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Exogenous Sonic Hedgehog modulates the pool of GABAergic interneurons during cerebellar development

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Abstract

All cerebellar GABAergic interneurons derive from a common pool of precursor cells residing in the embryonic ventricular zone (VZ) and migrating in the prospective white matter (PWM) after birth, where both intrinsic and extrinsic factors contribute to regulate their amplification. Among the environmental factors, we focused on Sonic hedgehog (Shh), a morphogen well known to regulate neural progenitor cell proliferation. We asked if and how exogenous Shh treatment affects the lineage of cerebellar GABAergic interneurons. To address these issues exogenous Shh was administered to embryonic and postnatal organotypic slices. We found that Shh is able to expand the pool of interneuron progenitors residing in the embryonic epithelium and in the postnatal PWM. In particular, Shh signalling pathway was highly mitogenic at early developmental stages of interneuron production, whereas its effect decreased after the first postnatal week. Gene expression analysis of sorted cells and in situ hybridization further showed that immature interneurons express both the Shh receptor Patched and the Shh target gene Gli1. Thus, within the interneuron lineage, Shh might exert regulatory functions also in postmitotic cells. On the whole our data enlighten the role of Shh during cerebellar maturation and further broaden our knowledge on the amplification mechanisms of the interneuron progenitor pool.
Introduction
All cerebellar neurons derive from two anatomically and neurochemically segregated germinal zones: the ventricular zone near the IV ventricle, where Ptf1-a^+ progenitors generate GABAergic phenotypes (Purkinje cells – PCs, nucleo-olivary neurons of deep cerebellar nuclei – DCN and all inhibitory interneurons) [1-3] and the rostral rhombic lip (RL), where Atoh1-expressing precursors progressively generate glutamatergic neurons (projection DCN neurons; unipolar brush cells, granule cells) [3-9]. These two germinative epithelia disappear around birth. However, dividing ventricular zone (VZ) precursors emigrate into the cerebellar prospective white matter (PWM), whereas those of the RL move along the pial cerebellar surface, where they form the external granular layer (EGL) [10, 11].

Progenitor amplification in both embryonic and postnatal germinal zones is sustained by a number of factors, one of which is Sonic hedgehog (Shh). This morphogen is secreted by Purkinje cells from E17.5 [12-15] and has a well-established role in the proliferation of granule cell progenitors (GCPs) in the EGL [12, 16, 17]. The Shh receptor Patched (Ptc) [18], its agonist Smoothened (Smo) [19] and the transcriptional target gene Gli [13, 20] exert specific functions in different phases of GCP development, in both normal and pathological conditions, such as medulloblastoma [21-23].

In addition, it has been recently demonstrated that Shh secreted by the choroid plexi in the embryonic cerebrospinal fluid is critically involved in the amplification of both radial glial cells and early VZ-derived GABAergic progenitors [15]. At later developmental stages, the same morphogen delivered by PCs maintains the PWM niche and sustains the proliferation of neural-stem-cell-like primary progenitors able to generate both CD15^+ precursors and Ptf1-a^+ progenitor cells [24]. Whereas CD15^+ cells are parenchymal astrocyte precursors, PWM Ptf1-a^+ cells are proliferative progenitors of GABAergic interneurons [24] that start expressing Pax-2 during their last S phase [24-26].

Here we want to further unveil the relationships between Shh signalling and GABAergic interneuron development by asking whether an exogenous administration of this ligand can affect interneuron generation in vitro. To clarify this point we treated organotypic cerebellar cultures derived from either embryonic or postnatal cerebella with the recombinant amino-terminal active fragment of Shh (SHH-N). By this approach we showed a role for this morphogen in the amplification of the interneuron progenitor pool. Moreover, we provided evidence that immature Pax-2^+ inhibitory interneurons express components of the Shh signaling pathway, such as Ptc and Gli1.
Materials and methods

Experimental animals

The experiments were performed on C57BL/6 mice (Harlan, San Pietro al Natisone, Italy) and Pax-2-GFP (green fluorescent protein) mice, which express enhanced GFP under the control of the Pax-2 promoter (a generous gift from Dr. M. Busslinger, Vienna Biocenter, Vienna, Austria [27]). Cerebella were removed aseptically from embryonic day (E) 16 embryos and from postnatal day (P) 2, P7, P13 mice, cryoanesthesized (P2 and P7) or anesthetized (P13) by intraperitoneal (i.p.) administration of ketamine (100 mg/kg) (Ketavet; Bayer) supplemented by xylazine (5 mg/kg) (Rompun; Bayer). Embryos were obtained by caesarean section from mouse dams anesthetized by intraperitoneal injection of ketamine and xilazine. All procedures were in accordance with the European Communities Council Directive European Communities Council (2010/63/EU), the NIH guidelines and the Italian Law for Care and Use of Experimental Animals, (DL26/14), and were approved by the Italian Ministry of Health and the Bioethical Committee of the University of Turin.

Cerebellar slice cultures

Postnatal cerebellar slice cultures were prepared by cutting 300 µm parasagittal sections using a McIlwain tissue chopper, whereas entire coronal E16 cerebella primordia were used for embryonic explants. 8 to 10 slices or embryonic explants were plated on culture inserts (BD Becton Dickinson, Franklin Lakes, NJ, USA) and placed in a 6-well plates containing 1 ml serum-free slice culture medium. Cultures were kept at 37 °C in 5% CO₂. Culture medium consists of Eagle’s basal medium with Earles’s salts (Life Technologies, Carlsbad, CA, USA) supplemented with glutamine (2 mM, Gibco, Carlsbad, CA, USA), glucose (32 mM, Sigma-Aldrich, St. Louis, MO, USA), penicillin-streptomycin (20 U/ml, Gibco), bovine serum albumin (10 mg/ml; Sigma, Saint Louis, MO, USA), and B27 supplement (Gibco). Cultures were treated with SHH-N (3 µg/ml, R&D Systems, Minneapolis, MN, USA) w/o cyclopamine (Tocris Bioscences, Bristol, UK), and fixed after 24 hours (1 day in vitro -DIV-) or 48 hours (2 DIV). To label proliferating cells, bromodeoxyuridine (BrdU, 10 µM, Sigma Aldrich) was applied at 22 hours and washed out after 2 hours. Slices were fixed immediately thereafter or at 2 DIV (Fig. 1 a). Untreated slices incubated with BrdU were used as control.

Histological procedures

Slices and explants were fixed at 1 and 2 DIV using 4% paraformaldehyde (PFA) in phosphate buffer (PB) 0.12 M, pH 7.4 for 30 minutes and then rinsed 3 times in phosphate buffer saline (PBS). Under deep general anaesthesia, E16 Pax-2-GFP pregnant dams and postnatal P2 and P7 Pax-2-GFP mice were transcardially perfused with 4% PFA in 0.12 M PB. The brains were immediately
dissected, post fixed overnight at 4°C and transferred to 30% sucrose in 0.12 M PB. The cerebella were embedded in optimal cutting temperature compound (OCT, Zeiss, Mainz, Germany) and cut in 30 µm parasagittal cryostat sections. Sections were incubated overnight at 4°C with primary antibodies (dissolved in PBS, with 1.5% normal serum and 0.25% Triton X-100): chicken anti-GFP (1:500, Aves Lab, Tigard, Oregon USA), rabbit anti-Ptf1-a (1:1000, Professor Hoshino, National Institute of Neuroscience, Tokyo, Japan), rabbit anti-Pax-2 (1:200, Zymed, San Francisco, CA) and rat anti-BrdU (1:500, ABCAM, Cambridge, UK). The sections were exposed for 1 hour at RT to fluoresceinated secondary antibodies (1:200; Vector), anti-rat Cy3 (1:500, Jackson Laboratories, Bar Harbor, ME, USA), anti-rabbit Alexa 488 (1:500, Invitrogen, Carlsbad, CA, USA) or biotinylated secondary antibodies followed by streptavidin-Texas Red conjugate (1:200; Invitrogen). DAPI (4', 6 diamidino 2 phenylindole dihydrochloride, Fluka, Buchs, Switzerland, diluted 1:1000 in PBS) was used to counterstain cell nuclei. For BrdU immunostaining, the sections were incubated in 2N HCl for 20 minutes at 37°C, followed by 10 min in 0.1 M sodium tetraborate (pH 8.5) and then exposed to anti-BrdU antibodies overnight at 4°C and reacted with secondary antibody. Samples were mounted on slides with Tris-glycerol supplemented with 10% Mowiol (Calbiochem, La Jolla, CA) for microscope visualization.

**Fluorescence-activated cell sorting (FACS) of Pax-2-GFP cells**

To prepare cells for flow cytometric analysis, embryonic and postnatal cerebellar cells were isolated under sterile conditions and meninges carefully removed. Tissues were mechanically disrupted and incubated with 0.5% trypsin (Gibco) and 0.1% DNase (Sigma Aldrich) at RT for 3 minutes. Subsequently, cells were triturated with a glass pipette, collected by centrifugation at 1000 rpm for 5 min and the pellet was resuspended at 1x10^6-10^7 cells/ml in “sorting” buffer (PBS with 0.6% glucose and 1% fetal bovine serum –FBS Gibco-). Cellular aggregates were removed by means of a cell strainer (70 µm, BD Biosciences, New Jersey, USA). Cell sorting, performed on a MoFlo™ XDP cell sorter (Beckman Coulter, Villepinte, Francia), was based on the measurements of GFP intensity. We selected three subpopulations with absent (GFP negative), low (GFP low) and high (GFP high) GFP fluorescence and collected them in 500 µl of supplemented FBS.

**RNA preparation and quantitative real time PCR (qRT-PCR)**

Sorted cells were collected by centrifugation at 1000 rpm for 5 min and total RNA was extracted from the pellet of each subpopulations with RNAspin Mini Kit (GE Healthcare, Bucking Hamshire, UK). DNase treatment was performed in column. The cDNA was prepared by reverse transcription of whole RNA sample for each subpopulation using “High Capacity cDNA Archive” kit (Applied
Biosystems, Foster City, CA, USA). The resulting cDNA was used as a template for amplification using Roche Universal probes (Roche, Indianapolis, IN, USA) performed on STEP ONE Real Time PCR System (Applied Biosystems). Reaction conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles 95°C for 15 s alternating with 60°C for 1 min. PCR amplifications were always run in duplicate. The following primer sequences were used: GAPDH forward (DQ403054.1) 5'-GAGCCAAACGGGTCACTA-3', GAPDH reverse 5'-CATATTTCTCGGTTCACAC-3', Pax-2 forward (NM_011037.4) 5'-AAGGCTTGGAGATTCAAGAC-3', Pax-2 reverse 5'-AACTAGGTGGCGGCTAGGC-3', PTF1-a forward (NM_018809.2) 5'-GGCTTGGCCATTGGCTACATT-3', PTF1-a reverse 5'-GGACTGTCTCTCCGAGG-3', GAD67 forward (D42051.1) 5'-AGGCAATCCGATTTTGA-3', GAD67 reverse 5'-GCCGATCTCATAGGTAAC-3', Gli1 (NM_010296.2) forward 5'-AGGAATTCGTGTGCCAT-3', Gli1 reverse 5'-TCCGACAGCCTCAAAG-3', Ptc1 forward (NM_008957.2) 5'-GGACCGGATCTATCGAC-3', Ptc1 reverse 5'-ACTTCGCTCTGCCCAG-3'. Control reactions without reverse transcriptase were performed to check for genomic DNA contamination. All samples were normalized with respect to the GAPDH reference gene and the relative expression was calculated as $2^{-\Delta CT}$, where $\Delta CT$ is the difference between the threshold cycle number of the analysed gene and that of GAPDH. Three independent experiments were performed. For the analysis of results, data from all ages (E16, P2, P7 and P13) were pooled together to compare expression levels of target genes either in the whole Pax-2+ population or in cells with low or high Pax-2 levels (Fig. 5 b-g). Additionally, data on Pax-2, Gli1 and Ptc1 were also plotted separately for each age (Fig. 5 h-j). Data are presented as means ± SEM.

Riboprobe Synthesis
Total RNA was extracted from fresh murine cerebella using Trizol (Invitrogen), treated with DNase I (Sigma Aldrich), then reverse transcribed using High Capacity cDNA Archive, according to the manufacturer's instruction (Applied Biosystems). cDNA fragments were amplified by PCR amplification using the following primers: Gli1 forward 5'-CACACCTGTCAGCACA-3' and Gli1 reverse 5'-TGGACCCCTAGCTCTCATA-3', Ptc1a forward 5'-CACCTGGACTCAGCACT-3' and Ptc1a reverse 5'-GTTACACCTCGCAATCAA-3', modified to insert the T7 and SP6 promoter at 5' and 3' of amplicons. Digoxigenin-labeled sense and antisense riboprobes were obtained by in vitro transcription using T7 and SP6 transcriptase respectively, incorporating digoxigenin-NTPs (Roche). The probes were purified with AMBION columns (Invitrogen).
In situ hybridization and immunohistochemistry

Brains from transcardially perfused animals were processed as above and cut in 15 µm-thick parasagittal slices. Before hybridization, the sections were treated for 20 sec with Proteinase K (200 ng/ml; Sigma Aldrich), then incubated in 0.5% Triton X-100, followed by acetic anhydride in triethanolamine. Hybridization was performed with digoxigenin-labelled sense or antisense riboprobes overnight at 66 °C, after an incubation for 2 hr at RT with the pre-hybridization buffer (50% formamide, 5X SSC, 10% blocking agent). Stringency washes were performed the day after at the hybridization temperature in 0.2X SSC for 1 hr. After saturation with 10% blocking agent/MABS (0.1M of maleic acid, 0.15M NaCl and NaOH at pH 7.5) the slices were incubated with an alcaline phosphatase (AP) conjugated antibody (Roche, 1:5000 in 10% blocking agent/MABS) for 1 hour at RT. Hybridization was revealed by incubation with NBT-BCIP solution (4-nitroblue tetrazolium chloride-5-bromo-4-chloro-3-indoyl-phosphate, Sigma Aldrich). Before proceeding with immunohistochemistry, samples were incubated 5 min at RT with 0.3% H2O2 to block endogenous peroxidase activity and then immunolabeled as previously described. Colour development was performed using 3,3’-diaminobenzidine (DAB) and the slides were mounted with Mowiol. Sense probes were used to reveal unspecific labelling (not shown).

Data analysis and statistical evaluation

Histological preparations were examined with Leica TCS SP5 confocal microscopy and digital images were processed with Adobe Photoshop CS2 (Adobe Systems, Mountain View, CA). Quantitative evaluation were made using ImageJ (National Institutes of Health, USA) and Imaris software package (Bitplane Scientific Solutions, Zurich, Switzerland). For each slice, the total number of cells (DAPI+), immature GABAergic interneurons (Pax-2+) and proliferating cells (BrdU+) were quantified on three fields of view at 60x in the PWM and on three adjacent confocal planes (4 µm apart). At least three slices were analysed for each experimental condition. For embryonic cultures, 6 to 8 fields of view in the outer or inner parts of the explants were evaluated. Experiments were performed in triplicate.

Statistical evaluation was performed with Graphpad Prism 6.00 software (La Jolla, CA, USA), applying the Student’s t test or the Mann-Whitney Rank Sum Test for real time analysis and ANOVA with Bonferroni’s multiple comparison post-hoc test for data from both cerebellar slice cultures and qRT-PCR. Data are presented as mean ± SEM. In all instance P<0.05 was considered as statistically significant.
Results

Exogenous Shh expands the pool of newborn GABAergic interneurons at earliest developmental stages

To test whether Shh regulates the proliferation of cerebellar GABAergic interneuron precursors, we cultured slices derived from E16, P2 and P7 cerebella for 24 or 48 hr in the presence or absence of SHH-N [16], without the Shh inhibitor cyclopamine [28], and pulsed them with BrdU for 2 hr (Fig. 1 a). Firstly, we focused on P2 animals, because the majority of interneurons is generated between P0 and P5 in mice [25]. During the same period, GCPs actively proliferate in the EGL in response to PC-secreted Shh [16, 29]. As a validation of the efficacy of SHH-N, we confirmed that the EGL was considerably thickened (24 hr, 70.59±9.85 µm in control slices vs. 226.60±22.06 µm in stimulated slices; Student’s t test P<0.001; 48 hr, 81.29±10.75 µm in control slices vs. 164.13±30.72 µm in stimulated slices; Student’s t test P=0.044; Fig. 1 b-e). Subsequently, we analysed cell proliferation in the PWM, where the GABAergic interneuron pool is amplified [25, 26, 30, 31]. After 1 DIV of exposure to SHH-N, the treatment resulted in 1.4 fold increase in the number of actively dividing cells (tagged by BrdU 2 hr before fixation) compared to control slices (Fig. 2 a, b, g; F2,25=8.48, P=0.024). This increment in cell proliferation was maintained at 2 DIV in the whole population that had proliferated during the last 24 hours (Fig. 2 d, e, g; F2,19=6.68, P=0.008).

We then asked whether the expansion of newly generated cells also included GABAergic interneuron progenitors, identified by the expression of the selective and specific marker Pax-2 [25, 32]. This marker physiologically labels the exit of PWM precursors from the cell cycle and their engagement in differentiation along the interneuron lineage [25, 26]. At 1DIV cells in active proliferation, positive for both Pax-2 and BrdU (double+/DAPI+ cells), doubled in the presence of SHH-N (Fig. 2 a, b, h; F2,25=5.35, P=0.015). A relevant increase in newly generated Pax-2+ cells was also confirmed at 2 DIV, and was further corroborated by significant increments of BrdU+ cells within the Pax-2+ cell population at all examined time points (Fig. 2 d, e, h, i; double+/DAPI+ cells, F2,25=9.27, P=0.020; double+/Pax-2+ cells, F2,25=7.54, P=0.031). All the detected accruements were reverted to control values by the addition of the Shh antagonist cyclopamine (Fig. 2 c, f, g, h, i; BrdU+/DAPI+ cells, 1 DIV, P=0.001; 2 DIV, P=0.041; double+/DAPI+ cells, 1 DIV, P=0.046, 2 DIV, P=0.001; double+/Pax-2+ cells, 1 DIV, P=0.033, 2 DIV, P=0.003). Thus, the generation of new GABAergic interneurons is boosted by SHH-N application.

Notably, Pax-2+ cells in active proliferation (S phase, 1 DIV) always constituted a limited proportion amongst both all proliferative cells (double+/BrdU+ cells 1 DIV, about 13% in control slices vs. 18% in stimulated slices) and interneurons (Fig. 2 i), thereby excluding their massive entry in the cell cycle in response to SHH-N and confirming the largely postmitotic feature of this population. These data further indicate that the increment in proliferation pertained mostly to Pax-2-negative precursors. Yet, the moderate increase in the fraction of double+ cells over the whole BrdU+ populations at 1 DIV suggests an anticipated acquisition of the interneuron phenotype in PWM precursors in response to SHH-N. Moreover, the disappearance of this effect during 2 days of
treatment (double+/BrdU+ cells 2 DIV: 16.26% in control slices vs. 18.52% in stimulated slices; \(F_{2,25}=7.39, P>0.05\)) indicates that on the whole SHH-N does not alter precursor specification.

Thereafter we checked whether SHH-N treatment could have similar effects at earlier developmental stages. We examined E16 cerebellar explants and distinguished between external and internal zones, which represent germinal epithelia and nascent PWM, respectively (drawings in Fig. 3). We found that actively dividing cells (BrdU+) were not significantly affected by 24 hr of SHH-N treatment, in both external and internal zones (Fig. 3 a, b, g, j, k, p). Conversely, 48 hr of SHH-N administration resulted in a 1.5 fold increase in the number of cells labelled by BrdU the day before. This expansion exclusively took place in the external zone of the explants (\(F_{2,23}=6.32, P=0.02\), Fig. 3 d, e, g), indicating that precursors responsive to SHH-N are only located at this site, and was reverted to control values by cyclopamine addition (\(F_{2,23}=6.32, P=0.015\), Fig. 3 f, g).

As for P2 data, BrdU+ cells displaying Pax-2 expression at 1 DIV represented a small fraction over all cells entering the cell cycle and the whole population of Pax-2+ cells (external zone: double+/BrdU+ cells, 6.43%; double+/Pax-2+ cells 2.33%, Fig. 3 i; internal zone: double+/BrdU+ cells, 10.15%; double+/Pax-2+ cells 3.20%, Fig. 3 r). At difference with later stages, these fractions were not affected by exposure to SHH-N or cyclopamine in either external or internal zones (double+/DAPI+ cells, 1 DIV external zone, \(F_{2,23}=0.65, P>0.05\); internal zone, \(F_{2,17}=2.67, P>0.05\); Fig. 3 a-c, h, j-l, q). Notably, however, an increased fraction of proliferative cells became Pax-2+ interneurons at the end of the culture period in the external zone (about 2.5 fold increase, \(F_{2,20}=8.86, P=0.012\)) and this effect was reverted by cyclopamine treatment (Fig. 3 d-f, h; \(P=0.002\)). A relevant increase in the proportion of newly generated interneurons was also evident in the internal zone (\(F_{2,13}=24.10, P=0.02\); Fig. 3 m, n, q), probably sustained by increased migration from external positions. Again, cyclopamine administration could abrogate this outcome (Fig. 3 o, q; \(P<0.0001\)).

Conversely, the fraction of double+/Pax-2+ cells in the internal zone remained unchanged (Fig. 3 r). In the internal zone, Pax2+/BrdU+ cells displayed a trend to decrease below control values in the presence of Shh + cyclopamine (Fig. 3 q, r). Nevertheless, this trend reached statistical significance only when double+ cells were confronted with the whole DAPI+ population (\(P=0.009\)). On the whole these data confirmed results obtained at P2 indicating that precursors in the external zone of the explants respond to exogenous SHH-N by increased proliferation and generation of Pax-2+ interneurons.

The outcome of SHH-N stimulation was completely absent when we performed the same experiments with P7 mice: neither changes in PWM precursor proliferation nor increment in newly generated interneurons were observed (\(P>0.05\); Fig. 4 a-c).
These *in vitro* data show that exogenous administration of SHH-N enhances the proliferation of PWM precursors, amplifying the number of GABAergic interneurons. These effects occur both before and soon after birth, though with distinct dynamics, whereas they disappear by the end of the first week of postnatal development.

**Shh pathway is active in immature Pax-2⁺ interneurons**

Previous functional studies showed that precursors of cerebellar GABAergic interneurons are influenced by endogenous Shh produced by the embryonic choroid plexi [15] or by postnatal Purkinje cells [24]. However, no direct evidence is available on the activity of the Shh pathway in Pax-2⁺ interneurons. To address this point we analysed GFP⁺ cells selected by fluorescence-activated cell sorting (FACS) from E16, P2, P7 and P13 Pax-2-GFP transgenic mice. The sorting report showed a wide range of fluorescence intensity ($10^2$-$10^5$) in GFP⁺ cells, indicating a relevant heterogeneity within this cell population (Fig. 5 a, R3 and R2 boxes). Indeed, we recognized two specific subpopulations of Pax-2⁺ interneurons at all considered ages: a population characterized by lower level of GFP intensity (defined "Pax-2 low", $10^2$-$10^3$ intensity of fluorescence; Fig. 5 a, box R3) and another one with a higher GFP signal (defined "Pax-2 high", $10^4$-$10^5$ intensity of fluorescence; Fig. 5 a, box R2). To better define both the entire GFP⁺ population and the two subpopulations, we performed a qRT-PCR analysis and quantified the expression of Ptf1-a and Gad67 genes, characterizing early and advanced stages of GABAergic interneuron development, respectively [1, 24, 26, 33]. Additionally, we analysed Gli1, as readout of the activation of the Shh pathway [34-36], and Ptch, as indicator of the capability of the cells to respond to Shh [37, 38]. The analysis of the whole GFP⁺ population showed that Gli1 is expressed to a relevant extent ($2^{-\Delta\text{CT}}=0.50\pm0.07$), being its mRNA levels about one third of those of the characterising transcription factor *Pax-2* ($2^{-\Delta\text{CT}}=1.61\pm0.19$; Fig. 5 b). The expression of *Ptch* ($2^{-\Delta\text{CT}}=0.17\pm0.04$) was also detected, whereas that one of both Ptf1-a ($2^{-\Delta\text{CT}}=0.044\pm0.02$) and Gad67 ($2^{-\Delta\text{CT}}=0.09\pm0.05$) genes were almost negligible (Fig. 5 b). To better understand these data and assess whether distinct Pax-2 levels really corresponded to cells with distinct features, we first confirmed that the expression levels of *Pax-2* were different in "Pax-2 low" and "Pax-2 high" subpopulations ($2^{-\Delta\text{CT}}$ *Pax-2*, 0.10±0.06 "Pax-2 low" vs. 3.12±0.39 "Pax-2 high"; Student’s t test $P<0.05$; Fig. 5 c). Both subsets displayed similar levels of *Ptf1-a* ($2^{-\Delta\text{CT}}$ *Ptf1-a*, 0.05±0.04 "Pax-2 low" vs. 0.04±0.05 "Pax-2 high"; Student’s t test $P>0.05$; Fig. 5 c) and a moderate expression of *Ptch* ($2^{-\Delta\text{CT}}$ *Ptch*, 0.20±0.09 "Pax-2 low" vs. 0.14±0.03 "Pax-2 high"; Student’s t test $P>0.05$; Fig. 5 f). Conversely, they significantly diverged in *Gad67* expression, indicating that these two subpopulations represent diverse
developmental stages of Pax-2+ interneurons (Fig. 5 d). In particular, in “Pax-2-low” cells Gad67 mRNA was close to zero, whereas the "Pax-2 high" subpopulation exhibited some levels of the transcript, indicating that it includes more mature cells (2-\text{dCT} Gad67, 0.002±0.01 "Pax-2 low" vs. 0.17±0.12 "Pax-2 high"; Student’s t test \(P<0.05\); Fig. 5 d). To understand if the activation of Shh pathway changed during the maturation of Pax-2+ interneurons, we quantified Gli1 mRNA in Pax-2 low/high subpopulations. Both subsets revealed significant Gli1 mRNA levels and suggest a slight decline, though not statistically significant, in more mature "Pax-2 high" cells compared to the "Pax-2 low" subset (2-\text{dCT} Gli1, 0.66±0.14 "Pax-2 low" vs. 0.34±0.12 "Pax-2 high"; Student’s t test \(P>0.05\); Fig. 5 g). All these expression patterns were confirmed when Pax-2, Ptch and Gli1 levels were analysed separately by age in “Pax-2-high vs. low” cells (Fig 5 h-j, main effect of population, Pax-2, \(F_{1,18}=112.1\), \(P<0.0001\); Ptch, \(F_{1,16}=1.78\), \(P>0.05\); Gli1, \(F_{1,16}=20.43\), \(P<0.001\). Notably, however, a reverted pattern with a higher expression in more mature cells was detected at P13 for Ptch and Gli1 (Fig. 5 i, j). On the whole these data indicate that 1) the Shh target gene Gli1 is expressed in Pax-2-positive interneurons; 2) Pax-2-positive cells comprise at least two diverse populations, representing inhibitory interneuron precursors at different stages of maturation.

Our data about Ptf1-a expression were confirmed in vivo by immunohistochemistry of cerebellar sections derived from E16 (Fig. 6 a-c), P2 (Fig. 6 d-f) and P7 (Fig. 6 g-i) Pax-2-GFP transgenic mice injected with BrdU 2h before sacrifice. In line with mRNA data, at all ages we found only few scattered cells double-labelled for Ptf1-a and (Pax-2)-GFP, showing that these markers are largely segregated in the interneuron lineage. In E16 cerebellar primordia double-labelled cells were located both in the ventricular/subventricular zone (Fig. 6 a, b) and in the cerebellar parenchyma (Fig. 6 a, c). Conversely, in P2 cerebella scattered Ptf1-a+/Pax-2-GFP+ cells were exclusively localized along the PWM (Fig. 6 d-f), where some of them were still proliferating (Fig. 6 e). Finally, in P7 mice very few double positive Ptf1-a+/Pax-2-GFP+ cells could be found and preferentially resided in the basal portion of the lobule (Fig. 6 g, h), from where more immature cells migrate toward the folial PWM (Fig. 6 g, i), suggestive of a possible maturation gradient proceeding from the base to the apex of the lobuli.

Using in situ hybridization, we next defined the expression pattern of Gli1 (Fig. 7 a-c, f-i, k) and Ptch (Figure 7 d, e) in developing Pax-2-GFP mice. Gli1 and Ptch mRNAs were detected in P2 cerebella in the EGL, PCL (likely corresponding to Bergmann Glia cells), and in PWM, in line with former studies [13, 17, 24, 32] (Fig. 7 a-c, k). To better characterize cells expressing Gli1 or Ptch,
we immunostained the tissue for GFP (Figure 7 a, b, d, e, j). Double Gli1+/Pax-2-GFP+ and Ptc1+/Pax-2-GFP+ interneurons were observed both in lobular PWM (Figure 7 a, d) and in deep PWM (Figure 7 b, e), confirming the presence of an active Shh signal in Pax-2+ interneurons.

At E16 the Gli1 mRNA was already present in cerebellum, though it appeared overall less expressed compared to postnatal ages (Figure 7 f-i). Gli1+ cells were detected all along the edge of the embryonic cerebellum, including the VZ/SVZ (Figure 7 f, g), as well as in scattered cells in the cerebellar parenchyma (Figure 7 f, h, i). The same expression pattern was observed for cells double-positive for Gli1 and Pax-2-GFP (Figure 7 f-i).

In summary our data show that Shh pathway is endogenously active in Pax-2+ cells located in germinal and migratory sites during both embryonic and postnatal cerebellar development.

**Discussion**

In this study we analysed whether an exogenous administration of Shh could affect the generation of GABAergic interneurons and whether the Shh pathway is activated in Pax-2+ cells in native conditions. We found that SHH-N can boost the production of Pax-2+ cells at embryonic and early stages after birth, whereas this effect is lost at the end of the first postnatal week. Furthermore, expression analysis of sorted Pax-2+ cells and in situ hybridization showed that Shh signalling is endogenously active in cerebellar interneurons during their development.

These experiments were conducted in organotypic cultures, which were already used to study the role of Shh in GCP proliferation [12, 16]. This model is easily amenable to experimental manipulations and has the advantage to mimic cell-to-cell interactions occurring in vivo, including those that crucially influence GABAergic interneuron production and differentiation [39, 40]. SHH-N treatment promoted the production of new Pax-2+ interneurons both in the external zone of E16 cerebellar primordia and in the PWM of P2 cerebellar slices, which represent the germinative sources of these inhibitory neurons [10, 11]. However, this occurred with distinct dynamics. Namely, at E16 two days of Shh treatment were required to evoke an increase in the number of double-positive Pax-2+/BrdU+ cells, whereas at P2 24 hours of administration were sufficient. The role of endogenous Shh in the amplification of the interneuron pool has been formerly shown at both embryonic [15] and postnatal stages [24]. Huang and colleagues demonstrated the action of choroid plexi-derived Shh in supporting the physiological expansion of inhibitory interneurons during embryonic development [15]. The same group demonstrated that Purkinje-derived Shh maintains the PWM niche postnatally, by sustaining the amplification of neural-stem cell-like
progenitors, the source of both GABAergic interneurons and astrocytes [24]. These experiments also showed that Shh signalling is involved in the proper generation of Ptf1-a+ cells and that it is active in these precursors, which precede in the lineage Pax-2+ interneurons [24]. Here, by an in vitro approach we further strengthen the evidence of the implication of Shh in the proliferation of precursors for inhibitory interneurons and in their subsequent progression toward a Pax-2+ fate. Moreover, we show for the first time that exogenous Shh can further enhance Pax-2+ interneuron production, indicating -at least in vitro- the existence of precursors potentially responsive to Shh, but not engaged by the endogenous ligand at the moment of treatment. The different temporal dynamics revealed by E16 and P2 slices also suggest a distinct stage-dependent availability of precursors apt to respond to exogenous Shh. At earlier stages, primary neural-stem cell-like progenitors could be all already engaged by endogenous Shh, as previously demonstrated [15]. Thus, some time could be required to generate relevant numbers of intermediate progenitors responding to exogenous Shh by increased Pax-2+ cell production. Conversely, in P2 slices Shh administration could have immediately affected the already expanded intermediate progenitor population that further proceeded along the interneuron lineage. Alternatively, these different dynamics could reflect different levels of endogenous Shh in embryonic and postnatal slices. In particular, the poor survival of PCs typically occurring at P2 [41] could determine low levels of endogenous Shh. In this condition SHH-N could immediately activate responsive cells not engaged by the low amount of the endogenous ligand. At embryonic stages surviving PCs could produce relevant levels of endogenous Shh saturating all responsive precursors at the earliest time points. Diverse endogenous levels of Shh could also account for differences in the effects of Shh inhibition in embryonic and postnatal slices. Postnatally, cyclopamine could antagonize exclusively exogenous Shh and bring values to control levels. The antagonist could instead counteract also theendogenous ligand when present (such as at embryonic ages), thereby reducing values below control levels. Indeed, this occurs in some instances in embryonic slices. Importantly, and in line with previous findings on Shh endogenous activity [24], the effects of exogenous Shh administration on Pax-2+ cell production disappear at P7, when most interneurons have been already produced [25, 31]. This observation suggests that Shh-responsive elements in the PWM are absent or rare at this age. Our expression data also showed the presence of Gli1 and Ptc1 mRNAs in Pax-2+ cells during the entire temporal window between E16 and P13 (Fig. 5 b) and that their expression remains overall stable in both “Pax-2-low” and “Pax-2-high” sorted subpopulations (Fig. 5 f, g, i, j). We previously showed that young postmitotic interneurons sojourn in the PWM for a rather long period, although they progressively acquire distinctive GABAergic traits, and they become competent to choose specific phenotypes in response to extrinsic cues [26]. It's conceivable
that these two Pax-2-expressing subpopulations selected by FAC Sorter represent two different stages of interneuron maturation in the PWM: the “Pax-2-low”, being characterized by cells exiting from the cell cycle and generically committed toward a GABAergic interneuron identity and the “Pax-2-high”, comprising cells at subsequent stages of differentiation. This is supported by their differential expression of Gad67 mRNA (Fig. 5 d). Conversely, Ptfl-a mRNA expression is noticeably weak and did not discriminate between the two subpopulations (Fig. 5 c), in agreement with its expression in actively dividing interneuron progenitors at Pax-2-negative stages [2, 24] (Fig. 6).

We also provide evidence that the Shh signalling pathway is active in Pax-2-positive cells by means of in situ hybridization. The expression pattern of the Shh receptor Ptch and that of the transcriptional target Gli1 have been already examined in postnatal and adult cerebella [13, 17, 18, 24, 42]. We strengthened these data showing the existence of double-positive Gli1/Pax-2-GFP⁺ and Ptch⁺/Pax-2-GFP⁺ cells in the lobular PWM and deep WM region (Fig. 7 a, b, d, e), confirming the presence of an active Shh signal in Pax-2⁺ interneurons. These data differ from previous findings obtained from genetic fate mapping of Gli1-expressing cells, showing the absence of Gli1 signal in Pax-2⁺ cells after P6 [24]. It is plausible that this discrepancy reflects differences in the applied experimental approaches (direct monitoring of Gli1 mRNA vs. Gli1 promoter activity). Yet, Gli1 mRNA levels as detected by both quantitative PCR and in situ analysis appear relevant in Pax-2⁺ precursors and suggest that Shh signalling might exert additional functions in cerebellar interneurons. Notably, this additional action could be in line with that of PC-derived Shh on other cerebellar cell types. Indeed, during the first postnatal weeks PC-derived Shh is known to switch its function from a support for proliferation of both oligodendrocyte progenitors and granule cells to a trigger for their differentiation [43, 44]. Thus, by secreting Shh PCs could modulate not only the amplification of the interneuron pool, but also aspects related to its maturation such as migration and/or the acquisition of specific phenotypes. The trend to display higher levels of Gli1 and Ptch in more mature Pax-2⁺ cells at P13 is in keeping with this hypothesis (Fig. 5 i, j). However, this remains to be assessed.

On the whole our data highlight the critical role of Shh in the generation of the interneuron progenitor pool in the PWM. This knowledge could be usefully applied to treat some cerebellar diseases associated to abnormal interneuron distribution and function [45, 46], or to reinforce processes of interneuron amplification in animal models in which the interneuron progenitor pool is precociously exhausted, as occurs in cyclin-D2 knockout mice [47, 48]. Indeed, Shh has been demonstrated to sustain cell cycle progression through the expression of cell cycle regulators, as
cyclin-D1 and D2 [29, 49]. In this view, Shh could be considered as one of the fundamental links between extrinsic and intrinsic factors controlling cerebellar development and maturation.

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References


cells of the cerebellum are produced in the rhombic lip and migrate through developing white matter. J Neurosci 2006;26:9184–95.


Figure Legends.

Fig. 1: Mitogenic effect of SHH-N on GCPs. a Schedule of SHH-N stimulation and BrdU treatment on organotypic culture. After 22 hr of culture in presence of SHH-N (3 µg/ml), organotypic slices were treated by 2 hr of BrdU. The analysis was performed at 24 and 48 hr after plating. b-e Representative pictures of SHH-N effect on EGL of postnatal cerebellum. Illustration of distribution of BrdU+ GCPs (red) and Pax-2+ interneuron precursors (green) after 24 and 48 hr of SHH-N stimulation. c, e In presence of SHH-N the thickness and the number of BrdU+ cells in the
Fig. 2 Effect of SHH-N on P2 organotypic slices. (a-f) Representative confocal images of P2 cerebellar organotypic slices show the distribution of Pax-2⁺ cells (green) and BrdU⁺ cells (red). Organotypic slices were maintained 1 and 2 DIV after plating in absence (CTRL, a, d) or presence of SHH-N (3 µg/ml, b, e) and SHH-N with cyclopamine (5 µM) (c, f). The graphs show the SHH-N proliferative response of BrdU⁺ PWM cells (g), and that one of newly generated BrdU⁺/Pax-2⁺ cells (h) at 1 and 2 DIV. The proliferative effect of SHH-N on BrdU⁺/Pax-2⁺ cells is present at 1 and 2 DIV also within the Pax-2⁺ cell population (i). Arrows in a-f indicate all BrdU⁺/Pax-2⁺ cells present in each confocal image and some of them are magnified in boxed insets in each figure. Nuclei are counterstained by DAPI (blue). Values for SHH-N and SHH-N+ cyclopamine were not different at the statistical level. ANOVA was performed with Bonferroni’s multiple comparison post-hoc test (n, fields of view = 9-12 for each experimental condition; *P<0.05, ** P<0.01, *** P<0.001). Double⁺, BrdU⁺/Pax-2⁺ cells. Scale bars: 40 µm.

Fig. 3 Effect of SHH-N on embryonic organotypic slices. Representative confocal images of cerebellar organotypic slices show the distribution of Pax-2⁺ cells (green) and BrdU⁺ cells (red) in the external zone (a-f) and internal zone (j-o) of E16 cerebellar primordium (indicated in the drawings). Organotypic slices were maintained 1 and 2 DIV after plating in absence (CTRL, a, d, j, m) or presence of SHH-N (3 µg/ml, b, e, k, n) and SHH-N with cyclopamine (5 µM) (c, f, l, o). The graphs representing the percentage between proliferating BrdU⁺ cells and total DAPI⁺ cells (g, p) illustrate a proliferative effect of SHH-N ligand only in the external zone. Conversely, in both areas SHH-N significantly increases the percentage of newly generated BrdU⁺/Pax-2⁺ on total DAPI⁺ cells (graphs % double⁺/DAPI⁺ cells, h, q). i, r show the values of double positive BrdU⁺/Pax-2⁺ on total Pax-2⁺; here SHH-N treatment exclusively affects cells located in the external region of the slices (graphs %double⁺/Pax-2⁺ cells, i). All these proliferative responses to SHH-N stimulation appear solely after 26 hr of BrdU treatment (2 DIV) and disappear in presence of cyclopamine (c, f, l, o and all graphs). Values for SHH-N and SHH-N+ cyclopamine were not different at the statistical level, unless differently indicated. Arrows indicate all BrdU⁺/Pax-2⁺ cells present in each confocal image and some of them are magnified in boxed insets in each figure. Nuclei are counterstained by DAPI (blue). ANOVA was performed with Bonferroni’s multiple comparison post-hoc test (*P<0.05, ** P<0.01, *** P<0.001). Double⁺, BrdU⁺/Pax-2⁺ cells. Scale bars: 40 µm.

Commento: Qui non abbiamo messo gli n. nei materiali e metodi abbiamo scritto: “6 to 8 fields of view in the outer or inner parts of the explants were evaluated”. Cosa mettiamo qui? n, fields of view = 6-8 for each experimental condition Va bene?
**Fig. 4** Effect of SHH-N on P7 organotypic slices. Organotypic slices were maintained 1 and 2 DIV after plating in absence (CTRL) or presence of SHH-N (3 µg/ml) and SHH-N with cyclopamine (5 µM). The graphs show the absence of SHH-N proliferative response of PWM cells (a) and newly generated BrdU+/Pax-2+ cells (b, c) at 1 and 2 DIV. ANOVA was performed with Bonferroni’s multiple comparison post-hoc test.

**Fig. 5** Gene expression of sorted Pax-2-GFP cells. a FACS analysis of postnatal Pax-2-GFP dissociated cells. Left graph displays an example of side scatter (SSC-Height) and GFP fluorescence intensity plot (Pax_GFP-Log_Height Comp), used to separate cells according to granularity and fluorescence emission, respectively. The R4, R3 and R2 gates include sorted cells with different fluorescence intensity, defined “negative”, “low” and “high” GFP sub-populations, respectively. In the right chart (counts vs GFP fluorescence intensity) is shown the number of events of each sub-population. The gates for negative and positive GFP cell population have been chosen with maximal stringency, to avoid cross contamination of the respective cell population. b-j Measure at different ages (E17, P2, P7 and P13) of transcript levels in Pax-2 “low” and “high” FAC Sorted cell populations using qRT-PCR. Histograms in b show level of expressed genes (Pax-2, Ptf1-a, Gad67, Gli1 and Ptch) of whole GFP+ population, obtained by collecting data of single GFP+ sub-population of all ages. The two sub-populations display no diversity in the transcript intensity of Ptf1-a (c), whereas are statistically significantly different in the Gad67 expression level (d). e illustrates the statistically significant higher expression of Pax-2 mRNA in the “high GFP” sub-population compared to the “low GFP” group, confirming the accuracy of sorting procedure. Ptch and Gli1 mRNA decrease in “high GFP” sample in comparison with “low GFP” one, though the differences are not statistically significant (f, g). Expression of Pax-2 (h), Ptch (i) and Gli1 (j) mRNA in “Pax-2 low” and “high” sub-populations at different ages (E17, P2, P7 and P13). Student’s t test and ANOVA test were performed (*P<0.05). Transcript levels, calculated as 2 ΔΔCT, were normalized to GAPDH mRNA and are presented as mean values of three independent experiments plus/minus standard error (SEM; n, samples = 4).
Fig. 6 Localization of Ptf1-a⁺ progenitors in embryonic and postnatal Pax-2-GFP transgenic mice. a–c illustrate the expression of Ptf1-a-positive interneuron progenitors (red) in the E16 primordium of a transgenic Pax-2-GFP cerebella, pulsed with BrdU (blue) 2 hours before sacrifice. Double positive Ptf1-a⁺/GFP⁺ cells, representing progenitors specified toward a GABAergic interneuron identity, are located both in the VZ/SVZ zone (b) and in the cerebellar parenchyma (c) and are characterized by a lower GFP expression (see inset in b). In postnatal P2 (d–f) cerebella, scattered Ptf1-a⁺/Pax-2-GFP⁺ cells are present at different levels along the PWM; these cells are generally characterized by lower GFP intensity (inset in e) and some of them are positive for BrdU administered 2h before sacrifice (e, f). Finally, in P7 cerebella (g–i) fewer numbers of double positive Ptf1-a⁺/GFP⁺ cells can be found in the basal portion of the folial PWM (h). PWM, prospective white matter; Scale bars: a= 100 µm; b, e=5 µm; c, f, h, i=10 µm; d, g=50 µm.

Fig. 7 In situ hybridization for Gli1 and Ptch expression followed by immunohistochemistry for Pax-2-GFP. a, b In situ hybridization for Gli1 expression in sagittal sections of P2 cerebellum followed by immunohistochemistry for Pax-2-GFP. Gli1 is expressed in the EGL, in the BG layer and in the PWM. Arrowhead in a indicates an example of Pax-2-GFP⁺ cell that express Gli1 in the lobular PWM (magnified in the inset). Presence of double-positive progenitors in deep PWM is shown at higher magnification in the boxed inset in a (b). Boxes in b show a further magnification of single double-labelled cells, indicated by arrowheads and arrow. The Pax-2-GFP staining (dark brown nucleus and cytoplasm) overlaps with the in situ hybridization signal for Gli1 (purple, cytoplasm) in the BG layer and PWM. The Gli1 signal in EGL is due to the presence of GCPs. c In situ hybridization for Gli1 expression in sagittal sections of P2 cerebellum. Gli1⁺ cells are present in the EGL, BG layer and in the PWM. The small panels show the boxed inserts at higher magnification, one for the EGL (indicated by *) and the other for the deep PWM. d, e Ptch1 is expressed in the EGL, BG layer and in the PWM. The arrowheads indicate examples of Pax-2-GFP⁺ cells that express Ptc1 in lobular PWM (d) and in deep PWM (e), magnified in boxed insets. f–i Weak levels of Gli1 expression are detected at E16 in the external layer and in the internal region of slices of the embryonic cerebellum. Higher magnifications of VZ (g), internal region (h) and more cortical region (i) in f show the presence of some double Gli1⁺/Pax-2-GFP⁺ cells, indicated by arrow and magnified in boxed insets. In j and k an example of Pax-2-GFP⁺ cell and Gli1⁺ cell, respectively. EGL, external granular layer; BG, Bergman Glia; PCL, Purkinje cell layer; PWM, prospective white matter; CP, choroid plexus; VZ, ventricular zone. Scale bars: a–e, f–i boxed insets in c=40 µm; boxed insets in a–e, g–i and j, k=10 µm.