

● PERSPECTIVE

## Considering calcium-binding proteins in invertebrates: multi-functional proteins that shape neuronal growth

Calcium is a critical second messenger molecule in all cells and is vital in neurons for synaptic transmission. Given this importance, calcium ions are tightly controlled by a host of molecular players including ion channels, sensors, and buffering proteins. Calcium can act directly by binding to signaling molecules or calcium's effects can be indirect, for example by altering nuclear histones which can lead to changes in gene transcription (Rishal and Fainzilber, 2014). All of these mechanisms come into play in developing axons as calcium is required for both axon pathfinding and branching. Furthermore, after neuronal injury, waves of calcium originating at the site of axon segmentation and propagating to the nucleus have long been known to be required for regeneration (Rishal and Fainzilber, 2014). These changes in intracellular calcium concentrations  $[Ca^{2+}]_i$  must be properly controlled or else new growth cones may fail to form and degeneration of the neuron may occur. While many of the molecular players involved in these calcium-dependent processes have been identified, calcium buffering proteins have often been undervalued for their role in regulating axon growth either during normal development or in the event of injury.

The EF-hand domain is the hallmark motif of proteins capable of binding calcium. Calcium-binding proteins typically contain one or more EF-hand domains though the local peptide context of each domain can alter their binding affinity and kinetics. In mammals, a small family of related calcium-binding proteins consisting of calbindin-D28k (CB), calretinin (CR), and secretogogin (SG), each contain 6 EF-hand domains. While less is known about the buffering function of SG; CR and CB have well-defined calcium buffering capacities (Schwaller et al., 2002). These proteins each have  $Ca^{2+}$  dissociation constants in the range of several hundred nanomolar. Since cellular calcium levels are typically below 100 nM, these buffers likely function during times of neuronal stimulation when calcium concentrations are increased due to calcium influx and calcium release from internal storage sites. Interestingly, mice deficient for CB or CR suffer from motor coordination defects which suggests that these proteins provide some fundamental support to neuronal circuit development (Airaksinen et al., 1997; Schiffmann et al., 1999). Despite extensive knowledge of the CB/CR/SG family's calcium buffering capacity from animal studies, little is known about their function in neuronal growth and development.

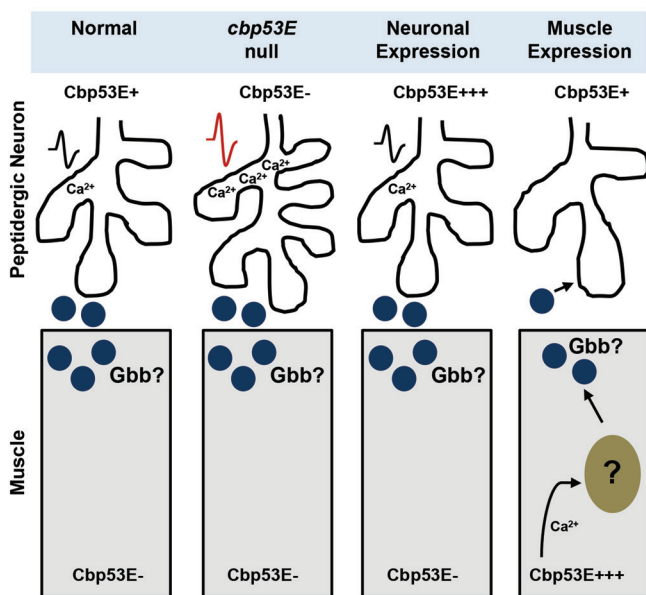
We have recently exploited the model organism *Drosophila* to investigate the role of calcium buffers in axon growth (Hagel et al., 2015). *Drosophila* have been used for decades to uncover genes involved in human development and there are now well established paradigms in *Drosophila* to study the dynamics of axon growth during development as well as after injury. We have focused on the CB/CR/SG family of proteins because unlike in mammals, the *Drosophila* genome encodes only a single protein with 6 EF-hand domains: Cbp53E (Reifegerste et al., 1993). Cbp53E is likely the evolutionary founder of the vertebrate CB/CR/SG calcium-binding family. Indeed, Cbp53E is nearly equally identical at the protein sequence level to all three mammalian family members. While empirical data must be acquired to conclude that the calcium-binding function of the mammalian family has been evolutionarily conserved in the *Drosophila* protein, the high sequence homology to well-characterized calcium-binding proteins suggests that speculation on this function is reasonable.

Like the CB/CR/SG proteins in the vertebrate brain, Cbp53E can be detected by immunofluorescence in a discrete but broad pattern

throughout the *Drosophila* central nervous system (Hagel et al., 2015). In addition, expression of Cbp53E is specifically enriched in larval axons leading out to the periphery. Interestingly, the synaptic localization of Cbp53E is dramatically specific at the larval neuromuscular junction (NMJ). Cbp53E expression is strongly detected in type II and type III peptidergic synapses at the NMJ, but not at all at type I glutamatergic synapses. Despite this unique synaptic localization pattern, *cbp53E* null mutants exhibit excessive axon branching in both classes of NMJ terminals. These results suggest that Cbp53E may function *via* two distinct mechanisms depending upon the synaptic properties of the neuron—a synaptic mechanism in peptidergic circuits, and a somatic mechanism in glutamatergic circuits. Further supporting this notion is the finding that postsynaptic overexpression of Cbp53E has no effect on glutamatergic axon terminal development, but results in a decreased growth of peptidergic NMJ terminals. Thus, the axon complexity of neurons with peptidergic synapses seems to be bidirectionally controlled by Cbp53E, while glutamatergic neurons are only sensitive to loss of Cbp53E.

It is also noteworthy that the reduced branching of type II and type III axon terminals is caused exclusively by postsynaptic muscle overexpression of Cbp53E (Hagel et al., 2015). Presynaptic neuronal overexpression has no effect on either peptidergic or glutamatergic NMJ's. Nevertheless, Cbp53E is functional in both compartments as both neuronal and muscle expression of Cbp53E is able to rescue all NMJ axon branching defects in *cbp53E* null animals. These results are seen despite the fact that endogenous Cbp53E is not detectable in the muscle. It is not yet clear what functional properties of Cbp53E may account for these differences in axon growth. Based on the calcium buffering properties of the mammalian CB/CR proteins, loss of function of Cbp53E is predicted to increase  $[Ca^{2+}]_i$  levels. Since CB/CR function primarily during neuronal activity,  $Ca^{2+}$  influx may also be affected by the presence or absence of Cbp53E. Calcium transients are tightly regulated and play important roles in shaping axonal architecture during normal development (Hutchins and Kalil, 2008). In mammals, augmented calcium transients correlate with changes in the size and shape of dendritic spines in CB knockout mice, indicating that these buffers can shape neuronal structure (Vecellio et al., 2000). Interestingly, in our targeted expression experiments, the NMJ system exhibited an unbalanced expression of Cbp53E on either side of the synapse, suggesting that the mechanism of action may be more complex than just changes in  $[Ca^{2+}]_i$  flux (Figure 1). The fact that Cbp53E can function in the muscle to affect the branching of the innervating neuron might point toward other muscle specific factors which are able to interact with Cbp53E. If this is true, then an important function of Cbp53E may be as a calcium sensor.

Calcium sensors are proteins that undergo conformational changes in response to changes in calcium concentration to enable binding to effector proteins. In mammals, the CB/CR/SG family of proteins is known to function as both calcium buffers and sensors (Schwaller, 2010; Alpar et al., 2012). Some of the known binding partners for the mammalian proteins include Ran binding proteins, voltage gated calcium channels and synaptic vesicle proteins involved in exocytosis. The full extent of signaling pathways affected by these interactions however, is unknown. Since Cbp53E is equally identical to all three vertebrate family members, the diversity in calcium sensor capacity may be consolidated in this evolutionary ortholog. It is possible therefore that multiple functions of Cbp53E are invoked in different scenarios (Figure 1). For example, presynaptic Cbp53E may buffer  $[Ca^{2+}]_i$  above a certain concentration such that excessive Cbp53E buffering does not adversely affect calcium transients and thus has no effect on axon growth. The postsynaptic effects on axon branching, however, may be elicited through Cbp53E binding to and altering the function of a component of retrograde signaling. Glass bottom boat (*gbb*) is the *Drosophila* ortholog of mammalian bone morphogenic protein (BMP) and is known to be secreted from the muscle to control neuronal growth (McCabe et al., 2003). Changes in calcium homeostasis induced



**Figure 1** Possible mechanisms of Cbp53E function in peptidergic neurons at the *Drosophila* NMJ.

Cbp53E is not normally expressed in muscle cells, so homeostasis is maintained by presynaptic expression of Cbp53E and other natural factors. In *cbp53E* null animals, presynaptic loss of Cbp53E may lead to increased calcium transients during neuronal activity which results in increased neuronal complexity. Presynaptic overexpression of Cbp53E has no effect on NMJ growth since calcium transients are properly controlled above a certain concentration by Cbp53E and other intrinsic neuronal factors. Muscle overexpression of Cbp53E, however, may invoke a Cbp53E calcium sensor function by binding factors which moderate retrograde signals such as Gbb/BMP to shape development of the innervating neuron. BMP: Bone morphogenetic protein; Gbb: glass bottom boat; NMJ: neuromuscular junction.

by Cbp53E could affect this process despite Cbp53E not normally being present in the muscle. In this way, both the calcium buffering and sensor functions of this protein may help determine the complexity of the NMJ.

In addition to shaping developing axons, calcium influx is also a vital component of axon degeneration and regrowth after neuronal injury. Interestingly, a hypomorphic allele of Cbp53E was examined in a *Drosophila* olfactory model of Wallerian degeneration and showed a delay in the degeneration of CNS axons (Avery et al., 2012). After axotomy, the axon cytoskeleton is normally destroyed through calcium-dependent proteases. If Cbp53E normally functions to dampen calcium currents, then Cbp53E functional hypomorphs would be expected to accelerate this process. The discrepancy between the experimental finding and the expected calcium buffering and/or sensor capacities of Cbp53E may highlight a difference between central and peripheral neurons. Peripheral circuitry has a much higher capacity for regeneration after injury than do central neurons. Perhaps Cbp53E binds different intrinsic factors in the CNS and PNS as speculated at the NMJ. Given the unique synaptic distribution of Cbp53E at the NMJ, these factors may even be localized differently within a single neuron such that loss of Cbp53E may delay distal axon degradation but still promote growth in the proximal compartment. Using a peripheral nerve injury model in *cbp53E* null animals and examining both axon degradation and regrowth in the same system will be a critical first step to teasing apart these mechanisms.

Understanding the exact molecular and cellular context of Cbp53E will be necessary for determining how it functions in normal neuronal development and acute axonal injury. Work in *Drosophila* is starting to uncover both pre- and postsynaptic mechanisms which may also be important in the mammalian CB/CR/SG orthologs. The speed, efficiency, and genetic malleability of the

*Drosophila* model strongly warrant its continued use to investigate this family of calcium-binding proteins. Indeed, *Drosophila* may also be an ideal system to conduct investigations into the utility of these proteins in altering axon growth and development in different diseases. Due to the strong evolutionary conservation of gene function, many researchers are now exploiting the benefits of *Drosophila* to model human diseases and to manipulate genes to rescue disease phenotypes. As such, Cbp53E and the mammalian counterparts may be molecularly engineered to enhance calcium-binding affinities and kinetics, or to anchor these proteins to sites of injury or specific neuronal compartments. These manipulations can be done to achieve specific phenotypic responses in individual disease states, but can also be used to shed light on basic mechanisms of neuronal growth and development. Using a complex organism such as *Drosophila* for these strategies will continue to provide great insight into these important multi-functional proteins.

Charles R. Tessier\*

Department of Medical and Molecular Genetics, Indiana University School of Medicine-South Bend, South Bend, IN, USA

\*Correspondence to: Charles R. Tessier, Ph.D., [crtesie@iupui.edu](mailto:crtesie@iupui.edu).

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orcid: 0000-0001-8931-6834 (Charles R. Tessier)

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## References

- Airaksinen MS, Eilers J, Garaschuk O, Thoenen H, Konnerth A, Meyer M (1997) Ataxia and altered dendritic calcium signaling in mice carrying a targeted null mutation of the calbindin D28k gene. *Proc Natl Acad Sci U S A* 94:1488-1493.
- Alpar A, Attems J, Mulder J, Hokfelt T, Harkany T (2012) The renaissance of Ca<sup>2+</sup>-binding proteins in the nervous system: secretagogin takes center stage. *Cell Signal* 24:378-387.
- Avery MA, Rooney TM, Pandya JD, Wishart TM, Gillingwater TH, Geddes JW, Sullivan PG, Freeman MR (2012) WldS prevents axon degeneration through increased mitochondrial flux and enhanced mitochondrial Ca<sup>2+</sup> buffering. *Curr Biol* 22:596-600.
- Hagel KR, Beriont J, Tessier CR (2015) *Drosophila* Cbp53E regulates axon growth at the neuromuscular junction. *PLoS One* 10:e0132636.
- Hutchins BI, Kalil K (2008) Differential outgrowth of axons and their branches is regulated by localized calcium transients. *J Neurosci* 28:143-153.
- McCabe BD, Marques G, Haghghi AP, Fetter RD, Crotty ML, Haerry TE, Goodman CS, O'Connor MB (2003) The BMP homolog Gbb provides a retrograde signal that regulates synaptic growth at the *Drosophila* neuromuscular junction. *Neuron* 39:241-254.
- Reifegerste R, Grimm S, Albert S, Lipski N, Heimbeck G, Hofbauer A, Pflugfelder GO, Quack D, Reichmuth C, Schug B, Zinsmaier KE, Buchner S, Buchner E (1993) An invertebrate calcium-binding protein of the calbindin subfamily: protein structure, genomic organization, and expression pattern of the calbindin-32 gene of *Drosophila*. *J Neurosci* 13:2186-2198.
- Rishal I, Fainzilber M (2014) Axon-soma communication in neuronal injury. *Nat Rev Neurosci* 15:32-42.
- Schiffmann SN, Cheron G, Lohof A, d'Alcantara P, Meyer M, Parmentier M, Schurmans S (1999) Impaired motor coordination and Purkinje cell excitability in mice lacking calretinin. *Proc Natl Acad Sci U S A* 96:5257-5262.
- Schwaller B (2010) Cytosolic Ca<sup>2+</sup> buffers. *Cold Spring Harb Perspect Biol* 2:a004051.
- Schwaller B, Meyer M, Schiffmann S (2002) 'New' functions for 'old' proteins: the role of the calcium-binding proteins calbindin D-28k, calretinin and parvalbumin, in cerebellar physiology. *Studies with knockout mice. Cerebellum* 1:241-258.
- Veccellio M, Schwaller B, Meyer M, Hunziker W, Celio MR (2000) Alterations in Purkinje cell spines of calbindin D-28 k and parvalbumin knock-out mice. *Eur J Neurosci* 12:945-954.