Cellular/Molecular

Activity-Dependent Neurotrophin Signaling Underlies Developmental Switch of Ca^{2+} Channel Subtypes Mediating Neurotransmitter Release

Takafumi Miki,[1] Hirokazu Hirai,[2] and Tomoyuki Takahashi[1,3]

[1]Laboratory of Molecular Synaptic Function, Doshisha University Graduate School of Brain Science, Kyotanabe 610-0394, Japan, [2]Department of Neurophysiology, Gunma University Graduate School of Medicine, Gunma 371-8511, Japan, and [3]Celluar and Molecular Synaptic Function Unit, Okinawa Institute of Science and Technology Graduate University (OIST), Okinawa 904-0495, Japan

At the nerve terminal, neurotransmitter release is triggered by Ca^{2+} influx through voltage-gated Ca^{2+} channels (VGCCs). During postnatal development, VGCC subtypes in the nerve terminal switch at many synapses. In immature rodent cerebella, N-type and P/Q-type VGCCs mediate GABAergic neurotransmission from Purkinje cells (PCs) to deep nuclear cells, but as animals mature, neurotransmission becomes entirely P/Q-type dependent. We reproduced this developmental switch in rat cerebellar slice culture to address the underlying mechanism. Chronic block of cerebellar neuronal activity with tetrodotoxin (TTX) in slice culture, or *in vivo*, reversed the switch, leaving neurotransmission predominantly N-type channel-dependent. Brain-derived neurotrophic factor or neurotrophin-4 rescued this TTX effect, whereas pharmacological blockade of neurotrophin receptors mimicked the TTX effect. In PC somata, unlike in presynaptic terminals, TTX had no effect on the proportion of Ca^{2+} channel subtype currents. We conclude that neuronal activity activates the neurotrophin–TrkB signaling pathway, thereby causing the N-to-P/Q channel switch in presynaptic terminals.

Introduction

At nerve terminals, Ca^{2+} entering through voltage-gated Ca2+ channels (VGCCs) triggers neurotransmitter release (Katz, 1969). Multiple VGCC subtypes including P/Q- ($Ca_V2.1$), N- ($Ca_V2.2$), and R- ($Ca_V2.3$) types mediate neurotransmitter release at immature synapses (Luebke et al., 1993; Takahashi and Momiyama, 1993; Wheeler et al., 1994; Wu et al., 1998). However, contributions of N- and R-type VGCCs to transmitter release decline during development at various types of synapses (Scholz and Miller, 1995; Rosato Siri and Uchitel, 1999; Iwasaki et al., 2000; Momiyama, 2003). In new born rodents, both at the cerebellar inhibitory synapse between Purkinje cells (PCs) and deep cerebellar nuclei (DCN) cells, and at the calyx of Held synapse in the brainstem, both N-type and P/Q-type VGCCs mediate transmitter release, but after 2 weeks, neurotransmission becomes entirely P/Q-type channel-dependent (Iwasaki et al., 2000). At the calyx of Held in $Ca_V2.1$ α1 subunit-ablated mice, N-type VGCCs are overexpressed and fully compensate for the lost P/Q-type channel currents (Inchauspe et al., 2004; Ishikawa et al., 2005). Nevertheless, these mice develop severe cerebellar dysfunction after the second postnatal week (Jun et al., 1999), implying that the N-to-P/Q-type channel switch may be essential for the functional maturation of cerebellum.

Presynaptic N-type and P/Q-type VGCCs have some distinct properties. During repetitive stimulation, P/Q-type Ca^{2+} currents undergo facilitation followed by inactivation (Cuttle et al., 1998; Forsythe et al., 1998; Tsujimoto et al., 2002), whereas N-type Ca^{2+} currents show monotonic inactivation (Inchauspe et al., 2004; Ishikawa et al., 2005). This P/Q-type Ca^{2+} current facilitation accounts for 40–50% of paired-pulse synaptic facilitation (Müller et al., 2008; Hori and Takahashi, 2009). In the cerebellum, firing frequency of PCs often exceeds 100 Hz (Llinás and Sugimori, 1980; Häusser and Clark, 1997; Womack and Khodakhah, 2002). The main inhibitory output from PCs is projected to DCN neurons, where excitatory inputs from mossy and climbing fibers converge to execute signal computations for motor control (Aizenman et al., 2003). In this neuronal circuit, the developmental N-P/Q switch in PC axon terminals may facilitate release of inhibitory transmitter in response to high-frequency inputs, thereby strengthening the inhibitory influence of PCs onto DCN neurons.

The developmental N-P/Q switch occurs widely at both excitatory and inhibitory synapses, but little is known as to the underlying mechanism. To address this issue, we have established a cerebellar slice culture preparation, where the developmental N-P/Q switch can be reproduced at the PC–DCN synapse. First, we determined whether the developmental switch is preprogrammed or induced secondarily by neuronal activity. Blockade

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Correspondence should be addressed to Tomoyuki Takahashi, Laboratory of Molecular Synaptic Function, Doshisha University Graduate School of Brain Science, 1-3, Todani, Tatara, Kyotanabe-Shi, Kyoto-Fu 610-0394, Japan. E-mail: ttakahas@mail.doshisha.ac.jp.

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of neuronal activity by TTX treatment reversed the N-P/Q switch and made neurotransmission predominantly N-type channel-dependent. The neurotrophin receptor (Trk) antagonist K252a mimicked this TTX effect, whereas coapplication with TTX of the TrkB ligands brain-derived neurotrophic factor (BDNF) or neurotrophin-4 (NT-4) rescued the TTX effect. As in slice culture, *in vivo* administration of TTX or K252a into pup cerebella reversed the N-P/Q switch and made PC-DCN transmission predominantly N-type channel-dependent. Thus, neuronal activity and ensuing activation of Trk receptors by neurotrophins likely underlie the developmental N-P/Q switch at presynaptic terminals.

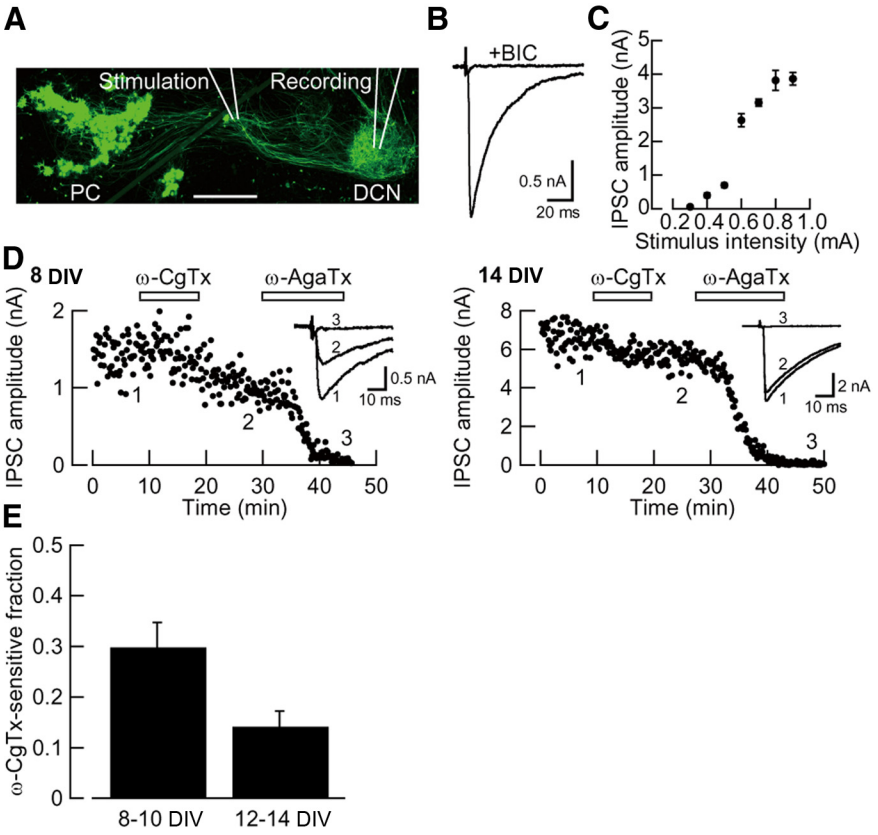

Figure 1. *In vitro* developmental decline in the ω-CgTx sensitivity of GABAergic synaptic transmission from PCs to DCN cells in slice culture. ***A***, EGFP-labeled PCs and their axons at 14 DIV in cerebellar slice culture infected with Lentivirus vector encoding EGFP that can be expressed under the control of a PC-specific promoter. IPSCs were evoked by stimulating PC axons and recorded from a DCN cell using glass electrodes. Scale bar, 300 μm. ***B***, IPSCs evoked in DCN cells by stimulating PC axons. Bath-application of bicuculline (BIC, 10 μM) abolished the IPSCs (superimposed). ***C***, Graded increase in the IPSC amplitude in response to stimulus intensity, with a maximal level reached at 0.8–0.9 mA. ***D***, Left, At 8 DIV, ω-CgTx (3 μM) reduced the amplitude of IPSCs by 37%. Subsequent application of ω-AgaTx (200 nM) abolished the remaining IPSCs. Right, At 14 DIV, ω-CgTx reduced the amplitude of IPSCs by 11%. Subsequent application of ω-AgaTx blocked the remaining IPSCs. Superimposed sample records are representative IPSCs at a holding potential of −60 mV before ω-CgTx application (1), after ω-CgTx application (2), and after ω-AgaTx application (3). In this figure, and Figures 2, 3, and 6, each data point represents the amplitude of an individual synaptic current. ***E***, The fraction of IPSCs blocked by ω-CgTx applications at 8–10 DIV and 12–14 DIV. The mean ± SEM derived from 7 to 10 cells are shown in bar graphs.

Materials and Methods

All experiments were performed in accordance with the guidelines of the Physiological Society of Japan.

Viral vector construct. Lentiviral vectors were designed to express EGFP under the control of a PC-specific promoter, L7-4N. The L7-4N promoter is a fusion promoter consisting of a minimal CMV sequence and a truncated (1 kb) PC-specific L7 promoter (Sawada et al., 2010). It exhibits much greater transcriptional activity than the original L7 promoter without compromising PC specificity (H. H., unpublished observation).

Viral vector production. The viral vector was produced by cotransfection of HEK293T cells (2.5×10^6 per dish) with a mixture of four plasmids (6 μg of pCAGkGP1R, 2 μg of pCAG4RTR2, 2 μg of pCAG-VSV-G, and 10 μg of vector plasmid pCL20c L7-4N-EGFP) using a calcium phosphate precipitation method as reported previously (Torashima et al., 2006). Briefly, cells were cultured in DMEM (Wako) supplemented with 10% fetal bovine serum. Sixteen hours after transfection, the culture medium was exchanged with fresh medium containing forskolin (10 μM). The medium containing vector particles were harvested once 64 h after transfection. Medium samples were filtered through membrane filters (0.45 μm), mixed with PEG-it Virus Concentration Solution (System Biosciences), and left overnight at 4°C. Viruses were collected from precipitates after centrifugation at 1500 × *g* the next day, and resuspended with PBS (−).

Preparation of cerebellar slice culture and solutions. Cerebellar slices were prepared according to the protocol of Gähwiler (1981) and Stoppini et al. (1991). In brief, P0 Wistar/ST rat pups of either sex were decapitated and brains were aseptically removed. Cerebella were dissected and meninges were carefully removed in cold Gey's balanced salt solution containing 5 mg/ml glucose and 1 mM kynurenic acid. Sagittal slices 350 μm thick were cut using a McIlwain tissue chopper, separated with fine forceps, and transferred onto transparent membranes (Millicell CM, Millipore). Slices were then cultured on a liquid layer of BME-based serum-free medium and maintained at 37°C in humidified 95% O_2 and 5% CO_2. Half of the medium was exchanged every 4 d. To inhibit glial proliferation, cytosine-β-D-arabinofuranoside (1 μM), uridine (1 μM), and 5-fluoro-2′-deoxyuridine (1 μM) were added to the medium after 4 d in culture. To visualize PCs, slice cultures at 1 DIV were infected with lentivirus-containing EGFP under control of a PC-specific promoter, L7-4N. The following drugs were applied to culture medium at 1 DIV until the day of recording: BIC (10 μM), TTX (1 μM), CNQX (10 μM), K252a (100 nM), and NGF family (NGF, BDNF, NT-3, and NT-4; 100 ng/ml, respectively). Before recording IPSCs, slices were moved from culture media to a recording chamber and washed for at least 30 min by superfusion with artificial CSF (aCSF) containing the following (in mM): 117.5 NaCl, 2.5 KCl, 6 $MgCl_2$, 2 $CaCl_2$, 10 glucose, 3 myo-inositol, 2 sodium pyruvate, 0.5 ascorbic acid, 1.25 NaH_2PO_4, and 26 $NaHCO_3$ (pH 7.4, when saturated with 95% O_2/5% CO_2). For recording GABAergic IPSCs, the aCSF contained CNQX (10 μM) and strychnine (0.5 μM) to block EPSCs and glycinergic IPSCs. Patch pipettes were filled with an internal solution containing the following (in mM): 157.5 CsCl, 9 NaCl, 10 HEPES, 1 EGTA, 2 Mg-ATP, and 5 QX314 Cl (adjusted to pH 7.3 with CsOH). Ca^{2+} currents were recorded in an external solution that contained (mM): 105 NaCl, 20 tetraethylammonium chloride (TEA-Cl), 2 $CaCl_2$, 6 $MgCl_2$, 2.5 KCl, 26 $NaHCO_3$, 10 glucose, 3 myo-inositol, 2 sodium pyruvate, 0.5 ascorbic acid, 1.25 NaH_2PO_4, and 0.0005 TTX (pH 7.4, when saturated with 95% O_2/5% CO_2). The pipette solution contained (mM): 80 Cs-gluconate, 30 CsCl, 40 HEPES, 10 TEA-Cl, 5 EGTA, 12 Na_2 phosphocreatine, 1 $MgCl_2$, 2 Mg-ATP, and 0.5 Na-GTP (adjusted to pH 7.3 with CsOH).

Immunostaining. At 12–15 DIV, cerebellar slice cultures were fixed in 0.1 M phosphate buffer containing 2% paraformaldehyde, 10% methanol, and 0.1% picric acid for 1 h (or 4% paraformaldehyde for 15 min).

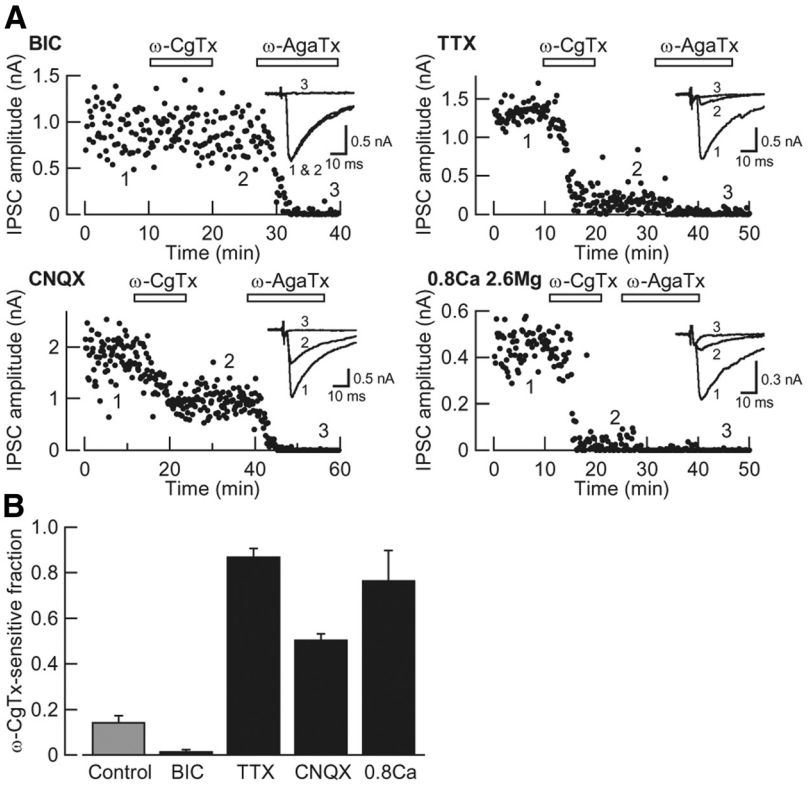

Figure 2. Activity-dependent regulation in the ω-CgTx sensitivity of GABAergic transmission in cerebellar slice culture. From 1 DIV to 12–14 DIV, slices were cultured in standard media containing bicuculline (BIC; 10 μM), TTX (1 μM), or CNQX (10 μM), or in low Ca^{2+} (0.8 mM) high Mg^{2+} (2.6 mM) media. IPSCs were recorded at 12–14 DIV after washing slices with standard aCSF for >30 min. ***A***, Top left, In a BIC-treated culture, ω-CgTx (3 μM) had no effect, but ω-AgaTx (200 nM) abolished IPSCs. Top right, In a TTX-treated culture, ω-CgTx attenuated IPSC amplitude by 89%. Subsequent application of ω-AgaTx abolished the remaining IPSCs. Bottom left, In a CNQX-treated culture, ω-CgTx attenuated the IPSC amplitude by 46%. Subsequent application of ω-AgaTx abolished the remaining IPSCs. Bottom right, In a cerebellar slice cultured in low Ca^{2+} (0.8 mM) high Mg^{2+} (2.6 mM) medium, ω-CgTx attenuated the IPSC amplitude by 95%. Subsequent application of ω-AgaTx abolished the remaining IPSCs. Sample records of representative IPSCs before ω-CgTx application (1), after ω-CgTx application (2), and after ω-AgaTx application (3) were superimposed. The holding potential was −60 mV. ***B***, The fraction of IPSCs blocked by ω-CgTx application in different culture conditions. Bar graphs show the mean ± SEM derived from 4 to 10 cells. Control data are the same as Figure 1*E* (gray bar).

Fixed cultures were processed for immunostaining as follows: (1) blocking in PBS(−) containing 4% skimmed milk and 0.2% Triton X-100 for 1 h, (2) application of primary antibodies in PBS(−) containing 0.1% bovine serum albumin and 0.05% Triton X-100 for 2 or 3 d at 4°C, (3) application of secondary antibodies in PBS(−) containing 20% goat serum and 0.05% Triton X-100 for 1.5 h, and (4) mounting with ProLong Gold antifade reagent (Invitrogen). We used primary antibodies to BDNF (rabbit polyclonal, Santa Cruz Biotechnology; diluted 1:50), calbindin D-28k (mouse monoclonal clone CB-955, Sigma-Aldrich, diluted 1:1000; rabbit polyclonal, Millipore; diluted 1:1000), VGLUT1 (guinea pig polyclonal, Millipore; diluted 1:500), GFAP (mouse monoclonal, Sigma-Aldrich; diluted 1:350), and TrkB (rabbit polyclonal, Santa Cruz Biotechnology; 1:50), along with goat secondary antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 555 (Invitrogen; diluted 1:500). In the primary antibody absorbing tests for evaluating the specificity of the BDNF, NT-4, or TrkB immunoreactivity, BDNF blocking peptide (Santa Cruz Biotechnology; final concentration 4 μg/ml), recombinant human NT-4 (Wako; final concentration 4 μg/ml), or recombinant human TrkB Fc chimera (R&D Systems; final concentration 28.6 μg/ml) was preincubated with primary antibodies overnight at 4°C. Confocal images were acquired using a Leica SP5 confocal microscope with a 20× objective lens. Excitation wavelengths were 488 nm (argon laser) and 543 nm (helium-neon laser). Emission wavelengths were 500–535 nm (for green) or >555 nm (for red). All immunostaining procedures were performed at room temperature (22–27°C), unless noted otherwise.

Drug infusion into immature rat cerebella in vivo. For implantation of a brain infusion cannula (Alzet brain infusion kit 1) connected to a mini osmotic pump (Model 1007D, Alzet) via a catheter tube (14 mm in length), Wistar/ST rats of either sex at P6 were anesthetized with isoflurane, skin over the cerebellum was cut, and using a 25 gauge needle, a hole was made in the skull over the cerebellum. A brain infusion cannula (0.36 mm in outer diameter, 1.5 mm in length) was inserted into the hole in the skull and fixed to the skull with dental cement. An osmotic pump was inserted into a subcutaneous pocket on the back of the rat. Before surgery, osmotic pumps were filled with TTX (159.6 ng) or K252a (93.5 ng) diluted in 100 μl of 0.9% saline at the final concentrations of 5 μM for TTX and 2 μM for K252a, respectively. The pumping rate of this osmotic pump was 0.423 ± 0.004 μl/h in isotonic saline at 37°C. TTX (16.2 ng/d) or K252a (9.5 ng/d) was infused into the cerebellar cortex for a maximum duration of 7 d. Control animals received osmotic pump infusion of 0.9% saline alone into the cerebellar cortex. To prevent pups from being killed by mother rats after surgery, pups were milk-fed four times per day.

Acute slice preparation. Sagittal slices of cerebellum (200 μm in thickness) were prepared from P12–P14 Wistar/ST rats of either sex killed by decapitation under isoflurane anesthesia. Before recording, slices were incubated for 1 h at 37°C in oxygenated aCSF. Neurons in slices were visually identified with a 20× water-immersion objective attached to an upright microscope (DM6000 CFS; Leica).

Recordings, drug application, and data analysis. Patch pipettes (2–3 MΩ) had a series resistance of 4–10 MΩ, which was compensated by up to 70% for a final value of 2–3 MΩ. Recording of IPSCs was made under voltage-clamp at a holding potential of −60 mV using a patch-clamp amplifier (Multiclamp 700B). Stimulation of synaptic input was made with a glass pipette filled with aCSF. The pipette was positioned in the vicinity of Purkinje cell axons to evoke GABAergic IPSCs every 10 s in DCN cells. Ca^{2+} currents in Purkinje cell somata were evoked every 20 s by a 20 ms depolarizing pulse from −80 mV holding potential to 0 mV under voltage-clamp. Synthetic ω-AgaTx (200 or 400 nM; Peptide Institute) and ω-CgTx (3 μM; Peptide Institute) were dissolved in oxygenated aCSF containing cytochrome *c* (0.25 mg/ml; Sigma-Aldrich) just before bath application. All records were low-pass filtered at 10 kHz and digitized at 20 kHz by Digidata 1320A with pClamp 9 software (Axon Instruments). Leak subtraction of Ca^{2+} currents was made by a P/N protocol (Takahashi et al., 1998). All values are given as mean ± SEM and significant difference was evaluated by Student's *t* test or ANOVA followed by Turkey–Kramer test. All experiments were performed at room temperature (18–23°C).

Results

Reproduction of the developmental N-P/Q channel switch in cerebellar slice culture

To investigate mechanisms underlying the developmental N-P/Q channel switch in presynaptic terminals (Iwasaki et al., 2000), we have established a cerebellar slice-culture preparation in which PCs and their axons are visually identified with EGFP labeling linked to the PC-specific L7-4N promotor (Fig. 1*A*). In cerebellar slices, cut from new born rats (postnatal day; P0) and maintained

for 8–15 DIV, bicuculline-sensitive, GABAergic IPSCs (IPSCs) were evoked in DCN neurons by stimulating PC axons (Fig. 1*B*). IPSC amplitude increased in response to graded stimulus intensities, indicating that multiple PC axons innervate a DCN cell (Fig. 1*C*). Therefore, we adopted maximal intensity for stimulation. In 8–10 DIV slices, bath-application of the N-type VGCC-specific blocker, ω-conotoxin GVIA (ω-CgTx, 3 μM), irreversibly attenuated the IPSC amplitude by 30 ± 5% ($n = 7$; Fig. 1*D*,*E*) as previously reported (Takahashi and Momiyama, 1993). The remaining IPSCs were then abolished with the P/Q-type-specific blocker, ω-agatoxin IVA (ω-AgaTx, 200 nM). The fraction of IPSCs mediated by N-type channels (N-IPSC) at 8–10 DIV is similar in magnitude to that in acute cerebellar slices from P8 rats (Iwasaki et al., 2000). Later, at 12–14 DIV, the N-IPSC fraction declined to 14 ± 3% ($n = 10$, $p < 0.05$; Fig. 1*D*,*E*) to a similar extent as in acute slices of P11–P14 rats (Iwasaki et al., 2000). Thus, the developmental N-P/Q channel switch for transmitter release was reproduced in this cerebellar slice culture preparation.

Neuronal activity regulates presynaptic N-type channels involved in neurotransmission

Using the cerebellar slice culture preparation, we investigated whether neuronal activity is essential for the developmental N-P/Q switch. We first tested the effect of bicuculline, as it increases firing rate of PCs by blocking tonic inhibition of PCs by ambient GABA (Häusser and Clark, 1997). When bicuculline (10 μM) was included in culture media from 1 DIV, IPSCs at 12–14 DIV became totally resistant to ω-CgTx, with the N-IPSC fraction being only 1.4 ± 0.9% ($n = 4$, $p < 0.05$; Fig. 2), as in acute slices from rats older than P16 (Iwasaki et al., 2000). Conversely, to completely block neuronal firing, we next tested TTX. When TTX was included in culture media (from 1 DIV), N-IPSCs accounted for 87 ± 4% ($n = 9$, $p < 0.001$) of total IPSCs at 12–14 DIV. Likewise, blockade of excitatory synaptic transmission by CNQX (10 μM) from 1 DIV increased the N-IPSC fraction to 50 ± 3%, ($n = 6$, $p < 0.001$). Thus, neuronal activity strongly reduces the contribution of N-type channels to transmitter release. Because neuronal activity induces Ca^{2+} influx via VGCCs into cell somata and neurites, we examined whether Ca^{2+} influx is essential for the N-P/Q switch, by reducing the Ca^{2+} concentration in culture media from 1.8 to 0.8 mM and increasing the Mg^{2+} concentration from 0.8 to 2.6 mM (from 1 DIV). This treatment also increased the N-IPSC fraction to 76 ± 13% ($n = 5$, $p < 0.001$; Fig. 2), suggesting that neuronal activity-dependent Ca^{2+} influx into cerebellar neurons reduces the contribution of N-type channels to transmitter release.

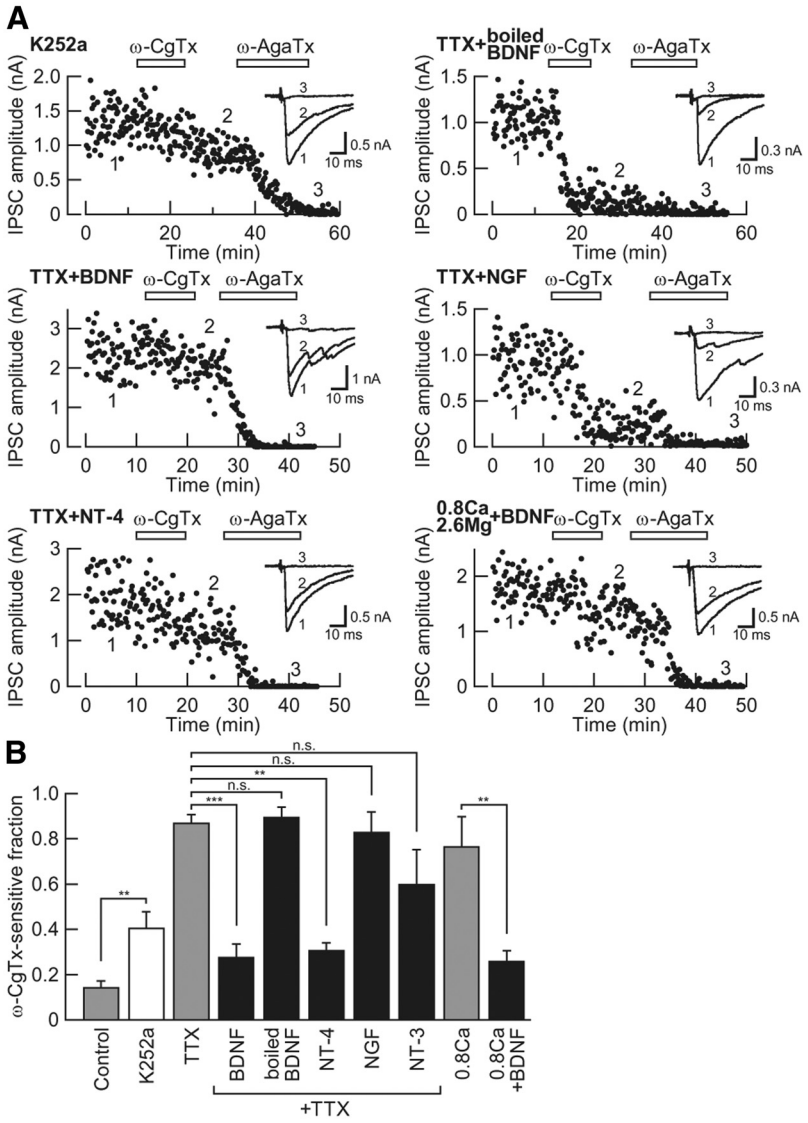

Figure 3. Involvement of TrkB in the activity-dependent decline of the ω-CgTx sensitivity of GABAergic transmission. Cerebellar slices were cultured with K252a (100 nM), TTX + neurotrophins (BDNF, NT-4, NGF, or NT-3; 100 ng/ml), or TTX (1 μM) + inactive (boiled) BDNF (100 ng/ml) in standard culture media from 1 DIV to 12–14 DIV, or cultured with BDNF (100 ng/ml) in culture media containing low Ca^{2+} (0.8 mM) and high Mg^{2+} (1.8 mM) from 1 DIV to 12–14 DIV. IPSCs were recorded at 12–14 DIV after washing slices with standard aCSF for >30 min. ***A***, Top left, After culture with K252a, ω-CgTx attenuated IPSC amplitude by 36% (cf. control). Middle left, After culture with TTX and BDNF, ω-CgTx attenuated IPSC amplitude by only 16% (cf. TTX alone). Bottom left, After culture with TTX and NT-4, ω-CgTx attenuated IPSC amplitude by only 23% (cf. TTX alone). Top right, In a TTX + boiled BDNF-treated culture, ω-CgTx reduced the amplitude of IPSCs by 92% (cf. TTX alone). Middle right, In a TTX + NGF-treated culture, ω-CgTx reduced the amplitude of IPSCs by 74% (cf. TTX alone). Bottom right, In a low Ca^{2+} (0.8 mM) high Mg^{2+} (2.6 mM) culture media treated with BDNF, ω-CgTx attenuated the IPSC amplitude by only 26% (cf. 0.8Ca). Sample records are IPSCs at −60 mV before (1), after ω-CgTx application (2), and after ω-AgaTx application (3). ***B***, The ω-CgTx-sensitive fraction of IPSCs in different culture conditions. The mean ± SEM derived from 4 to 12 cells are shown in bar graphs. Control, TTX, and 0.8Ca bars (gray) derived from Figures 1*E* and 2*B*. n.s.,Not significant; $^{**}p < 0.01$. $^{***}p < 0.001$.

Neurotrophins and Trk activity regulate N-type channels involved in neurotransmission

Ca^{2+} influx induced by neuronal activity triggers release of neurotrophins (Balkowiec and Katz, 2000; Tao et al., 2002), thereby activating the neurotrophin receptor Trks (Du et al., 2003). We investigated whether the neurotrophin signaling pathway might be involved in regulation of N-type channels mediating transmitter release in PC axon terminals, by introducing the Trk tyrosine

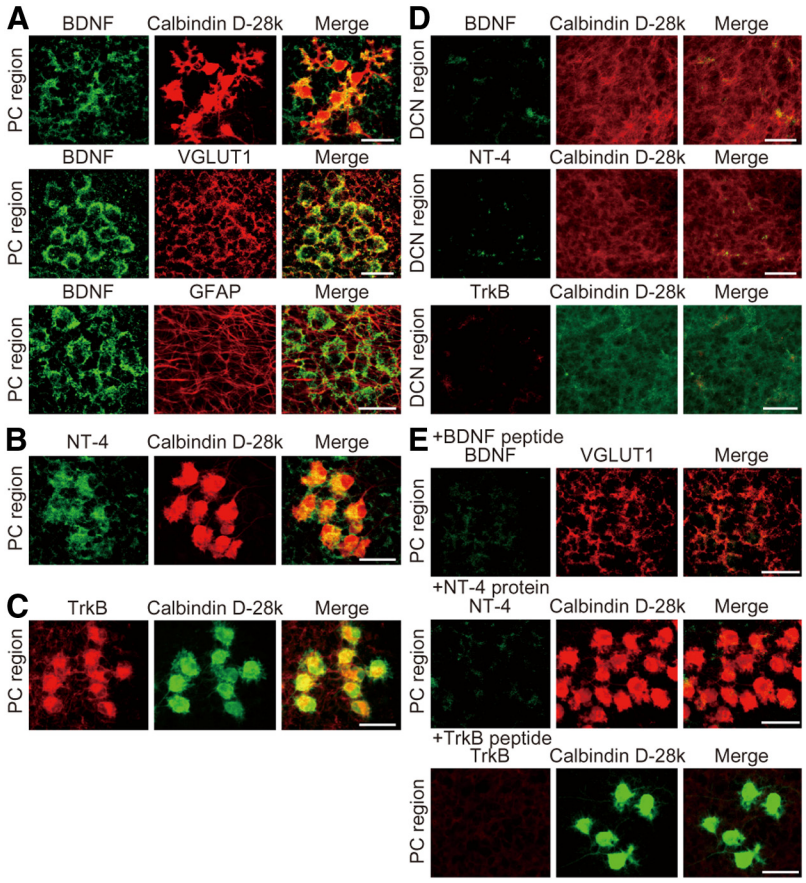

Figure 4. BDNF, NT-4, and TrkB immunoreactivities in cerebellar slice cultures. ***A***, Double-immunofluorescence labeling of BDNF (green, left column) with calbindin D-28k, VGLUT1, or GFAP (red, middle column). Merged fluorescence pictures are shown in the right column. Immunofluorescence signal of BDNF overlapped partially with that of calbindin D-28k in PC, and almost entirely with that of VGLUT1. BDNF immunofluorescence did not overlap with astrocytic GFAP immunofluorescence in the PC region. ***B***, NT-4 immunoreactivity (green, left) overlapped with calbindin D-28k immunoreactivity (red, middle) in the PC region. ***C***, Calbindin D-28k immunoreactivity (green, middle) overlapped with TrkB immunoreactivity (red, left) in the PC region. ***D***, In the DCN region immunofluorescence signals for BDNF, NT-4, and TrkB (green or red, left) were all weak, showing no overlap with calbindin D-28k immunofluorescence (red or green, middle). ***E***, Background signals in the PC region after absorbing primary antibodies with BDNF, NT-4, or TrkB peptides. Scale bars, 50 μm.

kinase inhibitor, K252a (100 nM), into culture media from 1 DIV. At 12–14 DIV, the N-IPSC fraction after K252a application was 40 ± 7% (n = 12; Fig. 3). That was significantly higher than control values (14 ± 3%, $p < 0.01$), suggesting that neurotrophin receptor activity contributes to downregulation of N-type channels in PC axon terminals. Neurotrophin receptors are classified as TrkA, TrkB, TrkC, and p75^{NTR} (Bothwell, 1995). To assess the type of receptor involved, we included different neurotrophins in culture media together with TTX. The TrkB ligand BDNF (100 ng/ml) plus TTX in culture media from 1 DIV, counteracted the effect of TTX, reducing the N-IPSC fraction from 87% (TTX alone) to 28 ± 6% ($n = 11$, $p < 0.001$), whereas boiled BDNF had no effect (89 ± 5%, $n = 4$, $p > 0.9$; Fig. 3). Likewise another TrkB ligand, NT-4 (100 ng/ml), when included in culture media with TTX from 1 DIV, counteracted the effect of TTX, with the N-IPSC fraction at 12–14 DIV being 31 ± 4% ($n = 4$, $p < 0.01$; Fig. 3). In contrast, the TrkA ligand NGF (100 ng/ml) or the TrkC ligand NT-3 (100 ng/ml), included in culture media with TTX, had no effect on N-IPSC fraction (NGF, 83 ± 9%, $n = 4$, $p > 0.9$; NT-3, 60 ± 16%, $n = 6$, $p = 0.16$; Fig. 3). As both of the TrkB ligands BDNF and NT-4, but neither the TrkA ligand NGF nor the TrkC ligand NT-3, counteracted the effect of TTX on the N-IPSC fraction, TrkB is most likely involved in downregulation of N-type channels mediating transmitter release. The upregulatory effect of low Ca^{2+} high Mg^{2+} media on N-IPSC fraction (Fig. 2) was also counteracted by BDNF (N-IPSC fraction, 26 ± 5%, $n = 5$, $p < 0.01$; Fig. 3), suggesting that TrkB signaling for N-type channel downregulation resides in downstream of Ca^{2+} influx into cerebellar neurons.

To further assess possible sites of neurotrophin action, we examined immunocytochemical localization of neurotrophins and TrkB in cerebellar slice cultures (Fig. 4). The BDNF immunofluorescence signal was prominent on PC where it overlapped partially with calbindin D-28k signal (Fig. 4*A*, top row) and almost completely with VGLUT1 signals (Fig. 4*A*, middle row), suggesting that it is likely localized in the glutamatergic terminals, such as those of parallel fibers (PFs). It showed little overlap with the GFAP signal in astrocytes (Fig. 4*A*, bottom row). Unlike BDNF, NT-4 and TrkB signals overlapped extensively with the calbindin D-28k signal (Fig. 4*B*,*C*), suggesting that they are expressed in PC somata. In the DCN region, BDNF, NT-4, and TrkB signals were all weak (Fig. 4*D*). These results are consistent with previous reports that BDNF is expressed in cerebellar granule cells (Das et al., 2001), and that NT-4 and TrkB are expressed in PC somata (Yan et al., 1997; Friedman et al., 1998). Thus, during postnatal development, BDNF released from PF terminals (Sadakata et al., 2004) together with NT-4 released from PCs may activate TrkB in PC somata, thereby facilitating the replacement of N-type with P/Q-type channels at cerebellar inhibitory presynaptic terminals.

Neuronal activity has no effect on the expression of VGCC subtypes in PC somata

If neuronal activity directly regulates *de novo* synthesis of N-type channels in PC nuclei, blockade of neuronal activity with TTX will increase N-type channel currents in PC somata. We examined this possibility by recording Ca^{2+} currents evoked in PC somata in cerebellar slices at 13–15 DIV pretreated with TTX (1 μM) from 1 DIV. Omega-CgTx (3 μM) and ω-AgaTx (400 nM) attenuated Ca^{2+} currents by 17 ± 4% ($n = 4$) and 69 ± 4% ($n = 4$), respectively (Fig. 5). Approximately 14% of Ca^{2+} currents remaining after ω-CgTx and ω-AgaTx applications were blocked by Cd^{2+} (100 μM; Fig. 5). This VGCC subtype current profile in PC somata after TTX treatment was essentially the same as that of controls ($p > 0.3$). Thus, chronic blockade of neuronal activity had no effect on VGCC subtype compositions in PC somata.

Neuronal activity and Trk activity are required for *in vivo* developmental downregulation of N-type channels that mediate transmitter release

Finally, we asked whether cerebellar neuronal activity, or neurotrophin/Trk signaling *in vivo* downregulates N-type channels

mediating PC-DCN IPSCs. To address this question, we infused TTX (16.2 ng/d) or K252a (9.5 ng/d) into the cerebellar cortex of pups every day from P6 to P12–P14 using an osmotic pump (Fig. 6*A*). In acute slices from control animals infused with saline, ω-CgTx attenuated the amplitude of IPSCs by 14 ± 6% ($n = 6$) as previously reported for normal rats at P11–P14 (Iwasaki et al., 2000). In slices from TTX-infused rats, ω-CgTx blocked IPSCs more strongly (53 ± 9%, $n = 7$, $p < 0.05$; Fig. 6*B*,*C*). Likewise, in slices from K252a-treated rats, ω-CgTx attenuated IPSCs by 63 ± 11%, ($n = 7$, $p < 0.01$). These results suggest that neuronal activity and Trk signaling play essential roles in the developmental N-P/Q switch in PC axon terminals.

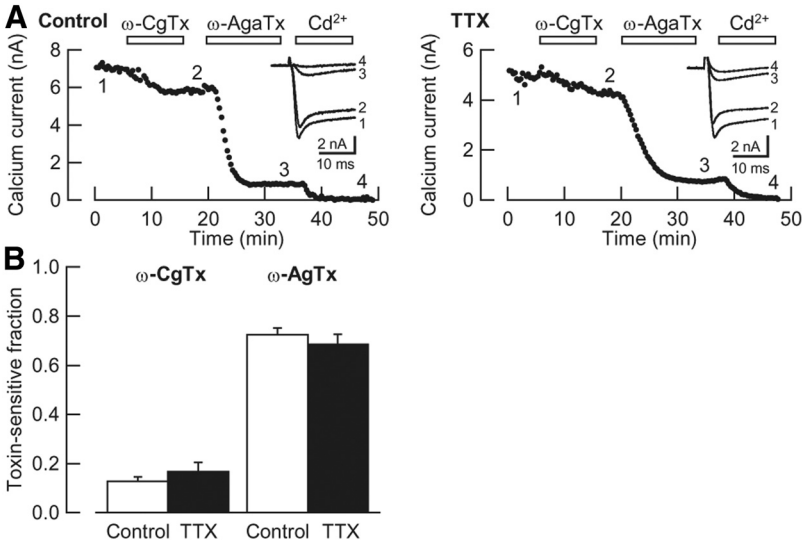

Figure 5. No effect of TTX treatment on composition of VGCC subtypes in Ca^{2+} currents recorded from PC somata in cerebellar slice cultures. ***A***, Left, In control, ω-CgTx (3 μM) and ω-AgaTx (400 nM) reduced the amplitude of Ca^{2+} currents by 15% (2) and 73% (3), respectively. A small fraction remaining after application of both toxins (12%), was abolished by Cd^{2+} (100 μM; 4). Right, In a TTX-treated culture, the percentages of the Ca^{2+} current amplitude attenuated by ω-CgTx (14%, 2), or ω-AgaTx (72%, 3) were similar to controls. Superimposed sample records are representative Ca^{2+} currents. Each data point represents the amplitude measured at the end of a voltage step. ***B***, The fraction of Ca^{2+} currents attenuated by ω-CgTx or ω-AgaTx in untreated control and TTX-treated cultures. The mean ± SEM derived from four cells are shown in bar graphs.

Discussion

Neuronal activity triggers developmental N-P/Q channel switch

In the early stage of animal development, N-type channels play essential roles in synaptogenesis (Jones et al., 1997), neuronal migration (Komuro and Rakic, 1992), and synaptic transmission (Luebke et al., 1993; Takahashi and Momiyama, 1993). However, at many types of synapses, the contribution of N-type channels to synaptic transmission decreases with time, both in culture (Scholz and Miller, 1995) and *in situ* (Rosato Siri and Uchitel, 1999; Iwasaki et al., 2000; Momiyama, 2003). At the PC–DCN synapse in acute cerebellar slices, the N-type-dependent fraction of IPSCs comprises 30–40% of total IPSCs at P8, but is reduced to 10% at P14, and becomes undetectable after P16 (Iwasaki et al., 2000). Likewise, at PC–DCN synapses in slice culture, the N-type-dependent fraction was 30% at 8–10 DIV, but was reduced to 10% at 12–14 DIV (Fig. 1), recapitulating postnatal development. When neuronal activity was chronically blocked with TTX, the developmental shift was apparently reversed in direction toward predominantly N-type channel neurotransmission, both in slice culture (Fig. 2) and *in vivo* (Fig. 6). Chronic blockade of excitatory transmission with CNQX likewise increased the N-type-dependent fraction of IPSCs, whereas a blockade of inhibitory transmission with bicuculline decreased it (Fig. 2). These results suggest an indispensable role of neuronal excitability for the developmental N-P/Q channel switch in presynaptic terminals.

Involvement of neurotrophins and Trk signaling in N-P/Q channel switch

Like TTX or CNQX, the Trk inhibitor, K252a, increased the N-type-dependent IPSC fraction, both in culture (Fig. 3) and *in vivo* (Fig. 6). The TrkB ligands BDNF or NT-4, when applied together with TTX, rescued the effect of TTX, whereas the TrkA ligand NGF or the TrkC ligand NT-3 had no such effect (Fig. 3). Neuronal activity triggers release of neurotrophins from neurons (Balkowiec and Katz, 2000), upregulates their expression (Tao et al., 2002), and translocates Trks between the cell surface and intracellular stores (Du et al., 2003). Ca^{2+} influx is essential for secretion (Balkowiec and Katz, 2000) and expression (Tao et al., 2002) of BDNF and activation of TrkB (Du et al., 2003). In this regard, reduction of Ca^{2+} influx by reducing the Ca^{2+}/Mg^{2+} ratio in culture media increased N-type channel-dependent neurotransmission (Fig. 2), consistent with the idea that developmental decline of N-type channel expression in the nerve terminal is mediated by BDNF and TrkB. In the cerebellum, BDNF and NT-4 were localized on PF terminals and PC somata, respectively, whereas TrkB was found on somata and dendrites of PCs (Fig. 4). Thus, BDNF may be released from PF terminals in response to GC firing and NT-4 is released from PC somata in response to PC firing, thereby making both neurotrophins to activate TrkB in PC somata for developmental downregulation of the N-type channel fraction in PC axon terminals.

Possible mechanisms of N-P/Q channel switch

It has been proposed that presynaptic Ca^{2+} channels compete for channel type-preferring slots (Cao and Tsien, 2010). This common slot model is consistent with the fact that P/Q-type Ca^{2+} currents lost by $Ca_V2.1$ knock-out are fully compensated by N-type VGCC overexpression (Inchauspe et al., 2004; Ishikawa et al., 2005). Moreover, developmental replacement of N-type Ca^{2+} currents by P/Q-type Ca^{2+} currents occurs without altering the total Ca^{2+} channel density (Iwasaki et al., 2000) at the calyx of Held presynaptic terminal. In cultured hippocampal neurons, overexpression of N-type channels displaces P/Q-type channels from the putative common slot, whereas overexpression of P/Q-type channels does not displace N-type channels (Cao and Tsien, 2010). If the presynaptic slot has such a preference, the developmental N-P/Q channel switch is likely caused by developmental downregulation of N-type channels, rather than upregulation of P/Q-type channels, in the slot.

How is TrkB signaling linked to developmental N-P/Q channel replacement in the PC axon terminals? At the calyx of Held,

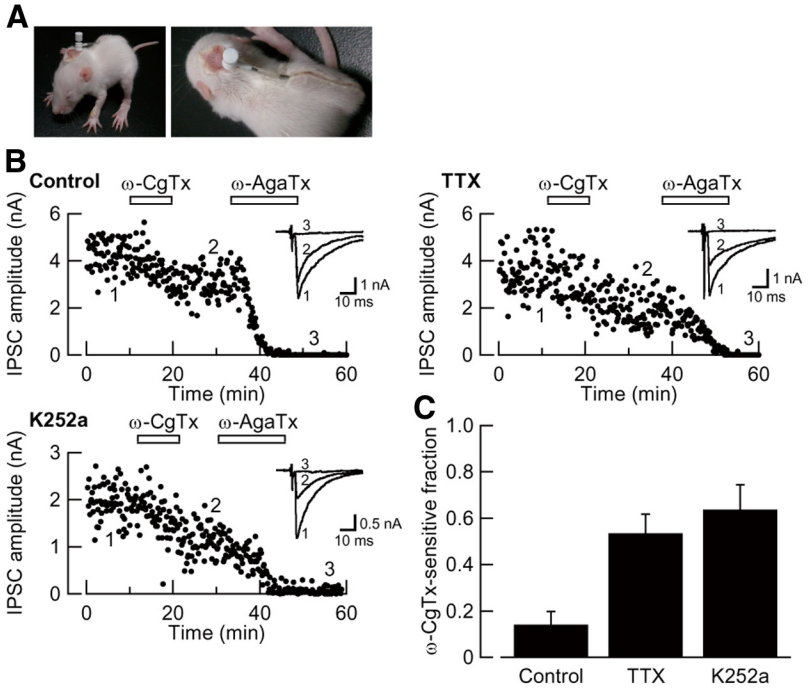

Figure 6. *In vivo* block of the developmental switch of VGCC subtypes mediating cerebellar GABAergic transmission. GABAergic IPSCs were recorded from DCN cells in acute cerebellar slices from P12–P14 rats, pretreated from P6, with vehicle (0.9% saline), TTX (16.2 ng/d), or K252a (9.5 ng/d) infused through a cannula into the cerebellum. ***A***, Photograph of a P13 rat implanted at P6 with an infusion cannula that is connected to a subcutaneous osmotic pump. ***B***, Top left, After vehicle treatment, ω-CgTx (3 μM) attenuated IPSC amplitude by 25%. ω-AgaTx (200 nM) blocked the remaining IPSCs. Top right, After TTX treatment, ω-CgTx attenuated IPSC amplitude by 46%. ω-AgaTx blocked the remaining IPSCs. Bottom, after K252a treatment, ω-CgTx attenuated IPSC amplitude by 48%. ω-AgaTx blocked the remaining IPSCs. Superimposed sample records are representative IPSCs at a holding potential of −60 mV before ω-CgTx application (1), after ω-CgTx application (2), and after ω-AgaTx application (3). ***C***, The ω-CgTx-sensitive fraction of IPSCs treated with vehicle (control), TTX, or K252a. The mean ± SEM obtained from six to seven cells (from three to four rats) are shown in bar graphs.

N-type and R-type Ca^{2+} currents are completely replaced by P/Q-type Ca^{2+} currents after P13 (Iwasaki et al., 2000). In contrast, in the bushy cell body of the calyx of Held in the anterior ventral cochlear nucleus, the proportion of VGCC subtype currents remains unchanged during development (Doughty et al., 1998). Likewise, in PC somata, TTX treatment had no effect on the proportion of VGCC subtype currents (Fig. 5). These results suggest a presence of Ca^{2+} channel type-specific sorting mechanism operating for presynaptic VGCC subtype replacement.

Although little is known as to the subtype-specific sorting mechanism for ion channel targeting, there are some hints. The $Ca_V2.2$ α_1 subunit of N-type channels has two splice variants, $Ca_V2.2a$ and $Ca_V2.2b$, in which only the former variant can interact with the active zone scaffold proteins CAST and Mint1 (Maximov and Bezprozvanny, 2002). However, it is unknown whether there is a regulatory mechanism for *de novo* synthesis of $Ca_V2.2a$. The light chain of microtubule-associated protein MAP1A is expressed near the active zone and can bind to a C-terminal domain of N-type channels, but not to P/Q-type channels (Leenders et al., 2008). However, it remains open whether MAP1A light chain 2 can be negatively linked to Trk signaling for the developmental downregulation of N-type channels in the nerve terminal. Collapsin response mediator protein 2 (CRMP-2) interacts with the C terminus of N-type channels with a high binding affinity and promotes cell surface expression of N-type channels (Brittain et al., 2009). Although its binding affinity to P/Q-type channels is unknown, CRMP-2 might be a candidate for the presynaptic slot. Thus, so far, the downstream mechanism of TrkB linked to presynaptic N-type channel regulation remains unsolved.

Physiological significance of N-P/Q channel switch

Although N-type and P/Q-type channels in the nerve terminal share many basic properties in common, they differ in modulatory characteristics (Ishikawa et al., 2005). N-type channels are more strongly inhibited by G-proteins than P/Q-type channels (Currie and Fox, 1997; Ishikawa et al., 2005), and are more often involved in asynchronous (Hefft and Jonas, 2005) and ectopic (Matsui and Jahr, 2004) transmitter release. P/Q-type channels, but not N-type channels, undergo facilitation during repetitive activation via binding to Ca^{2+} sensor proteins, such as calmodulin (DeMaria et al., 2001), NCS-1 (Tsujimoto et al., 2002), or VLIP-1 (Leal et al., 2012). At immature rodent calyces of Held, VGCCs show both activity-dependent facilitation and inactivation (Xu and Wu, 2005; Nakamura et al., 2008), but after the second postnatal week, facilitation becomes the dominant mode of activity-dependent VGCC modulation (Nakamura et al., 2008; Hori and Takahashi, 2009). Although the magnitude of the developmental decline in the contribution of N-type channels to synaptic transmission varies among synapses, complete N-P/Q replacement is observed at "detonator" type excitatory synapses, such as the calyx of Held (Iwasaki and Takahashi, 1998; Iwasaki et al., 2000) or neuromuscular junctions (Rosato Siri and Uchitel, 1999), and also at the major strong inhibitory synapses, such as PC-DCN or reticulothalamic synapses (Iwasaki et al., 2000). N-P/Q replacement and the ensuing increase in VGCC facilitation during postnatal development strengthens the efficacy of synaptic transmission in response to high-frequency inputs, thereby contributing to reliable neuronal processing at mature excitatory and inhibitory synapses.

Developmental roles of neuronal activity and neurotrophin signaling

During development neurotrophins promote neurogenesis, synaptogenesis, and synaptic strength (Huang and Reichardt, 2001). In mouse visual cortex, during early postnatal development, transgenic enhancement of BDNF release accelerates maturation and stabilization of GABAergic inhibitory system, causing precocious development of visual acuity and earlier termination of the critical period for visual cortical plasticity (Huang et al., 1999). In dark reared mice, BDNF overexpression sufficiently induces normal development of visual cortex (Gianfranceschi et al., 2003). Likewise, in GABAergic nerve terminals in cerebellar slice culture, BDNF treatment rescued reversed developmental switch of VGCC subtypes caused by neuronal inactivity. Thus, during postnatal development of animals, BDNF released in response to neuronal activity pro-

motes maturation of neuronal circuitries into strong, accurate, and stable signaling systems.

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