AWARD NUMBER: W81XWH-15-1-0076

TITLE: Atypical Opioid Mechanisms of Control of Injury-Induced Cutaneous Pain by Delta Receptors

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REPORT DATE: July 2018

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
July 2018	Annual	30 Jun 2017-29 Jun 2018

4. TITLE AND SUBTITLE
Atypical Opioid Mechanisms of Control of Injury-Induced Cutaneous Pain by Delta Receptors

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-15-1-0076

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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Stanford Junior University
1050 Arastradero Road, Bldg. A, Palo
Alto, CA 94304
MC: 5589

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Severe pain due to war-related injuries is difficult to treat, and current opioids (i.e. mu opioid receptor agonists such as morphine) cause unacceptable side effects including addiction. Injuries suffered most frequently by active military personnel include traumatic brain injury, nerve trauma, skin incision, and burn injury, and all these injuries are associated with acute cutaneous pain and/or mechanical allodynia/hypersensitivity. The goals of our research are to evaluate analgesics acting on delta opioid receptors (DORs) in animal models relevant to today's battlefield experience (Specific Aim 2), and elucidate the mechanisms by which DOR agonists, administered in skin and acting on mechanosensory dorsal root ganglia neurons, relieve pain (Specific Aim 1). We have determined the analgesic effect of two DOR agonists, deltorphin II and SNC80. We show that these compounds significantly elevate mechanical pain threshold, indicating their acute antinociceptive action. Furthermore, we found that in two models of injuries, namely skin incision and nerve trauma, a single injection of deltorphin II eliminates the mechanical hyper sensitivity caused by injury. We have also initiated studies aiming at identifying the peripheral sensory neurons that express DOR, a first step towards understanding the analgesic mechanism of action of DOR agonists. We are currently extending these findings by performing the other experiments described in our original proposal, without significant change in our plans and strategy. Importantly, our promising results support our hypothesis that DOR agonists, acting in the skin, represents an effective therapeutic strategy for blocking severe pain associated with injuries that can be suffered on the battlefield.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified		**19b. TELEPHONE NUMBER** *(include area code)*

TABLE OF CONTENTS

1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

This proposal will establish the mechanisms by which peripheral delta opioid receptors (DORs) inhibit mechanosensitive dorsal root ganglion (DRG) neurons, and test the hypothesis that DOR agonists can reduce pain in rodent models of injuries that soldiers can suffer on the battlefield.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Delta opioid receptor (DOR) agonists, cutaneous pain, acute pain, injuries suffered on the battlefield, injury-induced chronic pain, burn injury, incision injury, nerve injury, analgesia, mouse, intraplantar injections, pain behavior, electrophysiology, dorsal root ganglion neurons, histology, mechanism of action

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Specific Aim 1 – To resolve the mechanism of action by which peripherally administered DOR agonists inhibit neuronal activity.

Major Task 1: Electrophysiological analysis
Subtask 1: To establish the effect of DOR agonists on the activity of mechanosensitive DRG neurons using a largely intact ex vivo somatosensory system preparation. Months 1-30
Percentage of completion: 60%
Estimated time for completing Milestone #1 Demonstration that DOR agonists inhibit action potential firing in cutaneous mechanosensitive DRG neurons: 42 months (12 months later than the 30 months originally scheduled, originally due to delays in obtaining approval for our animal protocol and then due to move of Dr. Woodbury's laboratory from the University of Wyoming to the University of Utah)

Subtask 2: To resolve the molecular mechanisms by which DOR agonists inhibit DRG neurons in primary culture. Months 6-30
Percentage of completion: 90%
Estimated time for completing Milestone #2 Resolution of the mechanism of action of DOR agonists: 42 months (12 months later than the 30 months originally scheduled, due to delays in obtaining the electrophysiological equipment for performing these experiments)

Major Task 2: Characterization of DOR-expressing neurons in human DRG
Subtask 1: To characterize DOR-expressing neurons in human DRG by in situ hybridization Months 12-24
Percentage of completion: 70%

Estimated time for completing Milestone #3 Demonstration that DOR expression in human DRG is similar to that observed in mouse: 42 months (18 months later than the 24 months originally scheduled, due to problems with the high endogenous fluorescence of the tissue received from NDRI (this problem is now solved) and delays in obtaining tissues from NDRI)

Specific Aim 2 –To test the hypothesis that peripheral administration of DOR agonists can reduce acute cutaneous pain and chronic mechanical allodynia in rodent models of injuries that soldiers can suffer on the battlefield.

Major Task 3: Acute pain
Subtask 1: To evaluate the utility of DOR agonists for the treatment of acute cutaneous pain. Months 6-24
Estimated percentage of completion: 100%

Estimated time for completing Milestone #4 Demonstration of the efficacy of DOR agonists to reduce acute pain: 24 months (no change)

Major Task 4: Injury-induced mechanical allodynia: 90%
Subtask 1: To evaluate the utility of DOR agonists for the treatment of nerve trauma-induced mechanical allodynia. Months 6-30
Estimated percentage of completion: 100%

Subtask 2: To evaluate the utility of DOR agonists for the treatment of mechanical allodynia induced by incision injury. Months 1-24
Estimated percentage of completion: 100%

Subtask 3: To evaluate the utility of DOR agonists for the treatment of burn injury-induced mechanical allodynia. Months 12-36
Percentage of completion: 70%

Estimated time for completing Milestone #5 Demonstration of the efficacy of DOR agonists to reduce injury-induced mechanical allodynia: 36 months (as originally scheduled, the final experiments are ongoing)

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

1. Major Task 1: Electrophysiological analysis
Subtask 1: To establish the effect of DOR agonists on the activity of mechanosensitive DRG neurons using a largely intact ex vivo somatosensory system preparation.
Subtask 2: To resolve the molecular mechanisms by which DOR agonists inhibit DRG neurons in primary culture.

When performing electrophysiological studies in DRG neurons (see 2017 Annual Report), we have noticed that, contrary to the prevailing view, DOR agonists not only have presynaptic effects on DRG mechanosensory neurons, but also postsynaptic effects. Thus, DOR agonists not only reduced firing in DRG neurons, but also in connected second order neurons in the dorsal horn, suggesting DOR expression in these cells. This serendipitous finding had the potential to transform our understanding of delta opioid receptor analgesic mechanism of action. We thus verified that DOR is expressed by spinal neurons, using the histological techniques that we use to study DOR distribution and function in DRG (immunohistochemistry and in situ hybridization) (Figure 1, A-D). We first used DORGFP reporter mice and GFP immunolabeling to determine the DOR expression pattern in the spinal cord. We observed diffuse DORGFP expression throughout the spinal

cord grey matter, with a relatively brighter DORGFP+ band in lamina II (Figure 1A, left). This broadly-distributed DORGFP signal, which includes regions with limited primary afferent innervation, such as the lateral spinal nucleus, suggested the presence of DORGFP+ spinal cells. However, we could not unambiguously identify DORGFP+ cell bodies. To address this problem, we took advantage of the trafficking properties of DOR, wherein binding of agonists results in internalization and accumulation of the receptor in perinuclear lysosomes. Strikingly, treating DORGFP mice with the DOR agonist SNC80 uncovered the distribution of a very large number of DOR+ cell bodies, both in the dorsal and ventral horns (Figure 1A, right). SNC80-mediated internalization of DOR in tissue sections from DORGFP mice also enabled characterization of the DOR+ cells by immunohistochemistry.

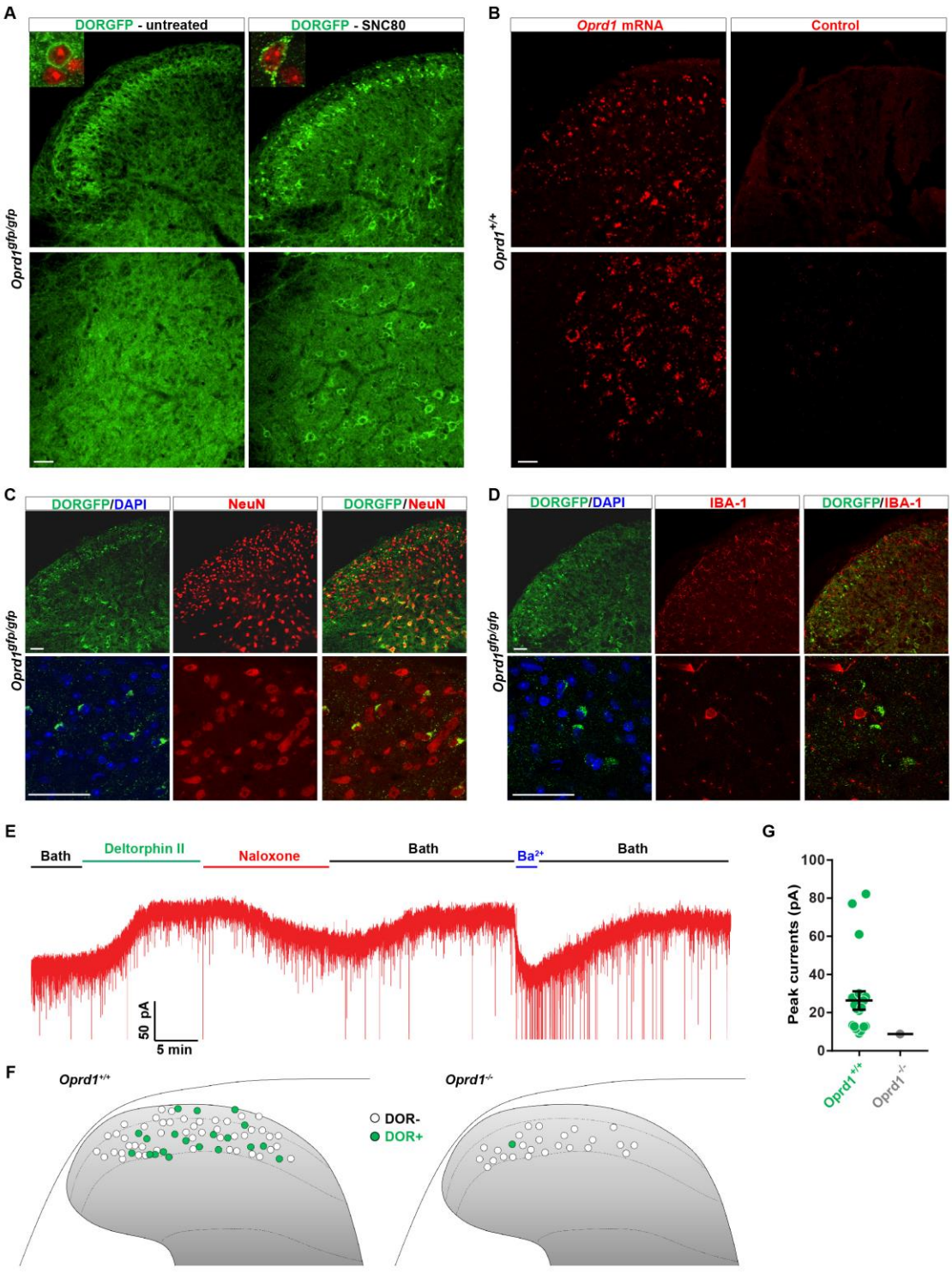

Figure 1. *DOR expression and function in second order spinal cord neurons of pain neural circuits. (A) Staining with anti-GFP antibody in spinal cord sections from either untreated or SNC80-treated DORGFP knockin mouse. Inset image shows DORGFP subcellular distribution at the cell plasma membrane (untreated) or in the cytosol (SNC80-treated). Red color corresponds to DAPI staining of the nucleus. (B) Oprd1 mRNA is detected in spinal cord sections from wild-type mice. The Oprd1 mRNA distribution pattern matches that of DORGFP+ cells. (C) Co-localization of DORGFP with the neuronal marker NeuN indicates that DORGFP+ cells are neurons. (D) DORGFP+ cells do not express the marker IBA-1 and therefore are not microglia. (E) Representative trace showing that the DOR agonist deltorphin II activates GIRK channels in spinal cord dorsal horn neurons in wild-type mice. Both the opioid receptor antagonist naloxone and the non-selective potassium channel blocker Ba2+ can block these deltorphin II-induced outward currents. (F) Schematic map showing the location of all recorded dorsal horn neurons in slices from wild-type or DOR knockout mice. Recorded neurons labeled in green presented deltorphin II-induced GIRK currents. (G) Quantification of GIRK peak current in deltorphin II-responsive neurons in wild-type and DOR KO mice shown in (F). Box plots in (G) show median, 25th and 75th percentile, and min and max values. Scale bars represent 50 μM*

For example, DORGFP+ cells coexpress the pan neuronal marker NeuN (Figure 1C), but not the microglial markers IBA-1 (Figure 1D), P2Y12, or CD11b (Figures S1A and S1B), indicating that DOR+ cells in the spinal cord are neurons. Labeling of the central terminals of CGRP+ and IB4+ nociceptors, and of PKC gamma interneurons, indicated that DORGFP+ neurons are particularly enriched at the ventral border of lamina II inner (lamina IIiv), which is defined by the presence of PKC gamma interneurons (Figures S1C and S1D). We next used in situ hybridization and electrophysiology in wild-type mice to test further the hypothesis that DOR is expressed by spinal neurons. Consistent with DOR expression pattern in DORGFP mice, Oprd1 mRNA is present in numerous neurons throughout the spinal cord grey matter of wild-type mice, mainly in small lamina II neurons, and in larger neurons in the ventral horn (Figure 1B).

In CNS neurons, postsynaptic opioid receptors are generally coupled to G protein-coupled inwardly-rectifying potassium channels (GIRK channels). We therefore performed whole cell patch clamp recordings of randomly-selected neurons in spinal cord slices of wild-type mice, focusing on lamina II. We bath perfused the DOR agonist deltorphin II, and monitored GIRK channels-mediated increases in holding currents. We found that deltorphin II induced an outward current in 29.4% (20/68) of recorded neurons (Figures 1E-G). Deltorphin II-responsive neurons were concentrated in lamina II inner, in agreement with the distribution of both DORGFP and Oprd1 mRNA. Naloxone, an opioid receptor antagonist, or barium (Ba2+), a potassium channels blocker, blocked the deltorphin II-induced currents (Figure 1E). To confirm the deltorphin II selectivity for DOR, we performed identical recordings in spinal cord slices from Oprd1 knockout mice. In only one out of 26 recorded neurons did we observe a small deltorphin II-induced GIRK current (8.8 pA), possibly due to deltorphin II-mediated activation of other opioid receptors in the absence of DOR (Figures 1F and 1G). Based on these results, we conclude that contrary to previous reports, DOR is not only expressed by primary afferent neurons, but also by numerous spinal neurons, both in the dorsal and ventral horns.

We next characterized the different populations of DOR+ spinal neurons. Superficial dorsal horn neurons, which originate from the Lbx1+ neuronal lineage, can be divided into TLX3+ excitatory and PAX2+ inhibitory neurons. Co-immunostaining revealed that 60.5% of the DORGFP+ neurons located in lamina II express TLX3, but that only 8.5% express PAX2 (Figures 2A, 2B and 2E). Furthermore, we crossed DORGFP mice with Vglut2Cre;Rosa26LSL-tdTomato and VgatCre;Rosa26LSL-tdTomato reporter mice and found that 85.0% of DORGFP+ lamina II neurons co-express the vesicular glutamate transporter 2 (VGLUT2), while only 24.6% co-express the vesicular GABA transporter (VGAT) (Figures 2C-E). Consistent with these anatomical results, whole cell recordings from VGLUT2/tdTomato+ or VGAT/tdTomato+ lamina II neurons in mice with a wild-type Oprd1 allele showed that 50.0% (8/16) of VGLUT2/tdTomato+ and 25.0% (4/16) of VGAT/tdTomato+ neurons displayed deltorphin II-induced GIRK currents, respectively (Figure 2F). For lamina II interneurons, neuro¬transmitter phenotype is thought to be correlated with action potential (AP) firing patterns; inhibitory neurons most commonly display a tonic AP firing pattern, while excitatory neurons generally show either delayed, gap or single AP firing patterns. Excitatory interneurons also display more negative resting membrane

potentials, compared to inhibitory interneurons. We found that 80.0% (16/20) of DOR+ neurons show a delayed, gap or single firing pattern (Figures 2G and 2H), and that DOR+ neurons displayed a more negative resting membrane potential (-72 mV), compared to DOR-negative neurons (-64 mV) (Figure 2I). Together, these results indicate that the great majority of DOR+ neurons in the dorsal horn are lamina II excitatory interneurons.

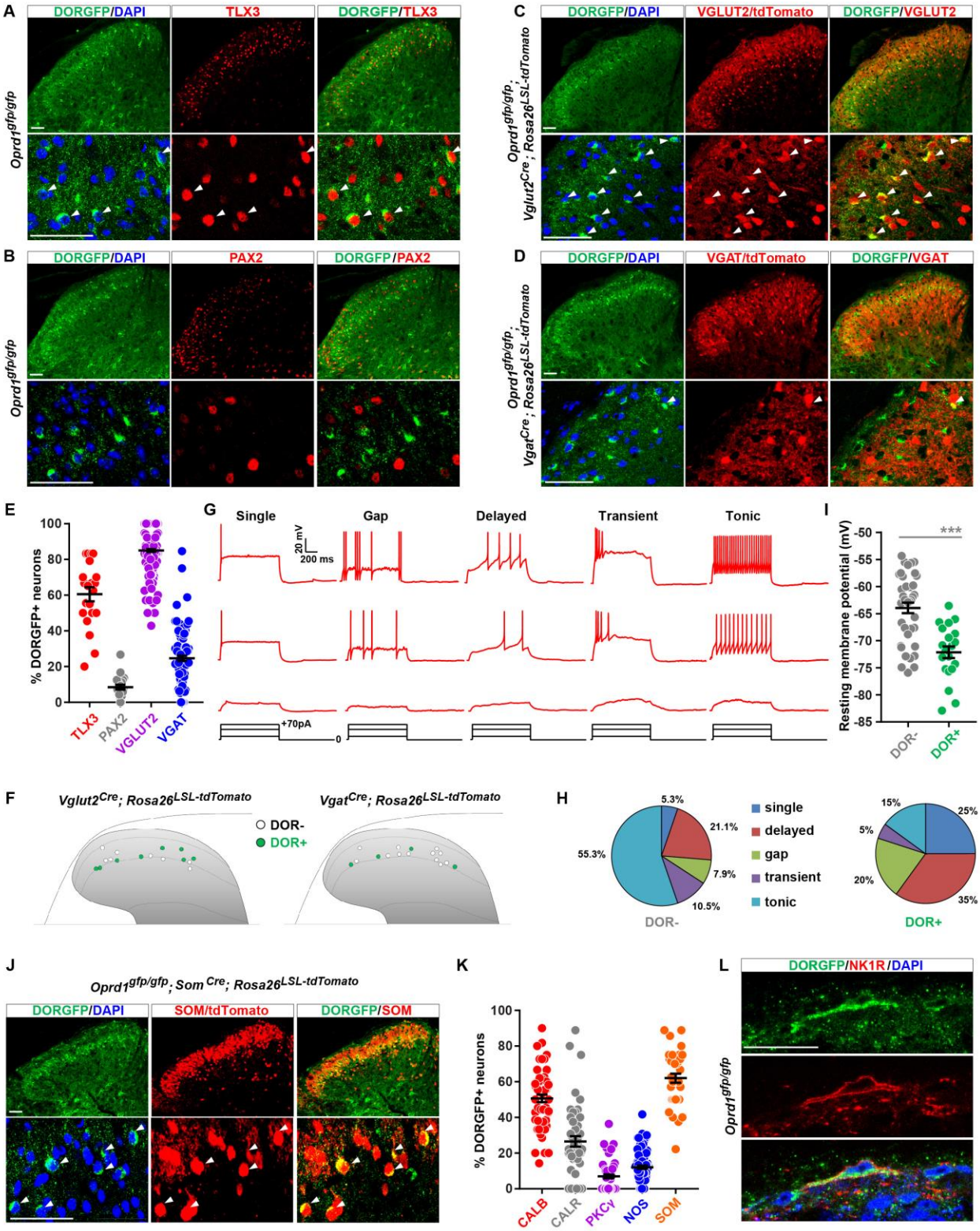

Figure 2. *Most DOR-expressing neurons in the dorsal horn are lamina II excitatory interneurons.*

(A) DORGFP+ neurons frequently co-express TLX3 (white arrowheads). (B) DORGFP+ neurons rarely express PAX2. (C) Most DORGFP+ neurons express VGLUT2/tdTomato (white arrowheads). (D) Few DORGFP+ neurons express VGAT/tdTomato (white arrowheads).
(E) Quantification of (A)-(D). Dots represent individual counts in spinal cord sections from 3-6 mice. (F) Schematic map of the location of VGLUT2+ or VGAT+ neurons that responded to deltorphin II (i.e., DOR+, green). n=16 neurons for each. (G) Whole cell recording indicated different AP firing patterns in deltorphin II-responsive neurons in wild-type mice.
(H) Proportions of DOR+ or DOR- neurons showing the different AP firing patterns.
*(I) DOR+ neurons have a more negative resting membrane potential compared to DOR- neurons. ***, $P < 0.001$ with unpaired t test. (J) DORGFP+ lamina II neurons frequently co-express somatostatin (white arrowheads). (K) Quantification of DORGFP+ neurons co-expression with multiple other neuronal markers. Dots represent individual counts in spinal cord sections from 3-6 mice. (L) DOR is expressed by a subpopulation of lamina I NK1R+ projection neurons.*
Data are presented as mean ± SEM in (E), (I) and (K). Scale bars represent 50 μM.

We further resolved the molecular identity of lamina II DORGFP+ excitatory neurons using antibodies or reporter mouse lines that distinguish dorsal horn interneuron subpopulations, including calbindin (CALB), calretinin (CALR), nitric oxide synthase (NOS), PKC□, and somatostatin (SOM) (Figures 2J, 2K and S2A-D). These experiments demonstrated that, among these markers, SOM is most often co-expressed by DORGFP+ lamina II neurons (62.0%). Although SOM+ neurons are distributed throughout lamina II, we found that the great majority of DOR+ SOM+ neurons are located in lamina IIiv, at the ventral border between laminae II-III (Figure 2J), intermixed with PKCgamma+ interneurons, which only rarely express DOR (Figures 2K and S2D). The rare DORGFP+ lamina IIiv neurons that co-express NOS likely correspond to a small population of SOM-negative inhibitory interneurons (Figures 2K and S2C). DOR+ excitatory interneurons also frequently co-express CALB, and to a lesser extent CALR, particularly when they are located more dorsally in lamina II (Figures 2K and S2A-B). To complete the characterization of DOR+ neurons in the dorsal horn, we also identified the few DOR+ neurons present in lamina I, in laminae III-V, and in the lateral spinal nucleus (LSN). Co-expression of DOR with neurokinin 1 receptor (NK1R), the receptor for substance P, in large lamina I and LSN neurons suggests that DOR is present in a subpopulation of glutamatergic projection neurons that relay pain information to the brain (Figure 2L). Finally, we determined that DOR is absent from lamina III, which contains parvalbumin+ (PARV) inhibitory neurons that gate mechanical hypersensitivity, but frequently is co-expressed with PARV in deeper dorsal horn neurons (laminae IV-V) (Figure S2E).

Given our finding that DOR is co-expressed with SOM in lamina II interneurons, we hypothesized that DOR+ lamina II neurons might also be part of mechanical pain circuits. To test this hypothesis, we first stimulated the hindpaw of DORGFP mice with a noxious mechanical stimulus and used Fos immunostaining to identify mechano-nociceptive dorsal horn neurons. Figures 3A and 3B show that 40.0% of DORGFP+ lamina II neurons were Fos+, among which 22.3% were also SOM+ (Figures S3A and S3B), indicating that DOR is indeed expressed by dorsal horn neurons that process cutaneous mechano-nociceptive information. Adelta and Abeta fibers, including myelinated mechanonociceptors (AMs) and low-threshold mechanoreceptors (A-LTMRs) are essential contributors to cutaneous mechanosensation. Using spinal cord slices with dorsal root attached, we found that the majority of DORGFP+ lamina II neurons receive mono- and poly-synaptic Adelta and Abeta inputs, but rarely C fiber inputs (Figures 3C and 3D). Additionally, these recorded lamina II DORGFP+ neurons extend a process dorsally, towards lamina I (Figure 3E), suggesting that these cells may relay mechanosensory myelinated afferent input to lamina I projection neurons.

DOR agonists are particularly effective at reducing pain provoked by mechanical stimuli, however, the mechanisms underlying these properties remain unclear. DOR agonist antinociceptive effects are observed following not only systemic but also intrathecal delivery of the drug, and were thought to result from an action on DORs present on primary afferent central terminals. Our finding that DOR is expressed by mechano-nociceptive dorsal horn neurons suggested that DOR agonists might also act centrally on DORs in SOM+

lamina II neurons, to reduce mechanical pain. To test this possibility, we deleted DOR selectively from SOM+ neurons, by crossing mice bearing conditional (i.e. floxed) Oprd1 alleles (Oprd1lox/lox mouse) with mice in which Cre recombinase expression is driven by the somatostatin gene (SomCre). We then evaluated the ability of intrathecal deltorphin II to decrease sensitivity to mechanical stimulation in the DOR conditional knockout mice (DOR cKO), by stimulating the mouse hindpaw with calibrated von Frey hairs or pinprick, and recording nociceptive withdrawal responses. We found that the deltorphin II-induced decrease in mechanical sensitivity observed in control littermates is lost in DOR cKO mice (Figure 3F). Similarly, in models of neuropathic and inflammatory mechanical hypersensitivity, deltorphin II anti-allodynic effect was profoundly reduced following deletion of DOR in SOM+ neurons (Figures 3G and S3G). As we did not observe expression of DOR in the SOM+ DRG neurons (Figures S3C and S3D), and very limited Som expression in ventral horn (Figures S3E and S3F), the effect is dorsal horn SOM+ neuron specific. This result suggests that intrathecal DOR agonists act, at least in part, on DOR expressed by SOM neurons to diminish mechanical sensitivity, and that action of the drug exclusively on presynaptic DORs expressed by DRG neurons is not sufficient to cause a significant antinociceptive effect. Finally, we also measured the effect of deltorphin II on heat sensitivity and found that the antinociceptive action of deltorphin II in the hotplate and tail immersion tests was intact in DOR cKO mice (Figure 3H).

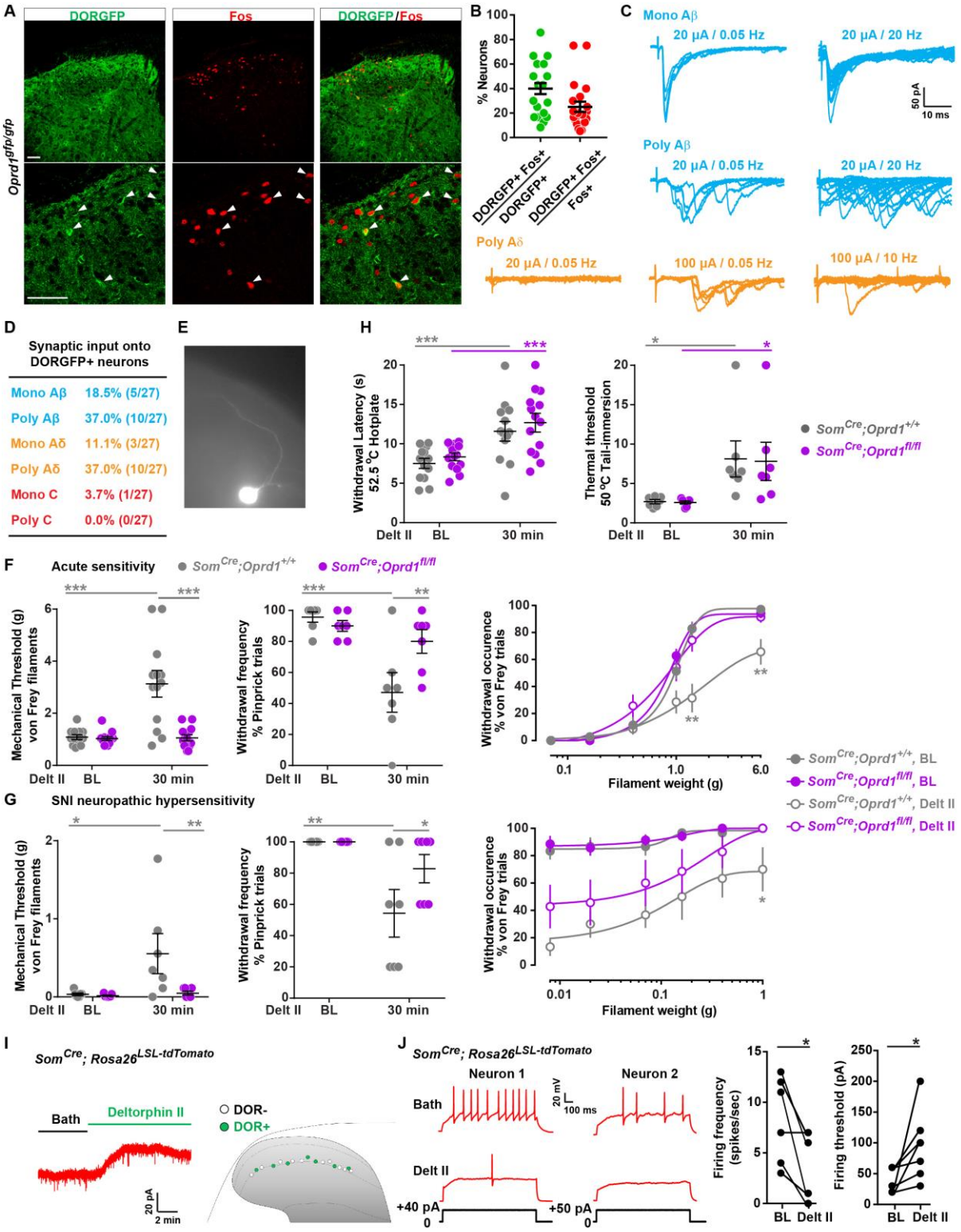

Figure 3. DOR Agonist Inhibits SOM+ Interneurons to Decrease Mechanical Pain.

(A) Noxious mechanical stimulation of the hindpaw of DORGFP mice induced Fos expression in DORGFP+ dorsal horn neurons (white arrowheads). (B) Quantification of (A). Dots represent individual counts in spinal cord sections from 3 mice. (C) Representative traces showing Aβ and Aδ fiber input to DORGFP+ neurons. (D) Summary of (C). n=27 neurons. (E) Morphology of a DOR+ neuron during recording. (F) Decreased effect of intrathecal deltorphin II (1 μg) against acute mechanical nociception in DOR cKO mice. Von Frey threshold test: n=13 control mice and 14 DOR cKO mice; pinprick and von Frey frequency tests: n=7 control mice and 7 DOR cKO mice. (G) Diminished anti-allodynic effect of intrathecal deltorphin II (15 μg) against SNI-induced

*neuropathic hypersensitivity in DOR cKO mice. Von Frey threshold and pinprick tests: n=7 control mice and 7 DOR cKO mice; von Frey frequency test: n=6 control mice and 7 DOR cKO mice. (H) The antinociceptive action of intrathecal deltorphin II (1 μg) against heat nociception (hotplate and tail immersion tests) is intact in DOR cKO mice. Hotplate test: n=13 control mice and 14 DOR cKO mice); tail immersion test: n=7 control mice and 7 DOR cKO mice. (I) Representative trace showing deltorphin II-induced GIRK currents (labelled in green) in tdTomato+ lamina II neuron in slices from SomCre;Rosa26LSL-tdTomato mice. (J) Deltorphin II reduces the AP firing rate and increases the AP firing threshold in SOM/tdTomato+ neurons (n = 7). *, P < 0.05, **, P < 0.01, ***, P < 0.001, (F-H), repeated measures, Two-way ANOVA + Bonferroni; (J), paired t-test.*
Data are presented as mean ± SEM in (B) and (F-H). Scale bars represent 50 μM.

To test the possibility that deltorphin II could decrease mechanical sensitivity by influencing SOM+ interneuron excitability, we next recorded from tdTomato+ lamina II neurons in spinal cord slices from SomCre;Rosa26LSL-tdTomato mice. Figure 3I shows that bath application of deltorphin II caused an increase in holding current in about half of the tdTomato+ recorded neurons. Furthermore, in the neurons in which we observed this hyperpolarization (i.e. deltorphin II responsive and thus DOR+), deltorphin II significantly decreased action potential firing (Figure 3J).

Collectively, these findings have important implications for this proposal as they establish that DOR agonists can act both peripherally on DRG neurons and on second order dorsal horn neurons, making this receptor an ideal target to reduce mechanical pain resulting from injuries suffered on the battlefield.

- Major Task 3: Acute pain
Subtask 1: To evaluate the utility of DOR agonists for the treatment of acute cutaneous pain.

We have completed behavioral studies to determine whether intraplantar injection of the two DOR agonists deltorphin II and SNC80 can reduce acute mechanical pain. As shown in Figure 4, we found that the intraplantar injection of both deltorphin II and SNC80 profoundly elevate the threshold for mechanical pain in the von Frey (Figure 4A and 4C) and pinprick tests (Figure 4B and 4D). Deltorphin II and SNC80 significantly reduced pain behaviors elicited by pinprick stimulation of the plantar surface of the injected hindpaw, compared to injection of the vehicle solution. These results suggest that SNC80 acts more rapidly, as soon as 20 min after the injection, while the effect of deltorphin is comparatively delayed. Together, these studies validate our hypothesis that peripheral DORs can be targeted to decrease acute cutaneous pain.

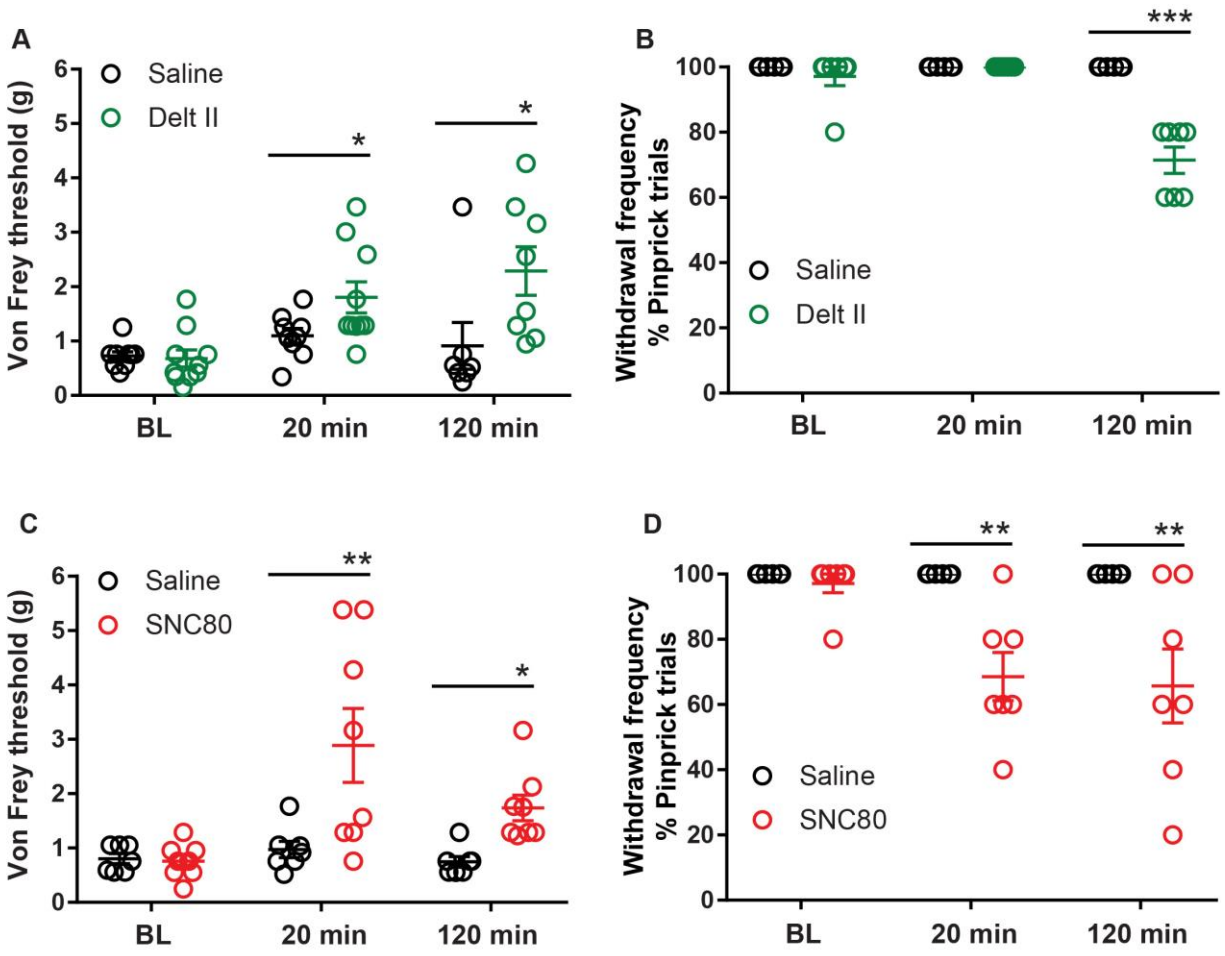

Figure 4.

Effect of the intraplantar DOR agonists deltorphin II (Delt II) and SNC80 on acute mechanical pain*. (A) and (C). Intraplantar injection of both deltorphin II (10 micrograms) and SNC80 (25 micrograms) elevated the mechanical sensitivity threshold measured with von Frey hair with the up-down method, indicating that activation of peripheral DORs can reduce acute cutaneous mechanical sensitvity. (B) and (D). Pinprick stimulation of the hindpaw plantar surface causes withdrawal responses at baseline, indicating acute mechanical pain (BL, left). A single intraplantar injection of deltorphin II (10 micrograms) or SNC80 (25 micrograms) increased mechanical threshold as evidenced by the decreased withdrawal frequency, indicating diminished pain. Data are represented as mean + SEM (error bars). Statistical analysis used a Repeated Measures ANOVA and Bonferroni posthoc test. * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001. n = 6-10 mice/ groups.*

- Major Task 4: Injury-induced mechanical allodynia

Subtask 3: To evaluate the utility of DOR agonists for the treatment of burn injury-induced mechanical allodynia.

We have performed behavioral studies to determine whether intraplantar injection of DOR agonist can reduce the mechanical hypersensitivity resulting from injuries, including skin incision, nerve trauma, or burn. We previously reported the remarkable analgesic effect of intraplantar DOR agonist against mechanical hypersensitivity resulting from nerve trauma or skin incision.We report here that SNC80 also reduces hypersensitivity resulting from burn injury. Figure 2 below shows that burn caused cutaneous mechanical hypersensitivity for several days, as measured with the von Frey test. Remarkably, a single intraplantar injection

of SNC80 was sufficient to almost completely eliminate this hypersensitivity, 120 min after the injection. We are presently completing these studies. Collectively, these data support our claim that activation of peripheral DORs is an efficient therapeutic strategy for reducing cutaneous mechanical pain that results from injuries that can be suffered on the battlefield.

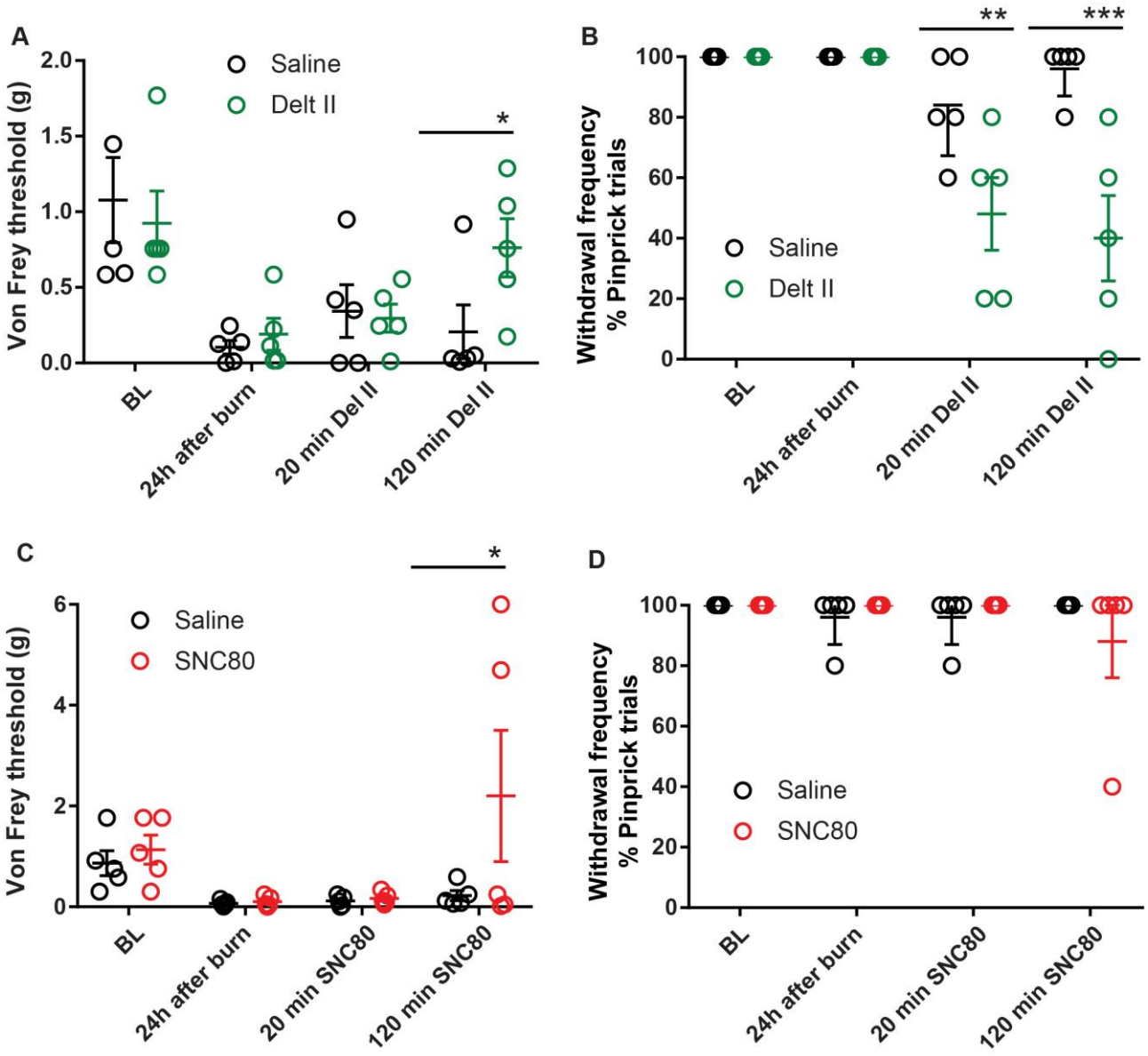

Figure 5. Effect of intraplantar DOR agonists deltorphin II and SNC80 on mechanical hypersensitivity induced by burn injury. *(A) and (C). Burn injury reduced mechanical sensitvity threshold measured with the von Frey test 24 hours after exposure to noxious heat (i.e. indicating pain) compared to baseline (BL). A single intraplantar injection of deltorphin II (10 micrograms) or SNC80 II (25 micrograms) increased this mechanical threshold, indicating diminished allodynia 120 min after injection. (A) and (C). (B) and (D). Deltorphin II, but not SNC80, additionally reduced burn-induced cutaneous mechanical hyperalgesia in the pinprick test. Altogether, these results suggest that DOR activation in the periphery efficiently limits hypersensitivity resulting from burn injuries. Data are represented as mean + SEM (error bars). Statistical analysis used a Repeated Measures ANOVA and Bonferroni posthoc test. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$. n = 5-10 mice/ groups.*

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

- **Amaury François, PhD, postdoctoral fellow, (electrophysiological studies, Major Task 1)**
  Amaury François attended the Society for Neuroscience meeting (November 11-15, Washington DC) and presented a poster on his work.

  Amaury François successfully transitioned to the next stage of his career and started a PI position at CNRS, the French National Center for Scientific Research (the equivalent in France of the NIH intramural program), on January 1 2018.

- **Dong Wang, PhD, postdoctoral fellow (histological and behavioral studies, Major Tasks 2-4)**
  Dong Wang attended the Stanford Neurosciences Institute Symposium (October 19th, 2017, Stanford University) and presented a poster on his work.
  Dong Wang was invited to present his work at the Department of Anesthesiology of Anhui Medical University (April 12, 2018) and the School of Life Science, University of Science and Technology of China (April 13, 2018) in the city of Hefei, China.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

The results were disseminated through publications and public seminars and conferences

Publications:

Wang D, Tawfik VL, Corder G, Low SA, François A, Basbaum AI, **Scherrer G**. Functional divergence of delta and mu opioid receptor organization in CNS pain circuits.
Neuron. 2018 Apr 4;98(1):90-108. PMID: 29576387.

François A, **Scherrer G**. Delta Opioid Receptor Expression and Function in Primary Afferent Somatosensory Neurons.
Handb Exp Pharmacol. 2017 Oct 10. PMID: 28993838

Public seminars and conferences:

Gregory Scherrer, PI

2018 Université de Strasbourg, Strasbourg, France, June 11.
"Neural substrates for pain experience and its modulation by opioids"

Pain Mechanisms and Therapeutics Conference, Sicily, Italy, June 3.
"Descending circuits modulating pain unpleasantness"

New York Stem Cell Foundation, Innovator Retreat, Montauk, NY, May 24.
"The neural basis of pain unpleasantness"

Rita Allen Foundation, Scholar Meeting, Princeton University, Princeton, NJ, May 22.
"Neural substrates of pain unpleasantness"

Indiana University, Stark Neurosciences Institute seminar. Indianapolis, IN, April 26.
"The neural basis of pain unpleasantness and its modulation by opioids"

McGill University, Killam Lecture, Montreal Neurological Institute. Montreal, Canada, April 24. "The neural basis of pain unpleasantness and its modulation by opioids"

University of North Carolina, Chapel Hill, UNC Neuroscience Center seminar. Chapel Hill, NC, Jan 25.
"Neural substrates of pain unpleasantness and its modulation by opioids"

Duke University, Dept. of Anesthesiology. Durham, NC, Jan 24.
"Neural substrates of pain unpleasantness and its modulation by opioids"

2017 American College of Neuropsychopharmacology (ACNP), Palm Springs, CA, Dec 6.
"Dissecting the functional organization of opioid receptors in pain neural circuits to optimize opioid analgesic therapies"

University of Virginia, International Symposium on Neuromodulation of Neural Circuits, Charlottesville, VA, Nov 10.
"Neural circuits and synaptic mechanisms for pain and opioid analgesia"

Napa Pain Conference, Napa, CA, Aug 19-20.
"Mechanisms of action of opioids: receptors, neurophysiology, and neural circuits"
"Neural circuits and molecular mechanisms underlying pain perception and opioid analgesia"

National Institute of Health, Strategic meeting: understanding the neurobiological mechanisms of pain to battle the opioid epidemic. Bethesda, MD, July 7. Invited by Drs. Collins and Volkow.

Temple University, Lewis Katz School of Medicine Center for Substance Abuse Research, Temple, PA, May 23.
"Neural circuits and molecular mechanisms for pain perception and opioid analgesia"

American Pain Society, Spring Pain meeting, Pittsburgh, PA, May 16.
"Functional interactions between delta and mu opioid receptors in pain circuits"

University of California, San Francisco, Wheeler Center for the Neurobiology of Addiction Retreat, San Francisco, CA, Mar 30.
"Opioid receptor mechanisms controlling neurotransmission in pain circuits"

Gordon Research Conference, Molecular Pharmacology, GPCRs: From Single Molecules to New Forms of Treatment, Lucca, Italy, March 15.
"Opioid receptor mechanisms controlling neurotransmission in pain pathways"

Scripps Research Institute, San Diego, CA, Feb 8.
"Neural circuits and molecular mechanisms for pain perception and opioid analgesia"

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

We obtained a no-cost extension of the award, during which we will be finalizing the experiments described in the Specific Aims 1 and 2, and will keep on disseminating our results.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Our results on the delta opioid receptor function in pain control at the level of the sensory peripheral neurons and spinal neurons profoundly transform our understanding of how opioid medication can be optimized to produce analgesia against cutaneous pain resulting from injuries suffered on the battlefield. Specifically, cutaneous application of delta opioid receptor agonists may decrease cutaneous hypersensitivity and may represent a more efficient and safer treatment option for these painful conditions compared to systemic mu opioid receptor agonist usage (e.g. morphine, oxycodone or fentanyl).

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Our results also have a profound impact in the broader Neuroscience and Pharmacology disciplines, by resolving the neural circuit organization underlying injury-induced cutaneous pain, and the receptor mechanisms by which opioids regulate neural activity in these circuits to produce analgesia.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*

- *adoption of new practices.*

We have been focused on executing the experimental plan and have not yet developed technology transfer initiatives

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:
- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Some translational experiments are still ongoing and the impact on society and technology will be assessed more accurately when these experiments will be completed

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

We do not have any change to Report.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

We do not have any actual or anticipated problem to Report.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report.
Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

N/A

Significant changes in use or care of vertebrate animals

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Wang D, Tawfik VL, Corder G, Low SA, François A, Basbaum AI, **Scherrer G**. Functional divergence of delta and mu opioid receptor organization in CNS pain circuits.
Published in Neuron. 2018 Apr 4;98(1):90-108. PMID: 29576387. *Acknowledgement of federal support: yes*

François A, **Scherrer G**. Delta Opioid Receptor Expression and Function in Primary Afferent Somatosensory Neurons.
Handb Exp Pharmacol. 2017 Oct 10. PMID: 28993838. *Acknowledgement of federal support: yes.*

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to Report.

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*
Nothing to Report.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to Report.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.
  Nothing to Report.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.
  Nothing to Report.

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

  Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name:	***Dr. Gregory Scherrer***
Project Role:	***PI***
Researcher Identifier (e.g. ORCID ID):	*gscherrer (eRA Commons)*
Nearest person month worked:	***4.8***
Contribution to Project:	Dr. Scherrer has overall responsibility for the proposed research. Specifically, he designs the proposed experiments, and analyze data and interpret the results.
Funding Support:	*Department of Defense; Anesthesia Department*

Name:	***Amaury Francois***
Project Role:	***Post-Doctoral fellow***
Researcher Identifier (e.g. ORCID ID):	*francois.amaury (eRA Commons)*
Nearest person month worked:	***5.5***
Contribution to Project:	Dr. François performs, analyzes, and interprets in situ hybridization studies (Specific Aim 1, subaim 1.b.), and behavioral experiments (Specific Aim 2), and electrophysiological experiments in DRG primary culture (Specific Aim 1, subaim 1.b.), Dr. François also presents the results to the research group
Funding Support:	*Department of Defense; Start-up funds from Dr. Scherrer*

Name:	***Dr. Dong Wang***
Project Role:	***Research Associate***
Researcher Identifier (e.g. ORCID ID):	*dong.wang2 (eRA Commons)*
Nearest person month worked:	***2.8***
Contribution to Project:	Histological and behavioral experiments
Funding Support:	*Department of Defense; Anesthesia Department*

Name:	***Dr. Daniel Berg***
Project Role:	***Post-Doctoral Scholar***
Researcher Identifier (e.g. ORCID ID):	*(eRA Commons)*
Nearest person month worked:	***1.5***
Contribution to Project:	Molecular characterization of mechanosensory dorsal root ganglion neurons expressing delta opioid receptors
Funding Support:	*Department of Defense; Anesthesia Department*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable;*

however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*