# Molecular mechanisms of axo-axonic innervation

Fabrice Ango, Nicholas Biron Gallo, Linda van Aelst

# Molecular mechanisms of axo-axonic innervation

Fabrice Ango[1], Nicholas Biron Gallo[2,3] and Linda Van Aelst[2]

[1]INM, University of Monpellier, INSERM, CNRS, Monpellier, France

[2] Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 11724, USA

[3]Department of Neurobiology and Behavior, Stony Brook University, Stony Brook, NY, 11794, USA

Corresponding author: Van Aelst, Linda (vanaelst@cshl.edu)

All authors contributed equally to: conceptualization, writing – original draft, and writing – reviewing and editing

## Abstract

One of the most intriguing features of inhibitory synapses is the precision by which they innervate their target, not only at the cellular level but also at the subcellular level (i.e axo-dendritic, axo-somatic, or axo-axonal innervation). In particular, in the cerebellum, cortex, and spinal cord, distinct and highly specialized GABAergic interneurons form precise axo-axonic synapses, allowing them to directly regulate neuronal output and circuit function. In this review, we summarize our latest knowledge of the cellular and molecular mechanisms that regulate the establishment and maintenance of axo-axonic synapses in these regions of the CNS. We also detail the key roles of the L1CAM family of cell adhesion molecules in such GABAergic subcellular target recognition.

## Introduction

The development of single-cell transcriptomic datasets reveals the extraordinary complexity and diversity of two broad types of neurons: (1) glutamatergic excitatory neurons, which are the central players propagating signals across diverse brain areas, and (2) GABAergic interneurons (GIs), which organize neural signal flow, both in time and space, that is critical for information processing associated with behavioral and cognitive functions [1,2]. Although GIs account for only 20% of total neurons, they seemingly have the greatest diversity in their morphological, neurochemical, and electrophysiological features [3]. In particular, one of the most striking features of GIs is their highly structured axonal arbors which target postsynaptic partners with exquisite subcellular synapse specificity [e.g., axo-dendritic, axo-somatic, or axo-axonic synapses] to control the input, integration, and output of their target cells [4]. A classic example of such synaptic specificity is provided by the so-called "axo-axonic" subtype of GIs which innervate the axon initial segment (AIS) of principal cells in different cortical and subcortical brain regions in addition to presynaptic sites of neurotransmitter release in the spinal cord. How the precise innervation of such discrete subcellular compartments is orchestrated during neural circuit formation remains an open question paramount to our understanding of neuronal network assembly and function.

The assembly of neuronal networks is achieved by means of several developmental events, from neuronal migration and axon guidance to cell-type innervation and subcellular synapse formation. For example, cell migration and axon growth are regulated by long and short-range guidance cues that provide spatial information for proper neuronal positioning and formation of neural process trajectories [5]. Axonal terminals integrate multiple sources of spatial information to accurately locate their postsynaptic targets with cellular and subcellular precision. Multiple classes of

molecules cooperate to ensure proper target recognition such as diffusible chemotropic cues, membrane-bound adhesive molecules, and extracellular matrix and repellent molecules. Most of these molecules were initially studied in isolation; however, recent work has begun to reveal key roles for intricate/complex molecular combinations of such factors *in vivo* in facilitating precise subcellular synaptic specificity. Here, we review recent advances in our understanding of the cellular and molecular mechanisms engaged by axo-axonic neurons to innervate discrete axonal compartments (i.e. AIS or axonal terminals) of their postsynaptic partner cells as well as compare and contrast the unique targeting strategies leveraged by axo-axonic GIs in several brain regions, including the cerebellum, cortex, hippocampus, and spinal cord (Figure 1).

## The axon initial segment as a strategic target to regulate firing

The AIS of neurons is a specialized subcellular domain located at the most proximal portion of the axon responsible for action potential initiation [6] and, as such, serves as a strategic point to regulate neuronal output and network activity [7]. The molecular organization of the AIS is dependent on a specialized scaffolding protein, ankyrin-G (AnkG), that acts as a hub to link the submembranous actin cytoskeleton to various transmembrane proteins, including ligand and voltage gated ion channels and cell adhesion molecules (CAMs) of the L1 immunoglobulin (Ig) family [8]. Members of this family, which include L1CAM, neurofascin (NF), NrCAM, and CHL1, all share a similar domain organization comprising six extracellular N-terminal Ig domains, five fibronectin type 3 domains, a single transmembrane segment, and a short C-terminal intracellular sequence. The extracellular domains of L1 family CAMs can mediate both homophilic binding and heterophilic interactions with soluble proteins and other CAMs that play critical roles in several developmental processes, including migration, neurite outgrowth, axon

guidance, and synapse formation. At the AIS, the interaction of L1 family members with AnkG is thought to increase the local concentration and oligomerization state of L1 family members at the cell surface to support high affinity cell adhesion activity [9]. The interaction of L1 proteins with AnkG is regulated by tyrosine phosphorylation of the FIGQY motif located in the C-terminus of L1 proteins. Tyrosine phosphorylation of this motif inhibits AnkG binding and decreases L1 proteins' local concentration by increasing their lateral mobility [10]. Hence, phosphorylation and dephosphorylation of the FIGQY motif could decrease or increase, respectively, cell adhesion via ankyrin. In addition to the distinctive expression of L1 family members in axonal compartments, expression of ankyrin isoforms are restricted to subcellular compartments. Specifically, AnkG is localized at the AIS of principal cells (i.e. purkinje cells (PCs) in cerebellum or principal neurons in cortex, hippocampus, or amygdala) while ankyrin-B (AnkB) is enriched at sensory terminals in the spinal cord [8,11]. Thus, the localization and interaction between L1 family members and ankyrin isoforms provide unique molecular tools to investigate subcellular-specific synapse localization, formation, and/or stabilization of axo-axonic cells, such as basket cells (BCs) in the cerebellum, chandelier cells (ChCs) in the cortex, and GABApre interneurons in the spinal cord (Figure 1).

## Chemotactic- and chemoaffinity-based mechanisms in Purkinje cell AIS innervation in the cerebellum

Over one hundred years ago, the first identification of axo-axonic synapses in the mammalian CNS was made by Santiago Ramòn y Cajal (1911) in the cerebellum. Ramòn y Cajal described a "paintbrush-like" pinceau synapse, in which multiple presynaptic axonal branchlets innervate the AIS of cerebellar purkinje cells (PCs). Subsequent studies determined that such connections arise

from the specific targeting of GI basket cell (BC) axon terminals to the cell body and AIS of PCs and have started to reveal the chemotactic mechanisms underpinning the formation of such subcellular synapse formation [12••] (Figure 2).

The presynaptic GI in pinceau synapses, BCs, are derived from dividing progenitors in the white matter of the postnatal cerebellum [13]. At the end of the first postnatal week, BCs complete their migration, settling into the molecular layer of the cerebellum immediately above the soma of neighboring PCs. BC axons then extend rostrocaudally above the PC layer and send multiple descending collaterals towards PC somata. The descending collaterals initially enwrap the somata of PCs, forming GABAergic synaptic contacts, before subsequently extending towards the AIS of PCs and forming pinceau synapses [12••]. Noteworthy, the strong directional growth of BC axons from PC soma to AIS requires an AnkG-dependent subcellular gradient of the L1 family member neurofascin 186 (NF186) which is most highly enriched at the AIS (Figure 2). This AnkG-bound form of NF186 is critical for pinceau synapse formation and/or stabilization, since depletion of AnkG or disruption of the NF186-AnkG complex is sufficient to inhibit formation and maintenance of the pinceau [12,14].

Recently, the presynaptic binding partner of PC-expressed NF186 on BC axon terminals was identified as the axon guidance receptor neuropilin-1 (Nrp1) (Figure 2). Such work found that Nrp1 is expressed on BC axons and directly interacts in *trans* with postsynaptic NF186 during target recognition [15•]. Notably semaphorin 3A (Sema3A), the canonical ligand of Nrp1, is only expressed by PCs during cerebellar development [16] where it triggers BC axon guidance towards the PC somata. At the PC somata, Sema3A stabilizes Nrp1 and facilitates its interaction with NF186 at the AIS [15]. Of note, Sema3A also induces BC axon terminal branching through a Fyn-

dependent mechanism to regulate the size of the pinceau synapse [17]. Interestingly, a recent study found BCs to be patterned along PC functional zones according to the size of their pinceau [18•]. Such distribution into size-specific zones is eliminated without PC output, pointing to an activity-dependent regulation of cerebellar pinceau synapses.

## Chandelier cells: key cellular players in axo-axonic synapse formation in the cortex

Unlike BCs in the cerebellum which form pinceau synapses at PC AISs, BCs in the cortex predominantly innervate the proximal dendrites and cell soma, but not the AIS, of neighboring excitatory PyNs. Instead, a very distinctive type of GI, called chandelier cells (ChCs), was found to form axo-axonic synapses exclusively onto the AIS of cortical PyNs. Although first described in the neocortex in the 1970s, ChCs have also been found in the hippocampus, piriform cortex, and amygdala [19-22]. Typified by their characteristic chandelier/candelabrum-like morphology, ChCs possess a unique, highly-branched axonal arbor that terminates in parallel arrays of short vertical strings of presynaptic boutons, known as cartridges [23,24] (Figure 3). Each of these cartridges selectively innervate neighboring PyN AISs, the site of action potential initiation, enabling ChCs to exert decisive control over the spiking of large PyN populations and directly regulate excitatory-inhibitory (E/I) balance [24, 25•, 26••].

Neocortical ChCs arise from multipotent NKX2.1-positive progenitors in the medial ganglionic eminence (MGE) and are primarily generated at the latest stages of embryonic cortical neurogenesis [27, 28••, 29]; albeit subsequent work indicates that a smaller population of ChCs is also produced as early as embryonic day 12 (E12) [30,31]. From their place of birth, nascent neocortical ChCs migrate along stereotyped routes on a defined schedule to ultimately settle at the

border of layers 1 and 2 (L1/2) and in L5/6 between postnatal day 3 (P3)-P7 [28] (Figure 3). From P7 onwards, ChCs begin to elaborate their axon with a dramatic increase in axonal branching/complexity first noted at P11/P12. This increase in axonal complexity continues through P14 before plateauing at around P28 [32••,33]. In regards to the timing of ChC axo-axonic synapse formation, neocortical ChC-PyN AIS synapses are established following a similar temporal schedule. Namely, ChC/PyN axo-axonic innervation in the murine neocortex was found to be minimal from P8-P11, but, starting at approximately P12, ChC/PyN AIS connectivity dramatically increases. Such a spike in axo-axonic innervation continues into the third/fourth postnatal week before plateauing at approximately P28, when, on average, each cartridge possesses four to nine boutons and is approximately 16-28 μm in length [32,34,35]. At this time, most PyN AISs in L2/3 of the murine somatosensory cortex are innervated by three to four ChC cartridges originating from distinct ChCs [35].

## Molecular mediators of ChC axo-axonic synapse development

While the mechanistic basis of functional ChC/PyN AIS connectivity still remains incompletely understood, recent studies have begun to identify and characterize key regulators of ChC cartridge/bouton development and axo-axonic synapse targeting/formation in the cortex (Figure 3). The first molecule implicated in ChC bouton morphogenesis was ErbB4, a receptor tyrosine kinase expressed by parvalbumin (PV)-positive interneurons, including ChCs. Loss of ErbB4 in ChCs using conditional *ErbB4* mice caused a decrease in ChC bouton density without impacting ChC cartridge density and length nor gross ChC morphology [29•]. Moreover, a reduction in the number of α2-containing $GABA_A$ receptor ($GABA_AR$) clusters at the AIS of PyNs was observed in such animals [36•]. Interestingly, concurrent work linked ErbB4's function(s) in ChC

morphogenesis to the Rac/Cdc42 guanine nucleotide exchange factor (GEF) DOCK7 [34]. This study found that DOCK7 acts as a cytoplasmic activator of ErbB4 and, most importantly, promotes ChC cartridge/bouton development by augmenting ErbB4 activation independent of its GEF activity. As seen for ErbB4 depletion, knockdown of DOCK7 in neocortical ChCs induced a marked reduction in the size and density of ChC boutons and a disorganization of ChC cartridge networks, while ectopic DOCK7 expression in ChCs dramatically increased ChC bouton size and density [34]. Finally, a decrease in ChC bouton number was more recently also reported in *Nkx2.1-CreER;ErbB4*$^{fl/fl}$ mice [37]. Noteworthy, in concurrence with the observed decrease in α2-containing $GABA_AR$ clusters at PyN AISs in *ErbB4* mutant mice, proper AIS $GABA_AR$ clustering/distribution itself is critical for AIS synapse formation. Specifically, use of the *Gabra2-1* transgenic mouse line, which harbors a mutation in the α2 subunit of $GABA_ARs$, found that these animals had a decreased number of VGAT-positive clusters at the AIS of cortical PyNs, indicating a reduction in axo-axonic innervation by ChCs [38].

More recent work leveraging RNA sequencing and whole transcriptome analyses of different classes of murine interneurons during peak synaptogenesis led to the identification of the nonsecretory growth factor FGF13 as a regulator of ChC synapse development. Knockdown of FGF13 in ChCs at P2 and at P14, when axon development is largely completed, was found to induce a significant decrease in the density of ChC presynaptic boutons [39•].

Despite the importance of ErbB4, DOCK7, and FGF13 in the regulation of neocortical ChC cartridge bouton/synapse development, it is worth noting that ChCs depleted of these molecules still make contact with PyN AISs, indicating that another/other molecule(s) must govern such a selective form of neocortical axo-axonic innervation. To this end, recent RNA interference (RNAi)

screening of PyN-expressed CAMs identified the pan-axonally expressed L1 family member L1CAM, and not AIS-localized NF186, as a key regulator of neocortical ChC/PyN axo-axonic innervation [32•]. Embryonic depletion of PyN L1CAM caused a marked decrease in the percentage of L1CAM-depleted PyN AISs innervated by ChCs at P28. In line with this, the number of VGAT and gephyrin puncta at the AIS, but not along the somatodendritic compartment, were concomitantly reduced in L1CAM-depleted PyNs, indicating that PyN L1CAM selectively regulates ChC/PyN AIS synaptic innervation. Noteworthy, L1CAM was found to be required for both the establishment of ChC/PyN AIS innervation at P11/P12 and for the maintenance of such synaptic contacts into adulthood. L1CAM's ability to direct ChC/PyN innervation selectively to the AIS results from its interaction with the AIS-localized AnkG-βIV-spectrin cytoskeletal complex as disruption of this complex was found to reduce ChC/PyN AIS contacts. From this, a model emerges in which AnkG/βIV-spectrin-clustered L1CAM at the AIS promotes high-affinity cell adhesion between ChC cartridges and PyN AISs, thereby facilitating neocortical ChC/PyN synapse formation/stabilization [32].

It remains to be seen whether L1CAM is also involved in/required for selective ChC/PyN AIS contact formation in the hippocampus and/or amygdala. Noteworthy, while NF186 is dispensable for neocortical ChC/PyN AIS innervation, recent studies have implicated NF186 in the regulation of axo-axonic synapse assembly in the murine hippocampus and amygdala. In cultured hippocampal neurons, NF186 expression was found to be necessary for the formation of AIS postsynaptic gephyrin clusters, and, *in vivo*, knockdown of NF186 in dentate gyrus granule cells (GCs) impaired both AIS gephyrin and $GABA_AR$ clustering [40,41]. In addition, a decreased number of GAD65-positive presynaptic terminals at the AIS of GCs was observed, suggesting an impairment of axo-axonic synapse formation. Likewise, in the basolateral amygdala (BLA),

knockdown of NF186 in principal neurons induced a drastic reduction of inhibitory synapses at the AIS of such neurons [42]. Interestingly, in this context, reduced axo-axonic synapse numbers in the BLA impaired synaptic plasticity and also modulated fear memory extinction, but not acquisition and consolidation, pointing to potential axo-axonic synapse-specific function(s) in the amygdala [42].

## Axo-axonic synapses in the spinal cord

In the ventral spinal cord, proprioceptive sensory terminals that make synapses onto motor neurons are the sole synaptic targets of the GABApre class of inhibitory interneurons. GABApre neurons gate sensory inputs onto motor neurons by directly regulating the release of neurotransmitters from presynaptic axon terminals [43] (Figure 4). The formation of this axo-axonic synapse is exclusively dependent on proprioceptive sensory terminals as ablation of sensory neurons induces the retraction of GABApre axon terminals that failed to form synapses with non-target cells [43].

Like in the cerebellum and cortex, a molecular recognition mechanism between synaptic partners through a lock and key-like mechanism has been identified for this specialized synapse. More specifically, a survey of Ig-domain-containing proteins conducted in sensory and GABApre neurons led to the identification of NB2 (Contactin5) and Caspr4 on sensory neurons and L1 family members NrCAM and CHL1 on GABApre neurons [44•]. Subsequent molecular genetic approaches in mice showed that an NB2/Caspr4 coreceptor complex on sensory terminals directs the formation of GABApre bouton synapses and that GABApre-expressed CHL1 and NrCAM are required for the formation of GABApre synapses with sensory neurons (Figure 4). Thus, sensory NB2/Caspr4 interact with GABApre CHL1/NrCAM to control synapse formation at the axonal termini of sensory afferents [44].

## Concluding remarks

Although the significance of intercellular communication within the CNS has long been appreciated, a growing number of studies highlight the importance of targeted/selective cell-cell signaling at discrete subcellular domains in proper neural circuit function. In particular, axo-axonic synaptic connectivity has gained recent attention given its powerful regulation of principal cell firing and E/I homeostasis in diverse cortical and subcortical regions. Central to axo-axonic synapses are GIs; namely, AIS-targeting BCs and ChCs in cerebellar and cerebral cortices and GABApre interneurons, which innervate sensory neuron presynaptic axon terminals, in the spinal cord. Recent work leveraging RNAi screening and transgenic mouse models has begun to elucidate the molecular mechanisms underlying the selective targeting of such subcellular axonal domains, highlighting key roles for the AnkG- and βIV-spectrin-based AIS cytoskeleton in addition to the L1 family of Ig CAMs. Among the identified molecules, the postsynaptically-localized CAMs NF186 and L1CAM have proven to be essential for proper AIS innervation in BC-PC pinceau synapse formation and ChC-PyN synapse establishment/maintenance. In the spinal cord, GABApre interneuron innervation of sensory neurons employs a trans-synaptic multi-protein complex, comprised of sensory neuron-expressed NB2 and Caspr4 and GABApre interneuron-expressed NrCAM and CHL1, for proper subcellular targeting. Noteworthy, while these examples of axo-axonic innervation involve distinct cell types and are found in vastly different regions of the CNS, some conservation of mechanism is apparent, given the shared reliance of all such connectivity on members of the L1 family of CAMs. Given the importance of axo-axonic signaling in proper neural circuit function, it is of little surprise that perturbations in such connectivity have been linked to neural disorders. For instance, altered regulation of PC firing activity has been found in movement disorders as well as cognitive dysfunction, including autism spectrum disorder

(ASD) [45]. ChC connectivity defects have also been tied to ASD, in addition to schizophrenia and epilepsy, likely due to imbalances in E/I homeostasis [46-49]. Lastly, in the spinal cord, deficits in presynaptic inhibition of sensory neurons by GABApre terminals has been observed in individuals with Huntington and Parkinson diseases [50]. In sum, these findings strongly implicate perturbed axo-axonic connectivity in neural disorders, such as schizophrenia, epilepsy, ASD, and movement-related diseases, but, most importantly, further underscore the clear need for additional research on this unique form of cell-cell communication to prevent and/or treat these debilitating conditions.

## Conflict of interest statement

Nothing declared

## Acknowledgments

We would like to thank members of the Van Aelst and Ango labs for their support and discussions. We further thank James Duffy for graphic art work. This work was supported by grants from the National Institutes of Health [R01MH119819 and R01NS116897] to L.VA., a fellowship from the National Institutes of Health [F31MH117871] to N.B.G., and ERA-Net NEURON/DECODE [18-NEUR-0004] to F.A.

## References and recommended reading


1. Tasic B, Menon V, Nguyen TN, Kim TK, Jarsky T, Yao Z, Levi B, Gray LT, Sorensen SA, Dolbeare T, et al.: **Adult mouse cortical cell taxonomy revealed by single cell transcriptomics**. *Nat Neurosci* 2016, **19**:335-346.

2. Paul A, Crow M, Raudales R, He M, Gillis J, Huang ZJ: **Transcriptional Architecture of Synaptic Communication Delineates GABAergic Neuron Identity**. *Cell* 2017, **171**:522-539 e520.

3. Kepecs A, Fishell G: **Interneuron cell types are fit to function**. *Nature* 2014, **505**:318-326.

4. Tremblay R, Lee S, Rudy B: **GABAergic Interneurons in the Neocortex: From Cellular Properties to Circuits**. *Neuron* 2016, **91**:260-292.

5. Colon-Ramos DA: **Synapse formation in developing neural circuits**. *Curr Top Dev Biol* 2009, **87**:53-79.

6. Clark BD, Goldberg EM, Rudy B: **Electrogenic tuning of the axon initial segment**. *Neuroscientist* 2009, **15**:651-668.

7. Klausberger T, Magill PJ, Marton LF, Roberts JD, Cobden PM, Buzsaki G, Somogyi P: **Brain-state- and cell-type-specific firing of hippocampal interneurons in vivo**. *Nature* 2003, **421**:844-848.

8. Jenkins PM, Kim N, Jones SL, Tseng WC, Svitkina TM, Yin HH, Bennett V: **Giant ankyrin-G: a critical innovation in vertebrate evolution of fast and integrated neuronal signaling**. *Proc Natl Acad Sci U S A* 2015, **112**:957-964.

9. Bennett V, Lorenzo DN: **An Adaptable Spectrin/Ankyrin-Based Mechanism for Long-Range Organization of Plasma Membranes in Vertebrate Tissues**. *Curr Top Membr* 2016, **77**:143-184.

10. Tuvia S, Garver TD, Bennett V: **The phosphorylation state of the FIGQY tyrosine of neurofascin determines ankyrin-binding activity and patterns of cell segregation**. *Proc Natl Acad Sci U S A* 1997, **94**:12957-12962.

11. Engelhardt M, Vorwald S, Sobotzik JM, Bennett V, Schultz C: **Ankyrin-B structurally defines terminal microdomains of peripheral somatosensory axons**. *Brain Struct Funct* 2013, **218**:1005-1016.

12. Ango F, di Cristo G, Higashiyama H, Bennett V, Wu P, Huang ZJ: **Ankyrin-based subcellular** •• **gradient of neurofascin, an immunoglobulin family protein, directs GABAergic innervation at purkinje axon initial segment**. *Cell* 2004, **119**:257-272.

**This study shows that the localization and signaling of AnkG-tethered NF186 contributes to the subcellular synaptic organization of pinceau synapses in the cerebellum. Purkinje cells deficient in AnkG show diffuse expression of NF186 outside the AIS, which promotes the growth of BC axons in ectopic locations without inducing synaptogenesis.**

13. Zhang L, Goldman JE: **Generation of cerebellar interneurons from dividing progenitors in white matter**. *Neuron* 1996, **16**:47-54.

14. Buttermore ED, Piochon C, Wallace ML, Philpot BD, Hansel C, Bhat MA: **Pinceau organization in the cerebellum requires distinct functions of neurofascin in Purkinje and basket neurons during postnatal development**. *J Neurosci* 2012, **32**:4724-4742.

15. Telley L, Cadilhac C, Cioni JM, Saywell V, Jahannault-Talignani C, Huettl RE, Sarrailh-Faivre C, Dayer A, Huber AB, Ango F: **Dual Function of NRP1 in Axon Guidance and Subcellular Target Recognition in Cerebellum**. *Neuron* 2016, **91**:1276-1291.

**This study shows that the axon guidance receptor NRP1 is the main molecular cue that orchestrates AIS innervation by controlling both BC axon pathfinding to Purkinje cell somas and subcellular target recognition by trans-synaptic interaction with NF186 at the AIS.**

16. Saywell V, Cioni JM, Ango F: **Developmental gene expression profile of axon guidance cues in Purkinje cells during cerebellar circuit formation**. *Cerebellum* 2014, **13**:307-317.

17. Cioni JM, Telley L, Saywell V, Cadilhac C, Jourdan C, Huber AB, Huang JZ, Jahannault-Talignani C, Ango F: **SEMA3A signaling controls layer-specific interneuron branching in the cerebellum**. *Curr Biol* 2013, **23**:850-861.

18. Zhou J, Brown AM, Lackey EP, Arancillo M, Lin T, Sillitoe RV: **Purkinje cell neurotransmission patterns cerebellar basket cells into zonal modules defined by distinct pinceau sizes**. *Elife* 2020, **9**.

**This study shows that pinceau synapses have different sizes and that their sizes are organized into zones around the well-established pattern of Purkinje cells (zebrin positive and negative zones). The size of the pinceau is determined non cell-autonomously by Purkinje cell GABAergic neurotransmission.**

19. Somogyi P: **A specific 'axo-axonal' interneuron in the visual cortex of the rat**. *Brain Res* 1977, **136**:345-350.

20. Szentagothai J, Arbib MA: **Conceptual models of neural organization**. *Neurosci Res Program Bull* 1974, **12**:305-510.

21. Jones EG: **Varieties and distribution of non-pyramidal cells in the somatic sensory cortex of the squirrel monkey**. *J Comp Neurol* 1975, **160**:205-267.

22. Somogyi P, Nunzi MG, Gorio A, Smith AD: **A new type of specific interneuron in the monkey hippocampus forming synapses exclusively with the axon initial segments of pyramidal cells**. *Brain Res* 1983, **259**:137-142.

23. Inan M, Anderson SA: **The chandelier cell, form and function**. *Curr Opin Neurobiol* 2014, **26**:142-148.

24. Wang Y, Zhang P, Wyskiel DR: **Chandelier Cells in Functional and Dysfunctional Neural Circuits**. *Front Neural Circuits* 2016, **10**:33.

25. Lu J, Tucciarone J, Padilla-Coreano N, He M, Gordon JA, Huang ZJ: **Selective inhibitory control of pyramidal neuron ensembles and cortical subnetworks by chandelier cells**. *Nat Neurosci* 2017, **20**:1377-1383.

**This study characterizes a subset of ChCs in the prelimbic cortex that selectively control PyNs projecting to the basolateral amygdala (BLAPC) compared to those projecting to the contralateral cortex (CCPC). Using optogenetics, this study found that activation of ChCs inhibits BLAPCs and BLA activity in freely behaving mice.**

26. Pan-Vazquez A, Wefelmeyer W, Gonzalez Sabater V, Neves G, Burrone J: **Activity-Dependent Plasticity of Axo-axonic Synapses at the Axon Initial Segment**. *Neuron* 2020, •• **106**:265-276 e266.

**Using voltage imaging at the AIS, this study found axo-axonic GABAergic transmission to be depolarizing during initial ChC axonal arborization and synapse formation. Manipulations of ChC and PyN activity during this time period and in adulthood revealed that ChC synapses follow homeostatic plasticity rules governed by the developmental switch in GABA polarity.**

27. Inan M, Welagen J, Anderson SA: **Spatial and temporal bias in the mitotic origins of somatostatin- and parvalbumin-expressing interneuron subgroups and the chandelier subtype in the medial ganglionic eminence**. *Cereb Cortex* 2012, **22**:820-827.

28. Taniguchi H, Lu J, Huang ZJ: **The spatial and temporal origin of chandelier cells in mouse** •• **neocortex**. *Science* 2013, **339**:70-74.

**This seminal study followed the developmental trajectory of ChCs, determining that they mainly derive from the ventral germinal zone of the lateral ventricle during late gestation and subsequently migrate along stereotyped routes and with a strict temporal schedule to position themselves in specific cortical layers. This report also shows that ChCs require the homeodomain transcription factor NKX2.1 for their specification.**

29. Fazzari P, Paternain AV, Valiente M, Pla R, Lujan R, Lloyd K, Lerma J, Marin O, Rico B: • **Control of cortical GABA circuitry development by Nrg1 and ErbB4 signalling**. *Nature* 2010, **464**:1376-1380.

**This study shows that Nrg1 and ErbB4, previously identified risk genes for schizophrenia, regulate the development of cortical inhibitory circuits by cell-autonomously controlling GABA interneuron connectivity. In particular, ErbB4 promotes the formation of axo-axonic inhibitory synapses on PyNs and does so likely via Nrg1.**

30. Sultan KT, Liu WA, Li ZL, Shen Z, Li Z, Zhang XJ, Dean O, Ma J, Shi SH: **Progressive divisions of multipotent neural progenitors generate late-born chandelier cells in the neocortex**. *Nat Commun* 2018, **9**:4595.

31. Kelly MKR, R.; Moissidis, M.; Kim, G.; Huang, Z.J.: **Multipotent radial glia progenitors and fate-restricted intermediate progenitors sequentially generate diverse cortical interneuron types**. *BioRxiv* 2019:1-48.

32. Tai Y, Gallo NB, Wang M, Yu JR, Van Aelst L: **Axo-axonic Innervation of Neocortical** •• **Pyramidal Neurons by GABAergic Chandelier Cells Requires AnkyrinG-Associated L1CAM**. *Neuron* 2019, **102**:358-372 e359.

**This study identifies the cell adhesion molecule L1CAM as a critical postsynaptic regulator of both the establishment and maintenance of ChC/PyN AIS innervation in the neocortex. Anchoring of L1CAM to the cytoskeletal AnkG/βIV-spectrin complex at the AIS is required for selective innervation of PyN AISs by ChCs.**

33. Steinecke A, Hozhabri E, Tapanes S, Ishino Y, Zeng H, Kamasawa N, Taniguchi H: **Neocortical Chandelier Cells Developmentally Shape Axonal Arbors through Reorganization but Establish Subcellular Synapse Specificity without Refinement**. *eNeuro* 2017, **4**.

34. Tai Y, Janas JA, Wang CL, Van Aelst L: **Regulation of chandelier cell cartridge and bouton development via DOCK7-mediated ErbB4 activation**. *Cell Rep* 2014, **6**:254-263.

35. Inan M, Blazquez-Llorca L, Merchan-Perez A, Anderson SA, DeFelipe J, Yuste R: **Dense and overlapping innervation of pyramidal neurons by chandelier cells**. *J Neurosci* 2013, **33**:1907-1914.

36. Del Pino I, Garcia-Frigola C, Dehorter N, Brotons-Mas JR, Alvarez-Salvado E, Martinez de Lagran M, Ciceri G, Gabaldon MV, Moratal D, Dierssen M, et al.: **Erbb4 deletion from fast-spiking interneurons causes schizophrenia-like phenotypes**. *Neuron* 2013, **79**:1152-1168.

**This study finds that deletion of ErbB4 from ChCs and BCs induces subtle but consistent synaptic defects that increase cortical excitability and oscillatory activity in addition to disrupting synchrony across cortical regions. Such perturbations are linked to impaired social behavior and cognitive function, increased locomotion, and atypical emotional responses.**

37. Yang JM, Shen CJ, Chen XJ, Kong Y, Liu YS, Li XW, Chen Z, Gao TM, Li XM: **erbb4 Deficits in Chandelier Cells of the Medial Prefrontal Cortex Confer Cognitive Dysfunctions: Implications for Schizophrenia**. *Cereb Cortex* 2019, **29**:4334-4346.

38. Hines RM, Maric HM, Hines DJ, Modgil A, Panzanelli P, Nakamura Y, Nathanson AJ, Cross A, Deeb T, Brandon NJ, et al.: **Developmental seizures and mortality result from reducing GABAA receptor alpha2-subunit interaction with collybistin**. *Nat Commun* 2018, **9**:3130.

39. Favuzzi E, Deogracias R, Marques-Smith A, Maeso P, Jezequel J, Exposito-Alonso D, Balia M, Kroon T, Hinojosa AJ, E FM, et al.: **Distinct molecular programs regulate synapse specificity in cortical inhibitory circuits**. *Science* 2019, **363**:413-417.

**This study utilizes RNA sequencing and whole transcriptome analyses of different classes of murine interneurons during peak synaptogenesis to identify interneuron subtype-specific synaptic molecules required for the proper targeting of synapses to the dendrites, soma, or AIS of PyNs. Such profiling demonstrates a key role for FGF13 in the regulation of ChC synapse development.**

40. Burkarth N, Kriebel M, Kranz EU, Volkmer H: **Neurofascin regulates the formation of gephyrin clusters and their subsequent translocation to the axon hillock of hippocampal neurons**. *Mol Cell Neurosci* 2007, **36**:59-70.

41. Kriebel M, Metzger J, Trinks S, Chugh D, Harvey RJ, Harvey K, Volkmer H: **The cell adhesion molecule neurofascin stabilizes axo-axonic GABAergic terminals at the axon initial segment**. *J Biol Chem* 2011, **286**:24385-24393.

42. Saha R, Knapp S, Chakraborty D, Horovitz O, Albrecht A, Kriebel M, Kaphzan H, Ehrlich I, Volkmer H, Richter-Levin G: **GABAergic Synapses at the Axon Initial Segment of Basolateral Amygdala Projection Neurons Modulate Fear Extinction**. *Neuropsychopharmacology* 2017, **42**:473-484.

43. Betley JN, Wright CV, Kawaguchi Y, Erdelyi F, Szabo G, Jessell TM, Kaltschmidt JA: **Stringent specificity in the construction of a GABAergic presynaptic inhibitory circuit**. *Cell* 2009, **139**:161-174.

44. Ashrafi S, Betley JN, Comer JD, Brenner-Morton S, Bar V, Shimoda Y, Watanabe K, Peles E, Jessell TM, Kaltschmidt JA: **Neuronal Ig/Caspr recognition promotes the formation of axoaxonic synapses in mouse spinal cord**. *Neuron* 2014, **81**:120-129.

**This study shows that axo-axonic synapse formation between sensory neurons and GABApre interneurons in the spinal cord is controlled in part by a select combination of neuronal cell adhesion molecules. Loss of these adhesion molecules, either individually or in combination, induces a reduction of axo-axonic synapse number.**

45. Beckinghausen J, Sillitoe RV: **Insights into cerebellar development and connectivity**. *Neurosci Lett* 2019, **688**:2-13.

46. Amina S, Falcone C, Hong T, Wolf-Ochoa MW, Vakilzadeh G, Allen E, Perez-Castro R, Kargar M, Noctor S, Martinez-Cerdeno V: **Chandelier Cartridge Density Is Reduced in the Prefrontal Cortex in Autism**. *Cereb Cortex* 2021.

47. Rocco BR, DeDionisio AM, Lewis DA, Fish KN: **Alterations in a Unique Class of Cortical Chandelier Cell Axon Cartridges in Schizophrenia**. *Biol Psychiatry* 2017, **82**:40-48.

48. Ribak CE: **Axon terminals of GABAergic chandelier cells are lost at epileptic foci**. *Brain Res* 1985, **326**:251-260.

49. Arellano JI, Munoz A, Ballesteros-Yanez I, Sola RG, DeFelipe J: **Histopathology and reorganization of chandelier cells in the human epileptic sclerotic hippocampus**. *Brain* 2004, **127**:45-64.

50. Knikou M: **The H-reflex as a probe: pathways and pitfalls**. *J Neurosci Methods* 2008, **171**:1-12.

## Figure Legends

**Figure 1. Axo-axonic synapses in the central nervous system**

**Left:** Schematic depicting the so-called "pinceau synapse" made by BC (green) at the AIS of PC (blue) in cerebellar cortex. The French word "pinceau" is used to describe the paintbrush-like shape of these synapses that are formed by multiple terminal BC axonal branchlets. **Center:** Schematic depicting a GABAergic ChC (red) positioned at the neocortical layer 1/2 (L1/2) border, specifically targeting the AIS of numerous PyNs in L2/3 and L5 (green). The axon terminals of ChCs, known as cartridges, are composed of a string of synaptic boutons that selectively innervate the AIS. **Right:** Schematic depicting SN (green) – MN (blue) innervation in the spinal cord. A GABApre (red) interneuron forms an axo-axonic synapse specifically at the presynaptic terminal of the SN. Abbreviations: BC, basket cell; PC, purkinje cell; ChC, chandelier cell; PyN, pyramidal neuron; AIS, axon initial segment; SN, sensory neuron; MN, motor neuron.

**Figure 2. Molecular mechanisms orchestrating pinceau synapse innervation in the cerebellum**

**Left:** BCs (green) develop a stereotyped axonal architecture consisting of a main branch, which extends parallel above the cell bodies of neighboring PCs, and multiple descending secondary branches which innervate the soma and AIS of PCs. **Right:** At the AIS of PCs, AnkG is required to maintain a high concentration of NF186, which is necessary for pinceau synapse formation. At BC terminals, Neuropilin-1 is stabilized at the cell surface by its cognate axon guidance ligand, Sema-3A, to facilitate interaction with NF186 and axo-axonic synapse formation. Abbreviations: BC, basket cell; PC, purkinje cell; AnkG, ankyrin-G; NF186, neurofascin-186; Sema-3A, semaphorin-3A.

**Figure 3. Neocortical ChC spatial positioning and molecular mechanisms governing cartridge/bouton morphogenesis and axo-axonic innervation**

**Left:** Schematic depicting the perinatal and early postnatal spatial positioning of superficial and deeper layer ChCs (red cells at layer 1/2 (L1/2) border with axons extending into L2/3 and in L5/6, respectively) produced by NKX2.1+ progenitors in the medial ganglionic eminence (MGE). **Right:** ChCs possess a highly-branched axonal arborization characterized by terminal strings of synaptic boutons, known as cartridges, that form axo-axonic synapses at the AIS of neocortical PyNs. ChC cartridge/bouton and synapse development relies on ChC ErbB4-DOCK7- and FGF13-mediated signaling. In addition, the AIS of neocortical PyNs has α2-containing $GABA_A$ receptors ($GABA_AR$-α2) necessary for ChC-mediated GABAergic neurotransmission, as well as a highly complex cytoskeleton comprising ankyrin-G (AnkG) and β4-spectrin. Among the many cell adhesion molecules (CAMs) present at the AIS of PyNs, AnkG-bound L1CAM is critical for ChC/PyN AIS innervation in the neocortex. For ease of visualization, innervation between a single ChC and one neighboring PyN is depicted. In vivo, single neocortical ChCs are able to simultaneously innervate hundreds of PyNs with each PyN AIS typically being innervated by 3-4 cartridges originating from distinct ChCs. Abbreviations: ChC, chandelier cell; AIS, axon initial segment; DOCK7, dedicator of cytokinesis 7; FGF13, fibroblast growth factor 13; PyN, pyramidal neuron.

**Figure 4: A multi-protein Ig CAM superfamily recognition mechanism regulates axo-axonic synapse specificity in the spinal cord**

**Left:** In the spinal cord, SN terminals in the dorsal root ganglion form excitatory synapses on MNs. The presynaptic axon terminals of SNs are exclusively targeted by GABApre interneurons. **Right:** Selective axo-axonic synapse formation between SNs (VGLUT1+) and GABApre

terminals is orchestrated by a complex of L1 family members, NRCAM and CHL1 on GABApre terminals, and a receptor complex of the Ig CAM superfamily proteins NB2/CNTN5 and CASPR4 on SN axon terminals. Shank 1A labels the postsynaptic site of MNs. Abbreviations: SN, sensory neuron; MN, motor neuron; NrCAM, neuronal cell adhesion molecule; CHL1, close homologue of L1; NB2/CNTN5, contactin 5; CASPR4, CNTN-associated protein 4; Ig, immunoglobulin.

## Conflict of Interest

The authors “Fabrice Ango, Nicholas Biron Gallo, and Linda Van Aelst” declare that there is no conflict of interest.

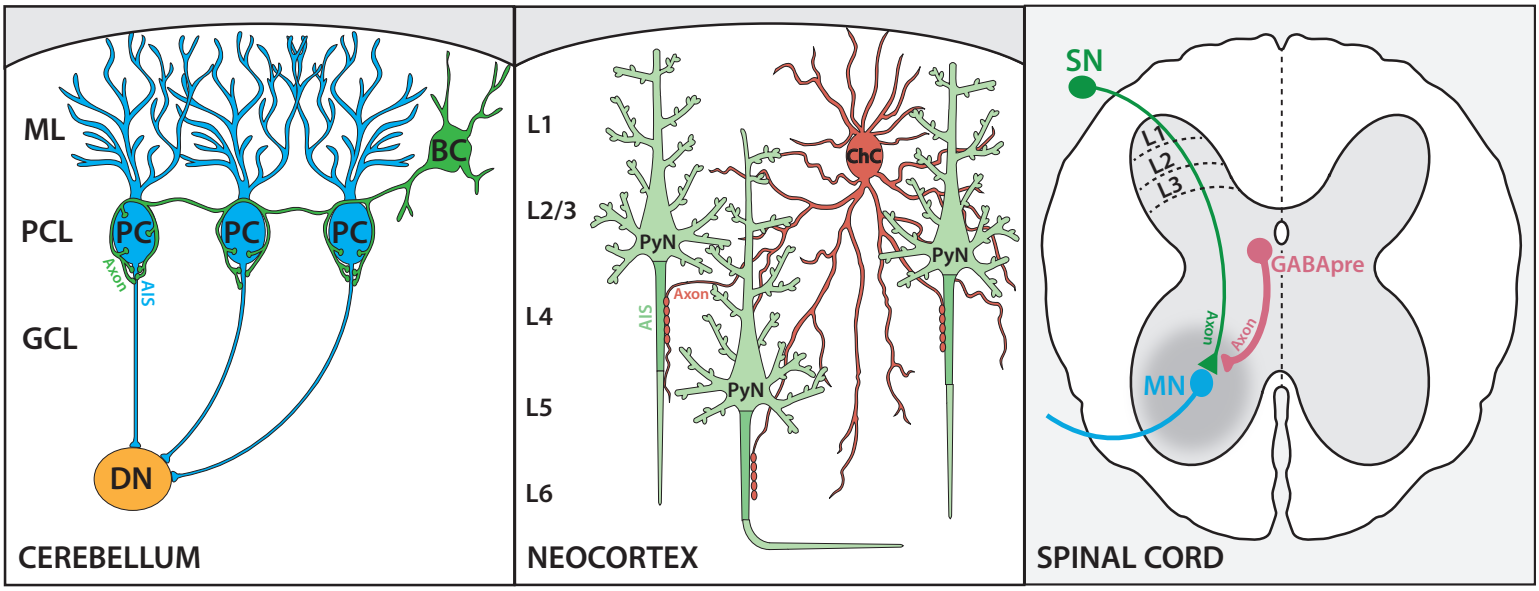


**Figure 1**

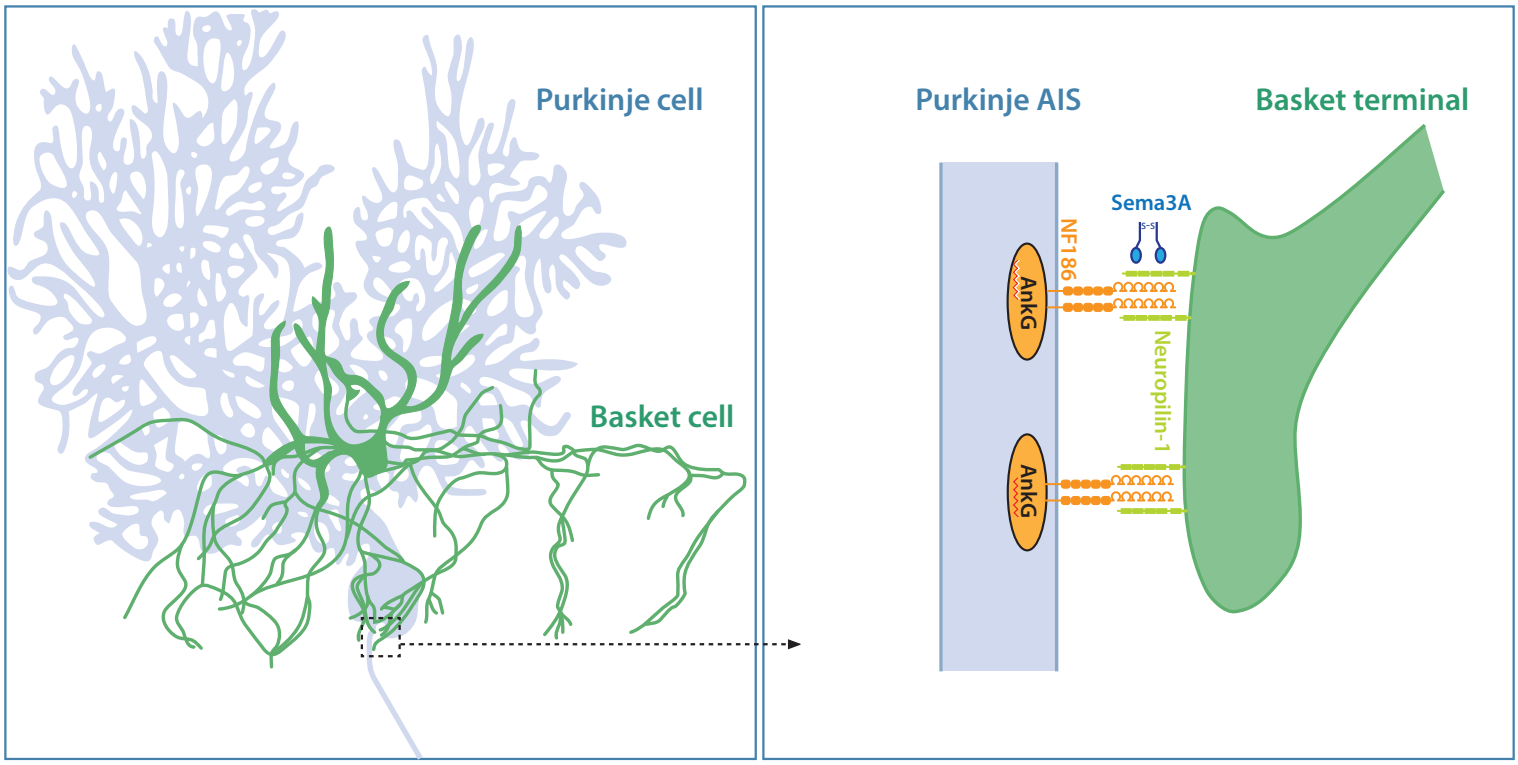


**Figure 2**

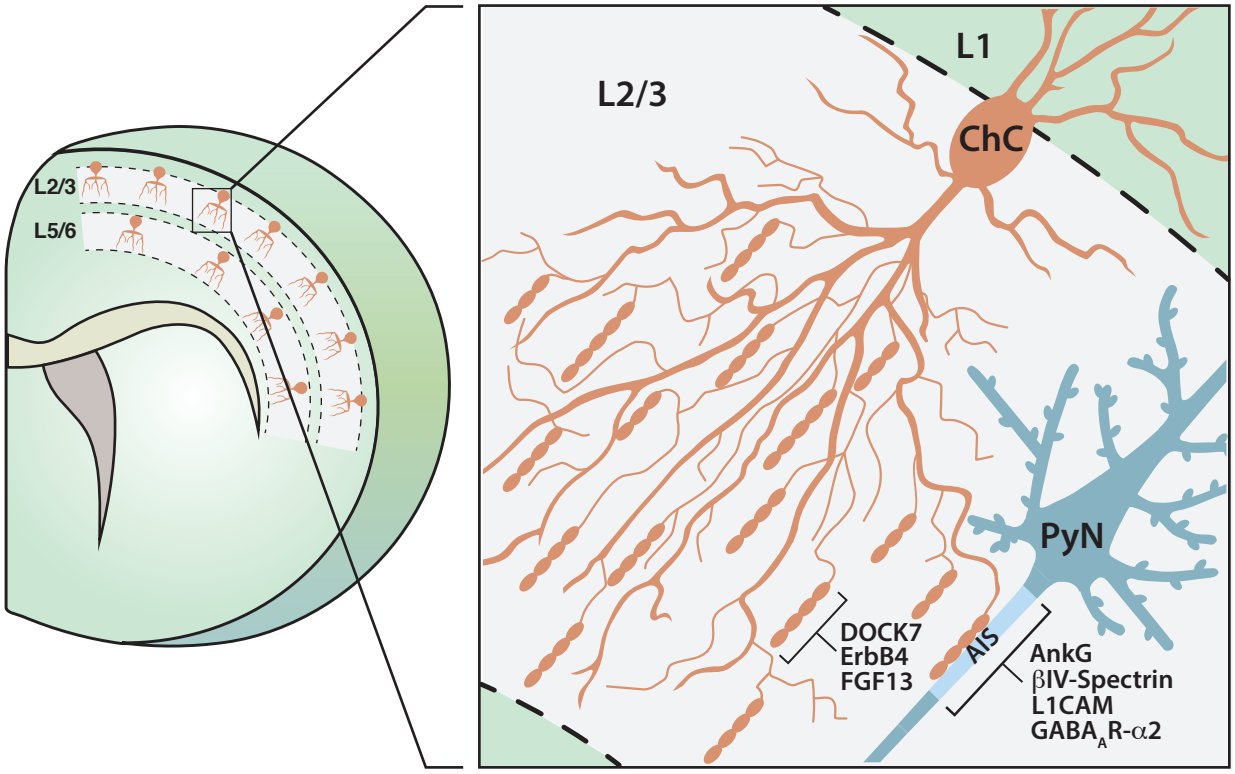


**Figure 3**

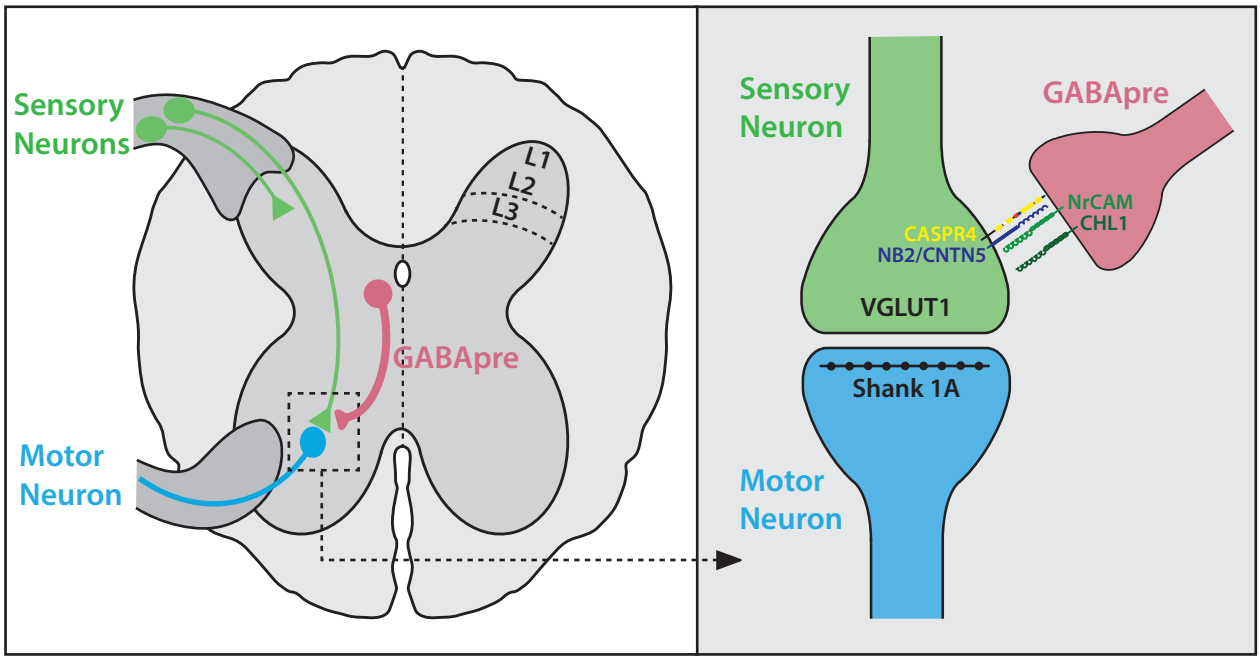


**Figure 4**