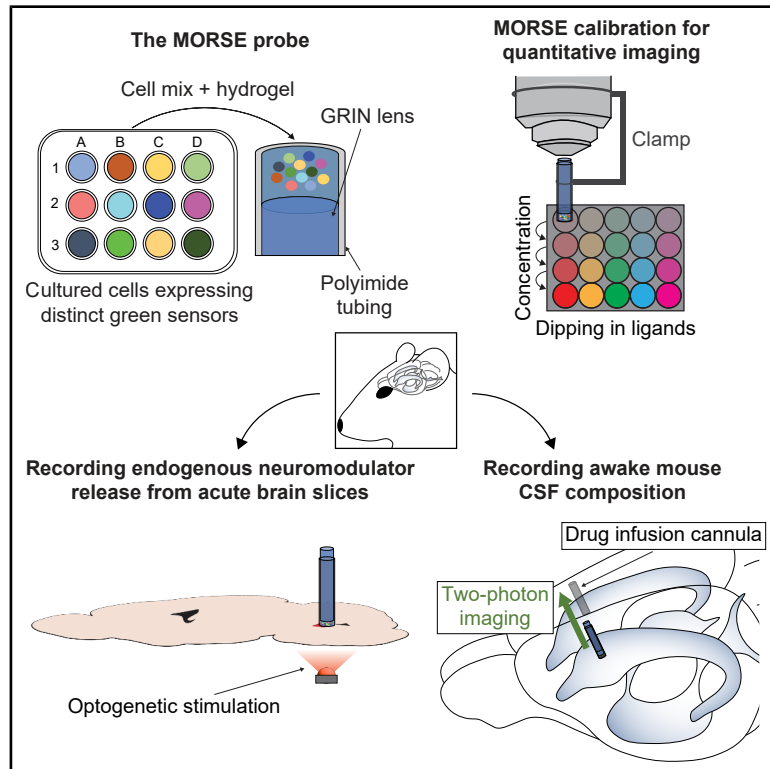


Simultaneous, real-time tracking of many neuromodulatory signals with multiplexed optical recording of sensors on a micro-endoscope

Graphical abstract



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In brief

Kalugin et al. present multiplexed optical recording of sensors on a micro-endoscope (MORSE), an approach to tracking many molecular signals simultaneously in brain extracellular fluids using spatial multiplexing of fluorescent sensors. They use this to quantitatively capture combinatorial neuromodulatory states in brain slices and cerebrospinal fluid of awake, behaving mice.

Highlights

- Dynamic optical tracking of absolute concentrations of 10+ ligands in brain and CSF
- Demixing sensor crosstalk reveals simultaneous release of NE and DA from LC slices
- Real-time *in vivo* CSF concentration profiling outperforms *ex vivo* measurements 100×
- Factors in lateral ventricle CSF rapidly reach the choroid plexus to modulate signaling

NeuroResource

Simultaneous, real-time tracking of many neuromodulatory signals with multiplexed optical recording of sensors on a micro-endoscope

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SUMMARY

Dozens of extracellular molecules jointly impact a given brain cell, yet we lack methods to simultaneously record many such signals in real time. We developed a probe to track ten or more neuropeptide and neuromodulator concentrations using spatial multiplexing of genetically encoded fluorescent sensors. Cultured cells, each expressing one sensor type, are immobilized at the front of a gradient refractive index lens for 3D two-photon imaging *in vitro* and *in vivo*. The sensor identity and detection sensitivity of each cell are determined via robotic dipping of the probe into wells containing various ligands and concentrations. Using this probe, we detected stimulation-evoked release of multiple neuromodulators in acute brain slices. We also tracked endogenous and drug-evoked changes in cerebrospinal fluid composition in the awake mouse lateral ventricle, which triggered activation of the choroid plexus epithelium. Our method introduces a new approach to quantitative, real-time, high-dimensional tracking of brain extracellular fluid composition.

INTRODUCTION

Brain cells exist in a rich environment of neuromodulators, peptides, hormones, and other molecular signals derived from both local and distant sources. Changes in these extracellular signals on timescales ranging from milliseconds to hours (or longer) underlie fundamental aspects of brain function and physiology.^{1,2} In vertebrates, time-varying levels of diverse signals travel over distances from microns to centimeters through brain interstitial fluid and cerebrospinal fluid (CSF) to engage dozens of distinct receptors in neurons, glia, immune cells, and brain barriers such as the choroid plexus (ChP) epithelium.^{3–11} Despite their complementary effects on various tis-

sues, rapid fluctuations in these signals have typically been studied for one signal at a time due to technical limitations.^{12,13} Systemic delivery of neurochemicals such as vasopressin (AVP) in autism spectrum disorder and L-DOPA in Parkinson's disease are useful treatments, but the precise trafficking routes and quantitative pharmacodynamics of such signals in target brain regions remain poorly understood.^{14,15} Moreover, we lack a mechanistic understanding of the synergistic effects of drugs or prescription medications on brain activity.¹⁶ Monitoring the coordinated dynamics of many molecular signals in brain fluids, while technically challenging, could yield profound insights into the nature of brain states and combinatorial therapeutics.

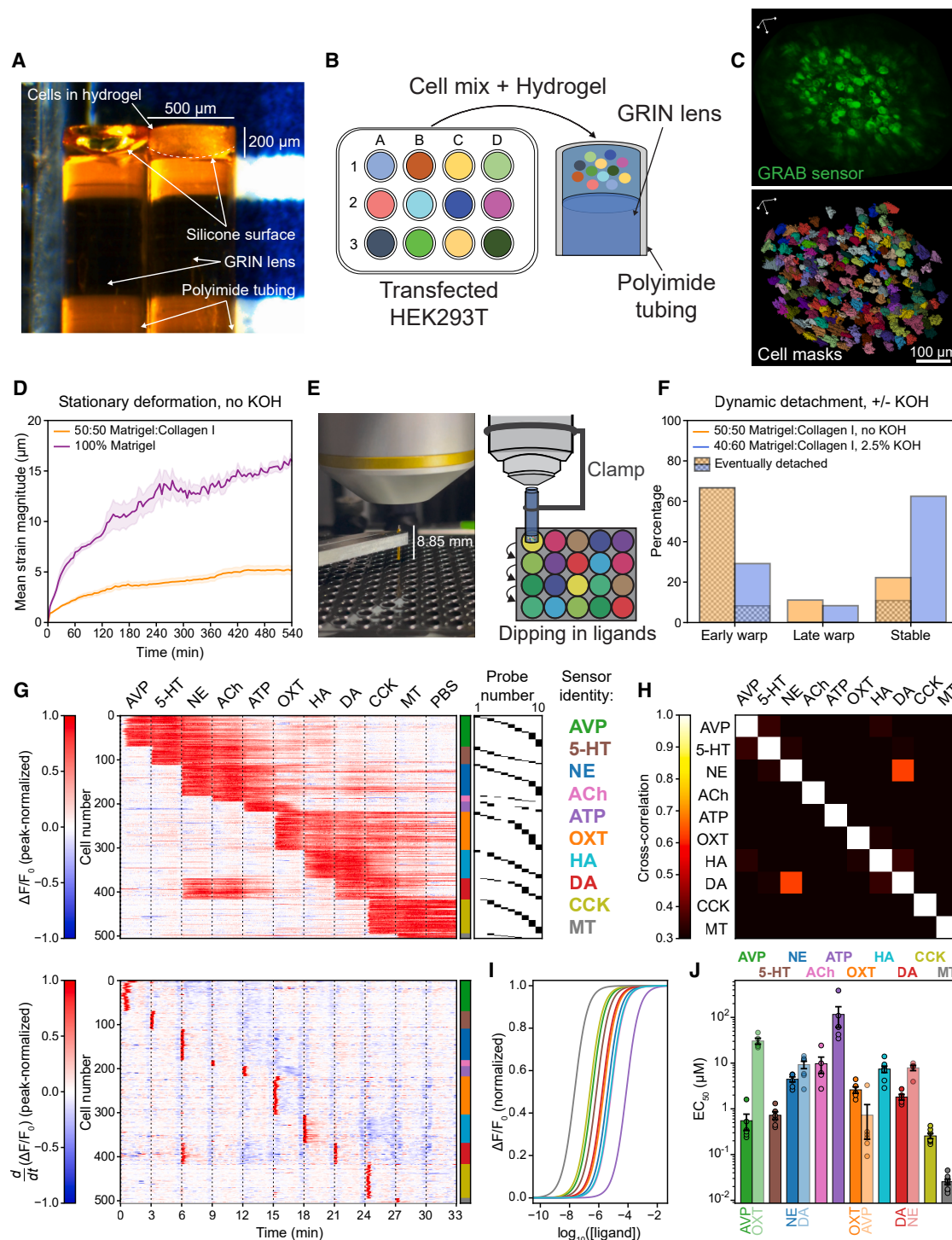


Figure 1. MORSE probe design

(A) MORSE probes before (left) and after (right) hydrogel deposition.

(B) Cultured HEK-293T cells are transfected with one plasmid at a time expressing a green GRAB sensor, pooled, mixed into an optimized hydrogel matrix, and attached to the front of a half-millimeter diameter GRIN lens in a receptacle of polyimide tubing and silicone adhesive.

(C) Top: 3D rendering of a two-photon imaging volume collected from a MORSE probe via the GRIN lens. Bottom: single-cell 3D masks. Scale bars: 100 μm .

(D) Quantification of warping across hydrogel recipes. Probes were prepared using hydrogels consisting of pure Matrigel and of a 50:50 mixture of Matrigel and collagen I ($n = 6$ probes per recipe) and imaged continuously for 9 h. Deformation (local strain vector magnitude) was averaged across the hydrogel volume (Figure S1C). Shading: $\pm$ SEM across probes.

(legend continued on next page)

Current molecular, electrochemical, and optical techniques to probe extracellular brain fluids each possess powerful capabilities and significant limitations. For instance, microdialysis collection followed by mass spectrometry allows up to hundreds of molecules to be measured simultaneously with exceptional sensitivity.¹⁷ However, such measurements substantially dilute *in vivo* fluids by introducing large volumes of dialysate (>100 nL/min) and almost always require many minutes for collection/analysis (but see Wells et al., 2024¹⁸), thereby precluding real-time readout and incurring analyte degradation in the time prior to *ex vivo* sample processing. Electrochemical methods such as fast-scan cyclic voltammetry provide more rapid readouts of signals *in situ* but are challenging to expand beyond the small set of molecules with measurable redox activity and suffer from significant crosstalk between ligands of related chemical structure.^{19–22} Aptamer-field-effect transistor devices are also capable of fast and sensitive molecular measurements but have so far been too fragile and prone to biofouling to measure complex biological fluids in living systems.^{23,24}

In recent years, genetically encoded indicators emerged as versatile tools for tracking molecular concentrations in the brain.¹³ Indicators for extracellular signals based on G protein-coupled receptors (GPCRs) have now been developed for dozens of neuromodulators and neuropeptides.^{25–32} These sensors can detect nanomolar concentrations at subsecond resolution *in vivo*. Nevertheless, genetically encoded indicators face two important challenges. First, they do not readily provide a quantitative readout of analyte concentration (even in the case of fluorescence lifetime sensors^{33,34}) due to the uncertainty of extrapolating *in vitro* calibrations to sensors expressed in brain tissue. Second, most sensors are green, and their broad fluorescence excitation and emission spectra limit the number of simultaneously measurable signals to 2–4 even in rare cases where spectrally distinct sensors are available.^{32,35}

To begin to address these challenges, we developed multiplexed optical recording of sensors on a micro-endoscope (MORSE)—an optical probe capable of simultaneously recording ten molecular signals in brain extracellular fluids *in situ* with nanomolar sensitivity and near-real-time, second-to-second readout. Our probe uses a stable, porous, and thin hydrogel (≤ 200 μm thickness) loaded with a mixture of cultured cell populations, each expressing a single green fluorescent sensor for a different neuromodulator or neuropeptide. The hy-

drogel is immobilized at the front of a half-millimeter diameter gradient refractive index (GRIN) lens. This allows 3D imaging of all green fluorescent sensors *in vitro* or *in vivo*, followed by determination of sensor identity and quantitative estimation of concentrations via post hoc robotic dipping of the probe into various fluid samples. Notably, our method relies on commercially available components, readily scales up to tens of sensors, and uses a standard two-photon microscope. We successfully applied this approach to decode dynamic concentrations of up to ten molecular signals in *ex vivo* biofluids, parenchymal interstitial fluid in acute brain slices, and CSF in awake mice. We additionally observed spontaneous fluctuations in CSF serotonin (5-HT) at a level sufficient to drive calcium elevations in the CSF-facing ChP epithelial cells. These findings demonstrate MORSE as a flexible approach for combining a broad range of genetically encoded fluorescent sensors for high-dimensional tracking of complex chemical environments.

RESULTS

MORSE probe development and characterization

Preparation of MORSE probes involved two fabrication stages. First, a standard half-millimeter GRIN lens was secured in polyimide tubing using silicone elastomer. The silicone was shaped to form a curved well at the front of the lens extending from the edges of the tubing (center thickness, ~ 200 μm ; Figure 1A, left). Next, HEK-293T cells were transfected with plasmids encoding one of up to 16 different green fluorescent protein (GFP)-based GPCR activation-based (GRAB) sensors for different neuromodulators and neuropeptides (Table 1; 10 of the 16 tested sensors demonstrated reliable ligand responses).^{31,36–45} To use the membrane proteins without interference from cell movement and other functions, we then treated the cells with high-dose colchicine to immobilize or kill them. We mixed these sensor cells together in a hydrogel consisting of extracellular matrix components collagen I and Matrigel, which provided a biocompatible, porous, yet stiff substrate.⁴⁶ We then applied this mixture to the front of the GRIN lens (Figures 1A and 1B). 3D two-photon imaging of the resultant hydrogel through the GRIN lens (30 planes/volume, $\sim 600 \times 600 \times 270$ μm^3 volume) demonstrated a uniformly distributed set of 100–200 green cells spanning the full thickness of the gel (Figure 1C, top). The total active probe preparation time

- (E) Left: the probe is secured on the microscope with a custom lens clamp above a 384-well plate of ~ 20 μL aliquots of solutions carrying various ligands at different concentrations. Right: the probe is robotically dipped into one well after another (3–5 min/well) during continuous 3D volumetric two-photon imaging. (F) Probes were prepared with or without 2.5% KOH lens surface coating, using hydrogels consisting of 50:50 ($n = 9$) and 40:60 ($n = 24$) Matrigel:collagen I. Probes were dipped into sequential wells (5 min/well over 2–7 h). Probes were labeled as early warp (>25 μm of 3D translation of the hydrogel in the first hour), late warp (>25 μm in the second hour), or stable (see also Figures S1C and S1D). (G) 506 single-cell masks (ROIs) were recovered from ten MORSE probes, each carrying ten GRAB sensor cell types and dipped into 20 μL volumes of high concentrations of the respective ligands: vasopressin (AVP, 10 μM), serotonin (5-HT, 100 μM), norepinephrine (NE, 1 mM), acetylcholine (ACh, 1 mM), adenosine triphosphate (ATP, 1 mM), oxytocin (OXT, 100 μM), histamine (HA, 1 mM), dopamine (DA, 100 μM), cholecystokinin (CCK, 10 μM), and melatonin (MT, 10 μM), followed by a wash well of phosphate-buffered saline (PBS). Top: heatmap depicting peak-normalized $\Delta F/F_0$ signals for each cell following background correction. Color bar (labeled per legend) and black raster indicate each cell's sensor identity and probe of origin, respectively. Bottom: peak-normalized time derivatives of the time courses shown in the upper heatmap. All ten sensors were activated (median: 9 active sensors per probe, average of 6.02 cells per sensor type per probe). (H) Cross-correlation of the mean time-derivative traces for each sensor group in (G), bottom (see also Figure S3A). (I) Consensus peak-normalized dose-response fits for each GRAB sensor to its on-target ligand from ten probes (see also Figure S3C). (J) EC₅₀ values. Error bars: $\pm$ SEM across $n = 5$ –8 probes per sensor.

Table 1. Summary of GRAB sensors used in this work

Sensor	Peak $\Delta F/F_0$ (epi)	EC ₅₀ (epi)	Peak $\Delta F/F_0$ (MORSE)	EC ₅₀ (MORSE)	References
AVP0.2	2.5 (AVP)	5 nM (AVP)	0.37 (AVP); 0.10 (OXT)	355 nM (AVP); 28.7 μ M (OXT)	current manuscript, developed by Huan Wang
OXT1.7	7 (OXT); 2 (AVP)	20 nM (OXT); 50 nM (AVP)	0.24 (OXT); 0.05 (AVP)	2.19 μ M (OXT); 247 nM (AVP)	current manuscript, developed by Lan Geng
5-HT2h	~5	~80 nM	0.36	687 nM	Deng et al. (2024) ³⁶
CCK0.5	5.8	25 nM	0.40	245 nM	current manuscript, developed by Huan Wang
NPY1.0	4.9	43 nM	–	–	Wang et al. (2023) ³¹
VIP1.0	3.6	19 nM	–	–	Wang et al. (2023) ³¹
NE2h	~4 (NE)	~190 nM (NE)	0.34 (NE); 0.15 (DA)	4.04 μ M (NE); 9.35 μ M (DA)	Feng et al. (2024) ³⁷
SST1.0	4.3	13 nM	–	–	Wang et al. (2023) ³¹
DA2.9	6.25 (DA)	–	0.27 (DA); 0.21 (NE)	1.70 μ M (DA); 8.20 μ M (NE)	Zhuo et al. (2024) ³⁸
gACh4l	~12	6.6 μ M	0.37	7.03 μ M	current manuscript, developed by Guochuan Li
ATP1.0-L	~10	~97 μ M	0.28	75.2 μ M	Wu et al. (2022) ³⁹
MT1.0	6	28 nM	0.30	22.0 nM	current manuscript, developed by Huan Wang
PTH1.0	~3.8	190 nM	–	–	Wang et al. (2023) ³¹
Ado2h	~5	14 μ M	–	–	current manuscript, developed by Bohan Li
eCB2.0	~3 (AEA)	~0.2 μ M (AEA)	–	–	Dong et al. (2022) ⁴⁰
HA1m	~6	380 nM	0.38	6.39 μ M	Dong et al. (2023) ⁴¹

Response parameters of the 16 GRAB sensors used in this work, compared between single-photon epifluorescence imaging in monolayer culture of HEK293T cells (epi) and two-photon imaging on a MORSE probe. Peak $\Delta F/F_0$: maximal change in fluorescence from basal to saturated state; EC₅₀: ligand concentration achieving half-maximal sensor activation. Blank cells represent values for which data were not available.

requires approximately 3 h, and the process is scalable to many probes in parallel with minimal additional time (probes prepared in the morning last the entire day).

To assess hydrogel stability, we first performed time-lapse 3D imaging of the hydrogel ($\sim 600 \times 600 \times 270 \mu\text{m}^3$ volumes, 30 planes/volume, ~ 0.5 volumes/s) while the probe was stationary in phosphate-buffered saline (PBS). GRAB sensors exhibited a wide range of baseline fluorescence intensities, so we first pre-processed each imaging volume using 3D cell segmentation (Cellpose2.0; Figure S1A).^{47,48} This generated a probability map of how likely each voxel was to belong to a fluorescent sensor cell—a measure largely independent of fluorescence brightness (Figure S1B). To quantify hydrogel deformation, we next performed nonrigid registration on these probability maps to a single reference time point using optic flow-based warping (Figure S1C).^{49,50} The small hydrogel volume (~ 40 nL) on the GRIN lens tip, when composed of pure Matrigel, exhibited substantial global and local deformations over tens of minutes. We minimized these deformations using an optimized mixture of Matrigel and collagen I, a more rigid extracellular matrix component (Figure 1D; Video S1).⁵¹

To determine the identity, crosstalk, and sensitivity of the sensor expressed in each cell, we performed time-lapse 3D imaging during several hours of sequential robotic dipping of the probe into several dozen wells of a 384-well plate containing $\sim 20 \mu\text{L}$ of various solutions (Figure 1E; Video S2). During this protocol, which involves frequent probe-liquid contact cycles, we

observed large translational hydrogel shifts and complete gel detachments, likely reflecting insufficient gel adhesiveness to the silicone coating (Figure S1D, left; Video S3). These deformations and detachments were strongly attenuated by increasing silicone surface hydrophilicity with potassium hydroxide (KOH) treatment prior to gel administration (Figures 1F, S1D, and S1E; Video S3).^{52,53}

Despite these improvements, slow residual hydrogel deformation occurred across tens of minutes. To account for this, we applied non-rigid deformations to the raw fluorescence volumes (Figure S2A). In a subset of probes, we prepared hydrogels with 10% of the cells expressing cytosolic tdTomato, performed two-color imaging, and compared the results of image alignment using the ligand-sensitive green GRAB sensor channel or the ligand-insensitive red channel (Figure S2B). Registration using Cellpose2.0 probability maps derived from the GRAB signals performed similarly to or better than those from tdTomato signals (Figure S2C; Video S4). Thus, segmentation probability map filtering offers a generalizable approach for extracting structural information useful for alignment in 3D two-photon imaging datasets containing diverse cell signal intensities.

To extract signals from individual cells, we next applied a constrained non-negative matrix factorization algorithm (Caiman) to define 3D masks based on correlated local fluorescence fluctuations.⁵⁴ We performed semi-automated curation of the resulting masks using a custom graphical user interface.⁵⁵ Many of the MORSE probes were imaged multiple times in several contexts

and experiments, such as in biological samples followed by *ex vivo* calibration. In these cases, we also matched cells across these recordings. To account for 3D rotations and deformations between different recordings of the same probe, we aligned representative single volumes from each recording using manually defined landmarks and matched overlapping cell masks (Figures S2D and S2E).^{56,57} With this approach, we could reliably track ~70–130 active sensor cells per probe across 2–8 sequential recordings in different contexts across up to 8 h in total (Figures 1C, bottom, S2F).

We began by dipping ten-sensor MORSE probes sequentially into solutions containing near-saturating concentrations of each of ten individual sensor ligands, one ligand at a time (Table 1; Figure 1G). This resulted in rapid, strong, and consistent patterns of elevated fluorescence in subsets of cells evenly spread throughout the gel in response to each ligand (Figures 1G and S3A; Video S5). Using hierarchical clustering on the rise time of the individual sensor cell signals, we could assign clear sensor identities across several hundred cells from ten representative MORSE probes (median of 9 active sensors per probe, interquartile range 8–9; average of 6.02 cells per sensor per probe) (Figure 1G, color bar). Significant crosstalk was detectable between dopamine (DA) and norepinephrine (NE) sensors as well as between AVP and oxytocin (OXT) sensors, as reported previously (Figures 1H and S3B).^{29,45}

Once the sensor identity of each cell was defined in this way, we next performed dose-response calibrations by dipping the probe sequentially into wells containing mixtures of ligands, with increasing concentrations of each ligand in successive wells (Video S6). The use of mixtures allowed us to more efficiently assess probe sensitivity to all ligands. We found that the sensors' half-maximal effective concentrations (EC_{50} , equivalent to a sensor's apparent dissociation constant, K_d) could be reliably estimated for all sensors (Table 1; Figures 1I and S3C). EC_{50} values could be very sensitive (e.g., tens of nanomolar range). Importantly, while each sensor exhibited a relatively broad range of peak response magnitudes across individual cells and probes (Figure S3B), peak-normalizing each cell's signal and averaging across cells expressing the same sensor produced precise dose-response relationships that were consistent across probes (Figures 1I, 1J, and S3C). This calibration allowed us to derive estimates of the concentrations of ligands at the probe (see later figures). When two sensors exhibited crosstalk (NE/DA; AVP/OXT), we first characterized the full extent of partial activation of each of the two sensors by each of the two ligands. We were then able to account for crosstalk effects by deriving closed-form analytical expressions for the absolute concentrations of each of the two interacting ligands based on simultaneous changes in both fluorescent signals (Figure S3D; see also STAR Methods and later figures).

Next, we assessed the effect of signal attenuation due to diffusion through the hydrogel. By measuring the response time course for each ligand concentration and assuming that ligand binding at the sensor is fast (τ on the order of 1 s or less in all GRAB sensors reported so far), we could estimate the diffusion time constant through the hydrogel layer. These diffusion constants varied across ligands, with most signals plateauing within 1 min (Figure S3E).^{31,36–45} We also assessed the dependence of

the diffusion constant on cell depth within the ~200 μ m thick hydrogel and found only a modest delay for cells located further from the front face of the probe (Figure S3F).

In summary, these *in vitro* experiments establish the feasibility of MORSE for quantitatively tracking ten or more green fluorescent sensors at once—an order of magnitude more sensors of a single color than is possible with current methods. In combination with our customized 3D image analysis pipeline, MORSE provides a promising platform for scalable multiplexed ligand sensing in fluids. We next applied MORSE to study the mouse brain in live slices and *in vivo*.

MORSE detects regionalized neuromodulator release in brain slices

We first tested MORSE's ability to detect endogenous release of multiple signals in brain slices. For this application, we created probes using seven of the most reliable sensors: NE, DA, 5-HT, acetylcholine (ACh), AVP, OXT, and histamine (HA). We gently pressed the probes gel-first against specific regions of the brain slice containing known neuromodulator-releasing inputs. Then, we collected 3D two-photon images while performing either (1) bath applications of potassium chloride (KCl) to depolarize the neurons and evoke neuromodulator release or (2) optogenetic activation of neurons.

We began by recording in the dorsal lateral geniculate nucleus (dLGN), the primary thalamic relay nucleus for visual information and a known site of complex neuromodulatory signaling. The dLGN is a major output target of the dorsal raphe nucleus (DRN) and exhibits dense focal enrichment of serotonergic axons from this source.⁵⁸ It is additionally innervated by noradrenergic projections from the locus coeruleus (LC).⁵⁹ We therefore selected the dLGN as a first site in which to test MORSE's ability to detect multiple endogenously released neuromodulators. We targeted the MORSE probes to the region by visualizing serotonergic axons in the dLGN after expressing a virus encoding tdTomato in DRN 5-HT neurons (Figure 2A). Bath application of 50 mM KCl activated GRAB sensors for NE, DA, and 5-HT (Figures 2B and 2C, top; Video S7). We directly confirmed sensor identities by subsequent bath application of the corresponding ligands on the slice. In a subset of experiments ($n = 4/9$), we additionally performed *in vitro* characterization of the probe by dipping it into wells of known solutions following the slice experiment to confirm the sensor type in each cell (Figure S4A; Video S8). Dipping the probe sequentially into wells containing NE, DA, or 5-HT evoked consistent responses in different subsets of several dozen cells matching the cells previously imaged on the slice (Figure 2B, right). These data highlight our capacity to record from this probe for 1–2 h in a brain slice, followed by accurate alignment to data subsequently collected during 1.5 h of dipping. The dipping also identified many additional cells expressing sensors for AVP, OXT, and HA. Most of these cells did not have matching cell masks in the slice experiment, indicating that they did not respond to bath application of NE, DA, or 5-HT, and that KCl did not elicit sufficient release of AVP, OXT, or HA from the slice to drive the respective sensors.

Due to the known crosstalk of NE binding to many versions of both the GRAB-NE and GRAB-DA sensors, we assessed whether the signal in the DA sensor could be driven by the

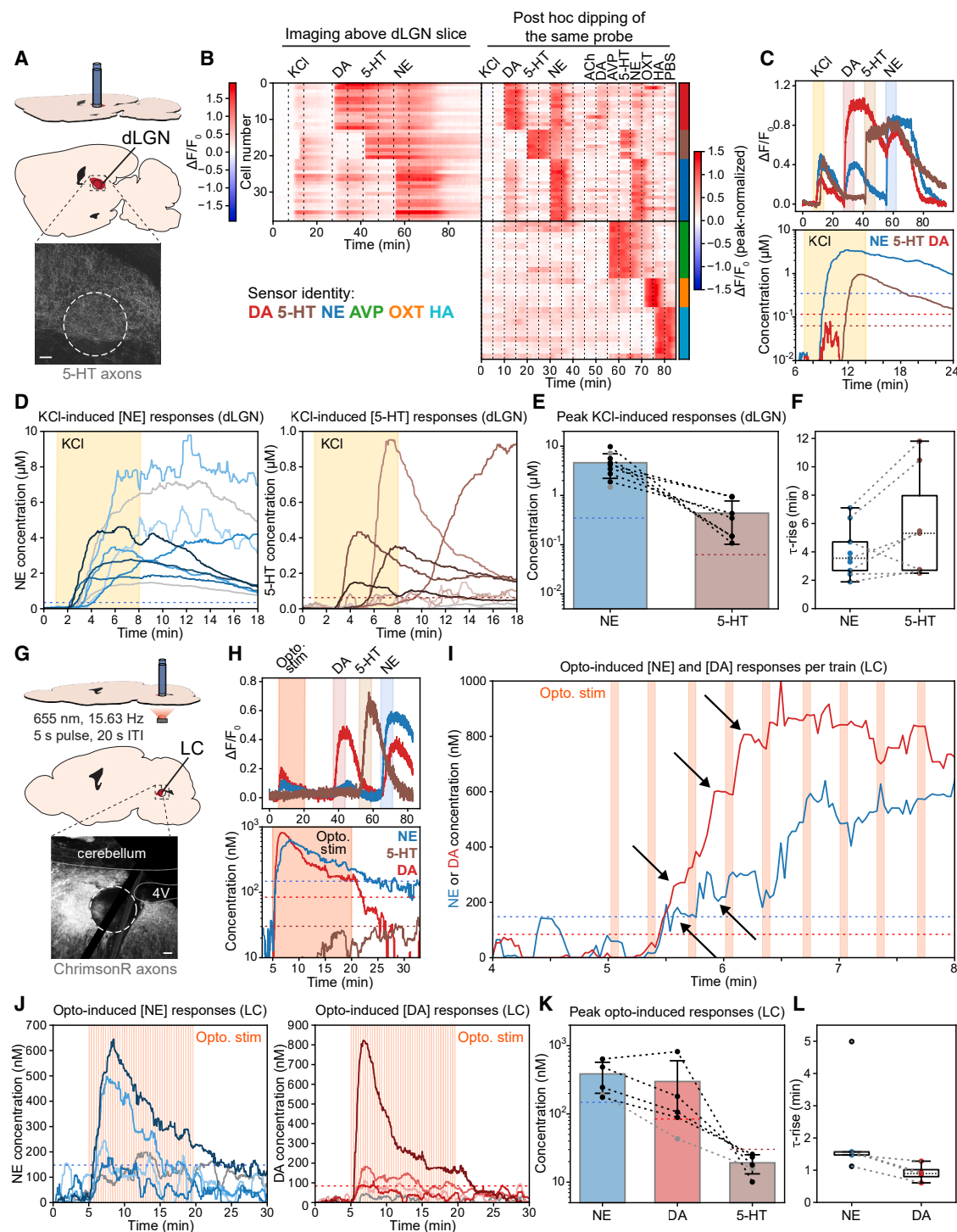


Figure 2. MORSE detects local release of multiple neuromodulators in live brain slices

(A) Dorsal lateral geniculate nucleus (dLGN) slice recording setup. Inset: two-photon image of tdTomato expression in dLGN. Dashed circle: probe location. Scale bars: 100 μm .

(B) Single-sensor cell traces from a seven-sensor (ACh, AVP, OXT, 5-HT, NE, DA, and HA) probe recorded above the dLGN (left heatmap and color bar) and subsequently dipped into standard solutions (right heatmap and color bar). The slice was sequentially superfused with high-KCl artificial CSF (aCSF) (50 mM), DA (50 μM), 5-HT (50 μM), and NE (50 μM). During dipping, the probe was dipped into these same solutions (interposed with dips into aCSF) and subsequently dipped into standard solutions in PBS (1 mM ACh, 1 μM DA, 1 μM AVP, 1 μM 5-HT, 10 μM NE, 1 μM OXT, and 1 mM HA). Lower half of right heatmap: cells that were inactive during the slice recording but responded to dipping. Color bar at right and legend indicate sensor identity.

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release of NE alone from the slice.²⁹ To do this, we calculated both sensors' predicted responses to both ligands using dose-response calibration parameters and obtained quantitative estimates of NE, DA, and 5-HT ligand concentrations at the probe over the course of each slice recording (Figure S3D, see STAR Methods). Responses to KCl at both the NE and DA sensors could be explained solely by the release of NE, rather than a combination of DA and NE (Figures 2C, bottom and S4B; Video S9).

Robust responses to KCl (defined as a peak-normalized $\Delta F/F_0$ signal increase exceeding the 95% confidence interval above the pre-wash-on baseline) were detected for NE in $n = 9/9$ slices and for 5-HT in $n = 7/9$ slices (Figure 2D). KCl-evoked peak concentrations were estimated at $\sim 4.61 \mu\text{M}$ for NE and $\sim 0.44 \mu\text{M}$ for 5-HT (Figure 2E). The rise time of activation of the 5-HT sensor was delayed relative to that of the NE sensor by an average of 111 s (Figure 2F). To correct for smoothing of the signal time courses caused by diffusion of ligands through the gel, we performed deconvolution using the hydrogel diffusion time constants estimated above (Figures S3E and S4C). This resulted in sharper and narrower peak responses for each ligand, suggesting that the true peak concentrations at the slice surface occurred up to 19% sooner and were up to 33% higher than those recorded at the sensors within the hydrogel (Figures S4D and S4E).

To further test the ability of MORSE to detect regional differences in neuromodulation, we also conducted experiments in the paraventricular nucleus of the hypothalamus (PVH). Abundant noradrenergic inputs from LC, as well as nucleus tractus solitarius (NTS) and ventrolateral medulla (VLM), are well established in the PVH, along with serotonergic fibers from the DRN.^{60–64} DA in the PVH has also been implicated in mediating certain sexual behaviors, but the quantities and mechanisms involved are unclear.^{65,66} MORSE probes were targeted to the PVH by visualizing the region after expressing a virus encoding yellow fluorescent protein (YFP) in local monoaminergic neurons of tyrosine hydroxylase (TH)-cre mice (Figure S5A). We used the same set of sensors for imaging in PVH as we did for imaging in dLGN, but this time we also assessed graded signal release by washing on increasing concentrations of KCl in a stepwise

fashion. KCl bath application here again activated the GRAB sensors for NE, DA, and 5-HT (Figures S5B–S5D).

Similar to our observations in the dLGN, quantitative estimation of NE and DA concentrations along with demixing of their combined effects at the DA sensor suggested that NE alone could explain the activation of both sensors in the PVH (Figures S5D and S5E; Video S10). KCl drove robust elevations in both NE and 5-HT in $n = 6/6$ experiments (Figure S5F). While NE sensor cells were reliably driven by 25 mM KCl, 5-HT sensor cells consistently required a higher dose of KCl (50 mM) to be fully activated (Figure S5G). The average NE concentration detected at the PVH in response to 50 mM KCl was $21.4 \mu\text{M}$ —nearly 5-fold higher than at the dLGN, consistent with anatomical findings in rats and macaque monkeys that the PVH receives a greater density of noradrenergic inputs than the dLGN (Figures S5F, S5H, and S5I).^{67,68}

As with the dLGN recordings, we also identified sizable populations of AVP, OXT, and HA sensor cells that were activated by dipping into the corresponding ligands but had no matching masks in the PVH slice recordings (Figure S5B). Thus, despite substantial prior evidence of peptidergic populations in the PVH capable of releasing ligands including AVP and OXT, we were unable to detect their release in response to KCl stimulation with MORSE.^{45,69–75} We speculate that this could reflect non-physiologic somatodendritic release of these molecules at concentrations below the detection limits of the sensors, insufficient quantities of these molecules reaching the probe to drive sensor responses due to their larger molecular weights and slower diffusion through brain tissue, and/or the presence of esterase and peptidase enzymes in the tissue. The effective distance between peptide release site and sensor could be improved by using thinner and more porous hydrogels, as well as sampling a wider range of tissue depths and locations within PVH. It may also be beneficial to shift from KCl stimulation to optogenetic or chemogenetic protocols optimized for maximum peptide release while blocking breakdown with peptidase inhibitors.

Next, we assessed MORSE's ability to detect more naturalistic neuromodulator release during recordings from the LC, a pontine nucleus that regulates wake states and arousal.^{76–78} While TH-expressing LC neurons play a major role in NE release,

(C) Top: mean $\Delta F/F_0$ trace for each sensor type in (B), left. Colored shading: ligand wash-ons. Bottom: estimated ligand concentration traces surrounding KCl delivery. Colored dotted lines signify 95% confidence bounds above pre-KCl baseline (NE: 348 nM [blue], DA: 116 nM [red]; 5-HT: 62.7 nM [brown]).

(D) Robust concentration increase in NE (left, 9/9 experiments) and in 5-HT (right, 7/9) in response to KCl (traces from the same experiment have matched hue). Gray traces: two experiments with peak 5-HT below the 62.7 nM threshold (colored dotted line).

(E) Quantification of the peak KCl-evoked NE and 5-HT concentrations. Dotted lines: robust response thresholds.

(F) Quantification of onset timing (τ -rise) of the time courses in (D).

(G) Locus coeruleus (LC) slice recording setup. Inset: two-photon image of tdTomato expression in LC. Dashed circle: probe location. ITI: inter-trial interval, 4V: fourth ventricle. Scale bars: 100 μm .

(H) Top: mean $\Delta F/F_0$ traces for each sensor type from one seven-sensor MORSE probe over LC, with optogenetic stimulation and ligand wash-on timings indicated by colored shading and sensors color-coded as in (B). The slice was optogenetically stimulated and then sequentially superfused with DA (50 μM), 5-HT (50 μM), and NE (50 μM). Bottom: estimated optogenetically evoked changes in ligand concentration. Dotted lines: 95% confidence threshold of robust responses (NE: 148 nM [blue]; DA: 84.3 nM [red]; 5-HT: 30.3 nM [brown]).

(I) Zoom-in of H, bottom, with individual optogenetic stimulation trains highlighted. Arrows highlight discrete evoked increments of 100–300 nM in both ligands, which reach a plateau over 15–20 s.

(J) Robust concentration increases in NE (left, 5/5 experiments) and in DA (right, 4/5) in response to optogenetic stimulation. Gray traces: one experiment with peak DA below the 84.3 nM threshold.

(K) Quantification of peak evoked concentrations in (J). All 5-HT responses were below threshold (dotted horizontal line).

(L) Quantification of τ -rise of time courses in (J). Error bars in (E) and (K): $\pm$ SEM across recordings.

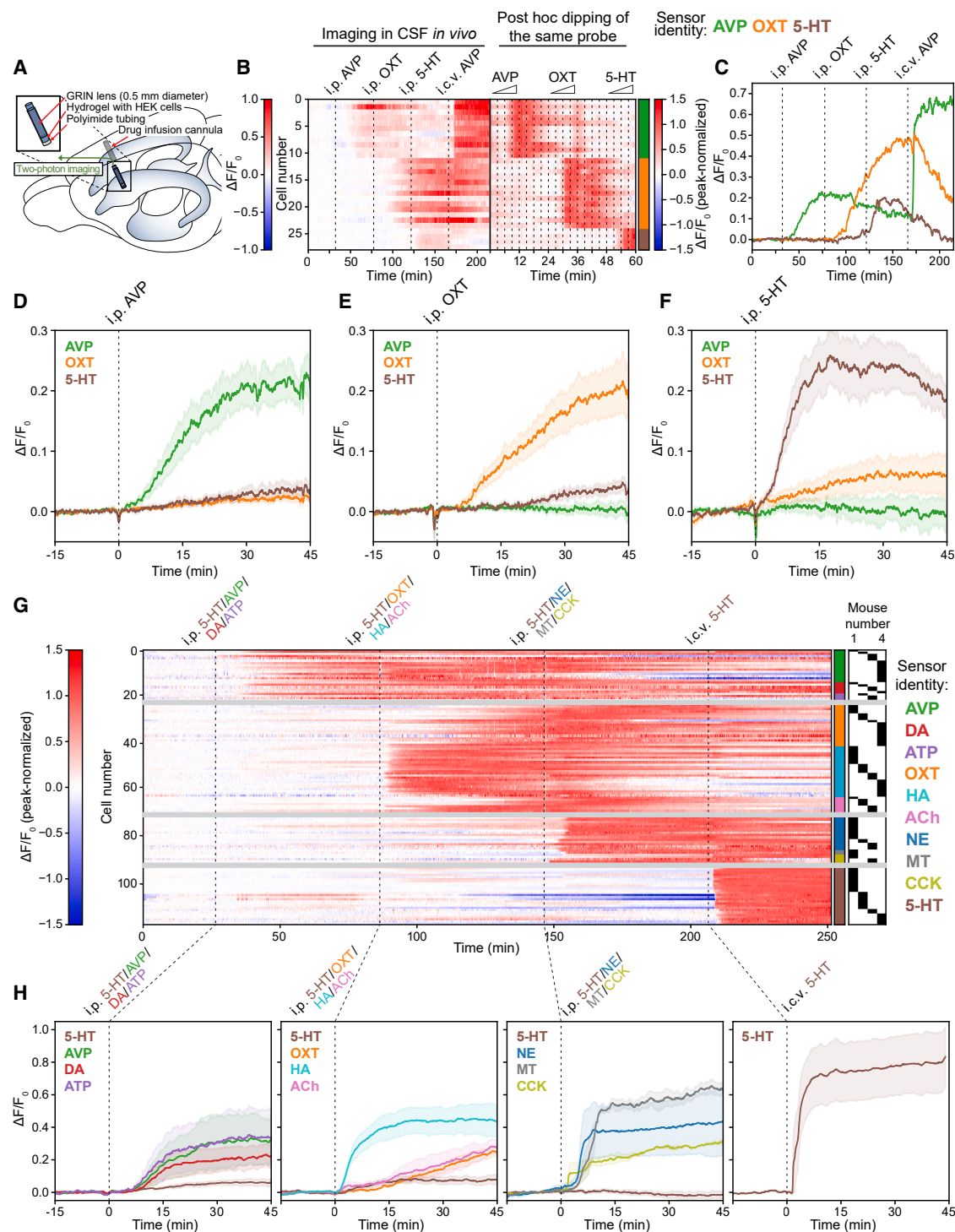


Figure 3. Three-sensor *in vivo* MORSE recordings in the lateral ventricle CSF of awake mice

(A) Schematic demonstrating chronically implanted cannulae in each lateral ventricle, one for MORSE probe insertion and the other for i.c.v. infusion. (B) Single-sensor cell traces from one three-sensor MORSE probe (AVP, OXT, and 5-HT) recorded in mouse CSF (left heatmap and color bar) and subsequently dipped into standard solutions (right heatmap and color bar). AVP (1 mg/kg), OXT (1 mg/kg), and 5-HT (3 mg/kg) were sequentially delivered i.p. in 200 μ L of PBS, followed by saturating i.c.v. delivery of either 500 μ M AVP, 500 μ M OXT, or 5 mM 5-HT (30 μ L at 5 μ L/min). The probe was then removed from the animal and dipped separately into standard solutions of the three ligands, increasing in 10-fold increments per well over a dynamic range of 100,000-fold (maximum concentration: 10 μ M AVP, 100 μ M OXT, 100 μ M 5-HT), with interposed dips in PBS. Right color bar and legend indicate sensor identity of each cell. (C) Mean $\Delta F/F_0$ traces for each sensor type from the left heatmap in (B). Dashed lines indicate i.p./i.c.v. injection times.

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recent studies suggest they also release DA in the hippocampus, midline thalamus, and basolateral amygdala.^{79–82} However, attempts to validate and quantify concurrent release of NE and DA have been hampered by their chemical similarity and aforementioned crosstalk in their detection by multiple modalities.^{19–21,32,38} To test for simultaneous release of NE and DA, we expressed a virus encoding the optogenetic actuator ChrimsonR in LC and nearby neurons and targeted MORSE probes expressing the same seven sensors to the region (Figure 2G).⁸³ Upon optical stimulation with 655 nm light to depolarize LC neurons, we detected activation of GRAB sensors for NE and DA (Figure 2H, top). Quantification of NE and DA release while accounting for sensor crosstalk demonstrated that both ligands were released simultaneously and with distinct time courses, with the DA concentration reaching its peak (248 nM, on average) before the NE concentration (347 nM; Figures 2H and S4F; Video S11). In fact, discrete 100–300 nM increments in the concentrations of both ligands were evident after each of the first several trains of optogenetic stimulation, suggesting that the inter-trial interval of 20 s was sufficient for released neuromodulators to equilibrate between the brain slice and the probe hydrogel (Figure 2I, arrows). Out of five brain slices with robust elevations in NE, four also had robust elevations in DA, while none had robust elevations in 5-HT (Figures 2J and 2K). Rise times of NE concentration were consistently delayed relative to DA concentration by 31 s, or 1–2 trains of optogenetic stimulation (Figure 2L).

Thus, MORSE probes can detect and demix multiple endogenous signals released from neurons. Moreover, these hydrogel probes remained stable for over an hour during slice recordings (46/48 probes) and over a subsequent 1.5+ h of post hoc dipping (15/17 probes).

Real-time, quantitative tracking of CSF composition with MORSE *in vivo*

To begin to test MORSE's ability to record signals in the awake brain, we focused on the CSF, which distributes endogenous signals and pharmaceuticals via the brain ventricles and subarachnoid space.^{2,84–86} AVP and OXT are two endogenous peptides implicated in a number of important central processes, including sociability, feeding, and physiologic regulation.^{69,70,74,75} Reduced CSF levels of AVP and OXT have been linked with certain features of autism spectrum disorder, including anxiety and social deficits, and intranasal administration of both molecules has undergone trials for improvement of such symptoms.^{15,87–89} Plasma 5-HT, known to exhibit substantial elevations in particular states such as viral illness, may also exert effects on the brain by inducing CSF secretion from the ChP.⁹⁰ However, the dynamic central

bioavailability of peripherally administered AVP, OXT, and 5-HT has never been systematically assessed.⁹¹

We prepared mice with a headpost and chronic cannula implantation in both lateral ventricles (Figure 3A). Cannula location and ventricular patency were confirmed with post hoc histology (Figures S6A–S6C).⁹² After at least 2 weeks of recovery, we opened the cannulae to confirm direct observation of pristine CSF. We acutely inserted a MORSE probe into the awake mouse lateral ventricle CSF via one cannula (without disrupting brain tissue) and a catheter for intracerebroventricular (i.c.v.) infusion into the other cannula. We then performed 3D volumetric two-photon imaging of the probe as described above. We began with MORSE probes expressing sensors for AVP, OXT, and 5-HT and delivered these ligands sequentially by intraperitoneal (i.p.) injection (1 mg/kg AVP, 1 mg/kg OXT, and 3 mg/kg 5-HT) followed by i.c.v. delivery of saturating concentrations of AVP ($n = 5$ experiments) or OXT ($n = 6$). Dipping characterization was then performed *ex vivo* to identify each sensor cell.

Upon peripheral delivery of each ligand, we reliably observed a specific corresponding sensor response in the CSF within at most 10–15 min, followed by wash-out (Figures 3B–3F; Videos S12 and S13). To assess AVP and OXT one at a time in CSF with a simpler fiber photometry setup, we replicated the analogous probe design on an optical fiber, utilizing one GRAB sensor at a time as reported recently (Figures S6D–S6F).⁹³ This approach demonstrated similar kinetics of CSF AVP and OXT time courses following i.p. injection as compared with the MORSE technique involving two-photon imaging (Figures 3D and 3E).

Next, we extended these recordings to the full set of ten sensors validated in Figure 1G. We sequentially delivered three i.p. injections of mixtures of four ligands at a time, with 5-HT present in every mixture (to assess repeatability), followed by a final saturating i.c.v. injection of 5-HT. Across four animals, we were able to successfully recover simultaneous signals from up to 9 distinct sensors per probe (median: 8.5 sensors; average of 3.8 cells per sensor) (Figure 3G). Each sensor demonstrated a response following i.p. injection of the respective ligand, with diverse response kinetics across ligands that likely reflect differential permeability from blood into CSF (Figure 3H).

We could also quantitatively estimate the concentration of one ligand by normalizing the corresponding sensor time course using saturating (i.c.v.) levels of the same ligand and mapping this normalized fluorescence to *ex vivo* dose-response curves. We found that i.p. delivery drove CSF levels in the tens of nanomolar (5-HT) or hundreds of nanomolar (AVP and OXT) ranges (Figures 4A, 4D, and 4G). To benchmark against conventional methods, we also performed identical i.p. injections in naive,

(D–F) Mean $\Delta F/F_0$ responses in AVP, OXT, and 5-HT sensor cells ($n = 11$ mice) from three-sensor MORSE probes in mouse CSF during i.p. injection of AVP (D), OXT (E), or 5-HT (F).

(G) Ten-sensor (AVP, DA, ATP, OXT, HA, ACh, NE, MT, CCK, 5-HT) MORSE probes were implanted in mouse CSF. Mice received three i.p. injections, each containing mixtures of four ligands (5-HT/AVP/DA/ATP at 3, 1, 1, and 50 mg/kg, respectively; 5-HT/OXT/HA/ACh at 3, 1, 10, and 10 mg/kg, respectively; and 5-HT/NE/MT/CCK at 3, 1, 10, and 1 mg/kg, respectively), followed by i.c.v. injection of 5-HT (5 nmol/ μ L in aCSF infused at 5 μ L/min over 6 min). Heatmap depicts peak-normalized $\Delta F/F_0$ signals for each individual cell in $n = 4$ mice. Color bar (labeled per legend) and black raster indicate each cell's sensor identity and probe of origin, respectively. Up to 9/10 sensors were successfully recorded in a single mouse (median of 8.5 active sensors per probe, average of 3.8 cells per sensor per probe).

(H) Mean $\Delta F/F_0$ responses of the four sensors corresponding to each multi-drug i.p. injection, or the single-ligand i.c.v. injection, in (G). Shading in (D)–(F) and (H): $\pm$ SEM across mice.

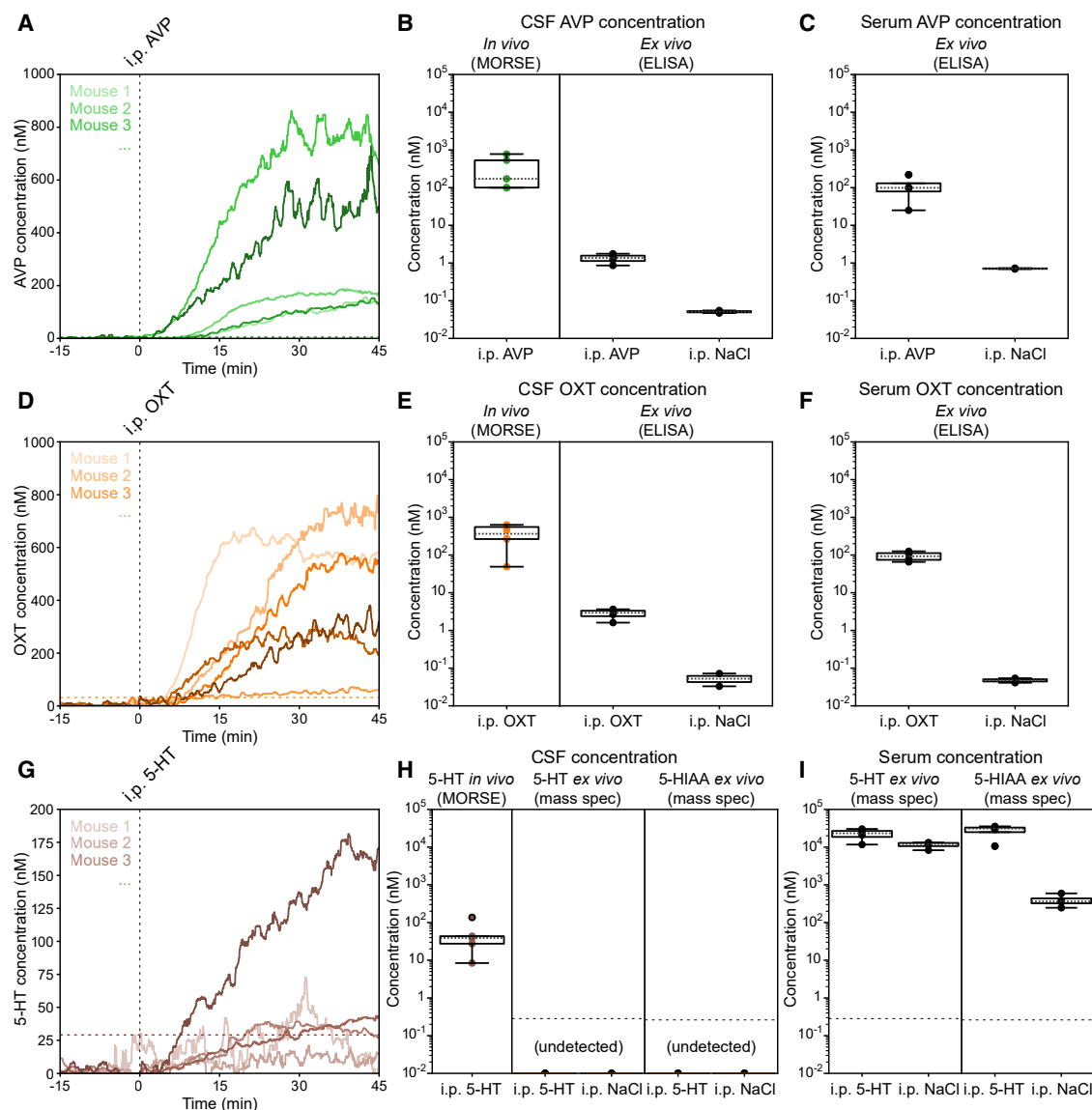


Figure 4. Real-time, quantitative estimates of AVP, OXT, and 5-HT concentrations in lateral ventricle CSF of awake mice following i.p. injection

Mice in Figures 3A, 3D, and 3G received i.c.v. infusions of AVP, OXT, or 5-HT, respectively, at the end of each recording to normalize i.p. responses for quantitative concentration estimation (Figure S3C).

(A) Changes in estimated AVP concentration in CSF upon i.p. injection of AVP (1 mg/kg) across five mice that later received i.c.v. AVP.

(B) Left: AVP concentrations in (A), 30 min after injection. Right: ELISA quantifications of AVP levels in CSF collected from naive mice 30 min following i.p. injection of AVP (1 mg/kg, $n = 3$) or saline control ($n = 2$).

(C) ELISA quantifications of AVP levels in serum (from the same animals as in B, right).

(D) Changes in estimated OXT concentration in CSF upon i.p. injection of OXT (1 mg/kg) across six mice that later received i.c.v. OXT.

(E) Left: OXT concentrations in (D), 30 min after injection. Right: ELISA quantifications of OXT levels in CSF collected from naive mice 30 min following i.p. injection of OXT (1 mg/kg, $n = 3$) or saline control ($n = 2$).

(F) ELISA quantifications of OXT levels in serum (from the same animals as in E, right).

(G) Changes in estimated 5-HT concentration in CSF upon i.p. injection of 5-HT (3 mg/kg) across five mice that later received i.c.v. 5-HT.

(H) Left: 5-HT concentrations in (G) at 30 min following i.p. injection. Middle/right: mass spectrometry quantifications of 5-HT (middle) or 5-HIAA (right) levels in CSF from naive mice, 30 min following i.p. injection of 5-HT (3 mg/kg, $n = 4$) or saline control ($n = 4$).

(I) Mass spectrometry quantifications of 5-HT (left) and 5-HIAA (right) levels in serum (from the same animals as in H, middle/right).

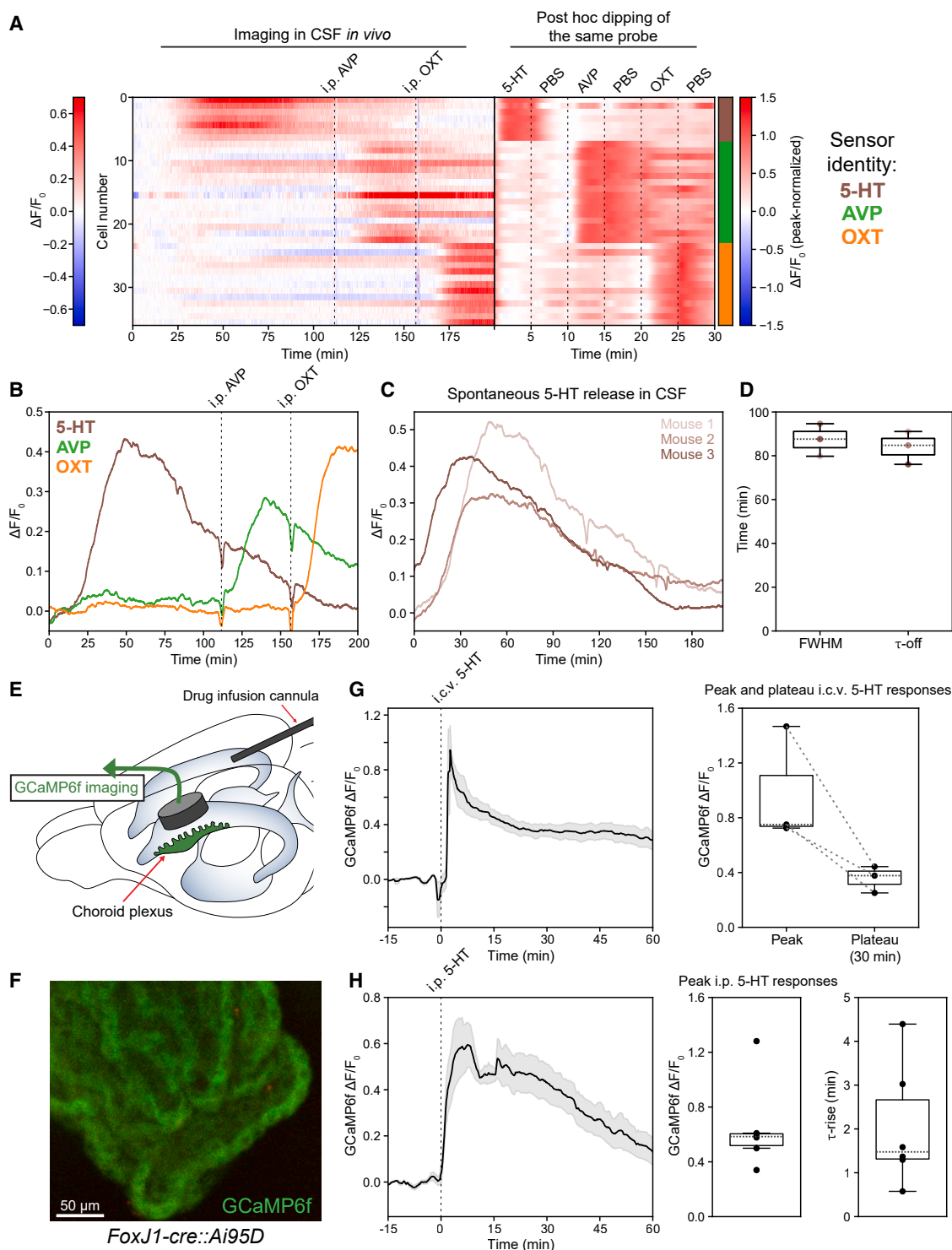


Figure 5. Spontaneous 5-HT fluctuations in lateral ventricle CSF

In a subset of three-sensor MORSE recordings ($n = 3$), putative spontaneous CSF 5-HT fluctuations were identified.

(A) Single-sensor cell traces from one three-sensor (AVP, OXT, and 5-HT) MORSE probe recorded in awake mouse CSF (left heatmap and color bar) and subsequently dipped into standard solutions (right heatmap and color bar). AVP and OXT were delivered i.p. (200 μ L, 1 mg/kg in PBS) at the indicated times. The probe was then removed from the animal and dipped into standard solutions of the three ligands in PBS (1 μ M AVP, 1 μ M OXT, and 1 μ M 5-HT), with interposed dips into PBS wash wells. Sensor identity of each cell is shown with the color bar on the right.

(B) Mean $\Delta F/F_0$ traces for each sensor type from the left heatmap in (A), with i.p. injection times indicated and sensors color-coded as in (A).

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unoperated mice, collected serum and CSF 30 min later, and performed ELISA or mass spectrometry to analytically quantify concentrations of AVP, OXT, 5-HT, and 5-hydroxyindoleacetic acid (5-HIAA)—the main breakdown product of 5-HT (Figures 4B, 4C, 4E, 4F, 4H, and 4I). We found that the i.p. injections drove elevated levels in serum, with AVP and OXT increases also detected in CSF. Notably, the levels quantified in these *ex vivo* CSF samples were over two orders of magnitude lower than those estimated *in vivo* by MORSE at the same 30-min time point following i.p. injection. Consistent with these concentration reductions being due to delayed *ex vivo* sample analysis, AVP concentration estimated by MORSE *ex vivo* by dipping in serum samples was similar to that obtained with ELISA (Figures S6G–S6J).

These experiments showcase the integrity of MORSE probes over >4 h in an *in vivo* setting, followed by subsequent hours-long *ex vivo* dipping for sensor identification. The probes can track pharmacologically relevant concentration changes in CSF over time while avoiding the challenges of *ex vivo* methods, such as contamination during collection and rapid analyte breakdown. Furthermore, multiplexed tracking of ten sensors simultaneously allows for *in vivo* studies of CSF pharmacodynamic interplay in combinatorial drug regimens, with a temporal resolution of tens of seconds and the potential for quantitative concentration readouts. The large reduction in estimated signal concentrations in CSF using standard methods of fluid profiling as compared with *in vivo* MORSE suggests that existing estimates of low CSF signal concentrations and assumptions about low accessibility of peripheral molecules to the CSF may need to be revisited.

MORSE detects endogenous CSF signals at concentrations that impact downstream signaling

To assess slow, endogenous changes in CSF composition during quiescence, we inserted a three-sensor MORSE probe (AVP, OXT, and 5-HT) into one cannula and imaged the probe with the mouse undisturbed on the running wheel for 2 h before administering several i.p. injections and then performing *ex vivo* dipping characterization. In a subset of experiments ($n = 3/7$), we observed a spontaneous elevation in CSF 5-HT that was evident in all 5-HT sensor cells (but none of the other sensor cells) within the first 60–90 min of the recording (Figures 5A and 5B). These fluctuations were of similar magnitude or larger than the signals we observed in CSF following i.p. 5-HT delivery (compare Figures 5A and 5B with 3F) and slowly rose and decayed over tens of minutes (Figures 5C and 5D). Fluctuations in CSF 5-HT on a timescale of several hours have been described in humans, but their

mechanisms and impacts are unknown.⁹⁴ One of the tissues that prominently contacts the CSF is the ChP epithelium, a sheet of cells within each ventricle that modulates CSF composition. The ChP is known to express high levels of the 5-HT_{2C} receptor, a 5-HT $G_{\alpha q}$ -coupled GPCR enriched on the apical (CSF-facing) surface of the ChP epithelium, which drives an increase in cytosolic calcium and secretory activity.^{95,96}

We wondered whether the magnitudes of these endogenous and i.p.-evoked elevations in CSF 5-HT might exert a meaningful impact on the ChP. To this end, we expressed the cytosolic calcium indicator GCaMP6f in the ChP epithelial cells (in adult *FoxJ1-cre::Ai95D* mice).⁹⁶ We implanted an imaging cannula with a glass window into one lateral ventricle and an infusion cannula into the other (Figures 5E and 5F).^{86,96,97} Four weeks later, awake mice received either i.p. (3 mg/kg, as in Figures 3F, 5A, and 5B) or i.c.v. (75–150 nmol or ~0.5–1 mM) delivery of 5-HT during 3D two-photon GCaMP imaging of the ChP epithelium (Figures 5G and 5H; Videos S14 and S15). Both routes of 5-HT administration drove similar amplitudes of calcium activity in the ChP epithelial cells, and both responses persisted for at least 60 min following delivery. These observations confirmed that changes in central (and possibly also peripheral) 5-HT can drive calcium increases in the ChP epithelium, likely resulting in downstream signaling.^{96,97}

Molecular transport through the CSF is tightly regulated, and the CSF components that we recorded with MORSE must reach receptors at the ChP epithelium, other CSF-facing barriers, or the brain parenchyma in order to drive downstream signaling. We selectively expressed GRAB sensors in the ChP epithelium to confirm that MORSE recordings estimate CSF signals in a manner that reflects their levels and dynamics immediately adjacent to this tissue. We began with i.c.v. delivery of a virus (GRAB-AVP0.2, OXT1.7, or 5-HT2h) expressing the same sensor as above and then implanted a lateral ventricle imaging window and infusion cannula (Figure 6A). 4 weeks later, awake two-photon imaging demonstrated membrane expression of each sensor in ChP epithelial cells (Figures 6B, 6D, and 6F; Video S16). We additionally injected a Texas Red-labeled 70 kDa dextran i.p. to label the vasculature and confirmed that GRAB sensor expression was largely concentrated in highly elaborate microvilli on the CSF-facing epithelial surface (Figure 6A, inset).⁹⁸

In subsets of mice, i.p. delivery of 5-HT, AVP, or OXT drove associated sensor signals in ChP with similar time courses as observed previously with MORSE (compare Figures 6C and 3F, 6E and 3D, 6G and 3E). Furthermore, i.p. 5-HT reached the apical ChP membrane (Figure 6C) fast enough to explain the

(C) Summary $\Delta F/F_0$ traces from 5-HT sensor cells in three separate mice demonstrating spontaneous 5-HT fluctuations.

(D) Quantification of dynamics (full width at half maximum, FWHM, and τ -off wash-out timing following peak signal) of spontaneous 5-HT signals summarized in (C) (same hues as in C).

(E) Setup for mouse ChP epithelium GCaMP6f imaging. 3D two-photon imaging of the ChP epithelial cells was performed via the cranial window, with the contralateral cannula optionally used for i.c.v. infusions during the recording.

(F) ChP epithelium expressing GCaMP6f in an awake *FoxJ1-cre::Ai95D* mouse imaged through the cranial window. Scale bar: 50 μ m.

(G) Left: mean $\Delta F/F_0$ GCaMP6f response to i.c.v. 5-HT delivery (5 nmol/ μ L in aCSF infused at 5 μ L/min over 3 min) in ChP epithelium of three mice. Right: comparison of peak and plateau (30 min time point) $\Delta F/F_0$ GCaMP6f responses in (G), left. Values from the same recording are connected by dotted lines.

(H) Left: mean $\Delta F/F_0$ GCaMP6f response to i.p. 5-HT delivery (2 mg/kg) in ChP epithelium of six mice. Middle: peak $\Delta F/F_0$ signals in each recording. Right: τ -rise onset timings of GCaMP6f time courses in (H), left. Shading in (G) and (H): $\pm$ SEM across mice.

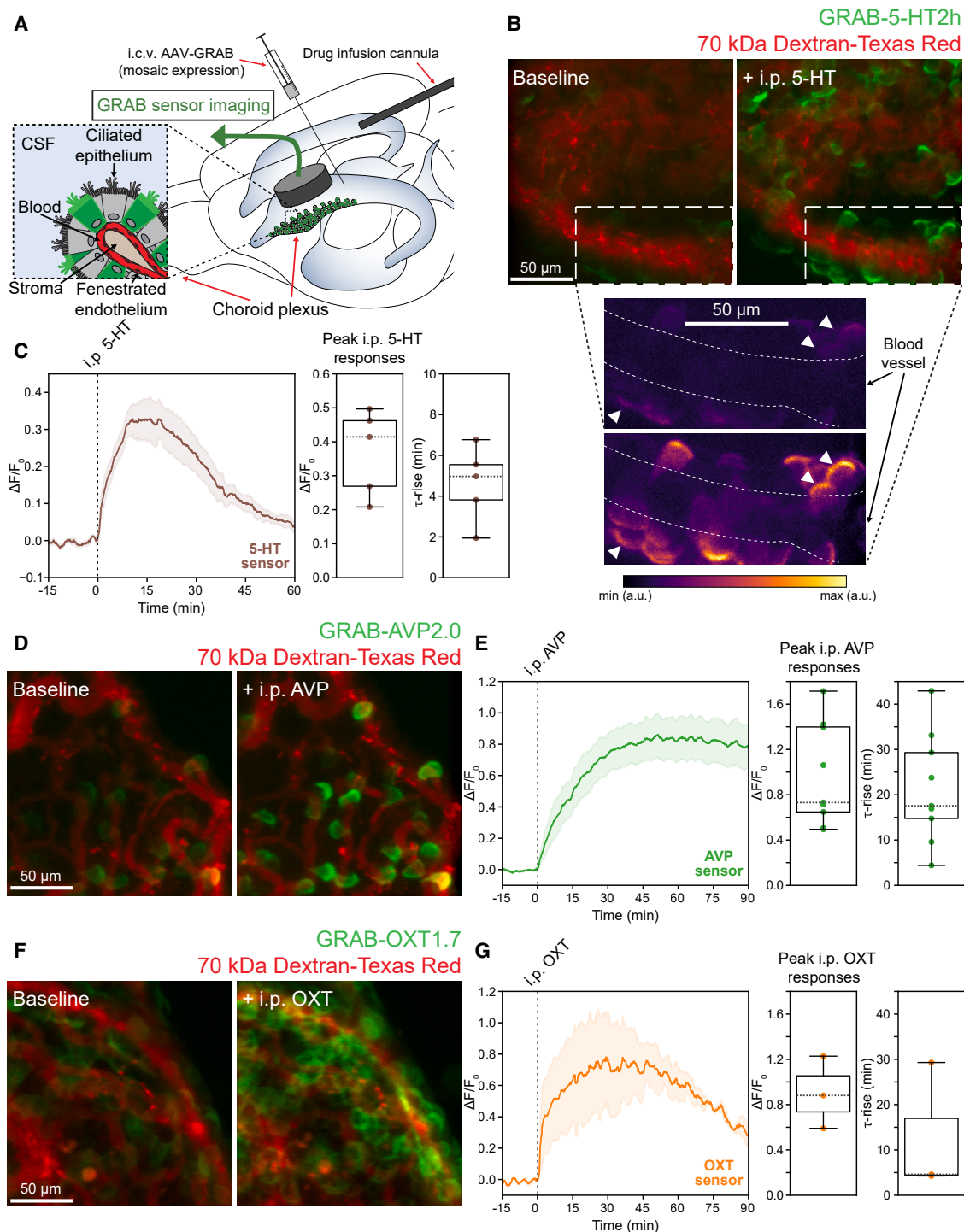


Figure 6. GRAB sensors expressed in the ChP epithelium directly report chemical signals in awake mouse CSF

(A) Setup for mouse ChP epithelium GRAB sensor imaging. 3D two-photon imaging of ChP epithelial cells expressing a GRAB sensor was performed via the cranial window, with the contralateral cannula used for i.c.v. infusions. Inset: zoom-in of ChP epithelium demonstrates mosaic pattern of GRAB sensor expression by i.c.v. adeno-associated virus (AAV) delivery.

(B) Top: GRAB-5-HT2h expression along the apical membranes of epithelial cells facing the CSF, away from the vasculature labeled with 70 kDa dextran-Texas Red. Bottom: closeup of dashed area in upper images, with green GRAB-5-HT2h channel shown in false color. White arrowheads indicate sensor activation on the apical epithelial membranes facing the CSF following i.p. 5-HT (2 mg/kg). a.u.: arbitrary units. Scale bars: 50 μ m.

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ChP calcium response (Figure 5H). Thus, levels of 5-HT following i.p. delivery or during endogenous fluctuations that we observed in CSF (Figures 5A–5D) were sufficient to drive calcium responses in ChP. These experiments demonstrate that MORSE exhibits sufficient sensitivity *in vivo* to detect signals of likely biological relevance.

DISCUSSION

To our knowledge, MORSE is the first demonstration of a probe that is both (1) capable of recording ten or more optical sensors simultaneously and (2) sufficiently compact (0.5 mm diameter, 0.2 mm² sensing area) for *in vivo* chemical tracking in the brain of an awake, behaving animal. Our approach is scalable due to the large number of sensor cells (>100) together with the ability to pair experiments with robotic dipping of the same probe into many standard solutions. We expect that the number of signaling molecules that can be studied in this fashion will substantially expand as the repertoire of genetically encoded sensors continues to grow, together with the additional scaling achievable by simultaneously expressing sensors of multiple colors in each cell.³² Some such sensors also possess measurable fluorescence lifetime signals, opening the possibility of chronic recordings over days and weeks.^{33,34} This approach is also extendable to other cell-based sensors such as cell-based neurotransmitter fluorescent engineered reporters (CNiFERS) as well as cell-free soluble sensors immobilized on microbeads or other carriers.^{99–101}

Our design possesses several limitations that could be improved in the future. First, we rely on a hydrogel to immobilize the sensor-expressing cells. A similar recent probe design, fiber-optic probes based on engineered cells (FOPECs), recorded a single GRAB sensor at a time *in vivo* using fiber photometry.⁹³ We optimized our fragile hydrogel significantly and combined several surface coatings at the lens-hydrogel interface to achieve reliable *in vivo* stability of ~10-fold smaller gel volumes. The resulting enhanced sensor sensitivity and time resolution (due to reduced signal diffusion and attenuation) allow detection of signal transients at a timescale of ~20 s (Figures 2I and S3E). Although our current design matches the fastest time resolutions (tens of seconds) of microfluidics-coupled microdialysis¹⁸ while avoiding the latter's challenges of continuous signal dilution in perfused fluid (>100 nL/min perfusion over hours vs. implantation of a single 40 nL hydrogel), it cannot yet resolve subsecond neuromodulator transients on the timescale of single action potentials. Another challenge with our approach is the reliance on cultured cells to express the sensors. We chose these because GRAB sensors, like many other receptor-based sensors, are transmembrane proteins that have been optimized and validated in cultured cells. Future strategies could replace the use of

cultured cells with sensor expression in liposomes or lipid bilayers.^{102,103} These could be purified and patterned directly on the lens face, resulting in a more stable probe-tissue interface that is essentially 2D.¹⁰⁴ Such a design would not be subject to hydrogel deformation and would likely have fewer barriers to signal diffusion as well as a reduced need for slow volumetric image sampling, potentially allowing faster and lower-concentration signals to be detected. Direct patterning of sensors on the lens would also facilitate further probe miniaturization, allowing more localized molecular release to be resolved, beyond the spatial scale of several hundred microns that we have demonstrated so far. Reducing immunogenic cultured cell epitopes, as well as potentially mixing immunosuppressant drugs directly into the hydrogel, would likely improve permeability at the probe-brain interface and increase the likelihood of their applicability in brain tissue in animal models and humans. While we did not assess the *in vivo* stability of our probes beyond several hours of imaging, previous hydrogel-based probes with similar recipes have been shown to remain functional for several days, though image quality may be compromised if the gel deforms or deteriorates over time.⁹³

We chose the CSF as the proof-of-principle brain fluid to study with MORSE *in vivo* because it is a readily accessible bulk fluid with important functions in brain-wide signaling and a major conduit for pharmaceuticals intended to reach the brain. Remarkably, we found that all of the ligands we delivered reached detectable concentrations at MORSE probes in CSF within 15 min of an i.p. injection at common doses used in the literature, suggesting that most molecules of sufficiently small molecular weight may cross from blood to CSF at appreciable quantities (see Figures 3G and 3H).⁸⁶ This is at odds with previous reports suggesting that some of the molecules we tested—particularly AVP, OXT, and 5-HT—“do not readily cross the blood-brain barrier.”^{91,105} and underscores the role of the CSF in the biodistribution of global signals to CSF-adjacent tissues (such as ChP and periventricular ependymal cells) and throughout the brain. Furthermore, fluctuations in concentrations of molecules in CSF are likely to be shaped by their processing in other fluid compartments. For example, the rapid degradation of 5-HT into 5-HIAA (Figures 3F and 4G–4I) by peripheral enzymes likely explains its rapid and transient appearance in CSF—a finding that cannot be captured by standard methods for analyzing fluid composition. Our findings also suggest an important new avenue for pharmaceutical testing: in addition to AVP and OXT, the clinical relevance of which was described previously, other potent molecules that entered CSF upon i.p. injection include DA and melatonin, which have long-standing therapeutic uses in Parkinson's disease and various sleep disorders, respectively.^{14,15,87–89} It also remains to be

(C) Left: mean $\Delta F/F_0$ GRAB-5-HT2h response to i.p. 5-HT delivery (2 mg/kg, 5 injections across 4 mice). Middle/right: peak $\Delta F/F_0$ signals (middle) and τ -rise onset timing (right) in each individual recording.

(D) GRAB-AVP2.0 expression along the apical membranes of epithelial cells, activated by i.p. AVP (1 mg/kg). Scale bar: 50 μ m.

(E) Left: mean $\Delta F/F_0$ of GRAB-AVP0.2 response to i.p. AVP (1 mg/kg, $n = 9$ injections across $n = 4$ mice). Middle/right: peak $\Delta F/F_0$ signals (middle) and τ -rise onset timings (right).

(F) GRAB-OXT1.7 expression along the apical membranes of epithelial cells, activated by i.p. OXT (1 mg/kg). Scale bar: 50 μ m.

(G) Left: mean $\Delta F/F_0$ GRAB-OXT1.7 response to i.p. OXT (1 mg/kg, $n = 3$ injections across $n = 2$ mice). Middle/right: peak $\Delta F/F_0$ signals (middle) and τ -on onset timings (right) from each animal. Shading in (C), (E), and (G): $\pm$ SEM across injections.

seen how all the barrier structures between blood and CSF—including the ChP as well as the circumventricular organs, meninges, blood-brain barrier of the neurovascular endothelium, and others—work together to shape the bioavailability of each signaling factor.¹⁰⁶

We additionally detected previously unstudied spontaneous fluctuations in CSF 5-HT and demonstrated that they are likely sufficient to drive intracellular calcium activity in the apposing ChP epithelial cells, suggesting that at least certain CSF-contacting tissues are readily poised to respond to these biologically meaningful signals.⁹⁴ This expands on previous studies highlighting roles for CSF in distributing signaling molecules mediating physiological transitions across circadian and diurnal rhythms, hunger and satiety, stress, and learning.^{3–11} Future studies can explore how neurons sense these CSF signals, either by sending projections directly into the fluid or by responding to molecules that have diffused into the interstitial fluid near the ventricle walls.^{107,108}

Multiplexed ligand tracking with MORSE also shows promise for studying high-dimensional dynamics of interstitial fluid composition in the brain parenchyma. As we demonstrated in our brain slice experiments, the probe can detect evoked NE, DA, and 5-HT release from brain slices in a quantitative manner that likely reflects the density of local neuromodulatory innervation. While previous studies using fast-scan cyclic voltammetry have performed similar quantitative measurements of monoamine release, they could not record all molecules simultaneously and did not allow for catecholamines NE and DA to be directly distinguished.¹⁰⁹

An essential yet unaddressed challenge in neuroscience is that neuromodulatory signals are often partially correlated but studied one at a time, even though various brain cell types integrate many of these signals simultaneously. Understanding the *dynamic patterns* of neuromodulatory concentrations across brain states is a new frontier, one that is critical to understanding how individual cells sense and adapt to changing brain states. Future efforts to miniaturize the MORSE probes, reduce their immunogenicity, and concurrently record the activity of nearby brain cells should further enhance the viability of our approach in diverse applications in brain tissue *in vivo*, beyond the CSF. Ultimately, these efforts will improve our understanding of how brain fluids change in composition across a range of timescales in a quantitative manner and allow for novel assessments of how sets of molecular signals act in concert to encode brain states and concurrently orchestrate diverse physiology and behavior.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Mark L. Andermann (manderma@bidmc.harvard.edu).

Materials availability

This study did not generate any new, unique reagents.

Data and code availability

- Individual sensor cell traces from all experiments have been deposited at Distributed Archives for Neurophysiology Data Integration (DANDI) at <https://dandiarchive.org/dandiset/001074> and are publicly available as

of the date of publication. Raw two-photon imaging and fiber photometry datasets are available from corresponding authors upon request.

- All original code is deposited on GitHub (<https://github.com/pkalugin/kalugin-et-al-morse>) as well as Zenodo at [10.5281/zenodo.21142692](https://doi.org/10.5281/zenodo.21142692) and is publicly available as of the date of publication.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

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AUTHOR CONTRIBUTIONS

P.N.K., M.K.L., and M.L.A. conceived and designed the study; P.N.K. and P.A.S. developed and tested probes and collected *in vitro* data with support from D.T. and A.L.; P.N.K. and C.I.M. performed slice experiments with support from Z.B.S.; P.N.K., P.A.S., M.P., Z.B.S., and D.C.G. prepared animals and collected *in vivo* tissue, and fluid data with support from S.X.Z.; P.N.K. and O.A. designed and implemented computational pipelines for image analysis and signal extraction with advice from M.L.A. and data annotation from P.A.S., Z.B.S., J.C.B., M.F.H., D.M.T., C.D.A., T.E.L., Y.C., and A.H.; H.W., L.G., G.L., B.L., and Y.L. developed and provided GRAB sensor constructs; P.N.K. and M.L.A. wrote the manuscript with support from P.A.S.; all co-authors edited the manuscript.

DECLARATION OF INTERESTS

M.L.A., P.N.K., M.K.L., P.A.S., and D.T. are co-authors of US Patent Office application number 63/481,367, docket number 01948-288001 concerning this work.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-Iba1	Wako	Cat#019-19741; RRID: AB_839504
Rat anti-GFAP (2.2B10)	Invitrogen	Cat#13-0300; RRID: AB_2532994
Alexa Fluor 488 goat anti-rabbit	Invitrogen	Cat#A-11008; RRID: AB_143165
Alexa Fluor 647 goat anti-rat	Invitrogen	Cat#A-21247; RRID: AB_141778
Bacterial and virus strains		
rAAV1-Syn-Flex-ChrimsonR-tdTomato	University of Pennsylvania Vector Core	Klapoetke et al. ⁸³
rAAV1-Syn-ChrimsonR-tdTomato	University of Pennsylvania Vector Core	Klapoetke et al. ⁸³
AAV5-GRAB-5-HT2h	University of Pennsylvania Vector Core	N/A
AAV5-GRAB-AVP2.0	University of Pennsylvania Vector Core	N/A
AAV5-GRAB-OXT1.7	University of Pennsylvania Vector Core	N/A
Chemicals, peptides, and recombinant proteins		
Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-free, LDEV-free	Corning	Cat#356231
Collagen I, High Concentration, Rat Tail	Corning	Cat#354249
Potassium hydroxide solution, 45 wt. % in H ₂ O	Sigma	Cat#417661
Colchicine	Sigma	Cat#C9754
[Arg8]-Vasopressin acetate salt	Sigma	Cat#V9879
Serotonin hydrochloride	Sigma	Cat#H9523
L-(–)-Norepinephrine (+)-bitartrate salt monohydrate	Sigma	Cat#A9512
Acetylcholine chloride	Sigma	Cat#A6625
Adenosine 5'-triphosphate magnesium salt	Sigma	Cat#A9187
Oxytocin acetate salt	Bachem	Cat#4016373
Histamine dihydrochloride	Sigma	Cat#H7250
Dopamine hydrochloride	Sigma	Cat#H8502
Cholecystokinin Octapeptide (sulfated) ammonium salt	Bachem	Cat#4033010
Melatonin	Sigma	Cat#5250
aCSF	Tocris	Cat#3525
Albumin from Bovine Serum (BSA), Alexa Fluor 555 conjugate	Invitrogen	Cat#A34786
Critical commercial assays		
DetectX Arg8-Vasopressin Enzyme Immunoassay Kit	Arbor Assays	Cat#K049-H1
DetectX Oxytocin Enzyme Immunoassay Kit	Arbor Assays	Cat#K048-H1
Deposited data		
Imaging dataset on DANDI	This paper	https://dandiarchive.org/dandiset/001074
Experimental models: Cell lines		
HEK-293T	ATCC	CRL-3216; RRID: CVCL_0063
Nanoluciferase/tdTomato Dual-Reporter HEK-293	Alstem	NRL01; RRID: CVCL_B7NY

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Mouse: C57BL/6J	The Jackson Laboratory	Cat#000664; RRID: IMSR_JAX:000664
Mouse: Crl:CFW(SW) Swiss Webster	Charles River	Cat#024; RRID: IMSR_CRL:024
Pet1-cre: B6.Cg-Tg(Fev-cre)1Esd/J	The Jackson Laboratory; Scott et al. ¹¹⁰	Cat#012712; RRID: IMSR_JAX:012712
TH-cre: B6.Cg-7630403G23RikTg(Th-cre)1Tmd/J	The Jackson Laboratory; Savitt et al. ¹¹¹	Cat#008601; RRID: IMSR_JAX:008601
Ai32: B6.Cg-Gt(ROSA)26Sortm32(CAG-COP4*H134R/EYFP)Hze/J	The Jackson Laboratory; Madisen et al. ¹¹²	Cat#024109; RRID: IMSR_JAX:024109
FoxJ1-cre: C57BL/6-Tg(FOXJ1-cre)F26Htm	Zhang et al. ¹¹³	MGI:5905134; RRID: MGI:5905134
Ai95D: B6J.Cg-Gt(ROSA)26Sortm95.1(CAG-GCaMP6f)Hze/MwarJ	The Jackson Laboratory; Madisen et al. ¹¹⁴	Cat#028865; RRID: IMSR_JAX:028865
Recombinant DNA		
pDisplay-GRAB-AVP0.2-ires-mCherry-CAAX	Yulong Li lab	N/A
pDisplay-GRAB-5-HT2h-ires-mCherry-CAAX	Yulong Li lab	N/A
pDisplay-GRAB-NE2h-ires-mCherry-CAAX	Yulong Li lab	N/A
pDisplay-GRAB-gACh4l-ires-mCherry-CAAX	Yulong Li lab	N/A
pDisplay-GRAB-ATP1.0-L-ires-mCherry-CAAX	Yulong Li lab	N/A
pDisplay-GRAB-OXT1.7-ires-mCherry-CAAX	Yulong Li lab	N/A
pDisplay-GRAB-HA1m-ires-mCherry-CAAX	Yulong Li lab	N/A
pDisplay-GRAB-DA2.9-ires-mCherry-CAAX	Yulong Li lab	N/A
pDisplay-GRAB-CCK0.5-ires-mCherry-CAAX	Yulong Li lab	N/A
pDisplay-GRAB-MT1.0-ires-mCherry-CAAX	Yulong Li lab	N/A
pDisplay-GRAB-NPY1.0-ires-mCherry-CAAX	Yulong Li lab	N/A
pDisplay-GRAB-VIP1.0-ires-mCherry-CAAX	Yulong Li lab	N/A
pDisplay-GRAB-Ado2h-ires-mCherry-CAAX	Yulong Li lab	N/A
pDisplay-GRAB-PTH1.0-ires-mCherry-CAAX	Yulong Li lab	N/A
pDisplay-GRAB-SST1.0-ires-mCherry-CAAX	Yulong Li lab	N/A
pDisplay-GRAB-eCB2.0-ires-mCherry-CAAX	Dong et al. ⁴⁰	Addgene plasmid #164611; RRID: Addgene_164611
pAAV-CAG-GRAB-5-HT2h	Yulong Li lab	N/A
pAAV-CAG-GRAB-AVP2.0	Yulong Li lab	N/A
pAAV-CAG-GRAB-OXT1.7	Yulong Li lab	N/A
Software and algorithms		
FV31S-SW	Olympus	https://evidentscientific.com/en/downloads

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Scanbox v.11.0	Neurolabware	https://scanbox.org
MATLAB 2021a, 2023b	MathWorks	https://www.mathworks.com/products/matlab.html ; RRID: SCR_001622
Python	Python Software Foundation	https://www.python.org ; RRID: SCR_008394
Cellpose2.0	Pachitariu and Stringer ⁴⁷	https://www.cellpose.org ; RRID: SCR_021716
Demons algorithm	Vercauteren et al. ⁵⁰	https://www.mathworks.com/help/images/ref/imregdemons.html
SimpleITK	Insight Software Consortium	https://simpleitk.org ; RRID: SCR_024693
Caiman	Giovannucci et al. ⁵⁴	https://github.com/flatironinstitute/caiman ; RRID: SCR_021533
Napari	Sofroniew et al. ⁵⁵	https://napari.org ; RRID: SCR_022765
Fiji 1.54f	Schindelin et al. ¹¹⁵	https://fiji.sc ; RRID: SCR_002285
Bigstitcher	Hörl et al. ⁵⁷	https://imagej.net/plugins/bigstitcher/
Bigwarp	Bogovic et al. ⁵⁶	https://imagej.net/plugins/bigwarp
Analysis scripts	This paper	https://doi.org/10.5281/zenodo.21142692
Other		
GRIN lens (singlet, 8.85 mm length, 500 μ m diameter)	GRINTECH	NEM-050-25-10-860-S-2.0p
Optical fiber with ferrule	Doric	MFC_400/470-0.37_6.5mm_MR1.25_FLT
Polyimide tubing	MicroLumen	Cat#220-I and 190-I
Hemocytometer	Carl Zeiss Microscopy	Cat#3715211/3715298
Kwik-sil	World Precision Instruments	KWIK-SIL
Kwik-cast	World Precision Instruments	KWIK-CAST
Multiphoton microscope	Olympus	FVMPE-RS
Multiphoton microscope	Neurolabware	http://neurolabware.com
Mai Tai HP DeepSee laser	Spectra Physics	https://www.spectra-physics.com/en/f/mai-tai-deepsee-ultrafast-laser
Insight X3 laser	Spectra Physics	https://www.spectra-physics.com/en/p/insight-x3-tunable-laser/insight@-x3-™-x3-™-tunable-femtosecond-lasers
Piezo microscope stage	nPoint	nPFocus250
Focus tunable lens	Optotune	https://www.optotune.com/products/focus-tunable-lenses/
10x air objective, 0.5 NA	Thorlabs	TL10X-2P
25x water objective, 0.95 NA, 8 mm WD	Olympus	XLSPN25XSVM2
Micro V-clamp	Thorlabs	Cat#VK250
Rack and pinion stage	Edmund Optics	Cat#36-347
655 nm LED	Luxeon Star LEDs	https://luxeonstar.com
Stainless steel cannula 3 mm diameter (custom 2.0 mm length)	Microgroup	Cat#304H11XX
AtmosLM cannula 12 mm	Amuza	Cat#PEG-12
AtmosLM dummy cannula 12 mm	Amuza	Cat#PED-12
Atmos LM cap nut	Amuza	Cat#AC-5
Stainless steel cannula 0.71 mm diameter (custom 7.0 mm length)	Microgroup	Cat#304H22XX
Stainless steel cannula 0.61 mm diameter plug (custom 7.0 mm length)	Microgroup	Cat#27XRND
PE-10 tubing	Fisher Scientific	Cat#BD427401

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Syringe pump	Harvard Apparatus	Cat#70-4504
UV curable glue	Norland products	Cat#7106

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Animal studies

Experiments were performed in accordance with National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committees and Institutional Biosafety Committees at Beth Israel Deaconess Medical Center and Boston Children's Hospital. Male and female adult mice, 2–12 months old, were used in this study. Wildtype C57BL/6J male and female mice aged 8 weeks used for lateral ventricle cannulation surgeries (22 male and 14 female mice aged 8–40 weeks), i.c.v. AAV-GRAB sensor injections (8 male and 3 female mice aged 8–24 weeks), and cranial window/contralateral side port implantation surgeries following i.c.v. AAV were ordered from The Jackson Laboratory (JAX). Swiss Webster mice used for CSF and blood collections (due to their larger brain ventricles) were ordered from Charles River (18 male mice aged 8–12 weeks). *FoxJ1-cre::Ai95D* mice were bred by crossing *FoxJ1-cre* mice with GCaMP6f-expressing *Ai95D(RCL-GCaMP6f)-D* mice (JAX# 028865) ordered from JAX (6 male and 3 female mice aged 8–60 weeks, all in C57BL/6 background).¹¹³

B6.Cg-Tg(Fev-cre)1Esd/J (or *Pet1-cre*) mice (JAX #: 012712), which express cre recombinase in *Pet1*+ serotonergic neurons, were injected with rAAV1-Syn-Flex-ChrimsonR-tdTomato in the dorsal raphe nucleus (DRN) to label serotonin neurons with tdTomato and used for brain slice experiments in Figures 2 and S4 (3 male and 3 female mice aged 8–20 weeks). *B6.Cg-7630403G23RikTg(Th-cre)1Tmd/J* (or *TH-cre*) mice (JAX#: 008601) were crossed with *B6.Cg-Gt(ROSA)26Sortm32(CAG-COP4*H134R/EYFP)Hze/J* (or *Ai32*) mice (JAX #: 024109) to generate mice with YFP expressed in tyrosine hydroxylase-expressing monoaminergic neurons. These mice were used for brain slice experiments in Figure S5 (5 male and 3 female mice aged 8–24 weeks). Wildtype C57BL/6J mice were injected with rAAV1-Syn-ChrimsonR-tdTomato in the locus coeruleus (LC) and used for optogenetic brain slice experiments in Figures 2 and S4 (5 male mice aged 8–16 weeks). All mice were given free access to food and water in an animal facility maintained at 70 ± 3°F and 35–70% humidity on a 12-hour light/12-hour dark circadian cycle. All experiments were carried out in darkness during the light cycle.

METHOD DETAILS

MORSE manufacture

Cell culture

Human Embryonic Kidney-293T (HEK-293T) cells (ATCC CRL-3216) and Nanoluciferase/tdTomato Dual-Reporter HEK-293 cells (AI-stem NRL01) were propagated at 37°C, 95% humidity, and 5% CO₂ in Denville 100 mm cell culture dishes (Thomas Scientific) stored in a standard tissue culture incubator. Cells were cultured in DMEM medium (high glucose GlutaMAX™, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS, R&D Systems) and 1% penicillin/streptomycin (P/S, Sigma). Dishes were passaged after reaching 80% confluence (approximately every 5 days), and media was replaced every 2–3 days. After being thawed, cells used for experiments were maintained in culture for up to 8 passages (~1.5 months) before being discarded.

Transfection

HEK-293T cells at ~80% confluence were detached from the culture dish using mechanical force, resuspended at a density of 300,000–500,000 cells/mL, and seeded in each well of a poly-D-lysine-treated (Sigma) 24-well plate (Corning), either directly on the plate surface or on a 15 mm glass coverslip (Warner Instrument Corp), at 150,000–170,000 cells/well. Each well was transfected with a plasmid encoding a different G protein-coupled Receptor Activation-Based (GRAB) sensor (pDisplay-GRAB-ires-mCherry-CAAX, gifts from Yulong Li) using a standard EFFECTENE® transfection kit (Qiagen). The transfection mixture was optimized separately for each plasmid according to the instructions provided by the supplier. In the end, an EFFECTENE®/DNA ratio (in $\mu\text{L}/\mu\text{g}$) of 25 was used for NPY1.0, HA1m, AVP0.2, and CCK0.5, while one of 6.25 was used for DA2.9, NE2h, gACh4I, 5-HT2h, OXT1.7, ATP1.0-L, eCB2.0, VIP1.0, MT1.0, Ado2h, PTH1.0, and SST1.0. In a subset of experiments, we also transfected Nanoluciferase/tdTomato Dual-Reporter HEK-293 cells with NE2h. All cells were incubated in the transfection mixture for 24 hours prior to direct imaging or MORSE probe preparation.

Cleaning and gluing of GRIN lenses and photometry fibers into tubing

To prepare a MORSE probe, a clean Gradient Refractive Index (GRIN) lens (singlet, 8.85 mm length, 500 μm diameter, GRINTECH NEM-050-25-10-860-S-2.0p) was carefully inserted into an 8 mm long piece of polyimide tubing (PI, internal diameter 0.56 mm, code 220-I, MicroLumen). All lenses were prepared on a hemocytometer under a dissection microscope to allow for exact measurements of probe dimensions (cat. no. 3715211/3715298, Carl Zeiss Microscopy). Once the lens was at least 2 mm into the tubing, an applicator was used to add a drop of Kwik-Sil (World Precision Instruments) – an optically clear room temperature vulcanizing (RTV) silicone elastomer – to the lens-tubing interface. The tubing was then slowly moved along the lens, allowing the Kwik-Sil to gradually

seep around the lens shaft until the tubing extended 0.3 mm beyond the front of the lens. The protruding tubing provided a small receptacle with a volume of ~ 0.2 nL in front of the lens face surrounded by tubing. Lenses were then laid flat, and the Kwik-Sil was allowed to harden at room temperature for at least 1 hour. Once the Kwik-Sil had hardened, two lines were drawn around the circumference of the PI tubing, 7 mm from the end of the receptacle; this would ensure the probe was inserted into a mouse's guide cannula (see below for details) to the correct depth. MORSE photometry probes were prepared in an analogous manner using 6.5 mm photometry fibers (400 μ m diameter, Doric MFC_400/470-0.37_6.5mm_MR1.25_FLT) and 6 mm pieces of PI tubing (internal diameter 0.48 mm, code 190-I, MicroLumen).

We also developed a method to clean and re-use the GRIN lenses and optical fibers between successive experiments. First, probes were left in 100% acetone (VWR) for at least 24 hours. To begin to remove the PI tubing, Cohan-Vannas spring scissors (item No. 15000-02, Fine Science Tools) were used to cut the PI tubing along the length of the probe. The GRIN lens or fiber was then removed from the cut tubing using ceramic forceps (11 cm, cat. no. 501209, World Precision Instruments) and dipped into a bath of 70% ethanol (Fisher). An applicator brush (cat. no. S379, Parkell) dipped in ethanol was used to scrub the remaining Kwik-Sil from the sides of the lens or fiber. Once the sides were clean, the lens or fiber was mounted with one face up on a stainless steel micro V-clamp (cat. VK250, Thorlabs) under a dissection microscope. A thin piece of lens paper (Fisherbrand) dipped in ethanol was used to clear debris from each lens or fiber face. GRIN lenses and photometry fibers were reused and reimaged in this fashion up to 10+ times and retired when they accumulated excessive scrapes and blemishes.

Pre-coating of GRIN and photometry fiber hydrogel receptacles

After being cleaned and glued into PI tubing, GRIN lenses were placed in a polystyrene foam holder with the receptacle face-up and cooled to 4°C in a cold room. To fill any air gaps remaining between the PI tubing and the lens, the lens face was then coated with a thin layer (~ 50 μ m thick) of Kwik-Sil, which was carefully spread out and shaped into a curved meniscus above the lens face. This Kwik-Sil layer was allowed to harden at room temperature overnight. Next, to activate the elastomer surface and make it more hydrophilic for binding to the hydrogel, the receptacle was filled with a drop of 2.5% potassium hydroxide solution (Sigma) for 1 hour at 37°C in the humidified cell culture incubator, rinsed 3 times in phosphate buffered saline (PBS, Thermo Fisher), and allowed to dry for 30 minutes at 37°C in a dehumidified incubator. Pre-coated lenses were then cooled back to 4°C in a cold room to prepare for hydrogel application. Photometry fibers were prepared in an identical manner.

Preparation and application of cell-laden hydrogel

After a 24-hour transfection, expression of the sensors was confirmed under an epifluorescence microscope (Zeiss Axio Observer D1). Cells were then treated with colchicine (1 μ M, Sigma), a microtubule polymerization inhibitor and chemotaxis blocker, for one hour to inhibit cell movement and proliferation. Colchicine-treated cells were then detached using TrypLE (Gibco) and centrifuged and resuspended in PBS twice (first for 8 minutes at 500 g, then for 10 minutes at 800 g) to remove any colchicine, debris, and culture media. After the second centrifugation, the supernatant was aspirated and cells were encapsulated in a hydrogel containing $\sim 60\%$ collagen I (stock concentration ~ 10 mg/mL, Corning) and $\sim 40\%$ MATRIGEL® (stock concentration ~ 20 mg/mL, Corning) by volume at a concentration of 10,000–50,000 cells/uL (for details of why this mixture was optimal, see below). To prepare this hydrogel, 200,000–1,000,000 cells were first pelleted in a 2 mL microcentrifuge tube (Eppendorf), which we found achieved optimally tight and dehydrated pellets. When preparing hydrogels with multiple types of sensor cells, the number of cells of each type included in the pelleted mixture was the total number of cells divided by the number of sensor types, so each sensor was represented at equal proportions, and the total number of cells was always consistent. In a subset of experiments, we additionally mixed in the tdTomato+ HEK-293 cells (see above) at a ratio of 1:10 to the total cell number. These cells were used as landmarks during two-photon acquisition and analysis (mCherry signal from GRAB plasmids was very dim at 980 nm excitation and thus did not present any confound to this approach). The pellet was then suspended in 11.5 μ L of stock collagen I (kept on ice) and 7.5 μ L of stock MATRIGEL® (thawed from -20°C on ice for 25–30 minutes), and the solution was thoroughly homogenized with a pipette. Because the polymerization of MATRIGEL® and collagen was temperature-sensitive, the cell/hydrogel solution was immediately placed on ice and transferred to a 4°C cold room.

In the cold room, the cell-laden hydrogel was drawn into a pulled glass capillary tube (Drummond 0.4 mm), and ~ 40 nL of the mixture was applied to each pre-coated GRIN lens receptacle under a dissection microscope. The lens was then transferred to a humidifying chamber (a closed container lined with Kimwipes soaked in ddH₂O) kept at 37°C in a tissue culture incubator for gel polymerization. Extracellular matrix polymerization was allowed to proceed for 10 minutes, forming a ~ 200 μ m thick hydrogel. Immediately after polymerization of the hydrogel and adherence to the distal end of the probe, each fully manufactured MORSE probe was immersed vertically in a separate 1.5 mL microcentrifuge tube (Eppendorf) containing the same media used to culture the cells (DMEM + 10% FBS + 1% P/S) for 1–2 hours. Finally, the probes were transferred to a PBS-filled 1.5 mL tube and allowed to soak for at least 15 min before being used in an experiment. MORSE probes were used to characterize the composition of biological fluids *in vitro* (i.e. dipping), *ex vivo* (i.e. brain slices), and *in vivo* (i.e. two-photon imaging in CSF). MORSE photometry probes were prepared in an identical manner, except that HEK-293T cells expressing just one sensor were used per probe. These were applied *in vivo* for fiber photometry recordings in CSF. All probes were prepared in the morning and were able to last an entire day of experiments.

Optimization of MORSE manufacture

We iteratively optimized each component of the MORSE manufacture protocol. This allowed us to increase probe stability from $\sim 10\%$ to $\sim 65\%$ in initial tests (Figure 1F), which improved further with skill refinement over time. In revision experiments, $>90\%$ probe

stability was achieved ($n=10/13$ *in vitro* dipping experiments, $n=9/9$ brain slice experiments, $n=13/14$ *in vivo* CSF experiments). Key steps in this optimization are detailed below.

HEK-293T cell transfection timeline

We compared the GRAB sensor expression in cells transfected for several durations between 12 and 48 hours. Cells transfected for longer than 24 hours accumulated increased quantities of bright green intracellular aggregates. We interpreted these inclusions as reflecting endoplasmic reticulum (ER) stress and excess GRAB sensor retention. This strong intracellular sensor expression, which is not exposed to extracellular fluid, overshadowed the signal from membrane-bound GRAB sensor expression, preventing these cells from meaningfully responding to extracellular ligands. Conversely, cells transfected for much less than 24 hours were difficult to visualize under a two-photon microscope. Thus, a 24-hour transfection was optimal.

Hydrogel composition

In optimizing our hydrogel's composition, we had to consider several competing factors. First, we needed a gel that was rigid enough to not warp excessively, but also permeable enough to support rapid coupling of surrounding fluid composition changes to sensor response. Second, a gel's composition affects its adhesive properties, so we needed to consider how each change that we made to prevent warping would affect the gel's attachment to the lens. We screened many different combinations of conditions and extracellular matrix (ECM) components (including MATRIGEL®, collagen I, fibronectin, and hyaluronic acid), before arriving at a 3:2 volumetric ratio of collagen I (stock concentration ~ 10 mg/mL): MATRIGEL® (stock concentration ~ 20 mg/mL). We also found that HEK-293T cells formed notably more stable hydrogels compared to HEK-293 cells at the same cell density and ECM component composition, presumably reflecting differences in membrane receptors and structural proteins in these closely related cell lines. For this reason, we kept the fraction of tdTomato-expressing HEK-293 cells in our probes to at most 10%.

At the molecular level, collagen I is a fibrous protein that forms a triple helix structure. This creates a rigid, yet porous matrix that bolsters connective tissues throughout the body.⁵¹ Therefore, collagen I is typically thought to confer a hydrogel with rigidity, strength, and inflexibility.¹¹⁶ This ECM was crucial in maintaining the structural integrity of MORSE and preventing hydrogel warping during recordings. However, hydrogels made up of more than 60% collagen I would more frequently fall off the GRIN lens face during *in vitro* dipping experiments. We interpreted this as likely due to the gels being too rigid and not adherent enough to the lens. In dipping experiments, the hydrogel must repeatedly break through the air-fluid interface in each successive well. The forces associated with overcoming this surface tension are applied directly to the gel. If the gel is insufficiently elastic, the force will be transmitted to the weakest part of the probe – the hydrogel-Kwik-Sil interface – causing detachment of the gel.

MATRIGEL® is a highly adherent ECM mixture containing laminin, collagen IV, entactin, and heparin sulfate proteoglycans – common components of basement membranes at epithelial tissue boundaries.¹¹⁷ This combination of ECM tends to create meshworks that are more elastic than collagen I matrices; a given concentration of MATRIGEL® has an elastic modulus that is $\sim 3\times$ lower than the same concentration of collagen I.¹¹⁸ Because it is derived from murine sarcoma cultures, MATRIGEL® also contains many globular proteins, including transforming growth factor and epidermal growth factor.¹¹⁹ These proteins, along with the heavily glycosylated proteoglycans, make MATRIGEL® appreciably stickier than collagen. Notably, despite its increased flexibility, MATRIGEL® may hinder molecular diffusion to a greater extent than collagen I due to viscous forces, which is a further advantage of our majority-collagen recipe.^{120,121} The addition of 40% MATRIGEL® provided needed flexibility to our hydrogel, allowing it to withstand repeated exposure to the air-fluid interface while remaining attached to the Kwik-Sil. However, gels with greater than 40% MATRIGEL® warped excessively. In sum, we carefully balanced structural rigidity with adhesiveness, elasticity and porosity to achieve an optimal mixture.

Pre-coating of GRIN lens and photometry fiber face

We initially deposited the cell-laden hydrogel directly onto the face of our lens after it was glued to the surrounding PI tubing. However, due to small gaps between the lens and the surrounding PI tubing, air often became trapped under the hydrogel. Then, while the still-liquid hydrogel was polymerizing in the incubator, the trapped air expanded and formed bubbles that forced their way upwards into the gel before it solidified, forming crescent-shaped gels that were difficult to use. We discovered that filling these gaps and sealing the lens face with a thin layer (~ 50 μm) of Kwik-Sil completely eliminated this problem. We found that it is best to perform this treatment at 4°C to ensure that the elastomer remains liquid for as long as possible as it fills the gaps, avoiding an analogous trapping of air bubbles. The Kwik-Sil coating does not noticeably impair cell visibility or image quality through the GRIN lens.

Adding Kwik-Sil to the front of the lens created another challenge: this silicone elastomer surface is hydrophobic and does not readily interact with the charged and polar groups in the proteins that make up our hydrogel, resulting in gel detachment. We tried several strategies to make the silicone surface more hydrophilic, including coating it with poly-D lysine, alginate, hyaluronic acid, polyacrylamide, chitosan, and polyethylene glycol, as well as treating it with oxygen plasma. Eventually, we settled on a relatively simple but effective solution that maintained gel adhesion to the GRIN lens: we pre-treat the Kwik-Sil coating with potassium hydroxide, which we hypothesize reacts with the Kwik-Sil to deposit polar hydroxide groups on the surface and roughens it to increase contact surface area, better anchoring the hydrogel to the lens surface.^{52,53}

Colchicine treatment

We began by treating cells with colchicine for 24 and 48 hours following transfection.¹²² However, these cells began developing bright intracellular aggregates spanning across the green and red fluorescence emission spectrum, likely reflecting large-scale intracellular protein denaturation indicative of excess toxicity. We found that a short one-hour treatment after transfection was sufficient to immobilize the cells for several hours of subsequent imaging without affecting sensor expression patterns or sensor function.

ChP imaging surgeries

For all surgical procedures performed for ChP imaging, male and female mice aged 8 weeks were administered meloxicam (10 mg/kg, subcutaneous), dexamethasone (1 mg/mL, intramuscular), and 0.9% saline (1 mL, subcutaneous) pre-operatively and anesthetized using 1–4% isoflurane in 100% O₂. They were then positioned in a stereotactic frame.

Intracerebroventricular (i.c.v.) injection of AAV5-GRAB

To infect the lateral ventricle ChP epithelium with a GRAB sensor, we first made an incision in the scalp and drilled a craniotomy at the coordinates +0.9 mm lateral and -0.6 mm posterior to Bregma. A pulled microcapillary tube (cat. no. P0549, Sigma Aldrich) was slowly lowered to -2.75 mm ventral to Bregma and used to administer 1 μ L of virus directly into the lateral ventricle. AAV5-CAG-GRAB-5-HT2h.D, AAV5-CAG-GRAB-AVP0.2, and AAV5-CAG-GRAB-OXT1.7 (University of Pennsylvania Vector Core) were administered at titers of 7.12×10^{11} gp/mL, 2.75×10^{12} gp/mL, and 1.28×10^{12} gp/mL, respectively (AAV5 has a known tropism to ChP epithelium when delivered i.c.v. in mice).⁹⁸ Mice were then sutured and given at least 1 week to recover. Cranial window and contralateral infusion cannula surgeries were then performed on these animals (see below).

Dorsal raphe nucleus (DRN) viral injection

To infect DRN serotonergic neurons with tdTomato, we used *Pet1-cre* mice and first made an incision in the scalp and drilled a craniotomy at the coordinates Bregma: AP: -4.5 to -5.1 mm, ML: 0 mm. A pulled microcapillary tube (cat. no. P0549, Sigma Aldrich) was filled with rAAV1-Syn-Flex-ChrimsonR-tdTomato virus (University of Pennsylvania Vector Core), lowered to the injection coordinates and injected into the dorsal raphe nucleus (200 nL at three locations, Bregma: AP: -4.55/-4.8/5.05 mm, DV: -2.9/-3.1/-3.3 mm, ML: 0 mm). Mice were sutured and given at least 2 weeks to recover prior to usage in brain slice experiments.

Locus coeruleus (LC) viral injection

To infect LC neurons with ChrimsonR and tdTomato, we used wildtype C57BL/6 mice and first made an incision in the scalp and drilled a craniotomy at the coordinates Bregma: AP: -5.6 mm, ML: ± 0.95 mm. A pulled microcapillary tube (cat. no. P0549, Sigma Aldrich) was filled with rAAV1-Syn-ChrimsonR-tdTomato virus (University of Pennsylvania Vector Core), lowered to the injection coordinates and injected into the locus coeruleus (200 nL at two locations, Bregma: AP: -5.6 mm, DV: -3.65 mm, ML: ± 0.95 mm). Mice were sutured and given at least 2 weeks to recover prior to usage in brain slice experiments.

Cranial window and contralateral infusion cannula surgeries

Cranial window surgeries using *FoxJ1-cre::Ai95D* or AAV5-GRAB-injected mice were performed as described in Shipley et al.⁹⁶ Briefly, a 3 mm diameter craniotomy was drilled over the left hemisphere (2.0 mm lateral and 0.2 mm posterior to Bregma), and the brain regions over the lateral ventricle were carefully aspirated. After hemostasis was achieved, a stainless steel cranial window with a sealed circular glass front face of 3 mm diameter was implanted over the large leaflet of the lateral ventricle ChP within the lateral ventricle. A stainless steel headpost was then affixed to the skin with VetBond (3M), and the cavity within the headpost was filled with C&B Metabond (Parkell). Mice were also outfitted with a custom-printed 3D imaging well (outer diameter of the base, inner diameter, height: 20 mm, 10 mm, 4 mm, respectively).

Optionally, a contralateral infusion cannula (“side port”) was also placed using the contralateral trans-occipital approach described previously.⁹⁷ Briefly, the neck muscles attached to the suboccipital bone were gently removed, and a stainless steel cannula (0.7 mm diameter, 12 mm length, Amuza AtmosLM PEG-12) was inserted into the contralateral lateral ventricle through an occipital craniotomy. A dummy cannula (stylet plugging the cannula while it is not in use to avoid leakage/infection) was then inserted and secured in place by a screw-on cap (Amuza AtmosLM PED-12 and Cap Nut AC-5). Mice were administered meloxicam (5 mg/kg, subcutaneous) and dexamethasone (4 mg/mL, intraperitoneal) daily for three days following surgery to ensure optimal recovery and minimize scarring around the implant. These mice were given at least 4 weeks to recover, after which they were used to infuse pharmaceuticals directly into the CSF while recording fluorescent signal from the ChP epithelium.

MORSE CSF recording surgeries

For all surgical procedures performed for MORSE CSF recordings, male and female mice aged 8 weeks were administered long-acting meloxicam (0.5 mg/kg, subcutaneous), dexamethasone (4 mg/mL, intramuscular), and 0.9% saline (1 mL, subcutaneous) pre-operatively and anesthetized using 1–4% isoflurane in 100% O₂. They were then positioned in a stereotactic frame.

Bilateral anterior lateral ventricle cannula implantation surgery

Bilateral craniotomies of ~ 1 mm in diameter were performed in wildtype C57BL/6 mice above both lateral ventricles (stereotactic coordinates: ± 1 mm lateral and -0.45 mm posterior to Bregma). To create an initial cannula tract, a beveled 21-gauge needle (PrecisionGlide, Fisher) was lowered -2.05 mm ventral to Bregma within each craniotomy. Custom cut stainless steel cannulae (22XX gauge, 7 mm length, Microgroup) were implanted to a depth of -2 mm ventral to Bregma and secured with Metabond. A headpost was then attached to the skin with VetBond, and the surgical site was sealed with Metabond. 7 mm-long pieces of stainless steel wire (27XRND, Microgroup) were used as dummy cannulae and secured with Kwik-Cast (World Precision Instruments). Mice were administered dexamethasone (4 mg/mL, intraperitoneal) daily for three days following surgery, as well as for two days prior to every MORSE recording, to minimize scarring and inflammatory response to the probe.

Sample collection

Terminal CSF and serum collection

Wild type Swiss Webster mice were injected with AVP (1 mg/kg i.p., Sigma), OXT (1 mg/kg i.p., Bachem) or a 0.9% sterile saline (Medline Industries Inc.) control (100 μ L total volume), or received a sham needle poke without injection. 30 minutes later, the mice were deeply anesthetized with 100–120 mg/mL ketamine / 10 mg/kg xylazine (i.p.). A pulled glass capillary tube (Drummond 0.4 mm) was then inserted into the cisterna magna and CSF was collected with gentle negative pressure. Next, a thoracotomy was performed, and blood was collected from the cardiac left ventricle using a 20-gauge needle attached to a syringe. The collected CSF and blood were stored in mini tubes (Micro sample tube Serum, cat. no. 41.1392.105, Sarstedt). CSF was then centrifuged at 3,000 g for 10 minutes at 4°C to remove any cellular debris. The supernatant was aliquoted and processed for ELISA or sent for mass spectrometry (see below), or stored at -80°C. Blood was allowed to coagulate on ice for 30 minutes and centrifuged at 2,000 g for 30 minutes at 4°C. The top two thirds of the serum volume, avoiding the coagulated pellet, was aspirated, aliquoted, and processed for ELISA or sent for mass spectrometry (see below), or stored at -80°C.

Imaging experiments

Pre-infusion

Mice with bilateral lateral ventricle cannulae were pre-infused as described in Fame et al.⁴ Briefly, two weeks after surgery, 15 μ L of aCSF (cat. no. 3525, Tocris) was infused into one cannula over three minutes. The contralateral cannula was then pre-infused two days later, and mice were given at least a week to recover before being used for experiments.

Habituation

Mice were habituated to being head-fixed on a 3D-printed running wheel for increasing periods of time starting from 30 min to 4–5 hours, every day for at least 1 week leading up to an experiment.

Post-hoc BSA infusion

Following an *in vivo* MORSE experiment, immediately preceding perfusion and histology (see below), mice with bilateral lateral ventricle cannulae were infused with BSA-Alexa Fluor 555 (cat. No. A34786, Invitrogen) for tracing of diffusion from CSF into brain parenchyma as described in Gahn-Martinez et al.⁹² Briefly, a 1% wt/vol solution of BSA-Alexa Fluor 555 was prepared in aCSF (cat. no. 3525, Tocris) and pre-filled into a polyethylene catheter (inner diameter 0.28 mm, outer diameter 0.61 mm, cat. no. BD 427401, Fisher Scientific), which was introduced into the lateral ventricle cannula previously used for the MORSE probe and sealed with Kwik-Cast. 10 μ L of BSA-Alexa Fluor 555 solution was infused at a rate of 5 μ L/min using a syringe pump (cat. no. 70-4504, Harvard Apparatus). 30 minutes from the start of the pump, the mice were perfused and brains were extracted.

Two-photon microscopy for MORSE recordings

Volumetric imaging of the cell-laden hydrogel in MORSE was performed through the GRIN lens using a resonant-scanning two-photon microscope (Neurolabware, Optotune, Scanbox v.11.0, 15.63 frames per second, 1,154 pixels x 512 pixels) equipped with an Insight X3 laser (Spectra-Physics). Probes were imaged at 980 nm excitation (<30 mW power measured below the objective) using a 10x super achromatic air objective (0.5 NA, 7.77 mm WD, $\sim$ 1000x500 μ m² FOV, Thorlabs). Volumes with 30 z-planes ($\sim$ 9 μ m between planes) were acquired at a rate of $\sim$ 0.5 vol/s. Both red and green fluorescence emission images were collected.

Probe dipping experiments

MORSE probes were calibrated and tested *in vitro* by sequential dipping into wells of a 384-well plate (cat. no. 89014-066, Nunc® Shallow Well Standard Height Plates, black, Thermo Scientific) containing experimental solutions or standards of known identity and concentration. The objective of these *in vitro* dipping experiments was to (i) spatially demultiplex sensor signals, (ii) establish quantitative dose-response curves for each ligand, and (iii) detect many molecules in synthetic and biological samples. To perform these recordings, the GRIN lens was placed in a stainless steel micro-V-clamp (cat. VK250, Thorlabs) attached to a rack and pinion stage (cat. no. 36-347, Edmund Optics, for vertical alignment) via a clamp mounted on the microscope arm (see Figure 1E; Video S2). This custom lens holder was secured in focus below the microscope objective with the probe aligned vertically and the hydrogel facing downward.

Motors on the microscope arm were programmed to move the front of the lens through sequential dips into the wells of the 384-well plate (20 μ L of fluid per well, 3–5 min/well and 15 sec/transfer, up to 80 wells/recording). Calibration wells contained buffered saline, near-saturating concentrations of one of up to 16 sensor ligands individually, or one of 6 logarithmically spaced concentrations of 6–8 ligands mixed together. These calibrations were performed at room temperature, and dose-response patterns may differ slightly from sensor responses to similar concentrations of ligand in the heated slice chamber or *in vivo*.

Acute brain slice preparation

Brain slices were prepared by deeply anesthetizing mice (12–20 weeks old, ad libitum fed) with isoflurane, followed by transcardial perfusion with ice-cold, carbogen-saturated (95% O₂, 5% CO₂) aCSF consisting of (in mM): 126 NaCl, 27.4 NaHCO₃, 2.5 KCl, 1.2 NaH₂PO₄, 1.2 MgCl₂, 2.4 CaCl₂, 10.5 glucose. The mice were then rapidly decapitated, and the brains were extracted. Upon removal, brains were immediately immersed in ice-cold, carbogen-saturated choline-based cutting solution consisting of (in mM): 92 choline chloride, 20 HEPES, 2.5 KCl, 1.25 NaH₂PO₄, 30 NaHCO₃, 25 glucose, 10 MgSO₄, 0.5 CaCl₂, 2 thiourea, 5 sodium ascorbate, 3 sodium pyruvate. aCSF osmolarity was measured at 310–320 mOsm/L and pH at 7.40. Next, 300 μ m-thick coronal (for PVH) or sagittal (for dLGN) sections were cut with a vibratome (Campden 7000smz-2) and incubated in carbogenated cutting solution at 35°C for 15 min. Slices were subsequently transferred to carbogenated aCSF at 35°C for an additional 30 min. Slices were then acclimated

at room temperature (RT, 20–24°C) for ~30 min until use. For experiments with wash-ons of high (50 mM) KCl, a second aCSF solution was made with 52.5 mM KCl and 77 mM NaCl to maintain osmolarity.

Before the experiment, a single slice was placed in the recording chamber under the two-photon microscope (NeuroLabware), where it was continuously superfused with fresh carbogenated aCSF via a peristaltic perfusion pump (Cole Parmer Masterflex 77120-52 C/L) at a rate of ~4 mL per min at RT. The target region on the slice was identified using live imaging with the two-photon microscope (locating tdTomato-positive axons in *Pet1-cre/DRN* AAV-Syn-Flex-ChrimsonR-tdTomato slices to find the dLGN, YFP-positive axons in *TH-cre::Ai32* slices to find the PVH, or tdTomato-positive axons in LC AAV-Syn-ChrimsonR-tdTomato slices to find the LC). A dissection microscope (Olympus) was then set up to observe the slice from an oblique angle, and a MORSE probe was attached to the two-photon microscope under the objective with a custom lens holder (see above). The probe was observed with live two-photon imaging and secured with the hydrogel in the focal plane of the objective, and then the whole microscope arm was lowered to press the MORSE probe against the slice region of interest. Correct targeting was achieved by direct observation of the slice through the angled dissection microscope as the probe was lowered onto it, and z-control on the microscope arm was used to position the end of the probe to an indentation of 100–150 μm below the slice surface. Two-photon imaging of the cell-based sensors pressed against the slice was then performed while delivering full-field optogenetic stimulation or superfusing aCSF containing different ligands onto the slice. The flow in the recording chamber was tested to confirm fluid exchange timings: at 4 mL/min pump setting, the time to completely wash out a pigmented solution from the chamber and replace it with clean inflowing aCSF was approximately 5 min. Optogenetic stimulation was achieved with a 655 nm LED (1 mW mm^{-2} , Luxeon Star LEDs) driven by an Arduino-controlled driver to deliver photostimulation trains. To protect the photomultiplier tube of the two-photon microscope, photostimulation pulses were timed to the start of each frame during the galvo mirror flyback time. At each pulse, the photomultiplier tube was blanked for 10 ms, with the LED on for the first 8 ms, achieving a stimulation frequency equivalent to the two-photon frame rate (15.63 Hz). Stimulation was delivered in this way for the first 5 s of each 20 s trial, with 45 consecutive trials per recording session.

In vivo MORSE experiments

At least one week after pre-infusion (see above), mice with bilateral lateral ventricle cannulae were head-fixed for two-photon imaging. Both dummy cannulae were acutely removed and a ~1 μL drop of CSF was allowed to accumulate at the top of each cannula. Only mice possessing optically clear CSF (indicating unobstructed cannula placement in a relatively healthy ventricle with minimal post-surgical bleeding) were used for recordings. A MORSE probe was removed from a PBS-filled 1.5 mL tube and carefully introduced into the cannula with better CSF flow, ensuring no air bubbles formed at the hydrogel-CSF interface. The lines drawn at 7 mm from the hydrogel end of the MORSE probe were used to guide the probe to the correct depth. The implanted probe was then sealed to the cannula with Kwik-Sil.

In a subset of experiments, a polyethylene catheter (inner diameter 0.28 mm, outer diameter 0.61 mm, cat. no. BD 427401, Fisher Scientific) pre-filled with serotonin solution was introduced into the contralateral ventricular cannula for i.c.v. infusions and sealed with Kwik-Cast. Two-photon imaging of the cell-based sensors was then performed through the GRIN lens while the mouse ran on a running wheel and was subjected to pharmacological (delivered i.p. and i.c.v.) stimuli. Each session involved a 30 minute acclimation period, followed by 3–4 hours of two-photon imaging. Drugs were mixed in PBS (Thermo Fisher) for i.p. injections of 100–300 μL volumes performed with an insulin syringe (Fisher) and mixed in aCSF (Tocris) for i.c.v. infusions in volumes of 15–30 μL at a rate of 5 $\mu\text{L}/\text{min}$ using a syringe pump (cat. no. 70-4504, Harvard Apparatus).

In vivo MORSE photometry experiments

At least one week after pre-infusion (see above), mice with bilateral lateral ventricle cannulae were head-fixed for photometry recordings. Both dummy cannulae were acutely removed and a ~1 μL drop of CSF was allowed to accumulate at the top of each cannula. Mice lacking optically clear CSF were not used for recordings. A MORSE photometry probe was removed from a PBS-filled 1.5 mL tube and carefully introduced into the cannula with better CSF flow, ensuring no air bubbles formed at the hydrogel-CSF interface. The probe was pushed all the way into the cannula until the fiber ferrule was flush with the cannula opening. The implanted probe was then sealed to the cannula with Kwik-Sil. Zirconia sleeves (Precision Fiber Products) were used to couple the fiber to a patch cord (2 m length, NA 0.57, Doric MFP_400/430/1100-0.57_2m_FC-MF1.25_LAF) that had been photobleached overnight.

All experiments were carried out using the fiber photometry setup described in Zhang et al.¹²³ Briefly, a 465 nm LED (~100 μW measured at the end of the patch cord, Plexon OPT/LED_Blue_TT_FC) excited the GRAB sensor for signal detection, and a 405 nm LED (~100 μW measured at the end of the patch cord, Plexon OPT/LED_Ultraviolet3_TT_FC) was used to collect an isosbestic control signal. Excitation light pulses were delivered at 50 Hz and included 6 ms of 465 nm LED, 4 ms of darkness, 6 ms of 405 nm LED, then 4 nm of darkness, controlled by Plexon LED drivers (OPT/1ch_LED_Driver) via the Nanosec photometry-behavioral system.¹²³ Light from both LEDs converged into the patch cord via a four-port fluorescence mini cube (Doric, FMC4_IE(400-410)_E(460-490)_F(500-550)_S). The fluorescence intensity emitted from the GRAB sensors was detected by a femtowatt photoreceiver (Newport+Doric FC Adapter, NPM_2151_FOA_FC). The recordings were conducted in darkness, and black heat shrink tubing was placed around the fiber-patch cord coupling region to prevent the LED light from being seen by the mice.

Photometry recordings of the cell-based sensors were then performed through the optical fiber while the mouse ran on a running wheel and was subjected to pharmacological (delivered i.p. and i.c.v.) stimuli. Each session involved a 30 minute acclimation period, followed by 2–3 hours of photometry recording. Drugs were mixed in PBS (Thermo Fisher) for i.p. injections (100–300 μL volume) performed with an insulin syringe (Fisher) and mixed in aCSF (Tocris) for i.c.v. infusions in volumes of 15–30 μL at a rate of 5 $\mu\text{L}/\text{min}$ using a syringe pump (cat. no. 70-4504, Harvard Apparatus).

The table below summarizes the pharmaceuticals injected i.p. and i.c.v. in the two-photon and photometry MORSE experiments:

Pharmaceutical	Dose(s)
5-hydroxytryptamine (5-HT, Sigma)	2–3 mg/kg (i.p.) 5 nmol/μL infused at 5 μL/min over 6 min (i.c.v.)
Arginine vasopressin (AVP, Sigma)	1 mg/kg (i.p.) 500 μM in aCSF infused at 5 μL/min over 6 min (i.c.v.)
Oxytocin (OXT, Bachem)	1 mg/kg (i.p.) 500 μM in aCSF infused at 5 μL/min over 6 min (i.c.v.)
Norepinephrine (NE, Sigma)	1 mg/kg (i.p.)
Dopamine (DA, Sigma)	1 mg/kg (i.p.)
Cholecystokinin (CCK, Bachem)	1 mg/kg (i.p.)
Acetylcholine (ACh, Sigma)	10 mg/kg (i.p.)
Melatonin (MT, Sigma)	10 mg/kg (i.p.)
Histamine (HA, Sigma)	10 mg/kg (i.p.)
Adenosine triphosphate (ATP, Sigma)	50 mg/kg (i.p.)

Two-photon imaging of lateral ventricle ChP

Imaging of *FoxJ1-cre::Ai95D* and i.c.v.-AAV5-GRAB-injected mice was performed as described in Shipley et al.⁹⁶ Briefly, mice were habituated to being head-fixed on a running wheel for 30–240 min/day for at least 1 week. On the day of the experiment, mice were head-fixed on the running wheel directly under a resonant-scanning two-photon microscope (Olympus MPE-RS Multiphoton microscope, 18.75 frames/s, 512x512 pixels/frame). i.c.v.-AAV5-GRAB-injected mice received an i.p. injection of 25 mg/kg of 70 kDa dextran-Texas RedTM (Thermo Fisher) 30 minutes before the recording to visualize the ChP vasculature. The imaging well on the mouse's head was filled with ultrasound gel, and a 25x, 0.95 NA, 8 mm WD objective (Olympus XLSLPLN25XSVM2) at 2.5x zoom (~203 × 203 μm²) was used to view the lateral ventricle ChP. Volume scanning was achieved by using a piezo microscope stage (nPFocus250, nPoint) moving axially in a sawtooth pattern.

FoxJ1-cre::Ai95D and i.c.v.-AAV5-GRAB were imaged at 940 nm excitation (Mai Tai DeepSee laser, Spectra Physics) and 3D volumes (81 z-planes, 5 μm/plane, stack volume 203 × 203 × 400 μm³) were collected at a rate of 1 volume/4.32 seconds for 2–3 hours. The power under the objective was measured to be between 34–45 mW. An i.p. injection of a ligand/antagonist was administered around 60–90 minutes into the recording. Optionally, in mice receiving i.c.v. injections, a polyethylene catheter (inner diameter 0.28 mm, outer diameter 0.61 mm, cat. no. BD 427401, Fisher Scientific) pre-loaded with a ligand was threaded 12 mm into the side port and secured using Kwik-Cast. 15 μL of the ligand diluted in aCSF was infused at a rate of 5 μL/min using a syringe pump (cat. no. 70-4504, Harvard Apparatus).

The table below summarizes the pharmaceuticals injected i.p. and i.c.v. in two-photon ChP imaging experiments:

Pharmaceutical	Dose(s)
5-HT (Sigma)	2 mg/kg in saline (i.p.); 5 nmol/μL in aCSF infused at 5 μL/min over 3 min (i.c.v.)
AVP (Sigma)	1 mg/kg in saline (i.p.)
OXT (Bachem)	1 mg/kg in saline (i.p.)

Post hoc tissue and fluid analysis

CSF and blood ELISA and mass spectrometry

Blood and serum samples were collected as described above. For vasopressin and oxytocin ELISA, the samples were processed further according to the manufacturer's protocols: DetectX® Arg8-Vasopressin Enzyme Immunoassay Kit, cat. no. K049-H1, Arbor Assays for AVP quantification and DetectX® Oxytocin Enzyme Immunoassay Kit, cat. no. K048-H1, Arbor Assays for OXT quantification. For mass spectrometry quantification of monoamines, which are small molecules less amenable to high-fidelity ELISA, the samples were sent to the VBI Neurochemistry Core at Vanderbilt University for quantification of serotonin, norepinephrine, dopamine, epinephrine, 5-HIAA, and DOPAC with detection limits of 0.050 pg/μL.

Perfusion and histology

Mice were deeply anesthetized with ketamine (100–120 mg/mL i.p.) and xylazine (10 mg/kg i.p.) or with Avertin (tribromoethanol, 125 mg/kg i.p.). A thoracotomy was performed, and the cardiac left ventricle was perfused with 10 mL of PBS followed by 10 mL of 4% paraformaldehyde or 10% formalin. Brains were extracted, allowed to fix in 4% paraformaldehyde or 10% formalin for 24 hours, and moved to 20% sucrose for 24 hours. 60 μ m-thick coronal sections were then prepared and stored in PBS.

Immunostaining and image acquisition

Histology was performed on a representative subset of mice used for *in vivo* experiments to confirm cannula placement and evaluate inflammation. Slices were blocked in 1% normal donkey serum, 0.05% sodium azide, and 0.05% Triton-X on a shaker at room temperature for 2 hours. Rabbit anti-Iba1 (1:200, cat. no. 019-19741, Wako) and rat anti-GFAP (1:200, cat. no. 13-0300, Invitrogen) antibodies diluted in the blocking solution were then used to stain the tissue, which was kept on a shaker at 4°C overnight. The tissue was then washed three times with PBS and stained with Alexa Fluor 488 goat anti-rabbit (1:500, cat. no. A-11008, Invitrogen) and Alexa Fluor 647 goat anti-rat (1:500, cat. no. A-21247, Invitrogen) for 2 hours on a shaker at room temperature. Stained slices were mounted on slides using VectaShield® HardSet™ with DAPI (cat. no. H-1500-10, Vector Labs). Slides were imaged using a VS120 slide scanner microscope (Olympus).

Two-photon image analysis

MORSE hydrogel deformation quantification and image stabilization

This process of MORSE hydrogel deformation quantification is diagrammatically outlined in Figure S1A. Due to the relatively slow (tens of seconds) timescales of the ligand calibration responses *in vitro* and *in vivo*, as well as the relatively slow progress of hydrogel warping, we began by creating temporally-downsampled (TDS) MORSE recordings by taking means of every 31–52 volumes (down to 1 volume every ~60–100 sec). For a limited subset of image planes from these TDS movies, we annotated individual cells by hand and trained a neural network model for 3D cell segmentation using Cellpose2.0.⁴⁷ We then applied this model to both the green (GRAB sensor) and red (tdTomato, in a subset of recordings) color channels of every TDS MORSE movie at every timepoint to generate probability maps estimating how likely each pixel is to be part of a cell (Figure S1B). Next, we performed nonlinear optic flow-based registration on these probability maps derived either from the green or red channel to a single reference timepoint, usually the middle timepoint of the movie (compute time ~5 sec/volume with GPU acceleration on Nvidia RTX3090 using the *imregdemons* function in MATLAB2023b, 4 Pyramid Levels, Accumulated Field Smoothing 2.0) (Figure S1C). To stabilize the recordings using these calculated hydrogel deformations, we linearly interpolated the resulting displacement fields up to the sampling frequency of the original raw movies (~0.5 vol/sec), applied the transformations to the raw movies, and corrected high-frequency intervolumetric translations by registering sets of 31–52 adjacent volumes using cross-correlation of the 3D Fourier transform to create aligned raw movies (compute time ~150 sec/stabilized volume using the *imwarp* function in MATLAB2021a on Intel Xeon E5-2650).⁹⁶ Stabilization success was quantified by reapplying the Cellpose2.0 model to temporally-downsampled versions of the aligned raw movies and calculating the cross-correlations of the GRAB signal probability maps at each timepoint to the first timepoint, restricted to the voxels identified as having at least a 50% likelihood of being part of a cell at the first timepoint (Figure S2C).

MORSE image segmentation and signal extraction

This process of MORSE single-cell trace extraction is diagrammatically outlined in Figure S2A. To estimate signals from single-cell regions of interest (ROIs), we generated temporally-downsampled aligned (TDSA) MORSE recordings by taking means of every 31–52 volumes of the stabilized raw movies from above and created Cellpose probability maps for these using the same neural network model as above. This time, we created maximum intensity projections of the probability maps over time and binarized them by identifying pixels that had greater than 50% likelihood of being part of a cell in at least one volume over the course of the TDSA movie. We masked out these pixels in the TDSA movie, spatially-downsampled the remaining pixels by a factor of 16 in the x and y dimensions, and performed 3D thin plate spline interpolation across the whole volume at each timepoint (python function *scipy.interpolate.RB-Finterpolator* with *degree=1* and *smoothing=100*), followed by Gaussian filtering in the x and y dimensions ($\sigma = 1.2$ pixels) and up-sampling back to the original spatial dimensions of the TDSA movie. We then subtracted this interpolated background from each TDSA movie to create the temporally-downsampled background-corrected aligned (TDSBA) movies.

For a preliminary estimation of cell masks, we applied the Caiman CNMF (constrained nonnegative matrix factorization) algorithm for fluorescence signal extraction from cells, which performs spatial and temporal demixing of distinct signal time courses in local regions, to the TDSBA movies (*p*=0 autoregressive order, *gnb*=0 global background components, *rf*=60 pix patch half-size corresponding to roughly 60*60 μ m² patches in the x-y dimension across the full z-thickness of the recording, *K*=30 cell components per patch, *gSig*=[10,10,1.25] pix half-widths of expected cells across the x-y-z dimensions corresponding to [10,10,1.25] μ m, *method_init*='greedy_roi' initialization method, *ssub*=*tsub*=1 indicating no spatial subsampling during initialization). Each movie was thus separated into 30 3D spatial bins of equal size evenly tiling the volume, with 30 putative cell signal ROIs extracted per bin for 900 total ROIs (compute time ~20 min/movie parallelized over 30 CPU cores on Intel Xeon E5-2650). ROIs possessing a minimum signal above a reasonable threshold (calculated as 1.96 times the standard deviation of the distribution of ROI baseline signals with negative values of background-corrected fluorescence signal, assumed to be a half-normal distribution) as well as high signal-to-noise ratio (calculated as the ratio of the maximal baseline-corrected signal at the ROI over time divided by the standard deviation of the signal around a Savitzky-Golay-smoothed version of the signal, with ROIs possessing ratio values above the 20–25th percentile of all ROIs included) were retained for downstream analysis. The ~450–500 ROIs that passed this step were then manually curated to distinguish

signals from individual cells using a custom GUI built in napari (masks were discarded or retained and possibly merged into groups, manual time ~ 2 hrs/movie).⁵⁵ Additional quality control was subsequently performed using another custom napari GUI to ensure that any previously discarded ROIs corresponding to true cells were retained (manual time ~ 1 hr/movie). The curated ROIs were then refitted to the TDSBA movies using Caiman to yield estimated cell masks and time courses (compute time ~ 2 hrs/movie on Intel Xeon E5-2650).

Finally, to apply these calculated cell masks back to the aligned raw movies, we first linearly interpolated the estimated background signals up to the sampling frequency of the aligned raw movies and subtracted them to create aligned raw background-corrected movies. We then linearly interpolated the estimated time courses for each cell mask in the TDSBA movies up to the raw sampling frequency and refitted them to the aligned raw background-corrected movies using Caiman, resulting in a final set of single-cell masks with time courses at native temporal resolution and corrected for background (compute time ~ 3 hrs/movie on Intel Xeon E5-2650).

MORSE cell matching across recordings

Many of the MORSE probes were imaged several times in multiple contexts *in vitro* and *in vivo*. We performed cell mask matching between different recordings of the same probe to track cell identities and activity patterns across recordings. Since the probes were moved and imaged in different orientations, we began by calculating transformations between recordings. We extracted the single reference volume used for registration of each TDS movie of a given probe (see above) and chose one of them as the target for cross-movie alignment. Using the BigStitcher plugin in ImageJ, we then manually rotated and translated each of the other reference volumes to best align with this alignment target volume (manual time ~ 10 min/probe).⁵⁷ For most probes, this was not enough for satisfactory alignment, as the recordings were performed with slight variations in vertical alignment of the probe relative to the microscope objective, and the hydrogels also exhibited some deformation in between imaging sessions. To address this, we further used the BigWarp plugin in ImageJ to manually match 30–40 anchor points between every pair of reference volumes aligned previously and produced a thin plate spline deformation field to achieve fine-scale alignment (manual time ~ 20 min/pair of volumes) (Figure S2D).⁵⁶ We then applied these deformation fields to the set of masks from the corresponding recordings of the probe and overlaid the outputs with the masks from the alignment target movie, successfully overlapping many masks with their correct partners (Figure S2E). Finally, we manually curated these overlapped mask matches to confirm correct cell identities using another custom GUI in napari (manual time ~ 2 hrs/pair of movies) (Figure S2F).

MORSE signal processing

We aimed to calculate dose-response parameters for each sensor to estimate quantitative concentrations of the ligands and unmix the dopamine-norepinephrine and vasopressin-oxytocin sensor crosstalk. To do this, we assumed that sensor-ligand binding kinetics were sufficiently rapid to model the process as a classic equilibrium reaction, with S representing the free sensor, L representing the free ligand, S·L the bound sensor-ligand complex, and K_d the dissociation constant between them¹²⁴:



In this case, the standard equilibrium expression, where [S], [L], and [S·L] are the concentrations of free sensor, free ligand, and bound sensor-ligand complex, respectively, holds:

$$K_d = \frac{[S][L]}{[S \cdot L]} \quad (\text{Equation 2})$$

If we assume that the S and S·L forms of the sensor have stable fluorescence brightnesses α and β , respectively, and that the recording also includes a ligand-independent background signal ϵ (all three dependent on imaging parameters such as excitation light intensity and detector gain), then we can express the fluorescent signal F as follows:

$$F = \alpha[S] + \beta[S \cdot L] + \epsilon \quad (\text{Equation 3})$$

If we assume that the total concentration of the sensor $[S]_0 = [S] + [S \cdot L]$ is constant, we can define fractional sensor occupancies as follows:

$$[S]_{\text{frac}} = \frac{[S]}{[S]_0} \quad [S \cdot L]_{\text{frac}} = \frac{[S \cdot L]}{[S]_0} \quad [S]_{\text{frac}} + [S \cdot L]_{\text{frac}} = 1 \quad (\text{Equation 4})$$

Finally, if we define baseline ligand-free signal $F_0 = \alpha[S]_0 + \epsilon$ and normalized brightness constant $\beta_{\text{norm}} = [S]_0(\beta - \alpha)/F_0$, we can express the fractional change in fluorescence as:

$$\frac{\Delta F}{F_0} = \frac{F - F_0}{F_0} = \frac{\alpha[S] + \beta[S \cdot L] - \alpha[S]_0}{F_0} = \frac{\alpha([S]_0 - [S \cdot L]) + \beta[S \cdot L] - \alpha[S]_0}{F_0} = \beta_{\text{norm}}[S \cdot L]_{\text{frac}} \quad (\text{Equation 5})$$

with β_{norm} equaling the maximal $\Delta F/F_0$ response to a saturating dose of L that brings $[S \cdot L]_{\text{frac}}$ asymptotically close to 1. This means that peak-normalizing the $\Delta F/F_0$ is equivalent to dividing by β_{norm} :

$$dFF_{peak-normalized} = \frac{\Delta F}{F_0} \bigg/ \left(\frac{\Delta F}{F_0} \right)_{peak} = [S \cdot L]_{frac} \quad (\text{Equation 6})$$

Writing out the equilibrium reaction in terms of fractional occupancies then yields:

$$[L] = K_d \frac{[S \cdot L]_{frac}}{1 - [S \cdot L]_{frac}} = K_d \frac{dFF_{peak-normalized}}{1 - dFF_{peak-normalized}} = K_d \frac{\Delta F/F_0}{\left(\Delta F/F_0 \right)_{peak} - \Delta F/F_0} \quad (\text{Equation 7})$$

This expression can be used to calculate K_d for each sensor by fitting a sigmoid curve to known concentrations $[L]$ and corresponding sensor responses $dFF_{peak-normalized}$. Notably, because complete sensor saturation may not have been achieved in all dose-response characterizations, the full fit involves two parameters, with K_d defined as before and F the predicted maximal $dFF_{peak-normalized}$ signal in the sensor (should be close to 1 if the sensor was subjected to a full dose-response and peak-normalized to a near-saturated signal):

$$[L] = K_d \frac{[S \cdot L]_{frac}}{1 - [S \cdot L]_{frac}} = K_d \frac{\frac{dFF_{peak-normalized}}{F}}{1 - \frac{dFF_{peak-normalized}}{F}} = K_d \frac{dFF_{peak-normalized}}{F - dFF_{peak-normalized}} \quad (\text{Equation 8})$$

Or rearranging:

$$dFF_{peak-normalized} = \frac{F}{1 + \frac{K_d}{[L]}} \quad (\text{Equation 9})$$

For completeness, the applied concentration of L , $[L]_0$, is the sum of free and bound L , i.e. $[L]_0 = [L] + [S \cdot L] = [L] + [S]_0[S \cdot L]_{frac}$ so for $[L]$ to be a faithful reflection of $[L]_0$, we must assume conditions of little buffering of free ligand by the sensor, i.e. $[S]_0 \ll K_d$. As a reference value, 100,000 copies of a given protein distributed across a spherical cell of 30 μm diameter equates to an average concentration of 12 nM; this corresponds to 35 molecules/ μm^2 of cell surface area – around double the density of extrasynaptic AMPA receptors in immature neurons.¹²⁵ For a probe composed of 10 sensors, this would correspond to a concentration of ~ 1 nM for each sensor averaged across the hydrogel – substantially lower than the K_d values of all sensors used in this study. Importantly, future applications of higher sensitivity sensors would require an explicit accounting for buffering.¹²⁶

In the case of bidirectional crosstalk of two ligands L_1 and L_2 at two respective sensors S_1 and S_2 , the model would include four ligand-bound states with distinct equilibrium constants:



In that case, the fluorescent signal at S_1 (and similar for S_2) is:

$$F_{S1} = \alpha_{S1}[S_1] + \beta_{S1L1}[S_1 \cdot L_1] + \beta_{S1L2}[S_1 \cdot L_2] + \epsilon_{S1} \quad (\text{Equation 11})$$

With analogous definitions of fractional occupancy and brightness, we then have:

$$\frac{\Delta F_{S1}}{F_{S10}} = \beta_{S1L1norm}[S_1 \cdot L_1]_{frac} + \beta_{S1L2norm}[S_1 \cdot L_2]_{frac} \quad (\text{Equation 12})$$

Normalizing by the saturating response of S_1 to its on-target ligand L_1 and defining $\gamma_{S1} = \beta_{S1L2norm}/\beta_{S1L1norm}$ as the ratio of the saturating response of S_1 to L_2 to the saturating response of S_1 to L_1 yields:

$$dFF_{S1-peak-normalized-L1} = \frac{\Delta F_{S1}}{F_{S10}} \bigg/ \left(\frac{\Delta F_{S1}}{F_{S10}} \right)_{S1-peak-L1} = [S_1 \cdot L_1]_{frac} + \gamma_{S1} * [S_1 \cdot L_2]_{frac} \quad (\text{Equation 13})$$

When each of the cross-talking ligands is applied individually, Equation 13 becomes equivalent to Equation 6, so the previous derivations can be used to fit each sensor's ligand-specific dissociation constants (K_{dS1L1} , K_{dS1L2}) and estimated maximal peak-normalized signals (F_{S1L1} , F_{S1L2}) similarly to Equations 8 and 9:

$$[L_1] = K_{dS1L1} \frac{dFF_{S1-peak-normalized-L1}}{F_{S1L1} - dFF_{S1-peak-normalized-L1}} \quad [L_2] = K_{dS1L2} \frac{dFF_{S1-peak-normalized-L2}}{F_{S1L2} - dFF_{S1-peak-normalized-L2}} \quad (\text{Equation 14})$$

Which can be rearranged:

$$dFF_{S1-peak-normalized-L1} = \frac{F_{S1L1}}{1 + \frac{K_{dS1L1}}{[L_1]}} \quad dFF_{S1-peak-normalized-L2} = \frac{F_{S1L2}}{1 + \frac{K_{dS1L2}}{[L_2]}} \quad (\text{Equation 15})$$

Furthermore, once these parameters are calculated, γ_{S1} can be directly derived:

$$\gamma_{S1} = \frac{\left(\frac{\Delta F_{S1}}{F_{S10}}\right)_{S1-saturating-L2}}{\left(\frac{\Delta F_{S1}}{F_{S10}}\right)_{S1-saturating-L1}} = \frac{F_{S1L2} * \left(\frac{\Delta F_{S1}}{F_{S10}}\right)_{S1-peak-L2}}{F_{S1L1} * \left(\frac{\Delta F_{S1}}{F_{S10}}\right)_{S1-peak-L1}} \quad (\text{Equation 16})$$

At this point, again assuming negligible buffering of either ligand at either sensor, concentrations can be derived from sensor signals by writing out the equilibrium reactions (Equation 2) in terms of fractional occupancies as follows:

$$[S_1]_{frac} + [S_1 \cdot L_1]_{frac} + [S_1 \cdot L_2]_{frac} = [S_1]_{frac} * \left(1 + \frac{[L_1]}{K_{dS1L1}} + \frac{[L_2]}{K_{dS1L2}}\right) = 1$$

$$[S_1]_{frac} = \left(1 + \frac{[L_1]}{K_{dS1L1}} + \frac{[L_2]}{K_{dS1L2}}\right)^{-1} \quad (\text{Equation 17})$$

Combining with Equation 13 and writing out for both sensors, this yields:

$$dFF_{S1-peak-normalized} = D_1 = \frac{F_{S1L1} \frac{[L_1]}{K_{dS1L1}} + \gamma_{S1} * F_{S1L2} \frac{[L_2]}{K_{dS1L2}}}{1 + \frac{[L_1]}{K_{dS1L1}} + \frac{[L_2]}{K_{dS1L2}}} \quad (\text{Equation 18})$$

$$dFF_{S2-peak-normalized} = D_2 = \frac{F_{S2L2} \frac{[L_2]}{K_{dS2L2}} + \gamma_{S2} * F_{S2L1} \frac{[L_1]}{K_{dS2L1}}}{1 + \frac{[L_2]}{K_{dS2L2}} + \frac{[L_1]}{K_{dS2L1}}}$$

Which has the solution:

$$[L_1] = \frac{K_{dS1L1} K_{dS2L1} (D_1 D_2 (K_{dS2L2} - K_{dS1L2}) + D_1 F_{S2L2} K_{dS1L2} - D_2 \gamma_{S1} F_{S1L2} K_{dS2L2})}{D_1 D_2 (K_{dS1L2} K_{dS2L1} - K_{dS1L1} K_{dS2L2}) + D_1 (\gamma_{S2} F_{S2L1} K_{dS1L1} K_{dS2L2} - F_{S2L2} K_{dS1L2} K_{dS2L1}) + D_2 (\gamma_{S1} F_{S1L2} K_{dS1L1} K_{dS2L2} - F_{S1L1} K_{dS1L2} K_{dS2L1}) + F_{S1L1} F_{S2L2} K_{dS1L2} K_{dS2L1} - \gamma_{S1} \gamma_{S2} F_{S1L2} F_{S2L1} K_{dS1L1} K_{dS2L2}} \quad (\text{Equation 19})$$

$$[L_2] = \frac{K_{dS1L2} K_{dS2L2} (D_1 D_2 (K_{dS1L1} - K_{dS2L1}) - D_1 \gamma_{S2} F_{S2L1} K_{dS1L1} + D_2 F_{S1L1} K_{dS2L1})}{D_1 D_2 (K_{dS1L2} K_{dS2L1} - K_{dS1L1} K_{dS2L2}) + D_1 (\gamma_{S2} F_{S2L1} K_{dS1L1} K_{dS2L2} - F_{S2L2} K_{dS1L2} K_{dS2L1}) + D_2 (\gamma_{S1} F_{S1L2} K_{dS1L1} K_{dS2L2} - F_{S1L1} K_{dS1L2} K_{dS2L1}) + F_{S1L1} F_{S2L2} K_{dS1L2} K_{dS2L1} - \gamma_{S1} \gamma_{S2} F_{S1L2} F_{S2L1} K_{dS1L1} K_{dS2L2}}$$

On top of that, because we perform identification and dose-response dipping recordings on all ligands at the same time, we can in principle determine the ratios between their $\Delta F/F_0$ peaks (i.e. their β_{norm} values), and thus in future recordings with this probe we would likely only need one ligand to be applied at saturating dose to estimate the expected peak responses of all the other sensors.

Importantly, all of the above derivations hold for the sensor at steady state, once the concentration throughout the hydrogel has equilibrated to the concentration in the outside fluid. However, in order to reach the sensor protein, each ligand must diffuse through the thin hydrogel layer. Modeling this diffusion process would allow us to obtain the transfer function of the hydrogel, which we could deconvolve from the observed signal to obtain a better estimate of ligand concentration in the surrounding fluid. It should be true that if the probe is placed into a relatively large volume of fluid with the ligand at a given concentration (as it happens during our dipping characterization), the ligand concentration inside the hydrogel will eventually plateau to that in the fluid. This diffusion process is expected to follow Fick's second law. If we define $[L](d,t)$ to be the ligand concentration at a sensor positioned at a depth d in the hydrogel over time, h as the total depth of the hydrogel, and $[L]_f$ as the constant concentration of the fluid that the probe is placed into at $t=0$, the solution to Fick's second law for a cylindrical hydrogel with one open end is the following¹²⁷:

$$[L](d,t) = [L]_f - 2([L]_f - [L](d,0)) \sum_{n=0}^{\infty} (-1)^n \frac{\cos\left[(2n+1)\frac{\pi}{2}\left(1 - \frac{d}{h}\right)\right]}{(2n+1)\frac{\pi}{2}} e^{-\left[(2n+1)\frac{\pi}{2}\right]^2 \frac{D_0 t}{h^2}} \quad (\text{Equation 20})$$

Rearranging, we get

$$\frac{[L]_f - [L](d, t)}{[L]_f - [L](d, 0)} = [L]_{\text{norm}}(d, t) = 2 \sum_{n=0}^{\infty} (-1)^n \frac{\cos \left[(2n+1) \frac{\pi}{2} \left(1 - \frac{d}{h} \right) \right]}{(2n+1) \frac{\pi}{2}} e^{-\left[(2n+1) \frac{\pi}{2} \right]^2 \frac{Dt}{h^2}} \quad (\text{Equation 21})$$

This is an infinite sum of exponentially decaying terms, with the $n=0$ term having the slowest decay and smallest factor in the denominator (although higher n components may have greater multiplicative weights depending on d and the factor in the numerator). In this case, fitting an exponential decay to this relationship will result in decay constant

$$\tau \approx \frac{4h^2}{\pi^2 D} \quad (\text{Equation 22})$$

The exact value will vary with d , but will always be independent of surrounding fluid concentration $[L]_f$.

It is critical to note that this τ calculated on $[L]_{\text{norm}}$ is not equivalent to an exponential fit on any linearly normalized version of the fluorescence signal since $[L]$ is nonlinearly related to $dFF_{\text{peak-normalized}}$ as described above. When L is applied on its own, $\ln([L])$ is approximately linearly related to fluorescence around the K_d of the sensor:

$$\ln([L]) = \ln \left(K_d \frac{dFF_{\text{peak-normalized}}}{1 - dFF_{\text{peak-normalized}}} \right) \approx \ln(K_d) + 4 * \left(dFF_{\text{peak-normalized}} - \frac{1}{2} \right) \quad (\text{Equation 23})$$

in which case $[L]$ can be estimated by taking the exponential of both sides of the equation, and that result can be used to assess diffusion:

$$[L] \approx K_d e^{4 * \left(dFF_{\text{peak-normalized}} - \frac{1}{2} \right)} \quad (\text{Equation 24})$$

However, this is not true where $[L]$ is substantially smaller or larger than K_d – in those cases, exponential fits on the result will be *underestimates* of diffusion speed (i.e. overestimates of τ) at low $[L]$ and *overestimates* of diffusion speed (i.e. underestimates of τ) at high $[L]$. In particular, this means that calculating probe response kinetics using saturating concentrations of $[L]$ will *underestimate* the magnitude by which the hydrogel slows down responsiveness if the nonlinearity is not correctly accounted for. Additionally, these relationships depend on the assumption that ligand binding kinetics at the sensor are much faster than diffusion through the hydrogel, which we assumed to be the case for most of the GRAB sensors we used based on published findings.^{25,29,31,36,45}

Now, given this concentration-independent delay constant τ relating the concentration measured at the sensor to the external concentration in the fluid immediately surrounding the probe, we can estimate the hydrogel's transfer function. Importantly, this depends on the assumption that the external concentration $[L]_e(t)$ (distinct from $[L]_f$ above because it is now varying over time) is uniform throughout the fluid facing the probe (likely true in CSF when concentrations are changing relatively slowly). In that case, we can model the hydrogel akin to a classic RC circuit, with the external concentration $[L]_e(t)$ analogous to the input voltage and the sensor concentration $[L](t)$ analogous to the voltage across the capacitor. This would imply that in the frequency domain,

$$[L](s) = H(s)[L]_e(s) = \frac{1}{1+\tau s}[L]_e(s) \quad (\text{Equation 25})$$

with transfer function $H(s) = 1/(1+\tau s)$ and corresponding impulse response

$$h(t) = \frac{1}{\tau} e^{-t/\tau} u(t) \quad (\text{Equation 26})$$

with $u(t)$ the Heaviside step function. Deconvolving this impulse response from $[L](t)$ (possibly with an appropriate Savitzky-Golay or Wiener filtering step to reduce noise) will then estimate $[L]_e(t)$ in situations that satisfy the above assumptions.

ChP image registration

Registration of the volumetric ChP images was performed as described in Shipley et al.⁹⁶ Briefly, due to rapid movement of the mouse, it was necessary in some recordings to account for intra-volume shifts of the tissue. This was achieved by aligning each plane of the volume to the brightest plane, which acted as an anchor, using Fourier cross-correlation. Next, volumes were registered to each other using cross-correlation of the 3D Fourier transform.⁹⁶

ChP signal processing

Following registration, a representative 3D subvolume of the recording was extracted by choosing x , y , and z dimension ranges that lacked any voxels cropped during registration. A mean projection of this volume across the z dimension was then produced, yielding a two-dimensional timelapse movie. A representative square region of interest spanning several ChP epithelial cells expressing the sensor of interest (GCaMP6f or a GRAB sensor) was selected and the mean green signal trace over the region across time was obtained. In recordings demonstrating slow signal decay prior to ligand administration, an exponential fit to the signal preceding ligand administration was obtained and subtracted from the trace to obtain the estimated change in fluorescence (ΔF) which was divided by the mean raw signal over the first 5 minutes of the recording to estimate the $\Delta F/F_0$.

QUANTIFICATION AND STATISTICAL ANALYSIS

In the case of two-photon recordings, processed single cell fluorescence signals from MORSE or tissue signals from ChP imaging were normalized to a baseline corresponding either to the mean signal over the 15-minute period preceding ligand administration (in the case of i.p. or i.c.v. injections) or to the mean signal of the lowest 10% of readings over the entire recording for each individual trace as follows:

$$\frac{\Delta F}{F_0} = \frac{F - F_0}{F_0} \quad F_0 = \text{mean}(F, 15 \text{ minute baseline}) \text{ or } F_0 = \text{mean}(F, \text{lowest } 10\%) \quad (\text{Equation } 27)$$

In the case of dipping recordings, processed fluorescence signals from MORSE that were normalized as above to obtain a $\Delta F/F_0$ signal often demonstrated a sharp upward jump in the signal at the start of every well, likely due to forces applied to the hydrogel as it crossed the air-fluid interface. This artifact was fitted with an exponential decay scaled by the jump in the $\Delta F/F_0$ signal between adjacent wells, which exhibited a consistent decay constant of ~ 30 sec (see [Video S3](#) for an example of such jumps). The artifact-corrected signal was then either fitted with an exponentially decaying background correction over tens of minutes of signal preceding sensor activation (single-dose sensor identification dipping as in [Figure 1G](#), with activation identified by a spike in the sensor's estimated first derivative – see below) or processed with a Butterworth filter with a passband in the 1/6000 to 1/50 Hz range to accentuate responses on the 3–5 minute timescale of the dipping process while discarding slowly changing background signal (slice and *in vivo* dipping characterizations in [Figures 2, 3, 5](#), and [S5](#), except for KCl dose-response dipping in [Figures S5B](#) and [S5C](#) which was not baseline corrected). Dipping characterization signals were subsequently reported in peak-normalized form (except for KCl dose-response dipping in [Figures S5B](#) and [S5C](#)), which was calculated as follows:

$$\left(\frac{\Delta F}{F_0}\right)_{\text{peak-normalized}} = \frac{\Delta F/F_0 - \text{mean}\left(\Delta F/F_0, \text{lowest } 10\%\right)}{\text{mean}\left(\Delta F/F_0, \text{highest } 10\%\right) - \text{mean}\left(\Delta F/F_0, \text{lowest } 10\%\right)} \quad (\text{Equation } 28)$$

We found that the most effective approach to clustering cells by sensor identity was to focus on the onset timings of each standard ligand administration (either dipping into an identification well or washing on a saturating ligand dose onto the slice), since different sensors and ligands exhibited variable wash-off kinetics. To do this, the peak-normalized $\Delta F/F_0$ signal calculated as above (with or without background correction or Butterworth filter) was processed with a Savitzky-Golay filter with a 1-minute window and polynomial order 1, which estimated the first derivative. This resulted in an estimate of sensor activation time, as the filtered signal had large values at the times when the signal was rising the fastest ([Figures 1G](#) (bottom) and [S3A](#)). We subsequently performed hierarchical density-based spatial clustering of applications with noise (HDBSCAN) on the derivative time courses.¹²⁸ This yielded high-quality clusters which were automatically matched to corresponding ligand identities by calculating the cross-correlation of the derivative time courses with the time courses of ligand administration. Calculating the means of the derivative time courses across each cell cluster and cross-correlating them with each other consistently demonstrated that only the DA2.9 dopamine and the NE2h norepinephrine sensors, as well as to a lesser extent the AVP0.2 vasopressin and OXT1.7 oxytocin sensors, exhibited crosstalk in their ligand responses ([Figures 1H](#) and [S3B](#)).²⁹ In the slice and *in vivo* recordings, we performed similar HDBSCAN clustering on the $\Delta F/F_0$ signals or peak-normalized $\Delta F/F_0$ signals to cluster the cell responses.

For the assessment of sensor K_d (i.e. EC_{50}) and saturation, $\Delta F/F_0$ signal from each cell from the dose-response dipping recording that was matched to a sensor identity from an identification dipping recording of the same probe was first corrected with an exponentially decaying background fit and then peak-normalized as above. Next, the mean of the sensor signal from the third minute of recording in a given well with a given concentration of the ligand was taken as the sensor's plateaued signal at that concentration. These values were fitted with a dissociation equation as per [Equation 9](#).

In a subset of slice and *in vivo* MORSE recordings, these sensor parameters were subsequently used to obtain quantitative estimates of molecular concentrations. For each recording, pooled signals for each sensor type were obtained by taking a median of the $\Delta F/F_0$ signal from all cells of that sensor type at each timepoint, and these were peak-normalized. In the case of the slice recordings, in which the saturating wash-on concentrations of NE, DA, and 5-HT were all 50 μM , the peak-normalization was performed to this reference concentration – in other words, the calculated K_d and F parameters for each sensor were used to estimate the $dFF_{\text{peak-normalized}}$ signal expected for a 50 μM administration of the ligand individually, and the signals were then scaled for their post-wash-on peaks to match this value. For *in vivo* AVP, OXT, and 5-HT quantification, the delivery of 15–30 μL of 500 μM stock AVP, 500 μM stock OXT, or 5 mM stock 5-HT in aCSF into a ~ 36 μL volume of endogenous mouse CSF translates to final concentrations of at least 100 μM AVP, 100 μM OXT, or 1 mM 5-HT, which is expected to saturate each of the sensors.¹²⁹ Therefore, in this case the signals were normalized to their peaks matching the F parameter of each of the sensors. A median filter with a 10–60 sec window was applied to these peak-normalized signals, and concentrations were estimated using either [Equation 8](#) in situations without crosstalk or [Equation 19](#) when crosstalk was accounted for. For each dataset of slice and *in vivo* recordings where concentrations were estimated, a threshold defining robust response was also defined. First, the standard deviation of each sensor's pooled peak-normalized $\Delta F/F_0$ signal over the period preceding any KCl/optogenetic stimulation or i.p. injection was

calculated. The maximum of those standard deviations was taken across the dataset and multiplied by 1.96 – this was defined as the 95% confidence threshold of robustness for that sensor. Finally, this was converted to a concentration using [Equation 8](#). These thresholds are plotted as horizontal dotted lines in [Figures 2, 4, S4, and S5](#).

We further used the dipping recordings to assess hydrogel diffusion parameters. To do this, we fitted an exponential rise curve to the first three minutes of each individual sensor cell's time course in each well of the recording and defined its time constant as the hydrogel diffusion time constant τ_{on} for that sensor cell at the ligand concentration in the given well ([Figure S3E](#)). We performed this analysis both on the raw $\Delta F/F_0$ signals for each cell as well as the estimated quantitative concentration time course obtained by applying [Equation 8](#), and we also assessed how time constants depended on sensor cell depth in the hydrogel ([Figure S3F](#)). We subsequently used these τ_{on} values to perform deconvolution as described above ([Figures S4C–S4E and S5H](#)).

In the case of fiber photometry measurements, the original signals were collected at 50 Hz frequency as described above. A median filter with a 2 second window (every 100 readings) was applied, and the signal was then fitted with an exponentially decaying background correction over tens of minutes of signal preceding sensor activation. Photometry signals were reported in $\Delta F/F_0$ format normalized to the 15 minutes preceding each i.p. injection, as in [Equation 27](#).

Outliers in box plots used throughout the paper are defined as values lying further than 1.5x the interquartile range from the box. All error bars and shading represent $\pm$ SEM as defined in figure legends.