Supplementary Methods

Primary cell cultures

Experiments were approved by the Animal Care and Use committee, The Salk Institute.

Primary astrocytes were prepared essentially as previously described1-3. Briefly, hippocampus from P0 or adult rat (older than 6 weeks) were dissected and meninges were carefully removed. They were digested in Eagle’s Balanced Salt Solution with Papain (for P0 tissue) or in DMEM with a mixture of 0.1% neural protease, 0.01% papain and 0.01% DNAse I (for adult tissue) at 37°C for 30 min. Then they were washed with PBS, mechanically dissociated and plated on tissue culture flasks in Modified Eagle’s medium (MEM, D-Val) containing N2 supplement, fetal bovine serum (FBS, 10%), and glucose (0.2 M). MEM with D-valine substituted for L-valine was used to inhibit fibroblast proliferation. Once the cells became confluent, the flasks were plated on a rotary platform for 48 hr. Supernatants were removed and replaced with fresh medium containing 20 μM cytosine arabinoside (AraC) for 72 hr followed by fresh medium for 24 hr. These steps could be repeated to further eliminate other cell types2,3, including putative neural stem cells. Under these conditions, the majority of cells were astrocytes, which were GFAP+ (95 ± 3 % and 81 ± 4 % for P0 and adult, respectively; n = 3) and/or S-100β+ (88 ± 4 % and 69 ± 17 % for P0 and adult, respectively; n = 3), without contamination by oligodendrocytes (RIP+) or neurons (MAP2ab+). Importantly, these astrocytes did not give rise to any detectable neurons (MAP2ab+) under our co-culture conditions.
Primary neurons were prepared from P₀ hippocampus as previously described¹,⁴,⁵ They were plated at 10K/cm² on confluent feeder layers or micro-islands of astrocytes from P₀ hippocampus in serum-free co-culture medium containing MEM, N2 supplement, sodium pyruvate (1 mM), glucose (0.2 M), L-glutamine (2 mM) and Ovabumin (0.1%, Sigma, St. Louis, Missouri).

Normal diploid skin fibroblasts were isolated from full-thickness skin explants taken from adult female Fisher 344 rats as previously described⁶. Cells were propagated in high glucose (4.5 g/l) DMEM containing 10% FBS. No detectable MAP2ab⁺, GFAP⁺, or RIP⁺ cells were found in these skin fibroblasts.

Culture of stem cells derived from adult hippocampus
The stem cells were originally isolated from hippocampus of adult Fisher 344 rats as previously described⁷,⁸. Intact hippocampal formations were dissected from female adult Fisher 344 rats (160-170 g). The tissue was diced into small fragments (<1 mm³) and then digested for 20-45 min at 37°C in DMEM containing a mixture of 0.1% neural protease, 0.01% papain and 0.01% DNAse I. Two to three times during digestion, the tissues were gently disrupted by pipetting through a 5 ml plastic pipette. Then tissues were rinsed three times with PBS and plated on polyornithine and laminin-coated plates in DMEM/F12 medium containing 10% FBS and antibiotics. The next day the medium was replaced with DMEM/F12 containing N2 supplement, L-glutamine (2 mM) and FGF-2 (20 ng/ml). The cells grew as attached cultures and were fed every three days with fresh medium and FGF-2 (20 ng/ml, final concentration). Cultures were passaged
by trypsinizing, and rapidly proliferating cells that could be passaged, frozen in 10% 
DMSO, thawed, and recultured were isolated. For establishing clonal cultures, cultures 
were dissociated to a single-cell suspension and replated at low clonal density (10^3 
cells/ml) in the presence of filtered conditioned medium from original cultures. Single 
clusters of dividing cells were transferred to 96-well plates (1 clone/well) using glass 
pipettes. The expanded cultures from single clones were infected with retrovirus to 
express GFP and selected as previously described^7. They were propagated on poly-
ornithine/laminin coated plates in DMEM/F12 medium containing N2 supplement, L-
glutamine (2 mM) and FGF-2 (20 ng/ml). These GFP^+ stem cells have been cultured for 
over six months and were used between passages 20 and 30 in this study.

**Co-culture with different types of primary cells**

Substrate cells were cultured on poly-lysine and collagen-coated coverslips until they 
were confluent. Fixed astrocyte feeder layers were prepared by light fixation with cold 
70% ethanol at –20°C for 30 min, followed by extensive washes. For Banker type of co-
cultures, astrocytes were cultured on the bottom of the 24-well plate, while laminin-
coated coverslips were placed above with support in the same well without contact^1.
Substrate cells were incubated in serum-free co-culture medium overnight before GFP^+ 
stem cells were plated at 5K/cm^2 or 15K/ cm^2 in parallel for all culture conditions. They 
were fed every other day with fresh co-culture medium. AraC (0.5 µM) was added at day 
6 to eliminate proliferating cells in the long-term cultures. Some cultures on laminin-
coated glass were treated with retinoic acid (0.5 µM) and FBS (0.5%) on days 0, 2 and 4 
to promote neuronal differentiation^8 and were fed with medium containing 0.5% FBS
afterwards. For some experiments, rhBDNF (20 ng/ml) was added every other day after
day 6 in co-culture.

**Immunocytochemistry and electron microscopy**

Cultures were fixed with 4% paraformaldehyde and incubated with primary antibodies
overnight at 4°C in TBS++ (0.1 M Tris-buffered saline with 5% donkey serum and 0.25%
TX-100). They were incubated with fluorescently labeled secondary antibodies (1:250;
Jackson Immunoresearch) in TBS++ for 90 min at room temperature. Primary antibodies
and dilutions were used as follows (ms, mouse; rb, rabbit): MAP2ab (1:250; ms; Sigma,
St. Louis, Missouri), RIP (1:50; ms; Hybromeda Bank), GFAP (1:500, guinea pig;
Advance Immuno, Long Beach, California), s-100β (1:500; rb; Sigma, St. Louis,
Missouri); GAP-43 (1:1000; rb; Chemicon, Temecula, California), GluR1 (1:1000; rb;
Upstate Biotech, Lake Placid, New York), NF200KD (1:1000; rb; Chemicon, Temecula,
California), synapsin (1:1000; rb; Calbiochem, La Jolla, California), synaptophysin
(1:500; ms; Calbiochem, La Jolla, California) and CaM kinase II α-subunit (1:100; ms;
Calbiochem, La Jolla, California). Images were taken on a Bio-Rad confocal system at
40x or 63x with individual filter sets for each channel and assembled in PhotoShop.

For electron microscopy (EM), both fluorescent and phase images of GFP+ cells
in selected fields were taken for later identification. The cultures were processed for EM
preparation as previously described. Serial thin sections (60 nm) with previously
selected fields were examined in a JEOL 100CXII electron microscope at 80 kV. The
GFP+ cells were identified based on location and morphology as compared to those in the
phase and fluorescent images. Quantitative analysis was carried out as previously described\(^9\).

**Electrophysiology and FM-imaging**

Electrophysiological recordings were carried out at room temperature in the external recording solution containing (in mM): NaCl 145; KCl 3; HEPES 10; CaCl\(_2\) 3; glucose 8; MgCl\(_2\) 2 (pH 7.30). The micropipettes (2-4 M\(\Omega\)) were filled with internal recording solution containing (in mM): potassium gluconate 136.5; KCl 17.5; NaCl 9; MgCl\(_2\) 1; HEPES 10; EGTA 0.2 (pH 7.30). Primary neurons and GFP\(^+\) neuron-like cells were identified by the absence/presence of green fluorescence under UV and by their distinct neuronal morphology (small cell body, uneven and long processes). The criteria were confirmed by immunostaining in parallel cultures. Whole-cell patch-clamp recordings were carried out with patch-clamp amplifiers (Axopatch 200, Axon Instruments) as previously described\(^4,5\). Signals were filtered at 2 kHz, digitized at 5 kHz, and analyzed with programs written in Microsoft Visual Basic. To examine the excitability of neurons, transient membrane currents from neurons in 14-15d cultures induced by stepping holding membrane potential from -70 mV to 0 mV (50 ms) were recorded. Then the cell was switched to current-clamp and currents (0.5-1 nA, 20ms, or 200 ms, 0.1Hz) were injected through the patch pipette to examine whether action potential could be induced. TTX (0.5 \(\mu\)M; Sigma, St. Louis, Missouri) was used to block voltage-dependent Na\(^+\) channels. Spontaneous synaptic currents were collected during a 10- 20 min period of continuous recording in voltage-clamp (\(V_m = -70\) mV) and analyzed. For the evoked responses, neurons were stimulated at 0.1 Hz by 1-ms step depolarization from -70 mV.
to +20 mV in voltage-clamp mode, and the responses from the paired and the stimulated neuron itself were recorded and analyzed. Bicuculline methiodide (25 µM; Sigma, St. Louis, Missouri) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 µM; Sigma, St. Louis, Missouri) were used to block GABAA and AMPA receptors, respectively.

Imaging experiments with FM4-64 were carried out as previously described10. Cultures (20-30 d) were stained for 45 sec with FM4-64 (20 µM; Molecular Probe, Eugene, Oregon) in the external recording solution with CNQX (10 µM) and DL-APV (50 µM; sigma, St. Louis, Missouri), followed by 15 min of perfusion with dye-free solution. External solution with 90 mM K+ (3 min) was used to destain the FM4-64 dye. Fluorescent images of FM4-64 were taken under 40X oil lens right after staining and destaining, respectively. The images were analyzed using programs written in MATLAB.


