Peripheral nerve injury-induced rearrangement of neural circuit in the spinal dorsal horn revealed by cross-correlation analysis

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Abstract

Peripheral nerve injury often induces abnormal pain states, such as hyperalgesia and allodynia. In this study, we attempted to elucidate how neurons are synaptically integrated into the neuronal circuitry in the spinal dorsal horn and how synaptic connectivity patterns among dorsal horn neurons are altered by peripheral nerve injury. Experiments were performed on 6–7-week-old ICR mice. Partial sciatic nerve ligation was performed. Transverse slices of the lumbar spinal cord were prepared. Spike activities were simultaneously recorded from multiple neurons in the superficial dorsal horn (SDH) using a multi-electrode array system, and cross-correlograms between spike trains of neuron pairs in the SDH were constructed. In sham-operated control mice, except for the flat cross-correlogram, the most common pattern was the central peak, followed by lagged trough, central trough, and lagged peak, in this order. The incidence of cross-correlograms with various patterns was significantly affected by peripheral nerve ligation. Particularly, the incidences of central peak and lagged peak were increased and that of lagged trough was decreased in peripheral nerve-ligated mice. Additionally, bath application of capsaicin, an agonist for transient receptor potential

vanilloid 1 receptor, increased the frequency of action potentials. The effects of capsaicin stimulation on the incidence of cross-correlograms with various patterns were significantly different between sham-operated control and sciatic nerve-ligated mice. The present observations seem to indicate that neurons in the SDH make excitatory and/or inhibitory synapses to the nearby neurons, and that synaptic connections among neurons in the SDH may significantly change after the development of neuropathic pain. Finally, cross-correlation analysis seems to be a useful technique for characterizing neuronal interactions in local neural circuits and for analyzing neuropathic pain-associated alterations in activities of local neural network.

Keywords: Cross-Correlation Analysis; Local Neuronal Circuit; Spinal Dorsal Horn; Neuropathic Pain

Highlights

- Peripheral nerve injury increased cross-correlograms with central and lagged peak.
- Peripheral nerve injury decreased cross-correlograms with lagged trough.
- Synaptic connections between dorsal horn neurons change in neuropathic mice.
- Effects of capsaicin differ significantly between control and neuropathic mice.
- Nerve injury seems up-regulate TRPV1 receptors prominently in excitatory neurons.

1. Introduction

Peripheral nerve injury often induces a state of abnormal pain known as neuropathic pain, which includes hyperalgesia and allodynia. Peripheral nerve injury-induced neuropathic pain has been associated with neural plastic changes in the spinal dorsal horn [21, 30].

Possible mechanisms for neuropathic pain include primary afferent fiber sprouting, synaptic rearrangement, and loss of inhibitory interneurons in the spinal dorsal horn among others [21, 30]. However, the exact nature of the changes in the neural circuitry of the spinal cord, which are responsible for neuropathic pain, remains unclear.

A considerable fraction of dorsal horn neurons is known to consist of local interneurons [31], which are either excitatory or inhibitory. These neurons play an important role in controlling the overall excitability of neuronal circuitry in the spinal dorsal horn and, therefore, in controlling the output of the spinal dorsal horn [31].

A number of electrophysiological studies have provided crucial information on the intrinsic properties of these interneurons [1, 12, 33]. More recently, a sophisticated technique using simultaneous whole-cell recording from two neurons in the spinal

dorsal horn provided detailed information on synaptic connection between these neurons [19, 34].

Elucidating how these neurons are synaptically integrated into the neuronal circuitry in the dorsal horn and how synaptic connectivity patterns among these neurons are altered by peripheral nerve injury would provide valuable insight into the neuronal mechanisms for neuropathic pain.

When the action potentials of two neurons are extracellularly recorded simultaneously, a cross-correlation analysis of spike trains of the two neurons will reveal changes in firing probability of one neuron relative to the spikes of the other neuron [16, 20, 23]. Thus, peaks and troughs of the cross-correlogram could provide information on the synaptic connectivity between two neurons [16, 20, 23]. This method has been applied to the investigation of synaptic connectivity between neurons involved in nociceptive processing [2, 4, 9, 24].

The multi-electrode array (MEA) system has been used as an increasingly important technique for studying neuronal activity in *in vivo* and *in vitro* conditions [28]. Simultaneous recording of multi-neuronal activities using the MEA system seems to

provide crucial information for characterizing neuronal interactions in local neural circuits.

In this study, we recorded spike activities from neuron pairs in the dorsal horn of the spinal cord slice preparation using the MEA system. Subsequently, we performed cross-correlation analysis between simultaneously recorded spike trains of two neurons. Additionally, capsaicin was bath-applied to stimulate the transient receptor potential vanilloid 1 (TRPV1) receptor, which is expressed in the nociceptive pathway in the spinal dorsal horn [6, 10, 27, 32], and the effects of capsaicin on cross-correlogram patterns were compared between sham-operated control mice and sciatic nerve-ligated mice. Such experiments would contribute to the understanding of mechanisms underlying peripheral nerve injury-induced pain at the neuronal circuit level.

2. Material and methods

2. 1. Animals

Experiments were performed on 6- to 8-week old male ICR mice. All animal experiments were approved by the institutional animal care and use committees at

Dokkyo Medical University. The care and use of the animals were in accordance with the National Institutes of Health guidelines on animal care and with the guidelines of the International Association for the Study of Pain [36].

2.2. Partial ligation of the sciatic nerve (PSL)

The mice were maintained in a temperature-controlled room under a 12 h/12h light/dark cycle. The sciatic nerve was partially ligated under sevoflurane anesthesia according to methods described by Seltzer et al [26]. In the sham-operated control mice, the sciatic nerve was exposed but not ligated (Sham-operated control group).

2.3. Behavioral assessment

To assess the effects of sciatic nerve ligation, we measured the frequency of withdrawal responses to 10 repetitive stimuli with von Frey filaments (Stoeling, Wooddale, IL) of 0.06 or 0.16 g force, according to the protocol outlined by Schwartz et al [25]. Behavioral assessments were performed every three days starting from three days before nerve ligation or sham operation. Sciatic nerve ligation increased the frequency of withdrawal responses, and this increase developed within 3 days after nerve ligation and persisted for several weeks (data not shown).

2. 4. Preparation of spinal cord slices

Electrophysiological recordings from spinal cord slices were performed after behavioral assessment on day 9 after sciatic nerve ligation or sham operation. On this day, the frequency of withdrawal responses was significantly increased by sciatic nerve ligation, indicating the development of mechanical allodynia (Ligation group).

Segments of the lumbosacral (L4–S1) spinal cord were removed under ketamine/xylazine anesthesia. A Vibratome (Dosaka EM, Japan) was used to cut transverse slices (450 μm) in Krebs solution at 4°C. The Krebs solution was equilibrated with 95% O_2 and 5% CO_2 and contained the following (in mM): NaCl, 113; KCl, 3; $NaHCO_3$, 25; NaH_2PO_4, 1; $CaCl_2$, 2; $MgCl_2$, 1; d-glucose, 11; pH 7.4.

2. 5. Extracellular recording with the MEA system

After a 1-h incubation period in Krebs solution at 37°C, the slices were mounted onto a recording chamber with MEA (Multi Channel Systems, Reutlingen, Germany), which was placed on a microscope stage and continuously perfused with Krebs solution. Electrodes were arranged 100-μm apart in an 8 x 8 pattern. The signals from the MEA

electrodes were sampled at 25 kHz and stored on the hard disk of a personal computer for offline analysis.

2. 6. Cross-Correlogram Analysis

Extracellular recordings obtained from electrodes placed on the superficial dorsal horn (SDH) were analyzed using a data analysis software package (Dataview, W. Heilter, University of St. Andrews, UK). Raw data usually contain spike activities of several neurons. Therefore, after spikes were detected by an amplitude threshold, they were sorted, based on their waveform with template matching, into clusters of single neurons. The extracted spike trains of single SDH neurons were stored and a cross-correlogram between simultaneous spike trains was compiled with Dataview. A peak or a trough was considered statistically significant if it deviated by at least three standard deviations from the baseline (99% confidence).

7. Statistical analysis

Results for the categorical variables were expressed as absolute and relative frequencies. The chi-squared test was used to assess associations between categorical variables. A post-hoc residual analysis was used for identifying the categories

responsible for a significant chi-squared statistic. P-values less than 0.05 were considered statistically significant.

3. Rsults

3. 1. Cross-correlation analysis between neuron pairs in the superficial dorsal horn

Figure 1 shows a representative cross-correlation analysis between neuron pairs in the SDH. Extracellular recordings were acquired from an electrode over which the SDH spread. Figure 1A shows an example of such traces of extracellular recording of action potentials from multiple neurons. Spikes were detected by an amplitude threshold and the detected spikes were sorted into clusters of single neurons based on their waveform with template matching. Superimposed spike traces in the upper inset show waveform of action potentials of two single neurons (neuron (a) and (b)). Two series of vertical lines in the lower inset indicate the time sequence of occurrence of action potentials of neuron (a) and (b). From these sequences, the cross-correlogram between neuron (a) and (b) was calculated (Fig. 1B). The ordinate of the cross-correlogram shows the number

of action potentials observed in neuron (b) at various times before and after neuron (a) generated action potentials. In this particular cross-correlogram, a clear peak exceeding the ± 99.9% interval of confidence (red dotted lines) is seen at 4–8 ms after the time when the neuron (a) generated an action potential. The lagged peak indicates that neuron (a) makes excitatory synaptic connection to neuron (b) [16, 20, 23].

3. 2. Various patterns of cross-correlogram between neuron pairs in sham-operated control mice

Figure 2 shows various patterns of cross-correlograms observed in sham-operated control mice.

A flat cross-correlogram was the most commonly observed pattern in sham-operated mice (61.1%, 157 of 257 neuron pairs, Fig. 2A). A flat pattern indicates that there is no synaptic connection between two neurons [16, 20, 23].

The next commonly observed pattern was a cross-correlogram with a peak straddling the origin (0 ms) of the horizontal axis (thereafter referred to as a central peak, 18.7%, 48 pairs, Fig. 2B). A central peak indicates that the recorded two neurons receive the common excitatory synaptic inputs from a third neuron [16, 20, 23].

In 11 pairs (4.3%), a cross-correlogram with a peak that is offset from the origin (0 ms) of the horizontal axis was observed (thereafter referred to as a lagged peak, Fig. 1B and Fig. 2C). A lagged peak indicates that one of the recorded two neurons makes excitatory monosynaptic connection to the other neuron [16, 20, 23].

In 23 pairs (8.9%), a cross-correlogram with a trough that is offset from the origin of the histogram was observed (hereafter referred to as a lagged trough, Fig. 2D). A lagged trough indicates an inhibitory monosynaptic input from a presynaptic neuron [16, 20, 23].

Eighteen neuron pairs (7.0%) exhibited a cross-correlogram with a central trough (Fig. 2E), being interpreted as sign of reciprocal synaptic inputs which are excitatory to the one and inhibitory to the other [16, 20, 23].

3. 3. Effects of sciatic nerve ligation on the incidence of various patterns of cross-correlograms

Figure 3A shows the frequency of various patterns of cross-correlograms in sham-operated control mice (left column) and sciatic nerve-ligated mice (right column). In the sciatic nerve-ligated mice, the incidence of a flat cross-correlogram was 53.3% (138 of

259 neuron pairs). The incidence of central peak, lagged peak, lagged trough, and central trough was 28.2% (73 pairs), 8.1% (21 pairs), 4.2% (11 pairs), and 6.2% (16 pairs), respectively.

The results of the chi-squared analysis showed a significant difference between sham-operated control and nerve-ligated mice (chi-squared (4) = 13.86, $p < 0.01$). Furthermore, a residual analysis revealed that the incidence of central peak was significantly increased in nerve-ligated mice (adjusted residual = 2.549, $p < 0.05$) and that the incidence of lagged peak was slightly increased in nerve-ligated mice (adjusted residual = 1.803, $p = 0.07$). In contrast, the occurrence of lagged trough was significantly reduced in nerve-ligated mice (adjusted residual = 2.153, $p < 0.05$).

3. 4. Effects of capsaicin on the incidence of various patterns of cross-correlograms in sham-operated control mice and sciatic nerve-ligated mice

Bath application of capsaicin, at the concentration of 1 μM for 60 s, increased the frequency of action spikes (data not shown). While the TRPV1 receptor expressing nociceptive pathway in the spinal cord was stimulated by capsaicin in sham-operated control mice, the occurrence of lagged peak significantly increased (chi-squared (4) =

38.52, adjusted residual = 5.919 $p < 0.01$, Fig. 3B, red upward triangle). The incidence of other patterns of cross-correlograms did not show any statistically significant changes.

Additionally, the effects of bath application of capsaicin were more prominent in sciatica nerve-ligated mice than in sham-operated control mice. In sciatic nerve-ligated mice, the occurrence of lagged and central peak was significantly increased (chi-squared (4) = 71.89, adjusted residual = 2.219, $p < 0.05$ for lagged peak and adjusted residual = 6.953, $p < 0.01$ for central peak, Fig. 3C, red upward triangles). On the other hand, the occurrence of central trough and flat cross-correlograms was significantly decreased (adjusted residual = 1.848, $p < 0.05$ for central trough and adjusted residual = 7.657, $p < 0.01$ for flat correlogram, Fig. 4B, red downward triangles).

4. Discussion

1. Main findings

In the present experiments, we observed that the incidence of cross-correlograms with various patterns was significantly affected by peripheral nerve ligation. Particularly, the

incidences of cross-correlograms with central peak and with lagged peak were increased and that of lagged trough was decreased in peripheral nerve-ligated mice.

Additionally, bath application of capsaicin increased the frequency of action potentials. Moreover, the effects of capsaicin application on the incidence of various patterns of cross-correlograms were significantly different between sham-operated control mice and sciatic nerve-ligated mice.

2. Cross-correlation analysis and spinal dorsal horn

Several previous studies have investigated synaptic interactions between two spinal dorsal horn neurons with simultaneously recorded cross-correlating spike trains [2, 4, 9, 24].

For example, Eblen-Zajjur et al. simultaneously recorded from two neurons in the spinal dorsal horn of anesthetized adult rats. Their cross-correlogram analysis revealed that a central peak was the most common pattern, and that central trough, bilateral peaks, lagged peak, or lagged trough were also observed in a smaller population [9]. Our present observations in sham-operated control mice are consistent with their results.

These are indicative that discharges of neighboring spinal dorsal horn neurons are strongly synchronized.

More recently, Roza et al. applied a MEA system to spinal cord slices prepared from young adult mice and recorded extracellular activities of neurons in the SDH [24]. They analyzed spike synchrony between simultaneously recorded two neurons by means of a cross-correlation analysis, and reported that peripheral nerve injury significantly increased the incidence of central peak. In line with this, our present results showed that sciatic nerve ligation increased the incidence of neuron pairs with central peak, which is indicative of common excitatory synaptic inputs to both neurons. These results suggest that peripheral nerve injury-induced allodynia and hyperalgesia are associated with a plastic enhancement of synchronous activity in the neural circuit of the spinal dorsal horn [9, 24].

Biella et al. performed cross-correlation analysis between neurons in the deep and in the superficial dorsal horn [2]. They showed that a cross-correlogram with lagged peak was observed in more than half of neuron pairs and that its incidence was significantly decreased in peripheral nerve-injured animals. They attributed the observed alterations

induced by peripheral nerve injury to plasticity in the spinal dorsal horn circuits, which includes the unmasking of somatotopically inappropriate synapses, to afferent fiber sprouting, and to synaptic rearrangements.

In contrast to the observations of Biella et al. [2], our cross-correlation analysis revealed that the incidence of lagged peak was less than 5 % in control mice and was slightly increased by peripheral nerve ligation. One of the reasons for this discrepancy may be attributable to the different relative locations of the two recorded neurons. While Biella et al. recorded two neurons, one located in the superficial lamina and the other located in deep lamina, we analyzed two neurons closely located in the superficial lamina. We used a MEA system with inter-electrode distance of 100 μm, and the activity of a single neuron was seldom recorded by two electrodes next to each other at the same time. Thus it is probable that the pairs of neurons we presently analyzed were located within a 100 μm distance from each other. Although we lack the information on the dorso-ventral, medio-lateral, and rostro-caudal relationship between two neurons under analysis, the present cross-correlation analysis contributes to the understanding of

how synaptic connections in a local neural circuit in the spinal dorsal horn change in response to peripheral nerve injury.

3. Increased central peak, increased lagged peak, and decreased lagged trough in nerve-ligated mice

Peaks and troughs of the cross-correlogram can be interpreted in terms of underlying synaptic connections. The presently observed peripheral nerve injury-induced changes could occur through addition/removal of synapses as well as through functional enhancement/attenuation of synaptic strength [11].

Peripheral nerve ligation-induced increase in central peaks may be attributed to sprouting of primary afferent fibers into the SDH and formation of new synapses by these sprouts [7]. It is also probable that neurons in the spinal dorsal horn sprout and make new synapses, which may underlie peripheral nerve injury-induced increase in lagged peaks [14, 29]. However, it is also suggested that peripheral nerve injury-induced changes are attributable to attenuating inhibition and to transforming silent synapses into active ones, but not to plastic changes of dorsal horn neurons [17, 18, 35]. Peripheral nerve injury-induced attenuation of synaptic inhibition probably results from

differential cell death of inhibitory neurons [22] and from reduced inhibitory synaptic efficacy, which is due to altered chloride ion homeostasis [8].

4. Capsaicin elicited action potentials in the spinal dorsal horn

TRPV1 receptors are expressed on unmyelinated and thinly myelinated primary afferent fibers [13], and is involved in nociceptive transmission in the spinal dorsal horn [5, 6]. TRPV1 receptors have been shown to be expressed in excitatory [27, 32] and inhibitory [10] neurons in the spinal dorsal horn. However, it is not yet fully elucidated whether and how TRPV1 receptors are involved in peripheral nerve injury-induced neuropathic pain.

We performed cross-correlation analysis between capsaicin-induced spike trains. In sham-operated control mice, capsaicin statistically significantly increased the incidence of lagged peak. The incidence of cross-correlograms with other patterns did not show significant changes. It is speculated that TRPV1 receptors located in excitatory neurons may play a pivotal role in nociceptive transmission in the spinal dorsal horn

Additionally, the effects of capsaicin were more prominent in sciatic nerve-ligated mice compared to sham-operated control mice. This may be attributable to peripheral

nerve ligation-induced upregulation of TRPV1 receptors [3, 15]. Although capsaicin only increased the incidence of lagged peak in sham-operated control mice, it increased the incidence of cross-correlograms both with lagged and with central peak in sciatic nerve-ligated mice. The present electrophysiological results seem to suggest that peripheral nerve injury upregulated TRPV1 receptors more prominently in excitatory than in inhibitory neurons in the spinal dorsal horn. TRPV1 receptor expressed in excitatory neurons play an important role in the peripheral nerve injury-induced sensitized state of neural circuit in the spinal dorsal horn.

5. Concluding remark

The present results indicate that peripheral nerve injury alters synaptic connectivity between two neurons in the SDH. Our results provide novel additional evidence for the enhancement of overall activity in the neural circuit of the spinal dorsal horn of sciatic nerve-ligated mice, which underlies peripheral nerve injury-induced allodynia and hyperalgesia.

Additionally, the results of the capsaicin experiments provide electrophysiological evidence that peripheral nerve injury-induced pain states are associated with different

changes in the expression level of TRPV1 receptors between inhibitory and excitatory neurons in the dorsal horn.

Finally, cross-correlation analysis between two spike trains recorded from the spinal dorsal horn provides a framework in which we can investigate how synaptic connectivity in local neural circuit is affected by peripheral nerve injury. Such analysis of synaptic activity in local circuits would further the understanding on nociceptive information modulation in the SDH.

Legends to Figures

Figure 1

Representative example of cross-correlation analysis between neuron pairs in the SDH.

A: A trace shows extracellular recordings of action potentials from multiple neurons recorded from an electrode over which the SDH of spinal slice spread. Spikes were sorted, based on their waveform with template matching, into two single neurons (a) and (b). Superimposed traces in the upper insets show waveforms of action potentials of neurons (a) and (b). The time of occurrence of each spike of two neurons (a) and (b) is indicated by vertical lines in the lower insets.

B: Cross-correlogram between neuron (a) and (b). The vertical axis indicates the time-varying probability of firing of neuron (b) relative to the time of firings of neuron (a). Bin width is 1 ms and bin number is 50. A red solid line and red dotted lines indicate mean and the 99.9% interval of confidence, respectively. This example was obtained from a sham-operated control mouse.

Figure 2

Various patterns of cross-correlogram between neuron pairs in the SDH of sham-operated control mice.

Bin width is 1 ms and bin number is 50 for all cross-correlograms.

Figure 3

A: Comparison of the proportion of various patterns of cross-correlograms between sham-operated control (Left column) and nerve-ligated neuropathic mice (Right column).

B & C: Effects of capsaicin on the incidence of various patterns of cross-correlograms in sham-operated control mice (B) and sciatic nerve-ligated mice (C).

Upward triangles indicate that the actual measurement of incidence is significantly larger than the expected values (chi-squared test and post-hoc adjusted residual analysis, $p < 0.05$). Downward triangles indicate that the actual measurement of incidence is significantly smaller than the expected values ($p < 0,05$).

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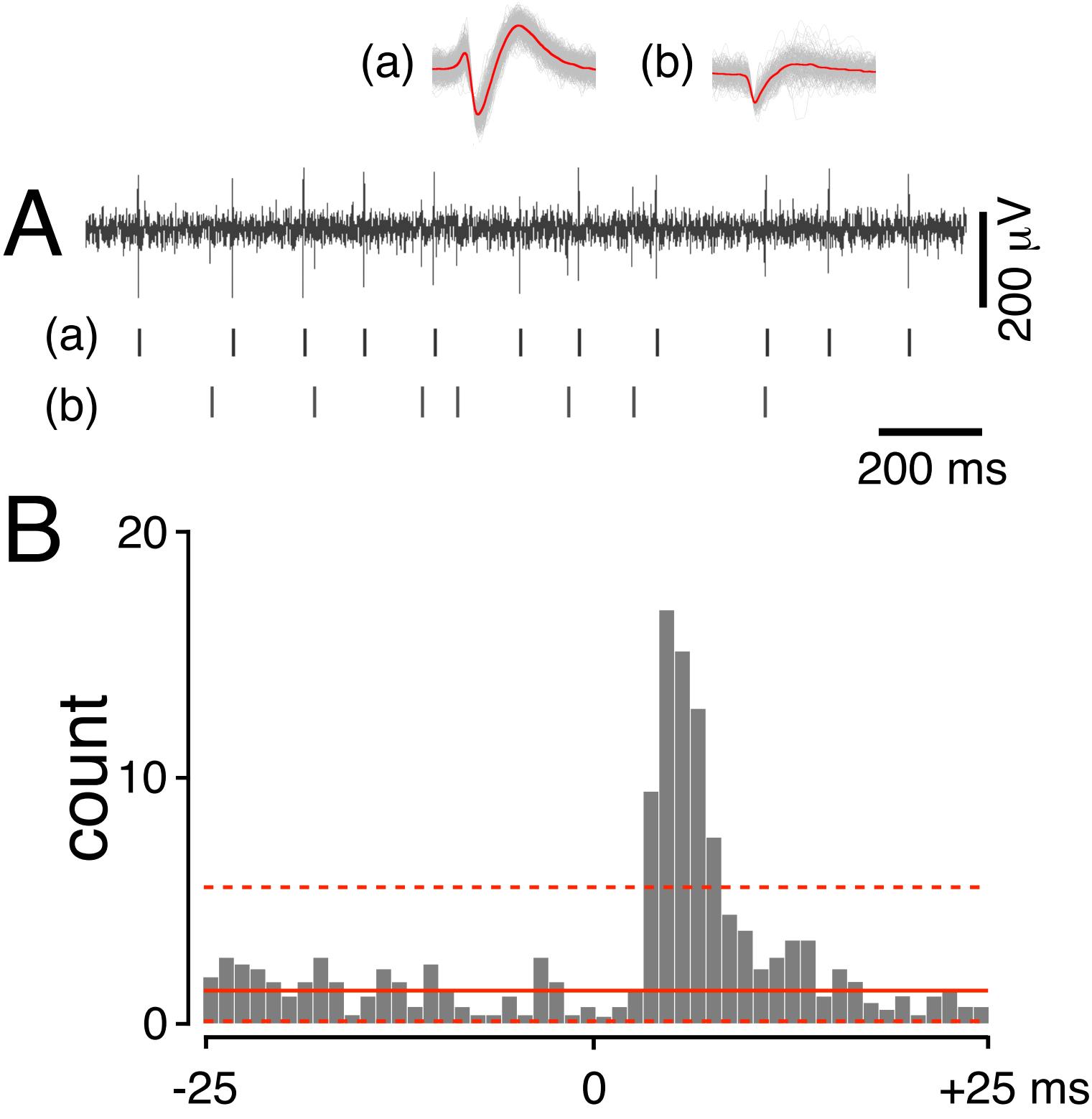

Figure 1

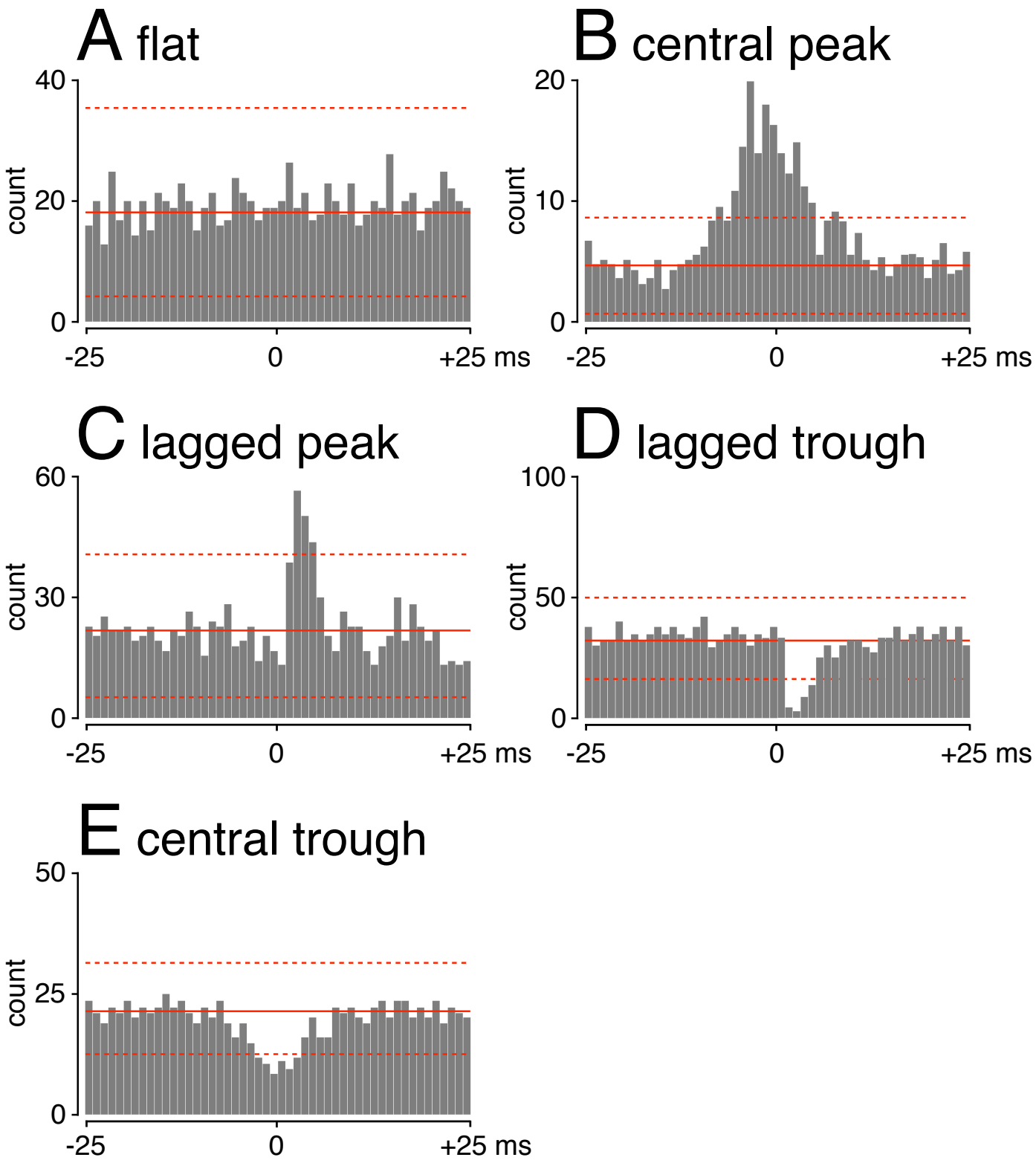
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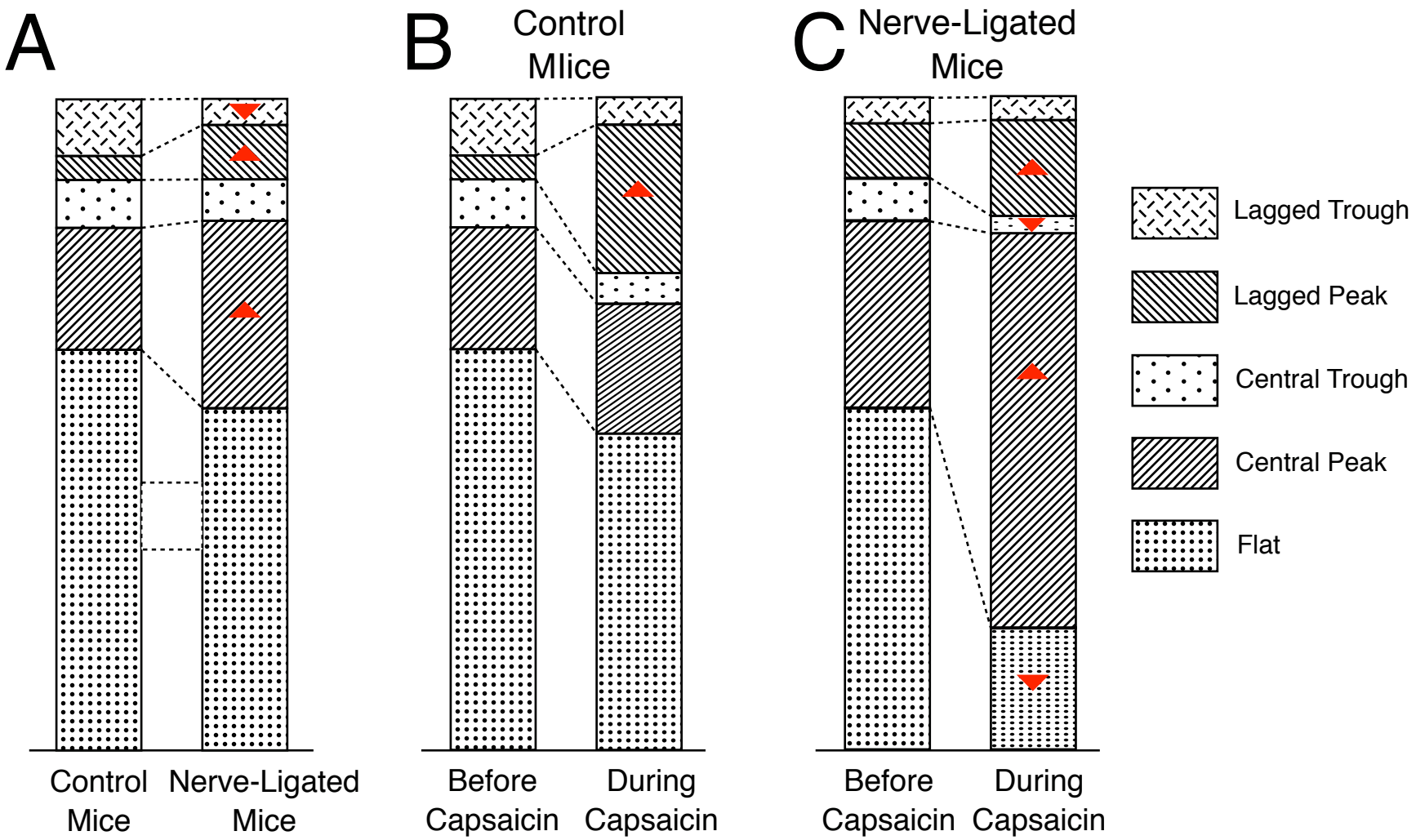
Figure 2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Nerve-Ligated
Mice
Before
Capsaicin
During
Capsaicin
Before
Capsaicin
During
Capsaicin

Figure 3