**Abstract**

Pain relief is the principal action of opioids. Somatostatin (SST), a growth hormone inhibitory peptide is also known to alleviate pain even in cases when opioids fail. Recent studies have shown that mice are prone to sustained pain and devoid of analgesic effect in the absence of somatostatin receptor 4 (SSTR4). In the present study, using brain slices, cultured neurons and HEK-293 cells, we showed that SSTR4 and δ-Opioid receptor (δOR) exist in a heteromeric complex and function in synergistic manner. SSTR4 and δOR co-expressed in cortical/striatal brain regions and spinal cord. Using cultured neuronal cells, we describe the heterogeneous complex formation of SSTR4 and δOR at neuronal cell body and processes. Cotransfected cells display inhibition of cAMP/PKA and co-activation of SSTR4 and δOR oppose receptor trafficking induced by individual receptor activation. Furthermore, downstream signaling pathways either associated with withdrawal or pain relief are modulated synergistically with a predominant role of SSTR4. Inhibition of cAMP/PKA and activation of ERK1/2 are the possible cellular adaptations to prevent withdrawal induced by chronic morphine use. Our results reveal direct intra-membrane interaction between SSTR4 and δOR and provide insights for the molecular mechanism that the anti-nociceptive property of SST in combination with opioids as a potential therapeutic approach to avoid undesirable withdrawal symptoms.

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**Introduction**

The functional consequences of GPCRs heterodimerization in a native system expressing these receptors endogenously, specifically in the central nervous system (CNS) are poorly understood. Opioid receptors (ORs), namely mu (μ), delta (δ) and kappa (κ), are the prominent members of the GPCRs super family [1,2]. The most indispensable function of ORs in CNS is to modulate pain. The activation of ORs in the presence of peptide produced endogenously or administered exogenously displayed distinct behavioural outcomes [3,4]. µOR is believed to mediate anti-nociceptive associated with morphine, while δOR appears to participate in acute and tonic pain models [2,3–7]. µOR is more efficient as an analgesic drug target due to its high expression at cell surface, however, reinstating δOR expression at neuronal membrane enhances receptor mediated analgesic effects [8]. These studies collectively suggest that ORs membrane expression is a prerequisite for receptors analgesic properties [8]. Interestingly, studies have also shown that knocking down δOR resulted in increased chronic pain and abolition of opioid mediated analgesic effects [9]. Furthermore, ORs functionally interact with other receptor of the family and display distinct pharmacological and signaling properties [10].

Like opioids, somatostatin (SST), is well expressed in the CNS and functions as a neurotransmitter and neuromodulator. In addition to exerting an inhibitory role on cell proliferation and hormone secretion, SST also plays a critical role in pain and inflammation [11,12]. Intrathecal or epidural application of SST analogue octreotide (OCT) induced analgesic effects in post-operative and neoplastic pain [13–15]. SST analogues have also been used successfully for pain relief in conditions like headache or in patients with terminal cancer, where opioids failed [11,12,16–20]. Further, results from animal studies favour the role of SST in morphine sparing and analgesia [13–15]. The biological function of SST is mediated by binding to five different receptor subtypes namely somatostatin receptor 1–5 (SSTR1–5) [21]. Previous studies have shown that amongst all SSTRs, SSTR4 is the only subtype that mediates analgesic effects of SST. Neurogenic and non-neurogenic inflammatory processes were significantly reduced upon administration of SSTR4 specific agonist in animal models [22]. Recently, SSTR specific knockout (ko) models have provided new insights for the role of SSTRs in certain pathophysiological conditions such as inflammation and analgesia [22,23]. Helyes et al., have described that SSTR4 ko mice are more susceptible to inflammation and exhibit sustained pain than wt mice [23].

OR and SSTR subtypes share >40% structural similarities, are coupled to pertussis toxin (PTX)-sensitive Gαi/o subunits and inhibit the second messenger cAMP [2,21,24–27]. Previous studies have also described that OR and SSTR subtypes functionally interact with each other in heterologous systems and modulate...
receptor pharmacology and trafficking [28]. Furthermore, SST analogues exhibit the displacement of opiate binding in rat brain membrane suggesting the ability of SST to bind and activate ORs [29–33]. These are compelling pieces of evidence supporting the notion that SSTR and OR subtypes might functionally interact in a native system. Clinically, opioids are still the first line of therapy and the most dependable analgesic drugs in pain treatment; however, they are associated with several side effects including dependence and withdrawal. Whether, the use of SST analogs in combination with opioids minimize such risk factors is not known. To test this hypothesis, the present study was undertaken to elucidate the molecular details and functional consequences of a possible crosstalk between SSTR4 and 8OR in rat brain slices, cultured neuronal cells and SSTR4/8OR cotransfected HEK-293 cells using morphological, biophysical and biochemical techniques.

As stated above, µOR is the prominent receptor subtype linked with pain relief and its pronounced analgesic effects, whereas, the role of δOR is also well appreciated in anti-nociception. In acute and chronic animal pain models, δOR agonists induced anti-nociceptive responses [36–38]. Mice lacking δOR are highly susceptible to pain and restoring δOR membrane expression is required to exert pain relieving and analgesic effect. δOR knockout mice displayed enhanced mechanical and thermal allodynia, and thermal hyperalgesia [9,39]. Therefore, in the present study we preferred δOR over µOR to ascertain whether SSTR4 enhances δOR function, expression and signaling pathways which has not been explored yet. Here, we provide direct evidence that SSTR4 and δOR exist in a complex and heterodimerization modulates signaling pathways associated with pain and withdrawal.

Materials and Methods

Immunohistochemistry

30 µm thick free floating brain and spinal cord sections were collected in Tris-buffer saline (TBS) and processed for immunocytochemistry as described earlier [40]. Sections were incubated in 5% normal goat serum (NGS) for 1 h followed by incubation with a mixture of SSTR4 antibody (1:400) and δOR antibody (1:250) (Santa Cruz Biotechnology, Santa Cruz, CA) for 16 h at 4 °C. Following three subsequent washes with TBS, sections were incubated with goat anti-rabbit (Alexa-488) and donkey anti-goat (Alexa-594) secondary antibodies (Invitrogen, Burlington, ON) for 1 h to visualize SSTR4 and δOR respectively. Sections were mounted and viewed under Leica Confocal microscope. All the Figure composites were constructed using Adobe Photoshop (San Jose, CA) and NIH ImageJ software. The protocols regarding animal care were followed in compliance with the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council and the University of British Columbia committee on Animal Care. The use of animals for the present study was approved by the University of British Columbia committee on Animal Care (Protocol # A06-0419).

Receptor constructs and cell lines

cMyc-δOR in pCDNA3.1+/Neo vector (hygromycin resistance) was purchased from TOP Gene Technologies (Montreal, Canada). Construct of HA-SSTR4 was made by using the pCDNA3.1+/Neo (neomycin resistance) as previously described [41,42]. Stable transfections of HEK-293 cells expressing HA-SSTR4 and/or cMyc-δOR were prepared by transfection reagent (Invitrogen, Burlington, ON) as described earlier [41,42].

Co-immunoprecipitation and western blot analysis

Co-immunoprecipitation (Co-IP) in tissue extract prepared from brain regions and cell lysate prepared from HEK-293 cells was accomplished as described previously [43,44]. Briefly, the brain tissue was homogenized in homogenization buffer and 250 µg of tissue protein was solubilized in 1 ml binding buffer (50 mM HEPES, 2 mM CaCl₂, 5 mM MgCl₂, pH 7.5) followed by treatment with SSTR4 specific agonist L-803087 (10 nM), δOR specific agonist SB-205607 (10 nM) alone or in combination for 30 min at 37°C. The lysates were than incubated with SSTR4 specific antibody (1:250) overnight at 4 °C followed by 2h incubation with protein A/G agarose beads (Calbiochem, EMD Biosciences, Darmstadt, Germany). The purified samples were fractionated by electrophoresis and transferred to PVDF membrane. Blocking of the membrane, incubation with δOR specific primary and secondary antibodies and detection by chemiluminescence were performed following ECL Western blotting detection kit as per manufacturer’s instructions (GE Healthcare, Piscataway, NJ). Membranes were developed using an Alpha Innotech FluorChem 9800 (Alpha Innotech Co., San Leandro, CA) gel box imager. Co-IP in cotransfected HEK-293 cells treated with receptor specific agonist alone or in combination was performed following similar steps. Cell lysates were immunoprecipitated with anti-cMyc antibody (1:500) and blotted with anti-HA specific antibody (1:500).

Western blot for signaling pathways was performed by using phospho-and total specific antibodies to detect extracellular signal-regulated kinases (ERK1/2 and 3), Phospho-inositol 3-kinase (PI3K), protein kinase B (AKT) (Cell Signaling Technology, Danvers, MA) and protein kinase A (PKA) (Santa Cruz Biotechnology, Santa Cruz, CA). Whole cell lysates prepared from control and treated cells were quantified by Bradford assay, fractionated by electrophoresis, transferred to PVDF membrane and blotted using antibodies following standard protocol as described earlier. Densitometry for quantification was done using FluorChem software (Alpha Innotech) [42,45].

Microscopic Photobleaching-fluorescence resonance energy transfer (Pb-FRET) analysis in mammalian cells and cultured striatal neurons

HEK-293 cells expressing cMyc-δOR/HA-SSTR4 were treated with SST-14 (1 µM), L-803087 (10 nM) and SB-205607 (10 nM) alone and in combination for 15 min at 37°C. Post treatment, cells were fixed and processed for immunofluorescence immunocytochemistry using monoclonal anti-HA and polyclonal anti-cMyc primary antibodies followed with FITC and Cy3 conjugated secondary antibodies to create donor and acceptor pair (Sigma-Aldrich, Inc., St. Louis, MO). Pb-FRET analysis was performed as described earlier [42,45]. The plasma membrane region was used to analyze the photobleaching decay on a pixel-by-pixel basis and FRET efficiency (E) was calculated [42,45].

Primary culture of striatal neurons was prepared from 15 days old rat embryonic brains as described earlier [46]. Pb-FRET analysis performed in neuronal culture was exactly similar, except the receptor expression was determined by using SSTR4 and δOR specific antibodies followed with FITC and Cy3 conjugated secondary antibodies to create donor and acceptor pair and processed for Pb-FRET analysis.

Receptor internalization

To study receptor internalization, HEK-293 cells stably transfected with cMyc-δOR/HA-SSTR4 were treated with SST-14 (1 µM), SB-205607 (10 nM) alone or in combination for 15
Receptor coupling to adenyl cyclase (AC)

Mono-and cotransfected HEK-293 cells expressing cMyc-δOR and/or HA-SSTR4 were processed for cyclic adenosine monophosphate (cAMP) assay as described [42]. Briefly, cells were treated with receptor specific agonists alone or in combination in presence of 20 μM Forskolin (FSK) and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) for 30 min at 37°C. Cells were then collected in 0.1 N HCl and cAMP was determined by immunofluorescence intensity was performed by using NIH, ImageJ software and the photograph composites were made by using Adobe Photoshop (San Jose, CA). Quantification of immunofluorescence intensity was performed by using NIH, ImageJ software.

Statistical analysis

The data presented in this study were analyzed using GraphPad Prism 4.0. The post hoc Dunnett’s test applied according the experimental conditions to compare with treatments. Significant statistical differences were taken at *p<0.05. Results are presented as mean ± SD unless otherwise stated.

Results

δOR colocalizes with SSTR4 in rat brain cortex, striatum and spinal cord

We first determined whether SSTR4 and δOR colocalize in brain region associated with different functions. Accordingly, colocalization of SSTR4 and δOR was determined in three different regions including the cortex, striatum and the spinal cord (Figure 1A). In cortical brain regions δOR and SSTR4-like immunoreactivity was selectively expressed in specific neuronal populations, displaying distinct morphology and a variable degree of colocalization. Three different neuronal populations, either positive to SSTR4 (green arrow), δOR (red arrow) and displaying colocalization (yellow arrow) were identified. In the cortex, putative glial cells (morphological identification) positive to SSTR4 were lacking δOR-like immuno-reactivity and were devoid of colocalization. In the striatum, two different neuronal populations, either positive to SSTR4 (green arrow) and displaying colocalization (yellow arrow) were observed. Unlike cortex, in the striatum, putative glial cells were strongly positive to δOR and displayed colocalization with SSTR4 (shown by *). In the spinal cord, SSTR4 and δOR were strongly expressed in ventral and dorsal horn, and displayed a region specific colocalization. Motor neurons in the ventral horn expressed strong SSTR4-like immunoreactivity in comparison to δOR. We observed that all δOR positive neurons colocalized with SSTR4 indicating that δOR expression was limited to SSTR4 positive neurons. In addition, some neurons were devoid of colocalization but expressed only SSTR4. Strongly positive δOR cell bodies in the dorsal horn (substantia gelatinosa) displayed a selective colocalization. In comparison, densely innervated nerve fibers in spinal cord rich in δOR immunoreactivity were lacking colocalization. These results provide first molecular basis for pharmacological and physiological interactions between these two receptor subtypes. The specificity of immunoreactivity was determined in the absence of primary antibodies and in presence of pre-immune serum or antigen adsorbed antibodies as described previously [40]. Taken together, consistent observations in three different brain regions revealed that all δOR positive neurons coexpress SSTR4, whereas, some neurons positive to SSTR4 were devoid of δOR like immunoreactivity.

Expression of δOR in SSTR4 immunoprecipitate prepared from rat brain cortex, striatum and spinal cord

In support of colocalization studies, Co-IP assay was performed to determine the δOR expression in SSTR4 immunoprecipitate. δOR was detected in SSTR4 immunoprecipitate prepared from cortical, striatal and spinal cord at the expected molecular size of ~90 kDa in basal as well as following treatment with receptor specific agonists (Figure 1B). Since no loading control is available for quantification in immunoprecipitate assay, the low expression of δOR seen in cortex and spinal cord upon treatment with SSTR4 agonist is not conclusive. These results support that SSTR4 and δOR exist in a heteromeric complex in CNS.

Constitutive heteromeric complex between δOR and SSTR4 in cultured striatal neuronal cells

Colocalization and Co-IP in cortical and striatal neurons can be taken in account to support interaction between SSTR4 and δOR in brain, however, heterodimerization is sensitive to distance, conformational dynamic and receptor orientation at the surface. We next determined the presence of heteromeric complex formation directly in striatal cultured neurons by Ph-FRET analysis. FRET signals were obtained from bleaching profile of donor molecules in presence or absence of acceptor molecules identified by using FITC- and Cy3- labeled antibodies. δOR and SSTR4 displayed colocalization in neuronal cells in a heterogeneous manner throughout the cell body with sparsely distributed colocalization in neuronal processes (Figure 2A). As illustrated in Figure 2B, high relative FRET efficiency of 23±3% in basal condition indicated a constitutive heterodimerization between δOR and SSTR4 in striatal neurons. In neuronal cells, the relative FRET efficiency of 15±2%, 19±3% and 18.5±2% was observed upon treatment with SST (1 μM), L-803087 and SB-205607 (10 nM each) respectively. Of note, simultaneous activation of SSTR4 and δOR displayed FRET efficiency of 21±3% which was relatively higher than the single receptor agonist treatment but comparatively less than the basal condition. As demonstrated in Figure 2C, the relative FRET efficiency was significantly variable at the neuronal cell body confirming heterogeneous population of heterodimers in neuronal cells. The relative FRET efficiency obtained from neuronal processes indicated by arrowheads was significantly lower despite receptor colocalization in comparison to the regions identified by arrows on neuronal cell bodies (Figure 2C). These results provide direct evidence for the association of δOR and SSTR4 as heterodimers in the native system. The loss of effective FRET efficiency in neuronal cells lacking colocalization supports the specificity and selectivity of receptor heterodimerization (Figure 2A; green arrows).
Interaction between δOR and SSTR4 in mammalian HEK-293 cells

Colocalization and co-immunoprecipitation studies in the rat brain cortex, striatum and spinal cord along with the Pb-FRET analysis in the neuronal cells indicated the formation of heteromeric complex between SSTR4 and δOR in native system. To overcome the complexity involved in examining native systems expressing multiple receptors in a single neuronal cell and brain slices, we next determined physical interaction between SSTR4 and δOR in stably cotransfected mammalian HEK-293 cells using Co-IP and Pb-FRET analysis. The membrane extract prepared from control and cells treated with SST (1 μM), L-803087 and SB-205607 (10 nM) alone or in combination was immunoprecipitated with cMyc-antibody (δOR) and immunoblotted with HA-antibody to detect SSTR4. Like brain regions, HA-SSTR4 was detected in the δOR immunoprecipitate at the expected size of SSTR4/δOR heteromeric complex (~90 kDa) in control and cells treated with the receptor specific agonists (Figure 2D). Importantly, the expression of SSTR4 in δOR immunoprecipitate was significantly diminished upon treatment with SST or SSTR4 specific agonist. Conversely, complex formation was stabilized in presence of SB-205607 alone and in combination with SSTR4 specific agonist L-803087. Immunoprecipitate of cotransfected cells prepared in absence of δOR or SSTR4 specific primary antibody was devoid of SSTR4 or δOR expression respectively (Figure S1). These observations confirm the specificity of the heterodimerization between SSTR4 and δOR in cotransfected cells comparable to in vivo system.

Receptor heterodimerization at cell surface is predominantly regulated by δOR

We next determined microscopic Pb-FRET analysis to demonstrate receptor heterodimerization at the cell surface in cotransfected HEK-293 cells (Figures 2E, S2 and S3). As illustrated in Figure 2E, Pb-FRET analysis showed a relatively high FRET efficiency of 16.40 ± 0.54% in the basal condition indicating constitutive heteromeric complex of SSTR4/δOR. Upon treatment with SST or L-803087, effective FRET efficiency was significantly decreased to 5.40 ± 0.27% and 6.69 ± 0.65% respectively. In contrast, relative FRET efficiency of 15.0 ± 0.7% was observed upon treatment with δOR (SB-205607) which was comparable to control. Furthermore, simultaneous activation of δOR and SSTR4 with SB-205607 and SST or L-803087 resulted in relative FRET efficiency of 12.1 ± 0.5% and 14.51 ± 0.71%, respectively (Figures 2E and S3). The relative FRET efficiency remained comparable irrespective of either SSTR4 or δOR being used as donor molecule in bleaching experiment. These results attest to the findings made in brain slices and neuronal cells and indicate the agonist dependent regulation of δOR and SSTR4 heterodimerization process. Furthermore, consistent with previous observations our results also indicate that activation of single interacting receptor is sufficient enough to trigger association or dissociation of heterodimers.

SSTR4 and δOR heterodimerization alter receptor specific trafficking properties upon coactivation in HEK-293 cells

SSTR4 or δOR like many other GPCRs exerts distinct role on signaling pathways when expressed at the cell surface or intracellularly. We next monitored and quantified SSTR4 and δOR membrane and intracellular expression in HEK-293 cells stably transfected with cMyc-δOR and HA-SSTR4 following treatment with receptor-specific agonists. Like neuronal cells, in basal condition SSTR4 and δOR are well expressed and displayed
strong colocalization at the cell membrane as well as intracellularly (Figure 3A). Upon treatment with δOR specific agonist SB-205607, δOR expression was reduced at the membrane, whereas, cells exhibited strong expression of SSTR4 at membrane comparable to control (Figure 3A and B). Agonist induced δOR Internalization resulted in weak colocalization with SSTR4 at the cell surface, accompanied with strong cytoplasmic colocalization of these two receptors. Importantly, permeabilized cells displayed two different populations intracellularly either colocalized or δOR (Figure 3A and C). Like δOR, the expression of SSTR4 at the cell surface was significantly diminished in the presence of SST and, consequently, enhanced
intracellularly when compared to control (Figure 3A, B and C). Interestingly, unlike the individual receptor activation, co-activation of SSTR4 and δOR in combination displayed colocalization at cell surface as well as intracellularly, without any discernible changes from control (Figure 3A, B and C). The present data demonstrates that receptor internalization induced by a single treatment is opposed by simultaneous activation of both receptors. Most importantly, these results elucidate that the unique property of individual receptors was changed or stabilized in cotransfected cells upon activation of both protomers, attributed to receptor heterodimerization. These results also strengthen the concept that SSTR4 mediated retention of δOR at the cell surface might enhance analgesic effect.

Changes in receptor coupling to AC
Receptor coupling to AC and the formation of cAMP through different heterotrimeric G-proteins is a characteristic of GPCRs functionality [47]. SSTR and OR subtypes negatively couple to AC and inhibit the formation of cAMP in a receptor-specific Gi dependent manner. To test whether activation of SSTR4 or δOR alone or in combination regulates cAMP in a distinct manner, cotransfected HEK-293 cells were treated with FSK with and without SST, SSTR4 and δOR specific agonists for 30 min and processed for cAMP. As shown in Figure 4A, FSK stimulated cAMP was inhibited by 26.2±0.83, 25.2±0.64 and 19.9±0.63% in the presence of SST, L-803087 and SB-205607, respectively. SST-14 (1 μM) in combination with SB-205607 (10 nM) induced an inhibition by 37.2±0.56% which was significantly higher than the single agonist treatment. Having established that SST, L-803087 and SB-205607 inhibit cAMP formation with enhanced inhibition in combination prompted us to determine the concentration dependent effect of SST (10⁻¹² – 10⁻⁶ M) alone or in combination with SB-205607 (10⁻² M) on cAMP inhibition (Figure 4B). The treatment of cells with increasing concentration of SST (10⁻¹² – 10⁻⁶ M) exhibited enhanced inhibition of cAMP in dose dependent manner. Interestingly, the inhibition of cAMP was significantly enhanced upon treatment with SST (10⁻¹² – 10⁻⁶ M) in combination with SB-205607 (10 nM) in comparison to SST (1 μM) treatment alone (Figure 4B). In contrast, increasing concentrations of SB-205607 (10⁻⁶ – 10⁻¹⁸ M) alone or in combination with SST (1 μM) also displayed cAMP inhibition but the efficiency of inhibition was significantly lower than SST (10⁻¹² – 10⁻⁶ M) alone or in combination with SB-205607 (10 nM) (Data not shown). Taken in consideration, these data strongly indicate that maximal inhibition achievable was significantly higher upon co-activation of the receptors in comparison to single receptor activation. Also, indicates that SSTR4 and δOR function synergistically and activation of SSTR4 in heteromeric complex is predominantly responsible for inhibition of FSK stimulated second messenger cAMP.

Negative regulation of cAMP formation is associated with the inhibition of PKA phosphorylation
Dolan et al, proposed a biphasic modulation of nociception and demonstrated the role of cAMP/PKA pathway in hypoalgesia and hyperalgesia [48]. To determine whether the inhibition of FSK stimulated cAMP also regulates PKA phosphorylation, mono and/or cotransfected cells were treated with receptor specific agonist alone and/or in combination for 15 min at 37 °C and cell lysate processed for PKA expression and phosphorylation. Upon treatment with receptor specific agonists in SSTR4 or δOR monotransfected cells, the status of phosphorylated PKA remained comparable to the control (Figure 4C). In comparison, PKA phosphorylation was inhibited in presence of SST, SSTR4 and δOR agonist in cotransfectants and cells displayed significantly higher inhibition of phospho-PKA upon combined treatment as indicated (Figure 4D). Furthermore, inhibition of PKA phosphorylation correlates with the suppression of FSK stimulated cAMP and strengthens the notion that cAMP/PKA function in an integrated manner. To demonstrate G protein dependency, we next determined the effect of Gi inhibitor PTX on PKA phosphorylation. Cotransfected cells pretreated with PTX displayed significantly high level of PKA phosphorylation in control or upon agonist specific treatments (Figure 4A). Taken together, these findings demonstrate a close association between cAMP/PKA, SSTR4/δOR heterodimerization and Gi dependency.

Receptor heterodimerization modulates ERK1/2 and ERK5 MAPKs signaling
Receptor oligomerization exerts crucial role in the modulation of multiple downstream signaling pathways with distinct physiological responses of cells. MAPKs, including ERKs, are linked to the induction and maintenance of neuropathic pain [49], accordingly, the status of ERK1/2 phosphorylation was determined in mono- and/or cotransfected cells. Monotransfected cells expressing SSTR4 displayed inhibition of phospho-ERK1/2 in the presence of SST and L-803087 in comparison to control (Figure 3A). In contrast, in cells expressing δOR, the status of phospho-ERK1/2 was comparable to the control in the presence of δOR agonist SB-205607 and interestingly cells were devoid of phospho-ERK1 isoform. In cotransfected cells, ERK1/2 phosphorylation was comparatively higher upon SSTR4 activation than δOR when compared to the control (Figure 3B). SST or SSTR4 specific agonist in combination with SB-205607 displayed enhanced ERK1/2 phosphorylation although relatively less in comparison to SST or SSTR4 agonist treatment alone. Our results further revealed significant differences in the phosphorylation of ERK1 in comparison to ERK2 isoform. To ascertain whether ERK1/2 is Gi sensitive or insensitive, cells were exposed to PTX prior to agonist treatment. As shown, phospho-ERK1/2 was highly expressed in basal condition without any significant changes in cells treated with receptor specific agonist (Figure 4B). These results are an indication of Gi dependency in cotransfected cells.

Pain hypersensitivity in response to nerve injury has been linked to the activation of ERK5 [50]. To determine whether crosstalk between SSTR4 and δOR and changes in the status of ERK1/2 are also involved in the regulation of ERK5 phosphorylation, mono- and/or cotransfected cells were processed for total and phosphorylated ERK5 expression. Monotransfected cells expressing SSTR4 displayed increased phospho-ERK5 in the presence of SST and L-803087, whereas, the status of phospho-ERK5 remained comparable with or without agonist treatment in cells expressing δOR (Figure 3C). In comparison, cells cotransfected with SSTR4/δOR, SSTR4 mediated activation of ERK5 was maintained with or without SB-205607 (Figure 3D). Interestingly, ERK5 phosphorylation was completely abolished in cells pre-treated with PTX (Figure 4C). These results uncovered Gi dependent ERK5 phosphorylation and signify that SSTR4 exerts differential effects on the regulation of ERK1/2 and ERK5 phosphorylation in mono- and/or cotransfected cells.

PI3K phosphorylation is receptor specific and dependent on Gi
Previous studies have shown that the inhibition of PI3K abrogates the anti-nociceptive effects of μOR and δOR agonists [51]. We next compared the status of activated PI3K in cells
expressing SSTR4 and/or δOR. As shown in Figure 6A, the status of activated PI3K was comparable to control in monotransfectant expressing SSTR4 upon treatment with SST or SSTR4 specific agonist. In contrast, phospho-PI3K expression was not detected in cells expressing δOR with or without agonist specific activation. In cotransfected cells, sustained activation of phospho-PI3K was observed in control as well as following treatment with SSTR4 or δOR specific agonist alone and in combination (Figure 6B). These results indicate that PI3K phosphorylation in cotransfected cells is predominantly SSTR4 dependent and comparable to SSTR4 monotransfected cells.

Activation of PI3K was abolished in cells treated with PTX indicating the Gi dependent effect on PI3K phosphorylation following either receptor activation (Figure S4D).

Changes in phospho-AKT as downstream effectors of PI3K are not associated with PI3K

The PI3K/AKT signaling pathway is linked to the development of neuropathic pain [52]. Whether PI3K and its downstream effector AKT function in a coordinated manner or distinctly, we next examined the status of AKT phosphorylation in cells expressing SSTR4 and/or δOR upon treatments as indicated.

Figure 6. Co-activation of SSTR4 and δOR retained receptors expression at the cell surface. (A) Representative confocal photomicrographs illustrating membrane and intracellular expression of SSTR4 and δOR in non-permeabilized (NP) and permeabilized (P) HEK-293 cells. Indicated color in red, green and yellow/orange (merged images) represents the expression of δOR, SSTR4 and colocalization respectively. In cotransfectants, the activation of SSTR4 (upper panel right) or δOR (lower panel left) preferentially promotes receptor internalization which was blocked upon combined agonist treatment. Histogram in panels B and C showed the quantification of receptor expression in non-permeabilized (NP) and permeabilized (P) conditions respectively performed by using NIH Image J software. Note the significant loss in expression of δOR (B, red histogram) and SSTR4 (B, green histogram) at cell surface upon treatment with receptor specific agonists as indicated. Intracellular expression of δOR (C, red histogram) and SSTR4 (C, green histogram) was significantly enhanced following treatments with SB-205607 and SST alone without having any discernible effects in combination (*, p<0.05). Scale bar = 10 μm.

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Figure 3. Co-activation of SSTR4 and δOR retained receptors expression at the cell surface. (A) Representative confocal photomicrographs illustrating membrane and intracellular expression of SSTR4 and δOR in non-permeabilized (NP) and permeabilized (P) HEK-293 cells. Indicated color in red, green and yellow/orange (merged images) represents the expression of δOR, SSTR4 and colocalization respectively. In cotransfectants, the activation of SSTR4 (upper panel right) or δOR (lower panel left) preferentially promotes receptor internalization which was blocked upon combined agonist treatment. Histogram in panels B and C showed the quantification of receptor expression in non-permeabilized (NP) and permeabilized (P) conditions respectively performed by using NIH Image J software. Note the significant loss in expression of δOR (B, red histogram) and SSTR4 (B, green histogram) at cell surface upon treatment with receptor specific agonists as indicated. Intracellular expression of δOR (C, red histogram) and SSTR4 (C, green histogram) was significantly enhanced following treatments with SB-205607 and SST alone without having any discernible effects in combination (*, p<0.05). Scale bar = 10 μm.

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Heterodimerization between SSTR4 and δOR PLOS ONE | www.plosone.org 7 January 2014 | Volume 9 | Issue 1 | e85193
Figure 4. Regulation of cAMP/PKA signaling pathways in receptor and agonist dependent manner. (A) Receptor coupling to adenylyl cyclase. SST, L-803087 and SB-205607 displayed significant inhibition of FSK stimulated cAMP in comparison to control. Data is representative of three independent experiments and presented as % inhibition upon treatment as indicated. (B) Concentration dependent inhibition of cAMP in HEK-293 cells. Treatment of cells with FSK alone was taken as 0% inhibition and treatment with forskolin and SST (1 μM) was considered as 100% inhibition. Note the significant increase in the efficiency of cAMP inhibition upon treatment with SST (10^{-12} - 10^{-6} M) in combination with SB-205607 (10 nM). (C and D) PKA phosphorylation in mono- and/or cotransfected cells. HEK-293 cells expressing SSTR4 and/or δOR were treated for 15 min at 37°C and cell lysate prepared was subjected to western blot analysis. In monotransfected cells, the status of phospho-PKA was comparable to basal upon receptor specific activation (C). In cotransfected cells, significant inhibition of PKA phosphorylation was observed which was further enhanced upon combined agonist treatment as indicated (D). Densitometric analysis for phospho-PKA was performed by using β-actin or total as loading control and data analysis was done by using ANOVA and post hoc Dunnett’s to compare against basal level (*, p<0.05).
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Figure 5. Receptors mediated changes in ERK1/2 and ERK5 phosphorylation. (A) HEK-293 cells expressing SSTR4 and/or δOR were treated as indicated for 15 min at 37 °C and cell lysate was subjected to Western blot analysis. In SSTR4 monotransfected cells, SST and SSTR4 agonist resulted in inhibition of phospho-ERK1/2 when compared to control, whereas, δOR activation was without any significant effect on ERK1/2 phosphorylation. (B) In cotransfected cells, SSTR4 activation displayed increased ERK1/2 phosphorylation. In presence of δOR agonist alone or co-expression, ERK1/2 phosphorylation was increased significantly compared with control. In cotransfected cells, δOR agonist alone or co-expression potentiated the activation of ERK1/2 phosphorylation in comparison to control. In Figure 6A-D, the expression of δOR or SSTR4 specific agonist (L-803087) resulted in increased ERK1/2 activation, indicating AKT phosphorylation is Gi dependent. (Figure 6C and D). In SSTR4 monotransfected cells, activated AKT was comparable to control upon treatment with SST and SSTR4 specific agonist (L-803087) (Figure 6C). In contrast, cell expressing δOR exhibit enhanced AKT phosphorylation in presence of SB-205607 when compared to control. These results are contrary to the pattern of PI3K expression in presence of SB-205607 when compared to control. These results are consistent with previous studies, we argue that activation of δOR preferentially distinguishes between homodimers of δOR from heteromeric complex of SSTR4 and δOR. In contrast to cotransfected HEK-293 cells, FRET efficiency in neuronal cells was relatively less when neuronal cells were treated with either SSTR4 or δOR agonist in comparison to control. Although, co-activation of both interacting protomers exhibit lower FRET efficiency, but was enhanced in comparison to SSTR4 activation alone. The selective and preferential heterodimerization in native and heterologous system revealed the possibility of artifact in FRET due to receptor overexpression. The membrane expression of ORs is critical for analgesic effect and studies have shown that μOR is more efficient in regulating nociception than δOR due to elevated membrane expression. In support, our results in cotransfected cells revealed that the presence of SSTR4 averts δOR internalization and retains the receptor at cell surface as a complex, whereas, activation of individual receptor displayed receptor trafficking like monotransfectants. These results indicate a close association between receptor trafficking and heterodimerization. Importantly, our results suggest that SSTR4 might enhance δOR mediated analgesic properties by retaining δOR at the cell surface.

Discussion

In the present study, we provide morphological, biochemical, and biophysical evidences supporting oligomerization between δOR and SSTR4 ex vivo and in vitro in rat brain, cultured striatal neurons and stably transfected HEK-293 cells. These observations suggest that heterodimerization of δOR and SSTR4 in fact creates a novel receptor complex with functional diversity. The consequences of cellular response upon activation of both interacting protomer are more pronounced than a single receptor in heterologous system. We also describe the heterogeneous complex formation between δOR and SSTR4 in neuronal cells despite strong receptor colocalization. The activation of SSTR4 and δOR in combination leads to a greater inhibition of cAMP/PKA and modulation of ERK1/2 and ERK5 than the activation of individual receptors. Importantly, while cell surface heterodimerization is essentially regulated by δOR, the signaling pathways are predominantly influenced by SSTR4. Our observations have uncovered that MAPKs (ERK1/2 and ERK5) and PI3K/AKT phosphorylation is regulated in Gi dependent and independent manner in cotransfected cells. This is the first comprehensive description providing receptor interaction and its functional consequences in modulation of pain related signaling pathways.

Despite strong colocalization of SSTR4 and δOR in neuronal cell body and processes, differences in relative FRET efficiency imply that receptor orientation and conformational dynamics at the cell surface is crucial for protein-protein interaction. Since neuronal cells expressed more than one receptor subtypes thus, the possibility of complex formation with other receptors cannot be ruled out from the discussion. However, future studies are warranted to determine the functional significance of heterogeneity in the receptor complex formation at neuronal soma and neuronal processes in synaptic transmission and plasticity.

The loss of FRET efficiency upon activation of SSTR4 with either SST or receptor specific agonist is in agreement with the concept of receptor complex dissociation and internalization. Consistent with previous studies, we argue that activation of δOR preferentially distinguishes between homodimers of δOR from heteromeric complex of SSTR4 and δOR. Consistent with previous studies, we argue that activation of δOR preferentially distinguishes between homodimers of δOR from heteromeric complex of SSTR4 and δOR. In contrast to cotransfected HEK-293 cells, FRET efficiency in neuronal cells was relatively less when neuronal cells were treated with either SSTR4 or δOR agonist in comparison to control. Although, co-activation of both interacting protomers exhibit lower FRET efficiency, but was enhanced in comparison to SSTR4 activation alone. The selective and preferential heterodimerization in native and heterologous system revealed the possibility of artifact in FRET due to receptor overexpression. The membrane expression of ORs is critical for analgesic effect and studies have shown that μOR is more efficient in regulating nociception than δOR due to elevated membrane expression. In support, our results in cotransfected cells revealed that the presence of SSTR4 averts δOR internalization and retains the receptor at cell surface as a complex, whereas, activation of individual receptor displayed receptor trafficking like monotransfectants. These results indicate a close association between receptor trafficking and heterodimerization. Importantly, our results suggest that SSTR4 might enhance δOR mediated analgesic properties by retaining δOR at the cell surface.

cAMP pathway in G-protein dependant manner is associated with nociception [53]. Increased cAMP is allied with behavioural symptoms of withdrawal and enhanced action potential in neurons [54]. Moreover, inhibitors of PKA alleviate withdrawal symptoms [55]. The synergistic activation of SSTR4 and δOR display pronounced inhibition of cAMP formation in comparison to the activation of SSTR4 or δOR independently. Increased cAMP formation is seen as a consequence of withdrawal upon chronic use of morphine, thus, our results demonstrate that SST in combination with opioids might maintain sustained inhibition of cAMP even during withdrawal [54,56]. Most importantly, significant inhibition of cAMP despite the loss in relative FRET efficiency upon activation of SSTR4 indicates that receptor coupling to AC and receptor heterodimerization are two independent processes. It is highly possible that this inhibition is attributed to SSTR4 homodimers. Previous studies have shown that PKA inhibitor H-89 reversed the mechanical hypalgesia induced by cAMP analogue [48]. Our results demonstrate an inhibition of PKA phosphorylation upon SSTR4 and δOR heterodimerization and provide direct physiologically relevant evidence that SSTR4 in concert with δOR might involve in inhibition of cAMP/PKA. In addition, the present results suggest that the inhibition of cAMP/PKA is Gi dependent because PKA phosphorylation was significantly upregulated in the presence of Gi inhibitor PTX.

MAPKs play critical role in pain progression [57,58].
Heterodimerization between SSTR4 and δOR

A) Monotransfected Cells
- SSTR4
- δOR

B) Cotransfected Cells
- SSTR4+δOR

C, D) Western Blot Analysis
- pAKT
- tAKT
- β-Actin

Percentage Over Control

Control, SST, L-803087, SB-205607

* indicates significant difference compared to control.
Figure 6. Changes in AKT phosphorylation are independent to PI3K activation. (A) HEK-293 cells expressing SSTR4 and/or δOR were treated as indicated for 15 min at 37°C and cell lysate prepared post treatment was subjected to western blot analysis to detect phospho and total-Pi3K and AKT expression. The status of phospho-Pi3K remained comparable to basal upon treatment with SST or SSTR specific agonist in cells expressing SSTR4. In contrast, δOR monotransfectant were devoid of phospho-Pi3K expression with or without receptor specific treatment. (B) Significant activation of phospho-Pi3K was observed in cotransfected cells at the basal level which remained comparable upon agonist treatments as indicated. (C) δOR monotransfected cells displayed significant increase in phospho-AKT expression upon treatment with receptor specific agonist in comparison to control, whereas, no discernible changes in phospho-AKT were observed in SSTR4 monotransfected cells. (D) In cotransfected cells, SSTR4 activation with SST or L-803087 alone or in combination with SB-205607 significantly enhanced AKT phosphorylation, with pronounced effect upon combined agonist treatment. Histograms illustrate densitometry for the blots for respective panels using β-actin or total as loading control. Data analysis was done by using ANOVA and post hoc Dunnett’s to compare against basal level (*, p < 0.05).

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Figure S1 Specificity of heterometric complex formation in HEK-293 cells. Cotransfected cells expressing SSTR4 and δOR were processed for Co-IP as indicated to determine the specificity of heterodimerization. Cells were treated with receptor specific agonist for 30 min at 37°C. The membrane fraction was isolated and solubilized with Tris-buffer and incubated with protein A/G agarose beads in absence of primary antibodies. The samples were electrophoresed, transferred to PVDF and incubated with δOR or SSTR4 (1:250) specific primary antibodies (overnight at 4°C). Note that no expression of δOR or SSTR4 was detected in the immunoprecipitate prepared from cotransfected cells. The absence of bands at the expected molecular weights in either control or treated condition indicates the specificity of heterodimerization. Data are representative of three independent experiments.

(TIF)

Figure S2 Microscopic Pb-FRET analysis in cells coexpressing δOR and SSTR4. (A) Representative photomicrographs illustrating HA-SSTR4 (green) and cMyc-δOR (red) and colocalization (yellow) in cotransfected HEK-293 cells. Microscopic Pb-FRET was performed as described in Material and Methods. (B and D) A selection of photomicrographs illustrating photobleaching profile taken from the cells incubated with the donor alone (B) and in the presence of acceptor (D). Histograms shown in panels (C and E) represent pixel by pixel analysis of time constant of donor in absence or presence of acceptor. The
shown in panels (Panels B and D), presence of acceptor comparison to control (Figure S2). Representative photomicrographs illustrating bleaching profile of the donor in the absence or presence of acceptor (Panels B and D), whereas, histograms shown in panels C and E represent pixel by pixel analysis of time constant of the donor alone or donor + acceptor respectively upon co-activation of the receptors. Data are representative of three independent experiments and 50–60 cells were analyzed per experiment. (TIF)

Figure S3 Changes in relative FRET efficiency upon Co-activation of SSTR4 and δOR. HEK-293 cells expressing HA-SSTR4 (green) and cMyc-δOR (red) were treated with SST-14 (1 μM) and SB-205607 (10 nM) in combination for 15 min at 37°C. Combined activation of SSTR4 and δOR with receptor specific agonists displayed loss in relative FRET efficiency in comparison to control (Figure S2). Representative photomicrographs illustrating bleaching profile of the donor in the absence or presence of acceptor (Panels B and D), whereas, histograms shown in panels C and E represent pixel by pixel analysis of time constant of the donor alone or donor + acceptor respectively upon co-activation of the receptors. Data are representative of three independent experiments and 50–60 cells were analyzed per experiment. (TIF)

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Author Contributions
Conceived and designed the experiments: RKS UK. Performed the experiments: RKS UK. Analyzed the data: RKS UK. Contributed reagents/materials/analysis tools: UK. Wrote the paper: RKS UK.

References
1. Jordan BA, Cvejic S, Devi LA (2000) Opioids and their complicated receptor complexes. Neuropepsychopharmacology 23: 55–S18.
2. Kieffer BL (1999) Opioids: first lessons from knockout mice. Trends Pharmacol Sci 20: 19–26.
3. Cooper A, Rice KC (2000) Role of delta-opioid receptors in biological processes. Drug News Perspect 13: 481–487.
4. Gaverez-Ruff C, Kieffer BL (2002) Opioid receptor genes inactivated in mice: the highlights. Neuropeptides 36: 62–71.
5. Hammond DL, Wang H, Nakashima N, Basbaum AI (1998) Differential effects of intracranially administered delta and mu opioid receptor agonists on formalin-evoked nociception and on the expression of Fos-like immunoreactivity in the spinal cord of the rat. J Pharmacol Exp Ther 296: 378–387.
6. Quock RM, Burton TH, Varga E, Hosohata Y, Hosohata K, et al. (1999) The delta-opioid receptor: molecular pharmacology, signal transduction, and the determination of drug efficacy. Pharmacol Rev 51: 503–532.
7. Stewart PE, Hammond DL (1993) Evidence for delta opioid receptor subtypes in rat spinal cord: studies with intracranial naltrinden, cyclic[D-Pen2, D-Pen5] enkephalin and [D-Ala2, Glu4]delalorphin. J Pharmacol Exp Ther 266: 620–628.
8. Buzas B, Cox BM (1997) Quantitative analysis of mu and delta opioid receptor gene expression in rat brain and peripheral ganglia using competitive polymerase chain reaction. Neuroscience 76: 479–489.
9. Gaverez-Ruff C, Noguchi G, Nakai N, Hever XC, Weibel R, et al. (2011) Genetic ablation of delta opioid receptors in nociceptive sensory neurons increases chronic pain and abolishes opioid analgesia. Pain 152: 1238–1248.
10. Jordan BA, Devi LA (1999) G-protein-coupled receptor heterodimerization modulates receptor function. Nature 399: 697–700.
11. Chrubasik J, Chrubasik S, Martin E (1993) Non-opioid peptides for analgesia. Acta Neurobiol Exp (Wars) 53: 289–296.
12. Penn RD, Pace JA, Kroin JS (1992) Octreotide: a potent new non-opiate analgesic for intrathecal infusion. Pain 49: 13–19.
13. Lanecranz I, Atkinson AB (1999) Results of a European multicentre study with Sandostatin LAR in acromegalic patients. Sandostatin LAR Group. Panunity 1: 105–114.
14. Schulz S, Schreier M, Schmitt H, Handel M, Prazrovicki R, et al. (1998) Immunocytochemical localization of somatostatin receptor sst2A in the rat spinal cord and dorsal root ganglia. Eur J Neurosci 10: 3700–3708.
15. Chrubasik J (1984) Low-dose epidural morphine by infusion pump. Lancet 1: 720–729.
16. Folds FF (1991) Pain control with intrathecally and peripherally administered opioids and other drugs. Anaesthesia Reanim 16: 287–298.
17. Folds FF, Nagashima H, Nguyen HD, Schiller WS, Mason MM, et al. (1991) The neuromuscular effects of ORG9426 in patients receiving balanced anesthesia. Anesthesiology 75: 191–196.
18. Sicentt F, Geppeppi P, Marabini S, Lemark B (1984) Pain relief by somatostatin in attacks of cluster headache. Pain 18: 359–365.
19. Williams G, Ball J, Bloom S, Joplin GF (1966) Improvement in headache associated with prolactinoma during treatment with a somatostatin analogue: an "N of 1" study. N Eng J Med 315: 1166–1167.
20. Molloyehi P, Rawal N, Gorth T, Jr., Olsson Y (1994) Intrathecal and epidural somatostatin for patients with cancer. Analgesic effects and postmortem neuropathologic investigations of spinal cord and nerve roots. Anesthesiology 81: 534–542.
21. Patel YC (1999) Somatostatin and its receptor family. Front Neuroendocrinol 20: 157–198.
22. Van Ooijen Bosch J, Torfi P, De Winter BY, De Man JG, Piekman MA, et al. (2009) Effect of genetic SSTR4 ablation on inflammatory peptide and receptor expression in the non-inflamed and inflamed murine intestine. J Cell Mol Med 13: 3203–3209.
23. Helges Z, PIeter E, Sandor K, Elekes K, Banovolsky A, et al. (2009) Improved defense mechanism against inflammation, hyperalgesia, and airway hyperreactivity in somatostatin 4 receptor gene-deleted mice. Proc Natl Acad Sci U S A 106: 13088–13093.
24. Casada Z, Dournaud P (2001) Cellular biology of somatostatin receptors. Neuropeptides 35: 1–23.
25. Kieffer BL (1995) Recent advances in molecular recognition and signal transduction of active peptides: receptor for opioid peptides. Cell Mol Neurobiol 15: 615–635.
26. Knapp RJ, Malatyuk E, Collins N, Fan L, Wang JY, et al. (1995) Molecular biology and pharmacology of cloned opioid receptors. FASEB J 9: 516–525.
27. Law PV, Yong YH, Loh HH (2000) Molecular mechanisms and regulation of opioid receptor signaling. Annu Rev Pharmacol Toxicol 40: 392–439.
28. Pfeiffer M, Koch T, Schroder H, Laugwisch M, Holt V, et al. (2002) Heterodimerization of somatostatin and opioid receptors cross-modulates phosphorylation, internalization, and desensitization. J Biol Chem 277: 19562–19572.
29. Hawkins KN, Knapp RJ, Gehlert DR, Lui GK, Yamamura MS, et al. (1988) Quantitative autoradiography of [3H]CTOP binding to mu opioid receptors in rat brain. Life Sci 42: 2541–2551.
30. Hawkins KN, Knapp RJ, Liu GK, Gulya K, Kazmerski W, et al. (1989) [3H-D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH2] ([3H]CTOP), a potent and highly selective peptide for mu opioid receptors in rat brain. J Pharmacol Exp Ther 248: 73–80.
31. Pelton JT, Kazmerski W, Gulya K, Yamamura HI, Hruby VJ (1986) Design and synthesis of conformationally constrained somatostatin analogues with high potency and specificity for mu opioid receptors. J Med Chem 29: 2370–2375.
32. Pugley TA, Lippmann W (1978) Effect of somatostatin analogues and 17-alpha-dihydroequilin on rat brain opiate receptors. Res Commun Chem Pathol Pharmacol 21: 153–156.
33. Schulz R, Wehmeyer A, Schulz K (2002) Opioid receptor types selectively cointernalize with G protein-coupled receptor kinases 2 and 3. J Pharmacol Exp Ther 300: 376–384.
34. Terenius L (1976) Somatostatin and ACTH are peptides with partial antagonist-like selectivity for opiate receptors. Eur J Pharmacol 38: 211–213.
35. Vincent SR, Johanson O, Hokfelt T, Meyerson B, Sach S, et al. (1982) Neuropeptide coexistence in human cortical neurones. Nature 298: 65–67.
36. Mika J, Przewlocki R, Przewlocka B (2001) The role of delta-opioid receptor subtypes in neuropathic pain. Eur J Pharmacol 415: 51–37.
37. Kovalevski CJ, Biaio D, Hruby VJ, Lai J, Ossipow MH, et al. (1999) Selective opioid delta agonists elicit antinociceptive supraspinally/spinal synergy in the rat. Brain Res 843: 12–17.
53. Skyba DA, Radhakrishnan R, Bement MKH, Sluka KA (2004) The cAMP pathway and pain: potential targets for drug development. Drug Discovery Today Disease Models 1: 115–119.

54. Xu JT, Tu HY, Xin HY, Liu XG, Zhang GH, et al. (2007) Activation of phosphatidylinositol 3-kinase and protein kinase B/Akt in dorsal root ganglia and spinal cord contributes to the neuropathic pain induced by spinal nerve ligation in rats. Exp Neurol 206: 269–279.

55. Skyba DA, Radhakrishnan R, Bement MKH, Sluka KA (2004) cAMP trafficking pathway and pain: potential targets for drug development. Drug Discovery Today 9: 271–282.

56. Fraser GL, Gaudreau GA, Clarke PB, Menard DP, Perkins MN (2000) Antihyperalgesic effects of delta opioid agonists in a rat model of chronic inflammation. Br J Pharmacol 129: 1661–1672.

57. Narita M, Ohnishi O, Nemoto M, Yajima Y, Suzuki T (2002) Implications of extracellular signal-regulated protein kinases 5 in spinal microglia and primary sensory neurons for neuropathic pain. J Neurochem 102: 1569–1584.

58. Dai M, Sumida T, Tagami M, Ide Y, Nagase M, et al. (2002) Suppressive effect of naloxone in cats. J Anesth 16: 211–215.

59. Bagley EE, Gerke MB, Vaughan CW, Hack SP, Christie MJ (2005) GABA transporter currents activated by protein kinase A excite midbrain neurons during opioid withdrawal. Neuron 45: 433–445.

60. Macey TA, Bobeck EN, Hegarty DM, Aicher SA, Ingram SL, et al. (2009) Extracellular signal-regulated kinase 1/2 activation counteracts morphine tolerance in the periaqueductal gray of the rat. J Pharmacol Exp Ther 331: 412–418.

61. Alter BJ, Zhao C, Karim F, Landreth GJ, Woolf CJ (1999) Nociceptive-specific activation of ERK in spinal neurons contributes to pain hypersensitivity. Nat Neurosci 2: 1114–1119.

62. Walker JM, Bowen WD, Atkins ST, Hemstreet MK, Coy DH (1987) Mu-opiate receptor specificity and functional significance. Pharmacol Rev 57: 289–298.

63. Walker JM, Bowen WD, Atkins ST, Hemstreet MK, Coy DH (1987) Mu-opiate receptor specificity and functional significance. Pharmacol Rev 57: 289–298.

64. Gulya K, Pelton JT, Hruby VJ, Duckles SP, Yamamura HI (1985) Conformationally restricted analogs of somatostatin with high mu-opiate receptor specificity. Proc Natl Acad Sci U S A 82: 236–239.

65. Notas G, Kampa M, Nifli AP, Xidakis K, Papasava D, et al. (2007) The inhibitory effect of opioids on HepG2 cells is mediated via interaction with somatostatin receptors. Eur J Pharmacol 555: 1–7.

66. Notas G, Kolios G, Mastrodimou N, Kampa M, Vasilaki A, et al. (2004) Cortistatin production by HepG2 human hepatocellular carcinoma cell line and distribution of somatostatin receptors. J Hepatol 40: 792–798.

67. Pitcher JA, Freedman NJ, Lefkowitz RJ (1998) G protein-coupled receptor kinases. Annu Rev Biochem 67: 653–692.

68. Freedman NJ, Lefkowitz RJ (1996) Desensitization of G protein-coupled receptors. Recent Prog Horm Res 51: 319–351; discussion 352–313.

69. Sterne-Marr R, Benovic JL (1995) Regulation of G protein-coupled receptors by receptor kinases and arrestins. Vitam Horm 51: 193–234.

70. Narita M, Inai S, Narita M, Kasukawa A, Yajima Y, et al. (2004) Increased level of neuronal phosphoinositide 3-kinase gamma by the activation of mu-opioid receptor in the mouse periaqueductal gray matter: further evidence for the implication in morphine-induced antinociception. Neuroscience 129: 515–521.

71. Cunha TM, Roman-Campos D, Lotofo CM, Duarte HL, Souza GR, et al. (2010) Morphine peripheral analgesia depends on activation of the PI3K-gamma/akt/nNOS/NO/KATP signaling pathway. Proc Natl Acad Sci U S A 107: 4442–4447.