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Neuroligins help dendrites keep up with the Joneses

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Abstract

A function for neuroligins in synapse formation has been controversial owing to conflicting data from *in vitro* work and knockout mice. A study now reconciles previous results, showing that cell-to-cell variability in neuroligin-1 expression, which mediates intercellular competition for presynaptic inputs, regulates synapse density and spinogenesis.

Synapse formation is an intricate process, involving the coordinated differentiation of pre- and postsynaptic compartments through recruitment of hundreds of proteins to new axodendritic contacts. These nascent contacts are subsequently stabilized through as yet unknown mechanisms. Both synapse formation and stabilization are mediated by trans-synaptic cell adhesion molecules (CAMs). An intensively studied family of trans-synaptic CAMs is the neuroligin (NL) family, which has been a focus of research since the discovery of NL mutations in autism spectrum disorders. Although it is clear that NLs regulate synaptic function, there has been a lively debate about whether NL1 regulates the initial formation of synapses or acts later in their activity-dependent stabilization and maturation. In this issue of *Nature Neuroscience*, Kwon and colleagues¹ use creative genetic approaches to systematically alter the expression of NL1 on a cell-by-cell basis and report results that reconcile conflicting data on this topic, revealing a new function for NLs in regulating synapse density through intercellular competition.

Until this report, results from studies using cell culture approaches and knockout mice were at odds regarding the function of NL1 in synapse formation. NL1 was the first adhesion molecule discovered to be sufficient for inducing the formation of functional presynaptic terminals in axons². Subsequent *in vitro* studies supported this action of NL1 in synapse formation. NL1 overexpression in neurons potently increases synapse density and function^{3–5}, whereas NL1 loss-of-function, using RNA interference, has the opposite effect⁴. Moreover, NL1 protein accumulates very rapidly, within seconds, at nascent axodendritic contacts, placing it at the right location and time to mediate the first stages of synapse formation⁶.

Despite these intriguing *in vitro* studies, there has been a clear lack of evidence that NL1 regulates synapse formation *in vivo*. Genetic deletion of NL1 alone, or in combination with NL2 or NL3, consistently results in normal synapse density and ultrastructure⁷. However, these manipulations do cause deficits in NMDA-type glutamate receptor (NMDAR)-mediated signaling^{8–9}, indicating that NL1 is essential for synaptic function, but not synapse formation, *in vivo*. Subsequent *in vitro* studies showed that chronic inhibition of NMDAR

activity prevents NL1 overexpression–induced increases in synapse density, suggesting that NL1 mediates activity-dependent maintenance, or validation, of pre-existing synapses rather than their initial formation³. While this model nicely reconciles several inconsistencies between *in vivo* and *in vitro* studies, it does not fully explain why non-neuronal NL1-expressing cells, which do not possess NMDARs, are capable of inducing rudimentary presynaptic specializations at contacts with neuronal axons², nor why antibody-mediated clustering of NL1 induces accumulation of postsynaptic proteins in the absence of changing synaptic activity⁶. Thus, the issue of whether NL1 influences the initial stages of synapse development has been a frustrating conundrum for the past few years.

In this issue, Kwon and colleagues¹ address an alternative possibility to reconcile these *in vitro* and *in vivo* discrepancies: NL1 might regulate synapse density through intercellular competition. If so, manipulating NL1 levels in individual neurons relative to their neighbors would alter synapse density, whereas changing NL1 levels throughout the neuronal network would not. To test this hypothesis, the authors used an elegant genetic strategy to manipulate the level of NL1 by varying degrees, in a variety of genetic contexts *in vivo*. Critically, this enabled, for the first time, a direct comparison of synaptic structure and function between neurons lacking NL1 and those in which NL1 levels varied from cell to cell, all in the same preparation. The authors used *in utero* electroporation to introduce short hairpin RNAs to knock down NL1 specifically in a sparse subset of cortical layer 2/3 pyramidal neurons. Comparison of spine densities and morphology between neurons in which NL had been reduced by knockdown and those of control cells revealed a marked reduction in the density of spines, as well as changes in spine length and spine head area. Genetic ablation of NL1 in knockout (*Nlgn1*^{-/-}) mice, however, failed to induce any significant changes in these measures. The ability to reproduce both the lack of change in synapse density in the *Nlgn1*^{-/-} mouse and the significant decrease in synapse density when NL1 is reduced in a subset of cells, in the same *in vivo* context (Figure 1), supports their alternate competition hypothesis and shows that the abundance of NL1 is a key determinant of synapse density *in vivo*, as well as *in vitro*.

Given that NMDARs are highly colocalized with NL1 during the initial stages of synapse formation⁶ and that their signaling is required for NL1-induced synaptogenesis³, the authors reasoned that NL1-dependent modulation of synapse number might occur as a secondary consequence of reduced NMDAR signaling. However, both sparse and global loss of NL1 produced similar deficits in NMDAR signaling, despite the lack of structural changes in synapse density observed with global loss of NL1. These experiments confirm earlier studies in which up- or downregulation of NL1 in nearly all systems tested was found to alter NMDAR-mediated synaptic currents^{3, 8, 9}. However, this new study is noteworthy in its clear demonstration of cell-autonomous regulation of NMDAR function by changes in the absolute level of NL1 that is distinct from non-cell-autonomous NL1-dependent regulation of excitatory synapse number (Figure 1).

Next the authors asked the critical question: does NL1 regulate the initial formation of synapses? Due to the inherent difficulty of studying the early stages of synapse formation in intact preparations, the authors used an elegant new paradigm in which glutamate uncaging reliably triggers the *de novo* formation of dendritic spines and synapses in acute brain

slices¹⁰. By quantifying the success rate of new spines formed in cortical neurons from wild-type mice following sparse knockdown or overexpression of NL1, compared to that in *Nlgn1*^{-/-} mice, the results were clear. The ability to form new spines was directly proportional to their relative levels of NL1, such that sparse knockdown of NL1 lowered, whereas overexpression increased, glutamate-induced spinogenesis. In another test of this model, the authors co-cultured neurons from *Nlgn1*^{-/-} and wild-type mice. Consistent with their competition hypothesis, *Nlgn1*^{-/-} neurons had reduced spine density when cultured with wild-type neurons. Pure cultures of either *Nlgn1*^{-/-} or wild-type neurons, however, displayed similar spine densities. Most importantly, this effect was proportional to the relative difference in NL1 levels between cells: the larger the ratio of wild-type to knockout neurons, the more marked the decreases in spine density on knockout neurons. Both approaches suggest that neurons compete with their neighbors to attract inputs from a limited number of presynaptic boutons and that NL1 abundance positively influences that competition.

These findings raised two important questions. First, does the endogenous level of NL1 vary between cells? If so, then can its ability to control spine density be recapitulated *in vivo*? By measuring mRNA using *in situ* hybridization in cortical layer 2/3 neurons, the authors found considerable variability in endogenous *Nlgn1* mRNA expression between neighboring cells. Next, to test their model *in vivo*, the authors systematically up- or downregulated NL1 in a sparse subset of neurons, on either a *Nlgn1*^{+/+}, *Nlgn1*^{-/-} or NL1 hemizygote (*Nlgn1*^{+/-}) background. Consistent with their previous findings, the greater the magnitude difference in NL1 expression between neighboring neurons, the more potent the effect on synapse density. Collectively, this study reveals that, in addition to regulating NMDAR function in a cell-autonomous manner, NL1-dependent effects on synapse formation and density exist that are only revealed when the level of NL1 differs across neurons. Thus, NL1 molecules likely compete for limited presynaptic neurexin ligands, enabling neurons expressing more NL1 to form synapses more readily.

In addition to its important contribution to our understanding of NL1, this study is noteworthy more generally in developing a new approach to study the function of specific genes in synapse formation. The long-standing discrepancies in the literature regarding the function of NL1 in synapse formation came about because investigators relied almost exclusively on neuronal cultures or knockout mice. Neuronal cultures are advantageous for examining the molecular mechanisms underlying synaptogenesis since individual synapses can be easily visualized and manipulated with temporal precision, but they suffer from loss of *in vivo* context, leading many investigators to discount all *in vitro* results. Conversely, knockout mice have been used for many years as the true test of the necessity of a gene in regulating synapse density, yet there is often extensive redundancy of function within gene families and compensation for the loss of genes that complicate interpretation of results from this approach. Knowing that relative levels of proteins are critical for synapse formation will now shift our emphasis on standard approaches toward examination of genetic manipulations in a variety of genetic contexts, both *in vivo* and *in vitro*, to facilitate comparison of sparse versus global manipulations of their abundance.

Although this is the first demonstration that NL1 mediates competition for synaptic inputs between neighboring neurons, the general idea that synapse density can be regulated by intercellular competitive mechanisms is not new. At the vertebrate neuromuscular junction, differences in synaptic activity between neighboring inputs determine their ability to successfully compete for space on the postsynaptic muscle¹¹. Moreover, reducing activity of individual young neurons in an active network decreases their synaptic inputs, whereas blocking synaptic activity across the entire network has no effect¹². Most relevant, though, is an elegant study by McClelland and colleagues¹³ that was the first demonstration that adhesion molecules can regulate changes in synapse density by means of intercellular competitive mechanisms. This report showed that relative levels of ephrin-B3 control synapse density, whereas global changes do not. This inter-neuronal competition requires binding to presynaptic EphB2 and inhibition of MAPK signaling and, like NL1-dependent regulation, only comes into play when the expression level of ephrin-B3 in surrounding neurons is varied.

The study by Kwon and colleagues¹ raises several important issues and questions. First, how and why do neurons express differing amounts of NL1? Our understanding of the regulation of NL1 transcription and translation is far from complete and may reveal complex mechanisms of regulation related to intercellular competition for synaptic inputs. Second, how do relative levels of NL1 in neighboring neurons regulate synapse density? The authors suggest that neurons may compete for binding to limiting amounts of presynaptic neurexins (Nrxns). Neurons that have more NL1 are better at attracting and/or maintaining contact with Nrxn-expressing presynaptic boutons; however, this hypothesis remains to be tested. Binding of NL1 to presynaptic Nrxns may also displace other Nrxn-binding partners¹⁴, yet the relative functions of the varied Nrxn binding partners remains mostly unexplored. Finally, how many other synaptogenic CAMs regulate synapse density through intercellular competition, and which of them coordinate with NL1? Addressing these questions is fundamental to discovering how neuroligin mutations that cause autism spectrum disorders⁵ alter connectivity in the developing brain. The present findings will undoubtedly intensify the competition among neurobiologists to unravel the complexity of CAMs and how they govern synaptogenesis.

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Figure 1. Competition-based model of synaptogenic adhesion

Relative levels of NL1 determine spine density through competitive cell-cell interactions.

(a) Neurons expressing similar levels of NL1 (red) exhibit normal NMDAR signaling (middle) and spine density (bottom). (b) Cell-autonomous reduction of NL1 expression (blue) in all cells in a neuronal network decreases NMDAR signaling without influencing spine density. (c) Reduction of NL1 in a subset of neurons (blue) likewise decreases NMDAR signaling but furthermore decreases spine density. Alterations in spine density arise only when there are differences in the expression of NL1 between neighboring cells (red versus blue).