

Distinct Temporal Expression of 5-HT_{1A} and 5-HT_{2A} Receptors on Cerebellar Granule Cells in Mice

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Abstract Serotonin plays an important role of controlling the physiology of the cerebellum. However, serotonin receptor expression has not been fully studied in the developing cerebellum. We have recently shown that cerebellar granule cells transiently express 5-HT₃ receptors. In the present study, we investigate expression of 5-HT₁ and 5-HT₂ receptors in the mouse cerebellum both during postnatal development and in juvenile mice. Here, we show for the first time that 5-HT_{1A} and 5-HT_{2A} receptors are present on cerebellar granule cells with a distinct temporal expression pattern: 5-HT_{1A} receptors are expressed only during the first 2 weeks, while 5-HT_{2A} receptor expression persists until at least 8 weeks after birth. Because of its prolonged expression pattern, we investigated the electrophysiological properties of the 5-HT_{2A} receptor. 5-HT_{2A} receptors expressed by cerebellar granule cells promote stability by reducing variability of the synaptic response, and they modulate the paired-pulse ratio of the parallel fibre–Purkinje cell synapse. Furthermore, pharmacological block of 5-HT_{2A} receptors enhances short-term synaptic plasticity at the parallel fibre–Purkinje cell synapse. We thus show a novel role for serotonin in controlling function of the cerebellum via 5-HT_{2A} receptors expressed by cerebellar granule cells.

Keywords Serotonin receptors · Cerebellum · Synaptic plasticity

Introduction

The cerebellum does not only play an important role in motor coordination and motor learning, but is also involved in cognitive processes. Several studies have discussed the involvement of the cerebellum in neurodevelopmental disorders such as autism [1–3] and schizophrenia [4–7]. These neurodevelopmental disorders are associated with a change in serotonin receptor expression in the cerebellum [8, 9]. It is known that the cerebellum of the rodent brain receives innervation of serotonergic fibres, originating mainly from the medullary and pontine reticular formation [10, 11]. These serotonergic fibres are predominantly found around the somata of Purkinje cells and in the overlying molecular layer, which contains the dendrites of Purkinje cells. However, during development of the cerebellum, the serotonergic system and its physiological significance are not fully understood.

We have recently shown that 5-HT₃ receptors are transiently expressed by cerebellar granule cells in mice during early postnatal development [12]. Pharmacological block of 5-HT₃ receptors impairs synaptic plasticity at glutamatergic inputs targeting Purkinje cells during this developmental period [12]. Furthermore, 5-HT₃ receptors regulate the morphological maturation of Purkinje cells [13]. 5-HT_{3A} receptor knockout mice show delayed climbing fibre elimination due to impaired plasticity at the parallel fibre–Purkinje cell synapse [13]. We hypothesized that other members of the serotonergic system in the cerebellum co-regulate cerebellar development and are functional at time points surrounding the transient expression pattern of 5-HT₃ receptors. This happens presumably by switching to other types of serotonin receptors expressed in the cerebellum during postnatal development and thereafter. Studies using autoradiography and immunohistochemistry have shown that multiple different 5-HT receptor subtypes are present on Purkinje cells, including the 5-HT_{1A}, 5-HT_{2A},

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5-HT_{2B}, 5-HT_{2C}, 5-HT_{5A} and 5-HT₇ subtypes [14–19]. On cerebellar granule cells, the expression of 5-HT receptors is less diverse. It has been reported that both 5-HT₃ and 5-HT₆ receptors are present on these cells [12, 18, 20, 21]. In addition, very low densities of 5-HT₁ receptors in the molecular and granule cell layer in the adult rat cerebellum have been found [22]. There is some evidence suggesting presence of 5-HT₂ receptors on dissociated cerebellar granule cells at P8 from rat cerebellum [23, 24]. Other studies done on adult rodents did not reveal the presence of 5-HT₂ receptors on cerebellar granule cells [18, 25].

The aim of the present study is to investigate the expression pattern and functional properties of 5-HT_{1A} and 5-HT_{2A} receptors in the mouse cerebellum. We show for the first time that functional 5-HT_{1A} and 5-HT_{2A} receptors are expressed by cerebellar granule cells during early postnatal development. 5-HT_{1A} receptors are transiently expressed during the first 2 weeks postnatally, while 5-HT_{2A} receptors remain present on granule cells until 10 weeks of age. We furthermore investigate the electrophysiological properties of 5-HT_{2A} receptors in the cerebellum and conclude that they modulate plasticity at the parallel fibre–Purkinje cell synapse.

Materials and Methods

Ethical Approval

Wild-type C57/Bl6 mice (Harlan, www.harlan.com) between the age of postnatal day (P) 2 and P90, both males and females, were used for this study. All experiments in this study were performed in accordance with the committee on animal bioethics of the University of Amsterdam, which specifically approved this study. All efforts were made to minimize suffering.

Immunohistochemistry

Brains of wild-type C57/Bl6 mice at P5 and P90 were isolated in ice-cold phosphate-buffered saline (PBS) and fixated in 4 % PFA in PBS for 6 h. Sagittal (P5) or coronal (P90) sections were cut from the cerebellum at 16 μ m and mounted on slides. Slides were washed for 3 \times 5 min in PBS, and blocking was performed in 4 % heat-inactivated foetal calf serum in PBS-0.25 % Triton (PBS-T). Primary antibody against the 5-HT_{2A} receptor (Cat no: 24288, Immunostar) was incubated at a dilution of 1:500 in PBS-T for 1 h at room temperature and 4 °C overnight. Excessive antibody was removed by washing 3 \times 5 min in PBS, and secondary antibody (goat anti-rabbit Alexa 488, A11008 Molecular Probes) was incubated at a dilution of 1:1,000 in PBS-T for 2 h. Slides were washed for 2 \times 5 min in PBS and a 4',6-diamidino-2-phenylindole (DAPI) staining was performed for 5 min (1:3,000 dilution of staining solution in PBS, original staining

solution stock 1 mg/ml, D9564 Sigma, in H₂O). Slides were washed 2 \times 10 min in PBS and embedded with FluorSave (345789-20 Calbiochem). Fluorescent pictures were taken using a Zeiss fluorescence microscope and MetaMorph acquisition software.

Electrophysiological Recordings

For whole-cell patch-clamp recordings, animals were killed by decapitation. Sagittal brain slices were cut using a vibrating blade microtome (Leica VT1200S) at a thickness of 300 μ m. During slicing, the brains were kept in cooled (4 °C) oxygenated artificial cerebrospinal fluid (ACSF) which was composed of the following (in mM): NaCl (120), KCl (3.5), CaCl₂ (2.5), MgSO₄ (1.3), NaH₂PO₄ (1.25), NaHCO₃ (25) and D-glucose (25), continuously bubbled with 95 % O₂ and 5 % CO₂ (pH=7.4). Brains from mice older than 3 weeks were sliced in modified ACSF, composed of the following (in mM): choline chloride (120), KCl (3.5), CaCl₂ (0.5), MgSO₄ (6.0), NaH₂PO₄ (1.25), D-glucose (25) and NaHCO₃ (25). During the experiments, slices were kept submerged at room temperature and continuously superfused with ACSF. Patch pipettes were pulled from borosilicate glass with a resistance of 2–3 M Ω for recordings from Purkinje cells and with a resistance of 8–10 M Ω for recordings from granule cells. Patch pipettes were filled with internal solution containing the following (in mM): K gluconate (110), KCl (30), EGTA (0.5), HEPES (10), Mg-ATP (4) and Na-GTP (0.5; pH 7.3 with KOH). Whole-cell recordings were made at room temperature using an EPC9 patch-clamp amplifier and PULSE software (HEKA Electronic, Lambrecht, Germany). Signals were filtered at 1–5 kHz and sampled at 10 kHz. Series resistance ranged from 2 to 11 M Ω and was compensated for at least 60 %. All recordings are corrected for liquid junction potential. Cells were voltage clamped at –70 mV.

Whole-cell patch-clamp recordings were made from granule cells (at P2–P68) with a second pipette connected to a picospritzer II (General Valve, Fairfield, NJ, USA) containing 100 nM of the 5-HT_{1A} agonist 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT; gift from Abbott Laboratories BV), 1 μ M of the specific 5-HT_{2B/C} agonist (S)-6-chloro-5-fluoro-1H-indole-2-propanamine (RO60-0175, gift from Abbott Laboratories BV), or 500 nM of the 5-HT₂ agonist 2,5-dimethoxy-4-iodoamphetamine (DOI, gift from Abbott Laboratories BV) in ACSF was positioned in the vicinity of the cell soma, and the drug was applied for 500 ms at 35–100 kPa. Antagonists were applied in the bath solution, and slices were preincubated in the antagonist for >30 min. We used 10 μ M *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridyl)cyclohexanecarboxamide (WAY 100,635, gift from Abbott Laboratories BV) to block 5-HT_{1A} receptors and 300 nM (R)-(+)- α -(2,3-dimethoxyphenyl)-1-[2-(4-

fluorophenyl)ethyl]-4-piperidinemethanol (MDL 100,907, gift from Abbott Laboratories BV) to block 5-HT_{2A} receptors.

Miniature postsynaptic currents from Purkinje cells were recorded in the voltage-clamp configuration in the presence of 0.5 μ M TTX (Latoxan, Valence, France) and analyzed as described before [26]. Miniature excitatory postsynaptic currents (mEPSCs) were recorded with additional 20 μ M bicuculline (Tocris Bioscience) in the bath solution, while miniature inhibitory postsynaptic currents (mIPSCs) were recorded with 20 μ M 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Tocris Bioscience) in the bath solution. Per cell, one or two traces of 5 min each were used for analysis, with at least 100 miniature synaptic events per cell which were visually verified. A cumulative distribution was made for each cell, and all distributions were averaged to get the final cumulative distribution as shown in Fig. 3. In this way, all cells contribute equally to the final distribution.

Glutamatergic synaptic currents in Purkinje cells were evoked by stimulation of the parallel fibres with a glass electrode filled with ACSF. Paired stimuli (100–400 μ A, 0.2 ms duration, interstimulus interval 50 ms) were delivered to the internal granule cell layer using a custom-made isolated bipolar current stimulator. Paired-pulse stimulations were delivered with a 20-s interval, and only recordings which were stable for at least 15 min were used to analyze the paired-pulse ratio (PPR). Recordings used for analysis were checked to have a gradual increase in amplitude upon a gradual increase in stimulus intensity and paired-pulse facilitation, both features of the parallel fibre–Purkinje cell synapse. The PPR was defined as the amplitude of the second EPSC divided by the amplitude of the first EPSC. The coefficient of variance (CV) was determined for cells that had a stable baseline for at least 50 stimuli in each condition. The CV was calculated for each cell by dividing the standard deviation by the mean of the EPSC amplitude.

For synaptic plasticity at the parallel fibre–Purkinje cell synapse, recordings were made from Purkinje cells as described above. Synaptic plasticity was induced using a low frequency stimulation protocol with a tetanus current injection of 8 Hz for 30 s [27], at a stimulus intensity which gave a postsynaptic current below the maximum induced postsynaptic current. The area of the EPSCs was used to analyze the postsynaptic responses. To investigate the time course of the synaptic plasticity onset, from some cells we recorded EPSCs throughout the complete time course (see example in Fig. 4c). In all cells, we recorded EPSCs at least during the 10–15 min after induction of plasticity. The analysis of these EPSCs is shown in Fig. 4d.

Statistical Analysis

Values are expressed as mean \pm standard error of the mean. Comparisons were made using the Student *t* test unless stated

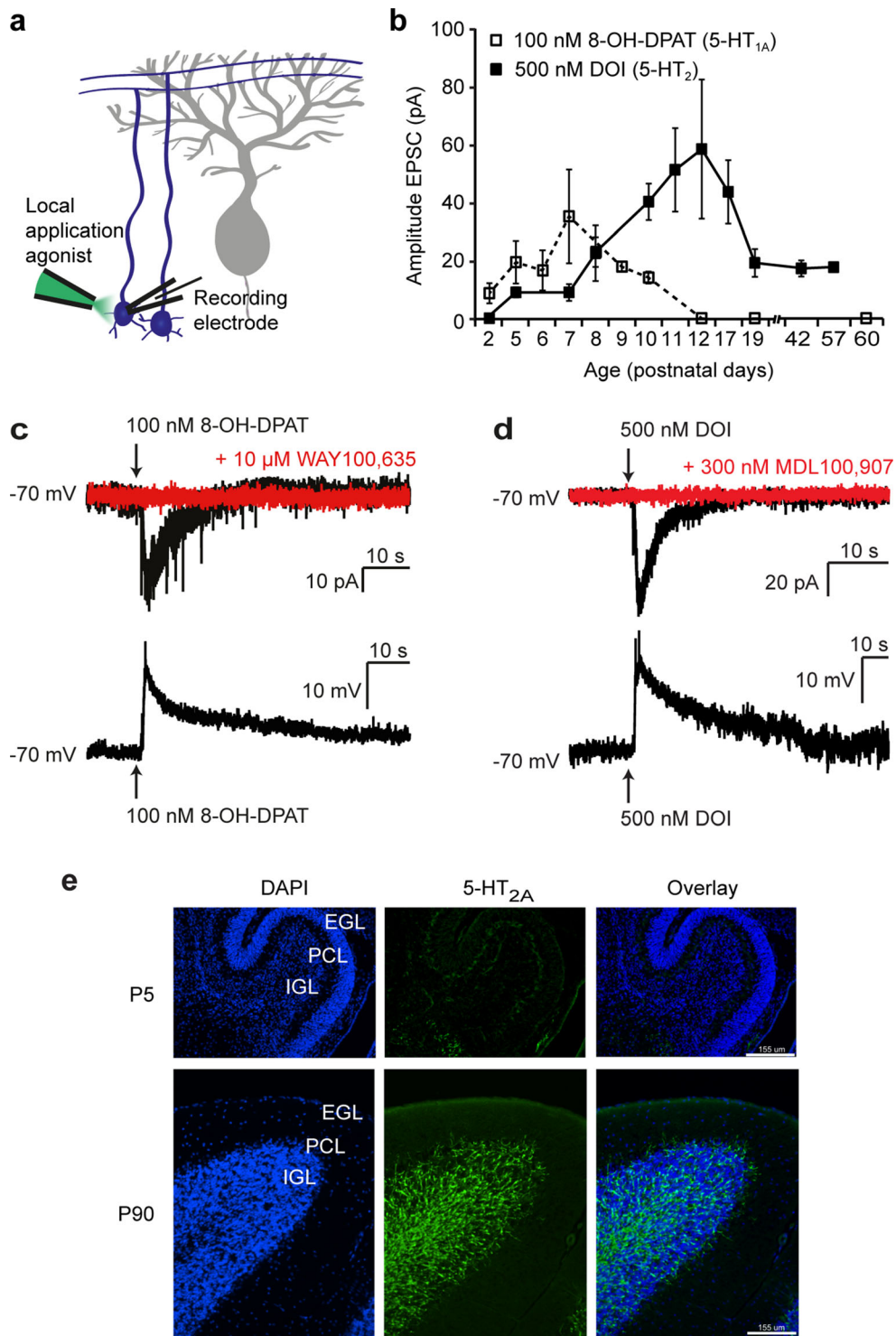
otherwise. A $p < 0.05$ was used to indicate a significant difference. Asterisks indicate $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

Results

Cerebellar Granule Cells Express 5-HT_{1A} and 5-HT_{2A} Receptors During Postnatal Development

The expression of 5-HT_{1A} and 5-HT_{2A} receptors on cerebellar granule cells during postnatal development was studied using whole-cell patch-clamp recordings and local application of selective agonists (Fig. 1a). A low concentration (100 nM) of the 5-HT_{1A} agonist 8-OH-DPAT was used to prevent activation of the 5-HT₇ receptors, for which 8-OH-DPAT has a low affinity [28]. Figure 1b shows that functional 5-HT_{1A} receptors are expressed by cerebellar granule cells during the first 10 days postnatally but not at later ages. Local application of 100 nM 8-OH-DPAT to granule cells at P6–P9 resulted in an inward current of 19.7 ± 2.2 pA ($n = 11$ cells, 4 mice, example trace in Fig. 1c, top) in voltage-clamp, and in a depolarization of 17.6 ± 3.5 mV ($n = 5$, example trace in Fig. 1c, bottom) in current-clamp. The 5-HT_{1A} receptor-mediated inward current in granule cells could be blocked in 6 out of 7 cells (3 mice) by bath application of 10 μ M of the 5-HT_{1A} specific antagonist WAY100,635 (Fig. 1c).

Conversely, 5-HT_{2A} receptors are expressed by cerebellar granule cells from 5 days after birth, with a peak in expression levels around the end of the second postnatal week and with lower but sustained expression at later ages until at least 10 weeks old. Local application of 500 nM of the 5-HT₂ agonist DOI to cerebellar granule cells resulted in an inward current of 49.7 ± 8.8 pA ($n = 19$ cells, 5 mice, example in Fig. 1c, top) at P10–P15 in voltage-clamp configuration and in a depolarization of 12.8 ± 2.7 mV ($n = 6$, example in Fig. 1c, bottom) in current-clamp configuration. As DOI cannot discriminate between the different 5-HT₂ receptor subtypes, 1 μ M RO60-0175, a specific 5-HT_{2B/C} agonist, was locally applied to the cerebellar granule cells. In contrast to DOI, RO60-0175 did not evoke an ion current at P10 ($n = 6$; data not shown). Application of 300 nM MDL 100,907, a 5-HT_{2A} receptor-specific antagonist, blocked the DOI-induced ion current at P11 ($n = 3$ cells, 3 mice, example in Fig. 1c, top) and at P42 ($n = 3$ cells, 3 mice). The expression pattern of the 5-HT_{2A} receptor was confirmed by immunohistochemistry using a specific 5-HT_{2A} receptor antibody (Fig. 1e). Because of the temporal restriction of 5-HT_{1A} receptors on cerebellar granule cells to the first 10 days postnatally and because of the sustained expression of 5-HT_{2A} receptors, the functional role of 5-HT_{2A} receptors on granule cells was studied in more detail.



Presynaptic 5-HT_{2A} Receptors Modulate Paired-Pulse Ratio and Stability at the Parallel Fibre–Purkinje Cell Synapse

To further investigate the presence of 5-HT_{2A} receptors presynaptically on excitatory synapses onto Purkinje cells, miniature postsynaptic spontaneous events were recorded from

Purkinje cells (Fig. 2a, with example trace in Fig. 2b) from control slices and from slices which were preincubated in 300 nM of the 5-HT_{2A} receptor antagonist MDL 100,907. Miniature excitatory postsynaptic currents (mEPSCs) were recorded in the presence of 0.5 μM TTX and 20 μM bicuculline. Both inter-event interval and amplitude

◀ **Fig. 1** Cerebellar granule cells express functional 5-HT_{1A} and 5-HT_{2A} receptors during postnatal development. **a** Schematic diagram of the electrophysiological recording configuration. **b** Amplitudes of inward currents recorded from granule cells, evoked by local application of 100 nM 8-OH-DPAT or 500 nM DOI at different ages during postnatal development. **c** Examples of a whole-cell patch-clamp recording from a granule cell in voltage-clamp (*top*) and current-clamp (*bottom*) at P8 showing an inward current and a depolarization of the membrane potential upon local application of 100 nM of the 5-HT_{1A} agonist 8-OH-DPAT. Also shown in *red* is an example trace of a recording in voltage-clamp after preincubation of the slice in 10 μM of the 5-HT_{1A} specific antagonist WAY100,635, which blocks the effect of local application of 100 nM 8-OH-DPAT. **d** Examples of a whole-cell patch-clamp recording from a granule cell in voltage-clamp (*top*) and current-clamp (*bottom*) at P11, showing an inward current and depolarization of the membrane potential upon local application of 500 nM of the 5-HT₂ specific agonist DOI. Also shown in *red* is an example trace of a recording in voltage-clamp after preincubation of the slice in 300 nM MDL 100,907, indicating no inward current upon local application of 500 nM DOI. *Arrows* in **b** and **c** indicate the start of drug application which lasts for 500 ms. **e** Immunostaining with DAPI and antibodies against 5-HT_{2A} receptors (obtained from Immunostar) at P5 and at P90, indicating presence of 5-HT_{2A} receptors both on Purkinje cells and in the granule cell layer, with higher expression levels in adult mice. *EGL* external granule cell layer, *PCL* Purkinje cell layer, *IGL* internal granule cell layer. *Scale bar* indicates 155 μm

distributions showed a difference in the presence of MDL 100,907 (both $p < 0.01$, Kolmogorov–Smirnov test, Fig. 2c). This confirms our earlier findings that 5-HT_{2A} receptors are located postsynaptically on Purkinje cells, and furthermore indicates presence of 5-HT_{2A} receptors presynaptically on excitatory synapses at Purkinje cells. Miniature inhibitory postsynaptic currents (mIPSCs) were recorded in the presence of 0.5 μM TTX and 20 μM CNQX. The inter-event interval distributions of mIPSCs recorded from Purkinje cells in control slices or slices preincubated with MDL 100,907 were different ($p < 0.001$, Kolmogorov–Smirnov test, Fig. 2d, top). This shows that 5-HT_{2A} receptors are presynaptically located at synapses of inhibitory inputs onto Purkinje cells. There was no difference in amplitude distributions of the mIPSCs, suggesting that 5-HT_{2A} receptors on Purkinje cells do not modulate postsynaptic GABA receptors (Fig. 2d, bottom).

Presynaptic 5-HT_{2A} receptors present on cerebellar granule cells were studied for their role in the short-term plasticity of the parallel fibre–Purkinje cell synapse. Whole-cell patch-clamp recordings were made from Purkinje cells while electrically stimulating parallel fibres in the internal granule cell layer (Fig. 3a, b). At P11–P14, short-term synaptic plasticity at the parallel fibre–Purkinje cell synapse was lower after pharmacological block of 5-HT_{2A} receptors. The average paired-pulse ratio was 1.67 ± 0.14 in control slices ($n = 5$ cells, 3 mice) and 1.27 ± 0.04 in the presence of 300 nM MDL 100,907 ($n = 5$ cells, 3 mice, $p < 0.05$, Fig. 3c). The effect of 5-HT_{2A} receptors on short-term plasticity at the parallel fibre–Purkinje cell synapse was also tested in 8- to 10-week-old mice (P57–P68). Here, the paired-pulse ratio was also lower after

pharmacological block of 5-HT_{2A} receptors, from 1.98 ± 0.17 in control slices ($n = 10$ cells, 5 mice) to 1.50 ± 0.08 in the presence of 300 nM MDL 100,907 ($n = 9$ cells, 5 mice, $p < 0.05$, Fig. 3c).

The stability of the EPSC at the parallel fibre–Purkinje cell synapse was investigated using the same electrophysiological recording configuration as described above (Fig. 3a, b). At P11–P14, the effect of a pharmacological block of 5-HT_{2A} receptors on synapse stability does not reach statistical significance, with a coefficient of variance of 0.33 ± 0.05 in control slices ($n = 5$) and of 0.25 ± 0.04 after blocking 5-HT_{2A} receptors with MDL 100,907 ($n = 5$, Fig. 3d). However, in 8- to 10-week-old mice (P57–P68), recordings from Purkinje cells in control slices had a higher variability of EPSC amplitude. The coefficient of variance had an average of 0.14 ± 0.02 in control slices compared to 0.076 ± 0.009 in slices preincubated in MDL 100,907 ($n = 9$, $p < 0.001$, Fig. 3d).

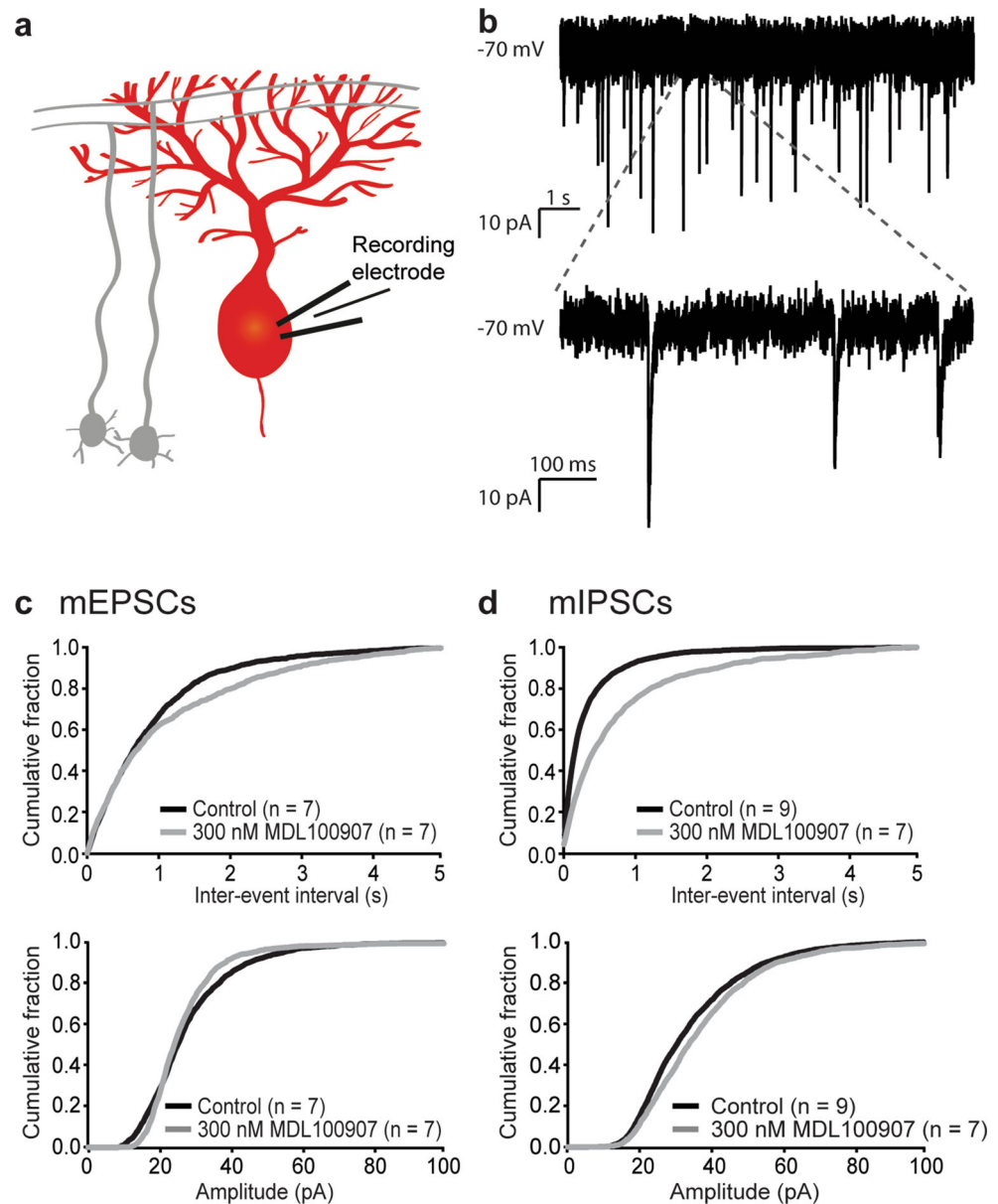
Block of 5-HT_{2A} Receptors Enhances Synaptic Plasticity

Given that 5-HT_{2A} receptors are expressed presynaptically and modulate the stability of synaptic transmission, we further investigated the role of the 5-HT_{2A} receptors in synaptic plasticity. As pharmacologically blocking 5-HT_{2A} receptors increased the stability of the EPSC at the parallel fibre–Purkinje cell synapse, we hypothesized that functional 5-HT_{2A} receptors impair presynaptic synaptic plasticity at the parallel fibre–Purkinje cell synapse. To test this, whole-cell patch-clamp recordings were made from Purkinje cells from 8- to 10-week-old mice while electrically stimulating the parallel fibres in the internal granule cell layer with a tetanus of 8 Hz during 30 s (Fig. 4a) in the absence and presence of 300 nM MDL 100,907 (Fig. 4b). Indeed, synaptic plasticity was increased after pharmacological block of 5-HT_{2A} receptors. The difference between control and MDL 100,907-treated slices became evident from 5 min after the tetanus protocol (example in Fig. 4c). A higher increase of the area of the EPSC 10–15 min after induction of plasticity by the tetanus protocol was found in slices treated with MDL 100,907 (Fig. 4d). Thus, we conclude that blocking 5-HT_{2A} receptors enhances short-term synaptic plasticity in 8- to 10-week-old mice.

Discussion

In this study, we show a novel way for serotonin to control development of the cerebellum, mediated via 5-HT_{2A} receptors present on cerebellar granule cells during postnatal development and until at least 8 weeks after birth. We show the electrophysiological effects of 5-HT_{1A} and 5-HT_{2A} receptors present on granule cells during postnatal development of the cerebellum. We further characterize 5-HT_{2A} receptor-

Fig. 2 Presynaptic 5-HT_{2A} receptors modulate the frequency of spontaneous mEPSCs and mIPSCs recorded from Purkinje cells. **a** Schematic diagram of the electrophysiological recording configuration of **b–d**. **b** Example trace (with magnification) of miniature inhibitory synaptic currents recorded from Purkinje cells at -70 mV. **c** Inter-event interval distributions and amplitude distributions of mEPSCs recorded from Purkinje cells show an increase in inter-event interval (a reduction in frequency) and a difference in mEPSC amplitude after application of 300 nM of the selective 5-HT_{2A} receptor antagonist MDL 100,907. **d** Inter-event interval distributions and amplitude distributions of mIPSCs recorded from Purkinje cells show an increase in inter-event interval (a reduction in frequency) but no difference in mIPSC amplitude after application of 300 nM of the selective 5-HT_{2A} receptor antagonist MDL 100,907



mediated currents and conclude that 5-HT_{2A} receptors expressed by granule cells are involved in mediating stability and short-term synaptic plasticity at the parallel fibre–Purkinje cell synapse.

Expression Pattern of 5-HT_{1A} and 5-HT_{2A} Receptors in the Cerebellum

We show for the first time expression of 5-HT_{1A} and 5-HT_{2A} receptors by cerebellar granule cells. The expression patterns of both 5-HT_{1A} and 5-HT₂ receptors on cerebellar granule cells mimic the known expression pattern of these receptors on Purkinje cells. Activation of these receptors leads to an inward current in voltage-clamp configuration and to a depolarization in current-clamp configuration.

Electrophysiological evidence indicates that expression of 5-HT_{1A} receptors by cerebellar granule cells peaks at P7, after which expression decreases, and no functional 5-HT_{1A} receptors are present from P12 onward. In Purkinje cells, 5-HT_{1A} receptor expression occurs during the first week postnatally, decreases thereafter, and no expression is detected in adult rodents [14, 29–31]. This makes the 5-HT_{1A} receptor the earliest serotonin receptor to be expressed in the cerebellum during postnatal development, and the expression pattern coincides with the expression pattern of serotonergic fibres in the cerebellar cortex. In rodents, serotonergic fibres appear in the white matter around birth. During the first postnatal week, serotonergic fibres extend into the internal granule cell layer, occasionally also penetrating the Purkinje cell layer. The serotonergic fibres extend further into the cerebellar cortex

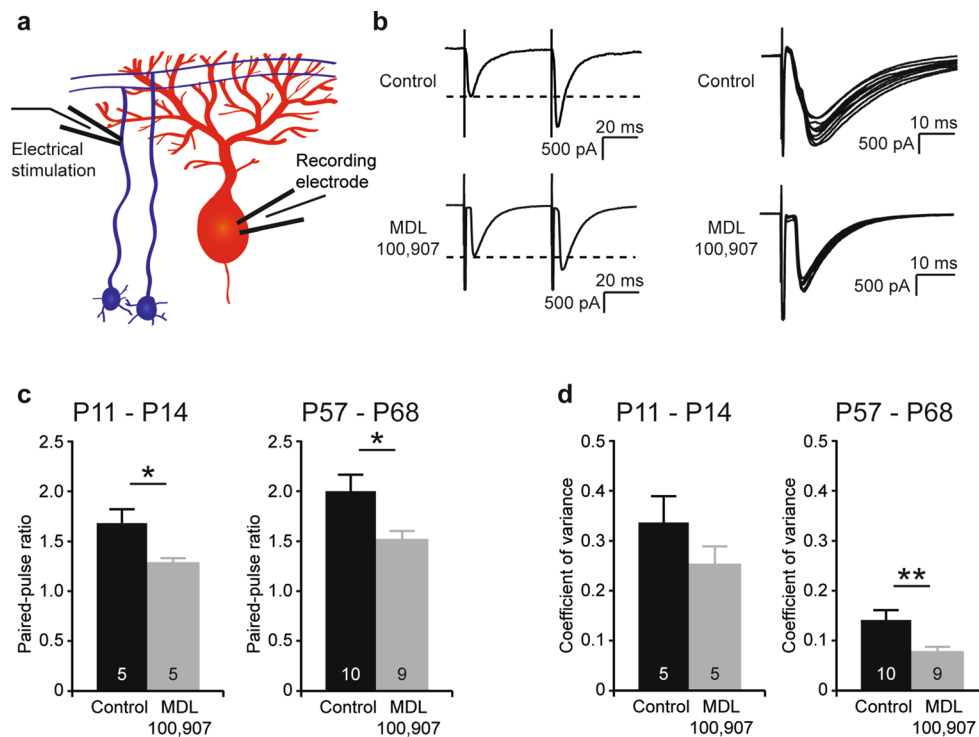


Fig. 3 Presynaptic 5-HT_{2A} receptors modulate stability of the parallel fibre–Purkinje cell synapse. **a** Schematic diagram of the electrophysiological recording configuration of **b–d**. **b** Examples of currents recorded from Purkinje cells at P11–P14 after electrically stimulating parallel fibres with a double-pulse protocol, and examples of ten consecutive currents recorded from Purkinje cells at P11–P14 after electrically stimulating parallel fibres, both in control slices and in slices preincubated with 300 nM MDL 100,907. **c** Pharmacological blockade of 5-HT_{2A} receptors

with 300 nM MDL 100,907 results in a reduced paired-pulse ratio at both P11–P14 and at P57–P68. **d** At P11–P14, the effect of pharmacological blockade of 5-HT_{2A} receptors with 300 nM MDL 100,907 on the coefficient of variance of EPSCs at the parallel fibre–Purkinje cell synapse does not reach significance. At P57–P68, blocking 5-HT_{2A} receptors results in a lower coefficient of variance of EPSCs at the parallel fibre–Purkinje cell synapse. The *numbers* in the bars of the graphs indicate the number of cells used for analysis

during the second and third postnatal week [32]. The first serotonergic effects in the cerebellum after birth are therefore likely to be mediated via 5-HT_{1A} receptors on Purkinje cells and granule cells. Their functional role at such an early age, after which they disappear, remains to be further investigated.

Functional 5-HT_{2A} receptors are not present on cerebellar granule cells before P5, after which their expression increases until it reaches a peak around 2 weeks after birth. Sustained expression of functional 5-HT₂ receptors on cerebellar granule cells is found until 8 weeks after birth. This also mimics 5-HT₂ receptor expression by Purkinje cells, which begins around P0–P5 and gradually increases until P21, as shown by immunohistochemistry [19, 33]. The effects of 5-HT₂ receptors during the postnatal development of the cerebellum were examined by recording spontaneous miniature events from Purkinje cells. Pharmacological block of 5-HT_{2A} receptors resulted in an increase in inter-event interval and a decrease in amplitude of the mEPSCs. These data confirm that the 5-HT_{2A} receptor is located both on Purkinje cells and on excitatory presynaptic inputs to the Purkinje cells. We conclude that the excitatory presynaptic cells expressing 5-HT_{2A} receptors are the granule cells, which we have shown to express 5-

HT_{2A} receptors using immunohistochemistry and electrophysiological experiments described above. Furthermore, an increase in inter-event interval of the mIPSCs was found after blocking 5-HT_{2A} receptors, indicating presence of 5-HT_{2A} receptors on presynaptic inhibitory inputs to the Purkinje cells. Of the inhibitory cells in the cerebellum, the Golgi cell is the only cell type known to express the 5-HT_{2A} receptor; however, these cells have no synapses on Purkinje cells [18]. Inhibitory basket and stellate cells do synapse on the Purkinje cell, but these cells are not known to be 5-HT sensitive [34]. A possible candidate is the Lugaro cell, which is sensitive to 5-HT and has 5-HT driven inhibitory synapses on Purkinje cells [34, 35].

Concluding Remarks

We have shown presence and functional consequences of 5-HT_{1A} and 5-HT_{2A} receptors on cerebellar granule cells in this study and recently also presence and functional consequences of 5-HT₃ receptors on cerebellar granule cells [12, 13]. All three serotonin receptors are expressed during the first 3 weeks after birth, but their specific temporal expression pattern is different [36]. 5-HT_{1A} receptors are expressed earliest during

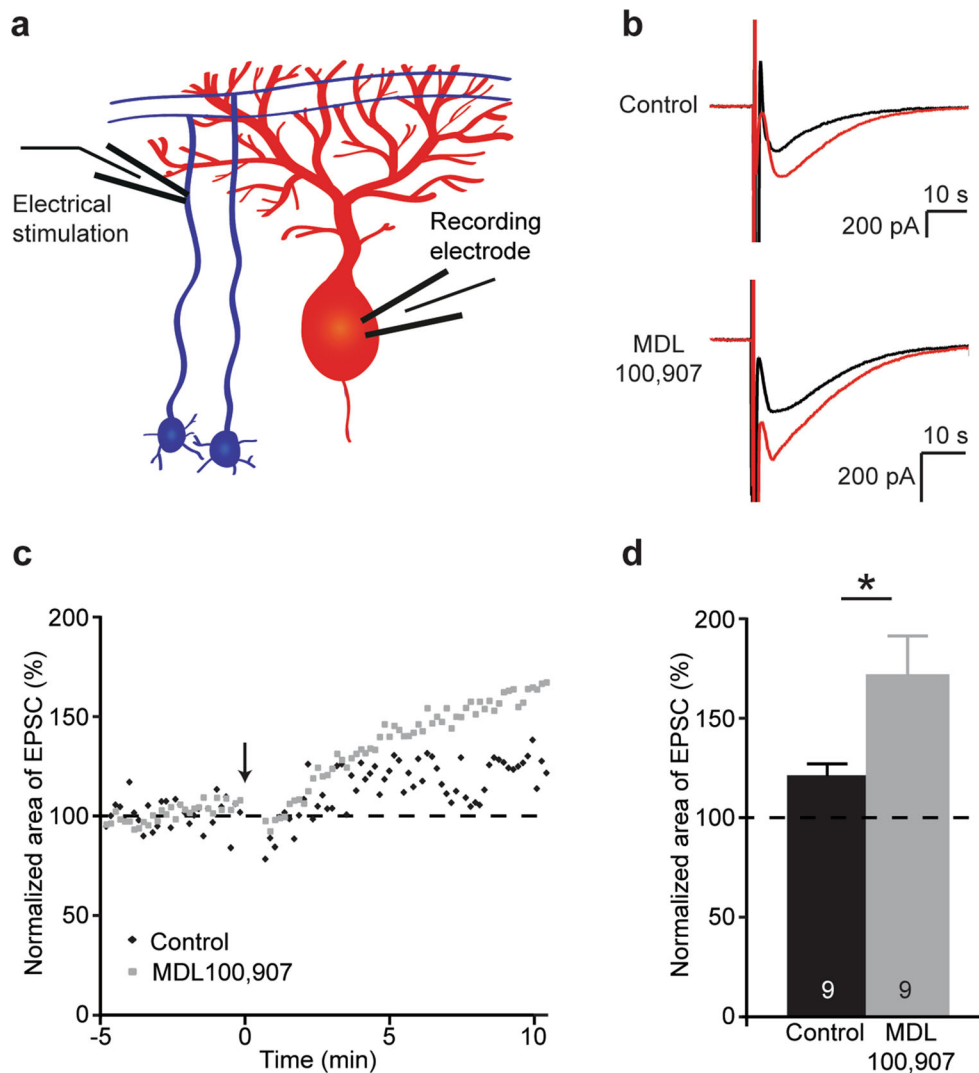


Fig. 4 5-HT_{2A} receptors modulate synaptic plasticity at the parallel fibre–Purkinje cell synapse in 8- to 10-week-old mice. **a** Schematic diagram of the electrophysiological recording configuration of **b–d**. **b** Examples of recordings from Purkinje cells while electrically stimulating the parallel fibres with a double-pulse protocol, from a control slice and from a slice which is preincubated with 300 nM of the 5-HT_{2A} selective antagonist MDL 100,907. *Black traces* are from recordings before inducing plasticity; *red traces* are from recordings 10–15 min after induction of plasticity using a 8 Hz, 30 s stimulation protocol. **c** Example of recordings from Purkinje cells from a control slice and from a slice which is

preincubated with 300 nM of the 5-HT_{2A} selective antagonist MDL 100,907 in which the area of the EPSC, normalized to the average before the tetanus protocol, is recorded over time. The *arrow* indicates the start of the tetanus to induce plasticity with a 8 Hz, 30 s stimulation protocol. **d** The area of EPSCs recorded 10–15 min after the plasticity stimulation protocol, normalized to the area of EPSCs before the plasticity stimulation protocol, indicates enhanced plasticity after blocking 5-HT_{2A} receptors with MDL 100,907. The *numbers* in the bars of the graphs indicate the number of cells used for analysis

the first postnatal week, and both 5-HT_{2A} and 5-HT₃ receptors are expressed during the second postnatal week, but only 5-HT_{2A} receptor expression remains until at least postnatal week 10. It has been shown before that functional interplays between serotonin receptors occur. Activation of 5-HT₂ receptors can potentiate 5-HT₃ receptor function in rat trigeminal ganglion neurons via involvement of G proteins and PKC [37]. 5-HT₂ and 5-HT₃ receptors have a similar rise of expression during the first postnatal week in rodents, but while 5-HT₃ receptor expression by cerebellar granule cells decreases after 2 weeks with no 5-HT₃ receptors left at 3 weeks

postnatally, 5-HT₂ receptors persist to be expressed by granule cells until at least 8 weeks postnatally. We assume that during the first 3 weeks, 5-HT₂ and 5-HT₃ receptors strengthen each other's function in postnatal development of the cerebellum, with 5-HT₂ receptors persisting in this function when 5-HT₃ receptors gradually disappear. Functional 5-HT₂ receptors reduce strength of synaptic activity and reduce stability at the parallel fibre–Purkinje cell synapse. Activity-dependent strengthening of the synapse could affect maturation, with 5-HT₂ receptor-mediated activity slowing down the maturational process.

Taken together, these results show that serotonin is in a powerful position to control the physiology of the cerebellum at all ages, ranging from newborn to adult, through distinct temporal expression of its receptors, not only on Purkinje cells but also on granule cells.

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References

- Fatemi SH, Halt AR, Realmuto G, Earle J, Kist DA, Thuras P, et al. Purkinje cell size is reduced in cerebellum of patients with autism. *Cell Mol Neurobiol.* 2002;22(2):171–5.
- Fatemi HS, Merz A, Realmuto GR. The roles of reelin, Bcl2, and serotonin in cerebellar pathology in autism. *J Dev Phys Disabil.* 2003;15(1):1–22.
- Allen G. The cerebellum in autism. *Clin Neuropsychol.* 2005;2(6):321–37.
- Arnold SE, Trojanowski JQ. Recent advances in defining the neuropathology of schizophrenia. *Acta Neuropathol.* 1997;92(3):217–31.
- Carper RA, Courchesne E. Inverse correlation between frontal lobe and cerebellum sizes in children with autism. *Brain.* 2000;123(4):836–44.
- Picard H, Amado I, Mouchet-Mages S, Olié J-P, Krebs M-O. The role of the cerebellum in schizophrenia: an update of clinical, cognitive, and functional evidences. *Schizophr Bull.* 2008;34(1):155–72.
- Andreasen NC, Pierson R. The role of the cerebellum in schizophrenia. *Biol Psychiatry.* 2008;64(2):81–8.
- Slater P, Doyle CA, Deakin JF. Abnormal persistence of cerebellar serotonin-1A receptors in schizophrenia suggests failure to regress in neonates. *J Neural Transm.* 1998;105(2–3):305–15.
- Eastwood SL, Burnet PW, Gittins R, Baker K, Harrison PJ. Expression of serotonin 5-HT_{2A} receptors in the human cerebellum and alterations in schizophrenia. *Synapse.* 2001;42(2):104–14.
- Takeuchi Y, Kimura H, Sano Y. Immunohistochemical demonstration of serotonin-containing nerve fibers in the cerebellum. *Cell Tissue Res.* 1982;226(1):1–12.
- Bishop GA, Ho RH. The distribution and origin of serotonin immunoreactivity in the rat cerebellum. *Brain Res.* 1985;331(2):195–207.
- Oostland M, Sellmeijer J, van Hooft JA. Transient expression of functional 5-HT₃ receptors by glutamatergic granule cells in the early postnatal mouse cerebellum. *J Physiol.* 2011;589(20):4837–46.
- Oostland M, Buijink MR, van Hooft JA. Serotonergic control of Purkinje cell maturation and climbing fibre elimination by 5-HT₃ receptors in the juvenile mouse cerebellum. *J Physiol.* 2013;591(7):1793–807.
- Matthiessen L, Kia HK, Daval G, Riad M, Hamon M, Vergé D. Immunocytochemical localization of 5-HT_{1A} receptors in the rat immature cerebellum. *Neuroreport.* 1993;4(6):763–6.
- Choi DS, Maroteaux L. Immunohistochemical localisation of the serotonin 5-HT_{2B} receptor in mouse gut, cardiovascular system, and brain. *FEBS Lett.* 1996;391(1):45–51.
- Maeshima T, Shutoh F, Hamada S, Senzaki K, Hamaguchi-Hamada K, Ito R, et al. Serotonin 2A receptor-like immunoreactivity in rat cerebellar Purkinje cells. *Neurosci Lett.* 1998;252(1):72–4.
- Cornea-Hebert V, Riad M, Wu C, Singh SK, Descarries L. Cellular and subcellular distribution of the serotonin 5-HT_{2A} receptor in the central nervous system of adult rat. *J Comp Neurol.* 1999;409(2):187–209.
- Geurts FJ, De Schutter E, Timmermans JP. Localization of 5-HT_{2A}, 5-HT₃, 5-HT_{5A} and 5-HT₇ receptor-like immunoreactivity in the rat cerebellum. *J Chem Neuroanat.* 2002;24(1):65–74.
- Li QH, Nakadate K, Tanaka-Nakadate S, Nakatsuka D, Cui Y, Watanabe Y. Unique expression patterns of 5-HT_{2A} and 5-HT_{2C} receptors in the rat brain during postnatal development: Western blot and immunohistochemical analyses. *J Comp Neurol.* 2004;469(1):128–40.
- Ward RP, Hamblin MW, Lachowicz JE, Hoffman BJ, Sibley DR, Dorsa DM. Localization of serotonin subtype 6 receptor messenger RNA in the rat brain by in situ hybridization histochemistry. *Neuroscience.* 1995;64(4):1105–11.
- Gérard C, Martres M-P, Lefèvre K, Miquel M-C, Vergé D, et al. Immuno-localization of serotonin 5-HT₆ receptor-like material in the rat central nervous system. *Brain Res.* 1997;746(1):207–19.
- Pazos A, Palacios JM. Quantitative autoradiographic mapping of serotonin receptors in the rat brain. I. Serotonin-1 receptors. *Brain Res.* 1985;346(2):205–30.
- Xu J, Chuang DM. Serotonergic, adrenergic and histaminergic receptors coupled to phospholipase C in cultured cerebellar granule cells of rats. *Biochem Pharmacol.* 1987;36(14):2353–8.
- Akiyoshi J, Hough C, Chuang DM. Paradoxical increase of 5-hydroxytryptamine₂ receptors and 5-hydroxytryptamine₂ receptor mRNA in cerebellar granule cells after persistent 5-hydroxytryptamine₂ receptor stimulation. *Mol Pharmacol.* 2005;43(3):349–55.
- Pazos A, Cortés R, Palacios JM. Quantitative autoradiographic mapping of serotonin receptors in the rat brain. II. Serotonin-2 receptors. *Brain Res.* 1985;346(2):231–49.
- van Hooft JA. Fast Green FCF (Food Green 3) inhibits synaptic activity in rat hippocampal interneurons. *Neurosci Lett.* 2002;318(3):163–5.
- Hansel C, Linden DJ, Angelo ED. Beyond parallel fiber LTD: the diversity of synaptic and non-synaptic plasticity in the cerebellum. *Nat Neurosci.* 2001;4(5):467–76.
- Sprouse J, Reynolds L, Li X, Braselton J, Schmidt A. 8-OH-DPAT as a 5-HT₇ agonist: phase shifts of the circadian biological clock through increases in cAMP production. *Neuropharmacology.* 2004;46(1):52–62.
- Daval G, Vergé D, Becerril A, Gozlan H, Spampinato U, Hamon M. Transient expression of 5-HT_{1A} receptor binding sites in some areas of the rat CNS during postnatal development. *Int J Dev Neurosci.* 1987;5:171–80.
- Matthiessen L, Daval G, Bailly Y, Gozlan H, Hamon M, Vergé D. Quantification of 5-hydroxytryptamine_{1A} receptors in the cerebellum of normal and X-irradiated rats during postnatal development. *Neuroscience.* 1992;51(2):475–85.
- Miquel MC, Kia HK, Boni C, Doucet E, Daval G, Matthiessen L, et al. Postnatal development and localization of 5-HT_{1A} receptor mRNA in rat forebrain and cerebellum. *Brain Res Dev Brain Res.* 1994;80(1):149–57.
- Lidov HGW, Molliver ME. An immunohistochemical study of serotonin neuron development in the rat: ascending pathways and terminal fields. *Brain Res Bull.* 1982;8(4):389–430.

33. Maeshima T, Shiga T, Ito R, Okado N. Expression of serotonin_{2A} receptors in Purkinje cells of the developing rat cerebellum. *Neurosci Res.* 2004;50(4):411–7.
34. Dieudonné S, Dumoulin A. Serotonin-driven long-range inhibitory connections in the cerebellar cortex. *J Neurosci.* 2000;20(5):1837–48.
35. Dean I, Robertson SJ, Edwards FA. Serotonin drives a novel GABAergic synaptic current recorded in rat cerebellar purkinje cells: a Lugaro cell to Purkinje cell synapse. *J Neurosci.* 2003;23(11):4457–69.
36. Oostland M, van Hooft JA. The role of serotonin in cerebellar development. *Neuroscience.* 2013;248:201–12.
37. Hu W-P, Guan B-C, Rub L-Q, Chen J-G, Li Z-W. Potentiation of 5-HT₃ receptor function by the activation of coexistent 5-HT₂ receptors in trigeminal ganglion neurons of rats. *Neuropharmacology.* 2004;47(6):833–40.