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Chronic pregabalin inhibits synaptic transmission between rat dorsal root ganglion and dorsal horn neurons in culture

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In this study, we have examined the properties of synaptic transmission between dorsal root ganglion (DRG) and dorsal horn (DH) neurons, placed in co-culture. We also examined the effect of the anti-hyperalgesic gabapentinoid drug pregabalin (PGB) at this pharmacologically relevant synapse. The main method used was electrophysiological recording of excitatory post synaptic currents (EPSCs) in DH neurons. Synaptic transmission between DRG and DH neurons was stimulated by capsaicin, which activates transient receptor potential vanilloid-1 (TRPV1) receptors on small diameter DRG neurons. Capsaicin (1 μM) application increased the frequency of EPSCs recorded in DH neurons in DRG-DH co-cultures, by about 3-fold, but had no effect on other measured properties of the EPSCs. There was also no effect of capsaicin in the absence of co-cultured DRGs. Application of PGB (100 μM) for 40–48 h caused a reduction in the capsaicin-induced increase in EPSC frequency by 57%. In contrast, brief preincubation of PGB had no significant effect on the capsaicin-induced increase in EPSC frequency. In conclusion, this study shows that PGB applied for 40–48 h, but not acute application inhibits excitatory synaptic transmission at DRG-DH synapses, in response to nociceptive stimulation, most likely by a presynaptic effect on neurotransmitter release from DRG presynaptic terminals.

Introduction

Neuropathic pain manifests itself in many forms, the majority of which do not respond adequately to conventional pain treatment. The gabapentinoid class of drugs, gabapentin (GBP) and pregabalin [PGB, (*S*)-3-(aminomethyl)-5-methylhexanoic acid], have proven effective in treatment of various forms of chronic pain, their effects being mediated through binding to the $\alpha_2\delta$-1 subunit,[1] which was first identified as an accessory subunit of voltage-gated calcium channels (VGCCs).[2] The $\alpha_2\delta$ subunits ha les in trafficking VGCC complexes to the cell surface,[3,4] and may also have a synaptogenic role,[5-7] which is blocked by GBP.[7] Gabapentinoid drugs also bind to $\alpha_2\delta$-2 subunits,[8,9] but not to $\alpha_2\delta$-3 [8] or $\alpha_2\delta$-4.[10]

The $\alpha_2\delta$-1 subunit is found to be upregulated in injured dorsal root ganglion (DRG) neurons in models of neuropathic pain.[11,12] Furthermore, evidence suggests that the contribution of $\alpha_2\delta$-1 to neuropathic pain states is significant.[13] We have shown previously that $\alpha_2\delta$-1 protein is upregulated in all DRG sub-populations and in both the superficial and deeper layers of the dorsal horn (DH) after spinal nerve ligation-induced nerve injury.[12] Our evidence indicates that the elevation of $\alpha_2\delta$-1 in the DH is a result of a presynaptic increase of $\alpha_2\delta$-1 in DRG terminals.[12] Prolonged treatment with PGB (8 d) reduced the nerve injury-associated pain behavior as well as the increased $\alpha_2\delta$-1 level in superficial DH but not in the DRG cell bodies.[12] This reduction of $\alpha_2\delta$-1 is likely to represent an effect of PGB on trafficking of $\alpha_2\delta$-1 to presynaptic terminals under neuropathic conditions.[12] The PGB analog, GBP, affects trafficking of the $\alpha_2\delta$-1 and $\alpha_2\delta$-2 subunits in cell lines,[14,15] and has been shown to inhibit the post-Golgi forward trafficking of $\alpha_2\delta$-2 by the interruption of a Rab-11-dependent recycling pathway.[4]

Previous work has identified actions at the cellular level for PGB and GBP, as well as widespread behavioral effects.[1,7,12,16] However, the mechanism of action of gabapentinoid drugs on the sensory networks involved in peripheral nociception, including those of the afferent DRG fibers and the initial processing in the DH, remains poorly defined. Previous work has hinted at a role of presynaptic VGCCs in the inhibitory action of GBP, as it caused a reduction of excitatory post-synaptic current (EPSC) amplitude in DH neurons after electrical stimulation of spinal cord slices.[17] In an effort to examine the action of PGB at DRG-DH synapses, we have used a DRG-DH co-culture system. Variations on this culture technique have been used previously in references 18–20, and allow the study of neurotransmission at the first sensory

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synapse, between nociceptive afferent neurons and superficial DH neurons.

Results

Stimulation of DRGs with capsaicin results in increased activity in DH neurons. The activity of DH neurons was assessed using both patch clamp recording and calcium imaging (**Fig. 1**). As has been reported previously, DRG neurons project axons which target DH neurons.[22] EPSCs in DH neurons were recorded both in monocultures and in DRG-DH co-cultures (**Fig. 1A**), at a holding potential of -60 mV, and in the presence of the antagonists of inhibitory synaptic transmission, strychnine (2 μM) and bicuculline (10 μM). At -60 mV, it was possible to record spontaneous activity (**Fig. 1B**), whose cumulative frequency distribution is shown in **Figure 1C**. When examining the total number of events, there was a small but statistically significant increase in the frequency of baseline spontaneous activity in DRG-DH co-cultures, compared with DH monocultures (**Fig. 1C**), although the mean inter-event interval was not affected (inset). This result may indicate that there are a small, variable proportion of DRG neurons firing spontaneously in these co-cultures.[23] The presence of synapses in DRG-DH co-cultures was confirmed immunocytochemically. **Figure 1D** shows axonal (left, axonal marker tau: red) and dendritic (middle, dendritic marker Map2: white) neurites that are intertwined (overlay, right). The synaptic marker synapsin (green) co-localizes with axonal (left) but not with dendritic staining (middle). As has been shown previously in references 19 and 20, the frequency of EPSCs was increased when capsaicin (1 μM) was applied to the co-cultures (**Fig. 1B and E**). An increase in EPSC frequency was not observed, however, when capsaicin was applied to DH monocultures (**Fig. 1E**).

We also employed Ca^{2+} imaging using Fura-2, as an index of neuronal activity, and this confirmed that the capsaicin-induced increase in EPSC frequency translated to an increase in intracellular [Ca^{2+}] in the DH neuron somata in DRG-DH co-cultures, but not in DH mono-cultures (**Fig. 1F and G**). About 70% of DH neurons in the co-cultures responded to capsaicin application with an increase in EPSC frequency (**Fig. 2A and B**), in agreement with previous work.[18] No change in mean EPSC amplitude was observed (**Fig. 2C and D**); the rise-times (**Fig. 2E**) and decay times (**Fig. 2F**) were also very similar. This suggests that capsaicin causes an increase in neurotransmitter release from DRG nerve terminals, rather than having more extensive synaptic effects.

Pharmacological characterization of EPSCs recorded in DH neurons. We then characterized these synaptic responses by examining the effects of various synaptic blockers on EPSCs recorded in DH neurons in the DRG-DH co-cultures (**Fig. 3**). In our study, nitroquinoxaline-2,3-dione (CNQX, 1 μM) produced a complete block of EPSCs (**Fig. 3A and C**), both in the presence ($p < 0.001$) and absence of capsaicin (data not shown), which was reversible upon washout (data not shown). This confirmed that 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid (AMPA) receptor-based excitatory neurotransmission was essential for the capsaicin-induced increase in EPSC frequency in DRG-DH co-cultures, as expected. However, application of the N-Methyl-d-aspartic acid receptor (NMDA-R) blocker, (2*R*)-amino-5-phosphonovaleric acid (APV, 50 μM) also resulted in a partial reduction in the capsaicin-evoked increase in EPSC frequency, in all cells tested (**Fig. 3B and C**; $p < 0.05$). Tetrodotoxin (TTX, 1 μM) also produced a partial reduction of the capsaicin-evoked increase in EPSC frequency in all neurons tested (**Fig. 3C**; $p < 0.05$). The decay time of EPSCs measured in the presence of capsaicin was also reduced by APV, reflecting the presence of an NMDA-R-mediated component (**Fig. 3D**; $p < 0.001$, K-S test).

Chronic PGB attenuates the capsaicin-induced increase in EPSC frequency in DH neurons. We have previously shown that chronic but not acute application of GBP (100 μM–1 mM) inhibited whole-cell VGCC currents in cell-lines and DRG neurons.[14,15] We, therefore, decided to test whether the GBP analog PGB would have an effect on spontaneous EPSCs, or on the increase in EPSCs evoked by capsaicin, in DRG-DH co-cultures (**Fig. 4**). Co-cultures were incubated with PGB for either 40–48 h (**Fig. 4A–D**), or briefly for 30 min (**Fig. 4E–H**), and this was removed immediately before recording. Following long-term PGB treatment, no difference was observed in baseline EPSC frequency (**Fig. 4B**). However during capsaicin application, a cumulative probability histogram revealed that the EPSC frequency was reduced in the presence of chronic PGB, in the same neurons (**Fig. 4C**). Furthermore, there was a 57% reduction in the capsaicin-mediated increase in mean EPSC frequency recorded from DH neurons in the chronic PGB-treated compared with control cells (**Fig. 4D**). In contrast, no significant difference in evoked EPSC frequency was observed between control cells and cells treated for 30 min prior to recording with a 10-fold higher concentration of PGB (1 mM; **Fig. 4E–H**) in baseline activity (**Fig. 4F**), activity during capsaicin (**Fig. 4G**), or in the capsaicin-mediated increase in mean EPSC frequency (**Fig. 4H**).

There was also no significant effect of either acute or chronic PGB on the mean amplitude of the EPSCs, either on baseline activity, or after capsaicin. For example the baseline amplitude was 37.8 ± 2.0 pA (n = 19 cells) under control conditions and 46.6 ± 9.1 pA (n = 18 cells) in the presence of chronic PGB. Similar data were obtained for the other conditions.

Discussion

Calcium channels, and in particular VGCC $\alpha_2\delta$-1 subunits, are expressed in both DRGs and DH neurons.[12,24,25] Therefore it is reasonable to expect that PGB might elicit an effect throughout the co-culture system used. However, the increase in $\alpha_2\delta$-1 that occurs in experimental neuropathic pain is restricted to injured DRG neurons and their terminals in the dorsal horn.[12] Although it is difficult to pinpoint the site of inhibition by PGB of nociceptive transmission, either specifically to the DRG neurons, or the interconnected DH neuronal network, several pieces of evidence suggest that DRG neurons are the main site of action of PGB in the present study. First, small DRG neurons have been observed to express a higher concentrations of $\alpha_2\delta$-1 than large DRG neurons.[21,26] Second, small nociceptive neurons have TRPV1

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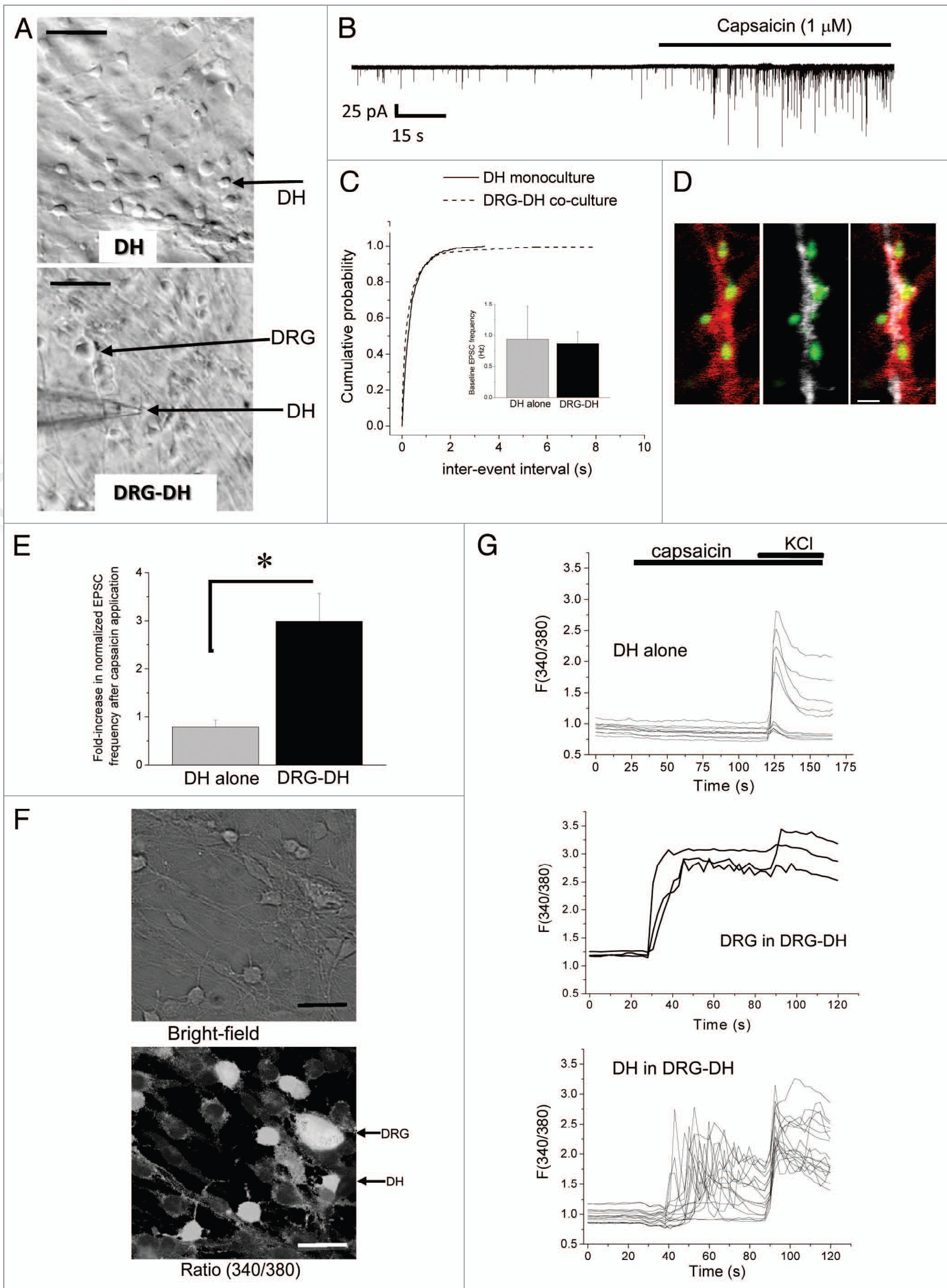
A
DH
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DRG
DH
DRG-DH
B
Capsaicin (1 μM)
25 pA
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DH monoculture
DRG-DH co-culture
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inter-event interval (s)
DH alone
DRG-DH
D
E
Fold-increase in normalized EPSC frequency after capsaicin application
DH alone
DRG-DH
F
Bright-field
DRG
DH
Ratio (340/380)
G
capsaicin
KCl
DH alone
DRG in DRG-DH
DH in DRG-DH
F(340/380)
Time (s)

Figure 1. Properties of DRG-DH co-cultures. (A) Brightfield micrographs showing DH monoculture (upper image) and DRG-DH co-cultures (lower image). Scale bars 50 μm. (B) Representative trace showing the increase in frequency of EPSCs in response to application of 1 μM capsaicin in a DRG-DH co-culture. Bar indicates time of application of capsaicin. (C) Cumulative frequency histogram of EPSCs in the 120 sec prior to capsaicin application, in DH monocultures (solid line, n = 422 events from five cells) and DRG-DH co-cultures (dotted line, n = 545 events from seven cells). Data are significantly different $p < 0.001$ (K-S test). Inset bar chart showing mean baseline EPSC frequency in 120 sec prior to capsaicin application, in DH monocultures (gray bar, n = 5) and DRG-DH co-cultures (black bar, n = 7); NS. (D) Immunofluorescence micrograph shows: Left part: co-localization (yellow) of synapses in DRG-DH co-cultures (synaptic marker synapsin, green) with axonal staining (τ, red). Middle part: synapsin (green) does not colocalize with dendritic Map2 (white). Right part: merged images; scale bar: 5 μm. (E) Bar chart showing average fold-increase in EPSC frequency in response to capsaicin application, in DH monocultures (gray bar, n = 5) and DRG-DH co-cultures (black bar, n = 14) *$p < 0.05$, Student's t test. (F) Micrographs showing a DRG-DH co-culture used in Fura-2 calcium imaging: bright-field (upper image); 340/380 nm fluorescence ratio (lower image). Scale bar 30 μm. (G) Fura-2 traces (340/380 nm) demonstrating elevation of intracellular Ca^{2+} in individual DH neurons in a DH monoculture in response to 50 mM KCl (top part) and elevation of intracellular Ca^{2+}, both in DRG neurons (middle part) and in DH neurons (bottom part) in a DRG-DH co-culture, in response to 1 μM capsaicin and 50 mM KCl (time of application in shown by horizontal bars above top part in G).

receptors on their cell bodies and presynaptic terminals,[27] which are activated by capsaicin.

In the present study, we observed that TTX produced a reduction of EPSC frequency, and although TTX is unlikely to prevent direct capsaicin-stimulated synaptic transmission from DRG neuron to DH neuron mediated by presynaptic TRPV1 receptors in this system,[27] it is also possible that capsaicin-induced generation of action potentials contributes to transmitter release, in addition to its direct depolarizing action on DRG terminals. Nevertheless the inhibitory effects mediated by TTX may also be network effects, acting upon relay neurons and other DH neurons in the network which then give rise to polysynaptic input onto the DH neuron being recorded. A further possibility is that some events remaining in the presence of TTX represent input from DRGs expressing TTX-resistant sodium channels. Although we did not record from DRG neurons, it is likely that there are only a small number of spontaneously active DRG neurons in these cultures,[23] which may contribute to the small increase in baseline activity observed in DRG-DH co-cultures.

We observed that chronic PGB (100 μM), applied for 40–48 h, reduced the capsaicin-evoked EPSC frequency increase recorded in DH neurons, whereas brief application of a 10-fold higher concentration of PGB had no significant effect on the same measures. The therapeutic range of PGB has been measured to be 30–50 μM.[28]

At the point of synaptic transmission between DRG and DH neurons, PGB might exert an effect on the ability of $\alpha_2\delta$-1 to traffic presynaptic calcium channels to synaptic boutons of DRGs with DH neurons, thereby influencing the number of VGCC complexes at the presynaptic membrane, thus regulating neurotransmitter release. This mechanism implies that, although capsaicin application may directly activate presynaptic TRPV1 receptors to promote transmitter release from DRG terminals, thus by-passing the involvement of VGCCs,[18] there may also be an involvement of activation of TRPV1 receptors in DRG somata and action potential-induced transmitter release, as discussed above. One alternative explanation is that gabapentinoid drugs also affect TRPV1 receptor trafficking via an action on $\alpha_2\delta$-1 subunits, but there is at present no evidence for this hypothesis. A further possibility is that chronic PGB affects the transmitter release process directly, and is therefore still effective to reduce capsaicin-mediated transmitter release, despite bypassing VGCCs.

In terms of alternative mechanisms of action of PGB, independent of VGCCs, recent work has highlighted gabapentinoid drugs to have roles not only in inhibiting the trafficking of VGCCs,[4,12,14] but also in disrupting excitatory synaptogenesis.[7] Therefore, PGB might also inhibit synapse formation in the DRG-DH cultures. Given that the co-cultures are cultured for 8–10 d in vitro before PGB is added, an extensive DRG-DH synaptic network will have developed. However, PGB might still play a role in the inhibition of synaptogenesis during the 40–48 h for which it is applied, although the lack of effect of chronic PGB on baseline EPSC frequency does not provide support for this hypothesis. It would be interesting to examine whether there is inhibition of synaptogenesis by exposure to PBG from the onset of cell culture.

The application of capsaicin to DRG-DH co-cultures has been employed in several studies, in an effort to model spinal output from nociceptive input.[18-20] The synaptic networks and cellular architecture that make up the dorsal laminae of the spinal cord have not been fully characterized,[29] and laminae I and II are populated with neurons that overlap with regard to their morphological and electrophysiological characteristics.[30,31] DRGs readily and extensively innervate DH neurons, allowing an in vitro approximation of the first sensory synapse. However, because only the superficial laminae of the DH are used, the system lacks the full synaptic networks present in vivo. In the present study, there was variability in terms of the baseline/spontaneous activity recorded in the DH neurons, and also to a lesser extent, the fold-increase in EPSC frequency elicited by capsaicin application. This can perhaps be attributed to variations in the sub-type of DRG and in the degree of innervation of DH neurons, as well as the local presence or absence of various types of DH interneuron in the vicinity of the DH neuron from which recordings are made. Such discrepancies have not been mentioned before, although it is well known that numerous types of DH interneuron are present in the superficial DH laminae,[30-32] and therefore variability is to be expected in such a heterogeneous population.

Similar experiments utilizing an in vitro DRG-DH culture system have been performed previously in references 18 and 20. One of these studies[18] did not observe any effect of the NMDA-R blocker, APV, unlike the results obtained here. The Mg^{2+} concentration of the bathing solution and the holding potential were the same as those used previously in reference 18. Potential explanations for the discrepancy between this and previous studies may be the handling and mixing of DRG and DH neurons in culture

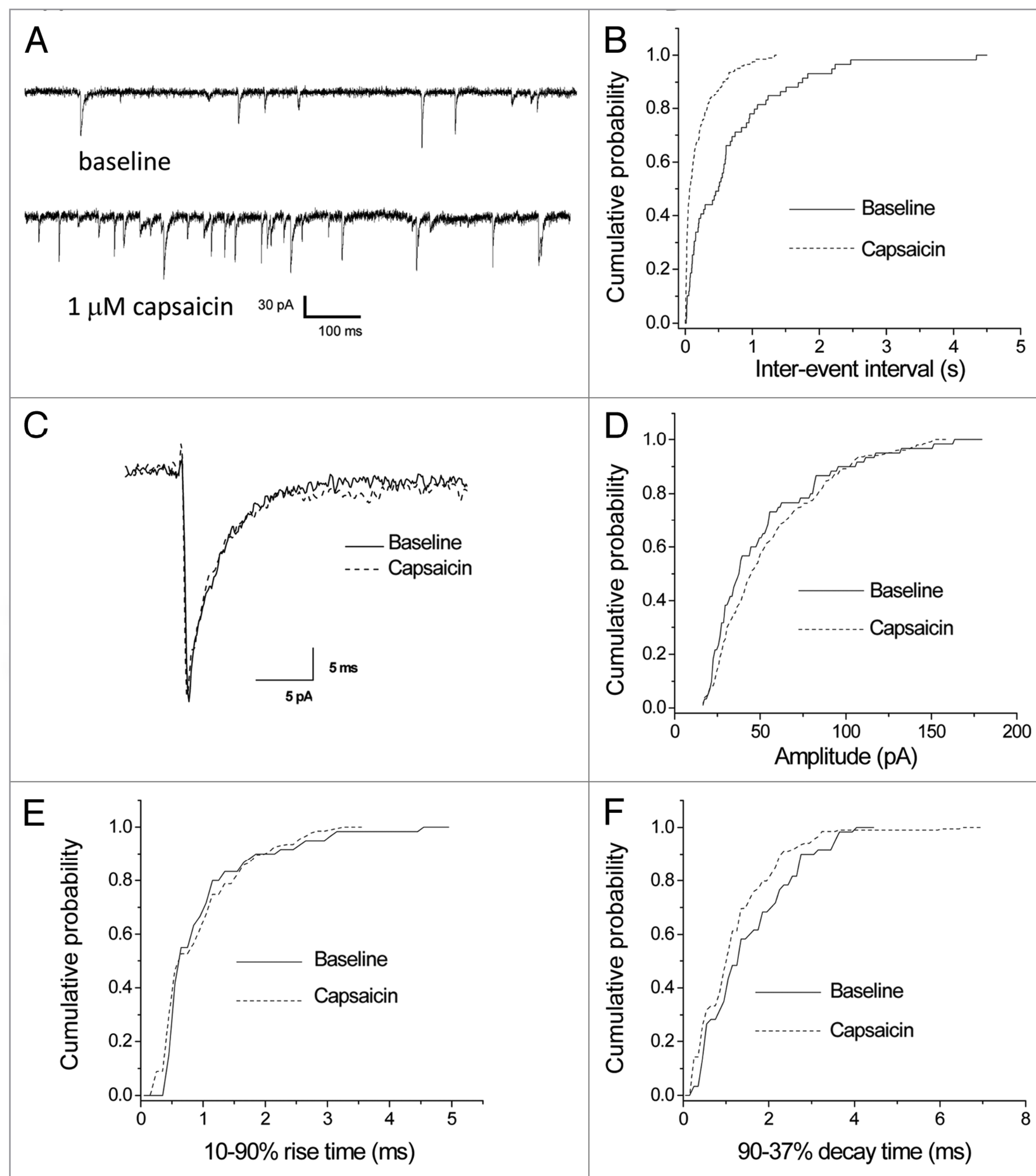

Figure 2. Effect of capsaicin on synaptic activity recorded in DH neurons in DRG-DH co-cultures. Acute application of 1 μM capsaicin caused an increase in the EPSC frequency of DH neurons in co-cultures with DRG neurons, without a corresponding change in other EPSC properties. (A) Representative traces showing spontaneous activity (upper trace) compared with capsaicin-evoked activity (lower trace). (B) Cumulative frequency histogram of inter-event interval showing increased EPSC frequency in the presence of capsaicin (data from a representative cell, 60 events before and 204 events after capsaicin application; $p < 0.001$ K-S test). (C) Overlaid averaged EPSCs in both conditions, each taken over 120 s, showing no difference in EPSC kinetics. (D–F) Cumulative frequency histograms showing: (D) EPSC amplitude, (E) EPSC 10–90% rise time, (F) EPSC 90-37% decay time, for the same cell, all NS using K-S test. In (B–F), solid lines represent spontaneous/baseline activity and dashed lines represent capsaicin-evoked activity.

(e.g., differences in the final DRG/DH ratio or in the amount of spinal cord taken in culture), as well as the use in the present study of bicuculline/strychnine to block inhibitory activity. Blockade of inhibitory GABAergic/glycinergic transmission can unmask synaptic connections made onto lamina I neurons that are normally inhibited in vivo.[33] This unmasked synaptic input is reliant on NMDA-R-activity. These neurons would be present in the DRG-DH culture system, and may well be similarly active in vitro. Variations in the extent of the effect of APV may be attributed to the heterogeneity of the neuronal population being sampled.

In conclusion, this work provides in vitro evidence that PGB has a long-term effect on the incoming nociceptive information from nociceptive afferent fibers, synapsing onto superficial DH.

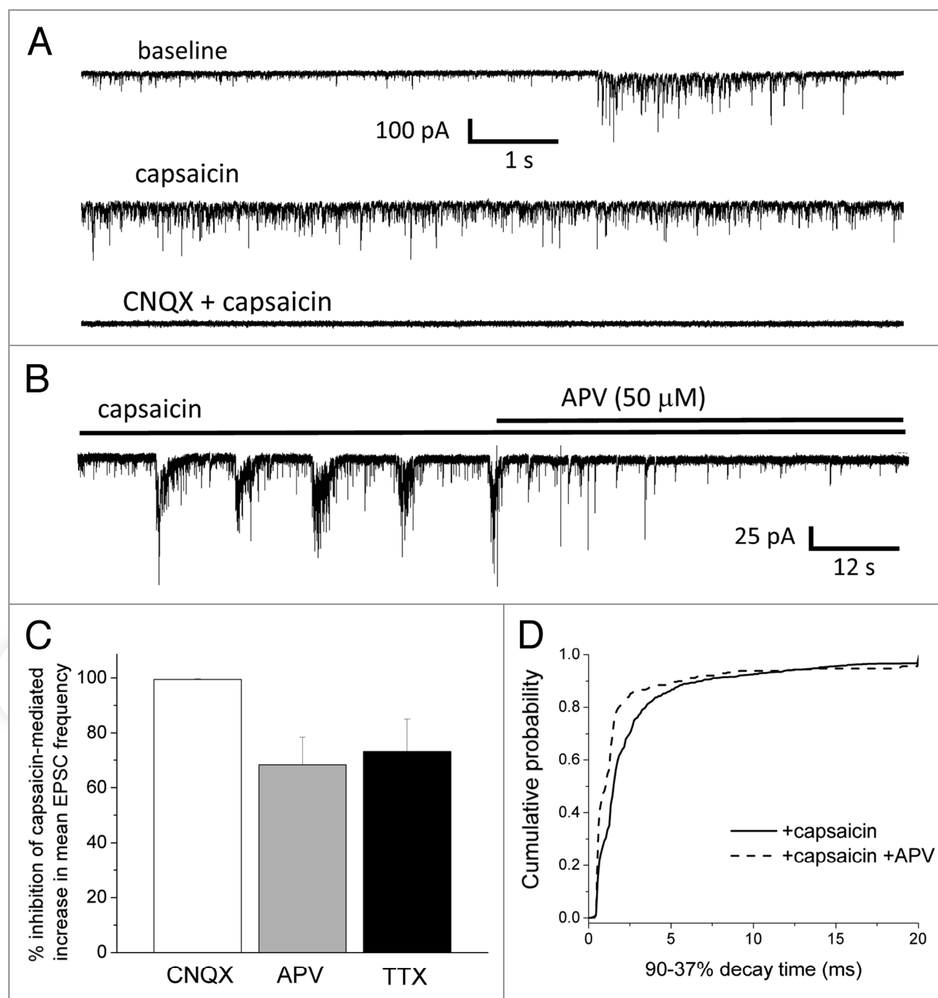

Figure 3. Effect of synaptic blockers on capsaicin-evoked EPSCs. (A) traces showing baseline EPSCs recorded in a DH neuron in a DRG-DH co-culture (top), the effect of capsaicin (1 μM, middle) and the subsequent effect of CNQX (1 μM, bottom). (B) Example of the action of APV (50 μM) on capsaicin-evoked EPSCs. (C) Bar chart showing % inhibition of capsaicin-evoked increase in EPSC frequency by CNQX (white bar, n = 9 cells) ($p < 0.001$, Student's t-test), APV (gray bar, n = 4 cells) and TTX (black bar, n = 5 cells) (both $p < 0.05$, Student's t-test, compared to their respective controls). (D) Cumulative frequency histogram of 90-37% decay time of EPSCs measured in the presence of capsaicin (solid line; n = 684 events from four cells) and after subsequent addition of APV (dashed line; n = 301 events from four cells). $p < 0.001$ (K-S test).

The results from this study support the growing literature that links an increase in DRG $\alpha_2\delta$-1 at presynaptic terminals in the superficial DH to phenotypes of allodynia and hyperalgesia.[11-13,34]

Materials and Methods

Cell culture. Time-mated female rats were killed by CO_2 exposure and cervical dislocation, according to Schedule 1 guidelines [Home Office Animals (Scientific procedures) Act 1986, UK]. Embryonic Sprague Dawley rats (E16–E18) were removed into cold modified Eagle's medium (MEM). Embryos were then decapitated. Spinal cords were removed to cold MEM, where the dorsal thirds were removed from each cord, and placed in 0.25% trypsin. DRGs were then excised and also placed in 0.25% trypsin. Tissue was digested for 30 min at 37°C, before being washed in warm medium and centrifuged at 1,000x rpm. Pellets were then re-suspended in seeding medium: MEM containing horse serum (5%); fetal bovine serum (5%); nerve growth factor (200 ng/ml) and gentamycin (40 ng/ml). Mechanical dissociation of the pellet produced a single cell suspension. Trituration of DRG and DH neurons was performed separately. After trituration, DRG and DH neurons were mixed, and placed onto poly-l-lysine and laminin-coated glass coverslips. The seeding medium was replaced after 24 h by Neurobasal medium containing B-27 supplement, NGF (200 ng/ml) and gentamycin (40 ng/ml). Cells were then cultured for 10–12 d before use in experiments, and the medium was changed every 4–5 d. For experiments, coverslips with either DH monocultures or DRG-DH co-cultures were secured in a perfusion chamber (Warner Instruments).

Drug application. For chronic PGB application, it was applied in the 40–48 h prior to recording. PGB (diluted from 100 mM stock solution in H_2O) was applied directly to DRG-DH

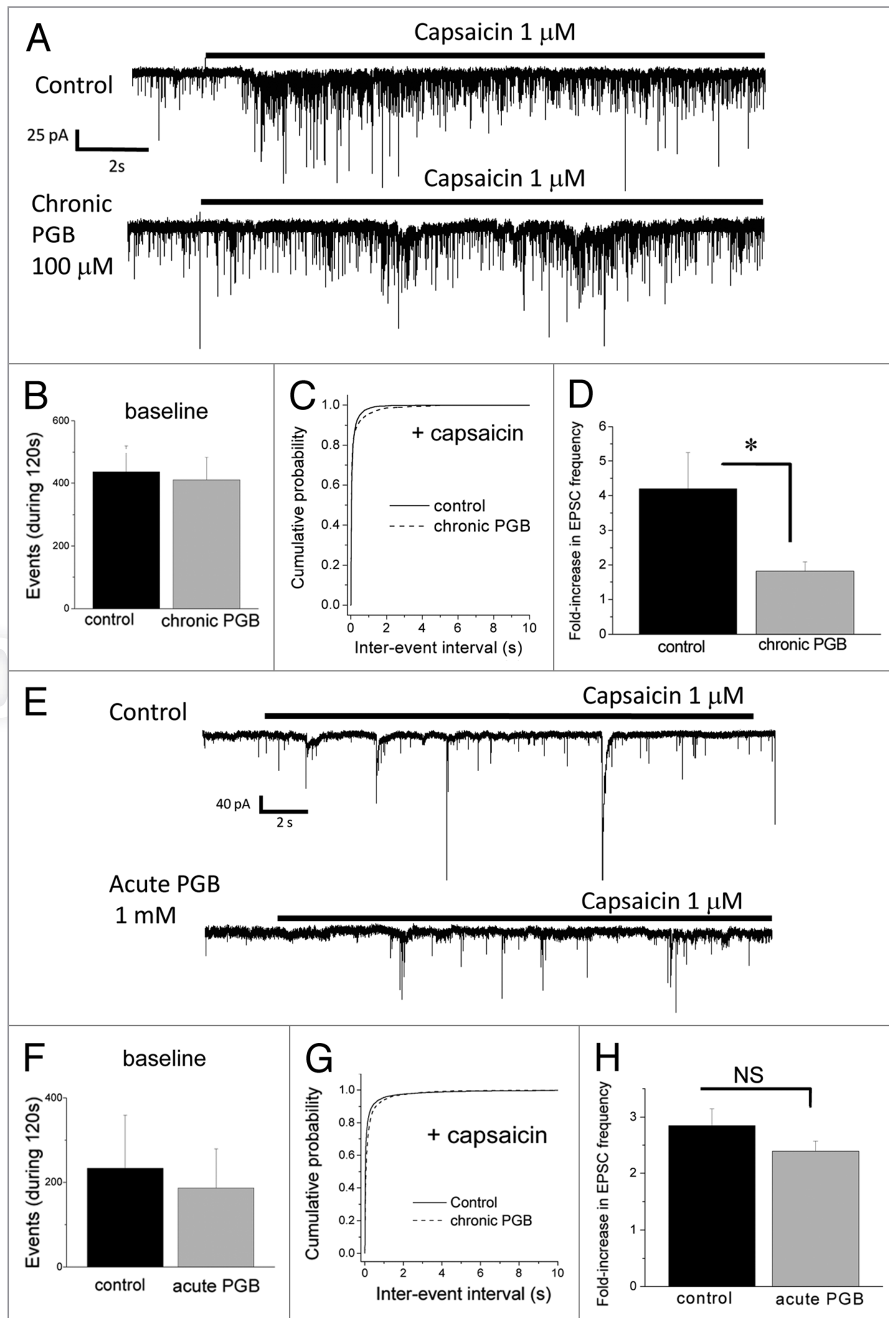

Figure 4. Effect of prolonged and brief PGB application on capsaicin-evoked EPSCs. Chronic PGB (40–48 h) attenuates the capsaicin-mediated increase in EPSC frequency. (A) Representative traces showing the onset of response to capsaicin application in control (upper) and chronic PGB-treated (lower) cells. (B) Mean baseline EPSC frequency measured as events during 120 sec for control (black bar, n = 19 cells), compared with chronic PGB-treated cells (black bar, n = 18 cells) NS, Student's t test. (C) Comparison of EPSC frequency during capsaicin application (shown as a cumulative histogram of inter-event interval) between control (solid line, 8,635 events from 19 cells) and chronic PGB-treated (dashed line, 5,545 events from 18 cells) co-cultures ($p < 0.001$, K-S test). (D) The data from (C) are expressed as capsaicin-evoked fold-increase in ESPC frequency when cultures were incubated chronically with 100 μM PGB (gray bar, n = 18), compared with control (black bar, n = 19) (*$p < 0.05$, Student's t test). (E) Representative traces showing the onset of response to capsaicin application in a control cell (upper trace) and a cell treated with PGB for 30 min (lower trace). (F) Mean baseline EPSC frequency measured as events during 120 sec for control (black bar, n = 19), compared with acute PGB (gray bar, n = 14); NS, Student's t-test. (G) Comparison of capsaicin application (shown as a cumulative histogram of inter-event interval) between control (solid line, 988 events from ten cells) and acute PGB-treated (dashed line, 774 events from five cells) co-cultures (*NS*, K-S test). (H) There was no significant reduction in the fold-increase in enhancement of EPSC frequency by capsaicin (gray bar, n = 14), compared with control (black bar, n = 19).

co-culture dishes. For sub-acute exposure, cells were incubated with 1 mM PGB for 30 min before recording. PGB was absent from the recording solutions in all cases.

Immunocytochemistry on DRG-DH cultures. Immunocytochemical detection in cultures was performed as described previously in reference 21. Briefly, co-cultures (10 d in vitro) were washed and fixed with 4% paraformaldehyde in Tris-buffered saline (TBS) for 5 min at room temperature, then washed and permeabilized (TBS with 0.02% Triton X-100, 15 min) before being blocked with 20% goat serum and 4% BSA in TBS for at least 30 min. Samples were then incubated with antibodies against axonal tau (1:200, host: rabbit, Chemicon), dendritic microtubule-associated protein 2 (MAP2, 1:1,000, host: chicken, Abcam) and synapsin (1:1,000, host: mouse, Synaptic Systems) at 4°C overnight, washed and incubated with biotinylated goat anti-mouse IgG (1:500; Invitrogen) for 2 h at 4°C, followed by streptavidin-Alexa Fluor 488 (1:500, Invitrogen), goat anti-rabbit Texas Red (1:500, Invitrogen) and goat anti-chicken Alexa Fluor 647 (1:500, Invitrogen) for 1 h at room temperature. After washing and DNA staining with 4',6-diamidino-2-phenylindole (DAPI; 300 nM, Invitrogen), samples were mounted in Vectashield (Vector Laboratories). Immunofluorescence labeling was detected with a LSM 510Meta (Zeiss) confocal microscope in optical sections of 1 μm thickness.

Calcium imaging. Fura-2 calcium imaging was performed to confirm that DRG stimulation using capsaicin led to a subsequent influx of calcium in DH neurons. Dye loading was performed at 37°C for 45 min with Hank's basal salt solution, containing (in mM): HEPES (10); $CaCl_2$ (2); $MgCl_2$ (0.1); glycine (0.005); fura-2 dye (0.002). Two washes (each lasting 5 min) were then performed in the same solution (but with fura-2 dye omitted), and cells were imaged immediately afterwards.

Imaging was performed using an Axiovert 200M microscope (Zeiss) and an ORCA-ER camera controller (Hamamatsu). Images were captured on a PC running Volocity 4 software (Improvision). Images were captured sequentially (0.5 Hz acquisition rate per paired image) at 340 and 380 nm excitation wavelengths, and a ratio image (340/380 nm) was generated offline for subsequent analysis. The elevation of intracellular Ca^{2+} in DRG and DH neurons was used as an indicator of cell activation.

Whole-cell electrophysiology. Excitatory post-synaptic currents (EPSCs) were recorded from DH neurons in the whole-cell configuration, using an Axopatch 200B patch clamp amplifier and the pClamp software suite (Molecular Devices). Patch pipettes were pulled from borosilicate glass (3–5 MΩ resistance) and filled with an internal solution containing (in mM): KCl (10); $MgCl_2$ (1); HEPES (10); EGTA (5); K-gluconate (125); Mg-ATP (3). The solution was adjusted to pH 7.25 using KOH and to 290 mosM with sucrose. The external solution contained (in mM): NaCl (140); KCl (5); $CaCl_2$ (1.3); $MgCl_2$ (0.5); HEPES (10); $MgSO_4$ (0.4); KH_2PO_4 (0.4); Na_2HPO_4 (0.6); $NaHCO_3$ (3); glucose (10). The solution was adjusted to pH 7.35 using NaOH and 310 mosM with sucrose. Experiments were performed at room temperature. Blockers of inhibitory synaptic transmission, bicuculline (10 μM) and strychnine (2 μM), were included in the recording solution.

The DH neurons were identified from their morphology, as they were small (10–20 μm diameter), multipolar (2–5 processes) and phase-dark. In contrast, DRG neurons were large (25–50 μm diameter), round, usually biopolar or pseudounipolar and phase-bright. DH neurons were patch-clamped and maintained at a holding potential of -60 mV. Recordings were made over a period of 240 sec, and data were digitized at 5 kHz. Background activity was measured for 120 sec, which formed the baseline activity used in analysis. Cells in which at least one ESPC was recorded over 120 sec were used in subsequent analysis. Capsaicin was then added and activity was measured for a further 120 sec. The fold-increase in EPSC frequency was then measured.

Data analysis. All recorded DH neurons, including those that did not respond to capsaicin (~30%), were included in the analysis. MiniAnalysis software (Synaptosoft) was used for analysis and ESPCs were detected with the threshold setting of 5x the root mean square of the background noise. Cumulative probability histograms were analyzed using Kolmogorov Smirnov (K-S) statistics, which uses the maximum difference between two population distributions as the test statistic. Mean data between cells were analyzed using Student's t-test, or paired t-test, as appropriate.

Materials. Pregabalin was a gift from Pfizer Global R&D, USA.

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Disclosure of Potential Conflicts of Interest

A.C.D. received a small grant from Pfizer Global R&D, and received pregabalin from Pfizer.

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