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## **Noradrenergic modulation of the parallel fiber-Purkinje cell synapse in mouse cerebellum**

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## ABSTRACT

The signals arriving to Purkinje cells via parallel fibers are essential for all tasks in which the cerebellum is involved, including motor control, learning new motor skills and calibration of reflexes. Since learning also requires the activation of adrenergic receptors, we investigated the effects of adrenergic receptor agonists on the main plastic site of the cerebellar cortex, the parallel fiber-Purkinje cell synapse. Here we show that noradrenaline serves as an endogenous ligand for both  $\alpha_1$ - and  $\alpha_2$ -adrenergic receptors to produce synaptic depression between parallel fibers and Purkinje cells. On the contrary, PF-EPSCs were potentiated by the  $\beta$ -adrenergic receptor agonist isoproterenol. This short-term potentiation was postsynaptically expressed, required protein kinase A, and was mimicked by the  $\beta_2$ -adrenoceptor agonist clenbuterol, suggesting that the  $\beta_2$ -adrenoceptors mediate the noradrenergic facilitation of synaptic transmission between parallel fibers and Purkinje cells. Moreover,  $\beta$ -adrenoceptor activation lowered the threshold for cerebellar long-term potentiation induced by 1 Hz parallel fiber stimulation. The presence of both  $\alpha$  and  $\beta$  adrenergic receptors on Purkinje cells suggests the existence of bidirectional mechanisms of regulation allowing the noradrenergic afferents to refine the signals arriving to Purkinje cells at particular arousal states or during learning.

## **ABBREVIATIONS**

$\alpha$ -ARs: alpha adrenergic receptors

$\beta$ -ARs: beta adrenergic receptors

CF-EPSCs: excitatory postsynaptic currents evoked by climbing fiber stimulation

EPSCs: excitatory postsynaptic currents

ISO: isoproterenol

LTD: long-term depression

LTP: long-term potentiation

NA: noradrenaline

PC: Purkinje cell

PF: parallel fiber

PF-EPSCs: excitatory postsynaptic currents evoked by parallel fiber stimulation

## **INTRODUCTION**

The activity of Purkinje cells (PC) is under the control of two types of glutamatergic fibers: the parallel fibers (PF) rising from the granule cells of the cerebellar cortex and the climbing fibers (CF) rising from neurons of the inferior olive in the brainstem. Besides these two major excitatory projections, the cerebellum receives a rich noradrenergic innervation that originates in the locus coeruleus (LC) and distributes to all parts of the cerebellar cortex and deep cerebellar nuclei (Bloom et al., 1971; Olson and Fuxe, 1971; Abbot and Sotelo, 2000; Schweighofer et al., 2004). Because of this widespread projection, noradrenaline (NA) is considered to modulate the neural circuits implicated in cerebellar dependent learning and memory (Bickford, 1993; Naka et al., 2002; Cartford et al., 2004). The involvement of NA in memory consolidation has been observed in several cerebellar dependent paradigms. For example, selective depletion of NA has been shown to suppress the adaptation of the vestibule-spinal and vestibule-ocular reflexes (Keller and Smith, 1983; Pompeiano, 1998). Lesion of the LC impairs the acquisition and performance of specific

locomotor tasks (Watson and McElligot, 1984; Bickford et al, 1992). Furthermore, blockade of postsynaptic  $\beta$ -adrenergic receptors ( $\beta$ -ARs) through systemic administration of propranolol alters the acquisition of the eyeblink conditioning in both rabbit and rat (Gould 1998; Cartford et al, 2002). At the cellular level, NA applied iontophoretically or via activation of the locus coeruleus induces depression of spontaneous discharges in Purkinje cells and potentiation of GABAergic transmission at basket cells - PC inhibitory synapses (Hoffer et al., 1971; Moises and Woodward, 1980; Mitoma and Konishi 1999; Kondo and Marty 1998; Saitow et al. 2000). These inhibitory responses are mediated by  $\beta$ -AR-induced enhancement of GABA release from molecular layer interneurons, thereby increasing the inhibitory tone on PCs (Lin et al. 1991; Llano and Gerschenfeld 1993).

Concerning the noradrenergic effect on the excitatory transmission, early reports indicated that  $\beta$ -AR activation facilitates the excitatory response to microiontophoretically applied glutamate in cultured embryonic chick Purkinje cells (Mori-Okamoto and Tatsuno, 1988; Mori-Okamoto et al., 1991). However, recent studies indicate that NA has an inhibitory effect on CF-PC synapse, acting presynaptically (on  $\alpha_2$ -ARs) to decrease the glutamate release at CF (Carey and Regehr, 2009). Whether NA plays any modulatory role on PF-PC synapse is still unclear. According to some authors, NA does not have any effect on PF-PC synapse whereas others have reported an inhibitory action of NA, mediated by  $\alpha_2$ -ARs, on the field potential evoked by PF stimulation (Mitoma and Konishi; 1999; Zhou et al., 2003; Carey and Regehr, 2009).

Thus, the aim of our study was to reveal (i) whether ARs are involved in the short-term control of the efficacy of the parallel fiber-Purkinje cell synapse and (ii) whether such a modification of synaptic transmission also occurs with the endogenous agonist noradrenaline. The results presented here show that activation of  $\beta_2$ -ARs by an exogenous agonist enhances the strength of the PF-PC synapse. Moreover, when NA is applied exogenously, the  $\beta_2$ -AR-mediated potentiation is masked by the inhibitory effect mediated by  $\alpha$ -ARs. These two opposing effects of ARs may serve to

decrease the background activity of PCs and enhance the effect of excitatory signals arriving via PF thus shaping both the dynamic and plastic properties of the intrinsic circuitry of the cerebellar cortex.

## **MATERIALS AND METHODS**

### **Slice preparations**

Experiments were performed on parasagittal slices prepared from the cerebellar vermis of CD-1 mice of either sex, 10-24 days old, as previously described (Tempia et al., 1998). Experiments were approved by the University Bioethical Committee of the University of Naples. Each animal was anaesthetized with isoflurane, USP (Abbott Laboratories, Illinois, USA) and decapitated. The cerebellar vermis was removed and rapidly immersed in ice-cold extracellular saline solution containing (mM): 125 NaCl, 2.5 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 20 glucose and the pH was maintained at 7.4 by bubbling with 95% O<sub>2</sub> – 5% CO<sub>2</sub>. Parasagittal cerebellar slices (200 µm thickness) were obtained using a vibratome (Vibroslice 752, Campden Instruments, Loughborough, UK) and kept for 30 minutes at 35 °C and then at room temperature.

### **Electrophysiology**

After a recovery period of at least 1 hr, an individual slice was transferred to a recording chamber and continuously perfused at room temperature (25 °C) at a rate of 1.5 ml/min with the saline solution saturated with the 95% O<sub>2</sub> – 5% CO<sub>2</sub>. The Purkinje cell soma was visually identified using a 40x water-immersion objective of an upright microscope (BX50WI, Olympus, Japan) and its upper surface was cleaned by a sodalime glass pipette (tip diameter 10–15 µm, filled with the extracellular saline solution). The same pipette was then placed in the molecular layer to evoke synaptic responses by delivering current pulses. Pipettes of borosilicate glass, with a tip diameter of 2–3 µm and a resistance between 2.0 and 3 MΩ, were used for patch-clamp recording. The intracellular solution had the following composition (mM): 130 potassium gluconate, 8 KCl, 2

MgCl<sub>2</sub>, 10 HEPES, 4 Na<sub>2</sub>ATP, 0.4 Na<sub>3</sub>GTP, 10 EGTA; the pH was adjusted to 7.3 with KOH. All recordings were performed at room temperature (22–25 °C) in whole-cell configuration, using an EPC-8 patch-clamp amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany). Voltage-clamp recordings were accepted only if the series resistance was less than 10 MΩ. The holding potential was set at –60 mV. Data were filtered at 3 kHz and digitized at 10 kHz using the filter and analog/digital converter of the amplifier. Digitized data were stored on computer disk using the Pulse software (HEKA Elektronik). Parallel fibers were stimulated every 20 seconds by an isolated stimulator (A/M Systems, Carlsborg, WA, USA) with a paired pulse protocol with 100 ms of interpulse interval. Data were analyzed by the commercial program Igor Pro (Wavemetrics, Lake Oswego, OR, USA). Drugs were bath applied by switching perfusion lines without altering the perfusion rate. The measured delay to reach the recording chamber was 90 s. In all analyses the time 0 of drug application was indicated as the time at which the drug reached the chamber. During recording the extracellular saline solution always contained gabazine (20 μM). In each Purkinje cell recording, only one adrenergic drug was applied and, to avoid interference of previous applications, at the end of the experiment the treated slice was discarded. Noradrenaline, clenbuterol, isoproterenol, atenolol and ICI-118,551 hydrochloride were purchased from Sigma-Aldrich (Milan, Italy); UK14,304, phenylephrine hydrochloride and gabazine were purchased from Abcam (Cambridge, UK). Prazosin hydrochloride, yohimbine hydrochloride and H89 hydrochloride were from Tocris Bioscience (Bristol, UK).

### **Immunohistochemistry**

Postnatal mice were perfused-fixed through the heart with 4% paraformaldehyde in phosphate-buffered saline (PBS). The cerebellum was dissected, postfixed and cryoprotected (for 24 h at 4°C in 4% paraformaldehyde in PBS, pH 7.4 and for 24 h at 4°C in PBS containing 30% sucrose). Tissues were embedded in O. C. T compound (Bio-Optica, Milan, Italy), cut on a cryostat and processed. Sagittal cerebellar slices (50 μm thickness) were permeabilized for 5 min in PBS-T (PBS

containing 0.1% tween) and then blocked with PBS-T and 10% normal goat serum (blocking solution) for 1 hr at RT. The slides were incubated for 48 h at 4°C with the primary antibodies (anti-rabbit  $\beta$ 2 adrenergic receptor, 1:400, Novus Biological, Milan, Italy; anti-mouse calbindin 1:700, Sigma Aldrich, Milan, Italy) diluted in blocking solution. After rinsing with PBS-T for 3 times, the fluorescent-labeled secondary antibodies (Alexa 488 anti-rabbit, 1:400, Invitrogen; Alexa 594 anti-mouse, 1:400, Invitrogen, Life Technologies, Milan, Italy) diluted in blocking solution were added. Control slides were incubated in the same solutions without primary antibodies and subsequently processed as above. Images were acquired with Leica microscope (Leica DM6000B).

## **2.4 Statistics**

Data analysis was performed with Excel (Microsoft) and IGOR Pro (WaveMetrics). All average results are reported as mean  $\pm$  standard error (SE) for n different cells. For each cell, all single EPSC events were measured. For drug applications, the control and effect values are the mean of 10 EPSCs respectively immediately before the arrival of the drug in the chamber, and at 5 minutes of drug perfusion. Statistics compared the mean baseline values obtained prior to drug application to the responses at 5 minutes of drug application, and included all cells that showed a stable baseline response. The response was then normalized to the baseline pre-drug average to give a percent change relative to the baseline response. Statistical significance was assessed using two-tailed, paired or unpaired, Student's t-test as appropriate.

## **RESULTS**

### **Activation of $\beta$ -ARs enhances the amplitude of the EPSC evoked by PF stimulation**

To determine whether the activation of  $\beta$ -ARs plays a role in modulation of the PF-PC synapse, we first examined the effect of isoproterenol (ISO), a non selective  $\beta$ -AR agonist, on the excitatory postsynaptic currents (EPSCs) evoked by parallel fiber stimulation. Since previous studies have



shown that these receptors enhance GABAergic activity impinging on PC, PF-EPSCs were evoked under specific experimental conditions that eliminated any possible contamination by GABAergic interneurons (Llano and Gerschenfeld, 1993; Mitoma and Konishi, 1999). Toward that end, we blocked GABA<sub>A</sub> receptors with gabazine (20  $\mu$ M) and also adjusted the intracellular solution so that Cl<sup>-</sup> was close to the equilibrium potential at the holding voltage of -60 mV. PF-EPSCs were evoked by placing a stimulating electrode in the molecular layer and these were distinguished from the synaptic response due to climbing fiber activation (CF-EPSCs) using the following two criteria: (i) the PF-EPSC amplitude could be finely graded by changing the strength of the stimulation, whereas the CF-EPSCs were “all or none”; (ii) with a paired pulse stimulation protocol (100 ms interpulse interval), the second PF-EPSC showed facilitation (paired pulse facilitation: PPF), while the second CF-EPSC showed depression (Perkel et al., 1990; Konnerth et al., 1990). The paired pulse stimulation to PFs was delivered every 20 seconds to avoid possible use-dependent plasticity. Under these conditions, bath application of ISO (1  $\mu$ M) significantly increased the evoked excitatory currents by 82% ( $\pm$ 17, n = 12 p<0.05; Fig. 1A, B, C and D) and returned to the original value after the wash out of ISO. To determine whether the potentiation of EPSCs induced by ISO was pre-or post synaptic in nature, we examined the effect of ISO on PPF. It is assumed that the potentiation is mediated by a presynaptic mechanism when it is associated with a decrease in PPF (Manabe et al., 1993). By contrast, a postsynaptic mechanism is most likely involved when the PPF remains unaltered. As shown in Fig. 1C, application of ISO did not induce any significant change in PPF suggesting a postsynaptic locus of potentiation.

### **Receptor subtype involved in the ISO-induced enhancement of PF-PC synaptic transmission**

To identify the subtype of  $\beta$ -adrenergic receptor involved in the response to ISO, we tested the effect of selective antagonists to  $\beta_1$  and  $\beta_2$ -ARs. Atenolol (1  $\mu$ M), a  $\beta_1$ -AR antagonist, did not inhibit the increase in PF-EPSC observed in the presence of ISO (n = 6, p< 0.001 vs control; p=0.87 vs ISO; Fig. 2A, B and G), whereas the  $\beta_2$ -adrenergic receptor blocker ICI-118,551 (1  $\mu$ M, n = 4)

blocked the ISO-mediated potentiation ( $p = 0.16$  vs control;  $p < 0.05$  vs ISO; Fig. 2C, D and G), suggesting that  $\beta_2$ -ARs are required for isoproterenol-dependent potentiation of PF-PC synapse.

Together the above findings led us to examine the effect of a selective  $\beta_2$ -AR agonist, clenbuterol, on PF-EPSC. As shown in Fig. 2E, F, and G, clenbuterol (10  $\mu$ M) treatment induced a transient increase of the PF-EPSC amplitude of 51% ( $\pm 21$ ,  $n = 5$ ,  $p < 0.05$  vs control;  $p = 0.35$  vs ISO) without altering the PPF (Fig. 2H).

### **Localization of $\beta_2$ -adrenoreceptors in the mouse cerebellum**

In order to reveal the cellular distribution of  $\beta_2$ -ARs, we performed immunofluorescent labeling of  $\beta_2$ -adrenoreceptors in cerebellar slices. As shown in Fig. 3,  $\beta_2$ -adrenoreceptor protein expression was high in Purkinje cell bodies, where it colocalized with calbindin, a commonly used marker for Purkinje cells. A strong immunolabelling was also seen in molecular layer, consistent with a localization at Purkinje cell dendrites.

### **The ISO-induced facilitation of PF-PC synaptic transmission is mediated by a protein kinase A-dependent pathway**

$\beta$ -adrenergic receptors are traditionally coupled to the stimulatory G protein ( $G_s$ ), which activates c-AMP and protein kinase A (PKA). PKA then engages a variety of signaling pathways that are responsible of cellular responses, including gene transcription and ion channel activity (Skalhegg and Tasken, 2000; Taylor et al., 2012). In cerebellar GABAergic interneurons, NA has been shown to activate PKA-dependent pathways resulting in the facilitation of inhibitory transmission between interneurons and PC (Mitoma and Konishi, 1999). We therefore examined the possibility that the ISO-induced enhancement of PF-EPSCs was mediated by PKA. As shown in Fig. 4, the facilitatory action of ISO on the PF-EPSCs was prevented by treatment with the protein kinase inhibitor, H89 (5  $\mu$ M,  $n = 5$ ,  $p =$  n.s. vs control). To determine whether the activation of postsynaptic PKA was necessary for the expression of  $\beta$ -AR mediated potentiation, we infused via the patch pipette PKI<sub>6</sub>-

22 (40  $\mu$ M) a membrane impermeable PKA inhibitor, into Purkinje cells. In the presence of PKI<sub>6-22</sub>, ISO had no effect on PF-EPSC amplitude (Fig. 4C, D and E;  $n = 7$ ;  $p = \text{n.s.}$ ). This suggests that ISO stimulates adenylate cyclase and activates PKA-dependent pathways in Purkinje cells, that are likely responsible for the facilitation of excitatory transmission between PF and PC.

### **Isoproterenol facilitates the long-lasting potentiation of PF-PC synaptic response**

Studies *in vivo* and *in vitro* suggest that NA has modulatory effects on synaptic plasticity in different brain areas including hippocampus, amygdala, cerebral cortex and cerebellum (Bear et al., 1986; Izumi and Zorumski, 1999; Mitoma and Konishi, 1999; Carey and Regehr, 2009; Holloway-Erickson et al., 2012; Zhou et al., 2013). In particular,  $\beta$ -AR activation has been shown to reduce the threshold for the induction of long-term potentiation (LTP) in the hippocampus, a brain structure critically involved in memory formation (Straube et al. 2003; Gelinis and Nguyen 2005). To determine whether  $\beta$ -AR stimulation can promote long-term synaptic plasticity of PF-PC synaptic response, we examined if ISO application was able to induce LTP at a train stimulus that was below threshold for LTP, i.e. 1 Hz for 2.5 min (Wang et al., 2014). Consistent with previous work (Lev-Ram et al., 2002), 1 Hz stimulation for 2.5 min failed to induce PF-LTP ( $n = 7$ ; Fig. 5A). In contrast, application of ISO (1  $\mu$ M) overlapping this weak stimulation was able to potentiate PF-EPSCs (to  $127\% \pm 7$  of baseline at  $t$  40 min,  $n = 5$ ,  $p < 0.01$  compared with baseline; Fig. 5B and C). Neither weak stimulation alone nor conjunction with ISO changed the PPF ratio (Fig. 5D).

### **Noradrenaline depresses the EPSC evoked by PF stimulation**

NA, which represents the physiological neurotransmitter released by noradrenergic fibers, can modulate the PF-PC synapse by acting through both  $\alpha$ - and  $\beta$ -ARs. However, it has been shown that the recruitment of these two classes of receptors can induce opposite effects on PC firing which depends on the concentration of NA in the bath (Basile and Dunwiddie, 1984). Specifically, it has been reported that NA can increase the spontaneous activity of PCs when applied at lower

concentration and decreases this activity when given at higher concentrations. These excitatory and inhibitory effects were linked to activation of  $\alpha$ - and  $\beta$ -ARs, respectively. To assess whether a similar mechanism is involved in the modulation of PF-PC synaptic transmission, we tested the PF-EPSCs amplitude in the presence of NA at concentrations ranging from 0.1 to 100  $\mu$ M. To avoid any possible contamination between repeated NA applications, we performed only one NA application in each cell. NA treatment, at concentrations between 0.3 and 100  $\mu$ M, produced a significant depression of the PF-EPSC, that did not recover following NA washout. This effect is summarized in the dose-response curve illustrated in Fig. 6D obtained from 24 cells and fitted with the Hill equation ( $EC_{50} = 0.46 \mu$ M and slope coefficient = 1.43). The maximum inhibition of the PF-EPSC amplitude was observed at 10  $\mu$ M corresponding to a decrease in EPSCs of 34.0% ( $\pm 5.5$ ,  $n = 6$ ,  $p < 0.05$ ; Fig. 6B and D). At this concentration (as was the case with the other effective concentrations) NA did not significantly alter the paired pulse facilitation ratio ( $4.0\% \pm 10.9$  increase,  $n = 6$ ;  $p = \text{n.s.}$ ; Fig. 6C).

### **$\alpha_2$ -AR agonists depress the EPSC evoked by PF stimulation**

The above results not only indicate that NA has a predominantly inhibitory effect on the PF-PC synapse but also suggest that the  $\alpha$ -ARs are the main candidates responsible for this depression. Since both  $\alpha_1$  and  $\alpha_2$ -receptors have been shown to be expressed in mouse cerebellum, predominantly in Purkinje cells and the molecular layers (Papay et al., 2004 and 2006; Hirono et al., 2008), we performed experiments in which noradrenaline was applied in the presence of either an  $\alpha_1$  (prazosin) or an  $\alpha_2$ -receptor (yohimbine) antagonist. Both prazosin (5  $\mu$ M;  $n = 4$ ; Fig. 7A and B) and yohimbine (5  $\mu$ M;  $n = 6$ ; Fig. 7C and D) attenuated the NA effect on EPSC without altering the PPF ( $p < 0.05$  vs NA; Fig. 7I and J). To further confirm the involvement of  $\alpha_1$  and  $\alpha_2$ -receptors in the modulation of PF-PC response, we examined the actions of  $\alpha_1$  (phenylephrine) and  $\alpha_2$ -receptor (UK 14,304) agonists on PF-EPSC amplitude in order to establish the pharmacological specificity

of NA's effect. As shown in Fig. 7E, F and I, application of phenylephrine (10  $\mu$ M) mimicked the depressant effect of NA on PF-PC response (33.3%  $\pm$ 6.8 decrease,  $n = 6$ ,  $p < 0.01$  vs control;  $p = \text{n.s.}$  vs NA). A similar reduction in synaptic response was observed in the presence of UK 14,304 (15  $\mu$ M) (40.2%  $\pm$ 6.5 decrease,  $n = 5$ ;  $p < 0.01$  vs control;  $p = \text{n.s.}$  vs NA; Fig. 7G, H and I), suggesting that both  $\alpha_1$  and  $\alpha_2$ -receptors signaling depresses the PF-PC synaptic transmission. However the depression induced by UK was associated with a trend of the paired pulse facilitation to increase by 23% ( $\pm 7$ ,  $p = \text{n.s.}$  vs control; Fig. 7J), indicating a potential presynaptic localization of the  $\alpha_2$ -AR subtypes.

## DISCUSSION

Our results demonstrate, for the first time, a significant role for both  $\alpha$  and  $\beta$ -ARs in the modulation of PF-PC synaptic transmission. The activation of these two classes of receptors leads to opposing physiological effects, a potentiation mediated by  $\beta_2$ -ARs and a depression mediated by  $\alpha_1$ - and  $\alpha_2$ -ARs. Our findings are consistent with previous studies in cultured cerebellar neurons showing a biphasic effect of NA on the response to glutamate - facilitatory by acting on  $\beta$ -ARs and inhibitory by acting on  $\alpha_2$ -ARs (Mori-Okamoto and Tatsuno, 1988; Mori-Okamoto et al., 1991). A similar bidirectional modulation has been also reported for evoked synaptic responses of hippocampal and cortical neurons that appeared to be facilitated by  $\beta_1$ -ARs and suppressed by  $\alpha$ -ARs (Mueller et al., 1981; Haggerty et al., 2013; Dodt et al., 1991; Kobayashi, 2007).

Here, we show that the activation of  $\alpha$ -ARs, particularly the  $\alpha_2$ -subtypes, produces an inhibitory influence on excitatory synaptic transmission, through a postsynaptic mechanism. The pharmacology of the postsynaptic decrease of glutamatergic response detected with electrophysiological method is consistent with the immunochemical localization of both  $\alpha_1$  and  $\alpha_2$ -ARs at the Purkinje cell dendrites (Papay et al., 2004 and 2006; Hirono et al., 2008). Since glutamate AMPA receptors are also concentrated at the dendritic membrane, it is likely that  $\alpha$ -ARs

may affect synaptic transmission by modifying these receptors (Baude et al., 1994; Nusser et al., 1994). Interaction between  $\alpha$ -ARs and postsynaptic AMPA receptors has already been reported in pyramidal cells of the rat prefrontal cortex, where stimulation of  $\alpha_2$ -ARs produces an inhibitory influence on excitatory synaptic transmission by the  $G_i$  signaling pathway (Ji et al., 2008). It is known that  $\alpha_2$ -ARs are coupled to the  $G_i$  protein, which negatively regulates the activity of adenylyl cyclase (AC). Therefore, stimulation of  $\alpha_2$ -ARs suppresses the production of cAMP and leads to a decrease in protein kinase (PKA) activity. A decrease in PKA activity has been shown to directly reduce the phosphorylation of AMPA receptors, which may lead to the reduction in synaptic responsiveness observed following  $\alpha_2$ -AR stimulation (Yi et al., 2013). However we cannot exclude an additional presynaptic involvement of  $\alpha_2$ -ARs since there was a tendency for PPF to increase of about 20% during the application of UK, a selective  $\alpha_2$ -AR agonist.

In contrast to the consistent depression of PF-PC synaptic transmission seen following activation of  $\alpha$ -ARs, stimulation of  $\beta$ -ARs with ISO evoked a significant increase of synaptic response that was fully reversible on washout of ISO and blocked by the specific  $\beta_2$ -AR antagonist ICI-118,551 but not by the  $\beta_1$ -AR antagonist atenolol. Based on our immunohistochemical and electrophysiological data, we assume that the ISO potentiation of the PF-EPSC was mainly mediated via postsynaptic  $\beta_2$ -ARs. This finding supports previous studies in Purkinje cells showing the enhancing effects of ISO on firing rate and postsynaptic response to glutamate (Mori-Okamoto and Tatsuno, 1988; Mori-Okamoto et al., 1991). The positive effect of  $\beta$ -AR agonists is linked with the activation cAMP and protein kinase-dependent pathway via activation of  $G_s$ . Recent reports have shown that  $\beta$ -ARs are also linked to the regulation of the AMPA-type glutamate receptor subunit GluA1 via stargazing and PSD-95 (Joiner et al., 2010). According to this model, once activated, PKA can phosphorylate GluA1 on S845, leading to postsynaptic accumulation of GluA1 and increased surface expression of GluA1 that results in an increased synaptic transmission (Esteban et al. 2003; Oh et al. 2006; Man et al., 2007).

Although our results indicate that both  $\alpha$ - and  $\beta$ -ARs play a role in the noradrenergic modulation of AMPA-mediated neurotransmission, the effect of  $\alpha$ -ARs prevails over  $\beta$ -ARs whenever NA was applied even at different concentrations. Consistent with the role of NA in the regulation of inhibitory drive, it has been found previously that NA increases the spontaneous activity of interneurons but also the release of GABA from presynaptic terminals leading to an enhancement in PC inhibition (Llano and Gerschenfeld, 1993; Mitoma and Konishi, 1999; Saitow et al., 2000). Thus, the main action of NA is to depress the activity of PC via inhibition of excitatory inputs from PF and CF and facilitation of inhibitory inputs from interneurons. In this context, the concomitant presence of the  $\beta$ -AR-mediated potentiation of the PF-PC synapse might dramatically enhance the effect and the plasticity of active PF excitatory inputs on PCs and consequently increase their signal-to-noise ratio. This type of neuronal processing may enable the noradrenergic neurons to selectively modify in a temporal coincidence detection manner only those synapses that are active when attentional- and/or arousing stimuli encoded by the locus coeruleus arrive (Schultz and Dickinson, 2000).

In addition, we demonstrate for the first time that activation of  $\beta$ -ARs facilitates the induction of postsynaptic LTP of the PF-PC synapse elicited by a subthreshold stimulation protocol, as opposed to the short-term potentiation we observed following application of ISO alone. Our results are consistent with previous studies showing that noradrenaline plays a permissive in LTP induction, via  $\beta$ -receptor activation, in all hippocampal subfields (Huang and Kandel, 1996, Thomas et al., 1996, Lin et al., 2003, Gelinias and Nguyen, 2005; Qian et al., 2012). Signaling by the  $\beta$ -ARs might support LTP at least in part because it induces accumulation of AMPA receptors at perisynaptic site. These receptors can be then recruited to postsynaptic sites upon additional stimulation (Oh et al., 2006). Signaling by the  $\beta$ -ARs might also affect LTP by other signaling pathways. For instance, it could stimulate phosphorylation of different NMDA receptor subunits by PKA, which enhances calcium permeability and thereby postsynaptic calcium influx during LTP induction (Leonard and Hell, 1997; Skeberdis et al. 2006). Finally,  $\beta$ -ARs can also act via the ERK/MAPK signaling

pathway, which is important for LTP induced by a single 1-s/100-Hz tetanus (Gelinas and Nguyen 2005; Gelinas et al. 2007).

It is currently hypothesized that synaptic plasticity, specifically long-term potentiation (LTP), in the neural circuits underlying behavior could provide a cellular substrate of memory storage (Bliss and Collingridge, 1993; Eichenbaum, 1996; Kandel, 2001). Consistent with this proposal, it has been demonstrated that memory of fear could be acquired and, perhaps, retained through the mechanisms of cerebellar LTP in the PF-PC synapse (Sacchetti et al., 2004). This fear conditioning-induced potentiation is postsynaptically expressed and displays some properties of LTP elicited *in vitro* by repetitive stimulation of parallel fibers (Zhu et al., 2007). Since emotionally arousing experiences are associated with activation of noradrenergic projections to different regions of the brain, such as cerebellum, we propose that activation of  $\beta$ -adrenoceptors by released noradrenaline may enhance the induction of LTP at the PF-PC synapse during learning, thus facilitating memory formation .

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## FIGURE LEGENDS

**Fig. 1.** The  $\beta$ -AR agonist isoproterenol potentiates synaptic transmission between PFs and PCs (A) Time course of PF-EPSCs evoked in a PC by paired pulse stimulation. Circles: EPSC1; squares: EPSC2. Isoproterenol (ISO) was bath applied at 1  $\mu$ M during the time indicated by the bar. (B) Representative superimposed traces of PF-EPSCs recorded before (control) and during the application of ISO, as indicated. Data are from the same PC shown in panel A. (C) Scatter plot showing the effect of isoproterenol on EPSC1, EPSC2 and paired pulse facilitation (PPF). Horizontal lines represent the mean percentage of change ( $\pm$ SEM from 12 experiments) at 5 min of isoproterenol application relative to the control (before isoproterenol application). Data points are represented as open circles. Asterisks indicate statistical difference from the control (\* $p$  < 0.05). (D) Comparison of EPSC1 amplitude before and at 5 min of isoproterenol application. Data are from individual Purkinje cells.

**Fig. 2.** Isoproterenol increases EPSC amplitude at the PF-PC synapse through activation of  $\beta_2$ -adrenergic receptors. (A) Effect on PF-EPSC of isoproterenol (ISO, 1  $\mu$ M) in the presence of the  $\beta_1$ -AR antagonist atenolol (ATE, 1  $\mu$ M). (B) PF-EPSCs from a representative experiment in control condition and in the presence of atenolol and ISO. (C) Effects of ISO on PF-EPSCs in the presence of the  $\beta_2$ -AR antagonist ICI-118,551 (ICI, 1  $\mu$ M) (D) Superimposed EPSCs recorded from the same Purkinje cell as in panel C, in control condition and in the presence of ISO and ICI. (E) Effect of clenbuterol (CLEN, 10  $\mu$ M) on PF-EPSC. (F) EPSCs recorded from a Purkinje cell before and during application of clenbuterol. (G) Scatter plot showing the mean ( $\pm$ SEM) percentage of change

of PF-EPSC1 amplitude relative to control measured at 5 min application of ISO,  $\beta$ -AR agonists and antagonists (\*  $p < 0.05$  and \*\*  $p < 0.001$  vs control). The # symbol indicates  $p < 0.05$  of ICI+ISO vs. ISO alone. (H) Scatter plot showing the mean (horizontal lines) percentage of change  $\pm$ SEM of paired pulse facilitation (PPF) in the presence of  $\beta$ -AR agonists and antagonists relative to control.

**Fig. 3.** Expression of  $\beta_2$ -ARs in the mouse cerebellar cortex. (A) Immunofluorescent detection showing expression of  $\beta_2$ -ARs in PC soma (arrow) and in the molecular (ML) and granular (GL) layers. (B) Identification of PCs using anti-calbindin antibody. (C) Merged image of  $\beta_2$ -ARs and calbindin. Note the co-localization in PC dendrites in the molecular layer. Scale bar = 75  $\mu$ m.

**Fig. 4.** Effect of the protein kinase inhibitor H89 on the isoproterenol-induced facilitation of PF-EPSCs. (A) Time course of EPSC amplitudes before and after application of ISO (1  $\mu$ M) and H89 (5  $\mu$ M). (B) Superimposed EPSCs obtained from a PC during the control period and after application of ISO and H89. (C) Time course of PF-EPSCs before, during and after a 10-min application of ISO, with the PKA inhibitor PKI<sub>6-22</sub> (40  $\mu$ M) in the patch-pipette. (D) Superimposed EPSCs from a PC loaded with PKI<sub>6-22</sub> before and during treatment with ISO. (E) Scatter plot of PF-EPSCs during application of ISO either with the PKA inhibitor H89 into the bath ( $n = 5$ ) or with PKI<sub>6-22</sub> in the patch-pipette ( $n = 7$ ). Horizontal lines represent the mean percentage of change ( $\pm$ SEM) relative to control.

**Fig. 5.**  $\beta$ -adrenoceptor activation promotes long-lasting potentiation of PF-EPSC when paired with a weak low frequency stimulation of parallel fibers. (A) Time course of EPSC amplitude evoked in one PC before and after a train stimulus of 1 Hz for 2.5 min. (B) Example of PF-EPSCs from one PC stimulated for 2.5 min at 1 Hz overlapping bath perfusion of ISO (1  $\mu$ M). (C) Representative recordings before (control) and after (t 40 min) treatment with ISO + 1 Hz stimulation. (D) Scatter plot showing the average (horizontal lines;  $\pm$ SEM) values of EPSC and PPF ratio from 5 cells treated with ISO + 1 Hz stimulation, expressed as percentage relative to control (\*\*  $p < 0.001$  vs control).

**Fig. 6.** Noradrenaline has an inhibitory effect on the PF-PC synapse. (A) Time course of the EPSCs



amplitude evoked by paired pulse stimulation of PF in a PC. Noradrenaline (10  $\mu$ M) was applied during the period indicated by the horizontal bar. (B) Superimposed traces of EPSCs recorded from the PC shown in panel A before (control) and after noradrenaline application. (C) Scatter plot of EPSC amplitude and PPF measured at 5 min of application of noradrenaline (10  $\mu$ M). Each line represents the mean  $\pm$ SEM (n=6) percentage of change relative to control. The asterisks indicate significant difference between the values determined under control conditions and under 10  $\mu$ M noradrenaline. (\*p< 0.05). (D) The mean ( $\pm$ SEM) EPSC1 amplitude was plotted as a function of the noradrenaline concentration and fitted with the Hill equation resulting in an EC50 = 0.46  $\mu$ M (Hill coefficient = 1.43).

**Fig. 7.** Noradrenaline induces depression of PF-PC synapse through the activation of both  $\alpha_1$ - and  $\alpha_2$ -adrenergic receptors (A) Effect on PF-EPSC of noradrenaline (NA, 10  $\mu$ M) in the presence of the  $\alpha_1$ -AR antagonist prazosin (PRAZ, 5  $\mu$ M). (B) EPSCs from a representative experiment in control condition and in the presence of PRAZ and NA. (C) Effects of NA (10  $\mu$ M) on PF-EPSCs in the presence of the  $\alpha_2$ -AR antagonist yohimbine (YOHI, 5  $\mu$ M). (D) Superimposed EPSCs recorded from the same Purkinje cell as in panel C, in control condition and in the presence of NA and YOHI. (E) Effect of the  $\alpha_1$ -AR agonist phenylephrine (PHE, 10  $\mu$ M) on PF-EPSC. (F) Example of EPSCs recorded from a Purkinje cell before and after application of PHE. (G) Effect of the  $\alpha_2$ -AR agonist UK 14,304 (UK, 15  $\mu$ M) on PF-EPSC. (H) Representative traces before and after application of UK. (I) Scatter plot of the mean (horizontal lines;  $\pm$ SEM) percentage of change of PF-EPSC1 amplitude relative to the control in the presence of  $\alpha$ -AR agonists and antagonists, as indicated (\* p< 0.05 and \*\* p< 0.001 vs control). The # symbol indicates p<0.05 vs. NA alone. (J) Scatter plot showing the mean (horizontal lines;  $\pm$ SEM) percentage of change of paired pulse facilitation (PPF) relative to the control in the presence of  $\alpha$ -AR agonists and antagonists.