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ORIGINAL PAPER

Chronic mild stress modulates 5-HT1A and 5-HT2A receptor expression in the cerebellar cortex of NC/Nga atopic-like mice

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Abstract Atopic eczema symptoms may worsen due to stress. In the present study, the cerebellar cortex of the atopic-like mouse NC/Nga was studied regarding the effect of chronic mild stress on expression of two well-characterized serotonergic receptors (R), 5-HT1A and 5-HT2A. In total 24 mice were used. Sixteen of these mice were subjected to unpredictable stressors for 12 weeks, and 8 mice were used as controls. In order to evoke an eczema, a mite antigen was applied to 16 mice from week 9 of the experiment. Thus, three groups of mice, stressed eczematous (SE), non-stressed eczematous (NSE) and stressed control (SC), respectively, were obtained. The expression of the 5-HT1AR was analyzed using quantitative immunohistochemistry. For evaluation of 5-HT2AR a semi-quantitative technique was used, the cell density and signal intensity being measured. The highest average value for 5-HT1AR expression, in the Purkinje cells, was recorded in the NSE group, while the lowest average was in the SC group. 5-HT1AR expression differed significantly between the groups. The highest average value for density of 5-HT2AR positive Purkinje cells was evident in the SE group, while the lowest was in the SC group, this difference between groups also being statistically significant. In addition, the signal intensity was highest in the SE group, with a difference compared to the other groups. In conclusion, chronic mild stress modulates serotonergic receptor expressions in the cerebellar cortex of atopic-like mice.

Keywords Atopic eczema · Cerebellum · Mouse · Purkinje cells · Serotonin receptors · Stress

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Introduction

Atopic eczema is an often severely itching disease. Scratching is of utmost importance for the genesis of the skin lesions, and coordination and motor functions may be involved in this behavior. Atopic eczema may be worsened by stress, and there is a vicious circle involving stress, scratching, and change of motor functions. A common animal model when studying pathological mechanisms in atopic eczema is the NC/Nga mouse, which acquires both macroscopic and microscopic features that mimic atopic eczema in humans [23]. This mouse strain, being well-characterized, has been previously used by our group to study the effect of chronic mild stress in this disease [21, 31].

There is a bilateral contact between the central neuroendocrine system and other organs including the skin [28, 29, 36]. In the neuroendocrine system the cerebellum has been mostly associated with coordination and motor functions. However, there are also reports of cognitive functions for this brain structure [13, 14, 22]. The cerebellum is particularly activated by combining motor and non-motor (sensory) tasks (e.g., active tactile exploration) [30], and is one of the brain areas that is activated during itching [11] and scratching [35].

The Purkinje cells are the only output neurons in the cerebellar cortex. They are key cells with important roles in

sensory-motor calibration [25]. These cells may be activated during the scratch reflex in the cat, the mossy fibers being responsible for conducting spikes from the Purkinje cells [3].

Profound changes in cerebellar neuromediator expression, including serotonergic changes, due to chronic stress have been reported [13]. In addition, one study [19] reported an increased functional connectivity between the cerebellum and the temporal poles in depression, also implying a substantial role for the cerebellum in the pathophysiology of depression.

Recent studies have indicated a connection between chronic inflammation and cerebellum, adding to the complex interaction between the neuroendocrine and the immune systems [6, 15, 32].

Serotonin (5-hydroxytryptamine; 5-HT) is an important mediator in the contact between the neuroendocrine system and the skin. The serotonergic system also has a role in inflammation, such as in atopic eczema [20, 29]. 5-HT exerts its effects via at least 21 subgroups of receptors (R) [5], of which the most characterized are the 5-HT1A and 5-HT2A [4]. Of these receptors 5-HT1AR is inhibitory while 5-HT2AR being excitatory in the nervous system [4]. Anti-inflammatory effects, in a contact allergy model, have been reported for an agonist of the 5-HT1AR [24] and an antagonist for 5-HT2AR [2]. Expression of several of the 5-HT receptors has been previously reported in the cerebellum [9, 26, 34].

We have earlier demonstrated a modulation of serotonergic receptors 1A and 2A in the hippocampus of atopic-like NC/Nga mice during chronic mild stress [31]. The aim of the present study was to extend these findings by investigating whether there was a modulation of expression of the serotonin 1A and 2A receptors in the cerebellum of mildly stressed NC/Nga mice, this mild stress which might be able to affect the scratching involved in atopic eczema. We focused on the Purkinje cell layer at the border between the molecular and granular layers in the cerebellar cortex, whose cells have earlier been reported to express the 5-HT1AR [26]. Should the balance between excitatory and inhibitory 5-HT receptors on these cells be altered, as found in the hippocampus [31], this might have marked impact on the firing of Purkinje cells.

Materials and methods

Animals

Twenty-four 6-week-old female NC/Nga mice were purchased from Charles River Laboratories, Germany. The mice were allowed to acclimatise for 1 week prior to the experiments, which were approved by the local Animal Ethics Committee.

Chronic stress and immunization

The chronic mild stress procedure was initially described by Lanfumey et al. [17] and has been used in our previous studies [10, 21, 31]. Briefly, mice that were subjected to stress were retained in a Scantainer box type 50-SCNT-Z11 (Scanbur AS, Köge, Denmark) in a conventional animal facility, fed with pellets (R70, Granngården, Malmö, Sweden) and tap water, while mice not exposed to stress were kept outside the box. Different stressors were used on a weekly basis, such as reversed light/dark cycle, one period of confinement to small cages for 12 h, two periods of cohabitation with foreign mice for 2 h, one period of continuous overnight illumination, one overnight period of wet soil, one period for 12 h of cage-tilting (30°) and one period (3 h), of food and water deprivation.

Mice were divided into 3 groups (8 mice per group), one group being stressed and sensitized (*stressed eczematous*, SE). The mice in this group were subjected to chronic mild stress for 12 weeks and eczema was induced from week 9 of the experiment through painting their ears with a mite antigen derived from Dermatophagoides pteronyssinus (Allergon, Ängelholm, Sweden) (10 mg/ml in phosphate buffered saline (PBS) and 0.5 % Tween 20). A non-sensitized control group was similarly stressed (*stressed control*, SC), and had their ears painted using the solvent. A second sensitized group was relieved from stress (*non-stressed eczematous,* NSE), being housed in a regular cage and the mice in this group were also exposed to the mite antigen from week 9. In contrast to the other groups, these non-stressed mice were maintained on a 12-h light/dark cycle under controlled temperatures between 18 and 22 °C and a humidity of 40–60 %.

Processing of cerebella

After 12 weeks the animals were sacrificed by cervical dislocation and the ear thickness was immediately measured using a spring-loaded micrometer (Kroeplin, Schluchtern, Germany), just before the cerebella together with the cerebra were dissected and preserved for further analysis. Plasma was immediately prepared from whole blood collected from the severed neck and frozen. Corticosterone levels were later measured using a corticosterone RIA kit (RS 490 11) from IBL (Hamburg, Germany) following the instructions of the manufacturer. There was no lag time between the stress protocol and terminating the mice.

The diameter of the ears was larger in the SE group 0.49 ± 0.11 mm (mean ± SD), compared to the NSE group 0.36 ± 0.13 ($p < 0.01$) and the SC group 0.23 ± 0.00 ($p < 0.001$).

The SE group had lower ($p < 0.05$) corticosterone values 496.4 ± 221.5 ng/ml compared to the other groups (NSE 738.6 ± 232.1; SC 805.2 ± 144.4 ng/ml).

The cerebella were subjected to immunohistochemistry to analyze changes in expression of serotonergic receptors. The samples were fixed in 4 % formalin with 0.2 % picric acid for 2 h at 4 °C. They were then rinsed with 0.1 mol/L phosphate buffer containing 10 % sucrose for at least 24 h. The specimens were embedded in Tissue-Tek (Sakura Finetek, Zoeterwoude, The Netherlands) and 14 μm thick sagittal sections (between 2.1 and 2.6 mm away from the midline) were processed using a Microm cryostat (Heidelberg, Germany). Sections were finally mounted on Super Frost Plus glass slides (Menzel-Gläser, Freiburg, Germany) and stored at −70 °C until staining.

Immunohistochemistry

A streptavidin–biotin technique was used with primary rabbit polyclonal antibodies against 5-HT2AR (24288; 1:300; ImmunoStar, Hudson, WI, USA) and a guinea pig antibody against 5-HT1AR (AB5406; 1:7,500; Chemicon, Temecula, CA, USA). Primary antibodies were incubated at 4 °C and overnight. Slides were then incubated with secondary biotinylated anti-rabbit (BA-1000) or anti-guinea pig (BA-7000) antibodies (1:200; Vector, Burlingame, CA, USA), respectively, for 40 min at room temperature, and finally the fluorochrome Cy2-labeled streptavidine (PA42001; 1:2,000; Amersham Pharmacia Biotech, Uppsala, Sweden), was added for 40 min at room temperature to enable visualization of staining.

To perform double staining designed to confirm that the serotonin receptor expressing cells in the cerebellar cortex were Purkinje cells, the biopsy sections were first incubated with the 5-HT1A and 5-HT2AR antibodies, followed by the secondary biotinylated antibodies and then streptavidin conjugate, Alexa Fluor 488 (1:1,000; S-32354; Life Technologies, Paisley, UK). The next day the sections were incubated with rabbit anti-calbindin D28k polyclonal antibody (214,002; 1:400; Synaptic Systems, Göttingen, Germany) (for 5-HT1AR) or guinea pig anti-calbindin D28k polyclonal antibody (214 004; 1:400; Synaptic Systems) (for 5-HT2AR), followed by Alexa Fluor 555 donkey anti-rabbit (1:1,000; A-31572) or goat anti-guinea pig, Alexa Fluor 568 (1:1,000) (both from Life Technologies), respectively.

As controls the primary antibodies were omitted or normal rabbit serum IgG (X 936, Dako, Glostrup, Denmark), or guinea pig serum IgG (006-000-003; Jackson ImmunoResearch, West Grove, PA, USA) were used at the same dilutions as the primary antibodies. This resulted in substantially decreased or abolished signals.

Finally, the sections were mounted with Kaiser's glycerol gelatine (Merck, Darmstadt, Germany) before being covered with glass slips.

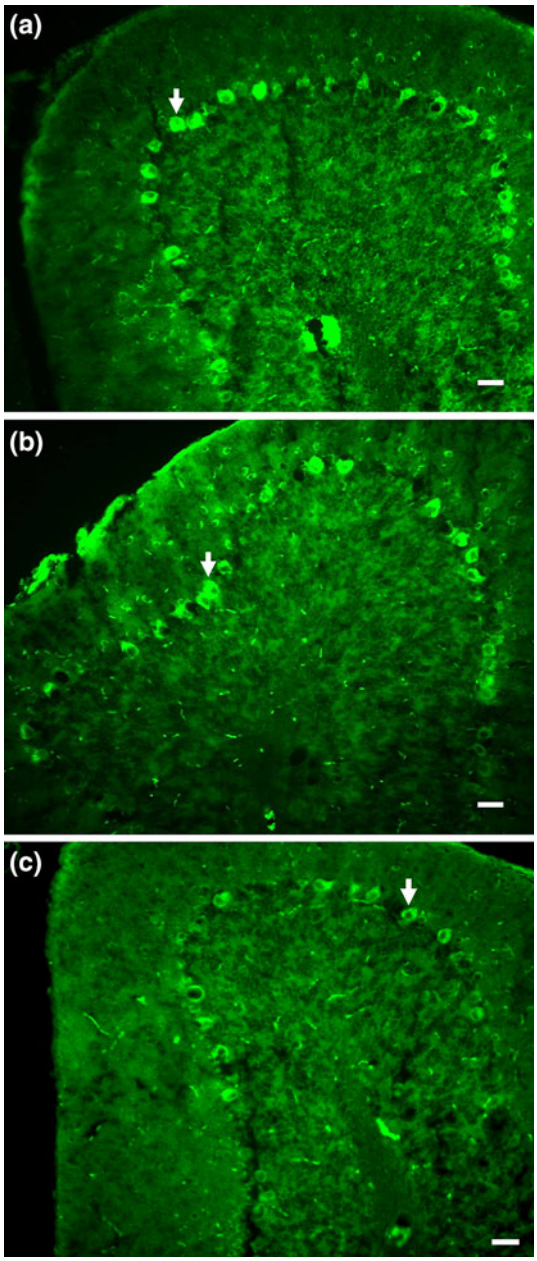

Fig. 1 Expression of 5-HT1AR in cerebellar cortex of NSE (**a**), SE (**b**) and SC (**c**) mice. *Arrows* indicate positive cells. *Scale bars* 20 μm

Microscopy

For evaluation of 5-HT1AR, an image analysis technique was used with an appropriate software (Easy Image Analysis, Bergström Instruments, Solna, Sweden). Labeled sections were examined using a fluorescence Zeiss Axioskop 2 MOT microscope (Stockholm, Sweden) at a magnification of 200×. Images of 4–6 sections were captured by a digital camera connected to a PC before being analyzed using the software. The area fraction (ratio of specifically immunoreactive area to total cortical area) in

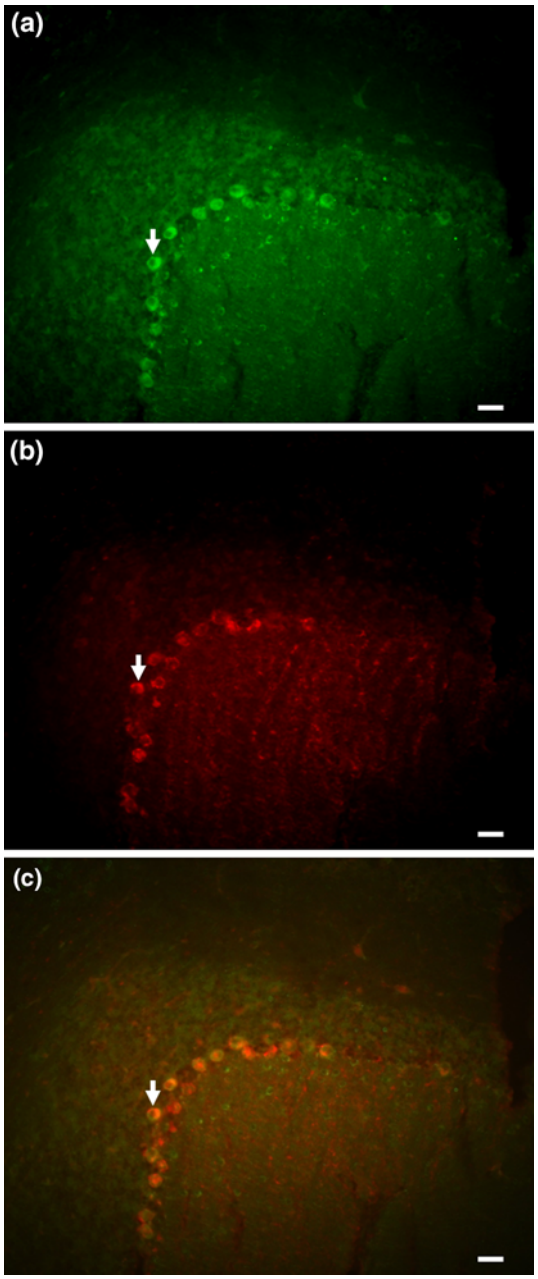

Fig. 2 Double staining showing co-localization of 5-HT1AR with calbindin. *Arrows* indicate a double-stained cell. 5-HT1AR (*green*, Alexa Fluor 488) (**a**), calbindin (*red*, Alexa Fluor 555) (**b**), 5-HT1AR+calbindin (**c**). *Scale bars* 20 μm

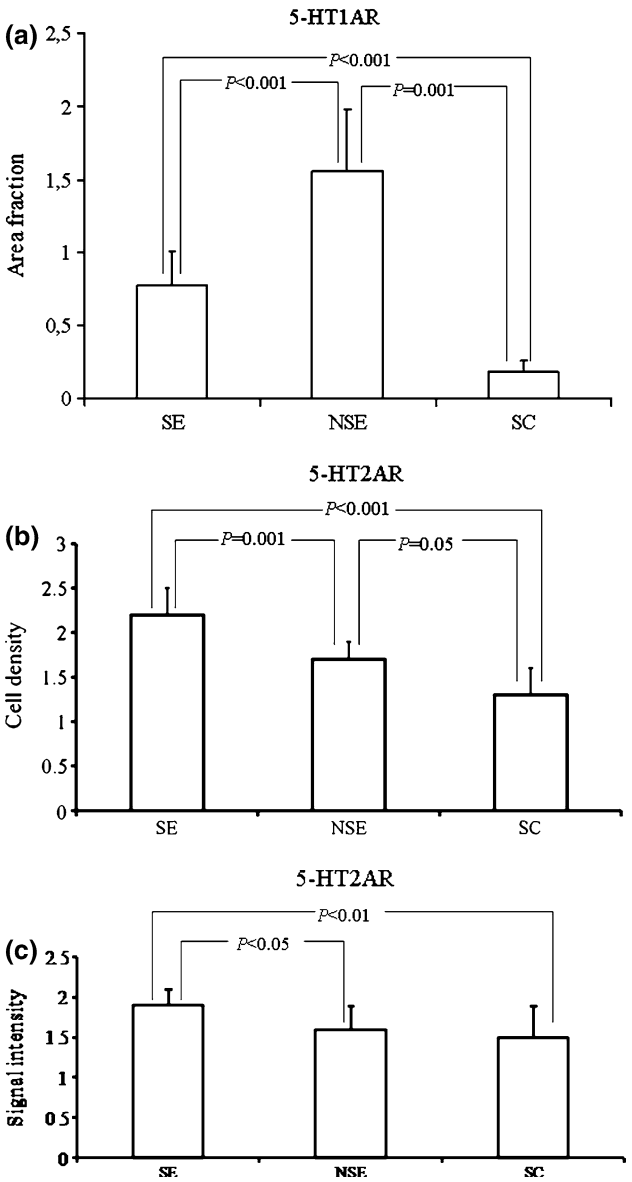

Fig. 3 *Graphs* depicting area fraction, in percent, for 5-HT1AR (**a**), and cell density (**b**) and signal intensity (**c**) for 5-HT2AR, from the different mice groups

percent was calculated. The threshold for immunoreactivity was set automatically by the program, and the areas outline in this way corresponded well to the subjective outline by the microscopic investigator (AR).

For 5-HT2AR expression a semi-quantitative technique was used, focusing on the density of immunoreactive Purkinje cells, being graded per visual field as: absent = 0, low = 1, medium = 2, and high = 3, and the intensity of the staining signal: none = 0, low = 1, moderate = 2, and high = 3. Slides were coded prior to analysis to permit blind evaluation by the investigator (AR). Also here, 4–6 microscopic fields per section were selected and used for quantification.

Statistical analysis

Multiple comparisons of continuous data were performed by analysis of variance, ANOVA. In the case of a statistically significant result in the ANOVA, comparisons between two arbitrary groups were made using the post hoc test proposed by Fisher to control for multiplicity [7, 27]. A p value of <0.05 was regarded as being statistically significant.

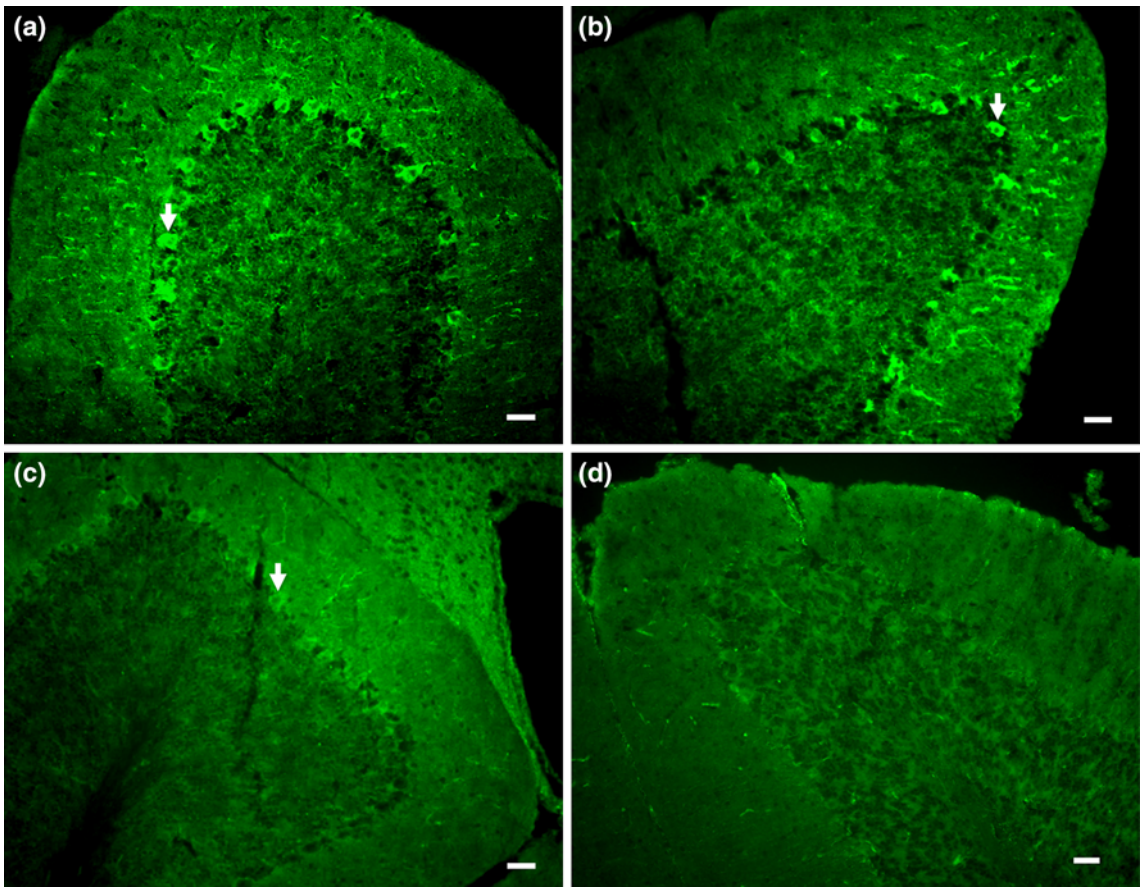

Fig. 4 Expression of 5-HT2AR in cerebellar cortex of SE (**a**), NSE (**b**) and SC (**c**) mice. *Arrows* indicate positive cells. Control without primary antibody, SE (**d**). *Scale bars* 20 μm

Results

5-HT1AR

Expression of 5-HT1AR was recorded on the Purkinje cells and occasionally also on some small interneuronal cells in the molecular layer (Figs. 1, 2). There was a difference ($p < 0.001$) in the 5-HT1AR area fraction between the SE 0.77 ± 0.24 % (mean ± SD) and NSE 1.56 ± 0.42 %, groups (Fig. 3). The lowest value was evident in the SC group 0.18 ± 0.08 %, also being significantly different ($p < 0.001$) from the other groups.

5-HT2AR

The 5-HT2AR was expressed in the Purkinje cells and sometimes in nerve fibers extending far out into the molecular layer (Figs. 4, 5). The density of the immunoreactive cells was highest in the SE 2.2 ± 0.3, compared to the other groups NSE 1.7 ± 0.2 ($p = 0.001$) and SC 1.3 ± 0.3 ($p < 0.001$), and there was also a difference ($p = 0.05$) between NSE and SC (Fig. 3).

The intensity of the fluorescence signal was also highest in the SE group 1.9 ± 0.2 compared to the NSE 1.6 ± 0.3 ($p < 0.05$) and SC 1.5 ± 0.4 ($p < 0.01$) groups.

Discussion

In the present study, chronic mild stress resulted in a reduced 5-HT1AR expression in SE compared to NSE mice. While inflammation *per se* leads to increased expression of this receptor, stress *per se* reduces expression. This latter effect might be due to a decreased synthesis of 5-HT1AR, but there are also other possibilities such as an internalization of the receptor.

Of course induction of inflammation *per se* by induction of eczema may be stressful for the animals. It has been reported that chronic inflammation may result in depression and changes in the hypothalamic–pituitary–adrenal (HPA) axis in humans [18, 36]. However, in the present study we are discussing the consequences of 'stress' as an external environmental factor.

The 5-HT1AR may be involved in the serotonergic control of growth of 5-HT fibers and/or the modulation of

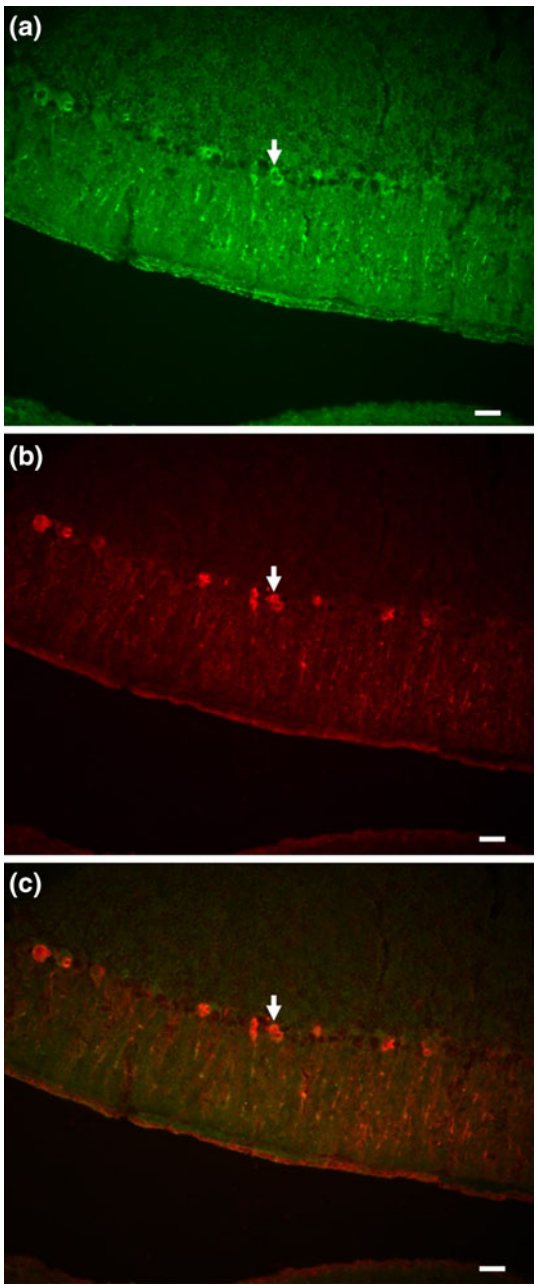

Fig. 5 Double staining showing co-localization of 5-HT2AR with calbindin. *Arrows* indicate a double-stained cell. 5-HT2AR (*green*, Alexa Fluor 488) (**a**), calbindin (*red*, Alexa Fluor 568) (**b**), 5-HT2AR + calbindin (**c**). *Scale bars* 20 μm

Purkinje cell activity [26, 33]. Functionally, 5-HT is able to modify the firing of these cells in the short term by changing the firing pattern from regular to burst firing [33]. This might have an impact on the motor pattern, including scratching behavior. In the long-term perspective, 5-HT may regulate dendrite formation in these cells via 5-HT1AR (stimulatory) and 5-HT2AR (inhibitory) [16].

The highest expression of 5-HT2AR was determined in the SE group, with the lowest in the SC group, indicating a role for the receptor both in inflammation and in chronic stress. These results also concord with our previous results from the hippocampal CA1 area [31]. In addition, our results are consistent with a recent study reporting an increase in 5-HT2AR in the cerebellum in a model of chronic stress in goldfish [12].

The functional impact of this modulation of the cerebellar serotonergic receptors during chronic mild stress remains to be elucidated. The Purkinje cells are critical to the output of the cerebellum to other brain areas [25]. In the Purkinje cell layer an expression of heat shock protein 70 was reported in an animal model of sustained muscular contraction [1], suggesting an involvement of these cells in muscle tension. Atopic dermatitis patients may exhibit increased muscular tension (see Ref. [8]), which may vary depending on the degree of anxiety/chronic stress, and here we might be dealing with a possible role for the cerebellum and its Purkinje cells.

Chronic skin inflammation, as in atopic eczema, may thus affect the function of the cerebellum through the serotonergic system. At least two mechanisms are conceivable: a chemical effect of blood carried inflammatory mediators on the cerebellum; or an effect of a changed movement pattern. Johansson et al. [15] demonstrated that skin or brain inflammation may have an impact on the cerebellum, illustrated by increased fusion between Purkinje cells in the cerebellum and transplanted bone marrow-derived stem cells.

In our study female mice were used. We cannot rule out the possibility of a hormonal impact on our results during the estrous cycle. It might also have been interesting to study male mice.

In conclusion, chronic mild stress modulates serotonin receptor 1A and 2A expression in the cerebellar cortex of atopic-like mice. Further studies of the connection between the cerebellum and the skin during inflammation, such as itching/scratching in atopic eczema, are warranted.

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References

1. Alvarez-Fischer D, Grundmann M, Lu L et al (2012) Prolonged generalized dystonia after chronic cerebellar application of kainic acid. Brain Res 1464:82–88
2. Ameisen JC, Meade R, Askenase PW (1989) A new interpretation of the involvement of serotonin in delayed-type hypersensitivity. Serotonin-2 receptor antagonists inhibit contact sensitivity by an effect on T cells. J Immunol 142:3171–3179
3. Arshavsky Yul, Orlovsky GN, Popova LB (1984) Activity of cerebellar Purkinje cells during fictitious scratch reflex in the cat. Brain Res 290:33–41

4. Azmitia EC (2001) Modern views on an ancient chemical: serotonin effects on cell proliferation, maturation, and apoptosis. Brain Res Bull 56:413–424
5. Bockaert J, Claeysen S, Bécamel C, Dumuis A, Marin P (2006) Neuronal 5-HT metabotropic receptors: fine-tuning of their structure, signaling, and roles in synaptic modulation. Cell Tissue Res 326:553–572
6. Bossù P, Cutuli D, Palladino I et al (2012) A single intraperitoneal injection of endotoxin in rats induces long-lasting modifications in behavior and brain protein levels of TNF-alpha and IL-18. J Neuroinflammation 9:101
7. Daniel WW (1995) Biostatistics: a foundation for analysis in the health sciences, 6th edn. Wiley, New York
8. De Mel S, Nordlind K, Holst M, Frohm-Nilsson M, Lonne-Rahm SB (2012) Polymorphisms in the serotonin transporter gene of patients with atopic dermatitis-association with personality traits related to high level of anxiety. Immunopharmacol Immunotoxicol 34:534–538
9. Geurts FJ, De Schutter E, Timmermans JP (2002) Localization of 5-HT2A, 5-HT3, 5-HT5A and 5-HT7 receptor-like immunoreactivity in the rat cerebellum. J Chem Neuroanat 24:65–74
10. Grip L, Lonne-Rahm SB, Holst M et al (2013) Substance P alterations in skin and brain of chronically stressed atopic-like mice. J Eur Acad Dermatol Venereol 27:199–205
11. Herde L, Forster C, Strupf M, Handwerker HO (2007) Itch induced by a novel method leads to limbic deactivations—a functional MRI study. J Neurophysiol 98:2347–2356
12. Hu X, Li Y, Hu Z et al (2012) The alteration of 5-HT2A and 5-HT2C receptors is involved in neuronal apoptosis of goldfish cerebellum following traumatic experience. Neurochem Int 61:207–218
13. Ito M (2002) Historical review of the significance of the cerebellum and the role of Purkinje cells in motor learning. Ann N Y Acad Sci 978:273–288
14. Ito M (2009) Functional roles of neuropeptides in cerebellar circuits. Neuroscience 162:666–672
15. Johansson C, Youssef S, Koleckar K et al (2008) Extensive fusion of haematopoietic cells with Purkinje neurons in response to chronic inflammation. Nat Cell Biol 10:575–583
16. Kondoh M, Shiga T, Okado N (2004) Regulation of dendrite formation of Purkinje cells by serotonin through serotonin1A and serotonin2A receptors in culture. Neurosci Res 48:101–109
17. Lanfumey L, Pardon M-C, Laaris N et al (1999) 5-HT1A autoreceptor desensitization by chronic ultramild stress in mice. NeuroReport 10:3369–3374
18. Lanfumey L, Mongeau R, Cohen-Salmon C, Hamon M (2008) Corticosteroid-serotonin interactions in the neurobiological mechanisms of stress-related disorders. Neurosci Biobehav Rev 32:1174–1184
19. Liu L, Zeng LL, Li Y et al (2012) Altered cerebellar functional connectivity with intrinsic connectivity network in adults with major depressive disorders. PLoS ONE 7:e39516
20. Lonne-Rahm SB, Rickberg H, El-Nour H, Mårin P, Azmitia EC, Nordlind K (2008) Neuroimmune mechanisms in patients with atopic dermatitis during chronic stress. J Eur Acad Dermatol Venereol 22:11–18
21. Lönndahl L, Lonne-Rahm SB, Nordlind K, Theodorsson E, El-Nour H (2010) Decreased innervation of eczematous skin in NC/Nga atopic mice during chronic mild stress. Immunopharmacol Immunotoxicol 32:147–152
22. Maeshima S, Osawa A (2007) Stroke rehabilitation in a patient with cerebellar cognitive affective syndrome. Brain Inj 21:877–883
23. Matsuda H, Watanabe N, Geba GP et al (1997) Development of atopic dermatitis-like skin lesion with IgE hyperproduction in NC/Nga mice. Int Immunol 9:461–466
24. McAloon MH, ChandraSekar A, Lin YJ, Hwang GC, Sharpe RJ (1995) Buspirone inhibits contact hypersensitivity in the mouse. Int Arch Allergy Immunol 107:437–438
25. Medina JF (2011) The multiple roles of Purkinje cells in sensorimotor calibration: to predict, teach and command. Curr Opin Neurobiol 21:616–622
26. Miquel MC, Kia HK, Boni C et al (1994) Postnatal development and localization of 5-HT1A receptor mRNA in rat forebrain and cerebellum. Brain Res Dev Brain Res 80:149–157
27. Montgomery DC (1991) Design and analysis of experiments, 3rd edn. Wiley, New York
28. Nalivaiko E, Sgoifo A (2009) Central 5-HT receptors in cardiovascular control during stress. Neurosci Biobehav Rev 33:95–106
29. Nordlind K, Azmitia EC, Slominski A (2008) The skin as a mirror of the soul: exploring the possible roles of serotonin. Exp Dermatol 17:301–311
30. Parsons LM, Bower JM, Gao J-H, Xiong J, Li J, Fox PT (1997) Lateral cerebellar hemispheres actively support sensory acquisition and discrimination rather than motor control. Learn Mem 4:49–62
31. Rasul A, El-Nour H, Blakely R et al (2011) Effect of chronic mild stress on serotonergic markers in the skin and brain of the NC/Nga atopic-like mouse strain. Arch Derm Res 303:625–633
32. Weberpals M, Hermes M, Hermann S et al (2009) NOS2 gene deficiency protects from sepsis-induced long-term cognitive deficits. J Neurosci 29:14177–14184
33. Williams SR, Christensen SR, Stuart GJ, Häusser M (2002) Membrane potential bistability is controlled by the hyperpolarization-activated current I(H) in rat cerebellar Purkinje neurons in vitro. J Physiol 539:469–483
34. Yew DT, Yeung L-Y, Wai MS-M, Mak YT (2009) 5-HT 1A and 2A receptor positive cells in the cerebella of mice and human and their decline during age. Micr Res Techn 72:684–689
35. Yosipovitch G, Papoiu AD (2008) What causes itch in atopic dermatitis? Curr Allergy Asthma Rep 8:306–311
36. Zachariae R (2009) Psychoneuroimmunology: a bio-psycho-social approach to health and disease. Scand J Psychol 50:645–651