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Organisation of sensitisation of hind limb withdrawal reflexes during the development of acute antigen-induced inflammation in the anaesthetised rabbit

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An acute noxious stimulus (20 % mustard oil) generates adaptive changes in withdrawal reflexes to individual muscles according to the site at which is applied (Harris and Clarke, 2003). In this study we have investigated whether inflammation causes similar patterns of changes in reflexes as those obtained with mustard oil.

Sixteen rabbits were immunised against ovalbumin using the 2-injection, 4-week regime described by Cooke (1988). At the end of the immunisation period, the animals were terminally anaesthetised with sodium pentobarbitone (average dose 42 mg kg^{-1}, followed by an infusion of 19 mg kg^{-1} h^{-1}). Reflexes were evoked in the medial gastrocnemius (MG) muscle by electrical stimulation of the skin at the heel, and in S.E.M. tendinosus (ST) and tibialis anterior (TA) muscles by electrical stimulation of the skin at the base of the toes. Reflexes were averaged and integrated by computer. After a control period of 1 h, 5 mg ovalbumin in 100 μl of 0.9 % saline was injected subcutaneously either at the heel or at the base of the toes. Injection sites were always distal to the position of the nearby stimulating electrodes.

Six hours after injection of ovalbumin at the toes ($n = 8$), the circumference of the foot at the injection point increased from a mean of 21 ± 0.6 mm (S.E.M.) to 23 ± 0.5 mm. Over the same time, the toes-TA and toes-ST reflexes increased to medians of 163 % (inter-quartile range, IQR 119–305 %) and 206 % (IQR 179–288) of pre-injection values respectively and the heel-MG reflex decreased to 33 % (IQR 19–63 %) of controls. When ovalbumin was injected at the heel ($n = 8$), the circumference of the foot at the point of injection increased from 10.0 ± 0.3 to 10.7 ± 0.4 mm over 6 h. The heel-MG and toes-ST reflexes increased to medians of 167 % (IQR 128–209 %) and 250 % (IQR 189–432 %) of pre-injection values respectively and the toes-TA reflex was 104 % (IQR 72–128 %) of controls. With the exception of the TA response to inflammation at the heel, all changes in reflexes were statistically significant (Friedman's ANOVA, $P < 0.03$). Alterations in reflexes were not readily apparent until 1 h after the induction of inflammation and persisted thence until the end of the recording period.

The acute inflammation generated by injection of antigen in pre-immunized rabbits (Cooke, 1988) represents a much more persistent noxious stimulus than that provided by mustard oil. Nonetheless, the pattern of changes in reflexes obtained with antigen-induced inflammation was exactly the same as that obtained with mustard oil, albeit expressed over a longer timecourse.

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Harris J & Clarke RW (2003). *J Physiol* **546**, 251–265.

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C153

Sources of fusimotor activation during apnoea induced by fentanyl in anaesthetised rats

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When fentanyl is given intravenously to anaesthetised rats they become apnoeic due to respiratory depression, and fusimotor neurones are activated with a delay, usually within a min (Gladden & Sahal, 2000; Gladden *et al.* 2001). Ventilating the rats promptly reduces the fusimotor activity, but if ventilation is stopped fusimotor neurones again become excited with a similar delay. This sequence can be reliably repeated providing that the opiate concentrations are maintained sufficiently to depress ventilation. In some preparations α-motoneurones are also activated, although later than the γ-motoneurones. Opiate-induced excitation of α-motoneurones is supraspinal in origin (Kuschinsky *et al.* 1977). Supposedly γ-motoneurones could be excited from the same source, but they may perhaps be responding directly to hypoxia. γ-motoneurones in spinalised preparations fire under hypoxic conditions -though note that spinalisation suppresses static γ-activity and activates dynamic γ-motoneurones (Alnaes *et al.* 1965).

Sprague Dawley rats were anaesthetised with urethane (1.7 g kg^{-1} I.P.). In-continuity recordings were taken from a nerve branch to the longissimus caudae muscle, and the S1 dorsal and ventral roots. Multi-unit records were rectified and averaged. Individual γ-and afferent spikes were identified by their shapes and appropriate latencies between the nerve and spinal roots. Following an initial dose of fentanyl (50 μg kg^{-1} I.V.) and before spinalisation, responses were gauged during 4 periods of 45 s of apnoea with at least 5 min recovery between -two periods before and one each after a laminectomy at Th2–3 and a hemisection of the cord. The earliest increases in spikes were detected in records from the ventral roots. Any spontaneous ventilation was suppressed with further doses of fentanyl. The animals were sacrificed after the experiments with an overdose of anaesthetic.

Before spinalisation the mean delay between the last respiration before the apnoea and the start of the increased output from the ventral roots was 29 s (S.E.M. 1.5; $n = 27$). After spinalisation the delay before any response was much longer (mean 70s ± 4.1; $n = 5$). In each individual experiment the delay after spinalisation was at least twice the longest delay before spinalisation, and in one case there was no response. Thus the excitation of γ-motoneurones with a short delay must be supraspinal in origin. This excitation survived contralateral hemisection ($n = 5$), but ipsilateral hemisection abolished it in one case and much attenuated it in another, suggesting that the descending pathway is mainly ipsilateral.

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Gladden MH & Sahal A (2000). *J Physiol* **527.P**, 136*P*.

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Kuschinsky K *et al.* (1977). *Naunyn-Schmiedeberg's Archiv of Pharmacol* **296**, 249–254.

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C154

Long-lasting depressions of cerebellar cortical Golgi cell firing rates are evoked by low (electrical) threshold cutaneous afferents

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We recently reported that somatosensory inputs from large areas of the body evoked long-latency, long-lasting depressions of spike activity in cerebellar Golgi cells (Holtzman *et al.* 2003) in anaesthetized rats. Here we ask which ascending pathways may contribute to these responses.

In deeply anaesthetized rats (Urethane, 1–1.2 g/kg, I.P.) single unit recordings were made from Golgi cells while various peripheral nerves were stimulated electrically. Hindlimb muscle nerves (Gastrocnemius and Hamstring), mixed nerves (Tibial at the ankle) and the principally cutaneous Sural nerve were exposed and mounted on pairs of silver wires in tubular electrodes, mounted in a mineral oil pool which was maintained at 37° C. The effectiveness of the stimulation (in terms of threshold of the most excitable fibres, T) was monitored by recording evoked volleys from the surface of the lumbosacral spinal cord (L2–3), which was exposed by laminectomy. At the end of the experiment all animals were killed by overdose of anaesthetic.

Nerves that contain a large proportion of cutaneous fibres (Sural and Tibial) evoked effects in most of the neurons tested: long-lasting depression of Golgi cell firing was elicited by stimulation at intensities $\geqslant$ 2T in 6/6 cells from sural and in 12/13 cells from tibial. In contrast, stimulation of the muscle nerves (Gastrocnemius and Hamstring) never evoked responses at intensities $\leqslant$ 2T. At higher stimulus intensities ($\geqslant$ 5T) responses were evoked in some neurons (9/15 and 5/8 cells, from Gastrocnemius and Hamstring, respectively). High (electrical) threshold cutaneous fibres are also likely to contribute to this Golgi cell response, since the firing rate depression was greater in response to stronger stimuli.

These results provide evidence that low threshold cutaneous afferents can generate long-lasting Golgi cell firing depressions, but that low threshold (group I) muscle afferents are unlikely to. The classical ascending spinocerebellar pathways (dorsal and ventral spinocerebellar pathways) are therefore unlikely to have mediated the Golgi cell responses. Higher (electrical) threshold fibres from cutaneous and muscle nerves also elicit these responses. Golgi cells are inhibitory interneurons that, by inhibiting granule cells, modulate transmission from mossy fibre input to the cerebellar cortex. A system that selectively depresses Golgi, but not Purkinje cell firing in response to cutaneous inputs from a wide region of the body is likely to have a substantial impact on this transmission. These data are not readily compatible with the conventional view that Golgi cells mediate feedback control of transmission through the mossy fibre system.

Holtzman T *et al.* (2003). *J Physiol* **547.P**, C139.

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C155

The effects of ionotropic glutamate receptor antagonists on cutaneous slowly adapting type I responses

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The role of Merkel cells in cutaneous sensory processes remains controversial. Chemical transmission, from Merkel cell to primary afferent nerve endings of slowly adapting type I units, has been suggested for some time (Iggo & Findlater, 1984). Slowly adapting sinus hair type I (St I) mechanoreceptors are selectively depressed, relative to other mechanoreceptors, by the broad spectrum glutamate receptor antagonist kynurenate (Fagan & Cahusac, 2001). This suggested that the Merkel cell, acting as a mechano-transducer, might transmit to the closely apposed nerve endings using an excitatory amino acid. In order to determine which class of glutamate receptors was involved in this process we tested a range of ionotropic glutamate antagonists.

Adult male Wistar-derived rats were humanely killed by anaesthetic overdose, first with 4 ml I.P. followed by 1 ml intracardiac injection of 25 % w/v urethane. A whisker pad was removed and individual vibrissae dissected out and placed in an organ bath superfused with synthetic interstitial fluid bubbled with a gas mixture of 95 % O2 and 5 % CO_2. Nerve strands from the deep vibrissal nerve were attached to a silver recording wire for single unit recordings. St I, slowly adapting sinus hair type II (St II) and rapidly adapting (RA) mechanoreceptors were identified by their characteristic firing properties. The vibrissa hair shaft was deflected mechanically for 5 s (500 ms onset and offset ramps, 4000 ms plateau). Firing was recorded continuously. Drugs were introduced into the organ bath at the rate of 1 ml/min, for 20 min.

The AMPA receptor antagonist CNQX (3–100 μM, $n = 4$), and NMDA receptor antagonists R-CPP (10–100 μM, $n = 3$) and D-AP5 (10–600 μM, $n = 4$) had no effect on St I responses. However, ifenprodil (3–100 μM, $n = 17$) had clear dose-dependent depressant effects on all activity. The static phase of responses was especially sensitive ($IC_{50} \approx 20$ μM). The depression was long-lasting, depending on the dose (mean recovery time ± S.D. after 10 μM was 63 ± 30 min). Other types of mechanoreceptors were less sensitive to ifenprodil. At concentrations of 50–200 μM, ifenprodil depressed St II ($n = 9$) and RA mechanoreceptor ($n = 3$) responses, but a particularly steep dose-response curve was apparent. The selective NMDA receptor subtype antagonist Ro 25–6981 (10 μM, $n = 2$) had little or no effect on St I responses.

These results suggest that AMPA/kainate receptors are not involved in St I responses. The lack of effect of the NMDA antagonists D-AP5 and R-CPP suggest that an unconventional NMDA receptor is involved. Previous work showed that the presumed (uncompetitive) ion channel NMDA antagonist MK-801 (dizocilpine) at 100 μM selectively depressed St I responses (Senok *et al.* 2001). In the present study ifenprodil had a selective and dose-dependent depressant action on St I responses. Ifenprodil is known as a non-competitive NMDA antagonist acting at the polyamine site, and furthermore is regarded as the prototypical NR2B subunit receptor antagonist. Recent work has shown that the expression of NR2A/B subunits is closely associated with Merkel cells, possibly including the nerve terminals (Senok *et al.* 2003). The apparent lack of effect of the more selective NR2B antagonist Ro 25–6981, could point to

NR2A, rather than NR2B, receptor subtype involvement in the Merkel cell mechano-transduction process.

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All procedures accord with current UK legislation

C156

Localisation of cyclooxygenase-3 in rat central nervous system

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Cyclooxygenase-3 (Cox-3) is a recently identified splice variant of the Cox-1 gene (Chandrasekharan *et al.* 2002) in which part of intron 1 is retained. Cox-3 shows cyclooxygenase activity but is inhibited by paracetamol-related drugs rather than traditional non-steroidal anti-inflammatories. Cox-3 was originally identified in canine cortex; in this study the localisation of Cox-3 has been studied in rat brain.

Wistar rats (250–300g) were humanely killed by decapitation under halothane anaesthesia and brains were rapidly removed and frozen on dry ice. All animal procedures conformed to UK legislation. Brains were sectioned either horizontally or coronally at 10μm and thaw mounted on poly-L-lysine coated slides. Immunohistochemistry was performed using an antibody raised against a peptide corresponding to the first 13 amino acids of the predicted retained intron 1 sequence (Chandrasekharan *et al.* 2002). Sections were post-fixed in 4% paraformaldhyde in phosphate buffer. Endogenous peroxidases were quenched by treatment with 0.5% H_2O_2 in methanol and non-specific binding was blocked in normal goat serum diluted 1:50 in 0.1M PBS. Sections were incubated in affinity-purified anti-Cox-3 antibody (1:2000) overnight at 4°C. Sections were rinsed and incubated in biotinylated goat anti-rabbit secondary antibody (1:2000) for 30 mins. Following a further 30 mins incubation in Vectastain ABC reagent, the substrate, diaminobenzidine tetrahydrochloride was added for 2 min. As a negative control, the primary anti-serum was replaced with immune-depleted serum at the same dilution under exactly the same conditions.

Control sections of rat brain showed no positive staining at all. Brains incubated in anti-Cox-3 antibody showed specific staining in neurons, that in many cases seemed to be nuclear or peri-nuclear. Areas of the brain in which positive neurons were identified included the hippocampal formation, deep but not superficial cortical layers and the spinal trigeminal nuclei. Specific neuronal staining was also identified in spinal cord, particularly in motor neurons. No staining was seen in white matter areas such as the corpus callosum.

These data indicate that Cox-3 protein is expressed in neurons within the rat nervous system. Glial expression was not seen in these preliminary studies. Cox-3 has been shown to be potently inhibited by drugs such as paracetamol, at lower concentrations than those that inhibit either Cox-1 or Cox-2. The analgesic action of paracetamol is thought to be exerted centrally; these data support the hypothesis that Cox-3 may be the central target of paracetamol and related drugs.

Chandrasekharan NV *et al.* (2002). *Proc Natl Acad Sci* **99**, 13926–13931.

All procedures accord with current UK legislation

PC131

Cyclooxygenase-1 in inflammation and nociception

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Cyclooxygenases (COX), and their products the prostaglandins, are important in inflammation and inflammatory nociception. COX-1 is found in primary afferent neurons that are thought to be nociceptive, and COX inhibitors alter neuronal processing of noxious inputs. We have used knockout mice to study COX-1 in inflammation and nociceptive processing.

Wild type (WT, $n = 12$) or COX-1 knockout (KO, $n = 12$) mice were injected intradermally with 100 μl of Freund's Complete Adjuvant (FCA, 2.5 μg/μl M. tub. in mineral oil) at two sites around the left tibiotarsal joint under brief inhalational anaesthesia (4% halothane in O_2). Mice were monitored for 20 days for paw swelling, mechanical allodynia using von Frey filaments, and thermal hyperalgesia. Animals were humanely killed by cervical dislocation on day 20. Paw circumferences and changes in nociceptive behaviour were compared to pre-injection values by ANOVA followed by Dunnett's or Bonferroni tests. Values given are means ± S.E.M.

All animals showed a rapid ipsilateral paw swelling apparent within 24 h that was maintained throughout the 20 days of the study (WT: 139% control, KO: 132% control at 24hrs; $P < 0.01$). The contralateral hind paws showed no swelling at any point in the study. KO mice had significantly less ipsilateral paw swelling than WT animals on day 6–20. This reduction in swelling was found principally in female rather than male mice (female KO: 78% of WT animals day 20, $P < 0.001$, $n = 5$ per genotype). There was no significant difference between the genotypes in either mechanical nociceptive threshold (WT: 2.6 ± 0.3g, KO: 3.4 ± 0.3g) or thermal withdrawal latency (WT: 14.3 ± 0.8 s, KO: 15.4 ± 0.8s) prior to FCA injection. All genotypes developed significant ipsilateral inflammatory mechanical allodynia within 24 h of FCA injection ($P < 0.01$). Male, but not female mice, also developed significant contralateral mechanical allodynia from day 8 −20 ($P < 0.01$). All genotypes developed significant ipsilateral, but not contralateral, thermal hyperalgesia within 24 h which was maintained for 20 days ($P < 0.01$).

These observations suggest that COX-1 contributes to the maintenance of chronic swelling in female mice. COX-1 may also be involved in secondary mechanical allodynia following FCA-induced inflammation.

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All procedures accord with current UK legislation

PC132

Inflammation alters cation chloride cotransporter expression in rat spinal neurons

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Neuronal responses to the neurotransmitter GABA are determined by the chloride gradient across the plasma membrane. During development, GABA has depolarising actions, due to outward chloride movement through $GABA_A$ channels. As neurons mature, the chloride gradient is reversed by a switch in the expression of the cation co-transporter from Na-K-Cl cotransporter (NKCC1) to K-Cl cotransport (KCC2). This renders GABA inhibitory, as opening of $GABA_A$ receptors results in an inward hyperpolarising chloride current. We have investigated the expression of KCC2 and NKCC1 in spinal neurons during inflammation, as a possible mechanism of altered neuronal excitability.

Male rats (200–240g) were briefly anaesthetised (4 % halothane in oxygen) and injected intradermally around the left tibio-tarsal joint with 100μl of Freund's Complete Adjuvant (FCA; M. tub. in mineral oil, made in house, 2.5μg/μl (w/v)). Controls were injected with the same volume of vehicle ($n = 4$). Animals were killed humanely by decapitation under halothane anaesthesia 4 days ($n = 4$) and 10 days ($n = 8$) after FCA injection. Expression of KCC2 and NKCC1 mRNAs was determined using *in situ* hybridisation in 10 μm transverse spinal cord sections, and was quantified by silver grain counting.

	NKCC1		KCC2	
	Left	Right	Left	Right
Control	168±5		183±11	
Day 4	229±8†	221±10†	220±7*	215±9
Day 10	205±7†	211±10†	167±18	163±22

Table 1. Expression of KCC2 and NKCC1 mRNA in superficial dorsal horn during inflammation. Values are means ±s.e.m. silver grain counts per neuron. *p<0.05, †p<0.01 ANOVA followed by t-tests (Bonferroni correction).

Rats showed significant ipsilateral paw swelling within 24 h of FCA injection, that was maintained for 10 days. On day 10 all rats also showed evidence of contralateral tibio-tarsal joint swelling. At 4 days, NKCC1 mRNA was significantly increased in superficial (layers I-II) laminae of the dorsal horn on both sides of the spinal cord (Table 1). Bilateral upregulation of NKCC1 was still significant at day 10. KCC2 mRNA expression was significantly upregulated in superficial dorsal horn at 4 days, ipsilateral to the inflammation, but returned to control values at day 10 (ANOVA followed by Bonferroni test). Neither NKCC1 or KCC2 mRNA levels were altered in deep dorsal horn (lamina IV-V). In acute and chronic inflammation it is possible that changes in spinal neuronal excitability can, at least in part, be attributed to an increase in NKCC1 expression, resulting in enhanced neuronal depolarisation to GABA.

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All procedures accord with current UK legislation