

1 **Quantum dot-based multiphoton fluorescent pipettes for**
2
3 **targeted neuronal electrophysiology**

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39 **Keywords:** Quantum dot, pipettes, electrophysiology, patch-clamp, fluorescence, Ca-sensing,
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42 **Abstract**

43 Targeting visually-identified neurons for electrophysiological recording is a fundamental
44 neuroscience technique; however, its potential is hampered by poor visualization of pipette tips
45 in deep brain tissue. We describe a technique whereby quantum dots coat glass pipettes
46 providing strong two-photon contrast at deeper penetration depths than current methods. We
47 demonstrate utility in targeted patch-clamp recording experiments and single cell electroporation
48 from identified rat and mouse neurons *in vitro* and *in vivo*.

49

50 Electrical recording from individual neurons in brain tissue using patch-clamp techniques
51 provides the most direct information on neuronal activity^{1,2}, and will be critical to success of the
52 brain mapping initiatives^{3,4}. Advances in genetic labeling of specific cell types open the
53 possibility of targeted patch-clamp recordings from individually-identified fluorescent neurons in
54 living brain tissue^{5,6}. However, direct access to neurons, both labeled and unlabeled, is hampered
55 by a lack of methods for visualizing thin pipettes tips as they are advanced through the brain to
56 contact the targeted neuron. Visualization, especially deeper within the brain, is currently
57 accomplished using two-photon (2P) imaging of fluorophores (*e.g.* Alexa Fluor dyes)⁵⁻⁷ that are
58 continuously expelled from the pipette during the approach, thereby creating a “shadow” around
59 a labeled or unlabeled neuron. Though such dyes have been successfully used for many years,
60 their applicability is still limited by low 2P excitation action cross-sections (absorption of two
61 photons of identical frequency) requiring potentially damaging higher laser powers,
62 susceptibility to photobleaching, and dye accumulation which causes increased background
63 fluorescence or light absorption, especially after multiple descents. As an alternative method for
64 targeted single-cell recordings, we developed a technique for robust fluorescent labeling of
65 standard borosilicate glass pipettes allowing their 2P visualization far deeper within brain tissue
66 than current methods.

67 From a photophysical perspective, their unique properties make semiconductor quantum
68 dots (QDs) ideal for this imaging challenge. These nanocrystals, whose photoluminescence (PL)
69 can be tuned *via* core size and composition, display desirable optical properties including high
70 quantum yields (ϕ), resistance to photo and chemical degradation, narrow and symmetrical PL

71 emission (full-width-at-half-maximum ~25-35 nm), broad absorption spectra coupled to large
72 one-photon ($\epsilon = 10^4\text{-}10^7 \text{ M}^{-1}\text{cm}^{-1}$) and some of the highest two-photon absorption cross-sections
73 ($\sigma_2 = 10^3\text{-}10^4$ Goeppert-Mayer or GM units) available^{8,9}. QD utility for 2P imaging in tissue has
74 been repeatedly confirmed⁸⁻¹⁰. Here, we show QD-labeled glass pipettes provide outstanding
75 contrast of the pipette tip even in deep brain for targeted electrophysiological recordings without
76 compromising electrical properties of the pipette or neuronal activity.

77 For optically targeting labeled neurons (typically expressing a red or green fluorescent
78 protein), we coated pipettes with green (ϕ 19%, 530 nm), yellow (ϕ 33%, 550 nm) or red (ϕ 45%,
79 625 nm) emitting CdSe-ZnS core-shell QDs (**Fig. 1a**). These QDs were cap-exchanged with
80 polyethylene glycol modified- or zwitterionic-terminated dihydrolipoic acid ligands for optical
81 characterization (**Supplementary Fig. 1**)¹¹ or diluted in hexane with native phosphine-
82 hexadecylamine ligands still present on their surface for pipette coating. We determined QD 2P
83 action cross-section ($\phi\sigma_2$) spectra using a two-photon spectrometer¹². QD $\phi\sigma_2$ were measured in
84 comparison to Alexa Fluor 488 (ϕ 92%), Alexa 546 (ϕ 79%), and Alexa 594 (ϕ 66%) dyes (**Fig.**
85 **1b-d**). Comparative $\phi\sigma_2$ at 880 nm were ~400 GM units for 530 QDs *versus* 8 GM for Alexa
86 488, 752 GM for 550 QDs *versus* 6 GM for Alexa 546, and 16470 GM for 625 QDs *versus* 12
87 GM for Alexa 594. Assuming a pipette could be uniformly coated with equal amounts of 625 QD
88 or Alexa Fluor 594 dye, and using a simplistic extrapolation of $(\phi\sigma_2)_{\text{QD}}/(\phi\sigma_2)_{\text{dye}}$ at equal 880
89 nm 2P excitation, the 625 QD probe should be >900X brighter.

90 To coat pipette tips with QDs, native QDs were first washed in organic solvent several
91 times to remove the excess synthetic ligands then dried down and re-solubilized in hexane. The
92 tip of the borosilicate pipette was then repeatedly dipped into the QD-hexane solution until a
93 desirable PL was reached (visualized under UV light). To prevent QDs from clogging the pipette
94 tip, we applied positive air pressure during the coating. Since native-capped QDs are completely
95 insoluble in aqueous solutions, they remain attached to the glass pipette, providing 2P contrast in
96 the presence of any physiological buffer, internal pipette solution or dyes. Comparing the
97 standard approach for pipette visualization using a soluble fluorescent dye against the QD-coated
98 pipette shows substantial intensity differences in the area of the pipette tip (**Fig. 1e,f**). When
99 Alexa Fluor 488 is ejected from the pipette, measured fluorescence intensity is lowest at the tip,
100 whereas QD-coated pipettes show the brightest fluorescence at the tip. This is ideal for

101 accurately determining pipette tip location in brain tissue, especially since this very structure will
102 first contact neuronal membranes. To determine the detection limits of coated pipettes in deep
103 brain tissue, we compared both methods in anaesthetized mice using 2P imaging and measured
104 the intensity of fluorescence signals down to 500 μm depth at various laser powers (**Fig.1 g-l**)^{5,13}.
105 While Alexa Fluor 594 fluorescence ejected from the pipette deteriorated rapidly below 300 μm ,
106 QD-coated pipettes were still clearly visible at penetration depths of 500 μm , while using 77%
107 less laser power (**Fig. 1i-l**). Even at the maximum excitation wavelength (800 nm), the Alexa
108 Fluor 594 signal was still lower compared to the QD coated pipettes (**Supplementary Fig. 2**).
109 Such extended imaging depths at lower laser power can expand experimental access *in vivo*.

110 To evaluate electrochemical and optical properties of QD-coated patch pipettes *in situ*,
111 we performed patch-clamp recordings in brain slices. The pipette resistance of QD-coated
112 pipettes did not differ from uncoated control pipettes, whereas the capacitance was slightly
113 decreased (**Fig. 2a-c**). QD-coated pipettes formed gigaseal contacts similarly to uncoated patch
114 pipettes^{5-7,13}, when using the standard “blow-and-seal” technique. We patched different
115 fluorescently-labeled cell types in brain slices, including hippocampal Ds-Red-labeled
116 cholecystokinin positive interneurons and GFP labeled parvalbumin-positive interneurons (**Fig.**
117 **2d, Supplementary Fig. 3a**). The fluorescence intensity of QD-coated pipettes was consistently
118 higher than the endogenously-expressed fluorescent markers. Indeed, sensitivity of the
119 photomultiplier detecting the QD-coated pipette signal needed to be scaled down to avoid
120 saturation at the laser power required for visualizing the fluorescent proteins. Basic
121 electrophysiological properties of neuron types patched with QD-coated pipettes were similar to
122 those recorded using uncoated pipettes (somatic firing, voltage responses to a series of positive-
123 negative current injections), confirming the QD coating did not interfere with neuronal
124 electrophysiological properties nor affected viability. Furthermore, 2P Ca^{2+} imaging from CA1
125 pyramidal neurons loaded with the Ca^{2+} -sensitive dye Oregon Green BAPTA-1 (OGB-1)
126 through the QD-coated pipette revealed normal dendritic and spine Ca^{2+} and voltage signals in
127 response to backpropagating action potentials (APs, **Fig. 2e**) as well as to direct synaptic
128 stimulation by 2P glutamate uncaging (**Fig. 2f and Supplementary Fig. 3b**)¹³.

129 Under *in vivo* conditions, we recorded with QD-coated patch pipettes from cortical L2/3
130 pyramidal neurons of anaesthetized mice expressing the genetically encoded Ca^{2+} indicator

131 GCaMP6 (**Fig. 3a** and **Supplementary Videos 2** and **3**). QD-coated pipettes could be clearly
132 visualized within the intact brain even after penetrating the dura. Spontaneous electrical activity
133 and corresponding somatic GCaMP6 Ca^{2+} signals were measured in the patched cells and
134 appeared normal. Recordings from channelrhodopsin-(ChR2) expressing interneurons using QD-
135 coated pipettes verified that activation of ChR2 with 470 nm light produced robust and precisely
136 driven firing as expected (**Fig. 3b**)^{6,14}. QD-coated pipettes also successfully electroporated⁷ with
137 Alexa Fluor 594 dye and a Ds-Red encoding plasmid into individually identified L2/3 pyramidal
138 neurons at ~300 μm depth (**Supplementary Fig. 4** and **Supplementary Video 1**). The challenge
139 of sequentially electroporating multiple cells *in vivo* did not alter the QD coating nor produced
140 QD adsorption to the brain parenchyma demonstrating the reliability of this method. Importantly,
141 GFP-expressing neurons were successfully electroporated *in vivo* at 760 μm depth using 625 QD
142 coated pipettes (**Fig. 3c-e** and **Supplementary Video 4**). Superb visibility of the QD coated
143 pipette tips also improved access to small cellular structures, such as local dendritic regions *in*
144 *vitro* (**Fig. 3f**).

145 In summary, we introduce a simple technique to fabricate permanently-labeled
146 fluorescent glass pipettes which facilitate visually targeted recordings from individual (labeled or
147 unlabeled) neurons at great depth and with high precision both *in vitro* and *in vivo*. Pipettes have
148 been labeled previously with fluorophores; however the dyes utilized did not provide the
149 required 2P properties for deep tissue imaging^{15,16}. Our approach is an alternative or complement
150 to the current “gold standard” method⁵⁻⁷ while removing the need to perfuse dye into the
151 extracellular space continuously which reduces visibility and contrast. High quality imaging with
152 QD-coated pipettes is possible even at depths of ~500-800 μm within *in vivo* brain tissue (**Fig. 1**
153 and **Fig. 3**). We note that the low intrinsic 2P properties of the currently used fluorescent proteins
154 expressed in labeled neurons may still require higher laser power for their visualization. QD
155 coating does not preclude use of fluorescent dyes, in fact, it allows the advantage of combining
156 both visualization modalities simultaneously for specific applications (*e.g.* for monitoring pipette
157 clogging or cell loading). Furthermore, narrow, size-tunable QD PL allows access to coatings of
158 various colors across the spectrum as experimentally required⁹. Critically, QD-coated pipettes
159 did not interfere with physiological functions monitored throughout our experiments for ≤ 3
160 hours, suggesting they can be used for a wide array of biological experiments. While we tested

161 QD coatings for electrophysiological recording pipettes in neuroscience, we expect that they can
162 be applied to coat any probe type wherever improved visualization in tissue is needed.

163
164 **METHODS.** Methods and any associated references are available in the online version of the
165 paper. *Note: Supplementary information is available in the online version of the paper.*

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177 **AUTHOR CONTRIBUTIONS.** B.K.A., M.B., J.J.M. and I.L.M. conceived the idea of using
178 QDs for coating patch pipettes. B.K.A. and J.K.M. performed and analyzed *in vitro* experiments.
179 G.L.G. and D.H. performed and analyzed *in vivo* experiments. J.J.M., K.S., J.B.D., A.L.H. and
180 I.L.M. produced the QDs or characterized them. I.L.M., B.K.A., G.L.G., D.H. and J.K.M. wrote
181 the paper with comments from all authors.

182
183 **COMPETING FINANCIAL INTERESTS.** B.K.A., M.B., J.J.M., K.S., J.B.D., A.H. and
184 I.L.M. have filed a patent application for production of QD-coated probes based on the results
185 reported in this paper. The rest of the authors declare no competing financial interest.

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216 **Figure Legends.**

217 **Figure 1.** QD photophysical properties and *in vivo* imaging. **(a)** Normalized absorption and PL
218 of QDs. Molecular extinction coefficients ϵ at wavelengths corresponding to the first excitation
219 peak are: 530 QD $159,092 \text{ M}^{-1} \text{ cm}^{-1}$ at 501 nm; 550 QD $120,000 \text{ M}^{-1} \text{ cm}^{-1}$ at 533 nm; 625 QD
220 $500,000 \text{ M}^{-1} \text{ cm}^{-1}$ at 610 nm. **(b-d)** 2P action cross-section spectra ($\phi\sigma_2$) in GM units for **(b)** 530-
221 , **(c)** 550- and **(d)** 625 QDs in phosphate buffered saline, superimposed over spectra of Alexa
222 Fluor 488, Alexa Fluor 546 and Alexa Fluor 594 in water, respectively. Inset in **d** shows the
223 enlarged spectrum of Alexa Fluor 594. **(e)** Image of a 625 QD-coated pipette (upper) and an
224 uncoated pipette ejecting Alexa Fluor 488 (lower). Intensity measurements were performed in
225 the white rectangles within the pipette tips. **(f)** Fluorescence intensity as measured in **(e)** for 625
226 QD, 550 QD and Alexa Fluor 488 dye. **(g, h)** Schematics of the **(g)** classic approach for pipette
227 visualization during shadow patching and the new **(h)** approach using QD-coated pipettes (red).
228 Alexa Fluor 594 filled pipettes **(i)** or 625 QD-coated pipettes **(j)** were imaged at different depths
229 (D) in the mouse brain at the indicated laser power (LP). Images are the average of 10 frames,
230 except for Alexa Fluor at $500 \mu\text{m}$ (100 frames average). Average gray value (10 frames) in
231 arbitrary units of either the Alexa Fluor 594 **(k)** or the 625 QD **(l)** pipette's fluorescence as a
232 function of laser power at 940 nm and depth.

233 **Figure 2.** Electrical properties of QD-coated patch pipettes. **(a-c)** Comparison of uncoated and
234 QD-coated patch pipettes, **(a)** resistance (unpaired t-test, $n = 7/6$, $P = 0.979$), **(b)** capacitance
235 (unpaired t-test, $n = 7/8$, $P = 0.020$), and **(c)** access resistance (one-way ANOVA test, $n = 4/7/6$
236 cells in 6 animals, $P = 0.454$). Black: mean \pm S.D. **(d)** 2P monitoring of QD-coated pipettes
237 (green, 530 nm QD) during patching of hippocampal neurons (red) in acute brain slices from a
238 BAC-CCK-Ds-Red mouse. Representative for 13 cells in 5 animals. Panels from left: 2P images
239 (1-3); voltage responses to positive and negative current injections (200 pA) in the same cell (4).
240 **(e)** Rat hippocampal CA1 pyramidal neuron loaded with Ca^{2+} sensor OGB-1 (green) through 625
241 QD-coated patch pipette (red) in acute brain slice. Circles: dendritic regions used for recording
242 backpropagation AP evoked Ca^{2+} signals induced by +50-150 pA current injections. Ca^{2+} signals
243 for each location are plotted on the right ($n = 1$). **(f)** Rat hippocampal CA1 pyramidal neuron
244 loaded with Alexa Fluor 594 (red) through a 550 QD-coated patch pipette in acute slice. Box
245 inset: dendritic region and 12 spines selected for 2P glutamate uncaging. Right, top: uncaging-
246 evoked excitatory postsynaptic potentials (gluEPSPs) at indicated spines with inter-spine
247 stimulation interval (IsSI) of 200 ms. Right, bottom: simultaneous glutamate uncaging at all 12
248 spines (IsSI = 0.3 ms) evokes dendritic spike (arrow, $n = 8/9$ dendrites in 4 neurons from 2
249 animals, patched with various QD-coated pipettes). Black: voltage trace, red: dV/dt trace.

250 **Figure 3.** Neuronal manipulations with QD-coated pipettes. **(a)** Left: GCamp6f expressing
251 cortical L2/3 pyramidal neuron (green) patched with 625 QD-coated pipette (red) *in vivo* at 207
252 μm depth. Right, GCamp6f Ca^{2+} signals (top) during spontaneous spiking activity (bottom).
253 Representative of $n = 5$ cells. **(b)** Left: mouse cortical interneuron (green) expressing CHR2-YFP
254 under the control of the vesicular gamma-aminobutyric acid (GABA) transporter (VGAT)
255 promoter, patched with 530 QD-coated pipette (green) *in vivo*. After recording spiking activity in
256 cell-attached mode, the cell was loaded with Alexa Fluor 594 (red). Right: 40 Hz sine wave-
257 modulated 470 nm LED light stimulation (top blue; delivered through 2P microscope optical

258 path) and electrical activity of the same patched neuron (middle: single trial trace; bottom: raster
259 plot of light evoked action potentials, 10 trials). **(c-e)** Deep layer targeting in Thy1-EGFP mouse.
260 **(c)** Top: Z-projection (80 μm) of targeted neuron soma (arrowhead) at 760 μm depth pre-
261 electroporation. Bottom: 3D reconstructed orthogonal view, corresponding to ~ 800 μm . Green:
262 GFP fluorescence, arrowhead: site of pipette contact to the neuron. **(d)** Targeted neuron during
263 electroporation. Red: 625 QD. 40 mW laser power at 940 nm. Frames are averaged 10x. **(e)**
264 Targeted neuron expressing DsRed (red) and GFP (green) 2 days post-electroporation. **(f)**
265 Fluorescence directed dendritic patching, representative of $n = 3$ dendrites in 2 animals. The
266 apical trunk of an *in vitro* CA1 pyramidal cell preloaded with Alexa Fluor 488 patched with 625
267 QD-coated pipette using fluorescent visualization. Dendritic patch formation (top). Synaptic
268 gluEPSPs (bottom) after uncaging at nearby spines.

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Supplementary Materials

Online Methods

Materials and Methods

Preparation of hydrophobic QDs. Native organic QDs¹¹ were washed twice to remove the excess ligands present from synthesis. QD samples in toluene or decane were precipitated by the addition of several milliliters of an acetone:methanol 50:50 mixture in a 15 or 50 mL Falcon tube. The QDs were then centrifuged to a pellet and the supernatant decanted and discarded. The pellet was dried under nitrogen and the QDs were again resuspended in hexane or toluene. This was followed by another round of washing and precipitation with drying under nitrogen for storage. The QDs were resuspended in hexane for probe coating.

Two-photon action cross-sections of QDs and Alexa Fluor dyes. Action cross sections were measured with an inverted microscope using a Ti:sapphire laser as an excitation source, as described earlier¹². Briefly, QD or dye solutions at micromolar concentration (or 0.1 μ M for 625 QD) were contained in coverslip-bottomed dishes (MatTek) and 2P excitation spectra from 710 nm to 1080 nm were obtained at a constant laser power at the sample of 0.5 mW. 530 QD, 550 QD, and 625 QD were measured in phosphate-buffered saline, Alexa Fluor 488, 546, and 594 were measured in water (for comparison to published values), and the 2P reference dye fluorescein was measured at pH 11. Spectra obtained from the buffers alone were used as background correction for the fluorophore spectra. No emission filters were used other than two short-pass filters (720/SP, Semrock). The absolute two-photon action cross section of the reference dye fluorescein was taken from Xu and Webb (1996)¹⁷, with corrections made for small differences in the quantum efficiency of the detector (avalanche photodiode detector model PDF; Micro Photon Devices) for the different emission wavelengths of the fluorophores.

317 ***Pipette coating with QDs.*** Borosilicate pipettes were pulled with a standard puller. Positive air
318 pressure was applied through the back of the pipette with a 10 ml syringe and submerged into
319 methanol to determine the bubble number¹⁸. After methanol evaporation, the tip of the pipette
320 was dipped into the QDs solution keeping the positive pressure to prevent clogging. After 0.5 to
321 2 seconds, the pipette was allowed to dry in the air to form a layer of QDs on the glass surface.
322 The coating procedure was repeated several times until reaching a desirable photoluminescence
323 determined under UV light. See **Supporting Protocol** for a stepwise QD coating procedure with
324 more details and some notes.

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326 ***In vitro and in vivo experiments***

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328 Note: all animal usage and all experiments were performed in strict accordance with institutional
329 IRB approval and met all applicable regulations.

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332 ***In vitro experiments***

333 ***Slice preparation.*** Acute transverse hippocampal slices were prepared from either 8-9 week-old
334 male Sprague-Dawley rats (400 μm thick slices) as described previously¹⁹, or from P18-24 BAC-
335 CCK-Ds-Red²⁰ or PV/GFP BAC²¹ mice of both sexes (300 μm thick slices), according to
336 methods approved by the Institute of Experimental Medicine, Hungarian Academy of Sciences,
337 in accordance with DIRECTIVE 2010/63/EU Directives of the European Community and
338 Hungarian regulations (1998. XXVIII. section 243/1998, renewed in 40/2013, II.14.). Briefly,
339 rats were deeply anaesthetized with isoflurane and transcardially perfused with ice-cold cutting
340 solution containing (in mM): sucrose 220, NaHCO_3 28, KCl 2.5, NaH_2PO_4 1.25, CaCl_2 0.5,
341 MgCl_2 7, glucose 7, Na-pyruvate 3, and ascorbic acid 1, saturated with 95% O_2 and 5% CO_2 .
342 Mice were deeply anaesthetized with isoflurane and decapitated without transcardial perfusion.
343 The brain was quickly removed and sectioned with a vibratome (VT1000A, VT1000S or
344 VT1200S, Leica). Slices were incubated in a submerged holding chamber (rat slices) or in an
345 interface chamber (mice slices) in artificial cerebrospinal fluid (aCSF) at 37°C for 30 min and
346 then stored in the same chamber at room temperature. For recording, slices were transferred to

347 the submerged recording chamber of the microscope where experiments were performed at 33-
348 35 °C in aCSF containing (in mM): NaCl 125, KCl 3, NaHCO₃ 25, NaH₂PO₄ 1.25, CaCl₂ 1.3,
349 MgCl₂ 1, glucose 25, Na-pyruvate 3, and ascorbic acid 1, saturated with 95% O₂ and 5% CO₂.

350

351 **Pipette property measurements.** Pairs of pipettes were pulled from the same borosilicate glass.
352 QD-coated and un-coated pipettes were filled with internal solution leaving a blocking bubble at
353 the tip of the pipette, then submerged into the aCSF-containing recording chamber. Pipette
354 capacitance was measured in voltage-clamp mode using 10 mV step command with a HEKA
355 Amplifier at 100 kHz filtering. After the removal of the blocking bubble from the pipette, the
356 pipette resistance was measured using the same protocol.

357

358 **Electrophysiology.** Cells were visualized using a Zeiss AxioExaminer epifluorescent microscope
359 equipped with infrared Dodt optics and a water immersion lens (63X, 0.9 NA, Zeiss). Current-
360 clamp whole-cell patch-clamp recordings were performed with a Dagan BVC-700 amplifier
361 (Dagan) in the active 'bridge' mode, filtered at 3 kHz and digitized at 50 kHz. Patch pipettes were
362 filled with a solution containing (in mM): K-gluconate 134, KCl 6, HEPES 10, NaCl 4, Mg₂ATP
363 4, Tris₂GTP 0.3, Na-phosphocreatine 14, pH 7.25. In some experiments (as indicated in the text)
364 the pipette solution was complemented with either 100 μM Alexa Fluor 488, 50 μM Alexa Fluor
365 594, or 100 μM Oregon Green 488 BAPTA-1 (OGB-1, for Ca²⁺ measurements; all dyes were
366 from Invitrogen). Series resistance was <30 MΩ.

367 **Two-photon imaging and uncaging.** Two ultrafast pulsed laser beams (Chameleon Ultra II;
368 Coherent) and a dual galvanometer-based two-photon laser scanning system (Prairie
369 Technologies) were used to simultaneously image neurons (at 880 or 920 nm) and to focally
370 uncage MNI-caged-L-glutamate (Tocris; 9-10 mM applied *via* pressure ejection through a 20-30
371 μm diameter pipette above the slice) at individual dendritic spines (at 720 nm)²². Laser beam
372 intensity was independently controlled with electro-optical modulators (Model 350-50,
373 Conoptics). All images shown are collapsed Z stacks of multiple images. Uncaging dwell time

374 was 0.2 ms; galvo move time was 0.1 or 200 ms. (see text). Linescan imaging was performed at
375 150-500 Hz.

376 **Data analysis.** Analysis was performed using custom-written macros in IgorPro (WaveMetrics).
377 Ca^{2+} and voltage signals were analyzed offline using averaged traces of 3-5 trials. Morphological
378 and distance measurements were performed using ImageJ (NIH) on two-dimensional maximal
379 intensity projections of 2 μm z-series collected at the end of the experiment. Only data obtained
380 in experiments meeting the standard technical criteria for successful recordings (GOhm seal
381 resistance, <30 MOhm access resistance) were included

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384 **In vivo experiments**

385 **Surgical procedures.** All *in vivo* mouse experiments were approved by the Animal Care
386 Committee of the University of Geneva. Adult (2-5 months old) C57/Bl6 wild type, VGAT-
387 ChR2 (YFP-Channelrhodopsin-2 expressing neurons under the control of the locus of the
388 vesicular γ -aminobutyric acid (GABA) transporter, VGAT) or Tg(Thy1-EGFP)MJrs/J (EGFP
389 expressing neurons under the control of a modified Thy1 promoter region) mice of both sexes
390 were used. All surgeries were conducted under isoflurane anesthesia (1.5%) in a custom made
391 stereotactic apparatus equipped with a thermic plate (37°C). Prior to the surgery, toe-pinch
392 nociceptive responses were assessed and mice received anti-inflammatory (2.5 mg/kg
393 dexamethasone i.m; 5 mg/kg carprofen s.c.), analgesic (0.1 mg/kg buprenorphine i.m.) and local
394 anesthetic (1% lidocaine s.c. under the scalp) drugs.

395
396 **Stereotactic injections of GCaMP6.** Two to 4 weeks prior to the electrophysiological
397 experiments, layer 2/3 cortical neurons of C57/Bl6 mice were labeled with the genetically
398 encoded calcium indicator GCaMP6 using a viral vector. The scalp was shaved and sterilized
399 with ethanol 70% and a betadine solution. A small skin incision was performed over the motor
400 cortex (1 mm anterior and 0.8 mm lateral to Bregma) and a small craniotomy was performed
401 with a dental drill to allow for virus injection. Glass capillaries (Drummond) were pulled (Sutter
402 Instrument P-97) and beveled to attain thin and sharp pipettes (outer diameter <30 μm). A
403 pipette was loaded with a suspension of the adeno-associated virus AAV1-Syn-GCaMP6f

404 (UPenn, 2.96e12 GC) and lowered into the motor cortex (250 μm deep). A 50 nL injection (10-
405 20 nL/min) was performed using a piston-based injection system (Narishige). After the injection,
406 the scalp was sutured and mice were left to recover for at least two weeks.

407
408 ***Craniotomy for in vivo recordings.*** The day of the electrophysiological recordings, the scalp
409 was removed. The exposed skull was cleaned and dried. The periostium was removed with a
410 scalpel and a custom-made titanium head bar was cemented to the bone with a thin layer of
411 cyanoacrylate glue and covered with dental cement. In order to create a well for the water
412 immersion objective of the microscope, 150 μL of 1% agarose (w/v) were dripped on the skull
413 and left to jellify. The border of the agarose drop was covered with dental cement to create a 1
414 mm deep recording chamber and the dental cement was allowed to cure for 10 minutes. A round
415 craniotomy of 1-1.5 mm diameter was performed over the motor cortex taking care of not
416 damaging the dura. The exposed dura was thoroughly rinsed with sterile saline to prevent
417 bleeding and to remove bone debris and kept moistened throughout the experiment. Once set, the
418 mouse was placed under the two-photon microscope and held by the titanium head bar with a
419 custom-built holder.

420
421 ***In vivo imaging.*** Two-photon imaging was performed using a scanimage r4.1 controlled
422 microscope equipped with a resonant scanner head (Thorlabs), two GaAsP photomultiplier tubes
423 (Hamamatsu 10770PB-40; filters: red and green channel) and a 16x 0.8 NA water immersion
424 objective (Nikon)⁷. The laser beam was tuned at 940 nm (Ti-Sapphire Coherent Ultra II
425 Chameleon) and light pulse (140 fs) dispersion was corrected with a group velocity dispersion
426 compressor (Chameleon PreComp). Maximal power used (measured in the air at the focal plane)
427 was <50 mW. To stimulate ChR-2 expressing neurons, the microscope was also equipped with a
428 470 nm LED illumination source controlled by ephus. Maximal power at the focal plane was 500
429 μW . During the pipette approximation to the targeted cell, images (256 x 256 pixels) were
430 acquired at 60 fps and online averaged (10 frames rolling window average).

431
432 ***In vivo electrophysiology and single cell electroporation.*** 4-6 $\text{M}\Omega$ (for electrophysiology) or 12-
433 15 $\text{M}\Omega$ (for single cell electroporation) borosilicate pipettes (Science Products GmbH)

434 were pulled with a two-step vertical puller (Narishige) and coated with QDs as described above.
435 Electrophysiological recordings were performed using an Axoclamp 200B amplifier (Molecular
436 Devices) controlled by Ephus. Pipettes were held a 30°- 40° angle with the cortical surface and
437 the tip of the pipette was positioned on the surface of the dura diagonally aligned to the targeted
438 cell. For the dura penetration, the pressure of the pipette was set to 150 mbar and reduced to 50
439 mbar once it was inside the brain. The pipette was diagonally advanced up to the targeted cell
440 and minor lateral or vertical adjustments were made to avoid blood vessels. Pipette resistance
441 was continuously monitored to check for clogging. GCamp6-, VGAT-ChR2- and Thy1-GFP-
442 expressing neurons were simultaneously visualized with the fluorescent pipette (red or green
443 QDs) and the tip of the pipette was carefully advanced to the center of the neuron and the
444 positive pressure was released after a 50% increase in the pipette resistance. Targeted single cell
445 electroporation was performed as previously described⁹ using an Axoporation 800A (Molecular
446 Devices). Borosilicate pipettes were filled with internal solution and 50 µg/µl of DsRed plasmid.
447 After seal formation, a single electroporation train was applied (1 s, 50 Hz, 500 µs pulse
448 duration, -7 V). To prevent brain damage, a maximum of 3 penetrations were performed at the
449 same brain location. Noticeably, QDs are readily adsorbed to the dura, therefore the fluorescence
450 of the pipette that pierced the dura was dimmer - on average - than the following ones. In spite of
451 this, QDs were never adsorbed to the brain parenchyma even after repeated pipette penetrations
452 or long recordings.

453

454 **Data analysis.** In statistical comparisons, differences were considered significant when $P < 0.05$.
455 Statistical analysis was performed using two-tailed unpaired t-test or one-way ANOVA. All data
456 were tested and met the assumption for normal distribution. . In all figures, symbols and error
457 bars represent mean \pm S.D. Experiments were not randomized or blind.

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462 **Methods-Only References**

463

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