Kainate-receptor-mediated sensory synaptic transmission in mammalian spinal cord

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Glutamate, the major excitatory neurotransmitter in the central nervous system, activates three different receptors that directly gate ion channels, namely receptors for AMPA (α-amino-3-hydroxy-5-methyl isoxozole propionic acid), NMDA (*N*-methyl-D-aspartate), and kainate, a structural analogue of glutamate. The contribution of AMPA and NMDA receptors to synaptic transmission and plasticity is well established[1]. Recent work on the physiological function of kainate receptors has focused on the hippocampus[2], where repetitive activation of the mossy-fibre pathway generates a slow, kainate-receptor-mediated excitatory postsynaptic current (EPSC)[3–5]. Here we show that high-intensity single-shock stimulation (of duration 200 microseconds) of primary afferent sensory fibres produces a fast, kainate-receptor-mediated EPSC in the superficial dorsal horn of the spinal cord. Activation of low-threshold afferent fibres generates typical AMPA-receptor-mediated EPSCs only, indicating that kainate receptors may be restricted to synapses formed by high-threshold nociceptive (pain-sensing) and thermoreceptive primary afferent fibres. Consistent with this possibility, kainate-receptor-mediated EPSCs are blocked by the analgesic μ-opiate-receptor agonist Damgo and spinal blockade of both kainate and AMPA receptors produces antinociception. Thus, spinal kainate receptors contribute to transmission of somatosensory inputs from the periphery to the brain.

Glutamate is the fast neurotransmitter between primary afferent fibres and dorsal horn sensory neurons[6]. Previous studies have shown that there are two components of transmission at these synapses: a rapid component blocked by the non-NMDA-receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and a slower component mediated by D(−)-2-amino-5-phosphonovaleric acid (AP5)-sensitive NMDA receptors[7]. Because CNQX blocks both AMPA and kainate receptors, this earlier work did not resolve the different contributions of these two receptor subtypes. To address this question, we obtained whole-cell patch-clamp recordings from neurons in the superficial dorsal horn of the spinal cord, which receive afferent inputs from primary sensory neurons in the

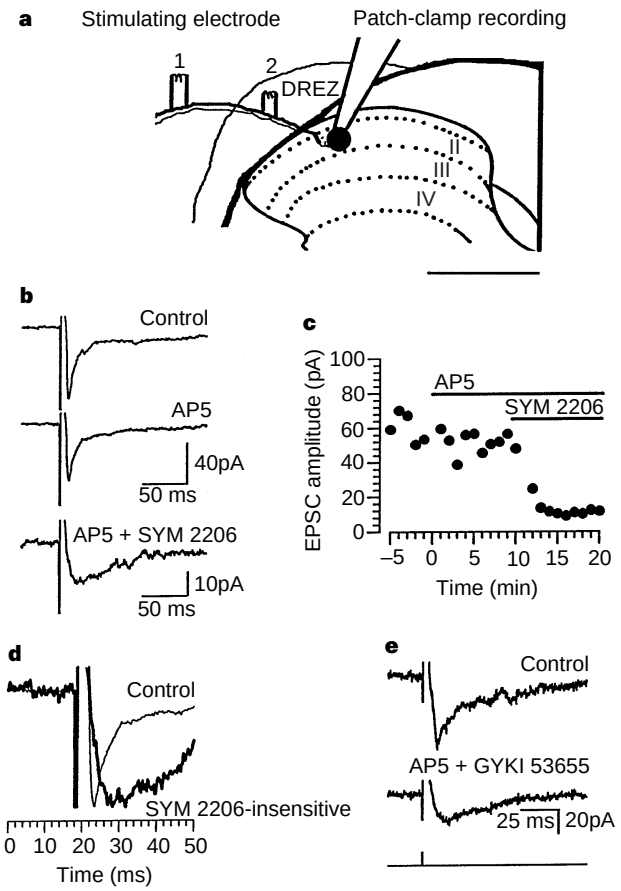

Figure 1 AMPA- and NMDA-receptor antagonists produce incomplete block of primary afferent EPSCs. **a**, Diagram of a spinal slice showing the placement of recording and stimulating (1, dorsal root nerves; 2, DREZ) electrodes. II, III and IV indicate laminae II, III and IV in the dorsal horn. Scale bar represents 1 mm. **b**, EPSCs recorded in control medium, 10 min after addition of AP5 (100 μM), and 10 min after addition AP5 and SYM 2206. **c**, Peak EPSC amplitude versus time for the traces shown in **b**; **d**, The SYM-2206-insensitive EPSC scaled to the peak of the control EPSC. **e**, EPSCs recorded in control medium and 10 min after addition of AP5 (100 μM) and GYKI 53655.

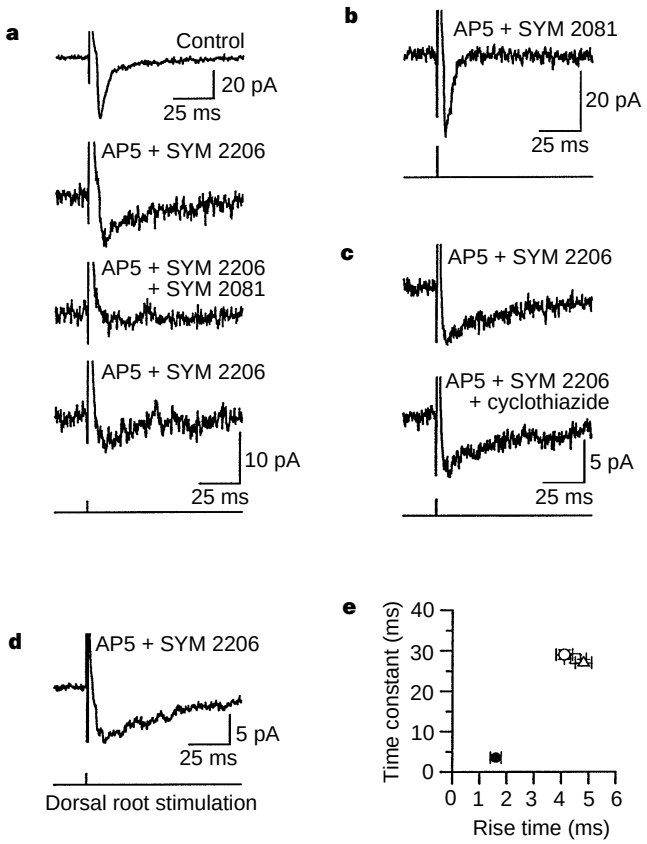

Figure 2 Kainate-receptor-mediated synaptic transmission. **a**, EPSCs in saline solution, in AP5 (100 μM) and SYM 2206 (100 μM) and AP5, SYM 2206 and SYM 2081 (1 μM), and partial recovery in AP5 and SYM 2206. **b**, EPSCs in AP5 and SYM 2081 (5 μM). **c**, SYM-2206-insensitive EPSCs were not affected by cyclothiazide (10 μM). **d**, An EPSC produced by dorsal root stimulation in the presence of AP5 and SYM 2206. **e**, Time constant of EPSC decay versus the rise time (10–90%) for EPSCs in GYKI 53655 (open circle) or SYM 2206 (triangle), with dorsal root stimulation in SYM 2206 (square) and minimal stimulation in saline solution (filled circle).

periphery[8,9]. Fast, monosynaptic EPSCs were induced by electrical stimulation delivered to the dorsal root entry zone (DREZ; Fig. 1a). We blocked NMDA receptors with the selective inhibitor AP5 (100 μM) and tested the action of two selective, non-competitive AMPA-receptor antagonists, GYKI 53655 and SYM 2206 (refs 10–13). Each drug was used at 100 μM, a concentration that produces maximal inhibition of AMPA receptors (half-maximal inhibitory concentration = 1–2 μM) but less than 20–30% inhibition of kainate receptors[10–13].

Superfusion of slices with AP5 plus GYKI 53655 (Fig. 1e; $n = 10$) or AP5 plus SYM 2206 (Fig. 1b–d; $n = 10$) reduced, but did not completely block, the EPSCs. In contrast, AP5 plus 10 μM CNQX or 2,3-dihydroxy-6-nitro-7-sulphamoylbenzo quinoxaline (NBQX) always produced almost complete inhibition of EPSCs ($n = 8$). For EPSCs studied before and after exposure to AMPA-receptor antagonists, the relative peak amplitude of the residual EPSC (the EPSC remaining after exposure to AMPA-receptor antagonists) was 31.3 ± 5.6% of control ($n = 20$ cells; 52.7 ± 7.0 pA before and 11.6 ± 1.7 pA after; 3 traces per cell for each condition). In two cases, the peak currents after superfusion of GYKI 53655 ($n = 1$) or SYM 2206 ($n = 1$) were small (2.6 pA and 3.8 pA). The rise time (10–90%) and decay time constant (τ) for residual EPSCs were significantly longer than for AMPA-receptor-mediated currents (Fig. 2e). To confirm that the residual currents were mediated by kainate receptors, we used SYM 2081, a selective kainate-receptor-agonist that causes potent receptor desensitization[14,15]. SYM 2081 (1 or 5 μM) significantly decreased or abolished the residual EPSCs in the presence of AP5 and SYM 2206 ($n = 3$; Fig. 2a), but did not affect the fast component of control EPSCs recorded in AP5 alone ($n = 4$; Fig. 2b). Cyclothiazide (10 μM), which potentiates currents mediated by AMPA receptors but not by kainate receptors[16], did not affect SYM-2206-insensitive EPSCs ($n = 5$; Fig. 2c). These results indicate that the residual EPSCs were mediated by kainate receptors.

Because stimulation of the DREZ might activate fibres from spinal glutamatergic interneurons and/or descending pathways[17], in addition to primary afferent fibres, we performed experiments in which the stimulating electrode was positioned on an attached dorsal root nerve (Fig. 1a). In all cases, this method evoked EPSCs with a component that was resistant to block by SYM 2206 (Fig. 2d; $n = 6$).

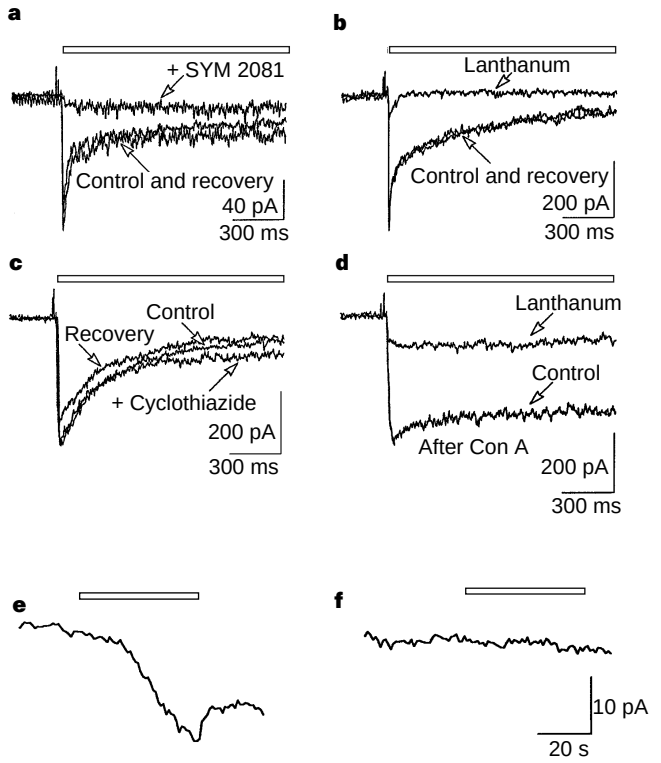

Figure 3 Kainate-receptor-mediated currents in spinal cultured neurons and a slice. **a**–**d**, Spinal cultured neurons; **e**, **f**, spinal splice. In **a**–**d**, GYKI 53655 was continuously superfused. **a**, Pre-exposure to 1 μM SYM 2081 inhibited the current evoked by 300 μM kainate (open bar; 76 ± 6% inhibition; $n = 5$. **b**, Kainate-evoked currents were inhibited by 15 μM lanthanum (77 ± 11% inhibition; $n = 3$). **c**, 5 μM cyclothiazide had little effect on kainate-evoked currents (101 ± 5% of control; $n = 4$). **d**, Receptor desensitization was reduced by pretreatment with conA ($n = 3$), but kainate-evoked current was still sensitive to inhibition by lanthanum. **e**, The current evoked by application of 10 μM kainate to a spinal slice in 100 μM SYM 2206, 100 μM AP5 and 2 μM Con A ($n = 8$). **f**, In the additional presence of 20 μM CNQX, the current produced in **e** was not observed ($n = 5$).

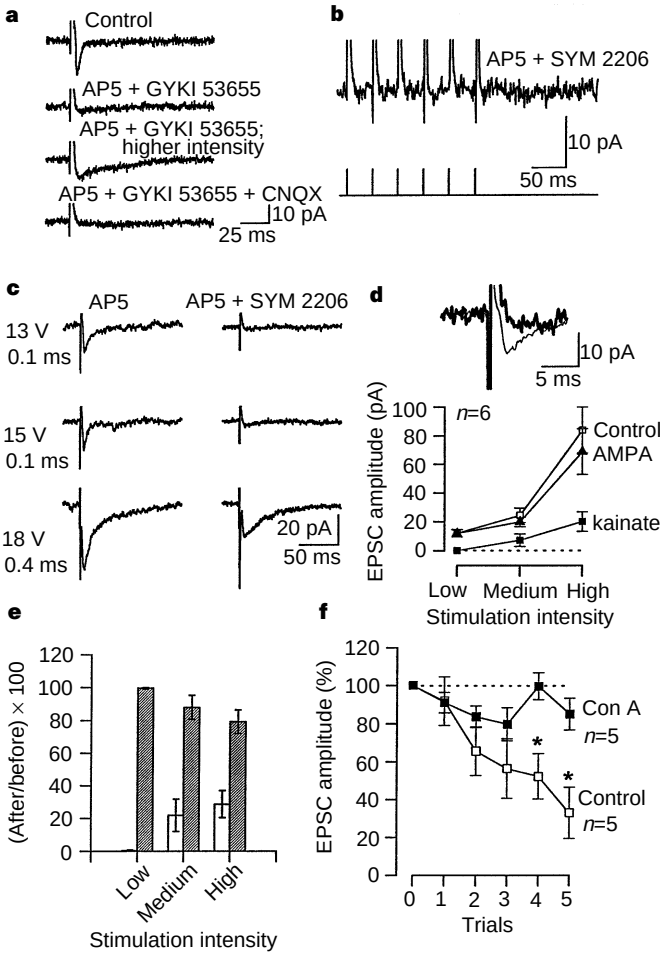

Figure 4 Kainate receptors reside at synapses receiving high-threshold afferents. **a**, Stimulation at low intensities induced GYKI-53655-sensitive AMPA currents (top two traces). At a higher intensity, a GYKI-53655-resistant current was induced and could be blocked by 10 μM CNQX (bottom two traces). **b**, Repetitive stimulation in the presence of AP5 and SYM 2206 did not elicit kainate currents. **c**, Single traces of EPSCs induced by stimulation at different intensities in 100 μM AP5 or AP5 plus 100 μM SYM 2206. **d**, Bottom, summary of within-cell data from **c** (AP5, $n = 6$, open squares; AP5 plus SYM 2206, $n = 6$, filled squares). Inset, the control EPSC at low-stimulation intensity, scaled to 30% of its original amplitude (thin line) and the EPSC in SYM 2206 (thick line); single traces are shown. **e**, SYM-2206-sensitive (AMPA; shaded bars) and -resistant (kainate; open bars) EPSCs as a percentage of control EPSCs. Differences between the means within each set are significant ($P < 0.01$). Responses to low intensity are different from responses to medium and high intensity by pairwise comparison ($P < 0.05$). AMPA component is the control ESPSC–kainate component. **f**, Pretreatment with conA (2 μM) reduced the decay of kainate currents during 1-Hz stimulation. Asterisk indicates $P < 0.05$.

Although the existence of kainate receptors in the spinal cord has been reported[18–20], their subunit composition and subcellular distribution remains unknown. In particular, it is not known whether spinal neurons express functional kainate receptors and whether these receptors co-localize with AMPA receptors at postsynaptic sites. Rapid applications of kainate to cultured spinal cord neurons evoked desensitizing currents in the presence of GYKI 53655 (Fig. 3). Evidence that these currents are mediated by kainate receptors[15] includes their blockade by SYM 2081 (Fig. 3a) and lanthanum (Fig. 3b), their insensitivity to cyclothiazide (Fig. 3c), and the reduction in their desensitization by exposure to concanavalin A (conA; Fig. 3d). Focal application of kainate (10 μM) to spinal slices also elicited currents during continuous exposure to NMDA- and AMPA-receptor antagonists and conA ($n = 8$; 16.4 ± 2.7 pA; Fig. 3e). However, in the present of CNQX (20 μM) these currents were blocked ($n = 5$; 0.8 ± 1.0 pA; Fig. 3f). Thus, kainate receptors are available on postsynaptic cells to produce the kainate-receptor-mediated EPSCs.

In young rats, sensory neurons in the superficial dorsal horn of the spinal cord receive both low- and high-threshold afferents[21,22]. To determine whether kainate and AMPA receptors co-exist at these synapses, we used low-intensity stimulation, which activates a minimal number of presynaptic fibres. If kainate and AMPA receptors always co-localize, then EPSCs evoked by minimal stimulation should include both AMPA- and kainate-receptor-mediated components. However, only rapidly decaying EPSCs, typical of the AMPA-receptor-mediated component, were observed following low-intensity stimulation ($n = 7$; 8.7 ± 1.7 pA; $\tau = 3.5 \pm 0.5$ ms). Addition of GYKI 53655 (Fig. 4a; $n = 2$) or SYM 2206 ($n = 3$) blocked these EPSCs completely, even when a brief train of low-intensity stimulation was delivered[3–5] (25 Hz, 6 pulses; $n = 5$; Fig. 4b). These results raise the possibility that kainate receptors might be displayed selectively at synapses formed by high-threshold afferents and not at synapses that receive low-threshold afferent inputs. Because accurate measurement of EPSC latency is difficult in thin-slice preparations, we studied the dependence of synaptic responses on stimulation intensity more systematically, using intensities that yield activation of different primary afferent fibre types in adult rats[23]. The amplitude of SYM-2206-resistant EPSCs increased disproportionately with stimulus intensity (Fig. 4c–e). Thus, a significantly greater percentage of the EPSC was resistant to block by SYM 2206 during stimulation at high intensities, which were sufficient to activate A_δ and/or C fibres *in vivo*, than was the case for lower intensity stimulation (Fig. 4e).

At mossy-fibre inputs to hippocampal CA3 neurons, the demonstration of kainate-receptor-mediated EPSCs required stimulation with brief repetitive impulse trains[3–5]. In contrast, at primary afferent synapses repetitive stimulation caused a frequency-dependent reduction in the amplitude of kainate-receptor-mediated EPSCs (0.05–5 Hz, data not shown; see Fig. 4f for 1-Hz stimulation). Pretreatment of primary afferent synapses with conA (5 μM; Fig. 4f) abolished this depression, indicating that the depression may result from cumulative desensitization of postsynaptic kainate receptors.

To determine whether kainate receptors contribute to sensory transmission in ascending pathways, we injected a retrogradely transported fluorescent tracer into the thalamus and, two or three days later, recorded from labelled cells in spinal slices. Consistent with the results of a previous study[24], many cells in the superficial dorsal horn were labelled (Fig. 5a). Recordings were obtained from four labelled cells (for 10–15 min), all of which exhibited kainate-receptor-mediated EPSCs upon stimulation of the DREZ ($n = 3$) or dorsal root nerves ($n = 1$) (Fig. 5b).

Opiates produce antinociceptive effects by inhibiting sensory transmission in the dorsal horn[6]. Damgo (1 μM), a selective μ-opiate-receptor agonist, blocked kainate-receptor-mediated EPSCs (Fig. 5c, d; $n = 5$). To study the role of kainate receptors in nociception further, we carried out behavioural experiments (Fig. 5e). Intrathecal administration of CNQX produced dose-dependent antinociception in the tail-flick and hot-plate tests. SYM 2206 had no effect in the hot-plate test but inhibited the tail-flick reflex at the highest dose. SYM 2081 was antinociceptive in both the tail-flick and the hot-plate tests. Latency in the cold-plate test[25] was not affected by any of the non-NMDA-receptor antagonists. The NMDA-receptor antagonists MK801 ($n = 6$; 7 nmol) and AP5 ($n = 4$; 25 nmol) increased latency in the cold-plate test, although motor side effects were also observed.

Our results show for the first time, to our knowledge, that spinal neurons express functional kainate receptors which contribute to synaptic transmission between primary afferent fibres and dorsal horn neurons. In contrast to previous results obtained with hippocampal mossy-fibre synapses, substantial kainate-receptor-mediated EPSCs were observed following individual, low-frequency, high-intensity stimuli. Low-threshold afferents appear to form synapses with few postsynaptic kainate receptors, whereas synapses that include both AMPA and kainate receptors are likely to receive inputs with a high activation threshold from, for example, C and/or A_δ fibres. Moreover, different subsets of postsynaptic gluta-

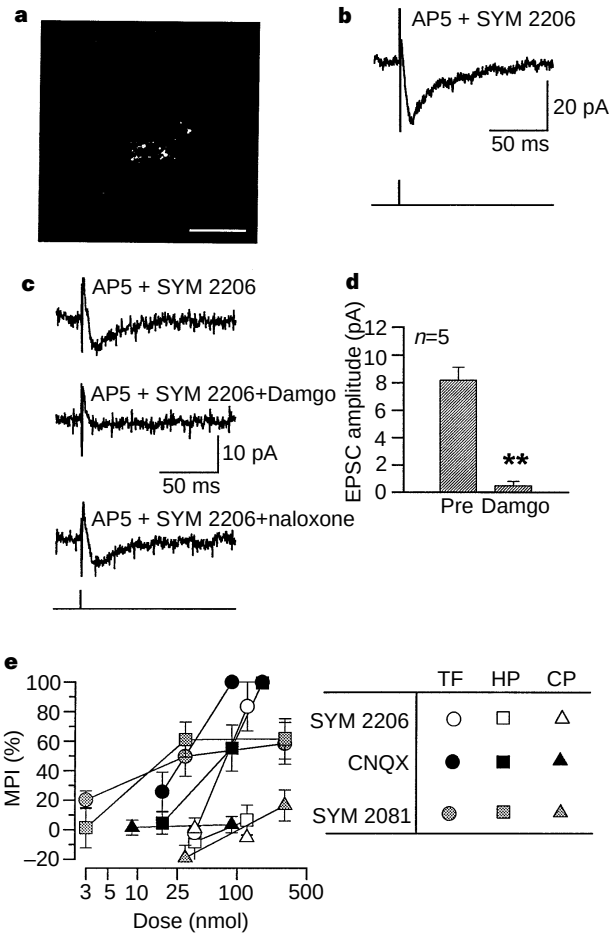

Figure 5 Kainate receptors contribute to ascending sensory transmission. **a**, A fluorescence photomicrograph of a dorsal horn cell that was labelled by fluorescent latex microspheres (light areas) 2 days after thalamic injection. Scale bar represents 20 μm. **b**, An EPSC from a labelled spinothalamic tract cell in 100 μM AP5 and 100 μM SYM 2206. **c**, EPSCs in AP5 plus SYM 2206, in AP5, SYM 2206 and Damgo (1 μM) and in AP5, SYM 2206 and naloxone (20 μM). **d**, Mean amplitude (±s.e.m.) of kainate EPSCs before and after application of Damgo in the presence of AP5 and SYM 2206 ($n = 5$). Double asterisk indicates $P < 0.001$. **e**, Antinociceptive effects of intrathecal administration of antagonists ($n = 3$–7 for each dose). TF, tail flick; HP, hot plate; CP, cold plate.

matergic receptor may subserve behavioural responses to noxious heat and cold. Although glutamatergic synapses mediate pain sensation at all intensities, recent studies[26,27] indicate that substance P and/or neurokinin A are important in sensing intense pain. Thus, it appears that both intensity and modality of pain are coded by different subtypes of postsynaptic receptor and by the release of different transmitters from primary afferent fibres. □

Methods

Spinal slices. Whole-cell recordings were made from spinal slices of rats at postnatal days 4–21 as described[28]. EPSCs were evoked at 0.05 Hz with a bipolar tungsten electrode (stimulus width 0.1 or 0.4 ms) placed at the DREZ or dorsal root nerve. Monosynaptic EPSCs were identified as described[28]. Only monosynaptic EPSCs were studied. Currents were filtered at 1 kHz and digitized at 5 kHz. Bicuculline methiodide (10 μM) and strychnine hydrochloride (1 μM) were added to the perfusion solution. Statistical comparisons were made using one-way analyses of variance (ANOVAs; Dunnett test for post-hoc comparison) or Student's *t*-test. $P < 0.05$ was considered to be significant. SYM 2206 and SYM 2081 were from Tocris–Cookson; other compounds were from RBI or Sigma.

Cultured neurons. Neurons were dissociated from the dorsal half of spinal cord slices and maintained for 7–14 days in culture using standard methods[15]. Whole-cell pipettes were filled with (in mM): 140 caesium glucuronate, 10 EGTA, 10 HEPES, 5 CsCl, 5 $MgCl_2$, 5 ATP and 1 GTP, pH 7.4. Drugs were dissolved in (in mM) 160 NaCl, 10 HEPES, 2 $CaCl_2$ plus 500 nM tetrodotoxin (pH 7.4), and applied by rapid local perfusion from a multibarrelled pipette as described[15].

Labelling with fluorescent dye. Rats were anaesthetized with halothane (2–3%) delivered through a nose cone (with 30% O_2 balanced with N_2) and were positioned in a stereotaxic apparatus. Fluorescent tracer (0.5–1 μl DiI, 0.2% in dimethylsulphoxide, or rhodamine latex microspheres) was injected into one side of the lateral and medial part of the thalamus[24]. Two days after injection, the somata of ascending projection cells were visualized under epifluorescent illumination with a rhodamine filter set.

Behavioural tests. The tail-flick reflex and hot-plate (50 °C) and cold-plate (0 °C) tests were measured as described[25]. Cold stimuli (0 °C) are believed to be noxious and to activate specific cold nociceptors[25]. During intrathecal injection, mice or rats were anaesthetized with halothane (2%). Injections of drugs (mice, 5 μl; rats, 15 μl) were made with the steel tip of a 30-gauge needle connected by a 1-ft length of flexible PE-10 tubing to a 50-μl syringe. Saline was used as a control. After the injection, it took 2–3 min for animals to recover. Data are presented as maximum possible inhibition (MPI) = (response latency − baseline response latency)/(cutoff time − baseline response latency) × 100.

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The oncogene and Polycomb-group gene *bmi-1* regulates cell proliferation and senescence through the *ink4a* locus

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The *bmi-1* gene was first isolated as an oncogene that cooperates with c-*myc* in the generation of mouse lymphomas[1,2]. We subsequently identified Bmi-1 as a transcriptional repressor belonging to the mouse Polycomb group[3–6]. The Polycomb group comprises an important, conserved set of proteins that are required to maintain stable repression of specific target genes, such as homeobox-cluster genes, during development[7–9]. In mice, the absence of *bmi-1* expression results in neurological defects and severe proliferative defects in lymphoid cells, whereas *bmi-1* overexpression induces lymphomas[4,10]. Here we show that *bmi-1*-deficient primary mouse embryonic fibroblasts are impaired in progression into the S phase of the cell cycle and undergo premature senescence. In these fibroblasts and in *bmi-1*-deficient lymphocytes, the expression of the tumour suppressors p16 and p19^{Arf}, which are encoded by *ink4a*, is raised markedly. Conversely, overexpression of *bmi-1* allows fibroblast immortalization, downregulates expression of p16 and p19^{Arf} and, in combination with H-*ras*, leads to neoplastic transformation. Removal of *ink4a* dramatically reduces the lymphoid and neurological defects seen in *bmi-1*-