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Hydrogen sulphide induces μ opioid receptor-dependent analgesia in a rodent model of visceral pain

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Abstract

Background: Hydrogen sulphide (H_2S) is a gaseous neuro-mediator that exerts analgesic effects in rodent models of visceral pain by activating K_{ATP} channels. A body of evidence support the notion that K_{ATP} channels interact with endogenous opioids. Whether H_2S -induced analgesia involves opioid receptors is unknown.

Methods: The perception of painful sensation induced by colorectal distension (CRD) in conscious rats was measured by assessing the abdominal withdrawal reflex. The contribution of opioid receptors to H_2S -induced analgesia was investigated by administering rats with selective μ , κ and δ opioid receptor antagonists and antisenses. To investigate whether H_2S causes μ opioid receptor (MOR) transactivation, the neuronal like cells SKNMCs were challenged with H_2S in the presence of MOR agonist (DAMGO) or antagonist (CTAP). MOR activation and phosphorylation, its association to β arrestin and internalization were measured.

Results: H_2S exerted a potent analgesic effects on CRD-induced pain. H_2S -induced analgesia required the activation of the opioid system. By pharmacological and molecular analyses, a robust inhibition of H_2S -induced analgesia was observed in response to central administration of CTAP and MOR antisense, while κ and δ receptors were less involved. H_2S caused MOR transactivation and internalization in SKNMCs by a mechanism that required AKT phosphorylation. MOR transactivation was inhibited by LY294002, a PI3K inhibitor, and glibenclamide, a K_{ATP} channels blocker.

Conclusions: This study provides pharmacological and molecular evidence that antinociception exerted by H_2S in a rodent model of visceral pain is modulated by the transactivation of MOR. This observation provides support for development of new pharmacological approaches to visceral pain.

Introduction

Visceral pain is the most common sign of acute and chronic gastrointestinal, pelvic and genitourinary diseases. As one of the most common causes of persistent disability, visceral pain represents a frequent reason for patients to seek medical treatment. Despite multiple therapeutic approaches, the treatment of visceral pain remains a significant challenge.

A complex network of signaling molecules mediates perception of visceral pain [1]. Hydrogen sulphide (H_2S) is a gaseous neuromodulator generated from L-cysteine

by the activity of two pyridoxal-5'-phosphate-dependent enzymes, the cystathionine γ -lyase (CSE) and the cystathionine β -synthase (CBS) [2-5], that exerts regulatory activities in the gastrointestinal tract [1,4]. In the central nervous system H_2S mediates the induction of hippocampal long-term potentiation [6-8] and the release of the corticotropin releasing hormone from the hypothalamus [9], enhances NMDA receptor-mediated responses [8] and protects against peroxynitrite-induced neuronal toxicity [10]. ATP-sensitive potassium (K_{ATP}) channels have been identified as important mediators of several effects exerted by H_2S [2,3,10]. Thus, glibenclamide, a K_{ATP} channels blocker, attenuates analgesic effect of H_2S in a

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model of visceral pain induced by colorectal distension (CRD) in healthy and post-colitis, allodynic rats [11,12].

Opioid receptors are G protein-coupled receptors (GPCRs) and the main receptors involved in the modulation of pain in mammals [13,14]. The principal opioid receptor subtypes, μ (MOR), δ (DOR) and κ (KOR), are all expressed in the spinal cord and in the brain contributing to the modulation of nociceptive transmission. In addition, the μ and κ opioid receptors are also expressed in the enteric nervous system. MOR is the preferred receptor for potent analgesics with high potential for abuse, such as morphine [14]. Endogenous opioids, including enkephalins, endorphins and opiates like etorphine, induce rapid μ receptor endocytosis in neurons and transfected cells [15,16], a process called internalization that is widely used as a marker of MOR activation [17,18].

Opioid receptors and K_{ATP} channels converge in regulating release of neurotransmitters, smooth muscle contractions and neuronal excitability with both signaling pathways being effective in attenuating perception of visceral painful sensations in animal models and patients [19,20]. Whether H_2S signaling integrates with the opioid system, however, is still unknown.

In the present study we provide evidence that antinociception exerted by H_2S in a rodent model of visceral pain is selectively modulated by the intervention of μ opioid receptors. By *in vitro* studies we demonstrated that a previously unrecognized neuronal circuit with H_2S -activated K_{ATP} channels transactivating the μ opioid receptor supports the analgesic activities of H_2S . These results identify new pharmacological targets in the treatment of chronic visceral pain.

Results

H_2S inhibits CRD-induced nociception

In all experimental settings two sequential distension-effect curves were constructed. The first distension-effect curve was used as a control, while the second was constructed in response to saline or specified drug. In all experiments animals were awake and no changes in the consciousness state were produced by Na_2S administration.

CRD (0.4-1.6 ml water) elicited a volume-dependent increase of the AWR scores which was rapid in onset, persisted for the duration of the distension period (Figure 1, panel A) and returned to the baseline immediately after the distension was stopped. In the fed animals CRD elicited a similar pattern of response (Figure 1, panel B). Injected intraperitoneally (i.p.) at the dose of 100 μ Mol/kg, Na_2S decreased the AWR score (Figure 1, panel C, $p < 0.05$ versus CRD alone) and determined a significant increase of colorectal compliance (data not shown) indi-

cating that H_2S induced a myorelaxant action on colonic smooth muscle cells. The antinociceptive effect of Na_2S was confirmed by analysis of spinal cFos mRNA expression. Thus, Na_2S administration abrogated cFos mRNA expression induced in the spinal cord by CRD (Figure 1, panel D, $p < 0.05$ versus control).

μ opioid receptors antagonism inhibits the H_2S -induced antinociception

The antinociceptive effect of Na_2S on CRD-induced pain was studied by pre-treating animals with selective opioid receptor antagonists. As illustrated in Figure 2, while the DOR antagonist NTI, and the KOR antagonist GNTI injected intracerebroventricularly (i.c.v.) had no effect on Na_2S -induced antinociception (Figure 2, panels A and B respectively, $p < 0.05$ versus CRD), the selective MOR antagonist CTAP injected i.c.v. reverted analgesia induced by Na_2S (Figure 2, panel C) without interfering with its myorelaxant activity (data not shown). Administering rats with NTI, GNTI and CTAP alone had no effect on CRD-induced nociception (data not shown). To confirm the above mentioned results by another method, we injected rats i.c.v. with oligodeoxynucleotide antisenses directed against each specific opioid receptor subtype. While pre-treating rats with mismatched antisenses failed to modulate Na_2S -induced analgesia (Figure 3, panel A, $p < 0.05$ versus CRD), the analgesic activity of H_2S on CRD-induced pain was abrogated by pre-treating animals with δ and μ opioid receptor antisenses (Figures 3, panels B and D respectively). In contrast, no effect was observed with the κ opioid receptor antisense (Figure 3, panel C, $p < 0.05$ versus CRD). All antisenses had no effect on colonic myorelaxation induced by Na_2S (data not shown). Finally, administering rats with antisenses alone had no effect on nociception induced by CRD (data not shown).

To determine whether the analgesic effect of Na_2S was modulated by K_{ATP} channels, we performed an experiment by using the K_{ATP} channel antagonist glibenclamide. The antinociceptive effect of Na_2S (Figure 4, panel A) was reverted by blocking the K_{ATP} channels with glibenclamide (Figure 4, panel B), while treating rats with glibenclamide alone failed to modulate nociception induced by CRD (data not shown).

H_2S induces MOR activation and internalization

To investigate the mechanisms by which Na_2S activates MOR, experiments were carried out in SKNMC cells, a neuron-like cell line that expresses functional μ opioid receptors. Agonist-induced activation of MOR results in conformational changes of the extracellular portion of the receptor that unmasks a specific epitope near to the N-

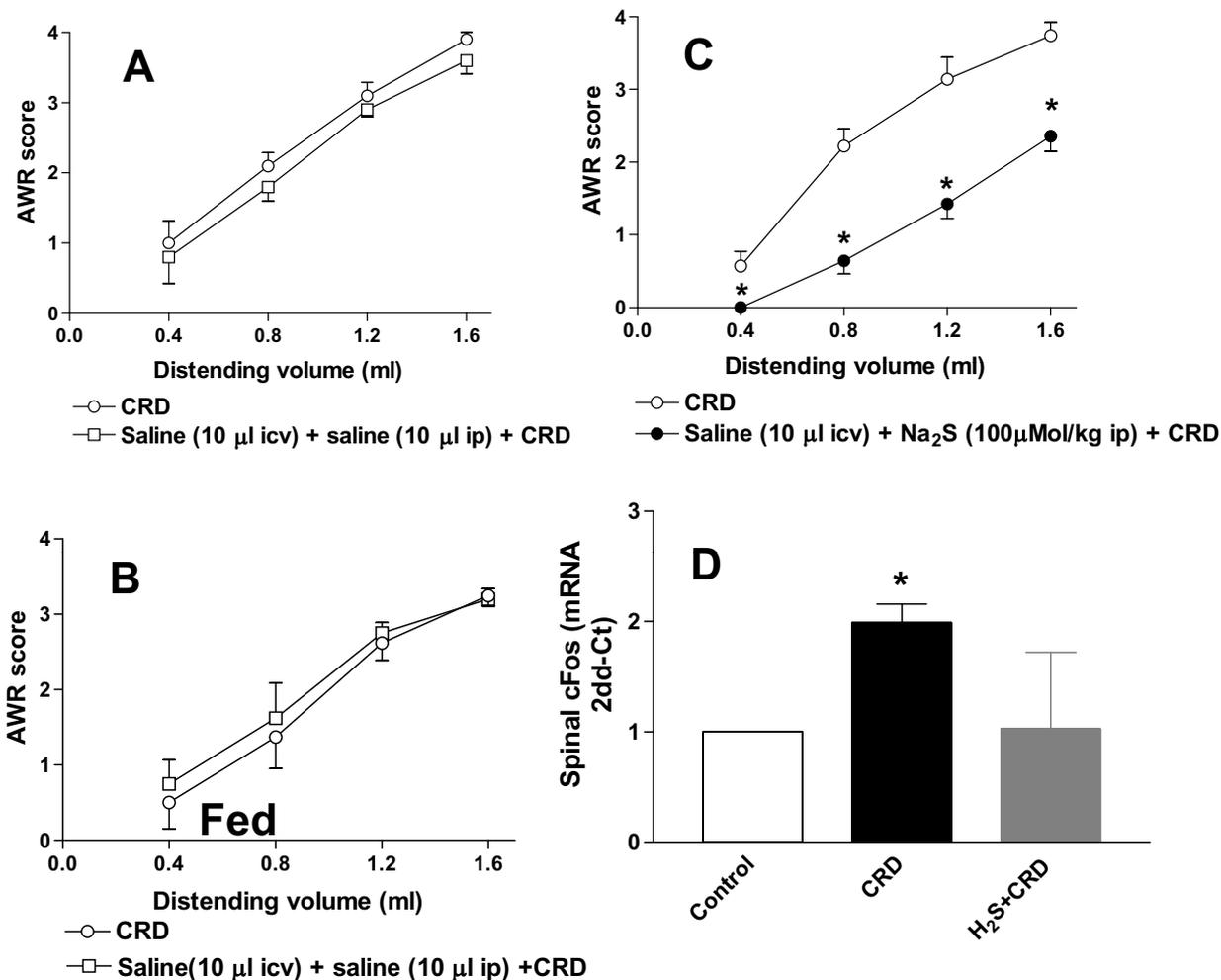


Figure 1 Na₂S induces antinociception. CRD induces a volume-dependent increase of the AWR score in both fasting and fed rats (panels A and B respectively) and Na₂S (100 μM/kg i.p.) causes a significant reduction of visceral sensitivity and pain (panel C). Data are mean ± SEM of 5 rats. *p < 0.05 versus CRD. CRD induces the increase of spinal cFos expression that is downregulated by Na₂S (panel D). Data are mean ± SEM of 5 rats. *p < 0.05 versus control.

terminus. By using a specific antibody that target this epitope, we have investigated whether Na₂S causes MOR activation. As illustrated in Figure 5, panels A and B, MOR activation was detected in cells exposed to either the μ receptor-selective enkephalin analog DAMGO and Na₂S, indicating that exposure to Na₂S induced an activity-dependent conformational change of the N-terminal region of the MOR. Further, exposure of SKNMCs to Na₂S caused the direct phosphorylation of MOR in the Ser(377) (Figure 5, panel C), a measure of the receptor activation, and exposure of cells to DAMGO also caused a robust induction of MOR phosphorylation in the serine residue, thought that the kinetic of the two effects was different (Figure 5, panel C). As expression of total MOR protein did not change (Figure 5, panel D), these results

demonstrated that exposure of SKNMCs to Na₂S induced a rapid and persistent phosphorylation of the μ opioid receptor in a site that is functionally linked to its activation.

Following its activation, MOR is rapidly internalized after its recruitment into a multiprotein complex with β arrestin. By co-immunoprecipitation experiments (Figure 5, panel E) we found that exposure of SKNMCs to DAMGO and Na₂S caused a robust induction of MOR association with β arrestin. By membrane fraction technique we found that DAMGO caused MOR internalization as shown by its disappearance from the plasma membrane and relocation into the cytosol fraction as early as 5 minutes of exposure (Figure 5, panel F). A similar pattern was observed in response to Na₂S, thought the

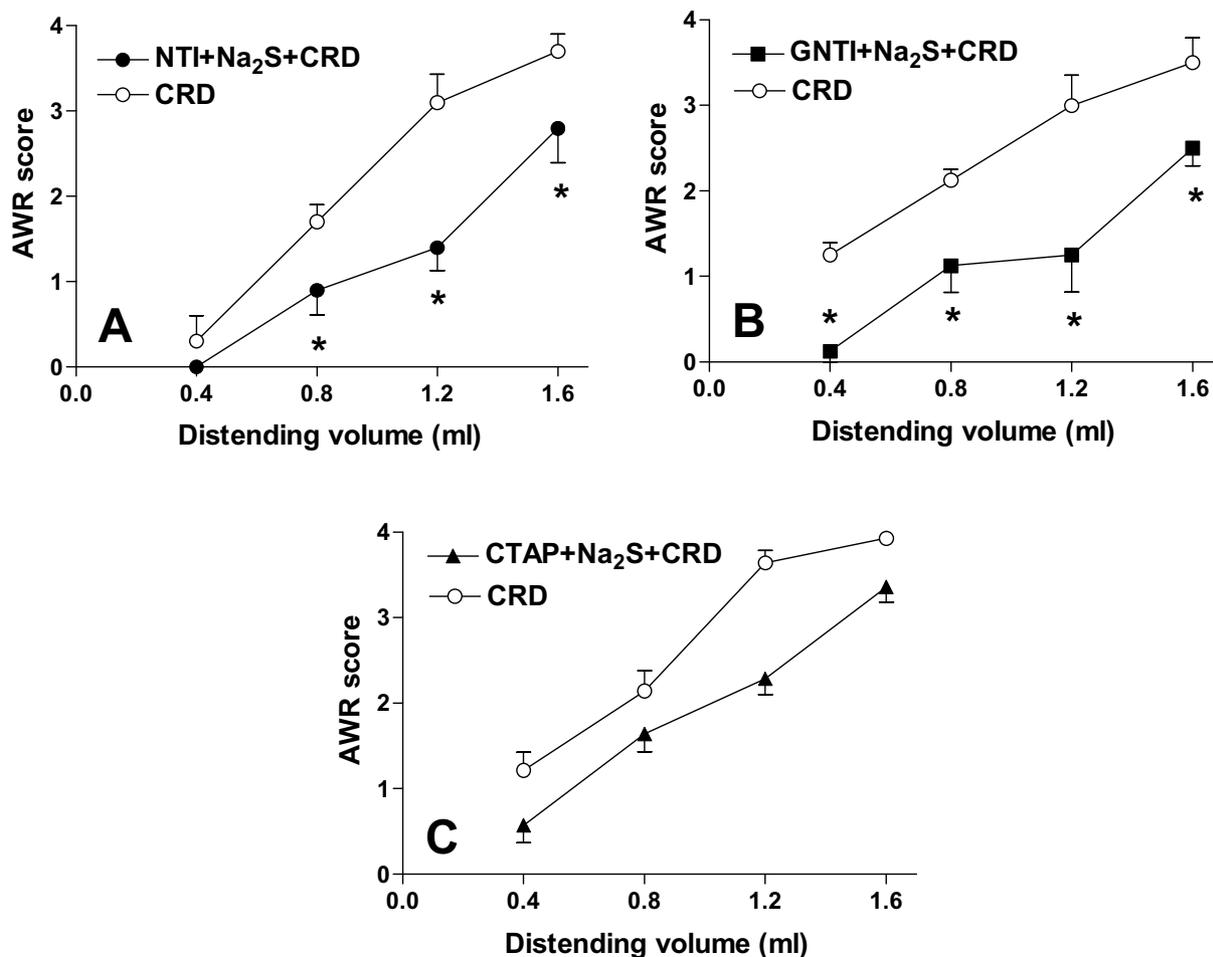


Figure 2 CTAP reverses the Na₂S-induced antinociception. Pre-treating rats with the selective μ opioid receptor antagonist CTAP (0.09 mg/kg i.c.v. thirty minutes before Na₂S; panel C) abrogates the antinociceptive effect of Na₂S (100 μ Mol/kg i.p.). In contrast, the selective δ opioid receptor antagonist NTI (4 μ g/kg i.c.v. five minutes before Na₂S, panel A) and the selective κ opioid receptor antagonist GNTI (0.08 μ g/kg i.c.v. three days before Na₂S, panel B) do not inhibit the analgesic effect of Na₂S, indicating that δ and κ opioid receptors have no effects on Na₂S-induced antinociception. Data are mean \pm SEM of 5 rats. * $p < 0.05$ versus CRD.

time course was slightly different (Figure 5, panel G). These findings were confirmed by confocal microscopy analysis (Figure 5, panels H-L). Thus, while resting SKNMCs exhibited MOR immunoreactivity predominantly at the cell surface (Figure 5, panel H), a massive translocation of receptor to the cytosol occurred in cells exposed to DAMGO (Figure 5, panel I) and Na₂S (Figure 5, panel L).

To further investigate whether activation of MOR by Na₂S occurs by direct receptor activation or is mediated by receptor transactivation, we challenged SKNMCs with the highly selective μ receptor antagonist CTAP. Results from these experiments demonstrate that while MOR activation induced by DAMGO was abrogated by CTAP, the antagonist had no effects on MOR activation induced

by Na₂S (Figure 6, panel A). Similarly, CTAP was effective in preventing MOR internalization induced by DAMGO but only partially prevented cytosolic MOR translocation induced by Na₂S treatment (Figure 6, panel B).

H₂S induces PI3K/AKT activation

Because H₂S induces AKT phosphorylation [21] and AKT is also activated in response to MOR activation by DAMGO [22], we have investigated whether Na₂S induces AKT phosphorylation in SKNMCs. Results of these experiments demonstrated that both DAMGO and Na₂S caused a long-lasting phosphorylation of AKT in Threonine 308 (Thre308), a marker of AKT activation (Figure 7, panel A). The induction of AKT phosphoryla-

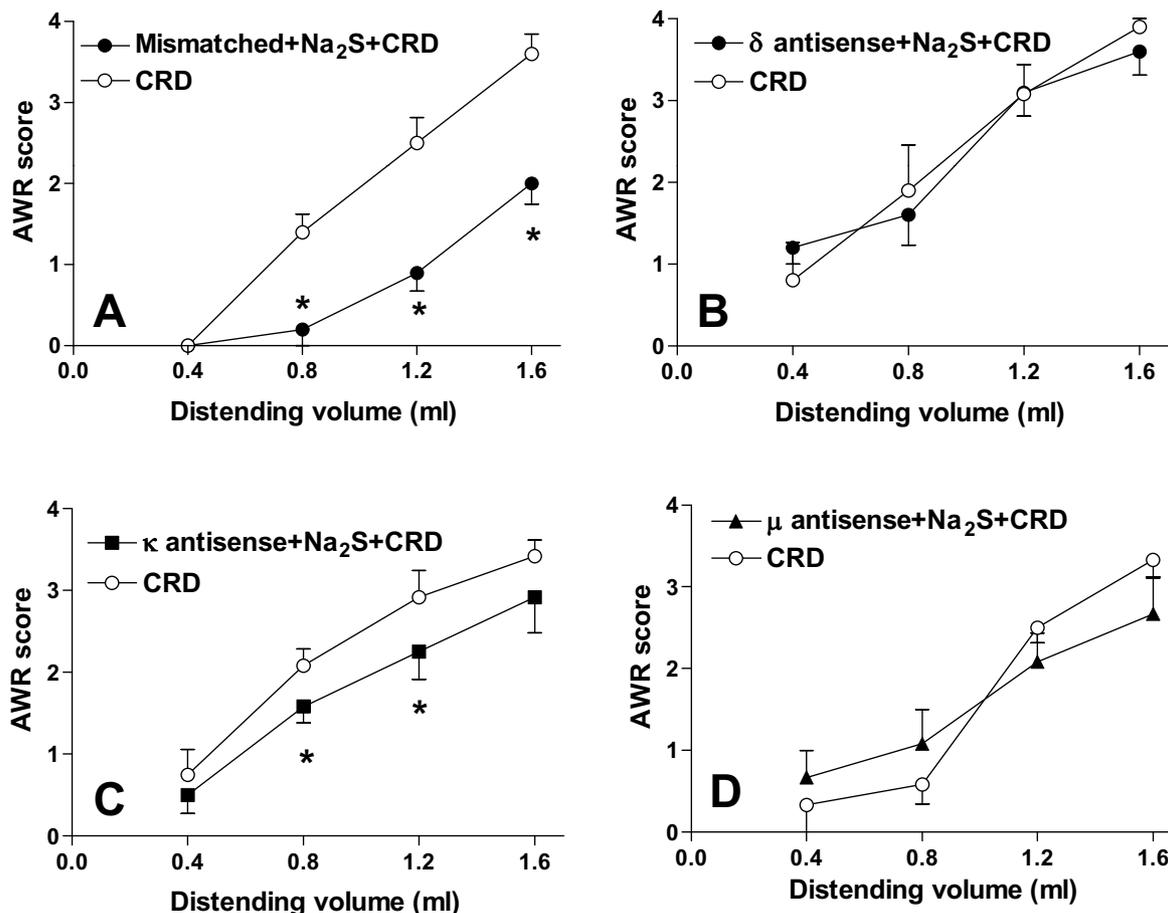


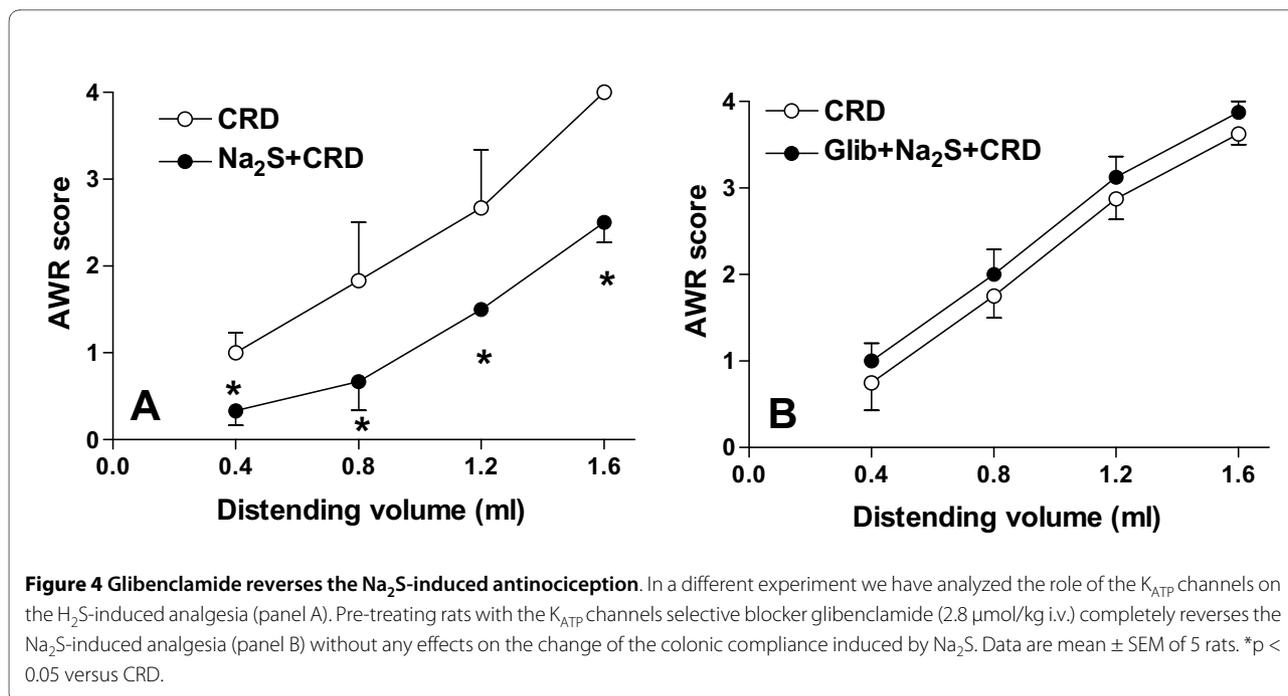
Figure 3 The selective antisense oligodeoxynucleotide probes against DOR and MOR reverse the Na₂S-induced antinociception. Pre-treating rats with both the mismatched antisense oligodeoxynucleotides (panel A) and the κ opioid receptor antisense oligodeoxynucleotides (panel C) does not modify the H₂S-induced decrease of the AWR score, confirming that KOR does not cause any change on the Na₂S-induced analgesia. In contrast, oligodeoxynucleotide probes against DOR and MOR reverse the antinociception caused by Na₂S (panel B and D respectively). Data are mean ± SEM of 5 rats. *p < 0.05 versus CRD.

tion by Na₂S was time dependent as further confirmed by an immunoassay that specifically detects AKT phosphorylation on Serine 473 (Ser473) (Figure 7, panel B). AKT phosphorylation induced by DAMGO was reversed by CTAP (Figure 7C). However, CTAP failed to inhibit AKT phosphorylation induced by Na₂S (Figure 7, panel C and D, p < 0.05 versus control).

To investigate the role of the PI3K/AKT pathway in Na₂S-induced MOR internalization, SKNMCs were pre-treated with the selective PI3K inhibitor LY294002 (50 μM). LY294002 had no effect on DAMGO-induced MOR internalization (Figure 8, panel A), but prevented MOR internalization induced by Na₂S (Figure 8, panel B). Moreover, LY294002 abrogated AKT phosphorylation induced by Na₂S (Figure 8, panel C).

SKNMCs express K_{ATP} channels subunits: glibenclamide inhibits MOR activation and AKT phosphorylation

Because glibenclamide abrogates analgesia induced by Na₂S suggesting the involvement of K_{ATP} channels, we have investigated whether SKNMCs express functional K_{ATP} channels. By RT-PCR we found that both the Kir6.2 and SUR1 subunits were expressed in the SKNMCs (Figure 9, panels A and B respectively) and by antagonism experiments we demonstrated that these channels were functionally active because glibenclamide (1 μM) inhibited MOR activation (Figure 9, panel C), MOR internalization (Figure 9, panel D) and AKT phosphorylation (Figure 9, panel E) induced by Na₂S.



Discussion

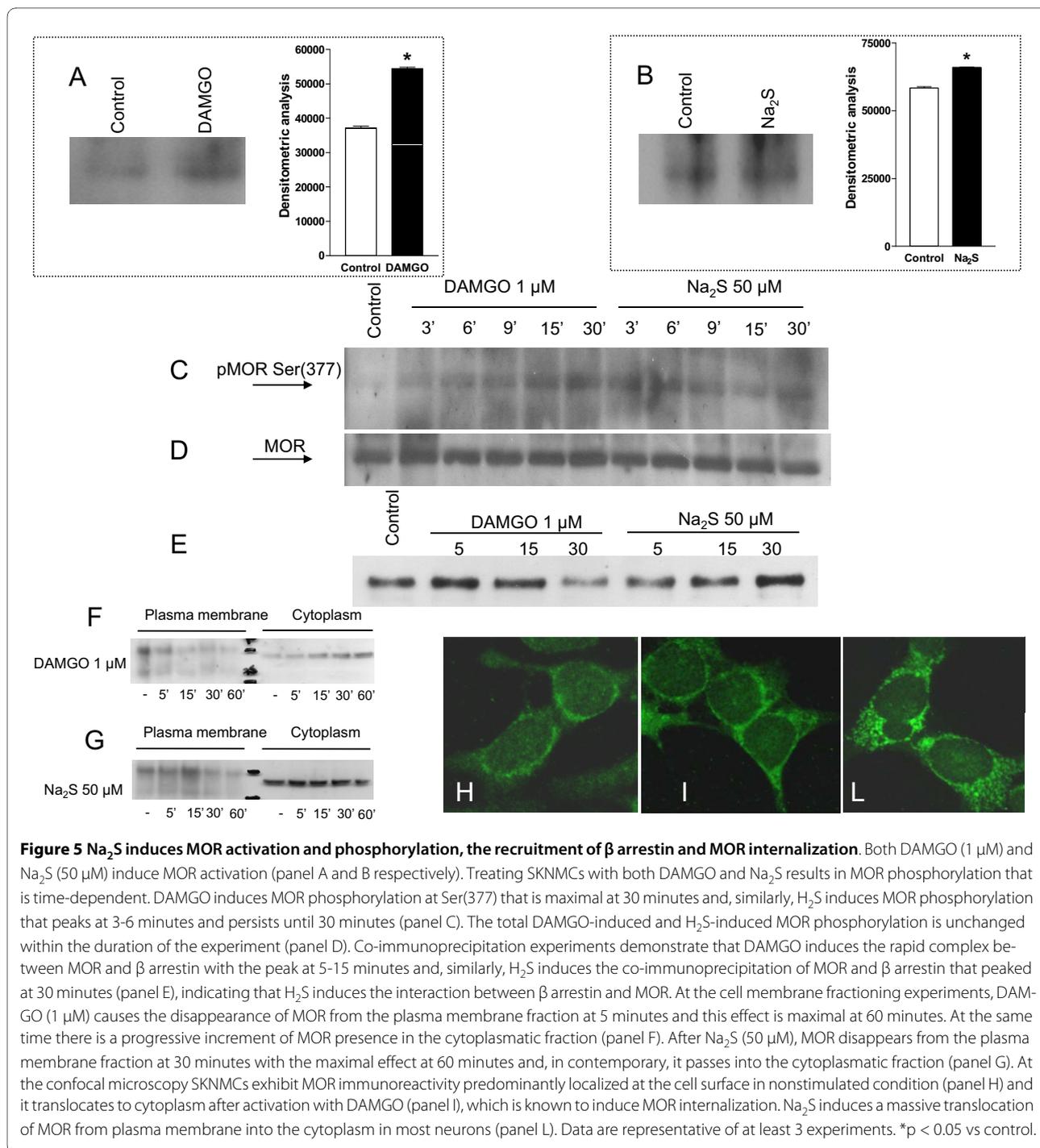
In this study we have demonstrated that H₂S induces μ opioid-dependent analgesia in a rodent models of visceral pain. Moreover, in a supplementary experiment, we have demonstrated that, in contrast to what previously reported on the effect of meal on visceral perception in humans [23-25], CRD induces a similar painful response in both fasting and fed animals, indicating that meal has no influence on visceral perception in this experimental setting. However, more experiments are needed to clarify this particular issue.

Several mechanisms might explain the antinociceptive effect of H₂S. *First*, a bluntness of sensorial functions that mimics a pain-free condition is unlikely because we did not observe any change in the consciousness of the rats during these studies. *Second*, as H₂S causes a relaxation of smooth muscle cells, H₂S could simply act as myorelaxant agent. However, this explanation seems unlikely, given that we have previously demonstrated that H₂S inhibited CRD-induced nociception at doses that did not modify the colorectal compliance [11]. A *third*, more likely explanation would be that the antinociceptive effect of H₂S is mediated by a direct inhibitory activity on colorectal afferent pathways. Consistent with this view, we found that administration of H₂S decreased spinal cord expression of cFos mRNA.

The widespread occurrence of the opioid receptors indicates that opioids have the potential for affecting multiple systems, including nervous, hormonal and immunological systems. Opioid receptors have specific

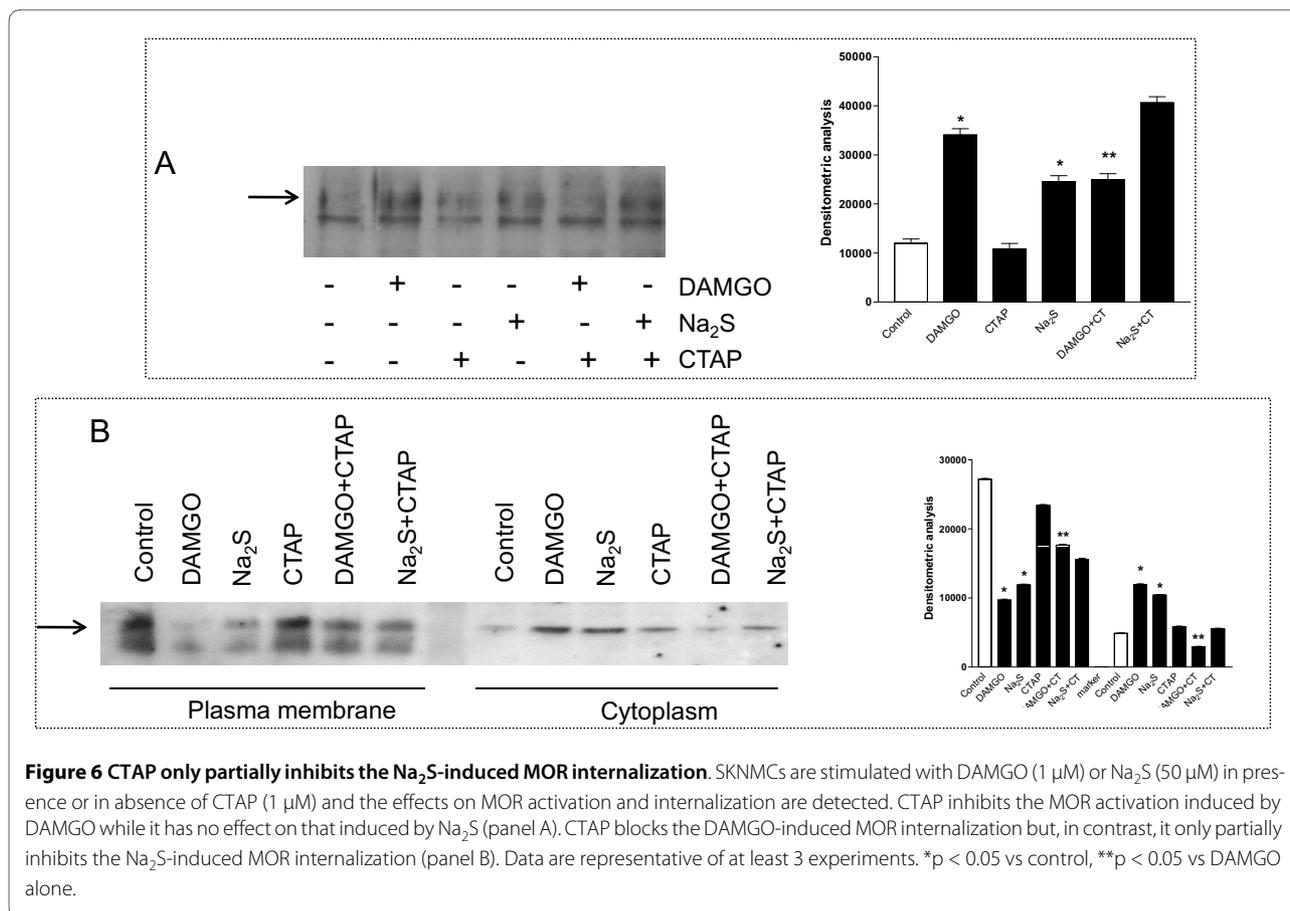
pharmacological profiles and physiological functions, maintain a certain degree of selectivity for various opioid ligands, and display unique patterns of expression in the nervous system, even though there is overlap in their binding affinity, distribution and function [26,27]. Agonists of μ opioid receptors produce analgesia, affect mood and rewarding behavior and alter respiratory, cardiovascular, gastrointestinal and neuroendocrine functions [27]. While the actions of μ opioid agonists are invariably analgesic, those of κ agonists can be either analgesic or anti-analgesic, the last effect being mediated by a functional antagonism on the action of μ receptor agonists. δ opioid receptor agonists also are potent analgesics in animals and, in isolated cases, have proved useful in human beings [27]. The main barrier to the clinical use of δ agonists is that the most available agents are peptides that do not cross the blood-brain barrier, thus requiring intraspinal administration. The ability to elucidate the roles of opioid receptor subtypes in the mediation of analgesia was first enhanced by the development of selective opioid receptor subtype antagonists direct against μ, κ and δ receptors and subsequently by the use of antisense probes to establish the relationship of the cloned receptors to opioid actions using sequences complementary to regions of specific exons of mRNA to down-regulate opioid receptor proteins.

In the present study we described for the first time that the analgesic effects of H₂S is reverted by central opioid antagonism. In particular, the selective μ antagonist CTAP, centrally administered, inhibits the H₂S-induced



analgesia while the selective κ and δ receptor antagonists have no effect. Moreover, when the selective, centrally administered antisense oligodeoxynucleotides have been used, the antisense oligodeoxynucleotides direct against μ receptors confirm the pharmacological data, suggesting that the μ opioid receptors are primarily involved in the mediation of H_2S -induced analgesia. In contrast, our pharmacological and antisense oligodeoxynucleotides

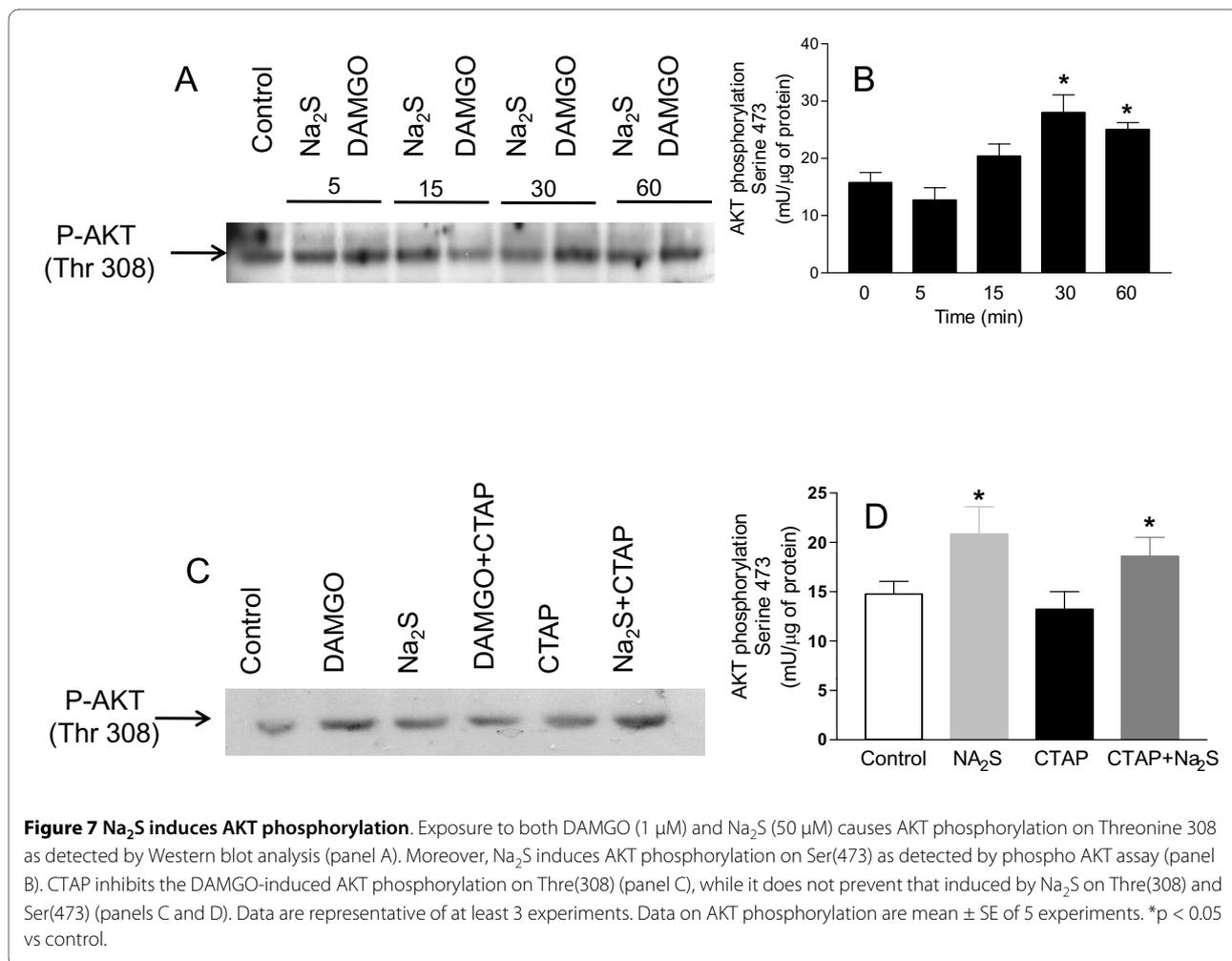
studies converge onto the indication that κ opioids receptors do not alter the H_2S -mediated effects on visceral sensitivity and pain. Previous pharmacological data indicating that activation of δ opioid receptors attenuates responses to noxious stimuli [28-31] were confirmed by studies conducted by using oligodeoxynucleotide probes direct against δ opioids receptors [32-34]. In our study, the selective δ opioid receptor antagonist NTI has no



effect on the H₂S-induced analgesia, while the oligodeoxynucleotide probes against DOR cause the reversion of the analgesic effect exerted by H₂S, suggesting a relatively minor contribution of δ opioid receptors to pain modulation by H₂S. However, the discrepancy between pharmacological and antisense data about the modulation of H₂S-induced analgesia by δ opioid receptors needs to be clarified by further studies.

Although hundreds of studies performed by using both pharmacological approaches and antisense probes focused on the different ability of the opioid receptors to cause analgesia, our data fit with the notion that MOR is identified as the most important opioid receptor linked with pain system so that the selective μ endogenous or exogenous agonists are invariably analgesic while selective μ opioid antagonists induce or exacerbate pain by blocking the effects of μ agonists in several experimental conditions. Because antisenses are highly selective and specific in downregulating one opioid receptor without interfering with the activity of other subtypes [35], these pharmacological and antisense studies converge in the indication that μ opioid receptors mediate H₂S-induced analgesia.

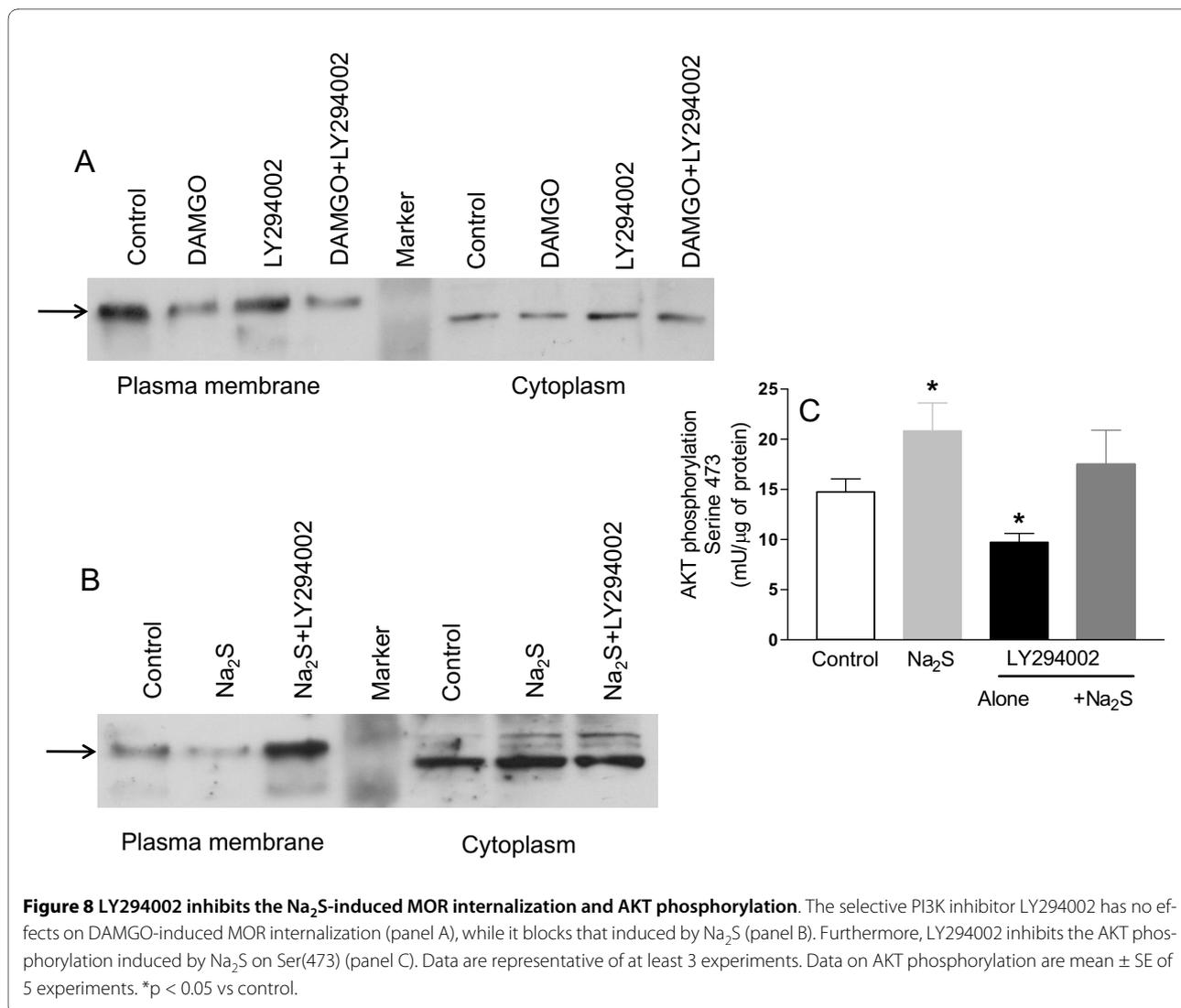
In the present study we have provided evidence that the analgesic activity of H₂S is mediated by the recruitment of μ opioid receptor. In addition to specific pharmacological antagonism exerted *in vivo* by CTAP and MOR antisense on antinociceptive activity of H₂S, results from *in vitro* pharmacological dissection of signaling pathways activated by H₂S are consistent in supporting the view that H₂S transactivates the μ opioid receptor. Exposure of SKNMCs to H₂S causes conformational changes of the extracellular tail of MOR that are known to be associated with an activated state of the receptor. These conformational changes of the N-terminus unmask a specific epitope that can be detected by an activation-state specific antibody [36,37]. Results of experiments carried out using this approach have revealed that exposure of SKNMCs to H₂S causes a change in the conformational status of MOR similar to that induced by the enkephalin analog DAMGO, a potent agonist of MOR. Further, and similarly to DAMGO, H₂S causes a robust, time- and concentration-dependent phosphorylation of MOR in Ser(377), a site that is specifically required to induce receptor activation and internalization by DAMGO. Previous studies have shown that among the 12° potential phosphorylation



sites present in the C-tail of MOR, only Ser(363), Thre(370) and Ser(375) are involved in MOR phosphorylation and linked to receptor activation [38]. DAMGO-induced MOR phosphorylation occurs at Thre (370) and Ser(375) [Ser(377) in human receptor] but only mutation of Ser(375) is reported to attenuate the rate and extent of receptor internalization [38].

One important observation we made is that phosphorylation of MOR's Ser(377) induced by H₂S is rapidly reversible. Because prolonged activation of μ opioid receptors leads to their phosphorylation, internalization, desensitization and down-regulation and represents one the main biochemical substrates of morphine tolerance, the fact that H₂S causes a short-lasting receptor phosphorylation and that rapid receptor phosphorylation (min) does not directly correlate with the relatively slow rate of desensitization (h) of MOR induced by morphine [27], suggests that this mediator is unlikely to play a role in long term desensitization of MOR and could still be a pharmacological target in situation of MOR desensitization

Mutational analysis has demonstrated that phosphorylation of Ser (375) or Ser(377) in the human receptor is critical for DAMGO-induced MOR internalization [38]. In the present study we have shown that exposure of SKNMCs to H₂S not only results in Ser(377) phosphorylation but also in MOR internalization. Similarly to DAMGO, H₂S induces a loss of cell surface expression of MOR as monitored by confocal microscopy and cell membrane fractioning technique. MOR internalization induced by H₂S is mediated by its recruitment to a protein-protein complex with β arrestin [18]. Previous studies have shown that once phosphorylated, the opioid receptor binds to β arrestin and is trafficked to clathrin-coated pits where it can subsequently be internalized into endosomes. Once internalized, endosomes containing receptors can be fused with lysosomes where receptors are proteolytically degraded or, alternatively, the receptors are dephosphorylated, resensitized and recycled back to membrane [39]. One of the main findings of the present study is that H₂S reproduces the same effects of DAMGO in terms of MOR phosphorylation, association

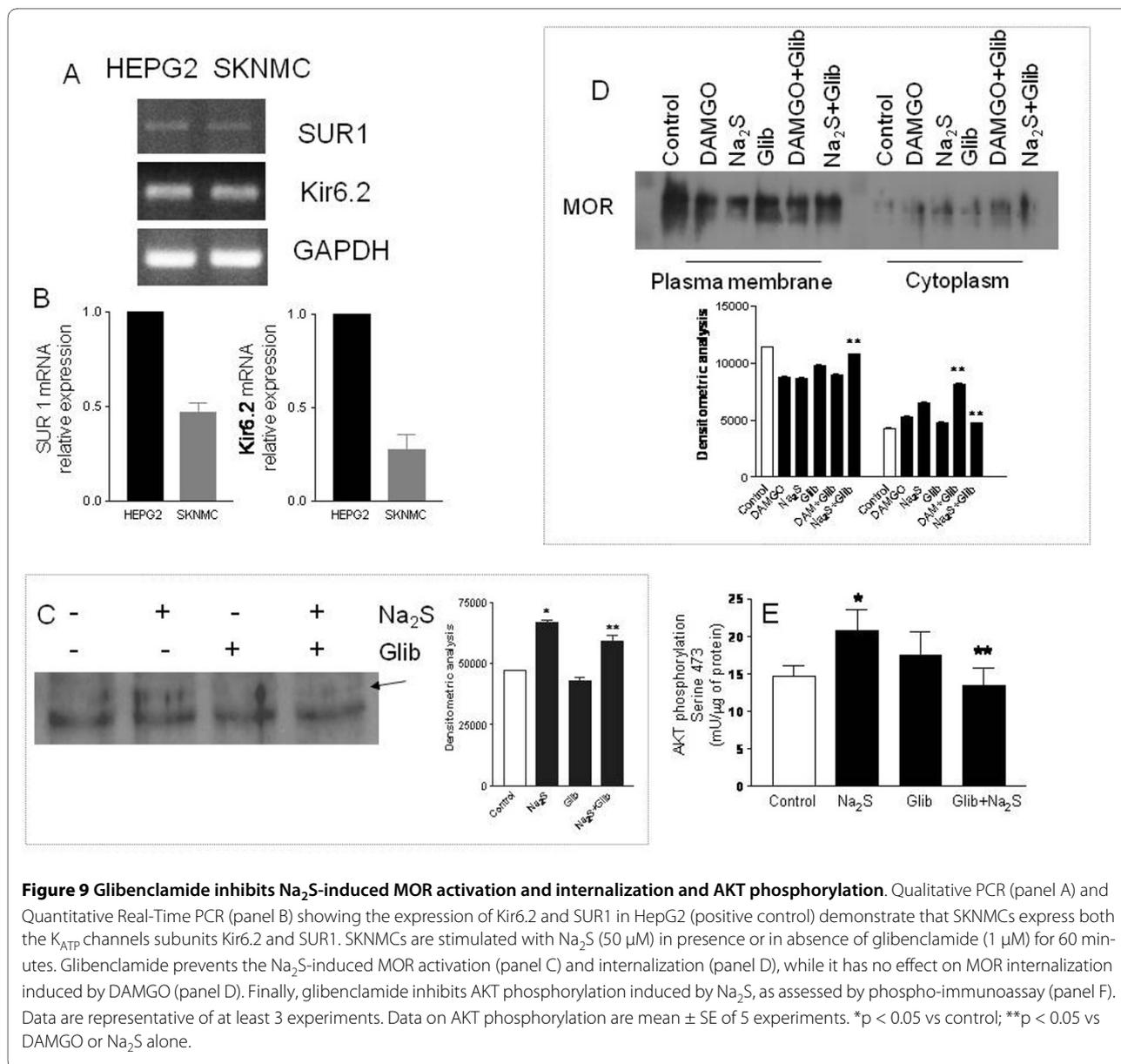


with β arrestin and internalization. However, H₂S induces a slower β arrestin recruitment and MOR internalization than DAMGO, providing evidence that it does not behave as a direct MOR agonist.

Results from mechanistic studies aimed at dissecting intracellular signals activated by H₂S in SKNMCs have shown that H₂S activates the PI3K/AKT pathway and induces AKT phosphorylation [21]. PI3K is a lipid kinase acting as a membrane-embedded second messenger [40] and AKT is a downstream target of the PI3K [41]. Activation of MOR by DAMGO induces AKT phosphorylation [42]. Our study confirms these observations and extend this effect to H₂S. However, while CTAP reverses AKT phosphorylation induced by DAMGO, it fails to inhibit the effects exerted by H₂S on AKT, indicating that, despite MOR trans-activation, H₂S-induced AKT phosphorylation is due to a direct effect of the gas on the PI3K/AKT pathway. The fact that inhibition of AKT

phosphorylation by the PI3K inhibitor LY294002 prevents MOR internalization induced by H₂S but not by DAMGO, indicates that H₂S directly activates the PI3K/AKT pathway and that activation of this pathway is hierarchically higher in the mechanism that leads to MOR activation by H₂S. These findings are consistent with the observation that activation and internalization of a GPCR can be regulated by activation of the PI3K/AKT pathway [43].

The mechanism through which H₂S targets the PI3K/AKT pathway involves K_{ATP} channels. Thus not only SKNMCs express SUR1 and Kir6.2, but blocking these channels with glibenclamide abrogates AKT phosphorylation and MOR activation and internalization induced by H₂S. This suggest a hierarchic order in the observed effects with H₂S acting as a K_{ATP} channels opener leading to activation of PI3K/AKT pathway and MOR activation and phosphorylation (Figure 10). Similar transactivation

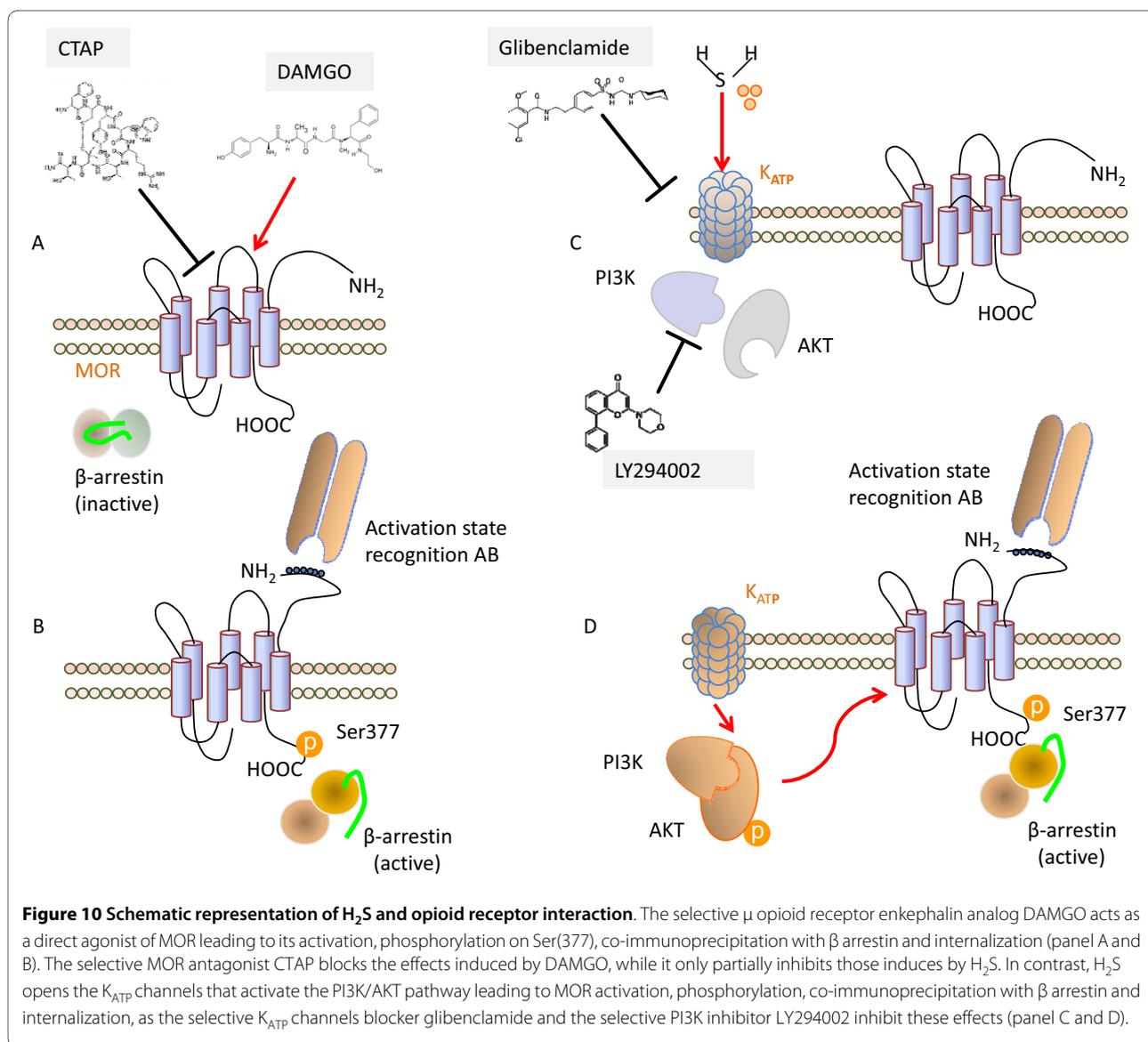


of opioid receptors by epidermal growth factor receptor has been recently described [44], however this is the first evidence of transactivation of MOR by activation of K_{ATP} channels.

Conclusion

This study demonstrates that, in a rodent model of visceral pain, H₂S-induced analgesia is mediated by μ opioids receptor activation as, *in vivo*, the selective antagonism of MOR by i.c.v. administration of both CTAP and antisense direct against MOR reverses the analgesic effects of H₂S. Moreover, pre-treating rats with the K_{ATP} channels selective blocker glibenclamide reverses the H₂S-induced analgesia. The *in vitro* studies

performed comparing the effect of the μ receptor-selective enkephalin analog DAMGO and H₂S confirm these data demonstrating that, in the neuronal-cell line SKNMC, both DAMGO and H₂S induce MOR activation and phosphorylation leading to interaction between MOR and β arrestin and MOR internalization. CTAP completely blocks MOR internalization induced by DAMGO while, in contrast, it partially inhibits MOR internalization induced by hydrogen sulphide. In addition, exposure to hydrogen sulphide causes the PI3K/AKT pathway activation and induces AKT phosphorylation. The selective PI3K inhibitor LY294002 does not interfere with the DAMGO-induced MOR internalization, while it causes the inhibition of the translocation



process of MOR from the plasma membrane to the cytoplasm induced by hydrogen sulphide as well as AKT phosphorylation induced by hydrogen sulphide. As glibenclamide reverted the analgesia induced by hydrogen sulphide, we hypothesize that the ATP potassium channels could modulate MOR activation induced by hydrogen sulphide. First we have demonstrated that SKNMCs express the ATP potassium channels subunits Kir6.2 and SUR1. Moreover, glibenclamide inhibits both MOR and AKT phosphorylation induced by hydrogen sulphide, demonstrating that activation of ATP potassium channels by hydrogen sulphide is a key process of these effects. On these basis we can speculate that hydrogen sulphide acts on the ATP potassium channels that induce the PI3K/AKT pathway that, on turn causes MOR activation and internalization (Figure 10). This study provides the first

evidence for a cross-talk between H₂S and the μ opioid receptors and paves the way to development of new therapeutic approaches to visceral pain.

Methods

Materials

Sodium sulphide (Na₂S) was used as donor of hydrogen sulphide and was from Sigma-Aldrich (S. Louis, MO, USA). Methylene blue, glibenclamide, naltrindole (NTI) 5'-guanidinonaltrindole (GNTI), 1 -D-Phe-Cys-Tyr- 2 -Trp-Arg-Thr-Pen-Thr-NH₂ (CTAP), mismatched and specific antisense oligodeoxynucleotide probes for opioid receptors, [1 -Ala 2 ,N-Me-Phe 4 ,Gly 5 -o1]enkephalin (DAMGO), ascorbic acid, salicylic acid, potassium hydroxide, trichloroacetic acid, pyridoxal-5'-phosphate

and calmodulin were from Sigma-Aldrich (S. Louis, MO, USA). Tissue Protein Extraction Reagent (T-PER) was obtained by Pierce Biotechnology (Rockford, IL, USA).

***In vivo* experiments**

Animals

Male, Wistar rats (200-250 g, Charles River, Monza, Italy) were housed in plastic cages and maintained under controlled conditions with 12-hour light/dark cycles (lights on at 07.00). Tap water and standard laboratory chow were freely available (Additional file 1). It has been demonstrated that the nutrients induce an enhancement of the colorectal sensitivity in both healthy subjects [23] and IBS patients [24,25]. To avoid the influence of the meal on colorectal perception and pain, food was withdrawn 12 hours before surgical procedures and CRD recordings in all *in vivo* experiments [11,12]. However, to verify whether meal could influence the perception of CRD-induced visceral pain, we performed a supplementary experiment on fed rats (n = 5). Experimental procedures were approved by our institutional animal research committees and were in accordance with nationally approved guidelines for the treatment of laboratory animals.

Surgical procedure

Rat were anesthetized by an i.p. injection of 70 mg/kg penthotal and were then mounted in a stereotaxic instrument. To perform the i.c.v. injection, a guide cannula (Alzet Brain Infusion Kit II, 3-5 mm) was inserted stereotaxically into the right lateral cerebral ventricle. The stereotaxic coordinates were 1,6 mm right laterally and 0,8 mm dorsoventrally from the bregma and 3,5 mm below the dura. Drugs dissolved in 10 µl saline were injected into the cerebral ventricle by insertion of an injection cannula (28 gauge stainless steel tube) connected to a catheter tube into the guide cannula which was connected to a syringe. In each injection 10 µl of vehicle or drugs were delivered manually into the ventricle over 3 min. At the end of each experiment, methylene blue solution was injected through the injection cannula to verify its correct placement in the right lateral ventricle. Rats exhibiting motor deficits after the surgical procedure were not used in the subsequent experiments.

CRD and behavioral testing

All experiments began 1 week after the surgical procedure. Distending procedure were performed as previously described (Additional file 2). The behavioral response to CRD was assessed by measuring the abdominal withdrawal reflex (AWR) as previously described [45,46] (Additional file 2).

Effects of H₂S on colonic nociception

The control group (n = 5) consisted of fasting rats that underwent surgical procedures but not CRD, while the CRD group consisted of fasting rats that underwent surgical procedures and two sets of CRD, the first acting as

control. To investigate whether H₂S administration modulates sensitivity and pain induced by CRD, rats were treated i.p. with vehicle (CRD group) or Na₂S, an H₂S donor, at the dose of 100 µMol/kg five minutes before CRD.

Effects of the opioid and K_{ATP} channels inhibitors

The role of the δ, κ and µ opioid receptors in the H₂S-induced antinociception was investigated by pre-treating rats with selective opioid receptor antagonists administered at final volume of 10 µl i.c.v.: NTI, a δ opioid receptor antagonist (4 µg/kg), was injected 5 minutes before Na₂S [47]; GNTI, a κ opioid receptor antagonist (0.08 mg/kg), was administered three days before Na₂S [48]; CTAP, a µ opioid receptor antagonist (0.09 mg/kg), was administered 30 minutes before Na₂S [49]. Control experiments were performed by injecting rats with NTI, GNTI and CTAP alone (n = 5 rats/group).

For antisense experiments rats were pretreated with antisense oligodeoxynucleotides direct against specific

Table 1: Primer used for antisense experiments

Probe sequence	Probe sequence
DOR-1 opioid receptor clone	
Exon 1 AS	TGT CCG TCT CCA CCG TGC
Exon 2 AS	ATC AAG TAC TTG GCG CTC TG
Exon 3 AS	AAC ACG CAG ATC TTG GTC AC
KOR-1 opioid receptor clone	
Exon 1 AS	GCT GCT GAT CCT CTG AGC CCA
Exon 2 AS	CCA AAG CAT CTG CCA AAG CCA
Exon 3 AS	GGC GCA GGA TCA TCA GGG TGT
MOR-1 opioid receptor clone	
Exon 1 AS	CGC CCC AGC CTC TTC CTC T
Exon 2 AS	TTG GTG GCA GTC TTC ATT TTG G
Exon 3 AS	TGA GCA GGT TCT CCC AGT ACC A
Exon 4 AS	GGG CAA TGG AGC AGT TTC TG
Mismatch	CGC CCC GAC CTC TTC CCT T

exons of DOR, KOR and MOR. A mismatched antisense was used as control (Table 1). All antisense oligodeoxynucleotides were administered i.c.v. in dose of 10 μ g in 10 μ l volume saline [50,52,53]. Treatment with antisenses was performed on day 1, 3 and 5 and the behavioral test was performed at day 6 [51] (Additional file 3).

The involvement of K_{ATP} channels in the analgesic effects of H₂S was assessed by pre-treating rats with glibenclamide, a selective K_{ATP} channel blocker, at a dose of 2.8 μ mol/kg injected intravenously (i.v.) for 20 minutes before Na₂S administration [11,12] (Additional file 4).

At the end of the CRD procedures, rats were sacrificed and spinal cords (L1-L5) collected for RT-PCR analysis of cFOS [54] (additional file 5) using the following sense and antisense primers: gtctggttcctctatgcag and taggtagtgcagctgggagat.

***In vitro* experiments**

The immortalized human neuronal SKNMCs were used for *in vitro* studies. Cells were grown in Minimum Essential Medium with Earl's salts supplemented with 10% FBS, L-glutamine, penicillin and streptomycin, and regularly passaged to maintain exponential growth.

For *in vitro* studies DAMGO was used at the dose of 1 μ M and Na₂S at the dose of 50 μ M. To determine whether H₂S induces MOR activation, SKNMCs were stimulated with DAMGO or Na₂S and MOR activation detected by Western blot analysis using a specific antibody raised against a specific epitope in the N-terminus of the receptor that becomes exposed in response to conformational changes induced by receptor activation [55]. This activation-state specific antibody exhibits enhanced recognition of activated receptor [36,37]. In addition, activation of MOR by H₂S was detected by Western blot analysis of receptor phosphorylation on Serine (Ser) (377). Finally, because MOR activation results in receptor recruitment to β arrestin, co-immunoprecipitation experiments were performed to investigate whether H₂S induces the formation of a protein-protein complex between MOR and β arrestin (Additional file 6).

Effect of H₂S on MOR internalization

To investigate whether exposure of SKNMCs to H₂S induces MOR internalization, cells were treated with the μ receptor-selective enkephalin analog DAMGO [15,16] and Na₂S alone or in combination with the MOR antagonist CTAP. Internalization of the receptor was assessed by Western blot analysis by measuring its translocation from the cell membrane fraction to the cytosol and by confocal microscopy (Nikon) using a specific anti-MOR immunofluorescent antibody (Additional file 7).

Effect of H₂S on AKT phosphorylation

SKNMCs were exposed to DAMGO and Na₂S up to 60 minutes and Western blot analysis performed on whole

cell lysates using a specific antibody that detected the phosphorylated form of AKT on Thre(308). AKT phosphorylation was also detected by measuring the AKT phosphorylated form on Ser(473) (phospho-AKT ELISA KIT, Biosource).

Activation of the PI3K/AKT pathway was tested by exposing SKNMCs to the selective PI3K inhibitor LY294002 (50 μ M) in the presence of DAMGO or Na₂S (Additional file 8).

Effect of K_{ATP} channels blockade

Expression of K_{ATP} channels in SKNMCs was evaluated by assessing the expression of Kir6.2 and SUR1 sub-units (Additional file 9). Qualitative and quantitative PCR were performed by using the following sense and antisense primers: hGAPDH: gaaggtgaaggctggagat and catgggtggaatcatattggaa; hSUR.1: gtccagatcatggaggctca and cagaa-gacagcccctgagac; hKir6.2: gtcaccagatccactcctt and ggggacttcaaatgttgcacat. The effects of glibenclamide (1 μ M) on AKT phosphorylation and MOR activation and internalization were determined (Additional file 9).

Densitometric analysis

All the densitometric analysis have been performed by using the *Image J* software.

Statistical analysis

Behavioral data are presented as mean \pm SE, with sample sizes of at least 5 rats per group. Statistical comparisons of unpaired data were performed by the Mann-Whitney test, while statistical comparisons of paired data were performed by the Wilcoxon signed rank test. Densitometric data have been analyzed with Turkey's multiple comparison test. Data on AKT phosphorylation are presented as mean \pm SE, with sample sizes of at least 5 experiments per group. An associated probability (p value) of less than 5% was considered significant.

Additional material

- Additional file 1 Animals.** This file describes the animals used.
- Additional file 2 CRD and behavioral testing.** This file describes the behavioral testing used in the *in vivo* studies.
- Additional file 3 Effects of the opioid receptors antagonism.** This file describes the methods used for blocking the opioid receptors.
- Additional file 4 Effects of K_{ATP} channels.** This file describes the method used for blocking the K_{ATP} channels.
- Additional file 5 Spinal cFOS expression.** This file describes the method used for determining spinal cFos expression.
- Additional file 6 Effect of H₂S on MOR.** This file describes the methods used to determine MOR activation
- Additional file 7 Effect of H₂S on MOR internalization.** This file describes the methods used to detect MOR internalization.
- Additional file 8 Effect of H₂S on AKT phosphorylation.** This file describes the methods used to determine AKT phosphorylation.
- Additional file 9 Effects of glibenclamide.** This file describes the methods used to determine the effects of K_{ATP} channels blockade.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ED and SF conceived the study and wrote the manuscript. S Cianetti wrote the manuscript. S Cipriani and AM carried out the *in vivo* studies and helped to draft the manuscript (Methods section). BR and MM carried out the *in vitro* studies and helped to draft the manuscript (Methods section). All authors read and approved the final manuscript.

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