptors by modulating their excitability, i.e. the likelihood that local depolarizations from sensory transduction channels like TRPV1 will lead to an action potential. There are many examples of ERK1/2 modulating excitability in other neurons (see below, Adams and Sweatt, 2002; Sweatt, 2004; Hu et al., 2006; Hu et al., 2007). In nociceptors, ERK1/2 activation downstream of the protease-activated receptor 2 (PAR2) is associated with membrane depolarization, a decrease in rheobase, and an increase in input resistance (Kayssi et al., 2007). All of these ERK1/2-dependent effects increase nociceptor excitability. PAR2 activation was also accompanied by inhibition of sustained voltage-gated K^+ currents, which may be related to the observations of ERK1/2 dependent modulation of excitability. There is also evidence that ERK1/2 may modulate voltage-gated Na^+ currents by direct phosphorylation of $Na_V1.7$ (Stamboulian et al., 2010). In this study, inhibition of basal ERK1/2 activity in cultured DRG neurons decreased action potential number in response to current stimulation, consistent with an effect on Na^+ currents. However, this was accompanied by a depolarizing shift in

the resting membrane potential. Although this is consistent with activation of ERK1/2 depolarizing the cell (Kayssi et al., 2007), it is unclear whether the effect of ERK1/2 inhibition in current clamp recordings is related to modulation of Na^+ or K^+ currents. To further complicate the picture, ERK1/2 has also been shown to regulate Ca^{2+} currents in DRG neurons, perhaps by direct phosphorylation in the case of the N-type Ca^{2+} channel, $Ca_V2.2$ (Fitzgerald, 2000; Martin et al., 2006; Woodall et al., 2008). Although the importance of specific phosphorylation sites cannot be determined without functional characterization of mutations at those sites in the context of the native cellular system, MEK inhibitor studies clearly show an effect of ERK1/2 activity on the excitability of nociceptors. Altogether, these data suggest that ERK1/2 activation by upstream signals can result in dramatic sensitization of nociceptors within minutes by modulation of sensory transduction as well as the transmission of nociceptive information.

In addition to rapid effects of ERK1/2 on cellular physiology, recent studies have identified links between ERK1/2 activity and transcriptional regulation within nociceptors. Inflammation and inflammatory mediators induce dramatic transcriptional changes within nociceptors that are likely to be important for long-term behavioral hypersensitivity (Woolf and Ma, 2007). Inflammation of tissues innervated by nociceptors increases the expression of substance P, CGRP, sodium channels, TRPV1, and BDNF in the soma of neurons. ERK1/2 is likely to participate in the signaling events that underlie this remodeling, since a number of inflammatory mediators feed into ERK1/2 signaling and have also been implicated in transcriptional regulation. For example, NGF regulates the expression of all of the molecules listed above (Pezet and McMahon, 2006). MEK inhibitors have been used in some cases, specifically implicating ERK1/2 in transcriptional regulation. CFA injection leads to ERK1/2 phosphorylation and BDNF expression within the same DRG neurons, and MEK inhibition prevents BDNF upregulation (Obata et al., 2003). This is likely related to NGF signaling since pERK1/2-positive neurons colocalized with TrkA and since intrathecal application of NGF in a separate set of experiments induced pERK1/2 and BDNF within the same neurons. ERK1/2 is also required for NGF-induced upregulation of TRPV1 expression in cultured DRG neurons (Bron et al., 2003). Another inflammatory mediator, IL-1β, has also been observed to exert transcription changes via ERK1/2, since upregulation of COX2 mRNA by IL-1β is prevented with MEK inhibitors (Amaya et al., 2009). Neuropathic pain also involves dramatic transcriptional changes, and ERK1/2 may be involved in these as well (Obata et al., 2003; Obata et al., 2004a). The exact downstream

molecules accounting for ERK1/2-dependent transcription in DRG neurons are not well understood. It is possible that ERK1/2 exerts its effects on transcription via CREB phosphorylation, which occurs in DRG soma after inflammation (Tamura et al., 2005) and is downstream of ERK1/2 activation in other neuronal systems (Adams et al., 2000; Mao et al., 2008). Other transcription factors may also be involved, Elk and SRF, although their role in sensory neurons during chronic pain is unstudied (Wickramasinghe et al., 2008; Kerr et al., 2010). Therefore, the pro-nociceptive effects of ERK1/2 activation in sensory afferents can occur rapidly by post-translational modifications of channels and can also occur by transcriptional regulation of genes that govern nociceptor function.

In spinal cord dorsal horn neurons as in nociceptors, ERK1/2 activation has both rapid post-translational effects on neuronal function and delayed but perhaps longer-lasting effects on transcription. ERK1/2 is activated by GPCRs, including mGluR5 and NK1R, receptor tyrosine kinases, such as TrkB, and NMDA and AMPA glutamate receptors (Ji et al., 1999; Karim et al., 2001; Kawasaki et al., 2004; Zhao et al., 2006; Hu et al., 2007; Ji et al., 2009). Several signaling cascades initiated by these cell surface receptors converge upon ERK1/2 in dorsal horn neurons, including cAMP/PKA, PLC/PKC, Src, PI3K, Ca^{2+} influx and Ca^{2+}-sensitive enzymes such as adenylyl cyclase 1 (AC1) and AC8, and probably others (Hu and Gereau, 2003; Hu et al., 2003; Kawasaki et al., 2004; Wei et al., 2006; Pezet et al., 2008; Ji et al., 2009). Several molecules acting downstream of ERK1/2 have been identified. One excellent example of ERK1/2 post-translational modulation involves the rapidly inactivating K^+ current, I_A. ERK1/2 activation with phorbol esters to activate PKC, AC activators to stimulate cAMP production and activate PKA, and group I mGluR agonists all rapidly decrease I_A (Hu et al., 2003; Hu et al., 2007). The use of MEK1/2 inhibitors confirms that these effects occur via ERK1/2 activation. Moreover, ERK1/2 modulation of I_A occurs through the specific modulation of $K_V4.2$, since $K_V4.2$ deletion or point mutations of candidate ERK1/2 phosphorylation sites prevents ERK1/2-mediated inhibition of I_A (Hu et al., 2006). Associated with these effects on K^+ currents, ERK1/2 modulates the intrinsic excitability of spinal cord neurons, and this modulation requires $K_V4.2$ (Hu and Gereau, 2003; Hu et al., 2006). Additionally, these electrophysiological effects are behaviorally relevant since $K_V4.2^{-/-}$ mice exhibit reduced behavioral sensitivity in the formalin test and in inflammation-induced mechanical hypersensitivity (Hu et al., 2006). This series of studies strongly suggests that the rapid post-translational modulation of $K_V4.2$ has functional implications on the cellular and behavioral level. Besides, modulation of $K_V4.2$,

ERK1/2 may exert rapid post-translational effects by increasing NMDA and AMPA currents perhaps by direct phosphorylation of the channels (Slack et al., 2004; Kohno et al., 2008; Ji et al., 2009).

ERK1/2 may also be able to modulate the activity and function of spinal cord dorsal horn neurons by transcriptional regulation. One identified pathway involves activation of the transcription factor CREB (Ji et al., 2009). Sciatic nerve injury, injection of formalin or capsaicin to the paw, and C-fiber stimulation all induce CREB phosphorylation in the spinal cord dorsal horn, and in each case CREB phosphorylation is reduced with MEK1/2 inhibition (Ji and Rupp, 1997; Kawasaki et al., 2004; Song et al., 2005). Several immediate early genes regulated by CREB have also been observed in spinal cord dorsal horn following noxious stimulation. For example, CFA-induced inflammation is associated with an increase in prodynorphin and NK1 expression, which is prevented with intrathecally-delivered MEK inhibitors (Ji et al., 2002). Other CREB-regulated genes are upregulated in models of pain, such as c-fos (Coggeshall, 2005; Ji et al., 2009), suggesting that ERK1/2 may coordinate transcriptional reprogramming of spinal cord dorsal horn neurons in pain states. However, direct evaluation of each putatively regulated gene target must be done. Perhaps a more fundamental question involves the functional importance of ERK1/2-mediated CREB activation. This might be addressed by testing whether ERK1/2 is required for the maintenance of hypersensitivity. If transcriptional changes dominated at later time points, MEK inhibition at these time points would either not reduce hypersensitivity at all or not decrease it immediately. In the CFA model, MEK inhibition long after established hypersensitivity is still able to reduce behavioral sensitization (Ji et al., 2002). However, the effects of MEK inhibition take 6-24 hours to evolve. This suggests that sustained ERK1/2 activation contributes to hypersensitivity but that this may involve processes with longer decay kinetics, such as those on the transcriptional level. Although this is suggestive, direct testing of the importance of ERK1/2-mediated transcription changes should be done to better understand the relative importance of post-translational and transcriptional effector mechanisms in spinal cord dorsal horn neurons.

The effects of ERK1/2 activation are less well-studied in other brain areas and in other cell types. Recently, the role of glial cells in pain has become an area of intense interest (Milligan and Watkins, 2009). As mentioned above, peripheral nerve injury induces ERK1/2 phosphorylation in both microglia and astrocytes in the spinal cord dorsal horn (Zhuang et al., 2005; Ji et al., 2009). Glial ERK1/2 activation may result in the release of inflammatory mediators, such as IL-

1β, IL-6, and TNFα, which have been linked with central sensitization (Kawasaki et al., 2008; Milligan and Watkins, 2009). Additionally, ERK1/2 activity in astrocytes has been associated with a downregulation of glutamate transporters, which may contribute to activation of extrasynaptic glutamate receptors and elevated activity in dorsal horn neurons (Sung et al., 2003; Milligan and Watkins, 2009). After spinal cord injury (SCI), activated microglia in the spinal cord caudal to injury show increased ERK1/2 phosphorylation, PGE_2, and COX-2 expression (Zhao et al., 2007). MEK1/2 inhibitors prevented all of these biochemical changes, reduced SCI-induced hyperresponsiveness of wide-dynamic range neurons, and reduced behavioral sensitization due to SCI. In other areas of the nervous system, pain-related ERK1/2 activation has been associated with increases in synaptic strength (Fu et al., 2008) and in CREB activation (Cao et al., 2009) with a presumed induction of immediate early genes. The molecular details of nociception-related ERK1/2 signaling within the brain as well as non-neuronal cells throughout the nervous system represents an area of promising future scientific investigation. More generally, ERK1/2 activity in many different cell types and areas of the nociceptive nervous system speaks to the widespread importance of ERK1/2 signaling.

ERK1 and ERK2 – Similarities and Differences

Although much is known about the function of MEK1/2-ERK1/2 signaling, little is known about the specific functions of each ERK isoform. As outlined above (Fig. 1), information gleaned from MEK1/2 inhibitors does not query potential isoform-specific functions since MEK1/2 activates both ERK1 and ERK2 (Pearson et al., 2001) and since MEK1/2 inhibitors are not selective for MEK1 versus MEK2 (Servant et al., 1996; Favata et al., 1998). As an alternative approach to pharmacologic inhibition, researchers have used transgenic expression of a dominant negative MEK1 (DN MEK) kinase (Shalin et al., 2004; Karim et al., 2006). In both pain models and fear conditioning, DN MEK transgenic mice express phenotypes that nicely parallel those observed with MEK1/2 inhibitors. Interestingly, when DN MEK1 is transiently transfected into sympathetic neurons or expressed in transgenic animals, ERK1 and ERK2 phosphorylation is equally reduced (Shalin et al., 2004; Karim et al., 2006). However, since this represents overexpression and may affect both endogenous MEK isoforms, it is difficult to conclude about preferential signaling between MEK and ERK isoforms. Therefore, neither DN MEK nor MEK inhibitor experiments are able to address whether ERK1 or ERK2 may have specific functions. Experiments evaluating ERK1/2 activation (phosphorylation) are also limited, since phospho-specific ERK antibodies bind to both

phosphorylated ERK1 and ERK2 due to high homology in their phosphorylation loop domains (Fig. 2). This limits immunostaining experiments which might reveal differences in ERK1/2 isoform phosphorylation in different cell types within tissues or at different subcellular locations. However, since ERK1 and ERK2 are different sizes, the activation states of each isoform can be resolved by Western blotting and have offered tantalizing clues about potential differences between isoforms.

Without evidence to the contrary, functional redundancy has been a working model in the field since ERK1 and ERK2 share a 90% cDNA homology (Boulton et al., 1991) and since the isoforms are coexpressed in many tissues throughout development (Krens et al., 2006b; Krens et al., 2006a; Di Benedetto et al., 2007). However, there is emerging evidence that ERK1 and ERK2 are functionally distinct. Genetic elimination of ERK1 or ERK2 in mice results in dramatically different phenotypes. ERK1 germline knockouts (*ERK1*$^{-/-}$) are viable, fertile, and live to adulthood (Pages et al., 1999). ERK2 germline knockouts (*ERK2*$^{-/-}$) are embryonic lethal due to defective mesodermal differentiation and placental development (Hatano et al., 2003; Saba-El-Leil et al., 2003; Yao et al., 2003). Further characterization of *ERK1*$^{-/-}$ mice has revealed a set of interesting gain-of-function phenotypes. On the systems level, ERK1 deletion actually enhances the association between morphine administration and a particular context, as observed using a conditioned place preference task (Mazzucchelli et al., 2002). This behavioral phenotype was correlated with enhanced LTP in nucleus accumbens as well as enhanced induction of immediate early genes by glutamate stimulation in cultured striatal cells (Mazzucchelli et al., 2002). Activation of ERK2 in cultured neurons was greatly enhanced by the loss of ERK1, raising the possibility that ERK1 functionally antagonizes ERK2 activity in some way. Additional experimentation revealed that *ERK1*$^{-/-}$ mice are also relatively hyperactive, spending significantly more time wheel running in their home cages, exploring more in a novel environment, and resisting immobility in the forced-swim task when compared with wild-type (WT) littermates (Selcher et al., 2001; Mazzucchelli et al., 2002; Engel et al., 2009). However, ERK1 deletion has no effect in fear conditioning or tests of anxiety such as the amount of time spent in the center of an open field apparatus (Selcher et al., 2001). Interestingly, no synaptic plasticity changes were observed in *ERK1*$^{-/-}$ hippocampus (Selcher et al., 2001; Selcher et al., 2003), in accordance with a lack of an effect in behavioral tests associated with hippocampal function.

Clearly, ERK1 plays important roles in different areas of the nervous system, but it

is unclear whether this is due to a specific requirement for ERK1 or ERK2 is also involved. As such, researchers have sought to examine the role of ERK2 in the same systems. However, this has been somewhat difficult due to embryonic lethality of *ERK2*$^{-/-}$. To circumvent these issues, a hypomorphic allele was identified in which targeted insertion of a *neo* cassette into the *mapk1* locus reduced ERK2 expression by ~30% (Satoh et al., 2007). This reduction was sufficient to significantly impair fear conditioning and performance in spatial learning tasks. Interestingly, there was no change in wheel running or open field exploration. Although ERK2 hypomorphs have not yet been tested in morphine conditioned place preference, each isoform appears to be required for different behavioral tasks. It is possible that a lack of a phenotype in the ERK2 hypomorph is due to incomplete reduction in ERK2 expression. To achieve complete elimination of ERK2 while circumventing embryonic lethality, conditional gene deletion strategies have been employed to varying success. Conditional deletion of ERK2 with a *hGFAP-Cre* line (Zhuo et al., 2001) resulted in the loss of ERK2 in most cells of the central nervous system but produced mice that survived to adulthood. Interestingly, these mice showed deficits in fear conditioning (Samuels et al., 2008), similar to ERK2 hypomorphs. However, since Cre expression occurred relatively early in development within multi-potent neural precursors, conditional deletion also reduced cortical size probably due to reduced proliferation of neural precursors and not as a result of cell death. Conditional deletion of ERK2 in neural crest progenitors with a *Wnt1-Cre* line led to embryonic lethality probably resulting from cardiac malformations (Newbern et al., 2008). Another attempt at conditional ERK2 deletion in the brain using a *Nestin-Cre* line resulted in unexpectedly incomplete elimination of ERK2 from hippocampus and hypothalamus, and not surprisingly there was no effect on several behavioral tests (Heffron et al., 2009). Another *Nestin-Cre* conditional knockout has been reported, but impaired proliferation of neural progenitors also occurs (Imamura et al., 2008). It is important to note that the effect of ERK2 deletion on the proliferation of neural progenitors is not shared by *ERK1*$^{-/-}$ mice, since brain structure is grossly similar between *ERK1*$^{-/-}$ mice and their WT littermates (Selcher et al., 2001). Thus, targeted deletion or disruption of ERK2 expression has significantly different results in nervous system development. From these studies, it seems likely that ERK1 and ERK2 also play distinct roles in learning and memory in the adult nervous system, although developmental effects of ERK2 may confound this interpretation. An inducible conditional deletion strategy could be employed in the future to rigorously test the role of ERK2 in these contexts.

Besides the different phenotypes incurred by deletion of ERK1 or ERK2, additional evidence of isoform-specific functions has been reported in other systems on the cellular level. In the NIH/3T3 fibroblast cell line, RNAi-mediated knockdown of ERK2 significantly reduces proliferation (Vantaggiato et al., 2006), which is consistent with observations in neural progenitors and is similar to the effects obtained by interfering with Raf-MEK-ERK signaling in fibroblasts (Seger and Krebs, 1995). Paradoxically, knockdown of ERK1 actually enhances proliferation. Knockdown of ERK1 is associated with an increase in immediate early gene activation following serum stimulation, while ERK2 knockdown is associated with the opposite effect. However, the role of ERK1 and ERK2 in fibroblasts is controversial, since a subsequent study found no “gain-of-function” effects of ERK1 knockdown (Lefloch et al., 2008). In fact, systematically varying the relative amounts of ERK1 and ERK2 knockdown led Lefloch, et al. to the model that ERK1 and ERK2 were functionally redundant, but that the differences in their expression levels within different cell types gave rise to apparent functional differences (Lefloch et al., 2008, 2009). Overall, these results suggest that ERK2, but not ERK1 plays, a predominant role accelerating fibroblast proliferation. Whether this is due to intrinsic functional differences or merely to relative expression levels remains to be determined. However, additional evidence supports the existence of intrinsic functional differences, since the rate of nuclear-cytoplasmic shuttling is different for ERK1 and ERK2 in NIH/3T3 cells (Marchi et al., 2008). ERK1 shuttles at slower rates than ERK2 due to sequence differences in the N-terminus. Moreover, there are other amino acid sequence differences that may impact MEK1/2 binding, ERK dimerization, and subcellular localization (see Fig. 2, Boulton et al., 1991; Zhang et al., 1994; Cobb and Goldsmith, 2000). These subtle differences in sequence may manifest in different protein-protein interactions that may be functionally important, between kinases and scaffolds for example. Indeed, ERK1 and not ERK2 interacts with the scaffold MP1 (Schaeffer et al., 1998). Therefore, differences between ERK1 and ERK2 on the cellular and molecular level are likely to underlie the different phenotypes observed with the deletion of ERK1 and ERK2.

What roles do ERK1 and ERK2 play in nociception and nociceptive sensitization?

Using MEK1/2 inhibitors and DN MEK mice, it has become clear that ERK1/2 activity throughout the pain neuraxis is required for behavioral sensitization. However, little is known about the relative importance of ERK1 and ERK2. By Western blot analysis, it appears that noxious stimulation, inflammation, and nerve injury all increase the phosphorylation state of both ERK1 and

ERK2 in most cases (Ji et al., 1999; Karim et al., 2001; Ji et al., 2002; Obata et al., 2003; Song et al., 2005). The one exception to this is in the amygdala in which ERK1 phosphorylation does not appear to change following formalin injection (Carrasquillo and Gereau, 2007). In other areas, ERK2 is generally more phosphorylated than ERK1. However, these biochemical observations are not a direct test of function, and represent a composite of ERK1/2 phosphorylation from tissue that is a heterogeneous mix of cell types. Given emerging evidence in other fields that ERK1 and ERK2 may even have opposing functions, potential isoform-specific roles in nociception and nociceptive sensitization is possible. If so, this may allow for a more specific therapeutic intervention which would target the important isoform in nociceptive sensitization without inhibiting unrelated functions of the less important isoform.

To test directly the role of ERK1 and ERK2 in nociception and nociceptive sensitization, I took a genetic approach that combined targeted deletion of ERK isoforms with a thorough behavioral and biochemical characterization of the mutant mice. Results of this study indicate that ERK1 is dispensable for behavioral sensitization in models of acute chemical nociception, long-term inflammatory pain, and neuropathic pain. It does not appear that ERK2 masks the effects of ERK1 deletion since ERK2 activation in pain models is unaltered by the loss of ERK1. Mice were also generated to lack ERK2 expression in small-diameter, unmyelinated nociceptors. Nociceptor ERK2 is required for acute chemical nociception and inflammation-induced hypersensitivity. Since ERK1 deletion had no effect in these models, altogether the results serve as good evidence that ERK2 and not ERK1 is required for nociceptive sensitization.

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Chapter 2: Genetic targeting of ERK1 suggests a predominant role for ERK2 in murine pain models

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Abstract

The extracellular signal-regulated kinase (ERK) isoforms, ERK1 and ERK2, are believed to be key signaling molecules in nociception and nociceptive sensitization. Studies utilizing inhibitors targeting the shared ERK1/2 upstream activator, mitogen-activated protein kinase kinase (MEK), and transgenic mice expressing a dominant negative form of MEK have established the importance of ERK1/2 signaling. However, these techniques do not discriminate between ERK1 and ERK2. To dissect the function of each isoform in pain, we utilized mice with a targeted genetic deletion of ERK1 (ERK1 KO) to test the hypothesis that ERK1 is required for behavioral sensitization in rodent pain models. Despite activation (phosphorylation) of ERK1 following acute noxious stimulation and in models of chronic pain, we found that ERK1 was not required for formalin-induced spontaneous behaviors, complete Freund's adjuvant-induced heat and mechanical hypersensitivity, and spared nerve injury-induced mechanical hypersensitivity. However, ERK1 deletion did delay formalin-induced long-term heat hypersensitivity, without affecting formalin-induced mechanical hypersensitivity, suggesting that ERK1 partially shapes long-term responses to formalin. Interestingly, ERK1 deletion resulted in elevated basal ERK2 phosphorylation. However, this did not appear to influence nociceptive processing, since inflammation-induced ERK2 phosphorylation and pERK1/2 immunoreactivity in spinal cord were not elevated in ERK1 KO mice. Additionally, systemic MEK inhibition with SL327 attenuated formalin-induced spontaneous behaviors similarly in WT and ERK1 KO mice, indicating that unrelated signaling pathways do not functionally compensate for the loss of ERK1. Taken together, these results suggest that ERK1 plays a limited role in nociceptive sensitization and supports a predominant role for ERK2 in these processes.

Introduction

Extracellular signal-regulated kinases (ERK), ERK1 and ERK2, are mitogen activated protein kinases (MAPK; Pearson et al., 2001) that have been identified as critical players in sensitization to noxious stimuli following peripheral inflammation and nerve damage (Ji et al., 1999; Karim et al., 2001; Ji et al., 2002; Ciruela et al., 2003; Obata et al., 2003; Song et al., 2005). A variety of acute noxious stimuli and chronic pain models result in ERK1/2 activation (phosphorylation) at many levels of the nociceptive sensory system including dorsal root ganglion, spinal cord, and amygdala (Ji et al., 2009). The use of inhibitors which block activation of both ERK1 and ERK2 by inhibiting their shared upstream MAP kinase kinases (MEK1/2) and transgenic expression in neurons of a dominant negative form of MEK1, which suppresses MEK1/2-ERK1/2 signaling, have demonstrated that ERK1/2 is necessary for nociceptive sensitization (Ji et al., 1999; Karim et al., 2001; Ji et al., 2002; Song et al., 2005; Hu et al., 2006; Karim et al., 2006; Seino et al., 2006; Ji et al., 2009).

Although much is known about MEK1/2-ERK1/2 signaling, little is known about the specific functions of each ERK isoform. Functional redundancy has been a working model because the isoforms are 90% homologous (Boulton et al., 1991) and no isoform-specific inhibitors currently exist. However, there is emerging evidence that ERK1 and ERK2 may be functionally distinct. ERK1 knockouts (ERK1 KO) are viable but exhibit behavioral abnormalities correlated with altered synaptic plasticity in striatum (Pages et al., 1999; Selcher et al., 2001; Mazzucchelli et al., 2002), whereas ERK2 knockouts are embryonic lethal at E8.5 (Krens et al., 2006). Alternative methods for targeting ERK2, including reduced expression from a hypomorphic mutant allele and conditional deletion in telencephalon, have revealed a requirement for ERK2 in several learning and memory paradigms (Satoh et al., 2007; Samuels et al., 2008). In cell culture, genetic targeting or RNA interference experiments suggest specific roles for ERK1 and ERK2 (Mazzucchelli et al., 2002; Vantaggiato et al., 2006). Evidence to support this hypothesis includes the observations that ERK1 exclusively interacts with the MEK-ERK signaling scaffold MP1 (Schaeffer et al., 1998) and the fact that differences in amino acid sequence between ERK1 and ERK2 occur in domains which may affect MEK1/2 binding, ERK dimerization, and subcellular localization (Boulton et al., 1991; Zhang et al., 1994; Cobb and Goldsmith, 2000). Indeed, ERK1 and ERK2 have different rates of shuttling between the cytoplasm and nucleus due to sequence differences in the N-terminus (Marchi et al.,

2008).

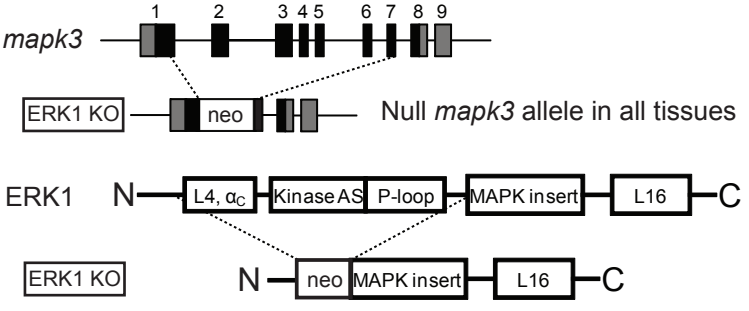
Currently, the importance of ERK1 in nociceptive sensitization remains unknown. Therefore, we tested the necessity of ERK1 in acute noxious sensitization and in models of chronic inflammatory and neuropathic pain using ERK1 KO mice. Although ERK1 is activated in these models, genetic deletion of ERK1 had a minimal impact on these ERK-dependent behaviors. Interestingly, ERK1 deletion increased basal ERK2 phosphorylation without affecting inflammation-induced changes in ERK2 phosphorylation. Our observations indicate that ERK1 is not required for nociceptive sensitization and suggest that ERK2 plays a predominant role.

Methods

Animals

All experiments were performed according to the guidelines of the National Institutes of Health and were approved by the Animal Care and Use Committee of Washington University School of Medicine. Mice were housed with a 12 h/12 h light/dark cycle and *ad libitum* access to food and water. Targeted deletion of ERK1 was achieved by homologous recombination in a process described in detail previously (Fig. 1; Nekrasova et al., 2005). Briefly, the targeting vector deleted exons 1-6 of *mapk3*, which includes the kinase active site and phosphorylation loop, and replaced them with a Neo cassette flanked with loxP sites. Embryonic stem cells from 129 Sv mice were transfected and selected for homologous recombination. Successfully targeted clones were injected into CD1 blastocysts, which were implanted in pseudopregnant CD1 females. Chimeras with germline transmission were obtained on the CD1 background. The *mapk3* mutation was subsequently transferred to the C57BL/6 background by backcrossing >10 times.

Figure 1: Schematic for targeted deletion of *mapk3*. Exons 1-6 of mapk3 were replaced with a neo cassette. On the protein level, mice homozygous for this null allele (ERK1 KO) either do not produce transcript or produce an inactive peptide without critical kinase domains (see Introduction, Fig. 2)



C57BL/6 *mapk3* mutant heterozygotes were crossed producing mice heterozygous for the *mapk3* mutant allele, homozygous for the mutant allele (ERK1 KO), and homozygous for the wild-type *mapk3* allele (WT). 7- to 9-week old ERK1 KO and WT male littermates from these crosses were used in all experiments. The experimenter was blind to the genotype and treatment in all behavioral experiments.

Measurement of Heat and Mechanical Thresholds

All behavioral tests were performed in rooms isolated from other activity and with white noise present. Tests were conducted at room temperature (~25°C) during the second half of the light cycle. Before testing, mice were allowed to acclimate to the behavioral room and apparatus for 2-3 hours. After acclimation, measurements of paw withdrawal thresholds to heat and mechanical stimuli were performed. Measurements were consistently made when mice were calm. Modular clear plexiglass behavior chambers (width=10 cm, length=10 cm, height=15 cm), which could be placed on different floor surfaces, were used with one mouse per chamber. Responses to heat were measured using a modified Hargreaves test (Hargreaves et al., 1988). Mice were placed in behavior chambers on a 390G Plantar Test Apparatus (IITC Life Science, Woodland Hills, CA) with a glass plate floor heated to ~30°C. Radiant heat was applied to the plantar surface of the hindpaw using a focused beam of light with an active intensity of 14% maximal, a cutoff of 20 sec, and idle intensity of 5% maximal. 100% beam intensity heats to ~250°C. To measure paw withdrawal latency, a timer was automatically initiated at the onset of heat application (active intensity), and upon paw withdrawal, the heat source was returned to idle intensity automatically stopping the timer. Five independent measurements, separated by 15 min alternating between right and left paws, were used to determine baseline withdrawal latency. For time points hours after paw injections, 2 independent measurements were averaged. On subsequent days, 3 independent measurements were used. To measure responses to mechanical stimuli, mice were placed in behavioral chambers on an elevated wire mesh floor. Calibrated von Frey filaments (North Coast Medical Inc. Morgan Hill, CA) were then applied through the mesh to the plantar surface of the hindpaw between the anterior and posterior foot pads, except for the spared nerve injury experiment in which filaments were applied to the lateral surface of the hindpaw in sural nerve territory. Filaments were pressed into the skin until the filament bent, held for approximately 1 sec, and then removed. Withdrawal of the hindpaw from the mesh indicated a positive withdrawal response. Each filament was applied

5 times at a frequency of approximately 0.5 Hz. The lightest filament (0.008 g) was initially used followed by progressively heavier filaments until the filament that evoked 3 withdrawals out of 5 applications was reached. The calibrated force of the filament was recorded as the paw withdrawal threshold. Three to five independent measurements were averaged to yield the baseline threshold for each hindpaw. Hours after paw injections, a single measurement was used, while on subsequent days, 3 independent measurements were averaged for each time point.

Formalin-Induced Spontaneous Behaviors

To measure spontaneous behavioral responses to formalin injection, mice were acclimated in behavior chambers with a plexiglass floor for at least 1 h. Mice were then injected subcutaneously with 10 µl 2% formalin (Sigma-Aldrich, St. Louis, MO) in sterile 0.9% NaCl into the plantar surface of the hindpaw and immediately returned to the behavior chamber. Total time spent licking and lifting the injected paw was recorded in five minute bins for one hour. In experiments with the MEK inhibitor, SL327 (Tocris Bioscience, Ellisville, MO), mice were allowed to acclimate as above, injected with SL327 or vehicle (DMSO), and then returned to the behavior chamber. Thirty minutes later, mice were injected into the plantar surface of the hindpaw with 3.5% formalin, returned to the behavior chamber, and video recorded from below with a webcam (Logitech) at a resolution of 960 x 720 for one hour without the experimenter present. Subsequently, videos were scored for time spent licking, lifting, and flinching the injected paw.

Formalin and Complete Freund's Adjuvant-Induced Hypersensitivity

Formalin-induced hypersensitivity to heat and mechanical stimuli was used as a model of persistent pain. Heat hypersensitivity to formalin was assayed by measuring baseline paw withdrawal latencies using a Hargreaves-style apparatus as described above on Day 1. On Day 2, mice were acclimated (2-3 h) and injected with 10 µl 2% formalin (in sterile 0.9% NaCl). Threshold measurements from the injected (ipsilateral) and uninjected (contralateral) paws were made at 1 h, 2 h, and 3 h post-injection. On subsequent post-injection days, mice were first allowed to acclimate (2-3 h), and then paw withdrawal thresholds were obtained. To measure mechanical hypersensitivity to formalin, a similar timecourse was performed except von Frey filaments were used as detailed above. Intraplantar injections of 10 µl 3.5% formalin (in sterile 0.9% NaCl) were made and subsequent threshold measurements were obtained. A higher concentration of formalin

was used in mechanical experiments because 2% formalin did not produce robust mechanical hypersensitivity in either WT or ERK1 KO mice (data not shown). Intraplantar injection of complete Freund's adjuvant (CFA, 10 µl, 1.0 mg/ml, Sigma-Aldrich) was used as a chronic inflammatory pain model. The same experimental design used in formalin-induced hypersensitivity experiments was used to assay mechanical and heat hypersensitivity following CFA injection.

Spared Nerve Injury Model

To measure mechanical hypersensitivity following spared nerve injury (Decosterd and Woolf, 2000), paw withdrawal thresholds from the lateral surface of each hindpaw were obtained using von Frey filaments with the up-down method (Chaplan et al., 1994). Baseline thresholds were measured on Day 1 following 2-3 h of acclimation on wire mesh. On Day 2, mice were anesthetized with pentobarbital, a small incision in the thigh was made, and the three main branches of the sciatic nerve were exposed. The common peroneal and tibial nerves were ligated with 8-0 silk suture and cut distal to ligation. 1 mm of distal nerve stump was removed, and the surgical wound was closed with 7 mm surgical clips. After 3 days of recovery, clips were removed. To measure mechanical hypersensitivity after surgery, mice were acclimated to the mesh (2-3 h), and mechanical withdrawal thresholds were measured from the sural nerve territory (lateral surface of hindpaw).

Sample Preparation and Western Blot Analysis

Mice were placed individually in cages and allowed to acclimate for 2-3 h to approximate behavioral testing conditions. For formalin and CFA experiments, mice were then injected subcutaneously into the plantar surface of the hindpaw with 10 µl of 3.5% formalin or vehicle (0.9% NaCl) in formalin experiments or 10 µl of CFA or 0.9% NaCl in CFA experiments. Mice were then sacrificed using a guillotine and spinal cords were rapidly removed by hydraulic extrusion. In dose-finding experiments with SL327, mice were acclimated as above, injected with SL327 or vehicle (DMSO), returned to their cage for 30 minutes, injected with 3.5% formalin, and sacrificed 3 minutes after paw injection. Spinal cord levels lumbar 3-6 (L3-L6) were isolated, and ipsilateral and contralateral sides were separated. For CFA experiments, the dorsal half was isolated and flash-frozen on dry ice. For formalin experiments, the entire dorso-ventral extent was flash-frozen. In experiments with uninjected mice, the lumbar enlargement was isolated and flash-frozen without further dissection. In naïve subjects, immediately following spinal cord extraction thoracic and lumbar dorsal root ganglia

(DRG) were dissected in ice-cold phosphate-buffered solution (PBS; Mediatech Inc. Manassas, VA), pooled into a single sample, and immediately homogenized. Frozen spinal cord and fresh DRG samples were homogenized using a dounce homogenizer in ice-cold buffer containing 20 mM Tris, pH 7.5, 1 mM EDTA, 1 mM $Na_4P_2O_7$, 25 µg/ml aprotinin (Sigma-Aldrich), 25 µg/ml leupeptin (Sigma-Aldrich), 100 µM PMSF (Roche Applied Science, Indianapolis, IN), 1 µg/ml microcystin LR (Enzo Life Science, Plymouth Meeting PA), and 1 mM Na_3VO_4 (Sigma-Aldrich) in Milli-Q distilled water. Protein concentrations were determined with the BCA protein assay (Pierce Biotechnology, Rockford, IL) and SmartSpec 3000 Spectrophotometer (Bio-Rad, Hercules, CA).

Protein homogenates were size separated by SDS-PAGE. For pERK/ERK or ERK/actin Westerns, 10 µg protein was used. For JNK and p38 Westerns, 20 µg of DRG homogenate was used, and 20-40 µg of spinal cord homogenate was used. For formalin and CFA experiments, precast Tris-HCl 4% stacking and 10% separating polyacrylamide midi-gels were used (Biorad). For experiments with naïve mice, 5% stacking and 10% separating polyacrylamide mini-gels were prepared based on the Laemmli system (Coligan et al., 2001). Gels were transferred to nitrocellulose membranes using a wet transfer system (Biorad). Membranes were blocked for 1 h at room temperature (RT) with Odyssey blocking buffer (LI-COR Biotechnology, Lincoln NE) and then simultaneously probed with two primary antibodies from different host species for 1 h at RT diluted in blocking buffer unless otherwise noted. Primary antibodies used include anti-pERK1/2 (mouse monoclonal, 1:1000; Cell Signaling, Danvers MA), anti-ERK1/2 (rabbit polyclonal, 1:1000; Cell Signaling), anti-actin (1:2000, mouse monoclonal; Sigma-Aldrich), anti-JNK (rabbit polyclonal, 1:1000; Cell Signaling), anti-pJNK (mouse monoclonal, 1:1000; Cell Signaling), anti-p38 (rabbit polyclonal, 1:1000; Cell Signaling), anti-p-p38 (mouse monoclonal, 1:500, primary incubation for 4 h at RT; Cell Signaling), anti-β-tubulin (mouse monoclonal, 1:20,000; Sigma). After several washes, membranes were then incubated for 1 h at RT with fluorescently-conjugated secondary antibodies goat anti-rabbit IRDye800 (1:20,000; LI-COR Biotechnology) and goat anti-mouse AlexaFluor680 (1:20,000; Invitrogen, Carlsbad, CA), which were detected with the Odyssey Infrared Fluorescence Imaging System (LI-COR Biotechnology) for simultaneous detection of 700 and 800 nm wavelength fluorescent emissions corresponding to mouse and rabbit primary antibodies, respectively. Densitometry of bands in 700 (pERK1/2, pJNK, p-p38, β-tubulin or actin) and 800 (ERK1/2, JNK, p38) channels was quantified using the Odyssey Infrared Fluorescence Imaging System software

(LI-COR Biotechnology).

Immunohistochemistry

Mice were placed individually in cages and allowed to acclimate for 2-3 h to approximate behavioral testing conditions. Three minutes after formalin injection (10 µl, 3.5% in 0.9% NaCl) mice were overdosed with Avertin anesthesia (1.25% working solution in 0.9% NaCl diluted from a stock of 1.0 mg/ml 2,2,2 tribromoethanol in tert-amyl alcohol, both from Sigma-Aldrich) by intraperitoneal injection. Mice were then transcardially perfused at a rate of 15-20 ml per minute with warm (~37°C) PBS and then 150 ml ice-cold paraformaldehyde (4% in PBS). Spinal cord was isolated, rinsed in PBS overnight at 4°C, then cryoprotected in 30% sucrose/PBS for 2 days at 4°C. Transverse 30 µm sections were obtained using a cryostat and collected in ice-cold PBS. Floating sections were then stained for pERK1/2 using DAB secondary detection. All incubations were performed with gentle agitation at RT, unless otherwise noted, with several washes between steps. Endogenous peroxidase activity was suppressed by incubating in 10% methanol, 0.3% H_2O_2 (in PBS) for 30 minutes. Sections were blocked with 1% normal goat serum/0.02% Triton X-100 in PBS (1% NGST) for 1 h, and then incubated in anti-pERK1/2 (1:3000 in 1% NGST, rabbit polyclonal; Cell Signaling) overnight at 4°C. Sections were removed from 4°C and allowed to warm to RT for 1 h, after which sections were incubated with biotinylated goat anti-rabbit IgG (1:200 in 1% NGST; Vector Laboratories, Burlingame, CA) for 1 h and then extrAvidin peroxidase (1:1000 in 1% NGST; Sigma-Aldrich) for 1 h. Finally, sections were incubated for 6 minutes with a 3,3'-diaminobenzidine (DAB) solution supplemented with nickel prepared from a DAB Substrate Kit (Vector Laboratories). Sections were placed on microscope slides and dehydrated by exposing slices to ethanol/water solutions with progressively increasing concentrations of ethanol and finally CitriSolv (Fisher Scientific, Pittsburgh, PA). Following dehydration, slides were coverslipped with distrene plasticizer xylene mounting media (Electron Microscopy Sciences, Hatfield, PA). Bright field and dark field images were captured with a 10x objective using an Olympus (Center Valley, PA) compound microscope with a Photometrics (Tucson, AZ) CoolSnap HQ monochrome camera. Bright and dark field images from the same field were then superimposed in Photoshop (version 7.0, Adobe, San Jose, CA) and the lamina II-III boundary was traced on the dark field image. The boundary was then applied to the bright field image, and the resultant image was used to count positively staining cell profiles in lamina I-II using ImageJ (version 1.42, NIH, Bethesda, MD). During imaging

and quantification, the experimenter was blind to genotype. Color images were obtained using a NanoZoomer automated microscope (Hamamatsu, Hamamatsu City, Japan).

Statistical Analysis

Statistical analysis was performed using Excel (Microsoft, Redmond, WA) and Prism (GraphPad Software, La Jolla, CA). Details of specific statistical tests are included in the results sections. Graphs showing means and standard error of the mean (S.E.M.) were graphed using Prism.

Results

Baseline sensory function remains normal in ERK1 KO mice.

Although a large body of evidence indicates that ERK1/2 is important in nociception, little is known about the relative contribution of each individual isoform. To assess the role of ERK1 in nociceptive sensory function, we utilized mice with a targeted deletion of *mapk3* (ERK1 KO), in which part of exon 1 and all of exons 2-6 are replaced with a pPGKneolox cassette (Nekrasova et al., 2005). Deletion of ERK1 was confirmed by Western blots of spinal cord and dorsal root ganglion (DRG) tissue obtained from ERK1 KO mice (Fig. 2*A,B*). As expected, ERK1 immunoreactivity was undetectable in ERK1 KO mice (Fig. 2*A,B*; unpaired t-test, *** $p<0.001$). Deletion of ERK1 had no effect on ERK2 protein expression (unpaired t-test). These data confirm previous findings that ERK1 protein is eliminated from the central nervous system (Selcher et al., 2001; Nekrasova et al., 2005) and extends these findings to the peripheral nervous system by demonstrating ERK1 deletion without altered ERK2 expression.

After confirming the absence of ERK1 in important nociceptive anatomic loci, we measured the baseline sensory function of ERK1 KO mice. The latency to paw withdrawal from a radiant heat source was comparable between ERK1 KO and WT mice (Fig. 2*C*). Additionally, paw withdrawal thresholds to mechanical stimulation with von Frey filaments in ERK1 KO mice were similar to WT littermates (Fig. 2*C*). In both cases, there was no statistically significant difference between WT and ERK1 KO mice (unpaired t-test). Both sensory tests rely on reflexive withdrawal of the hind limb to measure threshold responses. Since alterations in motor function secondary to ERK1 deletion could confound the results of these tests, we also screened the ERK1 KO mice for motor abnormalities. We found no difference in the performance of ERK1 KO and WT mice on the accelerating rotarod

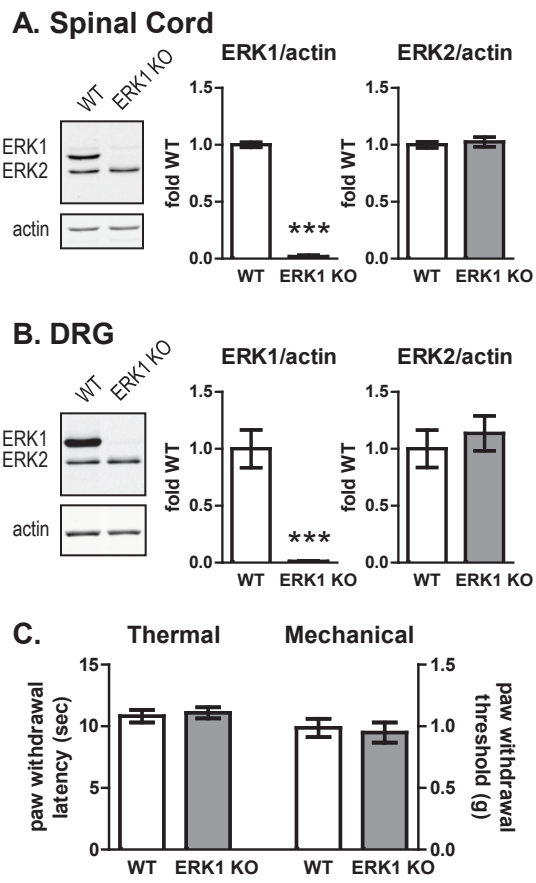


Figure 2: Disruption of *mapk3* eliminates ERK1 expression in spinal cord and dorsal root ganglion without affecting heat or light touch thresholds. A. Spinal cords were isolated from WT (n=13) and ERK1 KO (n=14) littermates and analyzed by Western blot for ERK1/2 and the loading control, actin. To quantify the intensity of ERK bands, integrated intensities of each isoform were divided by actin integrated intensities, and plotted as fold WT. **B.** DRG from WT (n=7) and ERK1 KO (n=8) were dissected and analyzed by quantitative Western blot as in A. In both spinal cord and DRG, there is a dramatic reduction in ERK1 expression without a significant change in ERK2 protein levels. **C.** No differences were observed in heat thresholds obtained by applying radiant heat to the hindpaws of WT (n=10) and ERK1 KO (n=10) littermates using a Hargreaves-style apparatus. No differences were observed in hindpaw mechanical withdrawal thresholds obtained with von Frey filaments from WT (n=14) and ERK1 KO (n=13) littermates. Error bars indicate S.E.M. (unpaired t-test; *** p<0.001).

task or in exploratory behaviors in an open field task (supplemental Fig. S1 at end of chapter). Overall, these results indicate that genetic disruption of ERK1 does not affect baseline withdrawal responses from heat or mechanical stimuli.

ERK1 is not required for 1st or 2nd phases of the formalin test.

Previous reports indicate that spinal ERK1 is activated (phosphorylated) within minutes after intraplantar injection of the noxious chemical formalin and that inhibition of spinal ERK1/2 using intrathecally-applied MEK inhibitors reduces the second phase of spontaneous formalin-induced

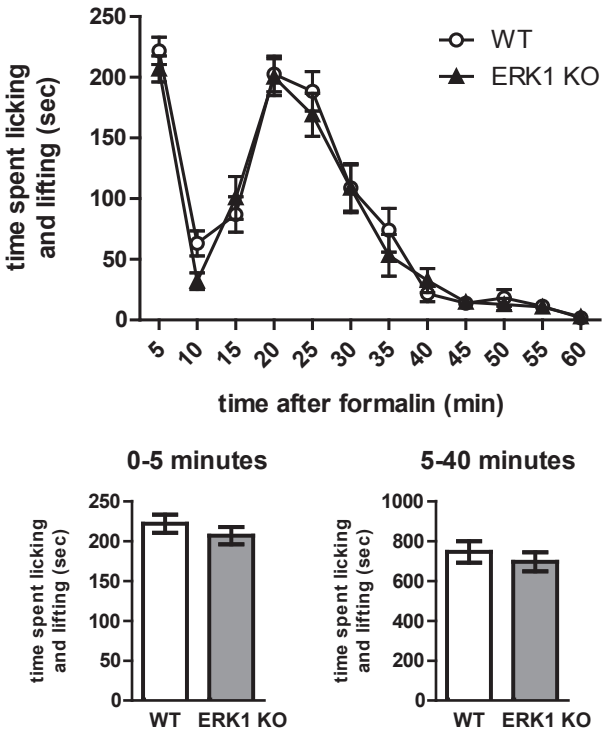


Figure 3: Formalin-induced spontaneous nociceptive responses are preserved in ERK1 KO mice. The amount of time spent in spontaneous nociceptive behaviors after 2% formalin injection (licking and lifting of the injected paw) was recorded from WT (n=16) and ERK1 KO (n=14) littermates and plotted in 5 minute bins. No statistically significant difference between WT and ERK1 KO was detected over the course of the 60-min trial or in total time spent in the 1st phase (0-5 min, lower left graph) or 2nd phase (5-40 min, lower right graph) of nociceptive behaviors. Error bars represent S.E.M. **Contributions:** This experiment was performed by Farzana Karim

nociceptive behaviors (Ji et al., 1999; Karim et al., 2001). Therefore, we hypothesized that ERK1 was necessary for full expression of spontaneous nociceptive behaviors following formalin injection. Surprisingly, ERK1 KO and WT littermates have indistinguishable formalin responses (Fig. 3). Both WT and ERK1 KO show a stereotypical biphasic response characterized by an immediate increase in licking and lifting (1st phase) which subsides momentarily but gradually increases and persists over the following ~30 minutes (2nd phase). There is no statistically significant difference between genotypes when considering the complete timecourse of the formalin response (2-way RM ANOVA: F=0.64, DFn=1, DFd=308, P value = 0.4304). Moreover, the amount of time spent in nociceptive behaviors in the 1st phase (0-5 min) and the 2nd phase (5-40 min) is similar between the genotypes (Fig. 3, unpaired t-test). Overall, these data indicate that even in the absence of ERK1, formalin can induce robust spontaneous behaviors which have previously been shown to require ERK1 and/or ERK2, suggesting a specific role for ERK2 in this process.

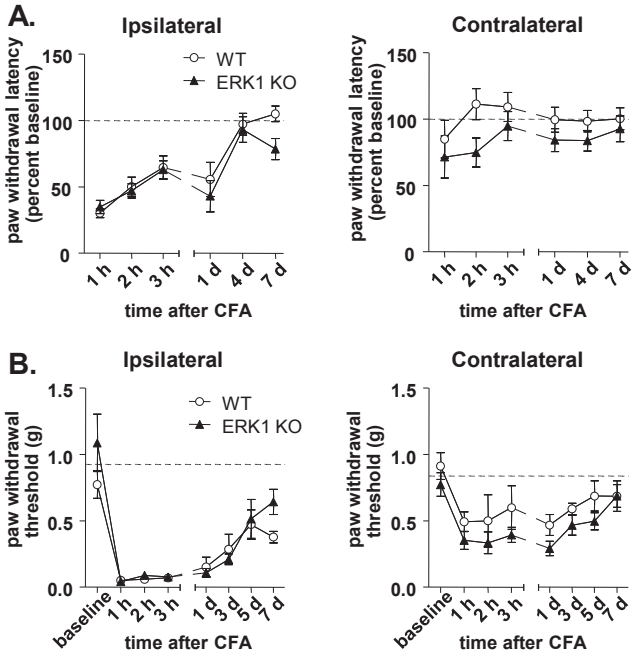


Figure 4: ERK1 is not necessary for CFA-induced hypersensitivity. A. To measure CFA-induced heat hypersensitivity, WT (n=10) and ERK1 KO (n=10) littermates were injected with CFA subcutaneously into the plantar surface of the hindpaw. Paw withdrawal latencies to a radiant heat stimulus were then obtained from both hindpaws, ipsilateral and contralateral to CFA injection, using a Hargreaves-style apparatus. Data were normalized to pre-injection baseline paw withdrawal latencies within subject and plotted as percent baseline. **B.** To measure CFA-induced mechanical allodynia, WT (n=6) and ERK1 KO (n=6) littermates were injected with CFA, and paw withdrawal thresholds to light touch were measured using von Frey filaments. In all cases, there was no statistically significant difference between genotypes. Error bars indicate S.E.M.

ERK1 KO mice develop robust hypersensitivity in long-term inflammatory and neuropathic pain models.

ERK1 and/or ERK2 contribute to the development and maintenance of chronic inflammatory and neuropathic pain (Ji et al., 2009). To directly assess the contribution of ERK1 from all tissues to CFA-induced hypersensitivity, ERK1 KO mice were injected with CFA into the hindpaw and examined for hypersensitivity to radiant heat and mechanical stimuli over time. ERK1 KO mice became robustly

hypersensitive to radiant heat applied to the paw ipsilateral to CFA injection (Fig. 4*A*). The degree of hypersensitivity in ERK1 KO mice was comparable to WT littermate controls (2-way RM ANOVA: F=1.38, DFn=1, DFd=90, P=0.2562). In the contralateral paw, there was no statistically significant hypersensitivity over time (2-way RM ANOVA: F=1.96, DFn=5, DFd=90, P=0.0923). Moreover, there was no statistically significant difference between the genotypes (2-way RM ANOVA: F=2.26, DFn=1, DFd=90, P=0.1504). CFA injection also produced dramatic hypersensitivity to mechanical stimulation that was indistinguishable between WT and ERK1 KO littermates (Fig. 4*B*). There was no statistically significant difference between genotypes in withdrawal thresholds in the ipsilateral paw (2 way RM ANOVA: F=0.86, DFn=1, DFd=70, P=0.3761) or the contralateral paw (2 way RM ANOVA: F=4.41, DFn=1, DFd=70, P=0.062). Overall, these data indicate that ERK1 KO mice show normal sensitization to mechanical and heat stimuli in the CFA model of long-term inflammatory pain.

The mechanisms driving nociceptive sensitization in neuropathic pain are often distinct from inflammatory pain. Indeed, the timecourse and anatomic localization of ERK1/2 phosphorylation

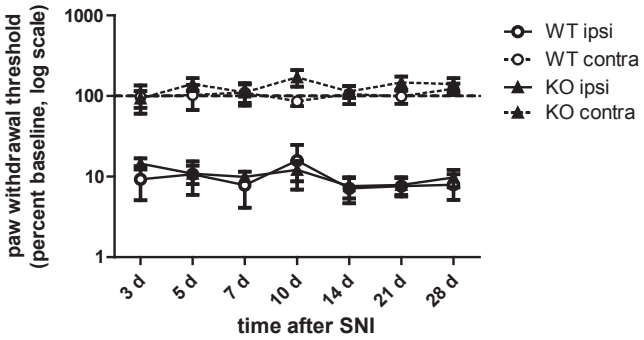


Figure 5: ERK1 KO and WT littermates develop similar mechanical hypersensitivity following peripheral nerve injury. One day after baseline measurements, WT (n=9) and ERK1 KO (n=10) littermates were anesthetized and the tibial and common peroneal branches of the sciatic nerve were ligated then transected, leaving the sural branch intact. Following the spared nerve injury surgery, paw mechanical withdrawal thresholds were measured ipsilateral (solid lines) and contralateral (dashed lines) to nerve injury. Baseline and post-injury withdrawal thresholds were determined by probing the lateral plantar surface of the paw, which is innervated by the sural nerve. Data are represented as percent baseline within each subject. Error bars indicate S.E.M. **Contributions:** This experiment was performed by Chengshui Zhao.

is different between neuropathic (Obata et al., 2004; Zhuang et al., 2005) and inflammatory (Ji et al., 2002; Obata et al., 2003) pain models. However, the relative importance of ERK1 or ERK2 in neuropathic pain remains unknown. To address this issue, we measured the responses of ERK1 KO mice in the spared nerve injury (SNI) model, in which two branches of the sciatic nerve are transected (tibial and common peroneal branches) leaving the third branch, the sural nerve, intact. In skin innervated by the sural nerve ipsilateral to SNI, ERK1 KO and WT mice developed equivalent, robust mechanical hypersensitivity (Fig. 5). In the contralateral paw, withdrawal thresholds remained unchanged relative to baseline. There is no statistically significant difference between ERK1 KO and WT littermates in withdrawal thresholds ipsilateral (2-way RM ANOVA: $F=0.05$, $DFn=1$, $DFd=102$, $P=0.8263$) or contralateral (2-way RM ANOVA: $F=0.89$, $DFn=1$, $DFd=102$, $P=0.3591$) to the nerve injury. These results indicate that ERK1KO mice are equally susceptible to SNI-induced mechanical hypersensitivity as WT littermates, suggesting that ERK1 is not required in this model of neuropathic pain.

ERK1 KO mice show reduced heat hypersensitivity after intraplantar injection of formalin.

Intraplantar injection of formalin produces well-characterized immediate spontaneous behaviors, which give way to hypersensitivity to heat and mechanical stimuli hours after the injection (Fu et al., 2001; Zeitz et al., 2004; Hu et al., 2006). Inhibition of ERK1/2 activation in the amygdala has no effect on immediate spontaneous behaviors but, instead, dramatically reduces long-term formalin-induced hypersensitivity (Carrasquillo and Gereau, 2007). Therefore, we reasoned that although there was no apparent role of ERK1 in formalin-induced spontaneous nociceptive behaviors, ERK1 may be involved in longer term formalin-induced hypersensitivity. To test this hypothesis, we injected ERK1 KO and WT littermates with formalin and measured responses to heat and mechanical stimuli. Interestingly, ERK1 KO mice showed attenuated heat hypersensitivity 1 and 2 hours after 2% formalin injection in the ipsilateral paw (Fig. 6*A*). A two-way repeated measures ANOVA confirmed this difference, indicating a significant main effect of genotype ($F=9.69$, $DFn=1$, $DFd=56$, $P=0.0076$) with a Bonferroni post-test revealing a significant difference at 1 h and 2 h time points ($p<0.05$). Neither WT nor ERK1 KO mice developed heat hypersensitivity in the contralateral paw. At the same time points after 3.5% formalin injection, ERK1 KO mice developed robust mechanical hypersensitivity ipsilateral to injection that was similar to WT littermates (Fig. 6*B*). Although 1 h and 3 h time points appear elevated in ERK1 KO mice, there is no statistically significant difference

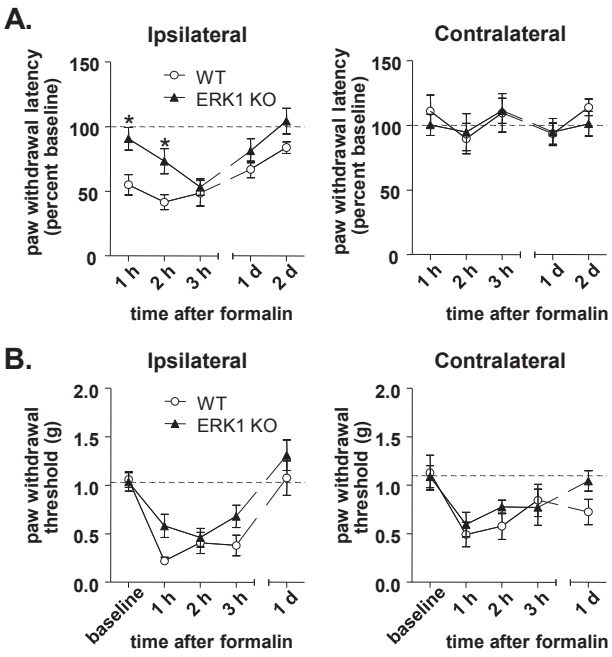


Figure 6: ERK1 contributes to the onset of heat hypersensitivity following intraplantar formalin injection. A. To assess the role of ERK1 in long-term formalin-induced heat hypersensitivity, WT (n=8) and ERK1 KO (n=8) littermates were injected with 2% formalin subcutaneously into the plantar side of the right hindpaw. Paw withdrawal latencies were measured before and after injection and are graphed as percent of pre-injection baseline. ERK1 KO mice have reduced hypersensitivity to heat at 1 and 2 h after formalin injection in the ipsilateral paw (2-way RM ANOVA with Bonferroni post-test; *p<0.05). **B.** To measure formalin-induced mechanical hypersensitivity, WT (n=8) and ERK1 KO (n=9) littermates were injected with 3.5% formalin. Paw withdrawal thresholds were obtained using von Frey filaments. There were no statistically significant differences between WT and ERK1 KO. Error bars reflect S.E.M.

between the genotypes (2 way RM ANOVA: F=3.81, DFn=1, DFn=60, P=0.0700). It is important to note that we used 3.5% formalin to measure mechanical hypersensitivity. In WT mice obtained from ERK1 heterozygous crosses, 2% formalin did not produce robust mechanical hypersensitivity (data not shown). Using 2% formalin, we observed no difference between ERK1 KO and WT littermates (data not shown). Overall, these data indicate that ERK1 KO mice do not fully express formalin-induced heat hypersensitivity, despite normal formalin-induced mechanical hypersensitivity.

Deletion of ERK1 elevates ERK2 phosphorylation without affecting ERK2 expression.

ERK1 and ERK2 are both phosphorylated by MEK1/2, giving rise to the possibility that eliminating one ERK isoform might alter the phosphorylation state of the remaining isoform. The effect of ERK1 deletion on ERK2 phosphorylation in spinal cord or DRG remains unknown. Therefore, we analyzed tissue from naïve WT and ERK1 KO mice by Western blot in order to size separate the isoforms and quantify

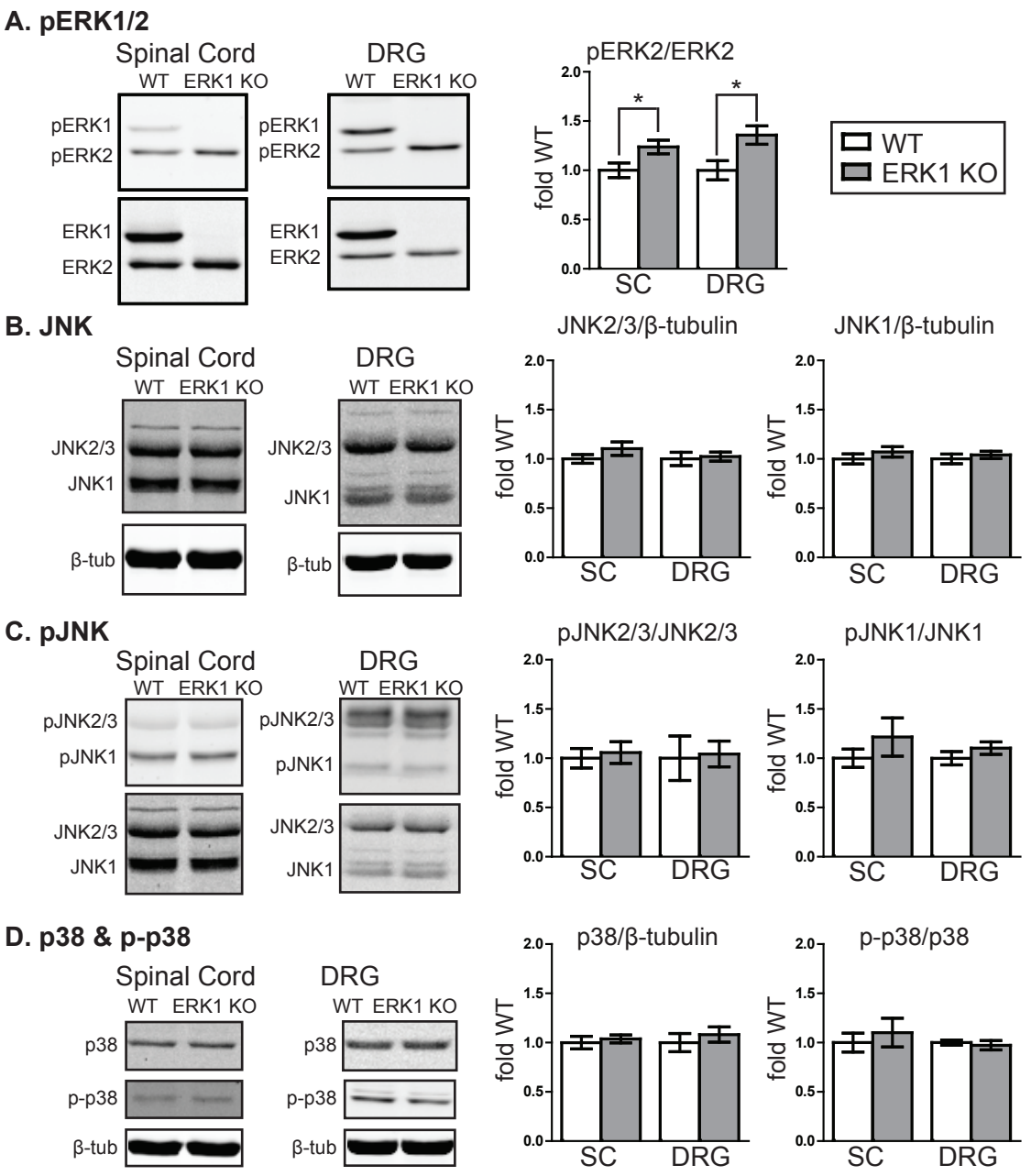


Figure 7: In spinal cord and DRG, basal ERK2 phosphorylation increases in the absence of ERK1. A. Spinal cord (SC) homogenates from naïve WT (n=7) and ERK1 KO (n=8) littermates were analyzed simultaneously for pERK1/2 and ERK1/2 by quantitative Western blot. Although there is no difference in total ERK2 expression in ERK1 KO spinal cord (see Fig. 1), ERK2 phosphorylation is significantly elevated. Similar results were observed in whole cell homogenates prepared from DRG (WT n=7, ERK1 KO n=8; unpaired t-test, * $p<0.05$). **B.-D.** The same spinal cord and DRG ho-

mogenates were used to evaluate the expression and phosphorylation of related MAPKs, JNK and p38. The integrated intensities of JNK2/3, JNK1, and p38 bands were divided by corresponding loading control β-tubulin bands, normalized to the mean WT intensity, and expressed as fold WT. To evaluate phosphorylation, pJNK2/3, pJNK1, and p-p38 bands were divided by their corresponding total protein bands from the same samples, normalized, and then expressed as fold WT. No significant difference was detected between WT and ERK1 KO mice in either spinal cord or DRG (WT SC n=7, ERK1 KO SC n=8, WT DRG n=5, ERK1 KO DRG n=6; unpaired t-test). WT data are represented with white bars, and ERK1 KO data are represented with grey bars.

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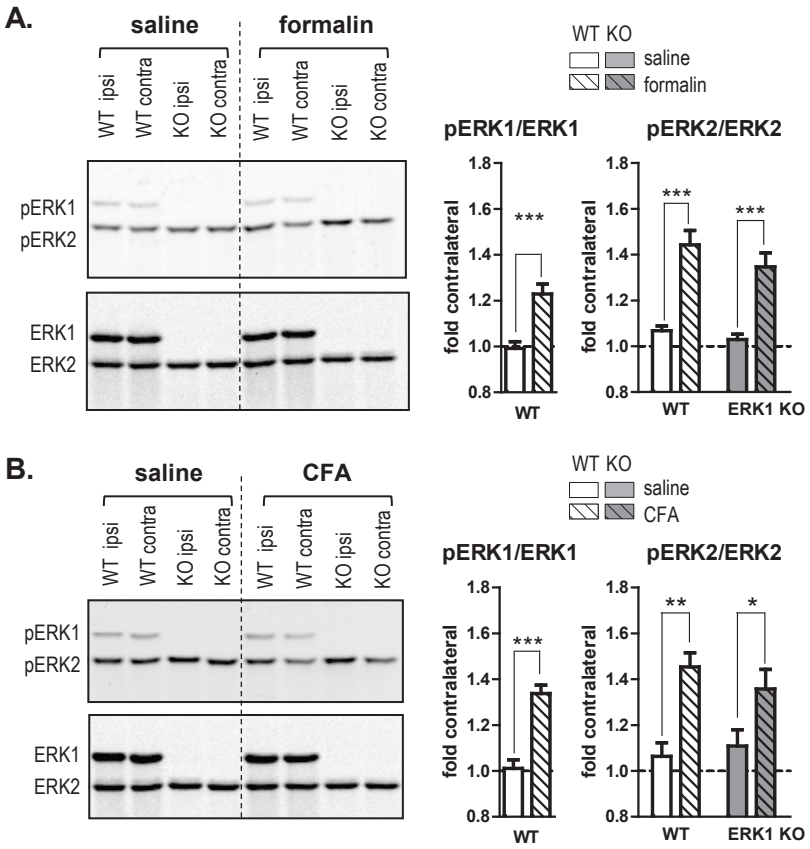
the basal phosphorylation state of each isoform independently (Fig. 7*A*). Interestingly, ERK1 KO mice have significantly elevated basal ERK2 phosphorylation compared with WT littermates in both spinal cord and DRG. It is important to note that in these tissues, total ERK2 expression remains unaltered (Fig. 2). To determine whether the activity of structurally related MAP kinases may be affected by ERK1 deletion, we analyzed spinal cord and DRG homogenates from naive WT and ERK1 KO littermates for JNK and p38 expression and phosphorylation (Fig. 7*B-D*). No significant difference was detected between ERK1 KO and WT homogenates, suggesting that altered basal p38 or JNK activity cannot account for the minimal behavioral impact of ERK1 deletion. Overall, these data do indicate that, in spinal cord and DRG, ERK1 elimination results in enhanced basal phosphorylation of ERK2.

Inflammation-induced elevation of pERK2 is equivalent between ERK1 KO and WT littermates.

Given that ERK2 phosphorylation is elevated in naïve ERK1 KO mice, we hypothesized that noxious stimulation would lead to hyperphosphorylation of ERK2. To test this hypothesis, ERK1 KO and WT mice were injected with either formalin or saline and sacrificed at the peak of formalin-induced ERK1/2 phosphorylation in spinal cord (3 min post-injection; Fig. 8*A*). After normalizing to the total ERK loading control, ipsilateral intensities were divided by contralateral intensities within each subject, to provide a within animal control. As previously reported (Karim et al., 2001), injection of WT mice with formalin significantly increased pERK1 and pERK2 as compared with WT saline-injected mice (Fig. 8*A*; for pERK1: unpaired t-test $p<0.001$; for pERK2: 2-way ANOVA $F=55.92$, $DFn=1$, $DFd=20$, $P<0.0001$). ERK1 KO mice also show significant elevation of pERK2 following formalin injection (2-way ANOVA $F=55.92$, $DFn=1$, $DFd=20$, $P<0.0001$). Interestingly, there is no



<u>Figure 8:</u> Inflammation-induced ERK2 phosphorylation is not elevated in ERK1 KO mice. A. ERK1 KO and WT littermates were injected subcutaneously into the plantar surface of the hindpaw with saline or 3.5% formalin (n=6 per group) and sacrificed 3 minutes later. The dorso-ventral extent of L3-L6 spinal cord was isolated and processed for pERK1/2 and ERK1/2 quantitative Western blot analysis. Ipsilateral pERK/ERK intensities were normalized to contralateral intensities from the same subject, and data were graphed as fold contralateral. Formalin injection led to significantly elevated pERK1 and pERK2 in WT mice. In ERK1 KO mice, the formalin-induced increase in pERK2 was similar to WT. **B.** ERK1 KO and WT littermates were injected subcutaneously into the plantar surface of the hindpaw with saline or CFA (n=5-6 per group). After 30 minutes, the dorsal halves of L3-L6 spinal cords were obtained and analyzed for pERK1/2 and ERK1/2 by Western blot as above. As with formalin injection, CFA injected WT mice had significantly elevated pERK1 and pERK2. Elimination of ERK1 had no significant impact on CFA-induced pERK2 elevation. Error bars represent S.E.M. (unpaired t-test or 2-way ANOVA with Bonferroni post-test;* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

significant difference between WT and ERK1 KO littermates in formalin-induced pERK2 increases (2-way ANOVA F=2.11, DFn=1, DFd=20, P=0.1617). These data suggest that ERK1 deletion does not affect the change in spinal ERK2 phosphorylation due to formalin-induced inflammation. Similar results were obtained when using the CFA model of inflammation (Fig. 8*B*). WT and ERK1 KO littermates were injected with CFA or saline and sacrificed at the peak time point of CFA-induced ERK1/2 phosphorylation (30 min post-injection; Ji et al., 2002). Western blot analysis of the dorsal portion of L3-L6 spinal cords indicated a significant increase in ipsilateral pERK1 for WT and pERK2 for both WT and ERK1 KO mice injected with CFA as compared with saline-injected mice (pERK1: unpaired t-test, $p<0.001$; pERK2: 2-way ANOVA, F=21.02, DFn=1, DFd=17, P=0.0003). This CFA-induced increase in pERK2 was not different between WT and ERK1 KO mice (2-way ANOVA F=0.13, DFn=1, DFd=17, P=0.7202). Similar results were obtained by normalizing pERK/ERK values to WT saline (supplemental Fig. S2, S3 at end of chapter). Using this alternative analysis, ERK1 KO mice have elevated pERK2 in all conditions compared to WT littermates. However, the relative increase in pERK2 due to formalin or CFA appears similar between WT and ERK1 KO, as is reflected in Fig. 7. Overall, these data indicate that ERK1 deletion does not affect inflammation-induced elevation of pERK2.

Formalin-induced pERK1/2 immunostaining is slightly reduced in ERK1 KO mice.

Although increases in pERK2 following noxious stimulation appear unaffected by ERK1 deletion, it is possible that the anatomic localization of pERK2 is altered which may reflect compensatory changes in nociceptive circuitry arising from the loss of ERK1 during development. To address this, ERK phosphorylation was measured by immunohistochemistry with a phospho-specific ERK1/2 antibody, since current antibodies cannot discriminate between pERK1 and pERK2 due to high sequence homology in the phosphorylation loop. ERK1 KO mice were injected with formalin and L3-L4 spinal cord sections were stained for pERK1/2. Interestingly, formalin-injected ERK1 KO mice had a similar pattern of pERK1/2 immunoreactivity to WT littermates (Fig. 9*A*). For both genotypes, positively-staining cell profiles and more diffuse fiber staining was evident in the superficial lamina of the dorsal horn ipsilateral to paw injection. The dorso-ventral or medio-lateral extent of pERK1/2 immunostaining was not appreciably different between genotypes. To quantify pERK1/2 staining, all pERK1/2-positive cell profiles found in the superficial dorsal horn (lamina I and lamina II), as defined by dark field illumination, were counted from serial sections separated by

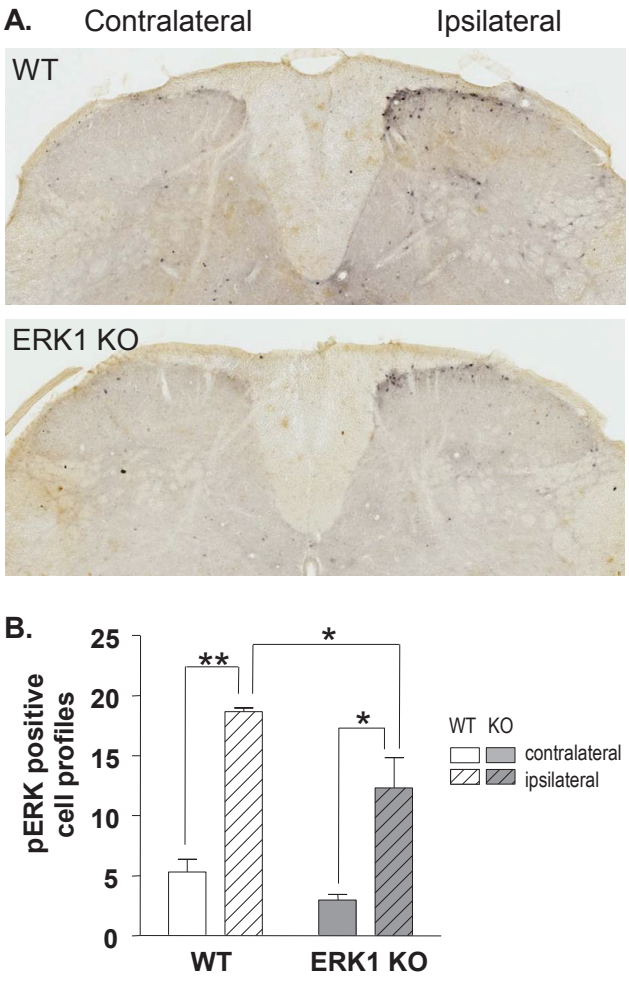


Figure 9: Noxious peripheral stimulation induces pERK1/2 immunoreactivity in superficial dorsal horn laminae despite the loss of ERK1. **A.** Three minutes following subcutaneous intraplantar injection of 3.5% formalin, WT and ERK1 KO littermates were sacrificed and analyzed for pERK1/2 immunoreactivity in lumbar spinal cord. On the side of the spinal cord ipsilateral to paw injection, pERK1/2-positive cell profiles appear in the superficial lamina of formalin-injected WT and ERK1 KO mice. Diffuse staining is also apparent, possibly due to ERK1/2 phosphorylation in dendrites or afferent fibers. In both WT and ERK1 KO, there is no gross difference in the anatomic pattern of pERK1/2 staining. **B.** To quantify results from A., pERK1/2 positive cell profiles in laminae I-II were counted from 6 sections per animal with 3 animals per group. The mean number of cell profiles for each subject was then used to calculate the mean number for the entire group. Formalin stimulation significantly increases pERK1/2-positive cell profiles in both WT and ERK1 KO littermates (2-way ANOVA with Bonferroni post-test; * $p < 0.05$, ** $p < 0.01$). Error bars reflect S.E.M.

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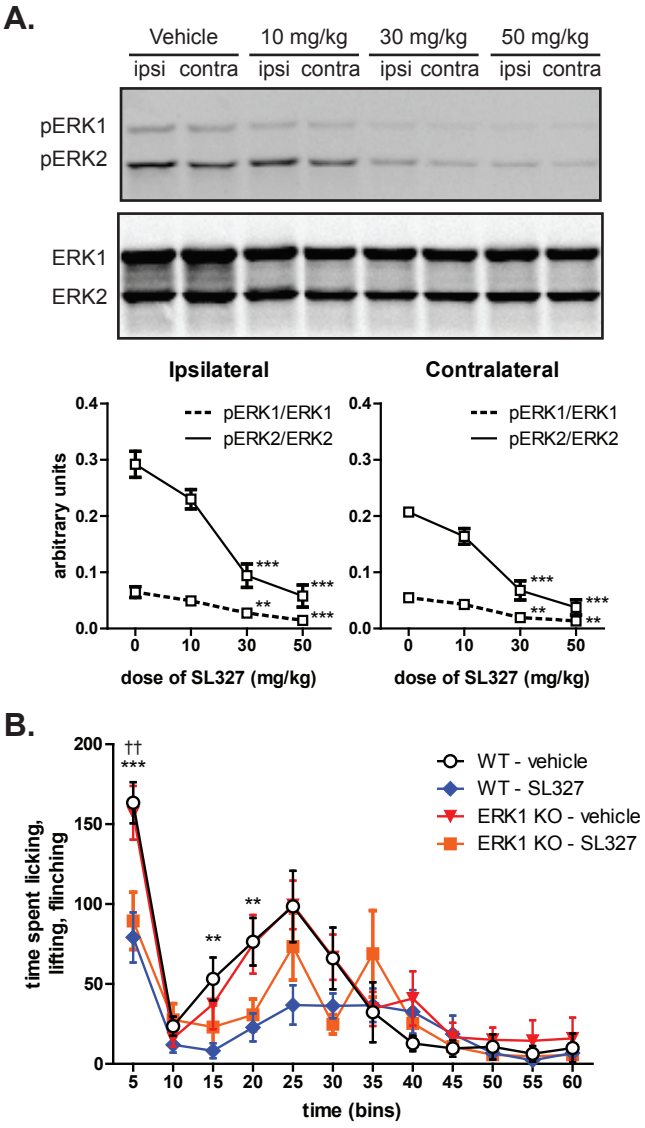
at least 90 μm (Fig. 9*B*). In both WT and ERK1 KO littermates, significantly more pERK1/2 positive cell profiles appear ipsilateral to formalin injection than contralateral (2-way ANOVA, F=56.68, DFn=1, DFd=4, P=0.0017). Interestingly, the number of pERK1/2-positive cell profiles ipsilateral to formalin injection is significantly reduced in ERK1 KO mice compared to WT littermates (2-way ANOVA, F=9.79, DFn=1, DFd=8, P=0.0141). This likely reflects the loss of ERK1 phosphorylation following formalin, which is normally seen in WT mice (Fig. 8). Importantly, in ERK1 KO mice

formalin-induced pERK1/2 immunostaining is not elevated and does not occur in unexpected laminae of the dorsal horn. Both observations would argue against the potential confounds of ERK2 hyperphosphorylation or developmental remodeling of nociceptive circuitry.

MEK inhibition attenuates formalin-induced spontaneous behaviors similarly in WT and ERK1 KO littermates.

Although we observed no change in p38 or JNK expression or phosphorylation with ERK1 deletion, it remains possible that other signaling pathways compensate for the loss of ERK1 in the context of nociceptive sensitization. To address this possibility, we sought to test whether MEK inhibition could reduce formalin-induced spontaneous nociceptive behaviors in ERK1 KO mice, as has been reported in WT mice (Karim et al., 2001). Previous studies have utilized intrathecal delivery of MEK inhibitors. Since ERK1 is eliminated in all tissues of the ERK1 KO and since ERK1/2 activity is important for nociceptive sensitization in areas besides spinal cord, a more appropriate test would involve systemic MEK inhibition. SL327 is a structural analogue of U0126 that is blood-brain barrier permeable and has previously been used to inhibit MEK in the brain by systemic intraperitoneal administration (Atkins et al., 1998). However, it has not yet been used in the formalin test and has not been evaluated in spinal cord. Thus, we began our study by determining the dose of SL327 required to maximally inhibit formalin-induced ERK1/2 phosphorylation in spinal cord (Fig. 10*A*). Pretreatment with both 30 and 50 mg/kg SL327 intraperitoneally 30 minutes before 3.5% formalin injection to the hindpaw significantly reduced pERK1 and pERK2 in spinal cord tissue ipsilateral and contralateral to the injected paw (1-way ANOVA: pERK1 ipsi $F=14.9$, $DFn=3$, $DFd=11$, $P=0.0012$; pERK2 ipsi $F=29.85$, $DFn=3$, $DFd=11$, $P<0.0001$; pERK1 contra $F=15.23$, $DFn=3$, $DFd=11$, $P=0011$; pERK2 contra $F=36.42$, $DFn=3$, $DFd=11$, $P<0.0001$). Therefore, we used 50 mg/kg SL327 to evaluate the effect of systemic MEK inhibition on the formalin test in both WT and ERK1 KO littermates (Fig. 10*B*). 50 mg/kg SL327 significantly attenuated formalin-induced spontaneous nociceptive behaviors in WT mice (2-way RM ANOVA $F=8.09$, $DFn=1$, $DFd=198$, $P=0.0108$). Importantly, no difference was detected between WT and ERK1 KO mice injected with SL327 (2-way RM ANOVA $F=0.91$, $DFn=1$, $DFd=187$, $P=0.3528$). Vehicle injected ERK1 KO and WT littermates also behaved similarly (2-way RM ANOVA $F=0.08$, $DFn=1$, $DFd=165$, $P=0.7795$), recapitulating results from Fig. 3 in animals injected with DMSO. Overall, these data indicate that systemic MEK inhibition reduces spontaneous nociceptive behavior in the formalin test regardless



<u>Figure 10:</u> Systemic MEK inhibition attenuates formalin-induced spontaneous behaviors similarly in WT and ERK1 KO littermates. A. Several doses of the blood-brain barrier permeant MEK inhibitor, SL327, or vehicle (DMSO) were administered intraperitoneally 30 minutes before hindpaw intraplantar formalin injection (3.5% in saline). Subjects (n=3 per dose) were sacrificed 3 minutes after formalin injection, and homogenates of spinal cord tissue ipsilateral and contralateral to the paw injection were analyzed for ERK phosphorylation. SL327 significantly reduced pERK1/ERK1 and pERK2/ERK2 at 30 mg/kg and 50 mg/kg (1-way ANOVA with Dunnett post-test comparing to vehicle (0) within ERK isoform; ** p<0.01, *** p<0.001). **B.** ERK1 KO and WT mice were injected with either vehicle (1 ml/kg DMSO) or 50 mg/kg SL327 intraperitoneally (WT - vehicle n=9, WT - SL327 n=11, KO - vehicle n=8, KO - SL327 n=8). After 30 minutes, formalin (3.5% in saline) was injected subcutaneously into the plantar surface of the hindpaw, and spontaneous nociceptive behaviors were measured. SL327 significantly attenuated formalin-induced nociceptive behaviors in both WT and ERK1 KO littermates (2-way RM ANOVA with Bonferroni post-test; stars comparing WT - vehicle and WT - SL327, ** p<0.01, *** p<0.001; crosses comparing KO - vehicle and KO - SL327, †† p<0.01). No significant difference was detected between WT and ERK1 KO mice injected with SL327.

of ERK1 deletion, suggesting that compensation from MEK-independent signaling pathways in ERK1 KO is negligible.

Discussion

Although it is clear that ERK1 and/or ERK2 drive behavioral sensitization in many pain models, little is known about the relative contribution of each isoform and whether they are redundant. This study is the first to address directly the role of ERK1 in rodent models of pain by characterizing the nociceptive function of ERK1 KO mice. We found that acute sensitivity to both noxious heat and the noxious chemical, formalin, remains intact despite the loss of ERK1 from all tissues. ERK1 deletion does not affect hypersensitivity due to chronic inflammation or nerve injury. Interestingly, long-term formalin-induced hypersensitivity to heat is slightly reduced by ERK1 deletion. Altogether, these data suggest ERK1 is not required for nociceptive sensitization.

Accumulating evidence from this study and elsewhere strongly suggests that spinal ERK2, and not ERK1, is critical for CFA-induced hypersensitivity. Targeting ERK1/2 nonspecifically, either with intrathecally applied MEK inhibitors or with transgenic neuron-specific expression of dominant negative MEK, attenuates CFA-induced hypersensitivity (Ji et al., 2002; Obata et al., 2003; Karim et al., 2006). Interestingly, RNAi-mediated ERK2 knockdown in spinal cord dramatically reduces CFA-induced behavioral sensitization (Xu et al., 2008). However, due to the partial reduction of ERK2 expression achieved with this method, there was a relatively small effect on ERK2 phosphorylation. ERK2 knockdown also caused an elevation of ERK1 phosphorylation in the spinal cord. In the physiologically normal context of isoform co-expression, spinal ERK1 is phosphorylated following CFA injection to the paw (Ji et al., 2002; Obata et al., 2003; Adwanikar et al., 2004; Xu et al., 2008), raising the possibility that ERK1 plays some role in behavioral sensitization. The current study directly addresses this question and demonstrates that ERK1 deletion has no effect on CFA-induced heat or mechanical hypersensitivity. Given the combined results of targeting MEK, ERK2 and now ERK1, it is likely that spinal ERK2 specifically drives CFA-induced hypersensitivity.

In the present study, we observed that ERK1 deletion is accompanied by increased ERK2 phosphorylation in the spinal cord and DRG of naïve mice. This could arise if ERK isoforms normally compete for binding and phosphorylation with their shared upstream kinase MEK. Without ERK1 expression, MEK binds and exclusively phosphorylates the remaining isoform

ERK2. Similar observations have been made in other systems, supporting such a model of MEK-ERK1/2 interaction. In NIH/3T3 cells, RNAi-mediated knockdown of one isoform elevates the phosphorylation state of the remaining isoform (Vantaggiato et al., 2006; Lefloch et al., 2008). However, the degree of isoform competition varies with context. *In vivo*, ERK1 deletion only affects ERK2 phosphorylation in certain non-neuronal tissues, such as the thymus (Nekrasova et al., 2005), but not in hippocampus, cortex, and striatum (Mazzucchelli et al., 2002). However, primary cultures of cortex or striatum from ERK1 KO embryos exhibit ERK2 hyperphosphorylation following stimulation with either glutamate or KCl without altered phosphorylation in untreated cultures. It appears that MEK-ERK1/2 interactions depend on cellular context, relative activity of MEK-ERK signaling, and perhaps developmental context given the differences between embryonic cultures and adult mice (Mazzucchelli et al., 2002).

In all studies utilizing isoform-specific knockout or knockdown, the functional importance of ERK isoform hyperphosphorylation is difficult to separate from the effect of deleting the targeted isoform. In ERK1 KO mice, ERK2 hyperphosphorylation in striatal cultures is correlated with augmented long-term synaptic potentiation in striatum and enhanced place preference conditioning for morphine (Mazzucchelli et al., 2002). Similar ERK2 hyperphosphorylation occurs in hippocampal cultures from ERK1 KO embryos (Mazzucchelli et al., 2002), but there is minimal impact of ERK1 deletion on hippocampal plasticity and, behaviorally, no effect on hippocampus-dependent tasks such as contextual fear conditioning and passive avoidance (Selcher et al., 2001). In the present study, elevated ERK2 phosphorylation is not associated with altered baseline withdrawal responses to noxious heat or mechanical stimulation, and there is no gain-of-function in nociceptive sensitization. Additionally, spinal pERK2 elevation above baseline following intraplantar formalin or CFA is similar between WT and ERK1 KO. This suggests that elevated basal ERK2 phosphorylation is unrelated to nociceptive sensitization. Basal phosphorylation may occur in non-nociceptive cells of the spinal cord, or it may be an artifact of tissue preparation or homogenization. In both cases, deletion of ERK1 would lead to ERK2 hyperphosphorylation only in this non-nociceptive-specific pool of ERK.

An alternative interpretation is that ERK2 hyperphosphorylation actually relates to nociception and functionally compensates for the loss of ERK1, masking a physiologic role for ERK1. We do not favor this interpretation for several reasons. First, knockdown of spinal ERK2

also increases basal ERK1 phosphorylation without affecting baseline sensory responses but with a profound effect on CFA-induced hypersensitivity (Xu et al., 2008). If ERK isoforms were interchangeable in the nociceptive spinal cord, then one would expect ERK1 to compensate for the loss of ERK2, which did not occur. Second, pERK1/2 immunohistochemistry is actually reduced in ERK1 KO mice, indicating that elevated pERK2 cannot make up for the loss of ERK1 in total pERK1/2 immunoreactivity. If isoforms were completely redundant, this net loss of pERK1/2 would be expected to affect formalin-induced spontaneous behaviors. Nevertheless, it remains difficult to exclude a role for ERK1 without directly testing ERK2 function. The converse is also true and, as such, the current study solidifies our understanding of spinal ERK2 function in the CFA model, as outlined above.

To our knowledge, the present study is the first to examine the role of ERK isoforms in the formalin model of chemical nociception. Moreover, our experiments with the systemically active MEK inhibitor, SL327, provide additional evidence that ERK1/2 activity is required for the behavioral response to formalin. Since SL327 exerts similar effects in WT and ERK1 KO mice, it seems likely that ERK2 is specifically required in this behavioral model, although this must be directly tested. It is tempting to hypothesize that the underlying cellular mechanisms of the formalin test would also rely upon ERK2 and not ERK1. These mechanisms may include sensitization processes in spinal cord that amplify incoming nociceptive information via enhanced synaptic strength and intrinsic excitability (Ikeda et al., 2006; Sandkuhler, 2009) both of which involve ERK1/2 (Ji et al., 2009). Formalin also increases primary afferent activity, which likely contributes to immediate, phase 1 behavioral responses (Puig and Sorkin, 1996). Since SL327 decreases the 1st phase of the formalin test and since ERK1 appears to be dispensable for formalin-induced spontaneous behaviors, our results predict that ERK2 specifically contributes to elevated afferent activity, perhaps by increasing excitability via direct phosphorylation of voltage-gated sodium channels (Stamboulian et al., 2010).

Noxious stimulation or inflammation induces hypersensitivity to heat which is largely due to peripheral sensitization of primary afferent nociceptors (Levine and Alessandri-Haber, 2007). Previous studies have demonstrated a role for nociceptor ERK1/2 in inflammation-induced peripheral sensitization using MEK inhibitors (Dai et al., 2002; Obata et al., 2003; Zhuang et al., 2004; Seino et al., 2006). ERK1/2 may contribute to heat hypersensitivity by modulating TRPV1 (Zhuang et al., 2004; Firner et al., 2006), which is a molecular sensor of noxious heat that is required

for CFA-induced heat hypersensitivity (Caterina et al., 2000; Levine and Alessandri-Haber, 2007). Our observation that ERK1 is not required for CFA-induced heat hypersensitivity suggests that ERK2 may specifically modulate TRPV1, a possibility which must be directly tested. Interestingly, our results also indicate that formalin-induced heat hypersensitivity is in part mediated by ERK1. The discrepancy between CFA- and formalin-induced heat hypersensitivity reported here may be the result of discrete parallel signaling pathways within nociceptive terminals that are sensitive to either CFA or formalin. Given that ERK1 KO mice develop heat hypersensitivity at later time points after formalin injection, it is also possible that ERK1 contributes to heat hypersensitivity but ultimately ERK2 is sufficient to induce sensitization.

Besides spinal cord and DRG, ERK1/2 is activated in many regions of the nociceptive system following noxious stimulation including the amygdala (Carrasquillo and Gereau, 2007). In amygdala, microinjection of MEK inhibitors does not affect acute formalin-induced spontaneous behaviors but does attenuate mechanical hypersensitivity. In the current study, we found that ERK1 deletion does not affect formalin-induced mechanical hypersensitivity. This fits well with our previous observations that formalin did not significantly induce ERK1 phosphorylation in amygdala (Carrasquillo and Gereau, 2007). However, since amygdala MEK inhibition affected both ERK1 and ERK2 phosphorylation, it was difficult exclude a role for ERK1. The current study extends our knowledge of the amygdala's role in nociception by providing evidence that ERK1 is not required for formalin-induced mechanical hypersensitivity.

Results from the present study indicate that ERK1 is not required for formalin-induced spontaneous behavior, chronic inflammatory and neuropathic pain models, suggesting a predominant isoform-specific role for ERK2. Isoform-specific functions may also occur in humans, since copy number variation of each isoform yields distinct neurological deficits. ERK1 deletion or duplication is associated with autism (Kumar et al., 2008), while reduced ERK2 expression is associated with microcephaly, cognitive deficits, and developmental delay (Newbern et al., 2008). The relevance to the human nervous system suggests that dissecting the roles of ERK1 and ERK2 will be important for understanding and treating chronic pain conditions.

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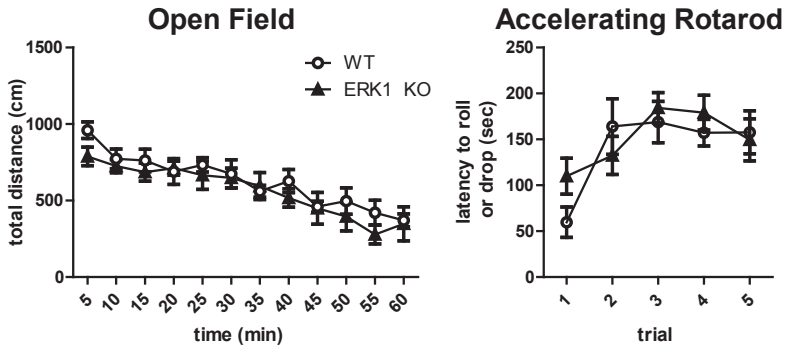
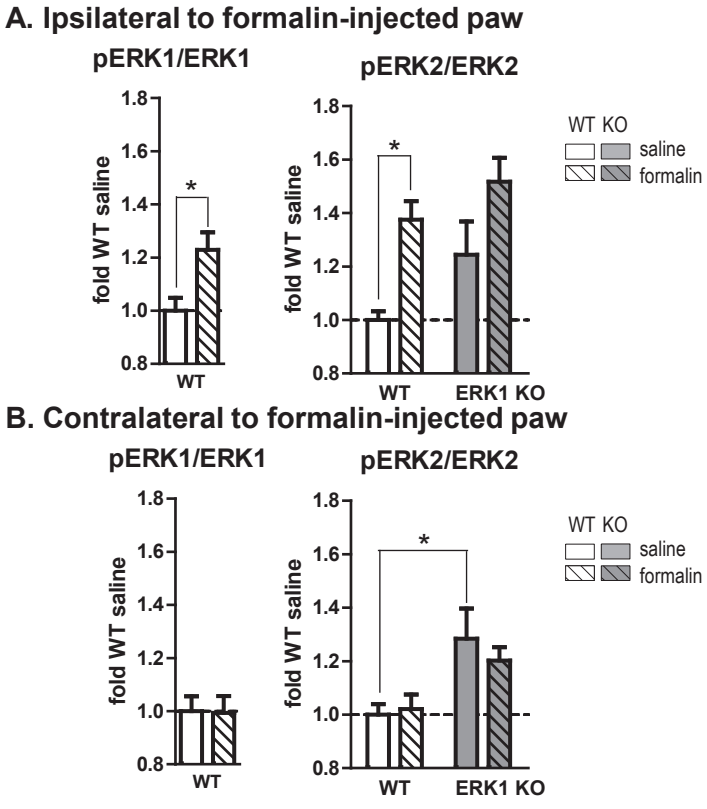


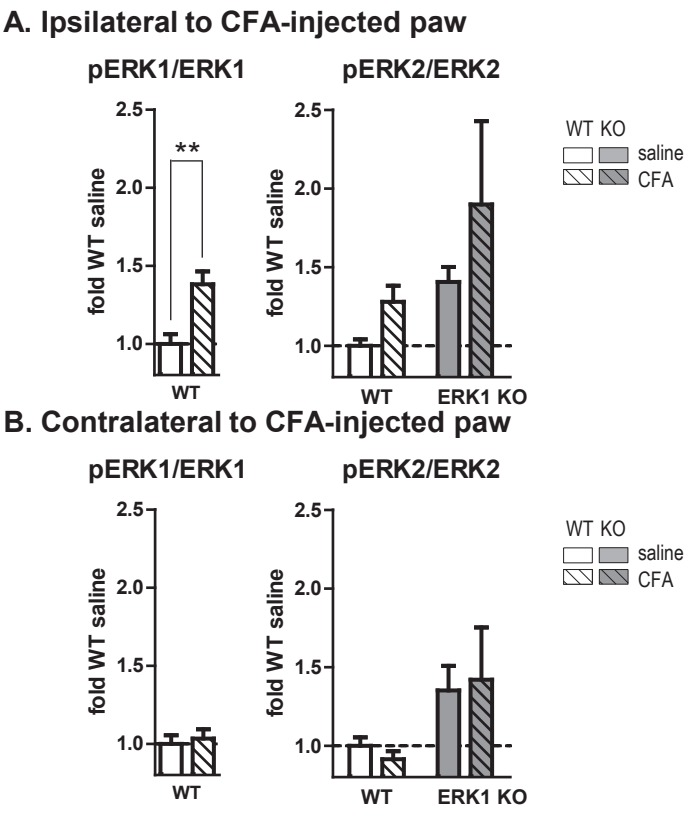
Supplemental Figure S1: ERK1 KO and WT mice have similar baseline locomotor behaviors. ERK1 KO (n=9) and WT (n=8) mice showed equivalent exploratory behavior on an open field task. Individual mice were placed into a box with a grid of infrared beams covering the horizontal space. Locomotor behavior was tracked for one hour. ERK1 KO mice (n=8) performed comparably to wild-type (WT) littermates (n=9) on an accelerating rotarod task. Untrained mice underwent 5 consecutive trials separated by 15 minutes, and latency to roll or drop from the rotating rod was recorded. For all graphs, mean values are plotted with error bars representing standard error of the mean (S.E.M.)

Supplemental Figure S2: Alternate analysis of formalin-induced ERK phosphorylation in spinal cord. pERK/ERK values from Fig. 7*A* were normalized to the average value of the WT saline group, in order to combine data across several Western blots. Ipsilateral (**A.**) and contralateral (**B.**) data were analyzed separately. In **A.**, an unpaired t-test indicates significant activation of pERK1 in WT mice. For pERK2/ERK2, a 2-way ANOVA indicates a



significant effect of genotype (F = 5.07, DFn=1, DFd=20, P=0.0357) and a significant effect of formalin (F = 14.32, DFn=1, DFd=20, P=0.0012) with a Bonferroni post-hoc test (* p<0.05). In **B.**, an unpaired t-test indicates no significant effect of formalin on pERK1/ERK1 in WT mice. For pERK2/ERK2, a 2-way ANOVA indicates a significant effect of genotype (F = 10.92, DFn=1, DFd=20, P= 0.0035) with no effect of formalin (F = 0.19. DFn=1 DFd=20, P=0.6691). The elevated pERK2/ERK2 in the contralateral spinal cord likely reflects the elevated basal pERK2 in ERK1 KO mice. A similar shift upwards in pERK2/ERK2 values for both saline and formalin injected ERK1 KO mice is observed in data from the ipsilateral spinal cord, although formalin induces pERK2/ERK2 elevation in both WT and ERK1 KO mice. Error bars indicate S.E.M.

Supplemental Figure S3: Alternate analysis of CFA-induced ERK phosphorylation in spinal dorsal horn. pERK/ERK values from Fig. 7*B* were normalized to the average value of the WT saline group**,** in order to combine data across several Western blots. Ipsilateral (**A.**) and contralateral (**B.**) data were analyzed separately. In **A.** an unpaired t-test indicates significant activation of pERK1 in WT mice due to CFA (** p<0.01). For pERK2/ERK2, a 2-way ANOVA indicates the effect of genotype is not significant (F = 3.84, DFn=1, DFd=17, P=0.0666). There is no significant effect of CFA (F = 2.18, DFn=1, DFd=17, P=0.1578). In **B.** an unpaired t-test indicates no significant effect of CFA on pERK1/ERK1 in WT mice. For pERK2/ERK2, a 2-way ANOVA indicates a significant effect of genotype (F = 5.72, DFn=1, DFd=17, P=0.0286) without a significant effect of CFA (F = 0.00, DFn=1, DFd=17, P=0.9612). The elevated pERK2/ERK2 in the contralateral



spinal cord likely reflects the elevated basal pERK2 in ERK1 KO mice. A similar shift upwards in pERK2/ERK2 values for both saline and CFA injected ERK1 KO mice is observed in data from the ipsilateral spinal dorsal horn. Error bars represent S.E.M.

Chapter 3: Nociceptor ERK2 drives peripheral sensitization and is required for epidermal innervation

This chapter is in preparation for submission:

Alter BJ, Satomoto M, O'Brien D, Morgan C, Golden JP, Kuner R, Landreth G, Gereau RW (2010) In Preparation.

Unless otherwise noted, all experiments were performed by Ben Alter.

Abstract

After injury or inflammation, kinases within nociceptive sensory neurons coordinate multiple extracellular signals to sensitize the cell to subsequent noxious stimulation, a process known as peripheral sensitization. Extracellular signal-regulated kinases 1 and 2 (ERK1/2) have been implicated in peripheral sensitization with the use of inhibitors and genetic manipulations which cannot specifically target the two isoforms. Previous studies, using mice that lack ERK1 in all tissues (*ERK1*$^{-/-}$), have suggested that ERK1 is not important in rodent models of pain. However, due to embryonic lethality in *ERK2*$^{-/-}$ mice the role of ERK2 in nociceptive sensory neurons remains unknown, since germline ERK2 deletion is embryonic lethal. To test the importance of ERK2 in nociceptors, we have conditionally deleted ERK2 in cells expressing $Na_V1.8$, a highly specific marker for small-diameter unmyelinated nociceptors. Interestingly, conditional ERK2 knockouts (*ERK2*$^{f/f}$*;*Na_V*1.8-Cre*) spent more time at cooler temperatures on the thermal gradient thermotaxis assay without any deficits in heat or mechanical sensation. Loss of ERK2 in nociceptors significantly attenuated the 2nd phase of the formalin test as well as mechanical hypersensitivity in the complete Freund's adjuvant (CFA) model of inflammatory pain. Previous work has shown no effect of ERK1 deletion in these models, suggesting that ERK2 is specifically required. Nerve growth factor (NGF), which is an inflammatory mediator involved in peripheral sensitization, produced dramatically less heat hypersensitivity in *ERK2*$^{f/f}$*;*Na_V*1.8-Cre* mice. Interestingly, ERK1 deletion exacerbates NGF-induced heat hypersensitivity. Since ERK1/2 signaling has been implicated in axon outgrowth and epidermal innervation and since we observed alterations in responses to NGF, it was important to evaluate the effects of ERK1/2 mutations on sensory neurons and their axons. Conditional deletion

of ERK2 partially reduced peptidergic innervation of the epidermis, while innervation was unaffected in *ERK1*$^{-/-}$ mice. There was no effect of ERK2 conditional deletion on the proportion of peptidergic cell bodies or the expression of the NGF receptor, TrkA. Partial loss of peptidergic innervation may contribute to diminished behavioral sensitization in *ERK2*$^{f/f}$*;Na*$_V$*1.8-Cre* mice. Overall, these data indicate that nociceptor ERK2 expression is necessary for epidermal innervation and rodent models of inflammatory pain.

Introduction

Nociceptors transduce noxious stimuli and transmit nociceptive information from peripheral tissues to the central nervous system (Hucho and Levine, 2007). In the context of injury or inflammation, nociceptors can become sensitized so that they respond to innocuous stimuli and respond more robustly to noxious stimuli. This process of peripheral sensitization involves intracellular signaling cascades within nociceptors which integrate input from multiple extracellular inflammatory mediators and orchestrate a variety of effector responses that include facilitation of transduction currents and enhancement of neuronal excitability. Kinases are components within these signaling cascades, and the mitogen activated protein (MAP) kinases, extracellular signal-regulated kinases (ERK) 1 and ERK2, are critical among them. ERK1/2 activation (phosphorylation, pERK1/2) occurs in the soma of small-diameter sensory neurons following heat, cold, and noxious mechanical stimulation (Dai et al., 2002; Mizushima et al., 2006). Injection of inflammatory mediators, capsaicin, formalin, or complete Freund's adjuvant (CFA) into peripheral tissues also increases pERK1/2 in both the soma and in peripheral terminals of afferents (Averill et al., 2001; Dai et al., 2002; Obata et al., 2003; Zhuang et al., 2004; Seino et al., 2006; Tsuda et al., 2007). Importantly, the use of inhibitors which block activation of ERK1 and ERK2 by inhibiting their shared upstream MAP kinase kinases (MEK1/2) reduces behavioral sensitization with concomitant reduction in primary afferent pERK1/2 (Dai et al., 2002; Obata et al., 2003; Zhuang et al., 2004; Seino et al., 2006). Although instructive, these studies cannot address the relative contribution of ERK1 and ERK2, since inhibitors do not selectively affect each isoform.

Although inhibitor studies do not address potential isoform differences, genetic strategies are yielding provocative new results suggesting that the functions of ERK1 and ERK2 may be quite different. Despite relatively high sequence homology (Boulton et al., 1991) and similar identified

downstream targets (English et al., 1999), deletion of ERK1 has no impact on viability (Pages et al., 1999; Nekrasova et al., 2005), while ERK2 deletion is embryonic lethal by E8.5 with deficits in mesodermal differentiation and trophoblast formation (Krens et al., 2006). Reduced expression of ERK2 due to a hypomorphic allele and conditional deletion of ERK2 in telencephalon results in learning and memory deficits that are not phenocopied in *ERK1*$^{-/-}$ mice (Selcher et al., 2001; Mazzucchelli et al., 2002; Satoh et al., 2007; Samuels et al., 2008). Interestingly, deletion of ERK1 actually enhances performance in conditioned place preference to morphine, which is associated with enhanced striatal plasticity (Mazzucchelli et al., 2002). The hypothesis that ERK1 and ERK2 are functionally distinct is supported by observations that ERK1 exclusively interacts with the scaffolding protein MP1 (Schaeffer et al., 1998) and that ERK1 and ERK2 shuttle between the nucleus and cytoplasm at different rates (Marchi et al., 2008).

Little is known about the specific roles of ERK1 and ERK2 in pain plasticity. A recent study has demonstrated that partial viral-mediated shRNA knockdown of ERK2 in spinal cord dramatically reduced CFA-induced heat and mechanical hypersensitivity (Xu et al., 2008). Deletion of ERK1 has no effect in this model, suggesting that ERK2 is the predominant isoform in modulating spinal plasticity in this model (Alter et al., 2010). However, the relative importance of ERK1 and ERK2 in nociceptors and peripheral sensitization remains unknown. To assess the role of ERK2 in nociceptors, we utilized the Cre-loxP system to delete ERK2 in small-diameter, unmyelinated sensory neurons expressing $Na_V1.8$ (Agarwal et al., 2004). Conditional deletion of ERK2 attenuated behavioral sensitization in several inflammatory pain models and partially reduced innervation of the epidermis. In parallel experiments, ERK1 deletion had minimal effects, except following the injection of nerve growth factor (NGF) in which case deleting ERK1 versus ERK2 produced opposing effects. Overall, our observations support an isoform-specific role for ERK2 in peripheral sensitization and epidermal innervation.

Methods

Animals

All experiments were approved by the Animal Care and Use Committee of Washington University School of Medicine and were performed in accordance with guidelines of the National Institutes of Health. Mice were housed with *ad libitum* access to food and water on a 12 h/12 h light/dark cycle.

7- to 10-week old male littermates were used in all experiments. The experimenter was blind to the genotype in all experiments.

Generation of ERK1 and ERK2 Mutant Mice

Conditional deletion of ERK2 in small-diameter, unmyelinated sensory neurons was achieved with a Cre/loxP approach. A targeting construct including exon 2 of *mapk1* flanked by loxP sites (flox) and the *neomycin* (*neo*) selection gene was transfected into 129 embryonic stem (ES) cells as described previously (Fig. 1*A*; Samuels et al., 2008). After selection for homologous recombination, ES cells were transfected with a Cre-expressing plasmid and screened for excision of *neo*. ES cells lacking Cre but containing the exon 2 flanking loxP sites (flox, f) were injected into C57BL/6 blastocysts. Germline chimeras were backcrossed two times onto the C57/BL6 background to generate homozygous floxed ERK2 mice (*ERK2$^{f/f}$*) mice. The generation of *Na$_V$1.8-Cre* mice was described previously (Agarwal et al., 2004). Briefly, the *Na$_V$1.8 (Scn10a)* locus was isolated from a bacterial artificial chromosome (BAC) genomic DNA library from 129 mice. A targeting construct was assembled containing the coding sequence for Cre recombinase, a polyadenylation tail, and an *ampicillin* (*amp*) resistance gene flanked by frt sites. Recombinogenic engineering was utilized, and clones positive for homologous recombination were transiently transfected with flp recombinase to remove *amp*. A combined restriction digest and southern blotting technique determined that Cre had been inserted 90 kb from the 5' end of the BAC. The engineered Cre-containing BAC was released from its vector with a NotI digest, purified, and then injected into oocyte pronuclei of the hybrid B6D2 mouse strain. Founder progeny were backcrossed eight times onto the C57/BL6 background. To generate *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice used in experiments, three successive crosses were performed: 1. *ERK2$^{f/f}$* X *Na$_V$1.8-Cre*, 2. *ERK2$^{f/+}$;Na$_V$1.8-Cre* X *ERK2$^{f/f}$*, 3. *ERK2$^{f/f}$;Na$_V$1.8-Cre* X *ERK2$^{f/f}$*. *ERK2$^{f/f}$* mice used in these crosses were backcrossed two times onto the C57/BL6 as outlined above. All subsequent breeding pairs were progeny of established *ERK2$^{f/f}$;Na$_V$1.8-Cre* X *ERK2$^{f/f}$* breeding pairs (cross 3). Therefore, mice used in all experiments are mixed C57/BL6 (~90%), 129, and DBA2. As such, great care was taken to include littermate controls in each experiment.

The generation of *ERK1$^{-/-}$* mice was described in detail elsewhere (Nekrasova et al., 2005). Briefly, exons 1-6 of *mapk3*, which includes the kinase active site and phosphorylation loop, were

targeted for replacement by homologous recombination with a Neo cassette flanked with loxP sites. Successfully targeted 129 Sv ES cells were injected into CD1 blastocysts. Chimeras with germline transmission were obtained on the CD1 background. The *mapk3* mutation was subsequently transferred to the C57BL/6 background by backcrossing >10 times. Pure C57BL/6 heterozygotes (*ERK1*$^{+/-}$) were crossed to produce *ERK1*$^{-/-}$ and *ERK1*$^{+/+}$ (WT) littermates used in all experiments.

Sample Preparation and Western Blot Analysis

Mice were sacrificed by decapitation and spinal cords were rapidly removed by hydraulic extrusion. Spinal cord levels L3-L6 were isolated and flash frozen on crushed dry ice. Brain regions (cerebellum, hippocampus, cortex) were dissected and flash frozen. Thoracic and lumbar DRGs were then dissected, pooled, and immediately homogenized using a dounce homogenizer. The homogenization buffer used for all tissues was kept on ice and consisted of 20 mM Tris, pH 7.5, 1 mM EDTA, 1 mM $Na_4P_2O_7$, 25 µg/ml aprotinin (Sigma-Aldrich), 25 µg/ml leupeptin (Sigma-Aldrich), 100 µM PMSF (Roche Applied Science, Indianapolis, IN), 1 µg/ml microcystin LR (Enzo Life Science, Plymouth Meeting PA), and 1 mM Na_3VO_4 (Sigma-Aldrich) in Milli-Q distilled water. Protein concentrations were determined with the BCA protein assay (Pierce Biotechnology, Rockford, IL) and SmartSpec 3000 Spectrophotometer (Bio-Rad, Hercules, CA). Protein homogenates (10 µg per sample) were size separated by SDS-PAGE with 10% separating gels prepared based on the Laemmli system (Coligan et al., 2001). Gels were transferred to nitrocellulose membranes using a wet transfer system (Biorad) and membranes were blocked for 1 h at room temperature (RT) with Odyssey blocking buffer (LI-COR Biotechnology, Lincoln NE). Two primary antibodies from different host species were applied to the blots (1 h at RT diluted in blocking buffer) followed by washes and then incubation for 1 h at RT with fluorescently conjugated secondary antibodies goat anti-rabbit IRDye800 (1:20,000; LI-COR Biotechnology) and goat anti-mouse AlexaFluor680 (1:20,000; Invitrogen, Carlsbad, CA) diluted in blocking buffer. Membranes were then visualized with the Odyssey Infrared Fluorescence Imaging System (LI-COR Biotechnology) for simultaneous detection of 700 and 800 nm wavelength fluorescent emissions corresponding to mouse and rabbit primary antibodies, respectively. Primary antibodies used include anti-actin (1:2,000, mouse monoclonal; Sigma-Aldrich), anti-β-tubulin (mouse monoclonal, 1:1,000; Sigma-Aldrich), anti-pERK1/2 (mouse monoclonal, 1:1,000; Cell Signaling, Danvers MA), and anti-ERK1/2 (rabbit polyclonal, 1:1,000; Cell Signaling). The intensity of bands in 700 nm (pERK1/2, β-tubulin, or actin)

and 800 nm (ERK1/2) channels was quantified using the Odyssey Infrared Fluorescence Imaging System software (LI-COR Biotechnology).

Behavioral Tests

All behavioral tests were performed during the second half of the light cycle at room temperature (~25°C). Tests were conducted in rooms isolated from other activity and with white noise present. Mice were allowed to acclimate to the behavior room and apparatus for 2-3 hours before testing began unless otherwise noted. In tests measuring reflexive responses to heat or mechanical stimuli, mice were only tested when calm in order to minimize variability.

Thermal Gradient

The thermal gradient apparatus is a continuous, rectangular metal surface with plexiglass walls and cover. Mice were initially acclimated to the behavior room in their home cages and then individually placed into the apparatus for a 2 hour testing session. On the first day of testing, the thermal gradient was turned off so that mice could explore the area with a room temperature floor. On the second day of testing, the thermal gradient was applied. On both days, webcam recordings were made, and location of mice in the apparatus was tracked using Anymaze. The amount of time spent in zones that are distributed evenly along the length of the rectangle was measured and plotted in 30 minute bins. Zone temperatures were measured with an infrared thermometer.

Open Field

Mice were acclimated to the behavior room in their home cages and then individually placed into a box with a grid of infrared beams covering the horizontal space. Locomotor behavior was tracked for one hour.

Rotarod

Mice were initially acclimated to the room. Untrained mice underwent 5 consecutive trials separated by 15 minutes, and latency to roll or drop from the rotating rod was recorded.

Formalin-Induced Spontaneous Nociceptive Behaviors

To measure spontaneous behavioral responses to formalin injection, mice were acclimated in

modular clear plexiglass behavior chambers (width=10 cm, length=10 cm, height=15 cm) with a plexiglass floor for at least 1 h, injected subcutaneously with 10 µl 5% formalin (Sigma-Aldrich, St. Louis, MO) in sterile 0.9% NaCl into the plantar surface of the hindpaw, and then returned to the behavior chamber. Behaviors were then video recorded from below with a webcam (Logitech) at a resolution of 960 x 720 for one hour without the experimenter present. Videos were scored for time spent licking, lifting, and flinching the injected paw.

Measurement of Heat Thresholds

Responses to heat were measured using a modified Hargreaves test (Hargreaves et al., 1988). Mice were placed in modular behavior chambers on a 390G Plantar Test Apparatus (IITC Life Science, Woodland Hills, CA) with a glass plate floor heated to ~30°C, except in experiments with CFA which were performed with a room temperature glass surface. From underneath the glass surface, a focused beam of light was used to heat the plantar surface of the hindpaw. At the initiation of the light beam, a timer automatically started and continued until the mouse withdrew its paw from the stimulus or until a 20 second cutoff time was reached. At withdrawal, the experimenter simultaneously stopped the light stimulus and the timer, recording the amount of time to paw withdrawal (latency). Light intensity was calibrated to achieve baseline paw withdrawal latencies between 10 and 15 seconds. After calibration with control animals (either WT or *ERK2*$^{f/f}$), the light intensity was kept constant within each experiment. In each experiment, mice were initially acclimated, and then baseline paw withdrawal latencies were determined by averaging five independent trial measurements separated by 15 min alternating between right and left paws. The day following baseline measurements, mice were allowed to acclimate, the right hindpaw was injected, and paw withdrawal latency measurements were collected every 15 minutes, alternating right and left hindpaws. Hour timepoint values were obtained by averaging two of these measurements. If measured, additional timepoints over the subsequent days were obtained by initially acclimating mice and then averaging 3 separate measurements. In all models, post-injection measurements were divided by baseline measurements within each subject to yield percent baseline data.

NGF-Induced Heat Hypersensitivity: After acclimation to the apparatus, baseline measurements were obtained, and then mice were injected subcutaneously into the plantar hindpaw with 0.2

μg NGF (2.5S purified from male mouse salivary glands; Harlan, Indianapolis, IN) in 10 μl sterile phosphate-buffered solution (PBS; Mediatech Inc. Manassas, VA).

Formalin-Induced Heat Hypersensitivity: After obtaining baselines, mice were injected subcutaneously into the plantar surface of the hindpaw with 5% formalin in 10 μl sterile saline (0.9% NaCl).

Mustard oil-Induced Heat Hypersensitivity: Following baseline measurements, mustard oil (0.75% in 10 μl light mineral oil; Sigma-Aldrich) was administered to the hindpaw by intraplantar injection.

CFA-Induced Heat Hypersensitivity: All measurements were conducted on a RT glass plate. Light intensities were adjusted to produce 10-15 second paw withdrawal thresholds in control mice at baseline. Using the RT glass, we found no difference between the baseline sensitivity of *ERK2$^{f/f}$;Na$_V$1.8-Cre* and *ERK2$^{f/f}$* littermates (data not shown), which was consistent with experiments using the warmed glass plate (Fig. 2). After collecting baseline data, mice were injected subcutaneously into the plantar surface of the hindpaw with 10 μl CFA (1.0 mg/ml, Sigma-Aldrich).

Measurment of mechanical thresholds and CFA-Induced Mechanical Hypersensitivity

To measure responses to mechanical stimuli applied to the plantar surface of the hindpaw, a set of calibrated von Frey filaments (North Coast Medical Inc. Morgan Hill, CA) was used. To allow access to the hindpaw, mice were placed in modular behavioral chambers on an elevated wire mesh and allowed to acclimate. Filaments were pressed into the skin between the anterior and posterior footpads until the filament bent, held for ~1 sec, and removed. Application of the same filament was repeated 5 times at a frequency of ~0.5 Hz. If the mouse withdrew its paw in 3 out of the 5 applications, the calibrated weight of that filament was recorded as the paw withdrawal threshold. For each measurement of paw withdrawal threshold, the lightest filament (0.008 g) was the first filament applied. If the mouse did not withdrawal 3 times or more, the next heaviest filament was applied 5 times, moving stepwise to heavier filaments until 3 out of 5 responses were obtained. To obtain the baseline threshold, three to five independent paw withdrawal threshold measurements were made and then averaged. On the following day, mice were allowed to acclimate, and CFA (10 μl of 1.0 mg/ml solution) was then administered by intraplantar injection into the hindpaw. Data points hours after injection consisted of single paw withdrawal threshold measurements, while data

points on subsequent days represented the averaged values of three independent paw withdrawal measurements.

Immunohistochemistry

Mice were euthanized with an overdose of Avertin anesthesia (1.25% working solution in 0.9% NaCl diluted from a stock of 1.0 mg/ml 2,2,2 tribromoethanol in tert-amyl alcohol, both from Sigma-Aldrich) by intraperitoneal injection. Footpad skin from the plantar surface of the hindpaw was collected into Zamboni's fixative (15% picric acid and 2% paraformaldehyde in PBS) and allowed to fix for 1-1.5 h on ice. Tissue was then washed with PBS and cryoprotected in 30% sucrose/PBS at 4 °C for at least 48 h. Meanwhile, mice were transcardially perfused first with a warmed 0.5% procaine/PBS solution to clear circulating blood and then with ice-cold 4% paraformaldehyde in PBS. DRG were dissected, washed in PBS, and then cryoprotected in 30% sucrose/PBS at 4°C for at least 48 h. 18 µm thick sections of DRG were collected. 30 µm thick sections of footpad skin were cut perpendicular to the surface of the skin. Staining for all markers and tissues was performed similarly using Coplin jars for blocking and washing steps. First, sections were rinsed with Tris-buffered saline (0.9% NaCl in 100 mM Tris, pH 7.5) and then blocked with 0.2% Difco milk (BD Biosciences), 1% bovine serum albumin (Sigma-Aldrich), 0.1% Triton-X-100 in TBS. Slides were rinsed with TBS, covered with primary antibodies diluted in tyramide signal amplification kit blocking reagent (Perkin-Elmer), protected with parafilm over the solution, and then placed in a humidified chamber to incubate at 4°C overnight. Slides were then washed with 0.1% TritonX100/TBS, rinsed with TBS, and incubated with secondary antibodies at RT for 1 h. Sections were finally counterstained with bis-benzimide (Invitrogen), coverslipped, and imaged using either a NanoZoomer automated microscope (Hamamatsu, Hamamatsu City, Japan) or a fluorescent compound microscope (Nikon 80i) with a monochrome camera (CoolSnapEZ). Primary antibodies used include anti-CGRP (goat polyclonal, 1:1,000; AbD Serotec, Raleigh NC), anti-TrkA (rabbit polyclonal, 1:300; Millipore, Billerica, MA), anti-βIII tubulin (rabbit polyclonal, 1:2,000, skin; Covance, Princeton, NJ), anti-βIII tubulin (mouse monoclonal, 1:1000, DRG; Millipore). Fluorescently conjugated secondary antibodies (all from Invitrogen) used were donkey anti-mouse Alexa488 (1:200, for βIII tubulin in DRG), donkey anti-rabbit Alexa555 (1:200, for TrkA), donkey anti-goat Alexa555 (1:200, for CGRP in DRG), donkey anti-goat Alexa488 (1:350, for CGRP in skin), and donkey anti-rabbit Alexa555 (1:200, for βIII tubulin in skin). For quantification, the experimenter was

blind to genotype. In DRG, sections were counted for total number of neurons (βIII tubulin-positive) and total number of neurons expressing the marker of interest. For each section, the percentage of total neurons expressing the marker (marker/βIII tubulin) was calculated. 2-5 sections were counted for each animal. In footpad skin, sections of thick skin with rete ridges were selected while viewing bis-benzimide counterstaining. The epidermal-dermal border was identified, traced on a live image in Metamorph (Molecular Devices), and its length measured. Fibers were then counted using the appropriate filter cube in live images, allowing the experimenter to follow fibers through multiple focal planes. The number of fibers was then divided by the length of the epidermal-dermal border and multiplied by 100 to yield fiber number per 100 μm length of epidermal-dermal border. 3-5 sections were analyzed per animal.

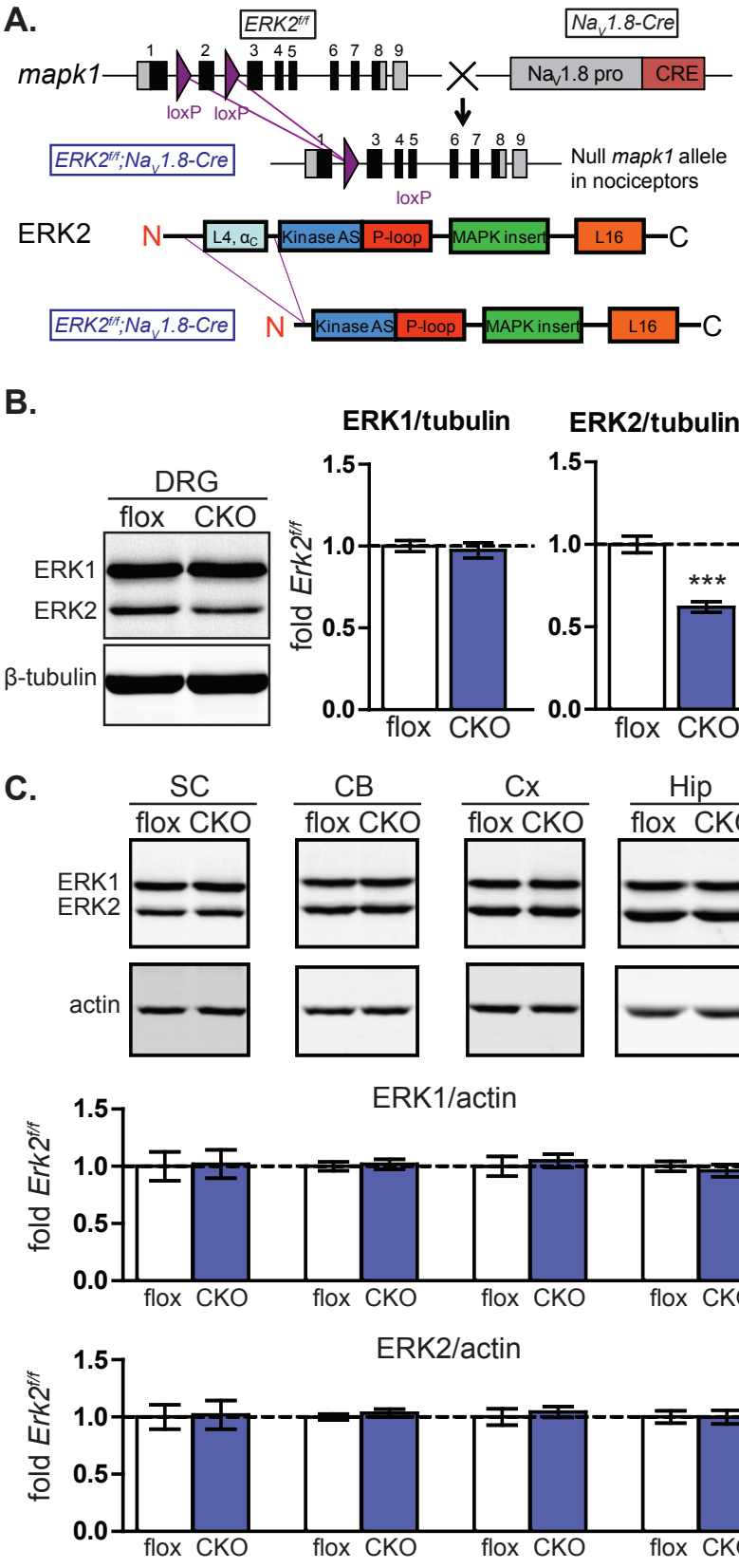
Statistical Analysis

Data analysis and graphing was performed using Excel (Microsoft, Redmond, WA) and Prism (GraphPad Software, La Jolla, CA). Details of specific statistical tests are included in the results sections.

Results

Conditional ERK2 deletion is tissue-specific.

To test the hypothesis that nociceptor ERK2 is required for peripheral sensitization, we generated conditional ERK2 knockouts with the Cre-loxP system (Fig. 1*A*). In one line, exon 2 of *mapk1* was flanked by loxP sites, as previously described (see Methods and Samuels et al., 2008). This was crossed with a Cre recombinase (Cre) expressing BAC transgenic line with the promoter elements from $Na_V1.8$ controlling Cre expression (see Methods and Agarwal et al., 2004). Since $Na_V1.8$ is expressed primarily in small-diameter, unmyelinated sensory neurons that respond to noxious stimuli (nociceptors), we expected progeny with the Cre transgene (*ERK2$^{f/f}$;Na$_V$1.8-Cre*) to lack ERK2 only in nociceptors. Compared to control littermates that lacked the Cre transgene (*ERK2$^{f/f}$*), *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice had a significant reduction in ERK2 protein in dorsal root ganglion (DRG, Fig. 1*B*, t-test). Since $Na_V1.8$-expressing nociceptors comprise a subset of sensory neurons found within the DRG, ERK2 expression in DRG homogenates is not completely abolished. The remaining signal in the ERK2 band is likely due to large- and medium-diameter sensory neurons which do


A.
ERK2f/f
Nav1.8-Cre
mapk1
1 2 3 4 5 6 7 8 9
loxP loxP
Nav1.8 pro
CRE
ERK2f/f;Nav1.8-Cre
1 3 4 5 6 7 8 9
loxP
Null mapk1 allele in nociceptors
ERK2
N
L4, αC
Kinase AS
P-loop
MAPK insert
L16
C
ERK2f/f;Nav1.8-Cre
N
Kinase AS
P-loop
MAPK insert
L16
C
B.
ERK1/tubulin
ERK2/tubulin
DRG
flox CKO
ERK1
ERK2
β-tubulin
fold Erk2f/f
1.5
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flox CKO
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Cx
Hip
flox CKO
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flox CKO
ERK1
ERK2
actin
ERK1/actin
fold Erk2f/f
1.5
1.0
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1.5
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Figure 1: ERK2 expression is specifically reduced in dorsal root ganglia of *Erk2$^{f/f}$;Na$_V$1.8-Cre* mice. A. Schematic for the generation of conditional ERK2 deletion in nociceptors. *Erk2$^{f/f}$* mice with loxP sites flanking exon 2 were crossed with transgenic *Na$_V$1.8-Cre* mice. In mice homozygous for the flox allele that also have the transgene (*Erk2$^{f/f}$;Na$_V$1.8-Cre*), this results in a null allele specifically in nociceptors with the flox allele present in all other cells. On the protein level, deletion of exon 2 eliminates L4 and α_C kinase domains that include a lysine required for catalytic activity (amino acids 52-103 using ERK1 numbering from Introduction Fig. 2). Thus, the coding sequence would either be inactive or not produce protein as has been observed by Western blot in other studies (Samuels et al. 2008). **B.** Tissue from *Erk2$^{f/f}$;Na$_V$1.8-Cre* (CKO) and *Erk2$^{f/f}$* (flox) littermates (n=7 each group) was obtained and analyzed for total ERK1/2 expression by Western blot analysis. The loading control was β-tubulin for DRG samples. The normalized integrated intensity of ERK2 was significantly reduced in *Erk2$^{f/f}$;Na$_V$1.8-Cre* mice (*** $p<0.001$) compared with littermate *Erk2$^{f/f}$* controls. No change was observed in the ERK1 band. **C.** Homogenates of tissue from other areas of the nervous system (SC = spinal cord; CB = cerebellum; Cx = cortex; Hip = hippocampus) were analyzed for ERK1/2 expression. There was no difference in ERK1 or ERK2 band intensities between *Erk2$^{f/f}$* and *Erk2$^{f/f}$;Na$_V$1.8-Cre* littermates, confirming tissue-specific deletion of ERK2 in sensory neurons.

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not express $Na_V1.8$ as well as non-neuronal cells present in the DRG. This is consistent with and supported by previous studies utilizing this transgenic *Na$_V$1.8-Cre* line to delete several different targeted genes exclusively in small-diameter, unmyelinated sensory neurons (Agarwal et al., 2004; Agarwal et al., 2007; Gangadharan et al., 2009; Stosser et al., 2010). Importantly, there is no effect of ERK2 conditional deletion on the expression of ERK1 (Fig. 1*B*). To confirm that ERK2 was not deleted in other areas of the nervous system, brain and spinal cord were analyzed by Western blot for ERK2 expression (Fig. 1*C*). No significant difference was observed between *ERK2$^{f/f}$;Na$_V$1.8-Cre* and *ERK2$^{f/f}$* littermate controls in any of the regions tested, including spinal cord, cerebellum, cortex, and hippocampus (t-test). As an important control experiment, we also compared *ERK2$^{f/f}$* with WT littermates and found that the presence of loxP sites in *mapk1* had no effect on protein expression in the DRG (data not shown). Overall these data indicate that *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice lack ERK2 expression in small-diameter, unmyelinated nociceptors.

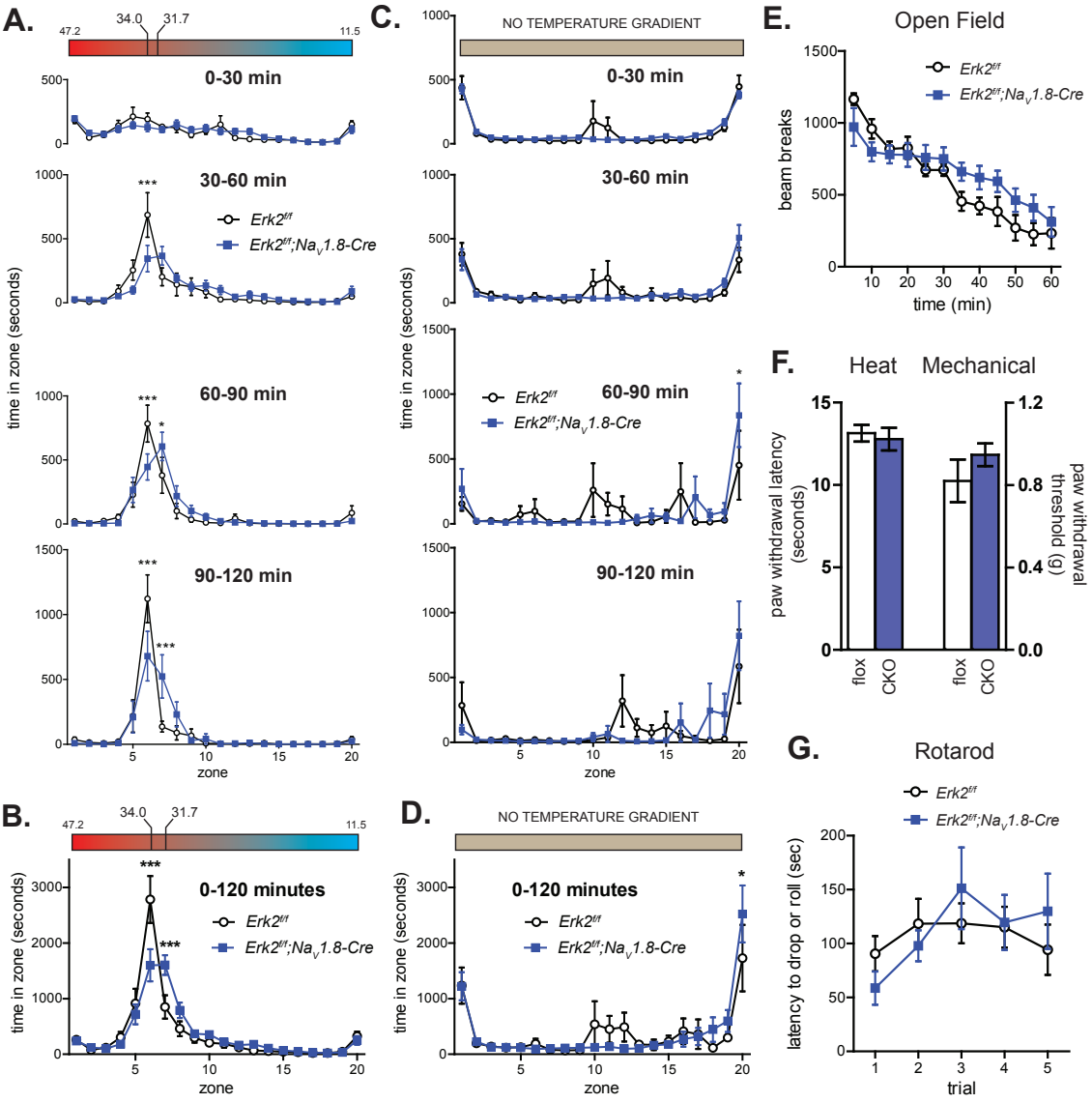

Figure 2: Conditional deletion of ERK2 impairs thermotaxis without affecting withdrawal from radiant heat or von Frey filaments. A.&B. *Erk2$^{f/f}$;Na$_V$1.8-Cre* (n=7) and *Erk2$^{f/f}$* (n=8) littermates were allowed to explore a thermal gradiant apparatus, consisting of a continuous metal surface with temperatures ranging from 47.2°C to 11.5°C. Locomotor activity was tracked for two hours. To analyze thermotaxis, 20 zones were created to subdivide the thermal gradient, and the presence time of each subject within each zone was determined. The temperature of each zone was measured using an infrared thermometer. Red and blue colored bars graphically represent the temperature gradient. At the beginning of the thermotaxis assay (0-30 minutes), presence time for both genotypes is distributed over many temperatures. Over time, *Erk2$^{f/f}$* mice spend an increasing

amount of time in the 34.0°C zone while *Erk2$^{f/f}$;Na$_V$1.8-Cre* mice prefer the cooler 31.7°C zone. In **B.**, presence time from the entire 2-hour experiment is summed and plotted as in **A. C.-D.** The same mice were allowed to explore the same appartus set to room temperature one day before the thermotaxis assay. Under these conditions, minimal differences were detected between *Erk2$^{f/f}$;Na$_V$1.8-Cre* and *Erk2$^{f/f}$* littermates, suggesting that differences in presence time in the thermotaxis assay (**A.-B.**) are related to temperature sensation and not simply exploratory behavior. For **A.-D.**, * p<0.05, ** p<0.01, *** p<0.001 (2-way ANOVA repeated measures, Bonferroni post-test). **E.** In the open field task, there was no difference between *Erk2$^{f/f}$;Na$_V$1.8-Cre* and *Erk2$^{f/f}$* (n=7) littermates (2-way ANOVA repeated measures), confirming that exploratory behavior is not altered in *Erk2$^{f/f}$;Na$_V$1.8-Cre* animals. **F.** *Erk2$^{f/f}$;Na$_V$1.8-Cre* (CKO; n=14) and *Erk2$^{f/f}$* (flox; n=14) mice have similar baseline responses to radiant heat in a Hargreaves assay. Mechanical withdrawal thresholds measured with von Frey filaments were also similar between *Erk2$^{f/f}$;Na$_V$1.8-Cre* (CKO; n=9) and *Erk2$^{f/f}$* (flox; n=9) littermates. **G.** *Erk2$^{f/f}$;Na$_V$1.8-Cre* and *Erk2$^{f/f}$* littermates (n=7) perform similarly on the accelerating rotarod, indicating that gross motor function is unimpaired due to deletion of ERK2.
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Conditional deletion of ERK2 affects thermotaxis without altering withdrawal responses to heat or mechanical stimuli.

To begin to evaluate the role of ERK2 in nociceptors, we sought to test baseline sensory function in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice. As an initial test of responses to thermal stimuli, we utilized the thermal gradient apparatus, which consists of an enclosed, rectangular area with a metal floor that is cooled on one end of the rectangle (11.6°C), heated (47.2°C) on the other end, and with a continuous range of temperatures between the two ends (Fig. 2*A-B*). *ERK2$^{f/f}$* and *ERK2$^{f/f}$;Na$_V$1.8-Cre* littermates were allowed to explore the area with the temperature gradient for two hours. *ERK2$^{f/f}$* mice initially (0-30 min) explore the length of the rectangle, resulting in an evenly distributed presence time across the zones. Over the next 90 minutes, *ERK2$^{f/f}$* mice spend more time in zones ranging in temperatures from 31°C to 36°C than in other zones. Interestingly, the presence time of *ERK2$^{f/f}$;Na$_V$1.8-Cre* littermates is shifted towards cooler temperatures. The presence time of *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice is more distributed, with less time spent in any one zone, than *ERK2$^{f/f}$* littermate controls. Within each 30 minute bin, a 2-way ANOVA indicates a significant main effect of zone and a significant interaction between zone and genotype ($P<0.01$) with the exception of the 0-30 min bin during which there is no significant interaction. Bonferroni post-tests indicate significant differences in presence time in the 34.0°C and 31.7°C zones. If presence time for the entire 2-hour test is summed (Fig. 2*B*), *ERK2$^{f/f}$;Na$_V$1.8-Cre* spend more time in the 31.7°C zone and less time in the 34.0°C zone than *ERK2$^{f/f}$* littermates (2-way ANOVA; significant interaction between zone and genotype, $F=4.42$ DFn=19 DFd=260 $P<0.0001$; significant main effect of zone, $F=42.60$ DFn=19 DFn=260 $P<0.0001$; Bonferroni post-test). Importantly, we observed only minimal differences between genotypes in presence time when there was no temperature gradient (Fig. 2*C-D*). These data were obtained using the same mice in Fig. 2*A-B*, which were allowed to explore the same apparatus without heating or cooling on the day before the thermotaxis assay. In both groups, significantly more presence time was observed at the ends of the apparatus, reflecting the tendency of mice to avoid open areas. Comparing *ERK2$^{f/f}$* and *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice, a statistically significant difference was detected in the amount of time spent at the end of the apparatus (zone 20) in the 60-90 min block (Fig. 1*C*) and in the summed data (Fig. 2*D*; 2-way ANOVA, Bonferroni post-test). This slight difference in exploratory behavior is not likely to explain the results in Fig. 2*A-B*. It is conceivable that a more distributed presence time over the temperature gradient, as seen with *ERK2$^{f/f}$;Na$_V$1.8-*

Cre mice (Fig. 2*A-B*), may be due to hyperactivity during exploration. However, as observed in Fig. 1*C-D*, *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice actually spend more time than *ERK2$^{f/f}$* littermates in zone 20 less time in other zones, suggesting that *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice are not hyperactive. To directly test this possibility, we assessed the open field behavior of *ERK2$^{f/f}$;Na$_V$1.8-Cre* and *ERK2$^{f/f}$* littermates (Fig. 2*E*). Mice were placed into the center of a large, enclosed, square-shaped area with a grid of infrared beams, and exploratory behavior was tracked for one hour. *ERK2$^{f/f}$;Na$_V$1.8-Cre* and *ERK2$^{f/f}$* showed no differences in activity (beam brakes) within the open field (2-way RM ANOVA). This suggests that hyperactivity or other alterations in exploratory behavior do not account for the effects observed on the thermal gradient apparatus. Overall, these data indicate that thermotaxis behavior in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice is altered, resulting in more presence time at cooler temperatures than *ERK2$^{f/f}$* littermates.

Given a cool shift in thermotaxis behavior, we next tested the response to noxious heat using a Hargreaves apparatus. Despite alterations in an innocuous temperature range (32-34°C), we observed no difference between *ERK2$^{f/f}$;Na$_V$1.8-Cre* and *ERK2$^{f/f}$* littermates in their responses to radiant heat (Fig. 2*F*, t-test). We then used von Frey filaments to assess mechanical sensitivity and found no effect of ERK2 conditional deletion (t-test). Since locomotor deficits may confound the interpretation of these and other behavioral models, we measured motor performance with an accelerating Rotarod (Fig. 2*G*). No significant difference was observed between *ERK2$^{f/f}$;Na$_V$1.8-Cre* and *ERK2$^{f/f}$* littermates (2-way RM ANOVA), indicating that conditional ERK2 deletion did not alter gross motor function. This indicates that conditional deletion of ERK2 in nociceptors does not confound reflexive tests, such as the measurement of heat and mechanical sensitivity (Fig. 2*F*), which require the ability to withdrawal the hindpaw to a given stimulus. Since there is no motor deficit, the above data indicate that deletion of nociceptor ERK2 does not affect baseline sensitivity to heat or mechanical stimuli.

ERK2 is necessary for peripheral sensitization.

Formalin injection produces spontaneous nociceptive behavior which responds to local anesthesia and is associated with elevated C-fiber afferent activity (Tjolsen et al., 1992; Puig and Sorkin, 1996), implicating peripheral sensitization in this model. Since ERK1/2 activity in nociceptive afferents has been implicated in peripheral sensitization in this and other models (Aley et al., 2001;

Dai et al., 2002; Obata et al., 2003; Zhuang et al., 2004; Seino et al., 2006; Tsuda et al., 2007), we sought to test the hypothesis that nociceptor ERK2 was required for formalin-induced spontaneous nociceptive responses. *ERK2$^{f/f}$;Na$_V$1.8-Cre* and *ERK2$^{f/f}$* littermates were injected with 5% formalin and nociceptive spontaneous behaviors were video recorded for one hour and subsequently scored for time spent licking, lifting, and flinching the injected paw. Control *ERK2$^{f/f}$* mice exhibited the stereotypical biphasic response to formalin injection (Fig. 3*A*) with an immediate behavioral response (phase 1, 0-10 minutes) which subsides and is followed by a longer lasting delayed response (phase 2, 10-60 minutes). Interestingly, the first phase of *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice was significantly higher than control *ERK2$^{f/f}$* littermates. In contrast, the second phase is significantly

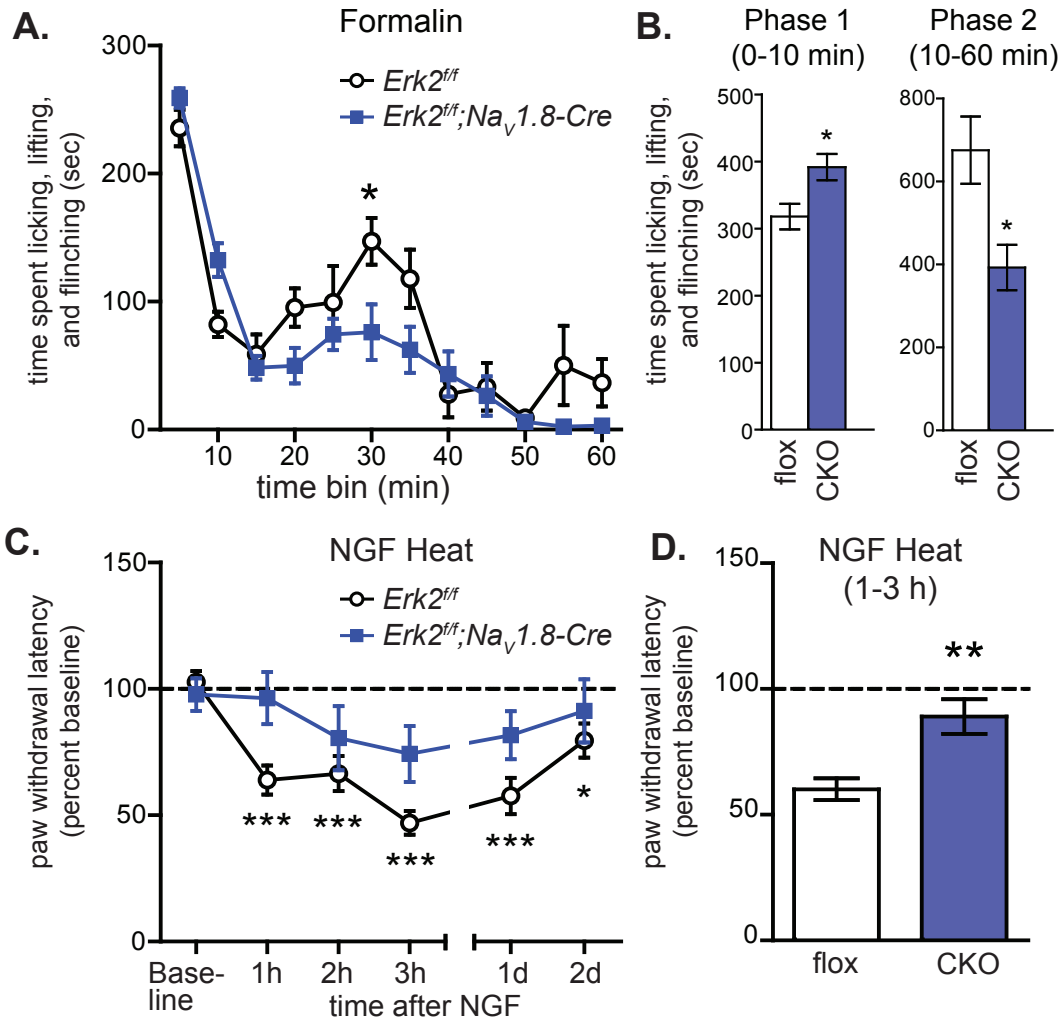

Figure 3: ERK2 is required for peripheral sensitization. A. *Erk2$^{f/f}$;Na$_V$1.8-Cre* (n=8) and *Erk2$^{f/f}$* (n=5) littermates were injected subcutaneously into the hindpaw with 5% formalin. The amount

of time exhibiting stereotypical nociceptive behavioral responses (licking, lifting, and flinching the injected paw) was recorded in five minute bins for an hour following injection. During early timepoints (0-10 min, phase 1), *Erk2$^{f/f}$;Na$_V$1.8-Cre* mice exhibit more spontaneous nociceptive behaviors than controls. Later in the test (10-60 min, phase 2), nociceptive behaviors are reduced in *Erk2$^{f/f}$;Na$_V$1.8-Cre* mice compared to *Erk2$^{f/f}$* littermates (2-way repeated measures ANOVA, * p<0.05 Bonferroni post-test). **B.** Dividing formalin-induced nociceptive behavior into first and second phases revealed a significantly increased response of *Erk2$^{f/f}$;Na$_V$1.8-Cre* mice (CKO) over *Erk2$^{f/f}$* (flox) littermates in the first phase. Paradoxically, *Erk2$^{f/f}$;Na$_V$1.8-Cre* mice had a significantly attenuated response in the second phase compared to controls (* p<0.05, t-test), suggesting that ERK2 is required for peripheral sensitization despite elevated first phase responses. **C.** Following intraplantar injection of the inflammatory mediator NGF (0.2 µg in 10 µl) paw withdrawal latency to radiant heat decreases in *Erk2$^{f/f}$* littermate controls, representing heat hypersensitivity (n=9, 1-way RM ANOVA with Dunnett post-test comparing to *Erk2$^{f/f}$* baseline; * p<0.05, *** p<0.001). *Erk2$^{f/f}$;Na$_V$1.8-Cre* mice do not develop significant hypersensitivity to NGF (n=11, 1-way RM ANOVA with Dunnett post-test comparing to *Erk2$^{f/f}$;Na$_V$1.8-Cre* baseline). There is also a significant difference when comparing the two groups (2-way RM ANOVA main effect of genotype). **D.** Acute NGF-induced heat hypersensitivity was calculated by averaging ipsilateral paw withdrawal latencies from 1 to 3 hours following injection. *Erk2$^{f/f}$;Na$_V$1.8-Cre* (CKO) mice had significantly less NGF-induced heat hypersensitivity than *Erk2$^{f/f}$* (flox) littermates (n=14 both groups; t-test, ** p<0.01).

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attenuated in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice compared to *ERK2$^{f/f}$* littermates. A 2-way repeated measures (RM) ANOVA confirmed this complicated result, indicating a significant interaction between genotype and time after formalin injection (F=2.80, DFn=11, DFd=121, P=0.0028). When dividing the behavioral response into separate phases (Fig. 3*B*), *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice exhibit a statistically significant increase in their initial response to formalin (phase 1) compared with *ERK2$^{f/f}$* littermate controls (t-test) and a statistically significant decrease in the duration of nociceptive behavior in the second phase compared to controls (t-test). The effect of ERK2 conditional deletion on the first phase likely suggests alterations in acute nociception, such as the initial detection of formalin and the afferent barrage that follows. The effect on the second phase suggests that ERK2 deletion impairs peripheral nociceptor sensitization. It is interesting that *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice exhibit reduced second phase behaviors despite elevated first phase behaviors and perhaps suggests multiple independent effects of ERK2 deletion within nociceptors.

To address the role of ERK2 in peripheral sensitization more directly, we turned to a model of heat hypersensitivity following injection of the inflammatory mediator NGF. NGF-induced heat hypersensitivity requires peripheral sensitization, involving the potentiation of heat transduction via modulation of TRPV1 (Huang et al., 2006). To test the requirement of nociceptor ERK2 for NGF-induced heat hypersensitivity, *ERK2$^{f/f}$;Na$_V$1.8-Cre* and *ERK2$^{f/f}$* littermates were injected with NGF, and changes in their responses to radiant heat were tracked over several days (Fig. 3*C*). Interestingly, *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice had significantly elevated paw withdrawal latencies compared with *ERK2$^{f/f}$* littermates (2-way RM ANOVA, F=5.11, DFn=1, DFd=72, P=0.0364), indicating reduced sensitization in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice. Indeed, *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice failed to develop statistically significant hypersensitivity relative to normalized baseline withdrawal thresholds, while control *ERK2$^{f/f}$* littermates did (1-way ANOVA, Dunnett post-test comparing to baseline within each group). In the contralateral paw, no significant difference between genotypes was noted (2-way RM ANOVA; data not shown). To directly evaluate sensitization processes on an intermediate timescale (hours), paw withdrawal latencies from the first three hours following NGF injection were averaged and plotted in Fig. 3*D*. *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice develop dramatically less heat hypersensitivity following NGF injection than *ERK2$^{f/f}$* littermates (t-test). Both analyses indicate that ERK2 is required for NGF-induced heat hypersensitivity over the complete timecourse of hypersensitivity and during the first few hours following injection. Taken with results from the formalin test, these data indicate that nociceptor ERK2 is required for peripheral sensitization.

Nociceptor ERK2 contributes to long-term inflammatory mechanical hypersensitivity.

Previous work has suggested that ERK1/2 sensitizes nociceptors in the CFA model of inflammatory pain (Obata et al., 2003). Given our findings that nociceptor ERK2 contributed to peripheral sensitization in the formalin and NGF models, we hypothesized that conditional deletion of ERK2 in nociceptors would attenuate behavioral sensitization following hindpaw injection of CFA. Control *ERK2$^{f/f}$* mice exhibited a dramatic reduction in paw withdrawal threshold to mechanical stimulation with von Frey filaments (Fig. 4*A*) that persisted for one week after CFA injection. One hour after injection, the paw withdrawal thresholds of *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice were lower than baseline and were similar to *ERK2$^{f/f}$* thresholds. However, at all timepoints afterwards, *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice had higher thresholds than *ERK2$^{f/f}$* littermates, indicating attenuated mechanical hypersensitivity (2-way RM ANOVA; significant main effect of genotype F=9.43, DFn=1, DFd=105, P=0.0078). In the

paw contralateral to CFA injection, we did not observe significant mechanical hypersensitivity, and there was no difference between *ERK2$^{f/f}$;Na$_V$1.8-Cre* and *ERK2$^{f/f}$* littermates (2-way RM ANOVA; data not shown). These results indicate that ERK2 in nociceptors contributes to mechanical hypersensitivity following inflammation.

Since CFA-induced mechanical hypersensitivity and NGF-induced heat hypersensitivity were attenuated in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice, we hypothesized that CFA-induced heat hypersensitivity would be attenuated by conditional ERK2 deletion. Surprisingly, *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice developed robust hypersensitivity to heat following CFA injection that was not different from that observed in control *ERK2$^{f/f}$* littermates (Fig. 4*B*, 2 way RM ANOVA). No significant hypersensitivity was observed in the paw contralateral to CFA injection, and there was no difference between genotypes (2 way RM ANOVA; data not shown). These data indicate that ERK2 is not required for heat hypersensitivity following CFA. Given the discrepancy between CFA and NGF models, we sought to define the role of nociceptor ERK2 in processes underlying heat hypersensitivity using additional, mechanistically distinct behavioral models. Hindpaw injection of formalin and mustard oil both produce robust heat hypersensitivity (Zeitz et al., 2004; Bautista et al., 2006), probably through activation of TRPA1 and subsequent peripheral sensitization (Bautista et al., 2006; McNamara et al., 2007). Interestingly, *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice develop heat hypersensitivity after hindpaw injection of either formalin (Fig. 4*C*) or mustard oil (Fig. 4*D*) that is equivalent to *ERK2$^{f/f}$* littermate controls (t-test). Overall, these data suggest that nociceptor ERK2 is specifically required in NGF-induced heat hypersensitivity but is not required in other models of heat hypersensitivity.

Conditional deletion of ERK2 elevates phosphorylation of ERK1.

Since conditional deletion of ERK2 had no effect in some models of heat hypersensitivity but profound effects in NGF-induced heat hypersensitivity, it seemed possible that ERK1 and ERK2 may have redundant functions and that ERK1 may compensate for the loss of ERK2 in some situations. To address this, we analyzed the expression and activation state (phosphorylation) of ERK1 in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice. As noted above (Fig. 1*B*), ERK1 expression in DRG homogenates is not affected by conditional deletion of ERK2. Using the same homogenates to measure ERK1 phosphorylation (pERK1) by Western blot, we found that *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice had significantly greater pERK1 in DRG than *ERK2$^{f/f}$* control littermates (Fig. 5*A*, t-test). There was no effect of

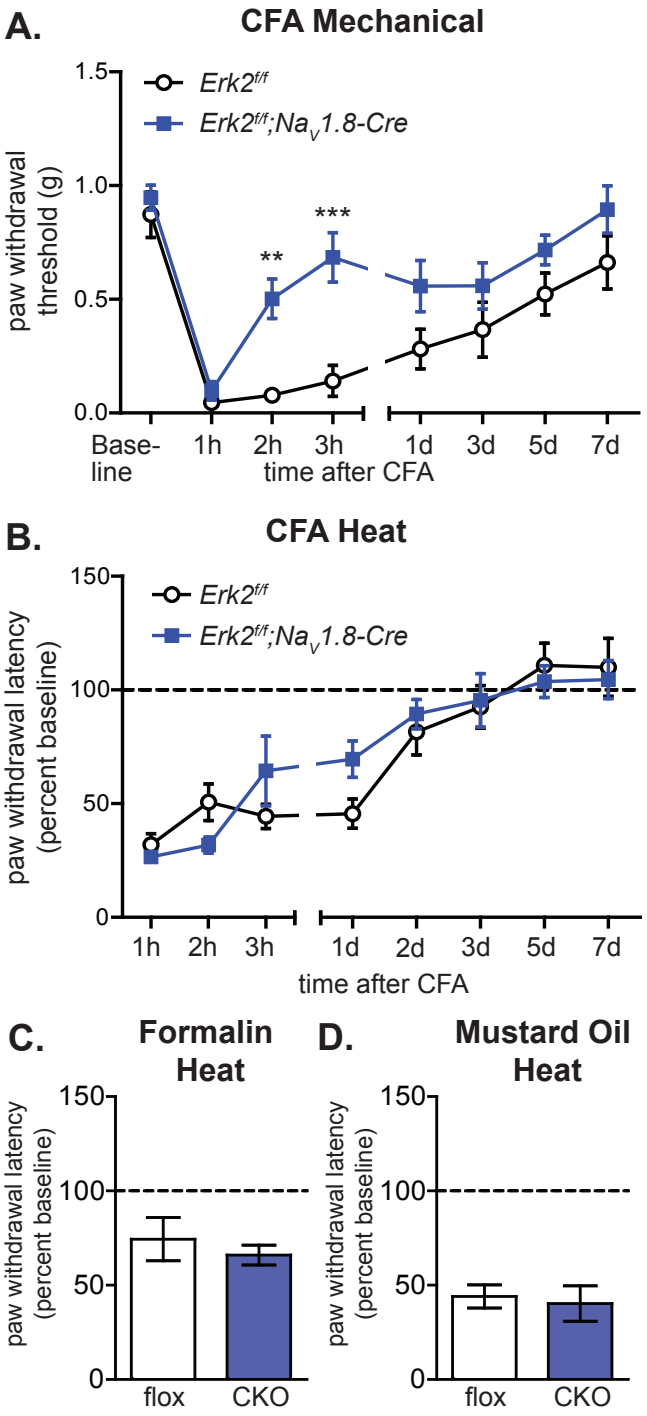


Figure 4: Deletion of ERK2 in nociceptors attenuates long-term inflammatory mechanical hypersensitivity. A. *Erk2$^{f/f}$* (n=8) and *Erk2$^{f/f}$;Na$_V$1.8-Cre* (n=9) littermates were injected with CFA (10 µg in 10 µl) subcutaneously into the plantar surface of the hindpaw, and paw withdrawal thresholds were measured with von Frey filaments. In the injected paw, *Erk2$^{f/f}$;Na$_V$1.8-Cre* mice have significantly less mechanical hypersensitivity than *Erk2$^{f/f}$* littermates (2-way repeated measures ANOVA with Bonferroni post-test; ** p<0.01, *** p<0.001). **B.** *Erk2$^{f/f}$;Na$_V$1.8-Cre* (n=10) and *Erk2$^{f/f}$* (n=9) littermates were injected subcutaneously into the paw with CFA (10 µg in 10 µl), and hypersensitivity to radiant heat was measured immediately following injection (1-3 hours) and then subsequently over several days. No significant difference was detected between *Erk2$^{f/f}$* and *Erk2$^{f/f}$;Na$_V$1.8-Cre* littermates (2-way ANOVA repeated measures). **C.** Formalin was injected subcutaneously into the hindpaw (5% formalin in 10 µl saline) of *Erk2$^{f/f}$* (flox, n=8) and *Erk2$^{f/f}$;Na$_V$1.8-Cre* littermates (CKO, n=10). No significant difference in formalin-induced heat hypersensitivity was detected between groups (t-test). **D.** Heat hypersensitivity evoked by injection of mustard oil (0.75% in 10 µl light mineral oil) is not different between *Erk2$^{f/f}$* (flox, n=5) and *Erk2$^{f/f}$;Na$_V$1.8-Cre* (CKO, n=4) littermates (t-test). **Contributions:** The experiment in A. was performed solely by Ben Alter. The

experiments displayed in B.&C. resulted from a collaboration between Dan O'Brien and Ben Alter. The experiment displayed in D. was a collaboration between Ben Alter and Maiko Satomoto.

ERK2 conditional deletion on pERK1 levels in spinal cord (Fig. 5*B*), which is consistent with our observations that manipulation of ERK2 expression in DRG did not affect ERK2 expression in spinal cord (Fig. 1*C*). It is worthwhile to note that in the quantification histograms ERK2 phosphorylation appears unchanged by conditional ERK2 deletion. This is actually due to the analysis method, in which the pERK2 band is normalized to the ERK2 band. Since total ERK2 expression is reduced in *ERK2$^{f/f}$;Na$_V$1.8-Cre* DRG, the total amount of pERK2 present is also reduced, which can be appreciated in the example image. The lack of an effect on pERK2/ERK2 also indicates that the proportion of phosphorylated ERK2 remaining in the DRG is not affected by the targeted deletion of ERK2 in nociceptors and suggests that ERK2 phosphorylation remains normal in cells that do not express Na$_V$1.8. However, within the Na$_V$1.8-expressing population, it seems likely that ERK1 is hyperphosphorylated and raises the possibility that ERK1 may functionally compensate for the loss of ERK2.

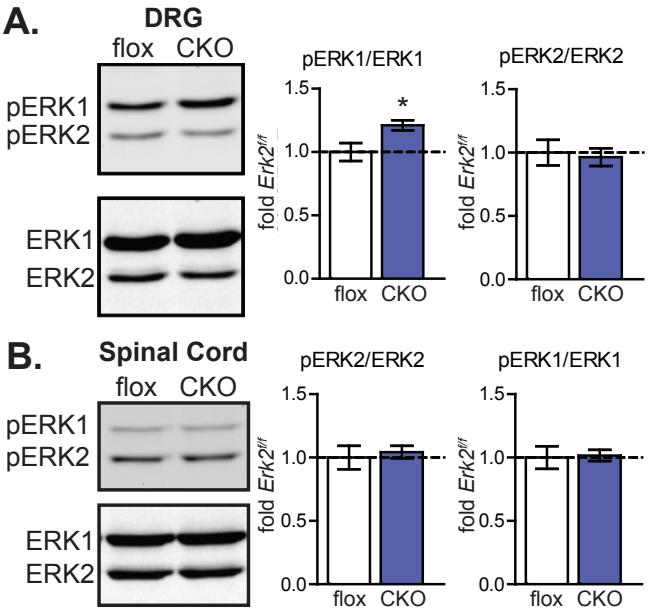


Figure 5: Deletion of ERK2 in small-diameter sensory neurons increases ERK1 phosphorylation. A. Whole cell homogenates from *Erk2$^{f/f}$;Na$_V$1.8-Cre* (CKO) and *Erk2$^{f/f}$* (flox) DRGs (n=7 animals) were analyzed by Western blot for ERK1/2 phosphorylation. The integrated intensities of phospho-specific bands were divided by intensities of total protein and then normalized to *Erk2$^{f/f}$* values. Reduced ERK2 expression in *Erk2$^{f/f}$;Na$_V$1.8-Cre* mice (see figure 1) is correlated with elevated ERK1 phosphorylation compared with *Erk2$^{f/f}$* samples (t-test, * $p<0.05$). No significant change in ERK2 phosphorylation was detected. **B.** No changes in ERK1 or ERK2 phosphorylation were detected in spinal cord homogenates from *Erk2$^{f/f}$* (flox) and *Erk2$^{f/f}$;Na$_V$1.8-Cre* (CKO) littermates (n=7)

ERK1 deletion exacerbates NGF-induced heat hypersensitivity.

If ERK1 could functionally compensate for the loss of ERK2 and if ERK1 and ERK2 were completely redundant, then it seems reasonable that deletion of ERK1 would produce similar effects as deletion of ERK2. Indeed, we previously observed that deletion of ERK1 from all tissues (*ERK1*$^{-/-}$) led to elevated ERK2 phosphorylation (pERK2) in DRG and spinal cord (Alter et al., 2010), which is analogous to the effects seen in *ERK2*$^{f/f}$*;Na*$_V$*1.8-Cre* DRG. However, on the behavioral level, ERK1 deletion had no effect on sensitization to formalin or CFA injection, suggesting that ERK1 cannot compensate for the loss of ERK2 in these models. Given these and our current results, it seems that basal ERK1 hyperphosphorylation is not behaviorally relevant. However, we had not previously tested the role of ERK1 in other models of heat hypersensitivity. To our surprise, ERK1 deletion had the opposite effect of ERK2 conditional deletion on NGF-induced heat hypersensitivity (Fig. 6*A*-*C*). Following intraplantar injection of NGF, *ERK1*$^{-/-}$ mice have significantly shorter paw withdrawal latencies than control WT littermates (Fig. 6*A*, 2-way RM ANOVA significant main effect of genotype F=4.52, DFn=1, DFd=88, P=0.045), indicating that *ERK1*$^{-/-}$ mice have exaggerated heat hypersensitivity. No significant difference between *ERK1*$^{-/-}$ and WT littermates was detected in the paw contralateral to NGF injection (2-way RM ANOVA; data not shown). After averaging the first three hours following NGF-injection (Fig. 6*B*), we observed a significant difference between *ERK1*$^{-/-}$ and WT littermates (t-test). For comparison, results from *ERK2*$^{f/f}$*;Na*$_V$*1.8-Cre* and *ERK2*$^{f/f}$ mice in the same test are presented again in Fig. 6*C*. Although performing statistical tests across these two experiments would be inappropriate given background strain differences, it is interesting to note the similarity between control groups in the magnitude of NGF-induced heat hypersensitivity. We also measured the effect of ERK1 deletion in mustard oil-induced heat hypersensitivity and found no difference between *ERK1*$^{-/-}$ and WT littermates (t-test). This is similar to the effect seen with conditional ERK2 deletion (Fig. 4*D*). Interestingly, from our previous study, we found that *ERK1*$^{-/-}$ mice had diminished formalin-induced heat hypersensitivity (Alter et al., 2010), which is different than the effect of conditional ERK2 deletion (Fig. 4*C*) in which *ERK2*$^{f/f}$*;Na*$_V$*1.8-Cre* and *ERK2*$^{f/f}$ littermates developed a similar degree of heat hypersensitivity. Therefore, in different models of heat hypersensitivity, manipulation of ERK1 or ERK2 produces distinct results. Following injection of NGF, conditional deletion of ERK2 attenuates heat hypersensitivity, while deletion of ERK1 exacerbates heat hypersensitivity. Overall, these results argue against functional redundancy and

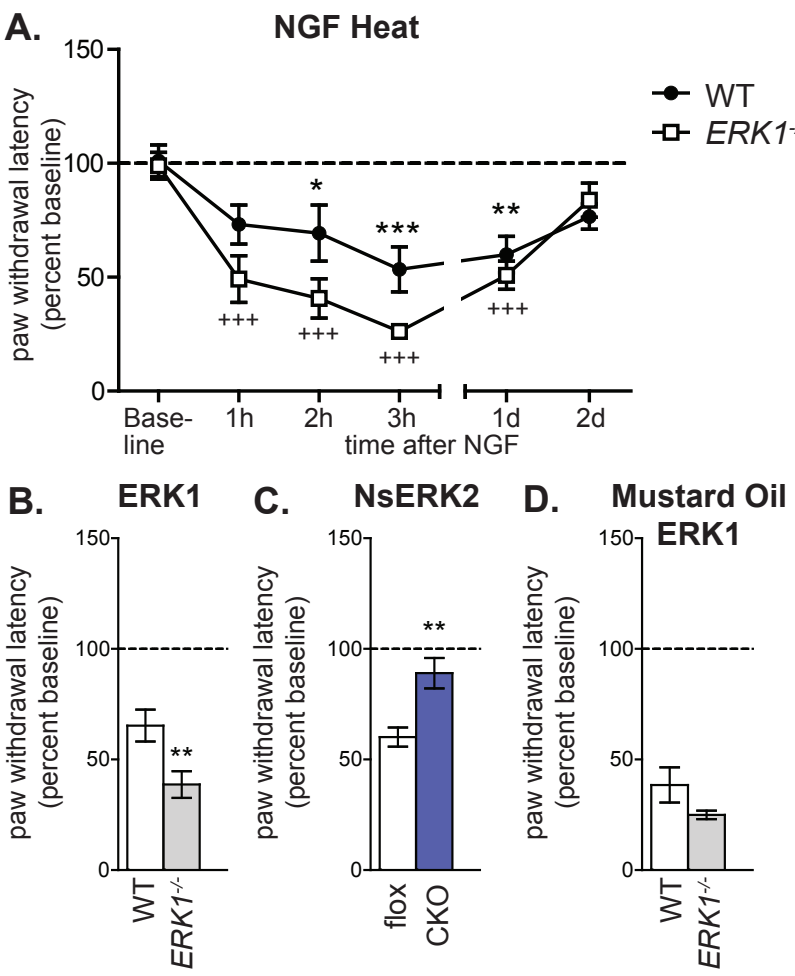
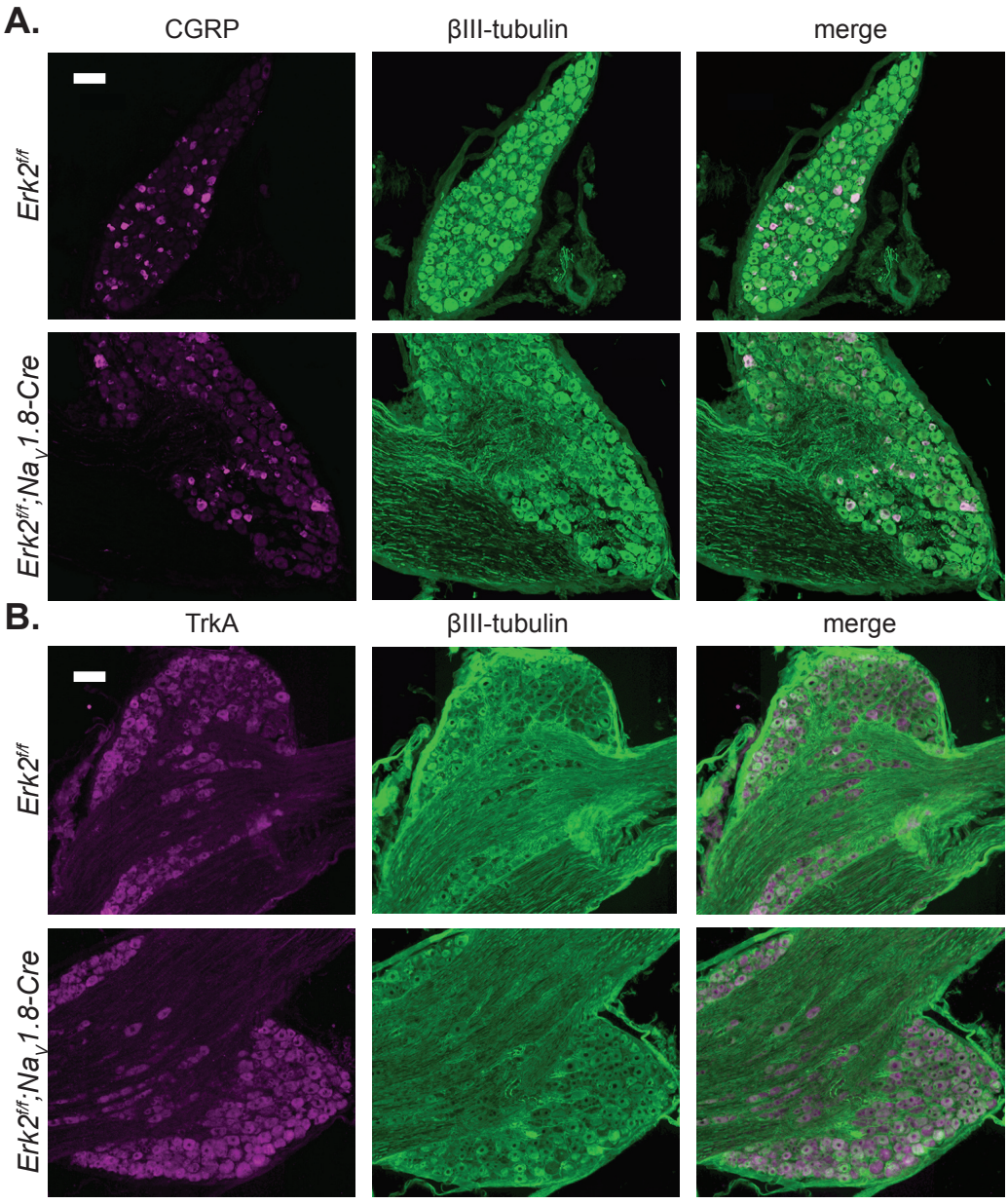


Figure 6: ERK1 deletion has the opposite effect of nociceptor ERK2 deletion on NGF-induced heat hypersensitivity. A. *ERK1*$^{-/-}$ mice (n=12) exhibited significantly exaggerated hypersensitivity due to NGF compared to WT littermate controls (n=12) in the paw injected with NGF (2-way RM ANOVA main effect of genotype). Although both groups develop significant hypersensitivity compared to baseline, paw withdrawal thresholds are significantly lower than baseline in *ERK1*$^{-/-}$ mice at earlier time points than WT littermates (1-way RM ANOVA with Dunnett post-test within each group; stars reflect WT test, pluses reflect *ERK1*$^{-/-}$ test; * p<0.05, ** p<0.01, *** p<0.001, +++ p<0.001). **B.** Acute heat hypersensitivity following NGF injection was calculated by averaging paw withdrawal latencies obtained 1-3 hours following injection. NGF induces significantly more heat hypersensitivity in *ERK1*$^{-/-}$ mice compared with WT littermates (n=12, t-test, ** p<0.01). **C.** Acutely following paw injection (1-3 h), *Erk2*$^{f/f}$*;Na*$_V$*1.8-Cre* mice (CKO) have significantly attenuated hypersensitivity to NGF compared with *Erk2*$^{f/f}$ littermates (flox; n=14 both groups; t-test, ** p<0.01, see Figure 1 for timecourse). **D.** Heat hypersensitivity evoked by injection of mustard oil (0.75% in 10 µl light mineral oil) is not different between *ERK1*$^{-/-}$ and WT littermates (n=5, t-test). **Contributions:** The experiments displayed in A.-C. were performed by Ben Alter. The experiment shown in D. resulted from a collaboration between Ben Alter and Dan O'Brien.


A.
CGRP
βIII-tubulin
merge
Erk2f/f
Erk2f/f;Nav1.8-Cre
B.
TrkA
βIII-tubulin
merge
Erk2f/f
Erk2f/f;Nav1.8-Cre

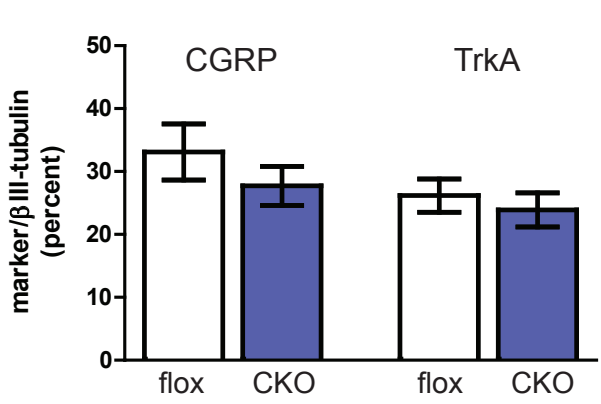

C.
CGRP
TrkA
marker/βIII-tubulin (percent)
50
40
30
20
10
0
flox
CKO
flox
CKO

Figure 7: Survival of peptidergic sensory neurons is unaffected by ERK2 deletion. A. Representative images indicate the presence of CGRP-positive cells within dorsal root ganglia of *Erk2$^{f/f}$;Na$_V$1.8-Cre* and *Erk2$^{f/f}$* littermates. **B.** TrkA immunoreactiivity is similar between *Erk2$^{f/f}$* and *Erk2$^{f/f}$;Na$_V$1.8-Cre* dorsal root ganglia. **C.** CGRP- and TrkA-positive cell profiles were counted from L4 dorsal root ganglia (n=5-6 animals). Counts were divided by number of cells immunoreactive for βIII-tubulin, a general neuronal marker. No significant difference was detected between *Erk2$^{f/f}$* and *Erk2$^{f/f}$;Na$_V$1.8-Cre* dorsal root ganglia (t-test), indicating that deletion of ERK2 does not affect DRG neuronal survival. Scale bar = 100 μm. **Contributions:** The experiments in this figure culminated from a collaboration between Maiko Satomoto and Ben Alter.

prompted us to further investigate the effects of ERK isoform deletion on NGF-induced heat hypersensitivity.

Peptidergic nociceptors are present and express TrkA normally despite conditional ERK2 deletion.

Nociceptors can be divided into subgroups based on the expression of neuropeptides, growth factor receptors, and sensory transduction channels (Hucho and Levine, 2007). Peptidergic nociceptors express calcitonin gene related peptide (CGRP) and the growth factor receptor TrkA. *In vivo*, TRPV1 expression is highly enriched within peptidergic neurons. Moreover, a growing body of data supports a model of inflammatory heat hypersensitivity involving NGF binding to TrkA with subsequent potentiation of TRPV1 function, although the precise intracellular signaling events downstream of TrkA remain controversial (Huang et al., 2006). Given the importance of peptidergic neurons to inflammatory heat hypersensitivity, it was important to determine whether these neurons were present and expressing the TrkA receptor. Therefore, L4 DRG from *ERK2$^{f/f}$;Na$_V$1.8-Cre* and *ERK2$^{f/f}$* littermates were stained for the peptidergic marker, CGRP, and for TrkA (Fig. 7). CGRP-positive cells were observed in *ERK2$^{f/f}$;Na$_V$1.8-Cre* DRG and there was no difference in the percentage of CGRP-positive neurons compared to *ERK2$^{f/f}$* littermates (Fig. 7*A,* 7*C*, t-test). This indicates that the proportion of peptidergic neurons is not altered by conditional deletion of ERK2. We also observed TrkA-positive cell profiles in the DRG of *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice (Fig. 7*B*). The proportion of TrkA-positive cells relative to the total number of neurons was also no different between genotypes (Fig. 7*C*, t-test). These results indicate that peptidergic cells are present and express the receptor for NGF in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice, suggesting that behavioral phenotypes are not due to a loss of peptidergic nociceptor

ERK2 and not ERK1 is required for peptidergic epidermal innervation.

Previous work has implicated ERK1/2 signaling in axon outgrowth *in vitro* (Markus et al., 2002a). *In vivo* knockout studies have shown that upstream molecules in the MAPK cascade and potential downstream targets for ERK1/2 are important in epidermal innervation, e.g. Raf isoforms and the transcription factor SRF (Zhong et al., 2007; Wickramasinghe et al., 2008). Given a reduction in behavioral sensitization in several pain models and a blunted response to NGF, it seemed possible that peripheral innervation required ERK2 signaling. To test this hypothesis, footpad skin from *ERK2$^{f/f}$;Na$_V$1.8-Cre* and *ERK2$^{f/f}$* littermates was stained for βIII-tubulin, which specifically labels axons. In *ERK2$^{f/f}$* thick skin (Fig. 8*A*), fine nerve endings could be observed extending from dermis into the epidermis. The vast majority of these free nerve endings are nociceptive, since they do not stain positive for tyrosine hydroxylase and are therefore not sympathetic (Rice et al., 1998). Fibers could also be seen in *ERK2$^{f/f}$;Na$_V$1.8-Cre* thick skin (Fig. 8*B*), without any gross structural differences. To quantify the density of epidermal innervation, random fields of thick skin were examined, the length of the dermal-epidermal border was measured, and the number of fibers crossing this border was counted (Fig. 8*C*). Interestingly, we observed a small but significant decrease in the number of βIII-tubulin-positive fibers in *ERK2$^{f/f}$;Na$_V$1.8-Cre* thick skin as compared with thick skin from *ERK2$^{f/f}$* littermates (t-test). This suggests that ERK2 partially contributes to nociceptor epidermal innervation. To determine whether a subset of fibers is affected, adjacent sections of skin were stained for the peptidergic marker, CGRP (Fig. 8*A-B*). Although CGRP-positive fibers were present in thick skin from *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice, a significant reduction in the density of fibers compared with control *ERK2$^{f/f}$* skin was observed (Fig 8*C*). Together, these results suggest that the decrease in innervation is restricted to peptidergic fibers, since the absolute number of fibers lost per unit length of dermal-epidermal border is the same for both CGRP- and βIII-tubulin-positive fibers.

Given the loss of peptidergic fibers following conditional deletion of ERK2, we sought to characterize epidermal innervation in *ERK1$^{-/-}$* mice. Footpads from *ERK1$^{-/-}$* and WT littermates were processed and stained for CGRP and βIII-tubulin (Fig. 9). Despite the loss of ERK1 in all tissues, fibers positive for both CGRP and βIII-tubulin were present in *ERK1$^{-/-}$* skin and were without obvious structural abnormalities. Quantification of the fiber density revealed that there was no difference between *ERK1$^{-/-}$* and WT skin in either total (Fig. 9*C*) or peptidergic (Fig. 9*F*) innervation, indicating that ERK1 is not required for innervation of the epidermis. Overall, these results indicate that ERK2

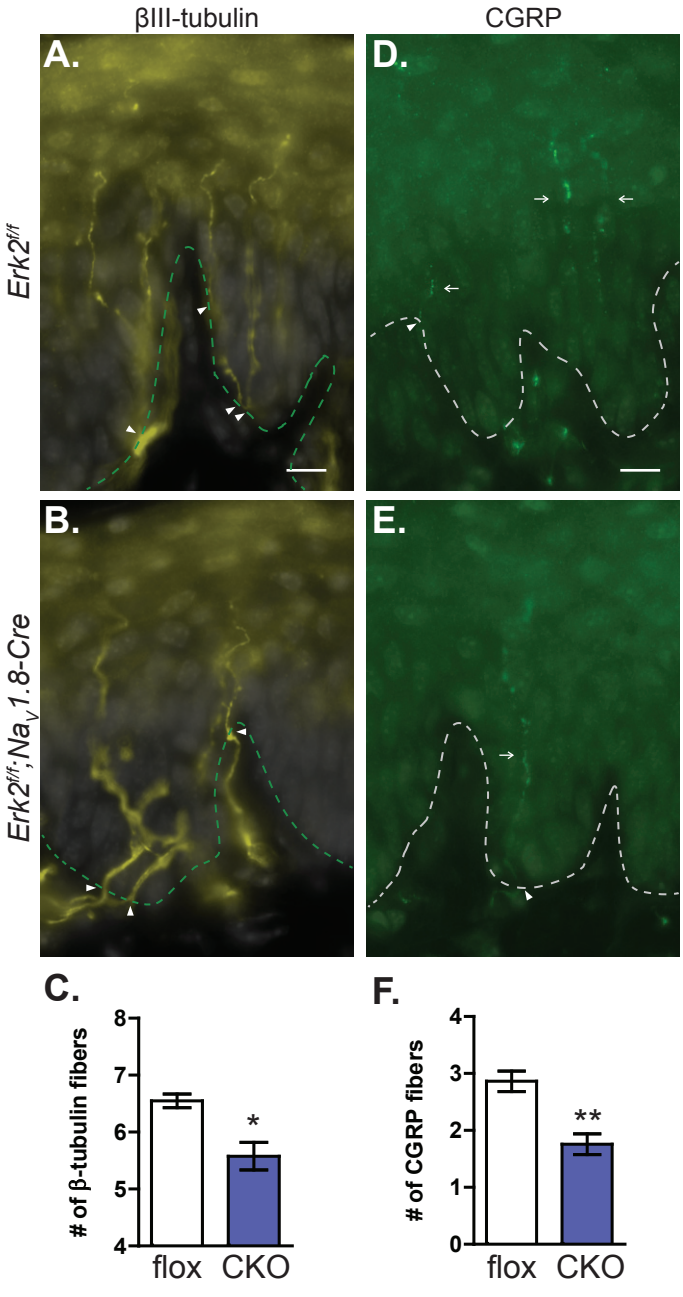


Figure 8: ERK2 is necessary for peptidergic innervation of the epidermis. A.-B. βIII-tubulin immunostaining (yellow) of glaborous skin from the hindlimb footpads of *Erk2$^{f/f}$;Na$_V$1.8-Cre* and *Erk2$^{f/f}$* littermates. Bis-benzimide staining is shown in gray. A green dashed line demarcates the dermal-epidermal border. Scale bar = 10 μm. **C.** βIII-tubulin-positive free nerve endings that crossed the dermal-epidermal border were counted (white arrowheads in A.&B.). In the same field, the dermal-epidermal border was traced and its length measured. The number of βIII-tubulin-positive fibers was then expressed as the number of fibers per 100 μm length of dermal-epidermal border. Significantly fewer βIII-tubulin-positive fibers are evident in *Erk2$^{f/f}$;Na$_V$1.8-Cre* mice as compared with *Erk2$^{f/f}$* littermates (n=4; * p<0.05, t-test). **D.-E.** CGRP immunostaining (green) of neighboring sections from A.-B. Bis-benzimide staining is shown in gray. Fibers that cross the dermal-epideraml border at this focal plane are noted with an arrowhead. Arrows mark fibers that extend into the epidermis but may not cross the dermal-epidermal border in this image. Scale bar = 10 μm. **F.** CGRP-positive free nerve endings from *Erk2$^{f/f}$* and *Erk2$^{f/f}$;Na$_V$1.8-Cre* skin were counted as in C. *Erk2$^{f/f}$;Na$_V$1.8-Cre* mice have significantly fewer CGRP fibers (n=4; ** p<0.01, t-test). The number of CGRP-postive free nerve endings lost

is similar to the number of βIII-tubulin-positive fibers lost, consistent with a reduction in peptidergic innervation and not simply a loss of CGRP expression.

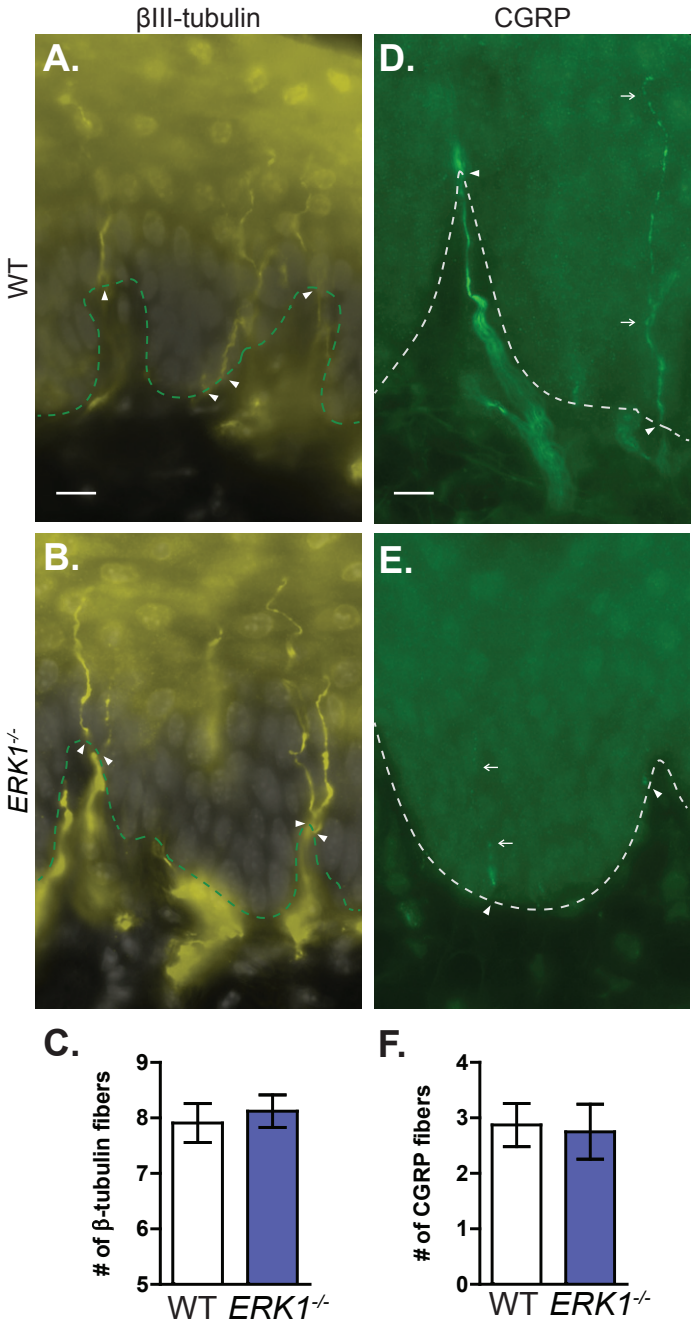


Figure 9: ERK1 is dispensible for peptidergic innervation of the epidermis. A.-B. βIII-tubulin immunostaining (yellow) of glaborous skin from the hindlimb footpads of *ERK1*$^{-/-}$ and WT littermates. Arrowheads indicate fibers crossing the dermal-epidermal border (outlined by a green dashed line). Overlayed in gray is bis-benzimide staining. Scale bar = 10 μm. **C.** No significant difference was detected between WT and *ERK1*$^{-/-}$ mice in the number of βIII-tubulin-positive fibers traversing the dermal-epidermal border (t-test, n=7 mice per genotype). **D.-E.** CGRP immunostaining (green) of neighboring sections from A.-B. Bis-benzimide staining is shown in gray. A dashed line indicates the dermal-epidermal border, and arrowheads highlight fibers crossing the border. Scale bar = 10 μm. **F.** WT and *ERK1*$^{-/-}$ have similar numbers of CGRP-positive fibers traversing the dermal-epidermal border (t-test, n=7 animals per genotype).

is partially required for peptidergic innervation of the epidermis. Importantly, ERK1 deletion has no effect on innervation density, indicating that ERK2 plays an isoform specific role in epidermal innervation.

Discussion

Accumulating evidence suggests that ERK1 and ERK2 play distinct roles. The present study is the first to address the role of ERK2 in nociceptors. Conditional deletion of ERK2 in nociceptors attenuated behavioral sensitization in several models, including the formalin test, NGF-induced heat hypersensitivity, and CFA-induced mechanical hypersensitivity. Attenuated behavioral sensitization was accompanied by a partial loss of peptidergic innervation of the epidermis, which likely contributes to the behavioral phenotypes. Additionally, we observed a significant increase in ERK1 phosphorylation due to the conditional deletion of ERK2. However, global ERK1 deletion had no effect on peripheral innervation and actually exacerbated NGF-induced heat hypersensitivity. Our previous study of ERK1 deletion indicated that ERK1 was not required for formalin-induced spontaneous behavior or CFA-induced mechanical hypersensitivity (Alter et al., 2010). Taken together, these results suggest that ERK2 and not ERK1 is required for epidermal innervation and peripheral sensitization.

Although we set out to test the role of ERK2 in acute sensitization of the nociceptor, it became clear that ERK2 was required for a more fundamental process of epidermal innervation. Previous studies have implicated ERK1/2 signaling in axon outgrowth *in vitro* using either MEK inhibitors or a constitutively active MEK1-ERK2 fusion protein (Robinson et al., 1998; Markus et al., 2002a; Markus et al., 2002b; Wickramasinghe et al., 2008). Conditional deletion of upstream MAP kinases Raf-1 and B-Raf using a neuronal *nestin-Cre* line reduced axonal branching in embryos (Zhong et al., 2007). However, direct *in vivo* evaluation of the importance of ERK2 in axon outgrowth of sensory neurons has been difficult since germline deletion of ERK2 is lethal early in development (Krens et al., 2006) and conditional deletion of ERK2 with *Wnt1-Cre* leads to late embryonic lethality (Newbern et al., 2008). To our knowledge, the present study provides the first *in vivo* evidence that ERK2 is required for epidermal innervation. Moreover, ERK1 is dispensible for epidermal innervation, suggesting that signaling pathways underlying axonal outgrowth rely exclusively upon ERK2. It is possible that ERK2 affects other functions associated with trophic support, such

as cell survival. However, the percentage of peptidergic neurons is unaltered in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice, suggesting that peptidergic neurons are not lost. It is possible that a total reduction in all DRG neurons might yield a similar percentage of CGRP neurons when in fact there is some neuronal loss. This is unlikely since the change in DRG CGRP and βIII-tubulin counts must be proportionately equivalent and, if so, one would expect a more significant reduction in βIII-tubulin fiber counts in the skin than we observed. Although our data suggest the requirement of ERK2 for peptidergic epidermal innervation we do not directly address the timeframe of this requirement. Since Cre activity begins at P0 in the BAC transgenic *Na$_V$1.8-Cre* line (Agarwal et al., 2004) and at that time epidermal innervation of the footpad has occured (Coggeshall et al., 1994; Jackman and Fitzgerald, 2000; Baccei and Fitzgerald, 2010), it is likely that peptidergic fibers are initially present in developing *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice but then die back. This would suggest that ERK2 is required for peptidergic fiber maintanence, which is certainly possible given that continued trophic support is required to maintain epidermal innervation in adult animals (Bennett et al., 1998). Overall, it seems likely that the loss of peptidergic fibers in *ERK2$^{f/f}$;Na$_V$1.8-Cre* skin is not due to cell loss but is instead due to failed maintanence of nociceptor axons, although a more detailed analysis over perinatal development may be warranted.

The partial loss of peptidergic innervation in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice may underlie some of the behavioral phenotypes we observed. Ablation of all Na$_V$1.8-expressing cells results in a dramatic reduction in the 2nd phase of the formalin test and significantly attenuates CFA-induced mechanical hypersensitivity (Abrahamsen et al., 2008), both of which are phenocopied with conditional deletion of ERK2 in the same cells. Additionally, ablation of TRPV1-expressing terminals with neurotoxic capsaicin treatments diminishes formalin-induced spontaneous nociceptive behaviors (Peterson et al., 1997; Chen et al., 2007), suggesting that the presence of peptidergic fibers is required for formalin-induced behavioral responses. Peptidergic fibers also express the receptor for NGF, TrkA. Loss of these fibers and the resulting loss of TrkA may explain the attenuated NGF-induced heat hypersensitivity we observed in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice. Peptidergic fiber loss may also account for alterations in thermotaxis behavior, in which conditional deletion of ERK2 increased presence time in 30-32°C zones and decreased presence time in 34-36°C zones relative to controls. Several TRP channels, which are activated in this innocuous temperature range, contribute to thermotaxis behaviors (Dhaka et al., 2006), including TRPM8, TRPV3, and TRPV4. Although the temperature

sensitivity for TRPV4 fits best with the temperature range affected in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice, deletion of TRPV4 actually shifts presence time towards warmer temperatures (Lee et al., 2005). Therefore, it seems more likely that conditional deletion of ERK2 in nociceptors affects TRPM8 or TRPV3 function, since deletion of either channel shifts thermotaxis towards cooler temperatures (Moqrich et al., 2005; Dhaka et al., 2007). Although controversial (Dhaka et al., 2008), TRPM8 expression has been reported in peptidergic neurons (Okazawa et al., 2004; Kobayashi et al., 2005; Takashima et al., 2007). Therefore, loss of peptidergic fibers may result in the absence of TRPM8 expression in the skin, compromising temperature sensation in the innocuous range. It is also possible that TRPV3 signaling is altered by loss of peptidergic fibers. Although TRPV3 is expressed in keratinocytes and not sensory neurons (Dhaka et al., 2006), loss of peptidergic fibers may prevent signaling downstream of TRPV3 and alter thermotaxis. Overall, our data indicate that the presence of ERK2-dependent peptidergic fibers seems to be critical in the formalin test, in NGF-induced heat hypersensitivity, and in thermotaxis.

Our analysis of epidermal innervation indicates that nonpeptidergic fiber density remains normal in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice, since the change in density of all fibers matches the change in CGRP-positive fibers. Given the continued presence of peptidergic fibers, it seems likely that ERK2 plays an important role in sensitizing this subpopulation of nociceptors. The deficit we observed in CFA-induced mechanical hypersensitivity supports this hypothesis. A recent study has found that nonpeptidergic, MrgD-expressing cells are required for CFA-induced mechanical hypersensitivity (Cavanaugh et al., 2009). Interestingly, ablation of these cells has no effect on CFA-induced heat hypersensitivity. Conditional deletion of ERK2 produces similar results. Loss of peptidergic neurons using neurotoxic capsaicin or resiniferotoxin treatments produces the converse effect -- inflammatory heat hypersensitivity is abolished while there is no effect on mechanical hypersensitivity (Chen et al., 2007; Cavanaugh et al., 2009; Mishra and Hoon, 2010). This suggests that the loss of peptidergic fibers in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice cannot explain the behavioral phenotype we observed in the CFA model. Taken together, these data support a model in which different subsets of nociceptors utilize ERK2 in different contexts. Nonpeptidergic neurons do not require ERK2 for epidermal innervation but do require ERK2 for inflammatory mechanical sensitization. Some peptidergic fibers clearly require ERK2 for epidermal innervation. Other peptidergic fibers do not require ERK2 for epidermal innervation and also do not require ERK2 in peripheral sensitization. The existence of this third

group is supported by our observations that, despite the loss of ERK2, CFA, formalin, and mustard oil can all induce robust heat hypersensitivity in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice. It is interesting to note that formalin-induced heat hypersensitivity is attenuated in mice lacking ERK1 (Alter et al., 2010). This fits well with the above model and implies that functional differences observed between ERK1 and ERK2 may be due to cell type specific expression of each isoform. Immunohistochemical experiments with careful controls for antibody specificity will be required to test this hypothesis. It is also possible that ERK1 and ERK2 are expressed in the same cell, but functional coupling or signaling microdomains restrict the involvement of one isoform or another.

The effect of conditional ERK2 deletion on peptidergic innervation raises interesting questions about the maintanence of innervation within the unaffected subgroups of nociceptors. Nonpeptidergic neurons lose their requirement for NGF and upregulate the trophic factor receptor Ret around birth and during postnatal development (Molliver et al., 1997). Previous studies have implicated Ret signaling in nonpeptidergic epidermal innervation, since conditional deletion of Ret with *Wnt1-Cre* reduced MrgD-postive fiber density in the epidermis (Luo et al., 2007) and deletion of the Ret co-receptor GDNF family receptor α 2 (GFRα2) decreased overall fiber density without affecting peptidergic innervation (Lindfors et al., 2006). Since deletion of either ERK isoform had no effect on nonpeptidergic epidermal innervation, it seems likely that Ret signaling does not utilize the MAPK pathway *in vivo*, although this should be directly tested. However, it is also possible that ERK1 and ERK2 may be redundant downstream of Ret, which could be evaluated in double knockout animals. In addition to nonpeptidergic fibers, many peptidergic fibers remained despite the loss of ERK2. This differential effect of ERK2 deletion on peptidergic fibers could be explained if different intracellular signaling cascades were required downstream of NGF-TrkA signaling. Another possibility would be that these fibers are maintained by other trophic factors. GFRα3 receptors have been observed in peptidergic fibers expressing TRPV1 (Malin et al., 2006), which might explain the survival of a subset of peptidergic fibers that could contribute to the preserved inflammatory heat hyperalgesia observed in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice.

The behavioral response to formalin includes spontaneous nociceptive behaviors that manifest in distinct early and late phases (Tjolsen et al., 1992). Conditional deletion of ERK2 in nociceptors paradoxically increases the first phase and decreases the second phase. As mentioned above, the decrease in the second phase may be attributed to reduced peptidergic terminals. However,

it is unclear how ERK2 deletion might increase the first phase response. Both phases require the expression of TRPA1, and *in vitro* data suggests that formalin chemically modifies and activates TRPA1 (McNamara et al., 2007). It is possible that the peptidergic fibers that remain in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice express TRPA1. In the conditional knockout, these fibers may have relatively greater afferent input than in normal mice, and therefore initial transduction of the noxious stimulus is actually elevated. However, since peptidergic fibers are diminished, nociceptor sensitization due to neurogenic inflammation is also diminished, resulting in a reduced second phase. At odds with this hypothesis is our observation that mustard oil-induced heat hypersensitivity is not affected by loss of ERK2. However, it is possible that the competing processes of elevated acute sensory transduction and diminished peptidergic sensitization preclude the observation of a phenotype following mustard oil injection.

One plausible mechanism for reduced NGF-induced heat hypersensitivity in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice is the partial loss of TrkA-expressing fibers. However, exaggerated NGF-induced heat hypersensitivity observed in *ERK1$^{-/-}$* mice cannot be explained by changes in innervation, since we observed no differences in CGRP- or βIII-tubulin-positive fiber density. Taken together, it is possible that the behavioral effects of ERK2 deletion may be due to acute functional changes, as well as structural differences. Assuming that behavioral phenotypes relate to acute sensitization processes, it is tempting to speculate about the apparently opposing functions of ERK1 and ERK2. It is possible that ERK1 competes with ERK2 for MEK activity and thereby partially inhibits NGF-induced sensitization, tuning the behavioral response to NGF. This hypothesis is supported by the observation that deletion of ERK1 increases the phosphorylation status of ERK2 in DRG (Alter et al., 2010). Similar gain-of-function effects of ERK1 deletion have been observed in cultured fibroblasts and cell lines (Vantaggiato et al., 2006) as well as in a morphine conditioned place preference paradigm (Mazzucchelli et al., 2002) and, in both cases, have been associated with hyperactivity of ERK2. Further experimentation investigating the activity of ERK1 and ERK2 downstream of NGF should elucidate these questions. Other explanations for the exaggerated response of *ERK1$^{-/-}$* mice to NGF could include the possibility that ERK1 acts through independent anti-nociceptive pathways or that global deletion of ERK1 affects other systems outside the nociceptor which enhance NGF-induced hypersensitivity indirectly. Inducible tissue-specific manipulation of ERK1 and ERK2 expression would help to address these provocative questions.

The current study indentifies an isoform-specific role for ERK2 in epidermal innervation within a subset of peptidergic fibers. Moreover, the loss of nociceptor ERK2 attenuates peripheral sensitization in the formalin test and in inflammatory mechanical hypersensitivity. Given the results of this study, it is possible that therapeutic inhibition of ERK2 may actually result in a peripheral neuropathy, although the importance of ERK2 in myelinated sensory axons remains to be determined. Further characterization of the role of ERK2 in pain will be important in understanding the pathophysiological mechanisms in chronic pain conditions and developing new therapeutic options to treat them.

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Chapter 4: Conclusions and Future Directions

A large body of evidence implicates ERK1/2 in nociception and nociceptive sensitization. Since ERK1 and ERK2 play distinct functional roles in other systems, I hypothesized that the ERK1/2 isoforms differentially contribute to sensitization of the nociceptive system. To test directly the requirement for ERK1 in all tissues, *ERK1*$^{-/-}$ mice were compared with WT littermates in rodent behavioral models of pain. Despite the activation of ERK1 following noxious stimulation, it appeared that ERK1 was dispensable for formalin-induced spontaneous nociceptive behaviors, inflammation-induced mechanical and heat hypersensitivity, and nerve injury-induced mechanical hypersensitivity. In contrast, conditional deletion of ERK2 in nociceptors (*ERK2*$^{f/f}$*;Na*$_V$*1.8-Cre*) attenuated formalin-induced spontaneous nociceptive behaviors and reduced inflammation-induced mechanical hypersensitivity. This suggests that ERK2 is required for behavioral sensitization in these models and that ERK1 cannot compensate for the loss of ERK2. Interestingly, injection of the inflammatory mediator NGF had the opposite effects in *ERK2*$^{f/f}$*;Na*$_V$*1.8-Cre* and *ERK1*$^{-/-}$ mice. NGF-induced heat hypersensitivity was diminished by conditional deletion of ERK2, while sensitization was actually exaggerated by the loss of ERK1. To better understand potential mechanisms underlying these behavioral effects, nociceptor microanatomy was evaluated by immunohistochemistry. Conditional deletion of ERK2 partially reduces nociceptor innervation of the skin. The partial loss is restricted to peptidergic nociceptors and is not associated with loss of peptidergic neurons. Deletion of ERK1 has no effect on nociceptor innervation.

Altogether, these results identify ERK2 specific roles on the behavioral level in several models of nociception and nociceptive sensitization. Additionally, characterization of mice lacking ERK2 in nociceptors revealed a role for ERK2 in epidermal innervation, providing *in vivo* evidence that ERK signaling contributes to axon growth and/or maintenance. These behavioral and structural functions appear to be specific to ERK2 since ERK1 is not able to compensate for the loss of ERK2. During this study, another group reported that partial knockdown of ERK2 in the spinal cord significantly attenuated inflammation-induced heat and mechanical hypersensitivity (Xu et al., 2008). Results from the current study support and extend these findings, indicating that ERK2 is the predominant isoform in nociceptors and spinal cord.

ERK2 is necessary for epidermal innervation in a subset of peptidergic fibers

Although the initial purpose for conditionally deleting ERK2 in nociceptors was to test the importance of ERK2 in peripheral sensitization, characterization of *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice revealed a partial loss of peptidergic fibers in the epidermis (see Chapter 3). Previous studies have implicated ERK1/2 signaling in axon growth assays *in vitro* (Markus et al., 2002). Although there have not yet been reports directly assessing the role of ERK1/2 *in vivo*, molecules upstream and downstream have been implicated. For example, the upstream kinases Raf-1 and B-Raf are required for cutaneous arborization (Zhong et al., 2007). A downstream transcription factor, SRF, is required for footpad epidermal innervation in Na$_V$1.8-expressing neurons (Wickramasinghe et al., 2008). The current study is consistent with the previous studies implicating Raf-MEK-ERK-SRF signaling in promoting epidermal innervation and provides *in vivo* evidence that ERK2 is specifically required.

Diminished peptidergic innervation may be due to a deficit in the initial elaboration of fibers into the epidermis, an exaggerated loss of fibers during axon pruning, or a deficit in the continued maintenance of innervation in mature skin. When considering these possibilities, it is helpful to compare the timeline of nociceptor development with the predicted onset of ERK2 deletion. By crossing the transgenic *Na$_V$1.8-Cre* line with a ROSA26 reporter line (Soriano, 1999), Cre-mediated excision at the ROSA26 locus was initially detected at P0 without any excision detected at E17 (Agarwal et al., 2004). Sensory neurons which develop into nociceptors are born in several waves of neurogenesis between E9 and E11 (Marmigere and Ernfors, 2007) well before the anticipated onset of Cre expression. Importantly, fiber loss due to the deletion of ERK2 was not associated with cell loss suggesting that the process of neuronal birth and naturally occurring cell death are unaffected in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice. Axon growth towards target tissues proceeds shortly thereafter in a proximodistal manner so that innervation of the footpad is well established before birth (Coggeshall et al., 1994; Jackman and Fitzgerald, 2000; Baccei and Fitzgerald, 2005). Since epidermal innervation occurs before birth, it is likely that peptidergic fibers are present in the epidermis of *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice before the onset of Cre expression. During this late embryonic period, the epidermis of the hindpaw is initially hyperinnervated with nerve endings reaching through the developing epidermis to the surface of the skin (Jackman and Fitzgerald, 2000). However, by E20 fibers have retracted towards the dermis and form a more mature configuration with a subepidermal plexus of nerve fibers from which fine nerve endings extend

into the epidermis. Although it is possible that ERK2 deletion affects this process of retraction, it is unlikely since there is probably a delay between Cre-mediated excision of ERK2 coding regions and a decrease in ERK2 protein levels due to the continued presence of previously transcribed ERK2. Additionally, upon gross examination, the subepidermal plexus found in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice appeared similar to that of control littermates. There is also evidence that trophic support in mature animals is required to maintain epidermal innervation, since infusion of NGF-sequestering antibodies into the skin results in ~40% reduction in innervation density (Bennett et al., 1998). From the current study, it is difficult to determine whether the loss of innervation results from a defect in initial fiber growth, pruning, or maintenance since all of these processes occur at time points near the expected onset of Cre expression. Analysis of epidermal innervation over the perinatal development will help to address this question.

Although the specific cellular mechanism underlying the fiber loss is not yet identified, it is reasonable to hypothesize it is related to a defect in NGF-TrkA signaling. NGF and TrkA are required for epidermal innervation during embryonic development (Patel et al., 2000). In mature animals, exogenous application of NGF into the skin induces ERK1/2 activation in the DRG (Averill et al., 2001). Additionally, the fibers that are lost in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice are peptidergic, CGRP-positive fibers. Since CGRP expression almost completely overlaps with TrkA expression in DRG (Averill et al., 1995), the lost fibers are likely to express TrkA. As mentioned above, interfering with NGF-TrkA signaling decreases epidermal innervation (Bennett et al., 1998). The magnitude of this decrease approximates the relative amount peptidergic fibers in the skin (Zylka et al., 2005). All of this data suggests that reduced peptidergic epidermal innervation is likely to due to impaired NGF-TrkA signaling. Results from the current study strongly suggest that ERK2 is downstream of TrkA activation and is important for peptidergic innervation. One way to test this hypothesis would be to determine whether ERK2 activation by NGF could increase epidermal innervation. Indeed, NGF overexpression in epidermal keratinocytes or elevations in NGF due to skin wounding result in hyperinnervation of the epidermis (Albers et al., 1994; Constantinou et al., 1994; Reynolds and Fitzgerald, 1995). Crossing *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice to the NGF overexpressing transgenic line or manipulating NGF content in the skin of *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice by injury or exogenous application would determine whether ERK2 was required for NGF-induced increases in epidermal innervation. The initial observation that ERK2 is partially required for peptidergic epidermal innervation raises

many interesting developmental questions which will be the topic of subsequent investigation.

The loss of one-third of peptidergic fibers in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice but the presence of other peptidergic fibers and all nonpeptidergic fibers suggests the existence of three distinct populations of nociceptors. One set of peptidergic fibers requires ERK2 for epidermal innervation. The remaining peptidergic fibers and nonpeptidergic fibers do not require ERK2 for epidermal innervation. It is likely that in the ERK2-dependent peptidergic fibers that ERK2 signals downstream of the TrkA receptor (see above). In ERK2-independent peptidergic fibers, it is also likely that NGF-TrkA signaling promotes their continued innervation of the epidermis. However, these fibers are not affected by the deletion of ERK2, which could be due to at least two reasons. The first is that ERK1 and ERK2 are redundant in this subset of neurons and that ERK1 is able to compensate for the loss of ERK2. This may explain the hyperphosphorylation of ERK1 in *ERK2$^{f/f}$;Na$_V$1.8-Cre* DRG homogenates (see below for discussion). A second possibility is that ERK1/2 signaling is not required for epidermal innervation in these neurons, but that other pathways emanating from TrkA are actually required. These may include PI3K-Akt or ERK5 signaling (Markus et al., 2002). Additional experiments using *ERK1$^{-/-}$;ERK2$^{f/f}$;Na$_V$1.8-Cre* double knockout mice might distinguish these two possibilities. The third set of nociceptors is the nonpeptidergic nociceptors. These fibers also do not require ERK2 for innervation of the epidermis. Previous studies have demonstrated that developing nonpeptidergic fibers require NGF-TrkA signaling for epidermal innervation since all fibers initially express TrkA (Patel et al., 2000). After downregulating TrkA and upregulating Ret (Molliver et al., 1997), maintenance of epidermal innervation may also switch from TrkA to Ret signaling. This has been supported with experiments showing diminished nonpeptidergic innervation in mice with conditional Ret deletion, although confounds such as cell loss and gastrointestinal defects makes these conclusions relatively tentative (Luo et al., 2007; Golden et al., 2010). Whether nonpeptidergic neurons require Ret or TrkA for epidermal innervation, the lack of an effect of ERK2 deletion indicates that either ERK1 and ERK2 are redundant in these fibers or neither isoform is required and that other cascades are important instead. It will be particularly interesting to determine the developmental timecourse of fiber loss in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice in relation to the nonpeptidergic fibers. If ERK2 contributes to the initial innervation of the epidermis, as is a possibility mentioned above, then it could be that the requirement for ERK2 signaling delineates small-diameter nociceptors before the switch in expression of TrkA to Ret in

developing nonpeptidergic fibers.

It is also possible that separate ERK2-dependent and ERK2-independent populations represent an artifact of conditional ERK2 deletion and that ERK2 is actually required for peripheral innervation of all fiber types. For example, “ERK2-independent” fibers may innervate the epidermis at an earlier developmental time point than “ERK2-dependent” fibers. Cre-dependent excision of ERK2 might then occur immediately before innervation by “ERK2-dependent” fibers. This would cause the loss of innervation in only a subset of fibers and appear as if the remaining fibers did not require ERK2. A way to test this possibility would be to use alternative Cre lines, such as the knock-in *$Na_V1.8$-Cre* line in which Cre expression occurs earlier at E13/14 (Stirling et al., 2005). This may be the ideal way to determine whether ERK2 is required for initial fiber innervation since this developmental time point roughly coincides with the beginning of trophic dependence (Mendell et al., 1999). However, it is also possible that ERK2 modulates naturally occurring cell death in addition to its functions in epidermal innervation, which could confound the interpretation of this experiment (Coggeshall et al., 1994; Mendell et al., 1999). Manipulations on a *$Bax^{-/-}$* background in which apoptotic cell death is diminished may provide a way around this potential confound, as has been successfully performed to analyze the function of NGF, TrkA, and Ret after developmental periods of naturally occurring cell death (Patel et al., 2000; Honma et al., 2010).

It will also be interesting to determine the molecular mechanisms associated with fiber loss in *$ERK2^{f/f}$;$Na_V1.8$-Cre* mice. *In vitro* experiments that analyze the mechanisms underlying axon growth may provide some insights. Axon growth depends on signaling events that occur both locally at the nerve terminal and distantly in the soma (Markus et al., 2002). MEK1/2 inhibitors applied only to axons and not cell bodies induce growth cone collapse in sympathetic neurons (Atwal et al., 2003), suggesting that ERK1/2 activity maintains growth cone structure. This may occur by phosphorylation of microtubule associate proteins either directly or via GSK3β (Markus et al., 2002; Goold and Gordon-Weeks, 2005). Modulation of microtubule assembly might then allow for regulation of growth cone structure. Transcriptional alterations also play a role in axon growth, and ERK1/2 signaling may be involved (Markus et al., 2002; Wickramasinghe et al., 2008). Indeed, retrograde ERK1/2 signaling has been reported in the sciatic nerve (Johanson et al., 1995), and it is thought that NGF-TrkA signaling endosomes may be transported to the soma where ERK may

initiate transcriptional changes via transcription factors such CREB, SRF, or NFAT (Markus et al., 2002; Wickramasinghe et al., 2008). However, a recent study with a two-compartment culture system found that NGF-TrkA signaling endosomes activate ERK5 instead of ERK1/2 (Watson et al., 2001). Moreover, NGF-mediated cell survival required ERK5 activity when NGF was applied only to the axons. In other studies implicating retrograde TrkA-ERK1/2 signaling, NGF was applied in single chamber cultures to both soma and axons (Wickramasinghe et al., 2008). In agreement with these findings, Watson et al. also found NGF-induced ERK1/2 phosphorylation, but this was only observed when NGF was applied to cell bodies. This suggests that ERK1/2 may not be involved in retrograde signaling to the soma. In support of this model, a nerve ligation study revealed that NGF injection to the paw actually decreased the amount of retrogradely transported pERK1/2 compared to baseline (Averill et al., 2001). Since conditional deletion of ERK2 resulted in a reduction in epidermal innervation without affecting cell survival, it will be very interesting to determine whether transcriptional changes occur in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice that might account for fiber loss. If transcriptional programs are unaltered in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice, it is likely that ERK2 promotes epidermal innervation locally by modulating cytoskeletal stability.

The results of this study indicate that ERK2 deletion results in a partial loss of peptidergic fibers in the epidermis. ERK1 deletion throughout development has no effect on epidermal innervation. This suggests that ERK2 plays an isoform-specific role in this process. Additional studies using double knockout animals must be performed to determine whether ERK1/2 activity is important for epidermal innervation in ERK2-independent fibers. In conjunction with this line of investigation, it will be interesting to interrogate other intracellular signaling cascades downstream of growth factor receptors in the fibers that are not affected by ERK2 deletion.

ERK1 and ERK2 do not modulate baseline responses to radiant heat or mechanical stimuli

Previous studies with MEK1/2 inhibitors have indicated that basal ERK1/2 activity does not appear to control responses to acute noxious heat or mechanical stimulation in naïve animals. Application of inhibitors by intraplantar injection or by intrathecal administration has no effect on baseline withdrawal responses to radiant heat or mechanical stimulation (Ji et al., 1999; Obata et al., 2003; Hao et al., 2008). Additionally, transgenic mice expressing a dominant negative MEK1 have normal baseline sensitivity to radiant heat (Karim et al., 2006). These findings fit well

with findings from the ERK mutants. Deletion of ERK1 in all tissues or ERK2 in nociceptors has no impact on baseline sensitivity to radiant heat or mechanical stimulation with von Frey hairs (see Chapters 2 and 3). This observation has important ramifications. First, it means that the loss of peptidergic fibers in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice does not affect baseline responses. Second, this frames the interpretation of changes in basal ERK1/2 phosphorylation seen in the isoform-specific mutant mice. In *ERK1$^{-/-}$* mice, ERK2 is hyperphosphorylated in the spinal cord and DRG of naïve animals (Chapter 2). In *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice, the converse occurs such that ERK1 is hyperphosphorylated in DRG (Chapter 3). Although interesting in considering the relationship between the isoforms (see below for discussion), it appears that these changes in phosphorylation in naïve animals have no affect on baseline withdrawal to heat or mechanical stimuli. The behavioral relevance of baseline hyperphosphorylation could be queried with MEK inhibitors in naïve animals. However, since there is no effect of MEK inhibitors on baseline withdrawal responses in WT animals with both ERK1 and ERK2, the relevance of this question to physiological nociception seems limited.

One behavioral finding that may be related to baseline hyperphosphorylation is the thermotaxis deficit observed in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice (see Chapter 3). This could occur by several mechanisms. Hyperphosphorylation of ERK1 could play some role in epidermal innervation, actively inhibiting the growth or maintenance of peptidergic fibers. However, this does not appear to be the case since ERK1 deletion in DRGs has no effect on peptidergic innervation. Another possible mechanism may be that ERK1 hyperphosphorylation in existent fibers within the epidermis sensitizes those fibers to warm stimuli. This may prompt *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice to spent more time at cooler temperatures. This seems less likely given that *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice do not have reduced withdrawal latencies to radiant heat, although there may be changes in relatively narrow temperature ranges which would allow for this potential mechanism. Alternatively, ERK2-dependent peptidergic fibers may contribute to thermotaxis behavior, perhaps by expressing TRPM8 which is activated in the innocuous cool range of temperatures. Loss of these fibers may result in increased time spent at cooler temperatures. Future experiments using the systemic MEK1/2 inhibitor, SL327 (Chapter 2), may address this problem. If hyperphosphorylation of ERK1 drives the thermotaxis behavior, MEK1/2 inhibition should normalize the behavior of *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice. If the cause of the altered behavior is due to structural changes, then the MEK1/2 inhibitor will have no effect on

the thermotaxis behavior.

Nociceptor ERK2 is required for inflammation-induced mechanical hypersensitivity

The combined analysis of *ERK1*$^{-/-}$ and *ERK2*$^{f/f}$*;Na*$_V$*1.8-Cre* mice indicated that ERK2 specifically drives peripheral sensitization in inflammation. *ERK1*$^{-/-}$ mice developed robust hypersensitivity to mechanical stimuli following CFA-induced inflammation (see Chapter 2). On the other hand, CFA-induced mechanical hypersensitivity was significantly attenuated in *ERK2*$^{f/f}$*;Na*$_V$*1.8-Cre* mice (see Chapter 3). Since ERK2 deletion is restricted to small-diameter, unmyelinated DRG neurons, the reduced mechanical hypersensitivity observed in *ERK2*$^{f/f}$*;Na*$_V$*1.8-Cre* mice is due to diminished peripheral sensitization. This behavioral phenotype is probably not due to the partial loss of peptidergic fibers, because ablation experiments suggest that peptidergic fibers are not involved in inflammation-induced mechanical hypersensitivity (Chen et al., 2007; Cavanaugh et al., 2009). Moreover, ablation of nonpeptidergic fibers specifically attenuates mechanical hypersensitivity without affecting thermal hypersensitivity (Cavanaugh et al., 2009), which is similar to the *ERK2*$^{f/f}$*;Na*$_V$*1.8-Cre* phenotype. This might suggest that ERK2 drives hypersensitivity to mechanical stimuli within nonpeptidergic neurons exclusively. Direct evaluation of this model could be achieved by deleting ERK2 specifically in nonpeptidergic cells, although such a Cre line does not yet exist. However, it seems unlikely that peptidergic neurons play no role in inflammation-induced mechanical hypersensitivity. Channels that are candidates for the mammalian mechanotransducer, such as ASICs or TRPA1 (Hu et al., 2006; Kerstein et al., 2009; Kwan et al., 2009; Smith and Lewin, 2009), are expressed more frequently in peptidergic neurons (Story et al., 2003; Kobayashi et al., 2005; Poirot et al., 2006). Moreover, facilitation of mechano-sensitive currents has been observed in peptidergic neurons in culture (Lechner and Lewin, 2009). Therefore, it seems very likely that peptidergic fibers contribute to CFA-induced mechanical hypersensitivity in addition to nonpeptidergic fibers. One potential molecular mechanism involves TRPA1. Considering that responses to mustard oil and the first phase response to formalin are not absent in *ERK2*$^{f/f}$*;Na*$_V$*1.8-Cre* mice, it is likely that TRPA1-expressing fibers are present in the epidermis despite the loss of ERK2. Additionally, both genetic deletion and pharmacological inhibition of TRPA1 has implicated it in mechanotransduction in the skin-nerve preparation and on the behavioral level in CFA-induced mechanical hypersensitivity (Eid et al., 2008; Kerstein et al., 2009; Kwan et al., 2009; da Costa et al., 2010). Since ERK1/2 has previously been shown to modulate TRPV1 (Zhuang et al., 2004),

a precedent has been set for acute modulation of other TRP channels by ERK1/2 in nociceptors. Therefore, it is quite possible that ERK2 modulates TRPA1 function and, thereby, contributes to mechanical hypersensitivity following inflammation. Although the partial loss of peptidergic fibers in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice precludes definitive answers, it seems likely that ERK2 drives sensitization of the remaining nociceptors, peptidergic and nonpeptidergic alike.

Nociceptor ERK2 and inflammatory heat hypersensitivity

Inflammatory heat hypersensitivity is thought to involve sensitization of peptidergic nociceptors, given the results of ablation studies (Chen et al., 2007; Cavanaugh et al., 2009; Mishra and Hoon, 2010) and the observation that TRPV1 is expressed predominantly in peptidergic neurons in mice (Zwick et al., 2002; Woodbury et al., 2004; Breese et al., 2005). However, there is likely to be a role for nonpeptidergic neurons in inflammatory states, since TRPV1 expression and function is upregulated in nonpeptidergic cells after several days of CFA-induced inflammation (Breese et al., 2005). This would argue for the importance of nonpeptidergic cells in longer term heat hypersensitivity, in processes involving altered protein expression. Acute hypersensitivity, developing within minutes to hours, is probably mediated by fast post-translation mechanisms, which to date have all involved TRPV1 (Huang et al., 2006). Although inflammatory heat hypersensitivity is attenuated by MEK inhibitors applied both intrathecally and peripherally (Ji et al., 2002; Obata et al., 2003; Zhuang et al., 2004), deletion of ERK1 in all tissues or deletion of ERK2 in nociceptors had no effect on CFA-induced heat hypersensitivity (see Chapters 2 and 3). There are several possible reasons for this. One would be that ERK1 and ERK2 serve redundant functions in the subset of nociceptors underlying CFA-induced heat hypersensitivity. Another possibility is that CFA-induced heat hypersensitivity does not actually involve ERK1/2 activity in the nociceptor, since the study that implicated peripheral ERK1/2 activity in the CFA model did so by intrathecally applying MEK1/2 inhibitors (Obata et al., 2003). This method of drug delivery certainly affects spinal cord dorsal horn since intrathecal administration of MEK1/2 inhibitors decreases spinal ERK1/2 activation following noxious stimulation (Ji et al., 1999; Karim et al., 2001; Ji et al., 2002). Therefore, intrathecal MEK inhibition may only be functionally relevant in spinal cord dorsal horn, in which case somatic ERK1/2 activation may not be important for behavioral hypersensitivity. Finally, it is also possible that other non-ERK1/2 dependent pathways are sufficient to produce inflammatory heat hypersensitivity. Deletion of both ERK1 and ERK2 from nociceptors may be a

way to address these different possibilities.

In contrast to CFA-induced heat hypersensitivity, NGF-induced heat hypersensitivity is attenuated in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice. This is somewhat surprising since CFA-induced heat hypersensitivity requires NGF-TrkA signaling (Pezet and McMahon, 2006). This discrepancy could be explained by the reduction in peptidergic innervation. With a normal complement of fibers, CFA-induced heat hypersensitivity requires NGF, but without some fibers, other inflammatory signaling molecules and pathways compensate. This could be tested by assessing the NGF-dependence of CFA-induced heat hypersensitivity in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice. Although the reason that NGF-induced heat is attenuated in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice remains unclear, evaluation of *ERK1$^{-/-}$* mice may shed some light on mechanism since there are no changes in epidermal innervation that make interpretation difficult. Interestingly, deletion of ERK1 actually exaggerates NGF-induced heat hypersensitivity. This suggests that the phenotypes observed following NGF injection may not be due to structural changes but instead may result from altered intracellular signaling in existent fibers. Although speculative, this leads to a model in which ERK1 and ERK2 are both downstream of TrkA activation, but that ERK1 antagonizes ERK2 function (see below for discussion). Assuming nociceptor ERK1/2 signaling is behaviorally relevant in CFA-induced heat hypersensitivity, there must also be distinct signaling pathways involving ERK1 and ERK2 that drive heat hypersensitivity. This separate “isoform-redundant” pathway may involve upstream G-protein coupled receptors such as adrenergic or bradykinin receptors. This could be directly tested by determining whether epinephrine- or bradykinin-induced heat hypersensitivity require ERK1, nociceptor ERK2, or either isoform by using the ERK mutants described in this study and double knockouts.

Finally, ERK1 deletion delays formalin-induced heat hypersensitivity (see Chapter 2). Interestingly, formalin-induced heat hypersensitivity is not altered by conditional deletion of ERK2 in nociceptors. This indicates that ERK1 may be preferentially activated downstream of TRPA1. However, mustard oil-induced heat hypersensitivity is unaffected by the loss of ERK1, which is at odds with the formalin result since both chemicals activate TRPA1. This apparent discrepancy may relate to the concentration of agonist, such that more concentrated formalin solutions would equivalently activate ERK1 and ERK2. In this case, the isoforms may then appear to be redundant. Overall, these studies indicate that ERK1 and nociceptor ERK2 are dispensable for CFA-induced heat hypersensitivity. However, testing the effects of double knockouts and evaluating the

responses of ERK mutants to additional inflammatory mediators will help to clarify whether either isoform can drive hypersensitivity or neither isoform is required. From these experiments, it seems likely that multiple isolated signaling pathways can lead to heat hypersensitivity. ERK isoforms probably participate in some of these pathways with a requirement for specific isoforms in some cases (e.g. NGF-induced heat hypersensitivity) and no requirement in others. Understanding the cellular and molecular basis for these seemingly insulated cascades represents an exciting area of future research

Nociceptor ERK2 is required for formalin-induced sensitization

Conditional deletion of ERK2 in nociceptors produced an unusual phenotype in the formalin test (see Chapter 3). The first phase was elevated while the second phase was reduced. Deletion of ERK1 in all tissues has no effect on either phase of the formalin test. The first phase is thought to represent the initial detection of formalin and formalin-evoked nociceptor activity (Tjolsen et al., 1992). The second phase results from peripheral and central sensitization. Interestingly, inhibiting or genetically targeting TRPA1 reduces both phases of the formalin test (McNamara et al., 2007). Moreover, systemic inhibition with the blood-brain barrier penetrant MEK1/2 inhibitor SL327 also reduces both the first and second phase of the formalin test (see Chapter 2). Therefore ERK1/2 activity on the whole increases 1st and 2nd phase responses, which would not explain the elevated 1st phase in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice. One potential explanation for the elevated 1st phase response of *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice is that structural changes not directly related to nociceptive plasticity cause elevated initial responses. This may occur if the partial loss of peptidergic fibers observed is also associated with an increase in the input from remaining fibers. Since mustard oil responses are normal in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice, it is likely that TRPA1 is present. Therefore, elevated input from TRPA1-expressing fibers may account for increased 1st phase behaviors. This could be tested by applying TRPA1 antagonists before formalin injection in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice.

Despite an elevated 1st phase, *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice have reduced 2nd phase responses. This could be due to reduced peptidergic innervation, since ablation of TRPV1 expressing fibers prior to formalin injection reduces the 2nd phase of the formalin test (Peterson et al., 1997; Chen et al., 2007). Formalin induces inflammation and the release of inflammatory mediators like substance

P, bradykinin, and many others (Damas and Liegeois, 1999). The loss of peptidergic fibers may reduce nociceptor activity downstream of these other inflammatory mediators. Moreover, the fibers themselves probably contribute to some amount of formalin-induced inflammation since nociceptor terminals release peptides into the skin as part of a neurogenic inflammatory response (Reeh et al., 1986). However, if TRPA1-positive fiber input is elevated, as proposed above, it seems likely that nociceptor input would be robust despite the reduction in neurogenic inflammation. In this case, it is likely that although TRPA1-expressing fibers are present, sensitization within these fibers is attenuated due to the loss of ERK2. This could be due to reduced sensitization of TRPA1 itself or by diminished excitability. Inducible deletion strategies may circumvent alterations in epidermal innervation and allow a direct test of this hypothesis.

Spinal ERK1 is not required for inflammation-induced hypersensitivity

As mentioned above, it is difficult to determine the exact cellular site of ERK1/2 action based on inhibitor experiments alone. However, during the course of the present study, a targeted knockdown of ERK2 in spinal cord was reported (Xu et al., 2008). In this study, viral vectors containing short-hairpin RNA sequences that targeted ERK2 were injected intraparenchymally into the dorsal horn of the lumbar spinal cord. This resulted in partial knockdown of ERK2 expression and a profound attenuation of CFA-induced mechanical and thermal thresholds. The results of the current study compliment and extend these findings. Although Xu et al. identified a role for ERK2 in the spinal cord, they did not directly address a potential role for ERK1 and, therefore, did not determine whether isoform specific functions existed in the nociceptive spinal cord. Since the current study found that ERK1 was not required for CFA-induced hypersensitivity, it seems very likely that ERK2, but not ERK1, is specifically required in the spinal cord. An important caveat is that the methods of deletion are very different, so comparisons are not without some degree of uncertainty. The ideal experimental tools would include RNAi-mediated knockdown of both isoforms or the development of a conditional ERK1 allele that could be compared more directly with *ERK2*$^{f/f}$ mice. Results from the current study suggesting the importance of ERK2 should motivate additional experimentation using identical methods to manipulate ERK isoform expression.

Competition Model

In other systems, isoform specific roles have been identified for ERK1 and ERK2

(see Chapter 1). In fibroblasts, reduction in ERK2 expression diminishes proliferation (Vantaggiato et al., 2006; Lefloch et al., 2008). The effect of targeting ERK1 is controversial. In some reports, reducing ERK1 expression paradoxically increases proliferation rates (Vantaggiato et al., 2006). Other studies have found either no effect of ERK1 targeting or similar but less pronounced effects and only in the context of ERK2 deletion (Lefloch et al., 2008; Voisin et al., 2010). Associated with these experiments, deletion of one ERK isoform resulted in the hyperphosphorylation of the remaining isoform. This led to the proposal of a competition model. In this model, ERK1 and ERK2 coexist within the same signaling microdomain. Each isoform can be phosphorylated by MEK1/2. However, ERK1 is less able to transmit the signal than ERK2. This is probably not due to differences in the catalytic domains of ERK1/2, since the phosphorylation loop and active site are highly homologous (see Chapter 1). Instead, domains regulating protein-protein interactions probably underlie this difference. For example, cytoplasmic-nuclear shuttling of ERK1 is slower than ERK2 due to amino acid differences in the N-terminus (Marchi et al., 2008). Binding to protein substrates may also be different, since a study comparing the crystal structures of ERK1 and ERK2 found subtle but potentially important structural differences in D-motif binding site and the Backside motif binding site (Kinoshita et al., 2008).

The competition model would explain several results observed both in the literature and in the current study. In the current study, deletion of one ERK isoform increased the phosphorylation state of the remaining isoform. This is predicted by the competition model and has also been observed in other systems (see Chapter 2). The model may also explain the behavioral effects of ERK deletion on NGF-induced heat hypersensitivity. Deletion of ERK2 attenuates NGF-induced heat hypersensitivity, suggesting that ERK2 is predominant. ERK1 is not able to compensate for the loss of ERK2, supporting the idea that ERK1 is not able to effectively transmit signals from TrkA. On the other hand, deletion of ERK1 relieves competition for MEK binding, increasing ERK2 phosphorylation and exaggerating heat hypersensitivity. It is important to note that this model is speculative. Genetic manipulations are not equivalent, and fiber loss in *ERK2$^{f/f}$;Na$_V$1.8-Cre* mice precludes definitive conclusions. However, the model could be tested by assessing NGF-induced ERK phosphorylation in the ERK mutant lines. It would also be interesting to determine whether the opposing effects of ERK isoform deletion observed on the behavioral level also occur on the cellular level, which is a fundamental assumption of the model. Ongoing experiments assessing

NGF-induced TRPV1 potentiation in the ERK mutants will address this issue. Additionally, isoform-specific antibody staining, with rigorous controls, will also help determine whether ERK1 and ERK2 are coexpressed in the same cell which would also be required for this model. The model also predicts that deleting both ERK1 and ERK2 might exacerbate deficits observed with only ERK2 deletion. This could be evaluated by characterizing mice that lack ERK1 and ERK2 in nociceptors.

Targeted ERK2 inhibition to treat chronic pain?

The results of the current study suggest that ERK2 is the predominant isoform driving peripheral sensitization. Since ERK1 has no effect in a variety of rodent models of pain, it seems logical to target ERK2 specifically to treat chronic pain conditions. There is some evidence to suggest that this may help to circumvent potential side effects of ERK1/2 inhibition. ERK1 is involved in regulating hepatocyte survival (Fremin et al., 2007; Fremin et al., 2009), directing erythropoetic development (Guihard et al., 2010), tuning adaptive immunity by modulating thymocyte development (Pages et al., 1999), and initiating adipogenesis (Bost et al., 2005). These metabolic and immune system functions may be negatively affected by nonselective inhibition of ERK1 and ERK2, suggesting that selective inhibition of ERK2 activity may be preferred. However, results from this study also indicate that nociceptor ERK2 is partially required for epidermal innervation. It will be important to determine whether ERK2 deletion affected innervation during development or whether continued ERK2 activity was required to maintain innervation. If ERK2 is required for fiber maintenance, systemic ERK2 inhibition may lead to denervation. This may be analgesic but may also create a neuropathic pain state. It is also hard to predict the effect of ERK2 inhibition in other fiber types in the peripheral nervous system. Further study will resolve some of these questions. It is important to note that not all nociceptor fibers are lost from the epidermis. As such, the results of the current study are fairly promising. On the behavioral level, targeted deletion of ERK2 attenuates inflammation-induced hypersensitivity. Moreover, ERK1 is not required in these models, suggesting that focused inhibition of ERK2 may be a novel approach to treating chronic pain.

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