Naloxone restored the TF and the off-cell pause in all nine cells.

Nicoll and colleagues have observed that opiates inhibit local inhibitory neurones at several central nervous system sites[11]. They and others[12] have proposed that the resultant disinhibition of projection neurones is a common function of central nervous enkephalinergic synapses. Morphine could produce analgesia by a similar disinhibition of off-cells, some of which we recently showed to project to the spinal cord[13].

The identity of the neural elements which inhibit the off-cell before the TF is not known. As we gave morphine systemically, it is not possible to specify where its initial action occurs. Furthermore, there is evidence that morphine, when given systemically, also has actions at sites outside the RVM (for example, the midbrain and spinal cord) which contribute to its analgesic effect[5,6]. However, naloxone microinjected into the RVM reverses the analgesic effect of systemic morphine[2], indicating that a significant part of the analgesic action of systemic opiates must be exerted directly on neuronal structures located within the RVM.

Off-cells in the RVM have the following characteristics which indicate that they inhibit nociceptor-driven neurones in the spinal cord: (1) they are driven by electrical stimulation of RVM near threshold for analgesia[14]; (2) they turn off just prior to tail-flick; (3) as we show here, their off-response disappears and reappears in parallel with the TF when opiate agonists and antagonists are administered; (4) they project to the spinal cord[13]; and (5) with stimulation of the midbrain periaqueductal grey, the threshold for excitation of off-cells and suppression of the TF is identical (unpublished studies in our laboratory). Thus, in all situations studied, tail-flick only occurs when off-cells pause. We conclude that off-cells are critical elements in pain modulation and that their disinhibition contributes to the analgesic action of systemically administered opiates.

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Evidence for a central component of post-injury pain hypersensitivity

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Noxious skin stimuli which are sufficiently intense to produce tissue injury, characteristically generate prolonged post-stimulus sensory disturbances that include continuing pain, an increased sensitivity to noxious stimuli and pain following innocuous stimuli. This could result from either a reduction in the thresholds of skin nociceptors (sensitization)[1,2] or an increase in the excitability of the central nervous system so that normal inputs now evoke exaggerated responses[3,4]. Because sensitization of peripheral receptors occurs following injury[5–7], a peripheral mechanism is widely held to be responsible for post-injury hypersensitivity. To investigate this I have now developed an animal model where changes occur in the threshold and responsiveness of the flexor reflex following peripheral injury that are analogous to the sensory changes found in man. Electrophysiological analysis of the injury-induced increase in excitability of the flexion reflex shows that it in part arises from changes in the activity of the spinal cord. The long-term consequences of noxious stimuli result, therefore, from central as well as from peripheral changes.

To investigate, in the unanaesthetized rat, the effects of an injury that would be expected to produce pain hypersensitivity in man, it has been necessary to perform the experiments in chronically decerebrated animals. Decerebration was performed under pentobarbital anaesthesia in eight rats. With body temperature control and orogastric tube feeding the decerebrate rats survived for several weeks[8]. Within 24 h of the decerebration the animals exhibited intact spinal and brain-stem reflexes. These included flexor withdrawal reflexes, vocalization and orientation to the site of the stimulus, following noxious stimulation of the hindpaw. The mechanical and thermal thresholds of the flexor reflex remained stable until a localized thermal injury to the lateral edge of the foot was produced. Figure 1 illustrates the fall in the mechanical threshold and thermal response latency resulting from the injury. The injury also increased the responsiveness of the reflex, from a brief flick (~1 s) following a suprathreshold stimulus pre-injury, to a sustained flexion (~40 s) post-injury. Peripheral tissue injury in the decerebrate rat produces, therefore, changes in the threshold and responsiveness of the flexion reflex that parallel the sensory disturbances found in man.

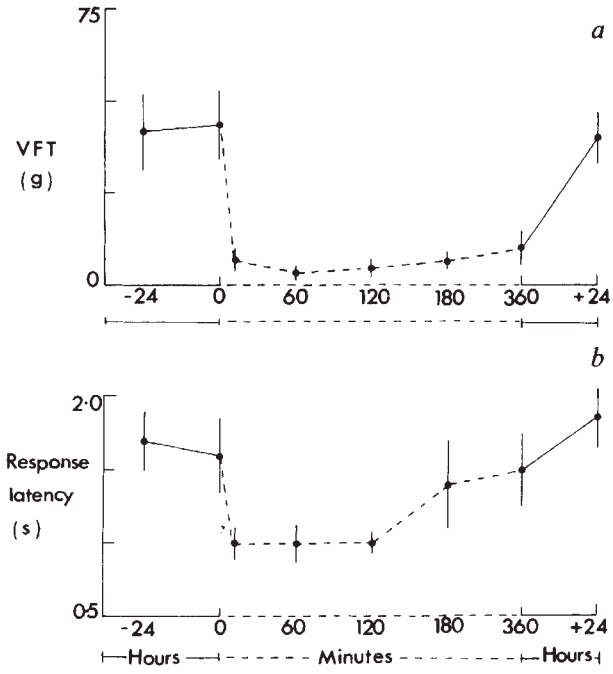

Fig. 1 Alterations in the threshold and responsiveness of the hindlimb flexion withdrawal reflex in eight chronic decerebrate rats produced by a thermal injury (radiant heat to lateral edge of foot, 75 °C for 60 s) at time 0. *a* Shows the reduction in the mechanical threshold, tested with Von Frey hairs (VFT) and expressed in grams, for eliciting flexion following stimulation of the dorsum of the foot adjacent to the site of injury, and its recovery to pre-injury levels within 24 h. *b* Shows the latency of response following immersion of the rats left hindpaw in water at 49 °C before and after that foot was subjected to a thermal injury. All results are expressed as means ± s.e.m. X-axis: solid, hours; dotted, minutes. The baseline mechanical thresholds of the decerebrate rats were lower than in intact animals but above the thresholds of high-threshold cutaneous mechanoreceptors[10]. The baseline thermal response latencies in intact and decerebrate rats were identical.

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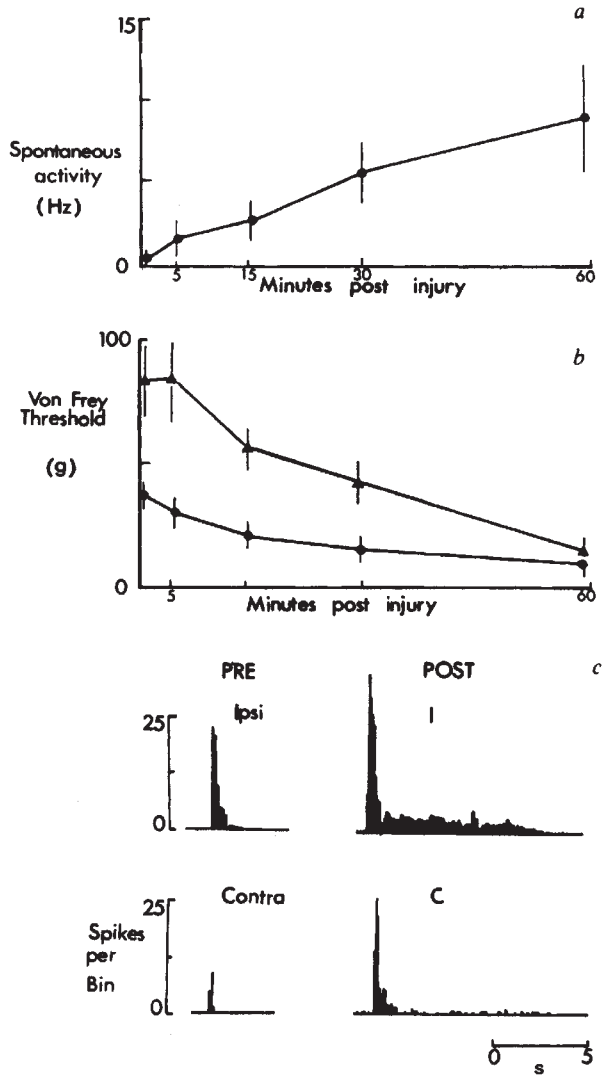

Fig. 2 *a*, Time course of the increase in the spontaneous activity of biceps femoris α motoneurone efferents ($n = 25$) after thermal injury to the ipsilateral foot at time 0. *b*, Mechanical thresholds required to elicit responses from the plantar surface of the ipsilateral (●) ($n = 25$) and contralateral (▲) ($n = 13$) foot determined by testing with Von Frey hairs. The ipsilateral tests were performed adjacent to but not on the site of the injury. *c*, Examples of responses produced in a single biceps femoris efferent by a standard pinch (150 g mm^{-2}) to the plantar surface of the ipsilateral and contralateral second toe, before a thermal injury (PRE) to the lateral side of the foot and 30 min after the injury (POST). Bin width was 500 ms. Note the bilateral increase in response amplitude and duration.

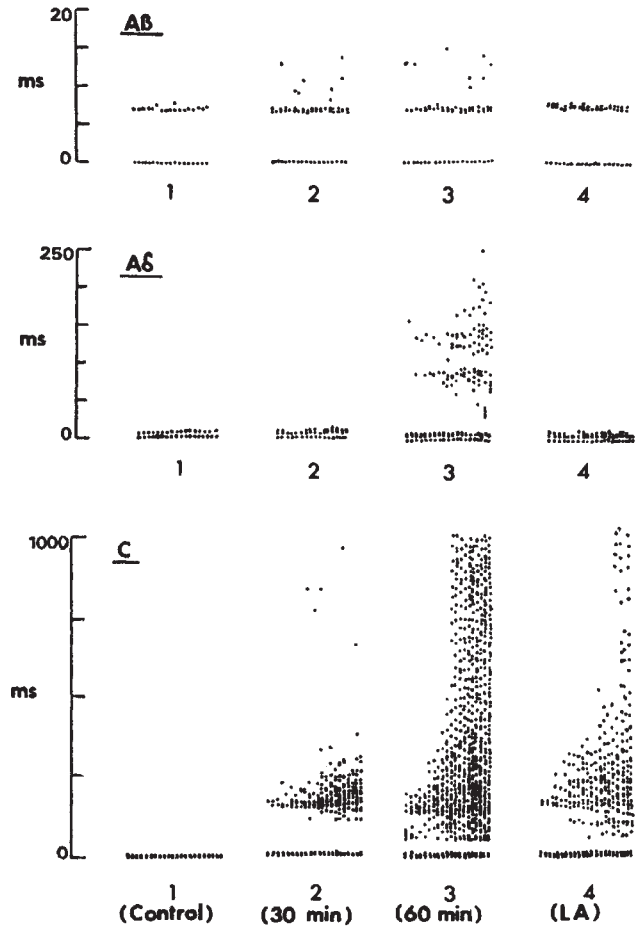

Fig. 3 Raster dot displays of a single biceps femoris unit activated by stimulation of the sural nerve before an ipsilateral thermal injury (control), 30 and 60 min post-injury and 10 min after the injured foot had been completely anaesthetized with Xylocaine. Each dot represents an action potential. The vertical scale is the latency of the response after the sural nerve stimulus. (The stimulus artefact is present at time 0), while the horizontal scale is real time (from left to right) with a new stimulus every 2 s. The sural nerve was stimulated at strengths which only activated Aβ fibres (100 μA, 50 μs), Aβ and Aδ fibres (250 μA, 50 μs) and Aβ, Aδ and C fibres (5 mA, 500 μs). Note the different timescales used for monitoring the Aβ, Aδ and C evoked responses. In the pre-injury state, only an Aβ input was evoked. Thirty minutes after the injury a C response begins to occur, while at 60 min both Aδ and C evoked bands of activity are present. (Note the progressive response increment or wind-up of the C responses.) Ten minutes following the local anaesthetic (administered 80 min post-injury) the sural C-evoked responses remain higher than before the injury although not as high as immediately before the local anaesthetic.

Electrophysiological experiments to examine the post-injury hypersensitivity of the flexion reflex were performed in 28 decerebrate-spinal adult Wistar rats. The animals were decerebrated under Althesin anaesthesia, which was then discontinued and the animals ventilated under gallamine paralysis. Spinalization was performed at T10–11. The nerve to the biceps femoris muscle and the sural nerve were exposed and covered with mineral oil. The biceps femoris nerve was cut distally, desheathed and small filaments teased out and placed on a recording electrode.

A total of 28 single biceps femoris α-motoneurone efferents were studied. The efferents had a low or absent spontaneous activity and high threshold mechanoreceptive cutaneous receptive fields on the ipsilateral foot which extended in 54% to the tail and contralateral foot[9]. Sixty-eight per cent of the units also fired in response to noxious thermal stimuli (<5 °C, >49 °C) applied to the ipsilateral foot.

A thermal injury localized to the lateral edge of the foot, identical to that used in the chronic decerebrate rats, was produced in all 28 animals. In 25 rats the injury was made ipsilateral to the recording site, in three it was contralateral. An immediate area of erythema developed in the skin subjected to the heat stimulus. By 5–10 min oedematous changes in the skin appeared and the swelling and the erythema slowly spread from the site of the stimulus to include most of the foot within 1 h.

The injury resulted in marked changes in the ipsilateral flexor efferents. Their spontaneous activity increased (Fig. 2*a*) and significant falls in the Von Frey hair thresholds for evoking a response from both the ipsi and contralateral mechanoreceptive fields occurred by 1 h (Fig. 2*b*). Associated with these changes was a bilateral increase in the amplitude of response to a standard pinch (150 g mm^2) and its duration (Fig. 2*c*). The afterdischarge to an ipsilateral pinch increased from 2.8 ± 0.3 s pre-injury to 14.2 ± 3 s post-injury ($n = 25$). Similar changes occurred to the

response evoked by a standard thermal stimulus (50 °C for 10 s). In addition to the changes in existing receptive fields, the size of the cutaneous receptive fields also increased in 72% of the units with an expansion to the ipsilateral hindquarter and the appearance of novel or larger contralateral inputs. One hour post-injury 84% of all units had bilateral receptive fields as opposed to 52% pre-injury. The number of units with contralateral thermoreceptive fields increased from 4% to 32%. Contralateral thermal injuries produced no changes in the responses of three animals so a generalized systemic effect of the injury is ruled out.

Sensitization of peripheral nociceptors adjacent to the injury[10,11] is probably responsible for some of the changes in the ipsilateral receptive fields of the flexor efferents, but the greater responses at a lower threshold from the contralateral fields and the expansion of the receptive fields, cannot be accounted for by such a sensitization. To examine further the injury-induced excitability change in the flexor efferents the responses to sural nerve stimulation were investigated. Thirty minutes after the injury, 10 of 18 animals tested exhibited a very substantial increase in sural -evoked response. The increased responses were most prominent to Aδ and C volleys. In five animals Aδ and C stimulation even began to evoke responses post-injury that had not been present before the injury (Fig. 3).

The increased responsiveness of the flexor reflex to natural and electrical inputs post-injury may be either the result of a summation of a continuing afferent input from the site of the injury with previously subthreshold inputs or the result of an injury-triggered increase in the excitability of the spinal cord. To test these possibilities the following experiment was performed on six single flexor efferents. The efferents had all exhibited an expansion of their receptive fields to the contralateral foot or tail by 1 h post-injury and in four these was also a marked increase in their sural evoked responses. Local anaesthetic (2% Xylocaine) was then injected into the ipsilateral foot to produce a complete sensory block of the entire foot and ankle region including the site of the injury and all surrounding inflamed tissue. The adequacy of the sensory block was tested by the development within 5 min of a failure to evoke any responses by natural or electrical stimulation. Ten to fifteen minutes after the sensory block the injury-induced contralateral receptive fields remained present in five of the six units and the sural-evoked response remained above pre-injury levels in three of four units (Fig. 3). Peripheral injury does therefore trigger an increase in the excitability of the spinal cord. Although the responsiveness of the efferents during the sensory block remained above pre-injury levels, in two units there was a small decline in the responses (Fig. 3). Either the excitability increase has a half life of about 10 min or an afferent input from the site of the injury contributes to the changes.

If the changes found here in the flexor reflex parallel changes in the sensory input to the brain then pain hypersensitivity following injury may be due to changes within the central nervous system as well as at the site of the injury. Precisely what neuronal mechanisms are responsible for the central changes requires further study but the changes in the responsiveness and functional connectivity of the spinal cord found here mean that its activity is determined by previous as well as by present inputs.

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Substance P in the ascending cholinergic reticular system

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The neocortex receives a major cholinergic innervation from magnocellular neurones in the basal forebrain[1–3]. However, an ascending cholinergic reticular system has also been postulated to arise from acetylcholinesterase (AChE)-containing neurones in the midbrain and pontine tegmentum[4–5]. Lesions of this region decrease both AChE and choline acetyltransferase (ChAT) in various forebrain areas[6,7], and recent immunohistochemical studies have identified a group of ChAT-containing cell bodies in the midbrain reticular formation and dorsolateral pontine tegmentum[8,9]. Here we have combined retrograde tracing with ChAT immunohistochemistry to demonstrate that this tegmental cholinergic cell group also directly innervates the cerebral cortex. Other immunohistochemical studies have indicated that the neuropeptide substance P is also present in certain cells in the laterodorsal tegmentum[10], and these too appear to project to the forebrain[11,12]. We have therefore performed immunohistochemistry for both ChAT and substance P and have discovered that a subpopulation of the ascending cholinergic reticular neurones contains substance P. Thus, peptide–cholinergic coexistence, previously noted in peripheral neurones, also occurs in the brain.

Indirect immunofluorescence techniques were used to identify the cholinergic and substance P-containing neurones of the midbrain and pontine tegmentum of young adult male rats (150–200 g, Wistar). To increase the levels of ChAT and substance P in cell bodies, the animals were given an injection of the mitotic inhibitor colchicine (80 μg in 20μl saline) into the third ventricle, 48 h before perfusion. The brains were fixed by perfusion through the ascending aorta with 50 ml of saline followed by 250 ml of 4% paraformaldehyde in 0.1 M phosphate-buffered saline. Brains were removed and postfixed for 2 h in the same solution and then rinsed for at least 48 h in buffered 15% sucrose. Coronal sections were cut at 25 μm thickness on a freezing microtome and collected in 50 mM Tris buffered saline (TBS).

Adjacent sections were processed for immunohistochemistry for ChAT and substance P. Indirect immunofluorescence of substance P was performed with an antibody raised in guinea pig[13], diluted 1:200 in TBS containing 0.5% Triton X-100 and 0.1% bovine serum albumin (BSA). Sections were incubated for 48 h at 4 °C in the primary antibody, rinsed in TBS and incubated at room temperature for 1 h in affinity-purified rabbit anti-guinea pig IgG labelled with rhodamine (TRITC; Zymed) diluted 1:20, rinsed in TBS and mounted in glycerol containing 5% *n*-propyl gallate, buffered with Tris-Cl to *p*H 8.

A well-characterized monoclonal antibody to ChAT[14] was used to identify the cholinergic cell bodies using a biotin–avidin immunofluorescence procedure. Sections were incubated at 4 °C for 48 h in ammonium sulphate-fractionated culture supernatant of hybridoma AB8[14] diluted 1:50 in TBS with Triton and BSA as above. After rinsing in TBS for 20 min the sections were incubated for 1 h at room temperature in biotinylated rabbit anti-rat IgG (Vector Laboratories), diluted 1:50, rinsed and then incubated in fluorescein isothiocyanate (FITC)-conjugated avidin (Vector), diluted 1:100 in TBS.

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