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Desensitization and trafficking of mu-opioid receptors in locus coeruleus neurons: Modulation by kinases

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Running Title - Kinase inhibition: MOPr desensitization and internalization

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Abbreviations; mu opioid receptors (MOPr), G-Protein Receptor Kinase (GRK), [Met]^{5}enkephalin (ME), Locus Coeruleus (LC), staurosporine (STP), arrestin3 knockout (ArrKO), artificial cerebro-spinal fluid (ACSF), Transgenic epitope Flag-tagged mu-opioid receptor (FlagMOPr)

Abstract

The phosphorylation of mu opioid receptors (MOPr) by G protein-coupled receptor kinase(s) (GRKs) followed by arrestin binding is thought to be a key pathway leading to desensitization and internalization. The present study used the combination of intracellular and whole cell recordings from rats and mice along with live cell imaging of epitope-tagged FlagMOPr from mouse locus coeruleus (LC) neurons to examine the role of protein kinases in acute desensitization and receptor trafficking. Inhibition of GRKs using heparin or a GRK2 mutant mouse did not block desensitization or alter the rate of recovery from desensitization. The non-selective kinase inhibitor, staurosporine did not reduce the extent of [Met^5]enkephalin (ME)-induced desensitization but increased the rate of recovery from desensitization. In the presence of staurosporine, ME-activated FlagMOPr was internalized but did not traffic away from the plasma membrane. The increased rate of recovery from desensitization correlated with the enhancement in the recycling of receptors to the plasma membrane. Thus ME-induced MOPr desensitization persisted and the trafficking of receptors was modified after inhibition of protein kinase(s). The results suggest that desensitization of MOPr may be an early step following agonist binding that is modulated by, but not dependent on kinase activity.

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Introduction

Mu opioid receptors (MOPr) belong to the G protein-coupled receptors (GPCR) superfamily and undergo homologous desensitization that is thought to result from phosphorylation of agonist-bound receptors by G protein-coupled receptor kinase(s) (GRKs) and the binding to arrestin (Gainetdinov et al., 2004; Schulz et al., 2004; Kenski et al., 2005). It is known however that MOPr are phosphorylated by multiple kinases (Johnson et al., 2005). Thus several kinase dependent mechanisms have been proposed to mediate desensitization. The activation of PKC has been shown to facilitate the desensitization induced by morphine but not DAMGO, whereas inhibition of GRK blocked the desensitization induced by DAMGO but not morphine (Bailey et al., 2004; reviewed in Kelly et al., 2008). Other studies have found that the inhibition of GRK2 activity in LC neurons had no effect on the acute desensitization induced by [Met^5]enkephalin (ME) (Quillinan et al., 2011; Dang et al., 2011). In order to reduce desensitization induced by ME, Dang et al., (2009) found that blockers of both GRK2 and ERK1/2 were required. It is also known that PLD_2/p38MAPK plays an important role in the internalization of MOPr and that can reduce the development of receptor desensitization (Koch et al., 2004; Yang et al., 2010). Thus, the specific kinase(s) that mediate acute desensitization remains an open question.

This study investigates the role of protein kinases in ME-induced desensitization and trafficking in LC neurons from rats and mice. The desensitization induced by ME in the LC from rat has been well characterized (Harris and Williams, 1991; Bailey et al., 2004; Dang and Williams, 2005; Virk and Williams, 2008). A transgenic mouse expressing FlagMOPr in LC neurons was utilized to study receptor trafficking in addition to desensitization (Arttamangkul et al., 2008). The non-selective kinase

inhibitor, staurosporine, was selected to study the role of serine/threonine protein kinases on MOPr desensitization. The results demonstrate that the inhibition of protein kinases did not prevent ME-induced desensitization but the rate of recovery from desensitization was enhanced. In addition, after treatment with staurosporine, ME induced receptor internalization but the internalized receptors remained close to the plasma membrane and recycled back to the plasma membrane more rapidly. The results indicate that the inhibition of multiple kinases in locus coeruleus neurons alters the trafficking of receptors but has little effect on acute desensitization.

Materials and Methods

All reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO), except SB203580 from Tocris (Ellisville, MO). Alexa Fluor® 594 succinimidyl ester was purchased from Invitrogen (Carlsbad, CA). M1-Alexa 594 was conjugated and purified by Bio-Spin® 6 Tris Columns (Hercules, CA). The nucleotide analog NaPP1 (1-(1,1-dimethyethyl)-3-(1-napthalenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine) was a gift of Dr. Kevan Shokat, University of California San Francisco, San Francisco, CA). 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).

Electrophysiolgy: Adult male Sprague-Dawley rats (150-250g, Charles River Laboratories, Wilmington, MA), FlagMOPr transgenic mice (Flag-Tg(+/-)MOPr, Arrtamangkul et al, 2008), and transgenic mice having a GRK isoform (GRK2as5) that is selectively inhibited by the nucleotide analog, NaPP1 (Quillinan et al., 2011) were used

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for electrophysiology experiments. Brain slices were prepared as described previously (Williams et al., 1984). In brief, rats and mice were anesthetized with isoflurane, the brain was removed and placed in ice-cold artificial cerebro-spinal fluid (ACSF) containing the following (in mM): 126 NaCl, 2.5 KCl, 1.2 MgCl2, 1.2 NaH2PO4, 2.4 CaCl2, 21.4 NaHCO3, 11 glucose and 0.03 (+)MK801. Horizontal slices were prepared (270 μm) using a vibratome (Leica, Nussloch, Germany) and incubated in warm (34˚C) oxygenated ACSF containing (+)MK-801 (10 μM) for at least 30 min. Glass electrodes (50-60 MΩ) filled with KCl (2 M) were used for intracellular recording of membrane potential. Whole cell recordings were made with pipettes (1.7-2.1 MΩ) with an internal solution containing (in mM): 115 methyl potassium sulfate, 20 NaCl, 1.5 $MgCl_2$, 10 HEPES, 10 BAPTA, 2 Mg-ATP, 0.5 Na-GTP, pH 7.3. Experiments were performed at 35˚C. Data was collected using Power Lab (Chart version 5.4, ADInstrument, Colorado Springs, CO) and acquired at 200 Hz.

Fluorescent-MOPr Internalization: FlagMOPr mice were used for all trafficking experiments (Arttamangkul et al., 2008). All data were collected from male and female transgenic hemizygous Flag-Tg(+/-)MOPr animals that were crossed with C57BL/6J (Jackson Laboratory, Bar Harbor, ME) or arrestin 3 knockout (ArrKO) mice (a gift from Dr. Robert Lefkowitz, Duke University Medical Center, Durham, NC). Experiments examining the role of arrestin3 used knockout (ArrKO) animals that were initially crossed with the Flag-MOPr transgenic animals. In a series of subsequent crosses animals that were hemizygous Flag-Tg(+/-)MOPr and homozygous arrestin 3 knockout(-/-) were obtained (Arttamangkul et al., 2008; Quillinan et al., 2011). Brain slices (200 μm) were prepared as those described for electrophysiological experiments.

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Slices were incubated in a solution containing M1-Alexa 594 (10 μg/mL, 45-60 min). The tissue was visualized with an upright microscope (Olympus, Center Valley, PA) equipped with a custom-built two-photon apparatus. Data were acquired and collected using Scan Image Software (Pologruto et al., 2003). A z-series was collected at 1 μm intervals for 15 μm. Drugs were applied by perfusion at the rate of 1.5 ml/min. All experiments were done at 35˚C.

Quantification of receptor internalization and reinsertion: Analysis was done off-line with Image J (NIH) software. Details of the analysis have been previously published (Arttamangkul et al., 2008). Briefly, the fluorescence in stacks of 15 images was summed. Five random ROIs away from neuronal staining were selected and averaged for background fluorescence. The average background fluorescence was then subtracted from the total fluorescent intensity of the whole frame. The fluorescent intensity obtained from slices before ME (30 μM) application was considered as total fluorescent receptors (C). After treatment with ME (30 μM, 15 min), the slice was treated with a calcium free ACSF containing ethylene glycol-bis(β-aminoethylether) N,N,N′,N′-tetraacetic acid (0.5 mM EGTA, 10 min) to strip all extracellular antibody binding. The fluorescence that was retained within the cells was considered to be internalized receptors (I). The percentage of internalization was calculated by (I/C)x100. In experiments where the return of receptors to the plasma membrane was measured, slices were perfused with ACSF for 10 or 45 min following treatment with ME (30 μM, 10 min) before the calcium free solution wash (additional 10 min). The remaining fluorescence after these recovery periods was calculated as described above.

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Drug application: Drugs were applied by perfusion at the rate of 1.5 ml/min. Slices were pre-incubated in staurosporine (10 μM) or SB203580 (10 μM) for 45 min before experiments followed by the presence of 1 μM inhibitors throughout. NaPP1 (1 μM) was applied to slices 10-15 min before experiments and was kept in test solutions the whole time.

Data analysis: Data analysis was performed with PRISM software (GraphPad, San Diego, CA, USA). All results are reported as the mean±SEM. Statistical significance was accessed using two-tailed, unpaired Student's *t*-test or two-way ANOVA with Bonferroni posttest. The level of significance was set at *P value* <0.05.

Results

Staurosporine had no effect on desensitization but increased the rate of recovery from desensitization.

A number of kinases are known to phosphorylate MOPr suggesting that simultaneous inhibition of several independent kinases may be required to affect desensitization (Dang et al., 2009). With this in mind a high concentration of a non-selective kinase inhibitor was used to examine the kinase dependence of desensitization. Initial experiments examined the action of staurosporine (10 μM) on the extent of and recovery from desensitization induced by ME. Intracellular recordings were performed in LC neurons in slices from rat (Figure 1A). ME (30 μM) applied for 10 min resulted in a peak hyperpolarization that declined during the application, an indication of desensitization. The decline in the peak hyperpolarization was not different in untreated and staurosporine treated slices (peak in control 35.1±1.0 mV decreased by

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26.7±4.0%, n=8; peak in staurosporine 29.8±1.2 mV decreased by 30.4±3.0%, n=8, *P value* = 0.7 Student's *t*-test). Unexpectedly, the recovery after desensitization measured by the hyperpolarization induced by ME (0.3 μM) was significantly increased (Figure 1A, *P value* <0.001 two-way ANOVA). The results indicate that the desensitization measured by the decline in the peak hyperpolarization using a saturating concentration of ME was not affected by a high concentration staurosporine while the rate of recovery from desensitization was facilitated.

The desensitization of MOPr was not blocked by staurosporine and inhibitors of GRK

Although staurosporine is a non-selective kinase inhibitor that is known to inhibit as many as 300 kinases particularly at the concentration used in this study (10 μM), it is not a potent inhibitor of GRKs (Karaman et al., 2008). Two approaches were used to determine if the inhibition of GRKs in addition to staurosporine sensitive kinases would affect acute desensitization. The first approach was to use the GRK2as5 transgenic mouse together with the specific nucleotide inhibitor NaPP1 in addition to staurosporine. A recent study demonstrated that acute desensitization induced by ME and the recovery after desensitization in slices prepared from morphine naive transgenic animals was the same in the absence or presence of NaPP1 (Quillinan et al., 2011). In this study intracellular recordings were made to examine desensitization and the recovery from desensitization in the absence and presence of the combined inhibitors, staurosporine (10 μM) plus NaPP1 (1 μM, Figure 1B). The peak hyperpolarization and decline from the peak induced by ME (30 μM, 10 min) was not different in the absence and presence of the inhibitors (control 28.3±1.7 mV a decrease of 31.2±1.6%, n=6;

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staurosporine+NaPP1 23.7±2.9 mV a decrease of 34.0±2.3%, n=6, *P value* = 0.4 Student's *t*-test). The recovery from desensitization was not significantly different from that of control, but trended toward being faster (Figure 1B, two-way ANOVA *P value* > 0.05).

The second approach used whole cell recordings in slices from the transgenic FlagMOPr animals where heparin (1 mg/ml) was included in the internal solution. Heparin is a polyanionic compound known to inhibit all forms of GRKs (Loudon and Benovic, 1994). The internal solution including heparin did not change the amplitude of the outward current induced by ME (0.3 μM, control 62.7 ±11 pA, n=6 *vs.*; heparin 43.5±5 pA, n=4, P value = 0.2, Student's *t*-test, Figure 1C). In addition the decrease in the peak current induced by ME (30 μM) was the same in control and heparin treated neurons (34.3±4.2%, n=4 for heparin and 42.1±3.2, n=5 for control, *P value* = 0.2, Student's *t*-test). The recovery from desensitization in heparin treated neurons was also not different from control (*P value* > 0.05, two-way ANOVA). The results indicate that heparin did not alter ME-induced acute desensitization or the recovery from desensitization LC neurons. Addition of staurosporine to the extracellular solution did not change the extent of decline in the peak current induced by ME (30 μM, 10 min; control 42.1±3.2% of the peak, n=5, heparin+staurosporine 43.5±2.1% of the peak, n=4, *P value* = 0.7, Student's *t*-test) but increased the rate of recovery from desensitization (Figure 1C, *P value* <0.05 two-way ANOVA). Thus experiments aimed at inhibiting the activity of many kinases did not change the extent of desensitization, and staurosporine appeared to have a strong effect on the recovery after desensitization.

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ME-induced MOPr trafficking changed after staurosporine treatment.

The effect of staurosporine to increase the recovery after ME-induced desensitization suggested MOPr trafficking could be modified. Staurosporine (10 μM, 45 min) caused a small reduction of ME (30 μM)-induced FlagMOPr endocytosis (control = 28.9±3.0%, n=11; staurosporine = 21.4±2.9%, n=9, *P value* = 0.09, Student's *t*-test). There was however a distinct change in the pattern of receptor distribution in the staurosporine-treated cells. The FlagMOPr formed clusters that remained closely associated with the plasma membrane (Figure 2 bottom panels). In addition, FlagMOPr-bound M1-A594 labeling in these clusters was protected from striping by the calcium-free solution. A high power scanning infrared image of the plasma membrane along with the labeled Flag-MOPr illustrate the localization of receptors following internalization induce by ME (Figure 3). In staurosporine-treated slices clusters of receptors that were not striped by the calcium-free solutions were located close to plasma membrane. The insensitivity to the striping procedure was taken to mean that they were internalized (Puthenveedu and von Zastrow, 2006), however it is also possible that they were not internalized but protected from the calcium-free solution.

To examine the effect of GRK/arrestin pathway in combination with staurosporine, the trafficking of MOPr was examined in FlagMOPr mice crossed with the arrestin3 knockout mice (FlagMOPr-ArrKO). Similar to previous reports (Arttamangkul et al., 2008; Quillinan et al., 2011), ME (30 μM)-induced receptor internalization in slices from the transgenic FlagMOPr-ArrKO was 29±2.8% (n=7), not different from that measured in the FlagMOPr animals (28.8±3.0%, n=11). After treatment with staurosporine (10 μM), labeled receptors in cells from FlagMOPr-ArrKO mice also formed clusters near plasma membrane (Figure 4). As was found in slices

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from FlagMOPr mice (control), the internalization of receptors in the FlagMOPR-ArrKO mice was decreased by treatment with staurosporine (staurosporine treated 19.0±3.0%, n=9, untreated 29.3±2.8%, n=7, *P value* = 0.03, Student's *t*-test). The time course of FlagMOPr trafficking after perfusion with ME (30 μM) of FlagMOPr and FlagMOPr-ArrKO mice indicate that receptors formed clusters as early as 3 min but in the presence of staurosporine they remained in the vicinity of the plasma membrane, whereas receptors in untreated slices moved into the cytoplasm (Figure 4). These experiments suggest that no additional effect on receptor trafficking was induced by staurosporine in slices from the arrestin3 knockout animals.

Staurosporine facilitated receptor re-insertion.

Given that the recovery from ME-induced desensitization was increased after treatment with staurosporine, the re-insertion of receptors onto the plasma membrane during the recovery from desensitization was examined. Following treatment with ME (30 μM, 10 min), slices were washed with ACSF solution and then the antibody M1-A594) was striped with the calcium-free solution (Figure 5A). The fluorescence associated with receptors that trafficked back to the plasma membrane would therefore be lost after this treatment such that a decline in fluorescence indicated the extent of receptor reinsertion. The internalization and re-insertion of FlagMOPr was examined in the presence and absence of staurosporine. Two time points of receptor reinsertion were measured (20 and 55 min). In control slices after a 55-min wash the cytoplasmic fluorescence decreased to 19.0±2.5% of control (n=8), whereas the cytoplasmic fluorescence decreased significantly more in slices treated with staurosporine (Figure 5A and B, 5.4±2.9%, n=8, *P* value < 0.01 two-way ANOVA). After a 20-min wash the

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fluorescence decreased to 19.0±2.5% in control (n=8) *vs*. 5.8±2.3% in staurosporine (n=8). These results indicate that in control slices some FlagMOPr recycled efficiently after the removal of ME whereas another pool was trapped or recycled more slowly. Treatment with staurosporine altered the extent, distribution and recycling of receptors demonstrating a role of kinases in agonist-dependent trafficking.

ME-induced desensitization was not affected by inhibiting p38MAPK.

Recent studies have reported an alternate pathway that regulates MOPr endocytosis (Macé et al., 2005, Yang et al., 2010). The activation of p38MAPK was proposed to be the kinase that regulated agonist-bound MOPr trafficking via phosphorylation of the small GTPase Rab5. It is suggested that internalization of MOPr can reduce desensitization via rapid recycling process. In this study acute ME-induced desensitization was examined using intracellular recordings in the absence and presence of the p38MAPK inhibitor SB203580. In the presence of SB203580 (10 μM), desensitization as determined by the decline in the peak hyperpolarization caused by ME (30 μM, 10 min) was not different from control (Figure 6A and B, control, a decrease of 29.5±2.9%, n=7; SB203580, a decrease of 30.5±7.1%, n=4, *P value* = 0.9, t-test). The recovery from desensitization was similar although more complete in the presence of SB203580. After 45 min the hyperpolarization induced by ME (0.3 μM) was 63.2±7.1% (n=9) of the initial amplitude in control and 82.6±3.6% in slices treated with SB203580 (n=8; *P value* < 0.05, two-way ANOVA).

The effect of SB203580 on the internalization of FlagMOPr was examined next. ME-induced FlagMOPr internalization in the presence of SB203580 was not different from control (control = 26.1±2.4%, n=6; SB203580 = 32.2±5.9%, n=7, *P value* = 0.4, t-

test). The fluorescent receptors were clustered throughout the cytoplasm as found in untreated slices (Figure 6C). In addition these receptors reinserted onto the plasma membrane to the same extent as untreated cells. After a 55 min wash the remaining cytosolic fluorescence was reduced to 14.9±6.9% of control (n=5) in SB203580 slices and 14.8±2.8 (n=9) of control in untreated slices. Thus in contrast to studies in HEK293 cells (Macé et al., 2005, Yang et al., 2010), the results of the present study suggest that the inhibition of p38MAPK alone had no effect on ME-induced FlagMOPr internalization in LC neurons.

Discussion

This study examined the role of protein kinases on MOPr desensitization, recovery from desensitization, internalization and reinsertion onto the plasma membrane. The results show that agonist-induced MOPr desensitization was resistant to the inhibition of many serine-threonine kinases. In the presence of the non-selective kinase inhibitors i.e. heparin and staurosporine, desensitization induced by ME was present in LC neurons. The rate of recovery from desensitization was however significantly increased in staurosporine-treated slices. This finding correlated with the observation of the qualitative change in receptor trafficking. Although the receptors were internalized, as judged by the insensitivity to the calcium-free wash, they clustered near the plasma membrane and readily recycled back to the membrane following the washout of ME. The results indicate that kinase activity may not be required for desensitization but is important in receptor trafficking and recovery after desensitization.

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The classical model of GPCR desensitization follows the sequential events that agonist-bound receptor is phosphorylated by GRK(s) to increase the affinity for arrestin and terminate signaling. Multiple phosphorylation sites on GPCRs are reported to be important for arrestin/receptor interactions (reviewed in Gurevich and Gurevich, 2006). One phospho-specific site, Ser375, on the C terminal tail of MOPr has been identified and proposed to be an initial site leading to receptor desensitization (Schulz et al., 2004; Doll et al., 2011). A more detailed quantitative analysis using mass spectrometry further demonstrates multiple phosphorylation clusters induced by different opioid agonists (Lau et al., 2011). Although these phosphorylated receptors are important for the arrestin binding and internalization process, the role in desensitization remain unclear. Studies in LC neurons and HEK293 cells demonstrate that MOPr internalization was not required for desensitization or recovery from desensitization (Arttamangkul et al., 2006; Doll et al., 2011; Dang et al., 2011; also see reviewed in Dang and Christie, 2011). Inhibition of GRK2 in LC neurons using GRK2as5 mutant mouse or a specific peptide inhibitor did not block ME-induced MOPr desensitization (Qullinan et al., 2011; Dang et al., 2011). Although other isoforms of GRK i.e. GRK3, GRK5 and GRK6 are able to phosphorylate MOPr (Kovoor et al., 1998), desensitization in LC neurons was not changed when heparin was applied intracellularly via the recording pipette. All isoforms of GRK are known to be sensitive to heparin (Benovic et al., 1989; Loudon and Benovic, 1994) and the protocol used to introduce heparin is known to inhibit desensitization of delta opioid receptors in NG108-15 cells and inositol 1,4,5-triphosphate signaling in dopamine neurons (Morikawa et al., 1998; Cui et al., 2007). The results therefore suggest a limited role of GRK(s) in the

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desensitization of MOPr and indicate that other kinases may be involved in the phosphorylation of MOPr.

The present study used a high concentration (10 μM) of staurosporine that has been shown to inhibit more than 300 different protein kinases (Karaman et al., 2008). Surprisingly, desensitization measured by the decline in the peak hyperpolarization or outward current induced by ME (30 μM) was not affected by staurosporine or staurosporine in combination with heparin. When desensitization was measured by the decrease in the response to ME (0.3 μM, EC50) after application of a saturating concentration, the extent of desensitization was attenuated but this may result from a more rapid recovery from desensitization in staurosporine treated tissues. Imaging experiments support this interpretation. Staurosporine caused FlagMOPr to form clusters that did not distribute into the cytoplasm and the receptors were reinserted on the plasma membrane more rapidly. Because staurosoprine is not a potent inhibitor of GRK, the concentration used in this study may allow receptors to form clusters via GRK/arrestin pathway. Another possible explanation is that arrestin could transiently bind to unphosphorylated receptors and initiate endocytosis (DeFea, 2011). The pronounced effect of staurosporine on MOPr redistribution however was not dependent on the level or the isoform of arrestin present in the cell. The increase in the rate of recycling in the staurosporine-treated cells may also explain a small reduction of the extent of internalized receptors.

The observation that desensitization was not blocked by the inhibition of many kinases suggests that the desensitized state of the receptor may occur prior to phosphorylation and trafficking. This could be a receptor conformation that is stabilized by an agonist but unable to activate G proteins efficiently. Recent structural

studies indicate that agonist-bound receptors have multiple affinity states, one of which could be a desensitized conformation (Rosenbaum et al., 2009). The intrinsic mechanism that underlies the desensitization state of ionotropic receptors, in particular the AMPA receptor, has been elegantly described (Armstrong et al., 2006; Sobolevsky et al., 2009). It is also possible that the interactions between MOPr and G proteins are dependent on the lipid environment and agonist dependent movement between lipid microdomains affects signaling (Zheng et al., 2008; Levitt et al., 2009). Taken together mechanisms beyond the classical concept of GPCR phosphorylation and arrestin binding are needed to explain acute MOPr desensitization.

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Authorship Contribution

Contributions: Participated in research design: SA, JTW
Conducted experiments: SA,H-WL, JTW
Contributed new reagents or analytical tools: EKL
Performed data analysis: SA,H-WL, JTW
Wrote or contributed to the writing of the manuscript: SA,H-WL, EKL, JTW

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Footnotes

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Figure 1. MOPr desensitization in the presence of protein kinase inhibitors. A (left), Example trace showing the desensitization and recovery from desensitization in a slice from rat that was incubated in staurosporine (10 μM, 45 min). A (right), Summarized results showing the recovery from desensitization. B (left), Example trace showing the desensitization and recovery from desensitization in a slice from GRK2as5 transgenic mouse that was incubated in staurosporine (10 μM, 45 min) and NaPP1 (1 μM). B (right), Summarized results showing the recovery from desensitization. C (left), Example trace showing the desensitization and recovery from desensitization in a slice from FlagMOPr mouse that was recorded with heparin (1 mg/ml) in the pipette. C (right), Summarized results showing the recovery from desensitization in control, with heparin and heparin and in the presence of staurosporine. * Denotes $P < 0.05$, two way ANOVA Bonferroni post-test.

Figure 2. Staurosporine causes a qualitative change in the distribution of internalized receptors from FlagMOPr mouse. Upper panel shows the sequential images of the untreated slice. Left, control staining with M1-A594 (45 min). Middle, after treatment of the slice with ME (30 μM, 15 min). Right, following treatment of the slice with calcium free solutions. Bottom panel shows the sequential images of the staurosporin treated slice. Left, control staining with M1-A594 after incubation in staurosporine (10 μM, 45 min). Middle, after treatment of the slice with ME (30 μM, 15 min). Right, following treatment of the slice with calcium free solutions.

Figure 3. High power image of staurosporine treated cells from FlagMOPr mouse illustrates the receptor clustering in cytoplasm near plasma membrane. Upper panel shows the images of a control slice. Left, Scanning IR image showing the location of the plasma membrane (arrow). Right, receptor staining with M1-A594 in the cytoplasm. Bottom panel shows images of the staurosporin treated slice. Left, Scanning IR image showing membrane of the cell. Right, the receptor staining with M1-A594, arrows show the tight location of receptors close to the plasma membrane.

Figure 4. Staurosporine causes MOPr clustering near plasma membrane of FlagMOPr-ArrKO mouse. Upper panel shows the sequential images of the untreated slice. Left, control staining with M1-A594 (45 min). Middle, after treatment of the slice with ME (30 μM, 15 min). Right, following treatment of the slice with calcium free solutions. Bottom panel shows the sequential images of the staurosporine treated slice. Left, control staining with M1-A594 after incubation in staurosporine (10 μM, 45 min). Middle, after treatment of the slice with ME (30 μM, 15 min). Right, following treatment of the slice with calcium free solutions.

Figure 5. Time course of FlagMOPr trafficking. Top Control, Middle after treatment with staurosporine (10 μM, 45 min), Bottom a slice from an arrestin knockout animal (FlagMOPr-ArrKO) after incubation with staurosporine (10 μM, 45 min).

Figure 6. Reinsertion of FlagMOPr following internalization. A (upper panel), images left to right; control, following treatment with ME (30 μM, 15 min), following a wasout period of 55 min; following treatment of the slices with calcium-free solution. A (bottom panel), the same experiment carried out in a slice that was incubated in staurosporine (10 μM, 45 min). B, Summarized results showing the amount of fluorescence remaining in slices following washout of ME (30 μM) for different periods. ** Denotes $P < 0.01$, two way ANOVA Bonferroni post-test.

Figure 7. The p38 MAP kinase inhibitor, SB203580, does not block desensitization or change internalization, but increases the extent of recovery from desensitization. A, Example trace showing the desensitization and recovery from desensitization in a slice that was incubated in SB203580 (1 μM, 45 min), * denotes $P < 0.05$, two way ANOVA Bonferroni post-test. B, Summarized results showing the decline and recovery of the hyperpolarization induced by ME (0.3 μM) following washout of ME (30 μM, 10 min). C, Example images show sequential steps of MOPr internalization and reinsertion. Left, control, Middle, after treatment with ME (30 μM, 15 min) followed with washout. Right, striping treatment of the slice with calcium free solution.

Figure 1

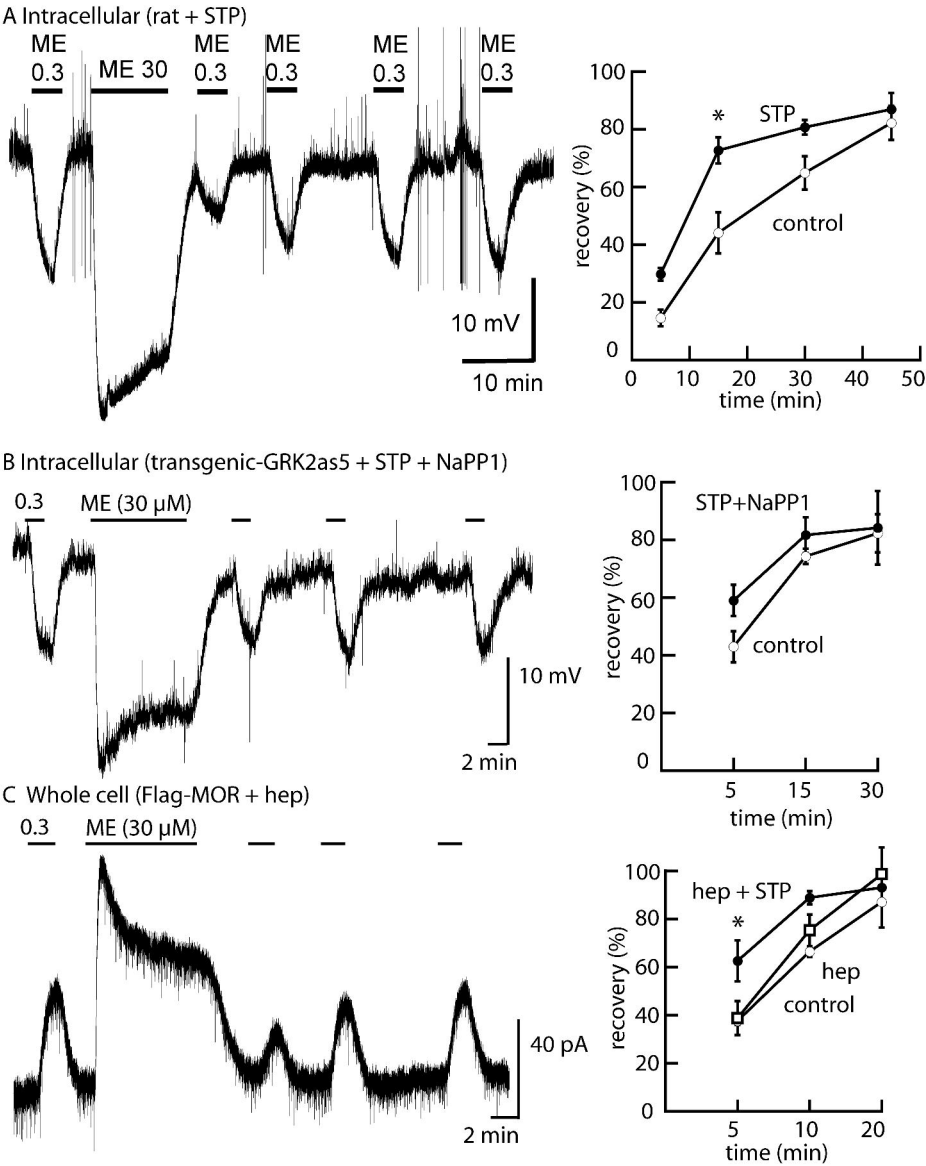

A Intracellular (rat + STP)
ME 0.3
ME 30
ME 0.3
ME 0.3
ME 0.3
ME 0.3
10 mV
10 min
recovery (%)
STP
control
time (min)
B Intracellular (transgenic-GRK2as5 + STP + NaPP1)
0.3
ME (30 µM)
10 mV
2 min
STP+NaPP1
control
recovery (%)
time (min)
C Whole cell (Flag-MOR + hep)
0.3
ME (30 µM)
40 pA
2 min
hep + STP
hep
control
recovery (%)
time (min)

Figure 2

Staurosporine modified trafficking of FlagMOPr

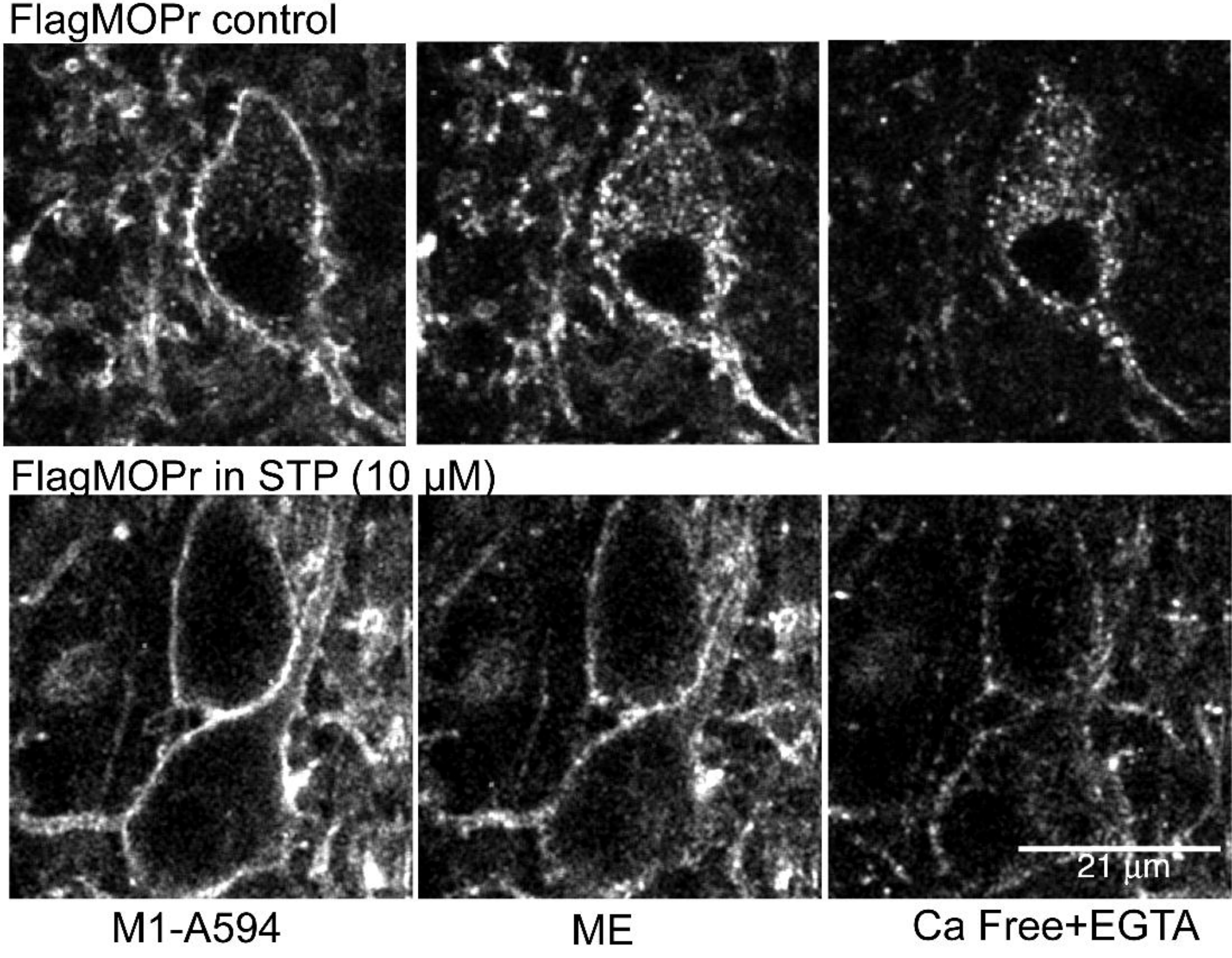


Figure 3

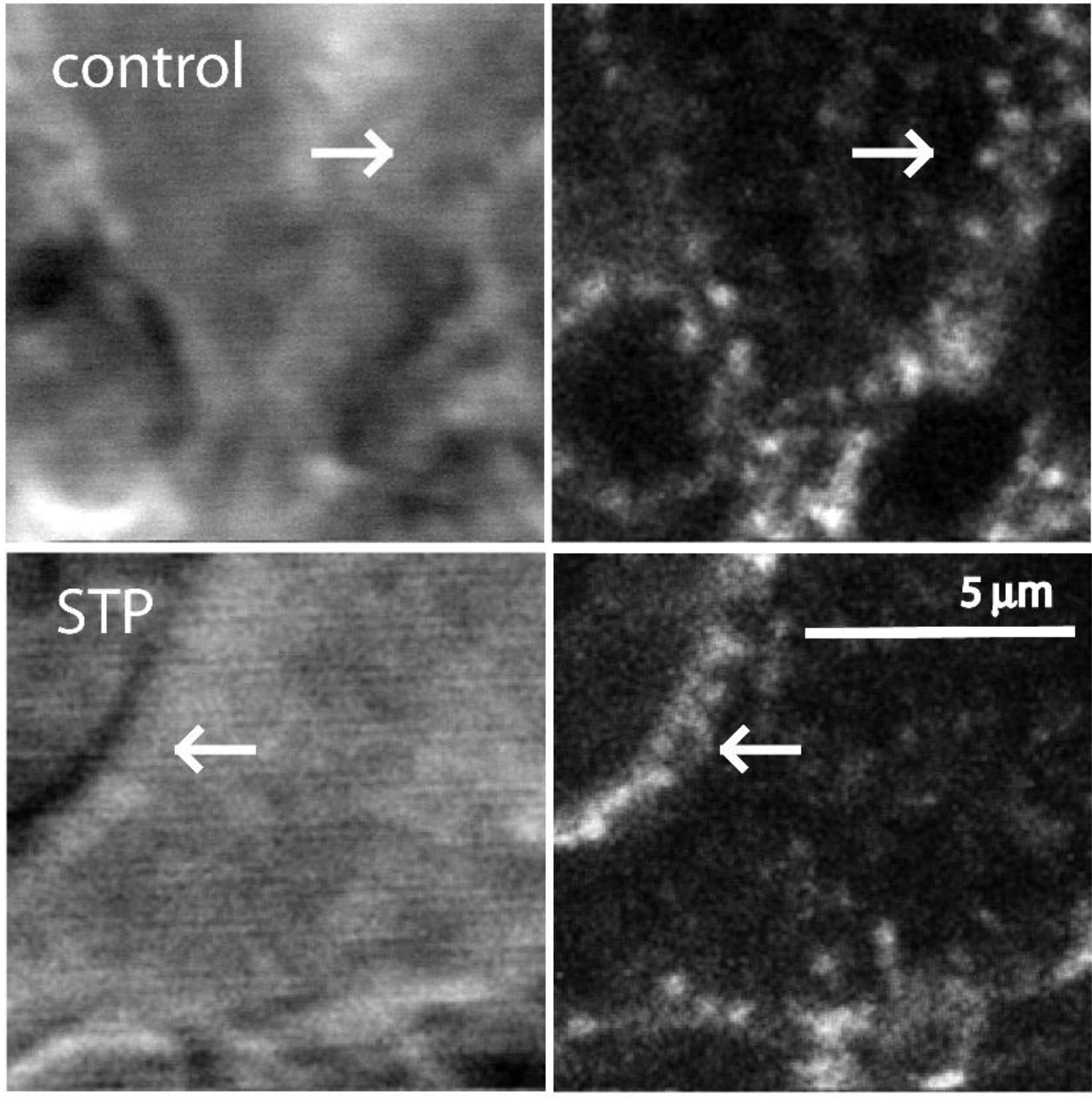
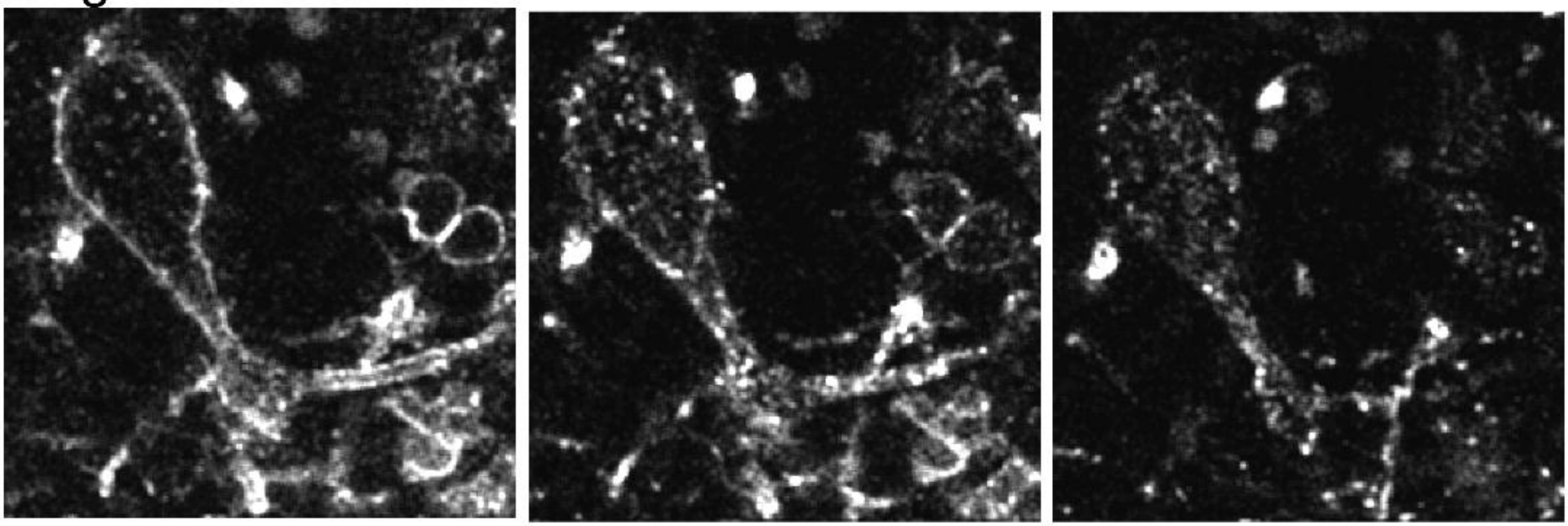


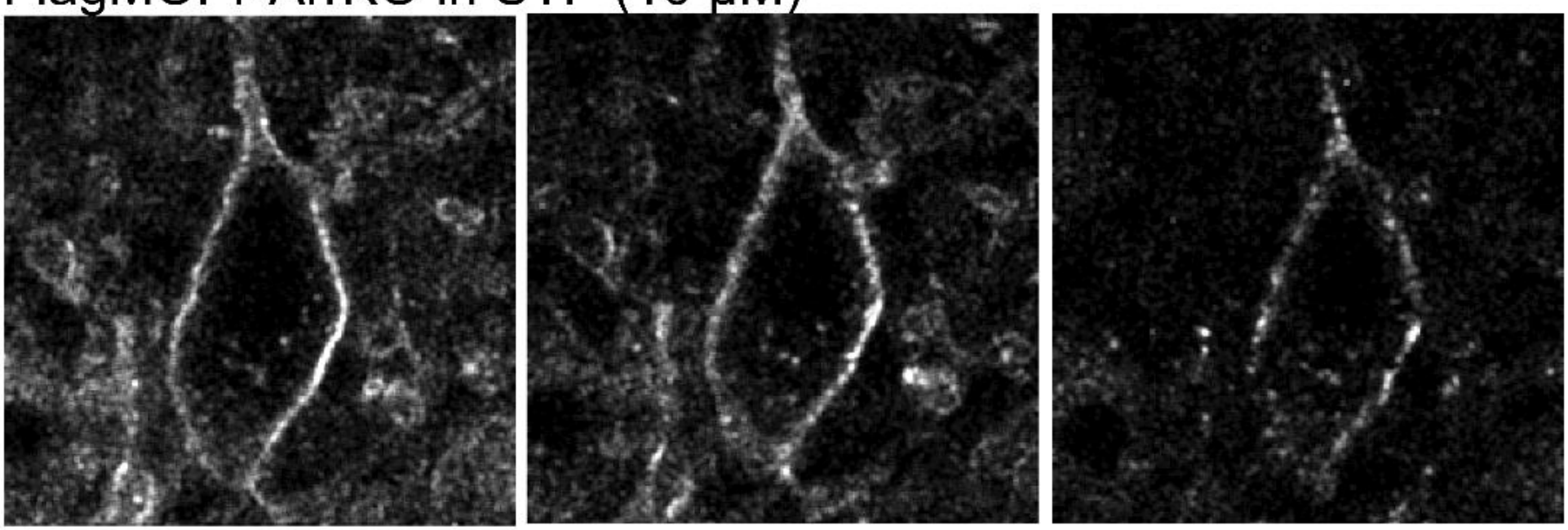
Figure 4

Staurosporine modified trafficking of FlagMOPr-ArrKO

FlagMOPr -ArrKO control



FlagMOPr-ArrKO in STP (10 μM)



M1-A594 ME Ca Free+EGTA

Figure 5

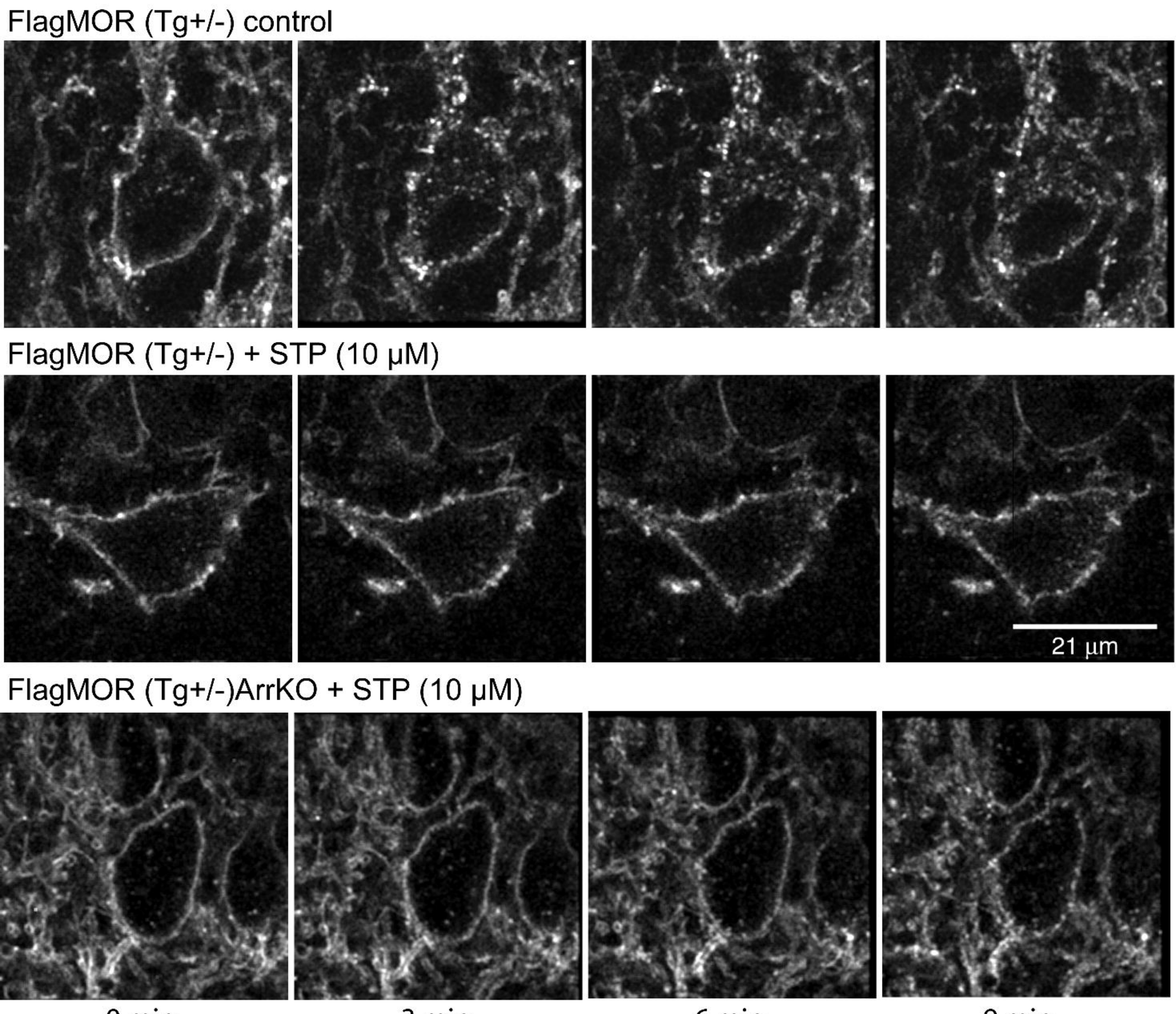
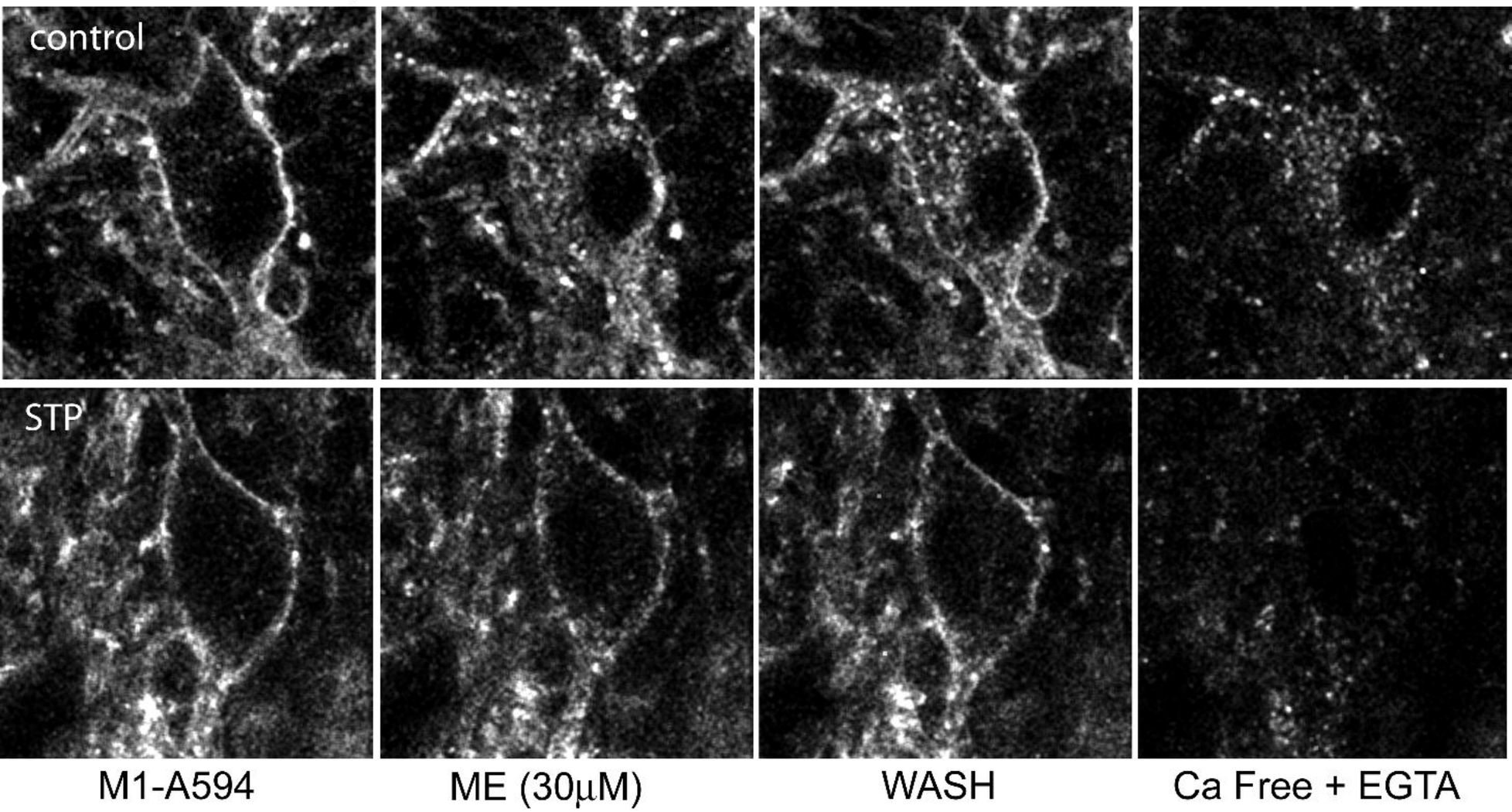

FlagMOR (Tg+/-) control
FlagMOR (Tg+/-) + STP (10 µM)
21 µm
FlagMOR (Tg+/-)ArrKO + STP (10 µM)
0 min
3 min
6 min
9 min

Figure 6


A) FlagMOPr Recovery
control
STP
M1-A594
ME (30μM)
WASH
Ca Free + EGTA

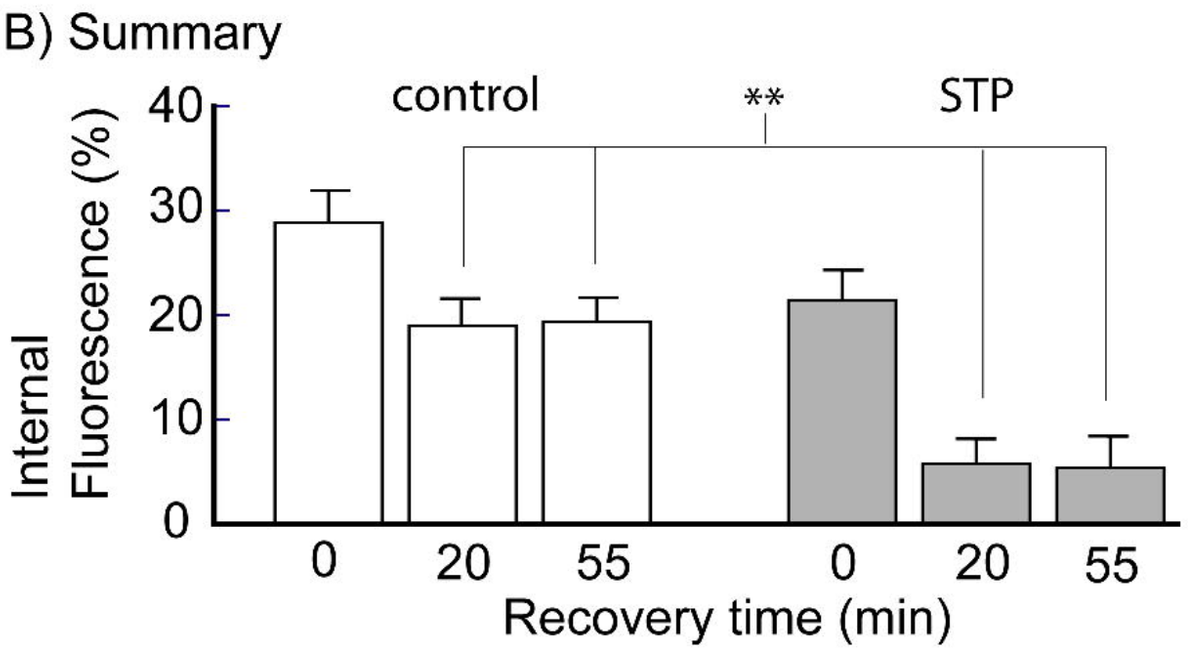
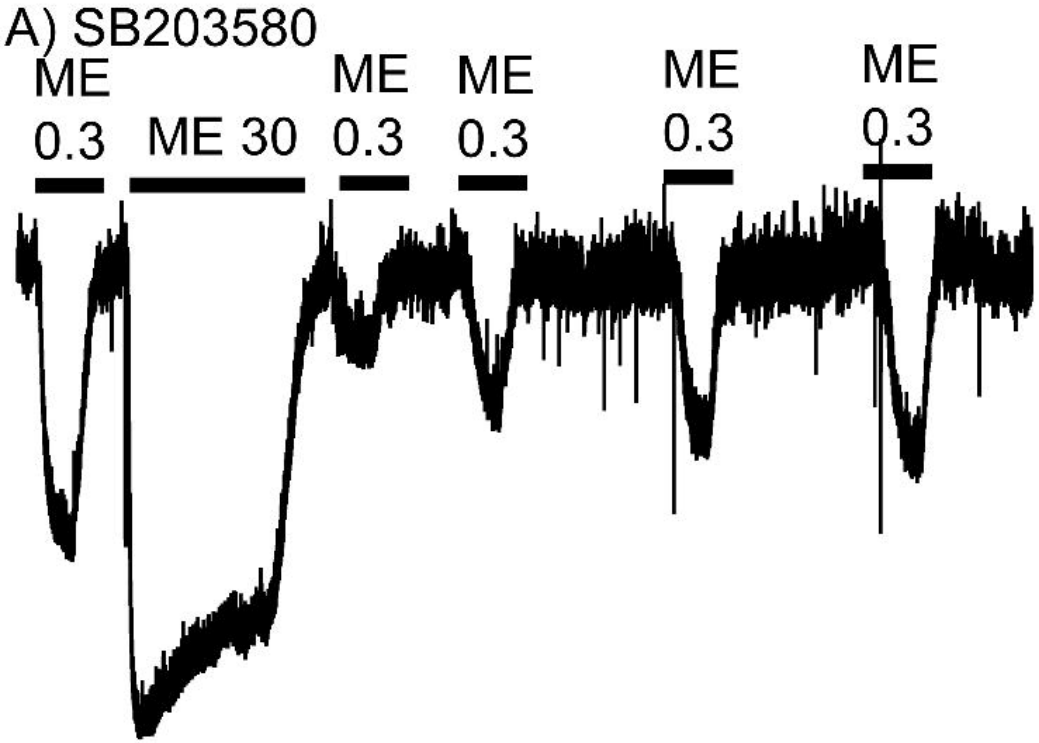
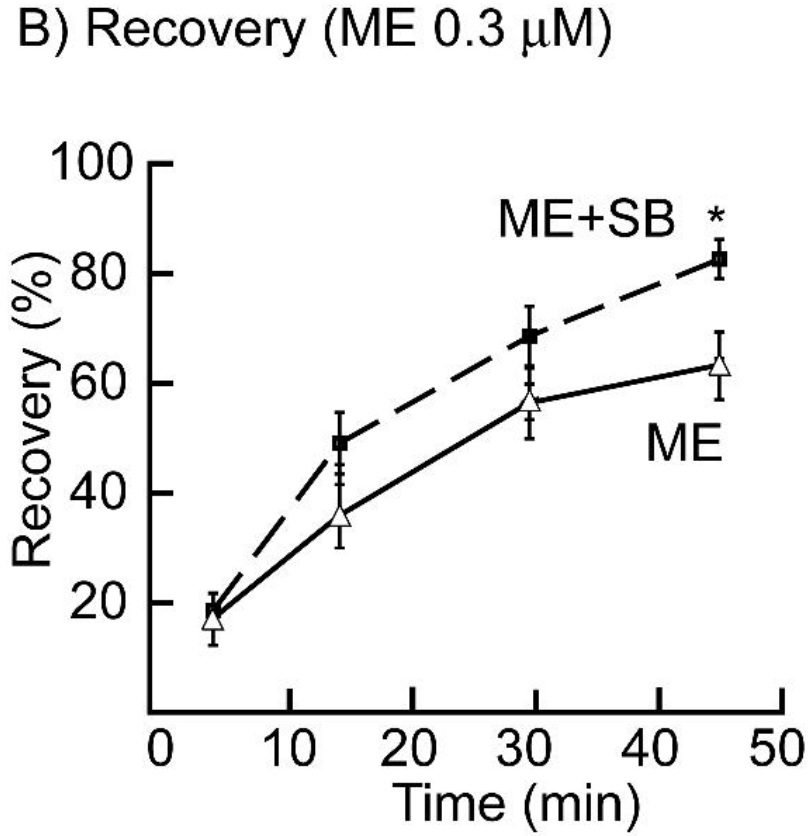

B) Summary
control
**
STP
Internal Fluorescence (%)
40
30
20
10
0
0
20
55
0
20
55
Recovery time (min)

Figure 7





C) FlagMOPr internalization in SB203580