C91

Glutamate-enhanced muscle spindle excitability - inhibition by PLD-coupled metabotropic glutamate receptor antagonists.

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50nm, clear synaptic-like vesicles (SLVs) are abundant in primary afferent mechanosensory endings. In muscle spindle primary endings, SLVs appear glutamatergic, and undergo exo- and endocytosis at a rate modulated by mechanical activity (Banks *et al.* 2000; Bewick *et al.* 2000). Exogenous glutamate increases stretch-induced spindle discharge but antagonists of ionotropic (kynurenate) and most metabotropic (MCPG, group I&II; CPPG & MAP4, group III) glutamate receptors do not abolish this effect, even applied together. We have now used antagonists of phospholipase D-coupled mGlu (PLD-mGlu) receptors (Albani-Torregrossa *et al.* 1999) to try to block exogenous glutamate effects and probe for endogenous glutamate release. Adult rats (>300gm) were killed by Schedule 1 methods (Animals (Scientific Procedures) Act, 1986) and 4th lumbrical nerve-muscle preparations excised. Stretch-induced spindle discharge recordings from the muscle nerve with Ag wire electrodes were used to count spikes in the first 1.5s of the 'hold' phase of 1mm stretch-and-hold cycles (~10% increase in muscle length), avoiding the initial 'dynamic' response. Data are expressed as percentages (mean ± SE, of *n* preparations) of the pre-drug count and the significance of differences determined by paired *t*-test. 100μM glutamate increased afferent discharge (137.4 ± 14.4% vs pre-drug spike count, *4*, P<0.05). As previously, MCPG (1mM) and CPPG (100nM) applied together were ineffective (156.8 ± 14.9%, *4*, P<0.03), excluding a Group I-III mGlu receptor-mediated effect. We next tested for the involvement of PLD-mGlu receptors. This receptor is designated outside the standard Group I-III classification, and is inhibited by the Group I agonist DHPG. DHPG (200μM) application reduced the glutamate-mediated enhancement (111.3 ± 7.6%, *4*, P>0.2). The effectiveness of the selective antagonist PCCG-13 (1μM) confirmed the involvement of PLD-coupled receptors (71.8 ± 8.6%, *6*, P<0.05). To test for endogenous glutamate release (e.g. by exocytosis from glutamatergic SLVs) activating the same pathway, PCCG-13 was then applied without exogenous glutamate. 1μM PCCG-13 alone reduced mean excitability (81.2 ± 13.1%, *6*), but this was not significant (P<0.1). However, 10μM PCCG-13 inhibited profoundly (24.7 ± 7.9%, *6*, P<0.01). These data support the hypothesis that muscle spindle SLVs release glutamate, activating PLD-mGlu receptors and enhancing afferent discharge. The functional significance of this mechanism is unclear, but it appears crucial for regulating mechanosensory transduction by stretch-sensitive channels.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C92

Why does apnoea trigger activation of γ-motoneurones in anaesthetised rats treated with high doses of fentanyl?

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Volunteers given alfentanyl in high doses developed muscle rigidity within a minute of the onset of apnoea, but prior administration of pure O2 ensured that they were not hypoxic (Benthousen et al., 1986). Streisand et al. (1993) found that volunteers avoided rigidity when given fentanyl so long as they remained conscious and could be prompted to breathe. Opiate-induced rigidity might thus be triggered by prolonged lack of respiratory movements rather than by hypoxia. In anaesthetised rats given fentanyl there is little or no rigidity because γ-motoneurones are depressed by the anaesthetic, but γ-motoneurones become excited within a minute of the onset of apnoea, activated by a descending pathway as are γ-motoneurones (Gladden & Breckenridge, 2004). If the rats were ventilated and given 10% O2 in N2, or 10%O2 with 6%CO2 in N2, no response was elicited from γ-motoneurones (Gladden et al., 2001). Further, whenever ventilation was stopped, any small spontaneous respiratory movements inhibited the response, suggesting that, as in humans, lack of respiratory movement rather than hypoxia initiated the response. Surprisingly, however, ventilation with pure N2 gave a comparable response to that elicited by apnoea. Simultaneous, in-continuity recordings were made from S1 or L6 dorsal and ventral roots and a nerve branch to the longissimus caudae muscle in Sprague Dawley rats anaesthetised with urethane (1.7g kg-1 i.p.). They were given fentanyl (50μg kg-1 i.v.) and ventilated. Additional doses of fentanyl were given as required to suppress any spontaneous respiratory movements. Tests of 45s of apnoea were alternated with tests of ventilation with pure N2 for 45s, allowing 5min for recovery between each test. Responses were assessed by rectifying and averaging the multiunit recording from the ventral root. The latencies of responses were the same in both cases (31±2.5s (mean ±S.E.M.) for apnoea; 31±2.4s for N2; n=11 in 4 experiments). The mean magnitudes of the responses were not significantly different (paired t test; mean % increase: apnoea 16±7.8 %; N2 22 ±5.5%). Coincident with this increased traffic in the ventral roots an increased frequency of γ-spikes was recorded in the muscle nerve branch. The same γ-motoneurones appeared to be activated in both conditions as judged by their spike shapes and conduction velocities, and the afferents that they excited. A possible explanation is that a falling pO2 acts as a stimulus initiating the descending drive to the γ-motoneurones.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C93

Modulation of fentanyl-induced inhibition of spinal reflexes by blockade of α_2-adrenoceptors: effects of acute inflammation.

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The inhibitory effect of intrathecal (i.th.) fentanyl on spinal reflexes in rabbit is reduced after blockade of spinal α_2-adrenoceptors with RX 821002 (100 μg i.th., Clarke et al., 1998). Thus, it appears that activity in spinally-projecting adrenergic neurones facilitates the efficacy of spinally-applied fentanyl. In the present study we have investigated the dose of RX 821002 necessary to produce this effect in decerebrated rabbits with and without acute inflammation of the ipsilateral metatarsophalangeal joints. Twelve rabbits were decerebrated under isoflurane (2 - 5 %)/N_2O anaesthesia. Of these, 5 had been immunized against ovalbumin over the preceding 28-day period (Harris and Clarke, 2003). Reflexes were evoked in the medial gastrocnemius (MG) muscle nerve by electrical stimulation of the skin at the heel using pulses of 10 mA, 1 ms. Responses were averaged and integrated by computer, and analyzed in 3 time bands relative to the stimulus: 5 - 20 ms (phase 1); 20 - 120 ms (phase 2) and 120 - 250 ms (phase 3): only data from phase 1 recordings are reported here. Fentanyl and RX 821002 were given alternately 30 - 40 min intervals. The dose of fentanyl was fixed at 3 μg kg^{-1} i.th., with the doses of RX 821002 at 3, 7, 20 and 70 μg kg^{-1} i.th. (cumulative dose 100 μg kg^{-1}) interposed between each injection of opioid. In the 5 immunized animals, this protocol was performed 3 - 6 h after injection of 5 mg ovalbumin in 100 μl of 0.9% saline at the ipsilateral metatarsophalangeal joints (Harris and Clarke 2003) to induce an acute inflammation. Experiments were terminated by i.v. injection of saturated KCl solution.In control animals before RX 821002 fentanyl inhibited MG reflexes to a median of 7% (inter-quartile range, IQR, 6 - 29%, n = 7) of pre-fentanyl values. After RX 821002 10 μg kg^{-1} cumulative dose, this effect was significantly decreased so that the opioid suppressed reflexes to a median of 63% (IQR 42 - 75%, Wilcoxon test, $p < 0.04$) of immediate pre-fentanyl values. In inflamed animals before RX 821002, fentanyl inhibited MG responses to a median of 75% (IQR 47 - 87%, n = 5) of pre-fentanyl levels, a significantly smaller effect than observed in non-inflamed animals (Mann-Whitney test, $p < 0.02$). After RX 821002 10 μg kg^{-1} cumulative, fentanyl reduced reflexes to a median of 28% (IQR 3 - 72%) of immediate pre-fentanyl values, not significantly different from the pre-treatment effect (Wilcoxon, $p > 0.1$).These data confirm that the effects of intrathecal fentanyl are supported by endogenous noradrenaline, but that this support is lost in conditions of acute antigen-induced inflammation.

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Supported by BBSRC.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C94

Spinal projections of back muscle spindle afferents revealed by spike-triggered averaging of focal synaptic potentials

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In studies of the reflexes of the back muscles it is not possible to rely on the classical methods of graded electrical stimulation of nerves to excite selected classes of afferents. We have therefore isolated single spindle afferents from longissimus dorsi (LD) by means of tungsten microelectrodes in dorsal root ganglia and have plotted their focal synaptic potentials (FSPs) in the spinal cord systematically by spike-triggered averaging (Taylor et al., 1978; Taylor et al., 1993).Cats were anaesthetised with halothane vapour in 50% oxygen-50% N2O and maintained on sodium pentobarbitone 45 mg kg^{-1} with 12 mg supplements. They were killed by anaesthetic overdose at the end of the experiment. Four heads of LD were detached from their insertions and attached to a servo puller. The cord was exposed from T12 to L5 and a longitudinal linear array of 6 glass-coated tungsten electrodes at 2 mm intervals inserted vertically into the cord 0.6 mm from the midline and centred on the root entry zone of L2, L3 or L4. Six identical amplifiers (x 8500, 5 Hz – 15 kHz) allowed averaging (1024 sweeps) triggered from the firing of a single LD spindle afferent, advancing the electrode in 50 or 100 μm steps. Moving the array longitudinally by 1 mm completed sampling at 1 mm intervals. In some cases the array was also moved in 100 μm steps laterally. Spindle primary and secondary afferents were distinguished by their maximum frequency of 1:1 response to vibration, 120 Hz being taken as the dividing line. They were also tested for the effects of succinylcholine (200 μg kg^{-1} IV) on responses to ramp and hold stimuli.The data are based on 43 primary-like and 13 secondary-like afferents from 18 cats. All afferents showed evidence of monosynaptic excitatory projection to the depths of the ventral horn. Fig. 1 shows an example for a secondary afferent. All FSPs were preceded by a terminal spike (Munson & Sypert, 1979). The delay from negative peak of the axon potential (recorded in the cord dorsum) to the onset of the FSP was 0.45 to 0.5 ms, which is appropriate for a monosynaptic effect. It was commonly observed, as in Fig. 1, that a single afferent projection was discontinuous in the rostro-caudal axis. Projections to the intermediate region were also observed, stronger for secondary than for primary afferents.

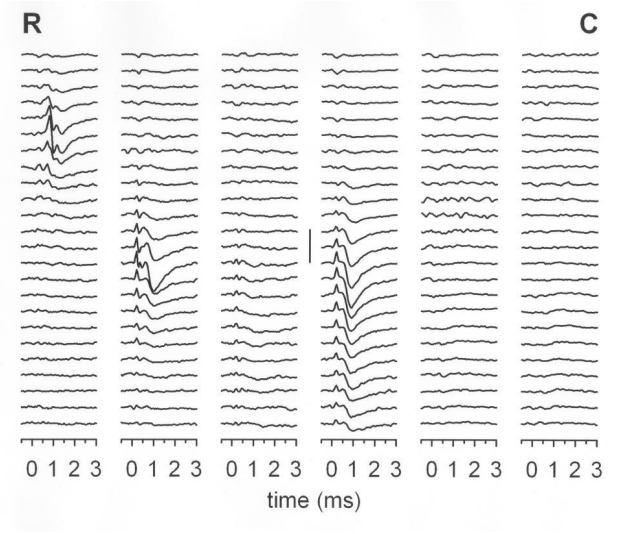

Figure 1. Example of averaged FSPs recorded from 6 electrodes (vertical columns) spaced at 2mm intervals. Traces start at 2.3 mm below the surface and are spaced at 50 μm depths. Vertical scale bar = 20 μV. R – rostral, C – caudal.

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Supported by the MRC UK

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C95

Transmitter content and connections of dorsal horn interneurons interposed in pathways from feline group II muscle afferents

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Dorsal horn interneurons in midlumbar segments are one of the major subpopulations of interneurons relaying information from group II muscle afferent fibres in spinal reflex pathways (Jankowska et al 2002). We used a combination of electrophysiological and immunocytochemical methods to study the axonal projections of these interneurons and the neurotransmitter content of their terminals.Adult cats (5) were deeply anaesthetised with chloralose (up to 50 mg kg-1 i.v.), following induction with sodium pentobarbital (40 mg kg-1 i.p.). Neuromuscular transmission was blocked (Pavulon 0.2 mg kg-1). All experimental procedures complied with national guidelines, including methods for ensuring anaesthesia was maintained during neuromuscular block, and can be found in Bannatyne et al (2003). Interneurons to be analysed were located in laminae IV-V, where stimulation of muscle nerves at intensities consistent with activation of group II muscle afferent fibres evoked maximal dorsal horn field potentials. Interneurons that were monosynaptically activated from these afferents were labelled intracellularly with rhodamine dextran and Neurobiotin. At the end of experiments the spinal cord was fixed by perfusion and the animals killed humanely. Sections containing axonal processes of 7 interneurons (to date) were incubated with antibodies raised against the following molecules: vesicular glutamate transporters 1 and 2 (VGLUT1 and 2) which are markers for glutamatergic terminals; glycine transporter molecule 2 (GlyT2) or gephyrin to reveal glycine-containing axons; or glutamic acid decarboxylase (GAD) to reveal GABAergic axons. Finally, labelled axons were processed for HRP histochemistry and reconstructed.Interneurons had axonal arbors in laminae IV-IX ipsilaterally and VI-VIII contralaterally. Immunocytochemical analysis of individual boutons showed that five neurons were glycinergic and two were glutamatergic. The population thus includes both excitatory and inhibitory cells. Our results confirm that this subpopulation of interneurons form boutons ipsilaterally in laminae VII and VIII where they may provide monosynaptic input to commissural cells interposed in pathways from group II afferents (Edgley et al 2003). They may also contact other groups of interneurons both ipsi- and contralaterally and form monosynaptic contacts with ipsilateral motorneurons; this is currently under investigation.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C96

Oscillatory firing patterns of putative cerebellar Lugaro Cells recorded *in vivo*

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Oscillatory activity is proposed to be important in cerebellar information processing. Cerebellar oscillations, of unknown origin, have been reported in the granule cell layer of awake rats (6-8 Hz, Hartmann & Bower, 1998) and primates (13-25 Hz, Courtemanche et al. 2002). It has been proposed that the Golgi cell-granule cell feedback loop generates oscillatory activity (Maex & De Schutter, 1998). We previously described the responses of Golgi cells to somatosensory stimuli in urethane-anaesthetised rats and showed that they do not respond with oscillatory firing (Holtzman et al., 2003). During these experiments we did find another class of neurone that discharges in an oscillatory way.The experiments were done in adult rats, under urethane anaesthesia (1000 mg/ kg, i.p.), which were killed by overdose at the end of the experiment. We observed the spikes of a class of large granular layer neuron with firing characteristics unlike those of Golgi cells. The spontaneous spikes of these cells had similar mean frequencies to Golgi cells but were continuous and extremely regular, giving interspike interval histograms that were approximately symmetrical and very narrow in comparison to Golgi cells. Individual neurons expressed preferred firing frequencies in the range of 6-20 Hz. Electrical stimulation of peripheral afferents, from bilateral face, fore- and hindlimbs, produced a brief silencing

of the spike discharges (duration 20-40 ms), after which spiking resumed. However, when spiking resumed, spikes occurred at preferred times related to the periodicity of the spontaneous firing. This oscillatory discharge lasted for 400-1900 ms, visible as peaks in a poststimulus time histogram, before desynchronising. These neurons were highly sensitive to the frequency content of a train of stimuli, falling silent at input frequencies >30 Hz. For input frequencies <30 Hz, the response was oscillatory but was frequency locked to the stimulus frequency, reverting to the preferred firing frequency after the stimulus train. Based on several lines of evidence, we suggest that these neurons may be Lugaro cells. They are large neurones located in the granule cell layer. These neurones fire as "oscillators with a preferred frequency" (5-15 Hz) *in vitro* (Dieudonne & Dumoulin, 2000) and inhibit Golgi cells. Our sample of putative Lugaro cells share broad peripheral receptive fields with Golgi cells, and we will provide evidence that the temporal dynamics of their oscillatory firing patterns may be suited to provide inhibition of Golgi cells.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C97

Thermal noxious input causes a widespread depression of cerebellar Golgi cell firing.

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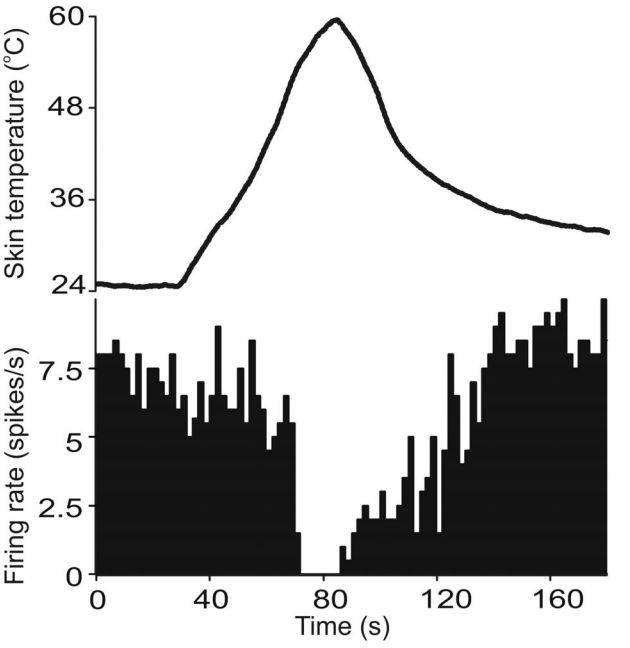
Golgi cells are large granular layer interneurones in the cerebellar cortex which exert feedback inhibitory control over granule cells, thus controlling information flow through the parallel fibre system. We recently reported that the spike activity of Golgi cells, but not Purkinje cells, is depressed by stimulation of peripheral afferents (Holtzman et al. 2003). These responses are evoked by low threshold cutaneous afferents and higher threshold muscle and cutaneous afferents from a wide receptive field (Phuah & Edgley, 2004). Evidence from spinal lesion experiments indicates that the ascending pathway is crossed and ascends in the ventral part of the lateral funiculus, which raises the possibility that the responses are mediated by the anterolateral system. If so, noxious stimulation should generate similar responses. Experiments were performed in rats under urethane anaesthesia (i.p. 1.2-1.5g/kg) which were killed at the end of the experiment with an opverdose. We used high-intensity radiant heat to selectively activate cutaneous nociceptors, but not mechanoreceptors, in the hindfoot pads. Skin surface temperature was estimated with a small k-type thermocouple placed on the skin. Extracellular single unit recordings were made from Golgi cells in the posterior lobe. The firing rate of all (16/16) of the Golgi cells tested was depressed when skin temperature was raised to 50-55°C (see figure). The onset of the response occurred at a mean temperature of 49.5°C SEM ±1°C), but there was no change at temperatures below 40°C. The response thus correlates with the threshold for nociceptor activation (Liu et al., 2003).Our results show that activation of thermal nociceptors reduces Golgi cell firing, consistent with the responses being mediated by the anterolateral system. This suggests that general arousal resulting from noxious stimulation is accompanied by a widespread disinhibition of transmission through the granule cell-parallel fibre-Purkinje cell system. Recent PET and fMRI studies in humans have implicated the cerebellum in various aspects of pain and nociception (Dimitrova et al., 2003). These metabolic changes could be the consequences of changes in cerebellar information flow mediated by Golgi cells.



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C98

Regulation of zif268 in spinal cord in a clinical model of persistent inflammation and hyperalgesia

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Pathological pain is associated with transcriptional alterations in key genes and induction of sustained neuronal plasticity (Ji & Woolf, 2001). The regulatory transcription factor, zif268 has been linked to plasticity underlying persistent cell modification and shown to be regulated by acute noxious stimulation (Nolan A 1987). The aim of this study was characterise the expression of zif268 in spinal cord in a clinical model of persistent inflammatory disease and hyperalgesia (Ley et al. 1989).Thresholds to noxious mechanical stimulation of each leg were measured in Newtons

(Rahman et al. 2002) in adult female sheep affected by unilateral lameness, clinically diagnosed as footrot, a bacterial infection of the digital tissues, and 12 healthy-control sheep. After establishing baseline thresholds, 34 lame sheep were treated according to standard clinical practice (foot bathing, pairing of the horny tissue surrounding the infected area, antibiotics (tetracycline, 20 mg/kg, i.m.)). Following treatment, thresholds were measured at set intervals for up to 30 days. Spinal cord tissues were collected from lame animals humanely killed before treatment (n = 6) and 3 days after treatment (n = 6), and healthy-control sheep (n = 6). Spinal cords were sectioned mid-line and processed for zif268 mRNA and protein using real-time RT-PCR and Western blotting, respectively. Data presented are mean ± S.E.M. and were analysed using ANOVA with post-hoc Tukeys test. Mechanical thresholds were significantly reduced on the limb affected by lameness compared to healthy-control animals (12.8 ± 0.8 N and 18.7 ± 1 N, respectively; $p < 0.001$), and compared to all other limbs (17.6 ± 0.9 N; $p < 0.001$). Hyperalgesia was significantly attenuated 2 days after treatment and had completely resolved by day 3 ($p < 0.01$). Zif268 mRNA and protein were found to be constitutively expressed in control sheep spinal cord. Significant bilateral up-regulation of zif268 mRNA (3.5-fold increase ipsilaterally and contralaterally; $p < 0.05$) and zif268 protein (190 ± 19% ipsilaterally and 160 ± 6 % contralaterally of control levels; $p < 0.05$) was detected in spinal cord recovered from lame sheep displaying hyperalgesia. Three days after treatment zif268 mRNA and protein expression had returned to control values.These results demonstrate that zif268 is elevated in spinal cord in response to persistent inflammation and hyperalgesia. This suggests that zif268 activity and downstream targets may be the trigger for long-lasting neuronal plasticity thought to underlie altered behaviours associated with persistent inflammatory pain.

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Supported by the BBSRC

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C99

Do serotonergic axons directly innervate cardiovascular neurons in rat nucleus tractus solitarius (NTS)?

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The NTS, a major site for blood pressure (BP) control, contains neurons receiving baroreceptor input. One neurotransmitter affecting cardiovascular control is serotonin (5-HT), which can modify NTS neuronal activity by several different 5-HT receptor subtypes (Sévoz-Couche et al. 2000). Here, we investigated whether barosensitive NTS neurons are directly innervated by serotonergic axons.In conscious rats expression of Fos was evoked by BP increases (125% baseline) due to 90 min i.v. phenylephrine infusions (Minson et al. 1997). After 2 hr the rats were anaesthetised (pentobarbitone, 100 mg kg^{-1} i.p) and perfused with fixative. Medullary sections were immunostained with peroxidase to reveal Fos and either 5-HT or serotonin transporter (SERT) and processed for light (LM; n=7 rats) or electron microscopy (EM; n=4). In another set of pentobarbitone anaesthetised (60 mg kg^{-1} i.p.), neuromuscularly blocked rats (Jones et al., 2002), NTS neurons responding to vagus (n=4 cells) or aortic nerve (AN; n=4) stimulation were juxtacellularly labelled with neurobiotin (Pinault, 1996). Filled neurons were detected with avidin-peroxidase and 5-HT, with immunohistochemistry in sections of fixed medulla.Raising BP induced strong Fos expression in many neurons in the baroreceptor region of caudal NTS. LM showed Fos-immunoreactive (IR) nuclei within a network of varicose 5-HT-IR or SERT-IR axons, suggesting that 5-HT terminals might appose barosensitive NTS neurons. EM showed that 5-HT-IR boutons only rarely formed asymmetric synapses on unidentified dendrites in the baroreceptor region. Thin glial processes were usually interposed between serotonergic terminals and NTS cell bodies, but in one case, a 5HT-IR terminal directly apposed a Fos-IR cell body without forming a morphologically-identifiable synapse. Intense SERT-IR occurred in intervaricose axons but terminals stained only lightly.Juxtacellular labelling revealed cell bodies and dendrites of neurons responding to vagus or AN stimulation and these received occasional or no close appositions from 5-HT-IR axons. The apposition density was so low that EM was not worthwhile.Our data indicate that, if 5-HT axons synapse on barosensitive NTS neurons, innervation is sparse and occurs mainly on dendrites. Our EM evidence suggests that glial cells may be involved in serotonergic transmission within the NTS.

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Supported by NH&MRC Australia and & Wellcome Trust UK.

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C100

Vagal reflexes acutely induced by instillation of diesel particulate into airways of anaesthetized rats

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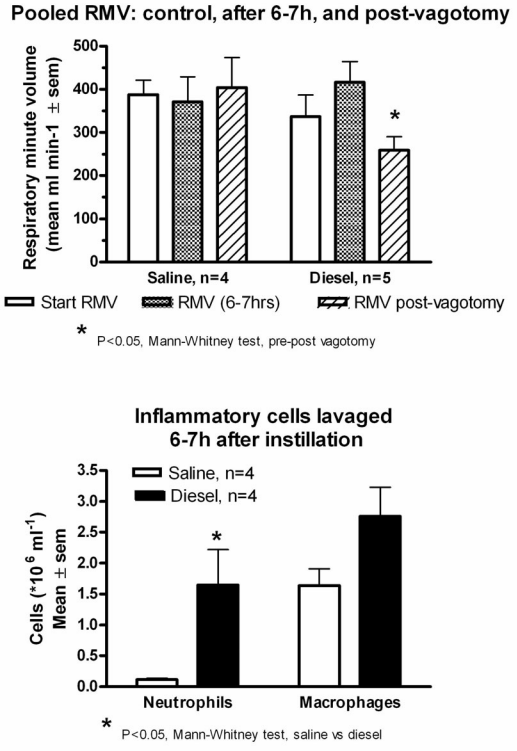
We investigated whether inflammatory reflexes (Tracey, 2002) can be evoked via the vagus nerve following instillation of diesel particulate into the upper airways of anaesthetized rats. Experiments were in accord with UK Home Office regulations. Adult male Wistar rats (mean s.e.m. body weight 335 21g, n=9) were anaesthetized with urethane (ethyl carbamate 25% w/v aqueous solution, 6 ml kg-1 i.p. single dose). Ventilation (respiratory minute volume, RMV) was measured via an electrospirometer connected to a tracheal cannula and recorded on a MacLab. Arterial blood pressure and blood gases were measured. Diesel particulate matter (Diesel): standard reference

material (SRM) 2975 from the National Institute of Standards and Technology (NIST) USA was dispersed in saline at a concentration of 1mg ml-1. Saline or diesel, 0.5ml, was instilled at the carina over 15s, and 6-7 h later the vagus nerves were sectioned in the neck. Ten minutes post-vagotomy the animal was killed humanely, the lungs removed, lavaged with saline and the neutrophil and macrophage content of the lavage measured.Results obtained (Figure 1) show that vagotomy significantly reduced RMV in animals that received diesel, but not those given saline. Neutrophils increased significantly in the lungs of diesel-treated rats, consistent with inflammation, but not in the saline controls. The increase in macrophages following diesel was not statistically significant.In conclusion, this evidence suggests that diesel particulate evokes an acute inflammatory reflex involving the vagus nerve, and further studies using this model will enable the mechanisms activated by diesel particulate, and the contribution made by afferent and efferent vagal fibres, to be determined.



Tracey KJ (2002) Nature 420, 853-859.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C101

Classification of cold responses in rat dorsal root ganglion (DRG) neurones

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Two cold-sensitive ion channels have been cloned recently, both belonging to the transient receptor potential (TRP) channel family. TRPM8 is menthol sensitive and is activated by gentle cooling (McKemy et al., 2002; Peier et al., 2002). ANKTM1 (or TRPA1) is menthol insensitive and requires stronger cooling for activation (Story et al., 2003). The super-cooling agent icilin activates both channels. Using calcium imaging in trigeminal ganglion neurones two types of cold responses were recently described based on temperature thresholds (Thut et al., 2003). However, the molecules underlying the two types of responses are not yet known.We have used $[Ca^{2+}]_i$ imaging on rat DRG neurones loaded with Calcium Green-1 (Reid et al., 2002) to classify cold responses based on the known pharmacology of cold-sensitive ion channels. Wistar rats were humanely killed by CO_2 inhalation followed by decapitation. Cells which responded to a cooling ramp from 36 °C to 14 °C with a fluorescence increase over its initial level ($\Delta F/F_0$) higher than 0.15 were considered cold-sensitive.Of a total of 579 neurones, 136 were cold-sensitive. Based on the effect of 100 μM menthol, these cells were classified as menthol-sensitive (MS, 92 cells) or menthol-insensitive (MI, 44 cells). Neurones were considered MS if they responded to menthol at 32 °C or their cold response was sensitised by menthol. Fluorescence signals during cooling ($\Delta F/F_0$ = 0.41 ± 0.27 compared to 0.24 ± 0.1, mean ± SD, $p < 0.001$, Student's t test) and temperature thresholds (25.3 ± 3.9 °C compared to 22.9 ± 2.7 °C, $p < 0.001$) were significantly higher for the MS group.Icilin sensitivity (50 μM at 32 °C) was restricted almost exclusively to MS cells (22/92 compared to 2/44 for the MI group, $p = 0.002$, χ^2 test) and significantly more MS than MI cells were sensitive to 2 μM capsaicin at 32 °C (61/92 compared to 22/44, $p = 0.02$, χ^2 test).In terms of separating cold responses into homogeneous groups, menthol sensitivity is a very efficient parameter: temperature thresholds, amplitudes of calcium signals and icilin sensitivity all follow the classification imposed by menthol sensitivity. The MS group is more sensitive to cooling and icilin and it is likely to express TRPM8. We have found no evidence that ANKTM1 is responsible for cold sensitivity in the MI group. All procedures accord with the principles of UK legislation.

McKemy et al. (2002) *Nature*, 416: 52
Peier et al. (2002) *Cell*, 108: 705
Reid et al. (2002) *J Physiol*, 545.2: 595
Story et al. (2003) *Cell*, 112: 819
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Funding was from the Volkswagen Foundation and the Physiological Society.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

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Temperature adaptation of the cold and menthol receptor TRPM8 depends on a membrane-delimited mechanism

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Innocuous cold is detected in skin thermoreceptors by activation of the cold and menthol receptor TRPM8. The activation threshold of the cold- and menthol-activated current shifts towards lower temperatures during sustained cooling (cold adaptation), due to an increase in $[Ca^2]_i$ (Reid *et al.*, 2002). Cold adaptation does not occur in outside-out patches (Reid & Flonta, 2002) indicating that it is not intrinsic to the channel, but depends on the cellular environment. To clarify cold adaptation mechanisms in TRPM8, patch clamp recordings were made in cold- and menthol-sensitive rat dorsal root ganglion (DRG) neurones, pre-selected by imaging of $[Ca^2]_i$ (methods described in Reid *et al.*, 2002; rats killed by CO_2 inhalation followed by decapitation), and in rat TRPM8 expressed in HEK293 cells.Cold adaptation of TRPM8 was intact in HEK cells, and therefore does not depend on a mechanism found only in sensory neurones. Cold adaptation was not preserved by keeping the cytoplasm intact: in amphotericin "perforated vesicle" outside-out patches from DRG neurones (Levitan & Kramer, 1990), cold adaptation was absent and thermal threshold was shifted dramatically to lower temperatures (perforated vesicle, 24.1 ± 2.4 °C; amphotericin whole cell in the same neurones before patch excision, 37.6 ± 0.9 °C; $n = 6$; both in 100 μM (-)-menthol), exactly as we have reported in conventional outside-out patches (Reid & Flonta, 2002). In contrast, in some inside-out patches, cold adaptation was intact (3 of 10 patches from DRG neurones, 3 of 9 patches from HEK cells), and the current in these patches decayed with a time constant of 26-94 s (DRG, 75.4 ± 12.9 s; HEK, 48.9 ± 38.9 s; mean ± SD), similar to that in intact neurones (62 - 69 s; Reid & Flonta, 2001).This indicates that cold sensitivity and cold adaptation of TRPM8 do not depend on an intact cytoplasm, but rather on the local integrity of the membrane around the channel; this is always disrupted in forming an outside-out patch but is often preserved in inside-out patch formation. The simplest mechanism that could account for these observations would be an accessory Ca^2 sensing protein interacting with TRPM8 in a membrane-delimited manner, and reducing its cold sensitivity on binding Ca^2 ; the drastic loss of cold sensitivity on outside-out patch formation could result from membrane or cytoskeletal disruption or loss of an accessory protein that sensitises TRPM8. All procedures accord with the principles of UK legislation.

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Rat TRPM8 was a kind gift of David Julius. Funding was from the Volkswagen Foundation and the Physiological Society.

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