A genetically encoded fluorescent sensor for \textit{in vivo} imaging of GABA

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Abstract (149 words)

Current techniques for monitoring GABA, the primary inhibitory neurotransmitter in vertebrates, cannot follow transients in intact neural circuits. We applied the design principles used to create iGluSnFR, a fluorescent reporter of synaptic glutamate, to develop a GABA sensor using a protein derived from a previously unsequenced Pseudomonas fluorescens strain. Structure-guided mutagenesis and library screening led to usable iGABASnFR variants (soluble protein: ΔF/F_{max} ~ 2.5, K_d ~ 9 µM, membrane localized protein: ΔF/F_{max} ~ 0.7, K_d ~ 30 µM).

iGABASnFR is genetically encoded, detects GABA release evoked by electric stimulation of afferent fibers in acute brain slices, and produces readily detectible fluorescence increases in vivo in mice and zebrafish. iGABASnFR enabled tracking of: (1) mitochondrial GABA content and its modulation by an anticonvulsant; (2) swimming-evoked GABAergic transmission in zebrafish cerebellum; (3) GABA release events during inter-ictal spikes and seizures in awake mice; and (4) GABAergic tone decreases during isoflurane anesthesia.

Introduction (3000 max, curr 3051 for Intro, Results, Discussion)

γ-Amino butyric acid (GABA) is a ubiquitous inhibitory neurotransmitter, reducing neuronal excitability by receptor-mediated hyperpolarization of membrane potential and shunting of excitatory currents. GABA_A receptors are hetero-pentameric Cl–-conducting channels. GABA_B receptors are hetero-dimeric metabotropic receptors that are coupled to multiple ionic currents by G_{i/o}-proteins, and are more sensitive to GABA than most synaptic GABA_A receptors.

Despite the clinical efficacy of drugs that alter GABAergic neurotransmission, the details of GABA release from neurons remain largely unknown. GABA signaling has traditionally been inferred from a combination of electrophysiology and pharmacology. Direct detection of GABA has required microdialysis followed by derivatization and quantification by HPLC and electrochemistry, but this technique has inherently poor spatiotemporal resolution (on the order of minutes and hundreds of microns).

While genetically encoded fluorescent sensors for calcium have been optimized for imaging neural activity, and sensors for the excitatory neurotransmitter glutamate have been developed, methods for directly imaging GABAergic signaling are lacking. A semi-synthetic sensor with organic fluorophores attached to the GABA_B receptor was reported, but its low affinity for GABA (400 µM) and dependence on two small molecules limit utility. Inhibitory neurotransmission can be inferred from the intracellular chloride concentration, [Cl]_, using Clomeleon. However, [Cl]_ does not change much during inhibition; the sensors conflate the activities of all ligand-gated (GABA, glycine) and voltage-gated Cl channels; and GABA_B receptors are G_{i/o}-coupled receptors that do not directly affect [Cl]_. Thus, direct detection is preferable for inference of GABAergic signaling in general, and is required for mechanistic studies of synaptic input-output transformations, as recently shown with glutamate release and presynaptic Ca^{2+} imaging.

We have generalized the development of genetically encoded sensors from bacterial periplasmic binding proteins (PBPs) by insertion of circularly permuted fluorescent proteins, including a glutamate sensor for in vivo imaging. Here we report development of Intensity-based GABA Sensing Fluorescence Reporter (iGABASnFR) and observe GABA uptake into mitochondria, and GABA release in cultured neurons and acute mouse brain slice. In vivo, we observed bulk
GABA transients in mouse visual cortex, and GABA release synchronized with inter-ictal spikes and seizures in a chemoconvulsant mouse model of epilepsy. In zebrafish we used iGABASnFR to correlate GABAergic signals across the cerebellum to motor output.

Results

Sensor engineering

There are many potential scaffolds for developing a GABA sensor. GABA_A and GABA_B receptors are inappropriate since the GABA-binding sites of the former are at protein-protein interfaces (rather than intramolecular) and the latter is difficult to express heterologously. Furthermore, over-expression of these receptors could alter cellular physiology. In theory, one could redesign the binding site of an existing sensor (e.g. iGluSnFR) to make it specific for GABA. Our attempts to do so failed (data not shown).

GABA is a prominent signaling molecule in plants. The plant pathogen Agrobacterium tumefaciens expresses two periplasmic GABA-binding proteins. We developed a sensor from Atu2422, but it has low (high-μM) affinity for GABA, and orders-of-magnitude higher affinity (low-μM) for alanine and proline (Supp. Fig. 1). Atu4243 has more stringent specificity and higher affinity for GABA (9 μM). Unfortunately, Atu4243 with either cpGFP or cp-superfolderGFP (cpSFGFP) inserted at various locations did not translocate to the membrane surface in cultured cells when cloned into the pDisplay vector (Invitrogen) (Supp. Fig. 2) and was abandoned.

Prior to the structures of Atu2422 and Atu4243 being published, a strain of Pseudomonas fluorescens (ATCC #BAA1781) was reported to take up GABA. We received a culture of this strain (CNG89; a gift from Dr. Catherine Guthrie) and sequenced its genome. We identified “Pf622” as a homologue of Atu4243 (Supp. Fig. 3) and cloned it into pRSET (Invitrogen) for heterologous expression in Escherichia coli. Its affinity was not in the low-nM range as expected, but rather ~110 μM as determined by isothermal titration calorimetry (Supp. Fig. 4). A fluorescent allosteric signal transducer, introduced by attaching an environmentally sensitive fluorophore, JF-585, via maleimide-thiol chemistry to a cysteine mutation at a residue in the hinge of the protein (V278C), resulted in a hybrid sensor with 240 μM affinity for GABA (Supp. Fig. 5). Stopped-flow analysis revealed atypical kinetics. Instead of a single exponential rise in fluorescence upon mixing of protein and GABA, the data is best fit by a double exponential (Supp. Fig. 6a,b), suggesting that multiple steps link GABA binding to fluorescence change (see below). Regardless, the rise time for Pf622.V278C-JF585 binding GABA is longer than it is for iGluSnFR binding glutamate (Supp. Fig. 6c).

Pf622 with cpGFP or cpSFGFP (whose substitution for cpGFP in iGluSnFR improved expression and photostability) inserted at any of several sites translocated to the membrane when cloned into pDisplay and expressed in mammalian cells (Supp. Fig. 7). We inserted cpSFGFP after residue 276 of Pf622 (Fig. 1a, Supp. Fig. 8), optimized binding protein-to-FP linkers, yielding a first-generation sensor (L1-LA/L2-AN), and solved its crystal structure in the unliganded/open state (Fig. 1b, Supp. Fig. 9, PDB accession 6DGV). The homology of Pf622 to Atu4243, for which both the unliganded/open and bound/closed structures are available, allowed us to test mutations to the hinge region of Pf622 expected to allosterically modulate affinity. Hinge mutation F101L increased affinity 10-fold (Supp. Fig. 10).
residue in Pf622 (N260A) that potentially interacted with cpSFGFP, and was also a potential glycosylation site, increased brightness in HEK cells (Supp. Fig. 11). Re-optimization of the linkers (L1-LAQVR, L2-AN), plus the SFGFP mutation F145W, resulted in a variant, called iGABASnFR, worthy of further characterization. iGABASnFR is Pf622 with cpSFGFP inserted after residue 276, with optimized linkers (L1-LAQVR, L2-AN), a hinge mutation (F101L), an interface/expression mutation (N260A), and an additional GFP mutation (F145W).

In vitro characterization

Purified iGABASnFR shows a maximum \( \Delta F/F \) of \( \sim 2.5 \) and a \( K_d \) for GABA of \( \sim 9 \mu M \) (Supp. Fig. 12a). It shows no affinity for other amino acids except very low affinity for glycine (500 \( \mu M \)), alanine (830 \( \mu M \)), and histidine (2.4 mM) (Supp. Fig. 12b). It has no affinity for similar four-carbon metabolites fumarate, malate, oxaloacetate, nor succinate (data not shown). As we characterized iGABASnFR in cellular systems, we made additional efforts to increase its affinity and \( \Delta F/F \). Mutation of a GABA-binding pocket residue to its Atu4243 homologue, F102Y, had minimal effect on GABA affinity and decreased \( \Delta F/F \). Meanwhile, the mutation Y137L increased \( \Delta F/F \), at the cost of poor expression. The double mutant F102Y.Y137L expressed well, exhibited higher \( \Delta F/F \) (3.5 vs. 2.5 for iGABASnFR) but lower affinity (70 \( \mu M \) vs. 9 \( \mu M \) for iGABASnFR; Supp. Fig. 12a). Addition of F102G to iGABASnFR (without changes to Y137) showed higher \( \Delta F/F \) (4.5) and affinity of 50 \( \mu M \). We created a non-binding variant by mutating a binding pocket residue (R205A). These variants were further characterized.

We profiled the iGABASnFR variants for binding to GABAergic drugs (Supp. Fig. 12c-f). Baclofen (\( \beta \)-(4-chlorophenyl)-GABA) affected all three iGABASnFR variants tested (as well as the negative control cpSFGFP, Supp. Fig. 12c), whereas vigabatrin (\( \gamma \)-vinyl-GABA, Sabril®) bound to only iGABASnFR.F102G. All three variants are also high-affinity sensors for muscimol (Supp. Fig. 12c-f, pink traces). Importantly, all other drugs showed much weaker binding to the sensors than GABA, allowing them to be used in conjunction with iGABASnFR imaging. The absorbance, excitation, and emission profiles of iGABASnFR show that fluorescence changes result from increased excitation at 485 nm when GABA is bound, and is reflected in the 2-photon cross sections of all three iGABASnFR variants (Supp. Fig. 13). The iGABASnFR variants are more sensitive to pH in the unbound state than the GABA-bound state (Supp. Fig. 14). Unlike other PBP-based sensors, the rate of fluorescence change for iGABASnFR as determined by stopped-flow kinetics was largely independent of the concentration of ligand used (Supp. Fig. 15).

GABA processing in mitochondria

After being transported into mitochondria (by an as yet unidentified transporter), GABA is degraded by GABA transaminase (GABA-T). Inhibition of GABA-T elevates synaptic levels of GABA\(^{22} \) and, of clinical significance, the GABA-T inhibitor vigabatrin is a prescribed antiepileptic\(^{23} \). We transfected cultured prostate cancer cells (LNCaP) with iGABASnFR.F102Y.Y137L (which does not bind vigabatrin; Supp. Fig. S12f), fused to an N-terminal mitochondrial matrix targeting motif from Cox8a (mito-iGABASnFR.F102Y.Y137L). Basal fluorescence was very low (Supp. Fig. 16), consistent with the observation that these cells contain little GABA\(^{24} \). Exogenous GABA increased fluorescence within the mitochondria as expected (Supp. Fig. 17a). GABA treatment increased fluorescence (Supp. Fig. 17b), reaching equilibrium after 48 hours, presumably reflecting both increasing mito-iGABASnFR expression.
and continued GABA uptake. The [GABA] dependence of mitochondria-localized iGABASnFR is hyperbolic (Supp. Fig. 17c). Vigabatrin treatment tends to increase iGABASnFR fluorescence in mitochondria (Supp. Fig. 17d). Transfection of mito-iGABASnFR.F102Y.Y137L expressing LNCaP cells with a plasmid encoding glutamic acid decarboxylase 1 (GAD67) resulted in increased endogenous GABA synthesis (Supp. Fig. 17e).

**Neuronal culture characterization**

HEK cells transfected with iGABASnFR variants cloned into a modified version of pDisplay lacking the HA tag (pMinDis) showed good membrane localization (Supp. Fig. 11). Primary culture of hippocampal neurons from P0 newborn rats infected with AAV2/1. hSynapsin1.iGABASnFR showed good membrane localization and brightness at 14 DIV, but the +F102G and +F102Y.Y137L variants were noticeably dimmer, and showed intracellular accumulation (Supp. Fig. 18). Perfusion of neuronal cultures with GABA revealed affinities for GABA similar to, but slightly weaker than, purified protein (iGABASnFR, 30 µM; +F102G, 42 µM; +F102Y.Y137L, 106 µM; Supp. Fig. 19).

Electrical field stimulation (50 Hz) of the cultured neurons produced fluorescence changes from single stimulus-evoked action potentials and increased until plateauing after 40 stimuli (Fig. 1c). The fluorescence change of iGABASnFR was restricted to the membrane (Fig. 1d), like SF-iGluSnFR, but with an order of magnitude lower maximal response (Supp. Fig. 20a). The iGABASnFR response within a field of view was also less uniform than SF-iGluSnFR (Supp. Fig 20b-e), consistent with GABAergic neurons representing a smaller fraction (~10%) of the neuronal population in hippocampal culture25.

**Hippocampal acute slice**

To confirm that iGABASnFR remains functional in organized brain tissue, we examined hippocampal acute slices prepared from mice injected with AAV2/1. hSynapsin1.iGABASnFR (see Supp. Methods). Although it was challenging to reliably record GABA release at individual synapses (data not shown), which was possible with iGluSnFR12,13, iGABASnFR displayed excellent signal-to-noise ratio following paired-pulse extracellular field stimulation in stratum radiatum. iGABASnFR responses to extracellular GABA transients were recorded from apical dendritic segments traced from the CA1 pyramidal cell body (Fig. 2a). Raising extracellular calcium increased fluorescence response amplitude (Fig. 2b,c), consistent with increased presynaptic release probability26.

**Somatosensory cortex acute slice**

We also probed the effect of iGABASnFR expression on postsynaptic signaling. Since the sensor is derived from a bacterial binding protein, it is unlikely to interact with GABA_A or GABA_B receptors. In slices of mouse somatosensory cortex, whole-cell patch-clamp recordings of uninfected and AAV.hSynapsin1.iGABASnFR-infected cells showed resting potentials near the expected -70 mV (Supp. Fig. 21a). Electrically evoked inhibitory and excitatory post-synaptic currents (IPSCs and EPSCs) in cells expressing iGABASnFR were statistically indistinguishable from uninfected cells (Supp. Fig. 21b,c), although there was high variability. The amplitudes of spontaneous PSCs showed similar distributions (Supp. Fig 21d). The frequency of spontaneous IPSCs was slightly higher (p = 0.038) in iGABASnFR expressing cells (Supp. Fig. 21e), but we
suspect this is a consequence of cell heterogeneity that would be normalized with higher sampling.

### Volume detection of GABA in mouse visual cortex

In the visual cortex, GABAergic inhibition\(^{27}\) and disinhibition\(^{28}\) contribute to context-dependent processing and can enhance the distinctness of sensory responses. Hypnotic effects of anesthetics have been associated with GABAergic transmission\(^{29}\). Volatile anesthetics decrease both glutamatergic and GABAergic synaptic transmission in cortex, with stronger effects on glutamate currents, leading to net inhibition of activity\(^{30}\). Anesthetics can therefore be used to determine whether iGABASnFR can detect decreases in cortical GABA release relative to basal rates, which may reflect tonic\(^{31}\) or ongoing phasic release.

To directly monitor cortical GABA in a time- and depth-resolved manner, we injected AAV2/1.\(^{hSynapsin1.iGABASnFR}\) into mouse primary visual cortex (V1) and measured fluorescence (with 1030 nm excitation) in a 150 µm x 150 µm x 450 µm deep volume over the course of 40 minutes. After 8 minutes, isoflurane anesthesia was administered, which is expected to suppress inhibition. Fluorescence decreased 20% during anesthesia treatment and slowly returned to baseline during recovery (Supp. Fig. 22a,b). In parallel experiments with the non-GABA-binding negative control (R205A), fluorescence decayed slowly over the observation period, with no recovery after removal of anesthesia (Supp. Fig. 22c).

### In vivo mouse model of epilepsy

Having established that iGABASnFR robustly detects synchronous release events, we tested it in small-scale, high-resolution, 2-photon neuropil imaging experiments. Focal neocortical epilepsy is typically accompanied by abnormal background electrocorticogram (ECoG) activity, including frequent inter-ictal spikes (occurring between seizures), which are dominated by GABAergic activity\(^{32}\). How seizures intermittently arise from this background is poorly understood.

Numerous mechanisms have been proposed, although the strongest evidence supports a failure of feed-forward inhibition\(^{33,34}\). This could, in principle, arise because interneurons enter a state of depolarization block in the face of over-excitation and fail to release GABA, or because shifts in the chloride reversal potential in principal cells resulting from intense GABA release render GABA\(_A\) receptor-mediated inhibition ineffective\(^{35}\). These hypotheses are not mutually exclusive, but important insights could be provided by imaging extracellular GABA during inter-ictal spiking and seizures. If extracellular GABA transients collapse rapidly at the onset of seizures, this would argue for depolarization block of interneurons, or another mechanism by which interneurons fail to be recruited or release GABA.

AAV2/1.\(^{hSynapsin1.iGABASnFR}\) (or variants) was injected into layer 2/3 of mouse V1 (Fig. 3a-d). Intracortical pilocarpine injection elicited focal epileptiform activity lasting up to 60 minutes\(^{36}\). This consisted of periods of inter-ictal spiking alternating with polyspikes and ECoG seizures lasting up to 1-2 minutes. iGABASnFR fluorescence reliably showed transients time-locked to inter-ictal spikes (Fig. 3e-g). Comparing across the different sensors, iGABASnFR and iGABASnFR.F102G gave the largest ΔF/F. iGABASnFR.F102Y.Y137L gave lower ΔF/F, and iGABASnFR.R205A was unresponsive (Fig. 3h).
During periods with stereotyped polyspikes, fluorescence peaks separated by <1 sec could be resolved with iGABASnFR.F102G (Fig. 4a,b). Discrete peaks coinciding with low-frequency ECoG components could still be seen during focal seizures lasting up to 1 minute (Fig. 4c). Fluorescence gradually decreased during seizures, slowly recovering during intervals of electrographic silence between seizures (Fig. 4c,d). As this slow decrease was not seen during inter-ictal events and polyspikes (Fig. 3e and Fig. 4a,b), it cannot be attributed to photobleaching. It is, however, consistent with iGABASnFR acidification (Supp. Fig. 14). Extracellular acidification during seizures has been abundantly documented, and has been proposed to contribute to seizure termination. The decrease in fluorescence (starting 1 s after the onset of the seizure, Fig. 4d) yielded a mono-exponential decay time constant of approximately 12.1 s, similar to the time constant for pH changes in astrocytes during seizures (~14.7 s, ref. 38). Fluorescence increased during periods of electrographic silence between seizures, consistent with pH return-to-baseline. (A mono-exponential curve fit from the end of one seizure to the start of the next (average time interval around 8.2 s) yielded a time constant around 16 s). Although this account of changes in fluorescence is consistent with a surge in GABA and changes in extracellular pH, further experiments using a GABA-insensitive pH indicator such as pHluorin, or an improved, pH-insensitive GABA sensor, would help to distinguish the two phenomena.

GABA in zebrafish cerebellum

As a final demonstration of iGABASnFR in vivo, we observed GABA transients in Danio rerio (zebrafish) cerebellum using light-sheet imaging in a fictive model of swimming (Fig. 5a). In zebrafish, fictive swimming triggers robust activation of GABAergic cerebellar Purkinje cells. We generated a transgenic zebrafish expressing iGABASnFR.F102Y.Y137L (available before iGABASnFR.F102G was identified) under the elavl3/HuC pan-neuronal promoter (Fig. 5b). During swim bouts, we observed reliable fluorescence changes in cerebellar neuropil regions containing cells onto which GABAergic Purkinje cells project (Fig. 5c,d, Supp. Video 1). Changes in fluorescence peaked roughly 200 msec. after swimming onset (Fig. 5e), with this region producing >8% fluorescence increases. This result indicates that iGABASnFR can reliably detect local GABA transmission that may play a critical role in motor control.

Discussion

To understand neural circuit function, numerous specific inputs onto neurons must be disentangled from their integrated output. Current sensor technologies, namely SF-iGluSnFR, GCaMP6 and GCaMP7, and jRGECO are sensitive enough to allow reliable detection of excitatory synaptic transmission, excitatory post-synaptic currents, and action potentials. Indicators for inhibitory synaptic transmission and inhibitory post-synaptic currents have lagged far behind. iGABASnFR offers the best performance of existing GABA indicators, and is the only one to be completely genetically encoded, facilitating in vivo use. Calcium and glutamate sensors have been iteratively optimized, both to improve overall performance and to match to precise requirements of specific settings. We expect further development of iGABASnFR, with altered affinity, kinetics, and improved signal-to-noise ratio. Different colors of iGABASnFR could also be developed, allowing simultaneous imaging in orthogonal chromatic channels, as has been done with iGluSnFr. More sensitive indicators will be required to facilitate single-spine GABAergic imaging and the separation of phasic from tonic signaling.
It is important to note the value of spatially resolvable readout of synaptic GABA release given the highly organized, cell type-specific nature of GABAergic innervation onto pyramidal cells. Imaging in different cellular compartments enables high-throughput study of GABA release from different populations of interneurons. Combined with patch-clamp electrophysiology and 
Ca\textsuperscript{2+} imaging with red-shifted indicators, iGABASnFR could allow direct study of local signaling requirements underlying important physiological phenomena such as depolarization-induced inhibition suppression.

In our data, GABA release does not collapse completely during seizures. However, our experiments do not rule out partial failure of GABA release when seizures start, due either to exhaustion of releasable GABA vesicles or to depolarization block in a subset of interneurons. Indeed, we imaged very superficial cortex, restricting the observation of GABA release to pyramidal neuron dendrites and layer 1 interneurons. Ultimately, to understand the role of GABA during seizures, more extensive experiments involving different interneuron types, simultaneous glutamate imaging, and controlling for pH is needed.

We hope that the demonstrated use of iGABASnFR in models of epilepsy and in the cerebellum of fictively behaving zebrafish will help elucidate the role of GABA in various circuits, during development, and in disease states. Aberrations in GABAergic signaling occur in Alzheimer's disease, Parkinson's, Huntington's, schizophrenia, and autism/ Rett syndrome.

iGABASnFR imaging in appropriate animal models will facilitate greater understanding of these mechanisms. iGABASnFR could also allow easy screening of candidate transporters such as the vertebrate mitochondrial GABA transporter. Applications outside of neuroscience are also possible, such as separating the critical roles of GABA in plant metabolism and signaling.

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Author contributions


Code availability
All analysis code used in this study is available upon request.

**Competing financial interests**

The authors declare no competing financial interests.


**Figure Legends**

**Fig. 1.** Sensor design and characterization in neuronal culture. a) Schematic of the primary sequence. *White:* IgG secretion signal (which gets cleaved). *Orange and pink:* GABA-binding protein Pf622, colored according to domain assignment in (b). *Green:* circularly permuted SuperFolderGFP. *Blue:* Myc epitope tag. *Red:* PDGFR transmembrane domain. b) Open, unliganded structure of a preliminary version of iGABASnFR. Grey spheres indicate approximate position of GABA, based on homology to the closed-liganded structure of Atu4243. Note that the transmembrane domain is included for illustrative purposes only, and was not part of the soluble structure. c) Averaged response (n=3 replicates, ± s.d.) of an ROI selected in a region of maximal ∆F/F to multiple stimuli (50 Hz). Black to light grey: 1, 2, 5, 10, 20, 40 field stimuli. d) Wide-field fluorescence and fluorescence difference images of iGABASnFR (top) and iGABASnFR.F102G (bottom) expressed in neuronal culture. ∆F/F heat map is shown for the maximum response to 10 field stimuli. Scale bar is 20 µm. Response was similar in 5 repetitions.

**Fig. 2.** Recording of stimulus-evoked extracellular GABA transients using iGABASnFR transfected acute brain slices. a) Experimental setup: concentric bipolar stimulation electrode (Stim.) placed in hippocampal CA1 stratum radiatum. Paired pulse stimulation (200 µsec pulse width) was given with a 300 msec inter-stimulus interval (1st stimulation at 2 seconds). At the same time the field EPSP was recorded in proximity to the imaging ROI and used to calibrate the stimulation amplitude (half-maximal). The segment, arising from the green cell visible in stratum pyramidale, was scanned under 2-photon excitation (910 nm) using a frame scan across a dendrite of interest (ROI; white box in a). b) *Left:* zoomed in image of ROI. Centre line and arrows to indicate region for X axis integration for linescan images. *Right:* Mean linescan of 10 stimulation trials recorded with a 30 second interval in 2 mM extracellular [Ca²⁺] (top) and 4 mM [Ca²⁺] (bottom). Dashed lines indicate onset of extracellular stimulation. Extracellular Ca²⁺ concentration was raised to increase pre-synaptic GABA release probability. c) Overlaid iGABASnFR response averages. blue, 2 mM Ca²⁺; red, 4 mM Ca²⁺. Black line represents a concurrently recorded field EPSP (fEPSP).

**Fig. 3.** iGABASnFR response during inter-ictal spiking. a) Experimental design. b) iGABASnFR expression in transduced area imaged after the end of the experiment, using immunofluorescence microscopy. Scale bar: 100 µm. c) iGABASnFR expression *in vivo* in cortical neuron processes 100 µm below the pia. Scale bar: 20 µm. d) Average iGABASnFR fluorescence image during inter-ictal activity. Scale bar: 50 µm. e,f) Simultaneous fluorescence (averaged in green square in d) and inter-ictal ECoG. Grey bar indicates the enlargement window in f.g). Time-course of iGABASnFR fluorescence and inter-ictal ECoG averaged from 68 events in one experiment. h) Comparison of average fluorescence transients obtained with different sensors. Similar results were observed in two additional animals.

**Fig. 4.** GABA sensor behavior during polyspikes and seizures. a) Trains of polyspikes (ECoG, bottom) evoked by pilocarpine and simultaneous iGABASnFR.F102G fluorescence (top). Similar results were observed in two additional animals. b) Average time-course of sensor fluorescence and ECoG during polyspike (mean ± s.d. indicated as shaded area from 5 seizures in one animal). c) iGABASnFR.F102G fluorescence during seizures. Note that the envelope of the fluorescence transients gradually decreases during seizures (mono-exponential decay time constant ~12.1 sec.), and then slowly recovers during periods of electrographic silence before the return of seizure activity (mono-exponential time constant ~16.0 sec.). This is a single
observation due to the sporadic nature of seizures. d) Average (black line) time course of sensor
fluorescence and ECoG during seizures (mean of 4 seizures, ± s.d. indicated as shaded area).

Fig. 5. GABA response measured with iGABASnFR.F102Y.Y137L fluorescence in a fictive
model of swimming in zebrafish larvae. See also Supp. Video 1, in which changes in
fluorescence within region (iii) are visible. a) Schematic illustration of the setup. An
immobilized zebrafish is placed under the light-sheet microscope and the fictive motor signals
from its tail are recorded during the imaging session. Visual stimuli below the fish move forward
(arrows) to induce swimming. b) Light sheet image of zebrafish cerebellum expressing
iGABASnFR.F102Y.Y137L under the elavl3 promoter. Three regions of interest (i, ii, iii) within
the neuropil (excluding cerebellar cell bodies) are indicated by dotted lines. Bright, clipped
regions at the top are optic tectum. c,d) Forward motion of visual stimuli (white boxes), fictive
motor signals (grey) and fluorescent signals from regions of interest indicated in (b). Traces in
dotted box in (c) are expanded in (d). e) Motor signals (left) and fluorescence changes (right)
averaged across 5 swim events. Shadows represent s.e.m. across swim events. Data shown is
from a single fish.
Online methods

Sequencing of the Pseudomonas fluorescens strain CNG89 genome

Genomic DNA was prepared using a QIAamp DNA mini kit (Qiagen). Genomic DNA was sequenced using a combination of Ion Torrent PGM and Illumina HiSeq technologies and assembled using Spades software with default parameters. Draft genome assembly (131 scaffolds; 141,337bp N50) was sufficient to facilitate tblastn of the Agrobacterium tumefaciens ATu4243 sequence to prioritize putative GABA-binding proteins. Complete genome assembly is ongoing and will be published elsewhere.

Cloning & mutagenesis

The gene for Pf622, lacking the periplasmic leader sequence, was amplified by PCR and cloned into pRSET-A (Invitrogen) by BamHI/EcoRI digest. Silent mutations were made to remove an internal PstI restriction site. The Pf622 gene was then re-cloned into a derivative of pRSET that has the N-terminal affinity and epitope tags replaced by a Arg-Ser encoding BglII site and a C-terminal Leu-Gln-His6 sequence (with PstI encoding Leu-Gln). This pRSET derivative was created to facilitate downstream cloning of iGABASnFR variants into AAV vectors. Genes for Pf622-cpSFGFP insertions were made by overlapping PCR.

Mutant screening

Mutations to iGABASnFR variants were made by the uracil template-based method. Mutations were initially targeted to the junction of Pf622 and cpSFGFP and expanded to include other residues as the sensor was improved. Mutated plasmids were transformed into T7 express cells (New England BioLabs), plated on LB+100 µg/mL ampicillin agar plates and grown overnight at 37°C. Colonies were picked into 2 mL 96-well plates containing 0.9 mL auto-induction media with 100 µg/mL ampicillin and grown overnight with vigorous shaking (400 RPM) at 30°C. Bacterial pellets were collected by centrifugation (3000g), and resuspended in 0.5 mL PBS by vortexing to wash away endogenous GABA, and pelleted again. The wash procedure was repeated five times, and pellets were frozen overnight at -20°C. Frozen bacterial pellets were then lysed by addition of 1 mL PBS and vortexing. Cellular debris was pelleted by centrifugation and clarified lysate removed for fluorescence assays by pipetting.

The green fluorescence (Ex 485 nm, Em 515 nm, 5 nm bandpass) of 100 µL of clarified lysate was measured in a Tecan Infinite M1000 Pro plate-reading fluorimeter. GABA was added to a final concentration of 1 mM and fluorescence measured again. Variants with increased ΔF/F over the starting construct were immediately re-assayed to estimate binding affinity, and winners were streaked out on agar plates, re-isolated, re-assayed, and sequenced.

Protein expression, purification, and in vitro characterization

Final variants of iGABASnFR variants were expressed as above, but on a larger scale (0.5 L) and pelleted by centrifugation, resuspended in PBS, and frozen overnight. Cells were lysed by thawing and sonication. Lysate was clarified by centrifugation at 35,000g and purification by immobilized metal affinity chromatography (IMAC) on a 5 mL Fast Flow Chelating Sepharose column (GE Life Sciences) with an elution gradient from 0 to 200 mM imidazole over 120 mL. Fluorescent fractions were pooled, concentrated by spin concentrator, and dialyzed 5x into PBS. Equilibrium binding affinities were determined by titration with serial dilutions of GABA (or
other compounds) into 0.2 µM protein solution in PBS. Kinetics of binding were determined by mixing equal volumes of 0.2 µM protein with varying concentrations of GABA in an Applied Photophysics SX20 stopped flow fluorimeter with 490 nm LED excitation and 510 nm long pass filter. Circular dichroism was measured with 20 µM protein in 0.1x PBS in an Applied Photophysics Chirascan with 1 sec data collection periods and 300 sec settling time at each temperature point. 2-photon cross sections were collected for 1 µM solutions of protein in PBS with or without 10 mM GABA, excited by pulses from a mode-locked Ti:Sapphire laser (Chameleon Ultra, Coherent, 80 MHz, 140-fsec pulse width, 1 mW power at the focus). Emission was detected by an avalanche photodiode (PDM Series, Micro Photon Devices) with a 550 nm filter (88-nm bandpass).

Prostate cell measurements

LNCaP cells were seeded in each well of a 96-well plate at a density of 25,000 cells/well in triplicate for each condition. Fluorescence was quantified by the IncuCyte ZOOM live-cell imaging microscope system (Sartorius), and analyzed using the IncuCyte ZOOM 2016B edition software. Four fields of view, predetermined by the software, were imaged for each well of the 96-well plate, every 4 hours. The total object integrated intensity values were compiled for each well at each time point. No ROIs were selected; instead, collected fields were quantified in their entirety. To determine total object integrated intensity values, cells were analyzed by the “top hat” background subtraction algorithm to subtract non-uniform background (Essen BioScience, IncuCyte Background Fluorescence Technical Note). Radius values for fluorescence masking were calibrated to 1.3x the radius of the largest cell-associated fluorescent object in the representative image set. Fluorescence (arbitrary units) values of images from control wells (i.e., untreated iGABASnFR transfected for GABA/vigabatrin experiments; iGABASnFR + empty vector transfected for GAD1 experiments) were thresholded to display a fluorescent object count of zero at the time of first scan. The above analysis parameters were applied across all images within an experiment. Fluorescence values for transfection controls (empty vector, i.e., no mito-iGABASnFR) were less than 0.1% but were nevertheless subtracted at each time point for the corresponding treatment conditions.

Primary rat hippocampal neuron cultures

A mixed cell culture (neurons and glia) was prepared from Sprague-Dawley rat pups (Charles River Laboratories). Briefly, P0 pups (either sex) were decapitated, and the brains were dissected into ice-cold neural dissection solution (NDS, 10 mM HEPES (Sigma) in HBSS (Invitrogen), pH 7.4). Cortical tissue was dissected and cut into small pieces to facilitate enzyme digestion. Cortex pieces were transferred using a large bore pipette into a 15 mL conical tube and incubated with enzyme digest solution (Papain, Worthington Biologicals) at 37°C for 30 min. After 30 min., the enzyme solution was removed, and Plating Media (MEM media containing 10% FBS) was added and tissue pieces were triturated resulting in mostly single cells. The cell suspension was filtered using a 45 µm filter. The filtered cell suspension was centrifuged, and the resulting cell pellet was re-suspended with Plating Media and counted. Cells were plated onto coverslips coated with poly-D-lysine (Sigma) and kept at 37 °C, 5% CO2 in Plating Media. For viral infection, 1 µL of 1E13 Genomic Copies/mL (GC/mL) was diluted into NbActiv4 and added on DIV3, with 50% of the media exchanged for fresh media every 4 days after that.
Imaging was performed on DIV14. Growth media was replaced with an imaging buffer containing 10 mM HEPES, 145 mM NaCl, 2.5 mM KCl, 10 mM D-glucose, 2 mM CaCl₂, 1 mM MgCl₂, pH 7.4. Images were collected on a Nikon Eclipse Ti2 wide-field fluorescence microscope with a Plan Apo 20x/0.75 NA objective. Fluorescence was excited with a 470 nm LED and emission collected through a 525 nm (36 nm bandpass) filter. Images were collected at 50 Hz. Field stimulation was achieved with a WPI stimulus isolator, 1 msec, 90mA, 50 Hz, platinum bar electrodes held in place with a bespoke holder.

**Hippocampal slice**

All animal procedures were conducted in accordance with the European Commission Directive (86/609/ EEC) and the United Kingdom Home Office (Scientific Procedures) Act (1986). Young C57BL/6J mice (3 - 4 weeks of age) male and female, were anaesthetised using isoflurane (5% induction, 1.5 - 2.5% v/v). Upon loss of pedal withdrawal reflexes, the animal was secured in a stereotaxic frame (David Kopf Instruments, CA, USA). Perioperative analgesics were administered (subcutaneous buprenorphine, 60 µg kg⁻¹) and the scalp was shaved and disinfected using three washes of topical chlorhexidine. A small midline incision was made and the skull was exposed. A craniotomy was performed over the right hemisphere using a high-speed hand drill (Proxxon, Föhren, Germany) equipped with a 0.4mm circular drill burr. The location overlaid the medial hippocampus (Stereotactic coordinates were 60% of the anterio-posterior distance from Bregma to lambda and 2.5 mm lateral to midline). Once exposed, a warmed aCSF variant (cortex buffer; in mM, 125 NaCl, 5 KCl, 10 HEPES, 10 glucose, 2 CaCl₂, 2 MgSO₄) was applied to the skull and cortical surface throughout the procedure.

Pressure injections of AAV2/1.hSynapsin.iGABASnFR (totalling 0.1 - 1 x 10¹⁰ genomic copies in a volume not exceeding 200 nL) were carried out using a pulled glass micropipette stereotactically guided to a depth of 1.3 mm beneath the cortical surface, at a rate of approximately 1 nL sec⁻¹. The total injection volume was delivered in three steps, reducing depth by 100 µm at each step. Once delivery was completed, pipettes were left in place for 5 minutes before being retracted. The surgical wound was closed with absorbable 7-0 sutures (Ethicon Endo-Surgery GmbH, Norderstedt, Germany) and the animal was left to recover in a heated chamber. Meloxicam (subcutaneous, 1 mg kg⁻¹) was subsequently administered once daily for up to two days following surgery. Mice were killed by transcardial perfusion with ice-cold sucrose-enriched slicing medium (in mM, 105 sucrose, 60 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 15 glucose, 1.3 ascorbic acid, 3 Na pyruvate, 0.5 CaCl₂ and 7 MgCl₂, saturated with 95% O₂ / 5% CO₂) after a 2 - 4 week incubation period and acute hippocampal slices were prepared for imaging and electrophysiological recordings as below.

Acute 350 µm thick hippocampal slices were obtained from 5-7-week-old C57BL/6J mice (Envigo, UK), as specified, in full compliance with national guidelines on animal experimentation. Slices were prepared in an ice-cold slicing solution described below, stored in the slicing solution at 34°C for 15 min before being transferred to a submersion chamber for storage in an extracellular solution containing (in mM) NaCl 119, KCl 2.5, MgSO₄ 1.3, NaH₂PO₄ 1, NaHCO₃ 26, CaCl₂ 2, glucose 10 (osmolarity adjusted to 295–305 mOsM with glucose). All solutions were continuously bubbled with 95% O₂/5% CO₂. Slices were allowed to rest for at least 60 min before recordings started.
For imaging of evoked GABA transients, we used a Femtonics Femto3D-RC imaging system, integrated with patch-clamp electrophysiology (Femtonics, Budapest) and linked on the same light path to two femtosecond pulse lasers MaiTai (SpectraPhysics-Newport) with independent shutter and intensity control as previously described. Cells were first identified as iGABASnFR-expressing using two-photon imaging at 910 nm and dendrites were followed from the CA1 pyramidal cell soma into stratum radiatum. Once a suitable dendritic segment was found a concentric bipolar stimulating electrode was lowered into position ~100 microns distant on the axis of the Schaffer collaterals in stratum radiatum. Dendritic iGABASnFR responses were recorded with a frame scanning ROI placed over a dendritic segment. Scans of 3.5 seconds duration were made at a sampling frequency of ~200 Hz with 2P excitation at 910 nm. During recording iGABASnFR responses were evoked by paired 100 μsec constant-current electrical stimulation of an amplitude that evoked a ~50% maximal field EPSP; recorded though a locally placed 1.5–2 MΩ resistance glass electrode filled with aCSF. Recorded scan data were analysed using Femtonics MES and traces expressed as ΔF/F.

**Brain slice electrophysiology**

All animal procedures were approved by the Institutional Animal Care and Use and Institutional Biosafety Committees at the HHMI Janelia Research Campus. Mice of either sex (n=4, C57BL/6J, Charles River, 4 weeks old) were bilaterally injected in the primary somatosensory cortex (1.2 mm posterior, 2.25 mm lateral, relative to Bregma) with 40 nL of AAV2/1.hSynapsin1.iGABASnFR (1E13 GC/mL) at two depths (300 and 600 µm). After 2-4 weeks, mice were deeply anesthetized with isoflurane, and the brain was quickly removed and transferred to an ice-cold sucrose solution containing (in mM) 150 Sucrose, 4 NaCl, 4 KCl, 1.25 NaH2PO4⋅H2O, 7 MgCl2, 25 NaHCO3, 10 D-Glucose, 0.5 CaCl2⋅2H2O (pH 7.3-7.4, 300-310 mOsm). Coronal brain slices were cut (300 µm thick) with a vibrating tissue slicer (VT 1200S, Leica Microsystems) and subsequently incubated at 37°C for 1 hour in recording solution consisting of (in mM) 124 NaCl, 2.5 KCl, 1.25 NaH2PO4⋅H2O, 1.3 MgCl2, 26 NaHCO3, 10 D-Glucose, 2 CaCl2⋅2H2O, 1 Na-Ascorbate (pH 7.3-7.4, 290-300 mOsm). The sucrose and recording solutions were constantly bubbled with 95% O2/5% CO2. Slices were then transferred to a custom-machined recording chamber, perfused with room-temperature recording solution and held down with a slice anchor (SHD-41/10, Warner Instruments).

Cells expressing iGABASnFR were identified on an upright epifluorescent microscope (Olympus BX-51 WI) equipped with an LED illumination system (470 nm, pE-2, CoolLED). Whole-cell voltage clamp recordings were performed with a Multiclamp 700b (Molecular Devices) amplifier. Patch-clamp pipettes were pulled on a horizontal puller (P-97, Sutter Instruments) to a tip resistance of 4 to 8 MΩ and filled with internal solution containing (in mM): 115 Cs-methanesulfonate, 15 CsCl, 3.5 Mg-ATP, 5 NaF, 10 EGTA, 10 HEPES, 3 QX-315 (pH: 7.3-7.4, 280-290 mOsm). For membrane voltage measurements in current clamp, we used 130 K methanesulfonate, 1 MgCl2, 1 Mg-ATP, 5 NaCl, 1 Na-ATP, 10 HEPES, 14 Phosphocreatine (pH: 7.3-7.4, 280-290 mOsm). Synaptic responses were evoked with short square pulses (0.1 ms duration, 10 s inter-stimulation interval) delivered to a bipolar stimulating electrode (TM33CCNON, World Precision Instruments) through a stimulus isolator (A365, World Precision Instruments). Pulse amplitude was set such that single evoked PSCs were reliably excited with minimum current (range: 0.3-0.6 mA). Series resistance was compensated.
at least 60%. Evoked EPSCs were recorded while holding the cell at -70 mV and IPSCs at 0 mV. Ten stimulus responses were recorded and averaged for each cell.

Spontaneously occurring excitatory and inhibitory PSCs were analyzed in recording segments between stimulation pulses. The PSCs peak times were manually identified, and amplitude was determined by subtracting the recording baseline averaged over the 10-50 ms time period before the PSC. Only PSCs with amplitudes > 20 pA were considered sufficiently distinct from recording noise (~10 pA, peak).

Statistical significance was computed using unpaired Student’s t-tests for normally distributed data (assessed with Shapiro-Wilk test for normality). Statistics were computed using Prism (Graphpad).

**Anaesthesia**

Parvalbumin-T2A-Cre mice (females, 6-8 weeks at the time of the surgery, Jax #012358) and VGAT-IRES-Cre mice (males, 8-12 weeks at the time of the surgery, Jax #028862) were anaesthetized using isoflurane in oxygen (3-4% for induction, 1.5-2% for maintenance), placed on a 37°C heated pad, and subcutaneously administered buprenorphine HCl (0.1 mg/kg) and ketoprofen (5 mg/kg). Each mouse’s head was gently restrained by a toothbar (David Kopf 923 B). A flap of skin and underlying tissue covering the parietal bones and the intraparietal bone was removed. The sutures of the frontal and parietal bones were covered with a thin layer of cyanoacrylate glue. A ring-shaped titanium headbar was glued over the left parietal cortex. We carefully drilled a ~4.5 mm craniotomy (centered in the middle of the left parietal bone) using a high-speed microdrill (Osada, EXL-M40). The dura mater was left intact. Glass capillaries (Drummond Scientific, 3-000-203-G/X) were pulled and beveled (30° angle, 20 µm outer diameter). Using a precision injector (Drummond Scientific, Nanoject III) we injected AAV2/1.hSynapsin1.iGABASnFR virus (30 nL, 5E11 GC/mL, 1 nL/sec, 300 µm deep) into 6-8 positions within the left visual cortex. The craniotomy was closed with a 4 mm round #1 cover glass and sealed with cyanoacrylate glue. Animals were imaged 5-15 weeks after surgery.

Volumetric raster two-photon imaging was performed, using a piezo objective mount to perform a 400 µm axial scan in 10 µm increments (volume period 12.05 sec.). Anaesthesia (1.5% isoflurane vol/vol) was initiated at the onset of frame 40 (8 min.), and stopped at the onset of frame 120 (24 min.). F0 was calculated by averaging the image intensity over the period prior to anaesthesia onset, for each depth.

**Seizure model**

All animal procedures were conducted in accordance with the European Commission Directive (86/609/ EEC) and the United Kingdom Home Office (Scientific Procedures) Act (1986). Male C57BL/6J mice (from Harlan, UK, 7-8-week-old) were anesthetized with isoflurane (5% induction, 1.5 - 2.5% v/v) and mounted in a stereotaxic frame (Kopf Instruments). Injection of 200 nL (100 nL/min) of AAV2/1.hSynapsin1.iGABASnFR variants (titer >10^{13} GC/mL, except for F102G variant 6 x 10^{12} GC/mL) into layer 2/3 of primary visual cortex (Antero-posterior: 2.8 mm from Bregma and Lateral: 2.4 mm, depth: 0.3 mm below the pia) was performed using a microinjection pump (WPI Ltd., USA), a 5 µL Hamilton syringe, and a 33-gauge needle (Esslab...
Two to three weeks later, a craniotomy was performed over the visual cortex, and electrocorticogram (ECoG) electrodes and a head-plate (Neurotar, Finland) were implanted under isoflurane anesthesia. After recovery, the mice were trained to tolerate brief sessions of immobilization via the head-plate. On the day of the experiment, pilocarpine (3.5 M, 0.2-0.3 μL) was injected into layer 5 (approximately 1-2 mm from the imaging area, depth 0.5 mm) close to the region of interest. Imaging was performed with a FV1200MPE multi-photon laser scanning microscope (Olympus), coupled to a Chameleon Ultra pulsed laser (Coherent, wavelength 908 nm). The ECoG was acquired via a MultiClamp 700B amplifier (Molecular Devices), time-stamped with the imaging data. ECoG data were band pass-filtered from 0.1 to 300 Hz and digitized at 1 kHz. The imaging frame rate was typically 10 Hz, and the region of interest was up to 0.25 x 0.25 mm. For the data shown here the fluorescence was averaged across the entire region of interest, or where iGABASnFR was expressed. To average the time-course of the GABA fluorescence transient occurring during inter-ictal spiking, a Shift and Mean algorithm was applied to blocks of frames straddling each spike.

Transgenic zebrafish

Transgenic zebrafish expressing iGABASnFR were generated as follows. A sequence of iGABASnFR.F102Y.Y137L was cloned downstream of the HuC/elavl3 promoter. This plasmid was injected into 2-cell stage embryos of Casper mutant zebrafish with mRNA of Tol2 transposase to generate founder (F0) transgenic zebrafish.

Imaging experiments were performed using 5-day old embryos. The zebrafish were immobilized and mounted to an imaging chamber as described previously. Briefly, the muscles of the zebrafish were paralyzed by a short (up to 30 seconds) bath incubation with alpha-bungarotoxin (1 mg/mL, Thermo Fischer Scientific, B1601) dissolved in external solution. After the fish became immobile, the zebrafish were further mounted to a custom-made chamber using 2% agarose (Sigma-Aldrich, A9414) and placed under a light-sheet microscope with a 16x objective lens (Nikon, CFI75 LWD 16xW). Imaging was performed using a 488 nm laser (180 μW) and a 525/50 emission filter (Semrock, FF03-525/50) with a frame rate of 30 frames/second.

To record swim signals from the axonal bundles of spinal motoneurons in the tail, we attached a pair of large barrel electrodes to the dorsal left and right sides of the tail. Signals were amplified by an amplifier (Molecular Devices, AxoClamp 700B) and recorded at 6 kHz using a custom software written in C# (Microsoft). For synchronization between the swim signals and neural activity images, camera trigger signals that initiate the acquisition of individual frames in the light-sheet microscope were simultaneously recorded with the swim signals. During the experiments, we projected red visual stimuli (red/black gratings with bars 2 mm thick) onto the bottom of the fish chamber. The speed of the moving visual stimulus alternated between 0 mm/s (stopped) and 2 mm/s (moving forward) every 10 seconds. This forward movement of visual stimulus reliably triggered swimming (Fig. 5c).

Statistical tests

Data are presented as mean with standard error (std. err.) or standard deviation (std. dev.), as noted in each case. All values of n are provided; no data were excluded. For comparisons between two datasets, a two-sided Student’s t-test was used.
**Data availability**

All data from this study is available upon request.

**Accession codes**

All constructs have been deposited at Addgene (112159-112180). Sequences have been deposited in Genbank (MH392466-8). Protein structure has been uploaded to Protein Data Bank (6DGV). AAV virus is available from Addgene.

**Online Methods References**


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**Figure a:** Bacterial protein Pf622 secreted from the cell.

**Figure b:** Structural model of the protein.

**Figure c:** Graph showing the change in fluorescence intensity over time (AF/F vs Time, sec.).

**Figure d:** Immunofluorescence images showing the localization of the protein.
Cranectomy, electrode implantation

Pilocarpine injection (200nL, 3M)

GABA sensor
ECoG

AAV2/1.hSynapticGABA
in V1

f

GABA sensor
ECoG

0.1 \( \Delta F/F \)

1 mV

1 s

g

GABA sensor
ECoG

0.1 \( \Delta F/F \)

0.5 mV

200 ms

e

GABA sensor
ECoG

0.1 \( \Delta F/F \)

1 mV

10 s

h

GABA sensor
ECoG

0.01 \( \Delta F/F \)

200 ms

N260A.F145W
N260A.F145W F102G
F101.N260A.F145W 102Y.Y137L
F101.N260A.F145W R205A
A genetically encoded fluorescent sensor for in vivo imaging of GABA

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Supplementary Figures

**Supp. Fig. 1.** Atu2422 sensor binding curves for alanine (orange), proline (red), and GABA (black). Approximate Kₐ’s 140 µM, 430 µM, 30 mM, respectively. This is a single titration.
Supp. Fig. 2. Atu4243 cpGFP and cpSFGFP fusions do not translocate properly to the membrane of HEK293 cells. Top row: insertion of cpGFP. Bottom row: insertion of cpSFGFP. From left to right: insertion after residue 31, 110, or 280 of Atu4243. All constructs are in the pDisplay vector. Scale bars, 20 µm. Similar results were observed when repeated one additional time.
Supp. Fig. 3. Primary sequence alignment of Pf622 to Atu4243. Numbering of Atu4243 based on PDB 4EQ7, which includes the periplasmic secretion leader sequence. Numbering of Pf622 based on recombinant cloning and expression, which excludes the periplasmic leader sequence. Residues in red indicate identical binding-pocket residues. With the exception of Pf622-F102/Atu4243-Y123 (orange), the binding pocket residues are identical. Pf622 lacks cysteines; Atu4243 has a disulfide bond between C129 and C137 (blue).

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Supp. Fig. 4. ITC of GABA binding to Pf622. Titration of 100 µM Pf622-His₆ with 3 mM GABA in PBS at 25°C. Protein was dialyzed for one week with four PBS exchanges. Origin software curve fitting to a single site indicates a $K_d$ for GABA of 130 µM. Curve fitting errors are listed in legend. Repetition from a separate protein purification yielded similar results, with a $K_d$ for GABA of 86 µM.
Supp. Fig. 5. Equilibrium titration of 0.2 µM Pf622.V278C-JF585 with GABA in PBS. Curve fit to a single-site binding isotherm indicates an affinity of 240 ± 20 µM. Data points are mean and error bars represent standard deviation of three technical replicates.
Supp. Fig. 6. Kinetics of Pf622.V278C-JF585 binding GABA. 0.2 µM protein was mixed with an equal volume of GABA-containing buffer in an Applied Photophysics SX-20 stopped-flow kinetics apparatus. JF585 was excited with a 572 nm LED and fluorescence emission was detected with a photomultiplier tube and a 610 nm long pass filter. a) Data fits poorly to a single exponential. b) Data fits well to a double exponential. c) Comparison of SF-iGluSnFR.A184V (binding 50 µM glutamate) and Pf622.V278C-JF585 (binding 50 µM GABA).
Supp. Fig. 7. Pf622-cpSFGFP fusions, when cloned into pDisplay, translocate to the membrane of transiently transfected HEK293 cells. Insertion after Pf622 residue: a) 156, b) 276, c) 278, d) 280. e) pMinDis.iGluSnFR. Scale bars, 20 µm. Single experiments (but representative of the entire culture dish.)
**Supp. Fig. 8.** Annotated amino acid sequence of iGABASnFR. Insertion of cpSFGFP is after residue D276 of Pf622. Residues RS near the N-terminus encode BglII, and residues LQ at the C-terminus encode PstI. Domains colored as indicated. Mutations included in iGABASnFR include:

- Affinity modulating hinge mutation: Pf622: F101L
- Pf622-SFGFP interface: Pf622: N260A
- Linker 1: SHNVY of SFGFP to LAQVR (SFGFP: S147L, H148A, N149Q, Y151R)
- Linker 2: SFGFP: F145W
- Linker 2: SVLAP of Pf622 to ANLAP (Pf622: S277A, V278N)
- Binding site mutation: Pf622: F102G/Y

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**IgG secretion signal**

**Pf622** 2-276
**SFGFP** 147-238 **Linker** SFGFP 1-14
**Pf622** 277-320
**Myc epitope**
**PDGFR transmembrane domain** 513-561

iGABASnFR

Binding site mutation indicated by orange arrow.

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**METDTLLLWLMLWMVGSTQDQRQAWAPFSKASGINTVQQDGPTDYGKLMVESGNQWDVVDVEADF**
**ALRAAEGGLLEPLDFSQVIQDKDFPFDSDHHGVGFLFSVFLLYNEGKLGASKPQDWTALFDTKTYPGKRALYKWPSPGVLEALLI**
**ADGVPADKLYPLLLDRAFKKDITKKDIVWGGGAQSOQLLASGEVSMGQQWNGRIHALQEDGAPVGSWSKQNLMADILVVPKGT**
**KNKAAAMKFLASASSAKQDDDFSLTAYAPVNIDSVQRQDMVVTADQKQRMANFKEHNVBDGSQVLADHYQQNP1GDNHP**
**VLLDHNLYSTQSVLSDKNRIKDHVYLFVDTAGITLGMDELYKQGGGKGKGKELPTGVVPLVLDGDVNGKFSVREGEB**
**QDANGIKITLKFETIKKLYVVPWPLTVTGWQCEFBRYPFDHMKQHDFFXAMPPGTVQERTISFPKDDQVTKTRAERVKEEGDLV**
**NRIELKGDPFKEGNLIGHKLYQVNLAPLRTAYVKQITLDFAYMAKNGPAILATRWNBEVVLQVLDQVEDQKLISEELNA**
**VQGDTQBVIVVVPSLPFKVYVVISAILALVVTLTISSLILMLWQQKPR**
Supp. Fig. 9. Structure of Pf622-276-cpSFGFP.L1LA/L2AN in the ligand-free state (PDB accession code 6DGV). Residues mutated in the optimization process are indicated with grey sticks: the junction between Pf622 and cpSFGFP (L1-LA), the junction between cpSFGFP and Pf622 (L2-AN), N149 and Y151 of cpSFGFP, and residues F101 (hinge) and N260 (Pf622-cpSFGFP interface) of Pf622.
Supp. Fig. 10. Effect of Pf622-F101 on affinity. a) In Atu4243, Y122 (grey sticks) is in the hinge between the two Venus-flytrap domains and interacts less with the N-terminal domain (pink) in the GABA-bound state (right, 3EUO) than in the ligand-free state (left, 3EQ7). b) Residue F101 in Pf622 is the homologue of Atu4243-Y122. Mutation of F101 results in variants with a broad range of binding affinities. F101L was chosen over F101T due to its aliphatic similarity to wild-type phenylalanine.
**Supp. Fig. 11.** Confocal images of HEK cells transfected with equal amounts of DNA encoding iGABASnFR variants under the *CMV* promoter (pMinDis). 20x objective. Pinhole adjusted to provide 1 AU, about 0.7 µm depth. All imaged adjusted to the same brightness/contrast. a) N260N.F145F. b) N260A.F145F. c) N260A.F145W (iGABASnFR). d) iGABASnFR.F102G. e) iGABASnFR.F102Y.Y137L. f) iGABASnFR.Y137L. Single replicates, but representative of the entire culture dish.
**Supp. Fig. 12.** *In vitro* titrations of purified iGABASnFR variants. a) GABA. iGABASnFR, black, $K_d$ 9 µM; +F102G, dark grey, $K_d$ 50 µM; +F102Y.Y137L, light grey, $K_d$ 70 µM. +R205A, black with white circles, $K_d$ > 2 mM. Mean and std. dev. shown. n = 3 technical replicates. b) Amino acids. Red, alanine, $K_d$ 830 µM; orange, glycine, $K_d$ 500 µM; green, histidine, $K_d$ 2.4 mM. No response was observed for cysteine, aspartate, glutamate, phenylalanine, isoleucine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, or tryptophan. (Data points of non-binders not shown for clarity.) c) Drugs binding cpSFGFP. Red, baclofen; orange, bicuculine; yellow, NNC 05-2090; green, CGP 54626; blue, CGP 46381; purple, SKF 89976A; pink, muscimol; brown, vigabatrin. d) Drugs binding to iGABASnFR. e) Drugs binding iGABASnFR.F102G. f) Drugs binding iGABASnFR.F102Y.Y137L. Titrations for neuro-modulating compounds are single titrations.
Supp. Fig. 12. cont

e) f)
Supp. Fig. 13. iGABASnFR spectra. a) absorbance, b) excitation, and c) emission profiles of iGABASnFR (black) or cpSFGFP (green). 0.2 µM protein in PBS with 1 mM GABA (solid) or without GABA (dashed). d) 2-photon cross section of iGABASnFR (black), iGABASnFR.F102G (dark grey), iGABASnFR.F102Y.Y137L (light grey). Single experiments.
Supp. Fig. 14. a) pH profile of iGABASnFR (black), iGABASnFR.F102G (dark grey), iGABASnFR.F102Y.Y137L (light grey). b) pH profile of non-binding iGABASnFR.R205A (black with white circles) or cpSFGFP (green). 0.2 µM protein in PBS with 10 mM GABA (solid) or without 10 mM GABA (dashed). Fluorescence was normalized to maximum value for each variant. c) Conversation of data from (a & b) into $\Delta F/F$ for each variant. Data points and error bars are mean and standard deviation of three technical replicates.
**Supp. Fig. 15.** Stopped-flow fluorescence of iGABASnFR variants binding GABA. 0.2 µM purified, dialyzed, bacterially produced soluble iGABASnFR was rapidly mixed with equal volumes of varying concentrations of GABA in an Applied Photophysics SX-20 stopped-flow spectrometer. Final concentration of iGABASnFR was 0.1 µM. Final concentration of GABA is half of the listed stock concentration. Top row, iGABASnFR; middle row, iGABASnFR.F102G; bottom row, iGABASnFR.F102Y.Y137L. Data points up to 1 sec. are average of 5 repetitions. Data points up to 100 sec. are average of 3 repetitions. *Left:* linear plot; *right:* log plot.

**Analysis:** Other periplasmic binding protein-based sensors have shown pseudo-first order kinetics, with rates of fluorescence change upon mixing linearly correlated with the concentration of ligand added\(^1,2\). The results with iGABASnFR are consistent with a two-step mechanism in which GABA-binding is followed by a slower change in fluorescence\(^3\). In time regimes relevant to *in vivo* GABA transients (<1 second), iGABASnFR and iGABASnFR.F102G appear to bind GABA faster than the F102Y.Y137L variant (\(t_{1/2}\) rise to the fluorescence value at 1 sec = 0.10 sec, 0.11 sec., 0.57 sec., respectively). Over longer timescales (observed up to 100 sec.), a second phase of fluorescence change occurs, implying a more complicated mechanism. While this characteristic is probably irrelevant to observations of synaptic GABA, and was not observed when the sensor was expressed on membranes in neuronal culture, it should be noted as a potential complicating factor if soluble iGABASnFR were used over long periods to analyze cytosolic GABA.

Supp. Fig. 15. cont.
Supp. Fig. 16. Responses of LNCaP cells transfected with mito-iGABASnFR.F102Y.Y137L to treatment with exogenous GABA (a-g, 0 µM, 5 µM, 10 µM, 50 µM, 100 µM, 500 µM, or 1000 µM). Similar results were observed with four biologically independent replicates. Quantification of fluorescence is plotted in Supp. Fig. 17c. Scale bar 300 µm.
Supp. Fig. 17. Transport of GABA into the mitochondria is detected by mito-iGABASnFR.F102Y.Y137L. a) LNCaP cells were transfected with mito-iGABASnFR.F102Y.Y137L in the presence of 100 µM GABA. 24 hours after transfection, cells were incubated with MitoTracker Red. Epifluorescence (60x objective) shows co-localization of the two signals. Left, green channel. Middle, red channel. Right, merged. Similar results were observed with five biologically independent replicates. b) LNCaP cells were transfected with mito-iGABASnFR and the media supplemented with GABA 24 hours post-transfection. Fluorescence was measured 2 hours after treatment, and every 4 hours thereafter. Data points are single measurements from an entire field of view. b) Time course of fluorescence for different GABA treatments (0, 1, 10, 100, 1000, and 10,000 µM GABA, light grey to black, respectively). c) [GABA] dependence of response was determined by averaging (mean) three time points (46, 50, 54 hours) within a single experiment. Error bars are standard deviation from those three time points. d) Fluorescence of LNCaP cells transfected with mito-iGABASnFR.F102Y.Y137L measured 26 hours post-transfection and 2 hours post-treatment with 100 µM GABA and/or 50 ng/µL vigabatrin, and every four hours after that (n=3 biologically independent replicates). Grey solid, untreated cells; black dashed, vigabatrin treated; grey dashed, GABA treated; black solid, GABA + vigabatrin treated. e) Fluorescence of LNCaP cells transfected with mito-iGABASnFR.F102Y.Y137L and a vector expressing glutamate decarboxylase 1 (GAD1, a.k.a. GAD67) relative to an empty vector control (n=6 biologically independent replicates). Fluorescence was measured beginning 50 hours post-transfection. Black, GAD1; grey, empty vector. For all plots, the fluorescence of a field of view (encompassing many cells) was quantified with thresholding using an automated algorithm (see Methods).
Supp. Fig. 17 cont.

b)

c)

d)

e)
Supp. Fig. 18. Membrane localization of iGABASnFR (and variants) on the surface of hippocampal neurons infected with AAV2/1.hSynapsin1 viruses of equal titer encoding the following variants of iGABASnFR: a) N260N.F145F. b) N260A.F145F. c) N260A.F145W (iGABASnFR). d) iGABASnFR.F102G. e) iGABASnFR.F102Y.Y137L. All images shown at same magnification. Scale bar 20 μm. Single experiments, but representative of all cells in the culture dish.
Supp. Fig. 19. Titration of membrane-localized iGABASnFR on the surface of hippocampal neurons infected with AAV2/1.hSynapsin1 viruses of equal titer encoding variants of iGABASnFR. a) Binding curves for iGABASnFR (black), +F102G (dark grey), +F012Y.Y137L (light grey). Affinities are 30 µM, 42 µM, 106 µM respectively. Average of three ROIs, ± s.d. b-d) Example ROIs of respective variants without GABA. e-g) Same ROIs with 1 mM GABA. Scale bar is 20 µm. The lower apparent ΔF/F for the +F102Y.Y137L variant is a result of the contribution of non-responsive, intracellular sensor to the background fluorescence signal. Similar results have been observed in five repetitions.
Supp. Fig. 20. Changes in fluorescence for SF-iGluSnFR.A184V (a-c) and iGABASnFR.F102G (d,e) in rat hippocampal culture 14 DIV in response to electrical field stimulation. a) Response of SF-iGluSnFR.A184V to multiple field stimuli (50 Hz). Black to light grey: 1, 2, 5, 10, 20, 40 field stimuli. For 1 and 2 stimuli, some trials induced a secondary glutamate response. Average (mean) of three trials shown. b) Fluorescence image of SF-iGluSnFR.A184V and corresponding c) heat map of $\Delta F/F$ in response to 10 AP. d) Fluorescence image of iGABASnFR.F102G and corresponding e) heat map showing $\Delta F/F$ in response to 10 AP stimulation. SF-iGluSnFR and iGABASnFR infected cultures were prepared and treated side-by-side. Scale bar 20 µm. Side-by-side comparison was performed only once, but similar results have been observed in at least independent five repetitions.
Supp. Fig. 20. cont.

b)  

d)  

e)
Supp. Fig. 21. Electrically evoked and spontaneous excitatory and inhibitory post-synaptic currents in somatosensory cortex of mouse. See Online Methods for details. a) Resting potential of uninfected and AAV \textit{hSynapsin1:iGABA\textsc{SnFR}}-infected cells (n=5 each). b) Raw traces of electrically evoked EPSCs and IPSCs selected by holding the cells at -70 mV or 0 mV, respectively (n = 12 uninfected cells, n = 10 iGABA\textsc{SnFR}-expressing cells). Traces are representative of 10 replicates each. c) Peak current amplitudes of evoked response in (b); data points are average of 10 technical replicates; Std. dev. are small and have been removed for clarity. d) Representative traces showing spontaneous IPSCs and EPSCs. e) Mean amplitudes of spontaneous PSCs greater than 20 pA observed in (d). f) Variance (standard deviation) of spontaneous PSCs greater than 20 pA observed in (d). g) Frequency of spontaneous PSCs. For each cell, the number of PSCs greater than 20 pA observed was divided by the 100 sec observation window to generate the PSC frequency. P-values determined by two-tailed, unpaired Mann-Whitney test.
c) **EPSC**  
\[ p = 0.6277 \]

\[ \text{current (pA)} \]

\[ \text{uninfected} \quad \text{iGABASnFR} \]

\[ \text{IPSC} \]
\[ p = 0.3463 \]

\[ \text{current (pA)} \]

\[ \text{uninfected} \quad \text{iGABASnFR} \]

**Supp. Fig. 21. cont.**

d) **EPSC**  
\[ \text{uninfected} \quad 25 \text{pA} \]

\[ 25 \text{pA} \]

\[ 550 \text{ms} \]

**IPSC**

\[ \text{uninfected} \quad \text{iGABASnFR} \]

e) **EPSC**  
\[ p = 0.9742 \]

\[ \text{Mean amplitude (pA)} \]

\[ \text{uninfected} \quad \text{iGABASnFR} \]

**IPSC**  
\[ p = 0.8212 \]

\[ \text{Mean amplitude (pA)} \]

\[ \text{uninfected} \quad \text{iGABASnFR} \]

f) **EPSC**  
\[ p = 0.9229 \]

\[ \text{EPSC variance (pA)} \]

\[ \text{uninfected} \quad \text{iGABASnFR} \]

**IPSC**  
\[ p = 0.9177 \]

\[ \text{IPSC variance (pA)} \]

\[ \text{uninfected} \quad \text{iGABASnFR} \]
Supp. Fig. 21 cont.
g)
Supp. Fig. 22. GABAergic signaling decreases under isoflurane anaesthesia in mouse visual cortex. a) Kymograph showing fluorescence intensity of iGABASnFR at multiple depths, normalized to the average intensity at all depths prior to anaesthesia. b) Averaged response at all depths across three animals (grey), with mean of three animals in black. c) Averaged response of the non-GABA-binding variant iGABASnFR.R205A, at all depths across four animals (grey), with the mean in black. Onset and removal of isoflurane treatment indicated by dashed red lines.