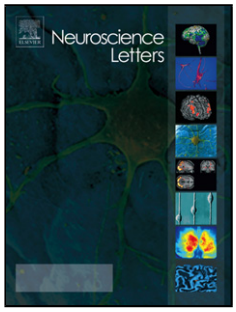

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Comparison of *ex vivo* and *in vitro* Actions of Gabapentin in Superficial Dorsal Horn and the Role of Extra-spinal Sites of Drug Action.

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Highlights

- Spinal actions of gabapentin studied *ex vivo* differ from those seen *in vitro.*
- Gabapentin increases sEPSC frequency in inhibitory neurons *ex vivo*
- Gabapentin decreases sEPSC frequency in inhibitory neurons *in vitro*
- This difference may relate to absence of descending pain control *in vitro.*
- Extraspinal actions are involved in gabapentin attenuation of allodynia.

Abstract

Although gabapentin (GBP) is a first-line treatment in the management of neuropathic pain, its mechanism of action is incompletely understood. We have previously shown, in rats made neuropathic following sciatic chronic constriction injury, that IP injection of 100mg/kg GBP decreases overall excitability of spinal cord slices obtained *ex vivo.* Excitability was assessed using confocal imaging to monitor the amplitude of K^+- induced increases in cytoplasmic Ca^{2+}. This decrease in excitability involved a reduction in the frequency and amplitude of spontaneous EPSC's (sEPSC) in putative excitatory *substantia gelatinosa* neurons and an increase in sEPSC frequency in putative inhibitory neurons. We used have whole-cell recording to compare these *ex vivo* actions of GBP with its acute *in vitro* effects on spinal cord slices obtained from neuropathic but drug-free rats. While GBP (100μM) decreased sEPSC amplitude and frequency in excitatory neurons *in vitro* in a similar fashion to effects observed *ex vivo*, sEPSC frequency in inhibitory neurons was decreased *in vitro* rather than increased. Acute *in vitro* application of GBP also failed to decrease the overall excitability of slices from neuropathic animals as monitored by

confocal Ca^{2+} imaging. Since spinal cord slices *in vitro* are disconnected from the periphery and higher brain centres, the GBP-induced increase in sEPSC frequency in inhibitory neurons previously reported and seen *ex vivo* must result from extra-spinal actions. It may be attributable to alterations in descending neurotrophic control of dorsal horn circuitry.

Key Words

Neuropathic Pain, alpha-2-delta, *substantia gelatinosa,* descending modulation, top down pain modulation

Highlights

- Spinal actions of gabapentin studied *ex vivo* differ from those seen *in vitro.*
- Gabapentin increases sEPSC frequency in inhibitory neurons *ex vivo*
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- This difference may relate to absence of descending pain control *in vitro.*
- Extraspinal actions are involved in gabapentin attenuation of allodynia.

1. Introduction

Neuropathic pain results from disease or lesion of the somatosensory system [1]. This chronic, maladaptive "disease of pain" neither protects from injury nor supports healing and repair. It can result from traumatic nerve, spinal cord or brain injury (including stroke) or can be associated with diabetic, HIV/AIDS, post-herpetic neuropathies or with multiple sclerosis [2]. Whereas opioids are effective in the management of nociceptive pain, there is no similar panacea for the treatment of neuropathic pain [3;4]. Although gabapentin (GBP) is a first line treatment [5], its mechanisms of action, at both the cellular and molecular level, are incompletely understood [4;6-10]. Actions of GBP within the somatosensory system are exerted at the spinal [11-14], thalamic [15], cortical [12;16] and possibly peripheral levels [17]. GBP is also known to act in brainstem and medulla to alter descending control of nociceptive processing at the spinal level [10;18-20] and to promote supraspinal modulation of affective qualities of pain [10].

In the rat spared nerve injury model of neuropathic pain, IP injection of 100mg/kg GBP rapidly and reversibly alleviates mechanical allodynia [21]. In our hands, using the chronic constriction injury (CCI) model, this dose of GBP attenuated mechanical allodynia without respiratory depression and only mild and transient somnolence [12]. Recordings from *substantia gelatinosa* neurons in spinal cord slices obtained *ex vivo* from GBP-treated animals revealed a decrease in the amplitude and frequency of spontaneous EPSC's (sEPSC's) in excitatory, delay firing neurons and an increase in frequency of sEPSC's in tonic firing, putative inhibitory neurons [12]. All of these actions would tend to reduce the overall excitability of the superficial dorsal horn. This was confirmed by the observation that depolarization evoked Ca^{2+}-responses were attenuated in *ex vivo* slices from animals that had previously received GBP. This reflects direct actions of GBP on superficial dorsal horn neurons and synapses and/or indirect actions as

a result of its interaction with descending control mechanisms [10;18-20] and/or actions on primary afferent neurons in the periphery. To distinguish between these possibilities, we compared our original *ex vivo* data [12] with the *in vitro* actions of GBP on spinal cord slices acutely isolated from rats subject to CCI. These are referred to henceforth as "neuropathic slices from neuropathic rats".

2. Materials and Methods.

Experimental protocols were approved by the Health Sciences Animal Welfare Committee at the University of Alberta which is responsible for maintaining standards set forth by the Canadian Council of Animal Care.

Male Sprague Dawley rats (19-23 days old) were anesthetized with isoflurane (5% induction, 2% maintenance) and subjected to sciatic CCI using polyethylene cuffs [22] and assessed for mechanical allodynia using von Frey filaments 10-14 days after surgery as described previously [23]. In sham operated animals, the sciatic nerve was exposed but not manipulated. Animals exhibiting a paw withdrawal threshold <6 g were assumed to be expressing allodynia and categorized as neuropathic.

Data from *ex vivo* spinal cord slices have been previously published and are presented again here with permission. In those experiments, Rats were euthanized with a high dose of urethane (1.5g/kg, IP). Following cessation of respiration and loss of ocular and nociceptive reflexes (paw pinch with forceps), the spinal cord was removed by laminectomy and transverse slices (300 μm) were cut at 4ºC using a HM 650V vibratome (Thermo Scientific). The slices were allowed to recover for 1hr at 37 ˚C prior to storage at room temperature before use.

Whole-cell patch-clamp recordings were made from lamina II (*substantia gelatinosa*) neurons ipsilateral to the sciatic injury. *Substantia gelatinosae* were identified by their

translucent appearance in the slice. Slices were superfused at room temperature (~22°C) with 95% O_2-5% CO_2 saturated aCSF which contained (in mM): 127 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 26 $NaHCO_3$, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 25 D-glucose, pH 7.4. Recording pipettes had resistances of 5-10 MΩ when filled with an internal solution containing (in mM): 130 potassium gluconate, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 10 EGTA, 4 Mg-ATP, 0.3 Na-GTP, pH 7.2, 290-300mOsm. Recordings were made using an NPI SEC-05LX amplifier in discontinuous, single electrode voltage-clamp or current clamp mode.

Data were collected from neurons that exhibited overshooting action potentials of at least 60 mV in amplitude. They were categorized by their discharge pattern in response to 800-ms square-wave depolarizing current pulses as either delay (Fig 1b) or tonic firing (Fig 2a). Data were not collected from neurons exhibiting transient, phasic or irregular firing patterns [23]. Membrane potential was set to −60 mV prior to delivery of depolarizing current commands. Delay firing neurons were assumed to be excitatory glutamatergic neurons whereas tonic firing neurons were assumed to be inhibitory GABAergic neurons [24;25].

Holding potential was set to -70mV for recording of sEPSC's. Mini Analysis Program (Synaptosoft, Decatur, GA, USA) was used to distinguish sEPSC from baseline noise and to generate event lists for cumulative probability plots. All detected events were then re-examined and accepted or rejected based on visual examination. Acceptable events had a sharp onset and exponential offset, a total duration of < 50ms and an amplitude at least double the baseline noise. Data for cumulative probability plots were obtained from the first 100 events observed during a 3 min recording period. In cells that displayed <100 events, all events recorded in the 3 min recording period were taken. Intracellular Ca^{2+} was imaged using an Olympus FV300 confocal microscope connected to an Argon laser (488 nm) as previously described [12]. Slices were

superfused at room temperature (22-24°C) with aCSF that was bubbled with 95% O_2 - 5% CO_2. Dorsal horn neurons were loaded with Fluo-4 acetoxymethyl ester (AM) (Fluo-4 AM) by pressure injection (25-50 mmHg for 20min) using a broken patch electrode (outer tip diameter of 5-15 µm) at a depth of 50 µm. A desired level of loading was achieved when at least 5 dorsal horn cells and their basic morphology were clearly visible in a given field of view of 200-350 µm diameter. Acquisition speed was 1.08 frames per second and fluorescence traces acquired from regions of interest using FluoView. The digital points corresponding to the fluorescence traces were exported to Microsoft Excel and Microcal Origin 2015 (Origin Lab Corp., Northampton, MA, USA) was used to generate plots of normalized fluorescence $((F_{max} - F_0)/F_0)$ in response to stimulation with 35mM K^+. Bar graphs of response amplitude were constructed in Microcal Origin 2015 (https://www.originlab.com/). Student's two tailed unpaired t-test was used to assess differences in $(F_{max} - F_0)/F_0$ between K^+ responses before and after superfusion of 100µM GBP. All experiments were performed blinded to the treatment of each rat until after analysis.

All chemicals were from Sigma, St. Louis, MO (USA) except GBP, which was from TCI America, Portland OR (USA).

3. Results.

3.1 Similarity of GBP Actions on Putative Excitatory Delay Neurons ex vivo and in vitro.

Figure 1a illustrates schematically the distinction between *ex vivo* and *in vitro* experiments. In our previous *ex vivo* experiments, animals were euthanized 45min after IP injection of 100mg/kg GBP and 30 min after Von Frey filament testing for attenuation of mechanical allodynia [12].

Figure 1b shows the discharge pattern evoked by current injections into a delay, putative excitatory neuron. Figure 1c illustrates our previous finding [12] that prior IP injection of GBP (100mg/kg) into neuropathic rats, prior to isolation of slices *ex vivo* and recording, produced a significant increase in the interevent interval (IEI) or decrease in frequency of sEPSC's in putative excitatory delay neurons. This effect was not seen in neurons from sham operated animals (Fig 1d). When 100 μM GBP was applied acutely to *in vitro* slices prepared from neuropathic rats, the IEI of sEPSC's was similarly increased (frequency decreased; Fig 1e). This effect was not seen in neurons in slices from sham operated animals (Fig 1f)

Figure 1g illustrates our previous finding that prior IP injection of GBP produced a significant decrease in sEPSC amplitude in putative excitatory delay neurons studied *ex vivo* [12]. A similar decrease in amplitude was seen in neurons from sham operated animals (Fig 1h). These effects were also seen following *in vitro* acute application of GBP to *in vitro* slices from both neuropathic and sham operated animals (Fig 1i and j). All effects of GBP on excitatory neurons seen *ex vivo* were thus replicated *in vitro.*

3.2 Differences between GBP Actions on Putative Inhibitory Tonic Neurons ex vivo and in vitro.

Figure 2a shows the discharge pattern evoked by current injections into a putative inhibitory, tonic neuron. Figure 2b illustrates previous data that show that prior IP injection of GBP (100mg/kg) produced a significant increase in frequency (decrease in IEI) of sEPSC's examined *ex vivo* in putative inhibitory tonic neurons [12]. Notably and by contrast, when 100μM GBP was applied to neuropathic slices *in vitro* sEPSC frequency was decreased (IEI increased, Fig 2d). In sham operated animals, GBP decreased the frequency (increased IEI) of sEPSC's both *ex vivo* and *in vitro* (Fig 2c and e).

The amplitude of sEPSC's in putative inhibitory tonic neurons was unaffected when GBP was administered *in vivo* (100mg/kg) and slices examined *ex vivo* (Fig 2f previous data from [12]) or when it was applied acutely *in vitro* (100μM) to slices from neuropathic rats (Fig 2h). The amplitude of sEPSCs in tonic neurons from sham operated animals was unaffected by GBP injection *in vivo* (Fig 2g) yet was slightly decreased when applied to neurons from sham operated animals *in vitro* (Fig 2i).

3.3 Differences Between in vitro and ex vivo Effect of GBP on Overall Dorsal Horn Excitability.

The observation that the effect of GBP in tonic putative inhibitory neurons depends on whether it is administered *in vivo* or *in vitro* suggest that sites of action outside the dorsal horn may be responsible for the increase in sEPSC frequency seen in these neurons *ex vivo* following *in vivo* administration (Fig 2b). This may be a consequence of increased descending inhibition [18;20] or decreased descending excitation [19] as a result of GBP actions in *locus coeruleus* and/or brainstem serotonergic nuclei. We have already shown using confocal Ca^{2+} imaging, that prior administration of GBP to neuropathic rats reduces the overall excitability of slices obtained *ex vivo* [12] (results reproduced as Fig 2j). By contrast, acute administration of GBP to neuropathic slices *in vitro* does not alter their overall excitability (Fig 2k). This result underlines the importance of extra-spinal mechanisms in mediating spinal actions of GBP *in vivo.*

4. Discussion and Conclusions

Spinal cord slices obtained *ex vivo* from GBP treated, neuropathic animals show reduced excitability compared to saline injected controls (Fig 2j). By contrast, excitability is unchanged when GBP is applied to neuropathic slices *in vitro* (Fig 2k). These findings are consistent with

the suggestion that a spino-bulbo-spinal pathway must be intact in order for the full effect of GBP to be observed [19] and with the finding that intracerebroventricular injections of GBP can effectively reverse both sensory and affective dimensions of pain in neuropathic rats [10].

4.1 Effects of GBP on Putative Excitatory Delay Neurons.

Despite this lack of effect of GBP on overall dorsal horn excitability *in vitro* (Fig 2k), the actions of GBP on putative excitatory delay neurons *in vitro* and *ex vivo* are similar. In both situations, the drug reduces sEPSC amplitude and frequency (Fig 1c, e, g and i). The decreases in frequency likely reflect actions of GBP that are selective for neuropathic neurons as the drug fails to affect sham operated neurons either *ex vivo* (Fig 1d) or *in vitro* (Fig 1f).

Whereas decreased frequency normally indicates a presynaptic site of drug action, decreased sEPSC amplitude may be attributable to both pre and postsynaptic effects. GBP also reduces sEPSC amplitude both *ex vivo* and *in vitro* in putative excitatory delay neurons from both neuropathic and sham operated animals (Fig 1g – j). Effects on sEPSC amplitude thus do not depend on neurons entering a neuropathic state and may reflect GBP-induced decreases in synaptic transmission previously described in unidentified, uninjured lamina II neurons [11;27].

4.2 Effects of GBP on Putative Inhibitory Tonic Neurons

The main difference between GBP actions in neuropathic rats *in vitro* compared to *ex vivo* are that it increases sEPSC frequency in putative inhibitory tonic neurons *ex vivo* (Fig 2b) yet decreases it *in vitro* (Fig 2d). This decrease *in vitro* was also seen in neurons from sham operated animals (Fig 2e) and again may reflect the previously described action of GBP on uninjured unidentified lamina II neurons [11;27].

In inhibitory neurons, sEPSC amplitude was unchanged by GBP both *in vitro* and *ex vivo* (Fig 2f and h) and in sham operated neurons *ex vivo*. (Fig 2g). The decrease in sEPSC amplitude seen in neurons from sham operated animals *in vitro* (Fig 2i) may also reflect the depressant action of GBP on unidentified dorsal horn neurons [11;27].

4.3 Differences Between ex vivo and in vitro Actions of GBP and the Role of Extra-spinal Sites of Drug Action

Because GBP has little or no effect on intrinsic excitability of dorsal horn neurons [12], its ability to decrease the frequency and amplitude of sEPSC's in excitatory neurons (Fig 1c and g) at the same time increasing sEPSC frequency in inhibitory tonic cells (Fig 2b) likely contributes to the decrease overall excitability observed *ex vivo* (Fig 2j). By contrast, in the *in vitro* situation (Fig 2k), the decreased sEPSC frequency in inhibitory neurons (Fig 2d) may counter the decreased synaptic activation of excitatory neurons (Fig 1c and g) so that no overall effect on excitability is seen (Fig 2k).

Since most of the actions of GBP involve decreases in Ca^{2+} channel current and decreased release of neurotransmitters [13;28-33], it is difficult to see how the drug could act directly to increase in sEPSC frequency in inhibitory neurons *in vivo* (Fig 2b) [12]. *In vitro* GBP decreases sEPSC frequency (Fig 2d). This may result from its actions on Ca^{2+} channels and their interaction with the release process in primary afferent terminals [32]. It is therefore likely that the increase in sEPSC frequency seen *ex vivo* in putative inhibitory tonic neurons(Fig 2b) [12], is a spinal manifestation of GBP effects in an intact nervous system. These override direct depressant effects of GBP on sEPSC frequency.

One possible concern with this conclusion is that the concentration of GBP reaching neurons *in vivo* is very different from that reaching neurons when it is applied acutely *in vitro*. This possibility is unlikely to explain the observation that GBP deceases sEPSC frequency in tonic neurons when applied acutely (Fig 2d) yet increases frequency when given *in vivo* (Fig 2b). Given that the primary action of GBP is to reduce neurotransmitter release, it is unlikely that any concentration of drug given *in vitro* would be able to increase transmitter release as is seen *in vivo.*

The role of serotonergic and noradrenergic pathways in controlling spinal nociceptive processing is well established [20;34-37]. Such processes may be especially relevant in regulation of neuropathic pain [20;38]. Descending inhibition of spinal nociceptive processing is mediated via both α_1 and α_2 adrenoceptors and $5HT_7$ receptors whereas serotonergic activation of metabotropic $5HT_2$ receptors and ionotropic $5HT_3$ receptors facilitates transmission [37;39-42]. The role of these mechanisms in the actions of GBP is illustrated by the observation that central depletion of central noradrenaline with 6-hydroxydopamine attenuates its anti-nociceptive action [43]. Moreover, GBP effects in *locus coeruleus* to either increase glutamatergic function [18] or to reduce GABAergic function [44] are thought to stimulate descending inhibition. Interestingly, α1 receptor activation causes selective depolarization of GABAergic but not of excitatory neurons in *substantia gelatinosa* [42]. This agrees with recent transcriptional studies of the dorsal horn where α1 adrenoceptors (*Adra1a, Adra1b* genes) are expressed mainly in GABAergic neurons [45]. It would be interesting to know whether activation of presynaptic α1-adrenoceptors also selectively increases the frequency of sEPSC's in these neurons. If so, it his might explain our observation that the presence of an intact nervous

system is required to see the increase in sEPSC frequency in inhibitory neurons following administration of GBP *in vivo* (Fig 2b).

If this is so, the time course of our experiments requires further consideration. Recordings from *ex vivo* slices are made 2-8h after the prior IP injection of GBP (Fig 1a). If drug action involves augmentation of descending inhibition, any noradrenergic effects on sEPSC's would need to persist for several hours after their initiation. Although the acute effects of noradrenaline in dorsal horn are well characterised [25;42;46;47], it remains to be determined whether it can exert long lasting neurotrophin-like effects on synaptic transmission as has been reported in cultured hippocampal neurons [48].

As well as supporting the role of descending pathways in the anti-allodynic action GBP, our findings lead us to the hypothesis that these pathways may exert tonic neurotropic control of spinal nociceptive processing.

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Author Contributions

SRAA Helped to design study, carried out all experiments and wrote first draft and contributed to revision of manuscript. PAS Instigated project, provided laboratory facilities, edited and revised manuscript.

Figure 1. Comparison of *ex vivo* and *in vitro* actions of GBP on sEPSC's in Putative Excitatory Delay Neurons.

a. Scheme to illustrate experimental design. For *ex vivo* experiments neuropathic or sham operated animals received 100mg/kg GBP prior to euthanasia and generation of slices. For *in vitro* experiments, neuropathic or sham operated animals were euthanized and the spinal cord slices subsequently exposed to 100μM GBP. **b.** Sample recordings of AP discharge in a putative excitatory delay neuron in response to a series of depolarizing current steps. Neuronal RMP was are held at -60 mV prior to current injection. **c.** Increase in sEPSC inter-event interval (IEI) in putative excitatory delay neurons recorded *ex vivo* from rats that had received 100mg/kg GBP or saline ($p<0.0001$). n's for GBP-injected = 6 neurons, 753 events. n's for saline-injected = 7 neurons, 1188 events. **d.** Lack of effect of GBP *ex vivo* on IEI in sham operated animals. n's for GBP injected 95 events in 5 neurons and for saline injected 75 events in 7 neurons. **e**. Increase in sEPSC IEI following 20min *in vitro* application of 100 μM GBP to delay neurons from neuropathic animals ($p<0.003$). Data from 5 neurons, 224 events before GBP and 414 events in presence of GBP. **f.** Lack of effect of GBP *in vitro* on IEI in neurons from sham operated animals. Data from 4 neurons, 233 events before GBP and 984 events in presence of GBP. **g.** Decrease in sEPSC amplitude in neuropathic delay neurons recorded *ex vivo* from rats that had received 100mg/kg GBP or saline ($p<0.0001$). n's for GBP-injected = 6 neurons, 753 events. n's for saline-injected = 7 neurons, 1188 events. **h.** Decrease in sEPSC amplitude in sham operated delay putative excitatory neurons recorded *ex vivo* ($p<0.0001$). n's for GBP-injected = 7 neurons, 98 events. n's for saline-injected = 5 neurons, 84 events. **i.** Decrease in sEPSC amplitude in delay neurons from neuropathic rats following 20min *in vitro* application of 100 μM GBP ($p<0.02$). Data from 5 neurons/slices, 229 events before GBP and 434 events in

presence of GBP. **j** Decrease in sEPSC amplitude in 4 neurons from sham operated exposed to GBP *in vitro***.** (237 events before GBP and 1002 events in presence of GBP). Panels **c,d,g** and **h** reproduced with permission from Alles *et al* [12]. *p* values obtained from Kolmogorov-Smirnov test.

Figure 1

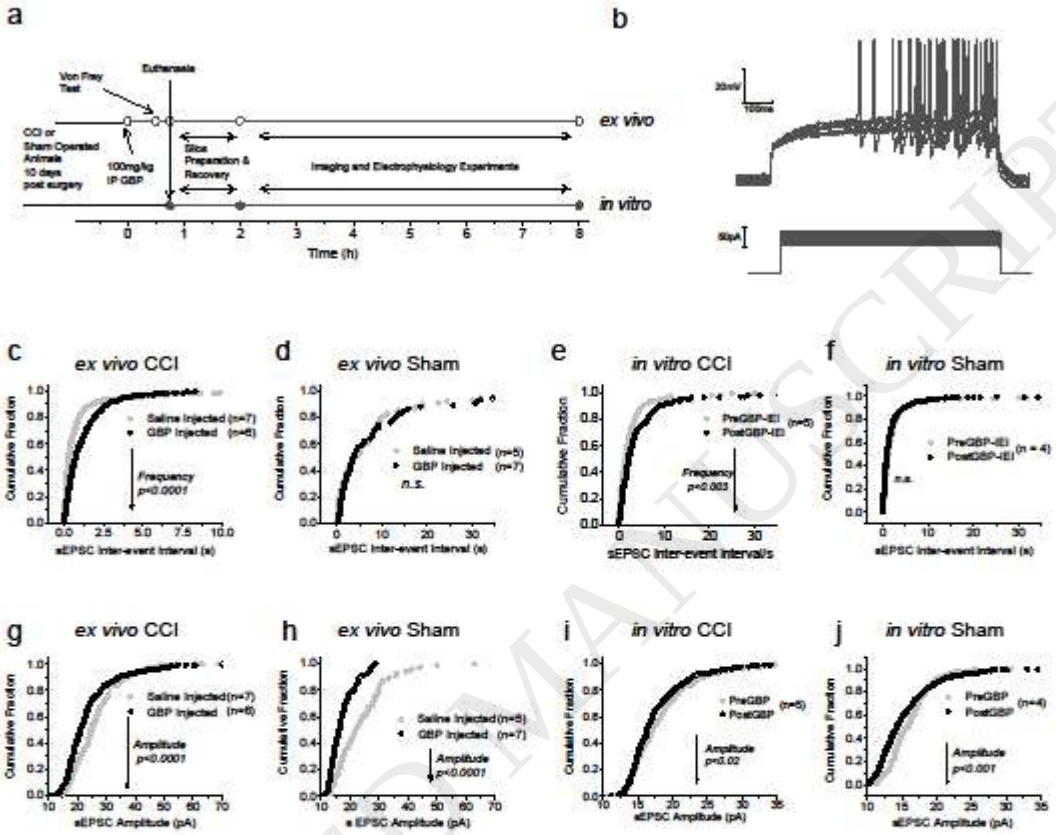

Figure 2. Comparison of *ex vivo* and *in vitro* actions of GBP on sEPSC's in Putative Inhibitory Tonic Neurons.

a. Sample recordings of AP discharge in a tonic neuron in response to depolarizing current steps. **b.** Decrease in IEI (increase in frequency) of sEPSC in tonic neurons recorded *ex vivo* from neuropathic rats that had received 100mg/kg GBP or saline. ($p<0.0001$). For GBP-injected = 11 neurons 636 events. For saline-injected = 10 neurons397 events. **c.** *ex vivo* effect of GBP on IEI of sEPSC's in tonic delay neurons from sham operated animals. For saline injected 7 neurons, 308 events , for GBP injected 5 neurons, 116 events. **d**. Increase in IEI (decrease in frequency) following 20min *in vitro* application of 100 µM GBP to 6 tonic neurons from neuropathic animals ($p<0.001$). 639 events before GBP and 963 events in presence of GBP ($p<0.01$). **e** Increase in IEI (frequency decrease) of sEPSC's in 4 tonic neurons from sham operated animals following *in vitro* exposure of to GBP 331 events before GBP, 127 events after GBP ($p<0.05$). **f.** No difference in sEPSC amplitude in tonic neurons recorded *ex vivo* from neuropathic rats that had received 100mg/kg GBP or saline. n's for GBP-injected = 10 neurons/, 753 events. n's for saline-injected = 11 neurons/, 1188 events. **g.** No difference in sEPSC amplitude in tonic neurons recorded *ex vivo* from sham operated rats that had received 100mg/kg GBP or saline. n's for GBP-injected = 5 neurons, 315 events. n's for Saline-injected = 7 neurons, 121 events. **h.** No difference in sEPSC amplitude in tonic neurons from neuropathic rats following 20min *in vitro* application of 100 µM GBP Data from 6 neurons, (229 events before GBP and 434 events in presence of GBP). **i.** Reduction in sEPSC amplitude in 4 tonic putative inhibitory neurons recorded from sham operated rats and exposed to 100µM GBP *in vitro*. 339 events prior to GBP 132 events in presence of GBP. Panels **b,c,f** and **g** reproduced with permission from Alles *et al*

[12].). *p* values for panels **b – i** from Kolmogorov-Smirnov test. **j.** Prior IP GBP *in vivo* reduces dorsal horn excitability *ex vivo* compared to saline injected animals. GBP, n = 4 rats, 12 slices, 107 cells; saline control, n = 3 rats, 8 slices, 80 cells (*p>0.001* two tailed unpaired t-test). **k.** Lack of effect of bath applied 100 µM GBP on the Ca^{2+} response to 35 mM K^+ of dorsal horn neurons from neuropathic animals. The average of at least 2 consistent normalized responses (dF/F_0) was obtained before and after application of 100 µM GBP for 10 min (*p>0.05* two tailed unpaired t-test) n=45 neurons, 6 slices. Bar graph in **j** constructed from data published previously in Alles *et al* [12] and used with permission.

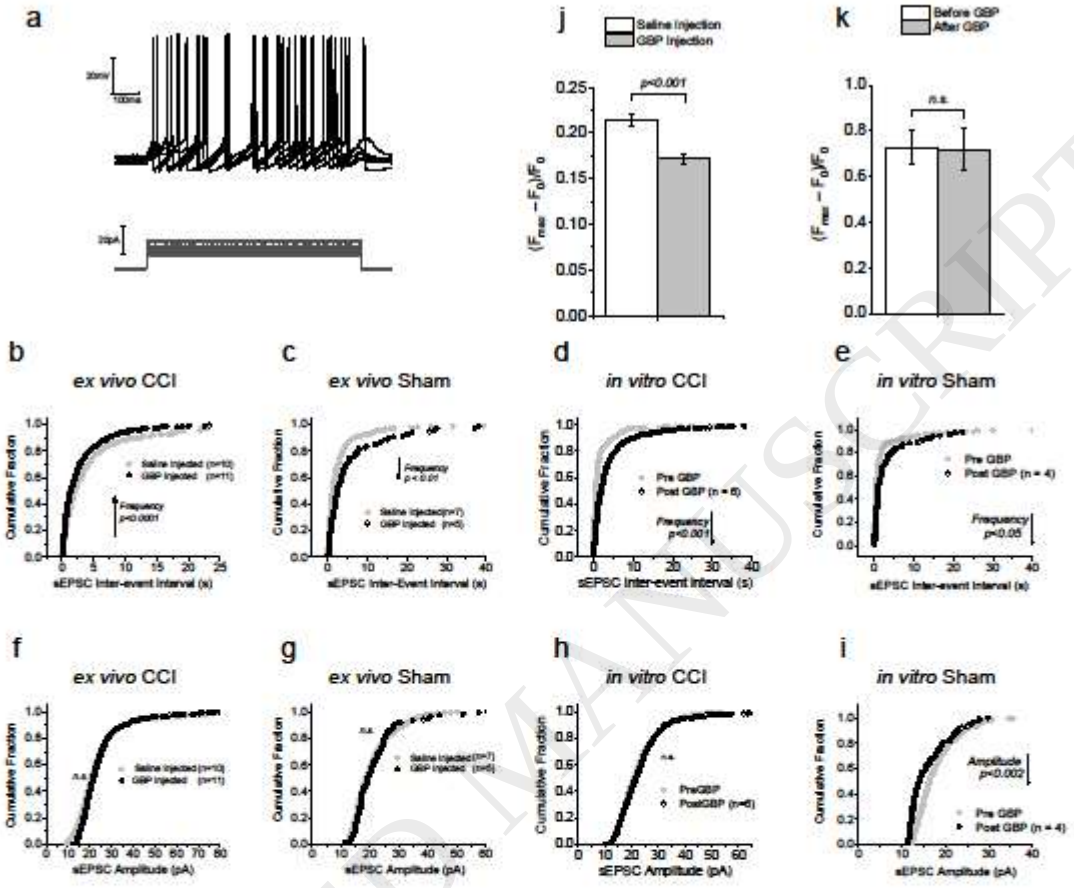

Figure 2

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