Cell-Specific Chemical Delivery Using a Selective Nitroreductase–Nitroaryl Pair

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Abstract: The utility of small molecules to probe or perturb biological systems is limited by the lack of cell-specificity. ‘Masking’ the activity of small molecules using a general chemical modification and ‘unmasking’ it only within target cells could overcome this limitation. To this end, we have developed a selective enzyme–substrate pair consisting of engineered variants of *E. coli* nitroreductase (NTR) and a 2-nitro-N-methylimidazolyl (NM) masking group. To discover and optimize this NTR–NM system, we synthesized a series of fluorogenic substrates containing different nitroaromatic masking groups, confirmed their stability in cells, and identified the best substrate for NTR. We then engineered the enzyme for improved activity in mammalian cells, ultimately yielding an enzyme variant (enhanced NTR, or eNTR) that possesses up to 100-fold increased activity over wild-type NTR. These improved NTR enzymes combined with the optimal NM masking group enable rapid, selective unmasking of dyes, indicators, and drugs to genetically defined populations of cells.
Small molecules are important tools for biological research. The ability to control the properties and activity of compounds through organic synthesis allows the generation of imaging agents such as fluorophores,\textsuperscript{1,2} sensors that bind analytes to measure the cellular environment,\textsuperscript{3,4} or pharmacological agents that interact with specific proteins to modulate cell-signaling pathways.\textsuperscript{5} Nevertheless, the use of small molecules is hampered by the absence of cell-specificity because most molecular probes act indiscriminately on all cells. This presents a major challenge when using these compounds in complex, multicellular organisms, which divide their physiological functions between multiple distinct and highly interacting cell types. One attractive solution to this problem is the use of selective enzyme–substrate pairs, where molecules are ‘masked’ with a disposable group that is stable to endogenous enzymes, but is rapidly removed by an exogenous enzyme that is expressed in defined subset of cells.\textsuperscript{6–8} This strategy combines the molecular specificity of small molecules with the cellular specificity of genetic manipulation.

There are two strategies for developing selective enzyme–substrate pairs for use in mammalian cells. The first approach involves making an existing delivery strategy cell-selective; we accomplished this previously by development of a selective esterase–ester pair.\textsuperscript{8} The other strategy involves utilizing an enzyme that is orthogonal to mammalian biochemistry and identifying a small masking motif that is removed efficiently by the action of the enzyme. An attractive enzyme system for this strategy is \textit{E. coli} nitroreductase (NTR), which is a flavoenzyme that reduces nitroaryl groups using NADH as a cofactor.\textsuperscript{9} NTRs have been used to activate fluorophores\textsuperscript{10–21} and toxins\textsuperscript{22–24} in cells and \textit{in vivo}, but a general masking strategy has not been described. Here, we use fluorogenic compounds to identify, evaluate, and optimize selective nitroreductase–nitroaryl pairs that allow facile delivery of diverse small molecules to defined cells.
RESULTS AND DISCUSSION

Synthesis and reactivity of bis(nitroaryl)-fluoresceins. Despite use of NTR in different applications, the literature on NTR-activated molecules is somewhat scattered. The identification of bacterial NTR enzymes began in the 1940s with the discovery that compounds containing nitroaromatic groups could selectively kill bacterial cells; this observation led to the development of new antibiotics such as nitrofurazone. In the 1970s, genetics led to the discovery of two genes in E. coli, nfsA and nfsB (nfs = nitrofurazone sensitivity), that encoded flavoproteins capable of reducing nitroaryl groups using NADH as a cofactor in the presence of oxygen, putting them in the category of oxygen-insensitive nitroreductases. In particular, the NTR encoded by nfsB has found utility in gene-directed enzyme-prodrug therapy (GDEPT) and targeted cell ablation in zebrafish. Several different nitroaryl groups have been used to mask fluorophores or luciferins to create indicators for nitroreductases but no systematic study of the reactivity of different nitroaryl groups with NTR has been performed. To add to the confusion, many of these same nitroaromatic motifs can be activated by endogenous oxygen-sensitive nitroreductases under extreme hypoxia; the absence of molecular oxygen allows multi-electron reduction of the nitroaryl to be catalyzed by these oxygen-sensitive reductases. Although oxygen-sensitive nitroreductases are widespread in both bacteria and multicellular organisms, oxygen-insensitive nitroreductases like the NTR encoded by nfsB do not appear to be found in higher eukaryotes. All of this prior work on NTR—antibiotics, GDEPT, targeted cell ablation, and selective unmasking in hypoxic tissue—supports the orthogonality of the oxygen-insensitive NTR encoded by nfsB in mammalian cells under normoxic conditions.

To find the best general masking group for NTR encoded by nfsB, we synthesized a series of potential fluorogenic substrates based on 2',7'-difluorofluorescein (Oregon Green), a photostable...
variant of fluorescein that is pH insensitive in the physiological range. Fluorescein-based substrates are useful as fluorogenic enzyme substrates because alkylation of the phenolic oxygens locks the molecule in a nonfluorescent lactone form resulting in low background signal. Although preparation of dialkylfluoresceins is complicated by competing alkylation the ortho-carboxyl group, we circumvented this issue by using reduced “leuco” fluorescein 1 as a synthetic intermediate to prepare novel substrates in a divergent fashion (Figure 1a, Supplementary Figure S1). Alkylation of 1 by different aryl bromides followed by oxidation with DDQ led to a high yield of the desired compounds. We prepared the following substrates: bis(4-nitrobenzyl)-Oregon Green, (NBOG, 2); bis(5-nitrofuranyl)-Oregon Green, (NFOG, 3); bis(5-nitrothiophenyl)-Oregon Green, (NTOG, 4); bis(2-nitro-N-methyl imidazolyl)-Oregon Green, (NMOG, 5). All of these substrates show low visible absorption and fluorescence background due to the formation of the nonfluorescent lactone form of the fluorescein dye.

We then tested this panel of substrates to find a structure that was unmasked rapidly by NTR but was stable to the endogenous intracellular reducing environment of mammalian cells. Measurement of enzyme kinetics with purified NTR enzyme showed the nitroimidazole 5 (NMOG) was the optimal substrate \textit{in vitro} (Figure 1a,b) with a $k_{\text{cat}}/K_M$ value of $8.1 \pm 0.8 \times 10^4$ M$^{-1}$s$^{-1}$ and a $K_m$ value of $1.6 \pm 0.2$ µM. NFOG (3) and NTOG (4) showed lower reactivity with $k_{\text{cat}}/K_M = 3.0 \pm 0.5 \times 10^3$ M$^{-1}$s$^{-1}$ and $6 \times 10^2$ M$^{-1}$s$^{-1}$, respectively, and the benzyl compound, NBOG (2), was largely unreactive. The 4-nitrobenzyl mask is arguably the most common NTR activated mask in the literature, making the higher reactivity of the nitroimidazole mask particularly noteworthy. Evaluation in cells overexpressing NTR mirrored the \textit{in vitro} experiments, with 5 showing the highest performance in HEK293 cells (Figure 1c). The intracellular NMOG unmasking requires NTR expression—mock transfection and incubation with
5 gave low cellular fluorescence (Figure 1c). Based on the rapid unmasking and low background fluorescence we selected NMOG (5) and its 2-nitro-N-methyl imidazolyl (NM) masking group for subsequent experiments.

‘Macro’ NTR engineering. We undertook a macro-engineering approach to improve the activity and expression of NTR in cultured neurons, reading out the enzymatic activity in cells using NMOG (5) and the enzyme expression levels using immunofluorescence (Figure 2a, Supplementary Figure S2). Fusion of NTR to a fluorescent protein improved expression, with mCherry giving a 3.5-fold increase in expression while preserving the activity per monomer. Because NTR is likely an obligate dimer\textsuperscript{35} we then explored different tandem-dimer configurations (tdNTR) to enhance stability and overall expression. Combining fusion of mCherry and the tandem dimer (tdNTR-mCherry) further enhanced both enzymatic expression and unmasking of NMOG fluorescence, with this construct showing a 12-fold increase in activity over wild-type enzyme (Figure 2a, Supplementary Figure S2). This fusion allows further testing of the cell-specificity of the unmasking using fluorescence microscopy. A co-culture of nitroreductase-expressing (NTR+) and untransfected (NTR−) cells incubated with NMOG (5) showed the Oregon Green fluorophore (green) is unmasked only in NTR+ cells expressing the tdNTR-mCherry fusion (red); NTR− cells bearing an epitope marker (blue) do not unmask the enzyme (Figure 2b, Supplementary Figure S3).

Cell-specific delivery of a novel Ca\textsuperscript{2+} indicator. We deployed this system to target a small-molecule calcium indicator to a specific cellular population. Existing calcium indicators, such as Fluo-4 (6, Figure 3a) have a fluorinated xanthene core structure similar to Oregon Green.\textsuperscript{4} However, 6 lacks the ortho-carboxyl group that is necessary for the molecule to adopt the fully colorless, nonfluorescent lactone form. We therefore synthesized a Fluo-4 derivative with the
requisite carboxyl group, which we called “Fluo-4XL” (Fluo-4 carboxyl, 7, Figure 3a, Supplementary Figure S4). We were also curious if the introduction of an ortho-substituent on the pendant ring would improve the performance of the indicator as suggested by the patent literature.\textsuperscript{36} We compared solutions of 6 and 7 with matched absorption at 490.5 nm ($A_{490.5} = 0.095$) in buffers containing different concentrations of free Ca\textsuperscript{2+} (Figure 3b). Both indicators showed low fluorescence of the apo state ($F_0$). We found the standard Fluo-4 to exhibit modest brightness in the Ca\textsuperscript{2+}-bound state with a quantum yield ($\Phi$) value of 0.14 ($\Delta F/F_0 = 150$) and $K_d = 390$ nM (Figure 3b, Supplementary Figure S5a). The Fluo-4XL indicator (7) showed a slightly lower affinity ($K_d = 610$ nM), presumably due to the electron-withdrawing nature of the additional carboxyl group. However, Fluo-4XL exhibited a substantially brighter Ca\textsuperscript{2+}-bound state with $\Phi = 0.40$ and $\Delta F/F_0 = 270$; this high $\Delta F/F_0$ was also observed under two-photon excitation (Figure 3b, Supplementary Figure S5b).

Delivery of small molecule indicators to specific cells in a mixed population has been hampered by the requisite carboxyl groups found in most ion chelator motifs, which require masking groups to cross the cellular membrane. Calcium indicators can be targeted to subcellular regions using self-labeling tags such as the SNAP-tag,\textsuperscript{37} but such compounds still load indiscriminately into all cells in a heterogeneous population. In addition to substantially improving the indicator properties, the extra carboxyl group in Fluo-4XL (7) makes it possible to fully mask the fluorophoric portion of the indicator, allowing unmasking of the dye only in defined NTR\textsuperscript{+} cells. We therefore synthesized a derivative of 7 bearing the nitroimidazole groups on the fluorophoric portion and standard acetoxymethyl (AM) groups on the BAPTA calcium-chelator moiety (8, Fluo-4XL NM/AM, Figure 3c). This cell-permeable derivative could be targeted to specific neurons expressing NTR in a co-culture with NTR\textsuperscript{−} cells bearing a surface epitope for
detection. As with the simpler NMOG substrate (Figure 2b), we observed fluorescence (green) only in the NTR+ cells (red) with no signal in the NTR– cells (blue). The green fluorescence signal was Ca\textsuperscript{2+}-sensitive and responded to field stimulation with the ability to detect single action potentials in cultured neurons (Figure 3d-f, Supplementary Figure S6, Movie S1).

**Cell-specific delivery of a masked cAMP analog.** We next extended the NTR–NM targeting method to target the membrane-impermeant protein kinase A (PKA) activator Sp-8OH-cAMPS (9), an analog of cyclic adenosine monophosphate (cAMP).\textsuperscript{38} We synthesized a NM-masked analog of Sp-8OH-cAMPS (10, Sp-(8-ONM)-cAMPS-NM, i.e., ‘Sp-NM’, Figure 4a, Supplementary Figure S7a). Because compound 9 is polar and cell impermeant, we surmised that addition of NM groups would allow 10 to diffuse through the cellular membrane, where it would be unmasked to yield trapped compound 9. To generalize the NTR–NM pair to additional cellular types, we tested this approach in MCF-10A cells, which were virally transduced with tdNTR and NTR expression validated by unmasking of NMOG (Figure S7b). cAMP analogs activate PKA signaling in MCF-10A cells, inhibiting phosphorylation of ERK.\textsuperscript{39} MCF-10A cells expressing either NTR or an empty plasmid were treated with either 10 or vehicle (DMSO/Pluronic F-127). The masked analog Sp-NM (10) activated cAMP signaling in NTR+ MCF-10A cells (MCF10A-tdNTR) but not in NTR– cells expressing the empty plasmid and a red fluorescent protein nuclear marker (MCF10A-H2B-RFP), as measured by increased phosphorylation of PKA substrates and decreased ERK phosphorylation (Figure 4b, Supplementary Figure S7c).

To investigate cell-specific unmasking of 10 in mixed cultures, we utilized a heterogeneous wound-healing assay. MCF10A-tdNTR (NTR+) and MCF10A-H2B-RFP (NTR–) cells were seeded at confluence in two-well IBIDI cell culture inserts in order to create a cell free gap
between two distinct cellular populations (Figure 4c). The cultures were starved for 18–24 h to synchronize the cells and limit proliferation. Upon insert removal, cultures were treated with vehicle or 100 µM Sp-NM 10 for 30 minutes, stimulated with assay media containing vehicle or 100 µM compound 10, and imaged over 24 h to measure migration of each cell population. Vehicle-treated cells migrated to close the gap over 18 h regardless of NTR expression, NTR+ cells treated with Sp-NM (10) showed substantially lower cell migration, and treatment with Sp-NM 9 did not inhibit migration in NTR– cells (Figure 4d and Movie S2). The difference in cell migration was particularly distinct in co-cultures of NTR+ and NTR– cells. The masked cAMP analog Sp-NM 10 inhibited migration specifically in the NTR+ population (Figure 4d, bottom panel, red nuclei), while NTR– (green nuclei) migrated freely.

‘Micro’ NTR engineering. The initial NTR fusion proteins showed improved expression and kinetics allowing unmasking of Fluo-4XL and Sp-8OH-cAMPS in specific cells. Both these compounds are cell impermeant once unmasked, however, allowing the use of relatively long incubation times. Other pharmacological agents are more cell permeable, which could necessitate a further improvement in enzyme kinetics. We thus engaged in a ‘micro’ engineering approach using directed evolution near the NTR enzyme active site. We used degenerate codons to mutagenize nitroreductase at seven active site positions and used NMOG to read out enzymatic activity in E. coli lysates in a high-throughput fluorescence format using a microplate reader (Figure 5a). Improved variants were then overexpressed, purified, and assayed to determine kinetic parameters (Figure 5b, Supplementary Figure S8a). We found three hotspots for improving the enzyme: positions 68, 70, and 124. Tryptophan mutations at positions Y68 and F124 resulted in significant improvements in $k_{cat}$ value and position F70 could accept the most diversity with six variants showing improved enzyme kinetics. Incorporation of these mutations
into the tdNTR-mCherry construct revealed that a single point mutant, F124W, showed the largest enhancement of $k_{\text{cat}}$ in vitro (Figure 5b) and in neurons (Figure 2a, Supplementary Figure S8b,c). Collectively, the ‘macro’ and ‘micro’-engineering efforts resulted in a tdNTR(F124W)-mCherry (enhanced NTR, or eNTR) that shows ~100-fold improvement in cellular activity (expression $\times$ kinetics) over the wild-type NTR enzyme (Figure 2a).

**Cell-specific delivery of NMDAR antagonists.** We then used this new eNTR enzyme variant to target an antagonist of the NMDA receptor (NMDAR) based on MK-801 (11), which has a binding pocket that has been mapped on the interior face of the NMDAR protein.\(^{41}\) Acylation of MK-801 abolishes pharmacological activity and intracellular targeting of MK-801 has recently been accomplished in acute brain slice using our previously reported selective esterase–ester pair system.\(^{42}\) We first synthesized nitroimidazole-masked MK-801, compound 12 (Figure 5c, Supplementary Figure S9a), which released MK-801 (11) in a rapid reaction catalyzed by NTR that was enhanced by the F124W mutation (Figure S9b). Using a short incubation with compound 12 (10 min), we observed potent inhibition of NMDAR function measured in a mono-culture of NTR+ neurons, and no inhibition in mono-cultured NTR– cells (Figure 5d, green and magenta). However, experiments using a co-culture NTR+ and NTR– showed no differential effect on the two cell types (Figure 5d, black). MK-801 (11) is cell permeable (logD = +1.8) and our results suggest that NTR releases sufficient MK-801 from NTR+ cells to block NMDA receptors in all cell types; this “bystander effect” is observed even with very sparse expression of NTR in a mixed culture (Supplementary Figure S10).

To remedy this bystander effect, we examined the medicinal chemistry literature\(^{43}\) to find a derivative of MK-801 with lower membrane permeability. A hydroxylated derivative of MK-801 (MK-801-OH, 13) shows similar binding to NMDAR as the parent MK-801 (11) in membrane
preparations ($K_i$ of 11/13 = 56 nM/260 nM), but was expected to show substantially decreased potency in intact slice experiments based on similar hydroxylated MK-801 analogs.\textsuperscript{43} The disparity between the membrane and slice assay suggested that introduction of extra hydroxyl groups on MK-801 decreases membrane permeability, as the pharmacological agents only have to cross membranes in the slice experiments. We synthesized the NM-masked derivative of MK-801-OH, compound 14 (Figure 5e). Repeating the neuronal cellular experiments with compound 14 showed a clear delineation in the NMDAR activity in NTR+ and NTR– cells, even in co-culture experiments (Figure 5f).

**CONCLUSION**

Small molecules are important tools for biology, yet their application to complex systems is often limited by indiscriminate delivery of chemical probes to all cells. The combination of molecular genetics and synthetic organic chemistry promises the ability to selectively measure and manipulate specific cell types in complex physiological environments using small molecules. Here, we provide a systematic evaluation of nitroaryl groups as substrates for the *E. coli* NTR enzyme encoded by *nfsB* (Figure 1), engineer the enzyme for improved activity against the best nitroimidazole mask (Figure 2, Figure 5), show the first enzyme-based cell-specific targeting of a small-molecule calcium ion indicator (Figure 3) and cAMP analog (Figure 4), and investigate how membrane-permeability can alter the bystander effect (Figure 5). This NTR–NM pair displays generality, allowing the masking of a diverse array of molecules and functional groups ranging from a relatively large (780.6 g/mol) Ca\textsuperscript{2+} indicator masked via an ether bond (Figure 3c), to a small (221.3 g/mol) NMDAR antagonist masked via a carbamate linkage (Figure 5). These results also further validate our approach of using fluorogenic enzyme substrates to identify and optimize selective enzyme–substrate pairs.
The prospect of cell-specific pharmacology is particularly interesting as biological investigations move increasingly from monolithic populations of cells in a dish to more complex mixtures in culture, in tissue, or in vivo. The selective enzyme–substrate approach compliments approaches that tether drugs to the cell surface, allowing pharmacological manipulation of targets inside cells. Importantly, our data reveals some additional design principles for cell-type specific pharmacology approaches, as many standard drugs that work on intracellular targets might be too permeable and may alter nearby cells through the bystander effect. This problem can be solved in many ways depending on the nature of the system under investigation; we utilized highly polar compounds (such as cAMP analog 10), and revisited the drug discovery literature to find pharmacological agents (e.g., 13) that exhibit high potency in homogeneous assays with partially purified protein but low potency in cell-based assays. Other solutions to this problem could include using covalent inhibitors to allow much lower compound concentrations, or perfusing the sample to increase the dilution of drugs that escape target cells. Our engineered NTR–NM system can thus be readily applied to release a wide variety of masked molecules to interrogate diverse biological systems.

**METHODS**

**Substrate comparison.** The initial velocity of fluorescence release ($\lambda_{ex} = 490 \text{ nm, } \lambda_{em} = 520 \text{ nm}$) of substrates 2–5 (10 µM to 0.078 µM, except for compound 2 where the highest concentration was 5 µM due to solubility) upon addition of 1 µg/mL nitroreductase (Sigma) was measured in triplicate on a FlexStation 3 platereader (Molecular Devices) in 10 mM HEPES pH 7.3, 1% DMSO, 100 µM NADH, 100 nM FMN. Data were fit using GraphPad Prism software to the Michaelis–Menten equation to extract kinetic parameters.
Vector construction. The *E. coli* nitroreductase (gene *nfsB*) sequence was used in nitroreductase vectors. See SI for full vector construction methods.

**HEK293 experiments.** HEK293T/17 cells were transfected with pCAG-NfsB-IRES-NLSmCherry using Lonza Nucleofection following manufacturer’s protocols. At 24 hours, substrates (11 µM) were added in Tyrode’s media with 0.1% DMSO and 0.02% pluronic-F127 (Sigma) and fluorescence was read as above.

**Neuronal assays of Fluo-4XL:** Dissociated hippocampal neurons were separately nucleofected with either SYN-tdNfsB-mCherry or a control construct bearing an extracellular HA epitope tag. Nucleofected cells were combined to generate mixed co-cultures of NTR+ (mCherry-positive) and NTR– (surface HA positive) cells. Experiments were performed two weeks later. NTR– neurons were labeled live via immunofluorescence by incubating coverslips in primary mouse anti-HA (1:1000, Covance) followed by secondary Alexa 647 anti-mouse (1:1000, Molecular Probes) antibodies (30 min each, in NbActiv4 media; 37 °C in the incubator). A 10 mM stock of Fluo-4XL NM/AM was prepared in fresh DMSO containing 20% pluronic F-127, and dissolved 1:1000 in NbActiv media (10 µM Fluo-4XL NM/AM final). Cells were incubated in this solution for 4 hours at 37 °C in the incubator. Cells were then rinsed 2× in a physiological buffer, and placed in a glass-bottom 24-well plate. Each well contained 500 µL of a physiological solution (in mM): 150 NaCl, 4 KCl, 2 MgCl₂, 2 CaCl₂, 10 HEPES, 10 glucose, pH 7.4, with 10 µM NBQX (to block AMPAR transmission), 10 µM CPP (to block NMDAR transmission), and 10 µM gabazine (to block GABA<sub>A</sub> transmission). Plates were transferred to an Olympus IX81 with 10X (0.4 NA) air objective lens, Cairn OptoLED illumination system, and a high-speed Andor Technology EMCCD camera (DU860_BV, 128×128 resolution, 500 frames/s, 1000 electron multiplying gain, 1× pre-amp gain, –60°C). A Grass S48 Stimulator (Grass Technologies) was
used for field stimulation, as previously described. Analysis was performed in custom MATLAB scripts.

**Western blotting.** MCF-10A cells were seeded at 2.5 × 10⁴ cells/well in a 48-well plate and cultured for 24 h. Near confluent cells were starved in non-supplemented DMEM/F12 Media overnight and subsequently treated with Sp-NM 10 in DMSO mixed with an equivalent volume of Pluronic (20% solution in DMSO) for 30 minutes. To measure phosho-ERK inhibition, cells were then stimulated with 20 ng/mL EGF for five min and lysed immediately in 1× lysis buffer (Cell Signaling Technologies) supplemented with protease and phosphatase inhibitors (Roche), diluted with sample buffer (BioRad) and analyzed by gel electrophoresis and Western blotting. To measure PKA activation, cells were lysed immediately after treatment with Sp-NM 10, and lysates were analyzed by gel and Western blot.

**MCF-10A Migration Assay:** Cells were plated in IBIDI μ-well 2-well inserts (IBIDI, Germany) at 10⁵ cells/cm². Cells were allowed to adhere for 6-10 h and then starved in non-supplemented DMEM/F12 media overnight. Inserts were removed to create a 500 μm cell free gap, and cells were treated with 100 μM Sp-NM 10 for 30 min. Migration was stimulated with the addition of DMEM/F12 media supplemented with 20 ng/mL EGF, 10 mg/mL insulin, and 2% horse serum. Wells were immediately imaged on an inverted microscope (Axiovert 200M; Zeiss, Germany) at 10× for 24 h at 30 min increments. Images were analyzed using Image J using a macro to quantify cell area and gap area (Supplementary Information).

**Screening of NTR variants.** Mutations were introduced into pRSET-NfsB at individual amino acids using degenerate NNS codons at positions S40, T41, Y68, F70, N71, G120, and F124 using Quikchange Multi Lightning (Agilent). For each site, 144 (probability 88% for full coverage) colonies in BL21Gold(DE3) (Agilent) were picked for further analysis.
Overexpression lysates were diluted 1:500 into PBS, and the reaction was initiated via addition of 0.2 µM NMOG (5) with 100 µM NADH (final concentrations) by a liquid handling platereader (Hamamatsu FDSS, green filter set). All colonies with rates of release of fluorescence greater than three standard deviations above wild-type NTR controls, as well as those with rates just under that threshold, were restreaked, reassayed, and sequenced.

**Masked MK-801 in cultured neurons.** Dissociated hippocampal neurons were separately nucleofected with either SYN-tdNfsB(F124W)-mCherry (NTR+) or a control construct bearing an extracellular HA epitope tag (NTR–). Nucleofected cells were combined to generate mixed co-cultures of NTR+ (mCherry-positive) and NTR– (surface HA positive) cells. Experiments were performed two weeks later. NTR– neurons were first labeled live via immunofluorescence by 30 min incubation in NbActiv media containing a primary mouse anti-HA (1:1000, Covance), followed by 30 min incubation in NbActiv media containing a secondary Alexa 647 antibody along with 3 µM Fluo2-AM-LR calcium dye (TefLabs), all at 37 °C. Masked drug-containing NbActiv media was transferred to 24-well plates, and warmed for 30 minutes in a 37 °C incubator during the second round of antibody staining. Neurons were incubated in drug-containing media for 10 minutes at 37 °C, then transferred to a glass-bottom 24-well plate containing 500 µL of a Mg-free resting solution consisting of (in mM): 150 NaCl, 4 KCl, 4 CaCl₂, 10 HEPES, 10 glucose, pH 7.4, with 10 µM NBQX (to block AMPAR transmission), 1 µM TTX (to block action potentials), and 10 µM gabazine (to block GABAₐ transmission). For each coverslip, one field of view was selected and imaged on an inverted IX81 microscope in multi-channel time-lapse imaging mode: TexRd (mCherry), Cy5 (surface HA epitope), and FITC (Fluo dye) channels were imaged once every 6 seconds. Following 30 seconds of baseline imaging, 1000 µL of a stimulation solution was added to produce a final concentration of 50 µM
NMDA + 50 µM glycine. Cells were imaged for 2 minutes. To minimize batch effects, each dose of each drugs was assayed consistently for 8 weeks of independent cultures. Cells were manually segmented and analyzed in custom MATLAB scripts.

**Bystander effect assay.** Mixed co-cultures of NTR+ and NTR– dissociated hippocampal neurons, prepared as above, were incubated with Fura 2 AM/LR, followed by NM-MK-801 (12) for 10 minutes. Assay was performed by addition of 50 µM NMDA and 50 µM glycine in 0 mM Mg²⁺, 4 mM Ca²⁺ Tyrode’s media.

For further information on synthetic methods, vector construction, culture methods, imaging assays, screening assays and other protocols, see SI Materials and Methods.

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**Competing Financial Interests**

The authors declare no competing financial interests.
Figure Legends

Figure 1. Identification of NM as an optimal NTR-reactive mask. (a) Synthesis and apparent kinetic parameters \((n = 3, \text{ mean } \pm \text{ SEM})\) of fluorogenic nitroreductase substrates 2–5 from leuco-fluorescein. (b) Michaelis-Menten plots of enzyme kinetics using substrates 2–5 with wild-type NTR \((n = 3, \text{ mean } \pm \text{ SEM})\). (c) Fluorescence release of fluorogenic substrates \((11 \mu \text{M})\) in HEK293 cells transfected with NTR \((n=3, \text{ dashed lines indicate SEM})\). Fluorescence from NBOG (2) signal is indistinguishable from mock transfected cells. 5* denotes incubation of NMOG (5) with mock transfected cells.

Figure 2. Engineering of NTR. (a) Improved activity of optimized NTR constructs, as measured by fluorescence release in cultured neurons transfected with NTR. Data represents analysis of >300 cells from each of 3 or more independent cultures \((\text{mean } \pm \text{ SEM})\). (b) Dye release from NMOG (5) within cultured neurons \((\text{green: released fluorescent dye; red: mCherry signal from tdNTR-mCherry transfected neurons; blue: antibody-labeled HA epitope tag marking NTR negative neurons; scale bar } = 100 \mu \text{m}}\).

Figure 3. Synthesis and targeted delivery of Fluo-4XL. (a) Structure of Fluo-4 and Fluo-4XL. (b) Comparison of the fluorescence emission spectra of \(\text{Ca}^{2+}\) indicators using samples with matched absorption. (c) Chemical structure of Fluo-4XL NM/AM (8). (d–f) Cell-specific loading of Fluo-4XL NM/AM (8) \((4 \text{ h})\) in dissociated hippocampal neurons and resulting fluorescence signal traces upon field stimulation \((\text{AP: action potential, } \Delta F: \text{maximum change in raw fluorescence values, } \text{“mCherry:Cy5 ratio”: mCherry signal divided by Cy5 signal from HA tag, } \Delta F/F_0: \text{ΔF divided by initial fluorescence (}F_0))\). Data represents 8 coverslips containing a total of 85 cells, with 42 designated NTR+ \((\text{mCherry:Cy5 } \geq 2.0)\) and 38 designated NTR– \((\text{mCherry:Cy5 } \leq 0.8)\). (d) Fluorescence change \((\Delta F)\) as a function of mCherry: Cy5 ratio. (e) Fluorescence traces upon field stimulation over time \((\text{mean } \pm \text{ SEM, gradient from black } = 160 \text{ AP to lightest gray } = 1 \text{ AP}, \text{ bottom trace } = \text{signal from NTR– cells})\). (f) Change in fluorescence over initial fluorescence \((\Delta F/ F_0)\) upon field stimulation \((\text{mean } \pm \text{ SEM})\). See also Movie S1, and Figure S6 for additional time points.

Figure 4: The masked cAMP analog Sp-NM 10 activates PKA signaling in a cell specific manner. (a) Structure and activation of masked cAMP analog \(\text{Sp-NM 10}\). (b) Treatment with Sp-NM 10 promotes PKA substrate phosphorylation \((\text{left})\) and inhibits ERK phosphorylation.
in NTR+ MCF-10A cells, but not in cells expressing empty plasmid (H2B-RFP). Data represent mean ± SEM of two independent experiments, each run in duplicate. Statistical significance was assessed using unpaired Student’s t test. ***P < 0.005. (c) Schematic describing a heterogeneous wound healing assay. (d) 100 µM Sp-NM 10 inhibits migration in homogeneous NTR+ cells (middle, right panel) but not homogeneous MCF-10A cells expressing the empty plasmid (top, right panel) over the period of 12 hours. In a heterogeneous culture of NTR+ and NTR− cells, Sp-NM 10 inhibits migration only in NTR+ (red nuclei) population (bottom, right panel). Yellow outlines represent cell edge at t = 0 h. Scale bar is 100 µm. (e) Quantification of cell migration in homogenous culture (top) and heterogenous culture (bottom). Data represent mean ± SEM of two independent experiments quantified at t = 12 h, each performed in triplicate, with each replicate containing 6–8 non-overlapping frames along the cell gap. Statistical significance was assessed using unpaired Student’s t test. ***P < 0.005

**Figure 5. Cell-specific delivery of MK-801 using eNTR.** (a) Active site residues40 investigated by saturation (NNS) mutagenesis (yellow: FMN cofactor; green: bound nitrofuran antibiotic. PDB 1YKI.47 (b) Fold improvement in $k_{cat}$ value (NMOG, 5) for mutations identified though high-throughput screening (n = 3, mean ± SEM). (c) Chemical structure of NM-masked MK-801 (12) and release by NTR. (d) Calcium response to masked MK-801 12 in NTR+ and NTR− co-cultured neurons (n = 8, mean ± SEM). (e) Chemical structure of NM–masked MK-801-OH (14) and release by NTR. (f) Calcium response to masked MK-801-OH 14 in NTR+ and NTR− co-cultured neurons (n = 8, mean ± SEM).

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FIGURES

Figure 1

a

\[
\begin{align*}
\text{Substrate} & \quad \text{R} & \quad k_{\text{cat}}/K_m (M^{-1} s^{-1}) & \quad K_m (\mu M) \\
2 (NBG) & \quad \text{NO}_2 & \quad 3.0 \pm 0.5 \times 10^3 & \quad 1.1 \pm 0.2 \\
3 (NFDOG) & \quad \text{NO}_2 & \quad 8.1 \pm 0.8 \times 10^3 & \quad 1.0 \pm 0.2 \\
4 (NTDOG) & \quad \text{NO}_2 & \quad \text{activity not detected} \\
5 (NMOG) & \quad \text{N} & \quad -d \times 10^2 & \quad -1
\end{align*}
\]

b

![Graph showing substrate concentration vs. reaction rate]

**Activity per monomer**

- NTR-mCherry
- 1x
- 3x
- 4.7x
- 4.7x
- NTR-mCherry
- NTR-tdNTR-(F124W)-mCherry
- 1x
- 2.6x
- 4x
- 4x
- 18x

**Relative enzyme activity**

- iNTR-mCherry
- 1x
- 3.9x
- 4.7x
- 4.7x

**Fluorescence (a.u.)**

![Graph showing fluorescence over time]

![Image of a and b]
Figure 3

6: R = H (Fluo-4)  
7: R = CO$_2$H (Fluo-4XL)  

6 (Fluo-4) $K_d = 390$ nM  
7 (Fluo-4XL) $K_d = 610$ nM  

$[\text{Ca}^{2+}]$  

$\Delta F$ (fluorescence)  

NTR expression (mCherry:Cy5 ratio)  

NTR+ cells  
NTR− cells  

Field stimulation (#AP)  

$\Delta F/F$
Figure 4

a) Chemical structures of Sp-NM (10) with NTR and H2B-RFP.

b) Graph showing % DNA act. in cell with NTR+ and H2B-RFP.

Step 1: Culture cells in insert.
Step 2: Remove insert and heat with caged molecule.
Step 3: Stimulate migration and image.

Cell type 1

Cell type 2

Vehicle

Sp-NM (10)

Vehicle

10

H2B-RFP

N.T.

***

% p-ERK

% p-ERK

**

**

Vehicle

10

H2B-RFP

N.T.

***
Figure 5

Graphical Table of Contents