Presynaptic GABA$_B$ receptors reduce transmission at parabrachial synapses in the lateral central amygdala by inhibiting N-type calcium channels

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The nocioceptive information carried by neurons of the pontine parabrachial nucleus to neurons of the lateral division of the central amygdala (CeA-L) is thought to contribute to the affective components of pain and is required for the formation of conditioned-fear memories. Importantly, excitatory transmission between parabrachial axon terminals and CeA-L neurons can be inhibited by a number of presynaptic receptors linked to Gi/o-type G-proteins, including α2-adrenoceptors and GABA$_B$ receptors. While the intracellular signalling pathway responsible for α2-adrenoceptor inhibition of synaptic transmission at this synapse is known, the mechanism by which GABA$_B$ receptors inhibits transmission has not been determined. The present study demonstrates that activation of presynaptic GABA$_B$ receptors reduces excitatory transmission between parabrachial axon terminals and CeA-L neurons by inhibiting N-type calcium channels. While the involvement of G$_{\beta\gamma}$ subunits in mediating the inhibitory effects of GABA$_B$ receptors on N-type calcium channels is unclear, this inhibition does not involve G$_{\beta\gamma}$-independent activation of pp60C-src tyrosine kinase. The results of this study further enhance our understanding of the modulation of the excitatory input from parabrachial axon terminals to CeA-L neurons and indicate that presynaptic GABA$_B$ receptors at this synapse could be valuable therapeutic targets for the treatment of fear- and pain-related disorders.
synaptic vesicles and subsequent release of neurotransmitter (i.e. glutamate), reducing the number of released vesicles but not release probability. In contrast, GABA<sub>B</sub>-receptor-mediated inhibition of release targets calcium influx through the voltage-gated calcium channels, resulting in a reduction in release probability. However, the intra-cellular signalling pathway responsible for GABA<sub>B</sub>-mediated inhibition of voltage-gated calcium channels has not been determined. As such, the present study sought to elucidate the intracellular mechanism by which the activation of presynaptic GABA<sub>B</sub>-receptors inhibits transmission between parabrachial axons and CeA-LC neurons.

Results

The axons of parabrachial neurons form perisomatic basket-synapses onto neurons of the lateral central amygdala (CeA-L). These synapses provide a significant, AMPA-mediated, excitatory input onto CeA-L neurons that is inhibited by the activation of pre-synaptic α<sub>2</sub>-adrenoceptors (by noradrenaline) and GABA<sub>B</sub>-receptors (by baclofen) (Fig. 1A,B; see also C,D). To determine the G proteins coupled to GABA<sub>B</sub>-receptors and α<sub>2</sub>-adrenoceptors located on parabrachial axon terminals, slices were pre-exposed to the sulphydryl alkylating agent N-ethylmaleimide (NEM) at a concentration that selectively inhibits signalling by pertussis-toxin sensitive Gi/o-type G proteins (50 μM, 10 min bath application). The pre-incubation of slices with NEM blocked the inhibitory effects of both baclofen and noradrenaline at these synapses (Fig. 1C,D, n = 5 and 4 respectively, p < 0.05 for both). Similarly, the α<sub>2</sub>-adrenoceptor antagonist yohimbine blocked the

Figure 1. GABA<sub>B</sub>-receptor and noradrenenergic inhibition of parabrachial-CeA-L EPSCs is blocked by pre-incubation with 50 μM N-ethylmaleimide (NEM) for 10 minutes prior to application of baclofen or noradrenaline. (A) Parabrachial-CeA-L EPSC amplitudes during the application of 2 μM baclofen (bcl); inset - average of control and baclofen responses. (B) Parabrachial-CeA-L EPSC amplitudes during the application of 10 μM noradrenaline (NAd); inset - average of control and noradrenaline responses. (C,D) Parabrachial-CeA-L EPSC amplitudes in control, and with subsequent application of NEM followed by baclofen (bcl) (C) and noradrenaline (NAd) (D). Inset - average traces in NEM (NEM) and NEM + baclofen (NEM + bcl) or noradrenaline (NEM + NAd) shown.
inhibitory effect of noradrenaline, but not the inhibitory effects of baclofen (n = 5, not shown), on transmission at this synapse (average 79.6 ± 6.0% inhibition in yohimbine compared to 75.8 ± 4.1% in control, n = 3 and 7 respectively). In addition, synaptic transmission was further inhibited by both the application of noradrenaline in the presence of baclofen (44.5 ± 7.0% inhibition, n = 5, data not shown) and the application of baclofen in the presence of noradrenaline (62.6 ± 7.0% inhibition, p < 0.05, n = 5, not shown). These results indicate that while GABAB-receptors and α2-adrenoceptors share a common class of G-proteins (Gi/o) they mediate their effects on vesicular release from parabrachial axon terminals via distinct signal transduction mechanisms.

The application of the myristilated Gβγ binding peptide mSIRK to terminals blocks the inhibitory effect of noradrenaline on vesicular release from parabrachial axons (Fig. 2A; see also7), indicating that Gβγ mediates the α2-adrenoceptor modulation of synaptic transmission between parabrachial axons and CeA-L neurons. In contrast, application of mSIRK failed to block the inhibitory effect of baclofen on synaptic transmission at this synapse (Fig. 2A; 71.1 ± 4.7% inhibition, p < 0.05, n = 4). However, mSIRK did block a baclofen-induced potassium current in neurons of the basolateral amygdala, demonstrating the Gβγ does mediate some of the effects produced by GABAB-receptor activation (Fig. 2B; n = 3). Therefore, while GABAB receptors can use Gβγ to influence downstream targets, the results of this experiment suggest that Gβγ might not be required for GABAB-mediated inhibition of synaptic transmission between parabrachial axons and CeA-L neurons.

Baclofen reduces transmitter release from parabrachial axons by reducing voltage-dependent calcium influx into terminals resulting in a decrease in the probability of action-potential-driven vesicle fusion and neurotransmitter release7, and the previous experiments indicated that this GABAB-mediated inhibition might not involve Gβγ signalling. Indeed, activation of GABAB receptors can produce a voltage-independent block of the N-type calcium channels via a Gβγ-independent activation of the pp60C-src tyrosine kinase12. To determine whether a similar intracellular signalling process was responsible for the GABAB-mediated inhibition of vesicle release from parabrachial axon terminals the specific N-type calcium channel antagonist ω-conotoxin CVID13 (200 nM) was applied to slices. This revealed that N-type calcium channels were largely responsible for mediating neurotransmitter release from the parabrachial axon terminals (69.6 ± 8.0% inhibition; Fig. 3A, n = 5, p < 0.5). In addition, application baclofen in the presence ω-conotoxin CIVD did not produce any further inhibition of synaptic transmission (Fig. 3A, 9.2 ± 22.3% change, n = 4). Thus, activation of presynaptic GABAB receptor on parabrachial axon terminals inhibits neurotransmitter release by inactivating N-type calcium channels. However, pre-incubation of the slices in a src-kinase antagonist (PP2; 10µM) did not block GABAB-mediated inhibition of transmission between parabrachial axons and CeA-L neurons (63.5 ± 6.2% inhibition p < 0.01, n = 8; Fig. 3B). Therefore, inhibition of neurotransmitter release from parabrachial axon terminals does not involve a voltage-independent block of the N-type calcium channels via a Gβγ-independent activation of the pp60C-src tyrosine kinase.

Figure 2. GABAB-mediated inhibition does not require Gβγ signalling. (A) Parabrachial-CeA-L EPSC amplitude recorded from synapses loaded with the myristlated compound mSIRK in control conditions and after the addition of noradrenaline (NAd) and baclofen (bacl). Below - average EPSCs in each condition. (B) Baclofen (bacl) activated post-synaptic currents recorded from CeA-L cells in control conditions and after mSIRK application.
Activation of presynaptic GABA_B receptors inhibits excitatory transmission between parabrachial axon terminals and CeA-L neurons. The results of the present study indicate that this inhibition involves the activation of Gi/o-linked G-proteins resulting in the inactivation of N-type calcium channels responsible for action-potential-driven neurotransmitter release. Further, the inactivation of N-type calcium channels within parabrachial axon terminals is not due to the activation of pp60C-src tyrosine kinase.

This study adds to the list of Gi/o-linked receptors that act presynaptically to inhibit synaptic transmission between parabrachial axons and CeA-L neurons. Presynaptic α2-adrenoceptors, GABA_B receptors, and Group II and Group III metabotropic glutamate receptors all inhibit the release of synaptic vesicles from axon terminals of parabrachial neurons within the CeA- LC. While these four receptors types link to Gi/o-linked G-proteins, they inhibit synaptic transmission in different ways. Binding noradrenaline to α2-adrenoceptors on parabrachial axon terminals activates Gi/o-linked G-proteins, releasing G_α and G_βγ subunits. Inhibition of the glutamate release from axon terminals then occurs via a direct action of G_βγ subunits on release machinery (i.e. SNARE complexes) to inhibit fusion of synaptic vesicles and subsequent release of neurotransmitter (i.e. glutamate). This mechanism reduces the number of vesicles released from the terminal without reducing release probability. In contrast, activation of GABA_B receptors, Group II or Group III metabotropic glutamate receptors reduces the probability of synaptic vesicle release from parabrachial axon terminals. While the mechanism by which metabotropic glutamate receptors influence release probability of parabrachial axon terminals remains to be determined, the present study demonstrates that GABA_B receptors reduce release probability at these terminals by inhibiting N-type calcium channels.

In the majority of instances, the activation of Gi/o-linked G-proteins inhibits the opening of presynaptic N-type calcium channels in a voltage-dependent manner. This voltage-dependent inhibition involves the binding of G_βγ subunits to α1-domain of N-type calcium channels to stabilise the closed state of the channel, making it ‘reluctant’ to open. In contrast, the present study found that application of the myristilated mSIRK did not prevent the GABA_B-mediated reduction in synaptic transmission between parabrachial terminals and CeA-L neurons. This result suggests that G_βγ subunits are not involved in the inhibition of N-type calcium channels in parabrachial axon terminals. That mSIRK was able to prevent α2-adrenoceptor-mediated inhibition of synaptic transmission at this synapse demonstrates the ability of mSIRK to infiltrate parabrachial axonal terminals. However, GABA_B receptors form a complex with G-proteins and N-type calcium channels, and the vesicular release protein syntaxin 1A form a complex with both G_βγ subunit and the α1-domain of N-type calcium channels. These two complexes facilitate the interaction between GABA_B receptors, G_βγ subunits, and N-type calcium channels, and quite possibly limit the ability of mSIRK to bind to G_βγ subunits before they bind to and inhibit N-type calcium channels. Therefore, involvement of G_βγ subunits in GABA_B-mediated inhibition of synaptic transmission between parabrachial terminals and CeA-L neurons warrants further investigation.
The present study did not find any evidence for a voltage-independent block of the N-type calcium channels via a Gβγ-independent activation of the pp60C-src tyrosine kinase. In addition, the results presented here demonstrate that GABAB-mediated inhibition of N-type calcium channels is not due to G-protein-triggered activation of a number of other kinases that are also blocked by PP2 (e.g. Lck, CSK, Eph-A2, FGFR-I, p38α MAPK, CKβ1 and RIP2).  

Nociceptive information carried by neurons of the parabrachial nucleus to neurons of the CeA-LC is critical for the formation of conditioned fear memories and is thought to play a key role in generating the affective components of the pain experience. Consistent with this, pharmacological interventions that alter synaptic transmission between parabrachial axon terminals and CeA-LC neurons influence nociceptive processing and fear-memory formation. For instance, activation of α2-adrenoceptors within the central amygdala appears to decrease the affective component of visceral pain, while blockade of α2-adrenoceptors within the central amygdala attenuates stress-induced analgesia. Further, the blockade of N-type calcium channels within the central amygdala inhibits the formation of conditioned-fear memories. The results of the present study reveal that GABAB receptors reduce synaptic transmission between parabrachial axon terminals and CeA-LC neurons by inhibiting N-type calcium channels. Therefore, it is likely that activation of GABAB receptors within the central amygdala will modulate both fear-memory formation and pain perception, making presynaptic GABAB receptors within the CeA-LC valuable therapeutic targets for the treatment of fear-and pain-related disorders.

Methods

Coronal brain sections were prepared from 21–28 day old Wistar rats. Rats were anaesthetized using isoflurane, decapitated, and their brains removed into ice cold artificial cerebrospinal fluid (ACSF) solution containing (in mM): 118 NaCl, 25 NaHCO3, 10 Glucose, 2.5 CaCl2, 1.2 NaH2PO4 and 1.3 MgCl2. Brains were sliced into 350 μm thick coronal sections using a Leica VT1000S vibratome at 0 °C, transferred to a chamber containing ACSF at 33–34°C for 30 min, then maintained for several hours in ACSF at room temperature. All procedures were performed with the approval of the Institutional Animal Ethics Committees of The University of Queensland and Charles Sturt University, and carried out in accordance with the approved guidelines.

Whole cell recordings were made from brain slices maintained at 32–33 °C in a recording chamber continuously perfused with oxygenated ACSF and visualized using IR/DIC techniques. Recording electrodes (3-5 MΩ) were filled with pipette solution containing (mM): CsMeSO4 135, NaCl 8, HEPES 10, Mg 2ATP 2 and Na3GTP 0.3 (pH 7.2 with CsOH, osmolality 290 mosm/kg). Whole cell voltage-clamp recordings were made using a patch clamp amplifier (Multiclamp 700A, Axon instruments, Foster City, CA). Current signals were filtered at 4-8 kHz and digitized at 20 kHz (National Instruments, USB-6221 digitiser), acquired, stored and analyzed on a Toshiba Satellite Pro L70 PC using Axograph software. Access resistance (5-15 MΩ) was monitored throughout the experiment. In all experiments, fast GABAergic transmission was blocked with picrotoxin (100 μM) added to the external ACSF solution.

Most drugs were added to the ACSF solution used to perfuse the slice i.e. picrotoxin (Sigma); CGP55845A, N-ethylmaleimide (NEM), yohimbine, prazosin, and NBQX (all from Tocris); and conotoxin CIVD (a gift from Prof David Adams). myristilated Gβγ binding peptide, mSIRK, (Tocris) was applied directly to the parabrachial terminals by puffer pipette for 10–15 min prior to recording. The src-kinase-specific antagonist PP2 (Tocris) was applied, in ACSF, to the slices for 30–90 min in a small incubation chamber before being removed to the recording chamber.

Excitatory post-synaptic currents (EPSCs) were evoked using a bipolar stimulating electrode placed onto the surface of the slice. Responses shown are averages of 10–50 individual trials. Student's t tests were used for statistical comparisons between groups (except where indicated). All results are expressed as mean ± s.e.m.

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**Author Contributions**
A.J.D. conceived and performed the experiments. J.W.C. and A.J.D. analysed and interpreted the results and prepared the manuscript.

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