

Methods

Cell Culture and Microscopy

Dissociated postnatal (P1-2) rat hippocampal neuron cultures, plated at a density of 230-460/mm², were prepared as previously described (1). In all experiments, neurons were used following 14-23 days *in vitro*. Prior to treatment, neurons were infected with a Sindbis virus encoding the protein synthesis reporter (coding sequence for a myristoylated, destabilized GFP flanked by the 5' and 3' untranslated regions of α -CAMKII) (1). For infection, cells were washed once with growth medium (Neurobasal A, supplemented with B27 and Gluta MAX-1), then incubated with virus (diluted in growth medium) for 10-20 min at 37^o C. Following infection, cells were washed again with growth medium, then incubated with conditioned media containing either vehicle, TTX alone (0.5 - 2 μ M), or TTX + CNQX (40 μ M) + APV (20 μ M) for 7-8 hrs.

For imaging, conditioned media was replaced with HEPES buffered saline (HBS) containing, in mM: 119 NaCl, 5 KCl, 2 CaCl₂, 2 MgCl₂, 30 Glucose, 10 HEPES, pH 7.4 along with the appropriate pharmacological treatment. Neurons were maintained at 37^o C in HBS for 1.5-2.5 hrs prior to imaging. For experiments involving acute treatment with CNQX/APV or α -LTX, treatment/vehicle was administered immediately following the first (baseline) image. For experiments examining BOTOX or anisomycin, treatments were delivered in HBS a minimum of 2 hrs prior to imaging. All neurons chosen for experiments had a pyramidal neuron-like morphology with one or two major dendrites emerging from the soma. In initial experiments (Fig.1), 1-2 pyramidal-like neuron(s) from each dish were identified under epifluorescence and the brightest neurons were chosen for imaging. In each experiment, all treatments were represented and identical acquisition parameters were used to acquire images from all neurons thus permitting relative intensity comparisons between neurons under different treatments. In subsequent

experiments (Fig. 2-4) where the rate of synthesis in a given neuron was examined, images were acquired with parameters that maximized the dynamic range of pixel intensity for the dendritic signal in each neuron. Images were acquired at 30- or 60-min intervals depending on the experiment, and neurons were maintained at 37^B C between images.

All images were acquired in 0.4 μ m sections on a laser-scanning confocal microscope. GFP was excited at 488 nm with an argon laser and emitted light was collected between 519-550 nm. Image analysis was conducted on maximal intensity z-compressed image stacks. To facilitate estimation of distance, the primary dendrite from each cell was linearized using Image J software and fluorescence intensity was measured as a function of both time and distance from the soma. To examine changes in the rate of reporter synthesis along the length of the dendrite, $\Delta F/F_0$ (with 1 added to both terms to eliminate the influence of zero values) was calculated and expressed as a function of distance from the soma. For group analysis, the average intensity was integrated across the length of distal dendritic segments (>125 μ m from the soma) for each time-point and expressed relative to either control cells (Fig. 1D) or relative to the baseline image (Fig. 1E- Fig. 4). Since it is not known whether different dendrites from the same neuron are completely independent with respect to protein synthesis, only one primary dendrite from each cell was used for analysis. Thus, “n” refers to both the number of dendrites and number of cells within a group. For statistical analysis, data were analyzed either by unpaired t-tests (2 groups) or by ANOVA (> 2 groups) followed by Fisher’s LSD, and are expressed as mean \pm SEM. Values that were not significant are indicated as NS.

Extracellular Glutamate Measurements

Extracellular glutamate levels were determined with a fluorimetric assay under conditions that closely mimic our imaging experiments. 2 dishes from each of 6 different preparations were treated

chronically with 1 μ M TTX for 8 hrs in conditioned media, which was then replaced with TTX-containing HBS. Neurons were maintained at 37°C and HBS samples from each dish were taken at incubation times of 1, 2, or 4.5 hrs. Extracellular glutamate content was determined with the Amplex Red fluorimetric glutamic acid assay according to the manufacturer's instructions. Assays were performed at 37°C in standard 96-well plates and read at 10-min intervals with excitation of 544 nm and emission read at 590 nm.

Metabolic Labeling

Neurons were treated with TTX (2 μ M) for 10 hr in conditioned media, then incubated with HBS+TTX for 1 hr. 15-min prior to labeling, neurons were treated with 40 μ M CNQX + 50 μ M APV (TTX + mini block) or vehicle (TTX alone). Cells were then labeled with 20 μ Ci [35 S]methionine/cysteine for 45 min in HBS. Neurons were maintained at 37°C throughout the labeling and pre-incubation procedure. Following labeling, cells were washed 3X with ice-cold PBS-MC (PBS + 1 mM MgCl_2 + 0.1 mM CaCl_2) and collected in lysis buffer containing, in mM: 100 NaCl, 10 NaPO_4 (pH 7.4), 5 EDTA, 5 EGTA, 50 NaF, 10 $\text{Na}_4\text{P}_2\text{O}_7$, 2 NaVO_4 , 10 lysine, 1% Triton-X, 0.1% SDS, 1 tablet Complete Mini protease inhibitor cocktail/7 ml, pH 7.4. The samples were centrifuged at top speed in a microfuge for 15 min to remove any insoluble material, then total protein concentrations were determined by a modified Lowry assay. Equal quantities of protein for each sample (5-10 μ g) were loaded and separated on 4-15% Tris-HCl gradient gels, then transferred to PVDF membranes. Blots were rinsed with 100% methanol and dried, then exposed to film for 2-7 days at room temperature until the autoradiographic signal was optimal. Films were scanned and unprocessed images were analyzed by densitometry using Image J. Equal loading was confirmed post-hoc by re-probing the blots with a monoclonal anti-tubulin antibody (1:500), followed by chemiluminescent detection. Statistical differences between conditions were

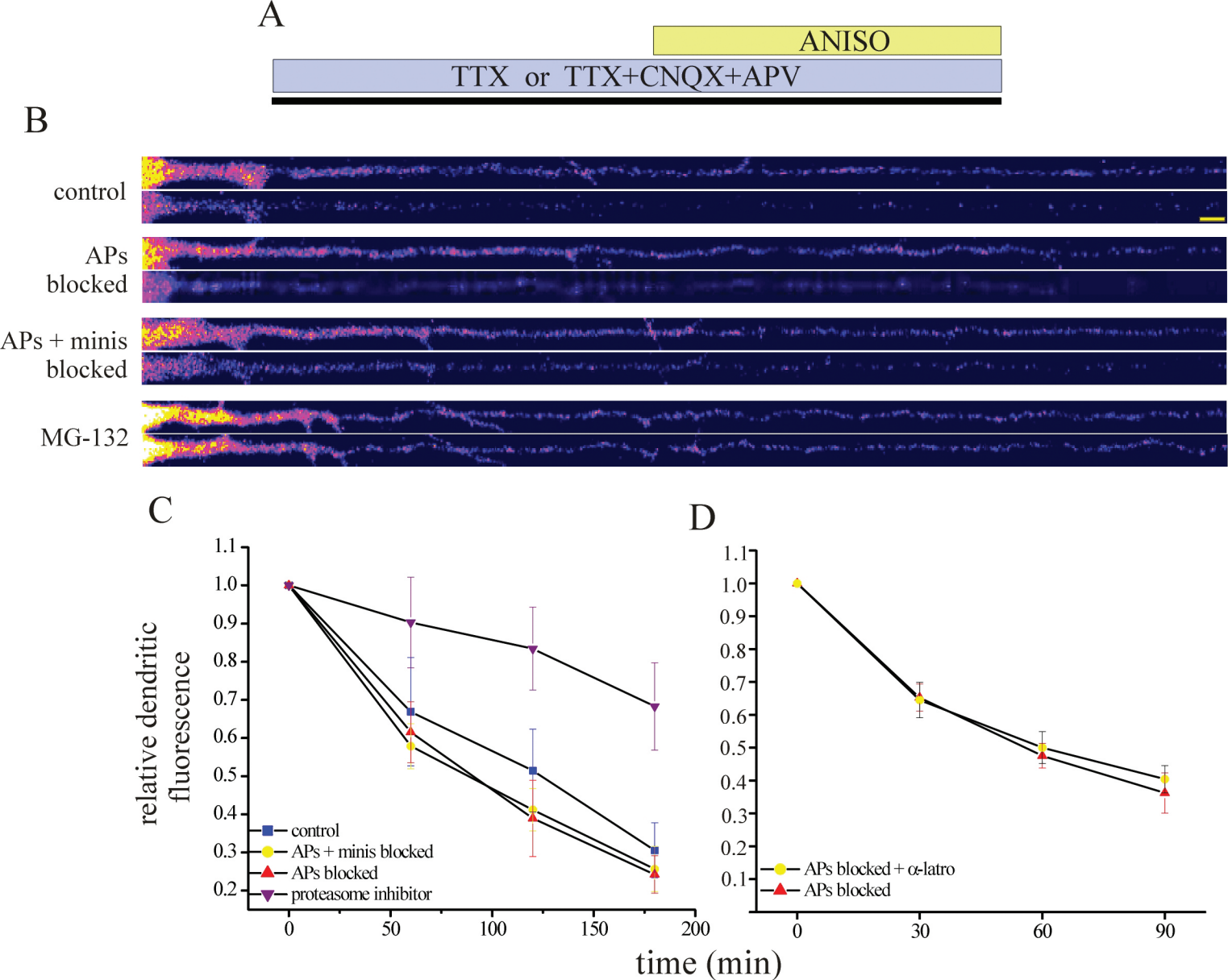
determined by a paired t-test on non-normalized values.

Electrophysiology

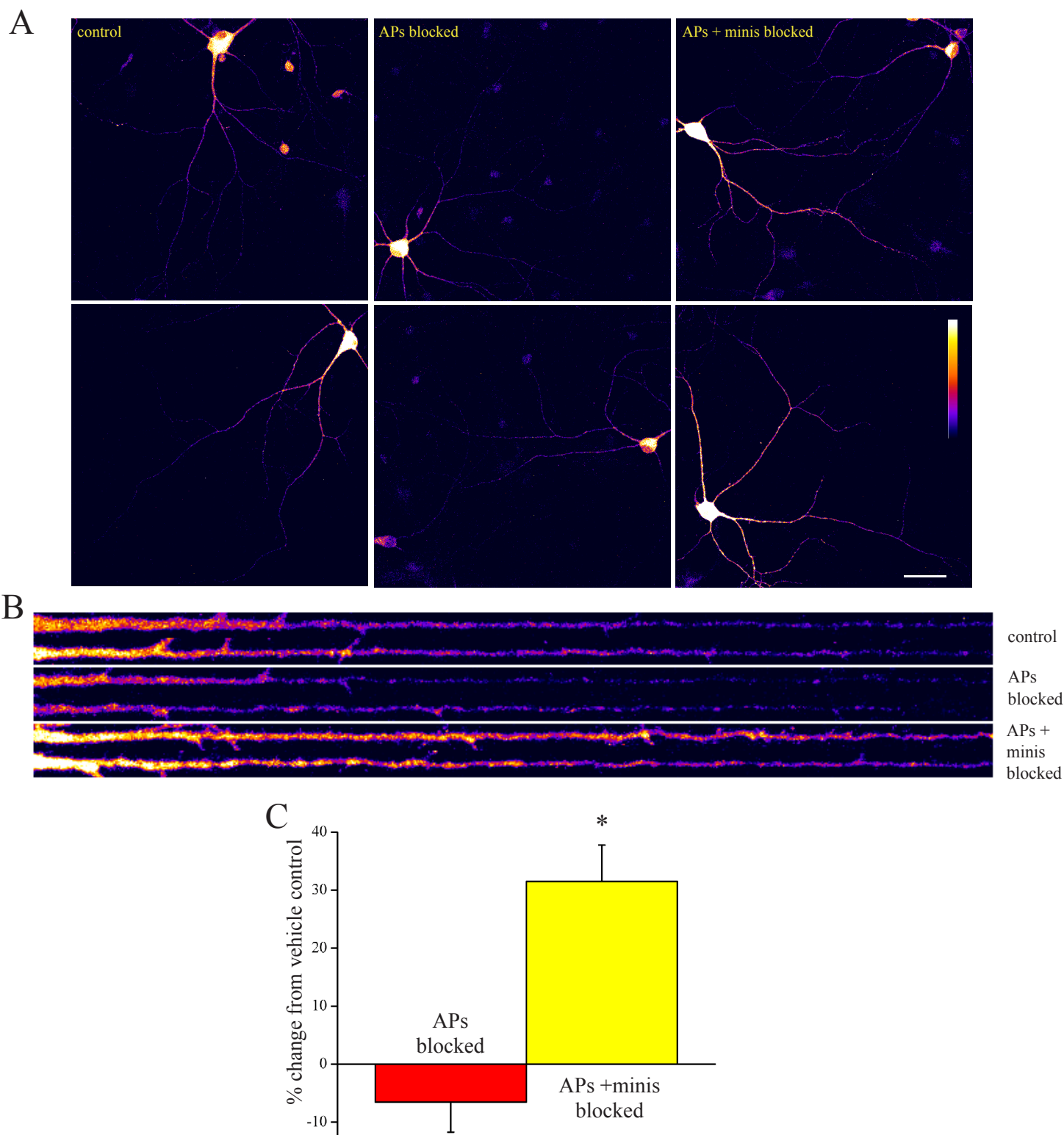
Whole-cell patch-clamp recordings were made with an Axopatch 200B amplifier from cultured hippocampal neurons bathed in HBS containing 1 μ M TTX and 50 μ M bicuculine. Whole-cell pipettes were filled with an internal solution containing, in mM: 100 cesium gluconate, 0.2 EGTA, 5 MgCl_2 , 2 adenosine triphosphate, 0.3 guanosine triphosphate, 40 HEPES, pH 7.2, with resistances ranging from 4-6 MO. Neurons with a pyramidal-like morphology were voltage-clamped at -70 mV and series resistance was left uncompensated. Only cells with < 10% change in series resistance were included for analysis. For analysis of BOTOX, dishes were pre-treated with either BOTOX (100 nM) or vehicle in HBS for 2-3 hrs at 37[°] C prior to recordings. For experiments using α -LTX, 10 μ M LaCl_3 was added to the HBS prior to recordings. After a baseline recording interval of 5-10 min, α -LTX (100 pM) was bath applied and mEPSCs were monitored for an additional 10-15 min period. mEPSCs were analyzed off-line using Synaptosoft mini analysis software.

Reference.

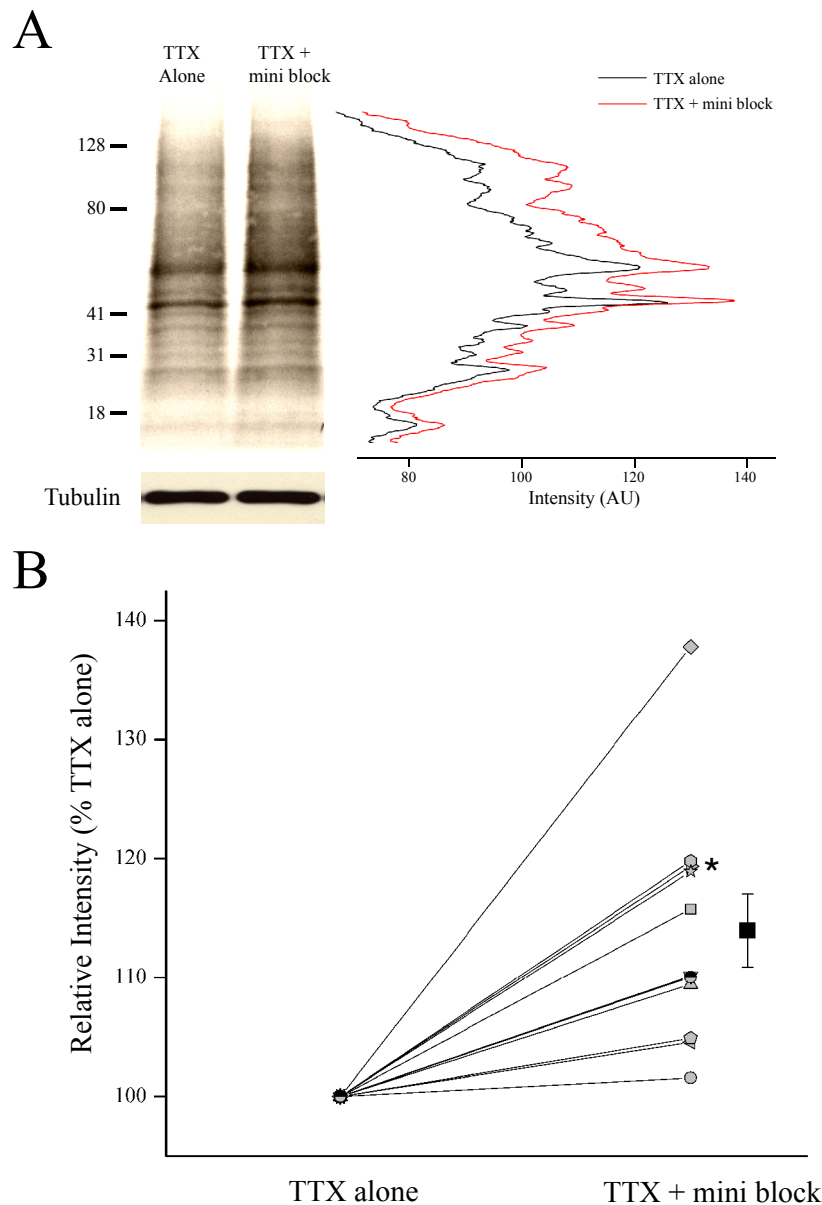
1. G. Aakalu, W. B. Smith, C. Jiang, N. Nguyen, E. M. Schuman, *Neuron* **30**, 489 (2001).



Suppl. Fig. 1. The effects of miniature synaptic events on the GFP reporter are due to translation, not degradation. (A) Experimental scheme: cultured hippocampal neurons were treated with either vehicle, TTX, or TTX+CNQX+APV for 9 hrs prior to imaging. At 7.0 hrs, the protein synthesis inhibitor anisomycin was applied. Image acquisition began 2 hrs later and continued at either 30- or 60-min intervals. One vehicle-pretreated group was treated with the proteasome inhibitor MG-132 (5 μ M) at the time of anisomycin application as a positive control. For experiments examining degradation in the presence of α -latrotoxin, it was applied immediately following acquisition of the baseline image. (B) Straightened dendrites from a time-lapse experiment in which neurons were treated as labeled. Shown are baseline images and those obtained 180 min later. Scale bar = 10 μ m. (C) Analysis of group data showing the change in reporter fluorescence levels for each group over time, as labeled. $n = 8$ for vehicle, APs blocked and APs + minis blocked groups, and $n = 6$ for the proteasome inhibitor group. Degradation rate did not differ between controls and either activity blockade condition (NS), but was significantly slowed by MG-132 ($p < 0.05$), indicating that the effects on GFP reporter levels observed following mini blockade are due to protein synthesis. (D) Analysis of group data showing the change in reporter fluorescence levels for APs blocked and APs blocked + α -latrotoxin groups ($n = 7$ for each group). Degradation rate did not significantly differ between the groups (NS).



Suppl. Fig. 2. Prolonged exposure to TTX is not required to observe the enhancement of local protein synthesis induced by mini blockade. Dishes of cultured hippocampal neurons were acutely exposed to one of 3 treatments: vehicle, TTX or TTX + GluR antagonists. Following a 2 hour treatment, dishes were fixed and the GFP signal in individual neurons was imaged and analyzed. **(A)** Two representative neurons and **(B)** their dendrites for each condition. Relative to controls, neurons acutely exposed to TTX showed lower levels of fluorescence in their dendrites. Neurons acutely exposed to TTX + GluR antagonists exhibited significantly higher levels of fluorescence than either the control or TTX alone groups. **(C)** Analysis of group data indicating that acute mini blockade (in the absence of prolonged TTX exposure) resulted in significantly higher levels of protein synthesis in distal dendrites relative to controls and TTX alone-treated neurons. N = 119 dendrites/neurons from a total of 5 dishes for each group. Scale bar in part A = 50 μ m.



Suppl. Fig. 3. Miniature synaptic events exert broad inhibition on synthesis of endogenous proteins. **(A)** Representative autoradiogram (left) and line-scan analysis (right) from metabolic labeling experiments in which cultured hippocampal neurons were treated chronically with TTX (2 μ M) followed by acute challenge with either CNQX+APV (TTX + mini block) or vehicle (TTX alone). No detectable autoradiographic signal was observed in control neurons treated with anisomycin (40 μ M) 1-hr prior to labeling ($n = 2$, data not shown). In the image shown, the contrast has been enhanced to allow for easier visualization of treatment differences; quantification of intensity was performed on unprocessed images. Approximate molecular weight standards (in kDa) for the autoradiogram is shown on the left; tubulin immunoreactivity (bottom) confirmed equal loading. **(B)** Shown are the individual results (grey symbols) and summary (black symbol) of eleven independent experiments in which the integrated autoradiograph signal between molecular weight markers of 200 and 18 kDa was compared between matched samples made from cultures treated with either TTX alone or TTX + mini block (as in part a). Intensity of the 35S signal was significantly ($p < 0.05$) greater when labeling was conducted under conditions where miniature events are blocked. Quantification of tubulin immunoreactivity confirmed equal loading for all samples (tubulin signal intensity: mean \pm SEM TTX + mini block /TTX alone = 1.016 ± 0.028). The experiment shown in part A is denoted by an asterisk.