Analysis of GPCR/Ion Channel Interactions

Christophe Altier and Gerald W Zamponi

Department of Physiology and Pharmacology, Hotchkiss brain Institute, University of Calgary, Calgary, T2N 4N1, Canada

ABSTRACT

Voltage gated calcium channels are key regulators of calcium homeostasis in excitable cells. A number of cellular signalling pathways serve to fine tune calcium channel activity, including the activation of G protein coupled receptors. Besides regulating channel activity via second messengers, GPCRs can also physically associate with calcium channels to directly regulate their functions, as well as the trafficking to and from the plasma membrane. Here we provide some methodologies that can be used to examine channel-receptor interactions and cotrafficking. While we focus on voltage gated calcium channels, the techniques described herein are broadly applicable to other types of channels.

Key words: calcium channel, G protein coupled receptor, luminometry, confocal microscopy

Introduction:

Calcium entry via voltage gated calcium channels (VGCCs) mediates a number of downstream effects the include activation of calcium-dependent enzymes, the release of neurotransmitters, cardiac muscle contraction and gene transcription (**1, 2**). Therefore, intracellular calcium levels must be precisely controlled. One such mechanism is the regulation of calcium channel activity by G protein coupled receptors (GPCRs), which upon agonist binding, activate G protein cascades and a plethora of intracellular signalling pathways that then have the propensity to regulate channel function (**3**). Our laboratory was the first to show that certain types of GPCRs, including members of the opioid receptor and dopamine receptor families, can also physically associate with N-type channels to not only regulate channel function, but also to regulate the density of channels in the plasma membrane. The latter is predicted to affect the amount of calcium entering into a given cell (**4-7**). We found that both

dopamine and nociceptin receptors can increase cell surface expression by enhancing channel trafficking to the plasma membrane, and furthermore, to specific subcellular compartments such as dendrites (**5, 8**). Once inserted into the plasma membrane, channel receptor complexes can be internalized into both endosomes and lysosomes upon prolonged agonist exposure (**9**). This has also been shown to be true for GABAB receptors (**10**). Altogether, the formation of channel receptor complexes provides a means for 1) optimizing signalling efficiency, 2) for receptor mediated channel targeting, and 3) for receptor mediated removal of channels from the membrane. The formation of channel receptor complexes is a novel means of regulating calcium channel function and density that can be examined with a variety of experimental approaches. Although there are numerous ways of addressing this issue, including FRET and BRET measurements, we shall focus here on techniques that have been successfully employed by our laboratory. It is worth noting however, that their applicability goes well beyond calcium channels and G protein coupled receptors and can be adapted to any type of ion channel. Moreover, the existence of channel-channel complexes is also beginning to emerge in the literature (**11**).

1. Materials

1.1. *Transfection*

1.1.1. HEK 293T cells are cultured in Dulbecco's Modified Eagle's Medium (DMEM) (invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco). Cells are trypsinized using ethylenediamine tetraacetic acid (EDTA, 1mM) trypsin solution (0.25%) (Gibco).

1.1.2. Calcium Phosphate solution for transfection: 250 mM CaCl2 (10.95g in 200mL H2O).

1.1.3. 2x HEPES buffered saline (HBS): 8g NaCl, 0.2 g Na_2HPO_4-$7H_2O$, 0.5 g HEPES. pH to 7.0 and bring up to 500 ml with distilled water.

1.2. *Immuno-luminometry*

1.2.1. Glass coverslips (12mm, Fischer Scientifics) treated with Poly-L-lysine (0.002% solution in Phosphate Buffered Saline. PBS, Sigma).

1.2.2. Phosphate Buffered Saline (PBS): 10X stock solution: 80.6mM Na2HPO4, 19.4mM KH2PO4, 27mM KCl and 1.37M NaCl in high purity dH_2O (adjust to pH 7.4 with HCl).

1.2.3. Paraformaldhyde: From 16% solution (EMS, Electron Microscopy Sciences), prepare a 4% (w/v) solution in PBS 1X.

1.2.4. White-bottomed microtitre plate (PerkinElmer).

1.2.5. Supersignal ELISA Femto Maximum Sensitivity Substrate (Pierce).

1.3. *Co-immunoprecipiation experiments*

1.3.1. Lysis Buffer: 20mM Tris-HCl (pH 7.5), 0.5% (v/v) triton-X-100, 0.005% sodium deoxycholate, 150mM NaCl, and protease inhibitor cocktail tablet (Roche).

1.3.2. Buffer B: 0.2% NP-40, 10mM Tris-HCl pH 7.5, 0.15M NaCl, 2mM EDTA.

1.3.3. Buffer C: 0.2% NP-40, 10mM Tris-HCl pH 7.5, 0.5M NaCl, 3mM EDTA.

1.3.4. Buffer D: 10mM Tris-HCl, pH 7.5.

1.3.5. 2X sodium dodecyl sulphate (SDS) sample buffer: 130 mM Tris-Cl, pH8.0, 20% (v/v) Glycerol, 4.6% (w/v) SDS, 0.02% Bromophenol blue, 2% DTT.

1.4. *Confocal microscopy*

1.4.1. HEK 293T cells are plated on MatTek dishes (MatTek Corporation, 14mm) before transfection.

1.4.2. Blocking solution: PBS+2% serum.

1.4.3. Hank's Balanced Salt Solution (HBSS): CaCl2.2H2O: 0.185g/L; MgSO4: 0.097g/L; KCl: 0.4g/L; KH2PO4: 0.06g/L; NaHCO3: 0.35g/L; NaCl: 8g/L; Na2HPO4: 0.047g/L; D-Glucose: 1g/L (Sigma).

1.4.4. Antibody solution: HBSS + 2% goat serum.

2. Methods

2.1. *Cell surface immuno-luminometry assays to measure surface expression of ion channels co-expressed with transmembrane receptors.*

2.1.1. Split cells at 60-70% confluence 8hrs before transfection and plate cells on 60 mm dishes. Transfect cells overnight using Calcium Phosphate transfection method, with cDNA encoding the different subunits of Voltage-Gated Calcium Channels: HA-tagged Ca_v-α_1 subunit (+ β + α_2-δ_1, ratio 2:1:1) alone, and in the presence of the receptor. Fourteen hours later, cells are washed with PBS, trypsinized using trypsin-EDTA (0,25% solution) and re-seeded in triplicate onto poly-L-lysine coated coverslips.

2.1.2. Cells are then incubated for 48hrs at 37°C. For each condition, a single replicate (3 coverslips) is treated with receptor agonist diluted in HBSS for 30min at 37°C. All cells are subsequently washed with PBS, and fixed with 4% paraformaldehyde (5min, RT).

2.1.3. Cells are then pre-blocked at RT for 30min in PBS containing 2% goat serum. At this stage, a second replicate (3 coverslips) from each condition is permeablized (7min) by the addition of 0.1% (v/v) triton-X-100 to the pre-blocking solution. Then cells are washed 2 times with PBS.

2.1.4. At that point mix solution A and B of Supersignal ELISA Femto Maximum Sensitivity Substrate (Pierce). Mixing the substrate 2 hrs before final reading will decrease background signal. 200 µl of substrate is added to a 24-well, white-bottomed microtitre plate (PerkinElmer) which is kept at RT in the dark.

2.1.5. Cells are then incubated in α-HA antibody (0.5µg/ml Roche) in PBS + 2% goat serum for 1hr at RT. Then cells are washed 3x10min in PBS+2%serum. The first wash consists of aspirating the solution from each coverslip. For next washes, single coverslips are transferred to a clean 24 well dish (containing PBS + 2% serum).

2.1.6. Cells are incubated in Horseradish Peroxidase HRP-conjugated goat α-rat IgG antibody (1:1000, Jackson ImmunoResearch Laboratories) for 40min at RT. Then cells are washed

4x20min in PBS+2% serum. The first wash consists of aspirating the solution from each coverslip. For next washes, single coverslips are transferred to a clean 24 well dish (containing PBS + 2% serum). One can also keep the coverslips in the same dish and use forceps to lift up the coverslip after each single wash (this way, antibody trapped under the coverslips will be washed).

2.1.7. Single coverslips are rapidly transferred into a 24-well, white-bottomed microtitre plate (PerkinElmer) containing substrate. At this point, the plate is covered with aluminum foil for 1min. The luminometry signal is detected at 492 nm using a Wallac 1420 Multilabel HTS counter (or any sensitive counterplate reader reading luminescence). As the HA-tag on the $Ca_v1.2$ channel construct is extracellular, signal intensity from un-permeabilized cells are used as a measure of $Ca_v1.2$ channel surface expression, and signal intensity from permeabilized cells are used as a measure of total cellular channel expression. The ratio of cell surface to total cellular channel expression allows for comparisons between different conditions and between similar conditions assayed from different batches of cells on different days (see Fig. 1).

2.2. *Co-immunoprecipiation experiments from rat or mouse brain.*

2.2.1. Preparation of rat brain Homogenate: Whole brains are removed from mice or rats and homogenized in ice cold lysis buffer. Samples are centrifuged (10,000xg, 10min at 4ºC), re-homogenized, centrifuged again, and the supernatant collected. Total protein in the supernatant is quantified (using DC Protein Assay, BioRad) and samples are used immediately in co-immunoprecipitation experiments.

2.2.2. 200µl of brain homogenate (100µg/sample) is pre-cleared with protein A-sepharose 4 fast flow beads (GE Healthcare) for 1hr at 4°C. Samples are centrifuged (2000xg, 5min at 4°C), and the pre-cleared supernatant collected. Pre-cleared supernatant is incubated with α-VGCC antibody (2µg) overnight at 4°C. 50µl of Protein A-sepharose beads, (or G-sepharose beads, according to the instructions of the antibody manufacturer) are added for 1hr and slurries are washed 2x with buffer B, 1x with buffer C, and 1x with buffer D. All immunoprecipitates are subsequently prepared in SDS sample buffer.

2.2.3. Proteins are separated by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and transferred to Polyvinylidene fluoride (PVDF) immobilon membranes (Millipore) for immunoblot analysis.

2.2.4. Membranes are pre-blocked overnight at 4°C in PBS/0.1% (v/v) Tween-20 (PBS-T) with 5% (w/v) milk prior to either overnight incubation (4ºC) or 2hrs incubation (RT) with appropriate receptor antibody.

2.2.5. Membranes are washed 3x10min with PBS-T and incubated for 45min (RT) with an HRP-conjugated goat α IgG antibody (targeted against the primary antibody IgG species) (Amersham, 1:5000).

2.2.6. Membranes are washed again with PBS-T (3x20min) and proteins are visualized by addition of ECL reagents and exposure to X-ray film.

2.3. *GST-pull down assays*

2.3.1. For pull-down experiments, Glutathione S-transferase (GST)-fusion proteins of the intracellular regions of the receptor are transformed using *Escherichia coli* BL-21 bacterial cells. Protein expression is induced by addition of 100 mM IPTG (Isopropyl-beta-thio galactopyranoside) for 2 hrs. Large scale bacterial sonicates of each protein are prepared according to pGEX vector manufacturer's instructions. (http://www6.gelifesciences.com/aptrix/upp01077.nsf/content/life_sciences_homepage).

2.3.2. Individual GST-fusion proteins are bound to glutathione-sepharose 4B beads (Amersham) by incubation of one volume of 50% beads (in PBS-T, 0.1%(v/v)) with 10 volumes of protein lysate (1hr, 4°C). Beads are washed 3x with PBS-T and incubated (3hr, 4°C) with rat brain homogenate (see above for preparation). Beads are washed 2x (15min, 4°C) with PBS-T. The resulting protein bound beads are prepared for western blot analysis by addition of 2x SDS sample buffer containing 0.1M DTT.

2.3.3. Proteins are separated by SDS-PAGE and transferred at 70V for 3hrs to PVDF immobilon membranes (Millipore) for immunoblot analysis. Immunoblotting is carried out as described above for co-immunoprecipitation experiments, using an α-VGCC antibody and an HRP-conjugated goat α IgG antibody (targeted against the primary antibody IgG species)(1:5000).

2.4. *Receptor-channel co-trafficking experiments using confocal microscopy*

2.4.1. HEK 293T are plated at 60-70% confluence on MatTek dishes coated with poly-Lysine 8hrs before transfection.

2.4.2. Cultured HEK 293T cells are transiently transfected overnight, using the calcium phosphate transfection method, with cDNA encoding different subunits of voltage-gated calcium channels cDNA. HA-tagged Ca_v-α_1 subunit (+ β + α_2-δ_1, ratio 2:1:1) and a full-length, YFP-tagged receptor. Fourteen hours following transfection, cells are washed with PBS and the medium is changed. Cells are incubated at 37 °C for 48-60hrs.

2.4.3. To visualize HA-Cav1 internalization in response to receptor stimulation, medium is removed and cells are immuno-stained for the HA-Ca_v1 channel using α-HA 1 μg/ml (3F10, Roche Molecular Biochemicals) in antibody solution (HBSS solution + 2% goat serum) for 30 min at 37 °C.

2.4.4. Cells are then washed with antibody solution (HBSS + 2% serum) and stimulated with receptor agonist at 37°C in the incubator for 30 min (agonist diluted in antibody solution).

2.4.5. Cells are washed with antibody solution 2x5 min at RT, fixed with 4% PFA for 10 min at RT then permeabilized with 0.05% Triton X-100 for 10min RT.

2.4.6. After 3x10min washes with blocking solution, cells are incubated for 45 min at room temperature, in the dark, with AlexaFluor594 coupled goat anti-rat antibody at a 1:1000 dilution (Molecular Probes). Then cells are washed 3x10min with blocking solution.

2.4.7. Image acquisition is obtained using the Zeiss LSM 510 META confocal microscope with a 63x 1.4NA oil immersion objective in the inverted configuration. The entire cell volume is imaged by a series of z-plane sections that resulted in a z-stack. In addition, for all confocal images a regular phase transmission image is obtained. Optical section thickness varies slightly between experiments but is typically near 0.3μm. Internalization is qualitatively confirmed, before quantitative analysis (using metamorph software), by the use of orthographic image projections (e.g X-Z plane projection), which can show the presence of internalized elements that are clearly localized to within intracellular regions.

2.4.8. Quantitative analysis is performed using Metamorph (Molecular Devices) and ImageJ (NIH). Images are background subtracted and then median filtered (2x2 pixels, effective 1 pixel radius) to remove impulse-type noise and boost signal-to-noise.

2.4.9. For calculation of the relative fluorescence ratio, we select 2 optical sections above and below the largest membrane cross-section, this will typically cover the effective cross-section of the cell. For each image plane, we manually trace the nucleus, the intracellular

region, and cell outline, and compute the integrated fluorescence intensity. Therefore, the intracellular fluorescence intensity is corrected for the presence of the nucleus.

2.4.10. For each cell, using a series of regions of interest (ROIs), we compute the internalization ratio as defined by Ri = I/M, where I is the integrated fluorescence intensity within the cell (corrected for the nucleus), and M is the fluorescence intensity in the membrane region as obtained by subtracting the total intracellular fluorescence (including the nucleus) from the total integrated intensity. In the case where clusters of cells are imaged, it is important to avoid multiple inclusions of cell membranes. Hence, the total fluorescence of the cluster is determined, and all intracellular compartments are integrated and subtracted from total integrated fluorescence of the cluster to obtain total membrane fluorescence (M) (see Fig. 2).

2.4.11. The cumulative intracellular fluorescence is corrected for the presence of the nuclei (I). Ri is computed for each optical section and a mean value for all five planes are used for each cell for subsequent pooling of data and statistical analysis. The ratio of these quantities is dimension-less and allows us to quantify the relative fluorescence intensity in the effective sections covering the membrane and intracellular regions without bias towards experimental variations including cell-to-cell protein expression level, detector gain, laser intensity, and cell shape.

Acknowledgments:

We thank Dr. Bourinet for creating an HA-tagged version of the Cav2.2 channel. GWZ is Scientist of the Alberta Heritage Foundation for Medical Research, and a Canada Research Chair. Work described in this chapter was supported by grants from the Canadian Institutes of health Research.

References:

1. Catterall, W. A. Structure and regulation of voltage-gated Ca2+ channels. Annu Rev Cell Dev Biol 16, 521-55 (2000).
2. Dolmetsch, R. E., Pajvani, U., Fife, K., Spotts, J. M. & Greenberg, M. E. Signaling to the nucleus by an L-type calcium channel-calmodulin complex through the MAP kinase pathway. Science 294, 333-9 (2001).
3. Tedford, H. W. & Zamponi, G. W. Direct G protein modulation of Cav2 calcium channels. Pharmacol Rev 58, 837-62 (2006).
4. Beedle, A. M. et al. Agonist-independent modulation of N-type calcium channels by ORL1 receptors. Nat Neurosci 7, 118-25 (2004).
5. Kisilevsky, A. E. et al. D1 receptors physically interact with N-type calcium channels to regulate channel distribution and dendritic calcium entry. Neuron 58, 557-70 (2008).
6. Kisilevsky, A. E. & Zamponi, G. W. D2 dopamine receptors interact directly with N-type calcium channels and regulate channel surface expression levels. Channels (Austin) 2, 269-77 (2008).
7. Evans, R. M. et al. Heterodimerization of ORL1 and opioid receptors and its consequences for N-type calcium channel regulation. J Biol Chem (2009).
8. Altier, C. et al. ORL1 receptor-mediated internalization of N-type calcium channels. Nat Neurosci 9, 31-40 (2006).
9. Dale, L. B., Seachrist, J. L., Babwah, A. V. & Ferguson, S. S. Regulation of angiotensin II type 1A receptor intracellular retention, degradation, and recycling by Rab5, Rab7, and Rab11 GTPases. J Biol Chem 279, 13110-8 (2004).
10. Tombler, E. et al. G protein-induced trafficking of voltage-dependent calcium channels. J Biol Chem 281, 1827-39 (2006).
11. Berkefeld, H. et al. BKCa-Cav channel complexes mediate rapid and localized Ca2+-activated K+ signaling. Science 314, 615-20 (2006).

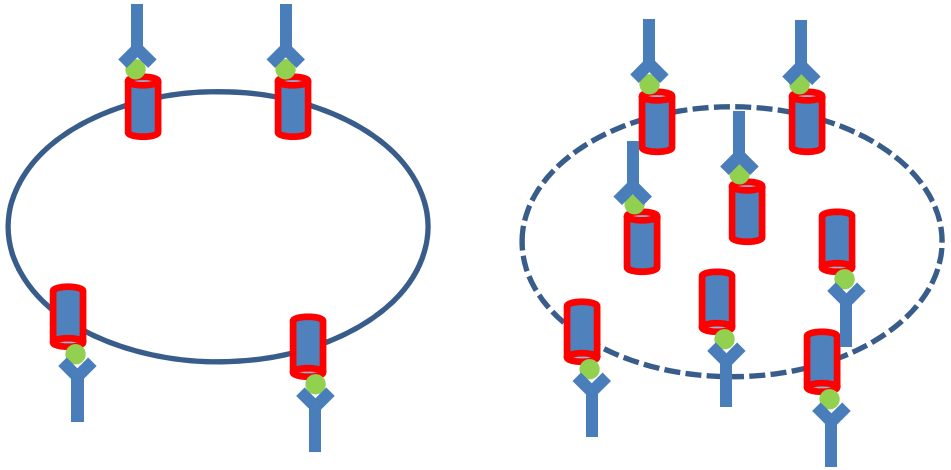
Figure legends:

Figure 1. A) Schematic representation of an immuno-luminometry experiment: in non permeabilized conditions only plasma membrane inserted membrane HA-tagged channels are detected (left). In permeabilized condition, all HA-tagged channels in the cell are detected (right). **B)** The ratio of surface and total signal gives the fraction of HA tagged channels expressed at the cell surface. The graph shows the surface expression of HA-Cav1.2 (L-type channel) alone or co-expressed with auxiliary subunits (i.e. β2a+α2-δ1 or β1b+α2-δ1).

Figure 2. A) Representative confocal image of tsA-201 cells transfected with a YFP tagged nociceptin receptor (ORL1-YFP) and an externally tagged N-type channel (HA-Cav2.2). To visualize HA-Cav2.2 internalization upon receptor agonist application, membrane inserted calcium channels were labeled with anti HA antibody (3F10, Roche Molecular Biochemicals) for 30 min at 37 °C. Cells were then washed and stimulated with 100nM nociceptin at 37 °C for 30 min. Cells were washed with PBS and fixed with 4% PFA for 5 min at room temperature and then, permeabilized with 0.05% Triton X-100. Cells were incubated for 45 min at room temperature with Alexa Fluor 594 coupled goat anti-rat antibody. Immunofluorescence microscopy was performed using a Zeiss LSM 510 META confocal microscope. White lines in the images depict the ROIs for the nucleus, cytoplasmic region, and plasma membrane. **B)** Relative cell surface expression of HA-Cav2.2 co-expressed with the ORL1 receptor, without or with application of the agonist nociceptin, measured by confocal microscopy and image analysis as described in the method.

Figure 1

A



B

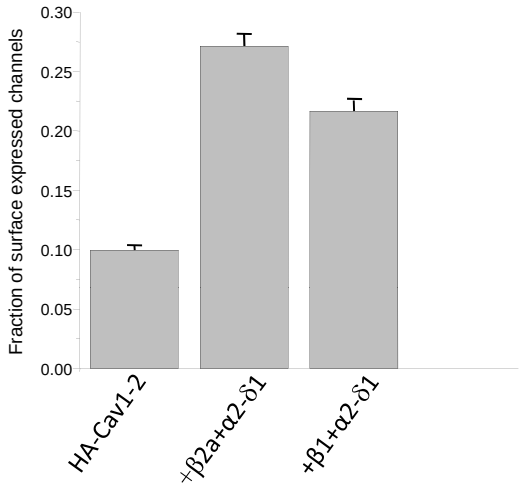
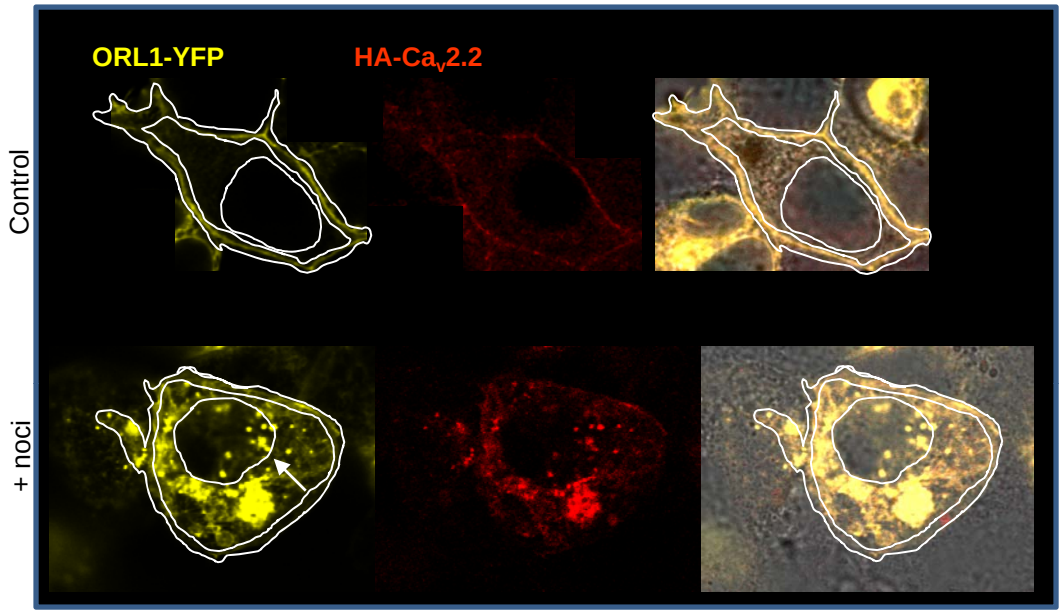
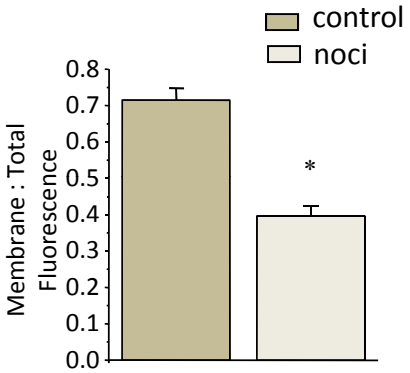

Fraction of surface expressed channels
0.30
0.25
0.20
0.15
0.10
0.05
0.00
HA-Cav1-2
+β2a+α2-δ1
+β1+α2-δ1

Figure 2

A


ORL1-YFP
HA-Cav2.2
Control
+ noci

B


control
noci
Membrane : Total Fluorescence
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*