A genetically encoded fluorescent biosensor for extracellular l-lactate

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L-Lactate, traditionally considered a metabolic waste product, is increasingly recognized as an important intercellular energy currency in mammals. To enable investigations of the emerging roles of intercellular shuttling of l-lactate, we now report an intensiometric green fluorescent genetically encoded biosensor for extracellular l-lactate. This biosensor, designated eLACCO1.1, enables cellular resolution imaging of extracellular l-lactate in cultured mammalian cells and brain tissue.
Traditionally, the conjugate acid-base pair of l-lactic acid and l-lactate have been considered a "waste" by-product of glucose metabolism. However, growing evidence suggests that l-lactate is better considered a crucial biological "fuel currency", that is shuttled from cell-to-cell. For example, the astrocyte-to-neuron lactate shuttle (ANLS) hypothesis states that astrocytes metabolize glucose to produce l-lactate which is then released to the extracellular environment and taken up by neurons. In neurons, l-lactate is converted to pyruvate which enters the citric acid cycle to produce the energy necessary to sustain heightened neural activity. The ANLS hypothesis remains controversial, with recent reports of evidence both for and against.

Investigations of such cell-to-cell l-lactate shuttles would be facilitated by a genetically encoded fluorescent biosensor that would enable high resolution spatially and temporally resolved imaging of the extracellular l-lactate concentration. Genetically encoded fluorescent biosensors are powerful tools for cell-based and in vivo imaging of molecules, ions, and protein activities, and many examples have been reported to date. However, despite remarkable progress in the development of such biosensors, including ones for intracellular l-lactate, no genetically encoded biosensors for extracellular l-lactate have yet been reported.

Here, we report the development of a genetically encoded fluorescent biosensor for extracellular l-lactate. This biosensor, designated eLACCO1.1, is the end-product of extensive directed evolution and structure-based mutagenesis followed by optimization of biosensor expression and localization on the cell surface. We confirm that eLACCO1.1 enables cellular resolution imaging of extracellular l-lactate in cultured mammalian cells and brain tissue.

Results

Development and characterization of a genetically encoded l-lactate biosensor, eLACCO1. Periplasmic binding proteins (PBPs) derived from prokaryotic organisms have proven to be particularly effective sensing domain for extracellular single fluorescent protein-based biosensors, with key examples that include glutamate, GABA, acetylcholine, and serotonin. To construct a prototype l-lactate biosensor, we inserted circularly permuted green fluorescent protein (cpGFP) into Thermus thermophilus TTHA0766 l-lactate binding periplasmic protein at 70 different positions (Supplementary Fig. 1). Positions of insertion sites in TTHA0766 were chosen by manual inspection of the protein crystal structure to identify loop regions that were solvent exposed and likely to undergo l-lactate-dependent conformational changes. The variant (designated eLACCO0.1) with the largest change in fluorescence intensity (ΔF/F = (Fmax − Fmin)/Fmin) upon l-lactate treatment had cpGFP inserted at position 191, and exhibited an inverse response (fluorescence decrease upon binding) with ΔF/F = 0.3. Efforts to create prototype biosensors by inserting cpGFP into the analogous regions of TTHA0766 homologs produced no variants with larger l-lactate-dependent changes in fluorescence intensity (Supplementary Fig. 2). Accordingly, we focused our efforts on further development of eLACCO0.1.

To develop variants of eLACCO0.1 with larger ΔF/F, we performed two rounds of linker optimization, followed by seven rounds of directed evolution by random mutagenesis of the whole gene, with screening for l-lactate-dependent change in fluorescence intensity (Fig. 1a). This effort ultimately produced the direct response variant eLACCO1 with ΔF/F of 6 (Fig. 1b–d and Supplementary Fig. 3) and a strict requirement for Ca2+ at concentrations greater than 0.6 μM (Supplementary Fig. 4a, b). The apparent dissociation constant (Kd) of eLACCO1 is 4.1 μM and 120 μM for l-lactate and d-lactate, respectively (Supplementary Fig. 4e). eLACCO1 showed a pH dependence similar to that of the GαMP66 Ca2+ biosensor, with pKd values of 6.0 and 8.7 in the presence and absence of l-lactate, respectively (Supplementary Fig. 4d). Relative to l-lactate, eLACCO1 was ~320, ~460, and ~1320 times less sensitive to the structurally similar molecules β-hydroxybutyrate, pyruvate and oxaloacetate, respectively (Supplementary Fig. 4c).

The X-ray crystal structure of eLACCO1. In an effort to obtain molecular insight into the structure and mechanism of eLACCO1, we determined the crystal structure of eLACCO1 in the l-lactate-bound state at a resolution of 2.25 Å (Fig. 1e and Supplementary Table 1). The overall structure reveals that the cpGFP-derived and TTHA0766-derived domains are closely associated via an extensive interaction surface that contains numerous molecular contacts. The TTHA0766-derived domain of eLACCO1 retains the same dimeric structure as TTHA0766 itself (Supplementary Fig. 5). The Hill coefficient of eLACCO1 is close to one, suggesting that the protomers in the dimer do not interact cooperatively (Supplementary Fig. 4c). Trp509 in the dimer interface forms a hydrophobic interaction with its symmetry-related self via π-π stacking. Mutagenesis of this residue abrogated l-lactate-dependent fluorescence presumably due to disruption of the dimeric structure, providing support for the conclusion that eLACCO1 functions as a dimer (Supplementary Fig. 5a).

Development and characterization of affinity-tuned eLACCO1. As the physiological concentration of extracellular l-lactate at rest is ~0.2 mM in the brain and 1 mM in serum, we expected that the affinity of eLACCO1 for l-lactate (apparent Kd ~4.1 μM) would be too high to respond to physiologically relevant concentration changes. To decrease the affinity for l-lactate, we introduced the structure-guided Tyr80Phe mutation to remove a hydrogen bond between l-lactate and eLACCO1. This produced the low-affinity eLACCO1.1 variant with apparent Kd of 3.9 mM (Supplementary Fig. 6a, b). A non-responsive control biosensor, designated deLACCO, was engineered by incorporating the Asp444Asn mutation to abolish l-lactate binding (Supplementary Fig. 6c, d).

With the affinity-optimized eLACCO1.1 variant in hand, we undertook a detailed characterization of its spectral properties. eLACCO1.1 has two absorbance peaks at 397 and 496 nm, indicative of the neutral (protoneated) and the anionic (deproto- nated) chromophore, respectively (Fig. 2a). Consistent with the absorbance spectrum, eLACCO1.1 displays excitation peaks at 398 and 493 nm and excitation at either peak produces an emission peak at 510 nm (Fig. 2b). To investigate the effect of Ca2+ on the biosensor functionality, we determined the dependence of the fluorescence intensity of eLACCO1.1 on l-lactate and Ca2+. These experiments revealed that the biosensor only functions as an l-lactate biosensor at concentrations of Ca2+ greater than 9 μM (Fig. 2c).

In vitro characterization of eLACCO1.1 revealed the brightness of the l-lactate-bound state to be ~80% and 42% of EGFP under one-photon and two-photon illumination, respectively (Table 1). eLACCO1.1 under one-photon excitation yields l-lactate-dependent decrease in excitation at the 398 nm peak and increase at the 493 nm peak (Fig. 2b). Under two-photon excitation (Fig. 2d), eLACCO1.1 also exhibited l-lactate-dependent ratiometric changes in excitation with an increase of the 924 nm peak and decrease of the 804 nm peak, corresponding to a shift from the neutral to the anionic state of the chromophore. The intensiometric l-lactate-induced two-photon-excited fluorescence change at 940–1000 nm (ΔF/ F = 8–10) is about twice as large as the one-photon-excited...
The tested variants are ranked from lowest (negative responses indicate inverse response) to highest response (ΔF/F, fluorescence increase upon binding) to L-lactate (ΔF/F = 0.3) while the template eLACCO0.1 decreased the fluorescence intensity (inverse response) in response to L-lactate. Nine rounds of the evolution led to eLACCO1 indicated with a magenta circle. Excitation and emission spectra of eLACCO1 in the presence and absence of 10 mM L-lactate. Excitation and emission peak is at 494 and 512 nm, respectively. D Schematic representation of eLACCO1 and its mechanism of response to L-lactate. Linker regions are shown in black and the two “gate post” residues in eLACCO1.1 (Supplementary Fig. 7). Hence, we turned to the combination of an N-terminal leader sequence and a glycosylphosphatidylinositol (GPI) anchor, both of which are derived from CD59. Ultimately, we optimized the biosensor functionality, we screened a number of linkers between eLACCO1.1 and GPI anchor. This experiment revealed that the 18 amino acid linker (GSTSGSGKPGSGEGSTKG) provides the best ΔF/F upon treatment with L-lactate (Fig. 3c, d). Bath application of 10 mM L-lactate resulted in no significant difference in ΔF/F in the presence versus absence of monocarboxylic transporter inhibitor AR-C155858, though the variability in ΔF/F was greatly decreased by this treatment (Fig. 3e). This result suggests that only the fraction of eLACCO1.1 on the cell surface contributes to the fluorescence response associated with changes in the extracellular L-lactate concentration. The decreased variability associated with AR-C155858 treatment is attributed greater consistency in extracellular lactate concentration due to blockage of lactate uptake into cells.

**Characterization of eLACCO1.1 in live mammalian cells.** We characterized cell-surface-targeted eLACCO1.1 in terms of several important parameters. eLACCO1.1, with an optimized linker between eLACCO1.1 and GPI anchor, robustly increased fluorescence intensity (ΔF/F of 3.3 ± 0.3, mean ± s.e.m., n = 26 cells) upon treatment with L-lactate (Fig. 4a, b). The control biosensor

**Targeting of eLACCO1.1 to the extracellular environment.** To test eLACCO1.1 as a genetically encoded fluorescent biosensor for extracellular L-lactate in a cellular milieu, we targeted it to the surface of mammalian cells by fusing it to various N-terminal leader sequences and C-terminal anchor domains (Fig. 3a). The widely used combination of the immunoglobulin κ-chain (Igκ) leader sequence and the platelet-derived growth factor receptor (PDGFR) transmembrane domain resulted in only intracellular expression with localization reminiscent of the nuclear membrane (Fig. 3b). We screened a range of leader sequences in combination with the PDGFR anchor, but did not discover any that led to robust membrane localization of PDGFR-anchored eLACCO1.1 (Supplementary Fig. 8). The control biosensor deLACCO showed no response to L-lactate, even at 100 mM (Fig. 2f). eLACCO1.1 responds to D-lactate with an apparent Kd of 21 mM, a concentration that is far greater than the 9.4 in the presence and absence of L-lactate, respectively (Fig. 2e).

**Fluorescence change (ΔF/F = 4, at 480–510 nm)** (Fig. 2b, d). A similar trend has been observed for some red fluorescent genetically encoded Ca2+ biosensors. The fluorescence of eLACCO1.1 is pH dependent, exhibiting pKₐ values of 7.4 and 9.4 in the presence and absence of L-lactate, respectively (Fig. 2e). The control biosensor deLACCO showed no response to L-lactate, and pH dependence that was similar to the lactate-free state of eLACCO1.1 (Supplementary Fig. 7). Investigation of the molecular specificity of eLACCO1.1 revealed that the decreased binding affinity extended to structurally similar molecules, with pyruvate and oxaloacetate causing negligible fluorescent response even at 100 mM (Fig. 2f).

**Fig. 1 Development of a genetically encoded L-lactate biosensor, eLACCO1.** a Schematic of the directed evolution workflow. Specific sites (i.e., the linkers) or the entire gene of template L-lactate biosensor genes were randomly mutated and the resulting mutant library was used to transform E. coli. Bright colonies were picked and cultured, and then proteins were extracted and ΔF/F upon addition of 10 mM L-lactate was determined. The variant with the highest ΔF/F was used as the template for the next round. b ΔF/F rank plot representing all proteins tested during the directed evolution. For each round, tested variants are ranked from lowest (negative responses indicate inverse response) to highest ΔF/F value from left to right. The first round of evolution used a library in which residues of the C-terminal linker were randomized. Screening of this library led to the discovery of eLACCO2.2. c A white R-lactate (ΔF/F = 0.3) while the template eLACCO1.1 decreased the fluorescence intensity (inverse response) in response to L-lactate. Nine rounds of the evolution led to eLACCO1 indicated with a magenta circle. d Excitation and emission spectra of eLACCO1 in the presence and absence of 10 mM L-lactate. Excitation and emission peak is at 494 and 512 nm, respectively.

**Fluorescence measurement** (Inverse fluorescence intensity and pH under physiological conditions.

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**Characterization of eLACCO1.1 in live mammalian cells.** We characterized cell-surface-targeted eLACCO1.1 in terms of several important parameters. eLACCO1.1, with an optimized linker between eLACCO1.1 and GPI anchor, robustly increased fluorescence intensity (ΔF/F of 3.3 ± 0.3, mean ± s.e.m., n = 26 cells) upon treatment with L-lactate (Fig. 4a, b). The control biosensor
deLACCO had similar membrane localization and, as expected, did not respond to L-lactate (Fig. 4a, b). eLACCO1.1 also exhibited an L-lactate-dependent change in the ratio of excitation at 365 and 470 nm, suggesting eLACCO1.1 could be applicable as both an intensiometric and a ratiometric biosensor (Supplementary Fig. 9). To test photostability, we continuously illuminated eLACCO1.1-expressing cells using one-photon wide-field microscopy (Fig. 4c). eLACCO1.1 showed photostability that is comparable to EGFP and cpGFP. To examine the on-rate kinetics of eLACCO1.1, we bathed eLACCO1.1-expressing HeLa cells in a solution containing 10 mM L-lactate. L-Lactate application induced the robust increase in the fluorescence with the on rate ($\tau_{\text{on}}$) of 1.2 min (Fig. 4d). Cell-surface-targeted eLACCO1.1 has an in situ apparent $K_D$ of 1.6 mM (Fig. 4e) and displays Ca$^{2+}$ and pH dependent fluorescence as shown in Supplementary Figs. 10 and 11. Stopped-flow analysis of purified eLACCO1.1 protein revealed that the off-rate kinetics are faster than the 1.1 ms dead time of the instrument used (Supplementary Fig. 12). To characterize the performance of eLACCO1.1 in neurons, we expressed eLACCO1.1 in rat primary cortical neurons. We observed that neurons expressing eLACCO1.1 exhibited bright membrane-localized fluorescence with some puncta apparent (Fig. 4f).

**Table 1** One- and two-photon photophysical parameters of eLACCO1.1.

|                      | eLACCO1.1 | EGFP  |
|----------------------|-----------|-------|
|                      | L-lactate free | L-lactate bound |       |
| Relative fraction of neutral chromophore ($\rho_N$) | 0.97 ± 0.07 | 0.57 ± 0.04 | 0.07$^a$ |
| Relative fraction of anionic chromophore ($\rho_A$) | N/A | 0.43 ± 0.03 | 0.93$^a$ |
| Neutral extinction coefficient (mM$^{-1}$cm$^{-1}$, $\varepsilon_N$) | 34 | 38 | N/A |
| Anionic extinction coefficient (mM$^{-1}$cm$^{-1}$, $\varepsilon_A$) | 78 | 89 | 56.0$^b$; 58.3$^c$ |
| Neutral quantum yield ($\phi_N$) | 0.20 ± 0.01 | 0.23 ± 0.01 | N/A |
| Anionic quantum yield ($\phi_A$) | 0.60 ± 0.03 | 0.78 ± 0.04 | 0.67$^b$; 0.76$^c$ |
| Molecular brightness ($\rho_A \times \varepsilon_A \times \phi_A$) | N/A | 29.9 | 34.9$^b$; 41.2$^c$ |
| Neutral 1PA peak (nm) | 398 | 397 | N/A |
| Anionic 1PA peak (nm) | 493 | 496 | 488$^b$ |
| Two-photon brightness $F_2$ (GM)$^d$ | 2.2 ± 0.3 (924 nm) | 16 ± 2 (924 nm) | 38 (911 nm)$^c$ |

Mean ± s.d. N/A, not applicable. Subscript A and N correspond to the anionic and neutral form of chromophore, respectively.

$^a$Calculated using the equation, log$_{10}($neutral)/anionic)=pH$-p$K_a$, where pH is 7.2 and p$K_a$ is 6.1 (ref. 16).

$^b$Data from ref. 16.

$^c$Data from ref. 17.

$^d$Two-photon brightness is calculated as $F_2=\rho_A \times \sigma^2_A \times \phi_A$ at the peak wavelength (in parentheses), where $\sigma^2_A$ is the peak two-photon absorption cross section.
The data indicate that eLACCO1.1 enables detection of extracellular lactate. Whiskers extend one standard deviation range from the mean represented as black bars. 10 mM (mean ± s.e.m., n > 10 neurons from 3 cultures, Fig. 4f). Attempts to use the previously reported Förster resonance energy transfer (FRET)-based biosensor Laconic for imaging of extracellular lactate were unsuccessful (Fig. 4b and Supplementary Fig. 13). Taken together, these results indicated that eLACCO1.1 could uniquely be useful for imaging of extracellular lactate concentration dynamics.

Two-photon imaging of lactate on astrocytes in acute brain slices. The ANLS hypothesis states that glial cells such as astrocytes can release lactate into the extracellular space and that this lactate is taken up by neurons to serve as an energy source. To determine whether eLACCO1.1 remains functional on the surface of astrocytes of mammalian brain tissue, we used two-photon microscopy to examine cortical acute brain slices prepared from mice injected with an adeno-associated virus (AAV) coding the c promoter and be resistant to proteolysis.

Imaging of endogenous lactate release from cultured glioblastoma cells. To determine if eLACCO1.1 can enable imaging of the release of endogenous lactate from cells, we targeted eLACCO1.1 to the surface of T98G glioma cells. Upon treatment with a high glucose concentration (25 mM), which is expected to stimulate the production of endogenous lactate, eLACCO1.1 on the surface of T98G cells underwent an increase in fluorescence consistent with increased secretion of lactate (Fig. 6a–c). In the presence of phloretin or AR-C155858, two inhibitors of the monocarboxylate transporter, the glucose-dependent fluorescence increase was diminished. In both the presence and absence of phloretin or AR-C155858, the control biosensor deLACCO showed no substantial change in fluorescence intensity, indicating that the observed fluorescence changes were due to the lactate-dependent response of eLACCO1.1. To image the production and export of endogenous lactate under a more physiologically relevant condition, we observed eLACCO1.1-expressing T98G cells treated with a physiological plasma concentration of glucose (5.6 mM, Fig. 6d). We first attempted to inhibit production of endogenous lactate by treating the cells with 100 μM GTP.
respectively. In the photobleaching curves, solid lines represent mean value and shaded area represent s.d. One-way ANOVA, continuously illuminated eLACCO1.1-expressing cells using one-photon wide-field microscopy. n = 26, 28 and 13 cells for eLACCO1, delACCO and eLaconic, respectively. Photobleaching curves (left) and time constants (right) for EGFP, cpGFP and eLACCO1.1 expressed on the cell surface. To test photostability, we continuously illuminated eLACCO1.1-expressing cells using one-photon wide-field microscopy. n = 25, 24 and 23 cells for EGFP, cpGFP and eLACCO1.1, respectively. In the photobleaching curves, solid lines represent mean value and shaded area represent s.d. One-way ANOVA, P > 0.05. Time-course of the fluorescence response of eLACCO1.1 on cell surface under L-lactate stimulation. To examine the on-rate kinetics of eLACCO1.1, we bathed eLACCO1.1-expressing HeLa cells in a solution containing 10 mM L-lactate. The plot was fitted with a single exponential function. n = 13 cells (mean ± s.e.m.). In situ titration of L-lactate on HeLa cells. Data were fitted with Hill equation. n = 11 cells (mean ± s.e.m.). Representative images of eLACCO1.1 expressed in primary cortical neurons before and after 10 mM L-lactate stimulation. eLACCO1.1 was expressed at relatively high levels, as indicated by bright membrane-localized fluorescence with some puncta apparent. Similar results were observed in more than 10 neurons from three independent experiments. Scale bars, 10 μm. In the box plots in b, c, the narrow part of notch with the horizontal line is the median; the top and bottom of the notch denote the 95% confidence interval of the median; the top and bottom horizontal lines are the 25th and 75th percentiles for the data; and the whiskers extend one standard deviation range from the mean represented as black filled circle. Source data of b–e are provided as a Source Data file.

Iodoacetate, an inhibitor of glyceraldehyde 3-phosphate dehydrogenase. Unexpectedly, this stimulation resulted in apparent aggregation of eLACCO1.1 (Supplementary Fig. 14). To avoid this iodoacetate-induced aggregation artefact, we turned to using NCI-737, an inhibitor of lactate dehydrogenase (LDH) (Fig. 6e, f). Treatment with NCI-737 caused a slight increase in the fluorescence response of deLACCO. In contrast, eLACCO1.1 showed a decrease in the fluorescence response upon NCI-737 treatment. The opposite effects of NCI-737 on the responses of eLACCO1.1 and deLACCO are consistent with the observed fluorescence changes being due to changes in the extracellular L-lactate concentration. Overall, these results demonstrate that eLACCO1.1 enables imaging of extracellular L-lactate release from glial cells with cellular resolution.

Discussion

This study describes the development of a genetically encoded extracellular L-lactate biosensor, designated eLACCO1.1. Screening of a library of variants with cpGFP inserted into different positions of Thermus thermophilus TTHA0766 L-lactate binding protein led to the identification of a biosensor prototype in which the L-lactate-dependent conformational change of TTHA0766 allosterically modulates the fluorescence intensity of cpGFP. Extensive directed evolution of the prototype led to the high-performance L-lactate biosensor, eLACCO1.1. Rational mutagenesis based on the crystal structure of eLACCO1 tuned its L-lactate affinity to be optimal for the physiological concentration range of extracellular L-lactate. An intense effort to target the affinity-tuned variant to the extracellular environment eventually produced eLACCO1.1 that enables cellular resolution imaging of extracellular L-lactate in cultured mammalian cells and brain tissue.

The choice of sensing domain is critically important to the development of any genetically encoded biosensor. Biosensors for extracellular applications (e.g., those with specificity for neurotransmitters like glutamate, GABA, acetylcholine, serotonin, dopamine, and norepinephrine) have generally used microbial PBPs or G-protein-coupled receptors (GPCRs) as the sensing domain. In this work, we chose to use Thermus thermophilus TTHA0766 L-lactate-binding PBP. Relative to other possible sensing domains that are normally found in the reducing environment of the cytoplasm, an advantage of microbial PBPs is that they naturally function in the oxidative environment of the periplasm. This property is likely beneficial to PBP-based biosensors for extracellular targets, since the protein must be trafficked through oxidative organelles such as the endoplasmic reticulum and Golgi apparatus and ultimately exposed to the oxidative extracellular environment. A gluconate operon repressor family transcription factor, LldR, has previously been used as the sensing domain for intracellular L-lactate biosensors. Attempts to use the Laconic LldR-based biosensor...
Fig. 5 Two-photon imaging of L-lactate on astrocytes in acute brain slices. a Schematic illustration of AAV injection into the somatosensory cortex for the brain slice experiments. ITR inverted terminal repeat, GFAP human glial fibrillary acidic protein promoter, WPRE woodchuck hepatitis virus posttranslational regulatory element, pA human growth hormone polyA signal. b Representative two-photon images of eLACCO1.1 expressed on astrocytes in brain slice before and after 10 mM L-lactate stimulation. Parallel experiments with deLACCO produced baseline fluorescence that was not significantly higher than background and did not produce a significant change in fluorescence relative to baseline ($\Delta F/F = 0.85 \pm 1.6, P = 0.25$, two-tailed paired t-test). Similar results were observed from more than 3 slices. Scale bar represents 50 μm. c Fluorescence traces of eLACCO1.1-expressing astrocytes in response to bath application of L-lactate (mean ± s.e.m.). 1 mM L-lactate ($n = 5$ slices from 4 mice), 2.5 mM L-lactate ($n = 5$ slices from 4 mice), or 10 mM L-lactate ($n = 6$ slices from 4 mice). d $\Delta F/F$ plots for eLACCO1.1 at each dose of L-lactate. $\Delta F/F$ was calculated by: $\Delta F/F = (F_x - F_b)/F_b$, where $F_x$ is the peak fluorescence intensity and $F_b$ is the baseline fluorescence intensity. 1 mM L-lactate ($n = 5$ slices from 4 mice, $P = 0.01$, two-tailed paired t-test), 2.5 mM L-lactate ($n = 6$ slices from 4 mice, $P = 0.02$, two-tailed paired t-test), or 10 mM L-lactate ($n = 6$ slices from 4 mice, $P = 0.01$, two-tailed paired t-test). The horizontal line is the median; the top and bottom horizontal lines are the 25th and 75th percentiles for the data; and the whiskers extend one standard deviation range from the mean represented as black filled circle. One slice data in the 2.5 mM group was omitted from the trace in c because it was not a complete time course, but $\Delta F/F$ of the slice could be measured and was included in d. Source data of c, d are provided as a Source Data file.

Fig. 6 Imaging of endogenous L-lactate release from glioblastoma cells. a Schematic representation of L-lactate metabolism in the presence of 25 mM of extracellular glucose. MCT monocarboxylate transporter, LDH lactate dehydrogenase. b Representative fluorescence images of T98G cells expressing eLACCO1.1 or deLACCO treated with 25 mM glucose. Scale bars, 20 μm. c $\Delta F/F$ versus time for T98G cells expressing eLACCO1.1 or deLACCO. $n = 7$ and 8 cells for eLACCO1.1 and deLACCO, respectively (mean ± s.e.m.). d Schematic representation of L-lactate metabolism in the presence of 5.6 mM of extracellular glucose. e Representative fluorescence images of T98G cells expressing eLACCO1.1 or deLACCO treated with 5.6 mM glucose. Scale bars, 20 μm. f $\Delta F/F$ versus time for T98G cells expressing eLACCO1.1 or deLACCO. $n = 8$ and 8 cells for eLACCO1.1 and deLACCO, respectively (mean ± s.e.m.). Source data of c, f are provided as a Source Data file.
for imaging of extracellular L-lactate were unsuccessful (Fig. 4b and Supplementary Fig. 13), consistent with the suggested importance of using sensing domains that are compatible with the extracellular environment.

Relative to GPCR-based biosensors, there are several technical advantages associated with development of PPB-based single FP-based biosensors. Of particular relevance to the methods used in this work, directed evolution with bacterial expression is applicable to PPB-based biosensors which can be expressed in soluble form, but is not applicable to integral membrane proteins such as GPCRs. Furthermore, these soluble biosensors can often be crystallized in order to resolve their structure at atomic resolution, providing insights that guide and accelerate engineering efforts. These insights ultimately help to produce biosensors with fluorescent responses that are substantially larger than those yet demonstrated for GPCR-based biosensors.

The mechanism of eLACCO1.1 must involve changes in the chromophore environment that are induced by conformational changes that accompany binding of L-lactate to the TTHA0766-derived domain5. Based on the pH dependence, these changes in the chromophore environment are stabilizing the brightly fluorescent deprotonated form of the chromophore. The structure of eLACCO1, which we assume to be representative of eLACCO1.1, reveals that the imidazole side chain of His195 is likely a key moiety for mediating changes in the chromophore protonation state (Fig. 1e). Further insight into the mechanism comes from examining the position of beneficial mutations discovered during biosensor optimization. In the first round of the directed evolution for optimization of the C-terminal linker, the introduction of the Asn439Asp mutation converted the biosensor from having an inverse response to having a direct response (Fig. 1b). Accordingly, we propose that the mechanism of eLACCO1.1 involves a conformational switch between two states: the L-lactate-free state where the protonated (dark) chromophore is stabilized by interaction with carboxylate side chain of Asp439 (and His195 is further away); and the L-lactate-bound state where the deprotonated chromophore (bright) is stabilized by interaction with the imidazole side chain of His195 (and Asp439 is further away). Consistent with this proposed mechanism, in the final round of the evolution, we discovered the Lys142Arg mutation that substantially improved ΔF/F (Fig. 1b). The eLACCO1 crystal structure reveals that Arg142 forms a salt bridge with Asp439 in the L-lactate-bound state. This salt-bridge may further limit residual lactate-bound state. This salt-bridge may further limit residual

As with many genetically encoded biosensors8,11, the maximal fluorescence response of eLACCO1.1 in acute brain slice (ΔF/F ~0.7) is smaller than that in HeLa cells (ΔF/F ~3) and cultured neurons (ΔF/F ~2). A previous study with the extracellular neurotransmitter serotonin biosensor iSeroSnFR similarly reported that its fluorescence response in acute brain slice (ΔF/F ~0.8) is smaller than that in HEK293T cells (ΔF/F ~8) and cultured neurons (ΔF/F ~6) upon treatment with 1 mM serotonin31. In addition to the smaller fluorescence response, eLACCO1.1 shows a relatively large variation of fluorescence response in acute brain slice (~6.7) compared to 5d). To develop a next generation eLACCO variant with improved performance in brain tissues, it might be necessary to combine bacteria-based high-throughput directed evolution with secondary neuron- or slice-based assessments to identify those variants that best retain their performance in brain tissues.

To date, the concentration and dynamics of extracellular L-lactate in brain tissue has typically been assessed using inserted enzyme-mediated electrodes14,29. Relative to electrodes, the inherent advantages of eLACCO1.1 are that it can be non-invasively introduced in the form of its corresponding gene, and it enables spatially-resolved imaging of L-lactate concentration dynamics. In addition, the targeted expression of eLACCO1.1 in specific cell types (e.g., astrocytes or neurons), will enable researchers to accurately determine which cell types are importing, and which are exporting, L-lactate. Accordingly, we anticipate that eLACCO1.1, and further improved variants, will play a central role in investigations of L-lactate shuttles, including the controversial ANLS hypothesis.

**Methods**

**General methods and materials.** Synthetic DNA encoding the lactate binding bacterial periplasmic protein TTHA0766 was purchased from Integrated DNA Technologies. Phusion high-fidelity DNA polymerase (Thermo Fisher Scientific) was used for routine polymerase chain reaction (PCR) amplifications, and Taq DNA polymerase (New England Biolabs) was used for error-prone PCR. The QuikChange mutagenesis kit (Agilent Technologies) was used for site-directed mutagenesis. Restriction endonucleases, rapid DNA ligation kits and GeneJET miniprep kits were purchased from Thermo Fisher Scientific. PCR products and products of restriction digests were purified using agarose gel electrophoresis and the GeneJET gel extraction kit (Thermo Fisher Scientific). DNA sequences were analyzed by DNA sequence service of the University of Alberta Molecular Biology Service Unit and Fasmac Co., Ltd. Fluorescence excitation and emission spectra were recorded on Saffire2 and Spark plate readers (Tecan).

**Engineering of eLACCO1.1.** The gene encoding cpGFP with N- and C-terminal linkers (LV and NP, respectively) was amplified using iGluSnFR gene as template, followed by insertion into each site of TTHA0766 lactate binding protein in a pBAD vector (Life Technologies) by Gibson assembly (New England Biolabs). Variants were expressed in E. coli strain DH10B (Thermo Fisher Scientific) in LB media supplemented with 100 μg mL−1 ampicillin and 0.02% L-arabinose. Proteins were extracted using B-PER bacterial protein extraction reagent (Thermo Fisher Scientific) and tested for fluorescence brightness and lactate-dependent response. The most promising variant, designated as eLACCO1.1, was subjected to an iterative process of library generation and screening in E. coli. Libraries were generated by site-directed mutagenesis using QuikChange (Agilent Technologies) or error-prone PCR of the whole gene. For each round, ~100–200 fluorescent colonies were picked, cultured and tested on 384-well plates under a plate reader. There were 9 rounds of screening before eLACCO1 was identified. Finally, the Tyr80Phe mutation was added to eLACCO1 to tune the lactate affinity using Q5 high-fidelity DNA polymerase (New England Biolabs). The resulting mutant was designated as eLACCO1.1.
Protein purification and in vitro characterization. The gene encoding eLACCO1.1, with a poly-histidine tag on the N-terminus, was expressed from the pBAD-HisB plasmid, lysed with a cell disruptor at 15,000 g for 30 min, and proteins were purified by Ni-NTA affinity chromatography (Agarose Bead Technologies). Absorption spectra of the samples were collected with a Lambda950 Spectrophotometer (PerkinElmer). To perform pH titrations, protein solutions were diluted into buffers (pH from 2 to 11) containing 10 mM Tris, 10 mM KCl, 10 mM CaCl2, and either no L-lactate or 10 mM L-lactate. Fluorescence intensities as a function of pH were then fitted by a sigmoidal binding function to determine the pK_a. For lactate titration, buffers were prepared by mixing a CaCl_2 (-) buffer (30 mM MOPS, 100 mM KCl, 1 mM CaCl_2, 100 mM L-lactate, pH 7.2) to provide L-lactate concentrations ranging from 0 to 100 mM at 25°C. Fluorescence intensities were plotted against L-lactate concentrations and fitted by a sigmoidal binding function to determine the Hill coefficient and apparent K_a. For CaCl_2 titration, buffers were prepared by mixing a CaCl_2 (-) buffer (30 mM MOPS, 100 mM KCl, 1 mM CaCl_2, 100 mM L-lactate, pH 7.2) and a CaCl_2 (+) buffer (30 mM MOPS, 100 mM KCl, 10 mM CaCl_2, 100 mM L-lactate, pH 7.2) to provide Ca_2+ concentrations ranging from 0 to 39 μM at 25°C. Rapid kinetic measurements for the interaction of eLACCO1 or eLACCO1.1 with L-lactate were made using an Applied Photophysics SX50 stopped-flow Reaction Analyzer. Fluorescence was detected by exciting at 488 nm with 2 nm bandwidth and collecting emitted light at 520 nm through a 10 nm path at room temperature. The dead time of the instrument is 1.1 ms. For K_a, 2 μM of purified protein sample saturated with 200 mM L-lactate and 1 mM CaCl_2 was dissociated 1:1 with 100 mM EGTA at room temperature. Graphpad Prism was used to fit a single exponential function to the dataset. For K_a, we performed time course measurements with a dead time of 1 s, so a baseline fluorescence in the saturated state was obtained as a negative control. All measurements were done in triplicates, and error ± represents the s.e.m.

To collect the two-photon absorption spectra, the tunable femtosecond laser (Jena LaserPhotonics, Hamamatsu City, Japan) by selecting an integral range ~ 8 nm to the left of the two wavelengths and averaged. To normalize to the total two-photon cross section, the two-photon absorption spectra were normalized to the fluorescence quantum yield at the registration wavelength. To normalize to the total two-photon cross section, a sigmoidal binding function to determine the Hill coefficient and apparent K_a was used with 100 mM EGTA at room temperature. Graphpad Prism was used to determine the fluorescence quantum yield at the registration wavelength. The purified eLACCO1 protein was further applied on Superdex 200pg (GE healthcare) size exclusion chromatography and the buffer was exchanged to TBS supplemented with 1 mM CaCl_2. The fractions containing the eLACCO1 protein were pooled and concentrated to ~20 mg mL^-1, and then incubated with 2 μM L-lactate for the crystallization trial. Initial crystallization of the eLACCO1 protein was set up using 384-well plate via sitting drop vapor diffusion against commercially available kits at room temperature. The eLACCO1 crystal used for the data collection was grown in 0.2 mM ammonium citrate dibasic and 20% w/v PEG 3350 by mixing 0.6 μL of reservoir solution with 0.6 μL of protein sample. The eLACCO1 crystals grew for 3–5 days and were cryoprotected with reservoir supplemented with 25% glycerol, and then frozen in liquid nitrogen. The crystals were collected at K = 0 of the x-ray Source beamline 23ID. The x-ray diffraction data were processed and scaled with XDS39. Data collection details and statistics were summarized in Table S1. The crystal structure of eLACCO1 was solved by molecular replacement approach implemented in Phaser program embedded in Phenix program package40, using the GFP structure (PDB 3860) and the lactate binding protein (PDB 2ZZV) as search models41-43. One molecule of L-lactate and one Ca_2+ were present in the asymmetric unit. The structure of eLACCO1 was refined against the missing residues and the structure was iteratively rebuilt in COOT and refined with Phenix program package44,45. The final model included the most of the residues except the N-terminal affinity tag and the glycine-rich linker to connect the original N- and C- termini of GFP. The final eLACCO1 structure contained one molecule of L-lactate and oneCa_2+ molecule of 2.5 mM concentration. The final R_free/R_ref were determined to 22.5% and refined to a final R_free/R_ref of 0.1484/0.1871 with high quality of stereochemistry. By generating symmetry mates, the eLACCO1 packed as a dimer in the crystal packing and shared a similar dimerization interface with TTHA0766 lactate binding protein (PDB 2ZZV).

Construction of mammalian expression vectors. For cell surface expression, the genes encoding eLACCO1.1, eLACCO, cpGFP, EGFP, and Laco(1) (a gift from Michael Brenner) for AAV production.

The X-ray crystallography. For crystallization, eLACCO1 cloned in pBAD-HisB with N-terminus 6×His tagged was expressed in E. coli DHB10 strain. Briefly, a single colony from freshly transformed E. coli was inoculated into 500 mL of modified Terrific Broth (2% Luria-Bertani broth supplemented with additional 1.4% tryptone, 0.7% yeast extract, and a cell K2PO4, 16 mM K2PO4, 0.8% glycerol, 0.2% (NH4)2SO4, and ampicillin sodium salt) and incubated by shaking at 37°C and 220 rpm for 8 h to reach exponential growth phase. Then, 1-aminobenzoic acid was added to 200 ppm to induce expression for another 48 h at 37°C and 250 rpm. Bacteria were then harvested and lysed by a sonicator (Qsonica) for 4 cycles of 150 s sonication with 2 s off between each 1 s sonication at 50 W power. After centrifugation at 13,000 g, the supernatant was purified by Ni-NTA affinity agarose bead (G-Biosciences) and eluted into PBS containing 250 mM imidazol. The eluted sample was further concentrated and desalted with an Amicon Ultra-15 Centrifugal Filter Device (Merck). The purified eLACCO1 protein was further applied on Superdex 200pg (GE healthcare) size exclusion chromatography and the buffer was exchanged to TBS supplemented with 1 mM CaCl_2. The fractions containing the eLACCO1 protein were pooled and concentrated to ~20 mg mL^-1, and then incubated with 2 μM L-lactate for the crystallization trial. Initial crystallization of the eLACCO1 protein was set up using 384-well plate via sitting drop vapor diffusion against commercially available kits at room temperature. The eLACCO1 crystal used for the data collection was grown in 0.2 mM ammonium citrate dibasic and 20% w/v PEG 3350 by mixing 0.6 μL of reservoir solution with 0.6 μL of protein sample. The eLACCO1 crystals grew for 3–5 days and were cryoprotected with reservoir supplemented with 25% glycerol, and then frozen in liquid nitrogen. The crystals were collected at K = 0 of the x-ray Source beamline 23ID. The x-ray diffraction data were processed and scaled with XDS39. Data collection details and statistics were summarized in Table S1. The crystal structure of eLACCO1 was solved by molecular replacement approach implemented in Phaser program embedded in Phenix program package40, using the GFP structure (PDB 3860) and the lactate binding protein (PDB 2ZZV) as search models41-43. One molecule of L-lactate and oneCa_2+ were present in the asymmetric unit. The structure of eLACCO1 was refined against the missing residues and the structure was iteratively rebuilt in COOT and refined with Phenix program package44,45. The final model included the most of the residues except the N-terminal affinity tag and the glycine-rich linker to connect the original N- and C- termini of GFP. The final eLACCO1 structure contained one molecule of L-lactate and oneCa_2+ molecule of 2.5 mM concentration. The final R_free/R_ref were determined to 22.5% and refined to a final R_free/R_ref of 0.1484/0.1871 with high quality of stereochemistry. By generating symmetry mates, the eLACCO1 packed as a dimer in the crystal packing and shared a similar dimerization interface with TTHA0766 lactate binding protein (PDB 2ZZV).

Imaging of eLACCO1 in HeLa, HEK293, and T98G cell lines. HeLa and HEK293FT cells were maintained in Dulbecco’s modified Eagle Medium (Nakalai Tesque) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) and 1% penicillin-streptomycin (Nakalai Tesque) at 37°C and 5% CO_2. T98G cells were cultured in DMEM/F12 (Nakalai Tesque) supplemented with 10% FBS, 1% penicillin-streptomycin, 1% non-essential amino acid (Nakalai Tesque) and 1 mM sodium pyruvate (Nakalai Tesque) at 37°C and 5% CO_2. Cells were seeded in 35-mm glass-bottom cell-culture dishes (Iwaki) and transiently transfected with the constructed plasmid using polyethyleneimine (Polysciences). Transfected cells were imaged using a 100X wide-field fluorescence microscope (Olympus) equipped with a PE-300 LED light source (CoolLED), a x40 objective lens (numerical aperture (NA) = 1.3; oil), an ImageEM X2 EM-CCD camera (Hamamatsu), Cellens software (Olympus) and a STR stage incubator (Tokai Hit). The filter sets used in live cell imaging had the following specification. eLACCO1.1, data set 1: Excitation 437/10 nm, emission 518/45 nm; eLACCO1, data set 2: Excitation 470/20 nm, emission 518/45 nm; and eLACCO1, data set 3: Excitation 545/20 nm, emission 518/45 nm; mCherry: excitation 545/20 nm, emission 561/45 nm; and cpGFP: excitation 470/20 nm, emission 518/45 nm.

NATURE COMMUNICATIONS | (2021) 12:7058 | https://doi.org/10.1038/s41467-021-27332-2 | www.nature.com/naturecommunications

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https://doi.org/10.1038/s41467-021-27332-2

9

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Fluorescence images were acquired with ImageX software (National Institutes of Health).

For imaging of lactate-dependent fluorescence, HeLa cells transfected with eLACCO1.1, deLACCO, or eLacO were washed twice with Hank’s balanced salt solution (HBSS; Nakalai Tesque) and then 2 mL of HBSS supplemented with 1 mM MgCl2, 5.6 mM D-glucose) supplemented with 20 mM HEPES and 10 mM L-glutamine was added to the imaging in the presence of DMSO (Wako), 1 μM AR-1558858 (Wako), 100 μM phloretin (Tokyo Chemical Industry), or 1 μM NCI-737 (a gift from Leonard Neckers). The fluorescence images were recorded for 2 h with the interval time of 15 min at 37 °C with time zero defined as the time point immediately preceding medium addition.

For imaging of Ca2+-dependent fluorescence, HeLa cells seeded onto coverslips were transfected with eLACCO1.1. Forty-eight hours after transfection, the coverslips were transferred into Attofluor cell Chamber (Thermo Fisher Scientific, Cat. #A7816) with modified Locke buffer (154 mM NaCl, 5.6 mM KCl, 10 mM MgCl2, 5.6 mM Mg(OH)2 solution) supplemented with 20 mM HEPES and 10 mM L-glutamate. Other bath solutions were supplemented with Ca2+ of 0, 0.05, 0.1, 0.5, 1, and 2 mM. Rapid change of bath solutions during the imaging was performed in a remove-and-add manner using a homemade solution remover.

For in situ pH titration, HeLa cells seeded onto coverslips were co-transfected with pEAMExt-eLACCO1.1 or deLACCO. Forty-eight hours after transfection, the coverslips were transferred into Attofluor cell Chamber with HBSS supplemented with 20 mM HEPES (Gibco, Cat. #15630130) and 10 mM 2-deoxy-D-glucose (Sigma-Aldrich Cat. #D8375-1G) and subsequently adjusted to their respective pH values. Rapid change of bath solutions during the imaging was performed in a remove-and-add manner using a homemade solution remover.

Cells are imaged on a Nikon Eclipse Ti-E epifluorescence microscope equipped with a 488 nm argon laser and a 543 nm He-Ne laser focused on the back aperture of a x60 oil total internal reflection fluorescence (TIRF) objective lens (NA 1.49, Nikon). TIRF setup was achieved by a Ti-TIRF-E Motorized Illuminator Unit (Nikon) to reduce the fluorescence intensity and to allow for camera offset. Reported values of ΔF/F were calculated as (sum of neurite pixel intensities SAT)/(sum of neurite pixel intensities APO) – 1.

Two-photon microscopy of eLACCO1 in acute cortical brain slice. Brain slices were imaged using a custom built two-photon microscope fed by a Ti:Sapphire laser source (Coherent Ultra II, ~4 W average output at 800 nm, ~80 MHz). Image data were acquired using MatLab (2013) running the open source scanning microscope control software ScanImage (version 3.81, HHMI/Janelia Research Campus). Imaging was performed at an excitation wavelength of 940 nm. The microscope was equipped with a primary dichroic mirror at 695 nm and green and red fluorescence was split and filtered using a secondary dichroic at 560 nm and two bandpass emission filters: 525–40 nm and 605–70 nm (Chroma Technologies). Time series images were acquired at 0.98 Hz with a pixel density of 512 by 512 and a field of view size of ~292 μm. Imaging was performed at room temperature. Brain slices were superfused with L-lactate (Sigma Aldrich) at concentrations of 1, 2.5, and 10 mM. ΔF/F was calculated by: ΔF/F = (Fb/F0), where Fb is the peak fluorescence intensity and F0 is the baseline fluorescence intensity. Regions of interest were selected based on identifying line processes via SR-101 fluorescence that did not shift focal plane during the duration of imaging.

Animal care. For experiments performed at University of Calgary, all methods for animal care and use were approved by the University of Calgary Animal Care and Use Committee and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. For experiments at HHMI Janelia Research Campus, all surgical and experimental procedures were in accordance with protocols approved by the HHMI Janelia Research Campus Institutional Animal Care and Use Committee and Institutional Biosafety Committee.

Statistics and reproducibility. All data are expressed as mean ± s.d. or mean ± s.e.m., as specified in figure legends. Box plots with and without notches are used for Figs. 4b, c, 3d, e, and 5d, respectively. In these plots, the horizontal line is the median; the top and bottom of the notch denote the 95% confidence interval of the median; the top and bottom of the box denote the 25th and 75th percentiles; the whiskers extend to the most extreme data points for the data; and the whiskers extend one standard deviation range from the mean represented as black filled circle. Sample sizes (n) are listed with each experiment.
No samples were excluded from analysis and all experiments were reproducible. In photobleaching experiments, group differences were analyzed using one-way ANOVA (SAS Pro 8). In eLACCO1.1 imaging (Fig. 3e) and brain slice experiments, the significant differences were analyzed using Student’s t-test (Graphpad Prism). Microsoft Excel software was used to plot for Figs. 1b, c, 2a, b, d, and 6c, f.

**Reporting summary** Further information on research data is available in the Nature Research Reporting Summary linked to this article.

**Data availability**
Structure coordinates of eLACCO1 have been deposited in the Protein Data Bank with a code of 7E9Y. pBAD-eLACCO1.1 (plasmid no. 167944), pBAD-deLACCO (plasmid no. 167945), pAEAXT-eLACCO1.1 (plasmid no. 167946), and pAEAXT-deLACCO (plasmid no. 167947) are available via Addgene [https://www.addgene.org/brow...e/28216216/]. pAAV-hsyn-eLACCO1.1 (plasmid no. 168788), pAAV-CAG-eLACCO1.1 (plasmid no. 168789), and pAAV-GFAP-eLACCO1.1 (plasmid no. 168790) are also available via Addgene [https://www.addgene.org/brow...e/28216308/]. Source data are provided with this paper.

**Code availability**
Custom code is available from the corresponding author, and at https://github.com/shucez/eLACCO_manuscript_TIRF_deltaF_F0.

Received: 10 June 2021; Accepted: 15 November 2021; Published online: 06 December 2021

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**Acknowledgements**

The authors thank the University of Alberta Molecular Biology Services Unit, Y. Shen, L. Zarnow, G. Nguyen, D. Qi and S. Harjo for technical support. We thank H. Yoshimura for the CD59 gene. We thank L. Neckers for the LDH inhibitor NCI-737. Work at the University of Tokyo was supported by the Japan Society for the Promotion of Science (Grants-in-Aid for Early-Career Scientists 19K15691 and Grants-in-Aid for Scientific
Research S 19H05633), Toyota Physical and Chemical Research Institute, and The Precise Measurement Technology Promotion Foundation. Work at the University of Alberta was supported by Natural Sciences and Engineering Research Council of Canada (NSERC) grant RGPIN-2018-04364 and Canadian Institutes of Health Research (CIHR) grant FS-154310. Work at Montana State University was supported by National Institutes of Health (NIH) grants U01 NS094246, U24 NS109107, and F31 NS108593. Y.W was supported by the Alberta Parkinson Society Fellowship and National Natural Science Foundation of China (Nos. 31870132 and 82072237).

Author contributions
Y.N. developed eLACCO1.1 and performed in vitro characterization and endogenous L-lactate imaging. R.S.M. and M.D. measured one-photon absorbance spectra and two-photon excitation spectra. C.M.R. and J.H. performed acute brain slice imaging. S.Z. and Y.W. worked on the crystallography of eLACCO1. S.Z. performed in situ pH titration. A.A. performed stopped-flow experiment. A.A. and K.P. performed the imaging of primary neurons and data analysis. Y.K. performed screening of leader sequence. M.-E.P produced AAV. M.J.L., K.P., J.S.B., G.R.G., and R.E.C. supervised research. Y.N. and R.E.C. wrote the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-27332-2.

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Peer review information Nature Communications thanks Juan Bolaños and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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