

SUPPLEMENTARY RESULTS

DA binding to BM3h-B7 and BM3h-8C8 is stable and reversible

The stability of dopamine (DA) binding to BM3h-B7 and BM3h-8C8 was tested by incubating each protein with various amounts of DA at room temperature and measuring the absorbance difference between 430 nm and 410 nm over two hours (Supplementary Fig. 1). During this time, a decline of less than 5% was observed when 1 μM sensor was incubated in the presence of excess DA (800 μM). Optical changes were greater when sensor was incubated with subsaturating concentrations of DA (up to 22% signal change for 1.3 μM DA incubated with BM3h-B7), consistent with the predicted effects of DA oxidation (known to take place under ambient conditions) on the partial saturation of the sensor. To test the reversibility of DA binding to BM3h-B7 and BM3h-8C8, we acquired absorbance spectra of the proteins alone and with 400 μM DA before and after filtering the solutions through a 30 kDa cutoff centrifugal filter. Successive steps of filtering and resuspension restored the original, ligand-free spectrum (Supplementary Fig. 2). Rate constants for binding and unbinding of DA to BM3h-8C8 were estimated to be $3 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ and 0.02 s^{-1} , respectively, at room temperature in phosphate buffered saline (PBS). Association was measured by rapid 1:1 mixing of protein (4.8 μM) and DA solutions (0.6-2.5 mM), followed by absorbance spectroscopy in a Hi-Tech KinetAsyst SF-61DX2 stopped-flow spectrometer (TgK Scientific, Wiltshire, UK). Dissociation was measured by fast 1:100 dilution of a solution containing 100 μM BM3h-8C8 and 50 μM DA, followed by absorbance recording in a Spectramax M2e spectrophotometer (Molecular Devices, Sunnyvale, CA).

Thermostabilization of BM3h-8C8

Mutations in the amino acid sequence of BM3h have been shown to reduce the protein's thermostability. This was observed during directed evolution of BM3h: the melting temperature (T_m , the midpoint temperature for thermal denaturation after 10 min) of WT BM3h was approximately 58 °C, while the T_m for BM3h-8C8 was approximately 48 °C. While this change did not significantly affect the protein's stability in physiologic buffer at room temperature, it did apparently reduce the yield of our bulk purification procedure. To improve thermostability of BM3h-8C8 before performing the fifth round of evolution, we introduced the mutation I366V, which has been shown previously to improve stability.¹ For BM3h-8C8 this improvement corresponded to a T_m increase by approximately 4 °C.

Histological analysis of injected rat brains

Histological analysis was performed on some of the experimental animals, following injection with contrast agents and MRI scanning (Supplementary Fig. 4). Brains were extracted, and frozen sections were obtained from regions near the cannula placement sites. Hematoxylin and eosin (H&E) staining was performed to evaluate tissue architecture, and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was performed to assay cell death in brain regions near the injection sites. Some mechanical disruption occurred in areas immediately surrounding the cannula implantation tracts, but the tissue appeared normal in other respects. The tissue was intact in brain regions that did not include the cannula tracts. To obtain quantitative in-

formation about cell death near the injection site, TUNEL positive and negative nuclei were tallied in a region of 0.5 mm x 0.5 mm near the cannula tip placement site in a representative histological image; this site had been administered an injection solution containing 500 μ M BM3h-8C8 and 500 μ M DA. Fewer than 4% of nuclei appeared to be TUNEL positive, indicating that widespread cell death was not induced by introduction of the agents used in our experiments.

BM3h expression in mammalian cells

As a preliminary test of the feasibility of applying BM3h-based sensors as genetically encoded reporters in mammalian cells and animals, we created mammalian codon-optimized versions of the BM3h and BM3h-8C8 genes using Gene Designer software (DNA2.0, Menlo Park, CA). The resulting sequences were synthesized by Blue Heron (Bothell, WA), cloned into a PCMV-Sport vector (Invitrogen, Carlsbad, CA), and transfected into the HEK293 cell line adapted for the Invitrogen Freestyle293 expression system. One day after transfection, cells were supplemented with 0-40 μ M hemin, to ensure an adequate supply of heme for expression and folding of BM3h. Cells were lysed after four days of expression. Proteins were purified from lysates by nickel affinity chromatography and analyzed by optical spectroscopy. Spectra of mammalian cell-expressed and recombinant bacterial BM3h were virtually identical, indicating correct folding of the proteins. Absolute BM3h expression levels were measured by performing CO assays on clarified HEK293 lysates, and the results were compared with BCA assay (Pierce, Rockford, IL) results indicating the total amount of protein present. BM3h accounted for 0.4% to 1.9% of total cell protein depending on the level of hemin supplementation dur-

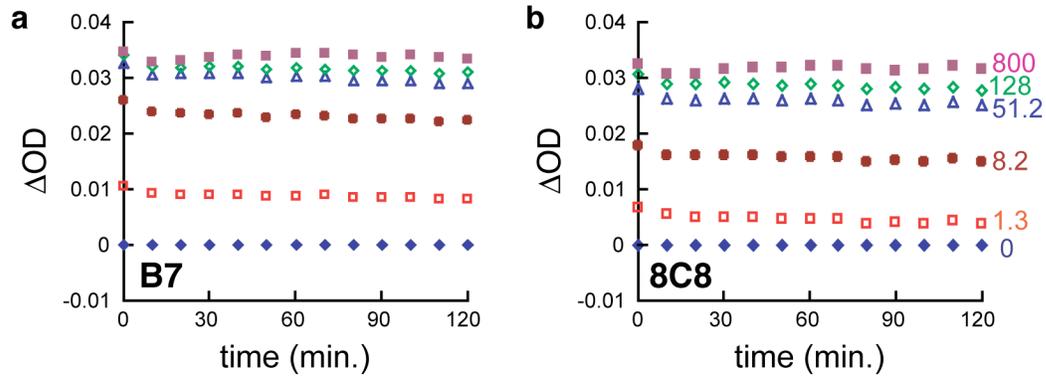
ing growth. Assuming a typical protein density of 150 mg/mL in living tissue, 1% of total protein would correspond to a tissue concentration of $\sim 25 \mu\text{M}$. This figure indicates cytosolic expression levels, but robust expression of secreted BM3h, appropriate for extracellular DA sensing, should also be feasible. High level expression of secreted proteins from mammalian cells has been described in the literature. One study reported yields of $\sim 5 \text{ mg/L}$ over a two-hour period from monolayer cultures containing approximately 10^5 cells per 1.5 mL well,² a figure likely to constitute well over 10% of total protein in the samples, and therefore significantly greater than the intracellular yields we obtained from BM3h expression in HEK293 cells. In mice, comparably high expression levels of several mg/L in serum of a secreted enzymatic reporter protein have been observed following adenoviral infection³ or genetically-modified xenograft implantation.⁴ Another point of reference related to attainable secreted protein expression levels is the protein content of the brain's extracellular space, which has been estimated at 1% by weight,⁵ equivalent to $200 \mu\text{M}$ of proteins averaging 50 kD.

SUPPLEMENTARY REFERENCES

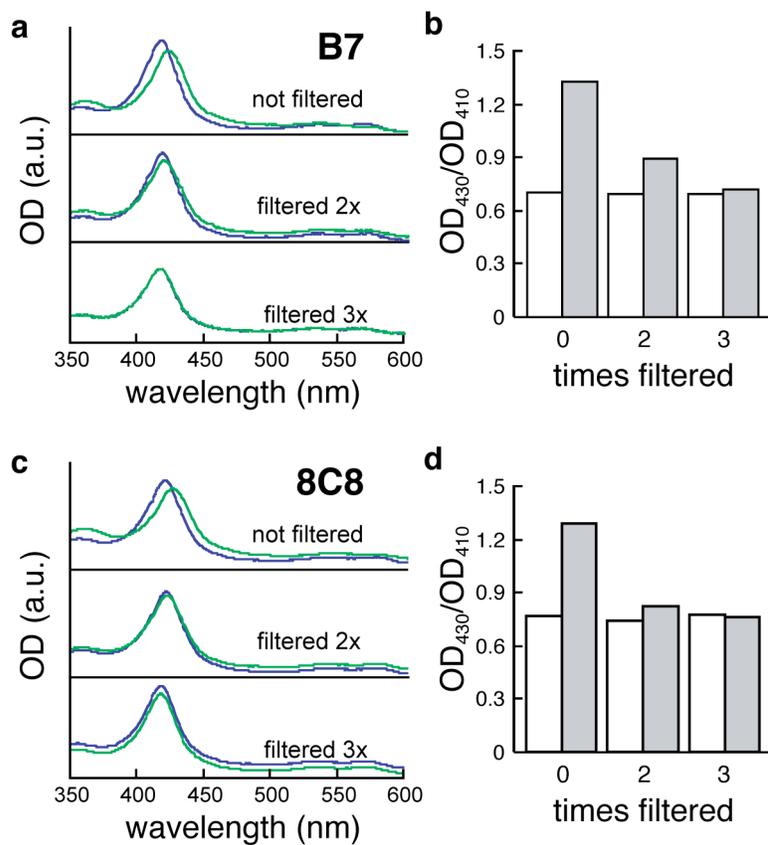
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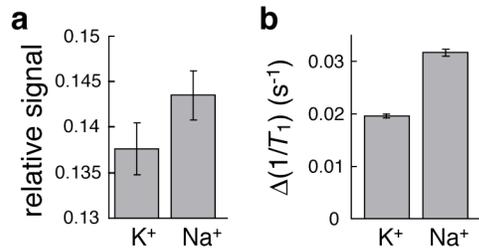
SUPPLEMENTARY FIGURES



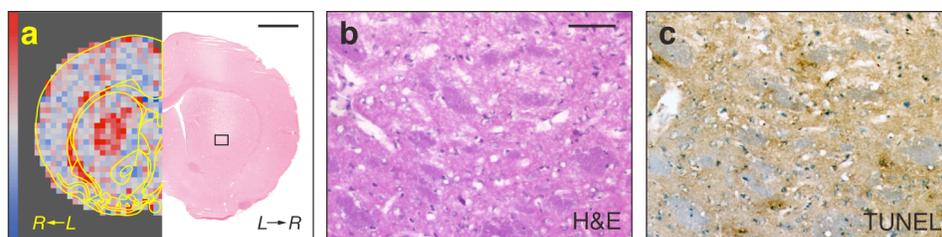
Supplementary Figure 1. Stability of BM3h complexes. Optical signatures of DA binding to BM3h-B7 (**a**) and BM3h-8C8 (**b**) are stable over two hours. Absorbance at 430 nm minus 410 nm collected over 2 hrs. for 1 μ M sensor incubated in the presence of 0-800 μ M DA (labels in color).



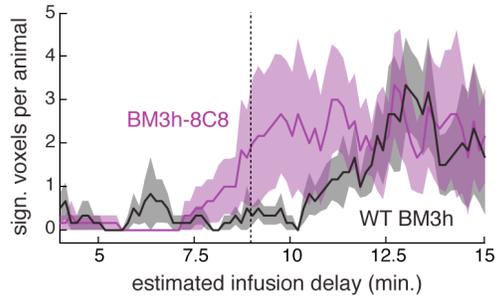
Supplementary Figure 2. DA binding to BM3h-B7 and BM3h-8C8 is reversible. **(a)** Absorbance spectra of 1 μ M BM3h-B7 alone (blue) or incubated with 400 μ M DA (green) before filtering (top), and after filtering twice (middle) or three times (bottom) through a 30 kDa cutoff filter. **(b)** Ratios of absorbance at 430 nm to 410 nm corresponding to the DA-free (white bars) and DA-incubated (gray bars) spectra in (a). Panels **(c)** and **(d)** display equivalent data for sensor variant BM3h-8C8.



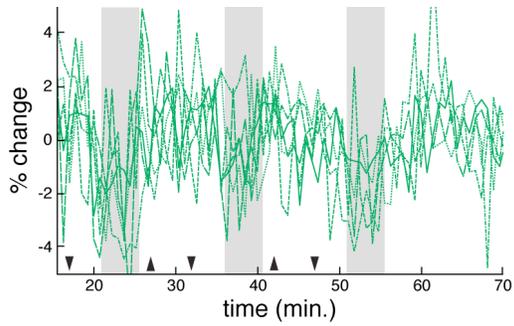
Supplementary Figure 3. BM3h-8C8 reports DA release from PC12 cells. Plots of signal change **(a)** and $\Delta(1/T_1)$ **(b)** are analogous to Fig. 4 in the main text, and were obtained using identical experimental procedures.



Supplementary Figure 4. Histological data from a rat injected with 500 μM BM3h-8C8 and 500 μM DA. Panel (a) shows a comparison of MRI and hematoxylin and eosin (H&E) stained brain hemi-slices, taken from near the injection site (~ 1 mm anterior to the cannula position). The pseudocolored MRI image (left) maps the percent change in MRI signal (relative to preinjection baseline) observed in this animal; the color scale (left edge) ranges from -25% to $+25\%$. The superimposed yellow brain atlas diagram⁶ demonstrates that the region of greatest signal change following this injection falls within the caudate nucleus of the striatum. The right side of panel (a) shows a corresponding H&E section from the same animal (scale bar = 2 mm) (H&E and MRI data are shown as mirror images, to facilitate comparison). A closeup view (20x) of the H&E stained section in the region of greatest contrast agent accumulation shows normal cellular staining (b). The scale bar denotes 100 μm , and the field of view shown in (b) corresponds to the small rectangle in the right half of panel (a). TUNEL staining was performed to assay cell viability in the injected ROI (c). Over 95% of visualized nuclei were judged to be TUNEL negative, indicating the overall health of the tissue.



Supplementary Figure 5. Statistical analysis of K^+ stimulation data with variable delay between infusion switching and estimated periods of high and low K^+ in the brain. Data acquired during three cycles of potassium stimulation were analyzed over a range of delays, and the number of voxels showing significant (t -test $p < 0.01$) signal decreases during modeled periods of high K^+ was tallied in 0.75 mm radius ROIs extending over three 1 mm slices around the BM3h-8C8 (purple) and WT BM3h control (black) infusion sites. The plot shows the average (solid line) and standard error (shading) across the six individual animals that contributed to the analysis. The horizontal scale spans a range from the dead time of the injection cannulae (4 min.) to the duration of an entire stimulus cycle (15 min.). The vertical dotted line specifies the delay (9 min.) used to generate figures and data cited in the main text.



Supplementary Figure 6. Time courses of MRI signal in the six individual animals contributing to the average in Fig. 5g. For each animal (represented by different line styles), signal was averaged over all voxels within ROIs of 2.7 mm diameter and three 1 mm slice thickness around BM3h-8C8 infusion sites, and determined to be correlated ($p < 0.05$) with K^+ stimulation blocks. As in Fig. 5g, gray vertical bars denote periods associated with highest K^+ stimulation, and arrowheads label timing of pump switches associated with transitions from low to high (up) and high to low (down) K^+ infusion conditions.