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Supporting Information

Selective and sensitive near-infrared fluorescent probe for acetylcholinesterase imaging

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1. General

All reagents and solvents were obtained from commercial suppliers (Sigma Aldrich, Acros, TCI) unless otherwise stated. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DPX 300 spectrometer (Bruker, Wissembourg, France). Chemical shifts are expressed in parts per million (ppm), and the residual solvent peak was used for calibration.[1] J values are expressed in Hz. Purification steps by semi-preparative HPLC were performed using Varian Kromasil C_{18} column (10 µm, 21.2 x 250 mm) with CH_3CN and 0.1% aq. TFA as eluents [0% CH_3CN (5min), followed by linear gradient from 0% to 60% (125 min) of CH_3CN] at a flow rate of 20.0 mL/min. Dual visible detection was achieved at 550 and 600 nm. High-resolution mass spectra (HRMS) were recorded either with a Thermo LTQ Orbitrap XL apparatus equipped with an ESI source. UV/Vis absorption spectra were obtained with a Varian Cary 50 scan spectrophotometer using a rectangular quartz cell (Varian, standard cell, Open Top, light path 10 x 10 mm, chamber volume: 3.5 mL). Fluorescence spectroscopic studies (emission/excitation spectra) were performed either on a Varian Cary Eclipse spectrophotometer with a semi-micro quartz fluorescence cell (Hellma, 104F-QS, light path: 10 x 4 mm, chamber volume: 1400 µL) or an ultra-micro quartz fluorescence cell (Hellma, 105.251-QS, light path: 33 mm, chamber volume: 45 µL) for uPA assays.

HPLC Separations: Several chromatographic systems were used for the analytical experiments and purification steps by semi-preparative HPLC: Analytic HPLC (Thermo Hypersil GOLD C_{18} column, 5 µm, 2.1 × 100 mm) with CH_3CN and trifluoroacetic acid (0.1% aq.; pH 2.0) as eluents [100% aq. TFA (5 min), then linear gradient from 0% to 100% (45 min) CH_3CN] at a flow rate of 0.25 mL/min. Triple UV/Vis detection was achieved at 220, 260, and 380 nm, and with the “Max Plot” (i.e., chromatogram at absorbance maximum for each compound) mode (220–700 nm). Semi-preparative RP-HPLC (Varian Kromasil C_{18} column, 10 µm, 21.2 x 250 mm) with CH_3CN and 0.1% aq. TFA as eluents [0% CH_3CN (5min), followed by linear gradient from 0% to 60% (125 min) of CH_3CN] at a flow rate of 20.0 mL/min. Dual visible detection was achieved at 550 and 600 nm.

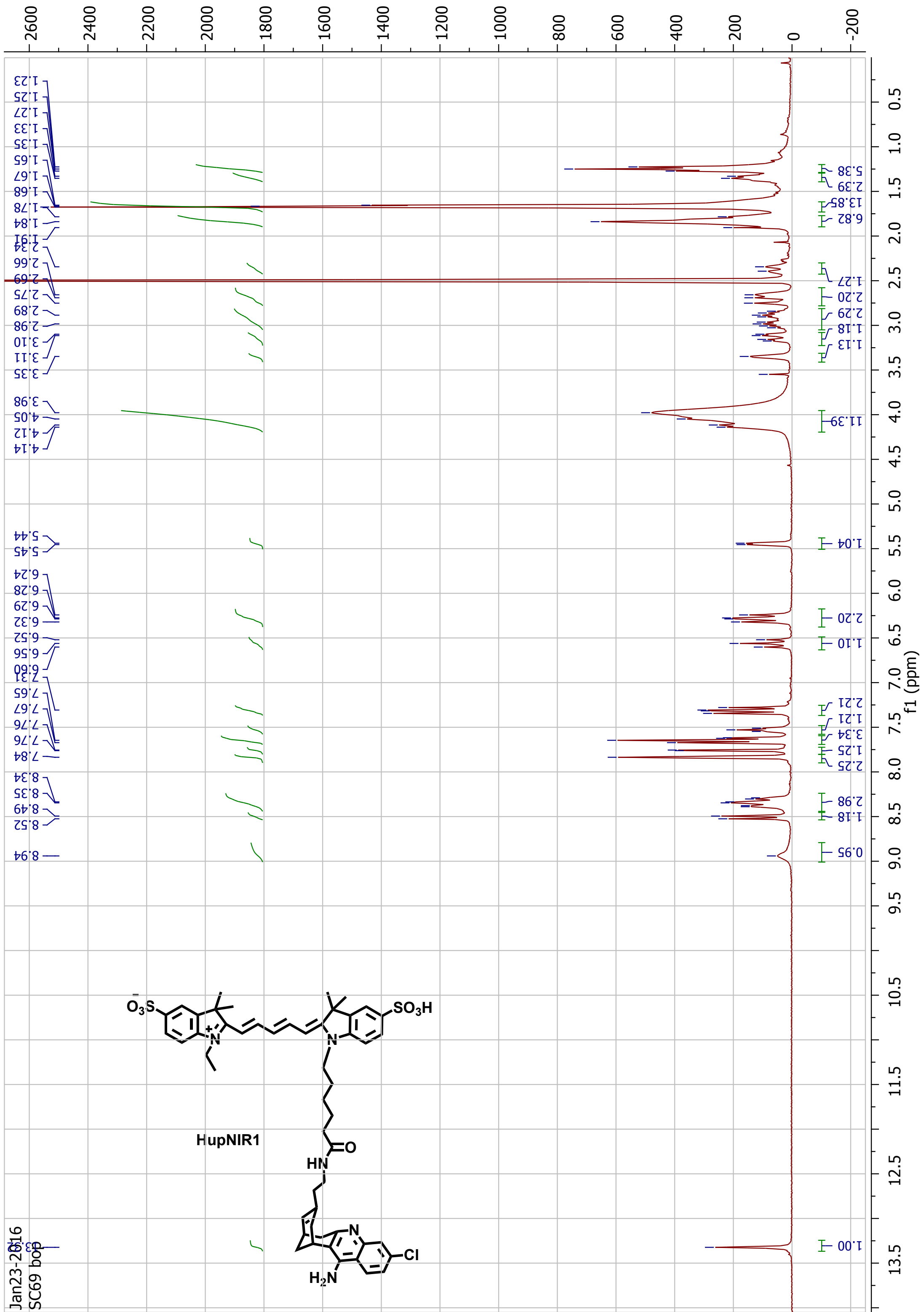
2. Chemistry

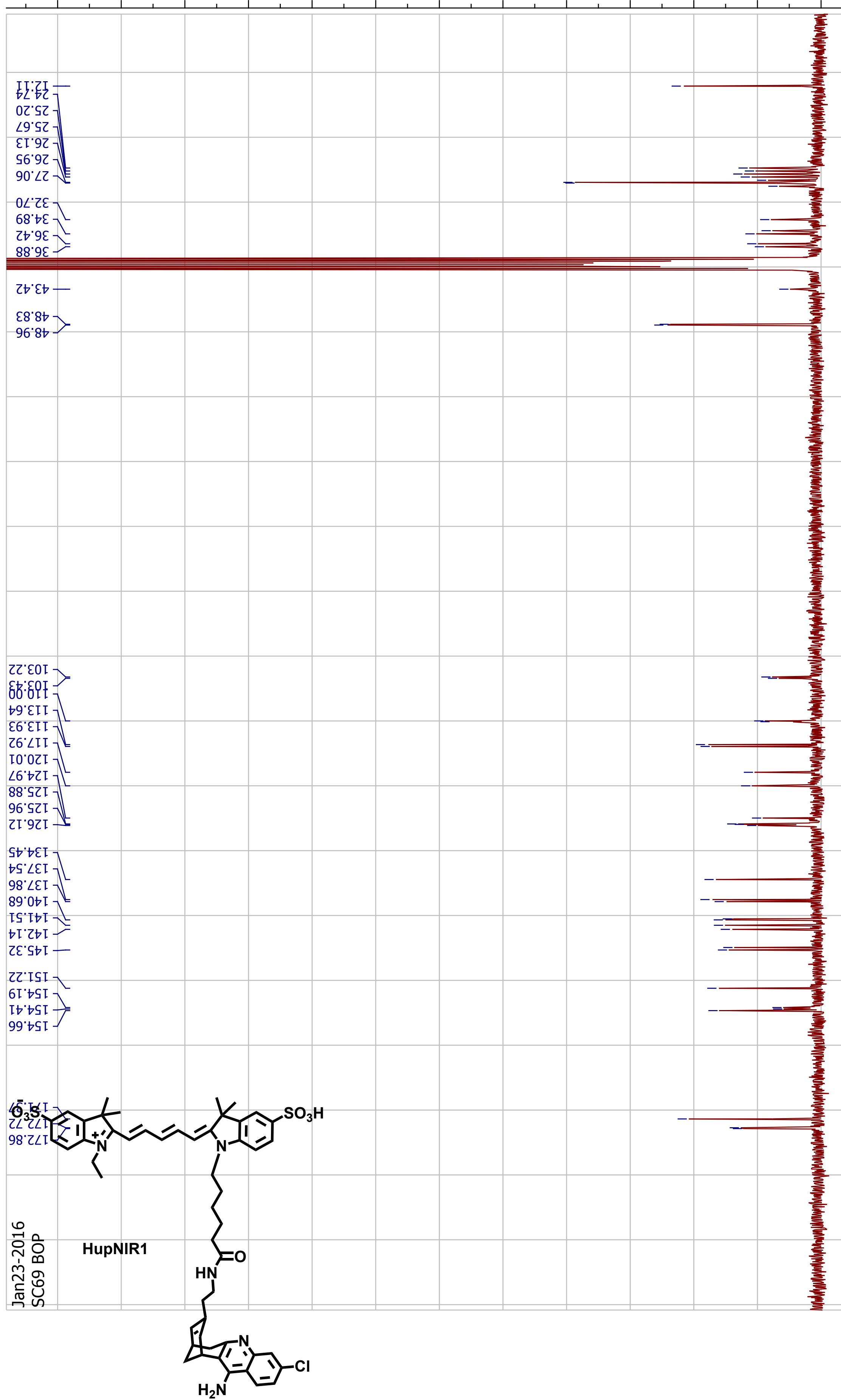
Synthesis of HupNIR1. A mixture of Cy 5.0 (39 mg, 0.059 mmol), BOP (31 mg, 0.070 mmol) and DIEA (51 µL, 0.295 mmol) in NMP (0.4 mL) was stirred for 1 h at room temperature. To this mixture was added huprine **Hup1** (20 mg, 0.065 mmol). The resulting mixture was stirred for 4 h at room temperature. Thereafter, the reaction mixture was quenched with acetic acid (17 µL, 0.295 mmol). Purification by semi-preparative RP-HPLC gave the desired product **HupNIR1** (14 mg, 20%) as a blue solid. ^{1}H NMR (300 MHz, DMSO-d_6) : δ 1.14–1.43 (m, 8 H), 1.55–1.74 (s, 12 H), 1.78–1.91 (m, 6 H), 2.34– 2.41 (m, 2 H), 2.59–2.78 (m, 2 H), 2.84–3.03 (m, 2 H), 3.14 (dd, J = 17.6, 4.9 Hz, 1 H), 3.35 (s, 1 H), 3.81–4.21 (m, 10 H), 5.45 (d, J = 4.8 Hz, 1 H), 6.28 (dd, J = 13.6, 10.0 Hz, 2 H), 6.56 (t, J = 12.2 Hz, 1 H), 7.31 (dd, J = 11.2, 8.5 Hz, 2 H), 7.53 (t, J = 5.6 Hz, 1 H), 7.62–7.67 (m, 3 H), 7.76 (d, J = 1.9 Hz, 1 H), 7.80 (s, 2 H), 8.35 (td, J = 13.3, 3.8 Hz, 3 H), 8.51 (d, J = 9.1 Hz, 1 H), 8.94 (s, 1 H), 13.32 (s, 1 H); ^{13}C NMR (75 MHz, DMSO-d_6) : δ 12.1, 24.7, 25.2, 25.7, 26.1, 26.6, 26.9, 27.0, 27.5, 32.7, 34.4, 34.9, 36.4, 36.9, 43.4, 48.8, 49.0, 103.2, 103.4, 110.0, 110.1, 113.6, 113.9, 117.9, 120.0, 125.0, 125.9, 126.0, 126.1, 134.4, 137.5, 137.9, 140.5, 140.7, 141.5, 142.1, 144.9, 145.3, 151.2, 153.2, 154.4, 154.7, 171.4, 172.7, 172.9; HRMS (ESI-) calcd for $C_{51}H_{57}N_5O_7S_2{}^{35}Cl$: 950.3388, found 950.3364

Synthesis of HupNIR2. A mixture of Cy 5.0 (39 mg, 0.059 mmol), BOP (31 mg, 0.070 mmol) and DIEA (51 µL, 0.295 mmol) in NMP (0.4 mL) was stirred for 1 h at room temperature. To

this mixture was added huprine **Hup2** (22 mg, 0.065 mmol). The resulting mixture was stirred for 4 h at room temperature. Thereafter, the reaction mixture was quenched with acetic acid (17 μL, 0.295 mmol). Purification by semi-preparative RP-HPLC gave the desired product **HupNIR2** (14.5 mg, 25%) as a blue solid. ^{1}H NMR (300 MHz, DMSO-d_6) : δ 1.17–1.28 (m, 10 H), 1.46–1.53 (m, 4 H), 1.72–1.89 (m, 5 H), 1.95 (t, J = 6.8 Hz, 2 H), 2.30 (d, J = 16.2 Hz, 2 H), 2.64–2.96 (m, 4 H), 3.16 (dd, J = 18.3, 5.4 Hz, 1 H), 4.00–4.22 (m, 4 H), 5.46 (d, J = 4.4 Hz, 1 H), 6.28 (d, J = 13.8 Hz, 2 H), 6.55 (t, J = 12.4 Hz, 1 H), 7.23–7.42 (m, 2 H), 7.79 (d, J = 1.8 Hz, 1 H), 7.83 (d, J = 1.3 Hz, 2 H), 8.34 (t, J = 13.1 Hz, 2 H), 8.51 (d, J = 9.1 Hz, 1 H), 8.94 (s, 1 H), 13.32 (s, 1 H); ^{13}C NMR (75 MHz, DMSO-d_6) : δ 12.1, 24.1, 24.7, 24.8, 25.3, 25.6, 25.7, 26.1, 26.6, 27.0, 27.1, 27.8, 28.2, 28.5, 32.9, 34.5, 34.9, 35.0, 36.1, 36.2, 38.0, 43.4, 103.1, 103.5, 110.1, 113.7, 113.8, 118.0, 120.0, 123.6, 123.9, 125.9, 126.0, 126.1, 136.7, 137.5, 137.7, 137.8, 140.5, 140.6, 141.5, 142.0, 142.1, 145.1, 145.2, 151.4, 154.3, 154.6, 171.5, 172.6, 173.0; HRMS (ESI-) calcd for $C_{53}H_{61}N_5O_7S_2{}^{35}Cl$: 978.3701, found 978.3679.

3. Copies of ^{1}H and ^{13}C NMR spectra

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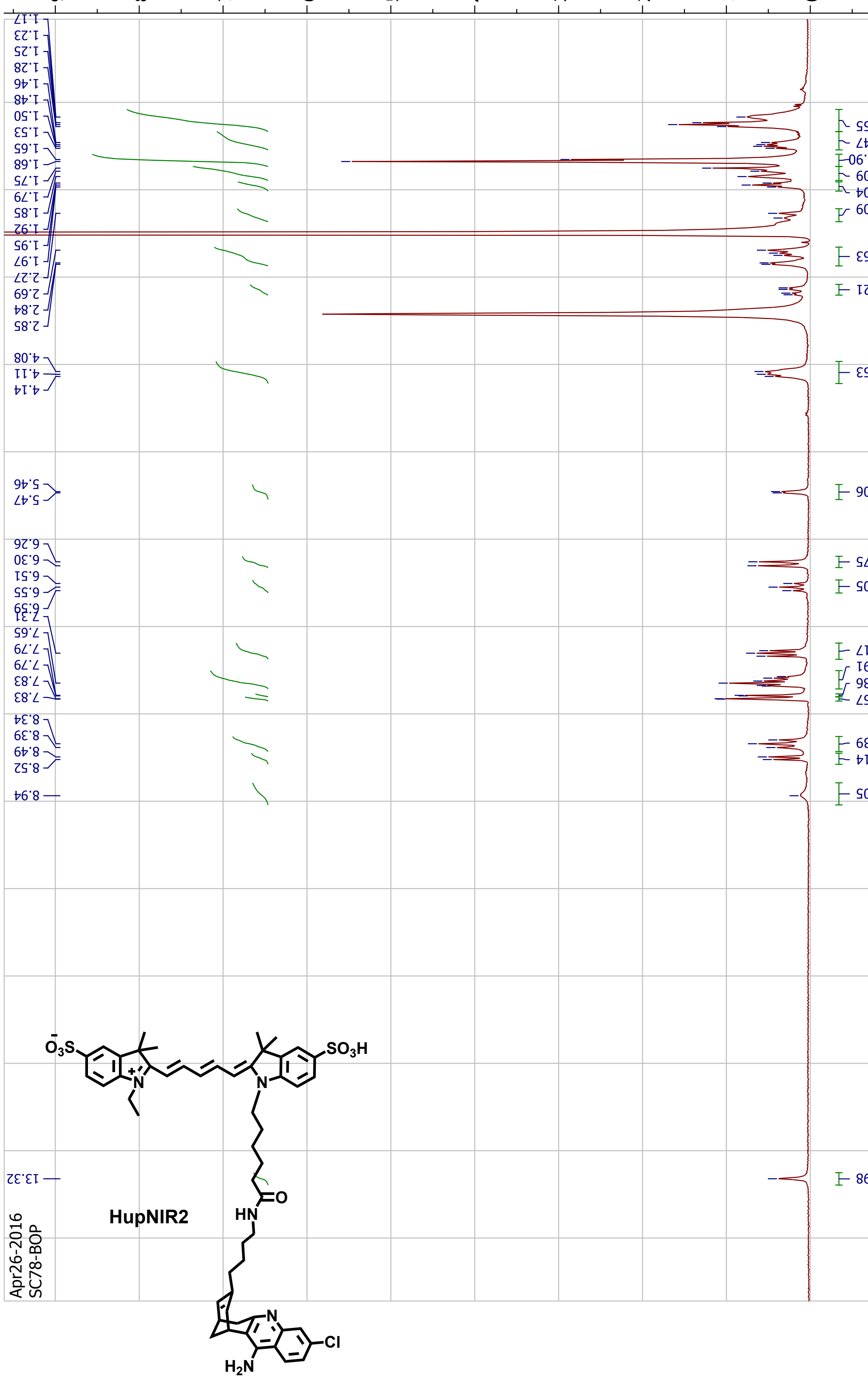
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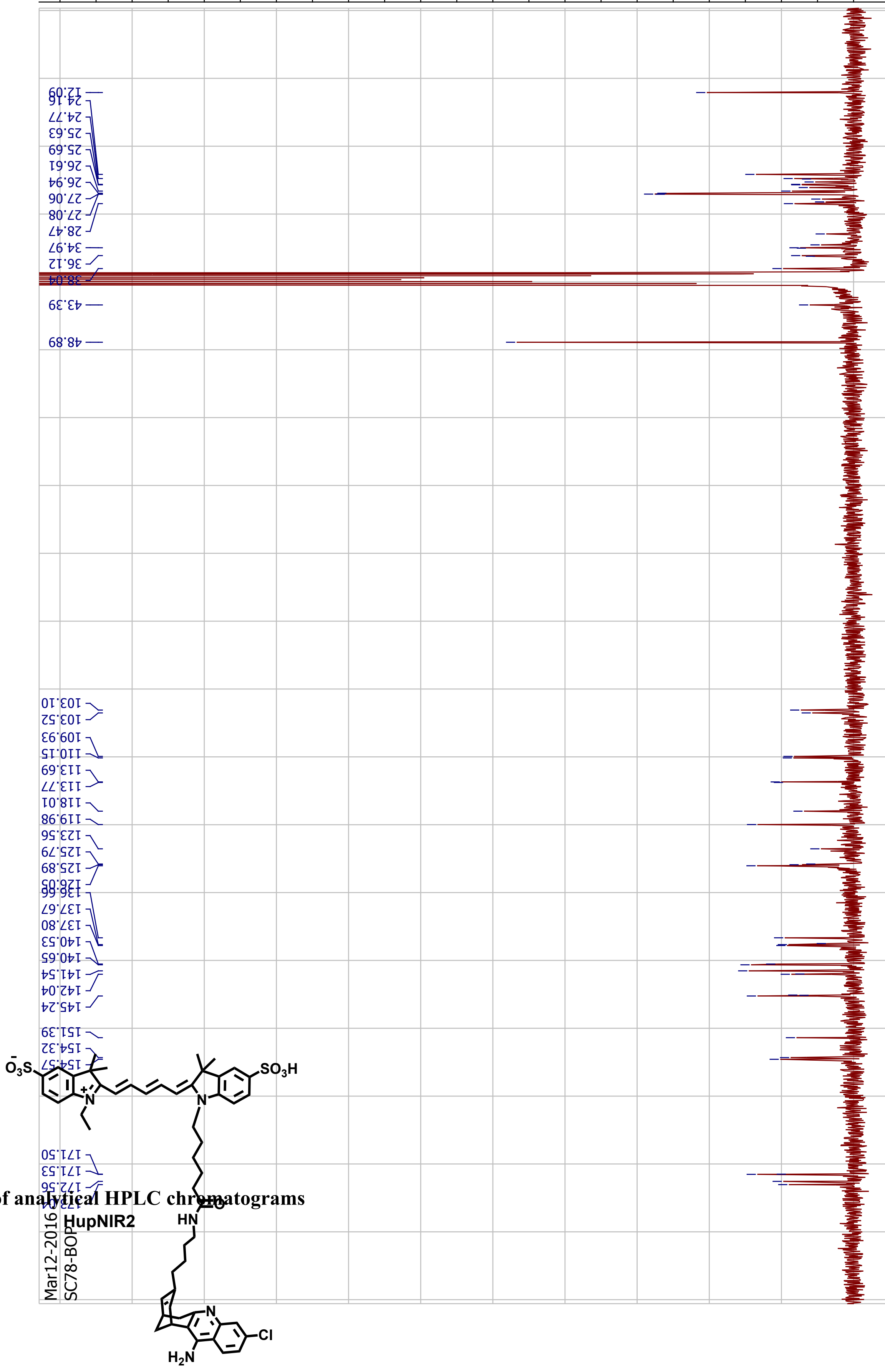
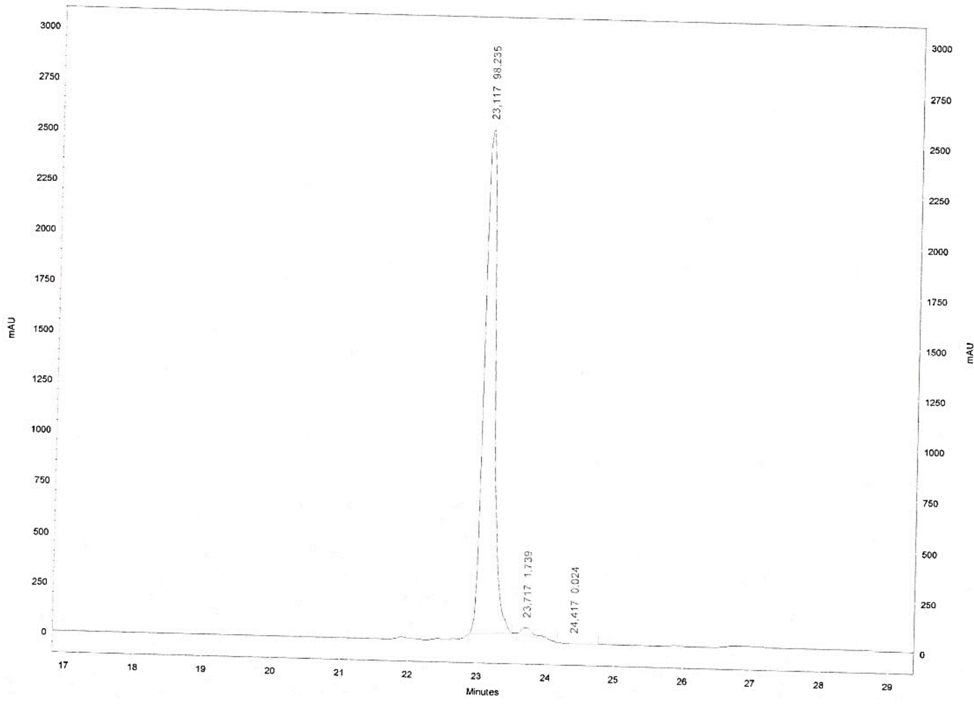
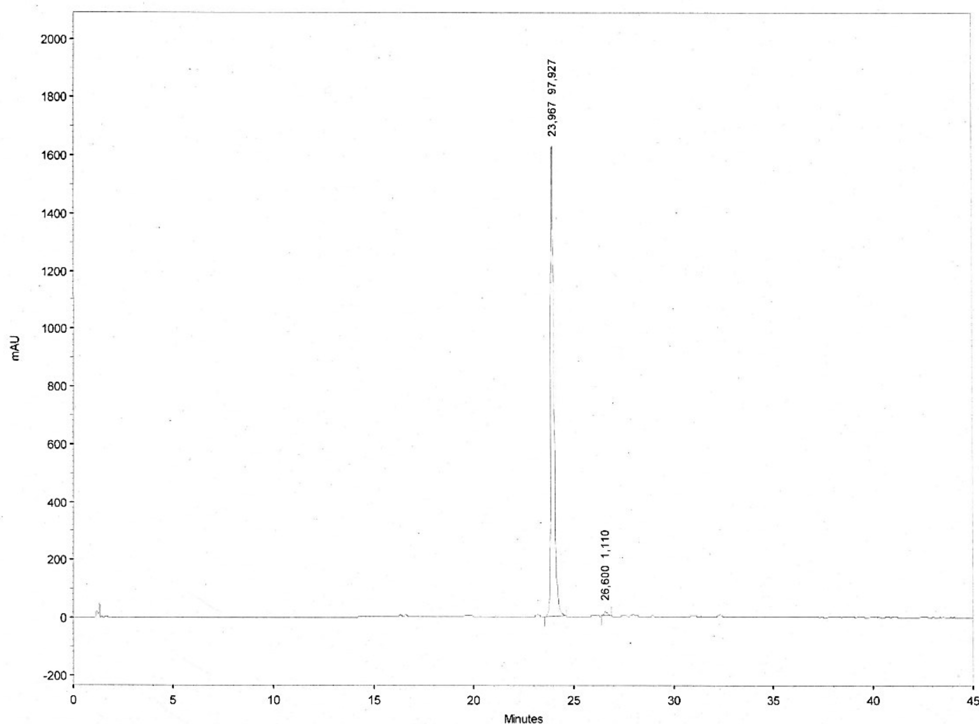


4. **Copies of analytical HPLC chromatograms**



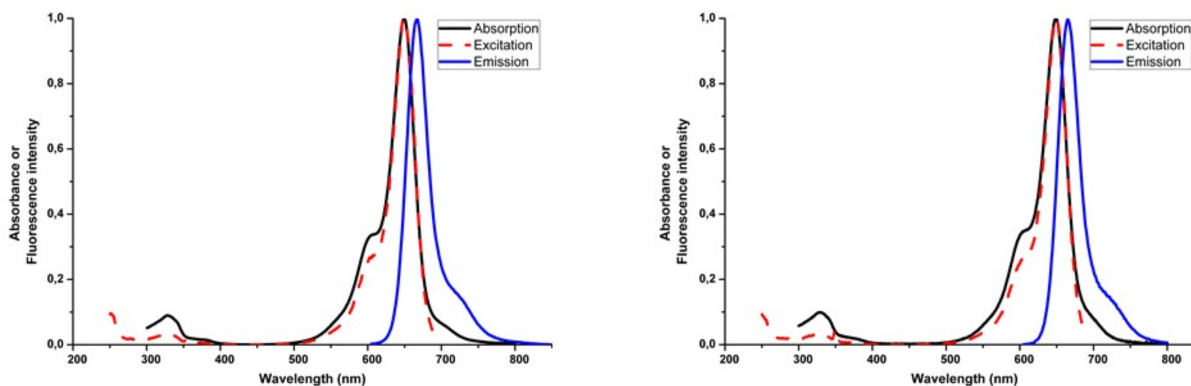
Analytical HPLC chromatogram of compound **HupNIR1**



Analytical HPLC chromatogram of compound **HupNIR2**

5. Fluorescence analysis

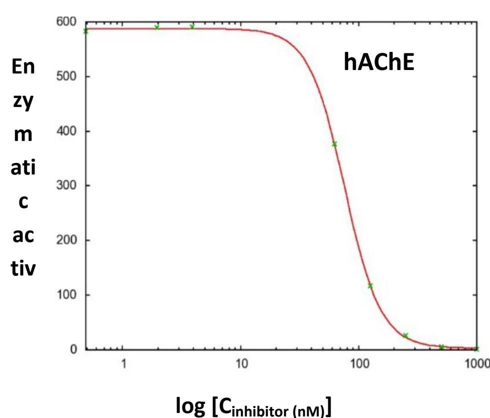
All absorption spectra were recorded (220–700 nm) at 25 °C in PBS. Excitation/emission spectra were recorded under the same conditions after emission/excitation at 595/700 nm (excitation filter: auto and emission filter: open, excitation and emission slit: 5 nm). Fluorescence quantum yields were measured at 25 °C by a relative method using sulfoindocyanine (Φ_F = 20% in PBS, pH 7.4) as a standard.[2, 3] The following equation was used to determine the relative fluorescence quantum yield: ΦF(x) = (AS/AX) (FX/FS) (nX/nS)2ΦF(s) where A is the absorbance (in the range of 0.01–0.1 AU), F is the area under the emission curve, n is the refractive index of the solvents (at 25 °C) used in measurements, and the subscripts s and x represent the standard and the unknown, respectively. The following refractive index values were used: 1.362 for EtOH and 1.337 for PBS.

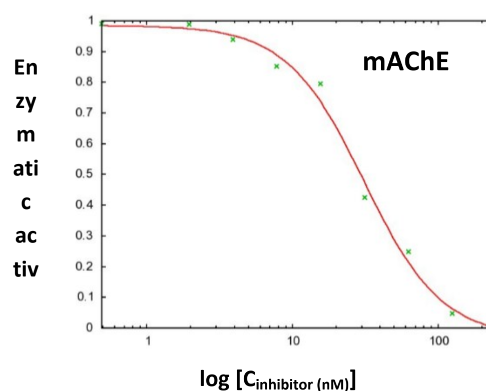


Normalized fluorescence spectra of **HupNIR1** (left) and **HupNIR2** (right), including absorption, excitation and emission (0.1 mM in PBS buffer, pH 7.4)

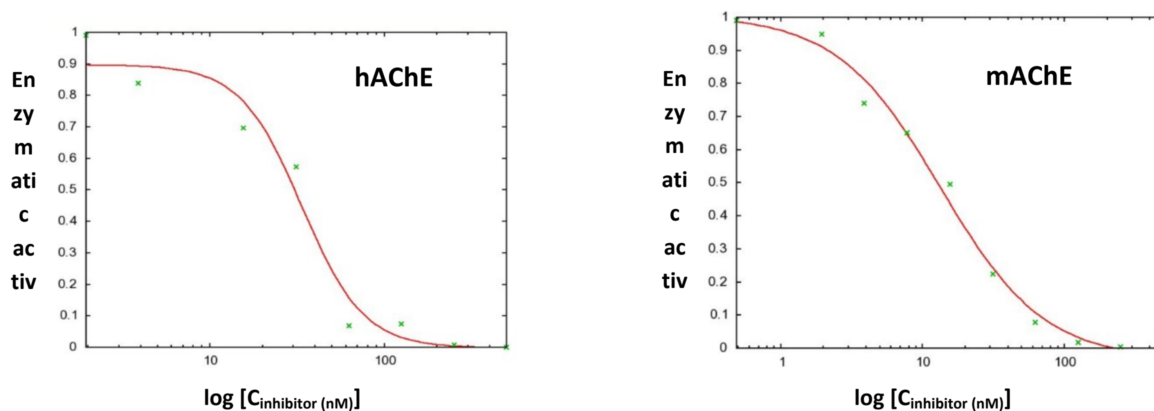
6. Biological assays

The inhibitory activity was evaluated by the method of Ellman using recombinant human AChE (*rh*-AChE), recombinant human BChE (*rh*-BChE) or mouse AChE (*m*AChE) and acetylthiocholine iodide or butyrylthiocholine (0.50 mM) as substrate. The reaction was performed in the presence of 125 pM of *rh*-AChE or *rh*-BChE in a final volume of 1 mL (tank) of 0.1 M phosphate buffered solution (pH 7.4), containing 15 µM Bovine Serum Albumin (BSA) and 300 µM 5,5'-dithiobis(2-nitrobenzoic)acid) (DTNB) solution used to produce the yellow anion of 5-thio-2-nitrobenzoic acid. The different inhibitory derivatives were pre-incubated with the enzyme at 25 °C for at least 30 min before measurement. One sample without inhibitor was always present to yield the 100% of cholinesterase activity. The rate of change of absorbance (ΔA/min), reflecting the rate of hydrolysis of acetylthiocholine was recorded at λ = 412 nm for 2 min (kinetic mode) using a UV-Vis Varien Cary 50 scan spectrophotometer. These experiments were generally done at least in duplicate and the values averaged. Data from concentration-inhibition experiments of the inhibitors were calculated by nonlinear regression analysis, which gave estimates of the IC_{50} (concentration of drug producing 50% of enzyme activity inhibition). Recombinant acetylcholinesterase was purified as previously described.[4] DTNB and acetylthiocholine were purchased from Sigma.





IC_{50} of **HupNIR1**



IC_{50} of **HupNIR2**

7. Confocal imaging of mouse tissues

All experiments were performed following French guidelines for laboratory animal handling approved by the Animal Committee of Université Paris Descartes in accordance with the European Community Council Directive of February 1, 2013 (82010/63/EE registration number CEEA34.EK/AGC/LB.111.12). B6D2, AChE1irr[5] and Thy1-mGFP (L17)[6] mice were used.

Preparation of the samples:

To visualize AChE in muscle, mice were killed by lethal injection of pentobarbital. Isolated anterior tibialis muscle were dissected from the leg, pinned on Rhodorsil (Rhône-Poulenc)-lined Plexiglas chambers (2 ml volume), and fixed with freshly prepared 4% paraformaldeyde (ElectronMicroscopy Sciences) in 10mM PBS for 1 h at room temperature. After washing with PBS, muscle fibers were teased apart. Groups of fibres were incubated in 0.1 M glycine in for 30 min at room temperature to quench the fluorescence and then washed 10 min. in PBS.

To visualize AChE in brain, mice were deeply anesthetized with pentobarbital and were subjected to transcardiac perfusion with a mixture of 2% paraformaldehyde and 0.2% glutaraldehyde. Sections from the neostriatum were cut on a vibrating microtome at 70 µm and collected in PBS. The sections were cryoprotected, freeze-thawed and stored in PBS until used.

Labelling of the tissues

Muscle fibres or brain sections were incubated for 1 hour with 1 µM **HupNIR1** or **HupNIR2** diluted from 1 mM stock solution (DMSO) in PBS containing 1% BSA and Alexa488 α-bungarotoxin (1 µM) to visualize nAChR when it is required. Tissues were washed 3 times, 10 min in PBS and mounted with Vectashield antifade mounting medium (Vector Laboratories).

Confocal imaging

NMJs were analyzed using a LSM 510 META microscope (Carl Zeiss), mounted on an inverted microscope, and controlled through the manufacturer-supplied software and workstation. Images were collected using an oil-immersion objective [Plan-Apochromat ×63/1.2 numerical aperture (NA)]. The pinhole aperture was set to 1 Airy unit. Images were digitized at 8-bit resolution into 1024 × 1024 pixel arrays. Labeling on brain sections was observed using a Confocal microscope Leica TCS SP5 AOBS/resonant scanner inverted. Images were collected using an oil-immersion objective [Plan-Apochromat ×63/1.2 numerical aperture (NA)]. The pinhole aperture was set to 1 Airy unit. The

fluorochrome was excited using a 633nm laser. Images were digitized at 8-bit resolution into 1024 × 1024 pixel arrays.

Data were analyzed using Fuji software[7] on a series of projections of maximal intensity. Images were then colored in Photoshop 5.0, schema was draw in Illustrator 5.0 and assembled with In Design 5.0.

8. Notes and References

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