In situ electrochemical generation of nitric oxide for neuronal modulation

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SUPPLEMENTARY INFORMATION
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Supplementary Information

In situ Electrochemical Generation of Nitric Oxide for Neuronal Modulation

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Supplementary Methods

Materials

FeCl₃ (99.8%, Sigma Aldrich), FeCl₂ - 4H₂O(99.8%, Sigma Aldrich), oleylamine (70%, Sigma Aldrich), sulfur (99.5 %, Alfa Aesar), potassium hexachloroplatinate (K₂PtCl₆, 99%, Sigma Aldrich), nitrosyl tetrafluoroborate (NOBF₄, 95%, Sigma Aldrich), dimethyl sulfoxide (DMSO, 99.9%, Fisher Scientific), 4-amino-5-methylamino-2',7'-difluorofluorescein, (DAF-FM, Sigma Aldrich), diethylamine NONOate diethylammonium salt (98%, Sigma Aldrich), sodium salicylate (C₆H₄(COONa)(OH), ReagentPlus®, ≥ 99.5%, Sigma Aldrich), sodium hypochlorite (NaOCl, reagent grade, 10-15% available chlorine, Sigma Aldrich), N,N-diethyl-p-phenylenediamine (DPD, 97%, Sigma Aldrich), potassium permanganate (KMnO₄, ≥ 99% Sigma Aldrich), sodium hydroxide (NaOH, Sigma Aldrich), sodium nitroprusside (Na₃[Fe(CN)₅(NO)]·2H₂O), sodium pentacyanonitrosylferrate (III) dihydrate (99-102%, Sigma Aldrich), ammonium sulfate ((NH₄)₂SO₄, 99.95%, Alfa Aesar) acetone (99.5%, VWR), toluene (99.5%, VWR), hexane (99.9%, Fisher Scientific), ethanol (Anhydrous, KOPTEC USP), and N,N-dimethylformamide (DMF, 99.8%, Sigma Aldrich) were purchased and used without additional treatment. Florine-doped tin oxide (FTO) electrodes were purchased from Delta Technologies. Tyrode's solution was composed of 125 mM sodium chloride (NaCl, ≥ 99 % Sigma Aldrich), 2 mM potassium chloride (KCl, ≥ 99 %, Sigma Aldrich), 2 mM magnesium chloride hexahydrate (MgCl₂·6H₂O, ≥ 99 %, Sigma Aldrich), 2 mM calcium chloride dihydrate (CaCl₂·2H₂O, ≥ 99%, Sigma Aldrich), 25 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES, ≥ 99.5 %, Sigma Aldrich), and 51 mM D-glucose (≥ 99.5%, Sigma Aldrich), with the pH was adjusted to 7.4.

XRD and TEM analysis

Powder X-ray diffraction (XRD) was conducted on a D-8 Advance X-ray diffractometer with Cu Kα radiation (λ = 1.54056 Å) to confirm the phase of the synthesized Fe₃S₄ nanocatalysts (Supplementary Fig. 3). For the analysis, 5 mL of precipitated ligand exchanged Fe₃S₄ nanocatalysts were dried in 60 °C oven. Transmission electron microscopy (TEM) images were obtained using a high-resolution transmission electron microscope (JEM-2100, JEOL, Japan) with an acceleration voltage of 200 kV. For the analysis, dispersed Fe₃S₄ nanocatalysts containing ethanol solution was dropped on the TEM grid and dried in an oven under 60 °C before analysis.
NH$_4^+$, H$_2$O$_2$, and H$^2$ quantification

In addition to NO, undesirable side products such as molecular hydrogen (H$_2$(g)), ammonium ions (NH$_4^+(aq)$), and hydrogen peroxide (H$_2$O$_2(aq)$) can be formed at the cathode from the following reactions (Eq. s1 to s3):$^1$ Therefore, we attempted to quantify these three side products.

\[
\text{NO}_2^-(aq) + 8H^+ + 6e^- \leftrightarrow \text{NH}_4^+(aq) + 2H_2O(aq) \quad E_0 = 0.897 \text{ V vs NHE} \quad \text{(s1)}
\]

\[
2H_2O(aq) + 2e^- \leftrightarrow H_2(g) + 2OH^- \quad E_0 = 0 \text{ V vs NHE} \quad \text{(s2)}
\]

\[
O_2(g) + 2e^- + 2H^+ \leftrightarrow H_2O_2(aq) \quad E_0 = 0.695 \text{ V vs NHE} \quad \text{(s3)}
\]

NH$_4^+$ in the Tyrode’s solution was quantified by using a salicylate method.$^2$ NH$_4^+$ in the Tyrode’s solution reacts with the reagents to generate 5-aminosalicylate and is then oxidized in the presence of sodium nitroprusside to form a greenish dye with an absorbance peak at 650 nm. First, a mixture of 2.5 M sodium salicylate and 300 µM sodium nitroprusside were prepared. A 1 % hypochlorite solution was prepared by mixing 0.4 M NaOH and 10-15 % sodium hypochlorite solution. Chronoamperometry measurements were then conducted with 2 mL of 0.1 M NaNO$_2$ containing Tyrode’s solution. 280 µl of the salicylate solution and 280 µl of 1 % hypochlorite solution were added in the electrolyzed solution right after electrolysis. The resulting solution was transferred into a plastic cuvette for UV-Vis analysis and absorbance was then analyzed by using an Ocean Optics spectrophotometer with a Flame-S-UV-Vis detector and a DH-mini-UV-Vis-NIR light source. For quantification, an external standard curve was obtained by using 0, 100, 250, 500, 750, and 1000 µM NH$_4^+$ standard solution (Supplementary Fig. 12). The Faradaic efficiency for NH$_4^+$ measured via the salicylate method in Tyrode’s solution was found in the range 10-20 % for both catalysts (Fig. 1k,l, and Supplementary Fig. 12)$^2$

In order to detect generated hydrogen gas, a one-compartment electrochemical cell was used with a two-electrode configuration. Nitrogen gas was introduced through the cathode side and then fed to an SRI Instruments Gas Chromatograph (GC). Using a TCD detector, the peak from hydrogen gas was identified. The peak area from hydrogen peaks was used to quantify hydrogen by an external calibration curve. For both nanocatalysts, negligible H$_2$ was observed via gas chromatography at measured voltage ranges, whereas significant H$_2$ was generated in the absence of NaNO$_2$, suggesting that hydrogen evolution was mostly suppressed by the NO$_2^-$ reduction reaction.
H₂O₂ in the Tyrode’s solution was quantified by using a colour assay method. H₂O₂ in the Tyrode’s solution reacts with (NH₄)₆Mo₇O₂₄·4H₂O to make a yellowish compound with a maximum absorption peak at around 350 nm. In order to get the external standard curve, we first prepared colour assay solutions containing 2.4 mM (NH₄)₆Mo₇O₂₄·4H₂O and 0.5 M H₂SO₄, and standard solutions containing 5, 12.5, and 25 µM H₂O₂. UV-Vis spectra were obtained by mixing 900 µL of assay solution and 100 µL of the prepared standard solution (Supplementary Fig. 13). No observable peaks were detected during electrolysis, indicating that the formation of H₂O₂ from molecular oxygen was not involved in the reduction reaction. Taken together, these findings support that NO was the major NO₂⁻ reduction product for both nanocatalysts.

**Cytotoxicity assay**

Fluorescent alamarBlue (Invitrogen) assays were conducted in 96 well plates (excitation: 545 nm, emission: 590 nm). For the assay involving nanocatalysts, HEK 293FT cells were incubated with 100 µg/mL of nanocatalyst in DMEM with 10% FBS. In the case of the assay with the NaNO₂ solution, cells were treated with various concentrations of NaNO₂ solution in DMEM with 10 % FBS for 2 hours each day. After each time interval, the alamarBlue reagent was incubated for 3 hours in a cell culture incubator, and then the supernatant was used for the measurement.

**NO generation methods for cGMP assay**

To investigate the effects of NO donor, 200 µL of DEA NONOate-containing Tyrode’s solution was added to the cultured neurons in Tyrode’s solution containing 200 µM 3-isobutylmethylxanthine (IBMX), and then neurons were incubated with NO donor for 5 min. The final concentration of DEA NONOate was 10 mM. For the control group, the same volume of Tyrode’s solution (200 µL) was added to the cultured neurons in Tyrode’s solution containing 200 µM IBMX. To observe the effects of electrochemically generated NO, the neurons cultured on glass coverslips were positioned in the immediate proximity of the Pt-Fe₃S₄ nanocatalyst-loaded FTO electrodes (3 × 5 × 0.2 cm³). NO was generated by applying –2.5 V vs Pt to the FTO electrodes for 5 min in Tyrode’s solution containing 5 mM NaNO₂ and 200 µM IBMX. To examine the effect of applied voltage on cGMP levels, control experiments were performed by
applying −2.5 V vs Pt to the FTO electrode for 5 min in 200 μM IBMX containing Tyrode’s solution without NaNO₂. Additionally, to investigate the effect of NaNO₂ on cGMP levels, another control experiment was carried out by incubating cultured neurons in Tyrode’s solution containing 5 mM NaNO₂ and 200 μM IBMX for 5 min.

**Immunohistochemistry analyses of c-fos expression.**

After NO stimulation or control experiments, all the mice kept in their home cages for 60 min to allow for c-fos expression. Mice were then euthanized by IP injection of Fetal Plus solution (100 mg/kg in saline) before performing transcranial perfusion with 4 % paraformaldehyde (PFA) in PBS. Extracted brains were then fixed in a 4 % PFA solution overnight at 4 °C. The fixed brains were washed with PBS three times and then sliced into 60 μm sagittal sections using a vibrating blade microtome (Leica VT1000S) with a FEATHER razor blade (Electron Microscopy Sciences, 72002). The slices were then placed in a Netwell insert (Corning, 3478) in a standard 12-well plate and incubated with 2.5 mL of the blocking buffer (0.3 % triton + 3 % normal goat serum (NGS) in PBS) for 1 hour at room temperature in the dark on an orbital shaker. The slices were then transferred to 2.5 mL of the primary antibody solution (500× dilution primary antibody + 3 % NGS in PBS) and incubated for 1 hour at room temperature in the dark on an orbital shaker. The slices were further incubated in the primary antibody solution overnight at 4°C. Following three washes with PBS, slices were transferred to the secondary antibody solution (500x dilution secondary antibody in PBS) and incubated for 3 hours at room temperature in the dark on an orbital shaker. Following another three washes with PBS, the slices were then transferred onto glass slides, and covered with mounting solution containing DAPI (Vectashield mounting medium with DAPI, H-1200, Vector Laboratories). Finally, the slides were covered with a coverglass, and sealed with nail-polish. A laser scanning confocal microscope (Fluoview FV1000, Olympus) with 20× (oil, 0.85 NA) objective was used for imaging. The c-fos quantification and cleaved caspase-3 analyses were conducted with z-stack images with a depth of 5 μm. Rabbit anti-c-fos (Cell Signaling technology, 2250s), rat anti-mCherry (Invitrogen, M-11217), and rabbit anti-cleaved caspase-3 (Cell Signaling Technology, 9661s) were used as primary antibodies. Goat anti-rabbit labeled with Alexa Fluor 488 (Invitrogen, A-27034), goat anti-rat labeled with Alexa Fluor 633 (Invitrogen, A-21094), and goat anti-rabbit labeled with Alexa Fluor 633 (Invitrogen, A-21070) were used as secondary antibodies.
**Immunohistochemistry analyses of foreign-body response.**

For evaluating the biocompatibility of the NO-delivery fibres, the fibres and stainless steel microwires (300 µm diameter, Good Fellow) were implanted into the VTA of either TRPV1⁺ mice or TRPV1⁻ mice. For TRPV1⁺ mice implanted with the fibres, NO stimulation was performed 3 days after the implantation. Tissue responses of the fibres and steel microwires were compared 7 days and 28 days after the implantation. Perfusion, staining, and imaging protocols for biocompatibility assays were identical to those used for c-fos analyses with the exception of the utilization of normal donkey serum instead of NGS for Iba1 assays. Goat anti-Iba1 (abcam, ab5076) and rabbit anti-GFAP (abcam, ab760) were used as primary antibodies. Donkey anti-goat labeled with Alexa Fluor 633 (Invitrogen, A-21082) and goat anti-rabbit labeled with Alexa Fluor 633 (Invitrogen, A-21070) were used as secondary antibodies.

**Fibre photometry analyses**

In order to establish the fibre photometry setup, a 473 nm diode laser (OEM Laser Systems, 50 mW peak power) was coupled to a ferrule rotary joint patch cable (Ø 400 µm core and Ø 1.25 mm ferrules, Thorlabs) utilizing a 20× microscope objective (0.45 NA, Olympus) integrated with the fibre launch (Thorlabs, MBT610D). The laser was then controlled by a function generator (400 Hz, square wave, Agilent) and the laser intensity out of the fibre tip was controlled to be approximately 100-200 µW. Before fibre photometry recordings, mice were anesthetized through an IP injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) mixture in saline and transferred to the photometry setup integrated with potentiostat and micropump for NO generation. Then, a fibre-optic cannula implanted into mice was connected to the photometry patch cord, which is linked to the fibre launch, with a ceramic split mating sleeve (Ø 2.5 mm, Thorlabs). The GCaMP6s fluorescence from NAc during NO generation was collected by the implanted fibre-optic cannula. The collected fluorescence was transmitted through a dichroic mirror (Thorlabs) and then filtered to avoid the remaining laser background with a longpass filter (Semrock) on a femtowatt silicon photoreceiver (NewFocus). The photoreceiver connected to a lock-in amplifier (8 ms time constant, Stanford Research Systems) was recorded by custom software written in Labview (acquisition frequency: 8.5 Hz). All the fibre photometry recordings were performed in the dark.
Supplementary Figure 1. Schlenk line setup for Fe₅S₄ nanocatalyst synthesis. a, A photograph of the Schlenk line and reaction flasks, and b-c, Photographs of reaction pots containing iron-oleylamine at 60 °C (b) and 240 °C, following the injection of sulfur solution (c). In panel (b), the left and right flasks contain sulfur and Fe-oleylamine mixtures, respectively.
Supplementary Figure 2. Ligand exchange procedure. Photographs of a, the Fe₃S₄ nanocatalysts in hexane, b, the mixture of hexane (with Fe₃S₄) and dimethylformamide (DMF), c, the mixture of hexane and DMF (with Fe₃S₄) following ligand exchange, and d, the Fe₃S₄ nanocatalysts in ethanol.
Supplementary Figure 3. X-ray diffraction spectra of Fe$_3$S$_4$ (red) and Pt-Fe$_3$S$_4$ (blue) nanocatalysts.
Supplementary Figure 4. High-resolution high-angle annular dark-field scanning transmission electron microscopy (HAADF-STEM) images (a-d) and bright-field STEM images (e-h) of the Pt-Fe₃S₄ nanocatalysts. Bright dots at the dark-field image and black dots at bright-field images are corresponding to Pt atoms. Scale bar: a,e,: 50 nm, b,f,: 5 nm, c,d,g and h,: 2 nm
Supplementary Figure 5. Cyclic voltammetry curves of iron sulfide nanocatalysts with two-electrodes (a) and three-electrodes system (b). Iron redox feature was observed at −1.9 V vs Pt and −1.0 V vs NHE. Based on the Pourbaix diagram and previous studies, the observed redox peaks could likely be attributed to the Fe$^{3+}$/Fe$^{2+}$ redox reaction$^{3-7}$. Each experiment was repeated three times independently with similar results.
Supplementary Figure 6. Ionic strength dependence. a, NO$_2^-$ concentration dependence. It was confirmed that the overall reduction current increased as added NO$_2^-$ increased, indicating NO$_2^-$ is actively involved in the reaction. The overall ionic strength was maintained at 200 mM by using a NaClO$_4$ electrolyte. b-c, The Supporting electrolyte effect is shown with NaClO$_4$ dependent cyclic voltammetry curves. (b) and chronoamperometry measurements (c). It was confirmed that conductive supporting electrolyte can also increase the reductive current, due to the lowered solution resistance. (a-c) Each experiment was repeated three times independently with similar results.
Supplementary Figure 7. pH dependence on NO$_2^-$ reduction reaction. a-b, Cyclic voltammetry curve (a) and chronoamperometry measurement (b) at different pH revealed that there was a negligible pH effect on the NO$_2^-$ reduction under near-neutral pH. These Faradaic currents were not significantly dependent on the pH of the solution, indicating that protons are unlikely to involved in the rate determining step of the overall denitrification reaction. (a-b) Each experiment was repeated three times independently with similar results.
Supplementary Figure 8. Quantification of Nitric Oxide (NO) generation. a, Electrochemical setup for NO quantification. b, Fluorescence of diethylamine NONOate (DEA NONOate) in Tyrode’s solution (excitation: 495 nm, emission: 515 nm). c, the Calibration curve for NO concentration between 10 µM to 600 µM obtained from saturated fluorescence in (b). All experiments were conducted in Tyrode’s solution. (b-c) The experiment was repeated two times independently with similar results.
Supplementary Figure 9. Faradaic efficiency toward nitric oxide from electrochemical NO$_2^-$ reduction reaction, catalysed by iron sulfide (a) and Pt decorated iron sulfide nanocatalysts (b). It was confirmed that generally Faradaic efficiency toward NO kept decreased over the electrolysis time at each potential, which might be due to the decaying process of NO. (a-b) Each experiment was repeated two times independently with similar results.
Supplementary Figure 10. Diffusion profile of electrochemically generated NO by Fe₃S₄ nanocatalysts. a-d, Voltage-dependent NO concentration distribution vs. the distance from the cathodes. e-h, Colour map images display time and distance-dependent NO distribution.
Supplementary Figure 11. Diffusion profile of electrochemically generated NO by Pt-Fe$_3$S$_4$ nanocatalysts. a-d, Voltage-dependent NO concentration distribution vs. the distance from the cathodes. e-h, Colour map images display time and distance-dependent NO distribution.
Supplementary Figure 12. NH$_4^+$ salicylate assay. a, Absorption spectra obtained for NH$_4^+$ solutions with concentrations between 0 µM and 1000 µM. b, Calibration curve obtained from absorption at 650 nm wavelength from spectra in (a). To quantify NH$_4^+$ during electrolysis, we conducted chronoamperometry measurements. c, Absorption spectra collected during electrolysis in the presence of Fe$_3$S$_4$ (red) and Pt-Fe$_3$S$_4$ (blue) nanocatalysts at –2.5 V and –2.0 V vs Pt, respectively. All experiments were conducted in Tyrode’s solution. The results are summarized in Fig. 1k and l. (a-c) Each experiment was repeated three times independently with similar results.
Supplementary Figure 13. H$_2$O$_2$ colourimetric assay. a, Absorption spectra obtained for H$_2$O$_2$ solution with concentrations between 5 µM and 25 µM. b, Calibration curve obtained from absorption at 350 nm wavelength from spectra in (a). c, Absorption spectra collected during electrolysis in the presence of Fe$_3$S$_4$ (red) and Pt-Fe$_3$S$_4$ (blue) nanocatalysts at –2.5 V and –2.0 V vs Pt, respectively. No peaks were observed at 350 nm. All experiments were conducted in Tyrode’s solution. (a-c) Each experiment was repeated three times independently with similar results.
Supplementary Figure 14. Active chlorine N,N-diethyl-p-phenylenediamine (DPD) assay. 

(a) Absorption spectra obtained for concentrations between 2.5 µM and 500 µM. The inset shows the calibration curve obtained from absorption at 515 nm. To quantify the active chlorine during electrolysis, we conducted chronoamperometry measurements.

(b-c) Voltage-dependent active chlorine Faradaic efficiency (FE) in electrolytic cells containing Fe₃S₄ (b) and the Pt-Fe₃S₄ nanocatalysts (c) at cathodes. These measurements indicate that oxygen evolution is likely the major counter-reaction. All experiments were conducted in Tyrode’s solution. (a-c) Each experiment was repeated two times independently with similar results.
Supplementary Figure 15. DEA NONOate concentration ($C_0$) dependent maximum of GCaMP6s relative normalized fluorescence intensity averaged across TRPV1$^+$ or TRPV1$^-$ HEK293FT cells (n = 300 cells for each point) during 400 s long measurements (mean ± standard error of the mean). Concentrations of DEA NONOate ranging from 10 µM to 10 mM were applied 30 s after the initiation of fluorescence measurement. $F_0$ value was calculated by averaging the fluorescent value of each cell during the initial 10 s of measurement.
Supplementary Figure 16. a-d, Times to activate the TRPV1\sup+ HEK293FT cells in the presence of Fe3S4 nanocatalysts (a, b) or Pt-Fe3S4 nanocatalysts (c, d) at various applied voltages. b and d are reproduced from a and c, respectively, to show the general trend between activation time of TRPV1\sup+ cells and their distances from the cathode. It should be noted that the frame rate of recording (1 s) in our system limited the more temporally precise evaluation, especially at more negative applied voltages. (a-d) The experiment was repeated three times independently with similar results.
Supplementary Figure 17. a-c, Effects of TRPV1 antagonist on NO-mediated Ca²⁺ influx. Individual (a, b) and averaged (c) GCaMP6s fluorescence traces for TRPV1⁺ HEK293FT cells (n = 300 cells for each trace) pre-incubated with 20 µM of TRPV1 antagonist 4-(3-Chloro-2-pyridinyl)-N-[4-(1,1-dimethylethyl)phenyl]-1-piperazinecarboxamide (BCTC), following NO delivery electrocatalysed by Fe₃S₄ at –1.75 V (a) and Pt-Fe₃S₄ at –1.5 V vs Pt (b) in the presence of 0.1 M NaNO₂.

d-f, Effects of TRPV1 expression. Individual (d, e) and averaged (f) GCaMP6s fluorescence traces for TRPV1⁻ cells (n = 300 cells for each trace) following NO delivery electrocatalysed by Fe₃S₄ at –1.75 V (d) and Pt-Fe₃S₄ at –1.5 V vs Pt (e) in the presence of 0.1 M NaNO₂.

g-i, Effects of NaNO₂. Individual (g, h) and averaged (i) GCaMP6s fluorescence traces for TRPV1⁺ cells (n = 300 cells for each trace) in the presence of Fe₃S₄ at –2.5 V (g) and Pt-Fe₃S₄ at –2.0 V vs Pt (h) without NaNO₂.

j-l, Effects of NH₄⁺. Individual (j, k) and averaged (l)
GCaMP6s fluorescence traces for TRPV1+ cells (n = 300 cells for each trace) following addition of 10 mM (j) or 1 mM (NH₄)₂SO₄ (k) containing Tyrode’s solution, respectively. The dotted lines indicate the time when voltages were applied or (NH₄)₂SO₄ solution was injected (a-l). Solid lines and shaded areas in (c, f, i, l) indicate the mean and standard error of the mean (s.e.m.), respectively. F₀ indicates the mean of the fluorescence intensity during the initial 10 s of measurement. (a, b, d, e, g, h, j, and k) The experiment was repeated three times independently with similar results.
Supplementary Figure 18. Effects of a reducing agent, ascorbate. a, Normalized GCaMP6s fluorescence averaged across 300 TRPV1+ HEK293FT cells during the first (left) and second (right) activation-and-recovery cycle. Activation of TRPV1+ cells was achieved by the NO release catalysed by Fe₃S₄ nanoclusters at –1.75 V vs Pt. When peak Ca²⁺ influx was observed, the voltages were turned off and sodium ascorbate was added into the solution to the final concentration of 33 mM (recovery). The entire reaction solution was carefully replaced with fresh Tyrode’s solution with 0.1 M NaNO₂ between the two cycles. Then, the recovered cells were re-activated at –1.75 V vs Pt. After peak Ca²⁺ influx, re-activated cells were recovered with the same method for recovering activated cells in the first cycle. b, Representative time-lapse images of Ca²⁺ responses in TRPV1+ cells during the first and second activation-and-recovery cycles in the presence of sodium ascorbate. Scale bar: 50 µm. F₀ indicates the mean of the fluorescence intensity during the initial 10 s of measurement. The experiment was repeated three times independently with similar results.
**Supplementary Figure 19.** Cyclic voltammogram of between the electrodes within the electrocatalytic fibre in 2 mM K$_2$PtCl$_6$ solution. Scan rate: 100 mV/s. The experiment was repeated three times independently with similar results.
Supplementary Figure 20. Chronoamperometry profiles of the NO-delivery fibre in the Tyrode’s solution containing 0.1 M NaNO₂. The current density for the electrodes within the electrocatalytic fibres was calculated based on the exposed surface area of the electrodes from the polymer cladding (diameter: 50 µm, and length: 300 µm) The experiments was repeated three times independently with similar results.
Supplementary Figure 21. **a-b**, Optical images of Pt-Fe₃S₄ nanocatalysts coated microwires of the NO-delivery fibre, positioned above the TRPV₁⁺ HEK293FT cells (a) or TRPV₁⁺ hippocampal neurons (b). **c-d**, Fluorescent images of TRPV₁⁺ cells (c) and neurons (d), positioned below the microwires in a and b, respectively. White dotted lines indicate the positions of microwires onto cells and neurons. **e-f**, Representative time-lapse images of Ca²⁺ responses in TRPV₁⁺ HEK293FT cells (e) and neurons (f) elicited by Pt-Fe₃S₄ electrocatalysed NO generation at −2.0 V vs Pt, indicating the localized activation of TRPV1 in the proximity of the microwires. Scale bar: 50 μm. (c-f) The experiment was repeated three times independently with similar results.
Supplementary Figure 22. Active chlorine assay on the miniature fibre electrode. a-b, Voltage-dependent active chlorine Faradaic efficiency (FE) in electrolytic cells containing Fe₃S₄ (a) and the Pt-Fe₃S₄ nanocatalysts (b) at cathodes. These measurements indicate that oxygen evolution is likely the major counter-reaction. All experiments were conducted in Tyrode’s solution. (a-b) These experiments were repeated two times independently with similar results.
Supplementary Figure 23. a, Four representative confocal images of the VTA of TRPV1+ mice implanted with NO-delivery fibres. Red fluorescence and green dots indicated the transduction area of TRPV1, which is tagged with mCherry, and anatomical locations of exposed microwires of the fibres, respectively (scale bar = 1 mm). The experiment was repeated four times independently with similar results. b, The overlay of four confocal images in a with the histological brain image (black line), which is reproduced from Paxinos and Franklin’s the Mouse Brain in Stereotaxic Coordinates (Academic Press, 2019) (scale bar = 1 mm).
Supplementary Figure 24. a-b, Representative low-magnitude (a) and high-magnitude (b) confocal images of GCaMP6s expression in VTA cell bodies (a: scale bar = 1 mm, b: scale bar = 200 μm). The experiment was repeated four times independently with similar results. c, The overlay of four confocal images with the histological brain image (black lines), which is reproduced from Paxinos and Franklin’s the Mouse Brain in Stereotaxic Coordinates (Academic Press, 2019). d-e, Representative low-magnitude (d) and high-magnitude (e) confocal images of GCaMP6s expression in VTA-NAc axons (d: scale bar = 1 mm, e: scale bar = 200 μm). The experiment was repeated three times independently with similar results.
Supplementary Figure 25. a-h. Representative confocal images of the GFAP expression near the implantation site of TRPV1⁺ mice subjected to NO stimulation and implantation of NO-delivery fibre (a,e), TRPV1⁻ mice implanted with NO-delivery fibre without NO stimulation (b,f), and TRPV1⁻ mice implanted with stainless steel wire without NO stimulation (c,g) 7 days (a-c) and 28 days (e-g) after implantation (scale bar = 100 μm). Percentages of the GFAP expressed area (mean ± s.e.m.) among the total area of the field 7 days (d) and 28 days (h) after implantation. i-p. Representative confocal images and average percentages of the Iba1 level near the implantation sites. Conditions are identical with a-h. One-way ANOVA and Tukey’s multiple comparison tests were performed for d, h, and l, and p (n = 6 mice, GFAP: 7 days - $F_{2,15} = 27.54$ p = 9.5×10⁻⁶, 28 days - $F_{2,15} = 5.75$ p = 0.014, Iba1: 7 days - $F_{2,15} = 17.83$ p = 0.00011, 28 days - $F_{2,15} = 1.69$ p = 0.22 * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001).
Supplementary Figure 26. a-b, Representative confocal images of cleaved caspase-3 positive cells (green) near the implantation site following NO generation (a) or after NaNO₂ injection without NO generation (b). Nuclear staining was performed with DAPI (blue). Scale bar: 50 µm. c, Percentages of cleaved caspase-3 positive cells among DAPI-labelled cells (mean ± s.e.m.) within 150 µm vicinity from the implantation site. No significant difference was found between the two groups, as assessed by one-tailed Student’s t-test (n = 5 animals for each group, p = 0.612).
Supplementary Figure 27. AlamarBlue assays of cell viability as a function of time in the presence of the nanocatalysts (left) or NaNO₂ solution (right) (mean ± s.e.m.). Nanocatalyst concentrations were 100 µg/mL. The cells in the control group were cultured without the nanocatalysts. No significant differences were identified (n=3 independent experiments, One-way ANOVA, Day 1: p = 0.052, F₂,₆ = 5.029, Day 2: p = 0.936, F₂,₆ = 0.067). For the NaNO₂ biocompatibility assays, cells were treated with various concentration of NaNO₂ solution in Dulbecco's modified Eagle medium (DMEM) for 2 h daily. No significant differences were observed for different NaNO₂ concentrations. (n=6, One-way ANOVA, Day 1: p = 0.411, F₃,₂₀ = 3.098, Day 2: p = 0.079, F₃,₂₀ = 2.623).
Supplementary Figure 28. Nanocatalyst physiological stability evaluation. Inductively coupled plasma - optical emission spectroscopy (ICP-OES) (PerkinElmer Optima 8000) was used to quantify the mass of iron on the implantable fibres. First, standard solutions, ranging between 1-100 ppm were prepared by diluting the ICP calibration standards (Sigma Aldrich). a, A linear fit was obtained after calibration. The experiment was repeated two times independently with similar results. b, Based on the calibration curve, the iron mass on the cathode (mean ± s.e.m.) was obtained from ICP-OES spectra. Four different groups of Pt-Fe₃S₄ loaded fibres were investigated; i) as-prepared fibres and the fibres explanted from the mouse brain ii) one-week, iii) one-month, iv) two months following implantation. (n= 4 fibres from 4 different animals for each point)
Supplementary Figure 29. a, Individual GCaMP6s fluorescence traces for 300 HEK293FT cells expressing TRPV4 (TRPV4\(^+\)) following the addition of a NO donor, DEA NONOate (10 mM), at 30 s (dashed line). b-c, Individual GCaMP6s fluorescence traces for 300 TRPV4\(^+\) cells following NO delivery electrocatalysed by the Fe\(_3\)S\(_4\) at \(-2.5\) V (b) and the Pt-Fe\(_3\)S\(_4\) at \(-2.0\) V vs Pt (c) applied at 30 s (dashed lines). Tyrode’s solution containing 0.1 M NaNO\(_2\) was used.
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