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Heterodimerization of ORL1 and Opioid Receptors and Its Consequences for N-type Calcium Channel Regulation*

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We have investigated the heterodimerization of ORL1 receptors and classical members of the opioid receptor family. All three classes of opioid receptors could be co-immunoprecipitated with ORL1 receptors from both transfected tsA-201 cell lysate and rat dorsal root ganglia lysate, suggesting that these receptors can form heterodimers. Consistent with this hypothesis, in cells expressing either one of the opioid receptors together with ORL1, prolonged ORL1 receptor activation via nociceptin application resulted in internalization of the opioid receptors. Conversely, μ-, δ-, and κ-opioid receptor activation with the appropriate ligands triggered the internalization of ORL1. The μ-opioid receptor/ORL1 receptor heterodimers were shown to associate with N-type calcium channels, with activation of μ-opioid receptors triggering N-type channel internalization, but only in the presence of ORL1. Furthermore, the formation of opioid receptor/ORL1 receptor heterodimers attenuated the ORL1 receptor-mediated inhibition of N-type channels, in part because of constitutive opioid receptor activity. Collectively, our data support the existence of heterodimers between ORL1 and classical opioid receptors, with profound implications for effectors such as N-type calcium channels.

ORL1 (opioid receptor-like 1) receptors (also known as NOP or nociceptin receptors) belong to the class of $G\alpha_{i/o}$-linked seven-helix transmembrane receptors (1, 2). They are structurally related to members of the classical opioid receptor family (*i.e.* μ-, δ-, and κ-opioid receptors) but do not interact with known opioid receptor agonists or antagonists. Instead, they are activated by the endogenous ligand orphanin FQ (also known as nociceptin), a 17-amino acid polypeptide (3). ORL1 receptors are expressed in both the central and peripheral nervous systems; the physiological effects of receptor activation include stimulation of food intake, reduced anxiety, reduced withdrawal symptoms, and when activated peripherally, analgesia (4–6). There is a functional cross-talk between ORL1 receptors and μ-opioid receptors such that chronic administration of the μ-receptor agonist morphine results in increased ORL1 receptor expression levels (7), whereas knock-out of the ORL1 receptor gene results in decreased morphine tolerance (8) without compensatory changes in opioid receptor expression (9). This may hint at the possibility of overlapping mechanisms controlling cellular expression levels of these two receptor subtypes.

We have recently shown that ORL1 receptors physically interact with N-type calcium channels and that this interaction results in two distinct consequences: first, an agonist-independent inhibition of N-type channels due to constitutive receptor activity (10), and second, receptor-mediated trafficking of N-type channels to and from the plasma membrane (11). Neither of these phenomena appeared to occur with μ-opioid receptors (11). ORL1 receptors have also been shown to heterodimerize with μ-opioid receptors (12), with dimerized receptors showing altered sensitivity to μ-opioid receptor agonists and receptor desensitization (12, 13). These findings suggest the possibility of larger, macromolecular signaling complexes involving multiple receptor types and voltage-gated calcium channels. Here, we show that ORL1 receptors also heterodimerize with the other members of the opioid receptor family and can co-internalize each other upon agonist exposure. Moreover, we show that, upon dimerization with opioid receptors, ORL1 receptor regulation of N-type channels is altered. Finally, we show that the ORL1 receptor can function as a molecular link that allows μ-opioid receptors to trigger N-type channel internalization. Considering the well documented involvement of both N-type channels and opioid receptors in the pain pathway, these functional interactions may provide novel avenues for regulating pain behavior.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—tsA-201 cells were cultured and transfected as previously reported (14). Cells were replated at ~8% confluence on either poly-L-lysine-coated glass coverslips or 35-mm glass-bottomed dishes (MatTek Corp.). Cells used for electrophysiology experiments were moved to 30 °C after transfection, whereas those used for confocal microscopy or Western blotting were maintained at 37 °C until used. In cells used for electrophysiological recordings, enhanced yellow fluorescent protein (YFP,[2] 0.5 μg; Clontech) was included as a transfection marker.

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[2] The abbreviations used are: YFP, yellow fluorescent protein; CFP, cyan fluorescent protein; PBS, phosphate-buffered saline; DRG, dorsal root ganglion; HA, hemagglutinin; DAMGO, [D-Ala[2],*N*-Me-Phe[4],Gly[5]-ol]enkephalin.

Molecular Biology—The C-terminally YFP-tagged ORL1, μ-, κ-, and δ-opioid receptor constructs were created by removal of the stop codon and insertion of the coding sequence into the N1-YFP vector (Clontech). N-terminally cyan fluorescent protein (CFP)-tagged full-length $Ca_v2.2$ channels were created by subcloning into the C1-CFP vector (Clontech). The N-terminally 3-hemagglutinin (HA)-tagged κ-opioid receptor construct was purchased from Missouri S&T cDNA Resource Center and subsequently subcloned into pcDNA3.1$^+$ to create an untagged version. Wild-type δ-opioid receptor (in pcDNA3.1$^+$) was also purchased from Missouri S&T cDNA Resource Center. The wild-type and N-terminally His_6/XPRESS-tagged ORL1 receptor constructs were cloned from human cerebellum cDNA and subcloned into appropriate expression vectors as described by us previously (10). Similarly, the N-terminally His_6/XPRESS-tagged μ-opioid receptor construct was created by subcloning into pcDNA3.1/HisB (Invitrogen). Construct accuracy was analyzed by sequencing and restriction digests. Cloning of the HA-tagged $Ca_v2.2$ construct was described by us previously (11). The wild-type μ-opioid receptor clone was donated by Dr. William Colmers at the University of Alberta.

Protein Biochemistry, Western Blot Analysis, and Enzyme-linked Immunosorbent Assays—Immunoprecipitation was carried out as follows. tsA-201 cells were grown for 48 h following transfection. Cells were washed with isotonic phosphate-buffered saline (PBS) and lysed on ice for 1 h using radioimmune precipitation assay lysis buffer (1× PBS, 1% Nonidet P-40, 0.1% SDS, and 0.5% sodium deoxycholate), and the lysate was harvested. Cell lysate was spun down at 15,000 × *g* (5 min), and the supernatant was collected and stored at −80 °C until used. Dorsal root ganglia (DRGs) were removed from all spinal levels of adult Sprague-Dawley rats and washed in ice-cold PBS. DRG tissue was lysed on ice for ~1 h in an extraction solution containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, and 0.5% sodium deoxycholate. The lysate was spun down at 15,000 × *g* (5 min), and the supernatant was collected and stored at −80 °C until used. The lysate was incubated overnight at 4 °C with the appropriate precipitating antibody and protein G-Sepharose beads. The beads were then gently spun down and washed with ice-cold PBS. An equal amount of SDS loading buffer was added to each sample, and samples were then boiled for 5 min before being resolved by SDS-PAGE (8–12% gels). Proteins were transferred to polyvinylidene difluoride membrane (0.4 μm) and probed overnight at 4 °C with the appropriate primary antibody in PBS + 0.1% Tween 20 and 5% powdered milk. After thorough washing with PBS + 0.1% Tween 20, the membrane was incubated at room temperature for 45 min with horseradish peroxidase-conjugated secondary antibody (1:5,000, PBS + 0.1% Tween 20 + 5% milk) before being washed again and exposed using the ECL detection method. Primary antibodies used were as follows: anti-XPRESS (1:2,500; Invitrogen), anti-HA (mouse, 1:1,000; Covance), anti-HA (rat, 1:1,000; Roche Applied Science), anti-μ-opioid receptor (guinea pig, 1:1,000, Millipore), anti-δ-opioid receptor (goat, 1:1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-κ-opioid receptor (rabbit, 1:1,000, Santa Cruz Biotechnology, Inc.), and anti-ORL1 generated by us as described previously (10). Enzyme-linked immunosorbent assays were carried out as previously reported (11).

Electrophysiology—Whole cell patch clamp recordings from tsA-201 cells were carried out as previously reported by us (15). The proportion of Gβγ-mediated and voltage-independent N-type calcium current inhibition was measured by application of a prepulse facilitation protocol as described.

Confocal Microscopy and Image Analysis—2–3 days after transfection, cells were subjected to either agonist or control treatment and then fixed using 4% paraformaldehyde for 10 min. If required, permeabilization was achieved using 0.05% Triton-X (in PBS) for 5 min followed by immunostaining (primary antibody, 1 h at room temperature, 3× wash in PBS + 3% bovine serum albumin; secondary antibody, 45 min at room temperature, 3× wash PBS + 3% bovine serum albumin). Images were acquired using a Zeiss LSM-510 META confocal microscope using a 63× 1.4 NA oil immersion objective in the inverted configuration. For quantitative analysis, on each image, regions of interest corresponding to the nucleus, the intracellular region, and the cell membrane were manually traced, and the integrated fluorescence intensity was measured. The internalization ratio was defined as *c*/*m*, where *c* is the integrated fluorescence intensity within the cell (corrected for the nucleus) and *m* is the fluorescence intensity in the membrane region as obtained by subtracting the total intracellular fluorescence (including the nucleus) from the cell's total integrated intensity.

RESULTS

ORL1 Receptors Biochemically Interact with Members of the Opioid Receptor Family—Because the majority of commercially available antibodies directed against opioid receptors are raised in rabbits, we created a number of tagged receptor constructs to circumvent the issue of secondary antibodies reacting with both precipitating and probing primary antibodies. Fig. 1*A* shows a series of co-immunoprecipitations between ORL1 receptors and different wild-type and tagged opioid receptor subtypes that were coexpressed in tsA-201 cells. As evident from the figure, ORL1 receptors were able to precipitate both the wild-type μ-opioid receptor and a μ-opioid receptor construct tagged with a His_6/XPRESS epitope (Fig. 1*A*). A His_6/XPRESS-tagged ORL1 receptor construct also co-immunoprecipitated with an HA-tagged κ-opioid receptor construct, irrespective of which receptor was targeted by the precipitating antibody. ORL1 and the δ-opioid receptor also precipitated together, with ORL1 as both the precipitating and blotting antibody. Negative controls consisted of the appropriate cell lysate incubated with protein G-Sepharose beads, with the precipitating antibody being omitted from the sample. When polyclonal antibodies were used to blot, a heavy band was evident at ~25 kDa. This band is likely due to nonspecific binding of lysate proteins to the protein G-Sepharose matrix and was still observed following preclearing of cell lysate. Such nonspecific binding to protein G-Sepharose has been observed by other groups (16). The band appears in both control and experimental lanes and is outside of the predicted molecular weight range for our proteins of interest; as such, it does not obfuscate interpretation of the blots. Fig. 1*B* shows a similar set of co-immu-

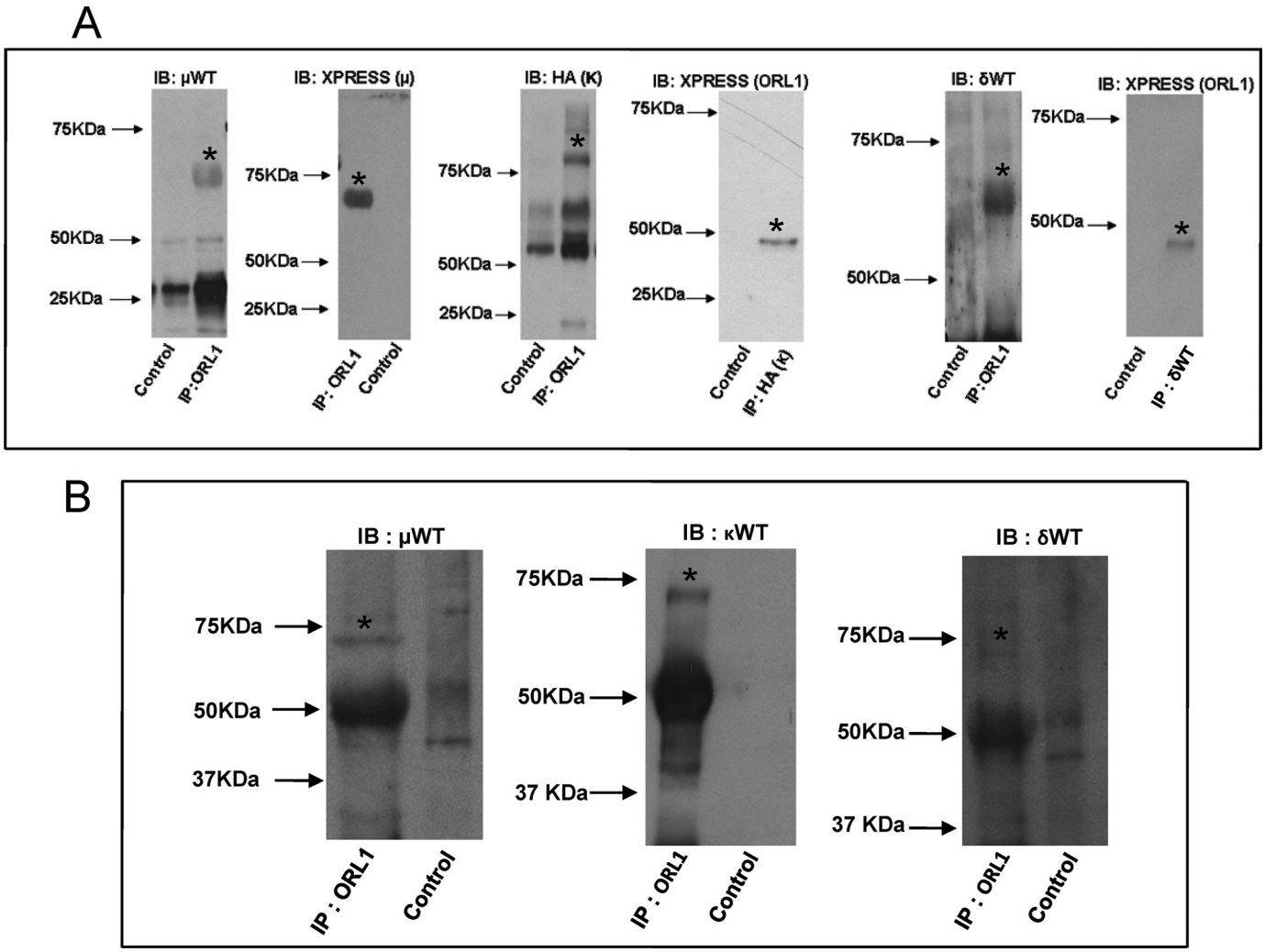

FIGURE 1. *A*, co-immunoprecipitation of ORL1 receptors and opioid receptor constructs from lysate of transfected tsA-201 cells. *IP* indicates the precipitating antibody, whereas *IB* indicates the probing antibody. The precipitating antibody was omitted from control samples. Molecular markers are indicated for each panel. *Asterisks* indicate the bands of interest. *WT*, wild type. *B*, co-immunoprecipitation of μ-, κ-, and δ-opioid receptors with ORL1 from lysate of adult rat DRGs. In all three cases, ORL1 was used as the precipitating antibody, with μ-, κ-, or δ-opioid receptor antibodies used to probe. *Asterisks* indicate the band of interest. As with tsA-201 cells, precipitating antibody was omitted from control samples. Bands appearing at ~50 kDa represent the heavy IgG chain remaining from the probing antibody. The reverse experiment was also performed, using opioid receptor antibodies to precipitate and ORL1 antibody to probe, and yielded similar results.

noprecipitations using lysate from adult rat DRGs. Consistent with our results from tsA-201 cells lysate, ORL1 receptors were able to precipitate wild-type μ-, δ-, and κ-opioid receptors from DRG lysate. Similar results were obtained using whole brain lysate from rats (data not shown). Taken together, these data indicate that stable biochemical complexes can be formed between ORL1 receptors and μ-, δ-, and κ-opioid receptor subtypes.

ORL1 and Opioid Receptors Co-internalize—Fig. 2 shows confocal images of tsA-201 cells transiently expressing YFP-κ-, YFP-δ-, or YFP-μ-opioid receptors, either alone or together with wild-type ORL1. In naïve cells expressing both receptors, the YFP signal was predominantly in the plasma membrane. Following a 30-min exposure to 1 μM nociceptin (at 37 °C), we observed a robust increase in cytoplasmic YFP signal for each of the three opioid receptor subtypes, which was not observed in cells where ORL1 was not coexpressed (Fig. 2, *A* and *C*). In the converse experiment, YFP-tagged ORL1 receptors were coexpressed with wild-type κ-, δ-, or μ-opioid receptor constructs. Cells were then exposed to the appropriate opioid receptor ligands ((±)-U50488 hydrochloride (Tocris), [D-Ala^2]deltorphin II (Tocris), or [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin (DAMGO; Sigma), respectively, for κ-, δ-, and μ-receptors) for 30 min at 37 °C. Again, this resulted in a significantly increased cytoplasmic YFP signal (Fig. 2, *B* and *D*), consistent with opioid receptor-mediated internalization of ORL1. These data fit with the biochemical analysis shown in Fig. 1 and support the existence of opioid receptor/ORL1 receptor heterodimers that can be trafficked together as a complex. As further confirmation of heterodimer formation, we transfected YFP-tagged μ-, κ-, or δ-opioid receptors with the His_6/XPRESS-tagged ORL1 receptor. Following the 30-min treatment with either vehicle or nociceptin (1 μM), cells were fixed and stained with the anti-XPRESS antibody (1:2,500) and anti-mouse Alexa 594 (1:5,000). These cells were then assessed for co-localization of the two receptors. For all three opioid receptors, a relatively high degree of co-localization was observed in both control and nociceptin-treated cells. These data are shown in Fig. 3 and further support

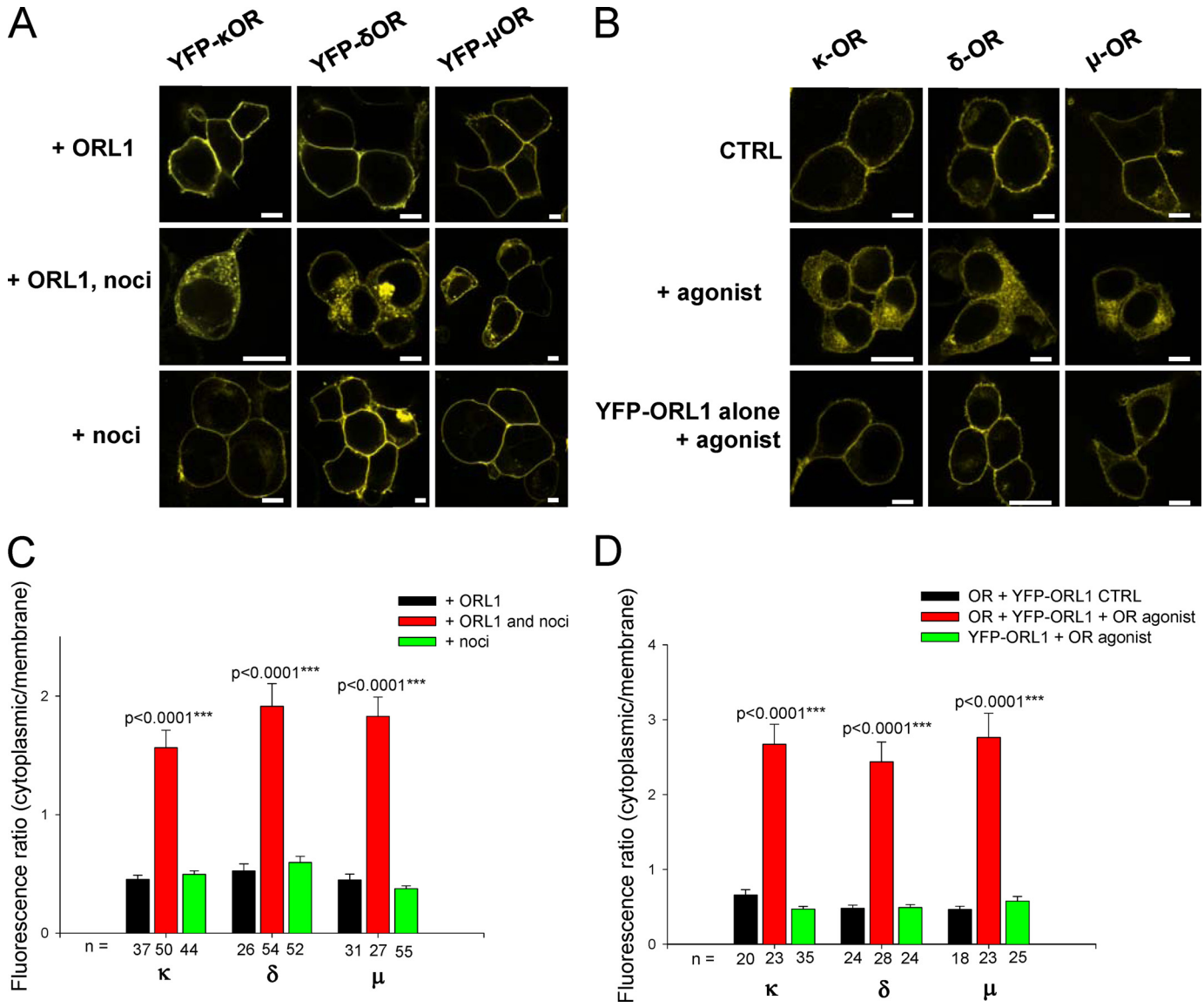

FIGURE 2. *A*, representative confocal images obtained from tsA-201 cells expressing YFP-tagged κ-, δ-, or μ-opioid receptors (*OR*), either alone or in combination with a wild-type ORL1 receptor construct. The *first row* of images is taken from cells expressing both receptors without agonist treatment. The *second row* shows cells subjected to a 30-min nociceptin (*noci*) treatment (1 μM at 37 °C). The *third row* shows nociceptin-treated cells lacking the ORL1 receptor. *Scale bars* represent 10 μm. *B*, confocal images of cells expressing YFP-tagged ORL1 receptors either alone (*bottom row*) or in conjunction with wild-type κ-, δ-, or μ-opioid receptors. The *top row* depicts naïve cells, whereas the *lower rows* depict cells exposed to the appropriate opioid receptor ligand (U50488, deltorphin, and DAMGO, respectively, all 1 μM at 37 °C for 30 min). *Scale bars* represent 10 μm. *CTRL*, control. *C* and *D*, means ± S.E. of cytoplasmic/membrane florescence ratios calculated for cells under all experimental conditions. *n*, numbers indicated below each *bar* represent the total number of cells analyzed under each condition. Significance was determined by Student's *t* test, as compared with the two-receptor control condition.

the notion that ORL1 and opioid receptors can exist as heterodimers.

Opioid Receptors Alter N-type Channel Regulation by ORL1—To investigate the consequences of ORL1 receptor/opioid receptor heterodimers on downstream effector systems, we examined the responses of N-type calcium channels, a well established target of both receptor classes (see Refs. 17–21), in cells expressing various combinations of receptors. Functional N-type calcium channels were reconstituted by expression of $Ca_v2.2$ along with auxiliary $\alpha_2\delta_1$ and β_{1b} subunits, and their electrical activity was examined using whole cell patch clamp recordings with 20 mM barium as the charge carrier. Cells were held at a potential of −100 mV, and currents were elicited by a step depolarization to +20 mV. A second depolarizing step to +20 mV was given immediately subsequent to a strong depolarization to +150 mV to assess the extent of voltage-dependent modulation (*i.e.* the amount of prepulse relief). Fig. 4*A* depicts representative current traces obtained with cells expressing N-type channels with either ORL1 receptors or ORL1 receptors coexpressed with the δ-opioid receptor before and after application of nociceptin. In cells expressing only ORL1 receptors, agonist application resulted in a pronounced inhibition of N-type channels that was largely reversible by the delivery of a strong depolarizing prepulse, consistent with membrane-delimited, Gβγ-mediated modulation of the channel. In the absence of agonist, a small degree of receptor-mediated, agonist-independent modulation could be observed (note the slight increase in current following the prepulse), consistent

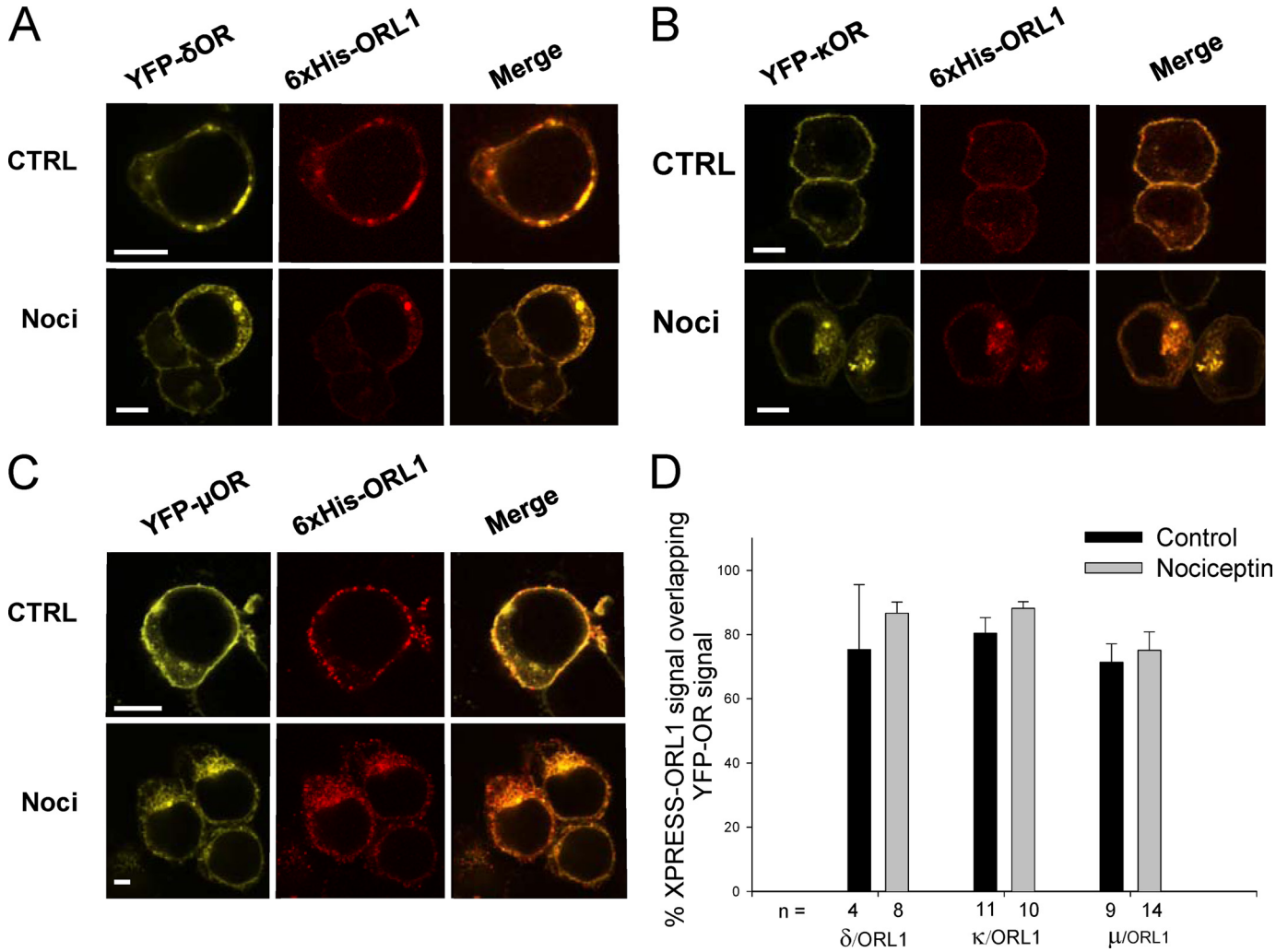

FIGURE 3. *A–C*, confocal images of tsA-201 cells coexpressing His_6/XPRESS-tagged ORL1 receptors (*red*) and YFP-tagged δ-, κ-, or μ-opioid receptors, respectively, under control conditions and following 30-min nociceptin (*Noci*) treatment (1 μM at 37 °C). Fluorescent channels are shown individually and as a merged image. *CTRL*, control. *D*, mean ± S.E. of the percentage of His_6/XPRESS-ORL1 signal overlapping with the YFP-opioid receptor (*OR*) signal under each condition. *n*, numbers indicated under each *bar* represent the total number of cells analyzed under each condition.

with our previous findings (10). In cells expressing both receptor subtypes, the effects of nociceptin were largely attenuated in comparison with cells expressing only the ORL1 receptor. Fig. 4, *B–D*, shows a quantification of these data. In the absence of κ-, δ-, or μ-opioid receptors, 100 nM nociceptin mediated an ~65% inhibition of N-type channel activity at a test potential of +20 mV. In contrast, nociceptin did not trigger G protein inhibition of N-type channels coexpressed with any of the opioid receptor isoforms (Fig. 4*B*). Coexpression of one of the three opioid receptor subtypes attenuated the nociceptin/ORL1 receptor-mediated inhibition in an opioid receptor subtype-dependent manner, with δ-opioid receptors blocking almost all of the actions of nociceptin (Fig. 4*C*), whereas μ-opioid receptors mediated a much smaller antagonism of the ORL1 response. This attenuation of the nociceptin response could in part be explained by a receptor-dependent, agonist-independent, tonic inhibition of N-type calcium channels that occurred simply by coexpression of the opioid receptor subtypes (Fig. 4*D*). As seen in Fig. 4*D*, expression of the μ-opioid receptor triggered only a small degree of constitutive channel inhibition, consistent with a low level of constitutive receptor activity (see also Ref. 22). In contrast, coexpression with either the δ-opioid receptor or the κ-opioid receptor triggered large tonic, agonist-independent G protein modulation, as evident from the 2-fold increase in peak current amplitude following the prepulse. Because the degree of prepulse relief is proportional to the amount of tonic G protein inhibition, these data suggest that the mere presence of these opioid receptor subtypes mediates an ~40–50% inhibition in current activity, thus precluding to a large extent further G protein inhibition via ORL1 receptor activation.

ORL1 Receptors Aid μ-Opioid Receptor-mediated N-type Channel Internalization—We have shown previously that ORL1 receptors physically associate with N-type calcium channels, thus allowing for ORL1 receptor-mediated internalization of the channels upon prolonged agonist exposure (11). In contrast, μ-opioid receptors did not appear to mediate N-type channel internalization upon treatment with DAMGO (11). Because ORL1 receptors are capable of heterodimerization with the μ-opioid receptors and can associate with N-type channels, we hypothesized that these three membrane proteins may form a larger macromolecular signaling complex, and if correct, any internalization of the μ-opioid receptors should

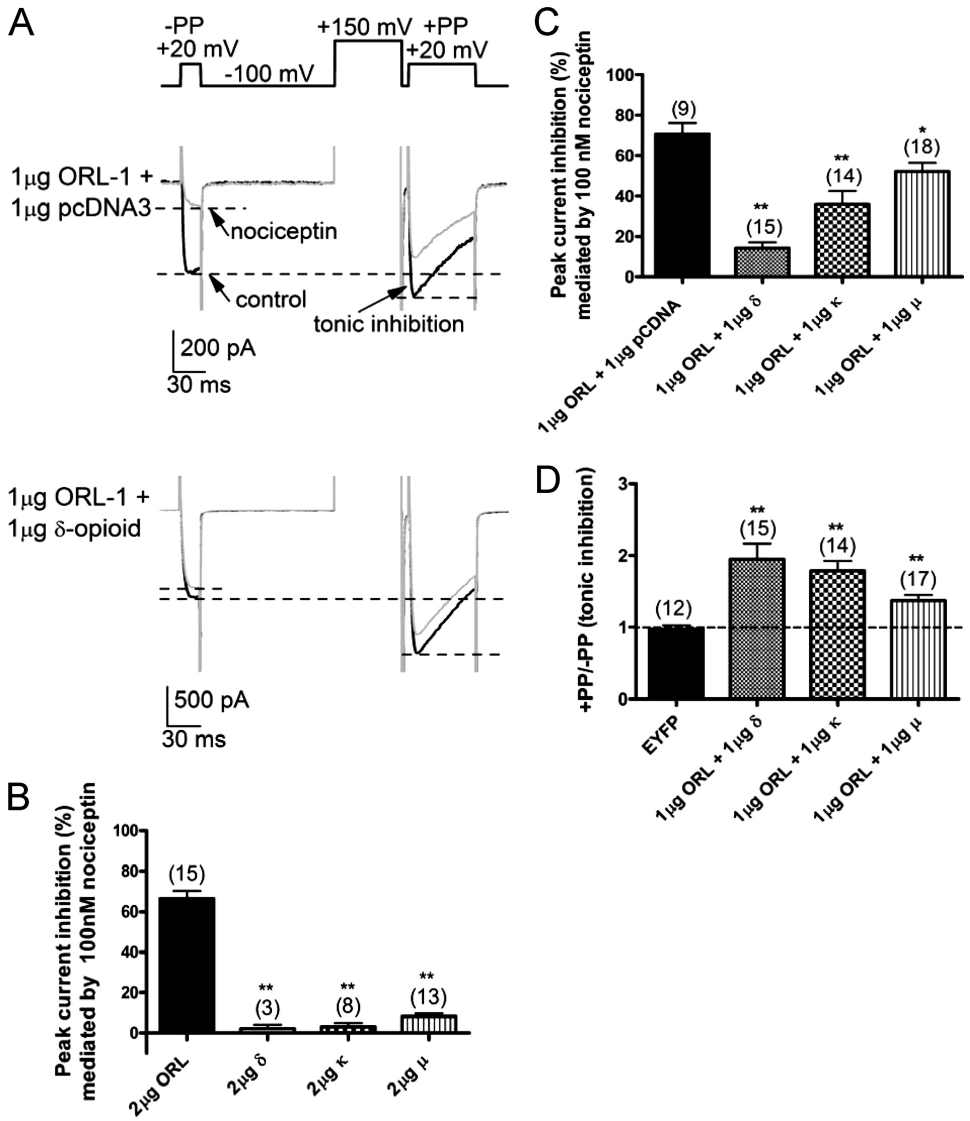

FIGURE 4. *A*, representative traces of voltage-clamp recordings of individual tsA-201 cell co-transfected with N-type calcium channel ($Ca_v2.2\ \alpha_1 + \beta_{1b} + \alpha_2\delta_1$) and 1 μg of ORL1 + 1 μg of pcDNA3 vector (*upper panel*) and 1 μg of ORL1 + 1 μg of δ-opioid receptor cDNAs (*bottom panel*) in response to 100 nM nociceptin. *B* and *C*, the peak current inhibition mediated by 100 nM nociceptin when N-type calcium channels were coexpressed with different combinations of receptors. **, $p < 0.01$ relative to 2 μg of ORL1 receptor for a given condition (*B*); *, $p < 0.05$, and **, $p < 0.01$ relative to coexpression of 1 μg of ORL1 + 1 μg of pcDNA3 for a given condition (*C*). *D*, voltage-dependent, agonist-independent inhibition (tonic inhibition) of N-type calcium channel revealed by the prepulse (*PP*) facilitation protocol. **, $p < 0.01$ relative to control condition, *i.e.* coexpressed with enhanced YFP (*EYFP*) only.

were coexpressed with $Ca_v2.2$ N-type channels carrying an extracellular HA epitope, and HA antibody reactivity was quantified in permeabilized and nonpermeabilized cells with or without agonist treatment, thus allowing us to measure cell surface expression relative to total protein expression via a fluorescence plate reader system. As shown in Fig. 5*D*, when coexpressed with the channels individually, ORL1 receptors were able to trigger internalization of N-type channels in a nociceptin dose-dependent manner. In the absence or ORL1 receptor expression, activation of μ-opioid receptors by DAMGO did not affect N-type channel surface expression, consistent with a lack of internalization. However, when both μ-opioid and ORL1 receptors were coexpressed, DAMGO application resulted in a dose-dependent loss of N-type channels from the cell surface. Hence, ORL1 receptors serve as a molecular link that allows μ-opioid receptors to regulate N-type channel surface expression.

DISCUSSION

The dimerization of G protein-coupled receptors is a well established concept (Ref. 24; for review, see Refs. 25 and 26); however, its impact on the activation and regulation of effector systems has been less clear. Here, we not only present evidence that opioid and opioid-like receptors can form heterodimers, but we also show that these interactions alter the functional modulation of N-type calcium channels by these receptors.

allow for co-internalization of both the ORL1 receptor and the channel. As shown in Fig. 5, *A* and *B*, coexpression of CFP-tagged N-type channels with wild-type ORL1 receptors and YFP-tagged μ-opioid receptors resulted in membrane localization of both fluorophores, which translocated to cytoplasmic compartments after a 30-min treatment with the agonist DAMGO. Triple labeling (*i.e.* CFP-N-type, YFP-μ-opioid receptor, and His_6/XPRESS-ORL1 receptor) confirmed a similar distribution of all three proteins before and after internalization, consistent with the idea that μ-opioid receptor activation triggers N-type channel internalization via ORL1 receptors (Fig. 5*C*). As independent verification of these results, we used an immunoluminometry approach as described by us previously (11, 15, 23). In this case, ORL1 and/or μ-opioid receptors

All members of the opioid receptor family have been shown to regulate N-type calcium channels when coexpressed in either tsA-201 cells or *Xenopus* oocytes (*i.e.* Refs. 18–21), with receptor activation resulting in voltage-dependent inhibition that is mediated by G protein $\beta\gamma$ subunits. For ORL1 receptors (10) and to a lesser extent μ-opioid receptors (22), agonist-independent modulation of channel activity has been reported likely because of constitutive receptor activity. Our data reveal a similar agonist-independent modulation of N-type channels by κ- and especially δ-opioid receptors. In the case of κ- and δ-opioid receptors, the tonic G protein inhibition was particularly large even upon transfection of as little as 1 μg of receptor cDNA. For comparison, in our previous work with ORL1 receptors, agonist-independent, tonic G protein inhibition necessi-

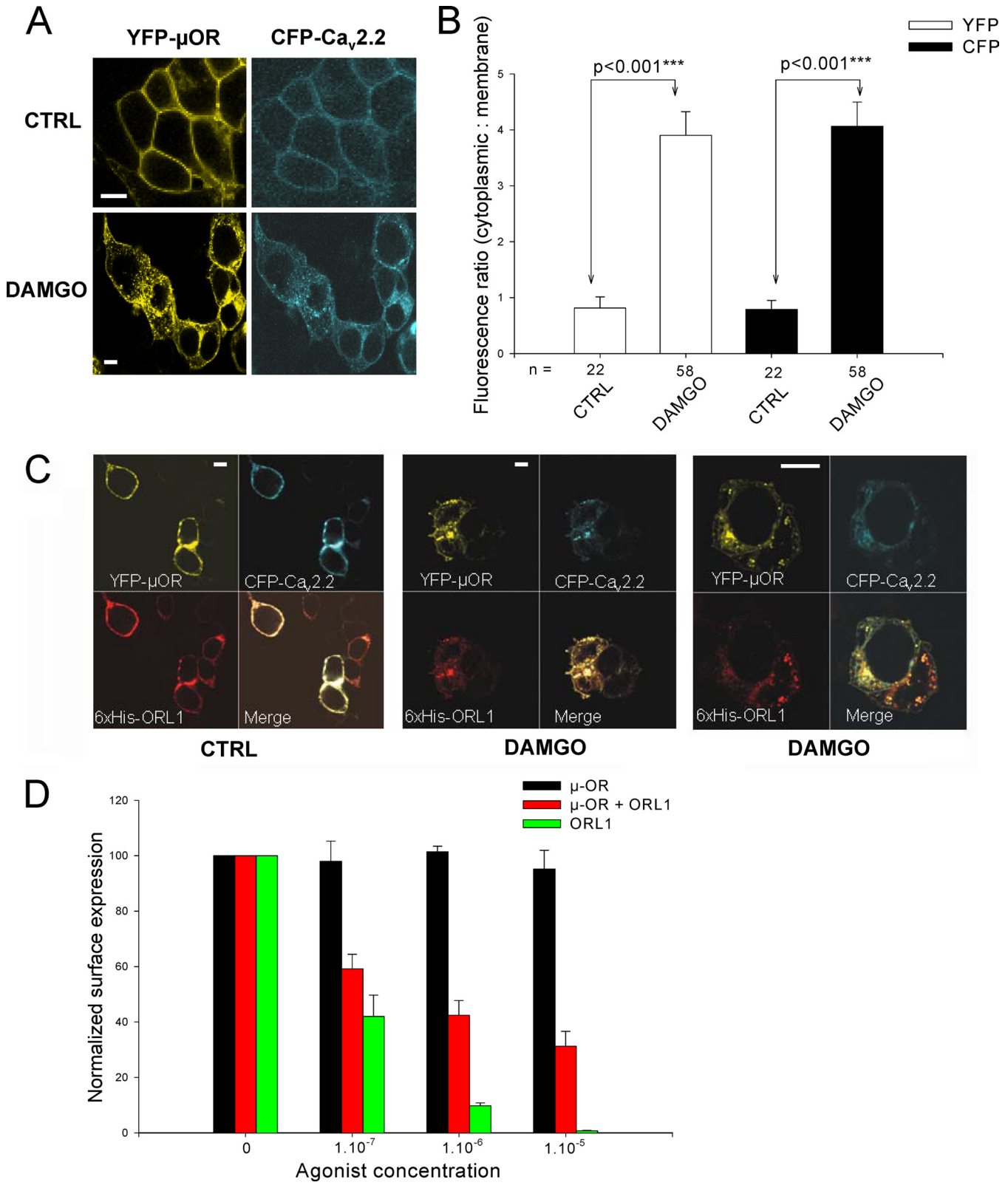

FIGURE 5. *A* and *B*, changes in cytoplasmic/membrane fluorescence distribution in tsA-201 cells coexpressing YFP-μ-opioid receptor (*OR*), wild-type ORL1 receptor, and CFP-tagged $Ca_v2.2$ (along with auxiliary $\alpha_2\delta_1$ and β_{1b} subunits) in response to DAMGO treatment (10 μM for 30 min at 37 °C). *Scale bars* are equal to 10 μm. *A*, representative confocal images from such cells under control (*CTRL*) and drug-treated conditions. *B*, the mean ± S.E. of both YFP and CFP fluorescence signals expressed as cytoplasmic/membrane fluorescence ratio. *n*, numbers indicated under the *bars* represent the total number of cells analyzed under each condition, with significance determined by Student's *t* test. *C*, confocal images showing tsA-201 cells expressing YFP-μ-opioid receptors, His_6/XPRESS-tagged ORL1 receptors, and CFP-$Ca_v2.2$ (along with auxiliary subunits). Each channel is shown separately, along with a merged image. *Scale bars* are equal to 10 μm. *D*, immunoluminometry analysis of channel internalization. Surface expression of HA-tagged $Ca_v2.2$ was measured by a fluorescent plate reader in tsA-201 cells expressing HA-tagged $Ca_v2.2$ (along with μ-opioid receptors, ORL1 receptors, or both) after treatment with increasing concentrations of DAMGO (μ and μ + ORL1 cells) or nociceptin (ORL1-only cells). Results are expressed as the mean ± S.E. Normalized surface expression data were collated from three experimental runs.

tated transfection of larger amounts (3–9 μg of receptor DNA) (10). Consistent with our previous work, only a small degree of tonic ORL1 receptor-mediated inhibition was observed in the present study when we used 1 μg of receptor DNA. Altogether, under identical transfection conditions, different opioid receptor subtypes appear to mediate distinct amounts of tonic G protein inhibition of N-type calcium channels, perhaps suggesting that they show differing levels of constitutive receptor activity. This in turn results in differential effects of various opioid receptor subtypes on the ability of ORL1 receptors to mediate N-type channel inhibition because the channels are, to a large extent, already tonically inhibited by Gβγ subunits before nociceptin application. In addition to precluding ORL1 receptor-mediated inhibition through tonic G protein modulation, it is also possible that the formation of receptor heterodimers alters receptor pharmacology and efficacy, as has been described for μ-opioid/ORL1 receptor dimers (12, 13), as well as for other types of G protein-coupled receptor heterodimers (27–29). For example, it is possible that nociceptin binding to ORL1 may be allosterically inhibited when the ORL1 receptor is part of a heterodimer, as has been demonstrated for μ- and δ-opioid receptor heterodimers (30). Indeed, the notion that κ- and δ-opioid receptors produced similar levels of tonic G protein inhibition (*i.e.* a similar degree of prepulse relief), and yet ORL1 receptor activation was more strongly attenuated in cells coexpressing the δ-opioid receptor subtype, may support such a mechanism.

The formation of heterodimers between the opioid and opioid-like receptors not only resulted in receptor co-internalization but also regulated N-type channel trafficking. We have shown previously that, unlike ORL1, μ-opioid receptors did not mediate trafficking of N-type channels to and from the plasma membrane (11), a notion that is confirmed by the immunoluminometry experiments presented here. However, when coexpressed with ORL1 receptors, activation of the μ-opioid receptors triggered internalization of the N-type channels, leading to co-localization of all three players in cytoplasmic compartments. This indicates the presence of ORL1/μ-opioid receptor/N-type channel complexes and suggests that the ORL1 receptors act as an intermediary, physically coupling the μ-opioid receptors to N-type channels. This receptor-induced channel internalization adds to an increasing body of evidence showing that various G protein-coupled receptors form signaling complexes with voltage-gated calcium channels and regulate their membrane expression (15, 23, 31–33). It is possible that δ- and κ-opioid receptors might mediate a similar trafficking effect, perhaps even in the absence of coexpressed ORL1 receptors, but this was not examined here and will be subject to further investigation.

It is well established that opioid and opioid-like receptors play a key role in controlling pain signaling in primary afferent fibers (34–38) by two primary mechanisms. First, these receptors activate G protein-coupled inward rectifier potassium channels, thus reducing neuronal excitability (39, 40); and second, they inhibit N-type calcium channels in nerve terminals within the dorsal horn of the spinal cord (41, 42). The formation of opioid/ORL1 receptor heterodimers thus has a potentially profound effect on nociceptive processing. Little is known about the precise distributions of these receptors in different types of afferent fibers and to what extent ORL1 and opioid receptors are co-localized in synaptic nerve terminals; however, the observation that knock-out of ORL1 receptors alters morphine tolerance (8) suggests that μ-opioid and ORL1 receptors show at least partial overlap in their cellular expression patterns. In this context, the heterodimerization of opioid and ORL1 receptors and the associated effects on N-type channel activity and trafficking may provide for a unique mechanism of fine-tuning pain transmission. Although not examined here, similar receptor dimer-dependent effects on G protein-coupled inward rectifier potassium channel activity are possible. Finally, it is worth noting that ORL1 receptors are expressed widely across the brain (43, 44), with receptor activity having been linked to physiological adaptations such as the regulation of food intake (45, 46), anxiety (47), and withdrawal symptoms (48, 49), providing yet further potential for physiological effects of ORL1/opioid receptor heterodimers and their formation of signaling complexes with N-type calcium channels.

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