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GABA receptors modulate trigeminovascular nociceptive neurotransmission in the trigeminocervical complex

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1 GABA (γ-aminobutyric acid) receptors involved in craniovascular nociceptive pathways were characterised by *in vivo* microiontophoresis of GABA receptor agonists and antagonists onto neurones in the trigeminocervical complex of the cat.

2 Extracellular recordings were made from neurones in the trigeminocervical complex activated by supramaximal electrical stimulation of superior sagittal sinus, which were subsequently stimulated with L-glutamate.

3 Cell firing evoked by microiontophoretic application of L-glutamate ($n=30$) was reversibly inhibited by GABA in every cell tested ($n=19$), the $GABA_A$ agonist muscimol ($n=10$) in all cells tested, or both where tested, but not by iontophoresis of either sodium or chloride ions at comparable ejection currents. Inhibited cells received wide dynamic range (WDR) or nociceptive specific input from cutaneous receptive fields on the face or forepaws.

4 The inhibition of trigeminal neurones by GABA or muscimol could be antagonized by the $GABA_A$ antagonist *N*-methylbicuculline, 1(S),9(R) in all but two cells tested ($n=16$), but not by the $GABA_B$ antagonist 2-hydroxysaclofen ($n=11$).

5 R(−)-baclofen, a $GABA_B$ agonist, inhibited the firing of three out of seven cells activated by L-glutamate. Where tested, this inhibition could be antagonized by 2-hydroxysaclofen. These baclofen-inhibited cells were characterized as having low threshold mechanoreceptor/WDR input.

6 GABA thus appears to modulate nociceptive input to the trigeminocervical complex mainly through $GABA_A$ receptors. $GABA_A$ receptors may therefore provide a target for the development of new therapeutic agents for primary headache disorders.

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Abbreviations: Baclofen, R(+)-baclofen hydrochloride; 2OH-saclofen, 2-hydroxysaclofen; GAD, glutamic acid dehydrogenase; LTM, low threshold mechanical; *N*-methylbicuculline, (−)-bicuculline metho-chloride/-bromide, 1(S),(9R); NS, nociceptive specific; PPE, plasma protein extravasation; SSS, superior sagittal sinus; TNC, trigeminal nucleus caudalis; WDR, wide dynamic range

Introduction

The pain in primary headache syndromes, such as migraine and cluster headache, is referred to the ophthalmic (first) division of the trigeminal nerve from pain-producing intracranial structures, such as the dura mater and large vessels, through the trigeminocervical complex (Goadsby, 2001). Modulation of trigeminal transmission in order to alleviate acute migraine requires an understanding of the pharmacology of the synapse onto second order trigeminal neurones. In recent years the pharmacology of this synapse has begun to be studied with most attention on serotonergic and glutamatergic transmission (Goadsby, 1999a). γ-Aminobutyric acid (GABA) is well known as an inhibitory amino acid neurotransmitter in the central nervous system (CNS) (Roberts, 1976) and may modulate nociceptive response in spinal cord (Roberts *et al*., 1986). Its role in trigeminovascular nociceptive transmission within the trigeminal nucleus has not been well characterized.

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Disordered central metabolism of the inhibitory neurotransmitter GABA has been implicated in migraine pathogenesis (Welch *et al*., 1975). Increases in the plasma concentrations of the excitatory amino acids L-glutamate and L-aspartate during migraine episodes have also been reported (Ferrari *et al*., 1990). Butalbital, which is commonly used for migraine and tension-type headache (Elkind, 1991; Raskin, 1988), may act through allosteric barbiturate binding sites on $GABA_A$ receptors. Progesterone and related steroids have been reported in some limited way to be effective in migraine treatment (Bradley *et al*., 1968; Lundberg, 1969; Singh *et al*., 1947); these approaches are not used widely in clinical practice (Lance & Goadsby, 1998). If effective, these compounds could exert their effects through $GABA_A$ receptor neurosteroid binding sites. More recently, clinical studies in migraine have indicated the efficacy of other potentially GABAergic drugs, such as valproate (Hering & Kuritzky, 1992; Jensen *et al*., 1994; Klapper, 1997; Mathew *et al*., 1995), topiramate (Potter *et al*., 2000), propofol (Krusz *et al*., 2000) and baclofen (Hering-Hanit, 1999). The action of valproate may be mediated through $GABA_A$ receptors (Cutrer & Moskowitz, 1996), whereas baclofen is a $GABA_B$

receptor agonist capable of crossing the blood brain barrier, although not all these compounds have exclusively GABAergic action. Expression of the proto-oncogene *c-fos* as a marker of nociceptive neuronal activity within the trigeminal nucleus caudalis is reduced by valproate (Cutrer *et al*., 1995) and allopregnanolone, a neurosteroid progesterone metabolite which modulates $GABA_A$ receptor activity through an allosteric binding site (Cutrer & Moskowitz, 1996).

Afferent fibres conduct nociceptive information to the trigeminal nucleus caudalis. $GABA_A$ and $GABA_B$ receptors are located at both peripheral and central sites. In the CNS, GABA immunoreactivity has been demonstrated in the spinal cord (Carlton & Hayes, 1990; Magoul *et al*., 1987). Both $GABA_A$ and $GABA_B$ receptors are present in the dorsal horn of rats (Bowery *et al*., 1987; Palacios *et al*., 1981) and humans (Bowery *et al*., 1987). Immunoreactivity to glutamic acid decarboxylase (GAD), the biosynthetic enzyme for GABA, has been demonstrated in the trigeminal nucleus caudalis (TNC) of the cat spinal dorsal horn (Basbaum *et al*., 1986). Ultrastructural localization indicated that inhibitory GABAergic controls in the TNC involve both pre- and postsynaptic mechanisms, and are probably mediated *via* direct contacts onto ascending projection neurones, as well as *via* synaptic contacts onto nociceptive primary afferent fibres (Basbaum *et al*., 1986).

Both $GABA_A$ (Roberts *et al*., 1986) and $GABA_B$ (Dickenson *et al*., 1985) receptors have been implicated in the modulation of pain. In this study we have characterized GABA receptors involved in craniovascular nociceptive pathways by the direct *in vivo* microiontophoresis of GABA receptor agonists and antagonists onto neurones in the trigeminocervical complex of the cat. We have used a paradigm involving activation of the trigeminal system in an animal model of trigeminovascular nociception that has functional similarities to primary headache syndromes (Goadsby, 1999b).

Methods

All studies were conducted and terminated under general anaesthesia in accordance with a project license issued by the Home Office of the United Kingdom under the Animals (Scientific Procedures) Act, 1986. Fifteen cats weighing 3.27 ± 0.37 kg (mean $\pm$ s.d.) were anaesthetized with α-chloralose (60 mg/kg i.p.; Sigma, St Louis, MO, U.S.A.) and prepared for physiological monitoring. Halothane (May & Baker-Rhone-Poulenc, Dagenham, U.K./Fluothane, Zeneca, Macclesfield, U.K.) (0.5–3% in a 40% oxygen/air mixture) was administered during surgical procedures and then discontinued during experimental protocols. A catheter was placed in the femoral artery for continuous measurement of blood pressure and heart rate and a second catheter placed in the femoral vein allowed for fluid and drug administration. Supplementary doses of α-chloralose in 2-hydroxypropyl-β-cyclodextrin (Research Biochemicals International (RBI), Natick, MA, U.S.A.) were given intravenously at a rate of 5–10 mg kg^{-1} h^{-1} (Storer *et al*., 1997). The cats were intubated after local anaesthesia with lignocaine hydrochloride (Intubeaze, Arnolds, Shrewsbury, U.K.) and fixed in a stereotaxic frame (Kopf Instruments, Tujunga, CA, U.S.A.). A Jackson/Foley urethral catheter was inserted to drain the bladder, providing more even temperature regulation, more stable control of blood pressure through control of bladder distension, and monitoring of urine output. Core temperature was monitored and maintained between 37–39°C using a rectal thermistor probe and a low radio noise-emitting homeothermic heater blanket system (Harvard Apparatus, Holliston, MA, U.S.A.). The cat was ventilated with a 40% oxygen/air mixture (Harvard Apparatus), end-tidal CO_2 was maintained between 2–4% and expired oxygen continuously monitored (Datex-Ohmeda, Helsinki, Finland). The depth of anaesthesia was monitored periodically throughout the experiment by testing for sympathetic (pupillary and cardiovascular) responses to noxious stimulation and withdrawal reflexes in the absence of neuromuscular blockade. If necessary supplementary anaesthetic was administered.

Surgery

A midline craniotomy (20 mm diameter) and C_1/C_2 laminectomy were performed allowing access to the superior sagittal sinus (SSS) and the recording site in the spinal cord. The sinus was isolated by dissecting the dura and falx cerebri adjacent to the sinus over approximately 15 mm. A small polyethylene sheet was inserted under the isolated sinus and tucked under the outlying dura. To prevent dehydration, and to provide electrical insulation to the cortex, a polypropylene dam was sealed to the bone around the craniotomy with dental acrylic (Vertex, Zeist, Netherlands) and filled with paraffin. Possible artefacts from arterial pulsation and respiratory movement were reduced by: bilateral pneumothoraces, held patent with polypropylene tubes; immobilization of the spine by clamping a thoracic spinous process to the stereotaxic frame; clamping the C_1 transverse processes to auxiliary ear bar holders on the frame, and clamping the remaining caudal portion of the dorsal C_2 spinous process to the frame.

Stimulation and recording

The isolated SSS was gently lifted onto a pair of platinum bipolar hook electrodes connected to a stimulus isolation unit (SIU5A; Grass Instruments, West Warwick, RI, U.S.A.). To activate primary trigeminal afferents, the SSS was supramaximally stimulated with stimulus-isolated square wave pulses from a Grass Instruments S88 stimulator (20–28 V, 250 μs, 0.1–1.0 Hz) after neuromuscular blockade with gallamine triethiodide (Concord, Essex, U.K.) (initially 10–15 mg kg^{-1} i.v., maintained with 5–10 mg kg^{-1} h^{-1}). Extracellular recordings were made using a microiontophoretic combination electrode consisting of a seven-barrelled glass pipette incorporating a central tungsten recording electrode with an exposed recording tip length of approximately 12 μm (Hellier *et al*., 1990). The recording electrode impedance was typically 400 kΩ when measured at 1 kHz in 0.9% saline. The dura above the recording regions on the surface of the spinal cord was reflected and held to the edges of the laminectomy with *N*-butylcyanoacrylate. After careful local removal of the pia mater the electrode was lowered into the cord substance around the C_2 roots in the area of the dorsal root entry zone. The electrode was advanced or retracted in the cord substance in 5 μm steps using a hydraulic microdrive (Kopf Instruments, Tujunga,

CA, U.S.A.). Tissue culture grade agar (3% (w v^{-1}) in saline; Sigma, St Louis, MO, U.S.A.) was set over the exposed cord after electrode insertion to prevent dessication of the cord tissue and to reduce further cardiovascularly related movements. Signal from the recording electrode was fed *via* a headstage amplifier (NL100AK; Neurolog, Digitimer, Herts, U.K.), AC preamplifier (Neurolog NL104A, gain × 1000), and noise eliminator (Humbug, Quest Scientific, North Vancouver BC, Canada) to Neurolog filters (NL125; bandwith approximately 300 Hz to 10 kHz), and then to a second stage variable amplifier (Neurolog NL106, gain approximately × 20 – × 90). This signal (total gain approximately × 20,000 – × 90,000) was fed to a gated amplitude discriminator (Neurolog NL201) and to an 80386 microprocessor based (Intel Corporation, Santa Clara CA, U.S.A.) personal computer *via* an A/D converter (LabMaster DMA, Scientific Solutions, Mentor, OH, U.S.A.) where the signal was processed and stored. A custom written programme (Microsoft C) was used to collect and analyse data. Filtered and amplified action potentials were displayed on an oscilloscope and fed to an audio amplifier (Neurolog NL120S) to assist the isolation of single unit activity from adjacent cell activity and noise. When discriminating between somatic and axonal recordings, a filter bandwidth of d.c. to approximately 30 kHz was used.

In order to record the response of single units to stimulation, post-stimulus histograms were constructed on-line and saved to disk. An analogue signal delay unit (Neurolog NL202) and averaging routine were used to construct averaged action potentials. Free-running neuronal activity, such as stimulated by local L-glutamate microiontophoresis, was analysed as cumulative rate histograms, where activity gated through the ampitude discriminator was collected into successive bins. During experiments electrophysiological data, blood pressure, heart rate and end-tidal CO_2 were processed and recorded on VHS magnetic tape (Pulse Code Modulator; Vetter, Rebersburg, PA, U.S.A.) for documentation and later review.

The location of recording sites was obtained by reference to lesions made by electrocoagulation and coordinates of recording electrode positions as determined by the micropositioner. Lesions were made by passing a current of 20 – 25 μA through the tungsten recording electrode for 20 – 30 s at the final electrode position (D.C.LM constant current lesion maker; Grass Instruments, Quincy, MA, U.S.A.). After termination of the experiment, tissue was fixed in neutral buffered 10% formalin, sectioned (40 μm) and Nissl stained with cresyl violet. The lesions were histologically identified and recording positions were drawn onto a representation of the cord at the level of C_2 (Figure 1).

Receptive fields

Cutaneous receptive fields were sought for most cells responding to superior sagittal sinus (SSS) stimulation. They were characterized as receiving low threshold mechanoreceptor (LTM) input if they responded non-noxious input such as brush or stroke on cutaneous receptive fields on the face or forepaws. They were characterized as nociceptive specific (NS) if they responded to noxious mechanical stimuli, such as pinch or pricking with a needle, or wide dynamic range (WDR) if they responded to both (Hu *et al*., 1981). These cells usually had increased firing in response to noxious stimuli.

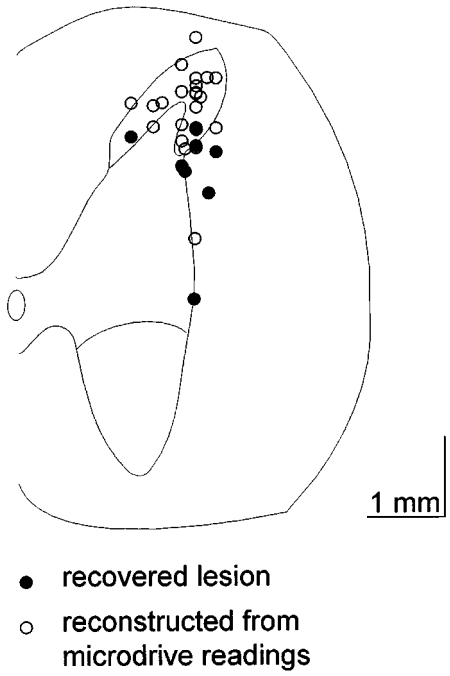

Figure 1 Location of recording sites. A transverse section through the spinal cord at the level of C_2 is represented. Lesions at the final recording sites in electrode tracts (●) from experiments were identified histologically, although not all lesions were recovered. Unmarked recording sites were reconstructed with reference to the positions of the lesion marks and microdrive readings (○). Although the units are only mapped to one side of the cord in the figure, they represent the results obtained from both the left-hand-side and right-hand-side of the spinal cord. Scale bar represents a distance on 1 mm in both directions.

Drugs

Micropipette barrels used for iontophoresis of test substances were filled with 1.0 M L-glutamate (L-Glu), monosodium, pH 8.0 (Sigma, St Louis, MO, U.S.A.); 0.2 M γ-amino-*n*-butyric acid, pH 3.5 (RBI, Natick, MA, U.S.A.); 0.1 M (−)-bicuculline methochloride/-bromide, 1(S),9(R) (*N*-methylbicuculline), pH 3.5 (RBI); 2-hydroxysaclofen (2OH-saclofen), pH 9.0 (RBI); 0.1 M muscimol, pH 3.5 (RBI); 0.1 M R(+)-baclofen hydrochloride (baclofen), pH 3.0, (RBI); saline (for controls), and 1.0 M NaCl (for automated current balancing). Muscimol, baclofen and *N*-methylbicuculline were ionised as cations and retained in the iontophoretic barrels with small negative currents (3 – 5 nA). L-Glu and 2OH-saclofen were ionized as anions and retained with small positive currents (3 – 5 nA). Ejection currents in directions opposite to the retaining currents were used (5 – 200 nA). Chloride or sodium ions ejected from the barrel containing saline were used as controls.

Microiontophoresis

After filling, the iontophoretic micropipette barrels had impedances 60 – 150 MΩ. A microiontophoresis current generator (Dagan 6400, Dagan Corporation, Minneapolis,

MN, U.S.A.) provided the current for ejecting test substances from the barrels. Retaining and balancing currents were used routinely (Bloom, 1974). The L-Glu ejection current was adjusted so cells had free-running activity within a physiological range at a submaximal rate of around 40 Hz, such that inhibition of the cell activity could be distinguished from random firing without causing excitotoxicity. Where L-Glu was applied in pulses the evoked firing rate was often higher. The zwitterionic GABA (γ-aminobutyric acid) was ionized as a cation, $NH_3(+)CH_2CH_2CH_2COOH$, at pH 3.5. Like the other cations used (muscimol, baclofen and *N*-methylbicuculline), it was ejected with positive currents in the order of (+) 5–200 nA and was retained with small negative currents in the order of minus (−) 3–5 nA. The GABA, muscimol and baclofen ejection currents were adjusted such that cell activity inhibited by these compounds could be reversed by the antagonists *N*-methylbicuculline and 2OH-saclofen.

Statistical analysis

Summary data are presented as the mean ± standard error of the mean. Neuronal responses to the test compound in any animal were compared with baseline firing rates using the critical ratio test (Nagler *et al*., 1973) that is based on the count data having a Poisson distribution, and employs the standard normal deviate (Armitage & Berry, 1994) to determine at the 5% level whether two firing rates differ. In practice this very closely approximates a 30% change from baseline. To analyse the entire cohort for experiments in which antagonists were employed an ANOVA with repeated measures was used (SPSS v9.0). The two levels were a time factor for the repeated observations and a with and without drug-of-interest factor. The *P* values were assessed at the 0.05 level and the Bonferroni test applied to comparisons between drugs.

Results

Animals from which data are reported had cardio-respiratory parameters that were normal for the anaesthetized cat. Blood gas parameters were measured at intervals throughout the experiment and were within normal limits: arterial blood pH 7.39 ± 0.01 and pCO_2 3.36 ± 0.07 kPa.

Neuronal characteristics

Extracellular single unit recordings were made from neurones in the trigeminocervical complex (Kaube *et al*., 1993). Cells were located +4 mm rostral to −4 mm caudal to the midpoint C_2 rootlets, 0–300 μm lateral to the dorsal root entry zone at a depth of approximately −700 μm to around −2000 μm below the (dorsal) cord surface. Cells responded with an increased probability of firing to electrical sagittal sinus stimulation with latencies consistent with A-δ fibres [fibre input/afferents] (typically 8–10 ms). Cell bodies were recorded, the recordings were characterized by their biphasic unfiltered action potential morphology (Fussey *et al*., 1970) and the reversible excitatory effect of L-glutamate on cell firing. Neurones identified as linked to stimulation of the superior sagittal sinus were then studied using L-glutamate, which produced firing rate of 39 ± 4 s^{-1} across the cohort of units studied.

Effect of control ejections

To examine for non-specific effects of the ejection, Na^+ or Cl^- ions were iontophoresed at comparable ejection currents to the test substances. For these cells ($n=4$) the firing rate under L-Glu was 15 ± 2 s^{-1}, during Na^+ ejection 14 ± 2 s^{-1} and during Cl^- ejection 18 ± 0.4 s^{-1}. There was no significant effect on cell firing across this cohort ($P>0.05$).

Effect of GABA

Cell firing evoked by microiontophoretic application of L-glutamate was reversibly inhibited by GABA in every cell tested ($n=19$; $P<0.001$) (Figure 2A). This effect was dependent on the current ejected (Figure 2B) which is directly proportional to the amount of GABA delivered iontophoretically (Bloom, 1974). The inhibitory effect of GABA could be antagonized by the $GABA_A$ antagonist, *N*-methylbicuculline, in all but two of the cells tested ($n=11$; $P<0.02$). The $GABA_B$ antagonist, 2-hydroxysaclofen, antagonized the effect of GABA in the two cells unaffected by *N*-methylbicuculline,

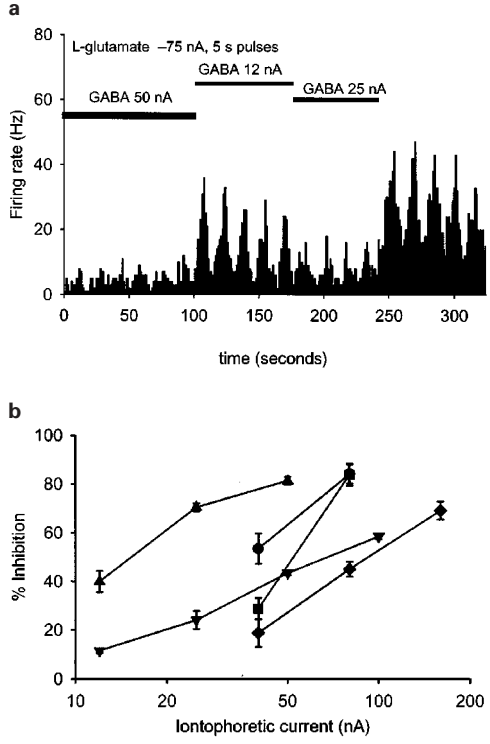

Figure 2 (A) Inhibition of L-glutamate excitation by GABA. L-Glutamate was applied in pulses at −75 nA for approximately 5 s followed by 15 s off (cycling throughout). Cell firing was suppressed during GABA microiontophoresis (bars) in a current-dependent fashion. Firing rate (ordinate, Hz) is plotted in 1 s bins on the abscissa (time seconds). (B) Current dependence of GABA inhibitory effect. Ejection of GABA in a range of currents (abscissa) produced a current dependent inhibition of L-glutamate-excited trigeminal neuronal activity (ordinate). Each data point represents a different range of currents tested repeatedly on individual cells ($n=5$) and plotted as mean ± s.e.mean

but not in six of the other GABA-inhibited cells ($P>0.05$) that were tested with this compound (Figure 3).

$GABA_A$ responses

The $GABA_A$ agonist, muscimol, reversibly inhibited trigeminal neuronal activity elicited by L-glutamate in all cells tested ($n=10$). Muscimol-inhibited L-glutamate induced firing could be antagonised by *N*-methylbicuculline in all cells tested ($n=5$) (Figure 4). Where tested, cells with $GABA_A$ receptors whose muscimol-inhibited L-glutamate-induced firing could be antagonized by *N*-methylbicuculline, were not affected by baclofen ($P<0.05$). Similarly, muscimol inhibited firing could not be antagonized by 2OH-saclofen. *N*-Methylbicuculline often also increased the level of background activity. GABA-inhibited cells received wide dynamic range (WDR) or nociceptive specific (NS) input from cutaneous receptive fields on the face or forepaws. The activation of cells by stimulation of receptive fields was checked by comparing unfiltered action potential morphology with that stimulated by SSS and L-Glu microiontophoresis (Figure 5). NS receptive fields accounted for 50% of those tested.

$GABA_B$ responses

Baclofen, a $GABA_B$ agonist, inhibited the firing of three cells activated by L-glutamate ($n=7$). Where tested, in two of the inhibited cells the inhibition could be antagonised by 2OH-saclofen (Figure 6). Both of these cells were characterized as having LTM/WDR input.

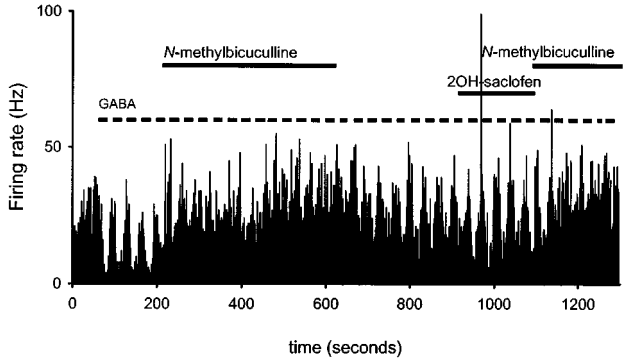

Figure 3 Inhibition of L-glutamate activated cell firing by GABA is $GABA_A$ mediated. Neuronal firing evoked by L-glutamate (ejected with a constant current of -50 nA) in a cell linked to SSS stimulation is inhibited by GABA applied at 37 nA in 10 s pulses (bars). This inhibition was antagonized by the iontophoresis of the $GABA_A$ antagonist, *N*-methylbicuculline, at 55 nA (bars) which often also increased the level of background activity. GABA elicited inhibition was not blocked by 2-hydroxysaclofen (2OH-saclofen; -55 nA). Firing rate (ordinate, Hz) is plotted in 1 s bins on the abscissa (time, seconds).

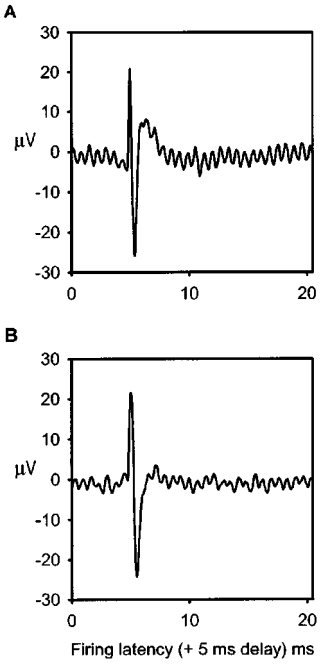

Figure 5 Original waveforms for recorded neurones. Waveforms from typical neurones studied are shown. (A) the firing of an L-glutamate driven SSS stimulated-linked cell is examined on a delay line over an average of 50 sweeps. (B) the waveform of the SSS-driven cell activity is plotted. The recorded element is classically bipolar and highly likely to be a cell body.

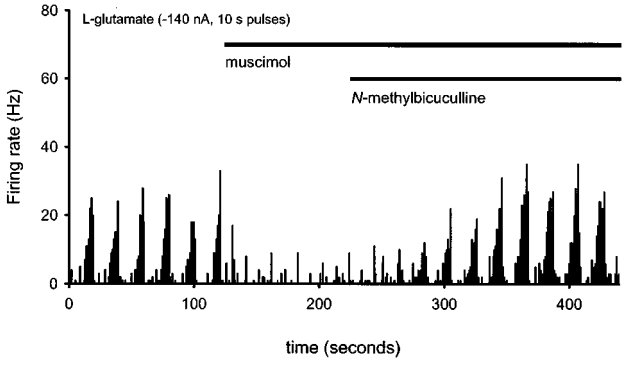

Figure 4 $GABA_A$ agonist muscimol inhibits SSS-linked trigeminal neurones. Neuronal firing evoked by pulses of L-glutamate onto a cell linked to superior sagittal sinus (SSS) stimulation is inhibited by the $GABA_A$ agonist, muscimol (20 nA). The inhibitory effect of muscimol is antagonized by the $GABA_A$ antagonist *N*-methylbicuculline at 20 nA. Firing rate (ordinate, Hz) is plotted in 1 s bins on the abscissa (time, seconds).

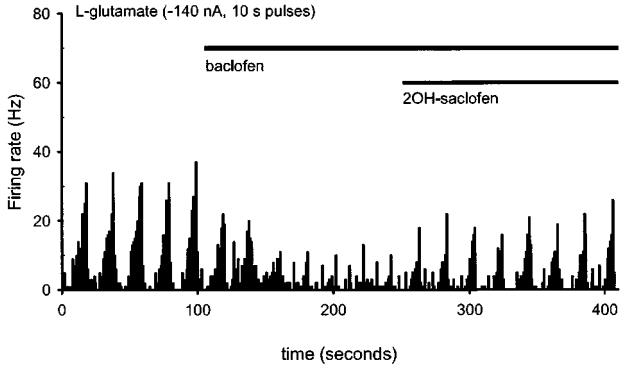

Figure 6 $GABA_B$ mediated inhibition of a trigeminal neuron. L-Glutamate driven superior sagittal sinus stimulation linked cell firing (-140 nA, 10 s pulses) is inhibited by the $GABA_B$ agonist, baclofen (20 nA). The inhibition could be reversed by the $GABA_B$ antagonist, 2OH-saclofen (20 nA).

Discussion

This study demonstrates that neurones within the trigeminocervical complex of the cat responding to a nociceptive stimulus, and activated by L-glutamate agonists, contain two distinct populations of GABA receptors corresponding to the $GABA_A$ and $GABA_B$ class. The largest part of the inhibition of trigeminal activation that we have observed can be accounted for by activation of the $GABA_A$ receptor activation. Most cells are inhibited by the $GABA_A$ agonist muscimol, an effect reversed by the $GABA_A$ antagonist *N*-methylbicuculline, but not by the $GABA_B$ antagonist 2-hydroxysaclofen. Many fewer units are inhibited by the $GABA_B$ agonist baclofen. Taken together the substantial GABAergic influence on trigeminovascular nociceptive neurones is mediated chiefly by $GABA_A$ receptors.

There are three main groups of GABA receptors ($GABA_A$, $GABA_B$, and $GABA_C$), which differ in their distribution and association with ion channels and second messenger systems. $GABA_C$ receptors have not yet been localized in the dorsal horn of the spinal cord (Bormann & Feigenspan, 1995). $GABA_A$ receptors are hetero-oligomeric transmembrane proteins that act as ligand-gated chloride ion channels. They contain distinct binding sites for benzodiazepines, barbituates, and other allosteric modulators of chloride ion flux (Macdonald & Olsen, 1994; Sieghart, 1992). The ionotropic $GABA_A$ receptors have a higher affinity for GABA than $GABA_B$ receptors and are specifically antagonized by (−)-bicuculline methochloride (Collins & Hill, 1974). Muscimol, originally isolated from the fairy mushroom, *Amanita muscaria* (L.) is a water-soluble archetypal $GABA_A$ receptor specific agonist (Krogsgaard-Larsen *et al*., 1983). Thus, our selection of muscimol and (−)-bicuculline metho-chloride/-bromide, 1(S),9(R) as an agonist and antagonist to study GABAergic effects in the trigeminocervical complex.

$GABA_B$ receptors preferentially bind the agonist baclofen (Bowery, 1993). The binding of either GABA or baclofen to $GABA_B$ receptors activates G-protein coupled Ca^{+2} and K^+ channels (Bonanno & Raiteri, 1993). The metabotrophic $GABA_B$ receptor-selective antagonists include phaclofen (Kerr *et al*., 1987), saclofen and CGP 35348 and CGP 55845A (Blake *et al*., 1993). 2-Hydroxysaclofen, a sulphonic analogue of baclofen, is a $GABA_B$ receptor antagonist that has higher selectivity and potency than phaclofen (Curtis *et al*., 1988; Kerr *et al*., 1988) and was thus suitable for use in this study.

In the spinal cord, $GABA_A$ receptor sites outnumber $GABA_B$ receptor sites, although this is not the case in the dorsal horn. There is a particularly high concentration of $GABA_A$, $GABA_B$ and benzodiazepine binding in lamina II with an extensive literature on the presynaptic control of nociceptive primary afferent input (Coggeshall & Carlton, 1997). *In situ* studies indicate that intrinsic neurones contribute significantly to the $GABA_A$ receptor population in the dorsal horn. Ultrastructural studies show that $GABA_A$ receptors usually participate in classical synapses and $GABA_A$-immunoreactivity has been found at post-synaptic specializations in the spinal cord (Alvarez *et al*., 1996; Coggeshall & Carlton, 1997). L-Glutamate is major source of excitatory transmission in the CNS (Seeburg, 1993). Pioneering studies of Salt & Hill (1982) demonstrated that local microiontophoretic application of L-glutamate could activate cells in the trigeminal nucleus caudalis of the rat. Input from the superior sagittal sinus, *via* afferents to the trigeminal nucleus, may be mediated by *N*-methyl-D-aspartate (NMDA) and non-NMDA L-glutamate receptors (Storer & Goadsby, 1999). Moreover, blood flow responses in the region of the trigeminal nucleus (Goadsby & Classey, 2000) and Fos expression in rat (Mitsikostas *et al*., 1998; 1999), are affected by blockade of glutamatergic activation. Furthermore, we have clarified that the neurons studied were activated by a trigeminovascular nociceptive input, from the superior sagittal sinus and had relevant trigeminal receptive fields. Thus, although strictly the data reflect GABAergic modulation of glutamatergic excitation, they are likely to reflect information about GABAergic modulation of the normally functioning trigeminal synapse.

To perform microiontophoretic experiments it was necessary to discriminate between somatic and axonal recordings, given that most receptor populations are not located on axons (Fries & Zieglgansberger, 1974). For this reason recordings made from axons may give false negative results when testing compounds of interest. The excitatory amino acid L-glutamate excites neurones by an effect on cell bodies and has no effect on axons, in contrast to electrical stimulation, which would excite both cell bodies and axons. L-Glutamate acts on NMDA receptors, as well as α-amino-3-hydroxy-5-methylisoxazole-4-propanoic acid (AMPA), kainate, and metabotrophic receptors. Action potential spike morphology can also be used to distinguish between activity recorded extracellularly at axons and that recorded at cell bodies. Axonal recordings being characterized by monophasic action potential morphology and cell body recordings by a biphasic action potential shape (Bishop *et al*., 1962; Grüsser-Cornehls & Grüsser, 1960; Hubel, 1960). For our studies we only recorded and reported data from units clearly identified as cell bodies.

GABA receptors are located either pre-synaptically, or post-synaptically, or on both membranes. The cells we examined in the trigeminocervical complex were activated by superior sagittal sinus stimulation and therefore involved in nociceptive neurotransmission. Since microiontophoretically applied L-glutamate directly stimulates the post-synaptic membrane receptors producing action potentials and this receptor mediated activity could be reversibly inhibited by local microiontophoretic application of GABA, muscimol and baclofen, the most likely location of GABA receptors involved in trigeminal nociceptive transmission is post-synaptic. Furthermore, the GABA receptor mediated inhibition could be reversed by local microiontophoretic application of the GABA receptor antagonist *N*-methylbicuculline, in the majority of cases, and by 2OH-saclofen in only a few cells. The effects of $GABA_A$ receptor activation in the TNC are therefore likely to be a result of post-synaptic inhibition since $GABA_A$ receptors are not readily found on pre-synaptic membranes (Liu *et al*., 1994). $GABA_B$ receptors are located both pre- or post-synaptically, or on both sides of the synapse, in several brain regions (Dutar & Nicoll, 1988). Given that baclofen inhibits the excitation by L-Glu it is likely that at least some of its effects are mediated by post-synaptic events, but this does not exclude the possibility that it may also have presynaptic actions. Moreover, recent evidence from the spinal cord suggests that $GABA_B$ receptor modulation of glutamatergic activation is preferential for C-

fibres over Aδ-fibres (Ataka *et al*., 2000). This would be consistent with the latency of activation of the neurones that we have recorded from, which is in the Aδ range, and thus with a predominant overall $GABA_A$ mediated inhibition of input to the trigeminocervical complex.

GABA agonist mechanisms have been implicated in both the prophylactic and acute treatment of migraine. Valproate, a well established migraine prophylactic medicine (Silberstein, 1996), enhances GABAergic neurotransmission by activation of the GABA synthetic enzyme, glutamic acid decarboxylase (Loscher, 1981), and by inhibition of the GABA degradative enzymes, GABA aminotransferase (Loscher, 1981; Maitre *et al*., 1978) and succinate semialdehyde dehydrogenase (van der Laan *et al*., 1979). Although valproate does not block mechanically induced cortical spreading depression in the cat (Kaube & Goadsby, 1994), it has been proposed that an increase in inhibitory GABAergic neurotransmission may suppress the pathophysiological events that underlie migraine aura (Cutrer *et al*., 1997). Valproate selectively reduces c-Fos-like immunoreactivity within lamina I and II_o of the spinal cord (Cutrer *et al*., 1995). Pre-treatment with bicuculline, but not phaclofen, reversed the effect of valproate and increased the number of c-Fos positive cells within lamina I and II_o of the spinal cord. However, bicuculline by itself decreased the number of c-Fos positive cells, indicating that more than one GABAergic mechanism can suppress *c-fos* expression. Allopregnanolone, a progesterone metabolite, which modulates $GABA_A$ receptor activity, blocks *c-fos* expression in the trigeminal nucleus caudalis (Cutrer & Moskowitz, 1996). Other neurosteroids, such as ganaxolone, which can modulate the $GABA_A$ receptor, are efficient inhibitors of plasma protein extravasation (Limmroth *et al*., 1996), but were ineffective in clinical trials (Data *et al*., 1998). These data suggest that GABAergic modulation that is to have anti-migraine effects may not necessarily have to impact upon dural plasma protein extravasation as a mode of action. Identification of neurones for study by stimulating the superior sagittal sinus has some advantages. SSS stimulation in humans is pain-producing (Feindel *et al*., 1960), while SSS stimulation in the cat leads to cranial release of peptides (Zagami *et al*., 1990) in the same pattern as seen in migraine (Goadsby & Edvinsson, 1993). SSS stimulation in the cat has been a good model for predicting anti-migraine effects in humans, and taken with the fact that the cells reported had trigeminal receptive fields, it is highly likely that GABAergic mechanisms play some role in the modulation of trigemino-vascular nociceptive transmission.

In this study we provide pharmacological evidence for the presence of $GABA_A$ and $GABA_B$ receptors on neurones in the trigeminocervical complex activated by a trigeminovascular nociceptive stimulus. Activation of these receptors also inhibited neuronal activity elicited by L-glutamate. $GABA_A$ receptors were found to be more prevalent and it is likely that at least a large proportion were post-synaptic. Given the polymeric structure of the $GABA_A$ receptor it is possible that regional differences in the sub-unit construction may enable targeting of central nervous system sites in pain that can reduce the unwanted effects of $GABA_A$ activation. Taken together with data on preventative and acute attack treatments of migraine, and data from other pre-clinical models, it is highly likely that GABAergic inhibitory mechanisms play a role in migraine pathophysiology, offering a further series of targets for the management if receptor sub-types can be determined and targeted.

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