Serotonergic control of the developing cerebellum

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Chapter 3

Serotonergic control of Purkinje cell maturation and climbing fibre elimination

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Serotonergic control of Purkinje cell maturation and climbing fibre elimination
by 5-HT₃ receptors in the juvenile mouse cerebellum
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Abstract

Functional 5-HT$_3$ receptors are transiently expressed by cerebellar granule cells during early postnatal development, where they modulate short-term synaptic plasticity at the parallel fibre - Purkinje cell synapse. Here, we show that serotonin controls maturation of Purkinje cells in the mouse cerebellum. Serotonin 3 (5-HT$_3$) receptors regulate morphological maturation of Purkinje cells during early postnatal development, and this effect is mediated by the glycoprotein reelin. Using whole-cell patch clamp recordings we further investigated physiological development of Purkinje cells in 5-HT$_{3A}$ receptor knockout mice during early postnatal development, and found abnormal physiological maturation, characterized by a more depolarized resting membrane potential, an increased input resistance, and the ability to fire action potentials upon a depolarizing current injection at an earlier age. Furthermore, short-term synaptic plasticity was impaired at both the parallel fibre - Purkinje cell and the climbing fibre - Purkinje cell synapse and both amplitude and frequency spontaneous miniature events recorded from Purkinje cells were increased. The expedited morphological and physiological maturation affects the whole cerebellar cortical network, as indicated by delayed climbing fibre elimination in 5-HT$_{3A}$ receptor knockout mice. There was no difference anymore between wildtype and 5-HT$_{3A}$ receptor knockout mice in any of the morphological or physiological properties described above at later ages, indicating a specific time window during which serotonin regulates postnatal development of the cerebellum via 5-HT$_3$ receptors expressed by granule cells.
3.1 Introduction

Both the anatomical and functional development of the cerebellum occurs for a substantial part postnatally (Altman and Bayer, 1996). At birth, no cerebellar-dependent behaviour can be detected and cells display an immature phenotype. During the first three weeks after birth, granule cells migrate from the external to the internal granule cell layer, and Purkinje cells fully develop their dendritic tree. Furthermore, connections between parallel fibres and Purkinje cells, and between climbing fibres and Purkinje cells are formed into functional synapses during this time. The rodent cerebellum is physiologically mature around four weeks after birth (Altman, 1972a; van Welie et al., 2011).

Purkinje cells are the sole output of the cerebellar cortex to the deep cerebellar nuclei. The morphological and physiological maturation of Purkinje cells is therefore of special interest (Kapfhammer, 2004). McKay and Turner (2005) described three stages of Purkinje cell maturation in the rat: an initial stable immature stage of minimal change from postnatal day (P) 0 to P9, a transitional stage in which the Purkinje cells undergo major morphological and physiological maturation, and from P18 a stable adult stage with only minor refinements. Functional parallel fibre - Purkinje cell synapses are formed at the end of the first postnatal week (Altman, 1972b). Parallel fibres form synapses at the distal dendrites of Purkinje cells. Climbing fibres wrap around the soma and proximal dendrites of Purkinje cells. Climbing fibres wrap around the soma and proximal dendrites of Purkinje cells and form their synapses there. In newborn rodents, a Purkinje cell is innervated by multiple climbing fibres, of which all but one will be removed during the first few postnatal weeks (Crepel, 1976). Parallel fibre input has a highly dominant role in climbing fibre elimination during development. In polyinnervated Purkinje cells, competition between different climbing fibres appears between P3 and P7 and continues during the second postnatal week (Scelfo and Strata, 2005).

5-HT$_3$ receptors are involved in postnatal maturation of pyramidal neurons in the cortex. Glutamatergic Cajal-Retzius cells express 5-HT$_3$ receptors up until the first two postnatal weeks (Chameau et al., 2009), during which they also synthesize and
secrete the glycoprotein reelin (D’Arcangelo et al., 1999). We have shown that reelin regulates the maturation of apical, but not basal, dendrites of layer II/III pyramidal neurons in the somatosensory cortex in a 5-HT$_3$ receptor-dependent manner. Specifically, dendritic complexity of these neurons in 5-HT$_{3A}$ receptor knockout mouse is increased, and the hypertrophy of dendritic arborization can be rescued by addition of recombinant reelin (Chameau et al., 2009).

Recently, we have shown that 5-HT$_3$ receptors are transiently expressed on glutamatergic granule cells in the cerebellum during the first three weeks after birth (Oostland et al., 2011). Interestingly, this coincides with the time window during which Purkinje cell dendrites develop (Altman and Bayer, 1996). In addition, it has been shown that granule cells synthesize and secrete reelin (Sinagra et al., 2008). We therefore hypothesized that serotonin modulates morphological and physiological maturation of Purkinje cells via 5-HT$_3$ receptors expressed on glutamatergic granule cells.

3.2 Methods

3.2.1 Ethical approval

Wildtype C57/Bl6 (Harlan) and 5-HT$_{3A}$ receptor knockout mice (Zeitz et al., 2002), were used for this study between the age of P5 and P72, both males and females. All experiments were performed in accordance with the committee on animal bioethics of the University of Amsterdam.

3.2.2 Electrophysiological recordings

For both single-cell electroporation and whole-cell patch clamp recordings animals were killed by decapitation between P5 and P72. Sagittal brain slices were cut using a vibrating blade microtome (Leica VT1200S) at a thickness of 300 μm. During slicing the brains were kept in cooled (4 °C) oxygenated ACSF which was composed of the following (in mM): NaCl (120), KCl (3.5), CaCl$_2$ (2.5), MgSO$_4$ (1.3), NaH$_2$PO$_4$ (1.25),
NaHCO$_3$ (25), glucose (25), continuously bubbled with 95% O$_2$ and 5% CO$_2$ (pH = 7.4). Brains from mice older than three weeks were sliced in modified ACSF, composed of the following (in mM): choline chloride (120), KCl (3.5), CaCl$_2$ (0.5), MgSO$_4$ (6.0), NaH$_2$PO$_4$ (1.25), glucose (25), and NaHCO$_3$ (25). During the experiments slices were kept submerged at room temperature and continuously superfused with ACSF. Electroporation pipettes were pulled from borosilicate glass and had a resistance between 7 - 12 MΩ. Pipettes were filled with biocytin hydrochloride (2 - 4 mg/ml, Sigma) dissolved in internal solution containing (in mM): Kgluconate (105), KCl (30), EGTA (5), CaCl$_2$ (0.5), HEPES (10), Mg-ATP (5), (pH 7.3 with KOH). The pipette was positioned in proximity of the membrane of the Purkinje cell, and the membrane was permeabilized by electrical stimulation with an Axoporator 800A (Molecular Devices, USA). Patch pipettes were pulled from borosilicate glass with a resistance of 2 - 3 MΩ and were filled with internal solution as described above. Whole-cell recordings from Purkinje cells were made 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 - 11 MΩ and was compensated for at least 70%. Cells were voltage clamped at -70 mV, corrected for liquid junction potential.

Action potential firing and input resistance were determined from whole-cell current clamp recordings from Purkinje cells during which a range of twelve currents was injected, with a duration of 1 s each and ranging from -100 pA to +175 pA. The amount of Purkinje cells which had showed > 1 action potential during any of these current injection steps were scored. Input resistance was calculated from the change in membrane potential at a current injection of -25 pA, during which Purkinje cells did not fire action potentials. Resting membrane potential was calculated from the input resistance of the cell and the amount of current necessary to inject in order to keep the cell at a holding potential of -70 mV using Ohm's law.

Miniature postsynaptic currents (mPSCs) from Purkinje cells in both wildtype and 5-HT$_{3A}$ receptor knockout mice were recorded in the voltage clamp configuration at P7 - P9 in the presence of 0.5 μM TTX (Latoxan, Valence, France) and analysed as described before (van Hooft, 2002). Per cell one or two traces of five minutes each
were used for analysis, with at least 100 miniature synaptic events per cell which were visually verified. Both distributions and average amplitudes and inter-event intervals were analysed per cell. The distributions and average values of each cell were then averaged over all recorded cells to get the final distributions and values as shown in figure 3.5. Glutamatergic synaptic currents in Purkinje cells were evoked by stimulation of either the parallel fibres or the climbing fibres with a glass-electrode filled with ACSF. Paired stimuli (100 - 400 μA, 0.2 - 1 ms duration, interstimulus interval 50 ms) were delivered either to the molecular layer or 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 minutes were used to analyse the paired-pulse ratio (PPR). The PPR was defined as the amplitude of the second EPSC divided by the amplitude of the first EPSC.

3.2.3 Organotypic slice cultures

Cell culture inserts (Falcon, 1 μm pore size) were coated with 66.7 μg/ml poly-L-lysine (Sigma) overnight at room temperature. Before use, inserts were rinsed 3 times with sterile H₂O and wells were filled with 1.8 ml culture medium, consisting of Neurobasal-A medium containing 10 μg/ml penicillin/streptomycin and supplemented with either the serum-free supplements B27 (1:50 vol/vol), N2 (1:100, vol/vol) and 2 mM L-glutamine, or supplemented with 25% horse serum, 30 mM glucose and 2 mM glutamax (all Invitrogen). Cerebellar slice cultures treated with G10 at P8 during two days in vitro in culture medium with 25% horse serum gave similar results as in the cultures without serum, indicating addition of the serum did not change the reelin-mediated maturation process of Purkinje cells. Western blot analysis showed that horse serum did not contain detectable amounts of reelin (data not shown). Eight-day old mice were killed by decapitation, after which brains were quickly dissected out and sagittal slices were made as described above. Slices were washed three times in sterile culture medium and placed on inserts, and excessive medium was removed. Slices were cultured for 1-2 days in vitro (Div) at 37 °C in a humidified atmosphere containing 5% CO₂. Culture medium from slices
in the experimental condition was supplemented with the N-terminal specific antireelin antibody G10 (Abcam) at a dilution of 1/1000 or the selective 5-HT$_3$ antagonist granisetron (100 nM, Sigma).

3.2.4 Morphological analysis

In order to reveal Purkinje cell morphology, Purkinje cells from lobex III - IX in the cerebellar vermis were filled with biocytin (Sigma, 2-4 mg/ml dissolved in internal solution as described above) during the whole-cell patch clamp recordings or with biocytin hydrochloride (Sigma, 4 mg/ml dissolved in internal solution as described above) during single-cell electroporation. Slices were fixed overnight in paraformaldehyde (PFA, 4% in 0.05 M PBS, pH = 7.4) at 4 °C and visualized using immunohistochemical methods. Slices were washed in PBS for 5 x 8 minutes, and endogenous peroxidase was inhibited by a 30 minute incubation in H$_2$O$_2$ (3% in PBS). After 60 minutes permeabilization in 2% Triton X-100 (2% in PBS), slices were incubated for two hours in avidin-biotin-peroxidase complex (Vectastain ABC Elite kit, Vector Laboratories). Biocytin was visualized as a dark brown substrate using a DAB (3,3’-diaminobenzidine-4 HCl, Sigma) reaction. Slices were washed 3 x 8 minutes before being mounted on a glass slide with mowiol 4-88 (Sigma; dissolved in 0.2 M Tris-HCl and glycerol, pH = 8.5). Images were made using a confocal microscope (Zeiss LSM 510) equipped with a 20x/0.75 objective and using the 543 nm line of an ArKr laser. These images were then used to make a 3D reconstruction of the Purkinje cells for morphological analysis. For the reconstruction, ImageJ (National institute of Health, Bethesda, MD; http://rsb.info.nih.gov/nih-image/) was used in combination with the NeuroMorpho plug-in. The different parameters were analysed using LMeasure software (Scorcioni et al., 2008). With these parameters, the dendritic complexity index (DCI) was calculated using the formula shown below, in which the branch tip order was defined for every branch tip as the number of branch points between the primary dendrite and the tip.

\[
DCI = \left( \frac{\sum \text{branch tip orders} + \# \text{of branch tips}}{\# \text{of primary dendrites}} \right) \times \text{total arbor length}
\]
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Sholl analysis was performed on the reconstructed Purkinje cells by plotting the sum of the length of the dendrites in bins of 10 μm at P9 or 20 μm at P35 as function of the distance from the soma using NeuronStudio software.

3.2.5 Western blot

For quantification of reelin levels we dissected the cerebellum from five wildtype and five 5-HT$_{3A}$ receptor knockout mice at P9 and homogenized these individually with a glass douncer in lysis buffer consisting of 320 mM sucrose, 10 mM HEPES and protease inhibitor mixture (Complete, pH 7.4, Roche). The homogenates were centrifuged at 510 x g for 5 minutes at 4 °C and the supernatant was centrifuged again at 10,000 x g for 10 minutes at 4 °C to discard undisrupted tissue. Samples were mixed with 5X sample buffer and proteins were denaturalized at 70 °C during 10 minutes. In total 20 μg of protein, quantified by a NanoDrop 2000 Spectrophotometer (Thermo Scientific), was loaded in triplicate and were separated by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) in a 6% Tris-glycine gel and transferred onto a nitrocellulose membrane. The membranes were allowed to dry overnight and then processed for immunodetection of reelin. Nonspecific binding sites on the nitrocellulose were blocked by immersion of the membranes in 4% nonfat dry milk (BioRad Laboratories) in Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl, pH 7.4) containing 0.1% (vol/vol) Tween-20 (TBST). Membranes were then incubated for 2 hours at room temperature with mouse anti-reelin G10 antibody (Abcam, diluted 1:1000 in the blocking solution), and mouse anti-β-actin (Sigma, 1:4000). Blots were then rinsed three times in TBST and incubated for 1 hour at room temperature with horseradish peroxidase-conjugated goat anti-mouse IgG (1:3000 in blocking solution). The protein bands were detected using the ECL Prime Western Blotting detection reagents (Amersham) and were visualized with the Odyssey 2800 (Li-Cor). Optical density (OD) levels of protein bands were quantified using ImageJ software, after which the reelin OD levels were compared to the OD levels of the β-actin bands within the same lane to obtain the OD ratio. At least two replicates were performed for each sample.
3.2.6 Statistical analysis

Values are expressed as mean ± standard error of the mean. Comparisons were made using the Student’s t-test unless stated otherwise. Fisher’s exact test was utilized to compare frequency distributions between wildtype and 5-HT$_3$A receptor knockout mice. Frequency and amplitude distributions of the miniature synaptic events were compared with a two-sample Kolmogorov-Smirnov test. p < 0.05 was used to indicate a significant difference. Asterisks indicate p < 0.05 (*), p < 0.01 (**) and p < 0.001 (***)

3.3 Results

3.3.1 Serotonin 5-HT$_3$ receptors control morphological maturation of Purkinje cells via reelin

Purkinje cells of both wildtype and 5-HT$_3$A receptor knockout mice were filled with biocytin during whole-cell patch clamp recordings to reveal their morphology. In nine-days old mice, the dendritic complexity index (DCI) of Purkinje cells was 77 ± 24% (n = 20) higher in 5-HT$_3$A receptor knockout mice than in wildtype mice (n = 15; p < 0.05; figure 3.1 A, B). The total dendritic length of Purkinje cells in 5-HT$_3$A receptor knockout mice was 35 ± 10% higher than in wildtype mice (p < 0.05). Sholl analysis indicated a significant difference in total dendritic length per concentric circle of 10 μm between wildtype and 5-HT$_3$A receptor knockout mice in the area between 40 and 60 μm from the soma (p < 0.05, figure 3.1 C). In five-week old mice, both the DCI (105 ± 15%; n = 13; figure 3.1 D, E) and total dendritic length (103 ± 8%; n = 13) of Purkinje cells were not different from those in wildtype mice (n = 8) and Sholl analysis did not reveal topological differences (figure 3.1 F).

The involvement of 5-HT$_3$ receptors in controlling the maturation of Purkinje cells was corroborated in organotypic slice cultures. Blocking 5-HT$_3$ receptors with 100 nM granisetron at P8 for two days in culture resulted in an increase of the DCI to 228 ± 42% (n = 6; p < 0.05; figure 3.2 B, D). The total dendritic length was increased to 187 ± 14% (p < 0.01) after treatment with granisetron. Sholl analysis revealed a topo-
Figure 3.1: Increased morphological complexity of Purkinje cells in 5-HT$_{3A}$ receptor knockout mice at P9 but not at P35.

A, Reconstructed Purkinje cells from wildtype and 5-HT$_{3A}$ receptor knockout mice at P9. B, Dendritic complexity index of Purkinje cells at P9 indicates an increased dendritic complexity in 5-HT$_{3A}$ receptor knockout mice as compared to wildtype mice. C, Sholl analysis indicates an increased dendritic length specifically at 30 - 60 µm from the soma in 5-HT$_{3A}$ receptor knockout mice at P9. D, Reconstructed Purkinje cells from wildtype and 5-HT$_{3A}$ receptor knockout mice at P35. E, The dendritic complexity index of wildtype and 5-HT$_{3A}$ receptor knockout mice does not reveal any difference at P35. F, Sholl analysis does not show any topological difference between Purkinje cells from wildtype and 5-HT$_{3A}$ receptor knockout mice at P35.
logical difference at 60-130 μm from the soma (p < 0.05; figure 3.2 E). Given the fact that 5-HT$_3$ receptors are expressed on reelin-producing granule cells, we next investigated whether reelin is involved in the dendritic maturation of Purkinje cells. After blocking the N-terminal region of reelin at P8 during one day in vitro in serum-free culture medium by treatment with anti-reelin antibody G10 the DCI was increased to 260 ± 44% (n = 17; p < 0.05; figure 3.2 C, F) and the total dendritic length to 185 ± 16% (p < 0.01). Sholl analysis indicated a significant difference in dendritic length 40-100 μm from the soma (p < 0.01; figure 3.2 G). Western blots were used to detect reelin protein levels in wildtype (n = 5) and 5-HT$_3$A receptor knockout mice (n = 5) at P9 and revealed that the levels of both full length reelin (p < 0.01) and the N-terminal of reelin (p < 0.001) were reduced in 5-HT$_3$A receptor knockout mice (figure 3.2 H, I).

Four different stages of morphological development in one-week old Purkinje cells were defined (figure 3.3 A), comparable to stage II - stage V as described by Altman (1972a). Stage I is defined as the most immature stage in which Purkinje cells have multiple primary dendrites in all directions. Stage II is a stage in which Purkinje cells still have multiple primary dendrites, but which are already polarised into one direction. Purkinje cells in stage III have only one primary dendrite, with an immature dendritic tree. Stage IV is the last stage before full maturation, in which Purkinje cells have one primary dendrite with an almost fully developed dendritic tree. Purkinje cells from 5-HT$_3$A receptor knockout mice were in a more mature morphological stage than Purkinje cells from wildtype mice (Fisher’s exact test, at P7: p < 0.001; P8: p < 0.001; P9: p < 0.001; figure 3.3 B). Taken together, these results indicate that there is a specific time window in 5-HT$_3$A receptor knockout mice during which Purkinje cell dendritic tree morphology is altered.

3.3.2 Abnormal physiological maturation of Purkinje cells in 5-HT$_3$A receptor knockout mice

Given the expedited morphological maturation of Purkinje cells in 5-HT$_3$A receptor knockout mice, we hypothesized that the physiological properties of Purkinje cells show a concurrent increase in speed of maturation. At P6 - P9, Purkinje cells from
Figure 3.2: Organotypic slice cultures show that morphological maturation of Purkinje cells is regulated by 5-HT\textsubscript{3} receptors via the glycoprotein reelin.

A, Example of a P8 Purkinje cell in control condition after two days in culture. B, Example of a P8 Purkinje cell after two days in culture with 100 nM granisetron, a selective 5-HT\textsubscript{3} receptor antagonist. C, Example of a P8 Purkinje cell after two days in culture with G10, an antibody against the N-terminal of reelin. Scale bars in A - C indicate 10 µm. D, The dendritic complexity index of Purkinje cells from cerebellar slices cultured at P8 after two days in culture shows an increased complexity of the Purkinje cells treated during the culture period with granisetron. E, Sholl analysis indicates an increase in dendritic length in granisetron-treated Purkinje cells in the area 60 - 130 µm from the soma. F, The dendritic complexity index from Purkinje cells from cerebellar slices cultured at P8 after one day in culture shows an increased complexity of the Purkinje cells treated during the culture period with G10. G, Sholl analysis shows an increase in dendritic length between 40 - 100 µm from the soma in the culture condition with G10. H, Western blots for whole-cerebellum homogenates from P9 wildtype and 5-HT\textsubscript{3,4} receptor knockout mice showing protein bands for full-length reelin, the N-terminal of reelin, and β-actin. I, Quantification of Western blot analysis indicates that the level of optical density (OD) of reelin is reduced in 5-HT\textsubscript{3,4} receptor knockout mice at P9. The numbers in the bars of the graphs indicate the number of cells used for analysis.
Figure 3.3: Expedited morphological development of Purkinje cells in 5-HT$_{3A}$ receptor knockout mice.

A, Purkinje cells from P8 mice show four different stages of morphological maturation: stage I is the most immature stage in which Purkinje cells have multiple primary dendrites in all directions, stage II is a stage in which Purkinje cells still have multiple primary dendrites, but which are already polarized into one direction, stage III are Purkinje cells which have one primary dendrite with a very immature dendritic tree, and stage IV is the last stage before the final maturation, in which Purkinje cells have one primary dendrite with an almost fully developed dendritic tree. B, Distribution of Purkinje cells in each morphological maturation stage in both wildtype and 5-HT$_{3A}$ receptor knockout mice at P7, P8, and P9 shows that Purkinje cells from 5-HT$_{3A}$ receptor knockout mice display a more mature morphology at an earlier age than Purkinje cells from wildtype mice. The numbers underneath the bars of the graphs indicate the number of cells used for analysis.
5-HT$_{3A}$ receptor knockout mice had a more depolarized resting membrane potential (-46.6 ± 2.4 mV) than Purkinje cells in wildtype mice (-60.7 ± 1.7 mV; p < 0.001; figure 3.4 A, B), and a higher input resistance (760.7 ± 95.9 MΩ; wildtype: 136.0 ± 16.1 MΩ; p < 0.0001; figure 3.4 C). In addition, at this age, the percentage of Purkinje cells firing action potentials upon a depolarizing current injection was significantly higher in 5-HT$_{3A}$ receptor knockout mice (80%; n = 25) than in wildtype mice (23%; n = 22; Fisher’s exact test; p < 0.001; figure 3.4 D). At P10 - P12, there was no difference between Purkinje cells in wildtype (n = 29) and 5-HT$_{3A}$ receptor knockout mice (n = 20) with respect to resting membrane potential or the percentage of cells firing repetitive action potentials upon a depolarizing current injection (figure 3.4 E - G), and comparable to previous studies (Crepel et al., 1987; Doughty et al., 1999; McKay and Turner, 2005; Fry, 2006). However, the input resistance in 5-HT$_{3A}$ receptor knockout mice (271.0 ± 41.4 MΩ) was still significantly higher than that in wildtype mice (170.7 ± 18.8 MΩ; p < 0.05; figure 3.4 H). Both the resting membrane potential (-69.0 ± 1.9 mV) and the input resistance (182.8 ± 32.1 MΩ) in 8 - 10 weeks old 5-HT$_{3A}$ receptor knockout mice (n = 18) were not different from those in wildtype (-69.2 ± 0.5 mV and 164.9 ± 21.8 MΩ, respectively, n = 17).

In order to assess the maturation of the input of Purkinje cells, miniature postsynaptic currents (mPSCs) from whole-cell voltage clamped Purkinje cells were recorded in wildtype (figure 3.5 A) and 5-HT$_{3A}$ receptor knockout mice (figure 3.5 B) between P7 and P9. Average amplitude and inter-event interval of the mPSCs were compared using the Mann-Whitney test. In wildtype mice, the average amplitude of the mPSCs increased with age, showing a developmental pattern. In 5-HT$_{3A}$ receptor knockout mice, the amplitude of the mPSCs was already high at P7 (p < 0.05 compared to wildtype P7), and did not show a further increase with age (figure 3.5 C). The inter-event interval of the mPSCs recorded from Purkinje cells in wildtype mice showed a developmental pattern between P7 and P9, revealing a decrease in the inter-event interval and thus an increase in frequency of the mPSCs at a later age. The average inter-event interval of the mPSCs in 5-HT$_{3A}$ receptor knockout mice was already low at P7 (p < 0.01 compared to wildtype P7), and remained low at P8 (p < 0.05 compared to wildtype P8) and P9 (n.s.; figure 3.5 D). Amplitude distributions were significantly
Figure 3.4: Purkinje cells in 5-HT$_{3A}$ receptor knockout mice display more advanced physiological maturation.

A, Example traces showing action potential firing upon a depolarizing current injection of +175 pA in current-clamp recordings from Purkinje cells in wildtype and 5-HT$_{3A}$ receptor knockout mice at P6 - P9. B, Purkinje cells from 5-HT$_{3A}$ receptor knockout mice show a more depolarized resting membrane potential than Purkinje cells from wildtype mice at P6 - P9. C, Input resistance of Purkinje cells from 5-HT$_{3A}$ receptor knockout mice at P6 - P9 is increased when compared to wildtype mice. D, Percentages of Purkinje cells with evoked action potentials in response to a depolarizing current injection of +175 pA show that more Purkinje cells from 5-HT$_{3A}$ receptor knockout mice at P6 - P9 display this physiological feature than Purkinje cells from wildtype mice at P6 - P9. E, Example traces showing action potential firing upon a depolarizing current injection of +175 pA in current-clamp recordings from Purkinje cells in wildtype and 5-HT$_{3A}$ receptor knockout mice at P10 - P12. At P10 - P12, there is no difference between Purkinje cells from wildtype and Purkinje cells from 5-HT$_{3A}$ receptor knockout mice in the resting membrane potential (F) or in the percentage of cells firing action potentials upon a depolarizing current injection of +175 pA (H), indicating an advanced physiological maturation of Purkinje cells in 5-HT$_{3A}$ receptor knockout mice only during a specific period of postnatal development. Input resistance of Purkinje cells was further decreased in 5-HT$_{3A}$ receptor knockout mice at P10 - P12 but still significantly different from the input resistance of Purkinje cells in wildtype mice (G). The numbers in the bars of the graphs indicate the number of cells used for analysis.
different between wildtype and 5-HT\textsubscript{3A} receptor knockout mice at P7 (figure 3.5 E; \(p < 0.001\)), but not at P8 (figure 3.5 G) and P9 (figure 3.5 I). Inter-event interval distributions were significantly different between wildtype and 5-HT\textsubscript{3A} receptor knockout mice at P7 and P8 (figure 3.5 F, H; both \(p < 0.001\)) but not at P9 (figure 3.5 J).

### 3.3.3 Delayed climbing fibre elimination in 5-HT\textsubscript{3A} receptor knockout mice

Given that the frequency of mPSCs in Purkinje cells from 5-HT\textsubscript{3A} receptor knockout mice is increased, we wondered whether this was due to an increase in parallel fibre inputs, climbing fibre inputs, or both. We have previously shown that acute block of presynaptic 5-HT\textsubscript{3} receptors at the parallel fibre - Purkinje cell synapse converts this synapse from a facilitating into a depressing one (Oostland et al., 2011). This finding was corroborated in 5-HT\textsubscript{3A} receptor knockout mice at P8, reflected by a decreased paired-pulse ratio (PPR) upon parallel fibre stimulation as compared to wildtype mice (\(p < 0.05\), figure 3.6 Aa, Ab, C). At P21, the PPR in wildtype mice (figure 3.6 Ba, C) and in 5-HT\textsubscript{3A} receptor knockout mice (figure 3.6 Bb, C) did not differ and showed a facilitating ratio as is a known feature of the mature parallel fibre - Purkinje cell synapse.

We next investigated the short-term synaptic transmission at the climbing fibre - Purkinje cell synapse by means of paired-pulse stimulation. The PPR at the climbing fibre - Purkinje cell synapse in wildtype mice increased with age, indicating maturation of the climbing fibre - Purkinje cell synapse (figure 3.6 Da, Ea, F). Compared to wildtype mice, in 5-HT\textsubscript{3A} receptor knockout mice the PPR was higher at P8 - P9 (\(p < 0.05\); figure 3.6 Db, F) and at P11 - P12 (\(p < 0.05\); figure 3.6 F), and showed a similar level to the PPR in wildtype mice at P24 (figure 3.6 Eb, F). During early postnatal development there is a competition between parallel fibres and climbing fibres for the available postsynaptic sites on Purkinje cells. Because the parallel fibre - Purkinje cell synapse is impaired in 5-HT\textsubscript{3A} receptor knockout mice, we hypothesized that this might be in favour of the total number of climbing fibres. Whole-cell patch clamp recordings of Purkinje cells were made and climbing fibres were activated with elec-
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Figure 3.5: Increased frequency and amplitude of spontaneous miniature postsynaptic currents in Purkinje cells from one-week old 5-HT$_{3A}$ receptor knockout mice.

A, Example trace of spontaneous mPSCs recorded at -70 mV from Purkinje cells in P7 wild-type mice. B, Similar example trace in P7 5-HT$_{3A}$ receptor knockout mice. C, Amplitude of mPSCs in Purkinje cells from wildtype and 5-HT$_{3A}$ receptor knockout mice indicate a significant difference at P7, but not at P8. D, Average inter-event intervals of the mPSCs recorded from Purkinje cells in wildtype and 5-HT$_{3A}$ receptor knockout mice indicate a difference at P7 and P8, but not at P9. E, G, I, amplitude distributions of mPSCs recorded from Purkinje cells show significant difference between wildtype and 5-HT$_{3A}$ receptor knockout mice at P7. F, H, J, inter-event interval distributions of mPSCs recorded from Purkinje cells show significant difference between wildtype and 5-HT$_{3A}$ receptor knockout mice at P7 and P8. The numbers in the bars of the graphs indicate the number of cells used for analysis.
Figure 3.6: Short-term synaptic plasticity at both the parallel fibre - Purkinje cell synapse and at the climbing fibre - Purkinje cell synapse is impaired in 5-HT_{3A} receptor knockout mice.

A - C, Paired-pulse recordings from parallel fibre - Purkinje cell synapses in wildtype mice at P8 in wildtype mice (Aa) and in 5-HT_{3A} receptor knockout mice (Ab) and at P21 in wildtype mice (Ba) and in 5-HT_{3A} receptor knockout mice (Bb). C, The paired-pulse ratio at the parallel fibre - Purkinje cell is reduces in 5-HT_{3A} receptor knockout mice at P8, but not at P21. D - F, Paired-pulse recordings from climbing fibre - Purkinje cell synapses at P8 in wildtype mice (Da) and in 5-HT_{3A} receptor knockout mice (Db) and at P24 in wildtype mice (Ea) and in 5-HT_{3A} receptor knockout mice (Eb). F, The paired-pulse ratio at the climbing fibre - Purkinje cell synapse is increased in 5-HT_{3A} receptor knockout mice at P8 - P9 and at P11 - P12, but there is no difference at P24. The numbers in the bars of the graphs indicate the number of cells used for analysis.
trical stimulations in the internal granule cell layer. A climbing fibre EPSC (figure 3.7 A) was defined as an EPSC which showed a step-wise change in amplitude in response to a gradual increase in stimulus intensity (figure 3.7 B), in combination with paired-pulse depression (as seen in figure 3.6 D - F). We found that the number of climbing fibres by which a Purkinje cell is innervated decreases over time in both wildtype and 5-HT$_{3A}$ receptor knockout mice (figure 3.7 C). At P5 and P6 there is an average of four climbing fibres per Purkinje cell in both wildtype and 5-HT$_{3A}$ receptor knockout mice. In wildtype mice, there is a transition stage between P7 and P12 during which the number of climbing fibres gradually decreases to just over one climbing fibre per Purkinje cell. This number is reduced to exactly one climbing fibre per Purkinje cell at P24 in all cells recorded. In 5-HT$_{3A}$ receptor knockout mice, this transitionary stage of climbing fibre elimination lasts longer, with still multiple climbing fibres innervating one Purkinje cell at P24 (p < 0.001, Mann-Whitney test).

In ten-week old (P68 - P72) 5-HT$_{3A}$ receptor knockout mice the number of climbing fibres innervating a single Purkinje cell was eliminated to 1.18 ± 0.10 (n = 17). Thus, climbing fibre elimination is delayed in 5-HT$_{3A}$ receptor knockout mice.

We additionally analysed the amplitudes of the smallest and the largest climbing fibre EPSC and the climbing fibre ratio. The amplitude of a single climbing fibre EPSC was calculated as the difference in amplitude between any EPSC and the previous recorded EPSCs. The climbing fibre ratio was defined as the amplitude of any given climbing fibre EPSC divided by the amplitude of the largest climbing fibre EPSC synapsing onto the same Purkinje cell. The number of climbing fibres (n) for which the climbing fibre ratio can be determined is n-1 for each Purkinje cell. Wildtype mice at P24 already had completed climbing fibre elimination, and therefore there is no climbing fibre ratio or amplitude of the minimum climbing fibre EPSC. The climbing fibre ratio remained higher for a prolonged time in 5-HT$_{3A}$ receptor knockout mice than in wildtype mice (figure 3.7 D). The minimum amplitude of the climbing fibre EPSC (figure 3.7 E) was significantly different at P5 but not at other ages. The maximum amplitude of the climbing fibre EPSC (figure 3.7 F) was different at young ages but not in older animals.

It thus seems in 5-HT$_{3A}$ receptor knockout mice there is not only expedited mor-
phological and physiological maturation of Purkinje cells, but that also the two glutamatergic inputs to the Purkinje cells are affected, as shown by impairment of both the parallel fibre and the climbing fibre input, and delayed climbing fibre elimination.

Figure 3.7: Climbing fibre elimination is delayed in 5-HT$_{3A}$ receptor knockout mice.
A, Example of a recording from a Purkinje cell with inputs from four different climbing fibres. B, Input-output graph of the same recording as in A, which shows a stepwise increase in amplitude of the climbing fibre EPSC upon a gradual increase in stimulus strength, indicating four climbing fibres innervating the recorded Purkinje cell. C, Number of climbing fibres in wildtype and 5-HT$_{3A}$ receptor knockout mice between P5 and P24 shows a delay in climbing fibre elimination in 5-HT$_{3A}$ receptor knockout mice. D, The climbing fibre ratio, defined as the amplitude of one climbing fibre EPSC divided by the amplitude of the largest climbing fibre EPSC within the same Purkinje cell, is increased in 5-HT$_{3A}$ receptor knockout mice at P7 and P8. E, There is no difference in the amplitude of the smallest climbing fibre EPSC between wildtype and 5-HT$_{3A}$ receptor knockout mice at P6 - P12. Only at P5 the amplitude of the smallest climbing fibre EPSC is smaller in 5-HT$_{3A}$ receptor knockout mice. At P24 there was only one climbing fibre innervating each Purkinje cell in wildtype mice, which we counted as the climbing fibre with the largest climbing fibre EPSC and thus there is no value for the amplitude of the smallest climbing fibre EPSC in P24 wildtype mice. F, The amplitude of the largest climbing fibre EPSC is decreased at P5, P7 and P8 in 5-HT$_{3A}$ receptor knockout mice, accounting for the increase in climbing fibre ratio as shown in C.
3.4 **Discussion**

The results in this study show a novel role for serotonin in the regulation of cerebellar postnatal development. This is mediated via 5-HT₃ receptors expressed by excitatory granule cells, which regulate the morphological and physiological maturation of Purkinje cells. The serotonergic modulation via 5-HT₃ receptors also affects the surrounding cerebellar cortical microcircuit as indicated by impaired short-term synaptic plasticity at the climbing fibre - Purkinje cell synapse and delayed climbing fibre elimination in 5-HT₃A receptor knockout mice.

3.4.1 **The role of 5-HT₃ receptors in spontaneous miniature events recorded from Purkinje cells**

Recently, we have shown that acute pharmacological block of presynaptic 5-HT₃ receptors leads to a reduction in frequency of mPSCs recorded from Purkinje cells from P9 wildtype mice (Oostland et al., 2011). In the present study we show that the frequency of mPSCs recorded from Purkinje cells from P7 5-HT₃A receptor knockout mice was increased compared to P7 wildtype mice. Thus, recordings from Purkinje cells in wildtype mice during pharmacological blockade of 5-HT₃ receptors for a short amount of time and recordings from Purkinje cells from 5-HT₃A receptor knockout mice give contradictory results. The increase in mPSC frequency reflects changes in both the parallel fibre - Purkinje cell synapse and the climbing fibre - Purkinje cell synapse. We have shown before that the parallel fibre - Purkinje cell synapse is impaired and turns into a depressing synapse after pharmacological blockade of 5-HT₃ receptors (Oostland et al., 2011). In the present study, we show a similar effect on the parallel fibre - Purkinje cell synapse in 5-HT₃A receptor knockout mice. Morphological and electrophysiological data demonstrate that the development of parallel fibre synapses is significantly correlated with the time course of the climbing fibre elimination (Scelfo and Strata, 2005). Given that a) the increase in mPSC frequency in 5-HT₃A receptor knockout mice may reflect a change in both parallel fibre and climbing fibre input, b) parallel fibre input is impaired in 5-HT₃A receptor knockout mice, and c)
parallel fibre input is necessary for climbing fibre elimination because of the competitive nature of this process, we hypothesized that the increase in mPSC frequency recorded from Purkinje cells from 5-HT$_{3A}$ receptor knockout mice results from an increase in climbing fibre input due to delayed climbing fibre elimination. This can explain the contradictory results described above, as pharmacologically blocking 5-HT$_3$ receptors for a short amount of time during recording does not affect climbing fibre elimination but only affects functioning of the parallel fibre - Purkinje cell synapse, leading to a decrease in mPSC frequency recorded from Purkinje cells (Oostland et al., 2011). Alternatively, an increase in parallel fibre - Purkinje cell synapse formation in 5-HT$_{3A}$ receptor knockout mice could account for the increase in mPSC frequency, although this would be accompanied by an increase in climbing fibre elimination, opposite to what we have observed.

### 3.4.2 Mechanisms of delayed climbing fibre elimination in 5-HT$_{3A}$ receptor knockout mice

In this study we show that climbing fibre elimination is delayed in 5-HT$_{3A}$ receptor knockout mice until at least three weeks after birth, and that this process is normalized between P24 and ten weeks postnatally. The course of climbing fibre elimination in the wildtype mice we have found in the present study is comparable to what has been described before (reviewed by Watanabe et al., 2011). Climbing fibre elimination is a developmental process which occurs in two stages. The early stage is characterized by homosynaptic competition between climbing fibres, and lasts up to P8 in rats (Crepel, 1981). The late phase consists of heterosynaptic competition between parallel fibres and climbing fibres, and typically lasts between P9 and P17 in rats (Crepel, 1981). In the present study, we find no difference in the number of climbing fibres innervating one Purkinje cell between wildtype and 5-HT$_{3A}$ receptor knockout mice at P5 and P6. Only from P7 onwards, climbing fibre elimination starts to be delayed in the 5-HT$_{3A}$ receptor knockout mice, resulting in multiple climbing fibre innervation in three-week old 5-HT$_{3A}$ receptor knockout mice. As our study is done in mice, whose developmental pattern is up to two days earlier than in rats, this
period of delayed climbing fibre is in concordance with the late phase as described by Crepel (1981) in rats. Thus, the delayed climbing fibre elimination we find in the 5-HT$_{3A}$ receptor knockout mice occurs during the period of heterosynaptic competition between parallel fibres and climbing fibres. This supports our hypothesis that presynaptic 5-HT$_3$ receptors at the parallel fibre terminal affect the climbing fibre elimination. However, we cannot exclude that a 5-HT$_3$ receptor knockout-unrelated change in climbing fibre elimination is involved.

Parallel fibre inputs to Purkinje cells can activate the mGluR1 receptor, which via the G$\alpha$q (Offermans et al., 1997), PLC$_4$ (Kano et al., 1998) and PKC$\gamma$ (Kano et al., 1995) signaling cascade in Purkinje cells plays a central role in elimination of surplus climbing fibre synapses, through still unknown mechanisms (Kano et al., 2008). The effects of an increased number of climbing fibres during early postnatal development on behaviour is unclear. It is known that persistent polyinnervation of Purkinje cells by climbing fibres leads to problems in motor learning in (young) adult mice (i.e. Kimpo et al., 2007). Other mouse models with delayed but not completely impaired climbing fibre elimination, such as the $\alpha$CaMKII knockout mouse line, do show motor learning deficits in adult mice (Hansel et al., 2006). In these $\alpha$CaMKII knockout mice, 49% of Purkinje cells were innervated by two climbing fibres at P21 - P28, while climbing fibre elimination was fully completed in adult mutants. However, these motor learning deficits can most likely be explained by a specific impairment of LTD induction in Purkinje cells. As 5-HT$_3$ receptors in the cerebellum are not expressed after three weeks postnatally we do not expect physiological or behavioural deficits in the 5-HT$_{3A}$ receptor knockout mice, and cerebellar-specific motor tests cannot be performed in mice younger than three weeks old.

### 3.4.3 Involvement of 5-HT$_3$ receptors in Purkinje cell development

Physiological and morphological postnatal development of Purkinje cells have been more closely examined in the past decade (reviewed by Kapfhammer, 2004). Purkinje cells from 5-HT$_{3A}$ receptor knockout mice have different electrophysiological properties than Purkinje cells in wildtype mice. They are able to fire action poten-
tials upon a depolarizing current injection of +175 pA at an earlier age and have a more depolarized resting membrane potential than in wildtype mice. Furthermore, input resistance is increased in 5-HT$_{3,A}$ receptor knockout mice. Data on the input resistance of Purkinje cells during postnatal development is inconsistent. McKay and Turner (2005) find an input resistance of Purkinje cells which decreases with age between P0 and P18, recorded in slices from rats. On the other hand, Fry (2006) finds in dissociated Purkinje cells from mice a decrease in input resistance from P5 to P7, followed by an increase between P11 and P18.

In addition to the role in electrophysiological maturation we show that 5-HT$_3$ receptors regulate dendritic maturation of Purkinje cells via the glycoprotein reelin, and we further investigated this by looking at the stage of morphological maturation between P7 and P9 in more detail. We defined four different stages of Purkinje cell dendritic development (see also Altman, 1972a) and the results from this study show that in 5-HT$_{3,A}$ receptor knockout mice Purkinje cells have a more mature morphology at an earlier age than Purkinje cells in wildtype mice, indicating advanced morphological maturation. However, we were unable to detect any morphological differences between Purkinje cells in wildtype and 5-HT$_{3,A}$ receptor knockout mice at five weeks old, showing that the dendritic morphology was normalized by that age. Thus, the serotonergic system, modulated by 5-HT$_3$ receptors and reelin, controls the time course of maturation in Purkinje cells.

In this study we have used 5-HT$_{3,A}$ receptor knockout mice, in which transgenic modifications are not restricted to one particular cell type or time window. The effects we have shown in this study could be affected by compensatory mechanisms. However, to the best of our knowledge the granule cell is the only cell type in the cerebellum to express 5-HT$_3$ receptors (Oostland et al., 2011). In order to investigate the morphology of Purkinje cells in a more time-specific manner, we used organotypic slice cultures in which we blocked 5-HT$_3$ receptors or reelin at P8 during one or two days. Using this time-specific method we found similar effects as in 5-HT$_{3,A}$ receptor knockout mice at P9.
3.4.4 5-HT\textsubscript{3} receptor-dependent regulation of dendritic morphology in cerebral cortex and cerebellum

Our group has shown before that 5-HT\textsubscript{3} receptors affect morphology of layer II/III pyramidal neurons in the somatosensory cortex both during early postnatal development and at P90 (Chameau et al., 2009). Furthermore, in P14 - P21 mice, pyramidal neurons with a more complex dendritic tree were found to have different electrophysiological properties, i.e. reduced spike frequency adaptation (van der Velden et al., 2012). Thus, the serotonergic system has a sustained effect on morphological properties of pyramidal neurons in the somatosensory cortex, while in the cerebellum the serotonergic system more specifically affects the time course of both morphological and physiological maturation. Interestingly, in both brain areas the 5-HT\textsubscript{3} receptors on glutamatergic cells are only present during the first three weeks postnatally, and in both cases the dendritic maturation is regulated via reelin (Chameau et al., 2009; Oostland et al., 2011). However, it is only in the cerebellum that the 5-HT\textsubscript{3} receptor-dependent regulation of dendritic morphology is limited to the early postnatal stages of development.

3.4.5 Concluding remarks

The role of the cerebellum has in recent years been extended from motor coordination and motor learning to involvement in neurodevelopmental disorders such as autism and schizophrenia. This cognitive function of the cerebellum is mediated by a.o. reelin and serotonin. In the cerebellum, decreased levels of reelin mRNA and protein have been found in brains from patients with autism (reviewed in Fatemi et al., 2005). Alterations in expression and distribution of serotonin receptors in the cerebellum have been implicated in schizophrenia (Slater et al., 1998; Eastwood et al., 2001). In order to gain a better understanding on the development of autism and schizophrenia and the role of the cerebellum therein, it is of importance to understand the role of reelin and serotonin in postnatal development of the cerebellum and its projections to the cerebral cortex. Efferent projections from Purkinje cells to
the cerebellar and vestibular nuclei occur during late embryogenesis and synaptic contacts are established around birth (Eisenman et al., 1991). The exact timing of the development of projections from the cerebellar nuclei to the thalamus is not known, but it is likely that these connections are made before P21 as it is during this neonatal and juvenile period that systematic changes take place and the cerebellar network is formed (Altman and Bayer, 1996). Thus, the involvement of serotonin and reelin via the transiently expressed 5-HT$_3$ receptors in postnatal Purkinje cell development during this critical period, even though we show in the present study normalization of climbing fibre input in young adult 5-HT$_{3A}$ receptor knockout mice, could be functionally relevant in neurodevelopmental disorders by changing Purkinje cell output. Future studies focussed on the development of efferent pathways from Purkinje cells to the cerebral cortex and the role of serotonin therein could shed more light on this topic.

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