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Rapid Communication

Src-family protein tyrosine kinase negatively regulates cerebellar long-term depression

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Abstract

Protein phosphorylation is a major mechanism for the regulation of synaptic transmission. Previous studies have shown that several serine/threonine kinases are involved in the induction of long-term depression (LTD) at excitatory synapses on a Purkinje neuron (PN) in the cerebellum. Here, we show that Src-family protein tyrosine kinases (SFKs) are involved in the regulation of the LTD induction. Intracellular application of c-Src suppressed LTD. We also show that application of a SFK-selective inhibitor PP2 recovered LTD from the suppression caused by the inhibition of mGluR1 activity. These results indicate that SFKs negatively regulate the LTD induction at excitatory synapses on a cerebellar PN.

Keywords: Purkinje neuron; Cerebellum; Synaptic plasticity; LTD; Src-family protein tyrosine kinase; mGluR1

Src-family protein tyrosine kinases (SFKs) are involved not only in the regulation of cell proliferation and differentiation, but also in the regulation of neuronal functions in the adult central nervous system (CNS). Five members of SFKs (Src, Fyn, Yes, Lck and Lyn) are expressed in differentiated, post-mitotic neurons in the CNS (Kalia et al., 2004). They regulate the activity of ion channels including ligand-gated channels, such as NMDA receptor (NMDAR), $GABA_A$ receptor and nicotinic acetylcholine receptor (Wang and Salter, 1994; Moss et al., 1995; Wang et al., 2004), as well as voltage-gated potassium channel (Fadool et al., 1997). They also affect slow EPSC mediated by metabotropic type 1 glutamate receptor (mGluR1) at parallel fiber to Purkinje neuron (PN) synapses (Canepari and Ogden, 2003).

Protein phosphorylation is involved in the regulation of synaptic plasticity, a persistent change in the efficacy of synaptic transmission assumed to be a cellular basis of learning and memory. Although involvement of serine/threonine phosphorylation of synaptic proteins has been extensively studied, recent studies have shown that tyrosine phosphorylation also plays important roles in the regulation of synaptic plasticity in the hippocampal CA1 region (Grant et al., 1992; Lu et al., 1998; Ahmadian et al., 2004; Huang and Hsu, 2006; Moult et al., 2006; Fox et al., 2007). The long-term depression (LTD) at excitatory synapses between parallel fibers and a PN is involved in some forms of motor learning (De Zeeuw et al., 1998; Ito, 2001), and the implication of protein tyrosine kinase (PTK) in its induction has also been reported (Boxall et al., 1996; Hartell, 2001; Ito, 2001). However, involvement of specific family of PTK such as SFKs has not been assessed. In this study, we examined whether SFKs are involved in the regulation of LTD in a PN.

At inhibitory synapses on a cerebellar PN, c-Src negatively regulates the induction of long-term potentiation known as rebound potentiation (Kawaguchi and Hirano, 2006). We first examined whether c-Src also affects the cerebellar LTD in a cultured PN. Methods of preparing cerebellar primary neuronal culture were similar to our previous study (Tsuruno and Hirano, 2007). Briefly, cerebella were dissected out from E20 Wistar rats and were incubated in Ca^{2+} and Mg^{2+}-free Hanks' balanced salt solution containing 0.1% trypsin and 0.05% DNase for 15 min at 37 °C. Neurons were dissociated by trituration and seeded on poly-D-lysine-coated coverslips in DMEM/F12-based medium containing 2% fetal bovine serum. A half of the medium was changed with the serum-free medium every 7 days, and cultured cells were used for experiments 3–4 weeks

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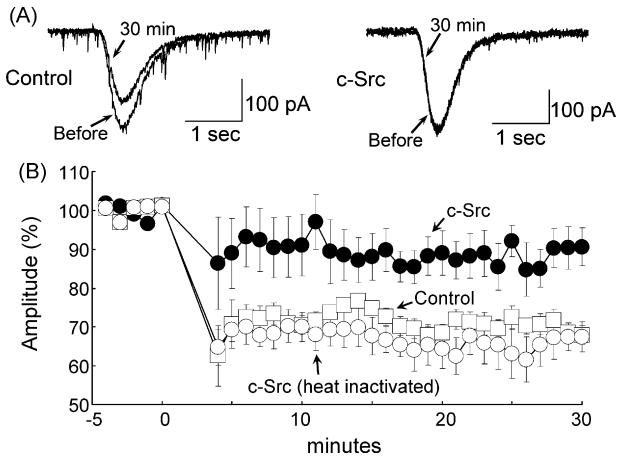

Fig. 1. c-Src suppressed LTD. (A) Representative glutamate responses before and 30 min after conjunction of depolarization pulses with glutamate application in a PN with (c-Src) or without (Control) intracellular c-Src. (B) Time courses of amplitudes of glutamate responses before and after conjunctive conditioning with intracellularly applied c-Src ($n = 6$), heat-inactivated c-Src ($n = 5$) or without application ($n = 6$).

after dissociation. All experiments were performed according to the guidelines for animal experimentation by the National Institutes of Health (United States) and Kyoto University. All procedures were approved by the local committee for handling experimental animals at the Graduate School of Science, Kyoto University.

Cerebellar LTD was monitored with the current responses to glutamate applied iontophoretically from a glass pipette containing 10 mM glutamate and 10 mM HEPES (pH 7.3) toward a primary or secondary dendrite of a PN. As described previously (Tsuruno and Hirano, 2007), currents were recorded using whole-cell patch clamp technique with an amplifier (EPC9, HEKA, Lambrecht, Germany) at room temperature (20–25 °C). The extracellular solution contained (in mM) 145 NaCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 10 glucose titrated to pH 7.4 with KOH. 1 μM tetrodotoxin (Wako, Osaka, Japan) was applied to suppress action potential and 20 μM bicuculline (Tocris, Bristol, UK) to suppress GABAergic IPSCs. A PN was visually identified by a large cell body and thick dendrites (Hirano and Ohmori, 1986). A patch pipette used to record from a PN was filled with the internal solution containing (in mM) 150 CsCl, 0.5 EGTA, 10 HEPES, 2 Mg-ATP (Sigma, St. Louis, MO) and 0.2 Na-GTP (Sigma) titrated to pH 7.4 with CsOH. The electrode resistance was 3–5 MΩ. The membrane potential of a PN was held at −70 mV unless otherwise stated. Only recordings with an input resistance of >100 MΩ and series resistance of <25 MΩ were accepted, and an experiment was terminated when a change of >20% was detected.

As shown previously (Linden et al., 1991; Tsuruno and Hirano, 2007), LTD was induced by the conjunction of depolarization pulses (0 mV for 3 s repeated 9 times at 0.05 Hz) with glutamate application, and monitored with the glutamate responsiveness. The amplitude of glutamate response 30 min after the conditioning was 68 ± 4% of the baseline (Mean ± S.E.M., $n = 6$, Fig. 1A and B). Intracellular application of c-Src (20 U/ml, Upstate, Charlottesville, VA) into a PN through a patch pipette was performed similarly to previous studies (Lu et al., 1998; Kawaguchi and Hirano, 2006). Application of c-Src suppressed LTD (91 ± 5%, $n = 6$, $P < 0.01$ by Students' t-test, Fig. 1A and B), while that of heat-inactivated c-Src did not (67 ± 4%, $n = 5$, Fig. 1B). We examined the effect of c-Src application on the mGluR1 activity, Ca^{2+} influx through voltage-gated calcium channel and synaptic transmission mediated by AMPA receptor (AMPAR), all of which are necessary for the LTD induction. They are reflected by the current induced by 3,5-dihydroxyphenylglycine (DHPG, Tocris), voltage-dependent Ca^{2+} current and miniature excitatory post-synaptic current (mEPSC), respectively (Table 1). Methods for measurements were described in our previous study (Tsuruno and Hirano, 2007). mGluR1-mediated current was recorded by iontophoretically applying an agonist DHPG from a pipette containing 2 mM DHPG and 10 mM HEPES (pH 7.3). The amplitudes of current measured in PNs in the presence of a drug were compared with the average amplitude recorded from 7 to 10 untreated control PNs in the same culture. Ca^{2+} current was recorded by depolarizing a PN to −10 mV for 80 ms. Application of c-Src did not cause any alteration in the basal properties. Thus, enhanced activity of c-Src suppressed LTD without affecting Ca^{2+} influx and responsiveness of mGluR1 and AMPAR, all of which are required for triggering the LTD induction in a PN.

We next examined the effect of inhibition of SFKs on the glutamate response in a PN. Extracellular application of PP2 (200 nM, Calbiochem, San Diego, CA), a selective inhibitor of SFKs, did not alter the glutamate response (94 ± 3%, $n = 6$, data not shown). Also, PP2 did not cause any alterations in the basal properties of PNs (Table 1).

Then, we examined whether inhibition of SFKs affects the LTD induction. Conjunction of the depolarization pulses with glutamate application induced LTD in the presence of PP2, but not to a further extent (65 ± 6%, $n = 5$, Fig. 2A). We next

Table 1
Effects of drugs and c-Src protein on basal properties of PNs

	AMPA–mEPSC				DHPG-induced current (%)	Ca^{++} Current (nA)
	Amplitude (pA)	10%–90% Rise (ms)	Half width (ms)	Frequency (Hz)		
Control	14.3 ± 2.8	2.0 ± 0.2	7.9 ± 0.8	2.8 ± 1.0 (10)	100 ± 51 (31)	5.8 ± 1.2 (10)
CPCCOEt	12.4 ± 2.2	2.0 ± 0.4	8.0 ± 1.1	4.3 ± 1.7 (10)*	1 ± 4 (10)*	5.6 ± 1.3 (10)
PP2	13.1 ± 2.7	2.1 ± 0.5	8.2 ± 1.0	3.1 ± 0.8 (10)	105 ± 53 (9)	6.1 ± 1.6 (10)
c-Src	13.8 ± 3.5	2.0 ± 0.3	8.0 ± 1.5	2.6 ± 1.2 (10)	93 ± 49 (10)	5.6 ± 1.8 (10)

N is presented in the parentheses. Data are presented as mean ± S.D. *Asterisks* indicate $P < 0.05$ (Dunnett test).

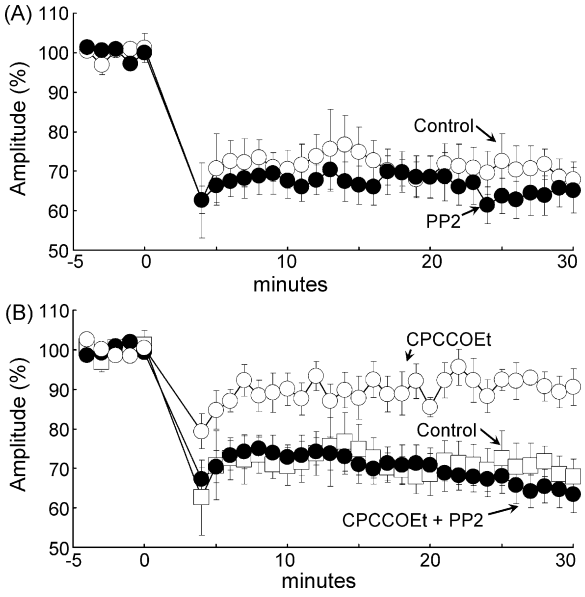

Fig. 2. Inhibition of SFKs facilitated the LTD induction. (A) Time courses of amplitudes of glutamate responses before and after conjunctive conditioning with ($n = 5$) or without ($n = 6$) PP2. (B) Time courses of amplitudes of glutamate responses before and after conjunctive conditioning with both CPCCOEt and PP2 ($n = 5$), with CPCCOEt alone ($n = 6$) or without either drugs ($n = 6$).

examined whether inhibition of SFKs recovers the LTD induction from a suppressed condition. Activation of mGluR1 is necessary for the LTD induction in a PN (Shigemoto et al., 1994). As shown in Fig. 2B, inhibition of mGluR1 with a selective inhibitor CPCCOEt (50 μM, Tocris) suppressed LTD ($91 \pm 5\%$, $n = 6$, $P < 0.01$, Fig. 2B). We confirmed that CPCCOEt reduced the amplitude of DHPG-induced current, supporting that CPCCOEt suppressed LTD through inhibition of the mGluR1 activity (Table 1). It was reported that the mGluR1 activity measured with the glutamate-induced Ca^{2+} increase in mGluR1-transfected COS1 cells is reduced to less than 20% with 50 μM CPCCOEt (Litschig et al., 1999). CPCCOEt did not affect Ca^{2+} current and the amplitude and time course of mEPSC, but slightly increased frequencies of mEPSCs for an unknown reason. When PP2 was applied with CPCCOEt, LTD was recovered ($63 \pm 5\%$, $n = 5$, $P < 0.01$, Fig. 2B). Thus, inhibition of SFK activity reversed the suppression of LTD caused by inhibition of the mGluR1 activity. One possibility is that PP2 recovers the mGluR1 activity suppressed by CPCCOEt. A previous study showed that PP1, an inhibitor of SFKs, increased mGluR1-mediated current in a PN (Canepari and Ogden, 2003). Thus, we examined whether PP2 enhanced the residual mGluR1 activity after the CPCCOEt application by monitoring DHPG-induced current. However, PP2 did not recover the DHPG-induced current from the inhibition by CPCCOEt ($1 \pm 2\%$, $n = 10$). Therefore, we considered that the mGluR1 activity might inhibit SFKs, which is required for the LTD induction.

There are two major phosphorylation sites involved in the regulation of the SFK activity. The phosphorylation of Tyr418 is required for c-Src activation, whereas the phosphorylation of Tyr529 inactivates c-Src. Phosphorylation levels of the two regulatory tyrosine residues in SFK were monitored with immunoblotting using the antibodies against phospho-Tyr418 and phospho-Tyr529 SFK in the cerebellar culture. As described previously (Kawaguchi and Hirano, 2006), cultured cells were treated with the external solution in the presence or absence of 50 μM DHPG for 10 min, followed by lysis in SDS sample buffer. Protein from littermate culture was subjected to SDS-PAGE and transferred to PVDF membrane. The membrane was immunoblotted using the rabbit polyclonal antibody against phospho-Tyr418 or phospho-Tyr529 SFK (Invitrogen, Carlsbad, CA) and HRP-conjugated secondary antibody (Chemicon). For sequential reprobing of the same blots, the membrane was stripped of the antibodies, and immunoblotted again using the mouse monoclonal antibody against total Src (Upstate, Billerica, MA) and HRP-conjugated secondary antibody. Signals were detected using the SuperSignal West Pico Substrate (Pierce, Rockford, IL). The signal intensity of phosphorylated SFK was divided by that of corresponding total Src for normalization. While the DHPG application increased phosphorylation of Tyr529 ($126 \pm 8\%$, $n = 5$, $P < 0.05$, paired t-test, Fig. 3), it did not increase phosphorylation of Tyr418 significantly ($114 \pm 10\%$, $n = 5$, $P = 0.23$, Fig. 3). These results indicate that activation of group I mGluR inhibits the SFK activity in the cerebellar culture. Taken together, our results suggest that the SFK activity negatively regulates the LTD induction and that the mGluR1 activation inhibits SFK in a PN.

Our previous study showed that c-Src suppresses rebound potentiation (Kawaguchi and Hirano, 2006), suggesting that SFKs might be generally suppressing long-term synaptic plasticity and keeping synaptic inputs at fixed levels in a PN. Integrins are upstream regulators of c-Src in rebound potentiation (Kawaguchi and Hirano, 2006). Our present results imply that mGluR1 might be also regulating c-Src in the LTD induction. The LTD at parallel fibers-PN synapses is induced by coincident mGluR1 activation and large Ca^{2+} influx caused by inputs from parallel fibers and a climbing fiber, respectively (Ito, 2001). It has been assumed that mGluR1 induces production of diacylglycerol (DAG) through activation of PLC. DAG activates PKCα cooperatively with Ca^{2+} ion, leading to the LTD induction. However, our previous study demonstrated that translocation of PKCα to the plasma membrane, which is necessary for its activation, is not significantly altered by activation of mGluR1 (Tsuruno and Hirano, 2007). We have shown here that SFK inhibition recovers the LTD induction from suppression caused by mGluR1 inhibition. We have also shown that mGluR1 activation inhibits SFK in the cerebellar culture. Thus, mGluR1

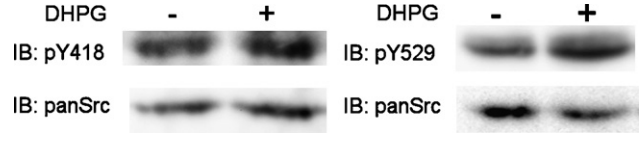

Fig. 3. DHPG increased phosphorylation of SFK at Tyr529 in the cerebellar culture. SFK was immunoblotted using the antibody against phospho-Tyr418 or phospho-Tyr529 SFK and reblotted with the antibody against total Src. The upper images show immunoblots using the phosphorylation-specific antibodies and the lower images show reblots using the antibody against total Src.

might contribute to the LTD induction through inhibition of SFKs. The down-stream targets of SFKs in the LTD regulation are unidentified, and should be addressed in the future study.

Striatal enriched tyrosine phosphatase (STEP) prevents induction of long-term potentiation by opposing Src-dependent upregulation of NMDAR in hippocampal CA1 neurons (Pelkey et al., 2002). We tried to examine whether suppression of LTD in a PN could be caused by the inhibition of protein tyrosine phosphatase, which counteracts the effects of c-Src. However, application of inhibitors of protein tyrosine phosphatase such as orthovanadate or phenylarsine oxide often induced unidentified large inward currents in cultured PNs, which hindered us from assessing their involvement in the cerebellar LTD. Previous studies showed that the LTD induced by group I mGluR activation in a CA1 neuron is accompanied by the reduction of both tyrosine phosphorylation and surface expression of GluR2 (Huang and Hsu, 2006; Moult et al., 2006). Thus, SFKs might suppress LTD through tyrosine phosphorylation of GluR2 in a PN. However, low-frequency stimulation of presynaptic fibers increases tyrosine phosphorylation of GluR2, which results in the reduction of its surface expression and induction of LTD in CA1 (Ahmadian et al., 2004; Fox et al., 2007). It has also been shown that tyrosine phosphorylation of C-terminal tyrosine residues in GluR2 results in the internalization of GluR2 in cortical neurons (Hayashi and Huganir, 2004). Thus, the effects of tyrosine phosphorylation of GluR2 could contribute to both potentiation and depression. There may be other target molecules of SFKs implicated in the regulation of LTD.

Previous studies using broad-spectrum inhibitors have shown that PTK activity is required for the cerebellar LTD in a PN (Boxall et al., 1996; Hartell, 2001), which is apparently controversial to our present results. The discrepancy would be ascribed to the target specificity of pharmacological agents used. Our study employed PP2, a selective inhibitor for SFKs (Hanke et al., 1996). In addition, we showed that the intracellular application of c-Src protein suppressed LTD, confirming that SFKs negatively regulate the LTD induction in a PN. Taken together, it might be possible that PTK other than SFKs positively regulates the LTD induction.

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References

Ahmadian, G., Ju, W., Liu, L., Wyszynski, M., Lee, S.H., Dunah, A.W., Taghibiglou, C., Wang, Y., Lu, J., Wong, T.P., Sheng, M., Wang, Y.T., 2004. Tyrosine phosphorylation of GluR2 is required for insulin-stimulated AMPA receptor endocytosis and LTD. EMBO J. 23, 1040–1050.

Boxall, A.R., Lancaster, B., Garthwaite, J., 1996. Tyrosine kinase is required for long-term depression in the cerebellum. Neuron 16, 805–813.

Canepari, M., Ogden, D., 2003. Evidence for protein tyrosine phosphatase, tyrosine kinase, and G-protein regulation of the parallel fiber metabotropic slow EPSC of rat cerebellar Purkinje neurons. J. Neurosci. 23, 4066–4071.

De Zeeuw, C.I., Hansel, C., Bian, F., Koekkoek, S.K., van Alphen, A.M., Linden, D.J., Oberdick, J., 1998. Expression of a protein kinase C inhibitor in Purkinje cells blocks cerebellar LTD and adaptation of the vestibulo-ocular reflex. Neuron 20, 495–508.

Fadool, D.A., Holmes, T.C., Berman, K., Dagan, D., Levitan, I.B., 1997. Tyrosine phosphorylation modulates current amplitude and kinetics of a neuronal voltage-gated potassium channel. J. Neurophysiol. 78, 1563–1573.

Fox, C.J., Russell, K., Titterness, A.K., Wang, Y.T., Christie, B.R., 2007. Tyrosine phosphorylation of the GluR2 subunit is required for long-term depression of synaptic efficacy in young animals in vivo. Hippocampus 17, 600–605.

Grant, S.G., O'Dell, T.J., Karl, K.A., Stein, P.L., Soriano, P., Kandel, E.R., 1992. Impaired long-term potentiation, spatial learning, and hippocampal development in fyn mutant mice. Science 258, 1903–1910.

Hanke, J.H., Gardner, J.P., Dow, R.L., Changelian, P.S., Brissette, W.H., Weringer, E.J., Pollok, B.A., Connelly, P.A., 1996. Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. J. Biol. Chem. 271, 695–701.

Hartell, N.A., 2001. Receptors, second messengers and protein kinases required for heterosynaptic cerebellar long-term depression. Neuropharmacology 40, 148–161.

Hayashi, T., Huganir, R.L., 2004. Tyrosine phosphorylation and regulation of the AMPA receptor by Src family tyrosine kinases. J. Neurosci. 24, 6152–6160.

Hirano, T., Ohmori, H., 1986. Voltage-gated and synaptic currents in rat Purkinje cells in dissociated cell cultures. Proc. Natl. Acad. Sci. U.S.A. 83, 1945–1949.

Huang, C.C., Hsu, K.S., 2006. Sustained activation of metabotropic glutamate receptor 5 and protein tyrosine phosphatases mediate the expression of (S)-3,5-dihydroxyphenylglycine-induced long-term depression in the hippocampal CA1 region. J. Neurochem. 96, 179–194.

Ito, M., 2001. Cerebellar long-term depression: characterization, signal transduction, and functional roles. Physiol. Rev. 81, 1143–1195.

Kalia, L.V., Gingrich, J.R., Salter, M.W., 2004. Src in synaptic transmission and plasticity. Oncogene 23, 8007–8016.

Kawaguchi, S.Y., Hirano, T., 2006. Integrin α3β1 suppresses long-term potentiation at inhibitory synapses on the cerebellar Purkinje neuron. Mol. Cell. Neurosci. 31, 416–426.

Linden, D.J., Dickinson, M.H., Smeyne, M., Connor, J.A., 1991. A long-term depression of AMPA currents in cultured cerebellar Purkinje neurons. Neuron 7, 81–89.

Litschig, S., Gasparini, F., Rueegg, D., Stoehr, N., Flor, P.J., Vranesic, I., Prézeau, L., Pin, J.P., Thomsen, C., Kuhn, R., 1999. CPCCOEt, a non-competitive metabotropic glutamate receptor 1 antagonist, inhibits receptor signaling without affecting glutamate binding. Mol. Pharmacol. 55, 453–461.

Lu, Y.M., Roder, J.C., Davidow, J., Salter, M.W., 1998. Src activation in the induction of long-term potentiation in CA1 hippocampal neurons. Science 279, 1363–1367.

Moss, S.J., Gorrie, G.H., Amato, A., Smart, T.G., 1995. Modulation of $GABA_A$ receptors by tyrosine phosphorylation. Nature 377, 344–348.

Moult, P.R., Gladding, C.M., Sanderson, T.M., Fitzjohn, S.M., Bashir, Z.I., Molnar, E., Collingridge, G.L., 2006. Tyrosine phosphatases regulate AMPA receptor trafficking during metabotropic glutamate receptor-mediated long-term depression. J. Neurosci. 26, 2544–2554.

Pelkey, K.A., Askalan, R., Paul, S., Kalia, L.V., Nguyen, T.H., Pitcher, G.M., Salter, M.W., Lombroso, P.J., 2002. Tyrosine phosphatase STEP is a tonic brake on induction of long-term potentiation. Neuron 34, 127–138.

Shigemoto, R., Abe, T., Nomura, S., Nakanishi, S., Hirano, T., 1994. Antibodies inactivating mGluR1 metabotropic glutamate receptor block long-term depression in cultured Purkinje cells. Neuron 12, 1245–1255.

Tsuruno, S., Hirano, T., 2007. Persistent activation of protein kinase Cα is not necessary for expression of cerebellar long-term depression. Mol. Cell. Neurosci. 35, 38–48.

Wang, K., Hackett, J.T., Cox, M.E., Van Hoek, M., Lindstrom, J.M., Parsons, S.J., 2004. Regulation of the neuronal nicotinic acetylcholine receptor by SRC family tyrosine kinases. J. Biol. Chem. 279, 8779–8786.

Wang, Y.T., Salter, M.W., 1994. Regulation of NMDA receptors by tyrosine kinases and phosphatases. Nature 369, 233–235.