

## Analgesia and hyperalgesia from GABA-mediated modulation of the cerebral cortex

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It is known that pain perception can be altered by mood, attention and cognition, or by direct stimulation of the cerebral cortex<sup>1</sup>, but we know little of the neural mechanisms underlying the cortical modulation of pain. One of the few cortical areas consistently activated by painful stimuli is the rostral agranular insular cortex (RAIC) where, as in other parts of the cortex, the neurotransmitter  $\gamma$ -aminobutyric acid (GABA) robustly inhibits neuronal activity. Here we show that changes in GABA neurotransmission in the RAIC can raise or lower the pain threshold—producing analgesia or hyperalgesia, respectively—in freely moving rats. Locally increasing GABA, by using an enzyme inhibitor or gene transfer mediated by a viral vector, produces lasting analgesia by enhancing the descending inhibition of spinal nociceptive neurons. Selectively activating GABA<sub>B</sub>-receptor-bearing RAIC neurons produces hyperalgesia through projections to the amygdala, an area involved in pain and fear. Whereas most studies focus on the role of the cerebral cortex as the end point of nociceptive processing, we suggest that cerebral cortex activity can change the set-point of pain threshold in a top-down manner.

It is well known that painful stimuli activate the insular cortex<sup>2</sup>, but less is known of how the output of the insular cortex might alter pain. Studies in humans and animals show that inhibition or lesion of this cortical area produces analgesia<sup>3–5</sup>, indicating that the insular cortex tonically produces hyperalgesia. Tonic depression of the nociceptive threshold could result from the activation of pronociceptive areas of the brain or from inhibition of the endogenous pain inhibitory system<sup>6</sup>. To examine the relationship between cortical activity and nociceptive threshold, we manipulated GABA neurotransmission in the RAIC and then tested nociceptive responses with the heat paw-withdrawal test. GABA levels were increased either by inhibiting GABA-aminotransferase-mediated degradation<sup>7</sup> using vigabatrin, or novel expression of glutamic acid decarboxylase 67 (GAD-67), one of the enzymes responsible for synthesizing GABA from glutamate.

Increasing GABA concentration unilaterally in the RAIC with vigabatrin (78 nmol in 200 nl), resulted in a clear and consistent bilateral analgesia, an effect not seen when the injections were in the surrounding brain areas (Fig. 1a, b). Injection of the GABA<sub>A</sub> antagonist bicuculline (200 pmol/200 nl) in the RAIC after vigabatrin normalized the nociceptive threshold, indicating that the effect of vigabatrin was receptor-mediated. Injection of the sodium-channel blocker bupivacaine (200 nl of a 0.25% solution) in the RAIC yielded similar results to vigabatrin (Supplementary Fig. 1a). The antinociceptive effect of vigabatrin seemed specific, because no motor or behavioural impairment was revealed in the open-field (no difference between vigabatrin-treated and saline-treated for any of the parameters;  $n = 6$  per group,  $P > 0.05$ ) and rotarod tests (Supplementary Fig. 1b). To determine whether a sustained increase in GABA would produce long-term analgesia, we injected the

defective herpes simplex virus (HSV) vector dvGAD-67 ( $5 \times 10^3$  defective particle units/1.5  $\mu$ l) encoding GAD-67. Infection of both neuronal and glial cells with dvGAD-67 leads to a continued synthesis and release of GABA<sup>8,9</sup> and resulted in bilateral antinociception (Fig. 1c, d) lasting for up to 10 days. Motor and general behaviours were unaltered (Supplementary Fig. 1b–d). Histological analysis confirmed that local expression of the LacZ reporter gene encoding  $\beta$ -Galactosidase (Fig. 1d) was confined to the RAIC and, with the exception of an occasional cell being labelled along the cannula tract, no labelling was found in other brain areas. Injection of a control viral vector expressing only LacZ and alkaline phosphatase had no effect (Fig. 1c), indicating that antinociception did not result from a non-specific event related to the injection of viral vectors.

The above behavioural results demonstrate that GABA-mediated neurotransmission is directly involved in the pain modulatory function of the RAIC. The long duration of the effect obtained with dvGAD-67 suggests that the antinociceptive action occurs through neural mechanisms that do not downregulate over time.

To determine whether the local increase in cortical GABA concentration changed the nociceptive threshold by an action on the descending pain inhibitory system<sup>6</sup>, we administered the non-selective  $\alpha$ -adrenoreceptor antagonist phentolamine (3 nmol in 10  $\mu$ l) intrathecally over the lumbo-sacral spinal cord through a chronically implanted catheter<sup>10</sup>. Phentolamine blocks descending inhibition mediated by noradrenergic bulbo-spinal projections, most of which originate from the locus coeruleus<sup>11,12</sup>. In all cases phentolamine reversed the antinociception induced by the increased cortical GABA concentration (Fig. 1e), indicating that the antinociceptive effect of inhibiting the RAIC involves an increased activity of noradrenergic bulbo-spinal projections. The dose of phentolamine used here was too low to alter the baseline nociceptive threshold (Fig. 1e).

We then examined projections from the RAIC that might modulate noradrenergic bulbo-spinal neurons. The RAIC was found to have bilateral projections to GAD-immunopositive cells in the caudal brainstem (Fig. 2a–d), including GABAergic cells in nucleus raphe magnus (Fig. 2c, d), many of which project to the locus coeruleus<sup>13</sup>. On the assumption that the output of the RAIC is glutamatergic, the inhibitory effect of RAIC activity on the locus coeruleus is probably mediated by the activation of GABAergic neurons contacted by RAIC afferents (Fig. 2a–d). Indeed, up to two-thirds of afferents onto locus coeruleus neurons are GABAergic<sup>13</sup> and, together with opioidergic and catecholaminergic afferents, GABAergic afferents constitute the main inhibitory input<sup>13</sup>. It is therefore likely that the analgesia seen after increasing GABA in the RAIC results from a decreased activity of cortical afferents to the peri-coerulear inhibitory neurons, leading to a net increase in the activity of noradrenergic bulbo-spinal neurons (see Fig. 4 for a summary diagram).

GABA acts on neurons through two different types of receptor. In the RAIC, GABA<sub>B</sub> receptors are concentrated on pyramidal neurons of cortical layer 5 (Fig. 2e, f), whereas GABA<sub>A</sub> receptors are located diffusely throughout all cortical layers on dendrites and soma (Fig. 2g). Because most lamina V neurons are projection neurons, we used retrograde and anterograde tracing to locate their targets. We found that a large contingent of RAIC neurons expressing GABA<sub>B</sub> receptors project to the ipsilateral basolateral nucleus of the amygdala ( $45.1 \pm 3.9\%$ ), and few GABA<sub>B</sub> neurons project to the peri-coerulear area.

These anatomical findings led us to propose that increasing the GABA concentration in the RAIC and then blocking GABA<sub>B</sub> receptors would selectively disinhibit projections to the amygdala, leaving other outputs silent and enabling us to determine the net effect of the disinhibited neurons on pain behaviour. The GABA<sub>B</sub>-selective antagonist saclofen (100 nmol/200 nl; see Supplementary

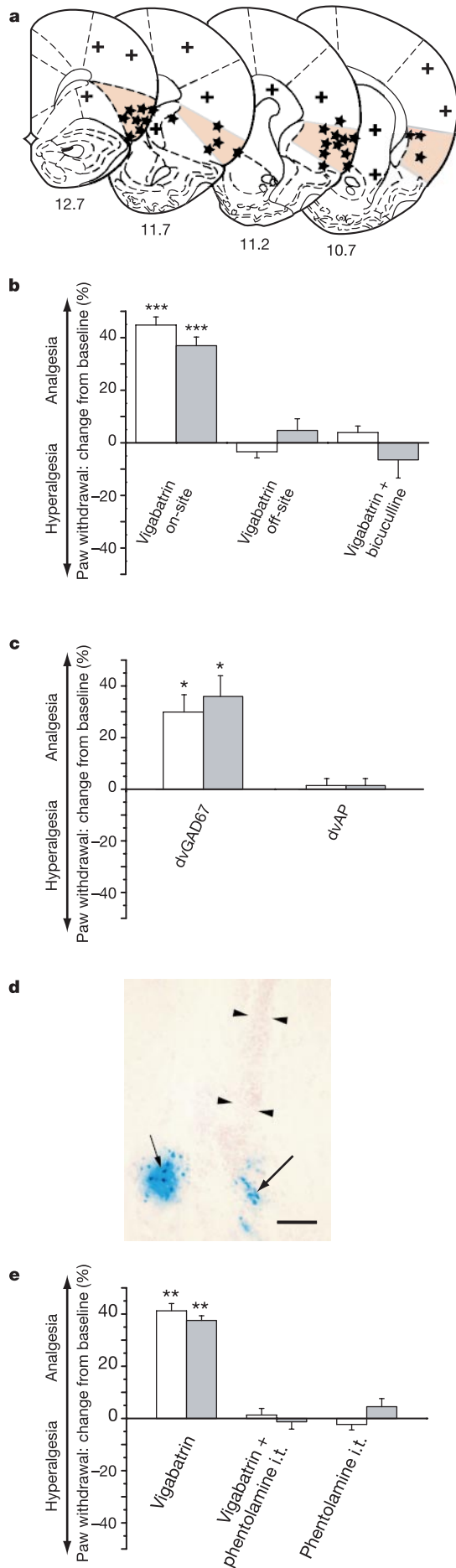


Fig. 2a for a dose–response curve) was microinjected into the RAIC 2 h after vigabatrin or 2–3 days after injection of dvGAD-67; the nociceptive threshold was then assessed. Rats that had become analgesic from vigabatrin or GAD-67 gene expression developed hyperalgesia, principally ipsilateral, immediately after the injection of saclofen (Fig. 3a, b). Fos immunocytochemistry confirmed increased activity in the amygdala (Figs 2h, i and 3c) associated with disinhibition of the GABA<sub>B</sub> cortical projection cells and the resulting hyperalgesia. Injection of saclofen alone into the RAIC did not alter the nociceptive threshold (Fig. 3b), indicating that the hyperalgesic effect of disinhibiting GABA<sub>B</sub>-receptor-bearing neurons in the RAIC might normally be opposed by other, unknown, neurons bearing GABA<sub>A</sub> receptors only.

We then determined that projections from the RAIC to the amygdala, rather than to the caudal brainstem, have a key function in the hyperalgesic state. Bupivacaine (0.25%, 200 nl) injected into the basolateral nucleus of the amygdala after injections of vigabatrin and then saclofen into the RAIC, completely abolished the hyperalgesia, and the nociceptive threshold reverted to the same analgesic state obtained after the injection of vigabatrin alone (Fig. 3a). Ensuing intrathecal injection of phentolamine (3 nmol in 10 µl) returned the nociceptive threshold from analgesic to normal (Fig. 3a). In contrast, injection of bupivacaine in the locus coeruleus did not alter RAIC induced hyperalgesia (Supplementary Fig. 2b), and blocking the amygdala with bupivacaine had no effect on the analgesia induced by vigabatrin in the RAIC (Fig. 3b).

The RAIC therefore acts on at least two independent subcortical systems to modulate the nociceptive threshold (Fig. 4). The first, from the RAIC to the locus coeruleus, influences the noradrenergic bulbo-spinal projections and is modulated predominantly by GABA<sub>A</sub> receptors. The second, from the RAIC to the amygdala, is modulated predominantly by GABA<sub>B</sub> receptors. Because GABA<sub>B</sub> receptors are metabotropic, their function is probably to keep cortical afferents to the amygdala under prolonged inhibition. However, the effect of GABA<sub>B</sub> receptor stimulation is conditional on a number of factors such as the resting potential and concentrations of intracellular chloride<sup>14</sup>, factors that significantly influence the outcome of GABA stimulation for cells expressing both types of receptor.

As the nociceptive threshold is reset by blocking RAIC activity with bupivacaine or by locally increasing the GABA concentration, this indicates that both of the above systems might be tonically active. However, tonic GABA release seems to contribute only moderately to the baseline activity of these projections because local injection of bicuculline alone did not significantly alter the nociceptive threshold (Supplementary Fig. 2c). This would imply that a lack of cortical GABA is unlikely to account for states of

**Figure 1** Cortical injection sites and nociceptive heat paw-withdrawal responses.

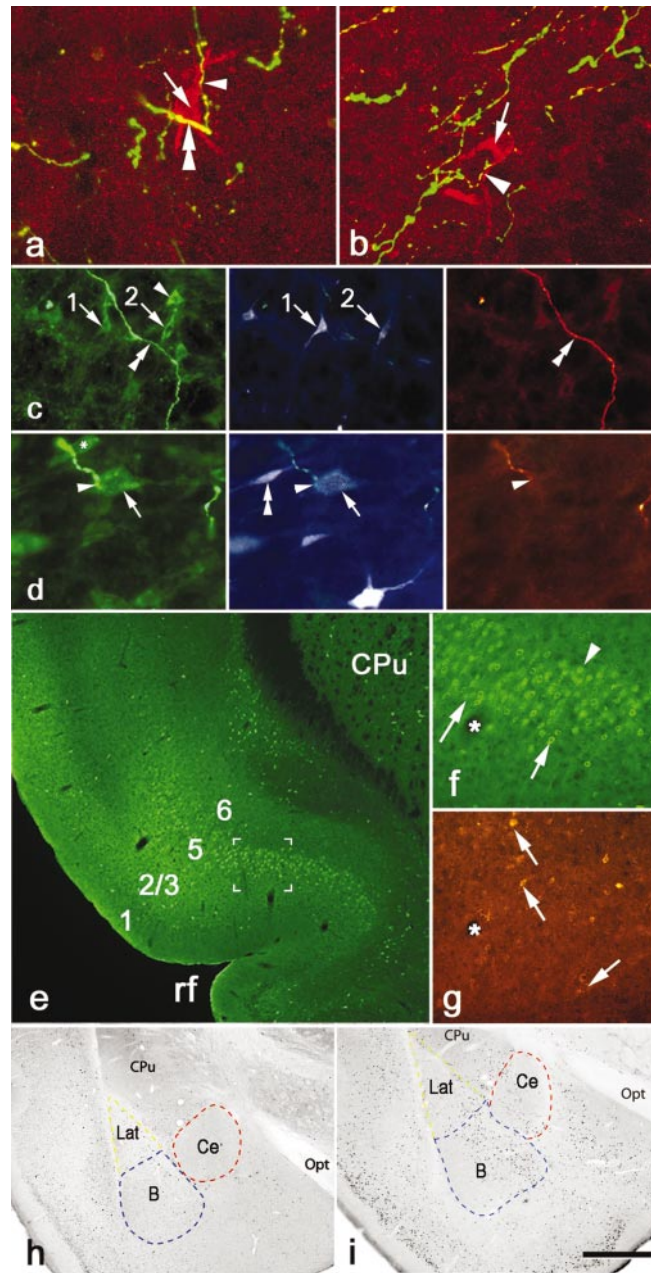
**a**, Drawings showing the location of the RAIC (shaded area) and representative on-site (asterisks) and off-site (plus signs) injections. Numbers represent rostro-caudal coordinates. **b**, On-site injection of vigabatrin produced a bilateral increase in withdrawal latency, whereas off-site injections had no effect. There was no statistical difference between left (white bars) and right (grey bars) paws. Injection of bicuculline restored the nociceptive threshold to normal. **c**, Injection of GAD-67-expressing vector (dvGAD-67;  $n = 6$ ) produced bilateral analgesia, whereas injection of the control vector expressing alkaline phosphatase (dvAP) produced no behavioural effect. White bars, left paw; grey bars, right paw. **d**,  $\beta$ -Galactosidase-stained section of dvGAD-67-injected RAIC, showing infected cells (arrows) adjacent to the cannula tract (arrowheads). **e**, Analgesia induced by vigabatrin in the RAIC was reversed by intrathecal (i.t.) phentolamine. Phentolamine alone had no effect. One asterisk,  $P < 0.05$ ; two asterisks,  $P < 0.01$ ; three asterisks,  $P < 0.001$ . Scale bar in **d**, 100 µm.

## letters to nature

hyperalgesia. In fact, a dysfunction of the noradrenergic system, which is a target of the RAIC, probably has a minor function in pain related to chronic inflammation or neuropathic injury, because the complete removal of this system does not, in the long term, alter the nociceptive threshold<sup>15</sup>. The direct projections from the RAIC to the caudal brainstem serotonergic cell groups could mediate

some of the pain-modulatory effects of the RAIC, including the hyperalgesia<sup>16</sup>.

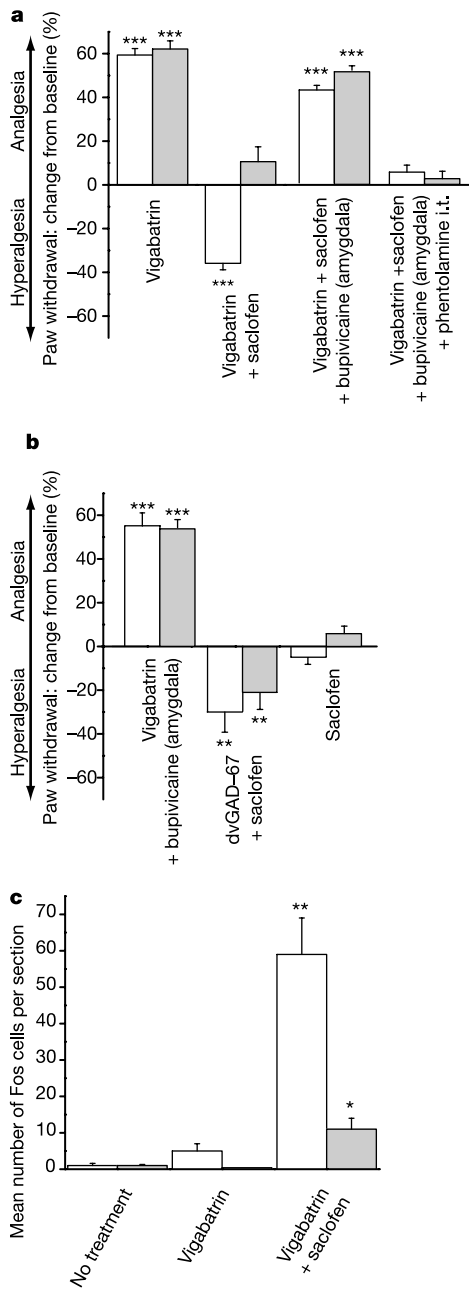
Our data indicate that if the RAIC is implicated in chronic pain it is through projections to the amygdala, the latter being part of a pro-nociceptive (pain-facilitating) circuit<sup>17</sup>. The preferential ipsilateral hyperalgesia after the injection of vigabatrin followed by



**Figure 2** Immunocytochemistry of the RAIC, amygdala and brainstem. **a, b**, Confocal images of GAD-67-immunopositive cells (red, arrows) near the locus coeruleus (**a**) and parabrachial nucleus (**b**) in apposition to labelled axons (green, arrowheads) after injection of biotin–dextran (BDA) into the RAIC. **c**, Nucleus raphe magnus GABAergic neurons (left, arrows 1 and 2) double-labelled with Fluoro-Gold (middle, arrows 1 and 2) from locus coeruleus injection, in apposition to a BDA-labelled fibre from the RAIC (left and right, double arrowhead). Single arrowhead, GABA-only-labelled neuron. **d**, High magnification of a nucleus raphe magnus GABA cell (left, arrow) double-labelled with Fluoro-Gold (middle, arrow) from the locus coeruleus in apposition to puncta from a BDA-labelled fibre (right, arrowhead) from the RAIC. The BDA fibre is also labelled green (left) and is seen faintly as bleed-through fluorescence in the middle panel (arrowhead). Asterisk in left panel, single-labelled GABA-immunoreactive neuron; double arrowhead in

middle panel, single-labelled Fluoro-Gold neuron. **e**, GABA<sub>B</sub>-immunopositive pyramidal neurons in lamina 5 of the RAIC. 1–6, cortical layers; rf, rhinal fissure. **f**, GABA<sub>B</sub>-immunostained neurons from area in **e** indicated by corners. Outer border (arrows) and inner border (arrowhead) of lamina 5 are indicated. **g**, GABA<sub>A</sub>-receptor immunostaining of the same area as **f**. Arrows indicate labelled cell bodies, asterisk indicates the same blood vessel as in **f**. **h**, Fos immunocytochemistry of the amygdala after injection of vigabatrin into the RAIC. **i**, After disinhibition of GABA<sub>B</sub> neurons in the RAIC there was an increased number of Fos-immunopositive cells in the basolateral nucleus of the amygdala (B) and to a smaller degree in the central nucleus (Ce). CPu, caudate putamen; Lat, lateral nucleus of the amygdala; Opt, optic tract. Scale bar, 40 μm for **a, b**; 110 μm for **c**; 40 μm for **d**; 350 μm for **e**; 175 μm for **f, g**; 1.4 mm for **h, i**.

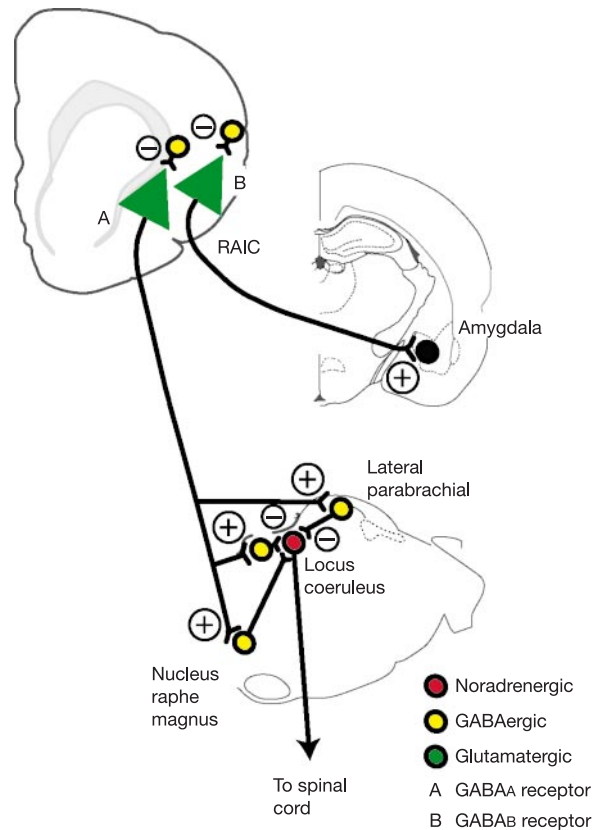
saclofen into the RAIC is not surprising, given previous reports that the amygdala affects morphine analgesia for the ipsilateral hemibody<sup>18</sup>. The present results indicate that the amygdala can enhance nociceptive responses in addition to its role in opiate analgesia<sup>18–20</sup>



**Figure 3** Nociceptive threshold and Fos immunoreactivity after drug treatment. **a**, Changes in nociceptive heat paw-withdrawal responses after the successive injection of four drugs. Each set of bars shows the behavioural response after the injection of the additional drug noted. Vigabatrin and saclofen were injected into the RAIC and the other drugs into the locations indicated. White bars, left paw; grey bars, right paw. **b**, Bupivacaine in the amygdala had no effect on the analgesia produced by vigabatrin alone in the RAIC. Hyperalgesia was also produced by injection of saclofen into the RAIC 2 days after injection of the GAD-67-expressing viral vector dvGAD-67. Injection of saclofen alone into the RAIC did not alter the nociceptive threshold. White bars, left paw; grey bars, right paw. **c**, Number of Fos-immunoreactive cells in the amygdala. White bars, basolateral nucleus; grey bars, central nucleus. One asterisk,  $P < 0.05$ ; two asterisks,  $P < 0.01$ .

and learned fear<sup>21</sup>. Anaesthesia of the amygdala alone did not alter the nociceptive threshold (Supplementary Fig. 2d), confirming previous reports<sup>18,21</sup> and showing that the amygdala does not function independently as a ‘pain generator’ but seems to be controlled directly by cortical afferents. The amygdala is a nodal point where reflexive and learned responses to threatening stimuli are orchestrated. The basolateral nucleus, to which the RAIC has a major projection, is involved in instrumental responses or operant conditioning<sup>22,23</sup>. In the present protocol, animals were conditioned to interrupt an applied heat stimulus by lifting their paw, a characteristic instrumental response. Rats did not display any signs of stress, such as defensive posture or analgesia encountered in fear reactions, associated with activation of the central nucleus of the amygdala<sup>23</sup>. Of the many brain areas to which the basolateral nucleus projects<sup>24</sup>, the nucleus accumbens is the most likely to initiate a behavioural response to aversive environmental cues<sup>25,26</sup>. Interestingly, the RAIC has a direct projection to the nucleus accumbens<sup>27,28</sup>, indicating that interactions between direct and indirect cortical afferents might occur there to modulate nociceptive responses.

Thus, the cerebral cortex modulates pain by acting on both pro-nociceptive and antinociceptive circuits. This dual effect is probably



**Figure 4** Summary of the main findings of the present study. From the RAIC, at least two independent projections modulate the nociceptive threshold, one to the amygdala and the other to the GABAergic neurons projecting to noradrenergic bulbo-spinal neurons. Both projections are modulated by local cortical GABAergic neurons. GABA<sub>A</sub> receptor stimulation (A) predominantly blocks the efferents to the peri-coerulear area, whereas GABA<sub>B</sub> receptor stimulation (B) blocks mainly the efferents to the amygdala. At least part of the projection from the RAIC to the peri-coerulear region terminates on GABAergic interneurons, which in turn are presumed to project to noradrenergic neurons in the locus coeruleus.

a defining feature of endogenous pain modulation<sup>17,21</sup>, and we speculate that an imbalance in the cortical output is likely to underlie some chronic pain states. □

## Methods

### Experimental animals

We used 252 male Sprague–Dawley rats (270–320 g; Bantin–Kingman). Procedures for the maintenance and use of the experimental animals conformed to the regulations of the UCSF Committee on Animal Research.

### Implantation of intracerebral cannulae

A stainless steel guide cannula (26-gauge, Plastics One) was cemented over a burrhole drilled over the RAIC (anterior–posterior (AP) 11.0, lateral (lat) 3.5 mm), and/or the amygdala (AP 6.20, lat 4.8 mm), and/or the locus coeruleus (AP –0.80, lat 1.2 mm), with interaural zero as the reference point. The guide cannula did not extend below the bone, to avoid any damage to the brain. Cortical injections were unilateral because stimulation of the RAIC with drugs was previously shown to produce bilateral effects<sup>4</sup>. On the day of testing (10 days after surgery), a 33-gauge bevelled injection cannula was inserted through the guide cannula to a distance 5.8, 8.0 or 6.0 mm below the cortical surface for the RAIC, amygdala or locus coeruleus, respectively. Drugs and viral vectors were injected over a 1-min period with a microinfusion pump.

### Intracerebral injections of tracers

Fluoro–Gold (4%; 10–40 nl) in double-distilled water or 10% biotin–dextran (Molecular Probes) in PBS pH 7.4 was injected through a micropipette (40- $\mu$ m tip). A period of 2–4 days was allowed for tracer transport.

### Nociceptive testing

A treatment-blind researcher conducted the behavioural experiments. Hindpaw responses to radiant heat were measured with a commercial heat paw-withdrawal device (Plantar Analgesia Instrument; Ugo Basile, Comerio, Italy) in accordance with a standard protocol<sup>15</sup>.

### Open-field test

The open-field test for motor and behavioural impairment was performed in accordance with a standard protocol<sup>15</sup>.

### Histology

Aldehyde-fixed brains were cut transversely (40  $\mu$ m slices) and kept in rostro-caudal order. Intracerebral drug injection sites were mapped on serial Nissl-stained sections. Histochemistry for  $\beta$ -galactosidase and acid phosphatase<sup>29</sup>, and immunocytochemistry, were performed in accordance with standard protocols. Guinea-pig anti-GABA<sub>A</sub> antiserum was provided by Dr Margeta-Mitrovic (Departments of Physiology and Biochemistry, University of California, San Francisco)<sup>30</sup>, and rabbit anti-*fos* antiserum was provided by Dr. D. Slamon (Department of Medicine, University of California, Los Angeles). Rabbit anti-GAD-67 (Ab5862) antiserum was purchased from Chemicon, and mouse monoclonal anti-GABAA (05-474) from Upstate Biotechnology.

### Stereology and analysis of immunolabelled cells

Counts of cells per unit area of the cortex or amygdala were performed with an unbiased counting method with the use of a computer-aided system (Stereo Investigator; MicroBrightField).

### Drugs

Vigabatrin, saclofen (Tocris Cookson), phentolamine (Sigma–Aldrich) and bupivacaine (AstraZeneca) were dissolved in 0.9% saline.

### Viral vectors

Double-cassette-defective HSV vectors (dvGAD-67 and dvAP) were generated from amplicon plasmids, pHCL–CGAD-67 encoding rat GAD-67 (obtained from A. Tobin, University of California, Los Angeles) and *Escherichia coli* LacZ, and pHCL–CAP encoding *E. coli* LacZ and human acid phosphatase<sup>29</sup>, with HSV-1 tsK as helper virus<sup>8</sup>. The titre of the defective vectors (in defective particle units) was determined by histochemistry with 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside. *In vitro*, infection of cultured cerebellar granule cells (CGCs)—predominantly glutamatergic—of cultured astrocytes resulted in both the expression of GAD-67 and the synthesis of GABA. GABA is released tonically from dvGAD-67-infected astrocytes and in a stimulus-evoked fashion from CGCs<sup>8,9</sup>. Expression from these vectors, with the CMV<sub>IE</sub> promoter/enhancer, continues for about 7–10 days *in vivo*. Control rats were injected with a defective HSV vector expressing alkaline phosphatase and LacZ.

### Statistical analysis

By using the outcome variable in each experiment, comparisons of treatment groups were made with Student's *t*-test or analysis of variance;  $P < 0.05$  was considered statistically significant. Post-hoc analysis of means and s.e.m. with Scheffé's *F*-test confirmed statistical significance.

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**Supplementary Information** accompanies the paper on [www.nature.com/nature](http://www.nature.com/nature).

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**Competing interests statement** The authors declare that they have no competing financial interests.

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