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Prostaglandin E2 is an endogenous modulator of cerebellar development and complex behavior during a sensitive postnatal period

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Abstract

Prostaglandins are lipid-derived molecules that mediate the generation of fever in the central nervous system. In addition to their proinflammatory role, prostaglandins also impact neuronal development and synaptic plasticity, sometimes in a sex-specific manner. The cerebellum has a high expression of prostaglandin receptors during development, but the role that these molecules play during normal cerebellar maturation is unknown. We demonstrate here that disrupting prostaglandin synthesis with cyclo-oxygenase inhibitors during a time-sensitive window in early postnatal life alters cerebellar Purkinje cell development in rats, resulting in initially increased dendritic growth in both sexes. We show that this results in later cerebellar atrophy in males only, resulting in a sex-specific loss of cerebellar volume. Further, although performance in motor tasks is spared, social interaction and the sensory threshold are altered in males developmentally exposed to cyclo-oxygenase inhibitors. This work demonstrates a previously unknown role for prostaglandins in cerebellar development and emphasizes the role that the cerebellum plays outside motor tasks, in cognitive and sensory domains that may help to explain its connection to complex neurodevelopmental disorders such as autism.

Keywords

autism; cerebellum; cyclo-oxygenase; dendritic; rat

Introduction

Prostaglandins are membrane lipid-derived compounds that function as paracrine, endocrine, and even autocrine signaling molecules as part of a wide variety of physiological functions (Ali *et al.*, 2006). The first step in prostaglandin production is the conversion of arachidonic acid to the precursor prostaglandin H2 (PGH2) by the membrane-bound enzyme cyclo-oxygenase (COX) (Rouzer & Marnett, 2009). PGH2 is converted to active prostaglandins by

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Conflict of interest

The authors declare that they have no conflicts of interest and are aware of no individuals or entity that would benefit from the studies described herein.

other enzymes to produce up to eight distinct prostanoids. COX exists in at least two isoforms, COX-1 and COX-2, and is the rate-limiting enzyme in prostaglandin production (Hla & Neilson, 1992). COX is the target of non-steroidal anti-inflammatory drugs, such as aspirin, which function by inhibiting the activity of either or both isoforms to reduce fever and treat pain and inflammation (Helleberg, 1981).

Prostaglandin E2 (PGE2) is the key determinant of fever and rises precipitously and immediately in the hypothalamus upon infection to initiate the febrile response. Prostaglandins also mediate brain functions other than fever, and affect hippocampal long-term potentiation (Yamagata *et al.*, 1993) and learning (Shaw *et al.*, 2003). In addition to these functions in adult brain, prostaglandins play a fundamental role in the development of the male preoptic area, a critical brain region for the control of sexual behavior. The preoptic area of male rats has a higher density of dendritic spine synapses than that of females, and we previously determined that the male increase in dendritic spines is mediated by PGE2 under the regulation of the gonadal steroid estradiol during early postnatal development (Amateau & McCarthy, 2002, 2004; Todd *et al.*, 2005; Burks *et al.*, 2007; Wright *et al.*, 2008). Treatment of neonatal males with COX inhibitors had enduring effects on synaptic profiles and adult sexual behavior. To our knowledge, these were the first reports that exposure to COX inhibitors significantly alters normal brain development.

In addition to the preoptic area (POA), PGE2 receptors (EP1–4) are expressed in a variety of other regions in the developing brain, including the cerebellum (Kaufmann *et al.*, 1997; Tai *et al.*, 1998). The function of these receptors has not been elucidated and, based on our previous finding in the POA, we were compelled to examine what role the inflammatory prostaglandins might play in the normal early postnatal development of this brain region. The cerebellum is a late-developing structure relative to other brain regions and is sensitive to environmental perturbation, particularly in males (Nguon *et al.*, 2005). Although best known for its role in motor control, the cerebellum also integrates sensory information (Parsons *et al.*, 1997), and recent work suggests that it is important for cognitive tasks such as working memory (Bellebaum & Daum, 2007) and social interaction (Berntson & Schumacher, 1980; Tavano *et al.*, 2007). We show here that, during a time-sensitive window, disrupting prostaglandin synthesis in the cerebellum results in increased growth in the Purkinje cell dendritic tree, as well as alterations in cerebellar volume and animal behavior later in development.

Materials and methods

Animals and developmental manipulations

Sprague–Dawley rats that were mated in our facility were allowed to deliver normally under standard laboratory conditions. All manipulations were approved by the Institutional Animal Care and Use Committee of the University of Maryland, Baltimore. The specific number of animals of each sex used is noted with the results of each experiment. Animals were weaned on postnatal day (PN)21 and housed in groups of three of like treatment and sex.

Subcutaneous treatments

From PN7 to PN13, male and female pups were treated once per day with subcutaneous injections of sesame oil vehicle or one of three prostaglandin synthesis inhibitors: nimesulide, indomethacin, or acetaminophen. A range of nimesulide doses was used (0.2, 1.0, or 5.0 mg/kg by body weight at a concentration of 0.2, 1, or 5 mg/mL, respectively) as noted in the individual experiments. The middle of this range was chosen based on the dose of nimesulide of 1.25–2 mg/kg used in clinical pediatrics. The indomethacin dose chosen was 50 μ g/kg, slightly lower than the 100–200 μ g/kg dose of indomethacin used to treat

patent ductus arteriosus in neonates. Each rat received, on average, 20 μg of indomethacin, close to the 25 μg used previously to induce changes in spinophilin in the preoptic area (Amateau & McCarthy, 2002). Finally, 200 μg of acetaminophen in 0.1 mL of sesame oil provided an average of 40 mg/kg acetaminophen per dose, which is the rectal dose of acetaminophen used in settings such as pediatric emergency rooms and intensive care units. The over-the-counter pediatric dose of acetaminophen by mouth is smaller, 10–15 mg/kg with a maximum daily dose of 75 mg/kg.

Intracerebellar injections

After pups were lightly anesthetized with a low dose of 2.5 mg ketamine plus 0.1 mg acepromazine, a sharp beveled needle attached to a hamilton syringe was inserted 2 mm at the base of the skull into the foramen magnum and 1 μL of drug or vehicle was slowly infused. Pups received two intracerebellar infusions on PN3 and PN5 for studies during the first postnatal week, on PN10 and PN12 for the second postnatal week, or on PN17 and PN19 for the third postnatal week. Animals were monitored under a heat lamp until recovery from anesthesia (pups were mobile, vocalized, and urinated at least once) and then returned to the dam. For intracerebellar injections, pups received 100 μg nimesulide in 1% dimethylsulfoxide in normal saline, or 2.5 μg PGE2 in normal saline vehicle.

Golgi–Cox impregnation and analysis

Brains were collected on PN14 and impregnated for 30 days using an established method (Glaser & Van der Loos, 1981) with described modifications (Mong *et al.*, 1999). Brains were cut in 100- μm -thick sagittal sections. Neurons were traced using MBF Bioscience Neurolucida under a Nikon 100 $\times$ oil objective. Neurons entangled with one another were excluded. Neuron subtypes were identified by morphology and location. Five neurons were traced for each cell type and cerebellar region examined, and the average of the five was calculated for the individual animals that were treated as the subjects for statistical analyses. Purkinje cells were identified by their location between the granule and molecular layer, and their large soma size, complex dendritic trees with many dendritic spines arranged in a sagittal array extending into the molecular layer that initially sprout from one to two apical dendrites, and single axon. Granule cells were identified by their location within the inner granule layer, small soma size, possession of a long axon extending into the molecular layer and three to five short dendrites. Basket cells were identified by the location of their cell body in the inner one-third of the molecular layer and stellate dendritic morphology with freely branching, dilated dendrites with no or very few spines. Stellate cells were identified by their location in the outer two-thirds of the molecular layer, and their freely branching stellate dendritic morphology with no identifiable dendritic spines.

The analysis of dendrite length incorporated the complete dendrite from soma to distal ends. For dendritic spine counts, spines were operationally defined as protrusions off the dendritic tree of $< 5 \mu\text{m}$ in accordance with established criteria (Amateau & McCarthy, 2002; Amateau *et al.*, 2004). Spines were counted only if they were visualized entirely in perpendicular profile, and those that projected at an angle to the z-axis were not analysed. The absolute number of spines was therefore greater than those included in this analysis. For Sholl analysis, concentric circles of increasing radius centered on the cell body were superimposed over traced neurons, and the number of intersections with each circle was counted. The Schoenen ramification index was calculated by dividing the maximum number of intersections by the number of primary dendrites. The Sholl regression coefficient was calculated by comparing the log of the number of intersections per area of the circle with the circle radius and calculating the regression coefficient using least squares fit.

Golgi image figure preparation

Cells impregnated by the Golgi–Cox method were viewed under a Nikon 40× objective in order to allow visualization of the entire dendritic tree. Images were captured with a Coolsnap CF camera (Photometrics). Images were then imported into Photoshop and adjusted for contrast, brightness, and color balance. All adjustments were applied to whole images and never to parts, and were applied to allow greater ease of distinguishing the characteristics of the printed images. In order to save space, images were cropped to include only the neuron in question.

Western blot

The posterior vermis of the cerebellar cortex was obtained and the cortex was carefully dissected away from the deep nuclei. Tissue was prepared and western analysis was used to quantify the relative amounts of spinophilin in neonatal and juvenile tissue as described previously (Amateau & McCarthy, 2002). The Phototype Chemiluminescence System (New England Biolabs) was used to detect spinophilin protein recognized with antisera at a concentration of 1 : 1000 (polyclonal rabbit, Upstate). Blots were exposed on Hyper-film-ECL (Amersham) for varying durations (1–15 min). The protein appeared as a band with a relative molecular mass of 120 kDa and the grayscale integrative area density was captured with a charge-coupled device camera and quantified with National Institutes of Health IMAGE software. Blots were dyed with 0.1% Ponceau S in 5% acetic acid to visualize other proteins. The integrative area density of Ponceau bands was measured and spinophilin integrative area densities were normalized to these results to control for differences in total protein loading. Representative portions of the original western blots are shown in Figs 2 and 5.

Cerebellar culture conditions

Whole cerebella from PN0 mixed-sex litters of rat pups were processed for primary dispersed mixed neuronal/glial cultures as described previously (Heitz *et al.*, 2008). The litter size ranged from 8 to 16 pups, and yielded between 15 and 30 plates. On average, each PN0 pup yielded approximately two plates. Cerebella were pooled during culturing such that the cells on each plate would be expected to be derived randomly from all pups that were killed during the culture protocol rather than from specific individual pups. Plates were maintained at 37 °C and 5% CO₂ for 14 days *in vitro* (DIV) (time of plating, DIV0). Cells were treated once per day from DIV7 to DIV13 with 1.5 nM PGE2 or saline vehicle. Cells were collected in lysis buffer at DIV14 for western blot. Individual plates were treated as subjects for statistical analysis.

Open field

In order to assess motor activity levels on PN60, animals were placed in an open field divided into a 5 × 4 grid for 10 min and the distance traveled as indicated by the number of times that all four paws crossed a boundary between boxes on the grid was recorded.

Negative geotaxis

In order to assess motor coordination, on PN13 an individual rat pup was placed on a ramp with a 30° incline, with the head pointing downwards. This triggers a righting reflex that causes pups to turn until their heads are pointing upwards. The time required to turn 180° was measured in seconds as described previously (Darba *et al.*, 2003).

Wire hang

In order to test motor strength and coordination, PN19 rats were held by the nape of the neck and their forelimbs placed on a 3-mm-thick wire that was 140 cm long and strung between

two poles at a height of 60 cm. The total time (measured in seconds) that each animal was able to hold on before falling onto a pad was recorded as described previously (Dean *et al.*, 1981). If animals reached a maximum hang time of 2 min they were removed from the wire.

Play behavior

In order to test the development of normal social interaction, play behavior was observed daily from PN28 to PN38. Groups of six to seven non-cagemate rats were observed and video-recorded in a neutral arena for two half-hour sessions per day. Groups included both sexes and treatments. Videos were reviewed for two separate 2-min intervals per half-hour session, and tallies of aggressive play events (initiation of chasing, pouncing, pinning, boxing, and biting), behaviors present in juveniles of both sexes but more so in males, were scored as previously described (Meaney & McEwen, 1986; Oleson *et al.*, 2005). The average number of play events initiated per 2-min interval was calculated for all animals.

Sensory threshold

On PN40, the sensory threshold was determined using von Frey filaments by a previously described method (Ren, 1999; Wang & Thompson, 2008). Briefly, the rat was habituated to being held with its hindpaws on the bench and its forepaws draped over the experimenter's hand. Stimuli were delivered to the dorsal surface of each hindpaw between the second and third toes by a set of calibrated Von Frey filaments (North Coast Medical). Each filament was tested five times at an interval of 5–15 s, and rats were watched to see if they withdrew their hindpaw upon stimulation. However, if the first three stimuli elicited hindpaw withdrawal, the fourth and fifth stimuli were not delivered. Testing began with the 20-g filament, which on average elicited withdrawal in two of five tests. Minimum detection was assessed by a descending series of filament forces until zero of five stimuli elicited withdrawal. Following the ascertainment of minimum detection, maximum tolerance was assessed by an increasing series of filament forces until three subsequent tests elicited withdrawal.

Object exploration

On PN62, rats were placed in the open-field apparatus for 5 min along with two objects: a novel object (mug) and a familiar object (box) to which they had been exposed for 5 min, 24 h previously, as previously described (Presti-Torres *et al.*, 2007). The time spent exploring both objects was measured. Exploration was defined as the animals sniffing the objects but not standing on the objects and rearing or touching the objects with their flanks. The object recognition index, the time spent exploring the novel object divided by the time spent exploring both objects, was compared in order to test memory. The total time spent exploring both objects was also recorded.

Volumetric analysis and cell count

Rats were killed by decapitation and brains sectioned into 100- μ m-thick coronal sections and stained with Nissl stain by a previously established method (Glaser and Van der Loos, 1981). The cerebellum in one of every five sections was traced using the Neurolucida system, and volumetric reconstruction was performed. One cerebellum (female treated with vehicle) was lost due to damage to the anterior portion during sectioning. The volume of the granule cell layer of vermal lobules vIX-X was also determined by the same method. Although coronal sections are not ideal for analyzing Purkinje cell morphology, they can be used to ascertain Purkinje cell numbers (Smith *et al.*, 1987). Purkinje cell bodies in vIX were therefore counted using a 40 $\times$ Nikon objective. Purkinje cell bodies were identified by their large size and location as a monolayer between the granule cell layer and molecular layer. Cell bodies in focus in the first focal plane were excluded.

Statistical analysis

All experiments with more than two groups were analysed with a one-way ANOVA followed by a Student–Neumann–Keuls or Tukey’s posthoc multiple comparison test to determine significance between groups. For analysis of groups segregated by both sex and treatment group, two-way ANOVA was used rather than one-way ANOVA. Experiments with only two groups were assessed by a Student’s *t*-test. All statistical tests used a value of $P < 0.05$ as the criterion for significance.

Results

Cyclo-oxygenase inhibitors increase dendrite length in cerebellar Purkinje cells

In the preoptic area, prostaglandins alter the dendritic spine density, allowing for masculinization of this brain region (Amateau & McCarthy, 2004). We first sought to determine whether the development of synaptic connections in the cerebellar cortex was also affected by the manipulation of prostaglandin production. Mixed-sex rat pups were treated subcutaneously with 1 mg/kg of the relatively selective COX-2 inhibitor nimesulide ($n = 5$ males, 5 females) or vehicle ($n = 5$ males, 5 females) during the second postnatal week. We then collected their cerebella on PN14 and visualized them using the Golgi method (Fig. 1A–D). As no sex differences were found at this point, regardless of treatment, the sexes were collapsed for analysis. Nimesulide increased the length of the dendritic tree in Golgi-impregnated cerebellar Purkinje neurons of the posterior vermis (two-tailed $t = 2.88$, $P = 0.020$, Fig. 1E) and hemispheres (two-tailed $t = 2.41$, $P = 0.041$, Fig. 1F). The nimesulide-induced increase in dendrite length was coupled to an increase in the overall number of spines per dendrite in the posterior vermis (two-tailed $t = 2.39$, $P = 0.044$, Fig. 1G) and lateral hemispheres (two-tailed $t = 2.38$, $P = 0.049$, Fig. 1H), compared with vehicle-injected controls. Thus, we saw a change in overall dendritic tree size, but not spine density, in the posterior vermis (vehicle, 0.097 ± 0.008 spines/ μm ; nimesulide, 0.0995 ± 0.009 spines/ μm ; two-tailed $t = 0.207$, $P = 0.12$) and hemispheres (vehicle, 0.107 ± 0.007 spines/ μm ; nimesulide, 0.111 ± 0.06 spines/ μm ; two-tailed $t = 0.42$, $P = 0.68$). Sholl analysis revealed changes in dendritic branching patterns. In the posterior vermis, dendritic branch density as a function of distance from the cell body was increased following nimesulide treatment (Fig. 1I; two-tailed $t = 2.82$, $P = 0.024$; Table 1). However, there was no difference in branching in the lateral hemisphere (Fig. 1J; two-tailed $t = 1.24$, $P = 0.26$; Table 1). In the anterior vermis of the same animals, these effects on dendrite length (vehicle, $3129.6 \pm 544.1 \mu\text{m}$; nimesulide, $2567.4 \pm 434.8 \mu\text{m}$; two-tailed $t = 0.98$, $P = 0.35$) and spine number (vehicle, 373.5 ± 79.0 ; nimesulide, 259.4 ± 74.2 ; two-tailed $t = 0.90$, $P = 0.39$) were not found.

There was no effect of COX inhibition on dendritic length of granule cells (vehicle, $43.1 \pm 15.8 \mu\text{m}$; nimesulide, $39.1 \pm 2.1 \mu\text{m}$; two-tailed $t = 0.38$, $P = 0.71$), inhibitory basket cells (vehicle, $333.7 \pm 15.6 \mu\text{m}$; nimesulide, $321.0 \pm 27.9 \mu\text{m}$; two-tailed $t = 0.55$, $P = 0.60$) or stellate cells (vehicle, $224.4 \pm 19.3 \mu\text{m}$; nimesulide, $205.0 \pm 17.8 \mu\text{m}$; two-tailed $t = 0.8$, $P = 0.22$). As these cell types do not have anatomically distinct dendritic spines, no analysis of spine number or density was performed.

Spinophilin content is increased by cyclo-oxygenase inhibition and decreased by PGE2

Spinophilin is a protein enriched in dendritic spines that is integral to proper synaptic function (Feng *et al.*, 2000). It is also a reliable semiquantitative indicator of the number of spines in many (Amateau & McCarthy, 2004; Schwarz *et al.*, 2008), but not all, (Fester *et al.*, 2009) brain regions. In order to determine if spinophilin is an appropriate proxy marker for dendritic spine number in the cerebellum, we again treated rat pups of both sexes with the COX inhibitor nimesulide at a low (0.2 mg/kg s.c.; $n = 4$ males, 4 females), medium (1 mg/kg; $n = 3$ males, 3 females), or high (5 mg/kg; $n = 4$ males, 5 females) dose or sesame oil

vehicle ($n = 2$ males, 4 females) once daily from PN7 to PN13. The posterior vermis of the cerebellum was collected on PN14, and the cerebellar cortex was carefully dissected away from the deep nuclei, and assessed for spinophilin content by western blot. As no sex differences were found at this point regardless of treatment, the sexes were collapsed for analysis. As predicted, based on our finding of increased dendritic spines in cerebellar Purkinje cells, there was a nimesulide-induced dose-dependent increase in spinophilin in the cerebellar cortex (ANOVA, $F_{3,25} = 4.40$, $P = 0.011$; Student–Neumann–Keuls posthoc high dose to low dose and vehicle, $P = 0.0028$; medium dose to low dose, $P = 0.089$, Fig. 2A). We next examined the effects of other COX inhibitors using the same treatment paradigm. Comparison of treatment from PN7 to PN13 with indomethacin ($n = 6$ males, 7 females), a non-selective COX-1 and COX-2 inhibitor, to vehicle ($n = 7$ males, 8 females) in the cerebellar posterior vermis produced results similar to nimesulide (two-tailed $t = 2.15$, $P = 0.019$, Fig. 2B), and treatment with acetaminophen, an over-the-counter pain killer that inhibits prostaglandin production by a different mechanism, also induced an *a-priori*-predicted increase in cerebellar posterior vermis spinophilin on PN14 as compared with vehicle ($n = 5$ males, 7 females for both vehicle and acetaminophen groups, one-tailed $t = 1.763$, $P = 0.026$, Fig. 2C).

In order to determine whether the effects of COX inhibitors were due to direct effects on the cerebellum as opposed to indirect effects in another brain region, we next treated animals by direct cerebellar injection through the foramen magnum. We first demonstrated that our injection technique was specific for the cerebellum by injecting the same volume of a diffusible dye (Fig. 2D). We then treated animals by direct injection of nimesulide ($n = 8$ males, 5 females) or vehicle ($n = 8$ males, 6 females) into the cerebellum on PN10 and PN12. Direct injection into the cerebellum produced the same increase in spinophilin on PN14 as seen with systemic treatment (two-tailed $t = 2.09$, $P = 0.047$; Fig. 2E).

Once we had determined that disrupting prostaglandin synthesis altered synaptic development in the cerebellum, we next sought to determine the effects of the prostaglandin PGE2. Cerebellar cultures generated on the day of birth from a mixed-sex litter and treated with PGE2 ($n = 9$ culture dishes) from DIV7 to DIV13 had decreased spinophilin protein compared with controls ($n = 10$ culture dishes) when spinophilin was measured by western blot on DIV14 (two-tailed $t = 2.11$, $P = 0.040$, Fig. 2F). In order to confirm that this also occurred *in vivo*, we next treated animals by direct injection of PGE2 ($n = 5$ males, 5 females) or vehicle ($n = 5$ males, 6 females) into the cerebellum on PN10 and PN12. Spinophilin was decreased in the posterior vermis of the cerebellum of PN14 animals following two intracerebellar injections of PGE2 compared with vehicle (two-tailed $t = 2.12$, $P = 0.047$; Fig. 2G).

Because of the highly dynamic nature of Purkinje neuron development, we then explored whether the effect of PGE2 on the cerebellum was temporally restricted. We compared cohorts of animals treated during the first or third week of postnatal life with the animals treated during the second week as discussed above. Again, as no sex differences were found at these time points regardless of treatment, the sexes were collapsed for analysis. We found that, whereas PGE2 administered during the second week of life decreased spinophilin, PGE2 administered during the first ($n = 5$ male vehicle, 5 female vehicle, 5 male PGE2, 5 female PGE2) or third ($n = 5$ male vehicle, 7 female vehicle, 5 male PGE2, 7 female PGE2) postnatal weeks had no effect on spinophilin in the posterior vermis ($F_{2,32} = 5.273$, $P = 0.0105$, Tukey's multiple comparison test, $P = 0.043$; Fig. 2H). We therefore conclude that the ability of PGE2 to alter the development of Purkinje neurons occurs during a sensitive period in the second postnatal week of life.

Inhibition of cyclo-oxygenase postnatally alters behavior in juvenile males but not females

We next wanted to determine if inhibition of prostaglandin synthesis alters behavior later in life. Animals were treated systemically with 500 μg nimesulide or vehicle ($n = 7$ males, 7 females for both groups) from PN7 to PN13 and examined for changes in motor behavior that are associated with cerebellar function. There was no effect of sex ($F_{1,42} = 0.79$, $P = 0.78$), treatment ($F_{1,42} = 1.58$, $P = 0.21$), or inter-action between the two ($F_{3,42} = 0.82$, $P = 0.36$) on the negative geotaxis righting reflex, a test of motor coordination (Fig. 3A). Females had more motor activity than males in the open field ($F_{1,42} = 6.07$, $P = 0.013$), but there was no effect of treatment ($F_{1,42} = 0.228$, $P = 0.64$) or interaction between the two ($F_{3,42} = 1.689$, $P = 0.20$; Fig. 3B). Finally, there was no effect of sex ($F_{1,42} = 2.376$, $P = 0.13$), treatment ($F_{1,42} = 0.159$, $P = 0.692$), or interaction between the two ($F_{3,42} = 0.007$, $P = 0.935$) in wire hang performance, a test of motor strength (Fig. 3C).

As the cerebellum is also associated with non-motor behavior (Allen *et al.*, 1997; Katz & Steinmetz, 2002), we then turned to complex behaviors such as play, attention and somatosensory sensitivity. Animals were treated systemically with 500 μg nimesulide or vehicle ($n = 7$ males, 7 females for both groups) from PN7 to PN13 and behavior tested from PN28 to PN38. The observation of spontaneous play behavior revealed a significant effect of treatment (ANOVA; $F_{1,26} = 16.00$, $P = 0.0008$) and sex ($F_{1,26} = 283.20$, $P < 0.0001$), as well as an interaction between the two ($F_{3,26} = 18.80$, $P = 0.001$). As expected, males exhibited higher levels of rough-and-tumble play than females. Nimesulide treatment reduced play frequency in males but had no effect on the already low levels in females (Fig. 4A). The sensory threshold for paw withdrawal was assessed with von Frey filaments on the same animals (Fig. 4B). The minimum withdrawal threshold was significantly affected by sex ($F_{1,26} = 9.25$, $P = 0.008$) and there was an interaction of sex and treatment ($F_{3,26} = 9.25$, $P = 0.008$), such that males had higher initial thresholds and these were reduced by nimesulide treatment to that of females. Again, treatment did not affect females, indicating a sex-specific effect of COX inhibition on play behavior and somatosensory sensitivity.

In order to determine if behavioral effects following peripheral injections were specific to the cerebellum, animals received direct intracerebellar injection of vehicle ($n = 11$ males, 13 females) or 100 μg nimesulide ($n = 11$ males, 13 females) on PN10 and PN12 and play behavior was again tested as above (Fig. 4C). Similar to peripheral injection effects, there was a significant effect of sex ($F_{1,42} = 112.711$, $P < 0.0001$) and an interaction between sex and treatment (ANOVA; $F_{3,42} = 4.105$, $P = 0.048$). Nimesulide treatment directly into the cerebellum reduced play levels in males but had no effect on females. We then tested the sensory threshold for paw withdrawal using von Frey filaments on the same animals (Fig. 4D) and again observed that the minimum withdrawal threshold was significantly affected by sex ($F_{1,42} = 7.78$, $P = 0.01$) and there was an interaction of sex and treatment ($F_{3,42} = 5.14$, $P = 0.029$). Object exploration was tested in the same animals, and a recognition index was calculated (female vehicle, 0.566 ± 0.022 ; female nimesulide, 0.615 ± 0.024 ; male vehicle, 0.498 ± 0.037 ; male nimesulide, 0.503 ± 0.046). There was a significant effect of sex ($F_{1,42} = 7.87$, $P = 0.007$), but not of treatment ($F_{1,42} = 0.704$, $P = 0.40$), or interaction between sex and treatment ($F_{3,42} = 0.458$, $P = 0.502$), indicating that treatment did not alter memory of novel vs. familiar objects. However, when the total time spent exploring either object was quantified, there was a trend toward a significant effect of sex ($F_{1,42} = 3.692$, $P = 0.062$), such that males spent more time exploring objects than females, and there was a significant interaction of sex and treatment ($F_{3,42} = 4.737$, $P = 0.035$; Fig. 4E), such that treatment with a COX inhibitor increased the total time spent exploring inanimate objects in males but not females. Treatment alone did not significantly alter total object exploration ($F_{1,42} = 1.814$, $P = 0.19$).

Cyclo-oxygenase inhibitors affect the cerebellum long-term and do not affect other regions

As we observed a sex difference in the effect of COX inhibition on behavior but had seen no sex-specific effects on dendritic morphology at PN14, we examined spinophilin levels and cerebellar volume as a proxy for changes in Purkinje cell dendrite size in animals treated daily with nimesulide ($n = 7$ males, 7 females) or vehicle ($n = 8$ males, 6 females) from PN7 to PN13 but not killed until PN40. In these older animals, there was no effect of sex ($F_{1,26} = 0.95$, $P = 0.34$) or treatment ($F_{1,26} = 2.10$, $P = 0.16$) on spinophilin content, but there was a significant interaction between sex and treatment ($F_{3,26} = 4.20$, $P = 0.049$). The spinophilin content was significantly reduced by COX inhibition in males but not females (Fig. 5A). This is in contrast to animals examined at PN14, when spinophilin was increased by COX inhibition in both males and females. We also examined the cerebellar volume in the same animals treated with systemic nimesulide, whose play behavior and sensory threshold had previously been evaluated. Males had a larger overall cerebellar volume than females ($F_{1,26} = 6.09$, $P = 0.02$), but there was an interaction between sex and treatment ($F_{3,26} = 4.63$, $P = 0.04$; Fig. 5B), such that males treated with nimesulide had smaller cerebella that did not differ from those of females regardless of treatment. There was no effect of treatment alone ($F_{1,26} = 2.77$, $P = 0.11$). In the granule cell layer, there was no significant difference in volume as a function of sex ($F_{1,26} = 2.74$, $P = 0.11$) or treatment ($F_{1,26} = 1.81$, $P = 0.19$) and no interaction between the two ($F_{3,26} = 0.002$, $P = 0.96$; Fig. 5C). Likewise, there was no effect of sex ($F_{1,26} = 1.28$, $P = 0.27$), treatment ($F_{1,26} = 1.04$, $P = 0.32$), or interaction ($F_{3,26} = 2.33$, $P = 0.14$) on Purkinje cell number (Fig. 5D).

As we saw differences in complex behavior, we also examined the effects of daily treatment with COX inhibitors from PN7 to PN13 on other brain regions at PN14. In the hippocampus, spinophilin was not affected by sex ($F_{1,17} = 0.16$, $P = 0.69$), nimesulide treatment ($F_{1,17} = 0.54$, $P = 0.47$), or an interaction between the two ($F_{1,17} = 0.06$, $P = 0.81$; Fig. 5E). Spinophilin in the cortex was again not affected by sex ($F_{1,28} = 0.36$, $P = 0.56$), treatment ($F_{1,28} = 0.97$, $P = 0.33$), or an interaction between the two ($F_{1,28} = 0.03$, $P = 0.87$; Fig. 5F). Also, spinophilin in the amygdala was not affected by sex ($F_{1,28} = 0.05$, $P = 0.83$), treatment ($F_{1,28} = 1.10$, $P = 0.30$), or an interaction between the two ($F_{1,28} = 0.44$, $P = 0.51$; Fig. 5G). We therefore conclude that the changes in cerebellar spinophilin are region and cell-type specific, rather than the result of global changes across the central nervous system.

Discussion

Prostaglandins were first described in 1935 as an unknown substance contained in human seminal fluid that acted as a powerful depressor of blood pressure (Goldblatt, 1935). The name was coined a year later based on the assumption, subsequently shown to be incorrect, that the prostaglandins found in seminal fluid were produced by the prostate. The prostaglandin PGE2 is chronically elevated during low-level illness, and acutely increased in response to pain, hypoxia/ischemia (Andreasson, 2010) or viral and bacterial infection (Blatteis, 2006). Prostaglandins also mediate brain functions apart from fever. In the brain, COX-2 is constitutively expressed but upregulated following high-frequency stimulation associated with long-term potentiation (Yamagata *et al.*, 1993), and COX-2 protein has been localized to dendritic spines (Kaufmann *et al.*, 1996). Inhibition of COX-2 reduces long term potentiation (LTP) induction in the hippocampal gyrus, and this effect is rescued with PGE2 (Chen *et al.*, 2002). COX inhibition also causes substantial deficits to spatial learning in the Morris water maze (Shaw *et al.*, 2003). In the visual cortex, RNA interference of the PGE2 receptors EP2 and EP3 causes significant increases and decreases in LTP, respectively (Akaneya & Tsumoto, 2006).

Our current findings as well as our previous work demonstrate that prostaglandins have equally critical roles in normal brain development. Here we show that suppression of baseline prostaglandin synthesis by treatment with COX inhibitors during the second week of postnatal life impairs normal cerebellar development by increasing the dendritic length and spine number on Purkinje cells in both sexes in the short term, but there is a loss of dendritic spines and cerebellar volume in males only over the long term. Although the cause of this initially increased dendritic growth followed by later atrophy is unknown, a similar phenomenon is observed in rats exposed to maternal stress *in utero* (Pascuel *et al.*, 2010). The effect of PGE₂ is limited to the second postnatal week, a time during which climbing fiber synapses with Purkinje cells are pruned so that by PN15 each Purkinje cell is contacted by a single climbing fiber (Crepel *et al.*, 1981). The second postnatal week is also when the bulk of cerebellar granule cells are born and begin to make contact with their Purkinje cell targets, their input organized into anatomically restricted rows of parallel fibers (Altman, 1972). Our results suggest that prostaglandins are important for pruning immature or inappropriate excitatory connections on Purkinje cells by parallel fibers from cerebellar granule cells or climbing fibers from the inferior olive during the second postnatal week. Disrupting prostaglandin synthesis with COX inhibitors altered this process by allowing for exuberant growth followed by later retraction. Conversely, increased prostaglandins locally within the cerebellum reduced spinophilin, a reliable marker of Purkinje neuron synaptogenesis.

We have previously shown that prostaglandin production in the POA is regulated by gonadal testosterone converted to estradiol within the POA by neuronal aromatase (Amateau & McCarthy, 2004). However, there is increasing evidence for local *de-novo* steroidogenesis in the brain that is not dependent upon gonadally synthesized precursors. In the rat cerebellum, immunoreactivity for estrogen receptor (ER) α and ER β is detected during early development in the cerebellum at greater levels than detected in adults (Pérez *et al.*, 2003). Steroid hormones including estradiol are produced locally in the cerebellum and alter dendritic spine number in cerebellar Purkinje cells during the second postnatal week (Sakamoto *et al.*, 2003). Future work will delve into the relationship between prostaglandins and estradiol in the cerebellum.

It remains unclear what, if any, role locally synthesized steroids in the brain are playing in the establishment of sex differences, and it is possible that these brain-derived steroids are important modulators of brain development in males and females. The cerebellum is not commonly considered to be a sexually dimorphic brain region, although a larger cerebellar volume in human males has been detected by some (Giedd *et al.*, 1996), but not others (Henery & Mayhew, 1989), and recent reports have demonstrated gonadal steroid-independent sex differences in the expression of cerebellar proteins such as calbindin (Abel *et al.*, 2011), Foxp2 (Hamson *et al.*, 2009) and JaridC (Xu *et al.*, 2008). In addition to these relatively subtle sex differences, there is a sex-based difference in developmental vulnerability, with males frequently developing greater pathology than females in response to the same insult (Dean & McCarthy, 2008). For example, normal male and female rats do not differ in performance on motor tasks traditionally used to gauge overall cerebellar function, such as the rotorod task. However, following perinatal exposure to polychlorinated biphenyls, a common industrial toxin that stunts the development of the Purkinje cell dendritic tree (Kimura-Kuroda *et al.*, 2007), males show a slightly greater reduction in cerebellar mass and fare considerably worse on rotorod performance (Nguon *et al.*, 2005). Male rats prenatally exposed to cocaine also show greater motor impairments later in life than females (Markowski *et al.*, 1998), which has been interpreted as a potential sign of greater cerebellar impairment. Ablation of the thyroid at birth causes a reduction in the volume of the granule cell layer and total granule cell number when measured at PN30 (Madeira *et al.*, 1988). Interestingly, this challenge actually seems to eliminate one of the

sex differences seen in normal animals: normal male rats have more granule cells than females, but hypothyroid males and females do not differ in their granule cell number because the males lose an even greater number of granule cells than the females. Here, we found that there were no sex differences apparent in the early responses to disrupted prostaglandin synthesis but, as animals matured, a selective vulnerability of males emerged. This was apparent in both behavior and in the morphology of the cerebellum, which was larger in mature males than females and selectively reduced in males treated with COX inhibitors during the preadolescent period. The high level of rough-and-tumble play by adolescent males is one of the most robust and reliable of sex differences across species (Olesen *et al.*, 2005). Similarly, males have higher somatosensory thresholds than females (Ren, 1999) and, in both cases, we found that treatment with COX inhibitors altered responses only in males. For play behavior this may simply reflect a basement effect below which females can go no further, but this would not be true for the sensory threshold. Likewise, the increased attention to objects in general displayed selectively by males treated with COX inhibitor cannot be attributed to constraints on the variable being measured. The source of the late-emerging sex difference and enduring consequences of preadolescent treatment with COX inhibitors are unknown but may reflect ongoing developmental processes that differ in males and females as is seen for many other brain regions (McCarthy *et al.*, 2008).

The increasing awareness of cerebellar pathology in disorders such as autism has highlighted an expanding view of cerebellar function. Autism spectrum disorder is defined by circumscribed interests as well as deficits in communication and social interaction. Abnormal sensory responses to normal tactile and auditory stimuli are frequently observed (Kern *et al.*, 2002). Although stereotyped motor behaviors and some minor deficits in fine motor control frequently develop in patients with autism (Estes *et al.*, 2011), the gross ataxia and poor postural control associated with cerebellar pathology are not present. However, pathology in the cerebellum has frequently been found in autistic patients (Courchesne *et al.*, 1987, 1994; Allen & Courchesne, 2003; Wills *et al.*, 2009), particularly in the cerebellar vermis (Courchesne *et al.*, 1988; Levitt *et al.*, 1999). This has led to an increasing appreciation of the function of the cerebellum outside motor control. The cerebellum is involved in multi-sensory integration (Kern, 2002) and has been implicated in emotional and cognitive processing (Konarski *et al.*, 2005). The amygdala is the dominant brain region associated with the regulation of play behavior (Auger & Olesen, 2009), and it has also been implicated in autism (Cauda *et al.*, 2011; Rudie *et al.*, 2011) but, as with most complex behaviors, the amygdala constitutes only one node in a network of relevant brain regions. The amygdala shares reciprocal connections with the cerebellum (Konarski *et al.*, 2005) and stimulation of the cerebellum of rats and cats by implanted electrodes causes activation of the medial amygdala (Heath *et al.*, 1978). Case studies from the 1970s, reviewed by Konarski *et al.* (2005), reveal that electrical stimulation of the cerebellar cortex in human epilepsy patients reduced feelings of anger, depression, and fear. Similarly, stimulation to the deep nuclei of the cerebellum, which receive inhibitory input from Purkinje cells, caused a sensation of fear or anger. Our findings of altered social behavior and somatosensory sensitivity following manipulation of cerebellar development thus conform to the increasingly well-understood role of the cerebellum in non-motor behavior (for further review, see Moulton *et al.*, 2010; Strick *et al.*, 2009; Stoodley & Schmahmann, 2010, 2009).

Here, we discovered that inhibition of prostaglandin synthesis did not alter motor output measured by traditionally cerebellar tasks such as the wire hang and negative geotaxis. We did not assess performance on a rotorod and so the potential for more subtle but undetected effects on cerebellum-dependent motor performance exists. Although we did not identify gross motor abnormalities in our animals, abnormalities in complex social behavior and attention as well sensory integration differences did emerge. In investigating complex

behaviors that arise from the network interaction of multiple brain regions, one must be cautious to distinguish between brain pathology that is a simple marker of behavioral dysfunction, rather than a true cause. Although it is known that prostaglandins alter development in the preoptic area, we demonstrate that the same changes in spinophilin found in the cerebellum are not found in regions such as the amygdala, hippocampus and cortex when COX inhibitors are administered systemically, showing that altering prostaglandin production at this time point does not lead to alteration in spinophilin expression in all brain regions. More importantly, we show that COX inhibitors injected directly into the cerebellum are sufficient to induce the same behavioral effects seen following systemic treatment. This makes a strong case for cerebellar pathology playing a causal role in the observed behavioral changes. Future work will elucidate the mechanism by which these changes occur in terms of the specific prostaglandin receptors responsible, the cells and subregions in which these receptors are expressed, and the changes in cerebellar physiology that occur between early postnatal treatment and later behavioral output. These findings fit well with the evolving understanding of cerebellar function and also demonstrate how a brain region once thought to be a humble motor center could be crucial to the genesis of neurodevelopmental disorders such as autism. As our understanding of cerebellar development expands, we will hopefully gain new insight into the mechanisms behind a human disease that is, at this time, one of medicine's most intriguing and urgent neurological mysteries.

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Abbreviations

COX	cyclo-oxygenase
DIV	days <i>in vitro</i>
ER	estrogen receptor
LTP	long term potentiation
PGE2	prostaglandin E2
PGH	prostaglandin H
PN	postnatal day
POA	preoptic area

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Fig. 1.

Postnatal inhibition of COX alters Purkinje cell dendritic development. Mixed-sex neonatal rat pups were treated daily with subcutaneous injections of vehicle (veh) (A and C) or the COX-2 inhibitor nimesulide (nim) (1 mg/kg; $n = 5$ males, 5 females for all groups) (B and D) from PN7 to PN13 and brains were subjected to Golgi-Cox impregnation on PN14. Scale bar: 25 μm (t -test, * $P < 0.05$). Total dendritic tree length (in μm) was measured. Inhibition of COX-2 activity resulted in an overall longer Purkinje cell dendritic tree in the posterior vermis (E) and lateral hemispheres (F). Total dendritic spine number was also increased in the posterior vermis (G) and lateral hemispheres (H). Spine density was unchanged, and there were no changes in the anterior vermis (see Results). Sholl analysis of dendritic geometry that provides information on the number of branch points relative to the distance from the cell body is displayed for the posterior vermis (I) and the lateral hemispheres (J).

Fig. 2.

Spinophilin content is increased by COX inhibition and decreased by PGE2. Changes in spinophilin, a 120-kDa protein enriched in dendritic spines, correlate with changes in dendritic spines and can be easily measured by western blot. The posterior vermis of the cerebellum of PN14 rat pups was collected. After visualization of antibodies specific to spinophilin, intraoptic density at 120 kDa was measured. Membranes were stained with Ponceau S as a loading control (LC) for total protein content, the integrative area density (iad) for membranes stained with Ponceau S was measured, and spinophilin values were normalized to total protein content. (A) Mixed-sex rat pups were treated systemically (daily s.c. injections) from PN7 to PN13 with low (0.2 mg/kg), medium (med) (1.0 mg/kg), or high doses (5.0 mg/kg) of the COX inhibitor nimesulide (nim), or a sesame oil vehicle (veh) ($n = 6-9$). Spinophilin was increased in the cerebellar cortex of PN14 animals (ANOVA, $**P < 0.01$, $\#P = 0.08$). Each lane on the western blot represents tissue from one animal. (B) Spinophilin was also increased in the cerebellar cortex of PN14 mixed-sex animals treated systemically from PN7 to PN13 with 50 $\mu\text{g/kg}$ indomethacin (indo), another COX inhibitor, over sesame oil vehicle ($n = 13-15$; $*P < 0.05$). (C) An over-the-counter inhibitor of prostaglandin production, acetaminophen (acet), also increased spinophilin in the cerebellar cortex of PN14 mixed-sex animals treated systemically from PN7 to PN13 with 200 μg acetaminophen over sesame oil vehicle ($n = 12$ for all groups; $*P < 0.05$). (D) Photograph of a rat brain following an intracerebellar dye injection of 1 μL ink. (E) Mixed-sex rat pups were treated with nimesulide (100 μg) or sesame oil vehicle on PN10 and PN12 ($n = 13-14$) by intracerebellar injections. Spinophilin was increased in the cerebellar cortex of PN14 animals ($*P < 0.05$). (F) P0 cerebella were collected and cultured as a mix of neuronal cell types including Purkinje cells, granule cells, and GABAergic interneurons. Daily PGE2

treatment (1.5 nM) from DIV7 to DIV13 has the opposite effect to COX inhibitors, resulting in a significant decrease in spinophilin content compared with saline vehicle ($n = 9-10$ culture dishes; $*P < 0.05$). (G) Mixed-sex rat pups were treated with intracerebellar PGE2 (2.5 μg) or vehicle ($n = 10-11$) on PN10 and PN12. Spinophilin was increased in the cerebellum of PN14 animals following two intracerebellar injections of PGE2 compared with vehicle ($*P < 0.05$). (H) Composite line graph comparing treatment effect of PGE2 seen in animals in G with mixed-sex animals treated with the same dose of PGE2 during the first and third postnatal week. Results are normalized to spinophilin levels measured in controls, set at 100%. Changes in spinophilin were significant during the second, but not the first and third postnatal weeks ($*P < 0.05$).

Fig. 3.

Inhibiting postnatal prostaglandin production does not alter motor output. Rat pups were treated by subcutaneous injection with the COX inhibitor nimesulide (nim) (500 μ g) or sesame oil vehicle (veh) on PN7 to PN13 ($n = 11-13$). (A) To test negative geotaxis, rats were placed nose down on a 30° ramp on PN13 and the time to turn nose up (in seconds) was recorded. There was no effect of sex, treatment, or interaction between the two. (B) Animals were placed in an open field divided into a 5 × 4 grid on PN60 for 10 min and the distance traveled as indicated by the number of times that all four paws crossed a boundary between boxes on the grid was recorded. Females had more total crossings than males (* $P < 0.01$). There was no effect of treatment or interaction between the two. (C) For the wire hang task, the forepaws of PN19 rats were placed on a thin wire suspended between two posts and the time to fall (in seconds) was recorded. There was no effect of sex, treatment, or interaction between the two.

Fig. 4.

Inhibiting postnatal prostaglandin production alters sensory threshold and social behavior in juvenile males but not females. (A and B) Male and female rat pups were treated daily by subcutaneous injection with the COX inhibitor nimesulide (nim) (500 μ g) or vehicle (veh) from PN7 to PN13. (A) Play behavior observed daily from PN25 to PN38 ($n = 7$ for all groups). Each incident of chasing, pouncing, boxing, biting, or pinning was counted as play, and incidents of play per 3-min interval were recorded. Males exhibited more play than females regardless of treatment and treatment with nimesulide reduced play in males but not females (ANOVA, $**P < 0.001$). (B) Following play behavior, von Frey filaments of increasing diameter were applied to the hindpaws of the same animals on PN40. Females

showed a lower withdrawal threshold, the minimum force (in grams) necessary for hindpaw withdrawal for three consecutive applications, than males regardless of treatment. Nimesulide treatment reduced the withdrawal threshold in males but not females (ANOVA, $**P < 0.01$). (C–E) Treatment by direct intracerebellar injection with the COX inhibitor nimesulide (100 μg) or sesame oil on PN10 and PN12 ($n = 11–13$). (C) The same effects on play behavior were seen with intracerebellar treatment as with systemic treatment (compare with A); males exhibited more frequent play events than females regardless of treatment ($**P < 0.01$); nimesulide reduced play behavior in males but not females ($*P < 0.05$). (D) Intracerebellar nimesulide treatment produced the same reduction in the minimum withdrawal threshold from von Frey filaments as systemic nimesulide treatment (compare with B); females reacted to a lower force than males ($**P < 0.01$), and nimesulide treatment reduced the withdrawal threshold in males but not females ($*P < 0.05$). (E) Animals were placed in an open field on PN62 and exposed to a novel object (mug) and an object to which they had been exposed the previous day (box) for 5 min. Although there was no difference in time spent exploring the novel vs. familiar object as described in the Results, male rats treated with nimesulide spent more total time examining both objects than all other groups ($*P < 0.05$). There was a trend towards a significant effect of sex ($\#P = 0.062$).

Fig. 5.

Inhibiting postnatal prostaglandin production alters development in the juvenile cerebellum but not the early postnatal amygdala, cortex, or hippocampus. (A) Animals were treated (subcutaneously) with 500 μ g nimesulide (nim) or vehicle (veh) ($n = 6-8$) from PN7 to PN13 and reared to PN40 before assay. Spinophilin was significantly reduced after nimesulide treatment in males only (* $P < 0.05$). (B) Rats were treated as in A ($n = 7$ all groups), and cerebella were collected at PN40 and processed for volumetric reconstruction. Male cerebella were significantly larger than those of females (* $P < 0.05$) at this time period and there was a trend towards a significant reduction in volume after treatment in males only (# $P = 0.07$). (C) The volumes of the granule cell layer in vermian lobules IX and X were measured and found to be unchanged. (D) The number of Purkinje cells in vermian lobule IXc was unaffected by treatment. (E) Rats were treated with 100 μ g of nimesulide (s.c.) from PN7 to PN13 ($n = 4-5$) and killed on PN14. No changes in spinophilin were found in the hippocampus based on sex, treatment, or an interaction between the two. (F) Rats were treated with 100 μ g of nimesulide (s.c.) from PN7 to PN13 ($n = 4-5$) and killed on PN14. No changes in spinophilin were found in the cortex based on sex, treatment, or an interaction between the two. (G) Rats were treated with 100 μ g of nimesulide (s.c.) from PN7 to PN13 ($n = 5$) and killed on PN14. There was no effect of sex, treatment, or an interaction between the two on spinophilin levels. iad, integrative area density.

Table 1

Sholl analysis of Purkinje cells following COX inhibition

Treatment	Cerebellar region	Sholl regression coefficient (<i>k</i>)	Schoenen ramification index (<i>M</i>)
Nimesulide	Posterior vermis	$-0.016 \pm 0.001^*$	16.18 ± 2.12
Vehicle	Posterior vermis	$-0.012 \pm 0.001^*$	16.63 ± 4.95
Nimesulide	Lateral hemisphere	-0.021 ± 0.007	15.14 ± 1.38
Vehicle	Lateral hemisphere	-0.015 ± 0.007	13.43 ± 2.98

Neonatal rat pups were treated daily with subcutaneous injections of vehicle or the COX-2 inhibitor nimesulide ($n = 5$ for all groups) from PN7 to PN13 and brains were subjected to Golgi-Cox impregnation on PN14. Cerebellar Purkinje cells were traced and subjected to Sholl analysis; concentric circles of increasing diameter centered around the cell body were drawn, and the number of dendrite intersections with the circle were counted. The Sholl regression coefficient is obtained by plotting the log of the number of intersections per circle area with the circle radius, and determining the slope. The slope measures the rate of change of branches in the dendritic tree. Nimesulide treatment increased the absolute value of the slope in the posterior vermis ($*P < 0.05$) but not the lateral hemispheres. The Schoenen ramification index is determined by dividing the maximum branch points by the number of primary dendrites. This did not differ by treatment in either region.