Available online at www.sciencedirect.com

SCIENCE DIRECT

European Journal of Pharmacology 506 (2005) 229–236

www.elsevier.com/locate/ejphar

Pharmacologically selective block of mu opioid antinociception by peptide nucleic acid antisense in absence of detectable ex vivo knockdown

Amynah A.A. Pradhan, Paul B.S. Clarke*

Department of Pharmacology and Therapeutics, McGill University, 3655 Prom. Sir William Osler Room 1325 Montreal, Quebec, Canada, H3G 1Y6

Received 1 July 2004; received in revised form 9 November 2004; accepted 12 November 2004
Available online 15 December 2004

Abstract

The goal of this study was to determine the neuroanatomical extent of mu opioid receptor knockdown in central nervous system (CNS) following intracerebroventricular (i.c.v.) administration of peptide nucleic acid antisense. Rats received subchronic i.c.v. injections of anti-mu opioid receptor antisense, mismatch or vehicle, and were tested for paw pressure latency following i.c.v. mu opioid receptor agonist ([D-Ala^2, N-Me-Phe^4, Gly-ol^5]-enkephalin; DAMGO) or delta opioid receptor agonist ((+)-4-[(*aR*)-*a*-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide; SNC80). The anti-mu opioid receptor antisense (but not mismatch) sequence abolished DAMGO-induced antinociception with no reduction in the delta opioid receptor-mediated response. In contrast, postmortem receptor autoradiographic analysis of CNS areas revealed no change in mu opioid receptor functional response ([^{35}S]GTPγS assay) or receptor labelling ([^{125}I]FK-33824 and mu opioid receptor immunoautoradiography). These results provide further evidence for antisense-induced knockdown at the behavioural level in the absence of clear changes at the tissue level.

Keywords: Opiate; Analgesia

1. Introduction

Peptide nucleic acids are synthetic deoxynucleotide analogs based on a pseudopeptide backbone (Nielsen et al., 1991). Their chemical properties confer several potential advantages for antisense applications. For example, peptide nucleic acid antisenses have high affinity for mRNA and poor tolerance for base mismatches (Dias et al., 1999; Larsen et al., 1999; Ray and Norden, 2000). Peptide nucleic acids are also highly resistant to nucleases and proteases (Demidov et al., 1994). The polyamide backbone of peptide nucleic acids is not only achiral but also charge neutral, minimizing interactions with proteins (Larsen et al., 1999), and at effective doses, peptide nucleic acids have not been associated with toxicity (Fraser et al., 2000a; Turner et al., 2003). An additional advantage is that peptide nucleic acids act independently of ribonuclease H, thereby avoiding nonspecific effects resulting from cleavage of nontarget mRNA (Stein, 2000).

As antisense agents, peptide nucleic acids have proven to be efficacious and target selective both in vitro (Aldrian-Herrada et al., 1998; Pooga et al., 1998; Cutrona et al., 2000; Turner et al., 2003) and in vivo (Tyler et al., 1998, 1999; Pooga et al., 1998; Fraser et al., 2000a; McMahon et al., 2001; Tyler-McMahon et al., 2001; Rezaei et al., 2001; Turner et al., 2003). Antisense effects have been reported in rodent brain and spinal cord, with evidence of CNS efficacy not only after central injection but even after systemic administration (Tyler et al., 1999; McMahon et al., 2001; Tyler-McMahon et al., 2001; McMahon et al., 2002; Turner et al., 2003).

The anatomical extent of protein knockdown following central peptide nucleic acid antisense administration is largely unknown (Tyler et al., 1998). To address this question in the present study, we used a peptide nucleic acid sequence that was previously shown to produce

* Corresponding author. Tel.: +1 514 398 3616; fax: +1 514 398 6690.
E-mail address: paul.clarke@mcgill.ca (P.B.S. Clarke).

0014-2999/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2004.11.018

profound behavioural effects, with a concomitant reduction in brain mu opioid receptor (~55%) in the PAG and hypothalamus (Tyler et al., 1998; McMahon et al., 2001). In the present study, mu opioid receptor protein knockdown was assessed not only by radioligand binding and immunohistochemistry but also by [^{35}S]GTPγS binding which is a potentially more sensitive measure (Fraser et al., 2000a). For greater anatomical resolution, mu opioid receptor abundance and function were assessed using tissue autoradiography.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (325–350 g; Charles River, St Constant, QC, Canada) were housed in groups of two in a temperature- and humidity-controlled animal colony lit from 7 a.m. to 7 p.m. Food and water were available ad libitum. All experiments were approved by the McGill University Animal Care Committee in accordance with the Canadian Council on Animal Care guidelines.

2.2. Surgery

Rats were anesthetised by intraperitoneal injection of ketamine/xylazine (80/16 mg/kg) solution (Bioniche, Belleville, ON, Canada and Novopharm, Toronto, ON, Canada) and placed in a stereotaxic device. Each animal was implanted with a 24-gauge guide cannula (Plastics One, Roanoke, VA, USA) extending into the right lateral ventricle of the brain (coordinates from bregma: AP, −0.8 mm; ML, 1.5 mm; DV, 4.1 mm) and fixed with dental cement. Rats were given dipyrone analgesic (100 mg/kg, Vétoquinol, Lavaltrie, QC, Canada) immediately following surgery. To prevent occlusion, guide cannulae were kept patent by stainless steel inserts which extended 0.5 mm beyond the cannulae tip. Rats were allowed 5–7 days to recover from surgery before random allocation into treatment groups.

2.3. Peptide nucleic acid antisense constructs

All peptide nucleic acid sequences were donated by Applied Biosystems (Framingham, MA). Peptide nucleic acid sequences were HPLC purified as TFA salts then converted to HCl salts by freeze drying from a dilute aqueous HCl solution. The completeness of the conversion was confirmed by ion exchange chromatography. The anti-mu opioid receptor peptide nucleic acid sequence (5′ -CAG CCT CTT CCT CT-3′) and the mismatch sequence (C**C**G C**A**T C**C**T C**T**T CT) were designed according to Tyler et al. (1998). Peptide nucleic acid sequences were reconstituted in a stock solution of sterile ddH_2O (1 mM) and stored at 4 °C. On each antisense treatment day, peptide nucleic acid antisense was diluted to 0.1 mM (1 nmol/10 μl) in Dulbecco's phosphate-buffered saline (DPBS; 0.5 mM $MgCl_2$, 2.7 mM KCl, 1.5 mM KH_2PO_4, 7.3 mM NaCl, 8.0 mM Na_2HPO_4), and the concentration was verified by determining the absorption of the solution at a wavelength of 260 nm. The following formula was used to quantify the peptide nucleic acid concentration: (A_{260}/extinction coefficient of the sequence)×dilution factor. The presence of soluble aggregates of peptide nucleic acid was also scanned for at 300 nm and was found to be negligible for all sequences used.

2.4. Intracerebroventricular injections

Antisense or vehicle (DPBS) was administered i.c.v. in daily bolus injections for 5 days. Antisense and opioid drugs were administered by the i.c.v. route to conscious rats through an indwelling 30-gauge injection cannula (Plastics One) connected via PE50 polyethylene tubing to a 100-μl Hamilton syringe. Solutions (10 μl) were injected over 1 min, and the injection cannula was left within the guide cannula for an additional 30 s.

2.5. Antinociceptive testing

Each rat was tested on only one occasion. The same investigator performed all antinociceptive testing. Acute mechanonociception was measured using an analgesy meter (Ugo Basile, Varese, Italy). Briefly, a rat was gently restrained by hand, and an increasing force was gradually applied to the right hind paw until the threshold force causing the rat to withdraw its paw was determined. A maximal cut-off force of 510 g was implemented for this study. Data are presented as percentage maximum possible effect (%MPE) calculated as follows: %MPE=[(response−baseline)/(cut-off−baseline)]×100%.

Animals were tested 18–20 h after the last antisense injection. In all experiments, baseline response thresholds were measured immediately before the administration of opioid agonist. The antinociceptive response to opioid agonists was measured at 15, 30, 45 and 60 min after drug treatment. ED80 doses of DAMGO (0.2 nmol) and SNC80 (400 nmol) were determined by Fraser et al. (2000b).

2.6. Preparation of tissue

Rats were decapitated 3 h after the hour-long test session, and the brains and spinal cords were rapidly removed, frozen in 2-methylbutane (−50 °C for 30 s) and stored at −40 °C. Brain and spinal cord sections were cryostat-cut at 20 μm. All sections were taken according to Paxinos and Watson (1997). Sections for the caudate putamen were cut between 10.7 and 7.7 mm above the interaural line. The thalamic and periaqueductal grey sections were taken between 6.44 and 4.2 and 3.2–1.2 mm above the interaural line, respectively. Brain stem sections were cut between 1.3

and 2.6 mm below the interaural line. Sections were thaw mounted onto gelatin-coated slides, air dried at room temperature for 10–15 min and vacuum dried with desiccant at 4 °C overnight. Slides were then stored at −40 °C until further use.

2.7. [^{35}S]GTPγS autoradiography

[^{35}S]GTPγS autoradiography was performed using a protocol modified from Hyytia et al. (1999). Sections were thawed at room temperature and rehydrated for 20 min in assay buffer containing 50 mM Tris–HCl, 5 mM $MgCl_2$, 100 mM NaCl and 1 mM EDTA (pH 7.4). Sections were then preincubated for 1 h with assay buffer plus 2 mM guanosine 5′ -diphosphate sodium salt (GDP; Sigma, St. Louis, MO, USA) and 1 μM 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, adenosine A(1) receptor antagonist, Sigma). The sections were incubated in plastic slide mailers for 1.5 h with assay buffer plus 2 mM GDP, 1 μM DPCPX, 1 mM dithiothreitol, 225 pM guanosine 5′ (γ-^{35}S-thio) triphosphate ([^{35}S]GTPγS, 1250 Ci/mmol, Perkin Elmer Life Science Products, Woodbridge, ON, Canada). Slide mailers were allocated to three incubation conditions: basal (i.e., no agonist present), agonist EC50 (with added mu opioid receptor agonist DAMGO 0.3 μM [Sigma]), agonist EC100 (10 μM DAMGO) and nonspecific (i.e., 10 μM unlabelled GTPγS [Sigma] with no agonist present). Sections were then rinsed in ice-cold buffer (50 mM Tris–HCl and 5 mM $MgCl_2$, pH 7.4, 2×5 min) and distilled water (2 s) then blow dried. Sections were exposed to X-ray film for 24 h in light-proof X-ray cassettes. Coexposure with [^{14}C] microscale autoradiographic standards (American Radiolabeled Chemicals, St. Louis, MO, USA) permitted quantification of the [^{35}S] radioisotope (Miller, 1991). The films were processed with D19 developer and GBX fixer (Kodak).

2.8. [^{125}I]FK-33824 autoradiography

[^{125}I]FK-33824 autoradiography was performed using a protocol modified from Fraser et al. (1999). [^{125}I]FK-33824 was donated by AstraZeneca R&D Montreal (specific activity 2200 Ci/mmol). Sections were thawed at room temperature and incubated at room temperature for 2 h in assay buffer comprising 50 mM Tris–HCl, 3 mM $MgCl_2$, 0.1% bovine serum albumin (pH 7.4) and a nonsaturating concentration of 0.03 nM [^{125}I]FK-33824. Nonspecific binding (NSB) was defined by the addition of the highly selective mu opioid receptor antagonist D–Phe–Cys–Tyr–D–Trp–Orn–Thr–Pen–Thr–NH_2 (CTOP, 1 μM; Tocris, Ellisville, MO, USA). Following incubation, sections were rinsed in ice-cold wash buffer (50 mM Tris–HCl, 3 mM $MgCl_2$; 3×5 min) and distilled water (2 s) then blow dried. Sections were exposed to Kodak X-OMAT AR X-ray film together with [^{125}I] microscale autoradiographic standards (Amersham Pharmacia Biotech, Piscataway, NY, USA) for 24 h in light-proof X-ray cassettes. The films were processed with D19 developer and GBX fixer (Kodak).

2.9. Immunoautoradiography

Immunoautoradiography of the mu opioid receptor was performed using a protocol modified from Grant and Clarke (2002). Sections were postfixed in an aqueous solution containing 6% paraformaldehyde, 20% absolute alcohol, 20% ethylene glycol, 10% glycerol and 0.32 M sucrose for 1 h at −20°C. After washing (2×5 then 1×30 min) in buffer (0.1 M phosphate buffer in 0.1 M NaCl (PBS)/0.3% Tween-20), sections were incubated in a blocking solution containing 30% skim milk powder (Carnation), 3% goat serum (Vector) and 0.05% NaN_3 for 2 h at room temperature. After washing with buffer (1×10 min), sections were incubated with rabbit polyclonal anti-mu opioid receptor antibody (1:5000; Neuromics Minneapolis, MN, USA) in 1.5% goat serum and 0.05% NaN_3 overnight at 4 °C. As a control, nonspecific binding was determined by incubating adjacent sections with 0.3 mM blocking peptide (NHQLEN-LEAETAPLP; Sheldon Biotech, McGill University, Montreal, QC, CANADA). After washing with buffer (1×5, 1×10, 1×30 min), the secondary antibody [^{125}I]-labelled goat antirabbit IgG (Perkin Elmer Life Science Products; specific activity 1200 Ci/mmol) was applied (8 pM) for 1 h at room temperature. This antibody was added to a solution containing 10% skim milk powder, 5% goat serum and 0.05% NaN_3. Sections were rinsed in ice-cold buffer (2×30 min), dipped briefly in distilled water then blow dried. Sections were exposed to Kodak X-OMAT AR X-ray film together with [^{125}I] microscale autoradiographic standards (Amersham Pharmacia Biotech) for 3 days in light-proof X-ray cassettes. The films were processed with D19 developer and GBX fixer (Kodak).

2.10. Quantitative image analysis

Film autoradiographs were quantified using an M4 MCID computer-based system (Imaging Research, St. Catherines, ON, Canada). Specific binding was determined by subtraction of nonspecific binding measured in adjacent sections. Agonist-stimulated [^{35}S]GTPγS binding was calculated by subtracting basal binding from agonist stimulated binding. Regions of interest were identified by reference to adjacent Nissl-stained sections.

2.11. Statistical analysis

Nonlinear regression analysis of concentration-response data (sigmoidal curve fit) was performed by GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA, http://www.graphpad.com). Multiple comparisons (i.e., *t*-tests with Bonferroni adjustment) and power analyses were performed using Systat v10.2 (SPSS, Chicago, IL, USA).

2.12. Overview of experiments

Four separate experiments were performed using different sets of animals. In each experiment, animals were pretreated with antisense or vehicle, tested behaviourally and sacrificed for in vitro analysis. Experiment 1 investigated target selectivity in vivo (i.e., delta opioid vs. mu opioid receptor-mediated antinociception), following which evidence of functional knockdown in vitro ([^{35}S]GTPγS assay) was sought in the caudate putamen and in brain areas that mediate mu opioid receptor antinociception. Experiment 2 tested whether behavioural knockdown was associated with changes in mu opioid receptor labelling ([^{125}I]FK-33824 and immunoautoradiography) in the brain. Experiment 3 investigated sequence selectivity in vivo (i.e., antisense vs. mismatch peptide nucleic acid). Experiment 4 tested for in vitro changes in the spinal cord that might account for the behavioural knockdown.

3. Results

3.1. Antisense abolished mu opioid receptor-mediated antinociception

In all four experiments, vehicle pretreated animals responded maximally or near maximally to the mu agonist DAMGO in the paw pressure assay. The peak drug effect occurred at 15 min post i.c.v. injection. In Experiments 1 and 3, pretreatment with anti-mu opioid receptor peptide nucleic acid antisense abolished this antinociceptive effect, as illustrated in Fig. 1. This effect was also seen in Experiments 2 and 4; thus, no DAMGO response was observed after antisense pretreatment (mean±SEM percent maximal possible effect, −1.6±4.1 and 3.3±5.8, respectively).

To test for target selectivity, antisense-pretreated rats were tested with the delta opioid receptor agonist SNC80 (Fig. 1A). The anti-mu opioid receptor antisense did not detectably reduce the response to this drug (P>0.2). In a test for sequence selectivity, pretreatment with a mismatch peptide nucleic acid sequence did not significantly alter the response to DAMGO (P>0.3, Fig. 1B). Lastly, pretreatment with antisense did not alter baseline antinociceptive responses (data not shown).

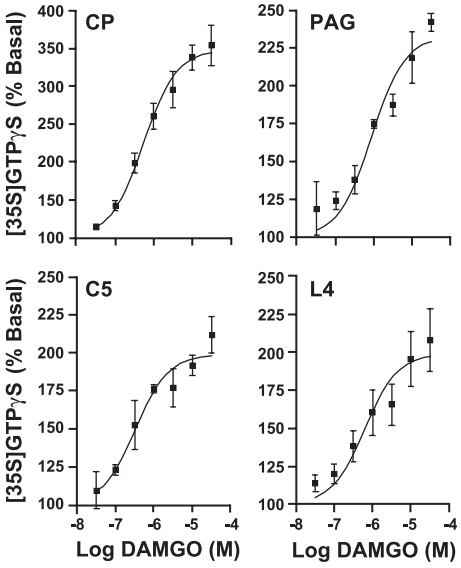

Fig. 2. Effect of DAMGO on [^{35}S]GTPγS binding in selected rat brain and spinal regions. DAMGO-stimulated binding in the caudate putamen (CP), periaqueductal grey (PAG), cervical segment 5 (C5) and lumbar segment 4 (L4). The y axis shows mean±S.E.M. specific [^{35}S]GTPγS binding expressed as a percentage of basal binding (i.e., absence of agonist; n=6–8 sections).

3.2. Anti-mu opioid receptor peptide nucleic acid antisense did not produce a detectable knockdown in CNS tissues

The functional response of the mu opioid receptor was determined in vitro using [^{35}S]GTPγS autoradiography. Based on an initial characterization (Fig. 2), an approximate EC50 and maximal concentration of DAMGO (0.3 and 10 μM) were selected for further testing in brain and spinal cord. The mu opioid receptor agonist DAMGO increased

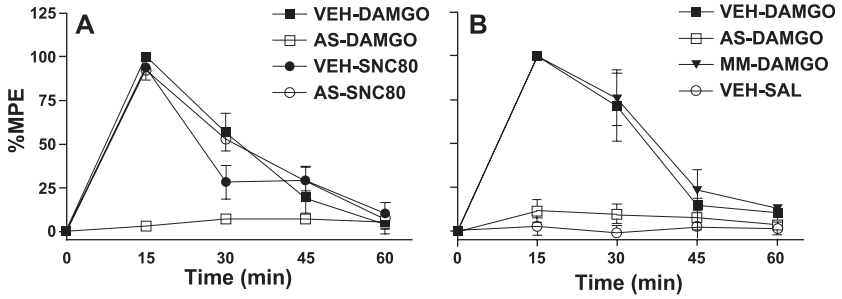

Fig. 1. Effect of antisense pretreatment on mu opioid receptor-mediated antinociception. Experiments 1 and 3 are depicted in panels (A and B), respectively. Depending on the experiment, rats were pretreated with antisense, mismatch or vehicle for 5 days followed by acute challenge with DAMGO (0.2 nmol), SNC80 (400 nmol) or saline. Peptide nucleic acid antisense targeting mu opioid receptors abolished the antinociceptive response to DAMGO (A and B). SNC80-induced antinociception was not affected by pretreatment with antisense (A). A three base pair mismatch sequence did not alter DAMGO-induced antinociception (B). The y axis represents the mean±S.E.M. response (n=4–8 rats/group) expressed as a percentage of the maximal possible antinociceptive effect. The x axis shows time relative to injection of challenge drug. Veh—vehicle; AS—peptide nucleic acid antisense; MM—peptide nucleic acid mismatch; sal—saline.

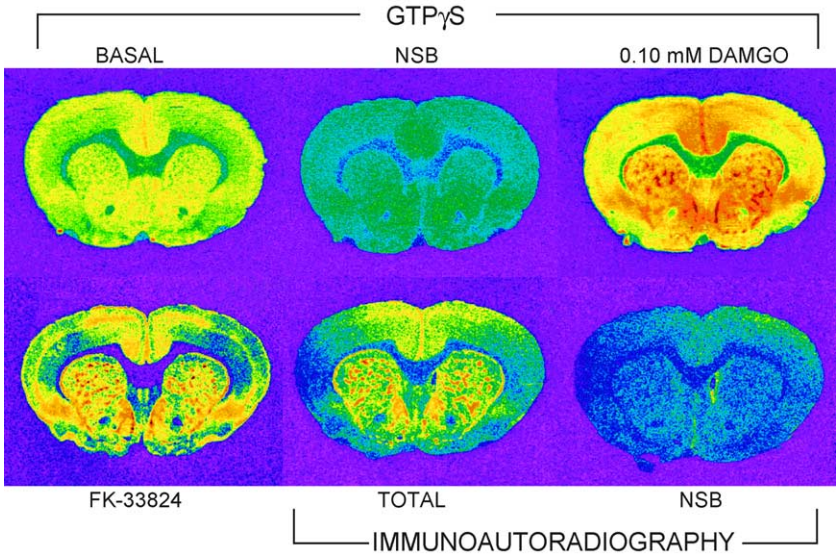

Fig. 3. Representative autoradiograms of DAMGO-stimulated [^{35}S]GTPγS binding, [^{125}I]FK-33824 and mu opioid receptor immunoautoradiography. In the [^{35}S]GTPγS assay, basal binding was determined in the absence of agonist, and nonspecific binding was determined in the presence of excess cold GTPγS. Nonspecific binding for [^{125}I]FK-33824 was determined by the addition of 1 μM CTOP and was virtually undetectable. Nonspecific binding for immunoautoradiography was determined by the addition of 0.3 mM of blocking peptide.

[^{35}S]GTPγS binding in mu opioid receptor-rich areas consistent with the pattern of conventional radioligand binding ([^{125}I]FK-33824) and immunoautoradiography (Fig. 3).

In all four experiments, the caudate putamen and the periaqueductal grey were assessed for in vitro changes in mu opioid receptor function. Antisense pretreatment produced no detectable change in DAMGO-induced [^{35}S]GTPγS binding in these regions, as represented in Fig. 4A and C. Subsequent analysis of other pain-related areas revealed no antisense effect, i.e., in thalamus, rostroventral medulla (Fig. 4B and D), cervical segment 5 and lumbar segment 4 regions (Fig. 4E and F).

Possible knockdown of brain mu opioid receptor abundance was determined using a mu opioid receptor-specific radioligand ([^{125}I]FK-33824). No change was detected after antisense pretreatment in the three areas assayed (i.e., caudate putamen, thalamus and periaqueductal grey, Table 1). Finally, no change in mu opioid receptor immunoautoradiographic labelling was detected (Table 1).

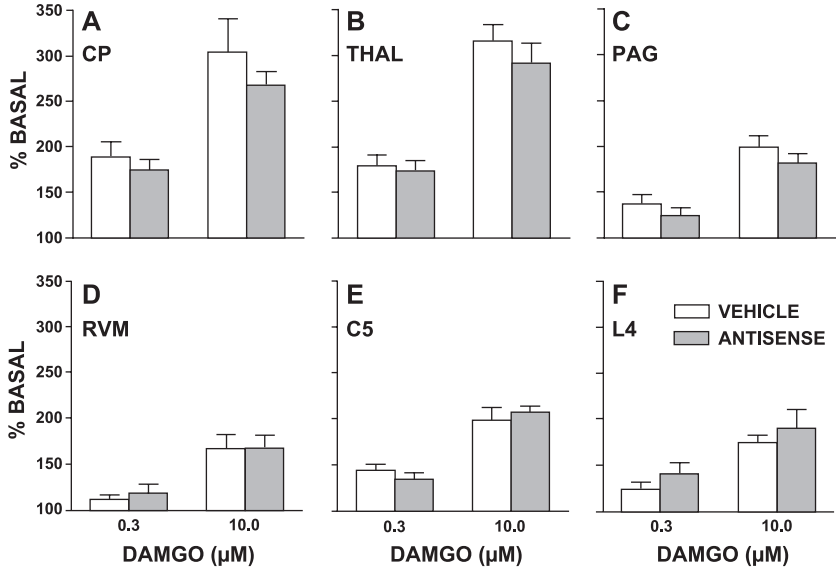

Fig. 4. Lack of antisense-induced knockdown of DAMGO-stimulated [^{35}S]GTPγS binding in rat CNS. Autoradiographic analysis of the response to EC50 (0.3 μM, open bars) and maximal (10 μM, dark bars) concentrations of DAMGO revealed no difference between antisense- vs. vehicle-pretreated rats in any area examined: (A) caudate putamen (CP), (B) thalamus (Thal), (C) periaqueductal grey (PAG), (D) rostroventral medulla (RVM), (E) cervical segment 5 (C5) or (F) lumbar segment 4 (L4). Panels (A–D) are derived from Experiment 1; panels (E–F) are from Experiment 4. The *y* axis shows mean±S.E.M. specific [^{35}S]GTPγS binding expressed as a percentage of basal binding (i.e., in the absence of agonist; n=4–8 rats/group).

Table 1
Autoradiographic labelling of mu opioid receptor with [^{125}I]FK-33824 and immunoautoradiography after vehicle or antisense pretreatment (mean±S.E.M.)

CNS area	[^{125}I]FK-33824		Immunoautoradiography	
	Vehicle	Antisense	Vehicle	Antisense
CP	1.27±0.11	1.44±0.18	0.05±0.02	0.08±0.03
Thalamus	1.14±0.12	1.07±0.14		
PAG	0.44±0.04	0.45±0.06	0.13±0.01	0.12±0.01

In each case, nonspecific binding (NSB) was subtracted from the total binding. Nonspecific binding for [^{125}I]FK-33824 and immunoautoradiography was defined by addition of 1 μM CTOP and 0.3 mM blocking peptide, respectively (n=6–8 rats/group).

Power analyses were performed on the most sensitive measures to determine the smallest detectable antisense effects. To this end, the effects of 10 μM DAMGO on [^{35}S]GTPγS binding were normalized (i.e., mean DAMGO effect of vehicle-pretreated group defined as 100%) and pooled across all four experiments. On this basis, we would have been able to detect a 23% or greater knockdown of the DAMGO response in the caudate putamen, whereas only a 3% reduction was actually observed. In the periaqueductal grey, a 17% knockdown in [^{125}I]FK-33824 binding would have been detectable, but instead, a 2% increase was observed in the antisense group.

4. Discussion

The main finding of the present study was the clear dissociation of behavioural and biochemical effects of peptide nucleic acid antisense targeted to mu opioid receptor. Thus, antisense treatment abolished DAMGO-induced antinociception with little or no detectable loss of mu opioid receptor protein or function in vitro.

It is likely that the behavioural knockdown represents a true antisense effect for the following reasons. First, the effect was sequence-dependent, as demonstrated by the mismatch control. Second, the peptide nucleic acid antisense effect appeared to be target selective in behavioural tests. Thus, we observed no knockdown of SNC80-induced antinociception, a response mediated by delta opioid receptor (Bilsky et al., 1995; Fraser et al., 2000b) and independent of mu opioid receptor (Fraser et al., 2000b). Sequence- and target-dependent effects have also been reported following intraparenchymal (Tyler et al., 1998) or systemic (McMahon et al., 2001) administration of the same sequence.

In trying to reconcile our negative in vitro findings with previous mu opioid receptor peptide nucleic acid studies, several procedural differences may be significant. For example, we administered antisense intracerebroventricularly, whereas in the earlier studies, it was administered either intraperitoneally or directly into brain tissue. We also gave the antisense daily, while in the previous studies, it was given less frequently (Tyler et al., 1998; McMahon et al., 2001). It is important to note that our animals were sacrificed within hours of behavioural testing to insure that the antisense effect was still present. It is unlikely that our assays were less sensitive than those used previously. Indeed, we assessed not only mu opioid receptor abundance (by radioligand binding and radioimmunohistochemistry) but also mu opioid receptor function using the [^{35}S]GTPγS assay which we previously found to be more sensitive to antisense treatment (Fraser et al., 2000a).

In view of the present mismatch between behavioural and biochemical responses, it is important to note that all of the CNS regions mediating mu opioid receptor antinociception were assayed in the present study. The caudate putamen was also examined because of its proximity to the site of antisense injection. All these areas are abundant in mu opioid receptor (Mansour et al., 1994, 1995), and therefore, the signals in these regions were large enough for changes to be detectable. Since both the peptide nucleic acid antisense and DAMGO were given intracerebroventricularly, changes in supraspinal mu opioid receptor were anticipated. However, no change was found in sites thought to mediate supraspinal antinociception by DAMGO (i.e., thalamus, PAG, and rostroventral medulla; Carr and Bak, 1988; Fang et al., 1989; Rossi et al., 1994). It is not known whether a significant concentration of peptide nucleic acid antisense would accumulate in the spinal cord after i.c.v. administration. Therefore, as a final check, spinal regions that might contribute to DAMGO-induced antinociception were assayed, with the same negative result.

The peptide nucleic acid antisense sequence used in this study is also complementary to several other rat transcripts (i.e., metabotropic glutamate receptor 6, ephrin B1 and succinate semialdehyde dehydrogenase), raising the possibility that our behavioural knockdown was not mu opioid receptor mediated. This possibility is unlikely for the following reasons. First, metabotropic glutamate receptor 6 is located only in the eye (Nomura et al., 1994). Second, ephrin B1 is known to decrease chronic inflammatory pain but is reported not to play a role in transmission of acute pain stimuli (Battaglia et al., 2003). Third, inhibition of succinate semialdehyde dehydrogenase expression would disrupt GABA metabolism and tend to produce a general behavioural disruption (Gupta et al., 2003). Importantly, the preservation of delta opioid receptor-mediated antinociception following peptide nucleic acid treatment renders all these possibilities unlikely.

It therefore appears that peptide nucleic acid antisense treatment suppressed expression of a behaviourally relevant mu opioid receptor population which was not detected in our in vitro assays. One possibility is that our antisense treatment differentially targeted splice variants of mu opioid receptor (Pasternak, 2001), but this is unlikely since our sequence targeted the 5' noncoding region of the mu opioid receptor transcript. A second possibility is that DAMGO-induced antinociception occurred via G-proteins that are not readily detected by the [^{35}S]GTPγS assay. However, it

remains to be explained why no knockdown of mu opioid receptor was observed in our receptor binding and immunohistochemical assays.

The present findings are reminiscent of our previous results using a peptide nucleic acid antisense sequence targeting delta opioid receptor (Fraser et al., 2000a). In the latter study, a marked inhibition of delta opioid receptor-mediated behavioural effects occurred, with only a small (25%) inhibition of delta opioid receptor-mediated [^{35}S]GTPγS response and no significant reduction in [^{3}H]-naltrindole binding in whole brain homogenates (Fraser et al., 2000a). A large discrepancy between behavioural knockdown and in vitro G-protein coupled receptor (GPCR) expression has also been reported for phosphodiester and phosphorothioate oligodeoxynucleotides (Weiss et al., 1993; Qin et al., 1995; Shah et al., 1997).

Such discrepancies may be especially surprising given that GPCRs, including the mu opioid receptor (Sora et al., 2001), are commonly associated with a receptor reserve (i.e., "spare receptors"). Hence, it has been proposed that newly synthesized receptors are especially susceptible to antisense treatment and contribute disproportionately to in vivo pharmacological responses (Qin et al., 1995; Hua et al., 1998; Van Oekelen et al., 2003). Consistent with this notion, our results show a clear and selective behavioural knockdown in the absence of readily detectable in vitro changes.

Acknowledgements

This work was supported by the Canadian Institutes of Health Research. We thank Applied Biosystems for their generous donation of all peptide nucleic acid sequences and to AstraZeneca R&D Montreal for the radioligand [^{125}I]FK-33824.

References

Aldrian-Herrada, G., Desarmenien, M.G., Orcel, H., Boissin-Agasse, L., Mery, J., Brugidou, J., Rabie, A., 1998. A peptide nucleic acid (PNA) is more rapidly internalized in cultured neurons when coupled to a retro-inverso delivery peptide. The antisense activity depresses the target mRNA and protein in magnocellular oxytocin neurons. Nucleic Acids Res. 26, 4910–4916.

Battaglia, A.A., Sehayek, K., Grist, J., McMahon, S.B., Gavazzi, I., 2003. EphB receptors and ephrin-B ligands regulate spinal sensory connectivity and modulate pain processing. Nat. Neurosci. 6, 339–340.

Bilsky, E.J., Calderon, S.N., Wang, T., Bernstein, R.N., Davis, P., Hruby, V.J., McNutt, R.W., Rothman, R.B., Rice, K.C., Porreca, F., 1995. SNC 80, a selective, nonpeptidic and systemically active opioid delta agonist. J. Pharmacol. Exp. Ther. 273, 359–366.

Carr, K.D., Bak, T.H., 1988. Medial thalamic injection of opioid agonists: mu-agonist increases while kappa-agonist decreases stimulus thresholds for pain and reward. Brain Res. 441, 173–184.

Cutrona, G., Carpaneto, E.M., Ulivi, M., Roncella, S., Landt, O., Ferrarini, M., Boffa, L.C., 2000. Effects in live cells of a c-myc anti-gene PNA linked to a nuclear localization signal. Nat. Biotechnol. 18, 300–303.

Demidov, V.V., Potaman, V.N., Frank-Kamenetskii, M.D., Egholm, M., Buchard, O., Sonnichsen, S.H., Nielsen, P.E., 1994. Stability of peptide nucleic acids in human serum and cellular extracts. Biochem. Pharmacol. 48, 1310–1313.

Dias, N., Dheur, S., Nielsen, P.E., Gryaznov, S., Van Aerschot, A., Herdewijn, P., Helene, C., Saison-Behmoaras, T.E., 1999. Antisense PNA tridecamers targeted to the coding region of Ha-ras mRNA arrest polypeptide chain elongation. J. Mol. Biol. 294, 403–416.

Fang, F.G., Haws, C.M., Drasner, K., Williamson, A., Fields, H.L., 1989. Opioid peptides (DAGO-enkephalin, dynorphin A(1–13), BAM 22P) microinjected into the rat brainstem: comparison of their antinociceptive effect and their effect on neuronal firing in the rostral ventromedial medulla. Brain Res. 501, 116–128.

Fraser, G.L., Labarre, M., Godbout, C., Butterworth, J., Clarke, P.B., Payza, K., Schmidt, R., 1999. Characterization of [125I]AR-M100613, a high-affinity radioligand for delta opioid receptors. Peptides 20, 1327–1335.

Fraser, G.L., Holmgren, J., Clarke, P.B., Wahlestedt, C., 2000a. Antisense inhibition of delta-opioid receptor gene function in vivo by peptide nucleic acids. Mol. Pharmacol. 57, 725–731.

Fraser, G.L., Pradhan, A.A., Clarke, P.B., Wahlestedt, C., 2000b. Supraspinal antinociceptive response to [D-Pen(2,5)]-enkephalin (DPDPE) is pharmacologically distinct from that to other delta-agonists in the rat. J. Pharmacol. Exp. Ther. 295, 1135–1141.

Grant, R.J., Clarke, P.B., 2002. Susceptibility of ascending dopamine projections to 6-hydroxydopamine in rats: effect of hypothermia. Neuroscience 115, 1281–1294.

Gupta, M., Hogema, B.M., Grompe, M., Bottiglieri, T.G., Concas, A., Biggio, G., Sogliano, C., Rigamonti, A.E., Pearl, P.L., Snead III, O.C., Jakobs, C., Gibson, K.M., 2003. Murine succinate semialdehyde dehydrogenase deficiency. Ann. Neurol. 54 (Suppl. 6), S81–S90.

Hua, X.Y., Chen, P., Polgar, E., Nagy, I., Marsala, M., Phillips, E., Wollaston, L., Urban, L., Yaksh, T.L., Webb, M., 1998. Spinal neurokinin NK1 receptor down-regulation and antinociception: effects of spinal NK1 receptor antisense oligonucleotides and NK1 receptor occupancy. J. Neurochem. 70, 688–698.

Hyytia, P., Ingman, K., Soini, S.L., Laitinen, J.T., Korpi, E.R., 1999. Effects of continuous opioid receptor blockade on alcohol intake and up-regulation of opioid receptor subtype signalling in a genetic model of high alcohol drinking. Naunyn-Schmiedeberg's Arch. Pharmacol. 360, 391–401.

Larsen, H.J., Bentin, T., Nielsen, P.E., 1999. Antisense properties of peptide nucleic acid. Biochim. Biophys. Acta 1489, 159–166.

Mansour, A., Fox, C.A., Thompson, R.C., Akil, H., Watson, S.J., 1994. mu-Opioid receptor mRNA expression in the rat CNS: comparison to mu-receptor binding. Brain Res. 643, 245–265.

Mansour, A., Fox, C.A., Burke, S., Akil, H., Watson, S.J., 1995. Immunohistochemical localization of the cloned mu opioid receptor in the rat CNS. J. Chem. Neuroanat. 8, 283–305.

McMahon, B.M., Stewart, J.A., Jackson, J., Fauq, A., McCormick, D.J., Richelson, E., 2001. Intraperitoneal injection of antisense peptide nucleic acids targeted to the mu receptor decreases response to morphine and receptor protein levels in rat brain. Brain Res. 904, 345–349.

McMahon, B.M., Stewart, J., Fauq, A., Younkin, S., Younkin, L., Richelson, E., 2002. Using peptide nucleic acids as gene-expression modifiers to reduce beta-amyloid levels. J. Mol. Neurosci. 19, 71–76.

Miller, J.A., 1991. The calibration of 35S or 32P with 14C-labeled brain paste or 14C-plastic standards for quantitative autoradiography using LKB Ultrofilm or Amersham Hyperfilm. Neurosci. Lett. 121, 211–214.

Nielsen, P.E., Egholm, M., Berg, R.H., Buchardt, O., 1991. Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. Science 254, 1497–1500.

Nomura, A., Shigemoto, R., Nakamura, Y., Okamoto, N., Mizuno, N., Nakanishi, S., 1994. Developmentally regulated postsynaptic localization of a metabotropic glutamate receptor in rat rod bipolar cells. Cell 77, 361–369.

Pasternak, G.W., 2001. Incomplete cross tolerance and multiple mu opioid peptide receptors. Trends Pharmacol. Sci. 22, 67–70.

Paxinos, G., Watson, C., 1997. The Rat Brain in Stereotaxic Coordinates, 3rd ed. Academic Press, San Diego, CA.

Pooga, M., Soomets, U., Hallbrink, M., Valkna, A., Saar, K., Rezaei, K., Kahl, U., Hao, J.X., Xu, X.J., Wiesenfeld-Hallin, Z., Hokfelt, T., Bartfai, T., Langel, U., 1998. Cell penetrating PNA constructs regulate galanin receptor levels and modify pain transmission in vivo. Nat. Biotechnol. 16, 857–861.

Qin, Z.H., Zhou, L.W., Zhang, S.P., Wang, Y., Weiss, B., 1995. D2 dopamine receptor antisense oligodeoxynucleotide inhibits the synthesis of a functional pool of D2 dopamine receptors. Mol. Pharmacol. 48, 730–737.

Ray, A., Norden, B., 2000. Peptide nucleic acid (PNA): its medical and biotechnical applications and promise for the future. FASEB J. 14, 1041–1060.

Rezaei, K., Xu, I.S., Wu, W.P., Shi, T.J., Soomets, U., Land, T., Xu, X.J., Wiesenfeld-Hallin, Z., Hokfelt, T., Bartfai, T., Langel, U., 2001. Intrathecal administration of PNA targeting galanin receptor reduces galanin-mediated inhibitory effect in the rat spinal cord. NeuroReport 12, 317–320.

Rossi, G.C., Pasternak, G.W., Bodnar, R.J., 1994. Mu and delta opioid synergy between the periaqueductal gray and the rostro-ventral medulla. Brain Res. 665, 85–93.

Shah, S., Duttaroy, A., Chen, B.T., Carroll, J., Yoburn, B.C., 1997. The effect of mu-opioid receptor antisense on morphine potency and antagonist-induced supersensitivity and receptor upregulation. Brain Res. Bull. 42, 479–484.

Sora, I., Elmer, G., Funada, M., Pieper, J., Li, X.F., Hall, F.S., Uhl, G.R., 2001. Mu opiate receptor gene dose effects on different morphine actions: evidence for differential in vivo mu receptor reserve. Neuropsychopharmacology 25, 41–54.

Stein, C.A., 2000. Is irrelevant cleavage the price of antisense efficacy? Pharmacol. Ther. 85, 231–236.

Turner, B.J., Cheah, I.K., Macfarlane, K.J., Lopes, E.C., Petratos, S., Langford, S.J., Cheema, S.S., 2003. Antisense peptide nucleic acid-mediated knockdown of the p75 neurotrophin receptor delays motor neuron disease in mutant SOD1 transgenic mice. J. Neurochem. 87, 752–763.

Tyler, B.M., McCormick, D.J., Hoshall, C.V., Douglas, C.L., Jansen, K., Lacy, B.W., Cusack, B., Richelson, E., 1998. Specific gene blockade shows that peptide nucleic acids readily enter neuronal cells in vivo. FEBS Lett. 421, 280–284.

Tyler, B.M., Jansen, K., McCormick, D.J., Douglas, C.L., Boules, M., Stewart, J.A., Zhao, L., Lacy, B., Cusack, B., Fauq, A., Richelson, E., 1999. Peptide nucleic acids targeted to the neurotensin receptor and administered i.p. cross the blood-brain barrier and specifically reduce gene expression. Proc. Natl. Acad. Sci. U. S. A. 96, 7053–7058.

Tyler-McMahon, B.M., Stewart, J.A., Jackson, J., Bitner, M.D., Fauq, A., McCormick, D.J., Richelson, E., 2001. Altering behavioral responses and dopamine transporter protein with antisense peptide nucleic acids. Biochem. Pharmacol. 62, 929–932.

Van Oekelen, D., Megens, A., Meert, T., Luyten, W.H., Leysen, J.E., 2003. Functional study of rat 5-HT2A receptors using antisense oligonucleotides. J. Neurochem. 85, 1087–1100.

Weiss, B., Zhou, L.W., Zhang, S.P., Qin, Z.H., 1993. Antisense oligodeoxynucleotide inhibits D2 dopamine receptor-mediated behavior and D2 messenger RNA. Neuroscience 55, 607–612.