Figures and figure supplements

Proneurotrophin-3 promotes cell cycle withdrawal of developing cerebellar granule cell progenitors via the p75 neurotrophin receptor

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Figure 1. Development of p75NTR in the rat cerebellum. (a) Low magnification images of sagittal sections through the entire cerebellum from postnatal day (P) 2 through P21 showing the abundant immunolabeling for p75NTR in the EGL, which decreases by P21. Size bar indicates either 200 μm or 400 μm, as indicated. (b) High magnification images of lobe 6 showing p75NTR labeling in the outer EGL. Arrows indicate the inner EGL where the neurons lack p75NTR. Size bar indicates 10 μm and is the same for all the images in B. (c) Western blot of cerebellum lysates from the indicated ages. Tissue was lysed with RIPA buffer containing protease inhibitors and 20 μg of total protein was separated on a 10% gel and probed for p75NTR. The gel was re-probed for actin as a loading control and is representative of 3 independent experiments.

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Figure 1—figure supplement 1. No differences in TUNEL labeling in the EGL between Ngfr−/− and WT mice. Sections from WT or Ngfr−/− mouse P7 cerebellum were processed for TUNEL labeling. Few labeled cells were detected and no difference between genotypes was observed.

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**Figure 2.** Expression of p75<sup>NTR</sup>, Ki67, and DCX in the P7 rat cerebellum. (a) Confocal image of p75<sup>NTR</sup> and the proliferation marker Ki67 showing colocalization in the external EGL. Note that p75<sup>NTR</sup> is also expressed in developing Purkinje cells. (b) p75<sup>NTR</sup> is downregulated in the inner EGL when DCX is expressed. eEGL – external External Granule Layer, iEGL – inner External Granule Layer, PCL – Purkinje Cell Layer. Size bar is 10 μm.

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Figure 3. Cell cycle withdrawal of GCPs in the EGL is delayed in the Ngfr-/- mice compared to wild type mice. (a) Low magnification images of the entire cerebellum showing incorporation of EdU at postnatal ages from P5 to P14. GCPs in WT mice began to decrease EdU incorporation at P7, which continued decreasing at P10 and P14 as the progenitors left the cell cycle. Mice lacking p75NTR continued to incorporate EdU at high levels at P7 and P10, and only began to decrease proliferation at P14. Size bar is 200 μm. (b) High magnification of EdU labeling in lobe 6 showing continued EdU incorporation Ngfr-/- mice compared to WT from P7 through P14. Nuclei labeled with Draq5 are shown in gray. Size bar indicates 10 μm. (c) Quantification of EdU labeling of WT and Ngfr-/- mice. EdU-labeled cells were counted across 150 μm in the EGL of lobe 6b and are graphed relative Figure 3 continued on next page.
Figure 3 continued

to the total number of cells labeled with Draq5. At least three brains per genotype were analyzed at each age. *significantly different from WT at p=0.0001. (d) Developmental expression of Ki67 from P2 through P14, confirming the increased expression of proliferation markers in the Ngfr-/- mice compared to WT from P7 through P14. Size bar indicates 10 μm. (e) Quantification of cells expressing Ki67 in lobe 6b. Labeled cells were counted across 150 μm in the EGL of lobe 6b and are graphed relative to the total number of cells labeled with Draq5. At least three brains per genotype were analyzed at each age. *significantly different from WT at p=0.0001. Data in graphs in c and e are expressed as mean +/- SEM of at least three independent experiments. Asterisks indicate significance by ANOVA with Tukey’s posthoc analysis, p values indicated below each graph. (f) Western blot showing the comparison of cyclin E1 expression in cerebellar lysates from WT or Ngfr-/- mice at the indicated postnatal ages. Differences in cyclin E levels between WT and knockout mice are evident at P7, P10 and P14. Blot is representative of three independent experiments. (g) Progressive increase in area of the cerebellum in Ngfr-/- mice compared to WT at P5, P7 and P10. **P7 Ngfr-/- significantly different from P7 WT at 0.038 by t-test **P10 Ngfr-/- significantly different from P10 WT at p=0.0174 by t-test.

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The following source data is available for figure 3:

Source data 1. Mean number of labeled cells of 3 slides for each animal, and statistical analysis for the graphs shown in 3c, 3e, and 3g.

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Figure 4. Mature neurotrophins have no effect on proliferation of cultured P7 rat GCPs in the absence or presence of Shh. GCPs from P7 rat were cultured with BrdU for 48 hr in the absence or presence of Shh with or without the different mature neurotrophins. BrdU labeling was analyzed by in-cell Western on the LiCor Odyssey. (a) GCPs with or without 20 ng/ml or 100 ng/ml of NGF. (b) GCPs with or without 20 ng/ml or 100 ng/ml of BDNF. (c) GCPs with or without 20 ng/ml or 100 ng/ml of NT3. (d) GCPs with or without 20 ng/ml or 100 ng/ml of NT4. Data are expressed as mean +/- SEM from three independent experiments. Asterisk indicates that Shh-treated cells increased BrdU incorporation compared to controls by ANOVA with Tukey’s posthoc analysis, p values are indicated below each graph.

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The following source data is available for figure 4:

Source data 1. Mean values for each experiment and statistical analysis for all graphs.

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Figure 5. ProNT3, but not proNGF or proBDNF, prevented Shh-induced proliferation of cultured GCPs. (a) GCPs were cultured from P7 rat cerebella with BrdU in the absence or presence of Shh with or without 4 or 8 ng/ml of proNGF, proBDNF or proNT3 for 48 hr. Cells were analyzed by in-cell Western for BrdU incorporation. (b) P7 rat GCPs were cultured for 48 hr with BrdU in the absence or presence of Shh, proNT3, or Shh + proNT3. Cells were fixed and immunostained for BrdU, and the number of labeled cells was counted, shown in the graph. (c) P7 rat GCPs were cultured without or with Shh, Shh+proNT3, or Shh+proNT3+anti-proNT3 for 48 hr and analyzed by in-cell Western for BrdU incorporation. Data in the graphs are expressed as mean values +/- SEM from at least 3 independent experiments. Asterisks indicate significance by ANOVA with Tukey’s posthoc analysis, with the indicated p value below each graph.

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The following source data is available for figure 5:

Source data 1. Mean values for each experiment and statistical analysis for all graphs in Figure 5.

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Figure 5—figure supplement 1. ProNT3 does not induce apoptosis of cerebellar neurons. Cerebellar neurons were cultured from P7 rat and treated overnight with proNT3. Cells were labeled with TUNEL and Dapi, and expressed as the ratio of TUNEL-positive cells to total cell number.

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Figure 5—figure supplement 2. Proneurotrophins induced death of hippocampal neurons. Hippocampal neurons were cultured from E18 rat embryos and treated overnight with 4 ng/ml of the indicated proneurotrophin, eliciting loss of approximately 30% of the cells, consistent with previous results (Friedman, 2000). After removal of the medium, cultured cells were lysed and intact nuclei were counted using a hemacytometer. Nuclei of dead cells either disintegrate, or if in the process of dying, appear pyknotic and irregularly shaped. In contrast, nuclei of healthy cells are phase bright and have clearly defined limiting membranes. Cell counts were performed in triplicate wells from 3 independent experiments. Data are expressed as the mean percentage of total surviving cells +/- SEM. Asterisks indicate values different from control at p<0.05 by ANOVA with Tukey's posthoc analysis. DOI: 10.7554/eLife.16654.013
Figure 6. ProNT3 requires p75NTR and SorCS2 to block Shh-induced GCP proliferation. (a) GCPs from wild type or Ngfr-/- mice were cultured with Shh, proNT3 or Shh+proNT3 for 48 hr in the presence of BrdU and analyzed by in-cell Western analysis of anti-BrdU. (b) Immunostaining for SorCS2 demonstrates expression of this co-receptor in the EGL, shown for P7, P10 and P14. Size bar indicates 10 μm. (c) GCPs from P7 rat were cultured with Shh, proNT3 and anti-SorCS2. Anti-SorCS2 reversed the effects of proNT3 on Shh-induced BrdU incorporation, but had no effect by itself or with either proNT3 or Shh alone. Data in the graphs are expressed as mean values +/- SEM from at least 3 independent experiments. Asterisks indicate significantly different from control by ANOVA with Tukey’s posthoc analysis, with p=0.0001. 
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The following source data is available for figure 6:

Source data 1. Mean values for each experiment and statistical analysis for graphs in 6A and 6c. 
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Figure 7. Expression and secretion of proNT3 in cerebellum. (a) Immunostaining of P7 and P14 rat cerebellum with an antibody to the pro domain of proNT3 shows the presence of proNT3 in Purkinje cells, labeled with calbindin. Note the abundant proNT3 labeling in the dendrites, especially apparent at P14. Nuclei labeled with Draq5 are shown in gray in the merged image. (b) Cultures of P7 rat cerebellum were treated with or without 25 mM KCl to depolarize the cells, and the media was analyzed by immunoprecipitation for NT3 followed by Western blot for proNT3, demonstrating that proNT3 can be secreted from cerebellar cells.

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Figure 7—figure supplement 1. Validation of the proNT3 antibody. Total brain lysates from Ntf3+/+, Ntf3+/-, and Ntf3-/- mice were immunoprecipitated with goat anti-proNT3 or goat control IgG, and probed with anti-proNT3 on a Western blot. ProNT3 produced from HEK cells was run for comparison. The proNT3 band is seen only in Ntf3+/+ and Ntf3+/- lysates IP’d with anti-proNT3. (b) Sections from P0 WT but not Ntf3-/- mice show immunostaining for proNT3 in cingulate cortex, as seen for NT3 mRNA by in situ hybridization (Friedman et al., 1991) and NT3-LacZ reporter expression (Vigers et al., 2000). DOI: 10.7554/eLife.16654.017
Figure 8. HDAC1 expression and regulation by Shh and proNT3. (a) Immunostaining for HDAC1 in WT and Ngfr-/- mice at P14. Size bar is 20 μm. (b) High magnification images of HDAC1 staining in the EGL in WT and Ngfr-/- mice at P14. Size bar is 10 μm. (c) Quantification of fluorescence intensity (mean gray value) of HDAC1 staining lobe 6b of the EGL at P14. *indicates significance at p=0.0024 by student’s t-test, 6 brains of each genotype were analyzed. (d) Western blot of cultured GCPs from P7 rat cerebellum treated as indicated and probed for HDAC1. (e) Quantification of Western blots from 3 independent experiments showing that treatment with Shh increased HDAC1 expression, which was reduced by proNT3 within 1 hr. ProNT3 alone had no effect on HDAC1 expression. * indicates significantly different from control at p=0.0034. (f) Regulation of Gli1 mRNA by Shh and Shh +proNT3. ** indicates significantly different from control, * indicates significantly different from Shh alone, p=0.004 by ANOVA with Tukey’s posthoc analysis.

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The following source data is available for figure 8:

Source data 1. Mean values for each experiment and statistical analysis for all graphs in Figure 8.

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Figure 9. Specific deletion of p75<sup>NTR</sup> from the EGL elicits increased GCP proliferation. (a) Ngfr<sup>fl/fl</sup> mice crossed with the Atoh1-Cre show lack of p75<sup>NTR</sup> specifically in the EGL while retaining p75<sup>NTR</sup> expression in Purkinje cells and meninges. Size bar indicates 20 µm. (b) Both Ngfr<sup>-/-</sup> and Ngfr<sup>fl/fl</sup>-Atoh1-Cre mice show increased Ki67 labeling in the EGL at P7, P10 and P14. Total nuclei labeled with Draq5 are shown in gray. Labeled cells were counted across 150 µm in the EGL of lobe 6b and are graphed relative to the total number of cells labeled with Draq5. (c) EdU labeling showing increased incorporation at P7, P10, and P14 in both Ngfr<sup>-/-</sup> and Ngfr<sup>fl/fl</sup>-Atoh1-Cre mice. Total nuclei labeled with Draq5 are shown in gray. Labeled cells were counted across 150 µm in the EGL of lobe 6b and are graphed relative to the total number of cells labeled with Draq5. At least three mice per genotype were analyzed at each age. Size bars in B and C indicate 10 µm. Data in all graphs are expressed as mean +/- SEM of at least three independent experiments. Asterisks indicate significantly different from WT by ANOVA with Tukey’s posthoc analysis with the p values below each graph.

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The following source data is available for figure 9:

Source data 1. Mean number of labeled cells of 3 slides for each animal, and statistical analysis for all graphs in Figure 9.

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Figure 10. The absence of \( p75^{\text{NTR}} \) during EGL development has persistent effects in the adult. (a) Cerebellar size of both \( \text{Ngfr}^{-/-} \) and \( \text{Ngfr}^{\text{fl/fl}}-\text{Atoh1-Cre} \) was increased in the adult compared to WT mice. * indicates significance at \( p=0.0003 \). (b) Motor balance on the rotarod was impaired in both \( \text{Ngfr}^{-/-} \) and \( \text{Ngfr}^{\text{fl/fl}}-\text{Atoh1-Cre} \). At least eleven mice per genotype were tested. Asterisk indicates significantly different from WT, # indicates \( \text{Ngfr}^{-/-} \) mice performed significantly worse than \( \text{Ngfr}^{\text{fl/fl}}-\text{Atoh1-Cre} \), \( p=0.0001 \) by ANOVA with Tukey’s posthoc analysis.

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The following source data is available for figure 10:

Source data 1. Mean values for each experiment and statistical analysis for all graphs in Figure 10.

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