CellPress

Glutamate Receptors: Not Just for Excitation

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https://doi.org/10.1016/j.neuron.2019.11.025

In this issue of *Neuron*, Fossati et al. (2019) report a new constellation of players regulating inhibitory synaptogenesis. They show that GluD1, through a non-canonical ionotropic-independent mechanism, controls GABAergic synapse formation via *trans*-synaptic interactions mediated by extracellular cerebellin-4. They identify ARHGEF12 and PPP1R12A as GluD1 intracellular interactors and downstream effectors.

While there is a wealth of information regarding the proteomic architecture of excitatory synapses and initiators of excitatory synaptogenesis (Scannevin and Huganir, 2000; Brose 2009), equivalent understanding of inhibitory synapses lags far behind. The postsynaptic scaffold of inhibitory synapses, gephyrin, is known to cluster GABA receptors, interact with the actin cytoskeleton and *trans*-synaptic adhesion molecules, and be targeted by intracellular signaling cascades (Tyagarajan and Fritschy, 2014). Yet, we know little regarding the mechanisms of inhibitory synapse formation and the molecules involved. In this issue of *Neuron*, Fossati and colleagues identify an unexpected regulator of inhibitory synaptogenesis and use it as an entry point to a mechanistic reveal and a treasure trove of interacting extracellular and intracellular molecular players that regulate inhibitory synapse formation (Fossati et al., 2019).

Ionotropic glutamate receptors, the chief excitatory receptors of the vertebrate central nervous system, canonically operate as nonspecific cation channels that open upon glutamate binding (Traynelis et al., 2010). In mammals, there are 18 genes for ionotropic glutamate receptors categorized into four classes based on their activation by specific agonists: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate, N-methyl-d-aspartate (NMDA), and delta. The two delta receptor subunits (GluD1 and GluD2, encoded by the genes *grid1* and *grid2*) are the least understood. GluD2 binds to both D-serine and glycine, but these ligands do not evoke current (Naur et al., 2007). Cell culture assays indicate that postsynaptic GluD1 induces formation of excitatory and inhibitory boutons in cortical neurons (Yuzaki and Aricescu, 2017) through an unspecified mechanism. Both GluD1 and GluD2 are thought to mediate *trans*-synaptic adhesion through interactions with neurexins and cerebellins (Cblns), best characterized at the parallel fiber onto Purkinje cell synapse in the cerebellum (Yuzaki and Aricescu, 2017). A deeper understanding of GluD1 function is of particular relevance to human health given that genetic association studies identify it as a risk gene for disorders such as schizophrenia, autism spectrum disorders, and depression (Yuzaki and Aricescu, 2017).

Fossati et al. use a sparse knockout approach to investigate the cell-autonomous effects of GluD1 removal without the confounds of compensatory mechanisms present in the *grid1* null mice. The authors employed *in utero* electroporation to fluorescently label a small subset of L2/3 neurons in mouse somatosensory cortex, with tdTomato as a cell fill and either PSD95.FingR-GFP or EGFP-gephyrin fusion proteins to label excitatory and inhibitory synapses, respectively. They observed that knockdown of GluD1 or knockout of its parent gene *grid1* both result in a decrease in the density of gephyrin clusters, which correspond to inhibitory synapses (Chen et al., 2012). Conversely, overexpression of GluD1 resulted in an increase in inhibitory synapse number. This implicates GluD1 as an important promoter of inhibitory synapse formation. In contrast, GluD1 knockdown had no effect on the density of spines or PSD95 puncta, but overexpression resulted in decreased spine density, suggesting that GluD1 is not important for the formation or maintenance of cortical excitatory synapses, but its upregulation may limit the number of these synapses.

Consistent with these results, knocking down GluD1 did not affect miniature excitatory postsynaptic current (mEPSC) amplitude or frequency recorded from L2/3 neurons, confirming that GluD1 is not critical for excitatory synapse formation or function. However, the frequency of miniature inhibitory postsynaptic currents (mIPSCs) was reduced with GluD1 knockdown. mIPSC amplitude was slightly increased in knockdown conditions, possibly in compensation for the reduction in inhibitory synapse number. A role for GluD1 at inhibitory synapses is further supported by its localization to postsynaptic inhibitory sites, shown by immunohistochemistry and immunoelectron microscopy.

To identify GluD1 domains required for its role in inhibitory synaptogenesis, the authors performed a proteomic analysis of predicted functional domains based on the homologous GluD2 protein. They deleted either the extracellular N-terminal putative Cbln binding domain or the C-terminal intracellular tail, which potentially mediates intracellular signaling. They also introduced point mutations that would disrupt Cbln binding, agonist binding (glycine or D-serine), or ion channel function. Surprisingly, they found that for its role as an inhibitory synaptogenic molecule, GluD1 requires the extracellular Cbln binding domain, an intact agonist binding domain, and the C-terminal domain. However, the mutation blocking ion flux through the transmembrane pore did not affect inhibitory synaptic density, demonstrating that at least in relation to its inhibitory synaptogenic role, GluD1 is a non-canonical glutamate

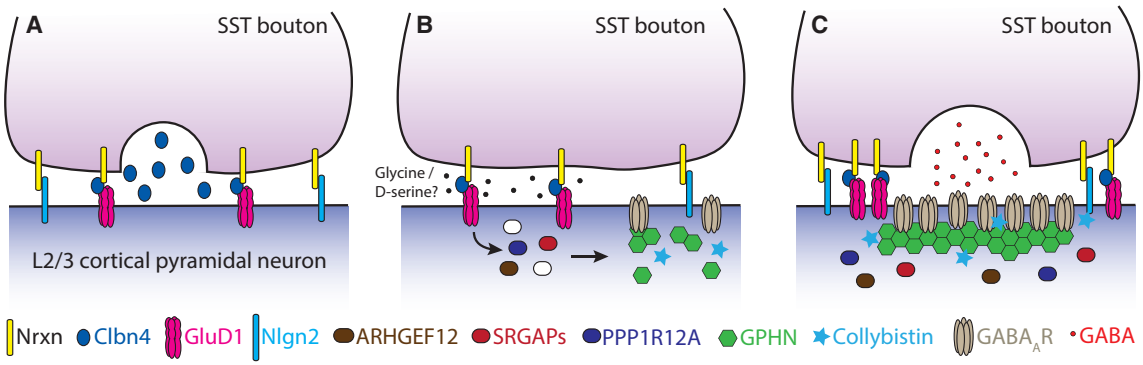

Figure 1. Formation of an Inhibitory Synapse

(A) Synaptic adhesion: somatostatin (SST) inhibitory synapse formation is initiated by *trans*-synaptic adhesion molecule bridges. Presynaptic neurexin (Nrxn) interacts with postsynaptic neuroligin2 (Nlgn2) present at all inhibitory contacts. SST boutons also secrete Cbln4, which bridges between Nrxn and postsynaptic GluD1 specifically at SST synapses.

(B) Signaling and recruitment: the cytoplasmic tail of postsynaptic adhesion molecules, like GluD1, recruits signaling molecules, such as ARHGEF12, PPP1R12A, and SRGAPs. Intracellular signaling pathways then initiate gephyrin clustering and recruit $GABA_A$ receptors.

(C) A mature inhibitory synapse where gephyrin and collybistin cluster $GABA_A$ receptors in opposition to the presynaptic active zone, where GABA is released.

receptor operating through non-ionotropic mechanisms. While the putative agonist binding domain of GluD1 is important for its function at inhibitory synapses, it is unknown if GluD1 actually binds glycine or D-serine. It is also unclear if GluD1's synaptogenic function as a *trans*-synaptic adhesion molecule is its only cellular function, or whether it plays additional roles mediated via ionotropic signaling. Fossati's immunohistochemistry with anti-GluD1 antibodies shows a significant number of GluD1 puncta that do not colocalize with gephyrin, suggesting GluD1 may be required at other synapse types or for other cellular roles.

To delineate intracellular pathways utilized by GluD1 for inhibitory synapse assembly, the authors performed immunoprecipitations with anti-GluD1 antibodies followed by liquid chromatography and tandem mass spectrometry to identify co-precipitating GluD1 interactors. Not surprisingly, the major interactors were all regulators of second messenger signaling cascades, specifically regulators of GTPases (rho guanine nucleotide exchange factor 12 [ARHGEF12] and SRGAP3) and regulators of protein phosphorylation (protein phosphatase 1 regulatory subunit 12A [PPP1R12A] and MRCKα). Using the same sparse knockdown strategy employed for GluD1, the authors knocked down several of these interactors, revealing that PPP1R12A or ARHGEF12 knockdown shows a cellular phenotype similar to that resulting from GluD1 knockdown. Double knockout of either *ppp1r12a* or *arhgef12* and *grid1* did not further reduce inhibitory synapse density as compared to the *grid1* knockout, suggesting that they are both downstream of GluD1 and implicating the regulation of GTPases and protein phosphorylation in the formation and regulation of inhibitory synapses.

Many proteins are known to play distinct roles in different cell types. While in cerebellar interneurons and hippocampal pyramidal neurons, GluD1 is required for the formation of excitatory synapses, Fossati et al. show that in cortical pyramidal neurons GluD1 is important for the formation of inhibitory synapses (Figure 1). It is yet to be determined whether downstream effectors such as ARHGEF12 or PPP1R12A work in concert with GluD1 regardless of synapse or neuron type or if these pathways are specific to inhibitory postsynaptic regulation. We have much to learn about the specificity of proteomic sets that distinguish inhibitory from excitatory synapses in different neurons, and potentially inhibitory synapses that differ in afferent source. For example, Cbln4 is specifically expressed at synapses innervated by somatostatin (SST) neurons, while Cbln2 is specific to synapses innervated by VIP neurons (Paul et al., 2017). Fossati et al. found that for regulating inhibitory synapse density, GluD1 requires its N-terminal putative Cbln binding domain, and indeed Cbln4, but not Cbln2, knockout phenocopies GluD1 knockout. This suggests that the constellation of GluD1 downstream targets identified by Fossati et al. may be important specifically for SST inhibitory synapses. Given that most inhibitory dendritic innervation is from SST neurons, it is curious that only 50% of gephyrin puncta are associated with GluD1. Either the extent of non-SST innervation to dendrites has been underestimated, or even within SST synapses there may be some heterogeneity.

While opening the door to a new universe of inhibitory synaptogenic regulators, Fossati et al.'s work highlights again the intricacies of synaptic structure and the complex combinatorial code that likely governs their formation in a partner-specific manner.

ACKNOWLEDGMENTS

Work in the author's lab is supported by the National Institutes of Health, the Office of Naval Research, and the JBP foundation.

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Insulin-like Peptides as Agents of Social Change

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https://doi.org/10.1016/j.neuron.2019.12.001

Many behaviors promote reproduction or food finding. These critical functions of behavior can conflict; successful reproductive strategies can grow populations to the point where food is depleted. In this issue of *Neuron*, Wu et al. (2019) show how the nematode *C. elegans* detects crowding to change feeding behavior by coupling pheromone sensing to signaling via insulin-like peptides.

The 18th century economist and philosopher Thomas Robert Malthus saw the seeds of disaster in the success of a society. As its ability to produce food increased, its population would increase, but the geometric nature of population growth would inevitably outpace a society's ability to feed itself. Malthus could not imagine a technological solution to what he saw as an ever-looming crisis of mass starvation. He instead fell back on his training as an Anglican minister and advocated temperance and abstinence as behavioral strategies by which humanity could save itself (Malthus, 1798). History shows that Malthus might have underestimated humanity's capacity for technological innovation and overestimated our capacity for self-regulation, but his exposition of the consequences of geometric population growth remains central to our understanding of ecology and evolution. In the animal kingdom, the best exemplars of Malthusian models of population growth are, perhaps, the nematodes, which combine a short generation time and a capacity for rapid generation of large broods. In this issue of *Neuron*, Wu, Zhang, and colleagues discover a behavioral mechanism and its molecular underpinnings by which nematodes mitigate the Malthusian consequences of their hyper-efficient reproductive strategy (Wu et al., 2019).

The nematode *C. elegans*, which is the subject of this study, experiences cycles of boom and bust. Most of its life is spent as a hardy and long-lived dauer larva seeking food, which, for *C. elegans*, is certain types of nutritive bacteria found on decaying vegetable matter. Upon finding food, the dauer larva completes development and becomes a sexually mature hermaphrodite with a prodigious capacity for reproduction; a solitary hermaphrodite can produce ten offspring per hour and more than 300 offspring in total. 72 h later, each of these offspring will reach adulthood and begin production of another massive brood. As the population is booming, the environment accumulates a class of nematode-specific pheromones, the ascarosides, which are used by *C. elegans* to sense population density. When some ascaroside pheromones reach a critical concentration, the next generation of *C. elegans* develops as dauer larvae, which then disperse to begin the cycle anew.

During the good times, i.e., when food is abundant and population density is low, *C. elegans* can be picky eaters. Previous work from Zhang and colleagues showed that *C. elegans* learns to avoid bacteria that make it sick, even if those pathogenic bacteria are initially more attractive than other, safer bacteria in the environment (Zhang et al., 2005). This learned avoidance of pathogenic microbes is powerful; transient exposure of juveniles to some pathogens can induce learned avoidance that lasts into adulthood (Jin et al., 2016), and recent studies show that learned pathogen avoidance can also be transmitted from generation to generation (Posner et al., 2019; Moore et al., 2019). Some of the neural mechanisms that mediate learned pathogen avoidances are known. Serotonin is critical for this phenomenon (Zhang et al., 2005) as are insulin-like