Final report

Illuminating the activation of glutamate receptors with fluorescent probes

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Contact partner: Dr. Andrew Plested
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Executive summary

Nerve cells form connections call synapses over which to pass information. Glutamate receptors are found throughout the brain, and are located in the cell membrane at excitatory synapses. Therefore glutamate receptor ion channels are important molecules for the normal function of the nervous system. The molecular structure of glutamate receptors is mostly well understood, but the dynamics of activation and the parts of the receptor that are exposed to the internal part of the cell are not well resolved.

A general strategy to examine protein function and dynamics is to incorporate fluorescent labels. Double labelling enables nanometer scale distances to be measured via spectroscopic approaches. In principle, if the target protein changes shape to get activated, movements should be detectable spectroscopically and this should allow observation of activity with light. Our goal with this project was to find a way to label glutamate receptors in cells and measure functional responses via electrophysiology and optical recording at the same time. Although this approach has been used on other ion channels, it’s application in ligand gated channels was very limited, and it had not been attempted for glutamate receptors before. In order to ensure that our results had wide applicability in neurons and brain tissue, we limited ourselves to genetically encoded methods, even though this path is much more challenging.

To achieve this aim, we constructed a unique, state-of-the-art recording set up, incorporating laser excitation, rapid perfusion of cells, electrophysiology, spectroscopic detection and an ultrafast camera, under full computer control.

To produce fluorescent glutamate receptors, we chose to pursue two novel methodologies - unnatural amino acid labelling and fluorescent protein fusions. We previously had success with unnatural amino acids, and were able to synthesise and specifically incorporate unnatural amino acids harbouring reactive handles. Unfortunately, we found that published labelling methodologies were either ineffective, non-specific in mammalian cells, or cytotoxic and therefore we could not achieve our desired specific labelling of glutamate receptors with organic dyes using this approach.

We had much more success by fusing fluorescent proteins inline using genetic engineering. With this approach, we could measure the state-dependent positions of the intracellular domains for the first time, and the control of the intracellular domains by the binding of glutamatergic ligands, an entirely unexpected result. Moreover, the positions of the intracellular domains that we were able to deduce, suggest that the “tail” of the receptor remains close to the membrane.

As part of our final goal, to develop a screen for modulatory drugs, we are performing experiments with the FMP screening unit, exploiting methods developed in this project. These ongoing experiments have potential commercial applications, and are also scientifically sensitive, and so are not described in this report.
Initial question and objective of the project

With this project, we aimed to produce optically-active glutamate receptors that gave a fluorescence signal related to their activity. The majority of investigations in this area are in vitro or in non-mammalian cells and at steady state. In contrast, we chose to develop methods that were applicable to live labelling in mammalian cells, in order to maximise applicability to brain tissue and receptor activity tracking.

Three broad aims were addressed. First, we aimed to use small organic dyes conjugated to unnatural amino acids to address structural mechanisms underlying glutamate receptor gating. Second, we wanted to exploit fluorescent proteins and fuse them inline with glutamate receptor domains, in order to measure resonance energy transfer. The first two aims were largely complementary. Organic dyes potentially offer larger signals, but with greater expense in terms of production of the relevant compounds, and the difficulty of determining labelling of bioorthogonal groups in cell culture. On the other hand, genetically-encoded fluorophores produce relatively constant labelling for essentially zero cost but are less bright and suffer from bleaching. It was unclear at the outset which method would work best. Finally, using these approaches, we wished to develop high-throughput fluorescence screening of drugs against the AMPA receptor.

Results and discussion, possible applications and subsequent projects

Aim 1 Unnatural amino acid handles and fluorophores

We have tried several approaches for labelling iGluRs at specific sites with organic dyes and fluorescent unnatural amino acids. Our confidence in incorporating azido groups in glutamate receptors, following the techniques established by Thomas Sakmar and his coworkers, initially led us to attempt to label Azido-phenylalanine in glutamate receptors. Unfortunately, labelling of azido groups in live cells proved unworkable, even with “fast-reacting” probes including dibenzocyclo-octyl-conjugated dyes, because of the slow kinetics and harsh conditions needed to provoke the reaction (data not shown). At the start of the project, candidates for bright, efficiently incorporated fluorescent amino acids were few. Therefore we decided to focus on strain-promoted inverse electron demand Diels-Alder cycloaddition (SPIEDAC), which had recently emerged as the conjugation method of choice, due to its fast and specific reaction profile.

Synthesis of unnatural amino acids and fluorophore tag for SPIEDAC

Strained alkynes and alkenes are relatively compact compared to other unnatural amino acids, when presented as part of derivatized Lysine residues. These residues can be genetically incorporated using modified Pyrrolysine RNA-synthetases from thermophilic archaeabacteria. Finally, dye coupling to a protein of interest can then in principle be achieved by conjugating fluorophores to tetrazine moities that react rapidly with bio-orthogonal groups. As well as their relatively fast (minutes rather than hours) reaction rates, a major advantage of tetrazine-endowed fluorophores is their "turn-on" fluorescence upon reaction with their target moiety.

However, the novelty of this approach meant that none of the required reagents were available commercially, even though the required genetically-encoded machinery of tRNAs and RNA-synthetases could be obtained (a gift from Jason Chin, MRC Cambridge). Therefore, we synthesised two unnatural amino acids, norbornene Lysine (Nor-Lys or Knor, VMR-01-09) and bicycloannone Lysine (BCN-Lys or Kbcn, VMR-01-22). The reactive fluorophore Tetrazine-TAMRA (Tet-TAMRA, VMR-01-24) and precursor Tetrazine-NH2 (VMR-01-19), were synthesized following synthetic procedures reported previously.

We confirmed that reaction of Tetrazine-TAMRA with the unnatural amino acids Knor and Kbcn was fluorogenic and occurring on the timescale of minutes in vitro (Figure 1).
With the unnatural amino acid-dye pair in hand, we attempted to perform fluorogenic tagging via of GluA2 expressed in HEK cells. This approach was reported for EGFR-1-GFP labeling in HEK-cells. We could rescue AMPA-type glutamate receptors with norbornene Lysine (Figure 2). Far smaller currents were obtained when including a commercially-available Ne-BOC-Lys derivative, suggesting that incorporation was selective (that is, not just random amino acid substitution). Two examples of selective incorporation (at different sites in the GluA2 AMPA receptor) are shown in Figure 2.

Figure 2 Selective incorporation of Norbornene Lysine in the AMPA receptor GluA2. Inclusion of the control unnatural amino acid, BOC-Lysine, which is an unfavourable substrate for the RNA-synthetase, failed to produce substantial rescue of functional receptors.
However, receptors expressed in this way that incorporated Nor-Lys could not be labelled by our in-house produced tetrazine fluorophore. Reasons for this failure may include - non-reactivity of the handle in cell culture, slow reactivity of the tetrazine-cyclo-octene pair in cellular environement, non-accessibility of the handle, steric interference between the dye and the surrounding receptor. We suspected that these problems could be addressed by incorporating Kbn instead, but could not achieve selective incorporation of this unnatural amino acid (data not shown).

The popularity of copper-free Click chemistry has led to the commercial production of tetrazine probes with multiple fluorescent dyes attached, as well as the relevant unnatural amino acids. Therefore, it is likely that we revisit this area in future and assess dye/handle compatibility in a more systematic manner. Likewise, fluorescent unnatural amino acids (such as 3-[(6-acetyl-2-naphthalenyl)amino]-L-alanine, ANAP) have shown increasing application in eukaryotic cells and ion channel research, suggesting that this avenue may also be profitable in future.

Although the original application contained 2 aims, the curtailment of funding (one postdoctoral position was cut from our original budget) and ongoing difficulties with Aim 1, particularly some failures to reproduce published work, led us to the conclusion that it was unrealistic to pursue Aim 1 further. Instead, we concentrated more profitably on aim 2.

**Aim 2 Fluorescence resonance energy transfer and patch clamp fluorometry**

A major part of the project was the construction of a custom-made setup for patch clamp recording of glutamate receptors. The fast kinetics of AMPA-type glutamate receptors require fast perfusion, in which we have considerable expertise. However, combining this technique with fluorescence excitation (from 4 diode laser wavelengths) via near-TIRF illumination and spectral recording (Fig. 4) required a new design of perfusion system and electrophysiology chamber. The four wavelengths were chosen to excite Cyan, green, yellow and red fluorescent proteins (CFP, GFP, YFP and RFP).

Another major challenge was to synchronise optical excitation in relevant wavebands, spectral recording and current recording. Previous work in this area used steady-state conditions, which forbade precise correlation of changes in fluorescence with relaxations of the receptors between their functional states (including active, desensitised and resting states). To do this, we synchronised two computers to control laser emission, image acquisition, current registration and the semi-automated inverted microscope. Control of the Olympus IX81 microscope was achieved by reverse engineering the serial control using a Python script and an Arduino microcontroller.

With this state-of-the-art setup we have achieved spectral FRET recording from whole mammalian cells at up to 200 Hz, using AMPA receptors harbouring genetically-encoded fluorophores.

In contrast to our difficulties with Aim 1, genetic encoding of fluorophores proved to be much more successfu. Glutamate receptors with inline inserted fluorescent proteins have been produced previously, but were not characterised in detail. In collaboration with Anders Kristensen (University of Copenhagen) we have extended previous work by making paired insertions in individual AMPA receptor subunits. So far, we have made AMPA-receptor fusions with mCerulean3 (CFP), green fluorescent protein (GFP), Venus (yellow fluorescent protein) and super-ecliptic pHluorin (SEP, a pH dependent variant of GFP). To date, we have not successfully fused any red fluorescent variant to the AMPA receptor. This failure is likely because of different maturation rates of the fluorescent protein and the section of the AMPA receptor chosen for insertion, because N- and C-terminal insertions in the AMPA receptor auxiliary protein, Stargazin, are tractable for mTAG-RFP-T, our red fluorescent protein of choice.
We performed FRET measurements on AMPA receptors harbouring fused blue and yellow fluorescent proteins. Here, the donor and acceptor fluorophores were fused to cytoplasmic sites. Donor lifetime was reduced in the presence of cyclothiazide, a modulator of AMPA receptor activity that binds to extracellular sites. Therefore, fluorescence measurements could reveal electrically silent conformational changes that propagate through the receptor from the extracellular domains to the cytoplasm.

Figure 5 (A) AMPA receptor with yellow and blue fluorescent protein inserts at the insertion sites I6 (pore loop) and I10 (C-tail). Membrane is a grey bar. (B) Cyclothiazide (CTZ) reduced FRET between the I6 and I10 sites, consistent with a separation of these two parts of the receptor. The reduction was specific because it was abolished by the S754Q mutation in the CTZ binding site. (C) Washing in CTZ revealed that the change in FRET was congruent with the functional profile. Adapted from Zacchariassen et al (2016).
We also used the membrane bound anion dipicrylamine (DPA) as a non-fluorescent quencher of YFP and recorded FRET during activation. In this case, we could detect distinct movements in different conformational states - that is, driven by glutamate activation.

Figure 6 (A) Geometry of quenching of YFP at the I6 and I10 sites in GluA2 by dipicrylamine (DPA, 5µM). (B) Simultaneous patch clamp and fluorescence measurement revealed that DPA provoked state- and position-dependent emission. (C) Summary of fluorescence changes. Adapted from Zacchariassen et al (2016).

Measurements of distances between the inserted fluorophores and their displacement from the membrane were combined using a theoretical model that we developed (see below) to give a 2-D map of the intracellular domains during activation and desensitisation.

Figure 7 Locations of the pore loop and carboxy terminus in the AMPA receptor GluA2 in three different functional states. Yellow box represents the insertion in the pore loop (‘I6’ site) and blue box shows the site of the C-terminal insertion (‘I10’ site). ‘P’ indicates a site of palmitoylation. Membrane indicated by a grey bar, and the extracellular side is upper. Adapted from Zacchariassen et al (2016).

Further experiments with other fluorophore insertion positions within the AMPA receptor and other components involved in excitatory synaptic transmission are ongoing.
Theoretical models of fluorophore energy transfer to a membrane quencher

In addition to detecting gating optically, we were able to use DPA in the membrane under voltage control as a spectroscopic ruler. Distance from the membrane plane for each state could be inferred thanks to saturation effects when fluorescent proteins are close to the membrane. We developed several theoretical models of DPA quenching, including a full 3-D Monte Carlo treatment in order to capture anisotropic aspects of energy transfer.

Figure 8 (A) DPA molecules adsorb to both the inner and outer faces of the plasma membrane. Their distribution depends on the applied voltage. (B) When the membrane is hyperpolarised, the extent of YFP quenching at short distances is limited by steric exclusion. (C) By applying scaling and background components, we found a unique solution for the triplet of fluorophore positions at the I6 site in GluA2 according to functional states. (D) For the YFP inserted below the pore domain, the range of movement from active to desensitised states is about 20 Å. Adapted from Zacchariassen et al (2016).

Anisotropy of resonance energy transfer can provide information about fluorophore orientation and physical environment, but can alter detection and interpretation of fluorescence data in unpredictable and undesirable ways. To understand possible anisotropic aspects that could include effects of DPA restriction within the membrane plane and potential conformational restriction of the donor fluorophore due to attachment to the receptor or proximity to the membrane, we devised a general description of the three dimensional system including dipole orientation for both DPA and YFP. The maximal effect of Anisotropy was to produce an effective reduction in the half-maximal energy transfer distance ($R_0$) that was incompatible with our data. One limitation of our approach was that the spheroid geometry of the HEK cell in the fast perfusion flow is expected to average out any anisotropic emission from the donor.
Figure 9 Comparison between a simplified 2-D treatment of YFP quenching by a plane of DPA molecules (left, isotropic case, as in Figure 8) and a full 3-D treatment (right) that includes dipole orientation to account for conformational restraint of the donor fluorescent protein. The overall effect of anisotropy is small but tends to reduce the half-maximal quenching distance ($R_0$), moving the hump of the difference curve towards the membrane, and into a position that is physically unobtainable by a donor with dimensions of YFP. Adapted from Zacchariassen et al (2016).

**High throughput screening**

Using knowledge derived from Aim 2, we have developed a high-throughput screening assay with the FMP screening unit, for extracting optical activity from glutamate receptors. At the present date, the details of this method and ongoing experiments remain scientifically sensitive, and have commercial potential, and so cannot be described here.
Development of the executed work including deviations from the original concept, problems in the project organization or technical execution

In the initial stages it became clear that the labelling of glutamate receptors according to bio-orthogonal chemical handles introduced through the unnatural amino acid incorporation was unworkable using contemporary technologies. The synthesis of the required unnatural amino acids, and the tetrazine-conjugated dye represented a major expense of time and effort and limited us to particular dye labels. The major drawback was that a) either incorporation was not efficient and specific (Bi-cyclo-nonyne-lysine) or b) we did not achieve specific labelling (Norbornene-Lysine) with tetrazine-based probes, despite clear evidence of unnatural amino acid incorporation. Further, given that the project funding was commensurate with a study of more limited scope, we opted to concentrate on genetic encoding of fluorophores, inserted inline within the AMPA receptor primary sequence.

Economic viability of the results, relevance of the work and future plans

The application of optical methods to neuroscience is rapidly increasing in sophistication and scope. This work indicates that native signalling molecules can be endowed with fluorescent proteins that enable fluorescent report of activation and other processes that are electrically silent. Despite their substantial size, the incorporation of fluorescent proteins derived from GFP can be functionally silent and have little effect on expression. The constructs developed here are a proof of principle for targeting any endogenous signalling protein of interest with optical activity.

It is expected that this work will provoke further projects that have commercial scope and potential.

Cooperation partners

Our main collaborator on this project was Anders Kristensen from the University of Copenhagen, Denmark. In 2012, we began testing some constructs from the Kristensen lab that showed ligand dependent FRET. These constructs provided a perfect test for our advanced PCF setup and formed the backbone of methods development. Our results from this collaboration were recently published in PNAS.

Further collaborators included Baron Chanda (Wisconsin, USA), Chris Ahern (Iowa, USA) and Teresa Giraldez (Tenerife, Spain). These collaborators fulfilled mainly advisory roles, assisting us to construct our PCF setup, and with chemistry for synthesis of unnatural amino acids, and labelling of receptors.

Theses

Ljudmila Katchan has written her PhD thesis based on the work described in aim 2. Robert Samhaber (FU Berlin) has written a Master’s thesis based on associated screening experiments at the FMP-Screening unit.

Publication

Zacchariassen LG†, Katchan L†, Jensen AG, Pickering DS, Plested AJR* & Kristensen AS* (2016) Structural rearrangement of the intracellular domains during AMPA Receptor activation PNAS in press †These authors contributed jointly. *Corresponding authors.

Data sharing and security

The data have been presented at several international conferences (GRC Ion Channels, 2016, Andrew Plested invited speaker and GRS Ion Channels, 2016 Ljudmila Katchan, invited speaker, Biophysical Society 2015).

DNA constructs produced from this work will be made freely available. In case of high demand, the constructs will be added to public repositories such as Addgene. Analysis software that were written for these projects is available on request. Primary data will be placed in long term storage through the FMP backup facility.
Press release

Contact- Silke Osswald, FMP-Berlin