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Antisense Ablation of Type I Metabotropic Glutamate Receptor mGluR<sub>1</sub> Inhibits Spinal Nociceptive Transmission

Marie R. Young,1 Gordon Blackburn-Munro,1 Tracey Dickinson,1 Melanie J. Johnson,2 Heather Anderson,1 Immaculate Nakalembe,1 and Susan M. Fleetwood-Walker1

1Department of Preclinical Veterinary Sciences, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Summerhall, Edinburgh EH9 1QH, United Kingdom, and 2Medical Research Council Brain Metabolism Unit, Edinburgh EH8 9JZ, United Kingdom

Electrophysiological and behavioral studies point to a role of group I metabotropic glutamate receptors (mGluR<sub>1</sub> and mGluR<sub>5</sub>) in mediating spinal nociceptive responses in rats. However, antagonists with a high degree of specificity for each of these sites are not yet available. We, therefore, examined the effects of antisense deletion of spinal mGluR<sub>1</sub> expression in assays of behavioral analgesia and of electrophysiological responses of dorsal horn neurons. Rats treated with an mGluR<sub>1</sub> antisense oligonucleotide reagent, delivered continuously to the intrathecal space of the lumbar spinal cord, developed marked analgesia as measured by an increase in the latency to tail-flick (55°C) over a period of 4–7 d. This correlated with a selective reduction in mGluR<sub>1</sub>, but not mGluR<sub>5</sub>, immunoreactivity in the superficial dorsal horn compared with untreated control rats, in parallel with a significant reduction in the proportion of neurons activated by the mGluR group I agonist 3,5-dihydroxyphenylglycine (DHPG), whereas the proportion of cells excited by the mGluR<sub>5</sub> agonist, trans-azetidine-2,4-dicarboxylic acid (t-ADA) remained unaffected. In contrast, rats treated with mGluR<sub>1</sub> sense or mismatch probes showed none of these changes compared with untreated, control rats. Furthermore, multiceptive dorsal horn neurons in mGluR<sub>1</sub> antisense-treated rats were strongly excited by innocuous stimuli to their peripheral receptive fields, but showed severe reductions in their sustained excitatory responses to the selective C-fiber activator mustard oil and in responses to DHPG.

**Key words:** metabotropic glutamate receptors; mGluR<sub>1</sub>; mGluR<sub>5</sub>; dorsal horn; nociception; antisense oligodeoxynucleotide probe

There is a large body of evidence supporting a role for the excitatory amino acid glutamate in mediating sensory information at the first central synapses in the dorsal horn of the spinal cord. Glutamate receptors in mammalian brain are classified into two functional groups: ionotrophic and metabotropic receptors (mGluRs), (Nakanishi, 1992). The metabotropic group of glutamate receptors, comprising eight receptors, is subdivided into three groups, according to their amino acid homology, pharmacological, and signal transduction profiles: group I (mGluRs<sub>1,5</sub>), group II (mGluRs<sub>2,3</sub>), and group III (mGluRs<sub>4,7,8</sub>) (Masu et al., 1991; Abe et al., 1992; Nakanishi, 1992; Pin and Duvoisin, 1995). The mGluRs, which couple via G-proteins to several signal transduction pathways, regulate neuronal excitability in the CNS by modulating a variety of ion channels (for review, see Saugstad et al., 1995). Many of the mGluRs have been shown to be present in the spinal cord (Shigemoto et al., 1992, 1993; Ohishi et al., 1993, 1995; Vidnáňszky et al., 1994; Annese et al., 1995; Romano et al., 1995; Boxall et al., 1996; Petralia et al., 1996; Valerio et al., 1996) where they appear to play a role in mediating nociceptive inputs in the dorsal horn of the spinal cord (Neugebauer et al., 1994; Young et al., 1994, 1995, 1997), as well as in the thalamus (Eaton et al., 1993). In particular (but not exclusively) group I mGluR<sub>1,5</sub> receptors have been implicated in nociceptive responses (Young et al., 1994, 1995, 1997; Fisher and Coderre, 1996a,b; Fundytus et al., 1998), and their actions may be mediated, at least in part, by protein kinase C (Young et al., 1995). This role appears to be more prominent after sustained noxious stimuli, such as intraplantar formalin and carrageenan-induced inflammation, where mGluR antagonists prolong the latency of behavioral nociceptive responses (Fisher and Coderre, 1996b; Young et al., 1997) or inhibit the sustained responses of dorsal horn neurons to mustard oil (Young et al., 1997). mGluR<sub>1,5</sub> agonists can increase the excitability of dorsal horn neurons (Morisset and Nagy, 1996) and facilitate responses to NMDA and AMPA receptor activation (both of which are likely to participate in processing sustained nociceptive inputs) (Bleakman et al., 1992; Cerne and Randic, 1992; Bond and Lodge, 1995; Jones and Headley, 1995). Similarly, administration of mGluR<sub>1,5</sub> agonists appears to interact with an NMDA (and/or AMPA) receptor-dependent mechanism to increase nociceptive behavioral responses to intradermal formalin injection and to noxious sensory stimuli (Coderre and Melzack, 1992; Meller et al., 1993, 1996; Fisher and Coderre, 1996b). In both electrophysiological and behavioral studies, agonists and antagonists with selectivity for mGluR<sub>1,5</sub> (as well as neutralizing antibodies) are effective (Young et al., 1995, 1997; Fisher and Coderre 1996a,b; Fundytus et al., 1998), and some of the agents used show partial selectivity for mGluR<sub>1</sub> over mGluR<sub>5</sub> (Hayashi et al., 1994; Sharp et al., 1994; Brabet et al., 1995; Kingston et al., 1995). Nevertheless, because the available pharmacological reagents are not wholly specific, we have taken the alternative strategy of antisense deletion of spinal...
mGluR, expression to gain an unequivocal assessment of its role in nociceptive processing.

MATERIALS AND METHODS

Animals and evaluation of analgesia. Adult male Wistar rats (weight 280–450 gm, Charles River, Kent, UK) were used. Measurements of tail-flick latency (using a modified Ugo Basile tail-flick unit made in house) of each rat (35–55°C to the base of the tail) were made for 3 d before, and recommencing 4 d after implantation of an indwelling intrathecal silicone cannula connected to an osmotic minipump for the continuous, quantitative administration of sense, antisense mGluR oligonucleotides (see below).

Oligonucleotide probes and implantation of osmotic minipump. The 21-base antisense oligodeoxynucleotides endcapped with phosphorothioate linkages (at the positions marked by *) were designed according to the primary sequence of the rat mGluR1 cDNA (Houamed et al., 1991; Masu et al., 1991). The sequence of the mGluR1 antisense probe was: 5’-GCGACGATTGTGCGAGA*T*-3’, targeted around the translation initiation site (nucleotides 11 to +10) and will clearly not differentiate between splice variants in the carboxyl tail region. The complementary mGluR1 sense probe used: 5’-T*T*CTTGGCCACAA-ATGGTCCGG*C*-3’, corresponds to the reverse order of nucleotides of the above. The mismatch probe was: 5’-T*T*CGGATCATTGG-GGGCAGC*A*-3’. None of the oligonucleotide probes shows internal complementarity (it is impossible among any other known sequences according to the GenBank Database. Custom synthesis, HPLC-purification, and gel filtration were performed by Oswel DNA Service (Southampton, UK), and probes were dissolved and aliquoted in sterile 0.9% saline, pH 7.4, to give 0.25 µg/µl final concentration for infusion, before being stored at −20°C until use.

For continuous infusion into the intrathecal lumbar spinal cord region, each rat was implanted with a minipump with cannula attachment, which was assembled the day before surgery. It consisted of an osmotic minipump [Alza Minipump, model 2001 (Palo Alto, CA); nominal infusion rate, 1 µl/hr] attached to two lengths of sterile cannulae: first, to the pump, a length of ~1 cm of vinyl cannula (internal diameter 0.76 mm, outer diameter 0.99 mm) and to this was fitted second, a length of ~6 cm of silicone cannula (internal diameter 0.64 mm, external diameter 1.20 mm) (Degania Silicone). The pump and cannulae were filled with one of the above solutions (or saline control) under sterile conditions, and then the cannulae were joined to the pump, avoiding air bubble formation, before being placed in sterile saline at 37°C overnight.

Surgery was performed under sterile conditions, with Sagatal (Rhone Merieux, Harlow, Essex, UK; 0.06 ml/100 gm, i.p.) anesthesia, followed by a maintenance level of halothane (Zeneca, Cheshire, UK). A minipump was placed intramuscularly at an interscapular site, and the caudally directed cannula was threaded through muscle close to the exposed region of the spinal column. A small area of muscle and vertebral bone was cleared from two dorsal thoracic segments (T10–T12), and the tip of the cannula was placed through a small incision, under the dura, at a dorsal spinal level 0.5 cm deep, with a pressure of ~45 nN, to within the region of the lumbar segments L3–L6. After resection of the wound, the rat was then kept for up to 7 d, and its behavior was monitored (see above) before it was either (1) used for an electrophysiologic recording experiment or (2) perfused and tissue taken for immunohistochemistry. A further group of rats was assessed behaviorally for 1 week subsequent to a 7 d infusion of antisense, to assess recovery. The position of the tip of the cannula with respect to the level of spinal segment was ascertained at the end of each experiment, and only data from animals with correct cannula placement (L3–L6) were used in analysis. No animal showing abnormal gait or paralysis during the 7 d period was included in the study.

Electrophysiological studies. To assess the physiological effects of the loss of receptor expression after mGluR, antisense treatment, neuronal responsiveness to ionophoretically applied mGluR agonists was investigated. Experiments were performed on 60 rats. Under initial halothane anesthesia, the jugular vein and trachea were cannulated. Intravenous a-chloralose (60 mg/kg) and urethane (1.2 mg/kg) with supplementary doses of a-chloralose (10 mg/ml) were given throughout the experiment as required. Core body temperature was maintained at 37–38°C by means of a thermostatically controlled water jacket. Animals inspired oxygen-enriched air. The animal was placed in a stereotaxic frame, and the thoracolumbar spinal column was supported using three pairs of swan-necked clamps. A laminectomy was performed at L2–L5, and agar (2% in saline at 37°C) was injected under the most rostral vertebra and over the exposed cord to increase mechanical stability. Above the recording region, a section of the now solidified agar was removed, the dura was removed, and liquid paraffin (37°C) was poured over the exposed cord. Extracellular recordings were made from single neurons in laminae III–V through the center barrel of a seven-barrelled glass microelectrode filled with 4 M NaCl (pH 4.0–4.5, tip diameter 4–5 µm, DC resistance 5–8 MΩ). The bandwidth of the recording amplifer was 1 Hz to 7 kHz. The following drugs were ionophoresed from the side barrels of the electrode: group I mGluR agonist: 3,5-dihydroxyphenylglycine (DHPG), 10 mM aqueous, pH 4.5; mGluR1 agonist: trans-azetidine-2,4-dicarboxylic acid (t-ADA), 10 mM aqueous, pH 8.0–8.5; and the AMPA receptor agonist: AMPA, 10 mM aqueous, pH 8.0–8.5. All compounds were obtained from Tocris Cookson, Bristol, UK. Retention currents of 10 nA were used to minimize drug leakage between tests. A remaining barrel contained 1 M NaCl, pH 4.0–4.5, for automatic current balancing, using a Neurophore BH2 ionophoresis system (Medical Systems, Great Neck, NY) and for current controls. The resistance of all side barrels was 20–30 MΩ.

Recordings were made from any multireceptive neuron encountered at a depth from the dorsal surface corresponding to laminae I–IV, as shown in previous studies using electrophoretic deposition of dye (Fleetwood-Walker et al., 1988, 1993). The cutaneous receptive fields of neurons were mapped by innocuous and noxious stimuli to the hindlimb. The use of a mechanized rotating fine brush to stimulate hair follicle (Aβ) afferents has been described previously (Fleetwood-Walker et al., 1985) and was qualitatively innocuous to human skin. Further characterization was performed using noxious radiant heat (30–48°C, rise time 5 sec, plate temperature for 10 sec) and noxious pinch. Approximately 90% of all the cells tested in normal animals also showed sustained responses to mechanical stimuli. The responses to ionophoresed agonists were then explored with drug ejection currents being increased in a stepwise manner, every minute in steps of 10 nA from 5 to 45 nA. The response of neurons to the chemical allogren mustard oil (allyl isothiocyanate; Sigma, Poole, UK; 7.5% in paraffin oil) was observed after being repeatedly applied to the receptive field area (normal) every 5 min until sustained activity was observed.

Statistical analysis of the proportion of cells activated by agonists in the different groups of rats (normal, antisense reagent-treated, or sense reagent-treated rats) was performed by Mann–Whitney U test.

Immunohistochemistry. To directly monitor the loss of receptor protein expression after 5 d of intrathecal treatment with the mGluR1 antisense oligonucleotide probe, immunohistochemical analysis of the lumbar spinal cord was performed. Animals taken for immunohistochemistry were deeply anesthetized with Sagatal (0.12 ml/100 gm, i.p.) and perfused transcardially with 0.1 M PBS (containing 3 mM sodium nitrite and 1000 U heparin, pH 7.4) before being perfused with 4% paraformaldehyde/0.1 M PBS. A laminctomy was then performed, and the spinal cord was removed, together with the brain, which were then post-fixed in the same solution for a further 4–5 hr, before being incubated in 25% buffered sucrose (straight 4°C) and then stored in cryoprotectant (25% glycerol and 20% glycerol, in 0.05 M PBS, pH 5.5) at −70°C. Transverse microtome sections (52 µm) were then cut from the frozen tissue, through lumbar segments L3–L6, and suitable sections of brain tissue were used as positive controls for the antibodies used. Tissue sections were stored in cryoprotectant at −20°C, until use. Sections were removed from the cryoprotectant as required, for processing for either mGluR1 or mGluR5 immunoreactivity. Unless otherwise stated, all solutions were made up in 0.1 M PBS, and all incubations were performed at room temperature with gentle agitation. In all steps involving antibodies, the tissue sections were washed twice, for 10 min each, with PBS between succeeding steps. Sections were incubated in 1% hydrogen peroxide (30 min; Sigma) to remove any endogenous peroxidase activity, followed by incubation in normal goat serum (1 hr) to block nonspecific binding. They were then incubated with polyclonal antipeptide antibodies raised to rat mGluR1 (1180–1199) (0.25 µg/ml, 48 hr, 4°C; Chemicon, Temecula, CA) or rat mGluR5 (1152–1175) (1 µg/ml, 48 hr, 4°C; Upstate Biotechnology, Lake Placid, NY) followed by biotinylated goat anti-rabbit IgG antibody (1: 200 in PBS, 1 hr; Vector Laboratories, Peterborough, UK). Sections were then incubated for 90 min with an avidin–biotin complex (Vectorstain Elite ABC kit, Vector Laboratories). A further wash with PBS was performed. Staining was visualized by diaminobenzidine tetrachloride (DAB; 0.2 mg/ml; Sigma) in the presence of 3% hydrogen peroxide (1 µg/ml) to enable visualization of the receptor protein precipitate. After a final wash in PBS, the sections were mounted onto poly-l-lysine-coated microscope slides, allowed to air dry.
Effects of mGluR1 sense, mismatch, and antisense oligonucleotide administration on behavioral nociceptive responses (tail-flick latency, in seconds, to noxious heat applied to the base of the tail). Statistically significant increases to tail-flick latency are indicated by * (p < 0.05; Mann–Whitney U test). Attenuation of behavioral nociceptive responses was observed 4–7 d after continuous infusion of the mGluR1 antisense oligonucleotide reagent (●) in saline to the lumbar spinal cord segments L3–L6, compared with saline-infused controls (○), sense (□), or mismatch (△) oligonucleotide-treated rats with similarly placed intrathecal cannulae. Values are means ± SEM (n = 6–14). Where error bars are not apparent, they fall within the dimensions of the symbol.

Figure 1. Effects of mGluR1 antisense, mismatch, and antisense oligonucleotide administration on behavioral nociceptive responses (tail-flick latency in seconds, to noxious heat applied to the base of the tail). Statistically significant increases to tail-flick latency are indicated by * (p < 0.05; Mann–Whitney U test). Attenuation of behavioral nociceptive responses was observed 4–7 d after continuous infusion of the mGluR1 antisense oligonucleotide reagent (●) in saline to the lumbar spinal cord segments L3–L6, compared with saline-infused controls (○), sense (□), or mismatch (△) oligonucleotide-treated rats with similarly placed intrathecal cannulae. Values are means ± SEM (n = 6–14). Where error bars are not apparent, they fall within the dimensions of the symbol.
Sustained activity caused by antisense (n-Mismatch) Acute chemical nociception 30.7
Innocuous brush 17.2 controls; declining over the next 5 min. This acute chemical nociceptive
shortly after application of mustard oil to the peripheral receptive
field (up to three applications, every 5 min). The average increase in ongoing activity of these
neurons after topical mustard oil application was from 0.3 ± 0.1 Hz before mustard oil to 10.1 ± 2.6 Hz
of sustained activity, measured as the mean over 10 sec taken 15–18 min after initial application (Fig. 2A).
Similarly, in the mGluR1 sense oligonucleotide-treated control animals (n = 5), seven of eight
multireceptive cells showed a sustained excitatory response, after three topical mustard oil applications [from 0.2 ±
1.0 Hz before mustard oil application to 7.4 ± 1.8 Hz sustained activity measured 15–18 min after initial
application (Fig. 2B)]. Six cells recorded from mismatch-treated animals (n = 6) (Fig. 2C) also showed marked
ongoing activation as in normals (0.1 ± 0.8 Hz before repeated mustard oil application and 7.3 ± 1.6 Hz
afterward). In contrast, in rats treated with the mGluR1 antisense oligonucleotide, which displayed greatly
attenuated behavioral nociceptive responses (n = 6), six of six dorsal horn multireceptive
cells showed no significant sustained response to repeated
application of mustard oil to their peripheral receptive
field (even with up to five applications); the average increase in
ongoing activity being from only 0.1 ± 0.1 Hz before mustard oil,
to only 0.5 ± 1.8 Hz (Fig. 2D). The sustained mustard oil-induced
firing in antisense oligonucleotide-treated animals was signifi-
cantly less than that in control or sense-treated animals (p < 0.05
by Mann–Whitney U test). All of the cells from which we re-
corded full sensory responses in normals, sense, and mismatch
animals also showed clear responses to DHPG. In the six cells
from antisense animals, from which we were able to gain ade-
quate records of both brush and some residual mustard oil
response, we found none that responded to DHPG.

### Table 1. Effect of mGluR1 antisense treatment on neuronal responses to DHPG and t-ADA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage of cells excited by agonist</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DHPG</td>
<td>t-ADA</td>
</tr>
<tr>
<td>Control (n = 13)</td>
<td>56 (25/45)</td>
<td>23 (6/26)*</td>
</tr>
<tr>
<td>Sense (n = 7)</td>
<td>53 (16/30)</td>
<td>21 (8/38)</td>
</tr>
<tr>
<td>Mismatch (n = 6)</td>
<td>55 (12/22)</td>
<td>27 (6/22)</td>
</tr>
<tr>
<td>Antisense (n = 7)</td>
<td>20 (13/64)**</td>
<td>25 (12/49)</td>
</tr>
</tbody>
</table>

Drugs were ionophoresed at 5–45 nA for 5 sec to 2 min. The numbers of cells responding, of those tested, are shown in parentheses, and the numbers of animals in each case are shown after the respective treatments. (*p < 0.05; **p < 0.01 compared to control response to DHPG in control, sense, and mismatch; χ² test).

### Table 2. Effects of sense, mismatch, and antisense mGluR1 oligonucleotide infusions on different sensory responses of dorsal horn neurons

<table>
<thead>
<tr>
<th>Stimulus-evoked response (Hz)</th>
<th>Normal</th>
<th>Sense reagent</th>
<th>Mismatch</th>
<th>Antisense reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stimulus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 8)</td>
<td>(n = 7)</td>
<td>(n = 6)</td>
<td>(n = 6)</td>
<td></td>
</tr>
<tr>
<td>Innocuous brush</td>
<td>17.2 ± 2.3</td>
<td>22.1 ± 5.7</td>
<td>14.5 ± 2.4</td>
<td>20.4 ± 5.6</td>
</tr>
<tr>
<td>Acute chemical nociception</td>
<td>30.7 ± 7.2</td>
<td>22.7 ± 7.7</td>
<td>20.9 ± 3.9</td>
<td>1.4 ± 0.5*</td>
</tr>
<tr>
<td>Sustained activity caused by</td>
<td>10.1 ± 2.6</td>
<td>7.4 ± 1.8</td>
<td>7.3 ± 1.6</td>
<td>0.5 ± 1.8*</td>
</tr>
<tr>
<td>repeated nociceptive stimuli</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The sensory responses monitored were the acute neuronal responses to innocuous
brush (mean taken over 10 sec) and the chemical algogen mustard oil applied to
the peripheral receptive field (mean taken over 10 sec, 20–40 sec after initial application),
as well as the sustained sensitized activity caused by repeated application of
mustard oil (mean taken over 10 sec, 15–18 min after the first three applications). Basal firing rates of neurons were all within the range 0–1.0 Hz and were no different
between treatments. Values are means ± SEM with numbers of neurons in parentheses. *Indicates significantly different from normal, sense, or mismatch reagent controls; p < 0.05, Mann–Whitney U test.

this, together with corresponding data from antisense, sense, and
mismatch-infused animals. A marked and significant reduction in
the proportion of cells responding to DHPG was seen after
antisense, but not the control reagents. The possibility that non-
equivalent samples of neurons may have been recorded in control
and antisense-treated animals cannot be excluded, but the pro-
portion of cells responding to t-ADA was not altered by any

treatment. Furthermore, ionophoretic application of AMPA
(10–40 nA) consistently caused very marked and rapid activation
of all cells tested, irrespective of whether they had been treated
with oligonucleotides or were untreated controls (data not shown).

Sensory responses

Although dorsal horn neurons displayed vigorous responses to
motor-driven innocuous brush, which was unaffected by mGluR1
sense, mismatch, or antisense oligonucleotide treatments (Table
2), the same neurons showed a greatly reduced ability to respond
to noxious chemical stimulation after the mGluR1 antisense oligonucleotide treatment.

In normal, untreated animals, neuronal activity increased
shortly after application of mustard oil to the peripheral receptive
field, quickly reaching a peak (at 20–40 sec) and then slowly
decreasing over the next 5 min. This acute chemical nociceptive
response (calculated as the mean value over 10 sec, 20–40 sec
after initial application) was not significantly altered in sense or
mismatch oligonucleotide-treated animals compared with un-
treated controls, whereas responses from antisense
oligonucleotide-treated animals were markedly reduced (p <
0.05; Mann–Whitney U test; Table 2). These observations concur
with the changes in acute thermal nociceptive responses (tail-
flick) seen in the behavioral experiments. Mechanical nociceptive
responses were not investigated quantitatively in the present

study.

In normal, untreated animals (n = 8), eight of nine cells showed a
sustained and incremental excitatory response to repeated,
topical application of mustard oil to their peripheral receptive
field (up to three applications, every 5 min). The average increase in
ongoing activity of these neurons after topical mustard oil
application was from 0.3 ± 0.1 Hz before mustard oil to 10.1 ± 2.6 Hz
of sustained activity, measured as the mean over 10 sec taken
15–18 min after initial application (Fig. 2A). Similarly, in the
mGluR1 sense oligonucleotide-treated control animals (n = 5),
seven of eight multireceptive cells showed a sustained excitatory
response, after three topical mustard oil applications [from 0.2 ±
1.0 Hz before mustard oil application to 7.4 ± 1.8 Hz sustained
activity measured 15–18 min after initial application (Fig. 2B)].
Six cells recorded from mismatch-treated animals (n = 6) (Fig.
2C) also showed marked ongoing activation as in normals (0.1 ±
0.8 Hz before repeated mustard oil application and 7.3 ± 1.6 Hz
afterward). In contrast, in rats treated with the mGluR1 antisense
oligonucleotide, which displayed greatly attenuated behavioral
nociceptive responses (n = 6), six of six dorsal horn multireceptive
cells showed no significant sustained response to repeated

topical application of mustard oil to their peripheral receptive
field (even with up to five applications); the average increase in
ongoing activity being from only 0.1 ± 0.1 Hz before mustard oil,
to only 0.5 ± 1.8 Hz (Fig. 2D). The sustained mustard oil-induced
firing in antisense oligonucleotide-treated animals was signifi-
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corded full sensory responses in normals, sense, and mismatch
animals also showed clear responses to DHPG. In the six cells
from antisense animals, from which we were able to gain ade-
quate records of both brush and some residual mustard oil
response, we found none that responded to DHPG.

### Immunohistochemistry and immunoblotting for mGluR1 and mGluR5

The specificity of antibody labeling was verified by analyzing the
distribution of mGluR1 and mGluR5 immunoreactivity in the
CNS. In the cerebellar cortex, the molecular and Purkinje layers
were strongly immunoreactive for mGluR5, whereas the granular
layer was less intensely labeled. The mGluR5 immunoreactivity in
the hippocampus was highest in the molecular layer, whereas
the molecular layer was less intensely labeled. The mGluR5
immunoreactivity in the hippocampus was highest in the molecular
layer, whereas there were many unlabeled cell bodies in the
CA1 and CA3 fields. These observations are entirely consistent
with previously published observations (Shigemoto et al., 1992, 1993; Romano et al.,
1995).

In lumbar spinal cord of control untreated animals, neural
elements strongly labeled for mGluR1 were distributed in lami-
nae I and II of the dorsal horn, to a lesser extent in deeper dorsal
horn, and also in the ventral horn around motoneurons (Fig.
3A,F). This pattern of immunoreactivity was unchanged in ani-
mals treated with the mGluR1 sense or mismatch oligonucleotide
probe (Fig. 3B,C). In contrast, animals treated with the antisense
Figure 2. Ongoing neuronal activity records showing typical excitatory firing responses of dorsal horn neurons to innocuous brush stimuli (■), to ionophoresis of DHPG (□), and to noxious mustard oil stimulation (●). A shows untreated control, B shows mGluR1 sense oligonucleotide-treated, C shows mGluR1 mismatch-treated, and D shows mGluR1 antisense-treated animals. All animals were tested for tail-flick responses before electrophysiological recording and were found to conform to the pattern displayed in Figure 1.
Figure 3. Effects of mGluR1 sense, mismatch, and antisense infusion on mGluR1 and mGluR5 immunoreactivity in lumbar spinal cord. A–D show typical representations of mGluR1 immunoreactivity in dorsal horn in control (saline), sense, mismatch, and antisense reagent-treated animals. E shows the virtual lack of immunoreactivity in control dorsal horn when the mGluR1 antibody was preabsorbed with membranes from mGluR1-overexpressing cells. F and G show mGluR1 immunoreactivity in ventral horn in control and mGluR1 antisense-treated animals. These results are typical of at least five animals in each case. Scale bars, 1.0 mm. H shows immunoblots using mGluR1 and mGluR5 antibodies after gel electrophoresis of lysates from spinal cord segments L3–L6 of (1) control, (2) antisense, (3) sense, and (4) mismatch-treated animals. The running positions of the molecular weight markers are shown in kilodaltons. Results are typical of three separate experiments.
oligonucleotide for mGluR₁, which had displayed attenuated behavioral nociceptive responses, showed a marked decrease in the intensity and distribution in immunoreactive labeling for mGluR₁ in the superficial layers of the dorsal horn (Fig. 3D). These losses were routinely observed throughout the extent of L3–L6, after cannula placement at any point within this range, thus giving a minimal extent of the extent of reagent diffusion. Under the present experimental conditions, the immunoreactivity for mGluR₁ that was associated with motoneurons in the ventral horn did not appear to be altered in the mGluR₁ antisense oligonucleotide-treated rats (Fig. 3G). Antibody specificity was demonstrated by preabsorption with membranes from COS 7 cells overexpressing recombinant rat mGluR₁a (Fig. 3E). Table 3 shows quantification of the mGluR₁ immunostaining as gray scale intensity measured across different laminae of the superficial dorsal horn, demonstrating significant reductions in inner (and to a slightly lesser extent, outer) lamina II of antisense-treated animals compared with the other conditions. This loss was confirmed independently in Western blots from polyacrylamide gel electrophoresis of spinal cord lysates (Fig. 3F). These showed a single mGluR₁-immunoreactive band at ~160–175 kDa that was clearly depleted in samples from antisense-treated animals compared with others. In contrast, mGluR₅ immunoreactivity (which was also concentrated in the superficial dorsal horn) showed no differences in the pattern of staining in animals treated with sense, mismatch, or antisense mGluR₅ reagents compared with normals (Table 3). In addition, Western blots showed the main band of mGluR₅ immunoreactivity (at ~160–180 kDa) was unaltered by mGluR₁ antisense treatment (Fig. 3H).

**DISCUSSION**

Various lines of evidence point to a role of mGluRs in spinal somatosensory processing and reflex responses. It appears that mGluRs of group I, II, and III may all have some role to play. The group I/II mGluR agonist, 1S,3R-ACPD (1S,3R-1-amino-1,3-cyclopentane dicarboxylic acid) causes postsynaptic increases in the excitability of dorsal horn neurons (Morisset and Nagy, 1996), and it has been suggested that not only group I, but also group II, mGluRs may contribute to this effect (Bond and Lodge, 1995). In fact, synergistic effects of group I and group II receptor agonists on second messenger production have been described (Schoepp et al., 1996). Group III and group II receptors at presynaptic sites act to inhibit synaptic inputs to ventral horn neurons (Jane et al., 1996) and may also modulate inputs to dorsal horn neurons. Correspondingly, mGluR₅ (group III) sites have been identified in primary afferent terminals (Ohishi et al., 1995).

Nevertheless, the present data, together with the other available information from electrophysiological and behavioral studies, suggest strongly that group I mGluRs play a crucial role in physiological nociceptive inputs (Neugebauer et al., 1994; Young et al., 1994, 1995, 1997; Fisher and Coderre, 1996a,b; Fundytus et al., 1998). Specifically, our pharmacological and antisense ablation data suggest strongly that a key role is played by mGluR₁. This is consistent with our previous evidence from partially selective pharmacological agents that suggested that mGluR₁ may play the predominant role (Young et al., 1995, 1997). Both the acute responses to mustard oil (a C-fiber selective activator) and the incremental activity resulting from its repeated application were severely inhibited by mGluR₁ ablation (Fig. 2; Table 2), with relative preservation of responses to innocuous brush. It is not possible from the present results to say whether mGluR₁ ablation has any specific influence on the mechanism of wind-up (increased excitability) per se, because the necessary prerequisite of C-fiber inputs is itself abrogated by the antisense strategy. The behavioral studies (Fig. 1) and previous reports with partially selective mGluR₁ antagonists and mGluR₁/mGluR₅ antisera (Young et al., 1997; Fisher and Coderre, 1996a; Fundytus et al., 1998) are entirely consistent with these observations in showing that prevention of spinal mGluR₁ function leads to inhibition of behavioral nociceptive responses.

However, mGluR₅ sites are also present in ventral horn, on or around motoneurons (Anneser et al., 1995; Alvarez et al., 1997; Boxall et al., 1998). Thus, effects of mGluR agents on ventral root potentials evoked by dorsal root stimulation (Boxall et al., 1996) are likely to represent a composite of actions in dorsal and ventral horn. Group I mGluR agonists increase ventral root potentials elicited by ionotropic GluR agonists (Ugolini et al., 1997), whereas intracellular recordings from motoneurons suggest that both postsynaptic facilitatory and presynaptic inhibitory effects are brought about by group I/II mGluR agonists (King and Liu, 1996). Presynaptic effects of group III mGluR agonists are also

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**Table 3. Image analysis quantification of immunohistochemistry for mGluR₁ and mGluR₅ in dorsal horn of animals treated with mGluR₁ oligonucleotides**

<table>
<thead>
<tr>
<th>Gray scale intensity (arbitrary units) in excess of dorsal column control</th>
<th>Control</th>
<th>mGluR₁ sense</th>
<th>mGluR₁ mismatch</th>
<th>mGluR₁ antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>mGluR₁</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lamina I</td>
<td>96.5 ± 1.1</td>
<td>95.4 ± 2.0</td>
<td>94.8 ± 1.5</td>
<td>86.8 ± 2.9</td>
</tr>
<tr>
<td>Lamina II</td>
<td>98.6 ± 0.6</td>
<td>97.2 ± 1.6</td>
<td>95.3 ± 1.3</td>
<td>84.5 ± 3.4* *** ****</td>
</tr>
<tr>
<td>Lamina II</td>
<td>80.3 ± 2.7</td>
<td>70.5 ± 5.0</td>
<td>75.0 ± 1.7</td>
<td>53.1 ± 3.3*** *** ****</td>
</tr>
<tr>
<td>Lamina III</td>
<td>23.3 ± 3.0</td>
<td>20.3 ± 2.2</td>
<td>25.9 ± 2.2</td>
<td>16.5 ± 4.8</td>
</tr>
<tr>
<td>Ventral horn</td>
<td>26.3 ± 2.0</td>
<td>22.7 ± 1.8</td>
<td>28.0 ± 6.3</td>
<td>25.7 ± 1.0</td>
</tr>
<tr>
<td>mGluR₅</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lamina I</td>
<td>70.6 ± 2.8</td>
<td>82.3 ± 2.5</td>
<td>77.2 ± 2.5</td>
<td>80.4 ± 2.8</td>
</tr>
<tr>
<td>Lamina II</td>
<td>66.7 ± 2.8</td>
<td>73.5 ± 2.9</td>
<td>58.3 ± 3.8</td>
<td>71.8 ± 4.5</td>
</tr>
<tr>
<td>Lamina III</td>
<td>43.3 ± 3.3</td>
<td>47.4 ± 3.0</td>
<td>36.9 ± 2.8</td>
<td>43.9 ± 3.7</td>
</tr>
<tr>
<td>Lamina III</td>
<td>17.9 ± 2.5</td>
<td>13.7 ± 2.3</td>
<td>17.4 ± 3.0</td>
<td>13.8 ± 2.4</td>
</tr>
<tr>
<td>Lamina III</td>
<td>32.0 ± 1.3</td>
<td>28.5 ± 2.3</td>
<td>26.7 ± 2.7</td>
<td>30.4 ± 2.0</td>
</tr>
</tbody>
</table>

The statistically significant differences were determined by Mann–Whitney U test (n = 15–16). *p < 0.05, **p < 0.01 compared with control; ***p < 0.05 compared with sense; and ****p < 0.05, *****p < 0.01 compared with mismatch.
prominent in motoneuron recordings (Cao et al., 1997). It is clear, therefore, that ventral horn effects of mGluR agents may potentially contribute to, or at least modify, the effects of mGluR manipulations in behavioral analgesia experiments. However, increased latencies or thresholds to nociceptive stimuli were measured in behavioral responses to intrathecally applied mGluR group I antagonists in the absence of any overt signs of motor insufficiency (Fisher and Codere, 1996a; Young et al., 1997). Similarly, although mGluR<sub>1</sub> knock-out mice display a disruption of complex coordination behaviors that may arise from cerebellar dysfunction, they possess well maintained muscle strength and can organize effective goal-oriented swimming behaviors as well as normal animals (Conquet et al., 1994). In the present study, there was no evidence for any deficit in motor coordination, gait, or locomotor activity in the mGluR<sub>1</sub> intrathecal antisense-treated animals, corresponding to the lack of change in ventral horn mGluR<sub>1</sub> immunoreactivity (Fig. 3<sub>F,G</sub>) after dorsally directed infusion of oligonucleotide. So, although it is not possible to unequivocally exclude a contribution of ventral horn effects to the behavioral results (and reflex indices of function here not tested), it is clear that the effects of mGluR<sub>1</sub> ablation (focused in the dorsal horn; Fig. 3, Table 3), as defined in the electrophysiological experiments (Table 2, Fig. 2), would alone be quite sufficient to explain the behavioral changes that we observed (Fig. 1) and have similarly been described in mGluR<sub>1</sub> knock-out mice (Corsi et al., 1996).

In conclusion, the present results demonstrate that the localized antisense ablation of mGluR<sub>1</sub> in dorsal horn (without affecting the congener mGluR<sub>5</sub>) results in a selective abrogation of neuronal responses to noxious stimuli (and perhaps also sensitization) without equivalent changes in non-nociceptive responses. Correspondingly, reflex behavioral responses to noxious thermal stimuli are attenuated in rats with antisense deletion of mGluR<sub>1</sub> in lumbar dorsal horn, in the absence of any signs of generalized motor deficit.

REFERENCES


Morisset V, Nagy F (1996) Modulation of regenerative membrane prop-


