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Dissemination Level	
PU = Public PP = Restricted to other programme participants (incl. the Commission Services) RE = Restricted to a group specified by the consortium (incl. the Commission Services) CO = Confidential, only for members of the consortium (incl. the Commission Services)	PU

A. Abstract / Executive Summary

LENS developed a two-photon microscope combined with multi-photon nanosurgery to investigate the reactive plasticity of the central neuron system *in-vivo*. Plasticity in the central nervous system in response to injury is a complex process involving axonal remodeling regulated by specific molecular pathways. LENS dissected the role of the growth-associated protein-43 (GAP-43) in the axonal structural plasticity by using, as a model, the climbing fibers (CFs). Single axonal branches were ablated by laser axotomy, avoiding collateral damage to the adjacent dendrite and the formation of a glial scar. Despite the very small denervated area, the injured axons consistently reshape the connectivity with surrounding neurons. At the same time, adult CFs react by sprouting new branches through the intact surroundings. Newly formed branches presented varicosities suggesting that new axons were more than just exploratory sprouts. Correlative light and electron microscopy reveals that the sprouted branch contains large numbers of vesicles accumulated most in varicosities in the close vicinity of Purkinje dendrites. By using an RNA interference approach, we found that downregulating GAP-43 causes a significant increase in the turnover of presynaptic boutons. In addition, silencing hampers the generation of reactive sprouts. Our findings show the requirement of GAP-43 in sustaining synaptic stability and promoting the initiation of axonal regrowth, providing a molecular read-out for structural plasticity in the brain.

These results are accepted for publication in PNAS.

B. Deliverable Report

1 Introduction

The central nervous system (CNS) is capable of remodeling in response to various stimuli, like physiological experiences associated with adaptation, learning and memory or pathological insults such as traumatic injuries [1]. The ability of adult neurons of the CNS to regenerate their axons in response to injury is limited in many neuronal types depending on both intrinsic and extrinsic factors [2-7]. Since axons in the CNS represent a challenging site for targeted manipulation and *in vivo* imaging, little is still known about their post-lesional reactive plasticity and how this is regulated by molecular mediators. A full characterization of this dynamic process is a prerequisite for realizing successful brain repair.

We investigated the reactive plasticity of an axonal terminal arbor by using the climbing fibers (CFs) as a model. These axons were shown to retain a high regenerative potential also during adulthood [8-10]. The injury paradigms commonly used in previous studies, i.e. mechanical severing or chemical treatments, exhibit limited specificity while producing massive degeneration [11]. In addition, post-mortem analysis provides just snapshots of fixed tissue, not allowing an unambiguous distinction between regenerating and unaltered fibers at the lesion site [12, 13]. Alternatively, *in vitro* studies lack the complexity of environmental cues that modulate the axonal response to injury.

Modern optical techniques have the potential to overcome these limitations [7, 14, 15]. The spatial localization and deep penetration of multi-photon absorption [16] can be used as a tool to dissect single neurites in the brain of adult mice *in vivo* [17]. The severed neuron can be imaged by two-photon fluorescence (TPF) microscopy [18, 19], so that the reactive plasticity of the injured process can be monitored in optically accessible parts of the adult CNS *in vivo*. Based on these technologies, this study aims at disclosing the real-time structural dynamics and synaptic reorganization of a severed axons in the cerebellar cortex of adult mice, defining the timescale and extent of degeneration and remodeling *in vivo*. Here we show that our optical approach allows ablating a single axonal branch avoiding collateral damage to the adjacent dendrite and the formation of a glial scar. We find that despite the very small denervated area, laser axotomy on single branches triggers axonal sprouting while eliciting synaptic remodeling in the surviving portion of the axon. Correlative light and

electron microscopy reveals that the new varicosities formed on the sprouted branch lie next to Purkinje dendrites, and contain a large numbers of vesicles.

The plasticity of mature CFs has been frequently associated with the high basal expression of growth-associated proteins such as GAP-43 [8, 20]. The involvement of GAP-43 in neuronal structural plasticity was demonstrated in many studies based on gene depletion or over-expression of its wild-type or mutated variants in cultured cells and transgenic mice [21, 22]. GAP-43 overexpression is sufficient to induce neurite formation and axonal sprouting in different regions of adult CNS [21, 23, 24]. However the requirement of GAP-43 in both physiological and post-injury axonal dynamics has never been investigated. Since homozygous knock-out mice are affected by a very low-survival rate during early postnatal period (> 95%) [25, 26], we opted for an RNA-dependent gene-silencing approach. By delivering RNA-interfering lentiviral vectors *in vivo* we downregulated this gene specifically in CFs and without affecting brain development. We found that downregulating GAP-43 profoundly affects the stability of varicosities and largely prevents the generation of reactive sprouts, providing a molecular read-out for structural plasticity in the brain.

2 Results

Long term structural plasticity of CFs was investigated by performing time-lapse TPF imaging on the cerebellar cortex of adult mice. The CFs were labeled by the green fluorescent protein (GFP) following lentiviral injection in the IO. Three weeks after the injection, an optical window was chronically implanted on the cerebellar cortex. The viral injection produces a sparse labeling of CF, with variable levels of GFP expression. A representative in vivo TPF image of a CF is shown in Fig. 1A. The CF terminal arbor is made by ascending branches which are closely abutting the PC dendritic arbor, and transverse branches (TBs) which are emerging perpendicularly from the ascending branches [27]. As previously reported [28], the ascending branches displays a high stability throughout the observation period of several days (see Fig. 1B).

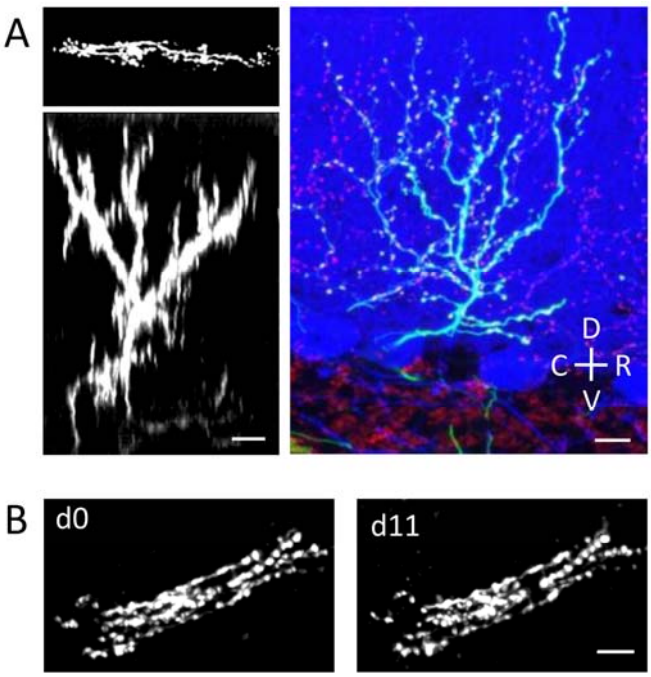

Figure 1: In vivo imaging of CFs. (A) The two panels on the left show the TPF transversal (maximum intensity z-projection of 60 images acquired from 0 to 120 µm deep below the pial surface) and sagittal view (digital rotation of the stack) of a single CF labeled by GFP expression in the cerebellar molecular layer. On the right panel, a confocal image of a single CF in a sagittal slice obtained from fixed cerebellum is shown for comparison. CFs were labeled by GFP expression (green); Purkinje cells

were labeled through immunofluorescent staining for Calbindin (in blue); CF varicosities in the molecular layer (together with some mossy fibers terminals in the granular layer) were labeled through immunofluorescent staining for VGlut2 (in red). D, dorsal; C, caudal; R, rostral; V, ventral. (B) Time-lapse images (TPF transversal view: maximum intensity z-projections) over a 12 days monitoring period showing the stability of CFs ascending branches.

Multi-photon laser axotomy was used to disrupt a single axonal branch. A highly localized damage was performed by irradiating a distal branch (ranging from 10 to 60 µm deep below the pia) of a labeled CF with a high energy dose of Ti:Sapphire laser. After laser irradiation the distal part of the lesioned axon undergoes a sequence of swelling, degeneration and disappearance (Fig. 2A). The specificity of this technique was explored by ablating an axonal branch and performing post-hoc immunostaining of the Purkinje cell (PC) dendrite adjacent to the axotomized CF. As shown in Fig. 2B we did not detect (N = 9) any visible degeneration of the PC dendrite in correspondence of the lesion site. Since during laser mediated brain injury microglial processes may rapidly converge on the site of injury [29, 30], we quantified the density of microglia one day after the lesion. We found that our injury paradigm allows dissecting single axonal branches without inducing significant gathering of microglial cells near the site of injury (Fig. 2C).

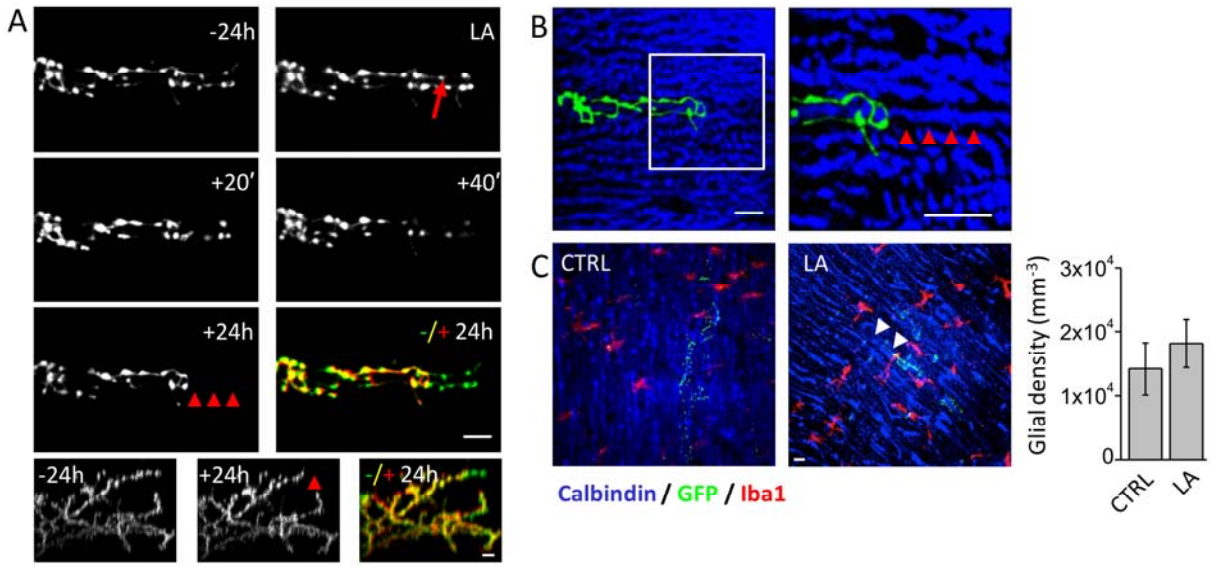

Figure 2: In vivo multi-photon laser ablation of single axons. (A) Time course of a distal branch of a CF (TPF transversal view: maximum intensity z-projections) before (-24h) and after laser axotomy (LA). The laser beam was focused on the axon where the red arrow points. The colored panel shows a superposition of the -24h (green) and +24h (red) frames. The red arrowheads at +24h and the temporal merge highlight the degeneration of distal portion. The bottom panels show sagittal views (digital rotation of the 3D TPF image) of the entire axonal arbor of the CF. (B) Confocal images of the same CF shown in panel (A) obtained from fixed cerebellum one day after laser axotomy. CFs (in green) are GFP labelled (maximum intensity z-projection from o to 88 µm below the pial surface); PCs (in blue) were labeled through immunofluorescent staining for Calbindin. The region in the white box of B is shown magnified 2× in the right panel (maximum intensity z-projection of 8 µm). The red arrowheads highlight the integrity of the PC dendritic arbor within the region of the laser focus. (C) Confocal images showing CFs (in green), PCs (in blue) and microglial cells labeled through immunostaining for Iba1 (in red) in a control region (CTRL) and around lesion site (LA). The white arrowheads in LA point at the region where the CF degenerated after laser axotomy. Scale bar, 10 µm. The graph on the right reports the density of microglial cells in CTRL (NCF = 9) and around the lesion site (LA, NCF = 8).

The ability of injured nerve cells to compensate for the synaptic loss by modifying their connectivity with surrounding neurons is investigated in terms of structural rearrangement of presynaptic boutons. After laser axotomy, the surviving portions of the main arbor show a significant increase in the fraction of dynamic varicosities, without an overall variation of their total number (Fig. 3A). The degeneration of CF distal portion may alter the interaction and signaling between PC and CF, triggering synaptic rewiring on the surviving portion of the CF. The CF arbor presents several transverse branches (TBs), thin filaments emerging perpendicularly from the main plane of the CF. The functional role of this pool of motile axons is still elusive. Previous studies proposed that dynamic TBs may be involved in regeneration or functional recovery [27, 28]. This suggestion was based on the analogies between the TBs and the axonal filaments, similarly poor in varicosities, present in CFs sprouting branches [9]. Our time-lapse observations show that in physiological conditions, TBs undergo rapid length changes in the time lapse of days (see Fig. 3B). Although the mean length and motility of the TBs protruding from the injured fiber are not affected by laser axotomy (Fig. 3B), CFs can react to injury by sprouting new transverse axonal branches a few days (1-4) after laser axotomy (Fig. 3C). The new sprouts are significantly longer and brighter than pre-existing TBs and do not stem from filaments present before axotomy. We find that sprouting of the injured CF can take place despite the very limited denervated area. No evident relation between the length of the degenerated axon and the length of the newly formed branch is observed.

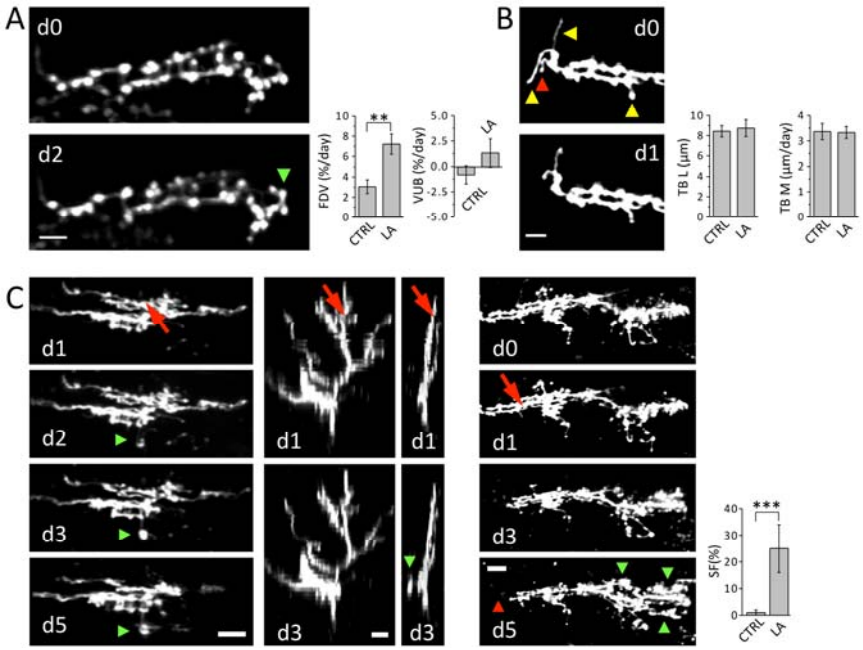

Figure 3: Reactive plasticity of CFs after laser axotomy (A) Time course of a portion of a CF showing the formation of a new varicosity (green arrowhead). The graphs compare average fractions of dynamic varicosities (FDV) and the varicosities unbalance (VUB) in control animals (FDV = 3.1 ± 0.7% per day; VUB = -0.79 ± 0.91% per day; NVar = 296, NCF = 6) and in CFs injured by LA (FDV = 7.2 ± 1.0% per day; VUB = 1.34 ± 1.39% per day; NVar = 433, NCF = 6). ** P < 0.01 (two-tailed t-test). (B) Time course of a portion of a CF displaying TBs disappearance (red arrowhead) and remodeling (yellow arrowhead). The graphs compare TBs length (TBL) and motility (TBM) in CTRL (TBL = 8.4 ± 0.5 µm; NTB = 62, NCF = 6; TBM = 3.4 ± 0.3 µm/day; NTB = 94, NCF = 6) and LA (TBL = 8.7 ± 0.8 µm; NTB = 55, NCF = 6; TBM = 3.3 ± 0.2 µm/day; NTB = 131, NCF = 6). (C) The images on the left column show the time course (from d1 to d5) of a CF after laser axotomy. The first image (d1) was acquired just before laser irradiation. The laser beam was focused on the axon where the red arrow points on d1. The green arrowheads at d2, d3 and d5 highlight the protrusion of a new branch. The images on the second and third columns show two orthogonal views (sagittal and coronal, respectively) of the same CF at d1 and d3. The right panel shows another example of laser induced

reactive plasticity. The first image (d0) was acquired one day before laser irradiation. The laser beam was focused on the axon at d1. The red and green arrowheads at d5 highlight the degeneration of distal portion and the protrusion of new branches, respectively. Scale bar, 15 μm. The graph compares the sprouting frequency (SF) in CTRL (SF = 1 ± 1%; NCF = 92, Nmice = 8) and LA (SF = 25 ± 9%; NCF = 24, Nmice = 15). *** P < 0.001.

3 Conclusions

We investigated the real-time dynamics of axonal remodeling after laser axotomy. The high selectivity of this injury paradigm has been demonstrated by disrupting a single axonal branch without perturbing the conjugated Purkinje cell dendrite. This highly localized damage does not trigger microglial migration towards the lesion site. Our optical approach thus allows the paired investigation of pre-and post-lesion axonal dynamics avoiding microglial activation and dendritic degeneration, in a myelin-free environment.

Previous works have shown that CFs are highly plastic and may expand or retract depending respectively on available denervated target or on target removal [8]. In this respect, we do not observe significant variation in the average length and motility of TBs in the injured axon, and at any time these filaments turned out to be buds of sprouted branches. Nevertheless, the surviving portion of the axotomized CF reacts by protruding new branches in the same direction of TBs. Indeed, the sprouted axon elongate in a region where PCs should be regularly innervated. We reconstructed the sprouted branch previously imaged *in vivo* with focused-ion beam/scanning electron microscopy. Three days after its appearance, the new axon contained the complete synaptic machinery. The newly formed varicosities lay next to Purkinje dendrites and gather a high density of vesicles, resembling developing sites for synapse formation.

A reshape in axonal connectivity is revealed by an increase in the turnover of varicosities on the portion of the injured CF not involved in remodeling events such as degeneration or sprouting. The focal lesion promotes synaptic reorganization on the entire CF, which possibly plays a compensatory role after damage.

Thus far we have shown that our approach can unravel the time-lapse dynamics of axonal degeneration, sprouting and synaptic remodeling. We wondered if one or few molecular promoters intrinsic to the CF were responsible for activating the sprouting program. The intrinsic ability of CFs to regrow has been often associated with the growth-associated protein GAP-43. Our *in vivo* observations in normally developed animals show that downregulating GAP-43 profoundly affects the stability of varicosities, eliciting an overall increase in the fraction of dynamic varicosities. This is consistent with the proposed role played by GAP-43 in the adult CNS in neurotransmitter release and synaptic plasticity [31-38].

The requirement of GAP-43 in axonal plasticity was further demonstrated in our injury paradigm. Although the average motility of TBs is not affected, nor we observe a general atrophy of these structures, downregulation of GAP-43 prevents the generation of reactive sprouts in laser axotomized CFs. Our results suggest that GAP-43 mediates the initiation of post-injury axonal outgrowth.

Axonal degeneration and modifications in GAP-43 expression profiles are associated with a plethora of neurological diseases, including amyotrophic lateral sclerosis, multiple sclerosis, epilepsy, diabetic neuropathy, schizophrenia and Alzheimer's and Parkinson's diseases [39-43]. In this respect, the results and techniques presented here may be helpful in assessing and validating new therapeutic strategies to prevent degeneration and promote axonal regrowth.

4 References/Publications

[1] D. V. Buonomano and M. M. Merzenich, "Cortical plasticity: from synapses to maps," *Annu Rev Neurosci,* vol. 21, pp. 149-86, 1998.

[2] M. H. Tuszynski and O. Steward, "Concepts and methods for the study of axonal regeneration in the CNS," *Neuron,* vol. 74, pp. 777-91, Jun 7 2012.

[3] P. J. Horner and F. H. Gage, "Regenerating the damaged central nervous system," *Nature,* vol. 407, pp. 963-70, Oct 26 2000.

[4] W. D. Snider, F. Q. Zhou, J. Zhong, and A. Markus, "Signaling the pathway to regeneration," *Neuron,* vol. 35, pp. 13-6, Jul 3 2002.

[5] F. Rossi, L. Wiklund, J. J. van der Want, and P. Strata, "Reinnervation of cerebellar Purkinje cells by climbing fibres surviving a subtotal lesion of the inferior olive in the adult rat. I. Development of new collateral branches and terminal plexuses," *J Comp Neurol,* vol. 308, pp. 513-35, Jun 22 1991.

[6] A. L. Hawthorne*, et al.*, "The unusual response of serotonergic neurons after CNS Injury: lack of axonal dieback and enhanced sprouting within the inhibitory environment of the glial scar," *Journal of Neuroscience,* vol. 31, pp. 5605-16, Apr 13 2011.

[7] M. Kerschensteiner, M. E. Schwab, J. W. Lichtman, and T. Misgeld, "In vivo imaging of axonal degeneration and regeneration in the injured spinal cord," *Nat Med,* vol. 11, pp. 572-7, May 2005.

[8] D. Carulli, A. Buffo, and P. Strata, "Reparative mechanisms in the cerebellar cortex," *Prog Neurobiol,* vol. 72, pp. 373-98, Apr 2004.

[9] F. Rossi, J. J. L. Vanderwant, L. Wiklund, and P. Strata, "Reinnervation of Cerebellar Purkinje-Cells by Climbing Fibers Surviving a Subtotal Lesion of the Inferior Olive in the Adult-Rat .2. Synaptic Organization on Reinnervated Purkinje-Cells," *Journal of Comparative Neurology,* vol. 308, pp. 536-54, Jun 22 1991.

[10] F. Rossi, L. Wiklund, J. J. L. Vanderwant, and P. Strata, "Reinnervation of Cerebellar Purkinje-Cells by Climbing Fibers Surviving a Subtotal Lesion of the Inferior Olive in the Adult-Rat .1. Development of New Collateral Branches and Terminal Plexuses," *Journal of Comparative Neurology,* vol. 308, pp. 513-35, Jun 22 1991.

[11] A. Buffo, M. Fronte, A. B. Oestreicher, and F. Rossi, "Degenerative phenomena and reactive modifications of the adult rat inferior olivary neurons following axotomy and disconnection from their targets," *Neuroscience,* vol. 85, pp. 587-604, Jul 1998.

[12] W. B. J. Cafferty, A. W. Mcgee, and S. M. Strittmatter, "Axonal growth therapeutics: regeneration or sprouting or plasticity?," *Trends Neurosci,* vol. 31, pp. 215-20, May 2008.

[13] O. Steward, B. Zheng, and M. Tessier-Lavigne, "False resurrections: distinguishing regenerated from spared axons in the injured central nervous system," *J Comp Neurol,* vol. 459, pp. 1-8, Apr 21 2003.

[14] D. H. Bhatt, S. J. Otto, B. Depoister, and J. R. Fetcho, "Cyclic AMP-induced repair of zebrafish spinal circuits," *Science,* vol. 305, pp. 254-8, Jul 9 2004.

[15] T. Misgeld and M. Kerschensteiner, "In vivo imaging of the diseased nervous system," *Nat Rev Neurosci,* vol. 7, pp. 449-63, Jun 2006.

[16] W. R. Zipfel, R. M. Williams, and W. W. Webb, "Nonlinear magic: multiphoton microscopy in the biosciences," *Nature Biotechnology,* vol. 21, pp. 1369-77, Nov 2003.

[17] L. Sacconi*, et al.*, "In vivo multiphoton nanosurgery on cortical neurons," *J Biomed Opt,* vol. 12, p. 050502, Sep-Oct 2007.

[18] F. Helmchen and W. Denk, "Deep tissue two-photon microscopy," *Nat Methods,* vol. 2, pp. 932-40, Dec 2005.

[19] K. Svoboda and R. Yasuda, "Principles of two-photon excitation microscopy and its applications to neuroscience," *Neuron,* vol. 50, pp. 823-39, Jun 15 2006.

[20] L. Kruger, C. Bendotti, R. Rivolta, and R. Samanin, "Distribution of GAP-43 mRNA in the adult rat brain," *J Comp Neurol,* vol. 333, pp. 417-34, Jul 15 1993.

[21] A. Buffo*, et al.*, "Targeted overexpression of the neurite growth-associated protein B-50/GAP-43 in cerebellar Purkinje cells induces sprouting after axotomy but not axon regeneration into growth-permissive transplants," *Journal of Neuroscience,* vol. 17, pp. 8778-91, Nov 15 1997.

[22] M. I. Mosevitsky, "Nerve ending "signal" proteins GAP-43, MARCKS, and BASP1," *Int Rev Cytol,* vol. 245, pp. 245-325, 2005.

[23] L. Aigner*, et al.*, "Overexpression of the neural growth-associated protein GAP-43 induces nerve sprouting in the adult nervous system of transgenic mice," *Cell,* vol. 83, pp. 269-78, Oct 20 1995.

[24] Y. Zhang*, et al.*, "Growth-associated protein GAP-43 and L1 act synergistically to promote regenerative growth of Purkinje cell axons in vivo," *Proc Natl Acad Sci U S A,* vol. 102, pp. 14883-8, Oct 11 2005.

[25] D. L. Maier*, et al.*, "Disrupted cortical map and absence of cortical barrels in growth-associated protein (GAP)-43 knockout mice," *Proc Natl Acad Sci U S A,* vol. 96, pp. 9397-402, Aug 3 1999.

[26] S. M. Strittmatter*, et al.*, "Neuronal pathfinding is abnormal in mice lacking the neuronal growth cone protein GAP-43," *Cell,* vol. 80, pp. 445-52, Feb 10 1995.

[27] I. Sugihara, H. Wu, and Y. Shinoda, "Morphology of single olivocerebellar axons labeled with biotinylated dextran amine in the rat," *J Comp Neurol,* vol. 414, pp. 131-48, Nov 15 1999.

[28] H. Nishiyama, M. Fukaya, M. Watanabe, and D. J. Linden, "Axonal motility and its modulation by activity are branch-type specific in the intact adult cerebellum," *Neuron,* vol. 56, pp. 472-87, Nov 8 2007.

[29] D. Davalos*, et al.*, "ATP mediates rapid microglial response to local brain injury in vivo," *Nature neuroscience,* vol. 8, pp. 752-8, Jun 2005.

[30] A. Nimmerjahn, F. Kirchhoff, and F. Helmchen, "Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo," *Science,* vol. 308, pp. 1314-8, May 27 2005.

[31] L. V. Dekker*, et al.*, "Inhibition of Noradrenaline Release by Antibodies to B-50 (Gap-43)," *Nature,* vol. 342, pp. 74-6, Nov 2 1989.

[32] R. L. Neve*, et al.*, "The neuronal growth-associated protein GAP-43 interacts with rabaptin-5 and participates in endocytosis," *Journal of Neuroscience,* vol. 18, pp. 7757-67, Oct 1 1998.

[33] T. Haruta*, et al.*, "Ca2+-dependent interaction of the growth-associated protein GAP-43 with the synaptic core complex," *Biochem J,* vol. 325, pp. 455-63, Jul 15 1997.

[34] B. M. Riederer and A. Routtenberg, "Can GAP-43 interact with brain spectrin?," *Mol Brain Res,* vol. 71, pp. 345-8, Aug 25 1999.

[35] C. Gianotti, M. G. Nunzi, W. H. Gispen, and R. Corradetti, "Phosphorylation of the Presynaptic Protein B-50 (Gap-43) Is Increased during Electrically Induced Long-Term Potentiation," *Neuron,* vol. 8, pp. 843-8, May 1992.

[36] G. M. J. Ramakers*, et al.*, "Temporal Differences in the Phosphorylation State of Presynaptic and Postsynaptic Protein-Kinase-C Substrates B-50/Gap-43 and Neurogranin during Long-Term Potentiation," *J Biol Chem,* vol. 270, pp. 13892-8, Jun 9 1995.

[37] G. M. J. Ramakers, R. K. McNamara, R. H. Lenox, and P. N. E. De Graan, "Differential changes in the phosphorylation of the protein kinase C substrates myristoylated alanine-rich C kinase substrate and growth-associated protein-43/B-50 following Schaffer collateral long-term potentiation and long-term depression," *J Neurochem,* vol. 73, pp. 2175-83, Nov 1999.

[38] G. Grasselli, G. Mandolesi, P. Strata, and P. Cesare, "Impaired Sprouting and Axonal Atrophy in Cerebellar Climbing Fibres following In Vivo Silencing of the Growth-Associated Protein GAP-43," *Plos One,* vol. 6, Jun 10 2011.

[39] L. Conforti, R. Adalbert, and M. P. Coleman, "Neuronal death: where does the end begin?," *Trends Neurosci,* vol. 30, pp. 159-66, Apr 2007.

[40] M. C. Raff, A. V. Whitmore, and J. T. Finn, "Neuroscience - Axonal self-destruction and neurodegeneration," *Science,* vol. 296, pp. 868-71, May 3 2002.

[41] G. B. Stokin*, et al.*, "Axonopathy and transport deficits early in the pathogenesis of Alzheimer's disease," *Science,* vol. 307, pp. 1282-8, Feb 25 2005.

[42] A. B. Oestreicher*, et al.*, "B-50, the growth associated protein-43: modulation of cell morphology and communication in the nervous system," *Prog Neurobiol,* vol. 53, pp. 627-86, Dec 1997.

[43] C. E. Teunissen*, et al.*, "Growth-associated protein 43 in lesions and cerebrospinal fluid in multiple sclerosis," *Neuropathol Appl Neurobiol,* vol. 32, pp. 318-31, Jun 2006.