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Separation of acute desensitization and long-term tolerance of μ-opioid receptors is determined by the degree of C-terminal phosphorylation

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Mu-opioid Receptor Desensitization and Tolerance

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Abbreviations: ME, [Met^5]-enkephalin; MOR, mu-opioid receptor; MTA, morphine treated animals .

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Abstract:

Phosphorylation of sites on the C-terminus of the mu opioid receptor (MOR) result in the induction of acute desensitization that is thought to be a precursor for the development of long-term tolerance. Alanine mutations of all 11 phosphorylation sites on the C-terminus of MORs almost completely abolished desensitization and one measure of tolerance in locus coeruleus neurons when these phosphorylation deficient MORs were virally expressed in MOR knockout rats. The present work identifies specific residues that underlie acute desensitization, receptor internalization and tolerance. Four MORs variants with different alanine or glutamate mutations in the C-terminus were examined. Alanine mutations in the sequence between amino acids 375 and 379 (STANT-3A) and the sequence between amino acids 363 and 394 having 4 additional alanine substitutions (STANT+7A), reduced desensitization and two measures of long term tolerance. Following chronic morphine treatment, alanine mutations in the sequence between 354 and 357 (TSST-4A) blocked one measure of long-term tolerance (increased acute desensitization and slowed recovery from desensitization) but did not change a second (decreased sensitivity to morphine). With the expression of receptors having glutamate substitutions in the TSST sequence (TSST-4E), an increase in acute desensitization was present after chronic morphine treatment but the sensitivity to morphine was not changed. The results show that all 11 phosphorylation sites contribute, in varying degrees, to acute desensitization and long-term tolerance. That acute desensitization and tolerance are not necessarily linked illustrates the complexity of events that are triggered by chronic treatment with morphine.

Significance

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This work shows that the degree of the phosphorylation sites on the C-terminus of the mu opioid receptor alters acute desensitization, internalization and measures of long term tolerance to morphine. The primary conclusion is that the degree of phosphorylation on the 11 possible sites of the C-terminus has different roles for expression of the multiple adaptive mechanisms that follow acute and long term agonist activation. Although the idea that acute desensitization and tolerance are intimately linked is generally supported, these results indicate that disruption of one phosphorylation cassette of the C-terminus, TSST (354-357) distinguishes the two processes.

Introduction

The C-terminus of the mu opioid receptor (MOR) has multiple phosphorylation sites that are implicated in the mechanisms of receptor desensitization and trafficking. Two cassettes on the MOR C-terminus were efficiently phosphorylated after treatment with a potent agonist (Wang et al., 2002; Lau et al., 2011; Chen et al., 2013; Just et al., 2013; Miess et al., 2018; Doll et al., 2011). When the residues from 375 to 379 (STANT) were mutated to alanine, arrestin recruitment and internalization were blocked (Lau et al., 2011). Residues in the second cassette ranging from 354 to 357 (TSST) were also efficiently phosphorylated, but internalization was unaffected. The electrophysiological consequences of alanine mutations in each cassette, was examined using viral expression in neurons of the thalamus and locus coeruleus (LC) in MOR knockout mice (Birdsong et al., 2015). The results indicated that mutations of all serine and threonine residues in the individual cassettes had small effects on acute desensitization. With mutations of the residues in both cassettes acute desensitization was reduced (Birdsong et al., 2015; Yousuf et el., 2015). The functional consequences of mutations in other potential phosphorylation sites (S-363, T-370, -383 and -394) have not been examined. Phosphorylation of S-363 is thought to be constitutive or PKC dependent while phosphorylation of T-

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370 can be induced by agonists (reviewed in Williams et al, 2013). There is no evidence showing phosphorylated forms at T-383 and T-394 by mass spectrometry (Lau et al., 2011). Acute desensitization measured in locus coeruleus neurons was almost completely eliminated in a knock in mouse where all 11 C-terminus phosphorylation sites or the 10 sites minus T-394 mutated to alanine (Kliewer et al., 2019). Likewise acute desensitization and the development of long term tolerance were blocked following viral expression of phosphorylation deficient MORs in locus coeruleus neurons of MOR knockout rats (Arttamangkul et al., 2018).

The present study examined acute desensitization in slices from untreated and morphine treated animals in each of four mutant MORs—STANT-3A (S375-T379 to AAANA), STANT-7A (S363A, T370A, 375-379 – AAANA, T383A, T394A), TSST-4A (354-375 to AAAA) and TSST-4E (354-357 to EEEE). Each receptor contained an N-terminus GFP tag that, when coupled with a fluorescent nanobody, was used to assess trafficking (Arttamangkul et al., 2018). Internalization of the STANT-3A and STANT-7A mutant receptors was blocked as was previously found in HEK cells (Birdsong et al., 2015). Acute desensitization was maintained in the STANT-3A and TSST mutant receptors and blocked in the STANT-7A receptors. Two measures of tolerance were examined following chronic treatment. First, the current induced by morphine was reduced in experiments with wild type and TSST-4A receptors whereas there was no change in slices expressing the STANT-3A and TSST-4E receptors. The second assay used the time course of recovery from acute desensitization as a measure of the development of tolerance. The recovery from acute desensitization was decreased in slices expressing wild type receptors following chronic morphine treatment (Dang & Williams, 2004; Quillinan et al., 2011; Arttamangkul et al., 2018) but in slices expressing each of the mutant MORs recovery was not different between untreated and morphine treated animals. The results suggest that

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phosphorylation of the C-terminus of the MOR contribute to the prolongation of the recovery from desensitization that is a hallmark of tolerance found in experiments from wild type animals.

Materials and Methods

Drugs - Morphine sulfate and morphine alkaloid were obtained from the National Institute on Drug Abuse (NIDA), Neuroscience Center (Bethesda, MD). Naloxone was purchased from Abcam (Cambridge, MA), MK-801, from Hello Bio (Princeton, NJ), UK14304 tartrate, from Tocris (Bio-Techne Corp. Minneapolis, MN). Potassium methanesulfonate was from Alfa Aesar (Ward Hill, MA). [Met^5] enkephalin (ME) was from Sigma (St. Louis, MO).

Morphine alkaloid was converted to salt form with 0.1 M HCl and made up a stock solution in water. The working solution was diluted in artificial cerebrospinal fluid (ACSF) and applied by superfusion. Naloxone (1 mM) was dissolved in water, diluted to 1 μM in ACSF and applied by superfusion. Bestatin (10 μM) and thiorphan (1 μM) was included in solutions containing ME to limit peptidase induced breakdown.

Animals– All animal experiments were conducted in accordance with the National Institutes of Health guidelines and with approval from the Institutional Animal Care and Use Committee of the Oregon Health & Science University (Portland, OR). Adult (180 – 300 g or 5-6 weeks) male and female Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). MOR-knockout Sprague-Dawley rats were obtained from Horizon (St. Louis, MO). Homozygous animals were bred in house.

Microinjection protocol- Microinjections of virus into the locus coeruleus was carried out as previously described (Arttamangkul et al., 2018). MOR-knockout animals (24-30 days) were

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anesthetized with isofluorane (Terrell®, Piramal Clinical Care, Inc., Bethlehem, PA) and placed in a stereotaxic frame. Viral particles containing adeno associated virus type 2 for the expression of mutant MORs (STANT-3A, AAV2-CAG-SS-GFP-MOR-STANT-WPRE-SV40pA, $2.06x10^{13}$ vg/ml), (STANT-7A, AAV2-CAG-SS-GFP-MOR- STANT-7A -WPRE-SV40pA, $2.06x10^{13}$ vg/ml), (TSST-4A AAV2-CAG-SS-GFP-MOR-TSST-4A-WPRE-SV40pA, $2.06x10^{13}$ vg/ml) and (TSST-4E, AAV2-CAG-SS-GFP-MOR-TSST-4E-WPRE-SV40pA, $2.06x10^{13}$ vg/ml). The N-terminus of each construct was fused to GFP with a human prolactin hormone signal sequence. All viruses were obtained from Virovek (Hayward, CA). Injections of 200 nl at the rate of 0.1 µl/min were done bilaterally at ±1.25 mm lateral from the midline and -9.72 mm from the bregma at a depth of 6.95 mm from the top of the skull using computer controlled stereotaxic Neurostar (Kähnerweg, Germany). Experiments were carried out 2-4 weeks following the injection.

Animal treatment protocols-Rats (5-6 weeks) were treated with morphine sulfate using osmotic pumps (2ML1, Alzet, Cupertino, CA) as described previously (Quillinan et al., 2011). Rats were anesthetized and the pumps were implanted subcutaneously.

Tissue preparation – Horizontal slices containing locus coeruleus (LC) neurons were prepared as described previously (Williams and North, 1984). Briefly, rats were killed and the brain was removed, blocked and mounted in a vibratome chamber (VT 1200S, Leica, Nussloch, Germany). Horizontal slices (250-300 µm) were prepared in warm (34°C) artificial cerebrospinal fluid (ACSF, in mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 1.2 NaH_2PO_4, 11 D-glucose and 21.4 $NaHCO_3$ and 0.01 (+) MK-801 (equilibrated with 95% O2/ 5% CO2, Matheson, Basking Ridge, NJ). Slices were kept in solution with (+)MK-801 for at least 30 min and then stored in glass vials with oxygenated (95% O2/ 5% CO2) ACSF at 34°C until used.

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Electrophysiology –Slices were hemisected and transferred to the recording chamber which was superfused with 34°C ACSF at a rate of 1.5 - 2 ml/min. Whole-cell recordings were made from LC neurons with an Axopatch-1D amplifier in voltage-clamp mode (V_{hold} = -60 mV). Recording pipettes (1.7 – 2.1 MΩ, World Precision Instruments, Saratosa, FL) used an internal solution of (in mM): 115 potassium methanesulfonate or potassium methyl sulfate, 20 KCl, 1.5 $MgCl_2$, 5 HEPES(K), 10 BAPTA, 2 Mg-ATP, 0.2 Na-GTP, pH 7.4, 275-280 mOsM. Recordings where the series resistance was <15 MΩ were terminated. Data were collected at 400 Hz with PowerLab (Chart version 5.4.2; AD Instruments, Colorado Springs, CO).

MOR-GFP Trafficking-Brain slices (240 µm) from the virally injected rats were prepared as previously described. Slices were visualized with an Olympus Macroview fluorescent microscope for GFP expression in the LC area and to visualize plasma membrane associated receptors slices were incubated in a solution of anti-GFP nanobody Alexa594 (Nb-A594, 10 µg/mL, 30-45 min, Arttamangkul et al., 2018). Images were captured with an upright microscope (Olympus, Center Valley, PA.) equipped with a custom-built two-photon apparatus and a 60x water immersion lens (Olympus LUMFI, NA1.1, Center Valley, PA). The dye was excited at 810 nm. Data were acquired and collected using Scan Image Software (Pologruto et al., 2003). Images were taken at a magnification where a single neuron filled the field of view. A z-series of 10 sections was collected at 1 µm intervals. With this protocol, the whole neuron was qualitatively compared. Drugs were applied by perfusion at the rate of 1.5 ml/min. All experiments were done at 35˚C.

Data Analysis - Analysis was performed using KaleidaGraph (Synergy software). Values are presented as mean±SD. Statistical comparisons were made using unpaired t-tests or a two-way ANOVA, as appropriate. Comparisons with $p<0.05$ were considered significant.

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Results

As in previous work, [Met]^{5}enkephalin (ME, 0.3 μM and 30 μM) was applied in each experiment. In slices from wild type animals, the initial current amplitude induced by ME (0.3 μM) was about 60% of that induced by the saturating concentration of ME (30 μM). The initial amplitude of the currents induced by ME (0.3/30 μM) measured in slices from virally expressed receptors varied but were similar in amplitude to the currents (in pA) measured in wild type animals (TABLE 1, ANOVA, Dunnett post hoc)

Trafficking

Previous results showed that virally expressed wild type receptors were efficiently internalized during the application of ME (30 μM, 10 min) whereas internalization of receptor where all phorphorylation sites on the C terminus were mutated to alanine was not observed (Arttamangkul et al., 2018). In the present study internalization of the receptors with specific mutations was examined using 2-photon microscopy. Each mutant receptor had an extracellular GFP-tag such that plasma membrane associated receptors were labeled with an anti-GFP nanobody conjugated with alexa594 (Figure 1). A z-stack of images of single cells was collected before and after treatment of slices with ME (30 μM, 10 min). There was a distinct qualitative change in the receptor distribution in slices with neurons expressing the TSST-4A (S/T354-357A) and TSST-4E (S/T354-357E) receptors. Before treatment with ME the receptors lined the plasma membrane and following treatment with ME receptors were internalized and the pattern became punctate (Figure 1). The distribution of STANT-3A (S/T375-377A, T379A) receptors was relatively unchanged. To quantify the increase in intracellular fluorescence the z-stack was compressed with a z-projection and a region of interest just inside of the plasma membrane of the soma was drawn (Supplemental Figure 1). The increase in fluorescence measured in arbitrary fluorescence units (AFU) in that region of interest following treatment with ME was taken as the

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internalized receptors. By this rough measure there was an increase in fluorescence with the TSST-4A (95% confidence level = 255±168 AFU, n-8) and TSST-4E (95% confidence level = 166±117 AFU, n=7) receptors and little change in the STANT-3A receptors (95% confidence level = 31±33 AFU, n=14). Internalization was statistically different between the STANT-3A and both TSST-4A and TSST-4E receptors (ANOVA, Student-Newman-Keuls post hoc). Given that the alanine mutations of the STANT sequence (375-379) largely blocks arrestin recruitment it was not surprising that the application of ME did not induce detectable internalization (Lau et al., 2011).

Acute Desensitization

The current induced by ME (0.3 μM) was measured before and following a 10 min application of ME (30 μM). The degree of desensitization was determined by the relative amplitude of the current induced by ME (0.3 μM) following a 5 min wash of the saturating concentration of ME compared to the amplitude of the initial current induced by ME (0.3 μM). Recovery from desensitization was determined by measuring the current amplitude induced by ME (0.3 μM) over time (Figure 2). As reported previously, recovery from desensitization in slices from wild type animals occurred over a period of 20-35 min.

Coupling efficiency using a partial agonist, morphine, to measure tolerance

The relative amplitudes of the currents induced by morphine and ME were compared (Figure 2D). Morphine (1 μM) was applied until the current reached a steady state. The solution was then switched immediately to a saturating concentration of ME (30 μM). The ratio of the morphine/ME current was determined in slices from untreated and morphine treated animals (Figure 2E). This protocol takes advantage of the fact that morphine is a partial agonist and therefore is more sensitive to changes in coupling efficiency and thus was used as a second measure of the tolerance induced by treatment of animals with morphine (Christie et al., 1987; Levitt, Williams, 2012).

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Animals were treated with morphine (80 mg/kg/day) for 6-7 days. Brain slices were prepared and maintained in morphine free solutions such that the preparations were in a state of acute withdrawal. First, the degree of tolerance was examined using the current induced by ME (0.3 μM) measured before and following the application of ME (30 μM, 10 min, Table 1). The recovery of the current induced by ME (0.3 μM) was examined over 20-35 min. As reported previously the recovery from desensitization was slowed in slices from morphine treated animals (Figure 2, Dang, Williams, 2004; Quillinan et al., 2011; Arttamangkul et al., 2018). Second, the current induced by morphine (1 μM) relative to the peak ME current was reduced in slices from morphine treated animals (Figure 2, $p<0.05$ Mann-Whitney U test). This simple assay is therefore a reasonable measure of tolerance and is particularly valuable for experiments where the viral expression of receptors in the knockout animals can be variable. A summary of the results with this measure is illustrated in the plots of the amplitude of the current induced by morphine (1 μM) against the current induced by ME (30 μM, wild type, Figure 3A). The current induced by morphine was smaller than that induced by ME in slices from morphine treated animals over a wide range of current amplitudes.

The same protocols were used to measure tolerance, desensitization and the recovery from desensitization in slices taken from animals expressing each of the mutant receptors.

STANT-3A

Application of a saturating concentration of ME resulted in desensitization of the STANT-3A receptor. The decline in current during the application of ME (30 μM, 10 min) was not different from that in slices from wild type animals ($p>0.05$, ANOVA, Dunnett post hoc). The decrease in the current induced by ME (0.3 μM) following the saturating concentration was reduced compared to the wild type (Figure 4, $p<0.05$ ANOVA Dunnett post hoc). This result was expected based on previous work

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in the mouse (Birdsong et al., 2015). The phosphorylation of S375 is thought to be an initial step leading to sequential phosphorylation events on the C-terminus. The observation that desensitization persisted indicates that this sequence is not the sole area that underlies acute desensitization.

In slices from morphine treated animals the decline from the peak current induced by ME (30 μM, 10 min) was not different from that in slices from untreated animals (Table 1, $p>0.05$, unpaired T-test). Likewise the recovery from desensitization was the same in slices from morphine treated and untreated animals (Figure 4C). Thus, in spite of the acute desensitization seen with this receptor, there was no apparent induction of tolerance as measured by an increase in desensitization or a prolongation of the time it takes to recover from desensitization. The ratio of the morphine/ME current was also not different between experiments carried out in untreated and morphine treated animals (Figure 4E). The current amplitudes (morphine/ME) for individual cells in slices from untreated and morphine treated animals overlapped over a range of current amplitudes. Thus by two measures, mutation of the STANT sequence in the MOR blocked the development of tolerance.

Finally, given that treatment of the slices with ME (30 μM, 10 min) did not induce detectable internalization, one conclusion from these experiments could be that trafficking and/or arrestin recruitment is a necessary step in the development of long-term tolerance.

STANT-7A

There was little or no decline in the current induced by ME (30 μM), or the ME (0.3 μM) current following washout of the saturating concentration in slices expressing the STANT-7A receptor (Table 1, Figure 5). Thus by the inclusion of 4 additional alanine mutations, the STANT-7A resulted in a near complete block of desensitization, as was found in STANT-3A receptors.

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In slices from morphine treated animals there was a substantial increase in desensitization as determined by the decrease in the current induced by ME (0.3 μM) 5 min following the wash of the saturating concentration (Table 1, Figure 5). The recovery from desensitization in slices from animals treated with morphine was near complete after washing for 10 min (Figure 5). The decline from the peak current induced by ME (30 μM, 10 min) was slightly smaller than in slices from morphine treated animals (Table 1, MTA=0.83±0.09, n=14, Untreated= 0.73±0.10, n=9, p=0.03, unpaired T-test). Chronic morphine treatment therefore resulted in a facilitation of acute desensitization measured by the decrease in current induced by ME (0.3 μM) that was short lasting and not present prior to the morphine treatment. Thus mutation of the 4 additional residues blocked acute desensitization in slices from untreated animals but did not eliminate at least one adaptive mechanism induced by chronic morphine. The results suggest that the 4 remaining phosphorylation sites play a role in the mechanisms induced by chronic morphine treatment.

TSST-4A

Acute desensitization and trafficking of this receptor closely resembled that measured in slices from wild type animals and experiments with wild type receptors expressed in the knockout animals (Figure 1 and 6). Following chronic morphine treatment, the decline from the peak current induced by ME (30 μM, 10 min) was not different from slices from untreated animals (untreated 0.53±0.13 of the peak n=7, MTA 0.60±0.05 of the peak n=5). There was also no difference in the decrease in the ME (0.3 μM) current or the time course or extent of recovery from desensitization following washout of the saturating concentration of ME (Figure 6B&C). Thus by one measure (acute desensitization) chronic morphine had no effect. However the current induced by morphine (morphine/ME) was reduced in slices taken from morphine treated animals (Figure 6D&E). Thus the second measure of tolerance was

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the same as that measured in slices taken from morphine treated wild type animals (compare Figures 2E and 6E) suggesting that the link between acute desensitization and the reduced sensitivity to morphine was broken.

TSST-4E

Previous work suggested that by mimicking phosphorylation of the TSST sequence with glutamic acid residues (TSST-4E), a desensitized state of the receptor was induced (Birdsong et al., 2015). The initial current amplitude induced by ME (0.3 μM) was not different from cells expressing TSST-4A or wild type receptors (TSST-4E=286±102 pA, n=7, WT=279±96, n=7, Table 1, $p>0.05$, ANOVA, Dunnett post hoc). Likewise the current induced by a saturating concentration of ME (30 μM) was not different (TSST-4E=400±123 pA, n=10: WT 451±95 pA, n=7, Table 1, $p>0.05$, ANOVA, Dunnett post hoc). The decline from the peak current induced by ME (30 μM) was greater in slices from morphine treated animals than in untreated controls (Figure 7D, Table 1 $p<0.05$, unpaired T test). The current induced by ME (0.3 μM) following the wash of the saturating concentration of ME (30 μM) was also smaller in slices from morphine treated animals than in untreated controls (Figure 7D, Table 1, $p<0.05$, unpaired T test). Finally, receptor internalization induced by ME (30 μM, 10 min) was similar to measured in wild type and TSST-4A receptors (Figure 1).

The results indicate that acute desensitization of the TSST-4E receptors in slices from morphine treated animals is facilitated but the recovery from desensitization is complete (Figure 7C, Table 1). Finally the plot of morphine versus ME current amplitudes illustrates the overlap in experiments in slices between untreated and morphine treated animals (Figure 3E, $p>0.05$, unpaired T test). Although acute desensitization was increased in slices taken from morphine treated animals, the recovery was complete and there was no change in the current induced by morphine suggesting that one measure of

tolerance of these receptors was blocked but acute desensitization was facilitated. As with the results obtained with the TSST-4A receptors acute desensitization and tolerance appear to be dependent on separate processes.

Discussion

The present study measured the opioid induced outward current mediated by GIRKs to examine the functional regulation of MORs by phosphorylation sites on the C-terminus. Alanine mutation of all phosphorylation sites (11S/T-A) on the C-terminus results in a dramatic decrease in acute desensitization and long-term tolerance to opioids (Arttamangkul et al., 2018; Kliewer et al., 2019). The present study used a series of MORs having selective alanine mutations in different areas of the C-terminus in order to determine the role of acute desensitization in the development of long term tolerance. The results show that each of the mutated receptors affected one or another aspect of MOR signaling. With one exception (STANT-7A), some degree of acute desensitization was present on all receptors and following chronic morphine treatment there was an acute, although transient, desensitization of even that receptor. The unexpected observation was that by using three measures of opioid action to determine the level of tolerance there were distinct differences between the mutant opioid receptors. Thus different phosphorylation sites on the C-terminus have functionally distinct actions following chronic morphine treatment.

Acute desensitization as a measure of tolerance.

Following chronic treatment of animals with morphine acute desensitization was augmented and recovery from desensitization was prolonged (Dang, Williams, 2004; Quillinan et al., 2011; Arttamangkul et al., 2018). Acute desensitization was facilitated in slices expressing TSST-4E as in

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wild type animals but not in STANT-3A, TSST-4A MOR variants. There was a transient facilitation in the STANT-7A variants but that is because there was no desensitization in slices from untreated animals, and the increase in desensitization was small, transient and to the same level as in the STANT-3A variants. Unlike the observation made in wild type animals, the delayed time course of recovery following chronic morphine treatment was not present in any of the mutated receptors. It therefore appears that each of the phosphorylation sites on the C-terminus are necessary for the both the augmented acute desensitization and slowed recovery from desensitization that is induced following chronic morphine treatment. Thus the full complement of phosphorylation sites appears necessary for the development of long term tolerance.

Uncoupling of signaling as a measure of long-term tolerance.

As a partial agonist morphine offers a sensitive assay for functional coupling (Christie et al., 1987; Dang, Williams, 2004; Levitt, Williams, 2012; Williams et al., 2014). In slices taken from animals treated chronically with morphine, the morphine-induced current was reduced (Christie et al., 1987; Quillinan et al., 2011). This measure of long-term tolerance was present in some but not all of the mutant MORs tested. With the notable exception of TSST-4E, the MOR mutations that blocked desensitization and internalization (STANT-3A, STANT-7A and Total Phosphorylation Deficient (TPD, Arttamangkul et al., 2018)) also blocked the decrease in the morphine induced current.

Phosphorylation cassettes

It is well established that alanine mutations in the STANT-3A sequence decreases arrestin recruitment and receptor internalization (Lau et al., 2011; Just et al., 2013). Measures of tolerance following

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chronic morphine were also blocked in receptors with the STANT-3A and STANT-7A mutations (this study).

Much less in known about how alanine mutations of the TSST sequence affect MOR function particularly following chronic morphine treatment. The TSST-4A receptors desensitize and internalize 'normally' with agonist application. A receptor binding assay that used a fluorescently labeled agonist, DERM-A594, in HEK cells was used to measure the change in agonist affinity following incubation with potent agonists, such as ME (Birdsong et al., 2013). There was a significant long term (1-2 hours) increase in agonist affinity that followed incubation for 20-120 min. The increased affinity was not affected after treatment with pertussis toxin, was present in arrestin2/3 knock cells and there was no change in antagonist affinity. The increase in agonist affinity was blunted in the TSST-4A receptors particularly with longer agonist incubation times. The results suggested that phosphorylation of the TSST sequence largely blocked one consequence of acute agonist desensitization. Glutamate substitutions in the TSST sequence (TSST-4E) increased agonist affinity in the absence of pre-incubation suggesting that the glutamate substitution largely mimicked the effect of phosphorylation of this sequence (Birdsong et al., 2013).

Previous work reported that there were two components of tolerance induced by chronic morphine treatment, one that was transient (60-90 min) and a second that was long term (>3 hours, Levitt, Williams, 2012). The striking difference between the TSST-4A and TSST-4E receptor function following chronic morphine treatment suggests an important role for these phosphorylation sites in the adaptive changes induced by chronic morphine treatment. Although the idea that acute desensitization and tolerance are intimately linked is generally supported, the results indicate that disruption of the normal role of this (TSST) sequence distinguishes the two processes. Following chronic morphine treatment, long term tolerance as measured by the decrease in the current induced by morphine was

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maintained with the TSST-4A mutants. There was however no decrease in the current induced by morphine in the TSST-4E mutants in slices taken from morphine treated animals. Thus this measure of long term tolerance to morphine was blocked. The adaptive changes in acute desensitization were different in that acute desensitization following chronic morphine treatment was increased in the TSST-4E receptors, as in wild type receptors. On the other hand, acute desensitization of the TSST-4A receptors was unchanged by chronic morphine treatment. The present results could result from two separate mechanisms that underlie acute desensitization as previously indicated (Dang et al., 2009).

Summary

Alanine mutations of different phosphorylation sites on the C-terminus were used to identify receptor dependent signaling by measuring acute desensitization, recovery from desensitization and internalization. Mutations of the STANT sequence blocked all three measures as well as measures of tolerance. Mutations in the TSST sequence had little effect on acute actions but had a modulatory effect on the expression of tolerance.

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Table 1. Summary of results. **I-ME (pA)** - The mean amplitude of the currents induced by ME (0.3 and 30 μM) in slices from wild type animals (WT) and each of the mutant MORs. There was no statistical difference from wild type (ANOVA, Dunnett post hoc) indicating that the sensitivity to ME in slices from wild type animals was similar to slices taken from animals expressing each of the mutant receptors. **Decline (10 min/peak)** – Paired comparison between experiments from untreated and morphine treated animals (MTA) showing the decrease in current induced by ME (30 μM) during a 10 min application for each of the mutant receptors. In slices from animals expressing the TSST-4E receptors the decline was increased in morphine treated animals (MTA, $p<0.05$, upaired T-test). **ME (0.3, post/pre)** – Paired comparison between slices from untreated and morphine treated animals. The ratio of the current induced by ME (0.3 μM) following desensitization normalized to the initial current for each of the mutant receptors. This ratio decreased in wild type, STANT-7A and TSST-4E ($p<0.05$, unpaired T-test).

Figure 1. Receptor imaging before and after treatment with ME (30 μM, 10 min). An anti-GFP nanobody conjugated with alexa594 was to image the (A) STANT, (B) TSST-4A and (C) TSST-4E receptors expressed in locus coeruleus before (top) and following ME (30 μM, 10 min, bottom).

Figure 2. Protocols used to measure two forms of tolerance using experiments were carried out in wild type animals. (A) Snake plot illustrates the phosphorylation sites on the C-terminus (yellow,T354, S355, S356, T357, S363, T370, S375,T376, T379, T383, T394). (B) Protocol used to determine acute desensitization and the recovery from desensitization. (C) Summary of results showing the time course of recovery from desensitization slices from untreated (black) and in morphine treated (red) animals

(MTA), blue dote indicate the results from the trace in B. (D) Trace shows the current induced by morphine (1 μM, 1) relative to that induced by ME (30 μM, 2). (E) Summary of the results measuring the relative current induced by morphine (1/2) in slices from untreated animals (95% confidence interval 0.518±0.047) and morphine treated animal (95% confidence level 0.37±0.045) indicating that the morphine induced current is smaller in slices from morphine treated animals. Mann-Whitney U test $P<0.001$. Blue dot indicates the results from the experiment in D.

Figure 3. Summary of results comparing the current induced by morphine (1 μM) plotted against the current induced by ME (30 μM) in individual cells in slices from untreated (black) and morphine treated (red) animals. A) Cells in slices taken from wild type animals. The current induced by morphine after treatment with morphine is smaller than that in untreated animals. B) Cells taken from animals following expression of the STANT mutant. C) Cells taken from animals following the expression of the TSST-4A mutant. D) Cells in slices taken from animals expressing the TSST-4E mutant.

Figure 4. Desensitization is decreased and tolerance is blocked in cells expressing the STANT mutant receptor. (A) Snake plot illustrates the sites in the STANT mutant having alanine mutations (red, S375. T376, T379). (B) An experiment taken from a morphine treated animal illustrating the desensitization and recover from desensitization of ME (0.3 μM). (C) Summary of results showing the recovery from desensitization in slices from untreated and morphine treated animals (MTA). Blue dot indicates the results taken from the trace in B. (D) Trace illustrating the morphine (1 μM) current relative to the ME (30 μM) current. Blue dot indicates the result from the experiment illustrated in D. (E) Summary of experiments plotting the current induced by morphine (1 μM) divided by that induced by ME (30 μM,

I-morphine/I-ME) indicating that the relative morphine current was unchanged in slices from morphine treated animals. Untreated 0.55±0.23, MTA 0.59±0.12 (95% confidence level).

Figure 5. Transient desensitization is induced following chronic morphine treatment in the STANT-7A mutant receptors. (A) Snake plot indicates the site (red, S363, T370, S375, T376, T379, T383, T394) with alanine mutations. (B) Illustrates the lack of ME induced desensitization in a slice from an untreated animal (left) and a morphine treated (right) animal. (C) Summary of results showing the transient desensitization that is induced by chronic morphine treatment (MTA, red). (D) Left, shows the decline from the peak current during ME (30 μM, 10 min) in slices from untreated (black) and morphine treated (red) animals. Right shows the ME (0.3 μM) current relative to the peak ME (30 μM) current in slices from untreated and morphine treated animals.

Figure 6. The TSST-4A mutant receptors distinguish tolerance measured by two assays, the increase in acute desensitization (blocked) and the decrease in sensitivity to morphine (present). (A) Snake plot illustrating the sites that were mutated to alanine (red, T354, S355, S356. T357). (B) An experiment illustrating the acute desensitization and recovery from desensitization. (C) Summarized results from showing the recovery from desensitization in untreated (black) and morphine treated (red) animals (MTA). Acute desensitization is insensitive to chronic morphine treatment. (D) An experiment in a slice taken from a morphine treated animal illustrating the decreased amplitude of the current induced by morphine. (E) Dot plot shows the relative current induced by morphine (I-morphine/I-ME) in slices from untreated (black) and morphine treated (red) animals indicating that the relative morphine current was less in slices taken from morphine treated animals. Untreated 0.55±0.14, MTA 0.30±0.13 (95% confidence level).

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Figure 7. Following chronic treatment with morphine, the TSST-4E mutant MOR separates induction of changes in acute desensitization (present) from the decrease in the sensitivity to morphine (blocked). (A) Snake plot shows the sites with glutamate substitutions (blue, T354, S355, S356. T357). (B) An example of acute ME induced desensitization and the recovery from desensitization in a slice from a morphine treated animal. (C) Summary of experiments showing the recovery from desensitization in slices from untreated (black) and morphine treated (red) animals (MTA). (D) Summarized results showing the decline from the peak current induced by ME (30 μM, 10 min) and the amplitude of the ME (0.3 μM) current relative to the peak current induced by ME (30 μM) in slices from untreated and morphine treated animals. (E) Dot plot of the relative current induced by morphine (1 μM) relative to the peak current induced by ME (30 μM, I-morphine/I-ME) showing that there was no change in the relative morphine current between slices from untreated and morphine treated animals. (Untreated 0.48±0.18, MTA 0.47±0.16, 95% confidence level)

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Table 1

	I- ME (pA)		**Decline (10 min/Peak)**		**ME (0.3 μM), Post/Pre**	
	0.3 μM	30 μM	Untreated	MTA	Untreated	MTA
WT	279±96 (7)	451±95 (7)	0.60±0.16 (7)	0.51±0.07 (9)	0.23±0.06 (7)	**0.13±0.08** (9)
STANT-3A	259±137 (9)	439±134 (9)	0.79±0.07 (7)	0.78±0.08 (7)	0.62±0.14 (10)	0.56±0.15 (9)
STANT-7A	290±138 (8)	435±143 (8)	0.73±0.10 (9)	0.83±0.09 (14)	0.82±0.15 (9)	**0.61±0.13 (13)**
TSST-4A	231±82 (8)	425±123 (8)	0.53±0.13 (7)	0.60±0.05 (10)	0.25±0.14 (6)	0.33±0.11 (5)
TSST-4E	286±102 (7)	400±123 (10)	0.65±0.10 (7)	**0.49±0.13** (10)	0.41±0.12 (7)	**0.18±0.13** (10)

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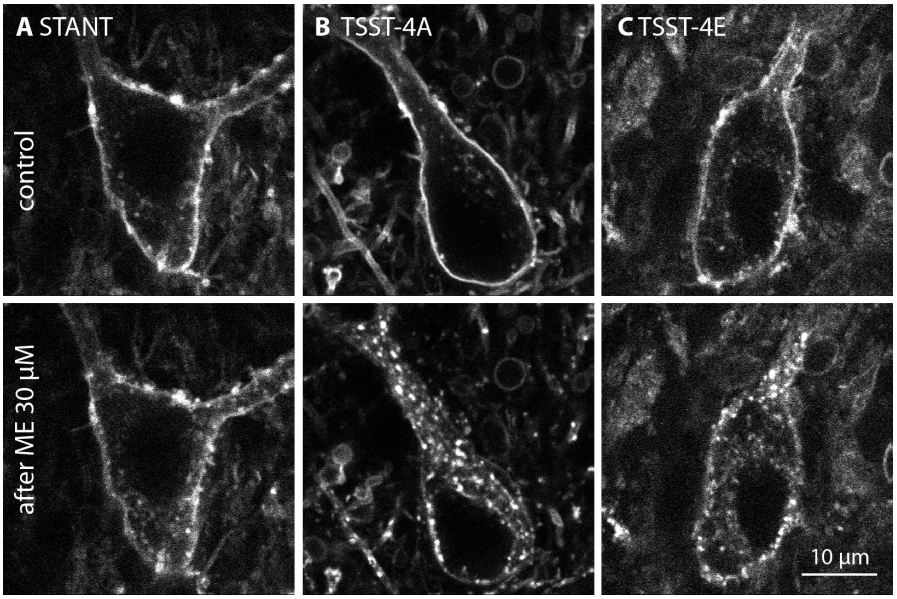

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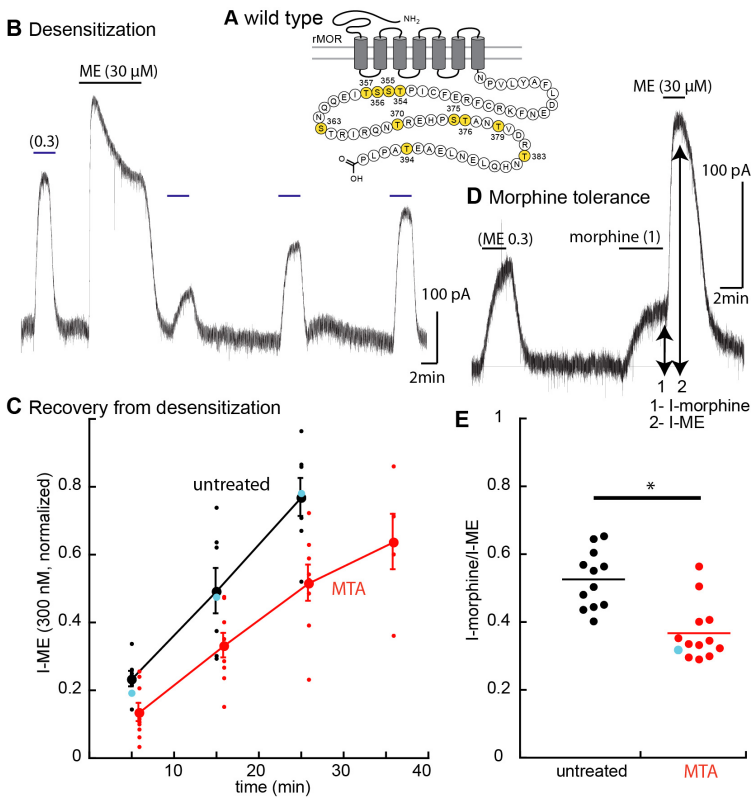

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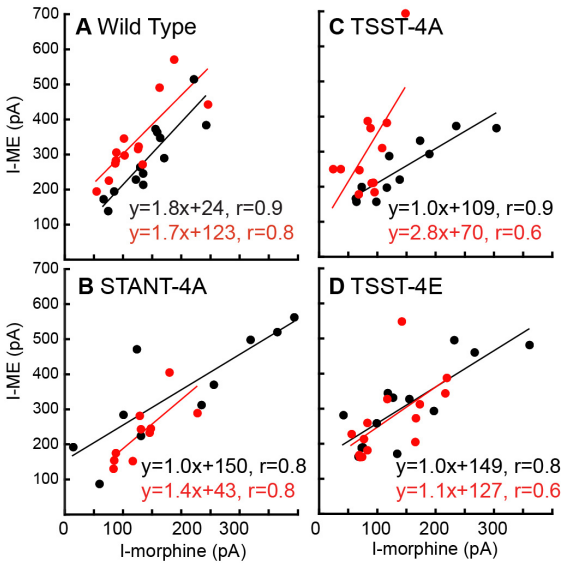

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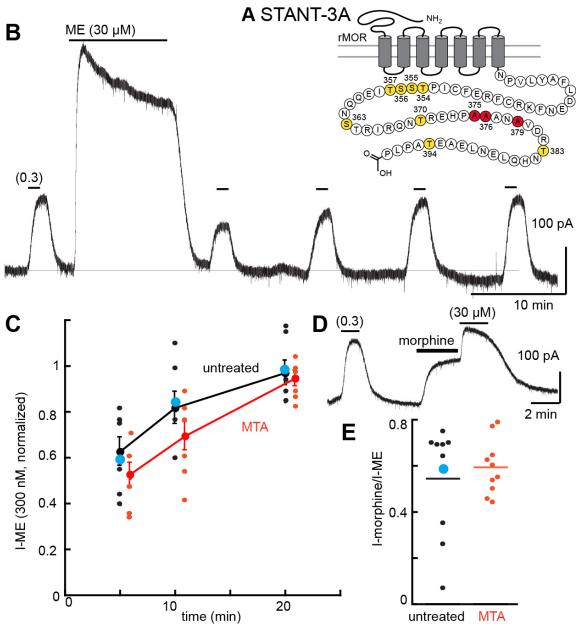

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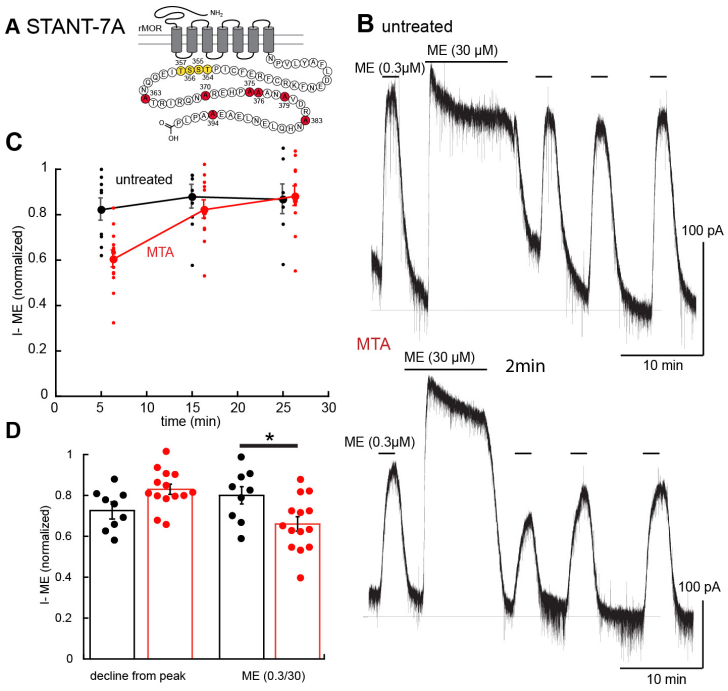

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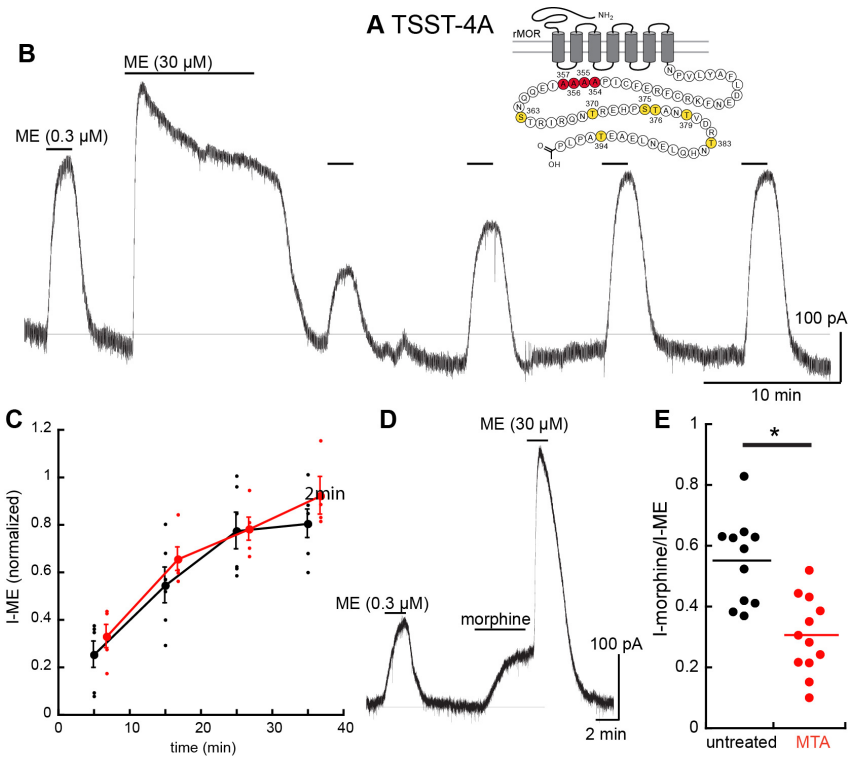

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