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Central amygdala activation of extracellular signal-regulated kinase 1 and age-dependent changes in inflammatory pain sensitivity in mice

Katelyn E. Sadler [a,b,c], Nathan M. Gartland [b,d], Jane E. Cavanaugh [b,c,d], Benedict J. Kolber [a,b,c,*]

[a] Department of Biological Sciences, Duquesne University, Pittsburgh, PA, USA
[b] Chronic Pain Research Consortium, Duquesne University, Pittsburgh, PA, USA
[c] Aging Research and Teaching Consortium, Duquesne University, Pittsburgh, PA, USA
[d] Division of Pharmacology, Duquesne University, Pittsburgh, PA, USA

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ABSTRACT

Aging populations are more sensitive to noxious stimuli as a result of altered somatosensory systems. In these experiments, we examined pain-like behaviors in young, middle-aged, and old mice during peripheral inflammation to determine if the same sensitivity exists in preclinical animal models. Immediately following injury, middle-aged and old mice exhibited more spontaneous pain-like behaviors than young mice, matching pain prevalence in clinical populations. Middle-aged and old mice also developed persistent mechanical hypersensitivity in the injured paw. Furthermore, old mice developed mechanical hypersensitivity in the noninjured paw suggesting age-dependent changes in central nociceptive systems. To address this end, pain-related protein expression was examined in the central nucleus of the amygdala, a limbic brain region that modulates somatic pain. Following injury, increased phosphorylation of extracellular signal-regulated kinase 1, a protein with known nociceptive functions, was observed in the right central nucleus of the amygdala of old mice and not middle-aged or young animals. These findings suggest that age-dependent changes in supraspinal nociceptive systems may account for increased pain-like behaviors in aging populations.

1. Introduction

As humans age, pain frequency and sensitivity increase, perhaps due to gradual changes in nociceptive sensory systems (Andersson et al., 1993; Bouhassira et al., 2008; Miaskowski, 2000; Patel et al., 2013). To date, very few basic science studies have investigated the changes that occur in nociceptive systems, specifically those in the brain, during natural aging; the focus of most published articles has been on peripheral nociceptive systems due to their direct and possibly easier-to-treat link to pain. However, in the context of persistent pain, the central nervous system and brain regions like the central nucleus of the amygdala (CeA) are critical for maintaining increased pain sensitivity and the affective abnormalities that often accompany these conditions (Veinante et al., 2013).

In the present study, we investigated the behavioral and molecular changes that occur in young, middle-aged, and old male C57Bl/6 mice following intraplantar formalin injection. Intraplantar formalin injection induces spontaneous pain-like behaviors in mice and rats that last for 60 minutes and mechanical hypersensitivity that lasts for several additional hours (Carrasquillo and Gereau, 2007; Fu et al., 2001; Kolber et al., 2010b). Both of these well-characterized behaviors are easy to measure and can reveal information about age-dependent changes in central and peripheral nociceptive modulation. Previous studies have revealed conflicting changes in spontaneous formalin behavior in both mice and rats, but no study has yet investigated if age affects mechanical sensitivity following formalin injection (Gagliese and Melzack, 2000; King-Himmelreich et al., 2015).

Formalin-induced mechanical hypersensitivity is dependent on higher brain structures including the CeA (Carrasquillo and Gereau, 2007). Specifically, group 1 metabotropic glutamate receptor (mGluR1 and mGluR5) signaling in the CeA is necessary for this behavior (Carrasquillo and Gereau, 2008; Kolber et al., 2010b). Through downstream activation of extracellular signal-regulated

* Corresponding author at: Department of Biological Sciences, Duquesne University, 600 Forbes Avenue, Pittsburgh, PA 15219, USA. Tel.: 412-396-5615; fax: 412-396-5907.
E-mail address: kolberb@duq.edu (B.J. Kolber).

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kinases 1 and 2 (ERK1/2), mGluR1/5 mediate CeA excitability after injury (Carrasquillo and Gereau, 2007; Kolber et al., 2010b; Neugebauer et al., 2003). Interestingly, hemispheric lateralization of this signaling cascade has been noted in inflammatory pain; both mGluR5 and ERK1/2 phosphorylation in the right CeA are necessary for formalin-induced mechanical hypersensitivity in mice, and pharmacological activation of mGluR5 (Crock et al., 2012; Kolber et al., 2010b) or ERK1/2 (Carrasquillo and Gereau, 2008) in the right CeA is sufficient to induce bilateral pain-like behaviors in uninjured mice. Unknown however, are the effects that age has on this signaling pathway prior to and following inflammatory insult.

Overall, the goal of these studies was to identify age-sensitive proteins involved in supraspinal pain modulation. By assessing both formalin-induced changes in behavior and concomitant changes in CeA protein expression, we identified ERK1 in the right CeA as a potential age-dependent modulator of inflammatory pain.

2. Materials and methods

2.1. Animals

Male C57Bl/6 mice were used for all experiments. “Young” mice were 2 months, “middle-aged” mice were 12 months, and “old” mice were 22 months, consistent with life history (Dutta and Sengupta, 2016). Animals were housed on a 12 hour light/dark schedule with ad libitum access to rodent chow and water. All protocols were in accordance with National Institutes of Health guidelines and were approved by the Animal Care and Use Committee at Duquesne University (Pittsburgh, PA; protocol #1410-14).

2.2. Behavior testing

Due to the nature of the following behavioral responses, different cohorts of animals were used for each pain test; spontaneous formalin responses were recorded through glass bottom cages while von Frey mechanical sensitivity testing was performed through wire mesh screen.

2.2.1. Spontaneous pain-like behaviors following formalin injection

Formalin testing was performed as previously described (Kolber et al., 2010b). Mice ($n = 6$) were bred in-house and/or acclimated to in-house conditions for at least 2 weeks after receipt from the National Institutes of Aging breeding colony. Briefly, animals habituated in 10 cm^2 × 15 cm Plexiglas boxes situated over a Logitech Pro 9000 HD camera for 1.5 hours. Low decibel white noise was played throughout the experiment to mitigate background sounds. The experimenter was present in the room for 30 minutes prior to start of testing to eliminate any sex-induced analgesic effects (Sorge et al., 2014). About 10 μL of 4% formalin (in 0.9% saline vehicle) was injected into the rear right paw; total time for injection was less than 1 minute per animal. Animals were returned to Plexiglas boxes as soon as the injection was complete. Animal behavior was recorded for 60 minutes following injection then scored offline by an experimenter blinded to age. Pain-like behaviors including licking, shaking, and biting of the injected paw were summed in 5 minutes bins across the entire 60 minutes test. Responses obtained during the first 10 minutes were attributed to peripheral mechanisms and those obtained from 10–60 minutes were attributed to central mechanisms.

2.2.2. Paw mechanical sensitivity testing

Peripheral mechanical sensitivity was assessed using calibrated von Frey filaments with the up/down method (Chaplan et al., 1994). Briefly, animals were ($n = 8–10$) habituated in 10 cm^2 × 15 cm Plexiglas boxes resting on a wire mesh grid for 1.5 hours. White noise was played throughout the experiment to mitigate background sounds. The experimenter was present in the room for 30 minutes prior to start of testing. Using calibrated von Frey filaments (0.02–2.56 g) and the up/down method, 50% withdrawal thresholds (the application forces that cause an animal to remove its paw during 50% of applications) were determined for the left and right paws of each animal by an experimenter blinded to age, as previously described (Chaplan et al., 1994). Each paw was assessed twice (>5 minutes between trials) then averaged to obtain a baseline withdrawal threshold. Paw mechanical sensitivity was then reassessed 60, 120, and 180 minutes following 10 μL injection of 4% formalin in the right rear paw.

2.2.3. Motor sensory assessment

Animals ($n = 6$) were placed through 6 different tests to assess age-dependent deficits in gross motor behavior and coordination. Each test was performed twice; scores were averaged across trials as previously described (Kolber et al., 2010a). Testing was done blinded to mouse age.

2.2.3.1. Walking initiation test. Mice were placed in the center of a 21-cm^2 square marked on a tabletop. The time for all 4 paws to exit the square was recoded.

2.2.3.2. Vertical pole descent test. Mice were positioned head-up at the top of a vertical metal pole (8-cm diameter, 55-cm height). The time it took each animal to turn 180° and climb down to the base of the pole was recorded; animals that slid down the pole or reached the bottom without changing direction were given a score of 120 seconds.

2.2.3.3. Ledge crossing test. Mice were placed on one end of 0.75-cm wide × 51-cm long Plexiglas ledge. The time animal remained on ledge was recorded (60 seconds maximum). If animal traversed entire length of ledge and returned to the starting point in less than 60 seconds, 60 seconds score was assigned.

2.2.3.4. Platform test. Mice were placed on a 3-cm diameter Plexiglas platform at the top of a 47-cm vertical pole. The time animal remained on the platform was recorded (60 seconds maximum).

2.2.3.5. Sixty and 90° inclined screen tests. Mice were positioned head-down at the bottom of a 47-cm high × 18-cm wide wire mesh screen inclined at either 60° or 90°. The time it took an animal to reorient and climb to the top of the screen and the total time on the screen (60 seconds maximum) were independently recorded.

2.2.3.6. Inverted screen hang. Mice were placed in the center of 47 × 18-cm wire mesh screen oriented at 60°. The screen was then inverted to 180° so that the mice were upside-down. The time the animal hung onto the screen was recorded (120 seconds maximum).

2.3. Western blotting

mGluR5 and pERK1/2 expression levels were assessed in the left and the right CeA of naïve 2-, 12-, and 22-month old animals and 3 hours following formalin injection in separate cohorts of 2-, 12-, and 22-month old animals. Mice were bred in-house and/or acclimated for at least 2 weeks after receipt from the National Institutes of Aging breeding colony or Hilltop Laboratories (Scottdale, PA, USA). To obtain CeA tissue, animals were decapitated, brains were removed, segmented into 500-μm thick coronal sections using a stainless steel brain mold (Stoelting), and punches were taken from the left and right CeA using a 1-mm diameter punch tool (Stoelting).

Punches were homogenized in ice-cold buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM Na4P2O7, 100 μM PMSF, 25 μg/mL aprotinin, 25 μg/mL leupeptin, Sigma Phosphatase Inhibitors II and III) then assessed for total protein content using the Pierce BCA protein assay kit (Thermo Scientific). About 8 μg of protein was separated on a 4%–15% gradient polyacrylamide gel and then transferred to a nitrocellulose membrane for immunoblotting.

Membranes were blocked in Odyssey blocking buffer for 1 hour, then coincubated with mouse anti-pERK1/2 (1:1,000, Cell Signaling) and rabbit anti-ERK1/2 primary antibodies (1:1,000, Cell Signaling; in 0.1% Tween 20 in Odyssey blocking buffer) for 1 hour. Blots were rinsed with 0.1% Tween 20 in Tris-buffered saline (TTBS) then incubated with goat anti-mouse Alexa 680 (1:20,000, Molecular Probes) and goat anti-rabbit IR 800 secondary antibodies (1:20,000, Rockland; in 0.1% Tween 20 in Odyssey blocking buffer) for 1 hour. Blots were rinsed in TTBS and then scanned on an infrared Odyssey imager. Immediately following successful imaging, blots were incubated in nitrocellulose stripping buffer (LI-COR) for 5 minutes. Following phosphate-buffered saline rinse, blots were reblocked in Odyssey blocking buffer for 5 minutes, then coincubated with mouse anti-β-tubulin (1:10,000, Sigma Aldrich) and rabbit anti-mGluR5 primary antibodies (1:500, Genemed Synthesis Inc; in 0.1% Tween 20 in Odyssey blocking buffer). Blots were rinsed in TTBS, then incubated with goat anti-mouse Alexa 680 (1:20,000, Molecular Probes) and goat anti-rabbit IR 800 secondary antibodies (1:20,000, Rockland; in 0.1% Tween 20 in Odyssey blocking buffer) for 1 hour. Following a final rinse in TTBS, blots were again scanned on an Odyssey imager. Image Studio Lite software (LI-COR, version 4) was used to assess band signal strength for ERK1, ERK2, pERK1, pERK2, mGluR5, and β-tubulin in each sample. pERK1/2 signals were normalized to ERK1/2 and mGluR5 was normalized to β-tubulin within a sample. Quantification was carried out in a blinded fashion.

2.4. Statistical analysis

Prism 6 software (GraphPad) was used to analyze all data. Results are expressed as means ± standard error of the mean. Two-way analysis of variance (ANOVA) with repeated measures was used to analyze data sets containing 2 independent variables and 1-way ANOVA was used to analyze data sets containing 1 independent variable; Bonferroni's post hoc tests were employed when significant main effects were found. A value of $p \leq 0.05$ was considered statistically significant for all comparisons.

3. Results

Acute peripheral inflammation is a common cause of pain throughout all stages of life. Pain variability and intensity during inflammation however, vary with age in human populations. In these experiments, both peripherally and centrally mediated acute pain–like behaviors were assessed in young (2 months), middle-aged (12 months), and old (22 months) mice in order to compare preclinical modeling to human responses across the life span.

3.1. Spontaneous pain-like behaviors following peripheral inflammatory injury vary with age

The formalin test was used to determine the effects of age on spontaneous nociceptive responses. To perform this assay, animals were injected with 4% formalin in the rear right paw; then, spontaneous pain-like behaviors (e.g., flicking, licking, biting) were recorded and tallied for the 60 minutes following injection. Typical biphasic response curves were observed in all age groups, however behavior times varied between ages during response progression (Fig. 1A; 2-way ANOVA, effect of time, $F_{(11, 165)} = 32.70$, $p < 0.0001$; time × age interaction, $F_{(22, 165)} = 1.986$, $p = 0.0082$; $n = 6$). When first (0–10 minutes) and second (10–60 minutes) phase responses were individually assessed, age-dependent differences were observed. During the peripherally mediated first phase, young animals spent significantly less time exhibiting pain-like behaviors than both middle-aged and old animals (Fig. 1B; 1-way ANOVA, $F_{(2, 15)} = 11.53$, $p = 0.0009$; Bonferroni post-test, 2 months vs. 12 months $p < 0.01$, 2 months vs. 22 months $p < 0.001$). During the centrally mediated second phase, however, all mice spent approximately the same total time exhibiting pain-like responses (Fig. 1C; 1-way ANOVA, $F_{(2, 15)} = 0.4610$, $p = 0.6393$). Old animals were more variable in their responses during this period (Fig. 1C), specifically demonstrating fewer pain-like behaviors than middle-aged animals 25–30 minutes following injection (Fig. 1A; Bonferroni post-test, 12 months vs. 22 months at 25–30 minutes $p < 0.05$). Overall, these results highlight age-dependent changes in peripheral processing of acute nociceptive stimuli, and suggest more complex age-dependent alterations in central nociceptive mechanisms.

3.2. Hind paw mechanical sensitivity varies with age and injury state

Age-dependent changes in both innocuous and noxious mechanical sensitivity have been reported in human somatosensory studies. To determine if C57Bl/6 naïve male mice also exhibit age-dependent alterations in mechanical sensitivity, 50% hind paw withdrawal thresholds were determined in young, middle-aged, and old naïve mice. Similar to the loss of sensitivity in aging humans, naïve old mice also exhibited decreased mechanical sensitivity, withdrawing their paws at significantly higher thresholds than young animals (Fig. 2; 1-way ANOVA, $F_{(2, 23)} = 4.315$, $p = 0.0256$; Bonferroni post-test 2 months vs. 22 months $p < 0.05$; $n = 8–10$).

Mechanical sensitivity was reassessed during acute inflammation to determine if animal age also affected this measure. Withdrawal thresholds for both the left (noninjected) and right (injected) paws were calculated prior to and following formalin injection. Interesting age-dependent changes in mechanical sensitivity were observed in both the left (Fig. 3A; 2-way ANOVA, time × age interaction, $F_{(6, 69)} = 3.674$, $p = 0.0032$, main effect of age, $F_{(2,23)} = 3.518$, $p = 0.0464$; $n = 8–10$) and right paws (Fig. 3B; 2-way ANOVA, time × age interaction, $F_{(6, 66)} = 2.503$, $p = 0.0309$; $n = 8–10$). As expected, significant decreases in withdrawal threshold were observed in the right (injected) paw; middle-aged and old mice developed dramatic mechanical allodynia that was maintained for 180 minutes following formalin injection (Fig. 3B; 2-way ANOVA, effect of time, $F_{(3, 66)} = 30.62$, $p < 0.0001$; Bonferroni post-test, 12 months baseline vs. 60 or 120 minutes $p < 0.001$, 12 months baseline vs. 180 minutes $p < 0.0001$, 22 months baseline vs. 60, 120, or 180 minutes $p < 0.0001$). Interestingly, mechanical allodynia also developed in the left (noninjected) paw of old mice and persisted for 180 minutes following formalin injection (Fig. 3A; 2-way ANOVA, effect of time, $F_{(3, 69)} = 2.125$, $p = 0.0343$; Bonferroni post-test, 22 months baseline vs. 60 minutes $p < 0.001$, 22 months baseline vs. 120 or 180 minutes $p < 0.01$).

3.3. Age-dependent changes in motor function and coordination are not responsible for altered nociceptive responses

After observing increased pain-like behavior in old mice, we performed basic physiological assessments in all age groups to determine if impaired functioning led to the observed changes in behavior. Naïve young, middle-aged, and old mice were placed through a motor sensory battery consisting of 6 different assays, weighed, and examined for gross anatomical variances. Both

middle-aged and old mice weighed more than young mice (Fig. 4A; 1-way ANOVA, $F_{(2, 15)} = 12.75$, $p = 0.0006$; Bonferroni post-test, 2 months vs. 12 months $p < 0.001$, 2 months vs. 22 months $p < 0.01$; $n = 6$). The only motor/coordination assays that revealed age-specific differences were the walking initiation test and the inverted screen hang. Specifically, middle-aged animals took a longer time to exit a 21-cm^2 square than young animals (Fig. 4B; 1-way ANOVA, $F_{(2, 15)} = 4.381$, $p = 0.0317$; Bonferroni post-test 2 months vs. 12 months $p < 0.05$; $n = 6$). Likewise, middle-aged animals also failed to remain on the inverted screen as long as young or old animals; old animals performed only slightly better than middle-aged animals, still not hanging onto the screen as long as young mice (Fig. 4C; 1-way ANOVA, $F_{(2, 15)} = 33.65$, $p < 0.0001$; Bonferroni post-test 2 months vs. 12 months $p < 0.001$, 12 months vs. 22 months $p < 0.001$, 2 months vs. 22 months $p < 0.05$; $n = 6$). Mice from all age groups had equal performances in the vertical pole descent, ledge crossing, platform balance, and 60° and 90° inclined screen tasks (data not shown). The only motor or coordination deficits suggested by these data are decreased walk initiation and ability to hold onto the inverted screen, both of which may have been related to increased weight gain in the older animals.

3.4. ERK1 activation in the right CeA occurs during peripheral inflammation in old mice

Formalin-induced mechanical allodynia is regulated, in part, by a specific signaling cascade within the right CeA (Carrasquillo and Gereau, 2007; Kolber et al., 2010b). To determine if the age-dependent changes observed in mechanical sensitivity (Fig. 3A and B) matched age-dependent changes in this molecular pathway, pERK1/2 and mGluR5 expression were assessed 180 minutes after formalin injection. Age-dependent increases in pERK1 expression were observed following peripheral injury (Fig. 5A and B). Specifically, old animals expressed more pERK1

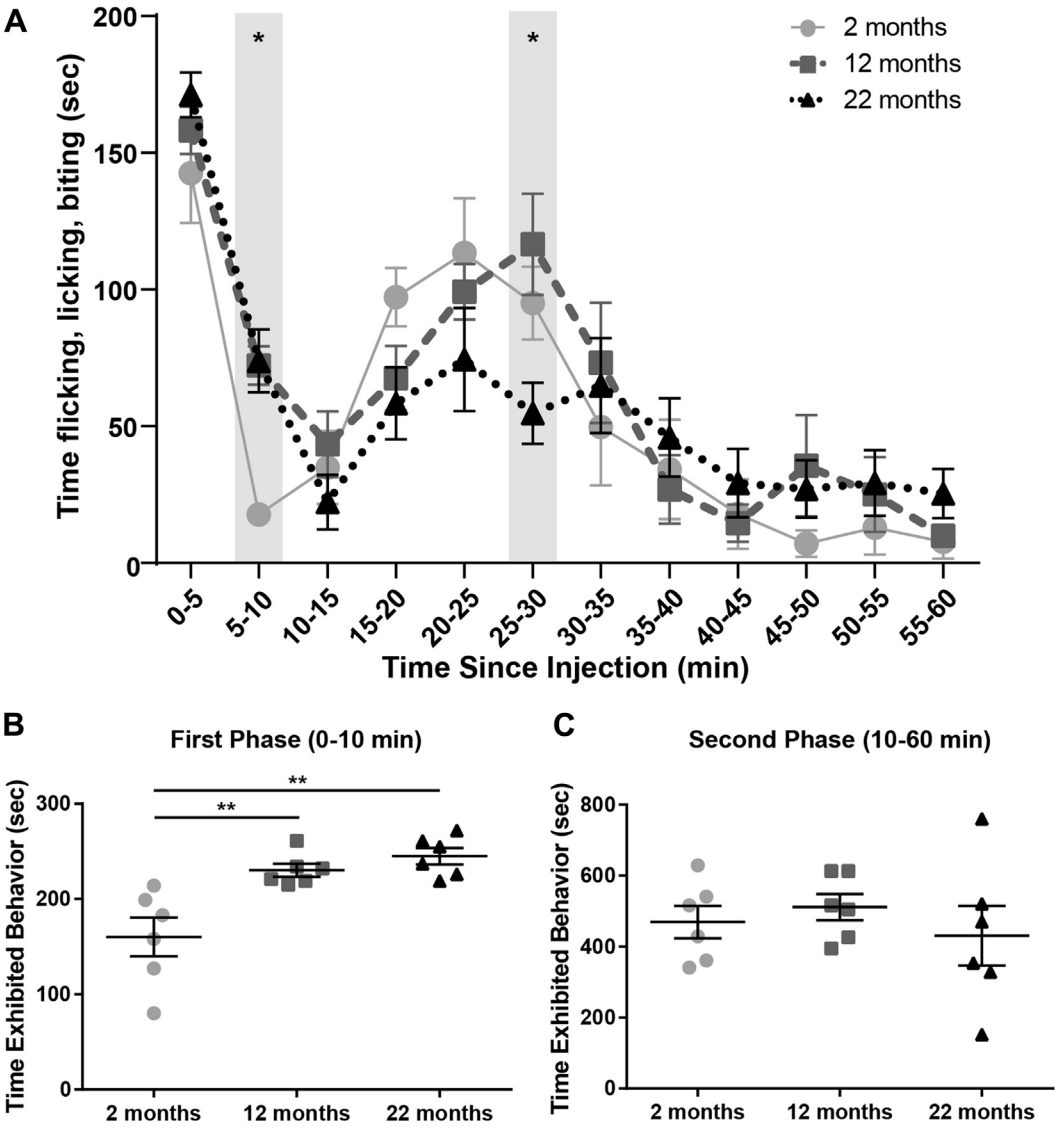

Fig. 1. Pain-like behaviors following peripheral inflammatory injury increase with age. (A) All ages exhibited typical biphasic response to injury, however age-specific responses varied significantly across time (2-way repeated measures [RM] ANOVA, effect of time $p < 0.0001$, time × age interaction $p = 0.0082$; $n = 6$). Specifically, young animals (2 months) displayed significantly fewer pain-like behaviors than old animals (22 months) during 5–10 minutes bin, and old animals displayed significantly fewer pain-like behaviors than middle-age animals (12 months) during 25–30 minutes bin (Bonferroni post-test 2 months vs. 22 months at 5–10 minutes $^{*}p < 0.05$, 12 vs. 22 months at 25–30 minutes $^{*}p < 0.05$). (B) During the first phase of the test, middle age and older mice exhibited increased pain-like behavior compared to young mice (1-way ANOVA, $p = 0.0009$; Bonferroni post-test, 2 months vs. 12 months $^{**}p < 0.01$, 2 months vs. 22 months $^{**}p < 0.01$; $n = 6$). (C) During the second phase of the test, mice exhibited overall equivalent pain-like behavior compared across ages (1-way ANOVA, $p = 0.6393$; $n = 6$).

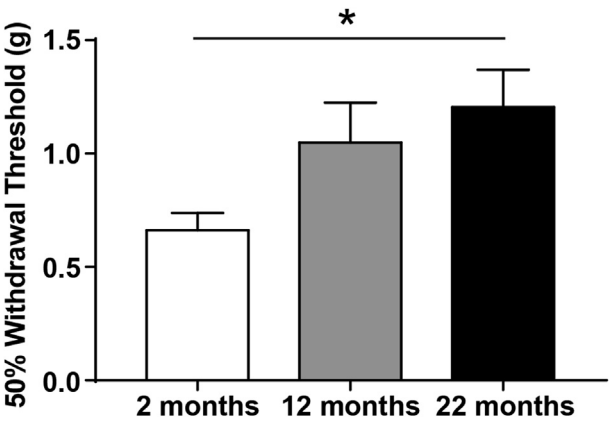

Fig. 2. Peripheral mechanical sensitivity decreases with age in naïve mice. Mechanical sensitivity was assessed in young, middle-aged, and old naïve male mice by probing both rear paws with calibrated von Frey filaments. About 50% withdrawal thresholds increased with age; old animals have significantly higher withdrawal thresholds (i.e., reduced mechanical sensitivity) than young animals (1-way ANOVA $p = 0.0256$; Bonferroni post-test, 2 months vs. 22 months $^{*}p < 0.05$; $n = 8–10$; L/R paw thresholds averaged for analysis).

in the right CeA than the left CeA (Fig. 5B; 2-way ANOVA, effect of age, $F_{(2, 45)} = 4.114$, $p = 0.0229$, effect of side of brain, $F_{(1, 45)} = 5.639$, $p = 0.0219$; Bonferroni post-test, 22 months left vs. right $^{*}p < 0.05$; $n = 6–9$). ERK1 phosphorylation does not differ between age groups in naïve conditions (young left CeA: 0.650 ± 0.118, young right CeA: 0.794 ± 0.146, middle-aged left: 0.641 ± 0.068, middle-aged right: 0.712 ± 0.150, old left: 0.805 ± 0.196, old right: 0.574 ± 0.060; 2-way ANOVA, no main effect of age $F_{(2, 14)} = 0.0393$, $p = 0.9616$, $n = 5–6$). Additionally, ERK2 phosphorylation levels did not differ between sides of the brain or between different age groups following formalin injection (Fig. 5C; 2-way ANOVA, no main effect of age $F_{(2, 12)} = 0.8485$, $p = 0.4476$; $n = 6–9$), or in naïve conditions (young left: 3.271 ± 0.326, young right: 3.275 ± 0.219, middle-aged left: 3.205 ± 0.214, middle-aged right: 3.411 ± 0.532, old left: 3.233 ± 0.495, old right: 3.062 ± 0.216; 2-way ANOVA, no main effect of age $F_{(2, 14)} = 0.07949$, $p = 0.9240$, $n = 5–6$).

mGluR5 is an upstream activator of ERK1/2 that is also required for the development of peripheral hypersensitivity. Based on the observed changes in pERK1, we hypothesized that age-dependent changes in mGluR5 expression would also coincide with age-dependent changes in mechanical sensitivity. We first assessed mGluR5 expression in naïve mice as endogenous levels of this protein affect mechanical sensitivity in the absence of injury. Older naïve mice trended towards decreased mGluR5 expression (mGluR5 expression 2 months = 0.006 ± 0.0012, 12 months = 0.006 ± 0.0004, 22 months = 0.003 ± 0.0006; 1-way ANOVA, $F_{(2, 18)} = 0.3655$, $p = 0.0507$; $n = 6$). About 180 minutes following formalin injection, however, CeA mGluR5 expression is increased to approximately the same level across all age groups, abolishing the age-dependent expression level trends that were suggested in naïve animals (Fig. 5D and E; 2-way ANOVA, effect of age, $F_{(2, 15)} = 0.2418$, $p = 0.7882$; $n = 6$). Notably, the most robust mGluR5 expression changes appeared to occur in the right CeA; both young and old mice exhibit significantly more mGluR5 in the right CeA than the left CeA (Fig. 5D; 2-way ANOVA, effect of side of brain, $F_{(1, 15)} = 35.48$, $p < 0.0001$; Bonferroni post-test, 2 months right vs. left $^{**}p < 0.01$, 22 months right vs. left $^{*}p < 0.05$).

4. Discussion

In this study, we investigated the effects of animal age on common acute nociceptive measures and the molecular changes in the CeA that accompany these behaviors. Specifically, spontaneous pain-like behaviors and mechanical sensitivity were assessed in young, middle-aged, and old male C57Bl/6 mice following an intraplantar formalin injection. These behavioral assessments allowed for the dissection of age-dependent changes in both peripheral and central nociceptive systems, the latter of which has received very little attention in basic science research. Pain-signaling pathways in the CeA were assessed across age, ultimately revealing phosphorylated ERK1 as a potential modulator of increased pain sensitivity in old mice.

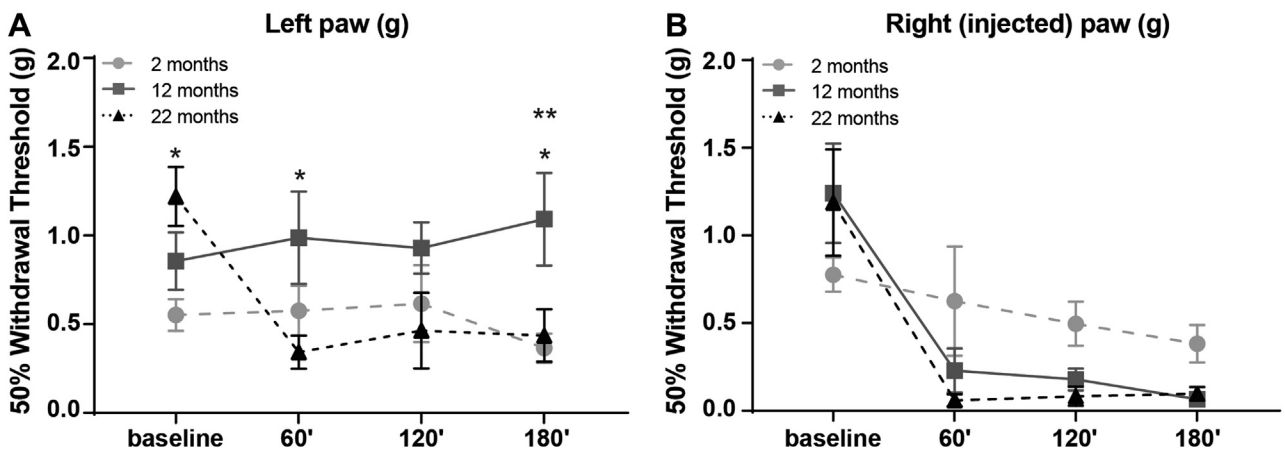

Fig. 3. Evaluation of mechanical hypersensitivity across age. Formalin-induced mechanical hypersensitivity was evaluated 60, 120, and 180 minutes after right paw formalin injection. (A) Hypersensitivity development in the noninjected left paw varied with age (2-way RM ANOVA, effect of age $p = 0.0464$, time × age interaction $p = 0.0032$; $n = 8–10$). Old mice quickly developed robust hypersensitivity that was maintained for 180 minutes (Bonferroni post-test for 22 months, baseline vs. 60 minutes $p < 0.001$, baseline vs. 120 or 180 minutes $p < 0.01$). This old-aged specific hypersensitivity was significantly different than the responses of middle-aged mice 60′ and 180′ following injection (Bonferroni post-test at 60′ 12 vs. 22 months $^{*}p < 0.05$, at 180′ 12 vs. 22 months $^{*}p < 0.05$). Young mice also had significantly lower withdrawal thresholds 180′ following injection when compared to middle-aged mice, however these thresholds did not differ significantly from baseline thresholds for this age group (Bonferroni post-test at 180′ 2 vs. 12 months $^{**}p < 0.01$, at baseline 2 vs. 22 months $^{*}p < 0.05$, for 2 months baseline vs. 60, 120, or 180 $p > 0.05$). (B) Hypersensitivity development in the injected right paw varied between the ages across time (2-way RM ANOVA, effect of time $p < 0.0001$, time × age interaction $p = 0.0309$; $n = 8–10$). Both middle-aged and old mice exhibited significant hypersensitivity 60, 120, and 180 minutes following injection (Bonferroni post-test for 12 months, baseline vs. 60 or 120 minutes $p < 0.001$, baseline vs. 180 minutes $p < 0.0001$; Bonferroni post-test for 22 months baseline vs. 60, 120, or 180 minutes $p < 0.0001$).

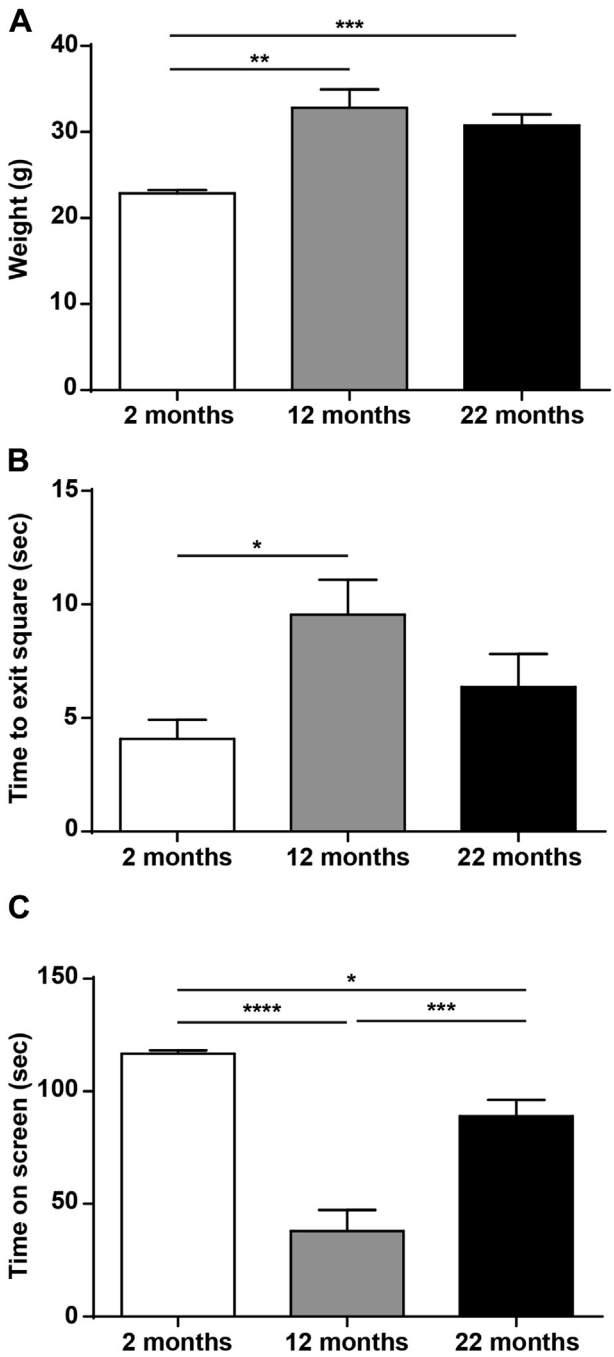

Fig. 4. Age-dependent evaluation of weight and motor function. (A) Middle-age and old mice weighed significantly more than young mice, however there was no weight difference between the older age groups (1-way ANOVA, $p = 0.0006$; Bonferroni post-test, 2 months vs. 12 months $^{***}p < 0.001$, 12 months vs. 22 months $^{**}p < 0.01$; $n = 6$). (B) Middle-aged mice took more time to exit a 21-cm^2 square during the walking initiation test than young mice (1-way ANOVA, $p = 0.0317$; Bonferroni post-test, 2 months vs. 12 months $^{*}p < 0.05$; $n = 6$). (C) Additionally, middle-aged mice were not able to hang upside-down as long as young or old mice during the inverted screen hang test; old mice performed worse on this assay compared to young mice but better than middle-aged animals (1-way ANOVA, $p < 0.0001$; Bonferroni post-test, 2 months vs. 12 months $^{****}p < 0.0001$, 12 months vs. 22 months $^{***}p < 0.001$, 2 months vs. 22 months $^{*}p < 0.05$; $n = 6$).

Although not the main focus of this paper, several peripherally mediated sensory changes were observed in these experiments. Prior to nociceptive insult, old mice exhibited low tactile sensitivity (i.e., increased mechanical withdrawal thresholds) compared to young mice. These data are consistent with previous reports in old C57Bl/6 mice (Garrison and Stucky, 2014) and geriatric populations, both of which show low-tactile sensitivity (Wickremaratchi and Llewelyn, 2006). However other reports demonstrate the opposite trend in old mixed strain mice (Weyer et al., 2016) and rats (Kitagawa et al., 2005), or no effect of age on tactile sensitivity at all (Wang et al., 2006). After the initiation of acute inflammatory pain, the hyposensitivity in middle-aged and old mice was reversed as both ages exhibited significant pain-like behaviors during the first phase of the formalin test and robust ipsilateral mechanical allodynia for at least 180 minutes following injection. These data suggest gradual increases in peripheral nociceptive sensitivity that first manifest in mid-life and persist through old age. Similar changes in pain sensitivity are also observed in human populations; many chronic pain disorders are first diagnosed in mid-life, however pain sensitivity continues to increase with age (Andersson et al., 1993; Bouhassira et al., 2008; Patel et al., 2013). The possible mechanisms responsible for altered peripheral nociceptive transmission are several fold; many others have demonstrated aging effects on peripheral afferent structure, channel protein expression, and resulting electrophysiological integrity (Garrison and Stucky, 2014; Wang et al., 2006; Weyer et al., 2016). Relevant to these studies, however, expression of TrpA1, the cation channel responsible for formalin-induced pain, is similarly increased in dorsal root ganglia of young and old mice at acute time points following complete Freund's adjuvant paw injections (Weyer et al., 2016). We expect that similar age-independent increases in TrpA1 occur following formalin injection, thus eliminating this target as the sole generator of age-dependent pain sensitivities. Additionally, the aging immune system cannot be ruled out as age is known to affect inflammatory responses and healing following injury (Arnardottir et al., 2014; Gagliese, 2009; Shaw et al., 2013).

Following formalin injection, old mice developed tactile hypersensitivity in the noninjected paw most likely as a result of altered central nociceptive systems. With efferent connections to key descending nociceptive regions including the periaqueductal gray and rostral ventromedial medulla, the CeA is well positioned to modulate both higher cortical components of pain and more rudimentary reflexive pain responses like the paw withdrawal behaviors measured in these experiments (Veinante et al., 2013). Previously, contralateral paw hypersensitivity was reported in young Swiss Webster and C57Bl/6 male mice following formalin injection; these behaviors were credited to mGluR5 and ERK1/2 signaling in the right CeA (Carrasquillo and Gereau, 2007; Kolber et al., 2010b). In line with one of these reports, we too observed increases in ERK1 phosphorylation in the right CeA 3 hours following formalin injection (Kolber et al., 2010b). Although ERK1 phosphorylation generally increased with age, the most robust increases in activation of this protein were observed in the right CeA of old mice; significant increases in right-lateralized pERK1 expression were not observed in young or middle-aged mice. To definitively contribute altered age-dependent nociceptive behaviors to increases in ERK1 phosphorylation, functional blockade of MEK, the ERK1/2 kinase should be performed in young, middle, and old age mice. Unfortunately, these experiments were beyond the scope of the current study. No age or side-dependent increases in ERK2 phosphorylation were observed 180 minutes following formalin injection. This is in contrast with previous reports in which young mice (both Swiss Webster and C57Bl/6 males) developed contralateral hypersensitivity and expressed increased levels of pERK2 in the right CeA 180 minutes following formalin injection (Carrasquillo and Gereau, 2007; Kolber et al., 2010b). Result differences may be due to formalin concentration (4% dose used in these studies was validated in unpublished study from our lab), animal strain, or animal source among other variables. Nonetheless,

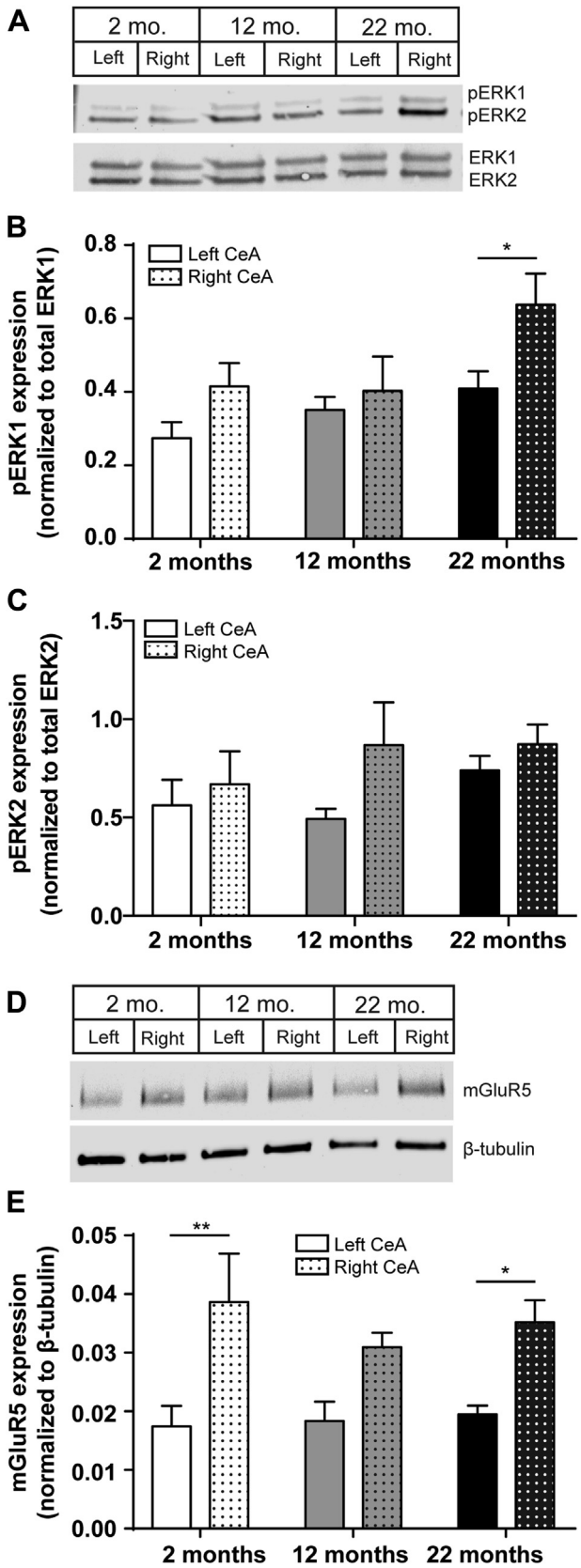

Fig. 5. Lateralized, age-dependent changes in CeA nociceptive signaling occur after peripheral formalin injection. Peripheral injury is known to increase ERK activation partially through the signaling cascade downstream of mGluR5. Western analysis was used to evaluate these proteins 180 after formalin testing. (A) Representative blot of pERK and total ERK in the left and right CeA of young (2 months), middle age (12 months) and old (22 months) mice. (B) Quantification of ERK1 activation showed significant age-dependent effects and that old animals express more pERK1 in the right CeA compared to the left CeA (2-way ANOVA, effect of age $p = 0.0229$, effect of side of brain $p = 0.0219$; Bonferroni post-test, 22 months left vs. right $^{*}p < 0.05$; $n = 6–9$). (C) No age-dependent changes were noted in pERK2 expression following formalin injection (2-way ANOVA, no main effect of age $p = 0.4476$; $n = 6–9$). (D) Representative blot of mGluR5 and loading control beta-actin in the left and right CeA of young (2 months), middle age (12 months) and old (22 months) mice. (E) Quantification revealed that following formalin injection, CeA mGluR5 expression is increased to approximately the same level across all age groups (2-way ANOVA, effect of age $p = 0.7882$; $n = 6$). Notably, most of the mGluR5 expression changes appeared to occur in the right CeA; both young and old mice exhibit significantly more mGluR5 in the right CeA than the left CeA (effect of side of brain $p < 0.0001$; Bonferroni post-test, 2 months right vs. left $^{**}p < 0.01$, 22 months right vs. left $^{*}p < 0.05$). Abbreviations: CeA, central nucleus of the amygdala; ERK1, extracellular signal-regulated kinase 1; mGluR5, metabotropic glutamate receptor 5; pERK1, phosphorylated extracellular signal-regulated kinase 1.

the finding of significant age-dependent changes in pERK1, and not pERK2, highlights the fact that these proteins, while similar, do not always perform parallel functions. Other functional differences between ERK1 and ERK2 have been found in peripheral nociceptive systems where ERK2 seems to play a larger role in modulating pain-like behavior and nociceptor development (Alter et al., 2010; O'Brien et al., 2015).

ERK phosphorylation in the CeA occurs downstream of multiple signaling cascades including group I metabotropic glutamate receptor (mGluR1/5) signaling pathways which can include protein kinase C (Carrasquillo and Gereau, 2007; Cheng et al., 2011) or protein kinase A (Fu et al., 2008). To determine if expression of mGluR5 was linked to age-dependent changes in ERK1 phosphorylation, and potentially nociceptive behaviors, mGluR5 levels were analyzed at baseline and 180 minutes following formalin injection. In the naïve condition, older animals trend towards decreased mGluR5 in the CeA, providing 1 potential central mechanism for the decreased baseline mechanical sensitivity observed in these animals. About 180 minutes after formalin injection, there was no significant difference in right CeA mGluR5 expression between young, middle age, or old mice, suggesting that other receptors and signaling systems are driving the changes in ERK1 activation.

To our knowledge, only 2 other papers have investigated the effects of age on signaling in the amygdala, a region that is not only important for acute pain modulation but also for the development and maintenance of chronic pain disorders which disproportionately affect older populations (Badowska-Szalewska et al., 2015; Hess et al., 1981; Veinante et al., 2013). These papers demonstrated age-dependent increases in expression of IL-β, an inflammatory cytokine, and opioid receptors in the amygdala, again suggesting that mGluR5 and MEK signaling pathways are not the exclusive modulators of age-dependent nociceptive behaviors (Badowska-Szalewska et al., 2015; Hess et al., 1981). Additional studies are required to determine if the observed behavioral and molecular changes are applicable across sexes and relative to chronic pain susceptibility and severity. Further studies are also needed to determine if changes in other central nervous system structures, especially those anatomically and functionally connected to the amygdala, contribute to age-dependent changes in pain sensitivity.

This basic science report, and others like it, is critical for understanding how aging affects central nociceptive systems. Overall, these data support the conclusion that gradual molecular changes in central regions like the CeA can lead to altered pain-related behavior in old animals.

Disclosure statement

The authors report no conflicts of interest. All animal protocols were in accordance with National Institutes of Health guidelines

and were approved by the Animal Care and Use Committee at Duquesne University (Pittsburgh, PA; protocol #1410-14).

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References

Alter, B.J., Zhao, C., Karim, F., Landreth, G.E., Gereau, R.W., 2010. Genetic targeting of ERK1 suggests a predominant role for ERK2 in murine pain models. J. Neurosci. 30, 11537–11547.

Andersson, H.I., Ejlertsson, G., Leden, I., Rosenberg, C., 1993. Chronic pain in a geographically defined general population: studies of differences in age, gender, social class, and pain localization. Clin. J. Pain 9, 174–182.

Arnardottir, H.H., Dalli, J., Colas, R.A., Shinohara, M., Serhan, C.N., 2014. Aging delays resolution of acute inflammation in mice: reprogramming the host response with novel nano-proresolving medicines. J. Immunol. 193, 4235–4244.

Badowska-Szalewska, E., Ludkiewicz, B., Spodnik, J.H., Krawczyk, R., Moryś, J., 2015. The influence of mild stressors on neurons containing interleukin-1β in the central (CeA) and medial (MeA) amygdala in the ageing process of rats. Acta Neurobiol. Exp. (Wars) 75, 279–292.

Bouhassira, D., Lantéri-Minet, M., Attal, N., Laurent, B., Touboul, C., 2008. Prevalence of chronic pain with neuropathic characteristics in the general population. Pain 136, 380–387.

Carrasquillo, Y., Gereau, R.W., 2008. Hemispheric lateralization of a molecular signal for pain modulation in the amygdala. Mol. Pain 4, 24.

Carrasquillo, Y., Gereau 4th, R.W., 2007. Activation of the extracellular signal-regulated kinase in the amygdala modulates pain perception. J. Neurosci. 27, 1543–1551.

Chaplan, S.R., Bach, F.W., Pogrel, J.W., Chung, J.M., Yaksh, T.L., 1994. Quantitative assessment of tactile allodynia in the rat paw. J. Neurosci. Methods 53, 55–63.

Cheng, S.-J., Chen, C.-C., Yang, H.-W., Chang, Y.-T., Bai, S.-W., Chen, C.-C., Yen, C.-T., Min, M.-Y., 2011. Role of extracellular signal-regulated kinase in synaptic transmission and plasticity of a nociceptive input on capsular central amygdaloid neurons in normal and acid-induced muscle pain mice. J. Neurosci. 31, 2258–2270.

Crock, L.W., Kolber, B.J., Morgan, C.D., Sadler, K.E., Vogt, S.K., Bruchas, M.R., Gereau, R.W., 2012. Central amygdala metabotropic glutamate receptor 5 in the modulation of visceral pain. J. Neurosci. 32, 14217–14226.

Dutta, S., Sengupta, P., 2016. Men and mice: relating their ages. Life Sci. 152, 244–248.

Fu, K.Y., Light, A.R., Maixner, W., 2001. Long-lasting inflammation and long-term hyperalgesia after subcutaneous formalin injection into the rat hindpaw. J. Pain 2, 2–11.

Fu, Y., Han, J., Ishola, T., Scerbo, M., Adwanikar, H., Ramsey, C., Neugebauer, V., 2008. PKA and ERK, but not PKC, in the amygdala contribute to pain-related synaptic plasticity and behavior. Mol. Pain 4, 26.

Gagliese, L., 2009. Pain and aging: the emergence of a new subfield of pain research. J. Pain 10, 343–353.

Gagliese, L., Melzack, R., 2000. Age differences in the response to the formalin test in rats. Neurobiol. Aging 20, 699–707.

Garrison, S.R., Stucky, C.L., 2014. Contribution of transient receptor potential ankyrin 1 to chronic pain in aged mice with complete Freund's adjuvant-induced arthritis. Arthritis Rheumatol. (Hoboken, N.J.) 66, 2380–2390.

Hess, G.D., Joseph, J.A., Roth, G.S., Forman, L.J., Meites, J., Rigter, H., McGaugh, J.L., 1981. Effect of age on sensitivity to pain and brain opiate receptors. Neurobiol. Aging 2, 49–55.

King-Himmelreich, T.S., Möser, C.V., Wolters, M.C., Olbrich, K., Geisslinger, G., Niederberger, E., 2015. Age-dependent changes in the inflammatory nociceptive behavior of mice. Int. J. Mol. Sci. 16, 27508–27519.

Kitagawa, J., Kanda, K., Sugiura, M., Tsuboi, Y., Ogawa, A., Shimizu, K., Koyama, N., Kamo, H., Watanabe, T., Ren, K., Iwata, K., 2005. Effect of chronic inflammation on dorsal horn nociceptive neurons in aged rats. J. Neurophysiol. 93, 3594–3604.

Kolber, B.J., Boyle, M.P., Wieczorek, L., Kelley, C.L., Onwuzurike, C.C., Nettles, S.A., Vogt, S.K., Muglia, L.J., 2010a. Transient early-life forebrain corticotropin-releasing hormone elevation causes long-lasting anxiogenic and despair-like changes in mice. J. Neurosci. 30, 2571–2581.

Kolber, B.J., Montana, M.C., Carrasquillo, Y., Xu, J., Heinemann, S.F., Muglia, L.J., Gereau Iv, R.W., Gereau Iv, R., 2010b. Activation of metabotropic glutamate receptor 5 in the amygdala modulates pain-like behavior 1/2 (ERK1/2). J. Neurosci. 30, 8203–8213.

Miaskowski, C., 2000. The impact of age on a patient's perception of pain and ways it can be managed. Pain Manag. Nurs. 1, 2–7.

Neugebauer, V., Li, W., Bird, G.C., Bhave, G., Gereau, R.W., 2003. Synaptic plasticity in the amygdala in a model of arthritic pain: differential roles of metabotropic glutamate receptors 1 and 5. J. Neurosci. 23, 52–63.

O'Brien, D.E., Alter, B.J., Satomoto, M., Morgan, C.D., Davidson, S., Vogt, S.K., Norman, M.E., Gereau, G.B., Demaro, J.A., Landreth, G.E., Golden, J.P., Gereau, R.W., 2015. ERK2 alone drives inflammatory pain but cooperates with ERK1 in sensory neuron survival. J. Neurosci. 35, 9491–9507.

Patel, K.V., Guralnik, J.M., Dansie, E.J., Turk, D.C., 2013. Prevalence and impact of pain among older adults in the United States: findings from the 2011 National Health and Aging Trends Study. Natl. Institutes Heal 154, 1–22.

Shaw, A.C., Goldstein, D.R., Montgomery, R.R., 2013. Age-dependent dysregulation of innate immunity. Nat. Rev. Immunol. 13, 875–887.

Sorge, R.E., Martin, L.J., Isbester, K.A., Sotocinal, S.G., Rosen, S., Tuttle, A.H., Wieskopf, J.S., Acland, E.L., Dokova, A., Kadoura, B., Leger, P., Mapplebeck, J.C.S., McPhail, M., Delaney, A., Wigerblad, G., Schumann, A.P., Quinn, T., Frasnelli, J., Svensson, C.I., Sternberg, W.F., Mogil, J.S., 2014. Olfactory exposure to males, including men, causes stress and related analgesia in rodents. Nat. Methods 11, 629–632.

Veinante, P., Yalcin, I., Barrot, M., 2013. The amygdala between sensation and affect: a role in pain. J. Mol. Psychiatry 1, 9.

Wang, S., Davis, B.M., Zwick, M., Waxman, S.G., Albers, K.M., 2006. Reduced thermal sensitivity and Nav1.8 and TRPV1 channel expression in sensory neurons of aged mice. Neurobiol. Aging 27, 895–903.

Weyer, A.D., Zappia, K.J., Garrison, S.R., O'Hara, C.L., Dodge, A.K., Stucky, C.L., 2016. Nociceptor sensitization depends on age and pain chronicity. eNeuro 3.

Wickremaratchi, M.M., Llewelyn, J.G., 2006. Effects of ageing on touch. Postgrad. Med. J. 82, 301–304.