Interferon-γ facilitates the synaptic transmission between primary afferent C-fibres and lamina I neurons in the rat spinal dorsal horn via microglia activation

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Abstract

Recent studies have demonstrated an important role of the pro-inflammatory cytokine interferon-γ in neuropathic pain. Interferon-γ is upregulated in the lumbar spinal cord of nerve-injured rodents and intrathecal injection of interferon-γ has been shown to induce neuropathic pain-like behaviours in naive rodents. A potential mechanism in the pathogenesis of neuropathic pain is a long-lasting amplification of nociceptive synaptic transmission in lamina I of the spinal dorsal horn. Here, we tested the effects of interferon-γ on the properties of the first synapse in nociceptive pathways in the superficial spinal dorsal horn. We performed whole-cell patch-clamp recordings in lamina I neurons in a spinal cord slice preparation with dorsal roots attached from young rats. We determined the effects of acute (at least 25 min) or longer lasting (4–8 h) treatment of the transversal slices with recombinant rat interferon-γ on spontaneous excitatory postsynaptic currents or on monosynaptic Aδ- and C-fibre-evoked excitatory postsynaptic currents, respectively. Prolonged treatment with interferon-γ facilitated monosynaptic C-fibre-evoked excitatory postsynaptic currents and this effect could be blocked by co-application of minocycline an inhibitor of microglial activation. In contrast, Aδ-fibre-evoked excitatory postsynaptic currents were not affected by the prolonged interferon-γ treatment. Acute interferon-γ application in the bathing solution did not change strength of monosynaptic Aδ- or C-fibre synapses in lamina I. However, the rate, but not the amplitude, of spontaneous excitatory postsynaptic currents recorded in lamina I neurons was decreased. This effect could not be blocked by the application of minocycline. Long-lasting treatment of rat spinal cord slices with interferon-γ induced an input specific facilitation of synaptic strength in spinal nociceptive pathways. Enhanced transmission between C-fibres and spinal lamina I neurons was mediated by the activation of microglial cells. We showed that the pro-inflammatory cytokine interferon-γ modifies the processing of information at the first synaptic relay station in nociceptive pathways.

Introduction

The protective role of acute pain is lost in chronic neuropathic pain states and patients are facing several challenges, such as the limited efficacy of clinical treatments.[1] Despite intense research, the underlying mechanisms of the transition from acute to chronic pain states are still poorly understood. Accumulating evidence from animal studies suggests that the interaction of neuronal with non-neuronal cells such as immune cells is causative in the development of chronic neuropathic pain states. Cytokines and chemokines are considered to mediate this communication.[2–4]

Resident immune cells of the nervous system (neuroglia) and T-cells infiltrating the spinal cord after

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peripheral nerve injury contribute to the development and maintenance of neuropathic and inflammatory pain in rodents.[5–10] T-cell-deficient mice show reduced neuropathic tactile allodynia.[5,9] This effect could be reversed by the adoptive transfer of T-cells from neuropathic animals.[9]

The canonical effector of differentiated T helper type 1 cells is the pro-inflammatory cytokine interferon-γ (IFNγ). In models of peripheral nerve injury, IFNγ is upregulated in the lumbar spinal dorsal horn in rats[5,11] and mice.[12] Intrathecal application of IFNγ initiates pain hypersensitive behaviour and persistent mechanical allodynia in rodents.[10,11,13–15] In addition, mice lacking IFNγ receptors show attenuation of the peripheral nerve injury-induced tactile allodynia.[5,15]

Long-lasting amplification of nociceptive synaptic transmission in the spinal cord dorsal horn is a potential mechanism in the pathogenesis of neuropathic pain[4,16] and plasticity at synapses between primary afferent nociceptive nerve fibres and neurons in lamina I of the spinal dorsal horn is a cellular model of injury-induced hypersensitivity[16–19] involving N-methyl-D-aspartate (NMDA) receptor signalling. Importantly, neuron-glia communication is required for activity-dependent long-term potentiation (LTP) in the superficial dorsal horn. The pro-nociceptive cytokines interleukin (IL)-1β and tumour necrosis factor (TNF), which can be released by glial cells, can modulate glutamatergic synaptic transmission at spinal synapses.[19,20] Moreover, spinal application of IL1-β or TNF is sufficient to induce LTP at synapses between C-fibres and lamina I neurones.[20] In contrast to these cytokines, investigations on the effect of IFNγ on the first central synapse in nociceptive pathways are missing. IFNγ receptors may be expressed on neurons and/or glial cells in the spinal cord dorsal horn.[13,15,21–23] A potential modification of synaptic strength by IFNγ is thus not necessarily mediated directly by binding to pre- and/or postsynaptic receptors. It is equally well possible that IFNγ acts on glial cells to trigger the release of neuromodulators, which then modify synaptic transmission and nociception. In the present study, we tested the effects of IFNγ on the synaptic transmission between primary afferent C- and Aδ-fibres and neurons in lamina I of the spinal dorsal horn in the absence and in the presence of microglial inhibition.

It is still unknown how pro-inflammatory cytokines like IFNγ may modify nociceptive transmission in spinal circuits. Lamina I neurons are not only innervated by primary afferent C- and Aδ-fibres but in addition receive input from many excitatory interneurons with cell bodies in laminae II and other lamina I local-circuit neurons.[24–27] Therefore, IFNγ may specifically modulate one defined input to the target cell or regulate various inputs differentially. For example, strengthening the primary afferent

nociceptive synaptic input and dampening the excitatory input from interneurons simultaneously would enhance the contrast in the spinal nociceptive network.

Here, we could show that prolonged IFNγ treatment induced an input-specific facilitation of synaptic strength at C-fibre synapses in spinal lamina I which was mediated by microglial activation demonstrating that the pro-inflammatory cytokine IFNγ modifies the processing of information at the first synaptic relay station in nociceptive pathways.

Materials and methods

All procedures were performed in accordance with European Communities Council directives on the use of animals for scientific purposes (2010/63/EU) and the rules of the Good Scientific Practice Guide of the Medical University of Vienna.

Spinal cord slice preparation

Under deep isoflurane anaesthesia male Sprague Dawley rats (age range: 20–30 days) were killed by decapitation. Then, a laminectomy with lumbar spinal cord removal was performed as described previously.[20] Briefly, 500 to 600 μm thick transverse slices with their dorsal roots attached were cut from the spinal segments L2 to L6 using a vibrating microslicer (DTK-1000, Dosaka, Kyoto, Japan). Slices were kept at 33°C in an incubation solution consisting of the following (in mM): 95 NaCl, 1.8 KCl, 1.2 KH_2PO_4, 0.5 $CaCl_2$, 7 $MgSO_4$, 26 $NaHCO_3$, 15 glucose and 50 sucrose, oxygenated with 95% O_2 and 5% CO_2, and with measured pH of 7.4 and osmolarity of 310 to 320 mosmol/l.

Patch-clamp recordings

A single slice was transferred to the recording chamber and constantly superfused with oxygenated recording solution at a rate of 3 to 4 ml/min. The solution was identical to the incubation solution except for the following (in mM): 127 NaCl, 2.4 $CaCl_2$, 1.3 $MgSO_4$ and 0 sucrose, osmolarity of 310 to 320 mosmol/l. Lamina I neurons were visualised using a ×40, 0.80 water immersion objective on an upright microscope (Olympus BX51WI, Olympus Optical, Japan) equipped with a video camera (PCO, Germany) and Dodt infrared optics[28] (Luigs & Neumann, Germany). Only neurons located not more than 20 μm from the dorsal border between the white and the grey matter were classified as lamina I neurons.[29] All recordings were performed in the whole-cell patch-clamp configuration at 31°C. Patch pipettes (2–4 MΩ) from borosilicate glass (Hilgenberg, Germany) were made with a horizontal micropipette puller (Model P97, Sutter Instrument, USA) and filled with a pipette solution consisting

of the following (in mM): 120 K-gluconate, 20 KCl, 2 $MgCl_2$, 20 Hepes, 0.5 Na_2-EGTA, 1 Na_2-ATP, 0.5 Na-GTP and 7.5 phosphocreatine, with measured osmolarity of 300 mosmol/l. The membrane potential was measured immediately after the whole-cell configuration was established and neurons with membrane potential less negative than −50 mV were discarded. Recordings were performed in voltage-clamp mode at a holding potential of −70 mV using a patch-clamp amplifier (Axopatch 200B), a digitiser (Digidata 1440A) and pCLAMP 9 acquisition software (all from Molecular Devices, USA). Signals were low-pass filtered at 2 to 10 kHz, sampled at 100 kHz and analysed offline with Clampfit 10 Software (Molecular Devices, USA). The liquid junction potential was not corrected. Throughout the experiment, series resistance was measured and neurons with values higher than 30 MΩ, and changes in series resistance more than 20% were excluded.

Evoked EPSCs

Electrical stimulation of the dorsal roots via a suction electrode connected to an isolated current stimulator (A360, World Precision Instruments, USA) elicited afferent-evoked excitatory postsynaptic currents (EPSCs) in lamina I neurons. The thresholds required to initiate an EPSC were determined and test pulses (0.1 ms pulse width) at stimulus intensities of two times the threshold values were given to record evoked EPSCs. To test for monosynaptic input, 10 stimuli at intervals of 1 Hz for C-fibre input and 10 Hz for Aδ-fibre input were given. EPSCs were identified as monosynaptically evoked by the absence of failures in response to the test pulses and a jitter in response latencies less than 10%. Afferent inputs were classified as C-fibre or Aδ-fibre-evoked, respectively, based on response threshold and conduction velocity.[30] C-fibres had a conduction velocity $\leq$ 1 m/s and Aδ-fibres > 1 m/s. Paired stimuli were given every 15 s with an interstimulus interval of 300 ms for C-fibres and 50 ms for Aδ-fibres. This 15-s recording period represents one trace. The paired pulse ratio (PPR) was calculated by averaging evoked EPSCs of nine traces and dividing the amplitude of the second monosynaptic Aδ- and C-fibre-evoked EPSC (eEPSC) by the amplitude of the first eEPSC.

Spontaneous EPSCs

Spontaneous EPSCs (sEPSCs) were recorded during the 15-s traces together with the evoked EPSCs. In each trace, the first 5 s contain the evoked EPSCs and were cut off offline using Clampfit 10. The remaining 10 s of nine traces (90 s in total) were used for analysis at indicated time points.

Drugs application

All drugs were applied to the bathing solution at known concentrations for the electrophysiological recordings. For prolonged treatment, recombinant rat IFNγ was applied to the incubation solution prior to in vitro recordings (2000 U/ml, PeproTech, Cat. No. 400–20). IFNγ was also added to the recording solution resulting in a total exposure time period of 4 to 8 h. To measure the acute effects on synaptic transmission, IFNγ (4000 U/ml) was added to the recording solution after 5 min baseline measurements for at least 25 min. For inhibition of microglial activation, minocycline hydrochloride (100 μM, Sigma) was added to the incubation solution 15 min prior to IFNγ incubation, and application was continued during ex vivo recording.

Data analysis and statistical analysis

For statistical analysis and for creating graphs, SigmaPlot 12.0 (Systat Software, USA) and GraphPad Prism 6 (GraphPad Software, USA) were used.

The peak amplitude of the first monosynaptic EPSC evoked by the paired pulses was measured and served as a quantification of synaptic strength. To analyse the effect of prolonged IFNγ treatment, neurons were recorded for 10 min, and the mean amplitude of the last nine EPSCs evoked by dorsal root stimulation was compared between treated and non-treated groups using the the Mann–Whitney rank-sum test. For group comparisons of more than two groups, the rank-sum analysis of variance (ANOVA) (Kruskal–Wallis test) was used followed by the Dunn's post hoc test.

For the investigation of an acute effect on the eEPSCs, a baseline of 5 min followed by at least 25 min of IFNγ treatment was recorded. The short-term effect of IFNγ was quantified as a percentage change in eEPSC amplitude in relation to the baseline. The mean amplitude of nine averaged test responses recorded prior to the IFNγ application served as controls. Drug effect was assessed by averaging the amplitudes of nine consecutive responses after 25 min of IFNγ application and by comparing the resulting means with the control values using the paired t-test.

To quantify potential effects of the prolonged or acute IFNγ treatment on the amplitude size and the event rate of the sEPSCs, the last 10 s of the recorded current traces were analysed by an experimenter blinded to the treatment groups using Mini Analysis Software 6 (Synaptosoft, USA). Time points used for statistical comparison were the same as for the analysis of evoked EPSC amplitudes. To test for an effect of prolonged IFNγ treatment the Mann–Whitney rank-sum test or the rank-sum ANOVA (Kruskal–Wallis test) followed by the Dunn's post hoc test was applied. The

acute IFNγ effect was analysed by comparing the sEPSC amplitude sizes and event rates of the last nine traces recorded prior to the drug application and the last nine traces after 25 min of IFNγ treatment by using the paired t-test.

If not mentioned otherwise, data are expressed as mean ± standard error of the mean (SEM) and p < 0.05 was set as the significance level.

Results

Prolonged IFNγ treatment facilitates synaptic strength at C-fibre- but not at Aδ-fibre synapses

The conventional IFNγ signalling by binding to its receptors IFNγR1 and IFNγR2 requires gene transcription via the downstream JAK-STAT pathway. To assess long-term effects of IFNγ, we incubated spinal cord slices with rat recombinant IFNγ (2000 U/ml) for at least 4 h prior to the whole-cell recordings. In order to prevent possible washout effects, IFNγ was also added to the superfusate during the recordings. The maximum exposure time to the cytokine (preincubation and recording time) was 8 h.

We first investigated whether prolonged IFNγ treatment had any effect on the synaptic strength between primary afferent C- or Aδ-fibres and spinal lamina I neurons.

Monosynaptic C-fibre-evoked EPSC amplitudes, as a measure for synaptic strength, significantly increased from 364 ± 117 pA in lamina I neurons of the control group (n = 15) to 1029 ± 226 pA in the IFNγ-treated neurons (n = 22; p = 0.009, Mann–Whitney rank-sum test, Figure 1(a) and (b)). To get information about the possible expression site of the observed change in synaptic strength, we analysed the PPR of the C-fibre-evoked EPSCs. We found that prolonged IFNγ treatment induced no changes in the PPR (change from 0.85 ± 0.07 in the control to 0.75 ± 0.05 in the IFNγ-treated group; p = 0.4, Mann-Whitney-Rank Sum test; Figure 1(d)), arguing against presynaptic mechanisms of synaptic facilitation.

IFNγ treatment had no effect on monosynaptic Aδ-fibre-evoked EPSC amplitudes (control group: 392 ± 65 pA, n = 22; treated group: 444 ± 141 pA, n = 14;
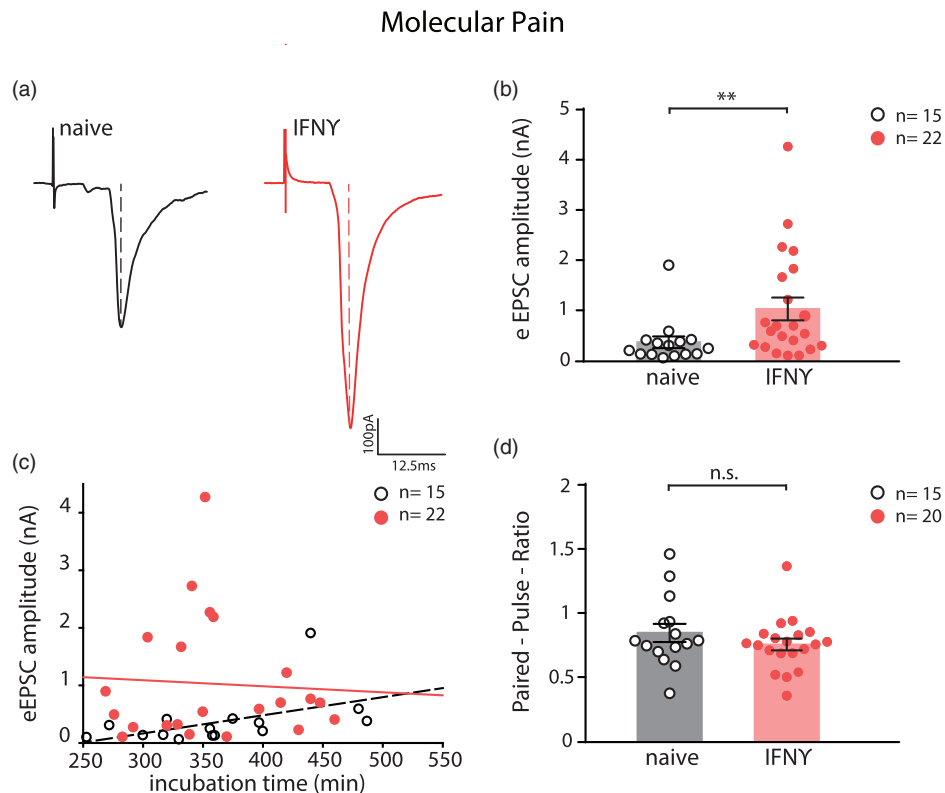
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Figure 1. Potentiated synaptic transmission at C-fibre synapses following prolonged IFNγ treatment. (a) Representative eEPSCs from naive group and IFNγ-treated group. Scale bar (100 pA; 12.5 ms). (b) Bar graphs show significant increase in eEPSC peak amplitude following prolonged IFNγ treatment (n = 22; p = 0.009, Mann–Whitney rank-sum test) when compared to naive group (n = 15). (c) No correlation between eEPSC amplitude and incubation time could be observed in the IFNγ-treated group (n = 22; R = −0.06). (d) The PPR remained unchanged following prolonged IFNγ treatment (p = 0.4, Mann–Whitney rank-sum test). IFNγ: interferon-γ; n.s.: not significant.
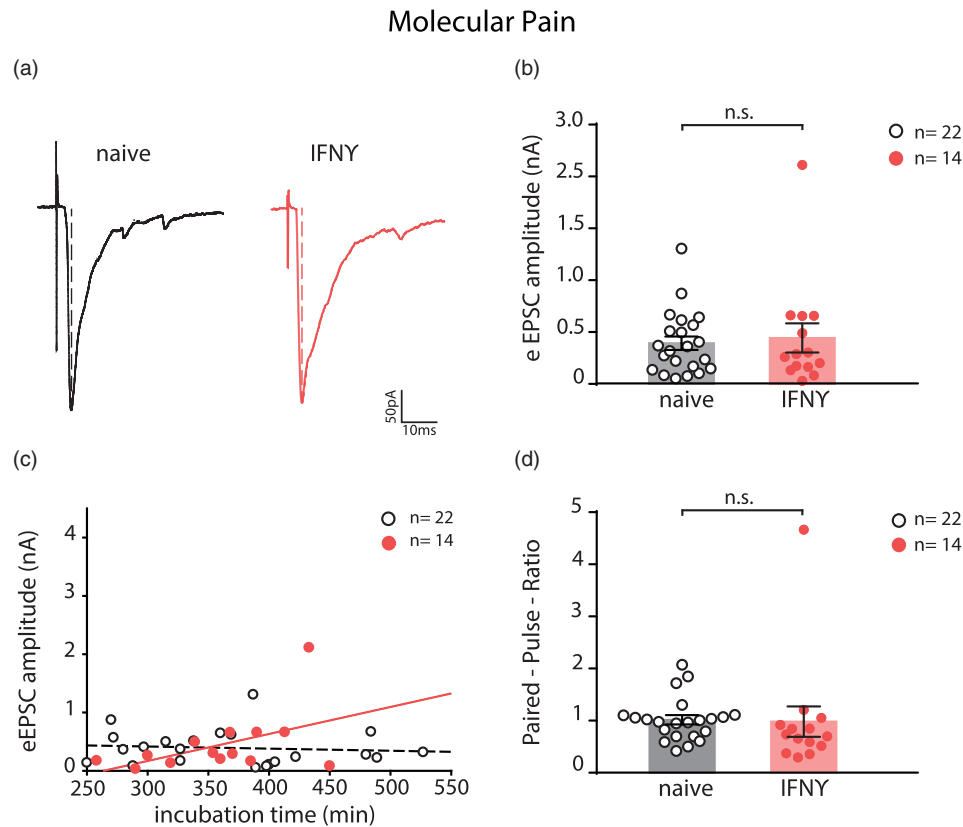
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Figure 2. Prolonged IFNγ treatment had no effect on synaptic strength at Aδ-fibre synapses. (a) Representative eEPSCs from naive group and IFNγ-treated group. Scale bar (50 pA; 10 ms). (b) Bar graphs of eEPSC peak amplitude show no alterations in the IFNγ-treated group (n = 14; p = 0.86, Mann–Whitney rank-sum test) when compared with the naive group (n = 22). (c) eEPSC amplitude does not correlate with the incubation time in the IFNγ-treated group (n = 14, R = 0.048). (d) The PPR remained unchanged following prolonged IFNγ treatment (p = 0.07, Mann–Whitney rank-sum test). IFNγ: interferon-γ; n.s.: not significant.

p = 0.86, Mann–Whitney rank-sum test, Figure 2(a) and (b)). The PPR of Aδ-fibre-evoked EPSCs was not different between the control (1.02 ± 0.09) and the IFNγ-treated group (0.98 ± 0.29; p = 0.07, Mann–Whitney rank-sum test; Figure 2(d)).

Since treatment duration ranged from 4 to 8 h, we tested a possible correlation between time and effect size. Neither C-fibre eEPSC amplitudes (correlation coefficient R = −0.06 and the coefficient for variation $R^2 = 0.03$, Figure 1(c)) nor Aδ-fibre eEPSC amplitudes (R = 0.48 and $R^2 = 0.23$, Figure 2(c)) correlated with the treatment duration.

These results suggest that prolonged IFNγ treatment induced an input specific, likely postsynaptic facilitation between C-fibres and spinal lamina I neurons.

IFNγ has no acute effect on synaptic strength

Even though the canonical IFNγ molecular pathway includes JAK-STAT signalling and gene transcription, there is evidence for alternative STAT-independent IFNγ signalling pathways, for example, involving mitogen-activated protein kinase and Ca^{2+}/

calmodulin-dependent protein kinase II.[31] In rat spinal cord slices, application of IFNγ for 2.5 min facilitated NMDA-induced currents in lamina II neurons.[32] Therefore, we further tested whether IFNγ additionally had an acute effect on synaptic transmission in lamina I neurons.

Following 5 min of baseline recordings, IFNγ (4000 U/ml) was applied to the superfusate for at least 25 min. This treatment had no effect on monosynaptic C-fibre-evoked EPSCs. Measured amplitudes were 96.9 ± 1.6% and 94 ± 4.7% of baseline (n = 7, p = 0.58, paired t-test; Figure 3(a) and (b)), respectively.

Likewise Aδ-fibre-evoked EPSC amplitudes did not change by IFNγ treatment (106.5 ± 2.8% and 93.8 ± 5.8% of baseline, n = 8, p = 0.06, paired t-test; Figure 3 (c) and (d)).

Recordings of lamina I neurons with stable recording conditions lasting up to 70 min provided no indication for any IFNγ mediated synaptic effects until the end of the recording period (data not shown). IFNγ induced no alteration in the PPR of C-fibre- or Aδ-fibre-evoked EPSCs (data not shown).
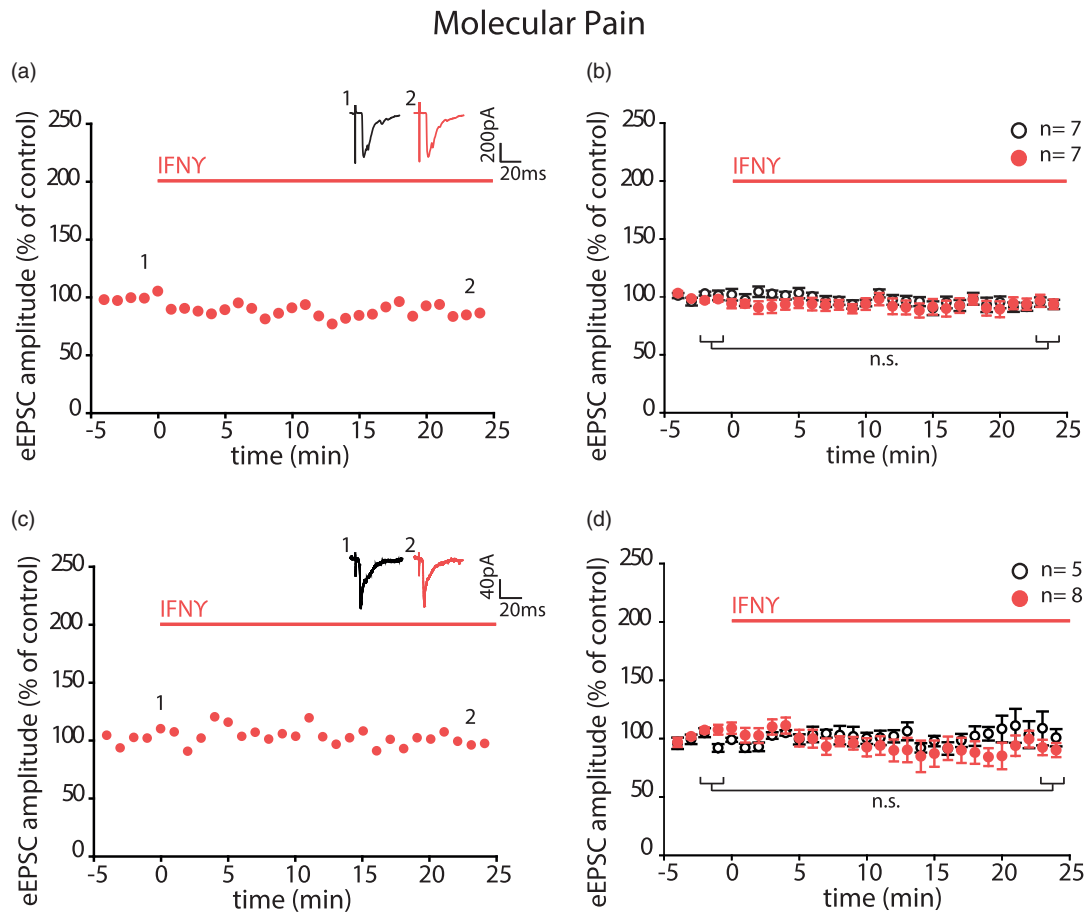
Figure 3. Short-term IFNγ treatment did not alter synaptic strength. (a and b) Recording of lamina I neurons with monosynaptic C-fibre input. eEPSC mean amplitudes were normalised to the baseline (5 min) and plotted against time. Period of IFNγ application is depicted as red horizontal line above. (a) Example of one representative neuron treated with IFNγ. Insets show original eEPSCs at indicated time points, prior treatment (1) and at the end of IFNγ application (2). (b) During 30 min of recording, control group (n = 7, non-filled black dots) shows stable recording. Last 2 min of IFNγ treatment show no changes compared to the last 2 min prior IFNγ application (n = 7, p = 0.58, paired t-test, filled dots). (c and d) Recording of lamina I neurons with monosynaptic Aδ-fibre input. (c) Treatment with IFNγ of one representative neuron, with insets illustrating original eEPSCs prior (1) and post (2) IFNγ treatment as in (a). (d) Again, control group (n = 5, non-filled black dots) shows no change during 30 min of recording; 25 min of IFNγ treatment had no effect on eEPSC amplitude size (n = 8, filled dots, p = 0.06, paired t-test). IFNγ: interferon-γ; n.s.: not significant.

Prolonged but not acute IFNγ treatment decreases sEPSC rate

IFNγ may not only affect monosynaptic transmission evoked by the stimulation of afferent fibres, but may also modulate the activity of other neurons synapsing to lamina I neurons. For example, IFNγ treatment increases spontaneous activity of spinal dorsal horn neurons of rodents in vitro and in vivo.[14,33] sEPSCs indicate the spontaneous neuronal network activity. We assessed the effect of IFNγ treatment on rates and amplitudes of spontaneous synaptic currents in lamina I neurons with monosynaptic C-fibre and/or Aδ-fibre input.

Prolonged IFNγ treatment significantly reduced the mean sEPSC rate from 11.0 ± 1.9 events/s in the control group (n = 32) to 5.7 ± 0.9 events/s in the treated group (n = 31, p = 0.02, Mann–Whitney rank-sum test; Figure 4(a) and (b)). The mean sEPSC amplitude remained stable and was 30.2 ± 1.3 pA in the control group (n = 31) and 30.1 ± 1.8 pA in the treated group (n = 32, p = 0.59, Mann–Whitney rank-sum test; Figure 4(c)). In contrast, acute IFNγ treatment had no effect on the mean sEPSC rate showing 8.3 ± 1.9 events/s in the control period and 8.6 ± 2.5 events/s after IFNγ application (n = 10, p = 0.8, paired t-test, Figure 5(a) and (b)). Mean sEPSC amplitude started with values of 40.6 ± 6.0 pA prior IFNγ application and remained stable throughout the 25 min recording at 38.8 ± 7.6 pA (n = 10, p = 0.11, paired t-test, Figure 5(c)).

These results show that prolonged IFNγ treatment induced a reduction in the sEPSC rate, suggesting that this effect could be mediated by a presynaptic mechanism.
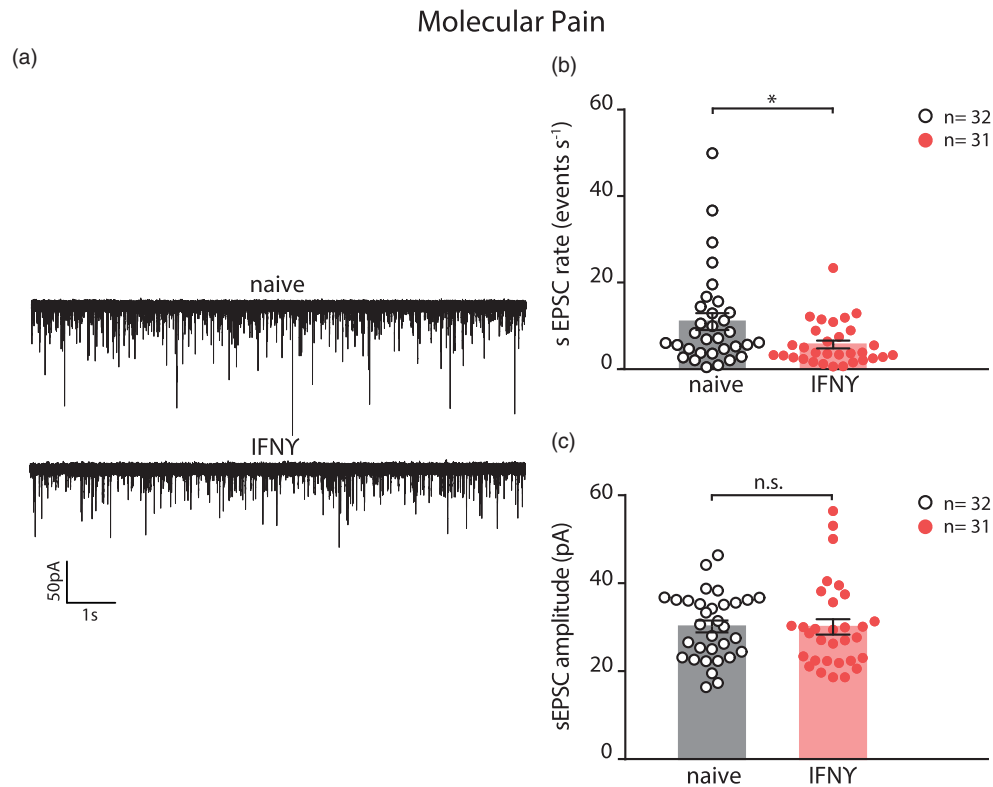
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(a)

(b)

Figure 4. sEPSC rate was decreased following prolonged IFNγ treatment. (a) sEPSC recording of one representative neuron each for the naive and the IFNγ-treated group. Scale bar (50 pA; 1 s). (b) Bar graphs show significant reduced mean sEPSC rate following prolonged treatment (n = 31) when compared to a naive group (n = 32, p = 0.02, Mann–Whitney rank-sum test). (c) Contrary to the sEPSC rate, the mean sEPSC amplitude showed no alterations following prolonged IFNγ treatment (n = 31, p = 0.59, Mann–Whitney rank-sum test). IFNγ: interferon-γ; n.s.: not significant.

Microglial inhibition abolishes C-fibre input specific synaptic facilitation

In the superficial spinal dorsal horn of rodents, IFNγ receptors are expressed by neurons[13,21] as well as by resting microglial cells.[15] The observed IFNγ induced input-specific synaptic facilitation between C-fibres and lamina I neurons could thus be mediated directly by neuronal IFNγ receptors or indirectly by IFNγ receptor activation in non-neuronal cells or both.

To assess whether microglia activation is involved in IFNγ-induced facilitation at C-fibre synapses, we applied the microglial inhibitor minocycline (100 μM) prior to IFNγ. This treatment abolished the IFNγ-mediated C-fibre-evoked synaptic facilitation at C-fibre synapses. Mean eEPSC amplitude was 291 ± 41 pA (n = 12) and was compared to the group only treated with IFNγ (1029 ± 226 pA, n = 22; p = 0.025, Kruskal–Wallis test with Dunn's post hoc test, Figure 6(a)). Minocycline treatment alone had no effect on synaptic transmission (control group: 291 ± 117 pA, n = 15; minocycline group: 567 ± 145 pA, n = 10; p = 0.25, Kruskal–Wallis test; Figure 6(a)).

The PPR did not show a significant change within these four groups (IFNγ group: 0.75 ± 0.05, n = 20; minocycline and IFNγ group: 0.67 ± 0.04 n = 10; naive group: 0.85 ± 0.07, n = 15; minocycline group: 0.81 ± 0.08, n = 9; p = 0.3, Kruskal–Wallis test; Figure 6(b)).

These results indicate that postsynaptic facilitation at C-fibres requires activation of microglia.

Microglial activation is not required for IFNγ mediated decrease in sEPSC rate

We then examined whether microglial activation is also required for the IFNγ-induced reduction in the rate of sEPSCs following prolonged treatment.

Statistical analysis using the Kruskal–Wallis test detected a significant effect in sEPSC rates within the four groups tested (p = 0.016), but post hoc comparison of the relevant groups (Dunn's test) showed no differences nor between the IFNγ-treated group (5.7 ± 0.9 events/s, n = 31) and the minocycline/IFNγ-treated group (6.4 ± 1.1 events/s, n = 11, p > 0.05) neither between the naive control group (11.0 ± 1.9 events/s, n = 32) and the minocycline-treated group (11.4 ± 2.1
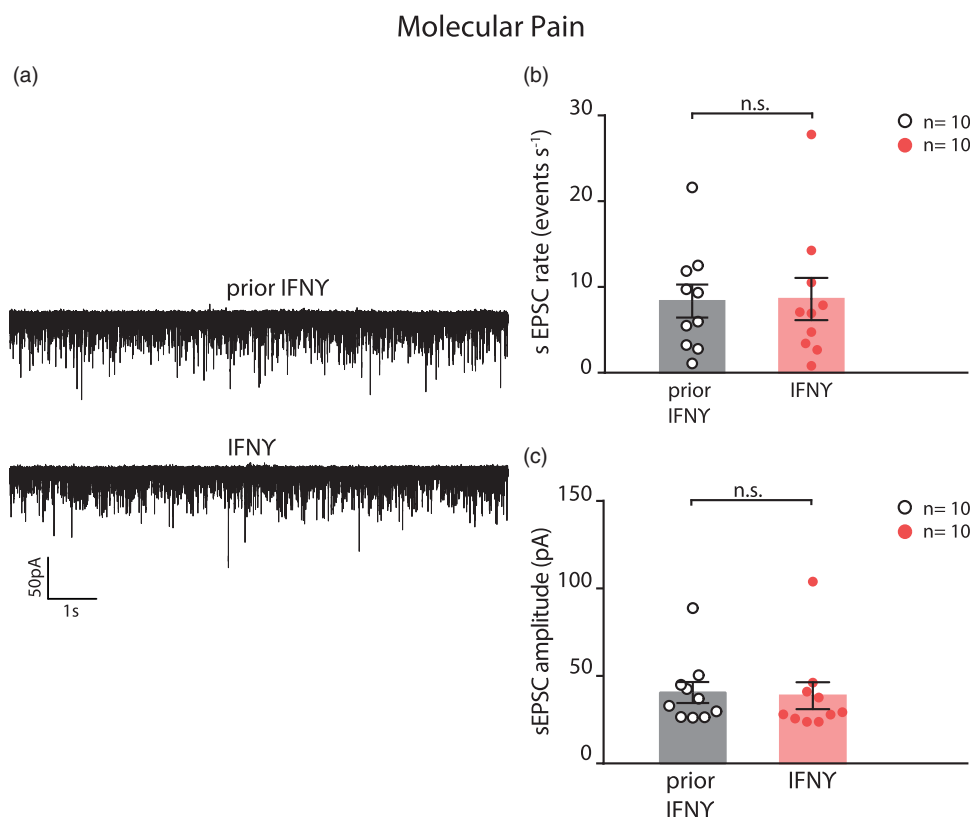
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Figure 5. Spontaneous activity was unaltered following short-term IFNγ treatment. (a) Recording of one representative neuron, illustrating spontaneous activity prior to IFNγ application and at the end of IFNγ treatment. Scale bar (50 pA; 1 s). (b) Bar graphs show no alteration in the mean sEPSC rate when comparing the averaged sEPSC rate of 2 min prior IFNγ treatment (n = 10) with the last 2 min of IFNγ treatment (n = 10, p = 0.8, paired t-test). (c) The mean sEPSC amplitude did not change following short-term IFNγ treatment (n = 10, p = 0.11, paired t-test). IFNγ: interferon-γ; n.s.: not significant.
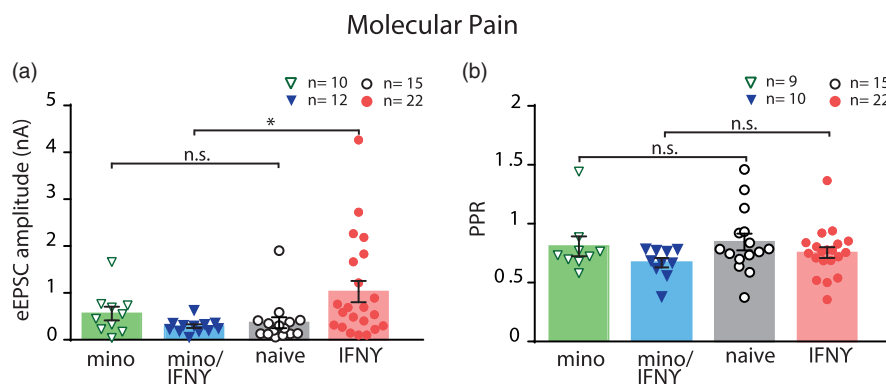
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Figure 6. Inactivation of microglial cells abolished the facilitative effect of IFNγ on synaptic strength at C-fibre synapses. (a) Bar graphs show no alterations in mean eEPSC amplitude between naive group (n = 15) and minocycline-treated group (n = 10, p = 0.25, Kruskal–Wallis test). Minocycline/IFNγ-treated group (n=12) shows a significant decrease in mean eEPSC amplitude when compared to the IFNγ-treated group (n = 22, p = 0.025, Kruskal–Wallis test and Dunn's post hoc test), hence illustrating the prevention of the IFNγ effect. (b) No alteration in PPR is shown within these four groups (p = 0.3, Kruskal–Wallis test). IFNγ: interferon-γ; n.s.: not significant.

events/s, n = 9, p > 0.05; Figure 7(a)). The mean sEPSC amplitudes of 30.1 ± 1.8 pA in the IFNγ group, 28.7 ± 1.3 pA in the minocycline/IFNγ group, 30.2 ± 1.3 pA in the naive group and 29.6 ± 2.7 pA in the minocycline group showed no significant differences within these four groups (p = 0.95, Kruskal–Wallis test; Figure 7(b)).

These data suggest that the reduction in the sEPSC rate after IFNγ treatment did not require activation of microglia.
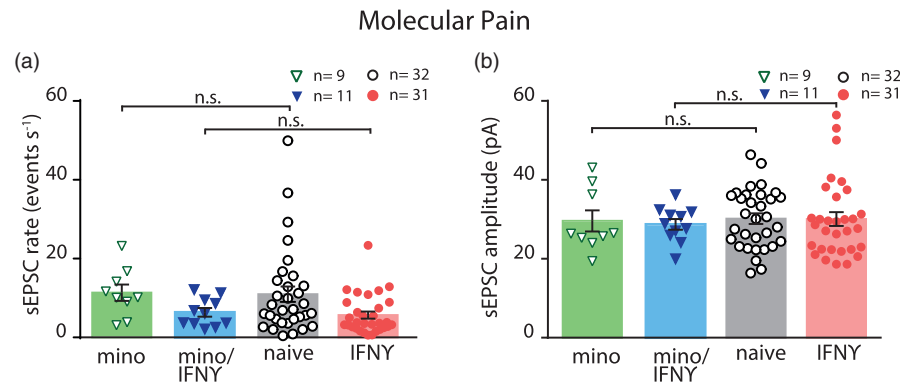
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Figure 7. Inhibition of microglial activation did not prevent the IFNγ effect on sEPSC rate. (a) Minocycline does not have an effect on the mean sEPSC rate as illustrated with the bar graphs comparing the naive group (n = 32) with the minocycline-treated group (n = 9, p > 0.05, Kruskal–Wallis test). Applying minocycline prior to IFNγ treatment (n = 11) shows no change when compared to IFNγ-treated group (n = 31, p > 0.05, Kruskal–Wallis test). (b) Bar graphs show no significant alteration in mean sEPSC amplitude within these four groups (p = 0.95, Kruskal–Wallis test). IFNγ: interferon-γ; n.s.: not significant.

Discussion

Here, we report effects of IFNγ on the transmission at the first synapse in nociceptive circuits in the rat spinal dorsal horn: First, prolonged IFNγ treatment facilitated the strength at C-fibre synapses but not at Aδ-fibre synapses. Second, IFNγ-mediated synaptic facilitation required microglial activation. Third, prolonged IFNγ treatment inhibited sEPSC rate of lamina I neurons. Fourth, IFNγ effect on sEPSC rate was independent of microglial activation. Acute IFNγ treatment up to 25 min had, in contrast, no impact either on the synaptic strength at C-fibre and Aδ-fibre synapses or on sEPSC.

Recent studies have demonstrated that the pro-inflammatory cytokine IFNγ, potentially released by infiltrated T-cells,[5,8,10] spinal cord astrocytes or neurons,[34] initiates persistent mechanical allodynia, a key symptom in neuropathic pain. This effect could be due to action at IFNγ receptors expressed by different cell types in the superficial dorsal horn of rodents. Receptor expression has been described at pre- and postsynaptic terminals,[13,14] astrocytes[10] and microglia.[15] A direct neuronal effect of IFNγ on α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) function has been shown in cultures of spinal dorsal horn[33] and hippocampal neurons.[35] The present results suggest that prolonged presence of IFNγ has an indirect, microglia-mediated effect on synaptic input to lamina I neurons. The increase in synaptic strength at C-fibres is most likely expressed postsynaptically as indicated by the unchanged PPR.

To differentiate a direct from an indirect effect of IFNγ on synaptic transmission, we applied minocycline, which is widely used to prevent microglial activation in vivo and in vitro.[10,15,32] But this drug has also potential effects on other cell types, especially on neurons.[36]

However, in our experiments, we found no direct effect of 100 μM minocycline neither on afferent fibre-evoked nor on spontaneous synaptic currents.

IFNγ can directly activate microglia in the spinal dorsal horn[15] leading to the release of glial mediators, such as the cytokines IL-β and TNF that modulate synaptic strength.[2–4] Another microglial mediator, the chemokine ligand 2 (CCL2) is involved in neuropathic pain processing.[37] Intrathecal injections of CCL2 induce mechanical hypersensitivity.[38,39] IFNγ treatment of rat spinal cord slices triggers CCL2 release from microglial cells, thereby enhancing NMDA inward currents in neurons of the superficial spinal laminae.[32] This could be a potential mechanism of presently identified IFNγ-mediated postsynaptic increase of synaptic strength at C-fibres.

Another main finding of this study was that the facilitation of the synaptic transmission induced by prolonged IFNγ treatment was input specific. IFNγ enhanced synaptic strength at C-fibre but not at Aδ-fibre synapses with spinal lamina I neurons. We recently reported that the chemokine CX3CL1 (fractalkine) likewise facilitates strength specifically at C-fibre but not at Aδ-fibre synapses contacting lamina I neurons.[40] The mechanisms leading to this input specificity are still unclear. Central terminals of C-fibres and Aδ-fibres may be differentiated by the range of released neurotransmitters. For example, studies reported that the brain-derived neurotrophic factor (BDNF) and its receptor TrkB is mainly expressed at peptidergic (substance P and calcitonin gene-related peptide (CGRP)) C-fibre synapses,[41,42] and BDNF caused hyperexcitability of lamina I neurons.[43] Another microglial mediator, CCL2, is also co-localised mainly with substance P and CGRP in spinal superficial laminae.[38] However, in the present study, a presynaptic modulation specifically at

C-fibre synapses is unlikely because the PPR was unchanged by IFNγ. It is not known if TrkB receptors or CC chemokine receptors type 2 are expressed selectively at postsynaptic membranes of C-fibre synapses at lamina I neurons. However, it has been demonstrated recently, for example, that postsynaptic AMPARs may be modulated in a synapse-specific manner in superficial spinal laminae. The transmembrane AMPAR regulatory protein TARPγ-2, which is necessary in a form of inflammatory pain-induced plasticity, is associated with AMPARs at synapses in lamina II but excluded from those at C-fibre inputs.[44] These findings reveal that synapses may differ in postsynaptic receptor function.

The canonical IFNγ molecular pathway includes JAK-STAT signalling and gene transcription resulting in delayed cellular effects. However, there is also evidence for alternative STAT-independent mechanisms.[31] Even STAT-dependent IFNγ signalling pathways via the cytoplasm have been described playing an essential role in the induction of NMDA-receptor-dependent long-term depression in the hippocampus.[45] In addition, behavioural, immunohistochemical and electrophysiological data are pointing to acute IFNγ effects in the spinal cord. Intrathecal IFNγ application increases spinal nociceptive reflexes,[46] evokes immediate biting behaviour in rats[13] and raises phospho-STAT1 levels in the spinal dorsal horn within minutes[15]. In addition, IFNγ treatment of spinal slices for 2.5 min facilitated NMDA-induced currents in superficial dorsal horn neurons.[32] We found, however, no evidence for immediate synaptic effects by IFNγ in lamina I neurons.

In contrast to the facilitatory effect of IFNγ on synaptic strength at C-fibre synapses, prolonged application of IFNγ reduced the spontaneous activity of lamina I neurons in the present study. This suggests that IFNγ may modulate distinct excitatory inputs to the same neuron differentially. Lamina I neurons express hundreds to thousands of excitatory synapses receiving input from primary afferents and local interneurons.[25,26] The amount of glutamatergic synapses contacting lamina I neurons from primary afferents and interneurons, respectively, could be very different ranging from at least half of the excitatory synaptic input to neurokinin-1-receptor-expressing projection neurons from peptidergic C-fibres to another population of lamina I neurons, which receive only very few contacts from peptidergic afferents or Aδ nociceptors.[47,48] This could contribute to the between-cell variability of our data. As a result, strengthening the input from nociceptive C-fibres to lamina I neurons while simultaneously dampening excitatory noise from other sources by IFNγ could be a potential mechanism of contrast enhancement in the spinal nociceptive network.

We found that the IFNγ effect on evoked EPSCs was mediated by the activation of glial cells. In contrast,

this was not the case for the IFNγ effect on the decreased rate of sEPSCs. In addition, the decreased rate and unchanged amplitude of sEPSCs suggest in this case a presynaptic expression of the IFNγ effect. Taken together, this points to fundamentally different mechanisms involved.

These findings substantiate the importance of neuro-inflammatory mediators for the signalling at the first synaptic relay station in nociceptive pathways.

Declaration of Conflicting Interests

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