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Measuring G protein-coupled receptor signalling in the brain with resonance energy transfer based biosensors

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Activation of a G protein-coupled receptor (GPCR) triggers downstream signalling pathways whose identity is determined not only by the genetic background of the cell, but also by the interacting ligand. Assays that measure endogenous GPCR signalling *in vivo* are needed to specify the intracellular signalling pathways leading to therapeutic *vs.* adverse outcomes in animal models. To this end, genetically encoded biosensors can be expressed *in vivo* with cell type specificity to report GPCR signalling in real time. Biosensor imaging is facilitated by novel microscopic and photometric techniques developed for imaging in behaving animals. The techniques discussed here herald a new wave of *in vivo* signalling studies that will help identify therapeutically relevant signalling, and design functionally selective drugs for neuropsychiatric diseases.

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Introduction

G protein-coupled receptors (GPCRs) are the largest receptor superfamily encoded by the mammalian genome, and regulate physiological functions in every tissue. In the last decade, the use of resonance energy transfer (RET) based biosensors to study GPCR signalling in live cells has dramatically expanded our understanding of GPCR signalling and pharmacology [1,2]. Many GPCRs display ligand-directed signalling, also known as 'functional selectivity' or 'biased agonism' [3]. These terms describe a phenomenon where different ligands acting at the same receptor trigger different sets of downstream effector pathways. We now know that the signalling cascades engaged by a given GPCR depend on several factors, including the identity of the ligand, the cell type and its protein complement, and the extracellular environment. Therefore it is not surprising that observations made in recombinant cell-based assays do not always translate to the *in vivo* context. In light of this complexity, there is a growing demand for tools that would allow endogenous GPCR signalling to be measured *in vivo*. In this short review, we will emphasize the value of measuring intracellular signalling *in vivo* in animals models and the emerging technologies that will make this possible for GPCRs in the brain.

Studying GPCRs *in vivo*: toward more physiologically relevant assays

The discovery that GPCRs can engage multiple signalling pathways has prompted the search for functionally selective ligands that can activate therapeutically relevant signalling while leaving pathways associated with 'on target' adverse effects unaffected [4,5]. Ligands able to dissociate G protein *versus* β-arrestin mediated signalling have been proposed for several clinical purposes. For example a G protein biased agonist at the μ-opioid receptor has completed phase 2 clinical trials for the treatment of post-operative pain [6,7,8], and β-arrestin biased ligands at the dopamine D2 receptor have been proposed for the treatment of schizophrenia [9] and L-DOPA-induced dyskinesia [10]. In addition to G protein *versus* β-arrestin functional selectivity, individual GPCRs may couple to multiple isoforms of Gα [11] and Gβγ [12] subunits and ligand-directed signalling at this level is also of interest.

At present, our ability to rationally design functionally selective ligands is hampered by the limited *in vivo* screening tools available to evaluate endogenous GPCR signalling and identify therapeutically relevant signalling pathways. GPCR signalling depends on a host of factors such that observations made in one experimental system cannot be easily generalized [13]. For example, in the case of the μ-opioid receptor the extent and even direction of bias (*i.e.* preference for G protein *versus* β-arrestin signalling) for a given ligand was dependent on the cell line used, and the expression level of signalling proteins including GRK2 or $G\alpha_{i/o}$[14••]. Another factor that can affect observations of ligand bias in cell-based assays is the kinetics of ligand binding and downstream effector activation [15•]. These examples further illustrate the need for more physiologically relevant experimental systems in order to better understand the physiological and pharmacological significance of functional selectivity.

Several elegant methods have been used to validate biased ligands and study the role of specific signalling

pathways *in vivo*. A simple way to validate potentially biased ligands is to immunoblot tissue taken from drug treated animals in order to identify phosphorylation 'barcodes' associated with the activation of different signalling pathways [16•]. Tissues from drug treated animals have also been analyzed to identify the transcriptomic signatures associated with biased ligands [17•,18•]. However, these *ex vivo* approaches tend to have poor temporal resolution and cell-type specific effects may be lost. Another approach has been to generate intrinsically biased dopamine D2 receptor mutants and express them in mice, thereby distinguishing the role of G proteins and β-arrestins in downstream signalling and in dopamine dependent behaviors [19•,20•]. This technique however is both technically demanding and to date has required receptor overexpression, which risks perturbing endogenous signalling dynamics. Alternatively, genetically encoded RET biosensors can report specific signalling processes in live cells with high spatial and temporal resolution, with minimal impact on endogenous signalling. Recently, RET biosensors have been used to study GPCR signalling in behaving animal models [21••,22••], providing a new tool to study intracellular signalling and agonist functional selectivity *in vivo*.

In vivo imaging with cell type specificity

Over the past decade the parallel development of genetically encoded neuronal activity indicators, *in vivo* gene delivery techniques and fluorescence imaging has made it possible to image neuronal firing at the single cell and circuit level in behaving animals. While these techniques have been applied primarily to the study of neuronal firing dynamics, the tools used can easily be adapted for studying GPCRs *in vivo* with genetically encoded biosensors.

Genetically encoded calcium indicators, and more recently voltage indicators have been used to measure neuronal firing *in vivo*. For example GCaMP type Ca^{2+} indicators have been widely used *in vivo* due to their brightness, high signal-to-noise and dynamic range [23,24]. The most recent variant, GCaMP6 has sufficient sensitivity to permit the imaging of Ca^{2+} transients in subcellular compartments *in vivo* [23,25••]. Voltage indicators, in contrast, can detect sub-threshold voltage changes and membrane hyperpolarizations, and as such offer an attractive alternative to Ca^{2+} indicators [26•]. Genetically encoded voltage sensors have been developed based on voltage-dependent Förster resonance energy transfer (FRET) between fluorescent proteins and rhodopsin voltage-sensitive domains. These FRET-opsin voltage sensors have ultrafast kinetics, high dynamic range and sufficient brightness for imaging *in vivo* [27••,28].

The brain is composed of a heterogeneous mixture of neurons and non-neuronal cells, and even within anatomically defined structures or circuits, neuronal subtypes co-exist. Thus a major challenge is to target the expression of biosensors to a defined cell type of interest, while maintaining the high expression levels required to image in light-scattering tissue. One approach is to engineer transgenic animals expressing a biosensor under the control of a cell type specific promoter [29••]. However, this approach can be costly and time consuming. Alternatively the Cre/lox recombination system offers a flexible way to achieve cell type-specific, robust expression of biosensors or other transgenes [30]. Animals expressing Cre recombinase under the control of a cell type-specific promoter can be crossed with transgenic lines expressing a reporter in a Cre-dependent manner [31]. Alternatively a Cre-dependent virus can be used to deliver the biosensor or other transgene in order to achieve structure- or circuit-specific expression [32]. Hundreds of cell type-specific and region-specific Cre driver lines have now been generated [33,34] and a variety of viral vectors capable of anterograde, retrograde and transynaptic tracing allow biosensor expression to be targeted to specific circuits and cell types [35,36]. Newer methods use multiple recombinases to target cell types based on the intersection of multiple genetic features, thus allowing biosensor expression to be targeted even more precisely to a cell population of interest [29••,37].

A variety of tools have been developed for *in vivo* functional imaging in the brains of behaving animals using one- and two-photon microscopy or photometric recording [38,39,40,41]. For cellular resolution imaging two-photon laser scanning microscopy (TPLSM) remains the gold standard [42]. The use of two-photon excitation provides optical sectioning and minimizes both autofluorescence and phototoxicity which allows cellular resolution imaging to be performed in tissue up to a maximum depth of 1 mm with conventional fluorophores [39], or 1.6 mm with far-red fluorophores [43]. Conventional one-photon techniques lack the optical sectioning capability of TPLSM and are better suited for imaging at the population level [44••]. With either one- or two-photon microscopy a microendoscopic lens or fiberoptic probe must be implanted for imaging in deep brain structures [45,46]. *In vivo* TPLSM is not only technically demanding, but is subject to two important limitations: animals must be head-fixed, limiting their behavioral repertoire [25••,47•,48], and low acquisition frame rates tend to compromise resolution or diminish the field of view [49•]. To circumvent the first limitation, miniaturized one- and two-photon microscopes have been mounted on the head, expanding the repertoire of behavioral tests that can be performed during imaging of small rodents [50,51••,52,53,54]. An alternative approach for functional recording in freely moving animals is to use thin fiber optic cables and time-correlated single photon counting to record 'bulk' fluorescence changes (*i.e.* with no spatial resolution). This population-level photometric approach has been used to record neuronal firing of defined cell

types during behavior [32,55,56••] or in response to pharmacological manipulations [57•,58]. Although photometry does not allow individual cells to be visualized, it does provide several advantages: high sampling rates can be achieved [59] and because the small diameter optic fibers cause minimal damage to surrounding tissue simultaneous recording from multiple brain areas is feasible [60].

Resonance energy transfer (RET) biosensors to study GPCR signalling *ex vivo* and *in vivo*

RET biosensors have been used extensively to characterize GPCR signalling in live cells, with exquisite spatial and temporal detail [1]. With the advent of brighter and more photostable fluorescent proteins recent studies have applied RET biosensors in more physiological models such as tissue sections and live animals [61•,62,63•,64].

GPCR signalling is of course a multi-step process, and biosensors have been designed to study events occurring either proximal to the receptor (*i.e.* receptor conformation changes or G protein activation) or at a more distal level (*e.g.* second messengers, downstream protein kinases). Unimolecular biosensors that report highly amplified signalling such as second messenger production or kinase activity are the most applicable *in vivo* since they provide strong signals and high signal-to-noise. An additional advantage is that these biosensors do not require overexpression of proteins (such as tagged receptors, G proteins or arrestins) which might alter cell physiology. Indeed, FRET based sensors have been successfully combined with one- and two-photon microscopy in organotypic brain slices, in order to reveal levels or activity of cAMP [62], cGMP [63•], PKA [61•,63•] and ERK1/2 [64]. New versions of these biosensors incorporating red-shifted FRET pairs will allow more robust FRET imaging in tissue [65,66,67••]. Imaging with FRET biosensors is based either on the ratio of emission spectra (ratiometric imaging) or on the lifetime of the donor fluorophore (FLIM). FLIM requires only the emission of one fluorophore to be measured and is thus well suited for *in vivo* imaging. A PKA sensor optimized for two-photon FLIM was recently expressed in live animals using a Cre-dependent adeno-associated virus and successfully used to measure PKA signalling with subcellular resolution in single neurons in brain slices [61•].

Two recent papers have shown that FRET based biosensors can indeed be expressed with cell type specificity, and imaged from deep brain regions in behaving animals [21••,22••]. In these studies, transgenic mice were generated with FRET based sensors of PKA or ERK1/2 expressed selectively in either D1 or D2 receptor expressing neurons of the dorsal striatum. A microendoscopic probe attached to a confocal laser scanning microscope was inserted into the striatum, and time-lapse FRET responses were analyzed for both single cells and whole fields of view during pharmacological manipulations or behavioral tests. To our knowledge, these studies mark the first published application of RET biosensors to detect GPCR downstream signalling in real time in behaving animals. These developments now render it feasible to characterize biased agonists acting in the living brain and to discern the physiological relevance of individual signalling pathways.

Conclusion

GPCR signalling dynamics are highly context dependent, highlighting the need for signalling assays that are more physiologically relevant. The requisite technology now exists to begin studying endogenous GPCR signalling in the brain. By combining the genetic targeting and *in vivo* functional imaging techniques described here with behavioral pharmacology, it may soon be possible to identify therapeutically relevant signalling pathways in the brain, to develop novel functionally selective therapeutics.

Conflict of interest statement

We have no conflicts of interest.

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