

## Development of low affinity, membrane targeted $\text{Ca}^{2+}$ sensors suitable for measuring presynaptic $\text{Ca}^{2+}$

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### Summary

1. Our aim is to measure near-membrane  $\text{Ca}^{2+}$  flux within presynaptic terminals of central neurons by modifying new genetically encoded  $\text{Ca}^{2+}$  sensors to develop tools capable of measuring localised  $\text{Ca}^{2+}$  signals.

2. We used standard recombinant DNA technologies to generate the DNA coding for a fusion construct of a modified fluorescent "pericam"  $\text{Ca}^{2+}$  biosensor with a presynaptic P2X7 receptor (P2X7R). The  $\text{Ca}^{2+}$  sensitivity of the biosensor was modified by rational site-directed mutagenesis of the calmodulin portion of the pericam.

3. Biosensor-receptor fusions were transfected into expression systems for evaluation. Expression studies in Human Embryonic Kidney-293 (HEK-293) cells showed that biosensor-receptor fusion construct delivered protein was localised exclusively to the plasma membrane, confirming that fusion did not affect the ability of the receptor to undergo normal protein synthesis and trafficking.

4. The  $\text{Ca}^{2+}$ -dependent fluorescence of the pericam portion of the fusion protein was also retained. Site-direct mutagenesis within the calmodulin moiety of the pericam significantly reduced the  $\text{Ca}^{2+}$  affinity of the complex. The dynamic range of the sensor following this modification is better matched to the higher  $\text{Ca}^{2+}$  levels expected within presynaptic  $\text{Ca}^{2+}$  micro-domains.

### Introduction

$\text{Ca}^{2+}$  is a ubiquitous cellular messenger controlling a diverse array of physiological processes from fertilisation through to gene transcription, muscle contraction, cell proliferation and migration, cell differentiation and ultimately, cell death. Tight control of the spatial, temporal and concentration profile of  $\text{Ca}^{2+}$  influx is therefore required to define specific functional roles in cells. This is important in the neuronal setting, especially within the presynaptic terminal where the release of transmitter is critically dependent upon small changes in  $\text{Ca}^{2+}$  concentrations<sup>1</sup>. The invasion of an action potential into the presynaptic terminal opens voltage-dependent  $\text{Ca}^{2+}$  channels allowing the rapid influx of  $\text{Ca}^{2+}$  ions, giving rise to a small local volume (microdomain) of elevated  $\text{Ca}^{2+}$ <sup>2-4</sup>.  $\text{Ca}^{2+}$  microdomains coincide with active zones that are areas of presynaptic membrane densely packed with  $\text{Ca}^{2+}$  channels and docked with neurotransmitter vesicles<sup>3</sup>. At these microdomains the  $\text{Ca}^{2+}$  concentration reaches

approximately  $100\mu\text{M}$ <sup>5</sup> within 800 microseconds<sup>6</sup>, over distances of less than  $1\mu\text{m}$  from the point of entry<sup>7</sup>.  $\text{Ca}^{2+}$  microdomains are determinants of neurotransmitter release<sup>2</sup> and play an important role in the modulation of synaptic strength<sup>8</sup>. Historically, it has been a challenging exercise to measure  $\text{Ca}^{2+}$  microdomains due to their small spatio-temporal profiles, with the most reliable data derived from modelling and simulation studies<sup>9</sup>. The difficulty in measuring  $\text{Ca}^{2+}$  microdomains means that a wide range of  $\text{Ca}^{2+}$ -specific signalling processes may go undetected by the current 'volume-averaged' methods routinely used. There is clearly a need for a biosensor that is capable of sensing  $\text{Ca}^{2+}$  microdomains. Genetically encoded  $\text{Ca}^{2+}$  sensors based on green fluorescent protein (GFP) provide an exciting opportunity to develop tools to measure these localised signals.

GFP derived from *Aequorea Victoria* jelly fish is a 238 amino acid protein with an apparent molecular weight of 27-30kDa<sup>10</sup>. Several GFP mutants with distinct spectral qualities have been established as sensors of cellular dynamics, for example, in monitoring local pH or  $\text{Ca}^{2+}$  concentration inside cells<sup>11</sup>. A powerful example of this technology has been the recent development of pericams<sup>12</sup> that consist of a single GFP variant sensitive to physiologically relevant substrates such as  $\text{Ca}^{2+}$  ions. To construct the pericams, circularly permuted enhanced yellow fluorescent proteins (cpEYFP) were used in which the amino and carboxyl portions had been interchanged and reconnected by a short spacer between the original termini. Calmodulin was fused to the C terminus of cpEYFP and its target peptide, M13, to the N terminus. The pericam was shown to be fluorescent with its spectral properties changing reversibly with the amount of  $\text{Ca}^{2+}$ . Of the three major pericams developed, ratiometric-pericam (RP) appeared to be most promising, in that due to its capacity for dual excitation nature, it has potential for quantitative imaging.

Insertions of genetically encoded sensor into host receptors that already have localisation signals offers a new strategy for measuring localised  $\text{Ca}^{2+}$ . Our goal is to measure  $\text{Ca}^{2+}$  microdomains within presynaptic terminals. The requirement therefore is a receptor that localises to the presynaptic membrane, but does not play a critical role in evoked release. The P2X7Rs are ligand-gated ion channels that are gated by ATP and other nucleotides<sup>13-15</sup>. Studies of expression patterns of P2X7R have confirmed localisation in presynaptic nerve terminals in both central and peripheral neurons<sup>16</sup>. Further, immunoreactivity studies in the

hippocampus indicate that P2X7Rs colocalise with the vesicular glutamate transporter, (VGLUT1), placing them within excitatory terminals<sup>17</sup>. This makes P2X7R an ideal tool for targeting Ca<sup>2+</sup> sensors to excitatory presynaptic nerve terminals in the hippocampus. Smart and colleagues have revealed that fusion of the purinergic P2X7R to GFP directed the complex to the plasma membrane in the simple HEK expression system<sup>18</sup>. Here we use this simple expression system to characterise a RP-P2X7R construct, determining its trafficking and Ca<sup>2+</sup> sensing ability.

As Ca<sup>2+</sup> concentrations within the presynaptic microdomains are thought to be in the 50-100µM range, the dissociation constant (Kd) of RP, reported to be 1.7µM, is therefore lower than required. As part of fine-tuning the Ca<sup>2+</sup> affinities of a previous Ca<sup>2+</sup> sensor, cameleons<sup>19</sup>, a number of mutations were performed in the calmodulin moiety of this construct to optimise the Kd for this reporting range. In particular a substitution mutation in the first Ca<sup>2+</sup> binding loop of calmodulin, where the 31<sup>st</sup> amino acid was changed from glutamic acid (E) to glutamine (Q), shifted the titration curve of cameleon-1 to the right reflecting an increase in the Kd of its low affinity component from 11µM to 700µM<sup>19</sup>. Here we use a similar strategy to lower the affinity of the RP, making it more suitable for measuring the high Ca<sup>2+</sup> concentrations expected in presynaptic microdomains.

## Methods

### Gene construction

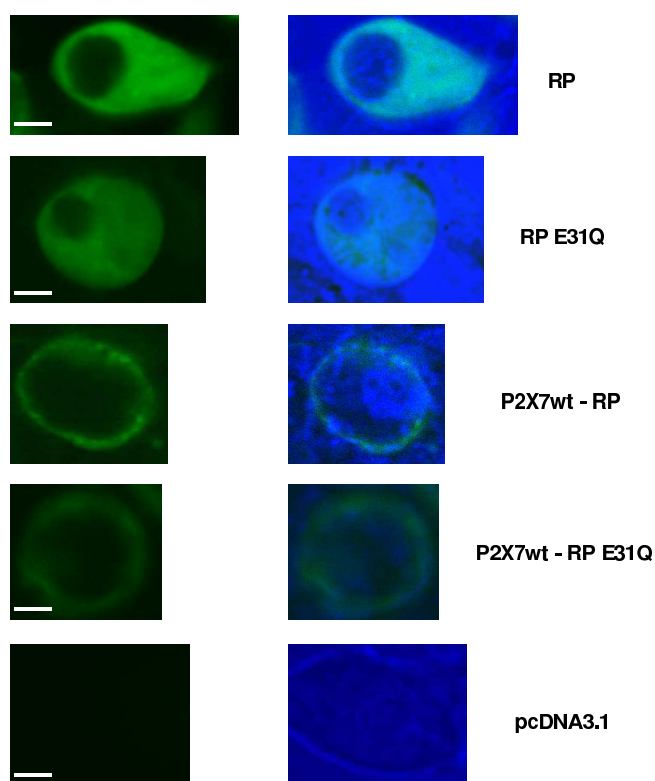
To incorporate the E31Q mutation into RP a total of three PCR reactions were performed. First the cDNA of the 5' portion of RP(E31Q) was amplified with a sense primer containing a HindIII restriction site and a reverse primer 5' C GGT GCC AAG TT G CTT TGT GGT GAT GG (with the base change introducing the mutation of interest being underlined). For both RP and RP(E31Q) a glycine-rich spacer sequence, GGA GGT GCA GGT AGT GGA GGT corresponding to Gly-Gly-Ala-Gly-Ser-Gly-Gly, was included upstream of the start codon in the forward primer. In the second PCR reaction, the cDNA of the 3' portion of RP(E31Q) was amplified with a forward primer: 5'CC ATC ACC ACA AAG C AA CTT GGC ACC G, and a reverse primer containing a XhoI restriction site. Finally the entire cDNA of RP(E31Q) was amplified with the HindIII and XhoI sites containing primers by using a mixture of the first and second PCR fragments as the template. The restricted product was cloned in-frame into the HindIII/XhoI sites of pcDNA3.1 (Invitrogen) vector. To generate the chimeric sensors, P2X7R-RP and P2X7R-RP(E31Q), a subcloning strategy was employed. The cDNA encoding P2X7R was amplified by using primers containing 5'NheI and 3'HindIII restriction sites. The restricted PCR fragments were ligated to the 5' end of RP or RP(E31Q) gene in pcDNA3.1 to yield the tagged Ca<sup>2+</sup> sensor constructs of P2X7R-RP and P2X7R-RP(E31Q).

### In vitro spectroscopy

To assess the spectral characteristics of the RP and RP(E31Q) proteins, HEK-293 cells were transfected with each DNA construct using Lipofectamine<sup>TM</sup>2000 (Life Technologies). Transfected HEK-293 cells were lysed 3 days post transfection and liberated cytosolic proteins (RP and RP(E31Q)) were collected. To determine the Kds of RP and RP(E31Q), a Ca<sup>2+</sup> Calibration Buffer Kit (Molecular Probes, C-3009) was used. The spectral properties of the proteins were measured using a Hitachi F-4010 Fluorescence Spectrophotometer. In separate experiments RP and RP(E31Q) were excited at 480nm, with an emission wavelength scan performed from 480 to 650nm. The solutions were maintained at pH 7.20 and 24°C. The Kd of both indicators (RP and RP(E31Q)) was calculated from a linearised (Hill) plot of fluorescence intensity as a function of Ca<sup>2+</sup> concentration. Data were generated by scanning the emission spectrum of the indicator in the presence of different Ca<sup>2+</sup> concentrations. All data for RP or RP(E31Q) were corrected for the fluorescence or a reference solution containing non-transfected HEK-293 cells (autofluorescence).

### Imaging

Fluorescence-based approaches were used to characterise the Ca<sup>2+</sup> sensors. Two or three days after cDNA transfection with Lipofectamine<sup>TM</sup>2000, HEK-293 adherent on poly-L-lysine-coated coverslips were analysed microscopically. Cells bathed in HEPES buffer (mM: NaCl 147, KCl 2, HEPES 10, Glucose 10, CaCl<sub>2</sub> 1 pH: 7.4) were imaged at 24°C on a laser scanning confocal (Biorad MRC-1024ES) employing an argon-ion laser, coupled to a Nikon Diaphot 300 microscope. Both the tagged and untagged RPs were illuminated at 488 nm, which excited the YFP portion of the constructs. YFP fluorescence emission of the RP was collected through a 510 long pass dichroic mirror and OG515 emission filter (>515nm). Fluorescence distribution patterns of tagged and untagged biosensors were achieved by collecting 512×512 pixel confocal images (slow scan rate – 1 s/image), a bright field or transmitted image, and a simultaneous image showing the colocalisation of the confocal signal with the transmitted image which revealed fluorescent cellular structures. Some images were taken as an average of 4 consecutive scans (Kalman algorithm) to smooth random noise fluctuations. For assessment of the Ca<sup>2+</sup> sensing ability of the Ca<sup>2+</sup> sensors the acquisition package 'Timecourse' was used. In any given field of cells several regions of interest (ROI) were defined. Examination of the Ca<sup>2+</sup> sensing properties of the RP, RP(E31Q) or chimeric constructs was performed in transfected HEK-293 cells being exposed to 2.5µM ionomycin (Sigma).



**Figure 1. Sub-cellular localisation of tagged and untagged  $Ca^{2+}$  sensor constructs expressed in HEK-293 cells.**

Images were captured using a laser scanning confocal microscope (488 excitation, >510 emission). Left panel represents the fluorescence images, displayed at identical gain and black level settings. Right panel is the co-registration of the fluorescence image with transmission light image of the same field. Transmission light images reveal the cell outline and presence of organelles. Scale bar = 5  $\mu$ m.

**A** Ratiometric Pericam (RP)

**B** Ratiometric Pericam E31Q (RP(E31Q))

**C** P2X7R-RP

**D** P2X7R-RP(E31Q)

**E** pcDNA3.1 vector (control) which shows no fluorescence

## Results and Discussion

### Development of a near-membrane $Ca^{2+}$ sensor

A fusion between a ratiometric pericam and a membrane targeted protein, the P2X7R was carried out using standard molecular biology techniques. This was revealed by imaging HEK-293 cells transfected with either the tagged (localised) or untagged (unlocalised) pericams and comparing fluorescence distribution patterns. As expected, untagged ratiometric pericams (RP) displayed a bright fluorescence intensity, which was confined to the cytosol but excluded from the nucleus (Figure 1A). Our targeting strategy of fusing the RP to P2X7R was tested next. P2X7R-GFP is known to localise to the plasma

membrane in the HEK expression system<sup>18</sup>. P2X7R-RP exhibits an identical expression pattern (Figure 1C) to that of P2X7R-GFP, suggesting that the normal P2X7R trafficking is not disrupted by the addition of RP. The signal sequence on the P2X7R therefore directed the ratiometric pericams exclusively to the plasma membrane. Confirming the locality of the sensors provides us with the potential for exclusively reporting near-membrane  $Ca^{2+}$  signals.

### Fine-tuning the $K_d$ of ratiometric pericams to measure high $Ca^{2+}$ concentrations

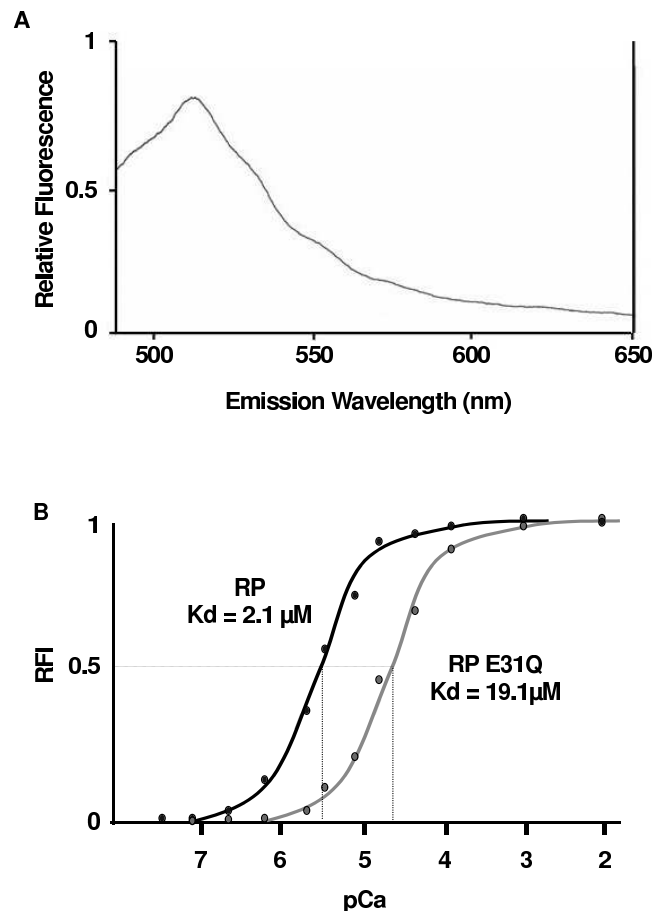
The high affinity of RP makes it unsuitable to accurately measure the large  $Ca^{2+}$  flux expected at presynaptic microdomains. Site-directed mutagenesis within the calmodulin portion of the  $Ca^{2+}$  sensor, cameleon-1, shifts the fluorescence- $Ca^{2+}$  relationship<sup>19</sup>. A single glutamic acid to glutamine mutation (RP(E31Q)) weakens the interaction between  $Ca^{2+}$  and its binding loop, decreasing the affinity of the sensor and increasing its dynamic range<sup>19</sup>. The mutation was introduced using a standard PCR protocol and it was confirmed by direct sequencing. RP and RP(E31Q) displayed virtually identical emission spectra, with an emission maximum at approximately 515nm, coinciding with the emission peak of the YFP portion of each pericam (Figure 2A). This observation confirms that the E31Q mutation did not alter the spectral characteristics of the protein. The E31Q mutation in RP altered the  $K_d$  from 2.1  $\mu$ M to 19.1  $\mu$ M effectively improving the dynamic range of 0.25  $\mu$ M-19  $\mu$ M to approximately 2  $\mu$ M-170  $\mu$ M (Figure 2B). RP(E31Q) expressed in HEK cells is uniformly distributed through the cytoplasm, but is excluded from the nucleus (Figure 1B). The P2X7R-RP(E31Q) construct was also generated and displayed an identical expression pattern to P2X7R-RP, limited to the plasma membrane (Figure 1D). The lower fluorescent signal seen with RP(E31Q) is consistent with its lower  $Ca^{2+}$  affinity (Figure 1C,D).

### Will the sensors be sensitive enough to measure $Ca^{2+}$ microdomains?

In turtle hair cells the  $Ca^{2+}$  concentration in  $Ca^{2+}$  microdomains was found to be at least 85  $\mu$ M<sup>7</sup>. Others have reported microdomain  $Ca^{2+}$  concentrations of 100-200  $\mu$ M, necessary to produce rapid neurotransmitter secretion<sup>5</sup>. With a dynamic range of 0.25  $\mu$ M-19  $\mu$ M the original RP indicator would be expected to saturate at these high concentrations. However, the RP(E31Q), with a dynamic range of 2  $\mu$ M-171  $\mu$ M, is ideally suited to the expected levels of  $Ca^{2+}$  concentrations within presynaptic microdomains.

### Sensing $Ca^{2+}$ in a mammalian expression system using genetically encoded sensors

HEK-293 cells transfected with each pericam were exposed to the ionophore ionomycin (2.5  $\mu$ M). Both the P2X7R-RP and P2X7R-RP(E31Q) sensors demonstrated an



**Figure 2. In-vitro properties of ratiometric pericams.**

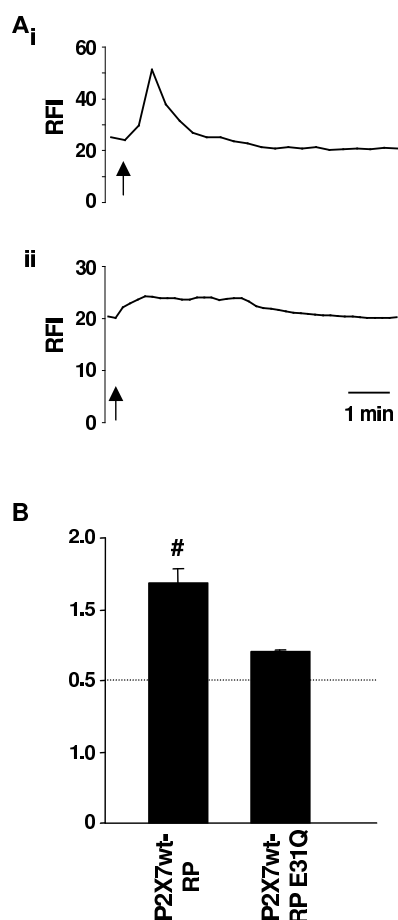
**A** Fluorescence emission spectrum of ratiometric pericam E31Q (RP(E31Q)). HEK-293 cells transfected with RP(E31Q) were lysed and the liberated cytosolic proteins containing RP(E31Q) were diluted in phosphor-buffered saline (PBS). Emission wavelength scans (490nm-650nm) were obtained using a spectrophotometer. The spectrum was measured at 24°C and pH 7.20, and the results corrected for with a reference solution identical in composition to the sample except for the absence of RP(E31Q). The results were similar to YFP spectra, showing an emission peak at approximately 515nm. A very similar emission spectrum was obtained for RP (results not shown) with a maximum peak at 510nm, indicating the maintenance of YFP spectral properties.

**B** Dose response curves of ratiometric pericams, showing relative fluorescence intensity (RFI) as a function of pCa ( $-\log_{10}[\text{Ca}^{2+}]_{\text{free}}$ ). The curves were generated by scanning the emission of the indicator (RP or RP(E31Q)) at 515nm. The concentration of free  $\text{Ca}^{2+}$  ions in solution was varied by cross dilution of  $\text{Ca}^{2+}$  ("high  $\text{Ca}^{2+}$  solution") and EGTA ("low  $\text{Ca}^{2+}$  solution") to produce a series of eleven solutions with increasing  $[\text{Ca}^{2+}]$  while keeping the concentration of the indicator (RP or RP(E31Q)) constant. The pH was kept at 7.2 during experimentation. The E31Q mutation shifted the dose response curve to the right, reflecting a change in Kd from 2.1 $\mu\text{M}$  for RP to 19.1 $\mu\text{M}$  for RP(E31Q).

increase in fluorescence intensity in response to application of ionomycin (Figure 3). As expected, P2X7R-RP showed a significantly larger change in fluorescence intensity than the lower affinity sensor, P2X7-RP(E31Q). These experiments demonstrate that the  $\text{Ca}^{2+}$  sensing abilities of the RP and RP(E31Q) are not altered when fused to the P2X7R and expressed in a mammalian cell line. Further studies are required to demonstrate this in primary neuronal cultures and other neuronal preparations.

*Will the sensor only measure  $\text{Ca}^{2+}$  from presynaptic  $\text{Ca}^{2+}$  microdomains?*

Using simulation studies, Fogelson and colleagues<sup>9</sup> predicted with a three dimensional model that  $\text{Ca}^{2+}$  enters the presynaptic terminal through discrete membrane channels and acts to release transmitter within 50nm of the entry point. In turtle hair cells the initial diameter of  $\text{Ca}^{2+}$  microdomains was found to be less than 1  $\mu\text{m}$ , as estimated by confocal microscopy<sup>7</sup>. Hence,  $\text{Ca}^{2+}$  microdomains can have very restricted spatial profiles and restricting even a sensor to the membrane may not guarantee localisation within  $\text{Ca}^{2+}$  microdomains (e.g. in neurons). However, one could still expect much better signal-to-noise ratios with a



**Figure 3. Responses of chimeric  $\text{Ca}^{2+}$  sensor constructs when expressed in HEK-293 cells to  $2.5 \mu\text{M}$  ionomycin.**

**A** Typical responses of HEK-293 cells expressing: (i) P2X7R-RP, (ii) P2X7R-RP(E31Q) upon application of ionomycin ( $2.5 \mu\text{M}$ ) as indicated by the upward arrows below each trace. RFI indicates relative fluorescence intensity. Application of ionomycin caused a transient increase in relative fluorescence intensity in each case, but the response of P2X7R-RP(E31Q) was smaller as compared to the response of P2X7R-RP. All experiments were performed in HEPES solution containing  $1 \text{mM}$   $\text{CaCl}_2$ . For each trace the horizontal bar indicates 1 minute.

**B** Collated responses of HEK-293 cells expressing each of the chimeric  $\text{Ca}^{2+}$  sensor constructs, P2X7R-RP ( $n=13$  cells), P2X7R-RP(E31Q) ( $n=9$  cells) to  $2.5 \mu\text{M}$  ionomycin.  $F/F_0$  indicates peak fluorescence intensity over basal fluorescence intensity. Responses are expressed as mean  $F/F_0 \pm$  s.e.m. #  $P < 0.05$  (one-way Anova) significantly different to P2X7R-RP(E31Q).

membrane-delimited sensor as opposed to a generalised cytosolic sensor. Therefore, a localised biosensor responding selectively to  $\text{Ca}^{2+}$  signals near the presynaptic membrane will provide a valuable tool to more accurately measure these signals. Further experiments will determine how close we can get.

## Conclusion and Future Directions

The  $\text{Ca}^{2+}$  microdomains within presynaptic nerve terminals are highly localised and the concentrations of  $\text{Ca}^{2+}$  within these regions are thought to be significantly larger than the global cytosolic concentration. By measuring 'volume-averaged' global signals in response to stimuli, important information about these compartmentalised functions remains undetected. In this study biosensors have been designed with characteristics suited to investigating  $\text{Ca}^{2+}$  microdomains within presynaptic terminals. We generated a fusion protein of a ratiometric pericam (with modified  $\text{Ca}^{2+}$ -sensing ability) and a P2X7 receptor, a protein known to localise to the presynaptic membrane of excitatory neurons. Fusion proteins successfully trafficked to the plasma membrane distribution of HEK cells and were capable of responding to changes in intracellular  $\text{Ca}^{2+}$ . Our next goal is to characterise these sensors in a neuronal setting, confirming localisation and  $\text{Ca}^{2+}$  sensing ability and refining these properties where necessary. Ultimately, we hope to study  $\text{Ca}^{2+}$  dynamics in brain slices derived from various mouse and rat models of relevant human diseases. The generation of genetically encoded fluorescent biosensors described is expected to continue to expand and provide exciting new insights into normal physiological and pathological processes in neurons.

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## References:

1. Reid CA, Bekkers JM, Clements JD. N- and P/Q-type  $\text{Ca}^{2+}$  channels mediate transmitter release with a similar cooperativity at rat hippocampal autapses. *J. Neurosci.* Apr 15 1998;18(8):2849-2855.
2. Augustine GJ. How does calcium trigger neurotransmitter release? *Curr Opin Neurobiol.* Jun 2001;11(3):320-326.
3. Llinas R, Sugimori M, Silver RB. Microdomains of high calcium concentration in a presynaptic terminal. *Science.* May 1 1992;256(5057):677-679.
4. Jarvis SE, Zamponi GW. Interactions between presynaptic  $\text{Ca}^{2+}$  channels, cytoplasmic messengers and proteins of the synaptic vesicle release complex. *Trends Pharmacol Sci.* Oct 2001;22(10):519-525.
5. Heidelberger R, Heinemann C, Neher E, Matthews G. Calcium dependence of the rate of exocytosis in a synaptic terminal. *Nature.* Oct 6 1994;371(6497):513-515.
6. Sugimori M, Lang EJ, Silver RB, Llinas R. High-resolution measurement of the time course of calcium-concentration microdomains at squid presynaptic terminals. *Biol. Bull.* Dec 1994;187(3):300-303.
7. Tucker T, Fettiplace R. Confocal imaging of calcium

- microdomains and calcium extrusion in turtle hair cells. *Neuron*. Dec 1995;15(6):1323-1335.
8. Zucker RS. Calcium- and activity-dependent synaptic plasticity. *Curr Opin Neurobiol*. Jun 1999;9(3):305-313.
  9. Fogelson AL, Zucker RS. Presynaptic calcium diffusion from various arrays of single channels. Implications for transmitter release and synaptic facilitation. *Biophys. J*. Dec 1985;48(6):1003-1017.
  10. Tsien RY. The green fluorescent protein. *Annu. Rev. Biochem.* 1998;67:509-544.
  11. Gerdes HH, Kaether C. Green fluorescent protein: applications in cell biology. *FEBS Lett*. Jun 24 1996;389(1):44-47.
  12. Nagai T, Sawano A, Park ES, Miyawaki A. Circularly permuted green fluorescent proteins engineered to sense Ca<sup>2+</sup>. *Proc. Natl. Acad. Sci. USA*. Mar 13 2001;98(6):3197-3202.
  13. North RA, Surprenant A. Pharmacology of cloned P2X receptors. *Annu. Rev. Pharmacol. Toxicol.* 2000;40:563-580.
  14. Paukert M, Hidayat S, Grunder S. The P2X(7) receptor from *Xenopus laevis*: formation of a large pore in *Xenopus* oocytes. *FEBS Lett*. Feb 27 2002;513(2-3):253-258.
  15. Rassendren F, Buell GN, Virginio C, Collo G, North RA, Surprenant A. The permeabilizing ATP receptor, P2X7. Cloning and expression of a human cDNA. *J. Biol. Chem.* Feb 28 1997;272(9):5482-5486.
  16. Deuchars SA, Atkinson L, Brooke RE, et al. Neuronal P2X7 receptors are targeted to presynaptic terminals in the central and peripheral nervous systems. *J Neurosci*. Sep 15 2001;21(18):7143-7152.
  17. Atkinson L, Batten TF, Moores TS, Varoqui H, Erickson JD, Deuchars J. Differential co-localisation of the P2X7 receptor subunit with vesicular glutamate transporters VGLUT1 and VGLUT2 in rat CNS. *Neuroscience*. 2004;123(3):761-768.
  18. Smart ML. Molecular Basis of P2X7R Pore Formation (Thesis). The laboratory of Biophysics and Molecular Biology. Department of Physiology, The University of Melbourne. 2002.
  19. Miyawaki A, Llopis J, Heim R, et al. Fluorescent indicators for Ca<sup>2+</sup> based on green fluorescent proteins and calmodulin. *Nature*. Aug 28 1997;388(6645):882-887.

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