Neurotrophic receptor p75^{NTR} mediates neuronal injury-associated synaptic dysfunction

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ABSTRACT

P75^{NTR} is a death receptor whose expression and interactions with adaptor proteins (TRAF6 and RIP2) is essential for the developing central nervous system (CNS) as they determine the proper neuronal architecture in the adult brain. P75^{NTR} is highly expressed during development but its level of expression is significantly downregulated in the adult CNS. However, neuronal injury or degeneration upregulates p75^{NTR}'s expression in adult nervous system including cerebellum. Given the numerous pathologies associated with cerebellar dysfunction, we aimed to determine the expression pattern of p75^{NTR} and, its downstream effector protein, TRAF6 in the adult brain and assess the number of synapses in the adult cerebellum in *ripk2*$^{-/-}$ transgenic mice following neurotoxin (cytosine arabinoside) induced neuronal injury using immunohistochemistry assays. Interestingly, we found that both p75^{NTR} and TRAF6 are expressed in the granular cell, Purkinje cell and molecular layers of the adult cerebellum and folia VI$_a$-VI$_b$, VII, VIII and X are among those displaying the highest levels of expression. Upon injury, we detected an increase in synaptic markers and synapses in *ripk2*$^{+/+}$ and *ripk2*$^{-/-}$. Additionally, impairment in the interaction between p75^{NTR} and RIP2 leads to an increase in synaptic markers in the adult cerebellum even in the absence of injury. These data suggest that p75^{NTR} play a role in modulation of synapses in adult cerebellum.

INTRODUCTION

Neurotrophins are growth factors that play a critical role in neuronal development, migration, survival, synaptic transmission and plasticity (1) and signal via neurotrophic receptor p75 (p75^{NTR}) and members A, B, and C of the Tropomyosin receptor kinase (1,2).

P75^{NTR} (also known as NGFR, TNFRSF16 and CD271) is a member of the tumor necrosis factor superfamily (TNFRSF) and its expression and interactions with other proteins is extremely important for the developing CNS as they determine the final number of neurons in the adult brain. Since p75^{NTR} lacks catalytic activity (3,4), it recruits intracellular components to play its role. Receptor-interacting protein 2 (RIP2) and TNF receptor associated factor 6 (TRAF6), for instance, are two well-characterized intracellular adaptor proteins that bind to p75^{NTR} and mediate p75^{NTR} signaling in the cerebellar granule neurons (CGNs).

P75^{NTR} downstream signaling cascades vary between different populations of de-

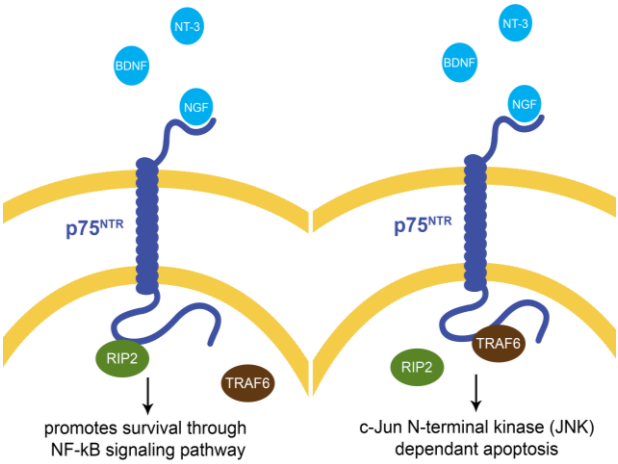

Figure 1. P75^{NTR}'s mechanism of action in the cerebellar granule layer. RIP2 and TRAF6 compete against each other for binding to two neighboring intracellular domains of p75^{NTR} (6). RIP2 binding to p75^{NTR} promotes survival through NF-kB signaling pathway whereas TRAF6 binding to p75^{NTR} leads to JNK-dependent apoptosis in these population of neurons (3,4).

veloping neurons. It has recently been shown, for example, that p75^{NTR} activation by neurotrophins, such as NGF, leads to apoptosis in hippocampal neurons (5) whereas neurotrophin binding to p75^{NTR} in the cerebellar granule neurons either promotes survival or induces apoptosis (6). Remarkably, it has been known since long time that the developing cerebellar granule neurons express and release large quantities of neurotrophins, such as Nerve Growth Factor (NGF) (5) but the underlying mechanism that determines the fate of CGN after NGF binding to p75^{NTR} was recently revealed; RIP2 and TRAF6 compete against each other for binding to the p75^{NTR} ICDs (6) (**Figure 1**).

As already mentioned, p75^{NTR} is widely expressed within the CNS during development but significantly downregulated in the adult CNS. However, injury or cellular stress to the adult nervous system often leads to reactivation of signaling pathways that are normally active during development and, as a matter of fact, upregulation of p75^{NTR}'s expression (**Figure 2**).

Although not responsible for movement initiation, cerebellum is involved in a variety of motor tasks, such as executive control, and cognitive functions, such as sensorimotor imagery, learning, memory (7) and time intervals' perception (8). Furthermore, the cerebellar granule neurons, on which we mainly focused in this study, control Purkinje cells' firing and play key role in acquisition and stabilization of motor learning and consolidation (9).

Based on the recent findings revealing the mechanism that determines p75^{NTR}-mediated response to neurotrophin binding in the developing cerebellum and the significance of p75^{NTR} upregulation in the CNS following seizures, lesions or other nervous damage, this study attempts to decipher the role of p75^{NTR} in the cerebellum of adult brain after injury. We examined the cerebella of mice lacking RIP2 after induced neuronal injury and found that p75^{NTR} plays a role in the modulation of synapses in the adult cerebellum, especially following neuronal damage.

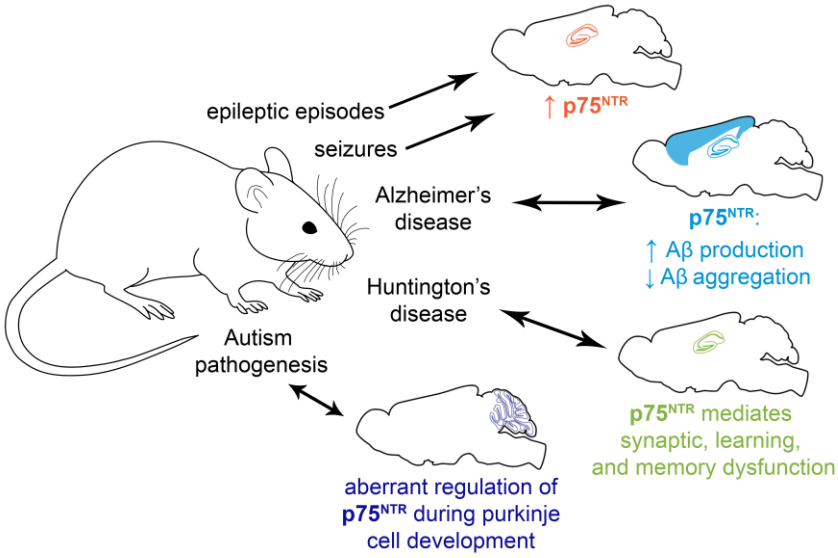

Figure 2. Role of p75^{NTR} in the injured brain. Several insults have been shown to upregulate p75^{NTR} expression; Hippocampal cells overexpress p75^{NTR} after epileptic episodes (17) or seizures (18). Furthermore, p75^{NTR} expression in adult brain has been associated with some neurodegenerative diseases such as Alzheimer (19) and Huntington's diseases (20). Given its critical role in development of the CNS, p75^{NTR} has been associated with some neurodevelopmental conditions as well; aberrant regulation of p75^{NTR} in Purkinje cell layer during development has been linked to autism pathogenesis (21).

MATERIALS AND METHODS

Tyramide Signal Amplification (TSA) protocol for p75^{NTR}, RIP2, TRAF6 Staining in adult mouse brain

Fixed wild type mouse brains were embedded in O.C.T. compound mounting medium for cryotomy (by VWR Chemicals), frozen overnight at -80 ^{o}C and sagitally sectioned in a Histology Cryotome at 20 μm. Sections were collected, washed in PBS and incubated in 0.3% H_2O_2 in PBS for 10 minutes.

Then, they were washed twice in Tris-NaCl-Tween buffer (TNT), blocked with 5% Normal Donkey Serum (NDS) containing 0.1% Triton in PBS for 1h and incubated overnight with primary antibodies at 4 ºC. Sections were washed in TNT and, at this point, samples incubated with primary antibodies against RIP2 were incubated with horseradish peroxidase (HRP) (1:1000) for 30 minutes in dark, washed in TNT and incubated for 10 minutes with Biotin + Tyramide amplification diluent (1:50). Next, all tissue sections were washed in TNT, incubated with appropriate secondary antibodies and DAPI for 2h in dark and washed in TNT once again. Finally, they were mounted on gelatin-coated slides and covered with a coverslip using a mounting medium.

Table 1. Dilutions of primary, secondary antibodies and DAPI used in the immunohistochemistry assays

Primary antibodies	Dilution
Goat antibody against $p75^{NTR}$	1:500
Mouse antibody against TRAF6	1:200
Guinea pig antibody against VGluT1	1:4000
Mouse antibody against PSD95	1:500
Rabbit antibody against Synaptophysin I	1:500
Secondary antibodies	
All secondary antibodies	1:1500
4′,6-diamidino-2-phenylindole (DAPI)	**1:1000**

Standard Immunostaining Protocol for the adult mouse brain

Fixed wild type mouse brains were embedded in O.C.T. compound mounting medium for cryotomy (by VWR Chemicals), frozen overnight at -80 ºC and sagittally sectioned

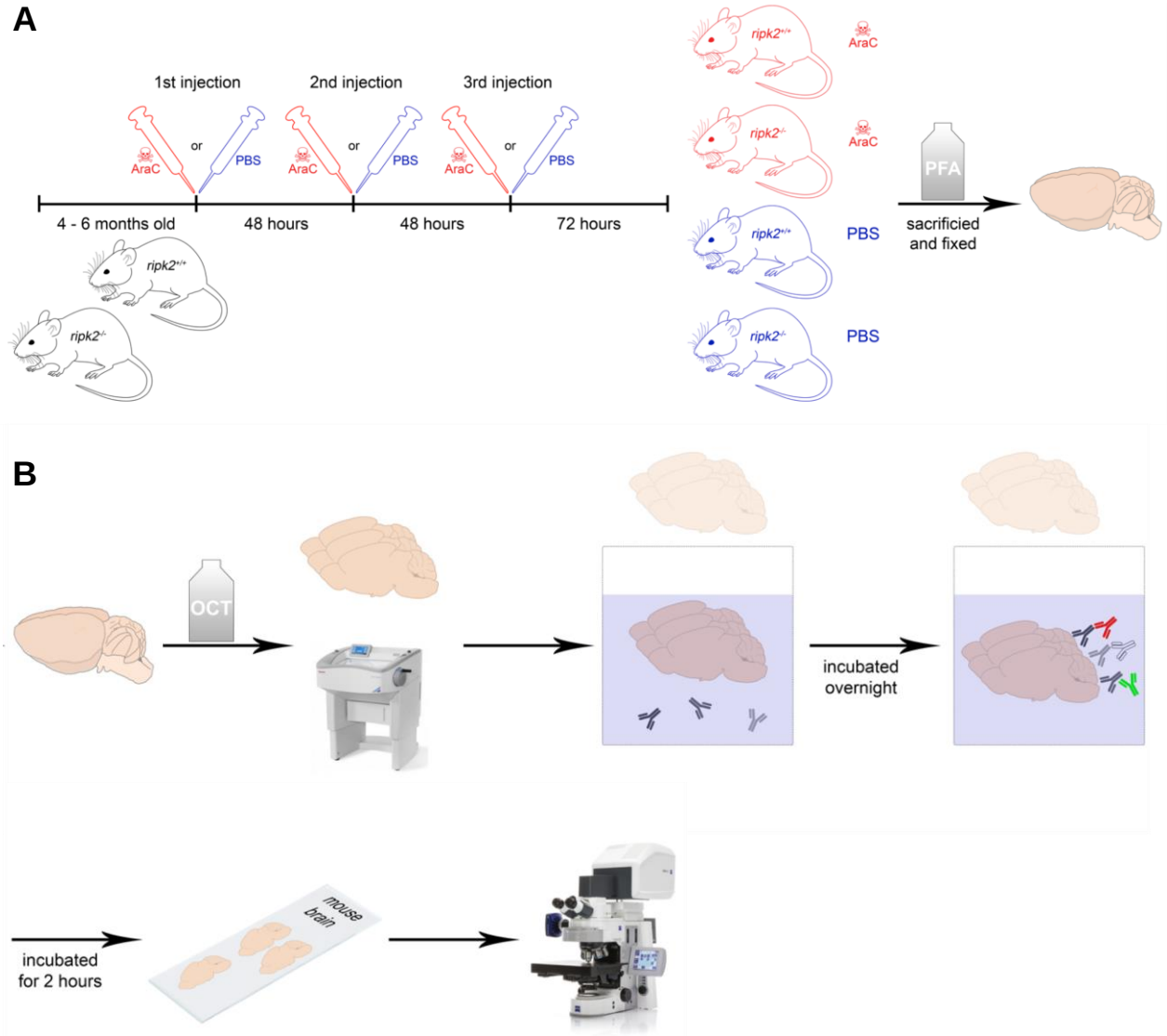

Figure 3. Experimental set-up. A. Experimental plan followed to generate the four animal groups (AraC and PBS treated *ripk2*$^{+/+}$ and *ripk2*$^{-/-}$ mice). B. Main steps of standard immunohistochemistry protocol: embed in OCT, cut sections in a cryotome, incubate with primary and secondary antibodies, mount sections on a slide, image slides using a confocal microscope.

in a Histology Cryotome at 25 μm for $p75^{NTR}$-TRAF6 staining and at 30 μm for the synaptic markers. Sections were collected, washed in PBS, blocked with 5% Normal Donkey Serum (NDS) containing 0.3% Triton in PBS for 1h and incubated overnight with primary antibodies (**Table 1**) at 4°C diluted in the blocking solution (1% NDS, 0.06% Triton). Then, sections were washed in PBS, incubated with appropriate secondary antibodies and DAPI (**Table 1**) for 2h in dark and washed in PBS once again. Finally, tissue sections were mounted on gelatin-coated slides and covered with a coverslip using a mounting medium.

Experimental set-up for the assessment of $p75^{NTR}$'s role in modulation of synapses in the adult cerebellum following induced neuronal injury

Wild type (*ripk2*$^{+/+}$) and global knock out (*ripk2*$^{-/-}$) 4 to 6-months-old mice were injected thrice with 400 mg/kg of either cytosine arabinoside (araC) to induce cerebellar injury (10,11) or PBS for the control group according to the experimental plan (**Figure 3**A). After 7 days from the first injection, mice were sacrificed and their brain was extracted and fixed in PFA. Then, fixed brains were stained for three synaptic markers: Vesicular Glutamate Transporter 1 (VGluT1), Postsynaptic Density Protein 95 (PSD95) and Synaptophysin 1 according to the Standard Immunohistochemistry Protocol (**Figure 3**B).

Image analysis and quantification

All slides were imaged with a Zeiss LSM confocal microscope. Images were analyzed using Fiji (ImageJ) version 2.0.0-rc-67/152e. We also made use of two Fiji (ImageJ) plugins; Image Stitching (12) was used for image mounting and Puncta analyzer v2.0 for synaptic dots quantification.

Statistical analysis

All tests were performed blind to the genotype. Data are expressed as mean value ± SE. Group comparisons were made using two-way ANOVA followed by Tukey's multiple comparison test. All data is included; no data points excluded unless significant outliers (positive for Grubb's test). Differences were considered significant when $p < 0.05$. Statistical analysis was performed and graphs were generated using Graphpad Prism 7.

RESULTS

Expression pattern of $p75^{NTR}$ and its intracellular effector protein, TRAF6, in the adult mouse cerebellum

We stained the cerebellum for $p75^{NTR}$ and TRAF6 and imaged it in sagittal midline sections of 3 and 6-months-old wild type mice and we found that both $p75^{NTR}$ and TRAF6 are present in the adult cerebellum. Notably, folia VI_a-VI_b, VII, VIII and X displayed the highest levels of $p75^{NTR}$ and TRAF6 expression both in 3-months-old (**Figure 4**) and in 6-months-old mice (data not shown). Additionally, we tried to stain for RIP2, the other intracellular effector protein, using the TSA protocol, but it did not work out (the antibody did not work, data not shown).

Assessment of $p75^{NTR}$'s role in modulation of synapses in the adult cerebellum after induced cerebellar injury

Firstly, quantification of Folia X's images of adult cerebellum stained for three synaptic markers, VGluT1 (excitatory pre-synaptic marker), PSD95 (excitatory post-synaptic marker) and Synaptophysin 1 (general pre-synaptic marker), showed that araC induced injury tends to increase the number of synapses in the cerebellum and the effect is greater in *ripk2*$^{+/+}$ compared to *ripk2*$^{-/-}$ mice both in the granular cell layer and in the molecular layer (**Figure 5**). Furthermore, *ripk2*$^{-/-}$ mice exhibited increased levels of synaptic markers compared to *ripk2*$^{+/+}$ mice in the absence of injury and, finally, the number of synaptic markers in the molecular layer (**Figure 5**A, C) was much higher than in the granular cell layer (**Figure 5**A, B).

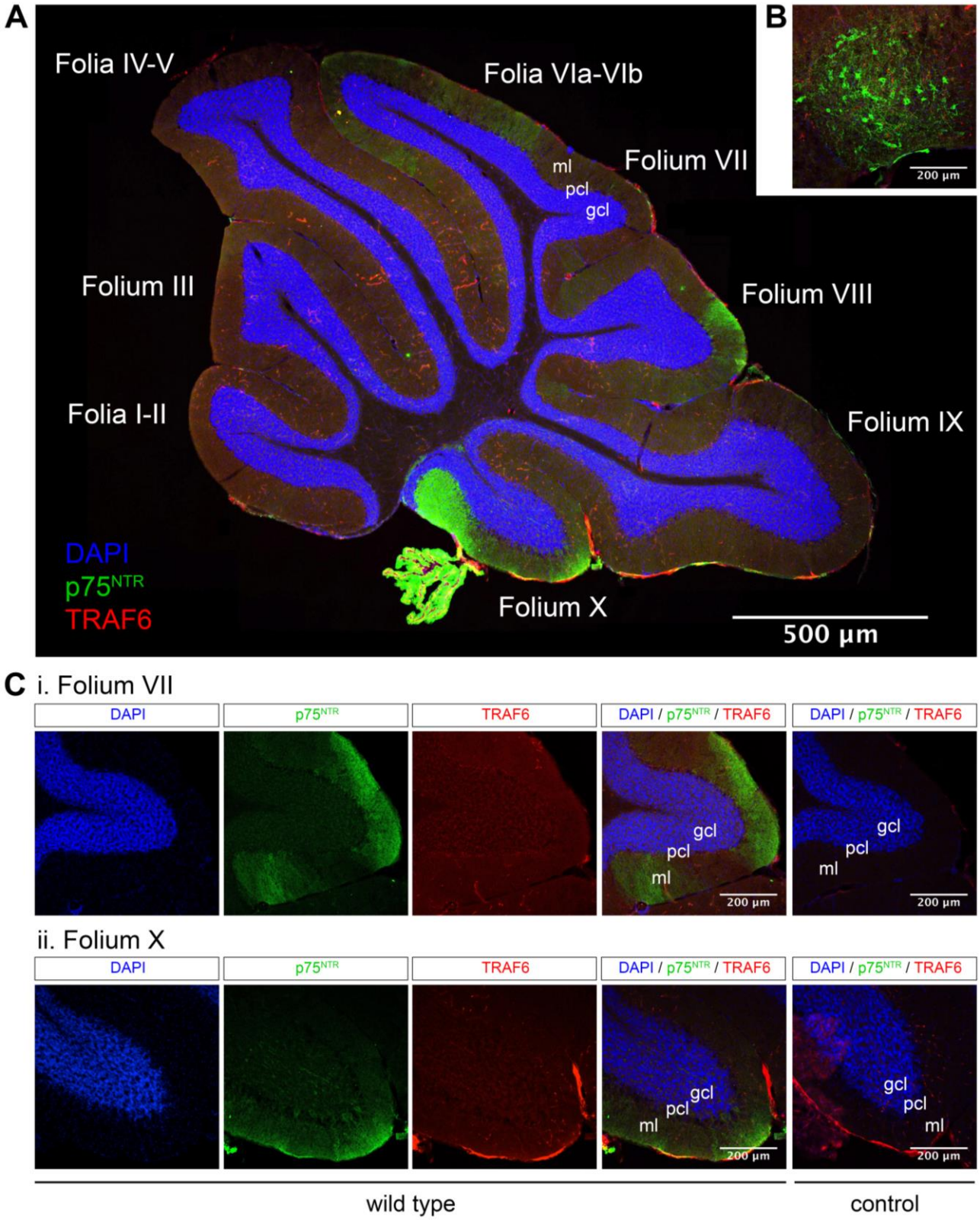

Figure 4. Expression pattern of p75^{NTR} and TRAF6 in the adult cerebellum. A. Expression pattern of p75^{NTR} and TRAF6 in the 3 months old adult mouse cerebellum. B. Basal forebrain cholinergic neurons express p75^{NTR} throughout life (18), that is why it was used as a positive control. C. Representative images of p75^{NTR} and TRAF6 expression in Folia VII and X, among folia that exhibit the highest levels of p75^{NTR} and TRAF6 expression. gcl: granular cell layer, pcl: purkinje cell layer, ml: molecular layer

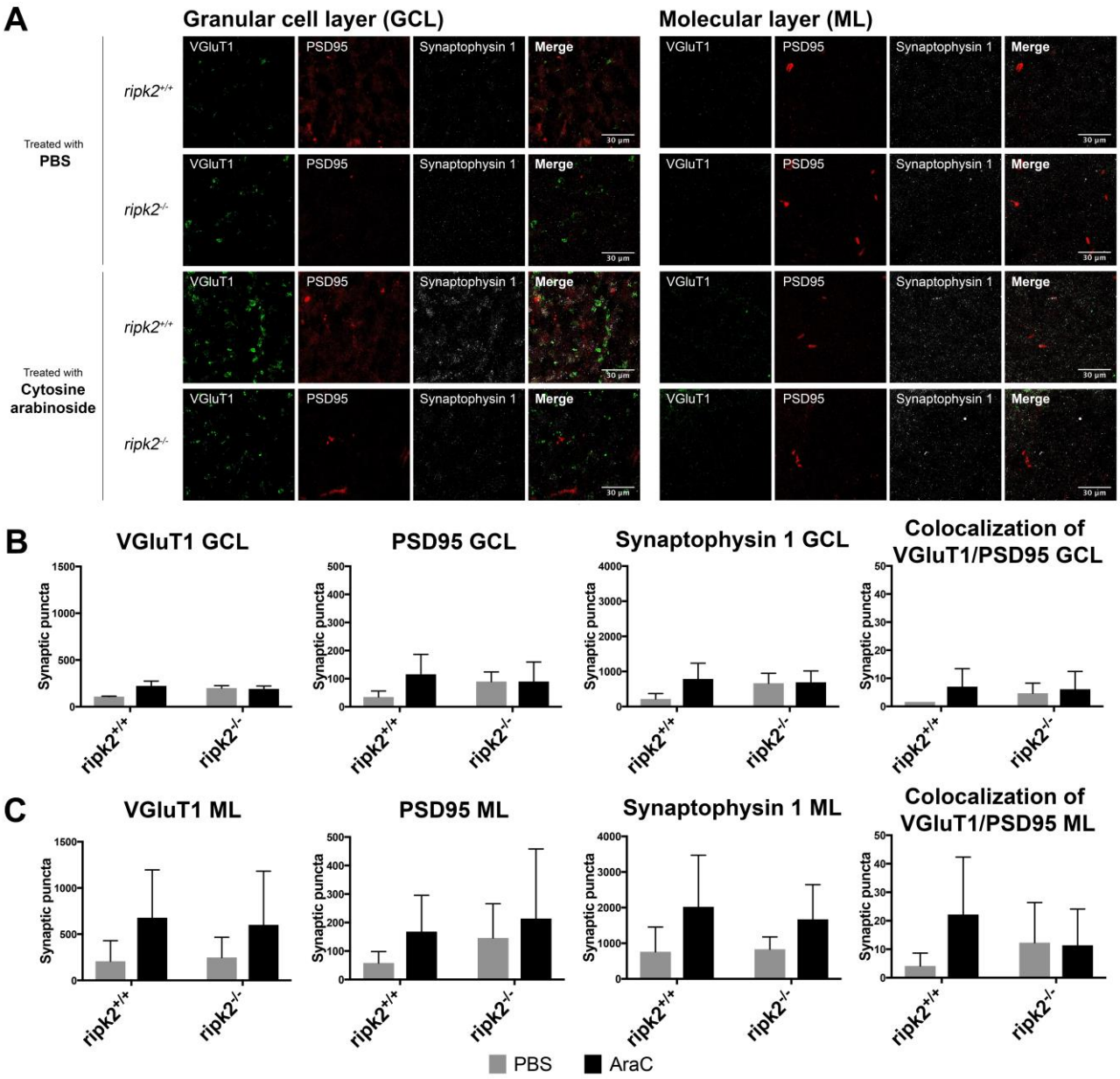

Figure 5. Images and quantification of synaptic markers in Folia X of the adult cerebellum after injury. A. Representative images of the synaptic markers from the granular cell (GCL) and the molecular layers (ML) of ripk2$^{+/+}$ and ripk2$^{-/-}$ mice treated with either AraC or PBS. B. Quantification of synaptic marker's images from the granular cell layer. C. Quantification of synaptic marker's images from the molecular layer. Results presented above indicate a trend but are not statistically significant ($p > 0.05$; two-way ANOVA followed by Tukey's multiple comparison test).

DISCUSSION

Notwithstanding much research on p75^{NTR}'s signaling pathways, our knowledge about the role of p75^{NTR} in the adult cerebellum remains scant. In this study, we determined the expression pattern of p75^{NTR} in the adult cerebellum and examined the impact of p75^{NTR} on the number of synapses in the normal and injured adult cerebellum.

Our data (**Figure 4**A) revealed the unique expression pattern of p75^{NTR} and TRAF6 in the adult cerebellum with folia VI$_a$-VI$_b$, VII, VIII and X being the ones displaying the highest levels of expression. This highly compartmentalized expression implies that certain neural structures express more p75^{NTR} and TRAF6 than others. Based on our current knowledge about the functional anatomy of the cerebellum (**Figure 6**), folia VI$_a$-VI$_b$, VII, VIII and X, as parts of the cerebrocerebellum and the vestibulocerebellum, are involved in movement planning and evaluation of sensory information for action and cerebellar cognitive functions, balance and eye movement. As a matter of fact, we

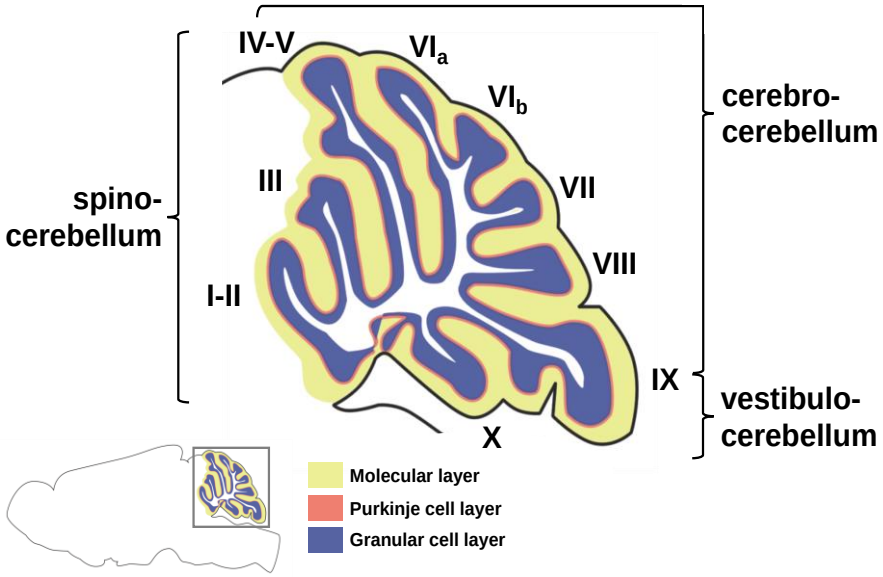

Figure 6. Functional anatomy of the cerebellum. Cerebellum consists of three main functional divisions: vestibulocerebellum (or Archicerebellum), spinocerebellum (or Paleocerebellum) and cerebrocerebellum (or neocerebellum) (22). A. Vestibulocerebellum is mainly involved in balance, spatial orientation and eye movement. B. Spinocerebellum regulates body and limb movements. C. Cerebrocerebellum evaluates sensory information regarding upcoming actions, participates in movement planning and in some cognitive functions.

propose that $p75^{NTR}$ and TRAF6 have a functional role in the adult cerebellum but this needs to be further investigated. As far as RIP2 is concerned, we were not able to stain for RIP2 in immunohistochemistry. However, it has been shown that *ripk2* mRNA is present in the adult cerebellum as well (6).

Furthermore, we assessed the number of synaptic markers in $ripk2^{+/+}$ and $ripk2^{-/-}$ mice and showed that impairment in the interaction between $p75^{NTR}$ and RIP2 simulated by knocking out *ripk2* gene (in $ripk2^{-/-}$ mice) leads to an increase in the synaptic markers both in the granular cell layer and the molecular layer of the cerebellum of PBS treated (control) mice (**Figure 5**B, C). Our findings are in good agreement with a previous suggestion that $p75^{NTR}$ plays an "anti-synapse" role in sympathetic neurons but insult or neuronal injury leads to reengagement of developmental synaptic programs modifying the number of synapses under normal physiological conditions in the adult brain (13).

Cytosine arabinoside at high concentrations induces apoptosis in cerebellar granule neurons (10,11). Thus, it was used to induce neuronal injury in a dose that was enough to trigger a signaling cascade yet not enough to widely kill the cerebellar granule neurons. Upon araC treatment, synaptic markers' expression marked an increase both in the granular cell (**Figure 5**B) and the molecular layer (**Figure 5**C) verifying *in vivo* the neuroprotective role of neurotrophin signaling against araC induced neuronal injury previously observed in immature cultured rat cerebellar granule neurons (14); We suggest that neurotoxin triggered a signaling cascade that led to synapse formation in order to promote survival.

Apart from the granular cell layer, where the granule neurons are located, we looked at the synaptic markers in the molecular layer, where they project, and found higher numbers of synaptic markers in the latter (**Figure 5**C). This finding was expected as granule neurons form excitatory synapses with Purkinje cell's rich dendritic trees and branches in the molecular layer.

Our findings imply that $p75^{NTR}$ plays a role in modification of cerebellar granule and Purkinje neurons synapses of the adult cerebellum and agree with similar findings concerning different brain regions; $p75^{NTR}$ modulates excitatory synapses in the hippocampus (15) and synaptic transmission in cultured rat cholinergic sympathetic neurons (16).

In summary, we described the compartmentalization of $p75^{NTR}$ and TRAF6 in the adult cerebellum and uncovered a role of $p75^{NTR}$ in the modulation of synapses suggesting that $p75^{NTR}$ death receptor has key functions in the adult cerebellum.

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