Nerve Growth Factor-Regulated Emergence of Functional δ -Opioid Receptors

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Sorting of intracellular G-protein-coupled receptors (GPCRs) either to lysosomes for degradation or to plasma membrane for surface insertion and functional expression is a key process regulating signaling strength of GPCRs across the plasma membrane in adult mammalian cells. However, little is known about the molecular mechanisms governing the dynamic process of receptor sorting to the plasma membrane for functional expression under normal and pathological conditions. In this study, we demonstrate that δ -opioid receptor (DOPr), a GPCR constitutively targeted to intracellular compartments, is driven to the surface membrane of central synaptic terminals and becomes functional by the neurotrophin nerve growth factor (NGF) in native brainstem neurons. The NGF-triggered DOPr translocation is predominantly mediated by the signaling pathway involving the tyrosine receptor kinase A, Ca^{2+} -mobilizing phospholipase C, and Ca^{2+} /calmodulin-dependent protein kinase II. Importantly, it requires interactions with the cytoplasmic sorting protein NHERF-1 (Na^+/H^+ exchange regulatory factor-1) and *N*-ethyl-maleimide-sensitive factor-regulated exocytosis. In addition, this NGF-mediated mechanism is likely responsible for the emergence of functional DOPr induced by chronic opioids. Thus, NGF may function as a key molecular switch that redirects the sorting of intracellularly targeted DOPr to plasma membrane, resulting in new functional DOPr on central synapses under chronic opioid conditions.

Introduction

Surface density and signaling strength of G-protein-coupled receptors (GPCRs) on mammalian cell membrane are actively regulated by receptor trafficking through endocytosis (internalization) and exocytosis, two trafficking processes dynamically determined by agonist stimulation and molecular properties of the receptors (Hanyaloglu and von Zastrow, 2008). The mechanisms of GPCR internalization mediated by receptor phosphorylation and β -arrestin association upon agonist binding have been well characterized and are consistent among many different types of GPCRs, whereas the cellular fates of internalized GPCRs are rather diverse. Constitutively, GPCRs can be sorted to the lysosomal pathway for degradation or to the recycling pathway for surface membrane insertion through exocytosis (Tan et al., 2004; Hanyaloglu and von Zastrow, 2008). A typical example of this constitutive sorting of GPCRs is opioid receptors: while internalized μ -opioid receptor (MOPr) is mostly directed to the recycling pathway for surface insertion, intracellular δ -opioid receptor (DOPr) is normally sorted to the lysosomal pathway for degradation or is constitutively retained in intracellular compartments,

resulting in a low level of functional DOPr on plasma membrane in many types of central neurons (Tsao and von Zastrow, 2000; Finn and Whistler, 2001; Wang and Pickel, 2001; Ma et al., 2006).

Recent studies show that the lysosomal sorting of DOPr is regulated by a GPCR-associated sorting protein (Whistler et al., 2002) and the predominant intracellular targeting of DOPr is retained by a specific domain in the cytoplasmic tail of DOPr, which is lacking in MOPr (Kim and von Zastrow, 2003). However, certain pathological conditions can alter the normal sorting of GPCRs and induce GPCR mislocalization with abnormal GPCR signaling, leading to a variety of diseases (Tan et al., 2004). For example, prolonged exposure to opioids induces exocytotic membrane trafficking of intracellular DOPr to surface membrane, resulting in new functional DOPr in central neurons involved in pain modulation and in drug addiction (Morinville et al., 2004; Hack et al., 2005; Ma et al., 2006; Zhang et al., 2006; Cahill et al., 2007; Bie et al., 2009). This mechanism of DOPr membrane trafficking has also been demonstrated in our previous confocal immunocytochemical study on brainstem neurons, which shows that chronic morphine recruits new functional DOPr on presynaptic terminals through DOPr translocation, with increased DOPr protein on plasma membrane and an increased number of DOPr-immunoreactive varicosities (presumably on presynaptic terminals) that appose postsynaptic membrane of cell bodies (Ma et al., 2006).

In contrast to the well characterized mechanism of GPCR internalization, little is known about the molecular mechanisms

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regulating the diverse sorting and membrane trafficking of intracellular GPCRs. Particularly, the molecular determinants that may alter the normal sorting destination of GPCRs and their cellular signaling under certain pathological conditions are unknown at present (Cahill et al., 2007; Hanyaloglu and von Zastrow, 2008). In this study, we used this morphine-induced DOPr as a functional measure of DOPr trafficking to investigate the mechanisms of its intracellular sorting and surface expression.

Materials and Methods

All procedures involving the use of animals conformed to the guidelines by the Institutional Animal Care and Use Committee of MD Anderson Cancer Center.

Animals and chronic morphine treatment. Male Wistar rats were treated with chronic morphine for 6 d by twice-daily injections of morphine solution with escalating doses (10–30 mg/kg, i.p.) as reported before (Bie et al., 2005; Ma et al., 2006). Saline was similarly injected in a separate group of rats as controls. Na^+/H^+ exchange regulatory factor-1 knockout (KO) (NHERF-1 $^{-/-}$) mice, originally generated and reported previously (Shenolikar et al., 2002), were inbred and genotyped onsite, and similarly treated with chronic morphine. DOPr $^{-/-}$ mice were purchased from The Jackson Laboratory.

Brain slice preparations. Neonatal rats (9–14 d), or NHERF-1 knockout and corresponding wild-type (WT) mice, were used for preparations of brainstem slices (250 μm thick) containing the nucleus raphe magus (NRM) for whole-cell recording as previously described (Bie et al., 2005). A single slice was submerged in a shallow recording chamber and perfused with preheated (35°C) physiological saline. Neonatal rats were used for better visualization of NRM neurons, and we have shown that the physiological and pharmacological properties of neurons from these young rats are indistinguishable from those of adult rats (Pan et al., 1997; Bie et al., 2005). The slice was generally treated with a specific inhibitor *in vitro* for a long (4 h) period, or a short (30 min) period for controls. The target specificity of those inhibitors at the concentration used has been documented previously (see supplemental material, available at www.jneurosci.org). Other drugs were applied through the bath solution.

Whole-cell recording and synaptic currents. Visualized whole-cell voltage-clamp recordings were obtained from identified NRM neurons with a glass pipette (resistance 3–5 M Ω) filled with a solution containing the following (in mM): KCl, 126; NaCl, 10; MgCl $_2$, 1; EGTA, 11; HEPES, 10; ATP, 2; GTP, 0.25; pH adjusted to 7.3 with KOH; osmolarity 280–290 mOsm/L. Electrical stimuli of constant current (0.25 ms, 0.2–0.4 mA) were used to evoke glutamate EPSCs or GABA IPSCs, as described in details previously (Bie et al., 2005; Ma et al., 2006). An inhibition by a drug was defined as a reduction of >10% of control in the amplitude of synaptic currents. Miniature EPSCs were obtained in 60 s epochs in the presence of tetrodotoxin (1 μM), and a sliding EPSC template custom defined with the acquisition software was used to detect and analyze the frequency and amplitude of miniature EPSCs.

Western blots. NRM tissues of rats (200–250 g) were harvested and standard Western blot experiments were performed as in our previous report (Ma et al., 2006). After tissue homogenization and lysis, samples were centrifuged and the supernatant was used for SDS-PAGE. Proteins were separated and electrotransferred onto nitrocellulose membrane. Samples were incubated overnight at 4°C with a primary antibody for nerve growth factor (NGF, 1:1000, Sigma, N6655, mouse), Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII, 1:200, Santa Cruz Biotechnology, sc-9035, rabbit), phosphorylated CaMKII (p-CaMKII) (1:200, Santa Cruz Biotechnology, sc-32289, mouse), or NHERF-1 (1:250, Santa Cruz Biotechnology, sc-71698, mouse), and for actin (1:200, Santa Cruz Biotechnology, sc-81178, mouse) or GAPDH (1:1000, Santa Cruz Biotechnology, sc-20357, goat) as controls (references to antibody specificity are available on the manufacturer's website). After washes, the blots were incubated with horseradish peroxidase-conjugated secondary antibody (1:10,000) (GE Healthcare) for 1 h and stained with 3,3'-diaminobenzidine tetrahydrochloride. The amount of protein loaded in each lane is 20 μg . Immunoreactive proteins were detected by the enhanced chemiluminescence (ECL) Advance Kit (GE Healthcare). The

intensity of bands was digitally captured and quantitatively analyzed with the software Kodak 1D (Eastman Kodak). The immunoreactivity of target proteins was normalized to that of actin or GAPDH.

Coimmunoprecipitation. NRM tissues were homogenized in a lysis buffer (0.1% Triton X-100, 150 mM NaCl, 25 mM KCl, and 10 mM Tris-HCl, pH 7.4, with protease inhibitors and phosphatase inhibitors). Cleared lysate was incubated overnight at 4°C with rabbit polyclonal anti-DOPr antibody (1:100, Millipore Bioscience Research Reagents, AB1560) and precipitated for 5 h by protein A-agarose beads (Sigma). Precipitates were washed extensively, and the proteins were separated on a 10% SDS-polyacrylamide gel and blotted to nitrocellulose membrane. The blots were incubated overnight at 4°C with monoclonal anti-NHERF-1 antibody (1:250, Santa Cruz Biotechnology). The membrane was washed with Tris-buffered saline and incubated with anti-mouse IgG horseradish peroxidase (HRP) (1:10,000, GE Healthcare). The membrane was then reprobed with goat polyclonal anti-DOPr primary antibody (1:250, Santa Cruz Biotechnology, sc-7492) to detect immunoprecipitated DOPr. The DOPr-blocking peptide (Millipore) had the sequence LVPSARAELQSSPLV and was incubated with the DOPr antibody for 45 min before use. For serine/threonine phosphorylation of DOPr, the membrane was incubated with monoclonal anti-phosphoserine/threonine antibody (1:2000; BD Biosciences, 612548; mouse), extensively washed, and then incubated with anti-mouse IgG HRP (1:10,000) before ECL detection was performed. The total amount of DOPr was detected by incubating the stripped membranes with goat polyclonal anti-DOPr antibody (1:250, Santa Cruz Biotechnology).

Synaptosome preparations. As in our previous report (Bie et al., 2009), NRM tissues from saline- and morphine-treated rats were gently homogenized in ice-cold 0.32 M sucrose buffer and centrifuged at 1000 $\times$ g (4°C). The supernatant was collected and centrifuged at 10,000 $\times$ g (4°C), and the synaptosomal pellet was resuspended in a lysis buffer with protease inhibitors. For total protein preparations (Ma et al., 2006), NRM tissues from saline- and morphine-treated rats were homogenized in the lysis buffer, the lysates were centrifuged at 14,000 rpm, and the supernatant was used for SDS-PAGE. Protein concentrations were determined by using the Bio-Rad protein assay kit.

Immunohistochemistry. Rats pretreated with morphine ($n = 6$) or saline ($n = 6$) were deeply anesthetized with 60 mg/kg sodium pentobarbital and perfused transcardially with 0.1 M PBS followed by 4% formalin. The brainstem was collected, postfixed in the same fixative for 4 h, and then cryoprotected in 30% sucrose in PBS for 3 d. Serial sections (30 μm , 15–20/rat) containing the NRM were cut from the fixed brainstem. The sections were incubated for at least 1 h in 0.01 M PBS with 0.3% Triton X-100 plus 5% normal donkey serum. Primary and secondary antibodies were diluted in 0.01 M PBS with 0.3% Triton X-100 plus 1% bovine serum. Sections were processed overnight at 4°C for double-labeling immunofluorescence using rabbit antibody directed against NGF (1:100, Santa Cruz Biotechnology, sc-548) and mouse monoclonal antibody against the neuronal marker NeuN (1:500, Millipore Bioscience Research Reagents, MAB377). Then, the sections were incubated with a mixture of FITC- (1:500, Jackson ImmunoResearch, Lot No. 87017) and Cy3-conjugated secondary antibodies (1:500, Jackson ImmunoResearch, Lot No. 88464) for 1 h. The stained sections were examined with a Nikon E600 fluorescence microscope (Nikon Instech), and images were captured with a CCD spot camera. Quantitative comparison of NGF-positive cells within the NRM between the two groups of rats was made manually in randomly selected sections.

Microinjection. As we described before (Bie et al., 2005; Ma et al., 2006), a rat was anesthetized with sodium pentobarbital (50 mg/kg i.p.) and restrained in a stereotaxic apparatus. A 26 gauge double-guide cannula (Plastics One) was inserted into the brain, aiming at the NRM (anteroposterior, -10.0 mm from the bregma; lateral, 0; dorsoventral, -10.5 mm from the dura) (Paxinos and Watson, 1986). The guide cannula was then cemented in place to the skull and securely capped. The rat was allowed to recover for at least 5 d before drug treatment. For *in vivo* treatment by microinjection, NGF (0.05 $\mu\text{g}/0.5 \mu\text{l}$), K252a (47 $\mu\text{g}/1 \mu\text{l}$), or saline was delivered into the NRM twice daily for 2 d through a 33 gauge double injector with an infusion pump at a rate of 0.2 $\mu\text{l}/\text{min}$. The

injection sites for the NRM were histologically verified afterward by injecting 0.5 μ l of a blue dye.

Statistical analyses and materials. General numerical data were statistically analyzed with paired or unpaired Students' *t* tests, or ANOVA where appropriate, and presented as mean $\pm$ SEM. Statistical analysis of miniature EPSCs was performed using the Statview software with the Kolmogorov–Smirnov test. Morphine sulfate, deltorphin, and naltriben were supplied by the Drug Program of the National Institute on Drug Abuse. The p75^{NTR}-blocking antibody was kindly provided by Dr. Moses Chao at New York University School of Medicine (New York, NY). Other drugs were purchased from Sigma-Aldrich or Tocris Bioscience.

Results

Chronic morphine induces new functional DOPr on presynaptic terminals of glutamate synapses

We first examined morphine-induced emergence of functional DOPr on glutamate synapses in brainstem neurons of the nucleus raphe magnus, which, under normal conditions, steadily express functional MOPr, but lack functional DOPr due to its predominant intracellular localization (Pan et al., 1997; Ma et al., 2006). In these neurons under whole-cell voltage-clamp recording in control slices taken from saline-treated rats ($n = 12$), the selective DOPr agonist deltorphin II (1 μ M) had no significant effect on the amplitude of evoked glutamatergic EPSC in any neuron tested (control, 136 ± 11 pA, deltorphin, 132 ± 11 pA, $n = 21$, $p = 0.30$) (Fig. 1*A*), indicating that functional DOPr is lacking on normal glutamate synapses in these neurons. By contrast, in neurons from chronic morphine-treated rats ($n = 15$), deltorphin (1 μ M) produced a reversible inhibition in the EPSC amplitude in every neuron tested (control, 170 ± 12 pA, deltorphin, 112 ± 8 pA, $n = 21$, $p < 0.01$) (Fig. 1*B*). The deltorphin inhibition was dose dependent, with a near-maximum inhibition of $34 \pm 2\%$ at 1 μ M and an EC₅₀ value of 32.4 nM (Fig. 1*C*), and it was completely abolished by the selective DOPr antagonist naltriben (50 nM) (control in naltriben, 183 ± 19 pA, +deltorphin, 172 ± 19 pA, $n = 9$, $p = 0.15$) (Fig. 1*D*). This indicates a selective DOPr-mediated effect, as we reported previously (Ma et al., 2006).

To determine the synaptic site of this newly emerged DOPr, we first used the paradigm of paired-pulse ratio (PPR), which has an inverse relationship with the probability of presynaptic transmitter release and has been widely used to determine a presynaptic involvement in a synaptic change (Dobrunz and Stevens, 1997; Bie et al., 2005). In those brainstem neurons of control slices, deltorphin failed to alter the PPR of glutamate EPSCs (control, 1.77 ± 0.06 , deltorphin, 1.73 ± 0.09 , $n = 10$, $p = 0.48$) (Fig. 1*E,F*). In neurons from morphine-treated rats, the baseline PPR was significantly decreased when compared to that of control neurons (1.25 ± 0.05 vs 1.77 ± 0.06 of controls, $p < 0.01$), indicating a significantly increased activity of glutamate synapses by the morphine treatment, an observation consistent with our previous study on the same neurons (Bie et al., 2005). In these neurons of morphine-treated rats, however, deltorphin significantly increased the EPSC PPR (control, 1.25 ± 0.05 , deltorphin, 1.68 ± 0.06 , $n = 10$, $p < 0.01$) (Fig. 1*E,F*), indicating a likely decrease in presynaptic glutamate release. To further confirm this mechanism, we analyzed the properties of spontaneous miniature EPSCs in the presence of tetrodotoxin (1 μ M). We found that the DOPr agonist, while having no effect on either the frequency or amplitude of miniature EPSCs in neurons of control rats, significantly decreased the frequency of miniature EPSCs without altering the amplitude in neurons from morphine-treated rats (frequency: control, 9.80 ± 0.51 Hz, deltorphin, 6.08 ± 0.42 Hz, $p < 0.01$; amplitude: control, 23.7 ± 1.4 pA, deltorphin, 23.3 ± 1.6 pA, $p = 0.51$, $n = 11$) (Fig. 1*G,H*). As we reported previously

(Ma et al., 2006), deltorphin remained ineffective on the postsynaptic membrane conductance in those neurons as well as in control neurons. These results suggest that chronic morphine induces new functional DOPr that appears on presynaptic glutamate terminals, and DOPr activation inhibits glutamate release.

NGF induces new functional DOPr on glutamate synapses

Recent studies, including ours, have shown that this morphine-induced functional DOPr results from exocytotic membrane trafficking of intracellularly localized DOPr (Ma et al., 2006; Zhang et al., 2006; Cahill et al., 2007). We began our search for the molecular determinant that might be responsible for this morphine-induced DOPr translocation to surface membrane by testing a number of compounds that activate the signaling pathways known to be upregulated by chronic opioids (Williams et al., 2001), in an attempt to trigger similar DOPr translocation in naive, normal neurons *in vitro*. Treatment of control slices with the adenylyl cyclase activator forskolin (10 μ M), the cAMP analog/protein kinase A activator 8-bromo-cAMP (1 mM), the protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (1 μ M), or neuropeptide cholecystokinin (300 nM) for up to 5 h all failed to induce deltorphin-mediated inhibition of glutamate EPSCs (data not shown).

However, in control slices ($n = 10$) treated with NGF (100 ng/ml) for a long period (4 h), deltorphin (1 μ M) produced a significant and reversible inhibition in the EPSC amplitude in 9 of 11 (82%) neurons generally surveyed (control, 207 ± 27 pA, deltorphin, 147 ± 19 pA, wash, 195 ± 33 pA, $n = 9$, $p < 0.01$; no significant effect on the remaining 2 cells) (Fig. 2*A,E*). The inhibition was reversed by the DOPr antagonist naltriben (50 nM) (control in naltriben, 180 ± 17 pA, +deltorphin, 171 ± 19 pA, $n = 5$ of a separate group, $p = 0.20$) (Fig. 2*B,E*). This NGF treatment itself did not appear to have a significant effect on basal EPSC amplitude when compared between control and NGF-treated slices ($p = 0.28$). Cotreatment of control slices ($n = 5$) with NGF (100 ng/ml) and the tyrosine receptor kinase (Trk) inhibitor K252a (200 nM) for the same time period blocked the deltorphin effect (control, 191 ± 39 pA, deltorphin, 186 ± 34 pA, $n = 5$, $p = 0.50$) (Fig. 2*E*); so did the cotreatment with NGF plus the specific TrkA receptor antagonist GW441756 (100 nM) (control, 124 ± 11 pA, deltorphin, 121 ± 10 pA, $n = 7$ from 7 slices, $p = 0.43$), but not with a blocking antibody (1:2000) to the other NGF receptor p75^{NTR} (control, 188 ± 26 pA, deltorphin, 127 ± 21 pA, $n = 5$ from 5 slices, $p < 0.01$) (Fig. 2*C–E*). In contrast, treatment of control slices ($n = 5$) with NGF (100 ng/ml) for a short period (30 min) was unable to induce functional DOPr on glutamate synapses (control, 157 ± 22 pA, deltorphin, 150 ± 25 pA, $n = 5$, $p = 0.45$). We also determined whether NGF administered in intact rats *in vivo* would induce similar appearance of functional DOPr on glutamate synapses. In brainstem slices from naive rats ($n = 6$) treated with twice-daily NGF (100 ng, i.p.) for 2 d, deltorphin (1 μ M) significantly and reversibly inhibited the EPSC in every neuron tested (control, 183 ± 27 pA, deltorphin, 118 ± 14 pA, wash, 171 ± 15 pA, $n = 6$, $p < 0.01$) (Fig. 2*F*).

Thus, it appears that long-term (at least hours) NGF treatment of both normal brainstem slices *in vitro* and naive rats *in vivo*, similar to the morphine treatment *in vivo*, is able to bring out functional DOPr on glutamate synaptic terminals by activating TrkA receptors.

NGF is responsible for morphine induction of DOPr

To determine whether NGF via TrkA receptors was responsible for morphine induction of DOPr, we first examined the effect of

Figure 1. Chronic morphine induces new functional DOPr on presynaptic terminals. **A, B**, Representative glutamatergic EPSCs in control, in the presence of DOPr agonist deltorphin II ($1 \mu\text{M}$), and after wash in a brainstem neuron of the NRM from a saline-treated rat (**A**) and from a morphine-treated rat (**B**). **C**, A dose–response curve for the deltorphin inhibition of EPSCs in brainstem neurons ($n = 7$ – 9 cells at each dose) from morphine-treated rats. Error bars here and in all following figures are SEMs. **D**, EPSCs in the presence of the DOPr antagonist naltriben (50 nM) and after addition of deltorphin in a neuron from a morphine-

blocking TrKA receptors on the DOPr function in slices from morphine-treated rats. In those slices ($n = 10$) after treatment with GW441756 (100 nM) *in vitro* for 4 h, deltorphin was no longer effective on EPSC amplitude in every cell tested (control, 160 ± 16 pA, deltorphin, 153 ± 17 pA, $n = 11$, $p = 0.18$) (Fig. 3A). The DOPr agonist also lost its inhibitory effect in the slices ($n = 8$) treated with K252a (200 nM) (control, 169 ± 9 pA, deltorphin, 159 ± 8 pA, $n = 8$, $p = 0.17$), but it remained effective after similar treatment of the slices ($n = 7$) with the blocking antibody to the $p75^{\text{NTR}}$ receptor (control, 159 ± 14 pA, deltorphin, 104 ± 12 pA, $n = 7$, $p < 0.01$) (Fig. 3A). Further supporting the NGF involvement in morphine-induced functional translocation of DOPr, incubation of the slices ($n = 7$) with an anti-NGF antibody ($1:4000$) for 4 h also abolished the DOPr effect (control, 184 ± 36 pA, deltorphin, 175 ± 32 pA, $n = 9$, $p = 0.38$) (Fig. 3A). Additional supporting evidence was obtained from Western blot experiments showing that brainstem tissues of the same nucleus, harvested from morphine-treated rats ($n = 7$), exhibited a significantly higher amount of NGF protein (normalized to actin proteins) when compared to brainstem tissues from control rats (to $248 \pm 42\%$ of control, $n = 7$, $p < 0.01$) (Fig. 3B). This upregulated NGF expression was also observed in brainstem neurons from morphine-treated rats ($n = 6$), with an increased number of neurons immunocytochemically stained for NGF when compared to those from saline-treated rats ($n = 6$) (saline, 19 ± 2 cells/section; morphine, 46 ± 4 cells/section, $n = 8$ sections for each group, $p < 0.01$) (Fig. 3C–G). Furthermore, we cotreated rats with the same regimen of morphine plus the TrKA receptor antagonist GW441756 ($0.29 \mu\text{g}$, i.p.) *in vivo*. In slices from those rats ($n = 5$), deltorphin could no longer inhibit the EPSC in any cell tested (control, 201 ± 41 pA, deltorphin, 192 ± 38 pA, $n = 5$, $p = 0.13$) (Fig. 3H). These results support the

treated rat. **E**, Representative EPSC pairs superimposed and scaled to the amplitude of the first EPSC in a neuron from a saline- and a morphine-treated rat. **F**, Group data of the deltorphin effect on the paired-pulse ratios of EPSCs in neurons from the two rat groups ($n = 10$ in each group). **G**, Distribution graphs of miniature EPSC frequencies and amplitudes in a neuron from a morphine-treated rat. **H**, Summarized deltorphin effects on the frequency and amplitude of miniature EPSCs in neurons of saline-treated rats ($n = 10$) and morphine-treated rats ($n = 11$). *** $p < 0.01$. Calibration: 50 pA and 10 ms.

Figure 2. NGF induces new functional DOPr on glutamate synapses. **A**, Representative glutamate EPSCs before (control), during and after (wash) application of deltorphen (1 μ M) from an NRM neuron in a normal slice taken from a naive rat and treated *in vitro* with NGF for a long period (4 h). **B**, EPSCs in naltrexone without and with deltorphen in an NGF-treated normal slice. **C**, **D**, EPSCs in a normal slice treated with NGF plus the TrKA receptor inhibitor GW441756 (100 nM, **C**) or plus an anti-p75^{NTR} receptor antibody (**D**). **E**, Group data of the deltorphen effects on EPSCs in normal slices treated with long-period NGF alone (control, $n = 9$), in the presence of naltrexone, or treated with NGF plus an inhibitor as indicated. **F**, Deltorphen inhibition of EPSCs from a naive rat treated with repeated NGF *in vivo*. * $p < 0.05$, ** $p < 0.01$. Calibration: 50 pA and 10 ms.

notion that NGF via its TrKA receptors is a critical signaling molecule for morphine induction of functional DOPr on glutamate terminals.

NGF induces functional DOPr on GABA synapses

We have previously reported that morphine also induces new functional DOPr through membrane trafficking on GABAergic terminals in these brainstem neurons (Ma et al., 2006). We then determined whether NGF induction of DOPr would also occur on GABA synapses in these brainstem neurons. We found that, in normal slices treated similarly with NGF for 4 h *in vitro*, deltorphen (1 μ M) produced a significant and reversible inhibition in the amplitude of GABA-mediated IPSCs in 12 of 20 (60%) neurons generally surveyed (control, 228 ± 29 pA, deltorphen, 135 ± 17 pA, $n = 12$, $p < 0.01$) (Fig. 4A, B). The averaged magnitude of inhibition was $39.7 \pm 3.0\%$. The DOPr antagonist naltrexone (50 nM) abolished this deltorphen effect (control in naltrexone, 181 ± 26 pA, +deltorphen, 179 ± 23 pA, $n = 6$, $p = 0.54$) (Fig. 4A, B). Acute or a short-period (<30 min) application of NGF (100 ng/ml) failed to induce functional DOPr on GABA synapses in control slices (control, 254 ± 24 pA, deltorphen, 241 ± 26 pA, $n = 10$, $p = 0.16$). In contrast, in slices from morphine-treated

rats and treated with the TrK receptor inhibitor K252a (200 nM) for 4 h *in vitro*, deltorphen was no longer effective on GABA IPSCs in every cell tested (control, 283 ± 56 pA, deltorphen, 273 ± 54 pA, $n = 10$, $p = 0.31$) (Fig. 4C). In similar slices of morphine-treated rats and kept *in vitro* for the same time period but without the TrK inhibitor, deltorphen significantly inhibited the GABA IPSCs (control, 287 ± 62 pA, deltorphen, 171 ± 40 pA, $n = 6$, $p < 0.01$). This indicates that NGF may have a broader role in regulating synaptic DOPr trafficking and functions on central synapses.

Signaling pathways for DOPr induction

TrKA receptors are known to signal through three main pathways: the phospholipase C (PLC γ) pathway, the phosphatidylinositol 3 (PI3)-kinase pathway, and the Ras/mitogen-activated protein kinase (MAPK) pathway (Sofroniew et al., 2001; Huang and Reichardt, 2003). We next determined which pathway was predominantly involved in the morphine induction of DOPr by treating slices from morphine-treated rats with a specific inhibitor for a long period (3–4 h). In those slices ($n = 10$) treated with the PLC inhibitor U73122 (2 μ M), deltorphen no longer produced any inhibition of glutamate EPSCs (control, 149 ± 23 pA, deltorphen, 140 ± 23 pA, $n = 11$, $p = 0.16$) (Fig. 5A). Treatment of the slices ($n = 7$) with wortmannin (1 μ M), a potent inhibitor of PI3-kinase, also antagonized the deltorphen effect (control, 206 ± 34 pA, deltorphen, 191 ± 30 pA, $n = 7$, $p = 0.12$), but not a similar treatment of the slices ($n = 7$) with the potent inhibitor of MAPK kinase U0126 (10 μ M) (control, 152 ± 24 pA, deltorphen, 95 ± 17 pA, $n = 7$, $p < 0.05$) (Fig. 5A). This suggests that both the PLC γ pathway and the PI3-kinase pathway are important for morphine induction of DOPr.

Given the Ca^{2+} dependence of vesicle fusion with plasma membrane involved in receptor exocytosis and Ca^{2+} dependence of agonist-induced rapid (in seconds) receptor trafficking (Lin and Scheller, 2000; Zhang et al., 2006), we further explored the Ca^{2+} -mobilizing PLC γ pathway to identify the downstream signaling molecules. The major products of PLC γ activation are diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3), and the latter regulates Ca^{2+} release from IP3-sensitive Ca^{2+} stores. Long-period treatment of slices ($n = 7$) from morphine-treated rats with the IP3 receptor inhibitor 2-APB (50 μ M) blocked morphine-induced DOPr function (control, 155 ± 18 pA, deltorphen, 152 ± 20 pA, $n = 7$, $p = 0.59$), as did similar treatment of the slices ($n = 11$) with another IP3 receptor inhibitor heparin (200 μ g/ml) (control, 137 ± 17 pA, deltorphen, 124 ± 16 pA, $n = 20$, $p = 0.12$) (Fig. 5B). The involvement of intracellular Ca^{2+} release was further supported by the observation that depleting intracellular Ca^{2+} stores with long-period thapsigargin (5 μ M) also abolished the DOPr function in those slices ($n = 10$) (con-

Figure 3. NGF is responsible for morphine induction of functional DOPr. **A**, Summarized deltorphan effects on EPSCs in slices of morphine-treated rats in control or after treatment *in vitro* with the indicated inhibitor. **B**, Representative Western blot lanes of NGF and actin proteins in NRM tissues taken from saline- and morphine-treated rats. **C**, **D**, Microscopic images of immunofluorescence labeling for NGF in NRM sections from saline (Sal)- and morphine (Mor)-treated rats. Calibration: 100 μ m. **E**–**G**, Images of labeling for NGF (red) and for neurons with the neuronal marker NeuN (green), and a merged image in NRM slices from a morphine-treated rat. Calibration: 20 μ m. **H**, EPSCs from a rat treated with morphine plus GW441756 *in vivo*. ** $p < 0.01$. Calibration: 50 pA and 10 ms.

trol, 154 ± 20 pA, deltorphan, 147 ± 18 pA, $n = 14$, $p = 0.17$) (Fig. 5B). In addition, morphine induction of DOPr appeared to be also dependent on Ca^{2+} entry through store-operated Ca^{2+} channels (SOCs), as long-period treatment of the slices ($n = 11$) with MRS1845 (5 μ M), a potent SOCC blocker, eliminated the deltorphan effect (control, 140 ± 13 pA, deltorphan, 127 ± 13 pA, $n = 13$, $p = 0.15$) (Fig. 5B). In contrast, in the slices ($n = 6$) similarly treated with long-period GF109203X (3 μ M), a selective inhibitor of PKC, which is the substrate of DAG, deltorphan retained its inhibition of glutamate EPSCs (control, 256 ± 76 pA, deltorphan, 164 ± 57 pA, $n = 6$, $p < 0.05$) (Fig. 5B).

Increased activity of CaMKII, a serine/threonine kinase, has been shown to drive rapid (in seconds) membrane insertion of AMPA glutamate receptors into synapses through interactions between GluR1 subunits of AMPA receptors and cytoplasmic proteins containing the PSD-95-Dlg-ZO1 (PDZ) domains (Hayashi et al.,

Figure 4. NGF mediates morphine induction of DOPr on GABA synapses. **A**, Superimposed GABA-mediated IPSCs in control and in deltorphan (1 μ M) in NRM neurons in a normal slice and in normal slices treated with NGF (100 ng/ml) alone or with NGF plus naltriben (50 nM) for 4 h *in vitro*. **B**, Group data of the deltorphan effects after treatment of normal slices with NGF or NGF plus naltriben. **C**, Representative GABA IPSCs in slices from morphine-treated rats and treated with the Trk receptor inhibitor K252a (200 nM) for 4 h *in vitro*. Note that use of KCl in the intrapipette solution with a holding potential of -60 mV resulted in a downward direction of the IPSCs. ** $p < 0.01$. Calibration: 50 pA and 20 ms.

2000; Collingridge et al., 2004). Considering the Ca^{2+} dependence of this DOPr induction, we were wondering whether Ca^{2+} -dependent activation of CaMKII was also required, although unlike the ligand-gated ion channel AMPA receptor, induction of membrane trafficking of DOPr, a GPCR, is a much slower (at least hours) process (Cahill et al., 2007). Nevertheless, in slices ($n = 10$) from morphine-treated rats and treated with the specific CaMKII inhibitor KN-93 (5 μ M) for a long period, the DOPr agonist was no longer effective (control, 191 ± 16 pA, deltorphan, 183 ± 18 pA, $n = 11$, $p = 0.45$) (Fig. 5C), whereas it inhibited EPSCs after similar treatment with the inactive analog KN-92 (5 μ M) (control, 164 ± 20 pA, deltorphan, 110 ± 13 pA, $n = 12$ from 10 slices, $p < 0.01$) (Fig. 5C). In further support of this CaMKII role, brainstem tissues taken from morphine-treated rats contained a significantly higher level of phosphorylated CaMKII proteins, the activated form of CaMKII, with the total CaMKII protein unchanged from controls (Fig. 5D,E).

As controls for the effects of all inhibitors we used, a short-period (30 min) treatment of slices from morphine-treated rats with those inhibitors failed to significantly alter morphine-induced DOPr function (see supplemental material, available at www.jneurosci.org).

Sorting of DOPr

Next, we sought other signaling proteins, downstream of CaMKII activation, which might account for altering the normal, intracel-

Figure 5. Morphine induction of DOPr involves the PLC–CaMKII pathway. **A–C**, Group data of delforphan effects on EPSCs in slices from morphine-treated rats in control or treated *in vitro* with the indicated inhibitor for a long period. **D**, Representative lanes of CaMKII, p-CaMKII, and GAPDH proteins in NRM tissues from saline- and morphine-treated rats. **E**, Summarized Western blot results of percentage changes in CaMKII and p-CaMKII proteins normalized to GAPDH. Thapsigargin. * $p < 0.05$, ** $p < 0.01$.

lular sorting fate of DOPr after prolonged exposure to morphine and NGF. One of the few such candidate proteins currently known is NHERF-1, also known as EBP50 (ezrin-radixin-moesin-binding phosphoprotein-50), which is a PDZ domain-containing scaffolding protein implicated in the assembly of protein complexes and in sorting of internalized β_2 -adrenergic receptors and κ -opioid receptor (KOPr) to the recycling pathway for receptor resensitization (Liu-Chen, 2004; Weinman et al., 2006; Hanyaloglu and von Zastrow, 2008). We first examined whether the morphine treatment would change NHERF-1 expression. In brainstem tissues taken from morphine-treated rats ($n = 8$), we found that the protein level of NHERF-1, normalized to GAPDH, was significantly increased to $152 \pm 18\%$ of control ($p < 0.05$) (Fig. 6A), raising the possibility that upregulated NHERF-1 protein changes the sorting fate of DOPr from normally intracellular targeting to exocytotic trafficking for membrane insertion and functional expression. This hypothesis was supported by our following experiments using coimmunoprecipitation (co-IP) to assess interaction between DOPr and NHERF-1 proteins. In control brainstem tissues, there was a low level of NHERF-1 protein that coprecipitated with DOPr (Fig. 6B,C), consistent with previous reports that NHERF-1 normally has low affinity to or interaction with the c-terminal of DOPr (Heydorn et al., 2004; Huang et al., 2004). However, in brainstem tissues from morphine-treated rats ($n = 10$), the amount of NHERF-1 protein that coprecipitated with DOPr was significantly increased [Fig. 6B (top two panels), C]. In contrast, after incubation of the DOPr antibody with a sequence-specific DOPr-blocking peptide (Patwardhan et al., 2005), no DOPr or NHERF-1 protein was detected in the precipitated preparations following the same IP procedure (Fig. 6B, bottom two panels), indicating a sequence-specific binding of the DOPr antibody used for DOPr co-IP. Additionally, this specific binding of DOPr by the DOPr antibodies is supported by the loss of DOPr immunostaining in DOPr KO mice (see supplemental material, available at www.jneurosci.org).

Demonstrating a specific role of TrK receptors in this increased NHERF-1–DOPr interaction, cotreatment of rats ($n = 5$) with morphine and the TrK receptor antagonist K252a *in vivo*

significantly inhibited the morphine-induced increase in NHERF-1–DOPr coprecipitation (Fig. 6C). In addition, morphine treatment also increased the serine/threonine phosphorylation of DOPr ($n = 13$ rats), which was blocked by cotreatment *in vivo* with morphine and K252a ($n = 5$ rats) (Fig. 6D). A similar increase in NHERF-1–DOPr coprecipitation was also observed in brainstem tissues from naive rats ($n = 6$) treated with NGF (50 ng/0.5 μ l) *in vivo* through microinjections into the brainstem nucleus (NHERF/DOPr ratio: saline, 0.36 ± 0.07 , NGF, 0.75 ± 0.07 , $p < 0.01$) (Fig. 6E). The NGF treatment of naive rats *in vivo* ($n = 6$ rats) also increased the amount of phosphorylated DOPr (pDOPr/DOPr ratio: saline, 0.40 ± 0.04 , NGF, 0.61 ± 0.04 , $p < 0.01$) (Fig. 6F).

DOPr in NHERF-1 knock-out mice

To further validate the specificity of DOPr–NHERF-1 interaction and its role in the DOPr trafficking, we examined the effects of morphine and NGF on DOPr induction in NHERF-1 KO mice we originally reported (Shenolikar et al., 2002). In saline-treated NHERF-1^{−/−} mice and corresponding WT mice, delforphan (1 μ M) was ineffective on the amplitude of glutamate EPSCs in the brainstem neurons (WT: control, 209 ± 14 pA, delforphan, 198 ± 14 pA, $n = 6$, $p = 0.38$; KO: control, 292 ± 8 pA, delforphan, 285 ± 7 pA, $n = 7$, $p = 0.37$) (Fig. 7A,E). In morphine-treated WT mice, functional DOPr, similar to that in morphine-treated rats, emerged as shown by delforphan inhibition of EPSCs (control, 173 ± 11 pA, delforphan, 109 ± 7 pA, $n = 6$, $p < 0.01$), but it was absent in the KO mice treated similarly with morphine (control, 234 ± 7 pA, delforphan, 253 ± 8 pA, $n = 11$, $p = 0.29$) (Fig. 7B,E). Moreover, the same long-period NGF treatment of brainstem slices from naive WT mice induced functional DOPr on glutamate synapses in 6 of 8 cells (control, 201 ± 18 pA, delforphan, 130 ± 13 pA, $n = 6$, $p < 0.01$), but failed to do so in any cell tested in slices from naive KO mice (control, 257 ± 8 pA, delforphan, 252 ± 8 pA, $n = 10$, $p = 0.46$) (Fig. 7C,E). In addition, morphine-induced DOPr function in WT mice was also abolished by long-period treatment of the slices *in vitro* with the TrK receptor antagonist K252a (200 nM) (control, 138 ± 6 pA, delforphan, 133 ± 7 pA, $n = 5$, $p = 0.36$) (Fig. 7D,E). The expression of DOPr protein in the NHERF-1 KO mice was not significantly changed from that in the WT mice (DOPr/actin ratio: WT, 0.75 ± 0.04 , $n = 5$; KO, 0.72 ± 0.06 , $n = 6$, $p = 0.72$) (Fig. 7F). These findings from NHERF-1-lacking mice further support our results from rats by showing that NHERF-1 is required for both morphine and NGF induction of synaptic DOPr.

Translocation of DOPr to surface membrane

To determine whether the emerged DOPr on synaptic terminals was indeed due to translocation of intracellular DOPr to surface membrane, we next examined the amount of DOPr protein on terminal membrane using brainstem preparations of synaptosomes, which contain mostly membrane structures of synaptic terminals with nearly no cell body contents and greatly reduced intraterminal contents (Ghijsen et al., 2003; Dunkley et al., 2008). In a previous study (Ma and Pan, 2006), we have shown that the

morphine treatment does not significantly change the total DOPr protein in the brainstem tissue. However, we found that, in brainstem synaptosomes from morphine-treated rats, the amount of DOPr protein, normalized to the specific synaptic terminal marker synaptophysin, was significantly increased when compared to controls, as revealed by Western blot analysis; this increase in synaptosomal DOPr was blocked by cotreatment of rats *in vivo* with morphine plus the Trk receptor antagonist K252a (Fig. 8A). Moreover, in rats treated with NGF *in vivo* that induced functional DOPr, the amount of synaptosomal DOPr protein was also significantly increased (Fig. 8A). Thus, it appears that the morphine and NGF induction of functional DOPr on synaptic terminals is likely due, at least partially, to DOPr translocation to surface membrane of synaptic terminals, consistent with our previous anatomical study of labeled DOPr with confocal microscopy on the same brainstem neurons (Ma and Pan, 2006) and with our recent report of morphine-induced increase in synaptosomal DOPr of amygdala neurons (Bie et al., 2009). This notion is also in line with the mechanism for membrane trafficking of postsynaptic DOPr on dorsal root ganglion cells (Zhang et al., 2006; Cahill et al., 2007).

If the appearance of functional DOPr was indeed due to translocation or trafficking of intracellular DOPr to surface membrane rather than other mechanisms, it should be blocked in conditions under which known processes required for exocytotic receptor trafficking and membrane insertion were disrupted. The endoplasmic reticulum (ER) and the Golgi apparatus are important membrane network for packing and transporting of cytoplasmic proteins in receptor trafficking (Tan et al., 2004). In slices ($n = 9$) from morphine-treated rats and treated for 4 h with brefeldin A (5 μ M), an inhibitor of protein transportation from ER to the Golgi apparatus (Donaldson et al., 1992), deltorphin (1 μ M) could no longer inhibit the EPSC (control, 165 ± 12 pA, deltorphin, 159 ± 13 pA, $n = 11$, $p = 0.22$) (Fig. 8B, G).

Furthermore, to inhibit receptor exocytosis, we used a fusion polypeptide, TAT-NSF81 (5 μ M), which we have previously shown to block exocytosis by inhibiting N-ethyl-maleimide-sensitive factor (NSF)-mediated disassembly of soluble NSF attachment protein receptor (SNARE) molecules (Matsushita et al., 2005). After the TAT-NSF81 treatment of slices from morphine-treated rats for 3 h *in vitro*, the slices were left in normal bath solution without the polypeptide for at least 20 min before EPSC recording. We found that TAT-NSF81 prevented deltorphin-induced inhibition of EPSCs in these slices ($n = 8$) (control, 153 ± 20 pA, deltorphin, 142 ± 17 pA, $n = 10$, $p = 0.47$) (Fig. 8C, G). By contrast, similar treatment with the corresponding but scrambled control peptide TAT-NSF81scr (5 μ M) failed to alter the deltorphin inhibition of EPSCs (control, 198 ± 23 pA, deltorphin, 135 ± 24 pA, $n = 7$, $p < 0.01$) (Fig. 8D, G). Next, we

Figure 6. Chronic morphine increases interaction between DOPr and NHERF-1 proteins. **A**, Western blot lanes of NHERF-1 and GAPDH proteins in NRM tissues from saline- and morphine-treated rats. **B**, DOPr immunoprecipitates (IP) immunoblotted for NHERF-1 and for DOPr in NRM tissues from saline- and morphine-treated rats in control (top two panels) and after preincubation of the DOPr antibody with a DOPr-blocking peptide (bottom two panels). **C**, **D**, Similar IP data of NHERF-1 and DOPr (**C**) and DOPr immunoprecipitates with immunoblotted phosphorylated DOPr (pDOPr) and total DOPr (**D**) in tissues from saline-treated rats and rats treated with morphine without or with K252a cotreatment *in vivo*. Also shown are changes in the ratios of NHERF-1 over DOPr and pDOPr over total DOPr proteins in the indicated three groups of rats. **E**, **F**, Similar DOPr precipitates immunoblotted for NHERF-1 and DOPr (**E**) and for pDOPr and total DOPr (**F**) in NRM tissues from naive rats treated with saline or NGF *in vivo*. Mor, Morphine. ** $p < 0.01$.

confirmed that TAT-NSF81 did not inhibit signaling by other neuronal receptors constitutively localized on the plasma membrane, using GABA_B receptor-mediated presynaptic inhibition as an example. In slices from morphine-treated rats, baclofen (10 μ M), a potent GABA_B receptor agonist, produced a large inhibition in the EPSC amplitude (control, 289 ± 57 pA, baclofen, 141 ± 39 pA, $n = 7$, $p < 0.01$) (Fig. 8E, G). This baclofen inhibition was largely due to activation of presynaptic GABA_B receptors on glutamate synaptic terminals (B. Bie and Z. Z. Pan, unpublished observation). In slices that were comparable but pretreated similarly with TAT-NSF81 (5 μ M), baclofen (10 μ M) induced a similar EPSC inhibition (control, 201 ± 23 pA, baclofen, 89 ± 12 pA, $n = 8$, $p < 0.01$) (Fig. 8F, G). There was no statistical difference ($p = 0.45$) in the baclofen-induced EPSC inhibitions with and without the polypeptide treatment, indicating that TAT-NSF81 does not have a significant effect on the synaptic function of preexisting GABA_B receptors on glutamate synapses. Thus, the recruitment of functional DOPr appears to require DOPr transportation from ER to Golgi and NSF-regulated vesicle exocytosis to the plasma membrane, two key processes for membrane trafficking of GPCRs (Tan et al., 2004). Figure 8H illustrates the proposed signaling pathways for chronic opioid-induced exocytotic trafficking and functional expression of DOPr on a central synaptic terminal.

Figure 7. NHERF-1 is required for morphine and NGF induction of functional DOPr. **A–D**, Representative glutamate EPSCs in control and in deltorphin ($1 \mu\text{M}$) in an NRM neuron from WT mice (left column) and NHERF-1 KO mice (right column) treated with saline (**A**), morphine (**B**), from normal slices treated with long-period NGF (**C**), or from a K252a-treated slice of a morphine-treated WT mouse (**D**). **E**, Summarized data of the deltorphin effects in the four treatment groups of WT and KO mice. **F**, Western blot lanes of DOPr and actin proteins in NRM tissues from WT ($n = 5$) and NHERF-1 KO mice ($n = 6$). ** $p < 0.01$. Calibration: 50 pA and 10 ms.

Discussion

We have shown that long-period NGF, applied to central neurons both *in vitro* and in animals *in vivo*, causes the emergence of new functional DOPr on central synaptic terminals likely by redirecting constitutively intracellular DOPr to surface membrane, a mechanism that may account for chronic opioid-induced synaptic DOPr function. In addition, we have identified the prominent signaling pathway for the NGF effect, from TrKA receptors to exocytosis for surface insertion. Thus, NGF may function as a key

molecular switch that alters the normal sorting of intracellular DOPr, redirecting intracellular DOPr to plasma membrane for functional expression under sustained opioid stimulation.

Classically, NGF has well known roles in neuronal survival and cell differentiation in the developing brain. Recent evidence has shown diverse trophic effects of NGF on the adult brain as well, particularly on structural modifications and synaptic efficacy in central synapses (Poo, 2001; Sofroniew et al., 2001; Chao and Bothwell, 2002). In cultured HEK293 cells, NGF or epidermal growth factor rapidly (within minutes) increases membrane insertion of TRP channels by PI3 kinase-dependent phosphorylation (Zhang et al., 2005). The current study shows that, in native brain neurons, NGF causes translocation and surface expression of intracellular DOPr, a member of the GPCR superfamily representing the largest population of membrane receptors responsible for most of signaling across plasma membrane in mammalian cells (Tan et al., 2004). Thus, NGF may have much broader regulating functions in receptor signaling of the adult brain under both normal and pathological conditions.

Recent studies increasingly suggest that sorting of intracellular GPCRs to either lysosomes for degradation or endosomes for recycling is diverse and highly regulated by cytoplasmic sorting proteins. However, our understanding of GPCR sorting is still in its infancy, as only a few GPCR sorting proteins have been identified to date, in contrast to the vast number of GPCRs in brain neurons. For constitutive recycling under normal conditions, the PDZ domain-containing NHERF-1 is responsible for recycling of β_2 -adrenergic receptors and KOPr (Liu-Chen, 2004; Hanyaloglu and von Zastrow, 2008), but has little interaction with DOPr as observed in heterologously expressing cell systems (Huang et al., 2004). Consistently, little NHERF-1-DOPr co-IP was observed in control, opioid-naïve tissues in the present study. In contrast to constitutive sorting of GPCRs, little is known about the molecular mechanisms by which the normal sorting of GPCRs is altered under pathological conditions. Our results of significant NHERF-1-DOPr interaction,

observed only after chronic morphine treatment and supported by results from NHERF-1 KO mice, indicate that NHERF-1 may possess a broader binding capacities and functional diversity in GPCR sorting and recycling under various conditions. It remains to be investigated how chronic opioids change NHERF-1-DOPr binding properties for their increased interaction, whether the increased DOPr phosphorylation we observed plays a role, and what specific C-terminal sequence of DOPr with certain phosphorylated sites is involved in the interaction.

The intracellular retention of DOPr was shown to be maintained by DOPr binding to vesicle luminal substance P (SP), and upon agonist stimulation, SP-bound DOPr in vesicles was rapidly (in seconds) inserted into plasma membrane in peripheral sensory neurons (Guan et al., 2005). However, that notion has been argued against by a recent study, using knock-in mice with a functional DOPr-eGFP fusion receptor in the same spinal neurons, showing that DOPr had no overlap with SP in expression and DOPr was trafficked independently of SP (Scherrer et al., 2009). Interestingly, NGF acutely (<60 min) increases the intracellular pool of ER-derived, Golgi-associated DOPr in cultured secretory PC12 cells *in vitro* (Kim and von Zastrow, 2003). This relatively acute NGF effect is in contrast to the long-term (4 h) NGF effect of DOPr translocation to surface membrane described in this study, and might serve as a parallel NGF effect that primes and replenishes the intracellular DOPr pool for surface trafficking. While we did not observe a significant increase in total DOPr protein after morphine treatment (Ma et al., 2006), upregulated NGF may increase the expression of many other downstream signaling proteins given its broad stimulating effect in neural protein expression, contributing to the NGF effect (Huang and Reichardt, 2003). For example, NGF treatment of PC12 cells increased expression of DOPr gene through epigenetic modifications (Chen et al., 2008). Previously reported, agonist- or depolarization-induced membrane trafficking of DOPr and TRP channels share a characteristic of rapid occurrence (within seconds to minutes) (Guan et al., 2005; Odell et al., 2005; Zhang et al., 2005), but clearly differ from the present finding of NGF-induced, slowly occurring (at least hours) translocation of synaptic DOPr. The latter may be indicative of extra layers of adaptive regulations at transcriptional and translational levels, and in the forward transportation of DOPr through the ER–Golgi–surface membrane secretory pathway.

Receptor exocytosis and insertion into surface membrane involve membrane fusion between plasma membrane and the membrane of receptor-containing vesicles (Pfeffer, 2007; Wojcik and Brose, 2007). The SNARE complex is a core execution component in the membrane fusion machinery. While much of our current understanding of SNARE-mediated fusion mechanisms has been derived from studies on fast (millisecond) synaptic release of neurotransmitters (Wojcik and Brose, 2007), emerging evidence suggests that re-

Figure 8. Morphine induction of DOPr involves DOPr translocation to surface membrane of synaptic terminals. **A**, Western blot lanes of DOPr protein and the synaptic terminal marker synaptophysin (synapsin), and their ratio changes in NRM synaptosomes from rats treated with saline, morphine, NGF, or morphine plus K252a *in vivo*. **B**, Representative EPSCs in a slice from a morphine-treated rat and treated *in vitro* with long-period brefeldin **A**. **C**, **D**, Deltorphan actions on EPSCs in slices from morphine-treated rats and treated *in vitro* with the exocytosis-inhibiting peptide TAT-NSF 81 (**C**) or with the corresponding but scrambled peptide TAT-NSF81scr (**D**) for 3 h. **E**, **F**, Effects of the GABA_B receptor agonist baclofen (10 μ M) on EPSCs in NRM slices from morphine-treated rats without (normal) or with the TAT-NSF81 treatment *in vitro*. **G**, Group data showing the effects of deltorphan and baclofen in NRM slices of morphine-treated rats in control or after long-period treatment with the agents indicated. $**p < 0.01$. Calibration: 50 pA and 10 ms. **H**, Diagram of the proposed signaling pathways for DOPr trafficking on a central synaptic terminal. Chronic morphine upregulates NGF (1), which activates the TrKA receptor (2) and its coupled PLC γ pathway (3) and PI3K pathway (downstream components to be investigated). PLC γ activation leads to production of IP $_3$ and increased intracellular Ca^{2+} through Ca^{2+} release from the IP $_3$ receptor (IP $_3$ R)-controlled Ca^{2+} store (4) and by Ca^{2+} influx through SOCC. DOPr is normally packed and transported through the ER/Golgi network and constitutively targeted to an intracellular pool (5). Increased intracellular Ca^{2+} activates CaMKII (6), which would lead to downstream changes in the expression of DOPr and NHERF-1 genes and proteins through yet unknown mechanisms of transcription and translation (7), resulting in an increased interaction between upregulated NHERF-1 and phosphorylated DOPr, and exocytotic translocation of intracellular DOPr to surface membrane. Interaction of DOPr-containing vesicles with the SNARE complex, whose function is regulated by NSF (8), causes membrane fusion for exocytosis and surface expression of functional DOPr (9).

ceptor trafficking and surface delivery also involve SNARE-dependent exocytosis (Lan et al., 2001). NSF, an ATPase associated with the SNARE complex, regulates exocytosis by disassembling the SNARE complex in the SNARE assemble/disassemble cycle necessary for normal SNARE functions (Zhao et al., 2007). We have developed the cell-penetrating peptide TAT-NSF81 that interrupts exocytosis by inhibiting NSF activity (Matsushita et al., 2005). Our observation of TAT-NSF81 blocking the surface recruitment of functional DOPr indicates that the DOPr recruitment likely requires Ca^{2+} -dependent, NSF/SNARE-mediated exocytosis. We noticed a reduction in EPSC amplitude after the TAT-NSF81 pretreatment, which possibly reflects its inhibitory effect on SNARE-dependent membrane fusion of synaptic vesicles for glutamate release. A short period of wash of the peptide appeared to partially recover its inhibition of glutamate release, but not the already translocated DOPr. In addition to regulating SNARE disassembly, NSF directly interacts with specific recycling sequences on the cytoplasmic terminal of receptors to regulate their membrane trafficking, including β_2 -adrenergic receptors and AMPA glutamate receptors (Zhao et al., 2007; Hanyaloglu and von Zastrow, 2008). Therefore, it is also possible that TAT-NSF81 blocks DOPr translocation by disrupting direct NSF–DOPr interaction.

Functional interaction between DOPr and MOPr has been well documented in previous studies using behavioral and genetic approaches (Kieffer and Gavériaux-Ruff, 2002). It likely involves diverse mechanisms at multiple levels, including synergistic interaction at the receptor level as DOPr–MOPr heterodimers in enhancing morphine analgesia (Gomes et al., 2004). Our current finding provides another potential mechanism by which NGF functions as a link between sustained MOPr stimulation and increased function of synaptic DOPr at the cellular level.

Proper GPCR localization and signaling are required for normal cell functions, and GPCR mislocalization and resultant abnormal signaling cause diseases (Tan et al., 2004). For example, NGF-mediated rapid membrane insertion of TRPV1 channels by PI3 kinase-dependent phosphorylation likely accounts for hypersensitivity of peripheral sensory neurons under inflammatory pain sensitization (Ji et al., 2002; Zhang et al., 2005). Given the widespread DOPr distribution and its membrane trafficking found in pain-related central neurons and recently in drug addiction-related brain areas (Ma et al., 2006; Zhang et al., 2006; Cahill et al., 2007; Bie et al., 2009), NGF-regulated membrane trafficking of DOPr may represent another leap in the ever-diversifying functions of this neurotrophin for GPCR signaling in the adult brain. In view of wide NGF involvement in many pathological conditions, such as tissue inflammation, nerve injury, drug addiction, behavioral stress, degenerative changes, and immune response-related diseases (Sofroniew et al., 2001), NGF control of GPCR trafficking may have broader functional implications as a potential cellular mechanism for synaptic alterations, abnormal cellular signaling, and system changes under those conditions (Poo, 2001; Collingridge et al., 2004; Hefti et al., 2006).

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