Chronic morphine reduces surface expression of δ-opioid receptors in subregions of rostral striatum

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Abstract

The delta opioid receptor (DOPr), whilst not the primary target of clinically used opioids, is involved in development of opioid tolerance and addiction. There is growing evidence that DOPr trafficking is involved in drug addiction, e.g., a range of studies have shown increased plasma membrane DOPr insertion during chronic treatment with opioids. The present study used a transgenic mouse model in which the C-terminal of the DOPr is tagged with enhanced-green fluorescence protein (eGFP) to examine the effects of chronic morphine treatment on surface membrane expression in striatal cholinergic interneurons that are implicated in motivated learning following both chronic morphine and morphine sensitization treatment schedules in male mice. A sex difference was noted throughout the anterior striatum, which was most prominent in the nucleus accumbens core region. Incontrast with previous studies in other neurons, chronic exposure to a high dose of morphine for six days had no effect, or slightly decreased (anterior dorsolateral striatum) surface DOPr expression. A morphine sensitization schedule produced similar results with a significant decrease in surface DOPr expression in nucleus accumbens shell. These results suggest that chronic morphine and morphine sensitisation treatment may have effects on instrumental reward-seeking behaviours and learning processes related to drug addiction, via effects on striatal DOPr function

Keywords

Introduction

Our understanding of G-protein coupled receptor (GPCR) trafficking has grown significantly in recent years. This is of interest because GPCR trafficking and localisation has important functional and hence therapeutic implications [1]. This is particularly true of the δ-opioid receptor (DOPr) [1, 2].

DOPr are typical Class A GPCRs that, together with the μ-opioid receptor (MOPr) and κ-opioid receptor (KOPr), comprise the classical opioid receptor family [3]. They are $G_{i/o}$-coupled receptors which upon agonist binding and activation mediate antinociceptive, anxiolytic and euphoric effects [2, 3]. While no DOPr-selective agonists are currently approved for clinical use, these effects make them an important class of potential novel therapeutics, particularly given that DOPr-selective agonists appear to exert fewer side effects than MOPr-active drugs [2].

Insight into dynamic DOPr trafficking and function arose two decades ago with the observation that, under homeostatic conditions, most DOPr's reside not at the cell membrane but rather within intracellular vesicles [4]. This finding has since been replicated in numerous studies using both pharmacological techniques and immunogold electron microscopy [1, 2], and provided the first indication that, unlike most GPCRs, DOPr's are not constitutively trafficked to the cell membrane. Indeed, DOPr's appear to undergo cell membrane-directed trafficking on an 'as-needed' basis via the regulated secretory pathway [2], and this stimulus-driven trafficking has recently been the subject of considerable study. Membrane trafficking and functional up-regulation of DOPr's has been observed both *in vivo* and *in vitro* following diverse pathological and pharmacological challenges, including chronic inflammatory pain [5], capsaicin treatment [1], chronic ethanol consumption, hypoxia and cancer [2]. The most extensively studied of these challenges, however, is chronic opioid exposure.

Initial evidence that DOPr's are trafficked to the cell membrane in response to opioids was provided by Cahill and colleagues, who proposed that increased DOPr function after chronic morphine exposure in rodents was attributable to increased membrane DOPr levels [6]. It was later demonstrated that this trafficking response is not specific to morphine challenge, and other opioids including methadone, etorphine and fentanyl likewise induce membrane DOPr accumulation following chronic treatment, an effect that appears to depend upon MOPr function [7]. Numerous *in vivo* rodent studies have since provided further histochemical and physiological evidence for this trafficking response to opioids in neurons in a number of

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regions, including studies of dorsal root ganglion (DRG) and dorsal horn of the spinal cord [6 − 8], periaqueductal grey (PAG) [9], nucleus accumbens (NAc) and dorsal striatum [10], nucleus raphe magnus [11], and amygdala central nucleus [12], indicating that this phenomenon is common and not limited to a single region within the nervous system.

This upregulation of cell membrane DOPr levels, and consequently DOPr function, carries important clinical implications. It raises the interesting notion that patients seeking the therapeutic effects of opioid drugs could first be treated with agents that modulate DOPr trafficking and function, essentially 'priming' patients for more effective use of these drugs. In this regard, it is significant that many of the regions noted above for DOPr trafficking are also important sites for the modulation of pain and anxiety. Given that chronic opioid exposure induces DOPr trafficking, upregulated DOPr's could also potentially be targeted by DOPr -selective agonists, to maintain effective analgesia in patients who have developed tolerance to the analgesic effects of opioids with their chronic use.

Recent studies have indicated that DOPr's may also play an important role in drug addiction. This is supported by the expression of DOPr's in circuits involved in reward processing - including the NAc and ventral tegmental area (VTA), as well as those involved in learning and memory processes - including the amygdala and hippocampus - thought to underlie addiction [3]. Inhibition of DOPr function in the rodent NAc and VTA reduces and augments, respectively, the self-administration of cocaine [13]. Similarly, infusion of the DOPr antagonist, naltrindole. into the central nucleus of the amygdala (CeA) reduces morphine and ethanol conditioned place preference [14]. More general evidence of a role for DOPr function in addiction has been provided by DOPr knock-out mice, which exhibit a reduced nicotine or morphine conditioned place preference relative to wild-type animals [3, 15]. Similarly, systemic administration of non-selective DOPr antagonists such as naltrindole has been shown to reduce self-administration of ethanol in rodents [16]. Taken together, these results suggest that DOPr's modulate drug-cue/context associations and drug-seeking instrumental behaviour.

Our recent work has provided evidence of a role for DOPr trafficking and function in cue-elicited instrumental reward-seeking behaviours. Pavlovian training - i.e. the learning of cue-reward associations - in mice induced a persistent upregulation of somatic membrane DOPr's in cholinergic interneurons (CINs) of the NAc shell subregion (NAc-S) [17]. That study exploited the ability to directly visualise DOPr using a transgenic mouse in which the DOPr

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is tagged at the C-terminal with eGFP, together with precise definition of the location of DOPr at the cell surface of CINs using immunohistochemistry of choline acetyltransferase (ChAT). The extent of this upregulation correlated with the magnitude of cue-elicited increases in reward-seeking instrumental behaviours, i.e. Pavlovian-to-instrumental transfer (PIT), while the administration of a DOPr antagonist directly into the NAc-S abolished this transfer effect [18]. Furthermore, this DOPr upregulation increased the variance in CIN firing, an effect enhanced by DOPr activation, and absent in mice not given Pavlovian training [17]. *In vivo*, this modulation of CIN firing activity is likely to have important consequences for the function of CINs as key modulators of local signalling within the striatum.

The above results indicate that dynamic DOPr trafficking and function in CINs of the NAc-S may mediate cue-elicited increases in reward-seeking behaviour following the learning of cue-reward associations. Several authors have argued that this behavioural phenomenon may reflect cue-elicited increases in instrumental drug-seeking behaviours in addiction [19, 20]. If so, this would indicate that the modulation of striatal DOPr trafficking may have important clinical consequences with respect to addiction. Indeed, this would be consistent with the evidence for DOPr function in drug-cue/context associations and drug-seeking instrumental behaviours discussed above.

Given the upregulation of membrane DOPr's with chronic opioid exposure and the potential role of DOPr's in CINs in learning mechanisms related to drug addiction, the current study investigated the effects of chronic morphine and morphine sensitisation treatments on CIN membrane DOPr's in the striatum using the methods described in [17]. We are not aware of other published studies on the effects of chronic morphine exposure either on surface expression of DOPr in striatal CINs, or studies using DOPr-eGFP mice to examine the effects of chronic morphine on DOPr trafficking. We found higher levels of membrane DOPr in CINs in subregions of the rostral striatum of male mice compared with female mice. Both continuous chronic exposure to morphine and a sensitisation treatment schedule produced either no effect or a reduction in surface membrane DOPr expression in male mice. These results suggest that chronic morphine regulates surface DOPr expression differently in striatal CINs compared with other neurons studied to date.

Methods

Animals

C57BL/6 knock-in mice expressing enhanced green fluorescent protein (eGFP)-tagged DOPr's were used for all molecular studies in which functional DOPr (Oprd1) fused to the eGFP gene is inserted into the wild-type Oprd1 locus, which provides fluorescent DOPr's with maintained cellular functions [21]. Mice used in chronic morphine experiments were all at least 8 weeks of age at the start of experiments; these were randomly assigned to morphine (n = 2 females, 5 males; however one female brain was lost during perfusion and processing so only results for males are presented below) or vehicle (n = 5 males) treatment. Those used for morphine sensitisation experiments were at least 6-8 weeks of age and were randomly assigned to morphine (n = 2 females, 4 males) or vehicle (n = 4 females, 2 males) treatment. For behavioural locomotor sensitisation experiments, 12 doubly heterozygous DOPr-eGFP x ChAT-cre mice expressing cre recombinase under the *CHAT* promoter were used. *CRE* insertion is predicted to have no behavioural impact without corresponding *LOX* site insertion; thus these mice were deemed suitable for the present experiment. 6 were assigned to morphine treatment (n = 4 males, 2 females) and 6 to vehicle (n = 4 males, 2 females); all were at least 8 weeks of age at the start of the experiment. All experimental procedures were approved by the University of Sydney Animal Ethics Committee.

Drug treatments

Chronic morphine treatments were administered using a sustained-release morphine formulation that slowly releases morphine from a depot over many days [9]. This was prepared by suspending morphine base in a vehicle emulsion composed of 10% mannide monooleate (Arlacel A) (Sigma, St. Louis, MO) and 40% light liquid paraffin (Biotech Pharmaceuticals, Victoria, Australia) in 0.9% v/v NaCl solution. This was administered via subcutaneous injection at 300 mg/kg of morphine or equivalent-volume vehicle every alternate day over 5 days. This protocol was shown previously to create a sustained release of morphine and induce tolerance and dependence in mice [9].

To induce sensitisation, morphine HCl (GlaxoSmithKline, Victoria, Australia) was dissolved in 0.9% v/v NaCl and administered at 20 mg/kg in 3 subcutaneous injections every alternate day over 5 days in a constant environment. Control mice received an equivalent volume (0.1 ml/20 g) of the vehicle (protocol modified from [15]).

Tissue Preparation and Immunohistochemistry

Mice were sacrificed 24 h after final morphine or vehicle injections in all chronic morphine experiments, and 30-60 min after final injections in morphine sensitisation experiments. Animals were anaesthetised with a 500 mg/kg intraperitoneal injection of sodium pentobarbitone (Lethabarb, Virbac Pty. Ltd., Australia) in 0.9% v/v NaCl solution. After confirming deep anaesthesia with tail and hindpaw reflexes, mice were perfused transcardially with ice-cold 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.3). Brains were extracted and post-fixed overnight at 4°C, before 30 µm-thick coronal sections containing the NAc-S, NAc core (NAc-C), anterior dorsolateral striatum (aDLS) and anterior dorsomedial striatum (aDMS) (+1.420 mm to +1.045 mm relative to bregma) were sliced.

To visualise DOPr-eGFP expressed by CINs, slices were rinsed in 0.1 M tris-buffered saline (TBS), before 5 min incubation in TBS solution containing 3% H_2O_2 and 10% CH_3OH. Following further rinsing, slices were incubated in 0.2% Triton X-100 in TBS for 20 min, followed by an additional TBS rinse and incubation with primary antibody. These were polyclonal Goat Anti-ChAT (1:300, Millipore, Billerica, MA), and polyclonal Rabbit Anti-eGFP (1:500, Life Technologies, Carlsbad CA) in TBS overnight at 4°C. Slices were then rinsed again in TBS before incubation in secondary antibodies for 1 h. These were Donkey Anti-Goat Cy3 (1:400; Jackson ImmunoResearch Laboratories, West Grove, PA), and Donkey Anti-Rabbit Alexa 488 (1:400, Invitrogen, Carlsbad, CA) in TBS. After final rinsing, all slices were mounted onto microscope slides, allowed to dry, covered with Vectashield 1000 (Sigma, St. Louis, MO) and coverslipped before starting microscopy.

Microscopy and Quantification

In order to compare relative CIN somatic membrane DOPr levels, double immunofluorescence microscopy was conducted on striatal sections from morphine and vehicle mice as previously described [17]. This was performed with a sequential laser-scanning confocal microscope (Olympus FV1000, Tokyo, Japan) and images were analysed using the open source software ImageJ (MacBiophotonics upgrade version 1.48v, Wayne Rasband, National Institutes of Health, Bethesda, MD). Images of striatal CINs were acquired (surface, 52.2 μm^2, optical magnification, 60X; digital zoom, 4; bit depth, 16; resolution, 19.6 pixels/µm) first for the ChAT signal (HeNe laser intensity 17%, HV 700, offset 31%) and then the corresponding eGFP signal (Ar laser intensity of 10%, HV usually 744, offset 44%). These were obtained for all ChAT-immunoreactive cells that possessed

distinct nuclei. In the chronic morphine experiments, this resulted in data acquisition for 380 neurons in the NAc-S, 304 in the NAc-C, 385 in the aDLS, and 402 in the aDMS across both hemispheres and all animals. For sensitisation experiments, 341 neurons were imaged in the NAc-S, 272 in the NAc-C, 298 in the aDLS and 356 in the aDMS.

To compare membrane DOPr levels, 2 regions of interest (ROI) were defined using the raw ChAT image of each neuron. ROI 1 delineated the CIN somatic membrane and comprised the intracellular-extracellular interphase defined by the borders of a ChAT stained nucleus (see Figure 2G in [17]). ROI 2 served as a background correction and was defined as the central nuclear region devoid of staining. Mean grey values of each ROI were then calculated using the overlapped DOPr-eGFP image, and ROI 2 subtracted from ROI 1 to calculate a single normalised mean grey value per neuron (i.e. ROI 1-ROI 2). This analysis was blind, with all image file names randomly renamed using a Microsoft Excel plug-in (Romain Bouju, Paris, France).

Locomotor Activity Measurement

To confirm that the morphine sensitisation treatments effectively induced sensitisation, mice underwent locomotor activity measurements to identify the sensitised locomotor-stimulant effects of morphine. All mice were habituated to the locomotor activity chambers during 2-hour periods across 3 consecutive days prior to starting drug treatment. On each treatment day, mice received an additional 1-h habituation to the chamber, followed by 20 mg/kg injections of morphine or equivalent-volume saline over 5 days as described above. After the injections, mice were placed in polypropylene buckets (diameter 29 cm, height 22 cm) with white polyvinyl chloride cylinders (diameter 11 cm, height 15 cm) in their centre. These created circular corridors around the cylinders in which the animals could run continuously. These buckets were placed in sound and light-attenuating wooden shells (48 x 73 x 50 cm) with infrared cameras mounted directly above. Locomotion was then recorded using the program Mot Men 2.8 (Motion Mensura Pty. Ltd., NSW, Australia) for Windows and the total travelled distance (mm) for each mouse per 5-min time bin was recorded for 2 h post-injection. Mean total travelled distance across these bins was then calculated. Video files for each recording session were captured for offline analysis.

Statistical Analyses

All data were plotted and analysed using the program GraphPad Prism 6 for Macintosh (GraphPad Software, La Jolla CA) with a Type I error rate of 0.05.

For molecular studies, between-subjects two-way analysis of variance (ANOVA) was used to compare pooled mean grey values of morphine or vehicle-treated neurons from all striatal regions simultaneously (posterior striatal regions were also included in chronic morphine analyses but excluded presently for brevity). Sidak's multiple-comparisons test was then used to identify significant differences in mean grey value between treatment types in each subregion, Male female comparisons were made using mean grey values from vehicle-treated neurons from sensitisation experiments, which were initially conducted on male and female mice to to restirctions in animal availabilty.

For locomotor activity measurements, mean total travelled distance values were grouped according to treatment type and treatment day and analysed using two-way ANOVA with Tukey's multiple comparisons test (MCT).

Results

Sexually Dimorphic DOPr's

Somatic membrane DOPr-eGFP immunofluorescence was observed in CINs throughout the striatum of DOPr-eGFP knock-in mice, consistent with previous reports [17]. Fig. 1 presents photomicrographs of dorsal striatum CINs. Exemplar images of male and female morphine-treated neurons are illustrated (Fig. 1a and 1b, respectively), wherein male neurons exhibit a bright 'ring' of DOPr-eGFP immunofluorescence at the somatic membrane, while female neurons express less membrane DOPr-eGFP (see Fig. 2). This was observed consistently in both chronic morphine and sensitisation experiments.

Normalised CIN somatic membrane DOPr-eGFP levels for vehicle-treated male and female mice are presented for the ventral and anterior dorsal striatum in Fig. 2. These indicate that females had significantly lower membrane DOPr-eGFP than their male counterparts in all regions except the aDMS. Two-way ANOVA revealed a significant main effect of sex ($F_{1, 649} = 57.3$; $P < 0.0001$) and region ($F_{3, 649} = 42.3$; $P < 0.0001$), and a significant interaction effect ($F_{3, 649} = 3.13$; $P < 0.05$). Simple effects analysis revealed that DOPr-eGFP levels were significantly lower in female mice relative to males in the NAc-S ($t_{649} = 6.34$; $P < 0.0001$)

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(Fig. 2a), the NAc-C (t_{649} = 3.23; $P < 0.01$) (Fig. 2b), and in the aDLS (t_{649} = 3.17; $P = <0.01$) (Fig. 2c) but not the aDMS (t_{649} = 2.49; $P > 0.05$) (Fig. 2d).

Effects of morphine on membrane DOPr's

As numbers of female mice in the chronic morphine vehicle treatment groups were small (see Methods), results for the effects of morphine in males alone are presented. Treating male mice with a sustained-release formulation of morphine over 5 days induced no significant change in membrane DOPr-eGFP levels in the NAc-S (Fig. 3a); however, it did produce a small, significant decrease in the NAc-C (Fig. 3b). In the anterior dorsal striatum, chronic morphine induced no significant membrane DOPr-eGFP changes in the aDLS (Fig. 3c) or the aDMS (Fig. 3d). Two-way ANOVA revealed no main effect of morphine treatment ($F_{1, 1880}$ = 0.176; $P > 0.05$), but a significant effect of striatal region ($F_{5, 1880}$ = 245; $P < 0.0001$) and a significant interaction effect ($F_{5, 1880}$ = 4.49; $P < 0.001$). Simple effects analysis confirmed that morphine had no effect in the NAc-S (t_{1880} = 0.653; $P > 0.05$) but elicited a significant reduction in membrane DOPr-eGFP in the NAc-C (t_{1880} = 2.90; $P < 0.05$). No effect was observed in the aDLS (t_{1880} = 1.53; $P > 0.05$) or aDMS (t_{1880} = 0.0570; $P > 0.05$).

The present study also investigated the effects of morphine sensitisation on CIN DOPr trafficking. Two-way ANOVA revealed a significant main effect of morphine treatment ($F_{1, 580}$ = 11.1; $P < 0.01$), and region ($F_{3, 580}$ = 37.5; $P < 0.0001$), but no interaction effect ($F_{3, 580}$ = 1.36; $P > 0.05$) in males. Simple effects analysis indicated that morphine sensitisation induced a significant reduction in membrane DOPr-eGFP relative to vehicle treatment in the NAc-S (t_{580} = 3.49; $P < 0.01$) (Fig. 4a). Conversely, morphine failed to produce any significant effect in the NAc-C (t_{580} = 0.756; $P > 0.05$) (Fig. 4b), aDLS (t_{580} = 0.829; $P > 0.05$) (Fig. 4c) or aDMS (t_{580} = 1.85; $P > 0.05$) (Fig. 4d).

Locomotor Sensitisation

As illustrated by Table 1, mice exhibited a clear sensitisation to the locomotor-stimulant effects of morphine, with a significant increase in locomotor activity observed across treatment days in morphine, but not vehicle-treated animals. ANOVA revealed a significant main effect of morphine treatment ($F_{1, 8}$ = 51.1; $P < 0.0001$) and treatment day ($F_{2, 16}$ = 13.2; $P < 0.001$) as well as an interaction effect ($F_{2, 16}$ = 11.6; $P < 0.001$). Tukey's MCT confirmed that mice receiving sensitisation treatments had a greater locomotor response on Day 5

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relative to Day 1 ($q_{16} = 9.63$; $P < 0.0001$) and Day 3 ($q_{16} = 2.61$; $P < 0.001$), indicating a significant sensitisation in morphine response across treatment days.

Discussion

Understanding of DOPr trafficking and function continues to grow rapidly and carries has potential clinical implications. In the present study, we found sexually dimorphic membrane DOPr expression in the rostral striatum; female mice exhibited significantly lower membrane DOPr levels than their male counterparts in most subregions. Furthermore, in contrast with numerous studies to date, we found that both chronic morphine and morphine sensitisation treatments failed to produce an up-regulation of cell membrane DOPr's in CINs in all subregions of the anterior striatum.

These sex differences are largely consistent with previous clinical and preclinical studies of sexually dimorphic opioid receptor expression. In both the medial amygdala [22] and hippocampus [23], lower membrane DOPr levels have been observed in female rats relative to males. ~~These differences presumably arise from the effects of ovarian steroid hormones.~~ In the rodent PAG, cell membrane MOPr expression and morphine analgesic potency fluctuate throughout the oestrus cycle and are lowest in those phases of the cycle characterised by high oestrogen levels [24]. A similar relationship may exist between the ovarian steroid hormones and DOPr trafficking and function, which would explain the lower membrane DOPr expression in females that we observed, However, other mechanisms could account for the differences.

The failure of chronic morphine to induce membrane DOPr accumulation in CINs is more difficult to explain. It contrasts with numerous prior studies that have observed membrane DOPr trafficking induced by chronic opioid exposure in various regions (see Introduction). Moreover, this response has also been noted in the rostral striatum itself, in both its ventral and dorsal divisions [10]. Hence it is unlikely that the present lack of accumulation is related to a regional process specific to the rostral striatum. It is more likely that some property unique to CINs accounts for this difference. This would be consistent with the fact that Lucido and colleagues did not distinguish cell types in their study [10], and were likely sampling mostly from cells other than CINs, which represent only 2-3% of striatal cells [17]. Lucido and colleagues [10] also reported no change in DOPr expression in frontal cortex,

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consistent with the interpretation that whether or not DOPr expression is increased after chronic morphine depends on the cell type.

It is unclear which property of CINs (or frontal cortex cells [10]) might preclude membrane DOPr accumulation. An important characteristic of CINs is their tonic activity, discharging spontaneously at a rate of 3-10 Hz [25]. Membrane depolarisation and consequent increases in intracellular Ca^{2+} via voltage-gated membrane Ca^{2+} channels have been shown to induce cell membrane DOPr trafficking in cultured DRG neurons [26]. Significantly, morphine causes hyperpolarisation and robust inhibition of CIN tonic firing activity [27], as well as reducing CIN N-type membrane Ca^{2+} channel conductance [28]. Thus it is possible morphine failed to induce the Ca^{2+}-dependent upregulation of membrane DOPr's in CINs observed in other cells studied to date because of decreasing intracellular Ca^{2+} levels. This would be consistent with the general trend of decreased membrane levels after morphine treatment in the present study. Another possibility is that DOPr accumulation in CINs is restricted to dendritic and/or axonal membrane regions rather than the somatic membrane sampled here. This would dovetail with the upregulation of DOPr's in the dendritic membranes of striatal cells observed by Lucido and colleagues [10]. This is clearly speculative, however, and further research will be necessary to determine why CINs failed to exhibit membrane DOPr accumulation with chronic morphine.

Although chronic morphine only caused a significant membrane DOPr reduction in the NAc-C and morphine sensitisation only in the NAc-S, both appear to have caused an overall trend towards decreased membrane DOPr's throughout the striatum. Hence the differential response of the NAc-C and NAc-S in both treatments appears to be a matter of degree, not kind. Differing sensitivity to morphine might account for these responses. In situ hybridisation studies have demonstrated that MOPr mRNA levels are greater in the NAc-S than NAc-C [29], suggesting morphine sensitivity may indeed vary across the striatum. This would be consistent with the larger magnitude of the DOPr reduction in the NAc-S relative to NAc-C. However, this does not explain why the NAc-S should respond significantly to morphine sensitisation alone and the NAc-C to chronic morphine. MOPr is expressed striatal CINs selectively in limbic versus sensorimotor zones [30] but its distribution in CINs of NAc-S versus NAc-C CINs is unknown. Moreover, it is not known if MOPr expression is required in the same neuron to alter expression of DOPr after chronic morphine exposure [1, 12]. It also remains unclear why both treatments should promote a unidirectional reduction

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in surface membrane DOPr's when chronic morphine treatment induces tolerance to the effects of morphine [9] and sensitisation treatments augment its effects [15].

Some authors have argued that the insertion of the eGFP tag itself disrupts the trafficking and localisation of DOPr's. Thus Wang et al. used immunostaining to show that the trafficking and distribution of DOPr-eGFP transfected into small-diameter DRG neurons differs from that of the endogenous DOPr [31]. Although the sexually dimorphic expression of DOPr's found here is broadly consistent with previous studies on DOPr expression, the possibility remains that DOPr-eGFP expressed by CINs is trafficked differently to wild-type DOPr, which could potentially explain the failure of chronic morphine to induce membrane DOPr accumulation.

The present results suggest that cell membrane accumulation of DOPr's is not a universal response to chronic opioid exposure and that the trafficking of DOPr's, or any GPCR, in response to a given stimulus exhibits cell-subtype specificity. This raises interesting questions as to how trafficking mechanisms for a particular receptor differ from one cell to another. Furthermore, they indicate that to predict an enhanced pharmacological response to DOPr agonists after chronic morphine exposure, one would need to demonstrate DOPr upregulation in a particular cell type of interest rather than a region as a whole. For example, to exploit upregulation of DOPr trafficking and function in antinociception it would be insufficient to show that the upregulation occurs in the dorsal horn without showing that it occurs specifically in those small-diameter neurons mediating nociception.

The present results also carry important behavioural implications. Given that DOPr function in NAc-S CINs is essential to PIT (see Introduction), it suggests that morphine sensitisation treatments might inhibit PIT via a reduction in membrane DOPr expression in the NAc-S. Thus morphine sensitisation treatment may have effects on instrumental reward-seeking behaviours and learning processes related to drug addiction, via its effects on striatal DOPr function. Further research will be necessary to determine whether morphine sensitisation does indeed alter PIT and other behaviours related to addiction, and if other cell types in addition to CINs exhibit the reduction in membrane DOPr's observed in this study.

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Table 1 Mean ± SEM mean total travelled distance (mm) in mice per 5-min time bin after 20 mg/kg morphine sensitisation (n = 6) or vehicle (n = 6) injections

Injection Day	Treatment	
	Vehicle	Morphine
Day 1	646 ± 172	13800 ± 2880 ****
Day 3	315 ± 64.4	23100 ± 2050 ***
Day 5	1540 ± 1150	48000 ± 8990

**** Significantly smaller than Day 5 at P < 0.0001

*** Significantly smaller than Day 5 at P < 0.001

Figure 1 Immunofluorescence images of striatal CINs. Images in the left column reflect ChAT immunostaining, those in the middle column reflect DOPr-eGFP immunostaining, and those in the right column are merged. Exemplar neurons from male and female DOPr-eGFP mice are shown in rows **a** and **b** respectively (note the dense 'ring' of DOPr-eGFP immunofluorescence in **a**). Scale bar, 20 μm.

Figure 2 Sexually dimorphic membrane DOPr-eGFP expressioNAc-Sn in CINs of mouse striatum. Mean ± SEM mean grey values of somatic membrane DOPr-eGFP immunofluorescence were calculated for CINs of the NAc-S (**a**), NAc-C (**b**), aDLS (**c**) and aDMS (**d**) of 2 male and 4 female vehicle-treated mice. **** $P < 0.0001$; ** $P < 0.01$.

Figure 3 Membrane DOPr-eGFP levels in striatal CINs following chronic morphine treatment. Mean ± SEM mean grey values of somatic membrane DOPr-eGFP immunofluorescence were calculated for CINs of the NAc-S (**a**), NAc-C (**b**), aDLS (**c**) and aDMS (**d**) of male mice following chronic treatment with 300 mg/kg morphine or equivalent-volume of vehicle. * $P < 0.05$.

Fig. 4 Membrane DOPr-eGFP levels in striatal CINs after morphine sensitisation treatments. Mean ± SEM mean grey values of somatic membrane DOPr-eGFP immunofluorescence were calculated for CINs of the NAc-S (**a**), NAc-C (**b**), aDLS (**c**) and aDMS (**d**) of male mice following 20 mg/kg morphine sensitisation treatment or equivalent-volume saline. ** $P < 0.01$.

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Figure
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