Molecular Pharmacology Fast Forward. Published on March 21, 2011 as doi:10.1124/mol.111.071456
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Kappa-opioid receptor inhibition of calcium oscillations in spinal cord neurons

Lakshmi Kelamangalath, Shashank M. Dravid, Joju George, Jane V. Aldrich, Thomas F. Murray

Department of Pharmacology, Creighton University, Omaha, NE 68178 (LK., SMD., JG., TFM).

Department of Medicinal Chemistry, The University of Kansas, Lawrence, Kansas 66045-7582 (JVA).

Molecular Pharmacology Fast Forward. Published on March 21, 2011 as DOI: 10.1124/mol.111.071456
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Running title: KOR regulation of Ca^{2+} oscillations

Corresponding author:

Dr. Thomas F. Murray

Department of Pharmacology, Creighton University School of Medicine

2500 California Plaza, Omaha, NE-68178

Phone: 402-280-2983

Fax: 402-280-2142.

E mail- tfmurray@creighton.edu

Manuscript: Figures: 8

Supplemental data: 1 figure

References: 40

Word document length: 30 pages

Word count: Abstract: 220, Introduction: 454, Discussion: 1031

Abbreviations: KOR, kappa opioid receptor; CNS, central nervous system; Dyn, dynorphin; DAMGO, [D-Ala2, N-MePhe4, Gly-ol]-enkephalin; DPDPE, [d-Pen2,d-Pen5]-enkephalin; Nor-BNI, norbinaltorphimine; VGlut, vesicular glutamate transporter; NMDA, n-methyl d-aspartate; TTX-tetrodotoxin; AMPA, alpha-amino-3-hydroxy-5-methyl-4- isoxazole-propionic acid; MK-801, (5*R*, 10*S*)-(+)-5-methyl -10,11-dihydro-5H-dibenzo-[*a*,*d*] cyclohepten-5,10-imine hydrogen maleate; APV, (2*R*)-amino-5-phosphonovaleric acid; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione; FITC, fluorescein isothio cyanate; Cy-3, cyanine 3.

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Abstract:

Mouse embryonic spinal cord neurons in culture exhibit spontaneous calcium oscillations from day in vitro (DIV)-6 through DIV-10. Such spontaneous activity in developing spinal cord contributes to maturation of synapses and development of pattern generating circuits. Here we demonstrate that these calcium oscillations are regulated by kappa opioid receptors (KORs). The kappa opioid agonist dynorphin (Dyn)-A (1-13) suppressed calcium oscillations in a concentration-dependent manner and both the nonselective opioid antagonist naloxone and the kappa selective blocker nor-BNI eliminated this effect. The KOR selective agonist U69593 mimicked the effect of Dyn-A (1-13) on calcium oscillations. A kappa specific peptide antagonist, zyklophin, was also able to prevent the suppression of calcium oscillations caused by Dyn-A (1-13). These spontaneous calcium oscillations were blocked by 1 μM tetrodotoxin indicating that they are action potential dependent. Although the L-type voltage gated calcium channel blocker nifedipine did not suppress calcium oscillations, the N-type calcium channel blocker ω-conotoxin inhibited this spontaneous response. Blockers of ionotropic glutamate receptors, NBQX and MK-801, also suppressed calcium oscillations revealing a dependence on glutamate-mediated signaling. Finally, we have demonstrated expression of KORs in glutamatergic spinal neurons and localization in a presynaptic compartment: consistent with previous reports of KOR mediated inhibition of glutamate release. The KOR mediated inhibition of spontaneous calcium oscillations may therefore be a consequence of presynaptic inhibition of glutamate release.

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Introduction

Periodic spontaneous network activity is a characteristic feature of the developing nervous system (Blankenship and Feller, 2010). In the traditional model of brain development it has been proposed that this form of spontaneous neural activity facilitates the refinement of wiring of the nervous system laid out by predetermined genetic programs. Spontaneous events have been observed in a wide range of developing brain structures in vivo, including the cerebral cortex, spinal cord and retina (O'Donovan, 1999; Feller, 1999). Spontaneous activity in the developing spinal cord contributes to motor neuron path-finding, maturation of synapses and development of pattern generating circuits (Blankenship and Feller, 2010). Spontaneous calcium transients have been implicated in regulating plasticity in developing neurons (Spitzer et al., 1995). Although there are basic similarities in the mechanisms responsible for spontaneous network activity in different brain regions, the signaling processes underlying these phenomena may vary with brain structure and developmental stage. Studies in isolated mouse spinal cord-limb preparations suggest that the depolarizing actions of GABA and acetylcholine are responsible for spontaneous network activity at early stages of development, while at later stages they become more dependent on glutamatergic neurotransmission (Hanson and Landmesser, 2003).

Opioid receptor activation in the central nervous system (CNS) has been shown to result in inhibition of neuronal firing and reduction of neurotransmitter release. Kappa opioid receptors (KORs) are widely expressed in the CNS and are specifically activated by endogenous opioids derived from prodynorphin (Chavkin et al., 1982). KORs and dynorphin mRNA initially appear during early embryonic development in both mouse and human CNS (Zhu et al., 1998; Brana et al., 1995).

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Dynorphin has been shown to inhibit excitatory transmission in the CNS either when exogenously applied (Castillo et al., 1996; Simmons and Chavkin, 1996;, Iremonger and Bains, 2009) or endogenously released (Wagner et al., 1993; Iremonger and Bains, 2009). Although the molecular mechanism for dynorphin inhibition of excitatory transmission remains to be fully understood, an increasing body of evidence suggests that G-protein-coupled receptor stimulated release of $G_{\beta\gamma}$ subunits directly inhibits the synaptic release machinery (Delaney et al., 2007; Iremonger and Bains, 2009).

Despite numerous reports of dynorphin mediated depression of synaptic transmission, little is known regarding its role and relevance in CNS development. We found that calcium oscillations driven by excitatory neurotransmission appear at DIV 6-8 in spinal cord neurons. We additionally observed that these spontaneous calcium oscillations in spinal neurons were suppressed through a KOR dependent mechanism. We further demonstrated that spontaneous calcium oscillations are dependent on glutamatergic neurotransmission and that the majority of the KORs are localized to glutamatergic spinal cord neurons. Although mu and delta opioid receptor mediated regulation of calcium dynamics has been described, this study represents the first report of KOR regulation of spontaneous calcium oscillations.

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Material and Methods:

Materials

Trypsin, penicillin, streptomycin, horse serum and soybean trypsin inhibitor were obtained from Atlanta Biologicals (Norcross, GA). Levobitz medium, neurobasal medium, B 27 and anti synaptophysin were purchased from Life Technologies (Carlsbad, CA). Deoxyribonuclease (DNase), poly-L-lysine, nor-BNI, DAMGO, DPDPE, naloxone, cytosine arabinoside, Fluoromount™ aqueous mounting medium were purchased from Sigma (St. Louis, MO). Pluronic acid and fluo-3 AM were purchased from Molecular Probes (Eugene, OR). VGLUT2 antibody (cat#MAB5504) and Cy 3 conjugated secondary antibody (cat# AP124CMI) were purchased from Millipore (Temecula, CA). PSD-95 antibody was obtained from Thermo Fisher Scientific (Rockford, IL). The antibody to KOR1 was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). ω-Conotxin, tetrodotoxin and nifedipine were purchased from Tocris Bioscience (Ellisville, MO). NBQX, MK-801 and D-APV were purchased from Ascent Scientific (Princeton, NJ). Dyn A (1-13) was purchased from American Peptide (Sunnyvale, CA). Dyn A (1-17), Dyn A (2-17) and zyklophin were synthesized as previously described (Patkar et al., 2005). U69593 was purchased from Sigma-Aldrich (St. Louis, MO).

Neuronal culture

Primary cultures of embryonic spinal cord neurons were harvested from embryos of Swiss-Webster mice on embryonic day 14. Briefly, pregnant mice were euthanized with CO_2, vertebral columns were extracted from mouse embryos and dissected using sterile fine tipped forceps to isolate spinal cords. Embryonic cords (10-12) were collected, meninges and dorsal root ganglia were removed from each cord and cords were minced to $1mm^3$ thick slices. Tissue was again macerated using a fire polished

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plugged pipette and digested with 0.025% trypsin for 25 minutes at 37°C. The cells were then dissociated by two successive trituration and sedimentation steps in soybean trypsin inhibitor and DNase containing isolation buffer, centrifuged and resuspended in neurobasal medium with 2% B-27, 1% penicillin-streptomycin and 0.5% L-glutamine, pH-7.4. The cells were strained using a 70 micron cell strainer (BD Falcon, San Jose, CA) to remove any undigested coarse particles. Cells were then counted using a hemocytometer counting chamber and plated onto Poly-L-lysine coated 96-well (9 mm) clear bottomed black culture plates at a density of $0.8x10^6$ cells/mL in Levobitz (L-15) medium supplemented with glucose, 2 % B-27, 2 % horse serum, 0.5% L-glutamine, 1% penicillin-streptomycin and 7.5% sodium bicarbonate. Culture plates were incubated at 37°C in a 5% CO_2 and 95% humidity atmosphere. Cytosine arabinoside (10 μM) was added to the culture medium on day 2 after plating to prevent proliferation of non-neuronal cells. The culture medium was changed twice weekly and replaced with serum free neurobasal medium. DIV 6-8 cultures were used for calcium oscillation experiments. For western blotting, cells were grown in a 12 well plate at $1.2x10^6$ cells/mL, and cell lysates were collected from DIV 1-9 cultures. For immunocytochemistry experiments, cells were grown on poly-L-lysine coated square coverslips (22 mm^2) inserted into 6 well plates at $1.0x10^6$/mL. All animal use protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Creighton University.

Intracellular Ca^{2+} monitoring.

Embryonic spinal cord neurons grown in 96-well plates (1.2×10^5 cells per well) were used for intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) measurements. The growth medium was removed and replaced with dye loading medium (100 μl per well) containing 4 μM fluo-3 AM and 0.04% Pluronic acid in Locke's buffer (154 mM NaCl, 5.6 mM KCl, 1.0 mM $MgCl_2$, 2.3 mM $CaCl_2$, 8.6 mM HEPES, 5.6 mM glucose and 0.1 mM glycine, pH 7.4). After 1 h of incubation in dye loading medium, the

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neurons were washed four times in fresh Locke's buffer (200 µl per well, 22^{0}C) using an automated microplate washer (Bio-Tek Instruments Inc, VT, USA) and then transferred to a FLEX Station^{TM} II (Molecular Devices, Sunnyvale, USA) benchtop scanning fluorometer chamber. The final volume of Locke's buffer in each well was 150 µl. Fluorescence measurements were carried out at 37^{o}C. The neurons were excited at 488 nm and Ca^{2+}-bound fluo-3 emission was recorded at 538 nm at 1.2 s intervals. After recording basal calcium oscillations (300-500 seconds), a 4X concentration of test compounds was added to the cells at a rate of 26 µl/s, yielding a final volume of 200 µl/well; the fluorescence was monitored for an additional period of 300-500 seconds.

Immunoblotting.

Immunoblot analysis was performed in cells plated in 12-well plates (1.2 x 10^6 cells per well) and grown for 1-9 days post-plating (DIV 1-9). After washing with ice cold Locke's buffer, cells were harvested in ice cold lysis buffer containing 50 mM Tris, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% NP-40, 0.1% SDS, 2 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 25 mM β-glycerophosphate. Phenylmethylsulfonyl fluoride (1mM) and 1X Protease inhibitor cocktail were then added and the lysate was incubated for 15 min at 4^o C. Cell lysates were then sonicated and centrifuged at 12,000×g for 15 min at 4^o C. The supernatant was assayed by the Bradford method to determine protein content. Equal amounts of protein were mixed with the Laemmli sample buffer and boiled for 5 min. The samples were loaded onto a 12% SDS-PAGE gel and transferred to a nitrocellulose membrane by electroblotting. The membranes were blocked in TBST (20 mM Tris, 150 mM NaCl, 0.1% Tween 20) with 5% BSA for 1 h at room temperature. After blocking, membranes were incubated overnight at 4 °C in primary antibody diluted in TBST containing 5% BSA. The blots were washed and incubated with the secondary antibody conjugated with horseradish peroxidase for 2 h, washed three times in TBST and exposed with ECL Plus for 4 min. Blots were exposed to Kodak

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Hyperfilm and developed. Immunoblot densitometry data was obtained using MCID Basic 7.0 Software® (Imaging Research, Inc. Cambridge, UK).

Subcellular fractionation

Subcellular fractionation was performed as described previously with minor modifications (Toda et al., 2003). Lysates were prepared from DIV-8 spinal neuronal culture in ice cold buffer containing 0.32 M sucrose, 10 mM HEPES and 1 mM EDTA, pH 7.4. Homogenates were cleared three times at 1,000 g for 10 min to remove nuclear fraction. The resulting supernatants were concentrated twice at 10,000 g for 20 min to obtain crude synaptosome fraction, and subsequently were lysed hypo-osmotically in 1% Triton containing buffer with gentle rotation for 1h at 4 ^{0}C. This lysate was centrifuged at 16,000 g for 30 min to obtain the PSD-95 enriched fraction (LP1). The resulting supernatant was then centrifuged at 200,000 g for 90 min to obtain the crude synaptic vesicle enriched fraction (LP2). The enrichment of LP1 and LP2 fractions were verified by immunoblotting, respectively, for PSD-95 and synaptophysin.

Immunocytochemistry (double immunofluorescence).

We performed a double immnofluorescence technique using antibodies to PSD-95 and synaptophysin to document the role of synapse formation in the development of spontaneous calcium oscillations. We also performed double immunofluorescence with antibodies to the vesicular glutamate transporter-2 (VGlut2) and KOR to demonstrate the localization of KOR in glutamatergic neurons. Cells (DIV-7) grown on 22 mm^2 coverslips inserted in 6 well plates were used. The cells were washed with phosphate buffered saline (PBS) (37^{o}C, pH-7.4) and fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature. After fixing, cells were washed, blocked and permeabilized by incubation for 30 minutes with PBS containing 6 % goat serum and 0.15% Triton X -100.

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Permeabilized cells were blocked in 3% serum and incubated with primary antibodies (anti synaptophysin and anti PSD-95 or anti-VGlut2 and anti-KOR) in 0.15% triton overnight at 4^{o}C. The cells were washed, blocked and incubated with Cy-3 and FITC conjugated secondary antibodies in 0.15% triton X-100 for 2 hours at room temperature in the dark. Cells were washed in PBS twice and coverslips were mounted on glass slides using Fluoromount. Confocal images were captured using an Olympus IX 71 spinning disk confocal microscope and Hamamatsu ORCA-ER digital camera.

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Results

Ontogeny of spinal neuron calcium oscillations:

Fluo3-AM loaded spinal neuron cultures were scanned in a Flexstation-II from DIV-1 onwards to study the ontogeny of spontaneous calcium oscillations. Spontaneous calcium oscillations were not detectable in spinal neurons up to DIV-3. Spinal cord cultures exhibited spontaneous calcium oscillations as early as DIV-4 and these calcium transients became more frequent and robust by DIV-6 (Fig. 1A&B). The calcium oscillations persisted through DIV-11. As previously demonstrated for cerebral cortical neurons (Dravid and Murray, 2004), the Ca^{2+} oscillations were highly synchronized in populations of neurons in culture and their mean frequency was 1.88 ± 0.01/min. The amplitude of the oscillations was somewhat variable among different cultures with basal amplitudes ranging between 3,000 and 8,000 fluorescence units. The synchronized nature of Ca^{2+} oscillations suggested that network activity played a key role in the generation of the transients. In order to examine the relationship between spontaneous calcium oscillations and synapse formation, we investigated the ontogeny of PSD-95 expression in spinal neuron cultures. We also examined the formation of synapses in spinal neurons using synaptophysin as a presynaptic marker and PSD-95 as a postsynaptic marker. PSD-95 is a multi-domain post-synaptic density protein that clusters glutamate receptors and associated signaling complexes and hence PSD-95 content is a primary determinant of the size and strength of synapses. Western blotting experiments with cell lysates collected from DIV1 to DIV-9 spinal cord neurons showed that the ontogeny for PSD-95 expression paralleled that of spontaneous calcium oscillations (Fig. 1C). PSD-95 was not detectable on DIV-1 or DIV-2; it appeared on DIV-3 and its expression continued to increase from DIV-4 to DIV-9 (Fig. 1C). In addition to the expression

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of PSD-95, using a double immunofluorescence technique we documented the development of synapse formation in spinal neuron cultures. Synapses were detected as yellow puncta indicating the colocalization of PSD-95 and synaptophysin in DIV-8 spinal neurons (Fig. 1D). These data are consistent with the hypothesis that spontaneous calcium oscillations require the development of neuronal connectivity and formation of functional synapses. Given that the spontaneous calcium oscillations became robust by DIV-6, DIV-6 through DIV-8 spinal cultures were employed for further studies.

Dyn A (1-13) induced suppression of spontaneous calcium oscillations in spinal neurons is opioid receptor mediated:

Dyn A (1-13) is a fragment of the full-length endogenous peptide dyn A (1-17) that acts as a kappa-selective ligand. Spinal cord neurons were treated with either Locke's buffer (control) or varying concentrations (1 nM to 100 μM) of dyn A (1-13) to examine its effects on spontaneous calcium oscillations. Dyn A (1-13) produced a concentration-dependent suppression of spontaneous calcium oscillations in spinal neurons (Fig. 2A). Nonlinear regression analysis of dyn A (1-13) concentration-response data yielded an IC_{50} of 28 nM (7-160 nM, 95% CI) (Fig.2B). To further evaluate the pharmacologic signature of the receptor mediating dynorphin-induced suppression of calcium oscillations, we compared the effects dyn A (1-17) and dyn A (2-17) on calcium oscillations (Fig. 3). Inasmuch as the first amino acid (tyrosine) is essential for imparting opioid receptor activity (Chavkin and Goldestein, 1981), the truncated dynorphin analog dyn A (2-17) is inactive at opioid receptors. A 1 μM concentration of dyn A (1-17) produced an almost complete suppression of calcium oscillations whereas 1 μM dyn A (2-17) was without effect (Fig.3). These data are therefore consistent with an opioid receptor mediation of the dyn A (1-13)-induced suppression of calcium oscillations in spinal neurons.

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To further establish the identity of the opioid receptor involved in the effect of dyn A (1-13) on spontaneous calcium oscillations, we tested the nonselective opioid antagonist naloxone (10 μM) against dyn A (1-13) (300 nM) challenge (Fig.4). Naloxone by itself had no effect on spontaneous calcium oscillations (Fig. 4B); however this pretreatment completely abolished the ability of dyn A (1-13) to suppress calcium oscillations (Fig.4C). The calcium oscillation frequency in the naloxone alone pretreated neurons did not differ significantly from the control level (Fig.4D). The naloxone antagonism of dyn A (1-13)-mediated suppression of calcium oscillations provided additional evidence for opioid receptor involvement in this effect.

Dyn A (1-13) induced suppression of spinal neuron calcium oscillations is mediated by kappa opioid receptors:

Next, we assessed the opioid receptor subtype involved in dyn A (1-13)-induced suppression of spinal neuron calcium oscillations. Inasmuch as dyn A (1-13) displays substantial affinity for mu and delta opioid receptors (Arttamangkul et al., 1995), we tested selective ligands for mu (DAMGO), delta (DPDPE) and kappa (U69593) receptors (Fig. 5B, C & D). At a concentration of 10 μM DAMGO and DPDPE were without effect (Fig. 5 B & C). In contrast, U69593 (300 nM) was able to completely suppress spinal neuron calcium oscillations (Fig.5D). An evaluation of the concentration dependence of U69593 suppression of calcium transients revealed an IC_{50} value of 38 nM (10-135 nM, 95% C.I) (Fig. 5F). These findings ruled out the possibility that the observed suppression of calcium oscillations produced by dyn A (1-13) involved either mu or delta opioid receptors. To confirm the kappa opioid receptor involvement in the actions of dyn A (1-13), we used the specific small molecule antagonist of kappa opioid receptor, nor-BNI (Fig. 6 A, C & D). Initially, nor-BNI was tested at a 3 μM concentration for its ability to block the suppression of calcium oscillations induced by 300 nM dyn A (1-13). Pretreatment with nor-BNI alone did not influence spinal neuron

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calcium oscillations; however, this concentration of nor-BNI abrogated the inhibitory response to dyn A (1-13). Next we generated a Schild plot derived from dyn A (1-13) concentration-response experiments performed in the absence and presence of a series of nor-BNI concentrations. The resultant Schild regression depicted in Figure 6D revealed a nor-BNI pA_2 value of 10.3±0.12, which is in close agreement with that of a previous study (Birch et al., 1987).

In addition to the small molecule antagonist nor-BNI, we also tested a selective peptide antagonist of kappa opioid receptors, zyklophin (Aldrich and McLaughlin, 2009), which is [N-benzylTyr^{1},(cyclo D-asp^{5},Dap^{8})] Dyn A-(1-11) amide (Fig. 6B). A 3 μM concentration of zyklophin completely eliminated the ability of dyn A (1-13) (100 nM) to suppress calcium oscillations. Together these observations underscore the involvement of kappa opioid receptors in the regulation of spontaneous calcium oscillations in spinal neurons.

Spontaneous spinal neuron calcium oscillations are dependent on propagation of action potentials and N-type calcium channels:

In order to determine whether spontaneous spinal neuron calcium oscillations were action potential dependent, we used tetrodotoxin (TTX, 1 μM) a voltage-gated sodium channel blocker. In agreement with an earlier report using cerebrocortical neurons (Dravid and Murray, 2004), TTX caused a complete elimination of the spontaneous calcium oscillations indicating that they are dependent on action potentials generated by activation of voltage-gated sodium channels (Fig. 7A). Action potential triggered neurotransmitter release involves both synchronous (phasic) and asynchronous components at select nerve terminals. Asynchronous release can complement phasic release and may contribute to postsynaptic responses under certain conditions (Hefft and Jonas, 2005; Iremonger and Bains, 2007). Asynchronus release from presynaptic nerve endings relies heavily on

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Ca^{2+} influx through N-type Ca^{2+} channels. The N-type Ca^{2+} channel antagonist ω-conotoxin at a 300 nM concentration inhibited spontaneous calcium oscillations (Fig. 7B), while neither the L-type voltage gated calcium channel blocker (L-type VGCC), nifedipine (1 μM) (Fig. 7G), nor the P/Q type calcium channel blocker, ω-agatoxin (100 nM), (data not shown) affected spontaneous calcium transients. The suppression of calcium oscillations by ω-conotoxin was concentration-dependent with an IC_{50} of 120 nM (7 nM- 2 μM, 95% CI) (data not shown). These data indicate that spontaneous calcium oscillations in spinal neurons are dependent on action potential propagation and Ca^{2+} influx through N-type calcium channels.

Spontaneous spinal neuron calcium oscillations are dependent on glutamatergic neurotransmission:

As the principal excitatory neurotransmitter in the central nervous system, we next sought to determine whether spontaneous calcium oscillations were driven by glutamatergic neurotransmission in spinal neurons. To assess the role of glutamate receptors we used the noncompetitive antagonist of NMDA receptors, MK-801 (3 μM), and the AMPA receptor antagonist, NBQX (1 μM) (Fig.7 C&D). Spontaneous calcium oscillations were completely abolished by addition of either of these glutamate receptor antagonists. A similar elimination of calcium transients was produced by the competitive NMDA receptor antagonist, APV (100 μM) (data not shown). The complete suppression of calcium oscillations by the glutamate receptor antagonists is consistent with glutamatergic neurotransmission serving as the primary driver for the spontaneous activity. Bicuculline ($GABA_A$ antagonist) and strychnine (glycine receptor antagonist) increased the amplitude and frequency of calcium oscillations respectively (Fig. 7 E&F). The effect of bicuculline and strychnine on spontaneous calcium oscillations suggests that in DIV-8 spinal neurons, the chloride gradient is similar to that of mature neurons allowing GABA and glycine to exert an inhibitory tone. These data indicate that in DIV-8

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spinal neurons spontaneous calcium oscillations are a manifestation of excitatory neurotransmission driven primarily by glutamate.

Colocalization of KOR and VGlut-2 in spinal cord neurons

In the spinal cord, glutamate is the transmitter in all primary afferents and in most intraspinal neurons that project to the brain (Broman, 1994). There are also glutamatergic interneurons in the spinal cord and antibodies against the vesicular glutamate transporters, VGlut1 and VGlut2, have been demonstrated to label these glutamatergic neurons (Todd et al., 2003). We used an antibody to VGlut2 to quantify the fraction of glutamatergic neurons in DIV-8 embryonic spinal cord cultures. Approximately 60% of the total neuronal population was labeled with the VGlut2 antibody indicating the relative abundance of glutamatergic neurons in this culture system (Fig. 8A). More than 90% of the spinal cord neurons were labeled with anti-KOR and a substantial proportion of the KOR immunolabeled neurons were colocalized with VGlut-2 suggesting the prevalent expression of KORs in glutamatergic neurons. This localization of KOR in embryonic spinal glutamatergic neurons is consistent with a presynaptic modulation of glutamate release.

Subcellular fractionation demonstrates the presynaptic localization of KORs

The degree of enrichment of LP1 and LP2 fractions separated by subcellular fractionation was confirmed by immunoblotting with anti-PSD-95 and anti-synaptophysin antibodies respectively. PSD-95 was enriched in LP1 fraction characteristic of the postsynaptic component and synaptophysin relative abundance was elevated in the LP2 fraction characteristic of the presynaptic fraction. Densitometric analysis of immunoblots from subcellular fractionation showed the relative abundance of KORs in crude synaptic vesicle fraction (LP2) as opposed to the PSD-95 enriched fraction (LP1) (Fig. 8B).

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An additional functional consequence of exposure of spinal neurons to the KOR ligand, U69593, was the suppression of neurite outgrowth (supplementary figure 1). Given the known trophic actions of endogenous glutamate (Mattson, 2008), we suspect that the suppression of neurite extension involves kappa opioid receptor inhibition of glutamatergic transmission. These preliminary data warrant further investigation to explore the role of kappa opioids in neurite outgrowth and development.

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Discussion

We have demonstrated that mouse embryonic spinal cord neurons in culture exhibit spontaneous calcium oscillations. These spontaneous calcium oscillations are driven primarily by glutamatergic neurotransmission. An interesting feature of our results is that kappa opioid receptors exert an inhibitory influence on spontaneous calcium oscillations in spinal cord neurons. We found that these spontaneous calcium oscillations were inhibited by exogenously applied kappa agonists (dyn A(1-13) and U69593). Opioid regulation of spontaneous calcium oscillations has been previously reported, but with discordant results. Spontaneous calcium oscillations exhibited by organotypic cultures of embryonic rat hippocampi were shown to be enhanced by the application of the mu opioid agonist DAMGO (Przewlocki et al., 1999), whereas DAMGO was shown to inhibit this phenomenon in GH_3 cells expressing only μ-opioid receptors (GH_3MOR) (Charles et al., 2003). However, in GH_3MORDOR cells (expressing μ and δ- opioid receptors in a 1:11 ratio) DAMGO's response changed from inhibitory to excitatory, and this was attributed to the differential coupling of μ-opioid receptors from G_i to G_q due to preferential δ-opioid receptor occupancy of G_i proteins (Charles et al., 2003). In a previous report, the δ-selective ligand DPDPE was shown to inhibit spontaneous calcium transients in cells expressing μ and δ-opioid receptors (Piros et al., 2000). Here we provide compelling evidence to demonstrate that the opioid receptor subtype involved in suppression of spontaneous calcium oscillations in spinal neurons is the kappa receptor. Spinal neuron spontaneous calcium oscillations were not affected by DAMGO or DPDPE. The inhibitory effect of kappa agonists on calcium oscillations was moreover completely blocked by the selective kappa receptor antagonists nor-BNI and zyklophin.

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Both KOR and MOR have been reported to be present from early fetal life in the rat and human brain and spinal cord (Xia and Haddad, 1991). Our results indicate that KORs become functional during embryonic development of spinal cord. This is in agreement with a report that KOR expression in spinal cord appears in the ventral cord at E15.5 and extends to the dorsal cord by E17.5 (Zhu et al., 1998). Given the evidence supporting the role of endogenous opioids in early development of the CNS, opioid receptors may be required for maturation of synapses and fine tuning of spinal circuitry (Hauser et al., 1987). Although no tonic kappa receptor-mediated inhibition of calcium oscillations was observed at this developmental stage in vitro, the possibility of endogenous dynorphins regulating oscillations in vivo cannot be ruled out. Several lines of evidence indicate that spontaneous calcium oscillations exhibited by DIV 6-8 embryonic spinal cord neurons were generated by network synaptic activity and are dependent on excitatory neurotransmission. TTX inhibited spontaneous calcium oscillations suggesting their dependence on action potentials. Calcium oscillations were also blocked by the NMDA receptor antagonist, MK-801, and the AMPA receptor antagonist, NBQX, suggesting a dependence on glutamatergic neurotransmission. These findings are consistent with previous reports in cultured cerebrocortical neurons (Murphy et al., 1992; Dravid and Murray, 2004), organotypic hippocampal slices (Przewlocki et al., 1999) and organotypic spinal cord slices (Sibilla et al., 2009). The observation that ω-conotoxin inhibited spontaneous calcium oscillations suggests that this phenomenon was also dependent on calcium influx through N-type calcium channels. Synapses between primary sensory afferents and intrinsic dorsal horn neurons are primarily glutamatergic (Jessell et al., 1986), and N-type calcium channels contribute to glutamate release at these synapses. These findings reinforce the dependence of spontaneous calcium oscillations on glutamatergic transmission. By blocking N-type calcium channels, ω-conotoxin may inhibit glutamate release and suppress spontaneous calcium oscillations. In contrast, L-type voltage gated

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calcium channels did not appear to play a role in the generation of spontaneous calcium oscillations. This finding is consistent with other studies monitoring spontaneous calcium oscillations in the presence of physiological concentrations of Mg^{2+} (Dravid and Murray, 2004). Previous reports examining calcium oscillations in organotypic spinal cord slices have also demonstrated that neither L- nor T-type channels were responsible for calcium oscillations (Sibilla et al., 2009). Our results are consistent with these earlier findings and point to N-type calcium channel involvement in the generation of spontaneous calcium oscillation activity triggered by the release of glutamate.

Opioids are known to depress synaptic neurotransmission either by inhibition of presynaptic calcium channels (MacDonald and Werz, 1986) or by activation of postsynaptic potassium channels (North et al., 1987; Wimpey and Chavkin, 1991), thereby hyperpolarizing neurons. A recent report, however, ruled out the involvement of either calcium or potassium channels in kappa receptor-mediated inhibition of dopamine release (Ford et al., 2007). Another study has moreover proposed that, once activated, kappa opioid receptors directly modulate presynaptic release machinery to reduce vesicular glutamate release (Iremonger and Bains, 2009). Consistent with the presynaptic mechanism of action reported for KORs, we found that KOR expression was enriched in spinal glutamatergic neurons. From both colocalization and subcellular fractionation studies, it is evident that KORs are present in spinal glutamatergic neurons with a relative abundance in the presynaptic compartment. This localization of KORs in embryonic spinal neurons is consistent with the ability of KOR ligands to modulate neurotransmitter release via a presynaptic mechanism of action (Rusin et al., 1997; Ford et al., 2007, Iremonger and Bains, 2009).

A compelling body of evidence has implicated the inhibitory effects of exogenously applied dynorphin on excitatory neurotransmission (Castillo et al., 1996; Simmons and Chavkin, 1996; Iremonger and Bains, 2009). Given the requirement of glutamatergic neurotransmission for

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spontaneous calcium oscillations, and the evidence that KORs are localized presynaptically in spinal glutamatergic neurons, a kappa opioid receptor inhibition of excitatory synaptic transmission represents the most parsimonious explanation for KOR regulation of spinal neuron calcium oscillations.

Significance

An increased production of the opioid peptide dynorphin has been reported following spinal cord trauma, and this has been implicated in neuropathic pain and pathophysiology associated with spinal injury (reviewed in Hauser et al., 2005). Recovery from such injuries is thought to recapitulate ontogeny and is facilitated by unmasking of subthreshold inputs to the surrounding healthy tissue (reviewed in Murphy and Corbett, 2009). This has been proposed to result from decreased inhibitory transmission, increased glutamate transmission and increased spontaneous activity; thus, promoting rewiring of lost synaptic connections. Accumulation of dynorphin in a spinal trauma site could therefore produce an inhibition of spontaneous activity and impede rewiring of synaptic connections.

Acknowledgements

We wish to thank Bridget Leuschen for her support in preparation of spinal cord neuronal cultures.

Authorship contributions:

Participated in research design: Kelamangalath, George, Aldrich and Murray.

Conducted experiments: Kelamangalath, Dravid and George.

Performed data analysis: Kelamangalath, Dravid and George.

Wrote or contributed to writing of the manuscript: Kelamangalath, Murray and Aldrich.

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Footnotes:
This work was supported by National Institute of Health Grant [RO1 DA 023924].

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Figure legends

Figure 1. Ontogeny of spinal neuron calcium oscillations. *A*. Spontaneous calcium oscillations emerged on DIV-4 and became robust by DIV-6. Traces shown are from a representative experiment repeated 6 times. *B*. Summary histogram representing the ontogeny of spinal neuron calcium oscillations. *C*. Ontogeny of spinal neuron calcium oscillations paralleled the ontogeny of PSD-95 expression. Data shown are from a representative experiment repeated 3 times. *D*. Confocal images show immnolabeling for PSD-95 (red), synaptophysin (green) and the regions of colocalization of PSD-95 and synaptophysin as puncta (yellow) to indicate synapses (scale bar, 10 μm) in DIV-8 spinal neurons.

Figure 2. Dynorphin A (1-13) suppresses spinal neuron calcium oscillations. *A*. Dyn A (1-13) suppressed spontaneous calcium oscillations in a concentration-dependent manner. Arrow indicates the time of addition of drug. *B*. Nonlinear regression analysis of the concentration-response data for dyn (1-13) suppression of calcium oscillations yielded an IC_{50} of 28 nM (95% CI, 7-160 nM). Each point represents the mean ± SEM (n=8) of oscillations following addition of dyn A (1-13). Experiment was repeated 8 times in independent cultures.

Figure 3. Dyn A (1-13) induced suppression of calcium oscillations is opioid receptor mediated. *A & B*. 1 μM Dyn A (1-17) produced a suppression of calcium oscillations whereas 1 μM Dyn A (2-17) did not affect calcium oscillations. *C*. Summary histogram of data represent the mean ± SEM (n=4) of calcium oscillations (for 300 seconds) before and after compound addition. *p<0.01, paired 't' test between the average number of oscillations before and after the addition of Dyn A (1-17) 1 μM and Dyn A (2-17) 1 μM.

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Figure 4. Dyn A (1-13) induced suppression of calcium oscillations is opioid receptor mediated. *A.* Dyn A (1-13) (300 nM) produced a suppression of spinal neuron calcium oscillations. *B.* The nonselective opioid antagonist naloxone (10 μM) alone had no effect on spontaneous calcium oscillations. *C.* Pretreatment with naloxone (10 μM) eliminated dyn A (1-13)-induced suppression of calcium oscillations. *D.* Summary histogram of data represent the mean ± SEM (n=5) of calcium oscillations (for 300 seconds) following compound addition. *p<0.01, unpaired 't' test between dyn A (1-13) 300 nM and dyn A (1-13) + naloxone (10 μM).

Figure 5. Kappa, but not mu or delta, opioid agonists inhibit spinal neuron spontaneous calcium oscillations .*B.* DAMGO, (mu agonist) and *C.* DPDPE (delta agonist) did not affect spontaneous calcium oscillations. *D*. U69593 (kappa agonist) induced suppression of calcium oscillations. *E*. Summary histogram of the data to show that activation of KOR mediates suppression of spinal neuron calcium oscillations. Data represent the mean ± SEM (n=5) of calcium oscillations before (empty bars) and after compound addition (filled bars). Only the KOR agonist, U69593, suppressed calcium oscillations. *p<0.05, paired 't' test between pre- and post-addition oscillations for U69593. DAMGO and DPDPE did not affect calcium oscillations. *F*. Nonlinear regression analysis of the concentration-response data for U69593-induced suppression of calcium oscillations yielded an IC_{50} of 38 nM.

Figure 6. Summary of kappa receptor antagonist block of dynA (1-13) inhibition of calcium oscillations *(A, B).*and Schild analysis of nor-BNI antagonism *(C, D*). Data in A and B are the mean ± SEM of calcium oscillations remaining after addition of respective compounds. Pretreatment with nor-BNI or zyklophin eliminated dynorphin-induced suppression of calcium oscillations [n=5 for nor-BNI experiment and n=6 for zyklophin experiment; *p<0.01, unpaired 't' test between dyn A (1-13) and nor-BNI + dyn A (1-13) 300 nM; dyn A (1-13) and zyklophin

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+ dyn A (1-13) 100 nM]. *C*. Dose-response curves for dyn A (1-13) in the absence and presence of fixed concentrations of nor-BNI.

D. Schild regression for nor-BNI antagonism of dyn A (1-13). Each data point represents the mean of 3 different experiments.

Figure 7. Spontaneous calcium oscillations are dependent on action potentials *(A)* N-type calcium channels *(B)* driven by glutamatergic transmission *(C&D)*, whereas the inhibitory influence of GABA and glycine are revealed with the antagonists bicuculline and strychnine respectively *(E&F)*. Spontaneous calcium oscillations are not dependent on L-type calcium channels (*G*). Summary histogram of these data *(H)*. Data are from a representative experiment performed in quadruplicate. Data represent the mean ± SEM (n=4) of calcium oscillations before (empty bars) and after compound addition (filled bars). Addition of TTX, MK-801, ω-conotoxin and NBQX significantly suppressed the occurrence of calcium oscillations, while addition of strychnine significantly increased the frequency of calcium oscillations. *$p<0.05$, paired 't' test between pre- and post-addition oscillations for each compound.

Figure 8. KORs are primarily localized presynaptically in embryonic spinal neurons. *A*. A large proportion of KORs are colocalized with VGlut2 in spinal neurons demonstrating the localization of KORs in glutamatergic neurons.(a) Confocal images show phase, VGlut2 (red) and KOR (green) immunolabeling separately with regions of colocalization that appear as yellow puncta (scale bar, 10 μm). (b) Boxed region in (a) shown in higher magnification (scale bar, 5 μm) separately and overlaid. *B*. Subcellular fractionation of embryonic spinal neurons in culture shows the relative abundance of KORs in the crude synaptic vesicle fraction (LP2 fraction). Data are from a representative experiment performed.

Figure 1

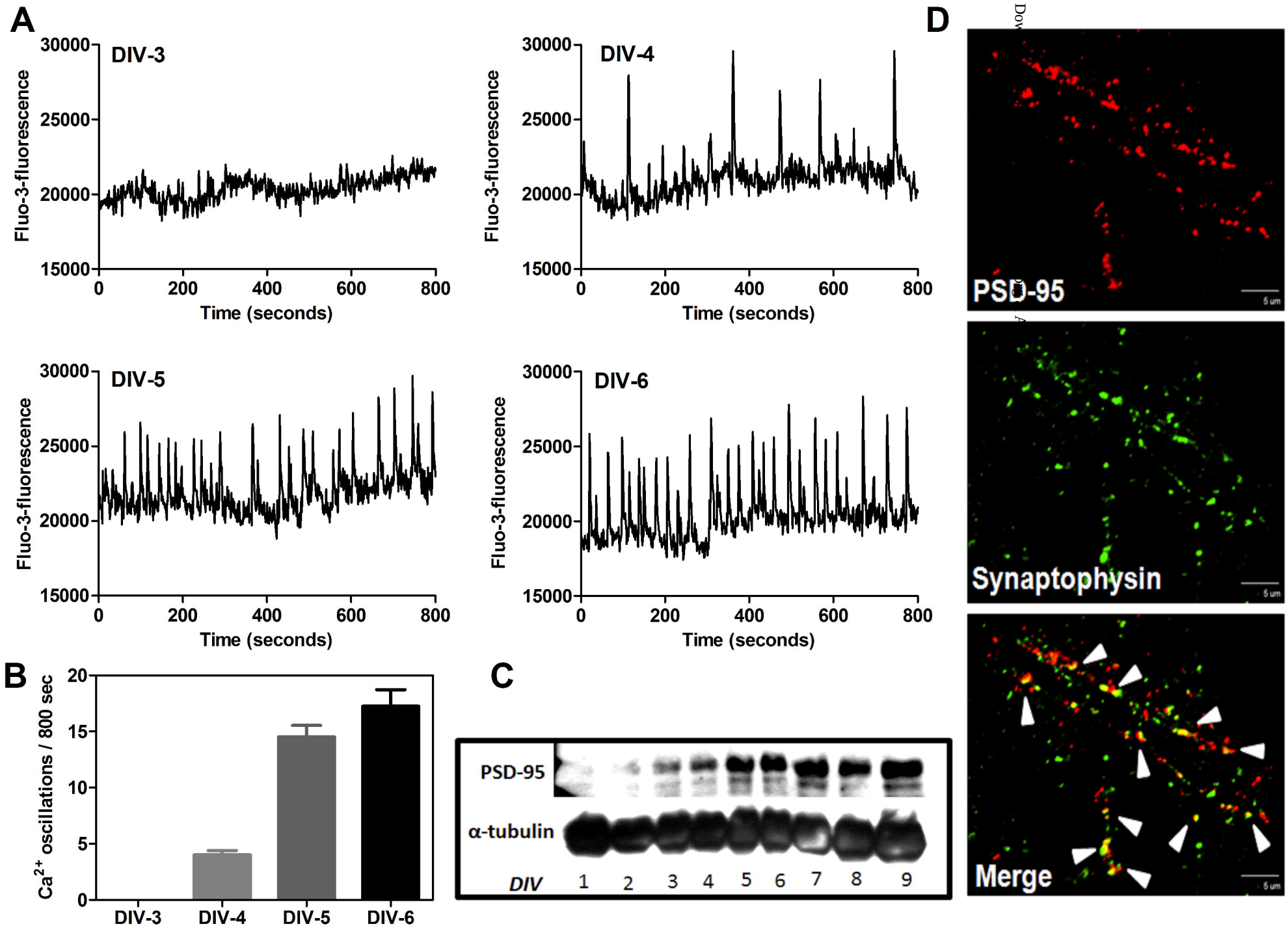

A
DIV-3
DIV-4
DIV-5
DIV-6
Fluo-3-fluorescence
30000
25000
20000
15000
0
200
400
600
800
Time (seconds)
B
Ca2+ oscillations / 800 sec
20
15
10
5
0
DIV-3
DIV-4
DIV-5
DIV-6
C
PSD-95
α-tubulin
DIV
1
2
3
4
5
6
7
8
9
D
PSD-95
Synaptophysin
Merge
5 um

Figure 2

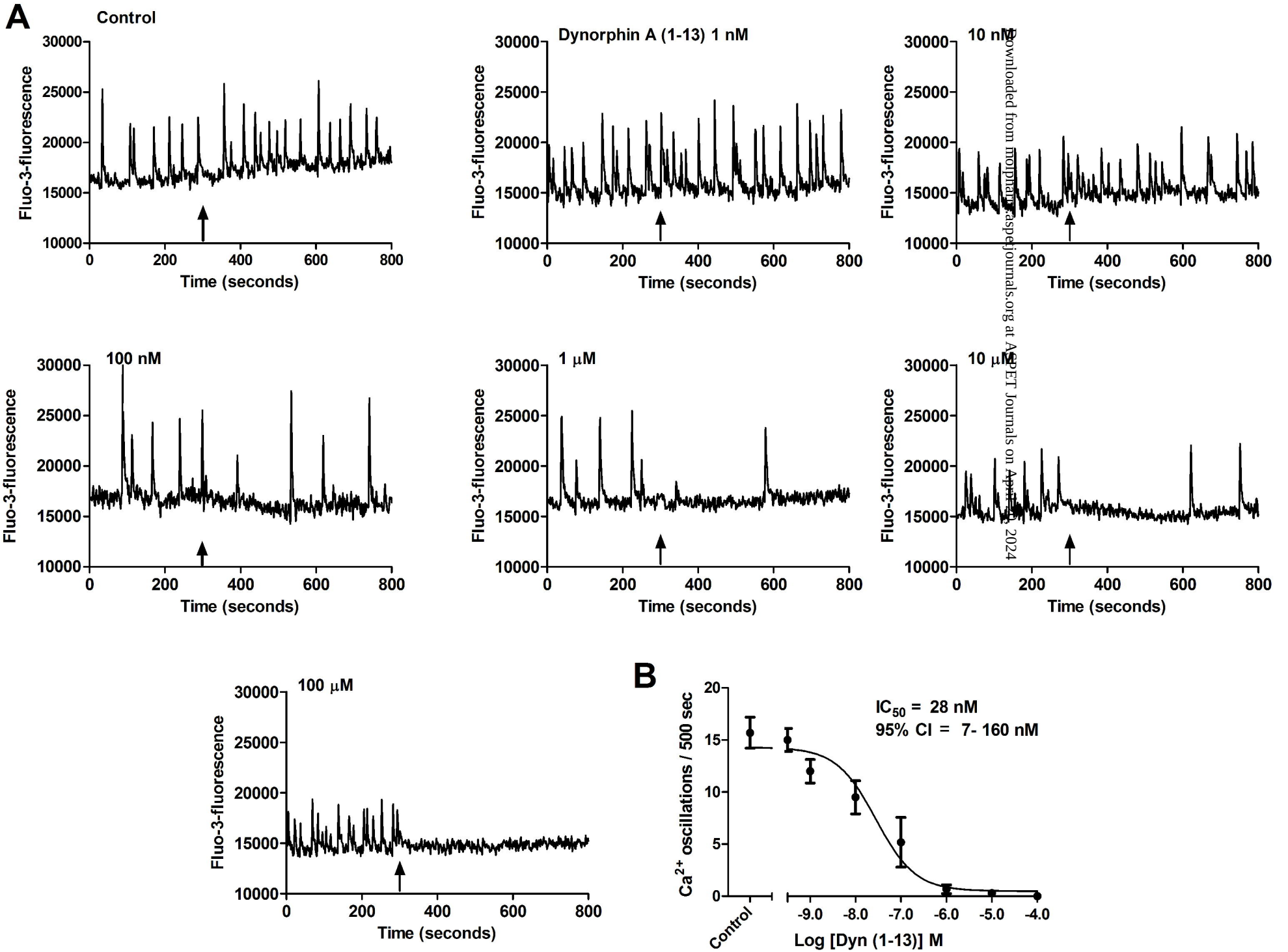


Figure 3

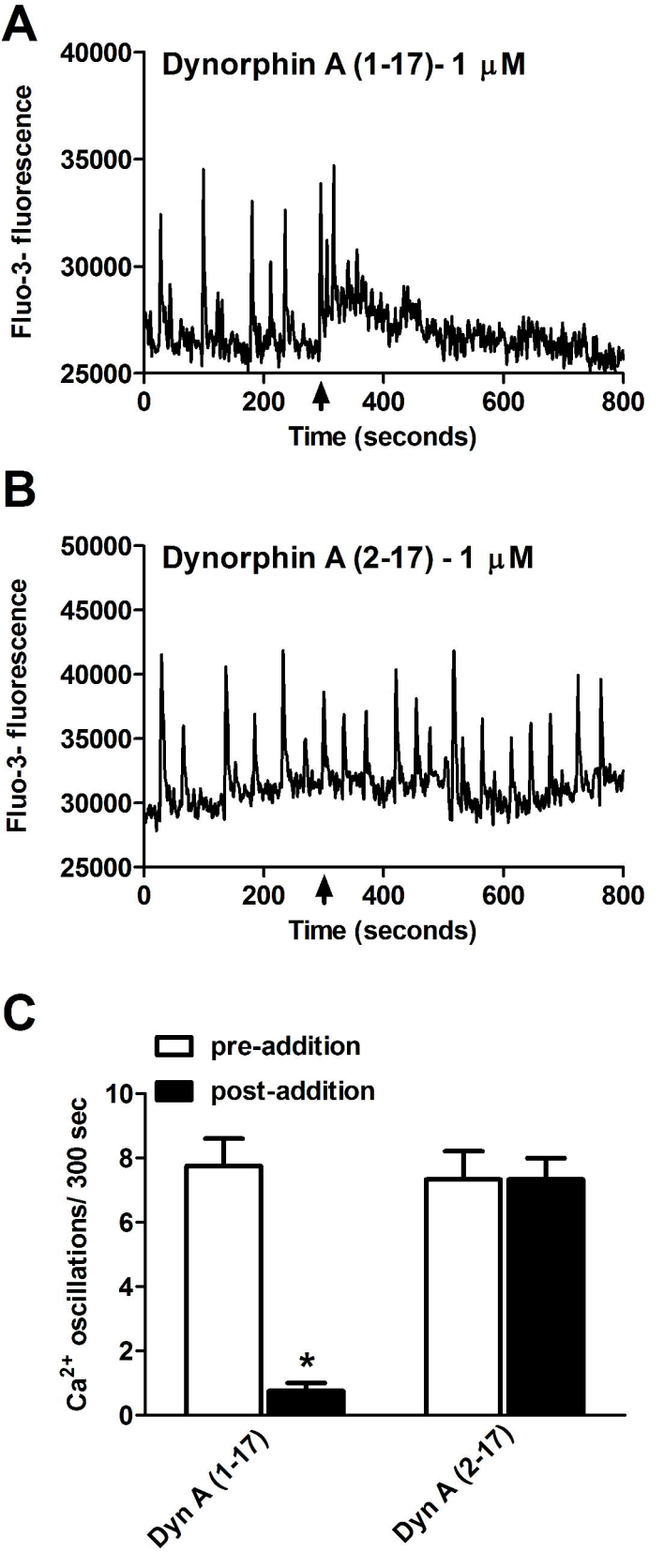

A
Dynorphin A (1-17)- 1 μM
Fluo-3- fluorescence
40000
35000
30000
25000
0
200
400
600
800
Time (seconds)
B
Dynorphin A (2-17) - 1 μM
Fluo-3- fluorescence
50000
45000
40000
35000
30000
25000
0
200
400
600
800
Time (seconds)
C
pre-addition
post-addition
Ca2+ oscillations/ 300 sec
10
8
6
4
2
0
*
Dyn A (1-17)
Dyn A (2-17)

Figure 4

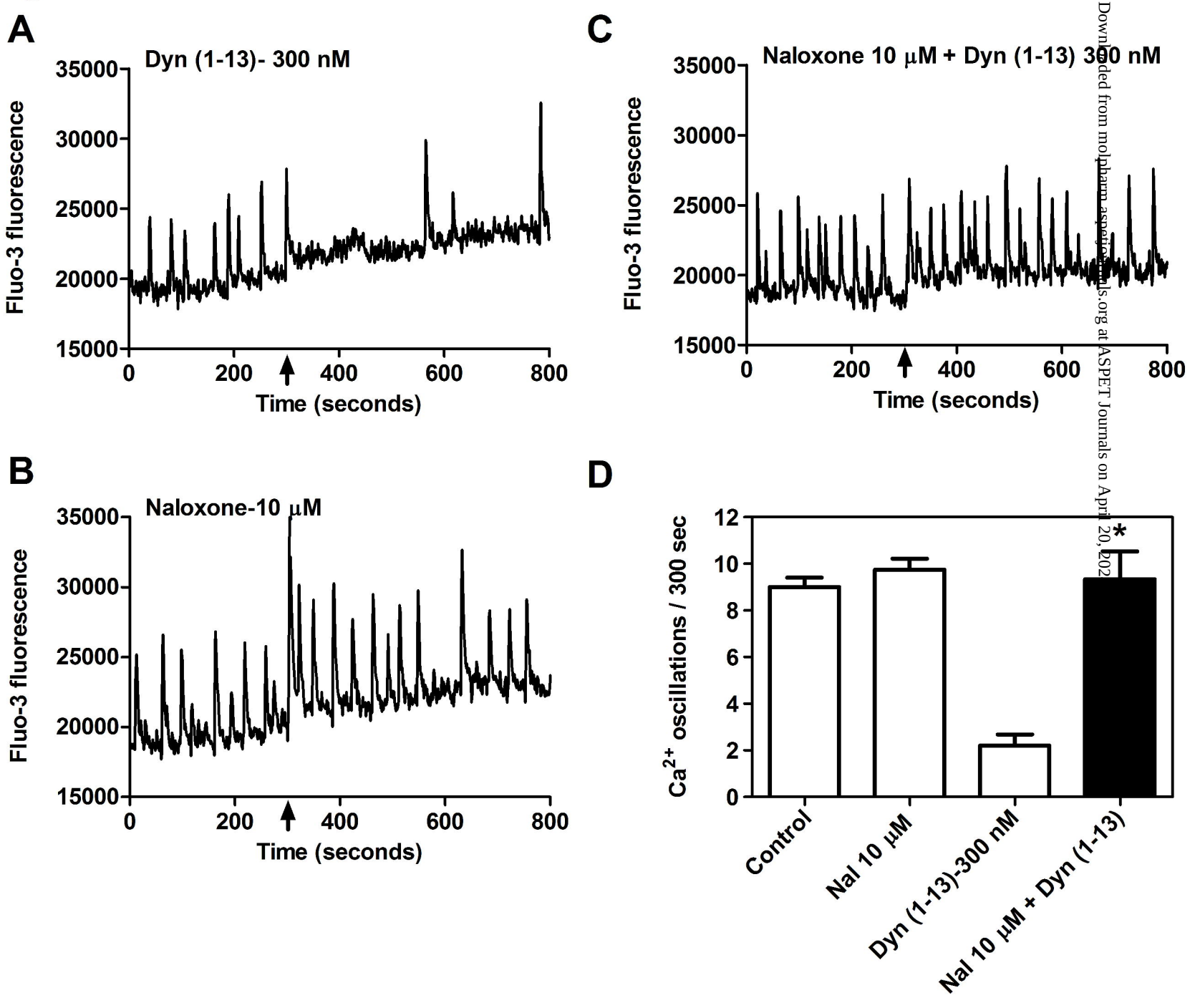


Figure 5

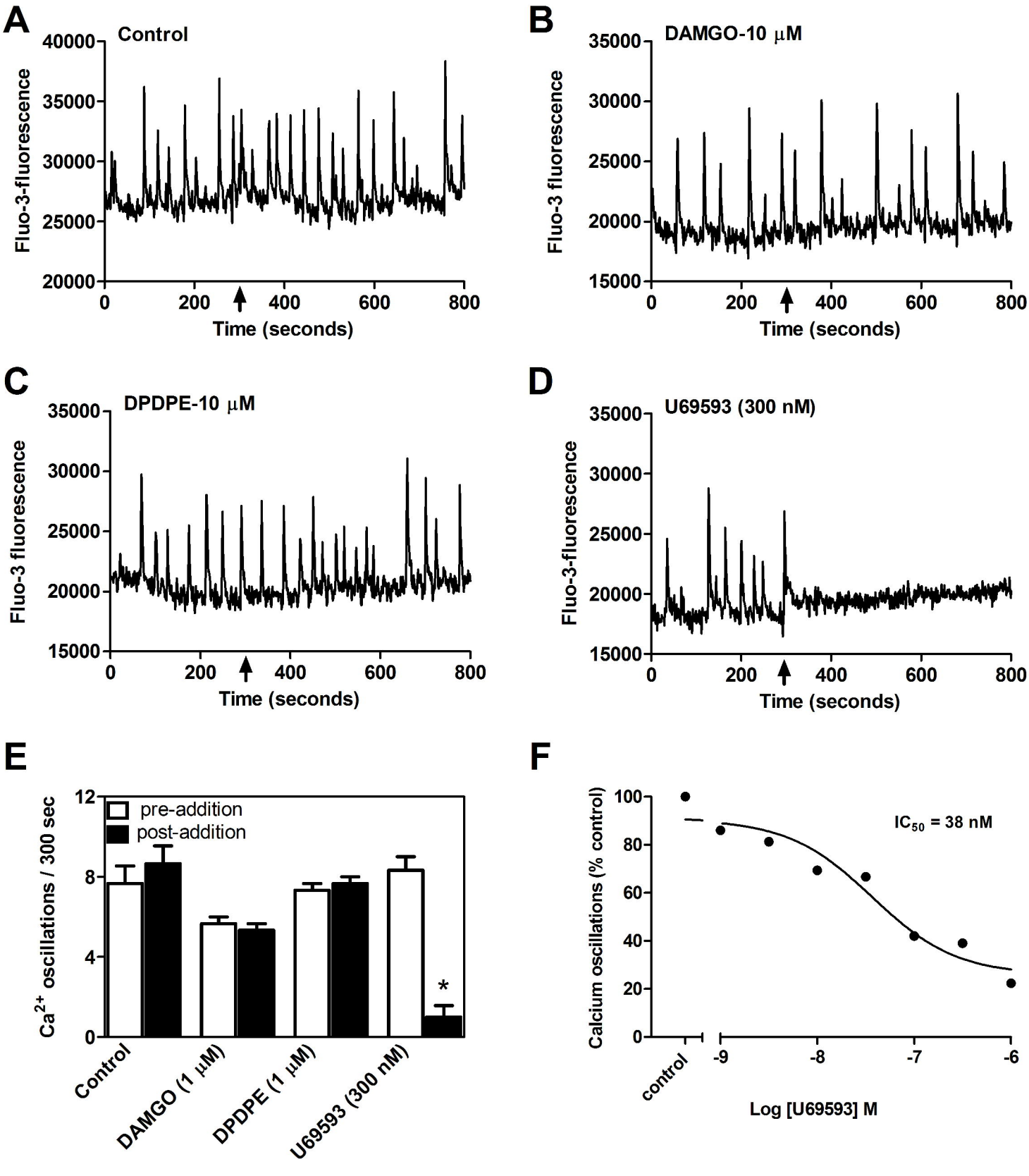
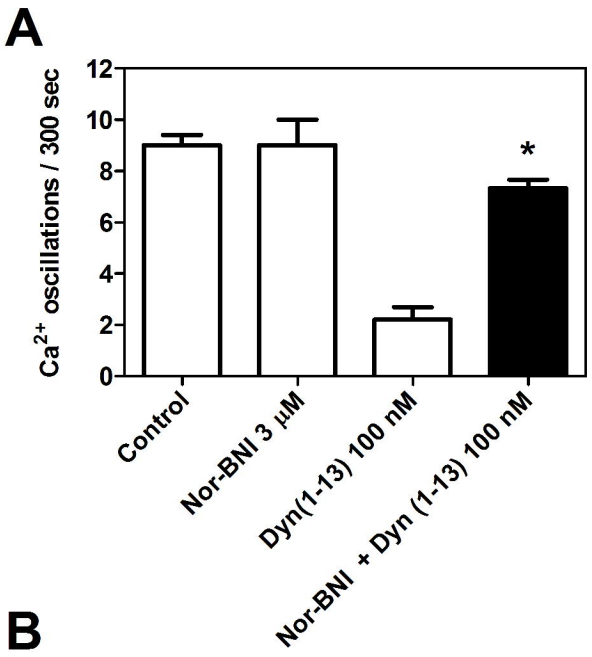
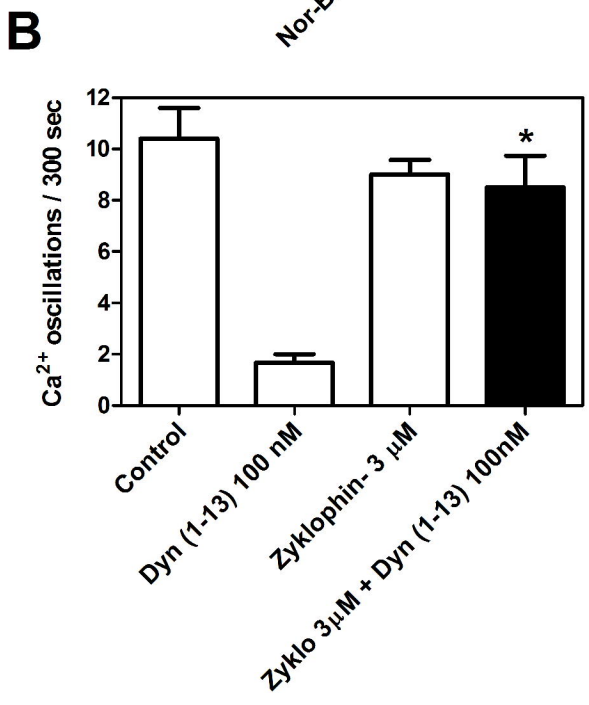
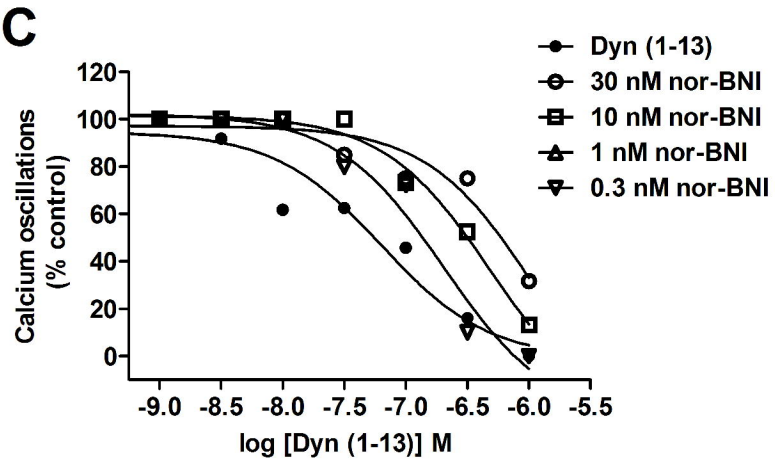

A
Control
Fluo-3-fluorescence
40000
35000
30000
25000
20000
0
200
400
600
800
Time (seconds)
B
DAMGO-10 μM
Fluo-3 fluorescence
35000
30000
25000
20000
15000
0
200
400
600
800
Time (seconds)
C
DPDPE-10 μM
Fluo-3 fluorescence
35000
30000
25000
20000
15000
0
200
400
600
800
Time (seconds)
D
U69593 (300 nM)
Fluo-3-fluorescence
35000
30000
25000
20000
15000
0
200
400
600
800
Time (seconds)
E
pre-addition
post-addition
Ca2+ oscillations / 300 sec
12
8
4
0
*
Control
DAMGO (1 μM)
DPDPE (1 μM)
U69593 (300 nM)
F
Calcium oscillations (% control)
100
80
60
40
20
0
IC50 = 38 nM
control
-9
-8
-7
-6
Log [U69593] M

Figure 6


A
Ca^{2+} oscillations / 300 sec
12
10
8
6
4
2
0
*
Control
Nor-BNI 3 μM
Dyn(1-13) 100 nM
Nor-BNI + Dyn (1-13) 100 nM


B
Ca^{2+} oscillations / 300 sec
12
10
8
6
4
2
0
*
Control
Dyn (1-13) 100 nM
Zyklophin- 3 μM
Zyklo 3μM + Dyn (1-13) 100nM


C
Dyn (1-13)
30 nM nor-BNI
10 nM nor-BNI
1 nM nor-BNI
0.3 nM nor-BNI
Calcium oscillations (% control)
120
100
80
60
40
20
0
-9.0 -8.5 -8.0 -7.5 -7.0 -6.5 -6.0 -5.5
log [Dyn (1-13)] M

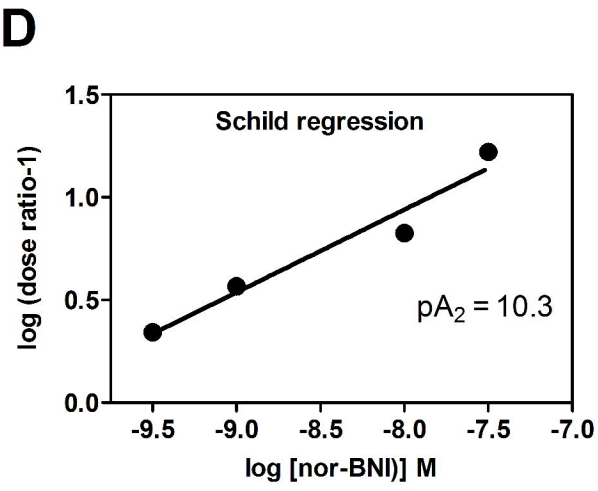

D
Schild regression
log (dose ratio-1)
1.5
1.0
0.5
0.0
pA_2 = 10.3
-9.5 -9.0 -8.5 -8.0 -7.5 -7.0
log [nor-BNI)] M

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Figure 7

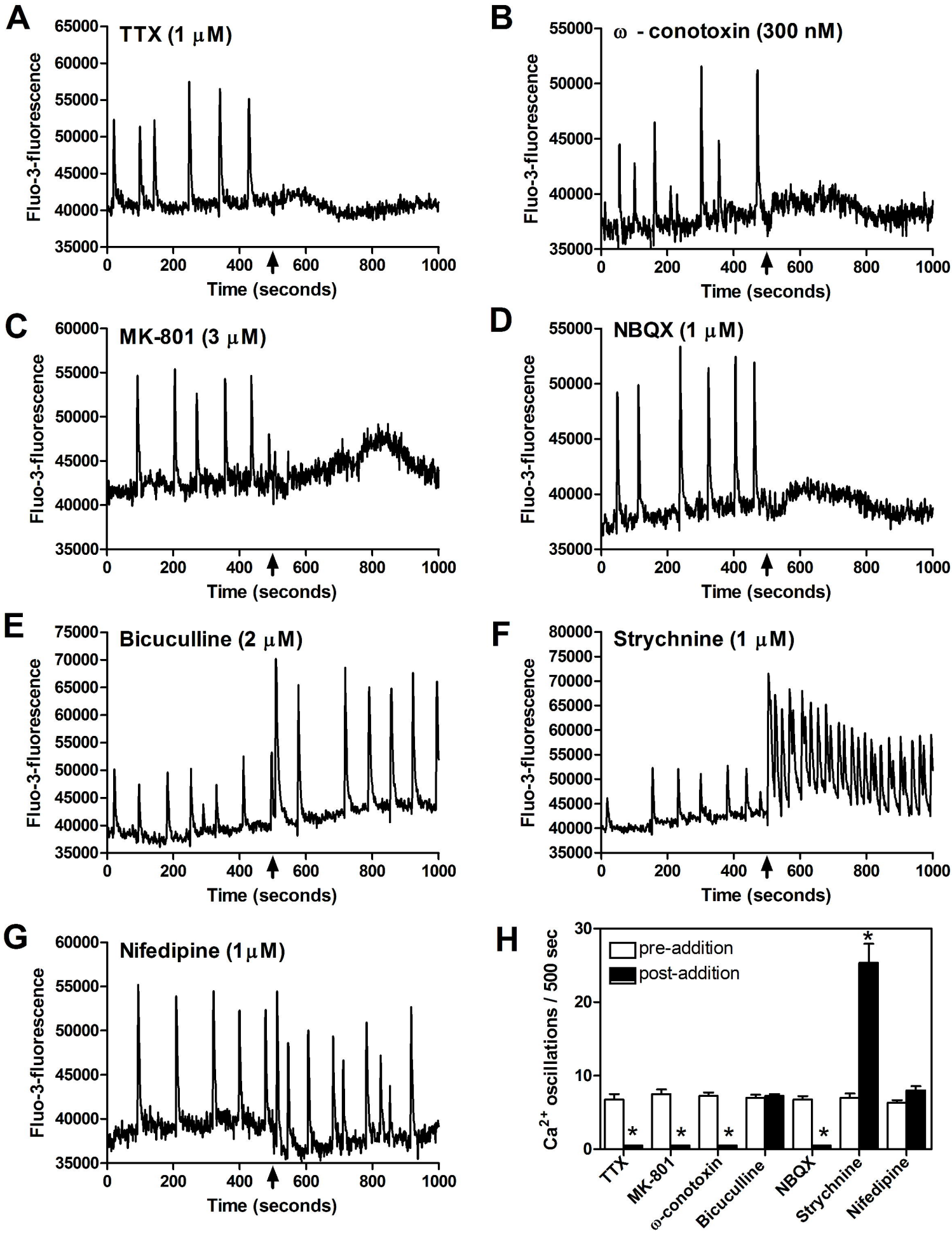
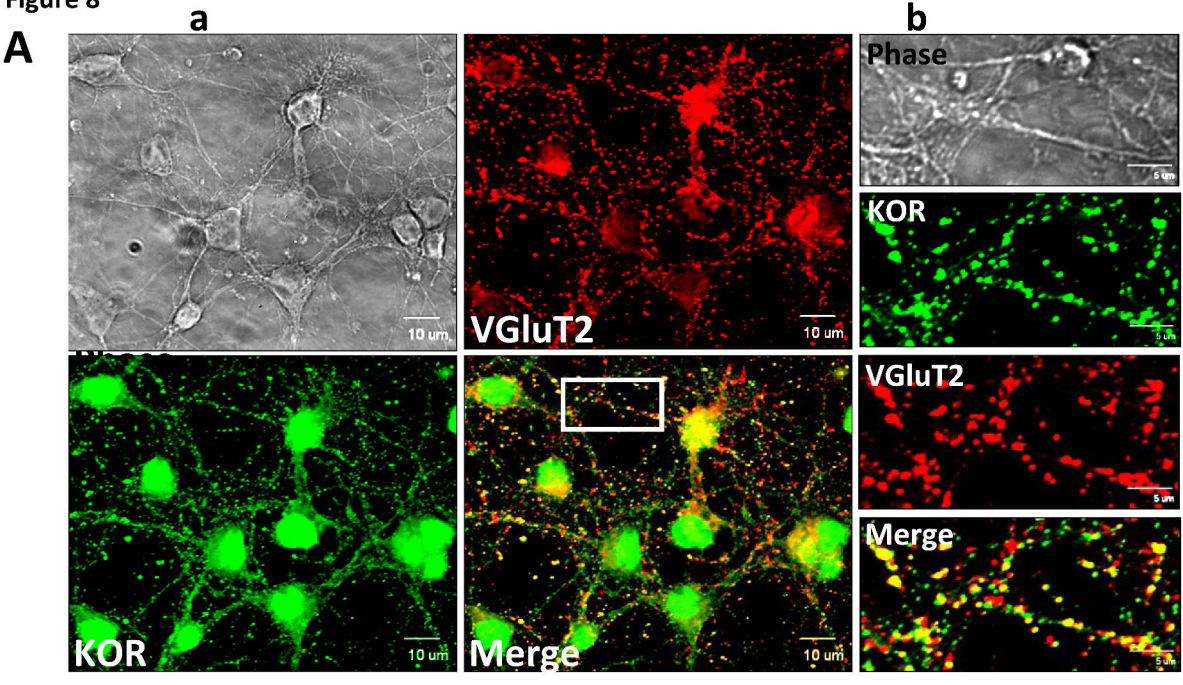


Figure 8


A
a
b
10 um
VGluT2
Phase
KOR
VGluT2
Merge
KOR
Merge
B
Initial homogenate
LP1
LP2
KOR-1
58 kDa
PSD-95
95 kDa
Synaptophysin
38 kDa
α-tubulin
52 kDa