

Brain-Derived Neurotrophic Factor Enhances GABA Release Probability and Nonuniform Distribution of N- and P/Q-Type Channels on Release Sites of Hippocampal Inhibitory Synapses

Pietro Baldelli,¹ Jesus-Miguel Hernandez-Guijo,² Valentina Carabelli,² and Emilio Carbone^{1,2}

¹Istituto Nazionale di Fisica della Materia Research Unit and ²Department of Neuroscience, Nanostructured Interfaces and Surfaces Center, I-10125 Turin, Italy

Long-lasting exposures to brain-derived neurotrophic factor (BDNF) accelerate the functional maturation of GABAergic transmission in embryonic hippocampal neurons, but the molecular bases of this phenomenon are still debated. Evidence in favor of a postsynaptic site of action has been accumulated, but most of the data support a presynaptic site effect. A crucial issue is whether the enhancement of evoked IPSCs (eIPSCs) induced by BDNF is attributable to an increase in any of the elementary parameters controlling neurosecretion, namely the probability of release, the number of release sites, the readily releasable pool (RRP), and the quantal size.

Here, using peak-scaled variance analysis of miniature IPSCs, multiple probability fluctuation analysis, and cumulative amplitude analysis of action potential-evoked postsynaptic currents, we show that BDNF increases release probability and vesicle replenishment with little or no effect on the quantal size, the number of release sites, the RRP, and the Ca^{2+} dependence of eIPSCs. BDNF treatment changes markedly the distribution of Ca^{2+} channels controlling neurotransmitter release. It enhances markedly the contribution of N- and P/Q-type channels, which summed to $>100\%$ (“supra-additivity”), and deletes the contribution of R-type channels. BDNF accelerates the switch of presynaptic Ca^{2+} channel distribution from “segregated” to “nonuniform” distribution. This maturation effect was accompanied by an uncovered increased control of N-type channels on paired-pulse depression, otherwise dominated by P/Q-type channels in untreated neurons. Nevertheless, BDNF preserved the fast recovery from depression associated with N-type channels. These novel presynaptic BDNF actions derive mostly from an enhanced overlapping and better colocalization of N- and P/Q-type channels to vesicle release sites.

Key words: neurotrophins; multiple probability fluctuation analysis; peak-scaled variance analysis; inhibitory synaptic transmission; N- and P/Q-type channels; RRP

Introduction

During neuronal development, brain-derived neurotrophic factor (BDNF) promotes the formation, maturation, and stabilization of both glutamatergic and GABAergic synapses in the CNS, regulating the balance between excitatory and inhibitory transmission. This is a fundamental step for neural circuit formation (Poo, 2001; Lessmann et al., 2003). To date, one of the most unclear aspects of the action of BDNF on the CNS concerns the molecular mechanism underlying the chronic effect on synaptic functions. In particular, it is not known how long-term exposures to BDNF can induce strong potentiation and increased connec-

tivity of inhibitory synapses in various brain regions (Vicario-Abejon et al., 2002). The available data on inhibitory synapses indicate that BDNF causes net increases in the frequency of miniature IPSCs (mIPSCs) and in the size of spontaneous and evoked IPSCs (eIPSCs) (Rutherford et al., 1997; Vicario-Abejon et al., 1998; Huang et al., 1999; Bolton et al., 2000; Baldelli et al., 2002; Yamada et al., 2002). Some of the above effects could be ascribed to an increased number of inhibitory neurons and to an enhanced arborization of dendritic and axonal processes (Vicario-Abejon et al., 1998; Kohara et al., 2003). This response, however, was clearly observed in hippocampal and cerebellar slices (Marty et al., 2000; Seil and Drake-Baumann, 2000) and hippocampal cultures of embryonic day 16 (E16) rat embryos (Vicario-Abejon et al., 1998) but was much less evident in E18 embryos (Sherwood and Lo, 1999; Bolton et al., 2000; Baldelli et al., 2002).

Most of the published data support a presynaptic site of action for BDNF that results in an increased size and intensity of GAD immunopositive terminals (Huang et al. 1999; Bolton et al., 2000; Yamada et al., 2002), an enhanced frequency of mIPSCs (Vicario-Abejon et al., 1998; Bolton et al., 2000; Baldelli et al., 2002), and a

Received Oct. 12, 2004; revised Feb. 16, 2005; accepted Feb. 16, 2005.

This project was supported by the Cavalieri-Ottolenghi foundation, by Italian Ministero dell'Istruzione, dell'Università e della Ricerca Grant 2003/249, and by a Marie Curie Fellowship to J.M.H.-G. (contract HPMF-CT-2000-00899). We thank Fabio Benfenati and Egidio D'Angelo for helpful discussions. We thank also Claudio Franchino and Brunella Tedesco for helping with cell cultures and preparation of solutions.

Correspondence should be addressed to Pietro Baldelli, Department of Experimental Medicine, Section of Human Physiology, University of Genoa, Viale Benedetto XV, 3, I-16132 Genoa, Italy. E-mail: pietro.baldelli@unige.it.

DOI:10.1523/JNEUROSCI.4227-04.2005

Copyright © 2005 Society for Neuroscience 0270-6474/05/253358-11\$15.00/0

higher paired-pulse depression (PPD) reflecting increased vesicle depletion (Baldelli et al., 2002). However, an increase in GABA_A receptor density has been also observed, suggesting the involvement of postsynaptic mechanisms (Yamada et al., 2002). Despite these well established findings, there is an impressive lack of information on how BDNF affects the elementary parameters controlling neurotransmitter release: probability of release [release probability (P_r) and vesicle release probability (P_{ves})], GABA_A receptor conductance, number of release sites, number of vesicles forming the readily releasable pool (RRP), and the distribution and role of presynaptic Ca²⁺ channels. This is a critical issue that would help to clarify the basic mechanisms of action of BDNF on developing neurons.

Here, using the variance analysis of mIPSCs (Traynelis and Jaramillo, 1998), the fluctuation analysis, and the cumulative amplitude profile of eIPSCs (Schneggenburger et al., 1999; Clements and Silver, 2000), we show that in inhibitory GABAergic synapses, BDNF primarily produces an increased P_r and P_{ves} , with little change in the number of release sites, the RRP, quantal content, and unitary conductance of GABA_A receptors. In line with previous reports indicating a BDNF-induced increase in Ca²⁺ channel synthesis and KCl-evoked synaptic activity (Baldelli et al., 2000, 2002), we bring here new evidence in favor of a BDNF-induced increase in P_r and P_{ves} associated with an enhanced contribution and “nonuniform” distribution of N- and P/Q-type channels to action potential-evoked IPSCs together with a new role of N-type channels in PPD. This represents a novel mechanism of the action of BDNF on the maturation of inhibitory synapses, which may result from a better coupling of presynaptic Ca²⁺ channels with vesicle release sites.

Materials and Methods

Cell culture and BDNF treatment. All experiments were performed in accordance with the guidelines established by the National Council on Animal Care and approved by the local Animal Care Committee of Turin University. Pregnant Sprague Dawley rats were killed by inhalation of CO₂, and E18 rats were removed immediately by cesarean section. Removal and dissection of the hippocampus, isolation of neurons, and culturing procedures were as described previously (Baldelli et al., 2000). The isolated hippocampal neurons were plated at low density (200 cells/mm²) and allowed to establish for 4 d, after which 15 ng/ml BDNF (Sigma, St. Louis, MO) was added every 3 d for the 3–4 weeks of the culture. When required, the cultured neurons were exposed to the tyrosine kinase B (TrkB)-specific inhibitor anti-TrkB IgG1 (10 μg/ml) (clone 47; Transduction Laboratories, Lexington, KY), which effectively inhibits BDNF binding to TrkB receptors (Balkowiec and Katz, 2000). All of the experiments were recorded in 14–21 d *in vitro* (DIV) neurons.

Current recordings, data acquisition, and analysis. Patch electrodes, fabricated from thick borosilicate glasses (Hilgenberg, Mansfield, Germany), were pulled and fire-polished to a final resistance of 2–4 MΩ. Patch-clamp recordings were performed in whole-cell configuration using an EPC-9 amplifier (HEKA Electronic, Lambrecht, Germany). mIPSCs and eIPSCs were acquired at 1–10 kHz sample frequency and filtered at one-half the acquisition rate with an eight-pole low-pass Bessel filter. Recordings with leak currents of >100 pA or a series resistance of >20 MΩ were discarded. Data acquisition was performed using Pulse programs (HEKA Elektronik). All of the experiments were performed at room temperature (22–24°C). Data are expressed as mean ± SEM for number of cells (n). Unpaired Student's t tests were used, and $p < 0.05$ was considered significant.

Solutions and drugs. mIPSCs and eIPSCs were recorded by superfusing the whole-cell-clamped postsynaptic neuron with Tyrode's solution containing (in mM): 2 CaCl₂, 150 NaCl, 1 MgCl₂, 10 HEPES, 4 KCl, and 10 glucose, pH 7.4. When required, solutions with higher Ca²⁺ concentrations (5–10 mM) were obtained by lowering the NaCl content. The unselective glutamate receptor antagonist kynurenic acid (1 mM) (Sigma)

was added to the Tyrode's solution to block excitatory transmission. Tetrodotoxin (0.3 μM) was added to block spontaneous action potential propagation. When recording eIPSCs, the external solution was supplemented with the selective GABA_B inhibitor (2S)-3-[[[(1S)-1(3,4-dichlorophenyl) ethyl] amino-2-hydroxypropyl] (phenylmethyl) phosphinic acid (5 μM). The neuron was constantly superfused through a gravity system, as described previously (Baldelli et al., 2002). The perfusion solution could be changed rapidly (50–60 ms) and applied for controlled periods. The tip of the perfusion pipette (100–200 μm) was placed 40–80 μm to the soma. The standard internal solution was (in mM): 90 CsCl, 20 TEA-Cl, 10 EGTA, 10 glucose, 1 MgCl₂, 4 ATP, 0.5 GTP, and 15 phosphocreatine, pH 7.4. K⁺ was substituted for Cs⁺ and TEA⁺ in the pipette solution to block outward K⁺ currents. N-(2,6-dimethylphenylcarbamoylmethyl) triethylammonium bromide (10 mM) was added to block Na⁺ currents activated during an eIPSC. Solutions containing nifedipine, ω-conotoxin-GVIA (ω-CTx-GVIA), and ω-agatoxin-IVA (ω-Aga-IVA) were prepared as indicated previously (Magnelli et al., 1998). BDNF (Sigma) was dissolved in distilled water containing 1 mg/ml BSA and maintained in stock solutions at –80°C.

Peak-scaled variance analysis. For the reasons discussed by Traynelis et al. (1993), analysis of mIPSCs was preferred to the analysis of eIPSCs to determine the conductance of postsynaptic receptor channels. Briefly, when averaged mIPSCs and eIPSCs are normalized and superimposed, it is immediately apparent that the rise time of evoked currents could also be twofold slower than miniature synaptic currents. Two possible explanations for this are (1) that inhibitory fiber stimulation activates several discrete release sites or (2) that there is multiple vesicle release at individual sites, even when evoked release is apparently synchronous. In either case, asynchronous transmitter release will increase the deviation of individual synaptic currents from the mean IPSC time course. Such current deviation could potentially introduce errors in the evaluation of postsynaptic single-channel conductance. On the contrary, the mIPSCs that result from the release of one or more transmitter packets presumably arise from nearly synchronous release of transmitter and receptor activation and are ideal for nonstationary fluctuation analysis.

The variance analysis of mIPSCs was performed as described by Traynelis et al. (1993) using MiniAnalysis programs (version 6.0.1, Synaptosoft, Leonia, NJ). The requirements for such analysis include the stability of the current decay time course throughout the recording and the absence of any correlation between decay time course and peak amplitude. For each recording, we used a number of events ranging between 40 and 120 events, eliminating all of the mIPSCs with the decay time distorted by double peaks or anomalous noise. Four to 20 min of continuous stationary activity was recorded in control and BDNF-treated neurons.

To isolate current fluctuations associated with the random channel-gating properties from those arising from variation in neurotransmitter release and number of postsynaptic receptors, the mean waveform obtained from the average of 40–120 traces was scaled to the peak of each individual mIPSC and subtracted (Traynelis et al., 1993; Nusser et al., 1997). The current–variance relationship for the decay of mIPSCs was calculated by dividing the decay phase ($10 \times \tau_{decay}$) into 10–30 sections based on equal fractional reductions in the amplitude. This resulted in sections that contained on average the same number of channel closures. Baseline variance (σ_B^2) was measured from postevent current (5.3 ± 3.3 pA²) and subtracted from the variance associated with each section. The single-channel current was calculated by fitting the relationship between peak-scaled variance $\sigma^2(t)$ and the mean amplitude $I(t)$ with the following equation: $\sigma^2(t) = iI(t) - (I^2(t)/N_{ch}) + \sigma_B^2$, where i is the weighted mean unitary current and N_{ch} is the number of channels open at the peak of mIPSCs. $I(t)$ is defined as follows: $I(t) = iP_o N_{ch}$, with P_o indicating the probability of postsynaptic channel opening. Scaling the average to the peak of mIPSCs provides a better estimate of the unitary postsynaptic current. However, this method is based on the assumption that the intrinsic stochastic variance is null at the peak of individual mIPSCs [i.e., that all postsynaptic channels participating to a given synaptic current are open at the peak of the current ($P_o = 1$)]. As discussed by Traynelis et al. (1993) and De Koninck and Mody (1994), several factors make the variance >0 at the peak of the synaptic current. In this case, the current–

variance relationship is skewed, and the only parameter that can be reliably estimated is the unitary current i (initial slope of the parabola).

eIPSCs and PPD. Monosynaptic GABAergic eIPSCs were investigated in pairs of 14–21 DIV cultured neurons. Presynaptic stimuli were delivered through a glass pipette of 1 μm tip diameter filled with Tyrode's solution placed in contact with the soma of the GABAergic interneuron in a loose-seal configuration (Baldelli et al., 2002). Current pulses of 0.1 ms and variable amplitude (6–24 μA) delivered by an isolated pulse stimulator (model 2100; A-M-System, Carlsburg, WA) were required to induce monosynaptic eIPSCs with short latency (2–4 ms). To ensure that only the synaptic contacts of the selected presynaptic neuron were stimulated by the extracellular stimulating pipette, for the analysis we used only those eIPSCs that were completely lost after a few micrometer displacements from the soma of the presynaptic neuron and preserved an all-or-none response to stimuli of graded intensity. The evoked currents remained stable for stimulation intensities two times the threshold. Stimulation intensity was set at 1.5 times the threshold for all experiments. The postsynaptic neuron was voltage-clamped and maintained at a holding potential of -70 mV. The current artifact produced by the presynaptic extracellular stimulation was subtracted in all of the eIPSCs shown. During long-lasting experiments in which the extracellular solution was changed several times, such as during dose-responses with different concentrations of extracellular calcium ($[\text{Ca}^{2+}]_o$), a correction factor of 5–14% was used to compensate for the slight amplitude rundown of eIPSCs.

Synaptic depression and recovery from depression induced by paired-pulse stimulation were studied by recording the eIPSCs associated with two consecutive stimuli separated by 20–800 ms interpulse intervals. The current traces used for the analysis were averages of eight consecutive responses repeated every 8 s. eIPSCs were inspected visually and rejected if spontaneous activity altered the recordings. PPD was calculated as follows: $\text{PPD} = 1 - (A_2/A_1)$, with A_1 and A_2 indicating the amplitude of the first and second eIPSCs. According to the activity-dependent depletion model of available release sites (Betz, 1970), A_1 is equal to the product of the number of release sites (N) multiplied by P_r and the quantum size (q): $A_1 = NqP_r$. Immediately after the first pulse, the number of release sites that remain effective is $N_2 = N - NP_r$, and the amplitude of the second eIPSCs is $A_2 = N_2qP_r = NqP_r(1 - P_r)$, if no facilitation is present and P_r remains unchanged during the two eIPSCs. Although these assumptions are often not valid, it follows that the PPD at brief interpulses furnishes an estimate of P_r : $\text{PPD} = 1 - (A_2/A_1) = P_r$.

We made no systematic measurements to verify these hypotheses in our experimental conditions. On the contrary, we had evidence that PPD at 20–50 ms significantly overestimated the P_r values calculated from the multiple probability fluctuation analysis illustrated below. Nevertheless, we found that variations in PPD within the same cell followed rather closely the variations in P_r . For instance, by changing extracellular Ca^{2+} from 2 to 5 mM, we obtained P_r changes of 28 and 29% in control and BDNF treated-neurons, respectively (see Fig. 3D), and changes in mean PPD of 24 and 25% in corresponding groups of neurons (see Fig. 6B). In addition to this, we never observed paired-pulse facilitation but rather homogeneous depressions to paired-pulse stimulations under conditions of high probability of release. Thus, although PPD could not be taken as an absolute indicator of P_r , changes in PPD appeared to be sufficiently well correlated to changes in P_r within the same cell. This is in line with recent observations on excitatory synapses for which average PPD is not found to be a reliable indicator of P_r in a cell population but rather to be linearly related to P_r within the same cell (Oleskevich et al., 2000).

Multiple probability fluctuation analysis. Analysis of the variance–mean (V – M) plots was used to estimate three main parameters describing the synaptic function (Clements and Silver, 2000): the average amplitude of the postsynaptic response to a vesicle of transmitter (Q_{av}), the average probability of vesicle release from a release site (P_{rav}), and the number of independent release sites (N). The three parameters were derived from the parabolic relationship between eIPSC variance (σ^2) and mean postsynaptic current amplitude (I_{av}): $\sigma^2 = AI_{av} - BI_{av}^2$, recorded under different release probability conditions by assuming that $I_{av} = NQ_{av}P_{rav}$ (Silver et al., 1998; Reid and Clements, 1999). A and B (the initial slope

and the curvature of the parabola, respectively) are free parameters that are adjusted to optimally fit the V – M plots and used to calculate a weighted mean of P_{rav} and Q_{av} and a lower limit for the number of independent release sites, N_{min} :

$$\begin{aligned} N_{min} &= 1/B \\ Q_{av} &= A/(1 + CV_i^2) \\ P_{rav} &= I_{av}(B/A)(1 + CV_i^2). \end{aligned}$$

CV_i is the coefficient of variation of mIPSC amplitudes at an individual release site. In our case, CV_i is 0.41 ± 0.04 ($n = 13$) in controls and 0.43 ± 0.04 ($n = 12$) in BDNF-treated cells (see Fig. 1D), indicating that the correction factor $(1 + CV_i^2)$ appears as a minor adjustment in both conditions (17–18%). The adjustment, however, introduces a small discrepancy between Q_{av} and mean mIPSC amplitudes (19.3 vs 25.2 pA in controls) (see Figs. 1E, 3E), which does not limit the conclusions of our analysis. The estimated lower values of Q_{av} in control and BDNF-treated cells are likely associated with the asynchronous release of unitary events, which produces eIPSCs of lower peak amplitudes than the linear sum of synchronized elementary events. In our case, BDNF did not specifically affect the mean latency associated with asynchronous release, which could introduce either delayed activation or prolonged decay phases of eIPSCs. In fact, BDNF had no effect on both the rising and the decaying phase of eIPSCs (see Results) and preserved the kinetic properties of mIPSCs (Baldelli et al., 2002). This implies that there is no serious need to correct the discrepancy between Q_{av} and mean mIPSCs when comparing effects in control and BDNF-treated neurons. Another possibility for the lower value of Q_{av} is that the mIPSC amplitudes are overestimated because of the occasional coincidence of two release events. Attempts to filter out double-peak events will not abolish all such paired events.

P_r was varied by changing $[\text{Ca}^{2+}]_o$ (2 and 5 mM) or adding Cd^{2+} to the external solution (0.5, 2, and 6 μM). I_{av} and σ^2 were calculated over a stable epoch of 30–150 events after the wash-in of each extracellular solution. Presynaptic stimulation continued during the wash-in at 0.1 Hz. After the solution exchange, the eIPSC amplitude remained stable throughout the subsequent analysis epoch. The variance attributable to the recording noise was estimated in the region before the test pulse and subtracted from the eIPSC variance. A zero point was included in each V – M plot to indicate that the noise variance was subtracted. In $\sim 10\%$ of epochs, the synaptic response decreased during the recording period, and the variance was calculated after subtracting a fitted regression line. This rundown correction was usually required under conditions in which P_r was high (Oleskevich et al., 2000). Data were rejected if the decrease was $>20\%$.

Cumulative eIPSC amplitude analysis during trains. To complete the set of parameters characterizing the synaptic function, we also estimated the RRP of synchronous release (RRP_{syn}) and the probability that any given vesicle in the readily releasable pool will be released. We indicated this quantity as P_{ves} to distinguish it from P_{rav} (Schneppenburger et al., 2002). RRP_{syn} was determined by summing up peak IPSC amplitudes during 15 repetitive stimuli applied at a frequency of 10 Hz. This analysis assumes that depression during the steady-state phase is limited by a constant replenishment of vesicles and that equilibrium occurs between release and vesicle replenishment (Schneppenburger et al., 1999). The number of data points to include in the linear fit of the steady-state phase was evaluated by adopting an objective criterion. Starting from the last data point (15th stimulus), we calculated the best linear fit including the maximal number of data points. We observed in all control ($n = 8$) and treated ($n = 8$) neurons that the cumulative amplitude profile showed the best linearity, after the first eight stimuli, in the range of 800–1400 ms. To estimate the cumulative eIPSC amplitudes, the last seven data points were then fitted by linear regression and back-extrapolated to time 0 (see Fig. 4B). The intercept with the y -axis gave RRP_{syn} , and the ratio between the first eIPSC amplitude (I_1) and RRP_{syn} furnished P_{ves} . Dividing RRP_{syn} by the mean amplitude of mIPSCs, we could also determine the total number of vesicles ready for release (N_{syn}).

Results

Rat embryo hippocampal neurons maintained in culture until 3 weeks of age were used to evaluate the effect of long-term exposure to BDNF on miniature and action potential-evoked IPSCs

(mIPSCs and eIPSCs). As reported previously (Baldelli et al., 2002), BDNF treatment (15 ng/ml added 4 d after plating and read every 3 d for the 3–4 weeks of culture) enhanced the spontaneous and KCl-evoked inhibitory transmission depending on the DIV of neurons. Here, we focused on 14–21 DIV neurons because of the high reproducibility and low fatigability of the synaptic response to action potential stimulation. This is an essential requisite for long-lasting electrophysiological protocols and for correctly determining the elementary parameters of transmitter release.

GABAergic synaptic activity recorded from most of the 14–21 DIV neurons (>97%) was characterized by action potential-evoked IPSCs with fast activation ($\tau_{\text{activ}} = 2.8 \pm 0.36$ ms) and slow decay ($\tau_{\text{decay}} = 61.3 \pm 0.8$ ms). These eIPSCs closely resembled those generated by hippocampal CA1 interneurons, the activity of which is controlled by N- and P/Q-type presynaptic Ca^{2+} channels, and are classified as slow GABA_A inhibitory responses (Wilson et al., 2001). Occasionally, we also observed fast eIPSCs with decay times in the order of 20 ms that were systematically discarded from the analysis. This, together with the observation that the GABAergic transmission in 14–21 DIV neurons was controlled by mixtures of N- and P/Q-type channels (see below and Ohno-Shosaku et al., 1994), suggested that our recordings derived from a rather homogeneous population of GABAergic interneurons characterized by slow-decaying inhibitory responses.

BDNF increases eIPSC amplitude and mIPSC frequency

Figure 1A depicts three typical eIPSCs recorded from 18 DIV hippocampal neurons that were maintained under control conditions, incubated with BDNF (15 ng/ml), or pretreated with BDNF plus the selective antibody for TrkB receptors, anti-TrkB IgG1 (10 $\mu\text{g/ml}$), which antagonizes the action of BDNF. BDNF caused a net increase in mean eIPSC amplitude (from 2.1 to 3.7 nA; $n = 18$; $p < 0.01$) that was effectively prevented by the anti-TrkB IgG1 (2.3 vs 2.1 nA; $n = 18$). Despite the increase in amplitude, BDNF had no significant effect on the time course of the eIPSCs, the activation and decay time constants of which remained nearly unchanged: τ_{activ} was 2.8 ± 0.36 pA and τ_{decay} was 61.3 ± 8.4 ms with controls ($n = 6$); τ_{activ} was 2.9 ± 0.38 pA and τ_{decay} was 57.6 ± 7.3 ms with BDNF ($n = 6$). The neurotrophin also induced a net increase in miniature frequency (from 1.0 ± 0.1 to 1.5 ± 0.2 Hz) (Fig. 1B,C) and a weak shift in the profile of the amplitude histogram toward events of higher amplitude (Fig. 1D), with no significant changes to the mean amplitude of mIPSCs (25.2 and 27.3 pA) (Fig. 1E). The action of BDNF on the amplitude of miniatures was more marked in 7–14 DIV neurons and probably depended on an accelerated maturation of functional synapses (Wang et al., 1995).

BDNF does not affect the unitary conductance of postsynaptic receptors

If BDNF does not alter the mean amplitude of miniature events in 3-week-old hippocampal neurons, it is likely that the neurotrophin preserves the unitary conductance and the density of postsynaptic GABA_A receptors. To examine this possibility, we estimated the unitary conductance of GABA_A channels by using the peak-scaled variance analysis (PSVA) of mIPSCs, which allows an estimate of the unitary current carried by single postsynaptic channels (Traynelis et al., 1993). Figure 2, A and B, summarizes the procedure followed to determine the variance, $\sigma^2(t)$, from a group of mIPSCs (see Materials and Methods).

In 14–21 DIV neurons, BDNF caused almost no changes in $\sigma^2(t)$, yielding a relationship between mean current, I , and peak-

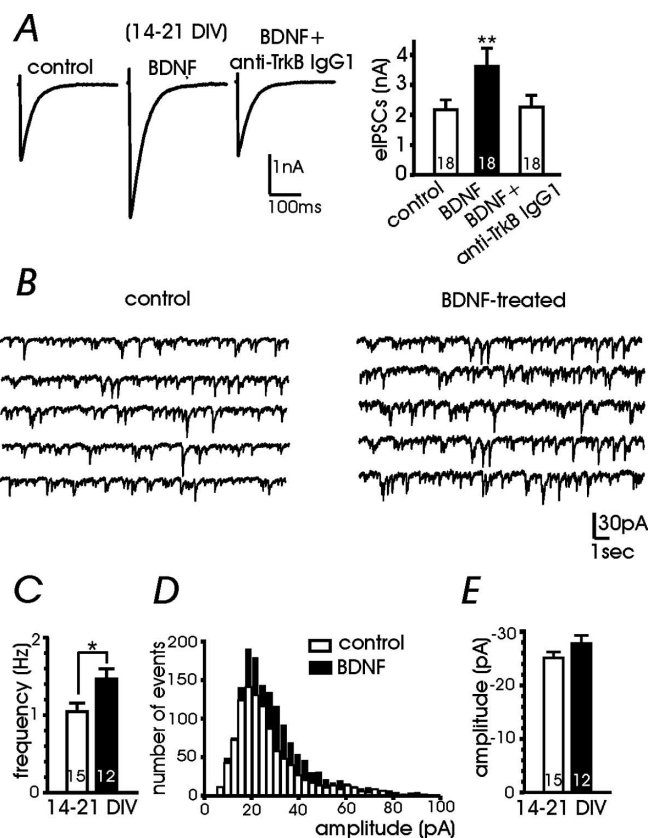


Figure 1. Effects of BDNF on eIPSC and mIPSC GABAergic currents recorded at the soma of whole-cell-clamped hippocampal neurons. **A**, Left, eIPSCs recorded from 14–21 DIV neurons under control conditions, with BDNF and BDNF plus anti-TrkB IgG1 ($V_h = -70$ mV). Right, Mean amplitude of eIPSCs in the conditions and number of neurons indicated; $**p < 0.01$ versus controls and anti-TrkB IgG1 plus BDNF using one-way ANOVA. **B**, Consecutive recordings of mIPSCs in 20 DIV control neurons (left) and 20 DIV BDNF-treated neurons (4 d without plus 16 d with BDNF) (right); $V_h = -70$ mV. **C**, Mean frequency of mIPSCs calculated from groups of 14–21 DIV control and BDNF-treated neurons. $*p < 0.02$ versus controls. **D**, Overlapped amplitude distributions of mIPSCs from the same two neurons in **B**. BDNF causes a consistent increase in the occurrence of events (filled bars). **E**, Mean amplitudes of mIPSCs calculated from groups of 14–21 DIV control and BDNF-treated neurons (the number of neurons for each condition is indicated in each bar).

scaled variance similar to controls (Fig. 2C). The plot was clearly parabolic in both cases and only slightly skewed toward higher amplitudes. The initial slope of the parabola yielded an estimate of the weighted single channel current: 1.8 and 1.7 pA for control and treated neurons, respectively. On average, we obtained mean unitary currents, i , of 1.8 ± 0.1 pA ($n = 15$) and 1.7 ± 0.1 pA ($n = 15$) in control and BDNF-treated neurons, respectively (Fig. 2D), corresponding to a mean single-channel conductance of 26.0 ± 0.9 and 24.6 ± 1.3 pS. A similar conductance was obtained when the amplitude of unitary current was either increased by lowering V_h to -100 mV (24.8 ± 1.2 pS; $n = 4$) or decreased by lowering intracellular Cl^- concentration ($[\text{Cl}^-]_i$) to 25 mM (23.0 ± 2.0 pS; $n = 5$) (Fig. 2D). Because the mean amplitude of mIPSCs was not altered by BDNF (Fig. 1E), we concluded that in 3-week-old hippocampal neurons, the density of GABA_A receptors was also unaffected by the neurotrophin. Similar conductance values were obtained for control and treated neurons in younger cultures (7–14 DIV; data not shown).

BDNF increases P_r , preserving the quantal size of vesicles

We then investigated how BDNF altered the elementary events responsible for the increased size of eIPSCs. For this, we used the

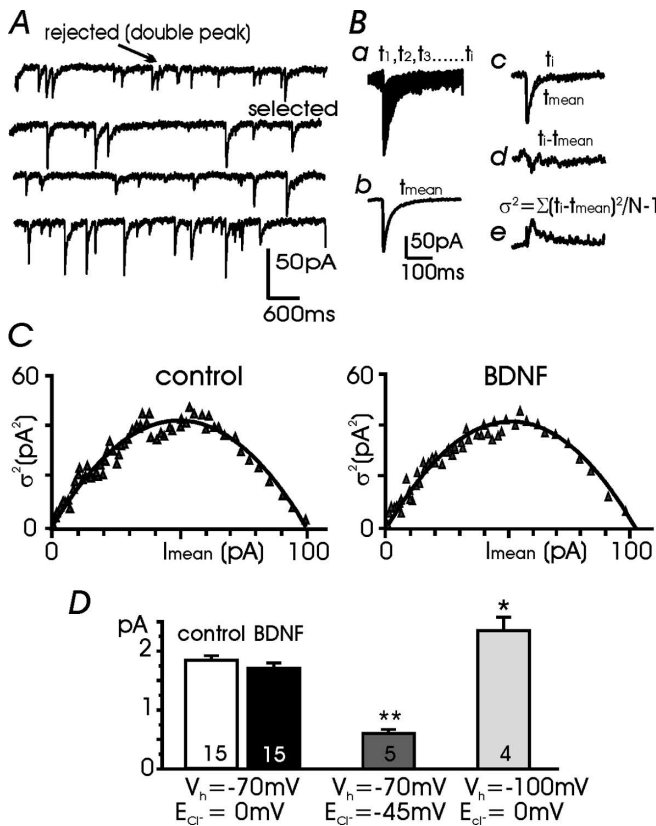


Figure 2. PSVA of mIPSCs. **A**, Consecutive miniature recordings used to select events without double peaks or anomalous noise. **B**, **a**, Eighty superimposed mIPSCs, selected as shown in **A**; **b**, mean waveform of selected events; **c**, an mIPSC superimposed to the scaled mean waveform; **d**, difference between the scaled mean waveform and the individual mIPSC; **e**, $\sigma^2(t)$ calculated by summing the squares of the difference traces divided by $N - 1$. **C**, Relationship between mean current and peak-scaled variance obtained from a representative control and BDNF-treated neuron. **D**, Mean unitary currents in control neurons and BDNF-treated neurons ($n = 15$) at $V_h = -100$ mV ($n = 4$) and at $[Cl^-]_i = 25$ mM ($n = 5$) (** $p < 0.01$ and * $p < 0.05$ vs controls). E_{Cl^-} indicates the Nernst equilibrium potential for Cl^- ions.

mean-peak fluctuation analysis (MPFA), which gives direct information about the average quantal release probability (P_{rav}), the number of independent release sites (N_{min}), and the average postsynaptic quantal size (Q_{av}). This crucial information was derived from the relationship between variance (σ^2) and mean amplitude of eIPSCs (I_{av}) recorded under different P_r conditions. We changed P_r by either elevating $[Ca^{2+}]_o$ from 2 to 5 mM or adding increasing doses of Cd^{2+} (0.5, 2, and 6 μM) to the extracellular medium (Fig. 3A₁, B₁, A₂, B₂). V–M plots were fitted with a simple parabola that furnished an estimate of N_{min} and Q_{av} , and P_{rav} was calculated as follows: $P_{rav} = I_{av}/N_{min}Q_{av}$. Q_{av} was derived from the initial slope of the parabola and corrected for the factor $1/1 + (CV_i)^2$, with CV_i indicating the coefficient of variation of mIPSCs at an individual site (see Materials and Methods). In the case of Figure 3C, the V–M plot for control and BDNF-treated neurons in 5 mM Ca^{2+} yielded the following: $Q_{av} = 19.4$ and 19.0 pA, $N_{min} = 227$ and 249, and $P_{rav} = 0.53$ and 0.78, respectively.

On average, these values were confirmed in control and BDNF-treated neurons ($n = 8$). There was a net 50% increase in P_{rav} at 5 mM Ca^{2+} (0.43 ± 0.05 vs 0.78 ± 0.04 ; $p < 0.01$) and a similar increase at 2 mM Ca^{2+} (0.34 ± 0.04 vs 0.60 ± 0.04 ; $p < 0.01$) (Fig. 3D). The mean eIPSC amplitude was $\sim 50\%$ greater in treated neurons (4.0 ± 0.9 vs 2.2 ± 0.7 nA; $p < 0.01$) (Fig. 3D), and there was no difference in the mean Q_{av} between control and

BDNF-treated neurons (19.3 ± 0.9 vs 19.7 ± 0.9 pA) (Fig. 3E). N_{min} was slightly larger in BDNF-treated with respect to control neurons (245 ± 27 vs 280 ± 25 ; $p < 0.05$), indicating a small but significant increase in the number of functioning release sites (Fig. 3E). It is worth noting that MPFA slightly underestimates the mean Q_{av} with respect to the mean mIPSCs (19.3 vs 25.3 pA in controls). As discussed in Materials and Methods, this depends on various causes that equally influence the recordings of control and BDNF-treated neurons and, thus, do not introduce serious errors to the present conclusions.

BDNF increases the recovery from depression during trains of stimuli

To further evaluate the effects of BDNF on the release probability and the size of the RRP, we analyzed the cumulative amplitude profile during high-frequency trains of stimuli (10 Hz for 1.5 s). As shown in Figure 4A, there was a significant depression of eIPSCs during trains in both control and BDNF-treated neurons. In the two cases, the cumulative profile of repeated eIPSCs showed a rapid rise followed by a slower linear increase of different steepness at later pulses (Fig. 4B). Assuming that the slow linear rise was attributable to the equilibrium between the release and the constant replenishment of vesicles, back-extrapolation of the linear portion to time 0 yielded the total release minus the total replenishment, corresponding to the RRP_{syn} (Schneeggenburger et al., 1999). As shown in Figure 4B, the RRP_{syn} was slightly enhanced in BDNF-treated neurons [5.5 ± 0.8 nA ($n = 8$) vs 6.7 ± 0.4 nA ($n = 8$)], whereas the mean amplitude of the first IPSC was almost doubled (2.0 ± 0.3 vs 3.9 ± 0.2 nA) (Fig. 4C). Because P_{ves} can be calculated as the ratio between I_1 and RRP_{syn} (see Materials and Methods), this implies that P_{ves} was also enhanced by BDNF (0.39 ± 0.04 vs 0.59 ± 0.05 ; $p < 0.01$). Figure 4D also shows that the number of vesicles (N_{syn}) forming the RRP_{syn} , obtained by dividing RRP_{syn} by the mean amplitude of mIPSCs, was only slightly increased by BDNF (211 ± 28 vs 243 ± 13 ; $p < 0.05$). This gives a number of readily releasable vesicles comparable with the number of active sites evaluated by MPFA (245 ± 27 vs 280 ± 25 ; $p < 0.05$). The analysis of cumulative amplitude profile further confirms the data obtained by MPFA and suggests that on average, one vesicle is ready for release at each active site.

The responses to trains of stimuli provide interesting information concerning the recovery from depression during repetitive stimulation. The time course of normalized eIPSCs during the train of stimuli (Fig. 4E) shows in fact that in control neurons, the depression can be approximated by a double exponential with a fast and slow time constant ($\tau_f = 76$ ms; $\tau_s = 720$ ms) and a markedly low asymptotic eIPSC amplitude (y_o) (Fig. 4F). BDNF dramatically changed these parameters. The initial phase of depression was faster, as expected from the increased P_{ves} ($\tau_f = 45$ ms), whereas the second phase was slower ($\tau_s = 1200$ ms), and y_o was approximately twofold larger with respect to control neurons. The slower rate of depression and the higher asymptotic eIPSCs are indicative of an increased recovery from depression with repetitive release, which may be associated with a higher degree of vesicle replenishment in BDNF-treated neurons, possibly induced by an enhanced level of residual presynaptic Ca^{2+} accumulating during repetitive stimuli (Dittman and Regehr, 1998; Stevens and Wesseling, 1998). This is an unexpectedly important feature of the action of BDNF, which compensates for the increased probability of release and allows sustaining high rates of neurotransmitter release by limiting vesicle depletion during repetitive stimulation.

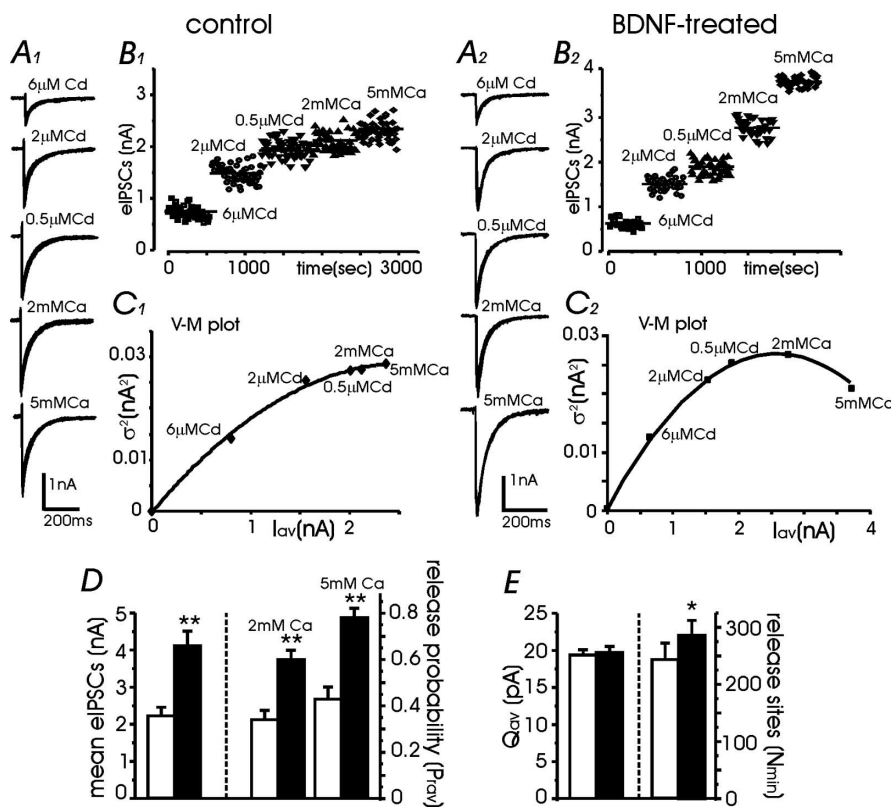


Figure 3. Multiprobability variance analysis used to estimate quantal parameters. *A₁*, *A₂*, Superimposed eIPSCs (8 traces for each condition) recorded in two representative control and BDNF-treated neurons at 0.1 Hz. Changing $[Ca^{2+}]_o$ (2–5 mM) or increasing Cd^{2+} (0.5, 2, and 6 μM) altered P_r . *B₁*, *B₂*, eIPSC amplitude versus time from the two representative neurons. *C₁*, *C₂*, The variance of the eIPSC amplitude is plotted against the mean for each epoch and fitted with a parabola to estimate Q_{av} and N_{min} . *D*, Mean IPSC and mean P_{rav} in control and BDNF-treated neurons (** $p < 0.01$ vs controls; $n = 8$). P_{rav} was calculated at 2 and 5 mM $[Ca^{2+}]_o$. *E*, Average quantal size (Q_{av}) and number of release sites (N_{min}) in control and BDNF-treated neurons (* $p < 0.05$ vs controls; $n = 8$). Q_{av} was obtained from the initial slope of the parabola corrected for $1/(1 + (CV_1)^2)$ (see Materials and Methods).

BDNF preserves the Ca^{2+} dependence of GABAergic transmission

An increased P_r after BDNF exposure could underlie different actions on presynaptic mechanisms. A possibility is that BDNF increases the Ca^{2+} dependence of synaptic transmission by increasing the efficacy by which Ca^{2+} flowing through presynaptic Ca^{2+} channels stimulates vesicles fusion. To examine this, we studied the dose–response relationship of eIPSC amplitude versus $[Ca^{2+}]_o$ in control and BDNF-treated neurons. In both cases, the eIPSCs increased steeply with $[Ca^{2+}]_o$ and saturated at >5 mM (Fig. 5*A₁*). BDNF mainly increased the size of eIPSCs without altering the shape of the dose–response curve. In other words, the slope coefficient (Ca^{2+} cooperativity) and the dissociation constant (K_D) remained unchanged ($n = 3.3$ vs 3.2; $K_D = 1.03$ vs 1.07 mM). As a consequence, the percentage increase in eIPSCs after BDNF treatment remained constant at different levels of $[Ca^{2+}]_o$, suggesting that potentiation of eIPSCs occurred regardless of the levels of $[Ca^{2+}]_o$ (Fig. 5*A₂*).

Having shown that BDNF does not affect the Ca^{2+} dependence of eIPSCs, we then examined whether BDNF could affect the Ca^{2+} dependence of PPD at different levels of $[Ca^{2+}]_o$ (see Materials and Methods). Under control conditions, with 2 mM external Ca^{2+} , the PPD at a 50 ms interpulse interval was ~ 0.49 and decreased to ~ 25 and 2% when $[Ca^{2+}]_o$ was lowered to 1 and 0.5 mM, respectively (Fig. 5*B₁*, *B₂*). At higher levels of $[Ca^{2+}]_o$, PPD increased moderately. As a result, the PPD of inhibitory synapses in cultured hippocampal neurons was steeply

Ca^{2+} dependent and remarkably high at 2 mM $[Ca^{2+}]_o$, a hallmark of central synapses operating near maximal P_r under physiological conditions. With respect to controls, BDNF-treated neurons displayed higher PPDs at all $[Ca^{2+}]_o$ levels but similar Ca^{2+} dependence (Fig. 5*B₃*).

Increased dominance of N- and P/Q-type channels on eIPSCs in BDNF-treated neurons

The increase in P_r observed regardless of the levels of extracellular Ca^{2+} in BDNF-treated neurons may have different origins. As already shown at the somatic level (Baldelli et al., 2000), it is possible that BDNF acts by selectively increasing the expression or changing the proportions of presynaptic Ca^{2+} channels coupled with exocytosis. To assay this possibility, we examined how selective Ca^{2+} channel antagonists affect action potential-evoked IPSCs and PPD in control and BDNF-treated neurons. As shown below, previous information on Ca^{2+} channel distribution derived from KCl-evoked IPSCs (Baldelli et al., 2002) furnishes a rather distorted view of presynaptic Ca^{2+} signaling involved in GABA release and thus should be taken as mainly qualitative of the action of BDNF.

An initial series of experiments was performed by sequentially applying nifedipine (3 μM), ω -Ctx-GVIA (1 μM), and ω -Aga-IVA (0.5 μM) on the same neuron, to block L-, N-, and P/Q-type channels.

Figure 6*A* shows the responses of two representative neurons, maintained under control conditions (left) or exposed to BDNF (right). As shown, nifedipine had reversible weak blocking effects on the eIPSCs in both control and treated neurons ($<3\%$), whereas ω -Ctx-GVIA and ω -Aga-IVA had irreversible blocking effects that either preserved 15% of the eIPSCs under control conditions or fully blocked the response in BDNF-treated neurons. Accounting for the small contribution of L-type channels, these data indicate that R-type channels contribute partially to synaptic transmission in control neurons and are absent in BDNF-treated neurons. Interestingly, by inverting the application of the two toxins in control neurons, we obtained comparable blocks of the eIPSCs (total block between 80 and 85%; see below), suggesting that N- and P/Q-type channels control distinct release sites in BDNF-untreated neurons and also that presynaptic Ca^{2+} channels are distributed in a “segregated” manner.

A second series of experiments was performed to evaluate precisely the contribution of each Ca^{2+} channel type to synaptic transmission by applying separately the Ca^{2+} channel antagonists on control and BDNF-treated neurons. As shown in Figure 6*B*, under control conditions (open bars), nifedipine induced a weak inhibition (2.7%; $n = 12$), whereas ω -Ctx-GVIA and ω -Aga-IVA blocked 34.7% ($n = 10$) and 46.8% ($n = 12$) of the initial synaptic current, respectively. A mean contribution of 10.9% was estimated for the R-channel. In BDNF-treated neurons, the action of nifedipine remained nearly unchanged (2.8%; $n = 11$), whereas the average inhibition by ω -Ctx-GVIA and

ω -Aga-IVA increased to 49.4% ($n = 10$; $p < 0.01$) and 66.5% ($n = 11$; $p < 0.01$), respectively, with a net increase of 42% in both cases. The total block of N- and P/Q-type channels increased from 81.5 to 116.9%, and R-type channels no longer contributed to the eIPSCs. We conclude that in BDNF-treated neurons, N- and P/Q-type channels assumed full control of synaptic inhibitory transmission and that together they control >100% the size of eIPSCs (supra-additivity). This is strong evidence that in BDNF-treated neurons, a percentage of release sites are controlled by mixed populations of N- and P/Q-type channels that are distributed in a nonuniform manner. Notice that supra-additivity and the switching from segregated to nonuniform distribution of presynaptic Ca^{2+} channels with BDNF were totally overlooked in a previous work using KCl-evoked IPSCs (Baldelli et al., 2002). In this case, the 1 s KCl stimulation overestimated the contribution of distal R-type channels and diminished that of N- and P/Q-type channels more proximal to the release sites. In particular, P/Q-type channels appeared to contribute less than R- and N-type channels under control conditions, whereas with action potential stimulation, their contribution is always maximal with and without BDNF (Fig. 6B).

BDNF increases the control of PPD associated with N-type channels

To gain a deeper insight into the action of BDNF, we next examined how N- and P/Q-type channels specifically affect PPD and recovery from depression. We found that in control neurons, PPD had large initial values (65.7% at $\Delta t = 20$ ms) (Fig. 7A,C), and recovery from depression was well approximated by a single exponential with the time constant $\tau_{rec} = 85.6$ ms (Fig. 7A,E, \square). PPD and recovery from depression remained almost unaltered when P/Q-type channels were mainly responsible for the eIPSCs (ω -Ctx-GVIA applied). PPD was 59.2% at $\Delta t = 20$ ms (Fig. 7A,C) and $\tau_{rec} = 98.9$ ms (Fig. 7A,F, Δ). On the contrary, the PPD markedly decreased (33.3% at $\Delta t = 20$ ms) (Fig. 7A,C) and the τ_{rec} was shortened ($\tau_{rec} = 49.5$ ms) (Fig. 7A,G) when N-type channels were mainly responsible of neurotransmission (ω -Aga-IVA applied). This suggests distinct roles of N- and P/Q-type channels in controlling GABA release in control neurons. P/Q-type channels dominate the control of PPD and determine the slow recovery of depression, which is related to vesicle replenishment and depends on residual Ca^{2+} at the presynaptic terminals (Dittman and Regehr, 1998). N-type channels control PPD less effectively but have

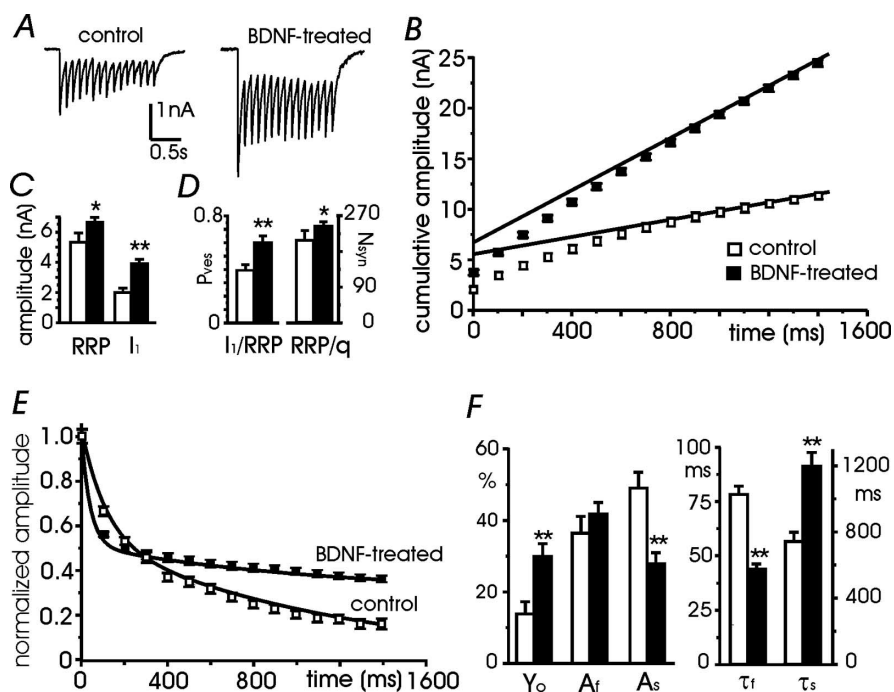


Figure 4. Estimate of the RRP_{syn} and P_{ves} through cumulative amplitude analysis. **A**, eIPSCs recorded during a train of repetitive stimuli of 10 Hz in a control (left) and a BDNF-treated (right) neuron. **B**, Cumulative eIPSC amplitude during 10 Hz trains in eight control and eight treated neurons. Data points in the range of 0.8–1.4 s were fitted by linear regression and back-extrapolated to time 0 to estimate the cumulative eIPSC amplitudes before steady-state depression (RRP_{syn}). **C**, The mean amplitude of the first eIPSC (I_1) is nearly doubled in treated neurons (filled bars; $n = 8$) versus control neurons (open bars; $n = 8$) (** $p < 0.01$ vs controls), whereas the mean RRP_{syn} is only slightly increased (* $p < 0.05$ vs controls). **D**, Mean P_{ves} (** $p < 0.01$ vs controls) and mean number of vesicles forming the RRP_{syn} (* $p < 0.05$ vs controls). **E**, Plot of eIPSC amplitude versus time during repetitive stimulation fitted with a double-exponential function: $I(t) = y_0 + A_f e^{-t/\tau_f} + A_s e^{-t/\tau_s}$. **F**, Mean values of the parameters obtained by the double-exponential fit of **E** ($n = 8$; ** $p < 0.01$ vs controls).

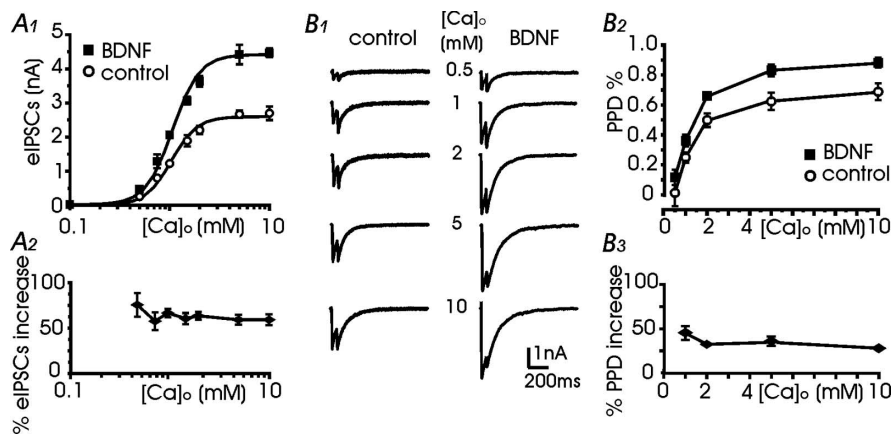


Figure 5. Ca^{2+} dependence of GABAergic transmission in control and treated neurons. **A₁**, Mean eIPSC versus $[Ca^{2+}]_o$ fitted with a Hill equation: $I = I_{max} [Ca^{2+}]_o^n / ([Ca^{2+}]_o^n + K_D)$. BDNF mainly increased the size of eIPSCs (I_{max}) without altering the slope coefficient (n) and K_D ($n = 3.3$ vs 3.2 ; $K_D = 1.03$ vs 1.07 mM; $I_{max} = 2.6$ vs 4.4 nA) ($6 < n < 32$). **A₂**, Percentage increase in eIPSCs after BDNF treatment remained constant at different $[Ca^{2+}]_o$ levels. Data were obtained from **A₁**. **B₁**, Response to paired-pulse stimulation (50 ms time interval) at increasing $[Ca^{2+}]_o$ levels in control and treated neurons. Each trace represents the average of six consecutive recordings (stimulation frequency, 0.066 Hz). **B₂**, Mean PPD versus $[Ca^{2+}]_o$ in control and treated neurons ($6 < n < 10$). **B₃**, Percentage increase in PPD in the presence of BDNF did not change between 2 and 10 mM $[Ca^{2+}]_o$. Data were obtained from **B₂**.

marked effects on the recovery of PPD, as proven by the faster rate of recovery supported by these channels.

BDNF had a marked effect on the contribution of N- and P/Q-type channels to PPD. PPD markedly increased with BDNF (85.9% at $\Delta t = 20$ ms) (Fig. 7B,D). Recovery from depression

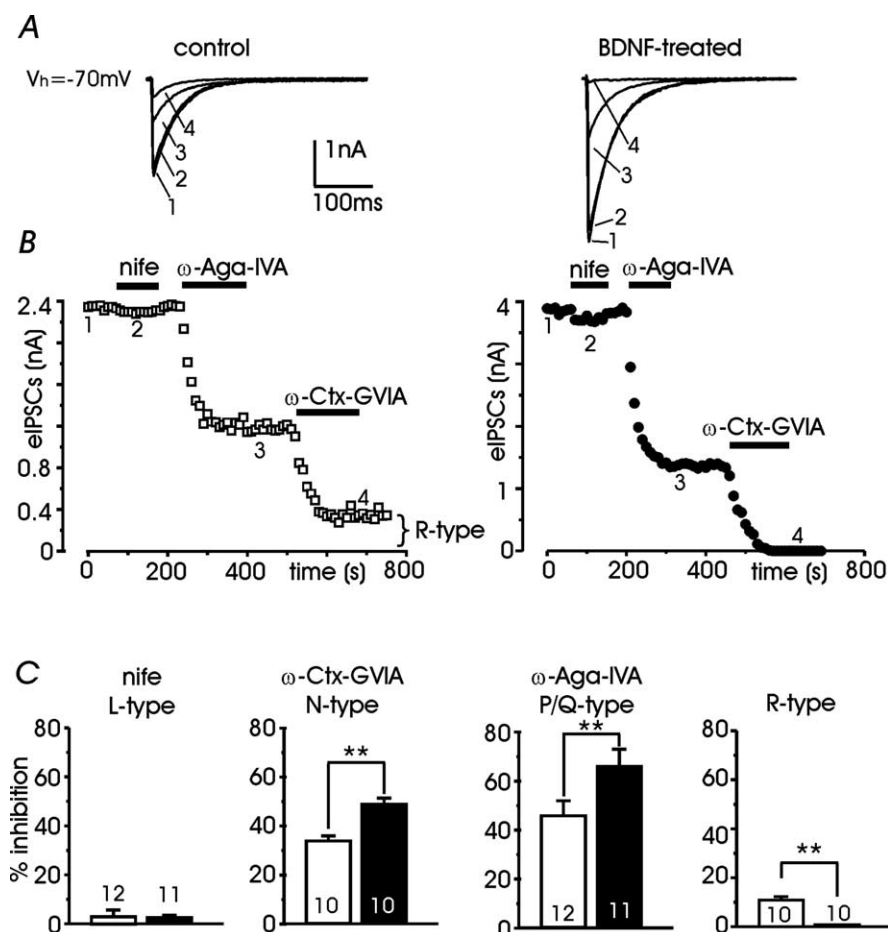


Figure 6. Pharmacological dissection of presynaptic Ca^{2+} channel types supporting eIPSCs. **A**, Examples of eIPSCs recorded before (1) during sequential application of $3 \mu\text{M}$ nifedipine (nife) (2), $0.5 \mu\text{M}$ ω -Aga-IVA (3), and $1 \mu\text{M}$ ω -Ctx-GVIA (4) in control neurons (left) and treated neurons (right). **B**, Time course of eIPSC amplitude relative to the same neurons shown in **A**. **C**, Mean percentage contribution of Ca^{2+} channel types to eIPSCs estimated by separately applying the three Ca^{2+} antagonists on control neurons (open bars) and treated neurons (filled bars); ** $p < 0.01$ vs controls.

was comparable with control neurons ($\tau_{\text{rec}} = 101.2$ ms) (Fig. 7B,E) but revealed a second, slower component responsible for the persisting PPD at $\Delta t = 800$ ms ($\tau_{\text{rec}} = 3$ s). In contrast to control neurons, N-type channel blockade had a clear effect on PPD. ω -Ctx-GVIA uniformly depressed the PPD by 25–30% at all interpulse intervals, causing the block of the slow phase of recovery from depression (Fig. 7B, \blacktriangle). The PPD at $\Delta t = 20$ ms decreased to 66.6%, which is comparable with the PPD of control neurons (59.2%) (Fig. 7D).

BDNF did not affect the rate of recovery from depression when only P/Q-type channels contribute to neurotransmitter release: τ_{rec} was 98.9 and 107.6 ms, respectively, for control and BDNF-treated neurons (Fig. 7F). Blockade of P/Q-type channels uncovered a PPD associated with N-type channels that was remarkably higher than in control neurons (52 vs 33.3% at $\Delta t = 20$ ms) (Fig. 7D), whereas recovery from depression remained similarly fast and complete after long interpulse intervals ($\tau_{\text{rec}} = 59.5$ ms) (Fig. 7G). Thus, the increased PPD induced by BDNF was mainly attributable to a shift of the contribution of N-type channels to sites of higher P_r , whereas both N- and P/Q-type channels contributed almost equally to the slow phase of depression.

As a final remark, it is interesting to note that although the rate of recovery from depression during paired-pulse stimuli was

poorly affected by BDNF (Fig. 7E), the same parameter was markedly increased during trains of repetitive stimulations (Fig. 4E). This apparent contradiction is attributable to the fact that the single stimulus used with PPD is unable to generate the presynaptic Ca^{2+} accumulation necessary to boost the recovery from depression achieved during trains (Dittman and Regehr, 1998; Stevens and Wesseling 1998). It is most likely that the enhancement of P_r during the depression induced by paired-pulse stimuli predominates, and that increasing vesicle depletion broadens the time necessary for the recovery of RRP.

Discussion

We have provided evidence that long-lasting exposures to BDNF produce a marked enhancement of eIPSCs in developing rat hippocampal neurons mainly by increasing P_r and P_{ves} . This, together with the slight enhancement of the RRP and the increased contribution of N- and P/Q-type Ca^{2+} channels to the eIPSCs, unequivocally points to a presynaptic site of action of BDNF.

Presynaptic versus postsynaptic site of action of BDNF

A presynaptic mechanism of action for BDNF on inhibitory synapses is suggested by previous electrophysiological and morphological data in rat hippocampal neurons that show increments of eIPSC amplitude with little change in the size of mIPSCs and the number of synaptic connections (Vicario-Abejon et al., 1998; Sherwood and Lo, 1999; Bolton et al., 2000; Baldelli et al., 2002). Our present data indicate that these effects are associated with an increase in P_r and P_{ves} , which most likely derive from a better redistribution of N- and P/Q-type channels at the sites controlling neurotransmitter release. This is new and different from what has been observed in other synapses, in which chronic exposure to BDNF either boosts the number of release sites (Rutherford et al., 1997; Huang et al., 1999; Marty et al., 2000; Seil and Drake-Baumann, 2000; Kohara et al., 2003) or enhances the number of docked vesicles (Collin et al., 2001; Tartaglia et al., 2001; Tyler and Pozzo-Miller, 2001; Carter et al., 2002).

Although most of the available data point to a presynaptic site of action of BDNF on neurotransmission, there is evidence for a marked increase in GABA_A receptors in young (7–10 DIV) hippocampal cultures chronically exposed to BDNF (Yamada et al., 2002). An increased density of functional GABA_A receptors should cause measurable increases in mIPSC amplitude that we observed only in 5–14 DIV neurons but not in 14–21 DIV neurons (Baldelli et al., 2002). Here, we extended our previous findings and show that the unitary conductance and the density of GABA_A receptors was unaffected by BDNF in 14–21 DIV neurons. Thus, most likely, overexpression of GABA_A receptors occurs predominantly at early stages of differentiation *in vitro*.

Higher P_r and recovery from depression: a strategy to enhance presynaptic efficiency

BDNF induces a marked increase in P_{ves} , but the size of the RRP is only slightly augmented (Fig. 4). This conclusion is in agreement with the observation that BDNF does not affect the size of the recycling pool (Yamada et al., 2002) and possibly of the RRP as well (Murthy and Stevens, 1999). An action on P_{ves} preserving the RRP is the opposite of what occurs in excitatory synapses, in which long-term exposures to BDNF enhance presynaptic activity by increasing the RRP (Collin et al., 2001; Tartaglia et al., 2001; Tyler and Pozzo-Miller, 2001; Carter et al., 2002). Thus, BDNF follows different strategies to enhance the rate of vesicle release in excitatory and inhibitory synapses, increasing either the RRP or P_{ves} .

An increased P_r (or P_{ves}) might have serious drawbacks during high-frequency stimulation in synapses that function at a high rate of release and saturating Ca^{2+} conditions, as in our case. Control eIPSCs undergo rapid depression during trains of stimuli (Fig. 4E), which would be worsened after a 50% increase in P_r . However, BDNF is able to slow down the rate of depression, maintaining high eIPSC amplitudes during trains. The twofold lower rate of depression observed with BDNF is possibly associated with an increased rate of vesicle replenishment attributable to an enhanced level of residual Ca^{2+} accumulating presynaptically during repetitive stimuli (Dittman and Regehr, 1998; Stevens and Wesseling, 1998). This new unexpected finding reinforces the presynaptic nature of the action of BDNF and can be associated with the BDNF-induced supra-additivity of presynaptic Ca^{2+} channels in controlling eIPSCs. BDNF may increase the amount of presynaptic terminals in which N- and P/Q-type channels are coexpressed and cooperate to control vesicle release, enhancing the possibility of intraterminal Ca^{2+} accumulation during high-frequency stimulation. Indeed, other presynaptic mechanisms could account for the higher rate of vesicle recycling induced by the neurotrophin. BDNF activation of TrkB receptors and increased intraterminal Ca^{2+} concentration activate mitogen-activated protein (MAP)-kinase and calmodulin kinase II, which phosphorylate synapsins at distinct sites (Benfenati et al., 1992; Jovanovic et al., 1996) and increase vesicle mobilization from the reserve to the RRP (Greengard et al., 1993). During high-frequency stimulation, the level of synapsin phosphorylation strictly correlates with synapsin dispersion and with the amount of vesicles sustaining repetitive release (Chi et al., 2001). Thus, BDNF could increase the rate of vesicle replenishment through direct (TrkB/MAP-kinase pathway) or indirect (Ca^{2+} /calmodulin kinase II pathway) synapsin phosphorylation, promoting vesicle mobilization from the reserve pool.

Presynaptic N- and P/Q-type channels as targets of the action of BDNF

Our data show clearly that BDNF changes both the distribution and contribution of N- and P/Q-type channels controlling the

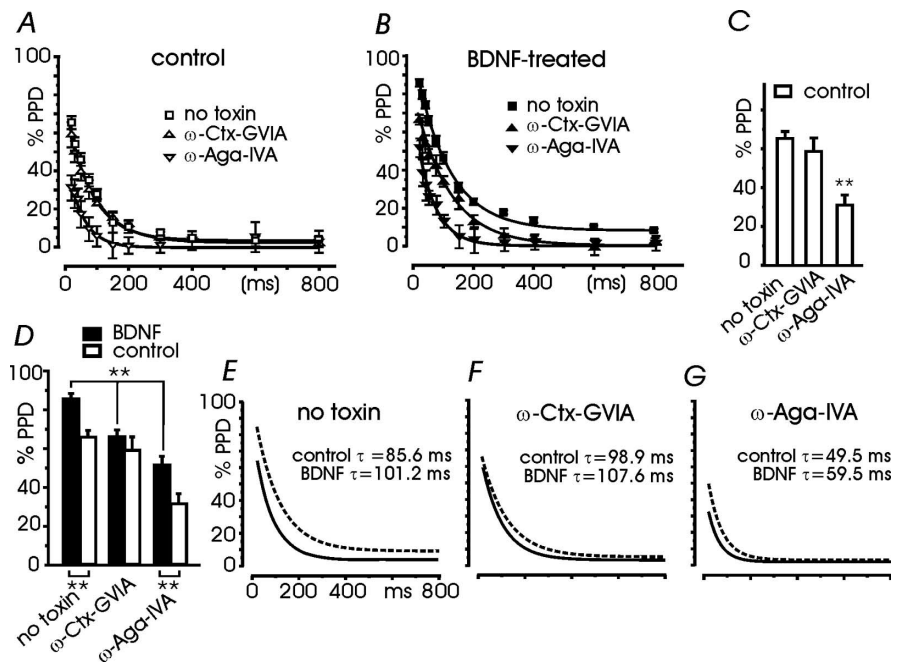


Figure 7. Pharmacological dissection of presynaptic Ca^{2+} channel types supporting PPD and recovery from depression. **A**, Percentage of PPD vs time in control neurons in the absence of toxins and in the presence of $1 \mu M$ ω -Ctx-GVIA and $0.5 \mu M$ ω -Aga-IVA. Intervent intervals ranged between 20 and 800 ms. Solid curves are monoexponential fits, with initial amplitude and τ_{rec} given in Results (for each point, $9 < n < 16$). **B**, Same as in **A** but in the presence of BDNF ($9 < n < 15$). **C**, Mean percentage of PPD calculated at $\Delta t = 20$ ms in control neurons under the indicated condition (** $p < 0.01$; $9 < n < 16$). **D**, Same as in **C** but in the presence of BDNF (filled bars; $9 < n < 15$). The open bars are taken from **C**, and statistical comparisons (** $p < 0.01$) are as indicated. **E–G**, Monoexponential fit of the percentage of PPD versus time taken from **A** and **B** for control neurons (solid lines) and treated neurons (dashed lines) in the absence of toxins (**E**) and in the presence of ω -Ctx-GVIA (**F**) and ω -Aga-IVA (**G**).

eIPSCs. The two channels control distinct release sites in BDNF-untreated neurons (segregated distribution), and their contribution is limited to 82% of the total eIPSCs. With BDNF, this percentage increases to 117%, increasing the percentage of release sites containing mixtures of the two channels (nonuniform distribution). This could represent a new form of synapse maturation in developing neurons induced by BDNF.

An appropriate colocalization of presynaptic Ca^{2+} channels at the vesicle release sites, rather than a generalized increase in Ca^{2+} channel density as the cause of the higher P_r with BDNF, is also supported by two other findings. First, neurotransmission is near saturation under control conditions, and P_r increases very little at >2 mM Ca^{2+} . Because saturation of Ca^{2+} fluxes through open Ca^{2+} channels occurs at >50 mM Ca^{2+} (Hess et al., 1986), it is evident that local $[Ca^{2+}]_i$ at the nanodomains formed by Ca^{2+} channels and docked vesicles (Augustine, 2001) is already maximal during control eIPSCs in 2 mM Ca^{2+} . Additional elevations of $[Ca^{2+}]_o$ or increased densities of distantly located Ca^{2+} channels would produce only minor changes. It is worth noting that saturation of eIPSCs occurs at values significantly smaller than the size of the RRP_{syn} (2.6 vs 5.5 nA in controls and 4.4 vs 6.7 nA with BDNF) and, thus, the number of available vesicles and Ca^{2+} entry are not limiting factors for the increase in P_r with BDNF. Most likely, the main limitation to P_r is the quantity of Ca^{2+} ions effectively entering the terminal through presynaptic Ca^{2+} channels, which may derive from a better colocalization of Ca^{2+} channels to the release active zones (Spafford and Zamponi, 2003). Second, BDNF enhances the expression of syntaxin 1 and synaptotagmin 1 (Wang et al., 1995; Vicario-Abejon et al. 1998; Tartaglia et al. 2001; Yamada et al. 2002). The two proteins bind selectively to the “synprint” region of N- and P/Q-type channels but not to R-type channels, which

lack the binding sequence (Catterall, 2000). Thus, an increased expression of vesicle-binding proteins would favor the colocalization of N- and P/Q-type channels rather than R-type channels, increasing the P_r .

A final consideration concerns the role that N- and P/Q-type channels play in the control of PPD and recovery from depression. As for other neurons (Mintz et al., 1995; Reid et al., 1997, 2003; Wilson et al., 2001), we observed nonuniform reduction of P_r after blockade of either N- or P/Q-type channels. In our case, P/Q-type channels appear to be strongly coupled with the release sites, and BDNF does not change the already high PPD associated with these channels (Fig. 7F). The increased contribution of P/Q-type channels to the size of eIPSCs may simply derive from an increased nonuniform distribution of these channels in terminals purely controlled by the N-type.

A relevant issue of the present study is that BDNF seems particularly effective in increasing the contribution of N-type channels to PPD, which is rather low in control neurons. The apparent contradiction that N-type channels contribute to the eIPSCs (35% block) and little to the PPD is attributable to the fact that PPD does not simply depend on vesicle depletion (P_r) but also on vesicle replenishment, which is Ca^{2+} dependent and determines the rate of recovery from depression (Dittman and Regehr, 1998). The inability of ω -Ctx-GVIA to affect PPD suggests that vesicle depletion induced by Ca^{2+} influx through N-type channels is well counterbalanced by an effective Ca^{2+} -dependent recovery from depression by the Ca^{2+} ions flowing through the same channel types. Notice that recovery from depression is particularly fast when N-types are the only channels supporting GABA release and BDNF does not affect this feature (Fig. 7G). This is line with recent observations on two species of mature inhibitory synapses possessing distinct populations of presynaptic Ca^{2+} channels (Poncer et al., 2000). The striatum oriens interneurons expressing only P/Q-type channels show marked PPD at all interpulse intervals, whereas the striatum radiatum interneurons, possessing only N-type channels, display facilitation at short intervals and weak PPD at longer intervals.

In conclusion, we have brought new evidence in favor of a novel action of BDNF on presynaptic Ca^{2+} channels that leads to a sharp increase in P_r and accounts for most of the increase in inhibitory postsynaptic signaling in developing hippocampal neurons. Maturation and stabilization of GABAergic synapses are expected to play a key role in balancing the activity of excitatory synapses during neuronal network formation (Vicario-Abejon et al., 2002). Our data suggest the possibility that a better colocalization of presynaptic Ca^{2+} channels with the vesicle fusion machinery may be a common mechanism adopted by inhibitory synapses to increase their strength in the hippocampus and other brain regions with chronic exposure to BDNF.

References

- Augustine GJ (2001) How does calcium trigger neurotransmitter release? *Curr Opin Neurobiol* 11:320–326.
- Baldelli P, Forni PE, Carbone E (2000) BDNF, NT-3 and NGF induce distinct new Ca^{2+} channel synthesis in developing hippocampal neurons. *Eur J Neurosci* 12:4017–4032.
- Baldelli P, Novara M, Carabelli V, Hernández-Guijo JM, Carbone E (2002) BDNF up-regulates evoked GABAergic transmission in developing hippocampal neurons by potentiating presynaptic N- and P/Q-type Ca^{2+} channel signaling. *Eur J Neurosci* 16:2297–2310.
- Balkowiec A, Katz DM (2000) Activity-dependent release of endogenous brain-derived neurotrophic factor from primary sensory neurons detected by ELISA *in situ*. *J Neurosci* 20:7417–7423.
- Benfenati F, Valtorta F, Rubenstein JL, Gorelick FS, Greengard P, Czernik AJ (1992) Synaptic vesicle-associated Ca^{2+} /calmodulin-dependent protein kinase II is a binding protein for synapsin I. *Nature* 359:417–420.
- Betz WJ (1970) Depression of transmitter release at the neuromuscular junction of the frog. *J Physiol (Lond)* 206:629–644.
- Bolton MM, Pittman AJ, Lo DC (2000) Brain-derived neurotrophic factor differentially regulates excitatory and inhibitory synaptic transmission in hippocampal cultures. *J Neurosci* 20:3221–3232.
- Carter AR, Chen C, Schwartz PM, Segal RA (2002) Brain-derived neurotrophic factor modulates cerebellar plasticity and synaptic ultrastructure. *J Neurosci* 22:1316–1327.
- Catterall WA (2000) Structure and regulation of voltage-gated Ca^{2+} channels. *Annu Rev Cell Dev Biol* 16:521–555.
- Chi P, Greengard P, Ryan TA (2001) Synapsin dispersion and recluster during synaptic activity. *Nat Neurosci* 4:1187–1193.
- Clements JD, Silver RA (2000) Unveiling synaptic plasticity: a new graphical and analytical approach. *Trends Neurosci* 23:105–113.
- Collin C, Vicario-Abejon C, Rubio ME, Wenthold RJ, McKay RD, Segal M (2001) Neurotrophins act at presynaptic terminals to activate synapses among cultured hippocampal neurons. *Eur J Neurosci* 13:1273–1282.
- De Koninck Y, Mody I (1994) Noise analysis of miniature IPSCs in adult rat brain slices: properties and modulation of synaptic GABA_A receptor channels. *J Neurophysiol* 71:1318–1335.
- Dittman JS, Regehr WG (1998) Calcium dependence and recovery kinetics of presynaptic depression at the climbing fiber to Purkinje cell synapse. *J Neurosci* 18:6147–6162.
- Greengard P, Valtorta F, Czernik AJ, Benfenati F (1993) Synaptic vesicle phosphoproteins and regulation of synaptic function. *Science* 259:780–785.
- Hess P, Lansman JB, Tsien RW (1986) Calcium channel selectivity for divalent and monovalent cations: voltage and concentration dependence of single channel current in ventricular heart cells. *J Gen Physiol* 88:293–319.
- Huang ZJ, Kirkwood A, Pizzorusso T, Porciatti V, Morales B, Bear MF, Maffei L, Tonegawa S (1999) BDNF regulates the maturation of inhibition and the critical period of plasticity in mouse visual cortex. *Cell* 98:739–755.
- Jovanovic JN, Benfenati F, Siow YL, Sihra TS, Sanghera JS, Pelech SL, Greengard P, Czernik AJ (1996) Neurotrophins stimulate phosphorylation of synapsin I by MAP kinase and regulate synapsin I-actin interactions. *Proc Natl Acad Sci USA* 93:3679–3683.
- Kohara K, Kitamura A, Adachi N, Nishida M, Itami C, Nakamura S, Tsumoto T (2003) Inhibitory but not excitatory cortical neurons require presynaptic brain-derived neurotrophic factor for dendritic development, as revealed by chimera cell culture. *J Neurosci* 23:6123–6131.
- Lessmann V, Gottmann K, Malsangio M (2003) Neurotrophin secretion: current facts and future prospects. *Prog Neurobiol* 69:341–374.
- Magnelli V, Baldelli P, Carbone E (1998) Antagonist-resistant calcium currents in rat embryo motoneurons. *Eur J Neurosci* 10:1810–1825.
- Marty S, Wehrle R, Sotelo C (2000) Neuronal activity and brain-derived neurotrophic factor regulate the density of inhibitory synapses in organotypic slice cultures of postnatal hippocampus. *J Neurosci* 20:8087–8095.
- Mintz IM, Sabatini BL, Regehr WG (1995) Calcium control of transmitter release at a cerebellar synapse. *Neuron* 15:675–688.
- Murthy VN, Stevens CF (1999) Reversal of synaptic vesicle docking at central synapses. *Nat Neurosci* 2:503–507.
- Nusser Z, Cull-Candy S, Farrant M (1997) Differences in synaptic GABA_A receptor number underlie variation in GABA mini amplitude. *Neuron* 19:697–709.
- Ohno-Shosaku T, Kazunari H, Sawada A, Yamamoto C (1994) Contributions of multiple calcium channel types to GABAergic transmission in rat cultured hippocampal neurons. *Neurosci Lett* 181:145–148.
- Oleskevich S, Clements J, Walmsley B (2000) Release probability modulates short-term plasticity at a rat giant terminal. *J Physiol (Lond)* 524:513–523.
- Poncer JC, McKinney RA, Gähwiler BH, Thompson SM (2000) Differential control of GABA release at synapses from distinct interneurons in rat hippocampus. *J Physiol (Lond)* 528:123–130.
- Poo MM (2001) Neurotrophins as synaptic modulators. *Nat Rev Neurosci* 2:24–32.
- Reid CA, Clements JD (1999) Postsynaptic expression of long-term potentiation in the rat dentate gyrus demonstrated by variance-mean analysis. *J Physiol (Lond)* 518:121–130.
- Reid CA, Clements JD, Bekkers JM (1997) Nonuniform distribution of Ca^{2+} channel subtypes on presynaptic terminals of excitatory synapses in hippocampus cultures. *J Neurosci* 17:2738–2745.

- Reid CA, Bekkers JM, Clements JD (2003) Presynaptic Ca^{2+} channels: a functional patchwork. *Trends Neurosci* 26:683–687.
- Rutherford LC, DeWan A, Lauer HM, Turrigiano GG (1997) Brain-derived neurotrophic factor mediates the activity-dependent regulation of inhibition in neocortical cultures. *J Neurosci* 17:4527–4535.
- Schneggenburger R, Meyer AC, Neher E (1999) Released fraction and total size of a pool of immediately available transmitter quanta at a calyx synapse. *Neuron* 23:399–409.
- Schneggenburger R, Sakaba T, Neher E (2002) Vesicle pools and short-term synaptic depression: lessons from a large synapse. *Trends Neurosci* 25:206–212.
- Seil FJ, Drake-Baumann R (2000) TrkB receptor ligands promote activity-dependent inhibitory synaptogenesis. *J Neurosci* 20:5367–5373.
- Sherwood NT, Lo DC (1999) Long-term enhancement of central synaptic transmission by chronic brain-derived neurotrophic factor treatment. *J Neurosci* 19:7025–7036.
- Silver RA, Momiyama A, Cull-Candy SG (1998) Locus of frequency-dependent depression identified with multiple-probability fluctuation analysis at rat climbing fibre–Purkinje cell synapses. *J Physiol (Lond)* 510:881–902.
- Spafford JD, Zamponi GW (2003) Functional interactions between presynaptic calcium channels and the neurotransmitter release machinery. *Curr Opin Neurobiol* 13:308–314.
- Stevens CF, Wesseling JF (1998) Activity-dependent modulation of the rate at which synaptic vesicles become available to undergo exocytosis. *J Neurosci* 21:415–424.
- Tartaglia N, Du J, Tyler WJ, Neale E, Pozzo-Miller L, Lu B (2001) Protein synthesis-dependent and -independent regulation of hippocampal synapses by brain-derived neurotrophic factor. *J Biol Chem* 276:37585–37593.
- Traynelis SF, Jaramillo F (1998) Getting the most out of noise in the central nervous system. *Trends Neurosci* 21:137–145.
- Traynelis SF, Silver RA, Cull-Candy SG (1993) Estimated conductance of glutamate receptor channels activated during EPSCs at the cerebellar mossy fiber–granule cell synapse. *Neuron* 11:279–289.
- Tyler WJ, Pozzo-Miller LD (2001) BDNF enhances quantal neurotransmitter release and increases the number of docked vesicles at the active zones of hippocampal excitatory synapses. *J Neurosci* 21:4249–4258.
- Vicario-Abejon C, Collin C, McKay RD, Segal M (1998) Neurotrophins induce formation of functional excitatory and inhibitory synapses between cultured hippocampal neurons. *J Neurosci* 18:7256–7271.
- Vicario-Abejon C, Owens D, McKay R, Segal M (2002) Role of neurotrophins in central synapse formation and stabilization. *Nat Rev Neurosci* 3:965–974.
- Wang T, Xie K, Lu B (1995) Neurotrophins promote maturation of developing neuromuscular synapses. *J Neurosci* 15:4796–4805.
- Wilson RI, Kunos G, Nicoll RA (2001) Presynaptic specificity of endocannabinoid signaling in the hippocampus. *Neuron* 31:453–462.
- Yamada MK, Nakanishi K, Ohba S, Nakamura T, Ikegaya Y, Nishiyama N, Matsuki N (2002) Brain-derived neurotrophic factor promotes the maturation of GABAergic mechanisms in cultured hippocampal neurons. *J Neurosci* 22:7580–7585.