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Refining the Roles of Neuroligins in Synapse Development and Function: A Reductionist Conditional Knock-out Approach

Hunter S. Shaw* and Christopher K. Salmon*

Centre for Research in Neuroscience, Department of Neurology and Neurosurgery, McGill University Health Centre, Montreal, Quebec H3G 1A4, Canada

Review of Chanda et al.

The human brain contains trillions of synapses, which need to be produced in the correct places, in the correct quantity, and with the correct properties for the brain to function normally. Trans-synaptic cell adhesion molecules play integral roles in this process, by initiating synapse formation, specifying synapse type, and coordinating synapse maturation and maintenance (Dalva et al., 2007). The neuroligins (NLs) and neurexins (NRXs) were one of the first pairs of synaptic adhesion molecules to be characterized, and their mutation was quickly shown to be associated with autism, and later with schizophrenia (Jamain et al., 2003; Sun et al., 2011). Yet, despite over 2 decades of inquiry, their precise role in synapse development remains elusive. NLs are the postsynaptic binding partners of presynaptic NRXs, and in rodents three NLs are highly expressed in the nervous system (Dalva et al., 2007). NL1 is localized at glutamatergic synapses, NL2 at GABAergic and glycinergic synapses, and NL3 at both glutamatergic and GABAergic synapses (Budreck and Scheiffele, 2007). Early work using RNAi-mediated knock-down and overexpression (OE) approaches suggested that NL1 and NL2 were necessary and sufficient for excitatory and inhibitory synapse formation, respectively (Chih et al., 2005). However, in a study using NL123 triple knock-out (TKO) mice, Varoqueaux et al. (2006) showed that NLs were not required for the initial formation of synapses in the brainstem but were essential for the maturation of synaptic currents. Since then, the discrepancies between these early studies have not been fully resolved, with recent work using conditional NL knock-outs (cKOs) suggesting that NLs may be involved in regulating both synapse number and maturation (Rothwell et al., 2014; Liang et al., 2015; Zhang et al., 2015; Jiang et al., 2017).

In a recent article, Chanda et al. (2017) undertook a systematic study of the role and functional redundancy of NLs in synaptic development. The authors employed dissociated mouse hippocampal and cortical neuronal cultures, using Cre-recombinase-mediated cKO of NL1, 2, and/or 3. Chanda et al. (2017) first examined NL123 triple cKO neurons, finding decreased frequency and amplitude of miniature postsynaptic currents (mPSCs), as well as decreased evoked PSCs, at both inhibitory and excitatory synapses. As expected from the above-mentioned work of Varoqueaux et al. (2006), and despite the alterations in synaptic physiology, Chanda et al. (2017) found no changes in synaptic puncta size or number or dendritic spine density between control and NL123 triple cKO neurons, suggesting that the synapse number was unaltered.

The decreased amplitudes of miniature and evoked PSCs clearly suggest defective maturation of synapses. According to the quantal theory of neurotransmitter release though, decreased mPSC frequency can indicate either deficits in presynaptic neurotransmitter release or changes in synapse number. Because the latter possibility was ruled out, as mentioned above, Chanda et al. (2017) tested whether decreased mPSC frequencies resulted from deficits in presynaptic release. They ruled out changes in presynaptic function in cKO neurons with multiple lines of evidence, leaving the cause of the reduced mPSC frequency unclear. Similarly unexplained decreases in mPSC frequency were found in other studies of NL KOs as well (Varoqueaux et al., 2006; Rothwell et al., 2014; Zhang et al., 2015). One possible explanation is that the number of silent synapses is elevated in NL KOs. Silent synapses would appear normal by immunological studies but would not contribute to mPSC frequency. Silent synapses, which are present normally during development,

Received Aug. 30, 2017; revised Oct. 27, 2017; accepted Nov. 1, 2017.

We thank Dr. Derek Bowie and Dr. David Stellwagen for insightful discussion.

*H.S.S. and C.K.S. contributed equally to this manuscript.

Correspondence should be addressed to Hunter S. Shaw, McGill University Health Centre, 1650 Cedar Avenue, Room L12-513, Montreal, QC H3G 1A4, Canada. E-mail: hunter.shaw@mail.mcgill.ca.

DOI:10.1523/JNEUROSCI.2492-17.2017

 0270-6474/17/3711769-03$15.00/0

can be unsilenced by the induction of long-term potentiation (LTP; Hanse et al., 1997). Interestingly, NL123 cKO mice have deficits in LTP (Jiang et al., 2017), which could explain the potentially elevated numbers of silent synapses suggested by the decreased mPSC frequency seen by Chanda et al. (2017) and others.

To further characterize the deficits in synapse maturation indicated by decreased PSC amplitudes, Chanda et al. (2017, their Fig. 7*D*,*E*) examined receptor content and found that the total charge transfer during PSCs evoked by puffing AMPA or GABA was lower in cKO cells than in controls. Decreased charge transfer may indicate shorter decay times of the evoked PSCs, which seems apparent in their example traces. This is consistent with a change in AMPA and GABA receptor subunit composition. GluA2-containing AMPA receptors give rise to EPSCs with slower decay kinetics compared with those lacking GluA2 (Geiger et al., 1995), and, furthermore, NL1/NRX1β binding preferentially recruits GluA2-containing AMPA receptors (Heine et al., 2008). Notably, Chanda et al. (2017) used GluA2 surface staining as a general indicator of glutamatergic synapse function and found that GluA2 puncta were smaller in cKO neurons than in control neurons, explaining the probable change in decay kinetics they observed. Although NL2 has not been shown to recruit specific GABA receptor subunits, gradual shifts in GABA receptor composition underlie the maturation of GABAergic currents during development (Fritschy and Panzanelli, 2014). In addition, gephyrin, the major binding partner of NL2 in the GABAergic postsynaptic scaffold, has been proposed to play differential roles in synapse assembly depending on its conformation and phosphorylation state (Tyagarajan and Fritschy, 2014). A role for NL2, via its interactions with gephyrin, in recruitment of specific GABA receptor subunits would further confirm the notion that NLs promote synapse maturation by influencing the subunit composition of neurotransmitter receptors.

Chanda et al. (2017) also questioned the functional redundancy of NLs by examining double KOs (dKOs) and single KOs (sKOs). Confirming previous work, they showed that NL1 is necessary for excitatory synapse development, NL2 for inhibitory synapses, and NL3 for neither. Importantly NL1 sKO neurons had deficits only in evoked, not in miniature EPSCs (mEPSCs), while the further loss of NL3 in NL13 dKO neurons decreased both evoked and mEPSC amplitude, suggesting that NL1 and NL3 may play different roles at the synapse. This is further supported by experiments in which NL1 OE increased evoked AMPA and NMDA receptor currents above basal levels in both control and triple cKO neurons, whereas NL3 OE only returned AMPA receptor currents to basal levels in cKOs. Divergent roles for NL1 and NL3 are likely underpinned by different complements of binding partners for the two molecules, which would alter their ability to recruit or stabilize synaptic components. In particular, *cis*-interactions of NLs with meprin/A5 protein/receptor protein tyrosine phosphatase μ domain-containing glycosylphosphatidylinositol-anchored proteins have recently been shown to differentially regulate NL function and may hold the key to the divergent roles of NL1 and NL3 at excitatory synapses (Elegheert et al., 2017).

Chanda et al. (2017) conclude that NL1, 2, and 3 are necessary for the maturation of synapses and have limited functional redundancy. In interpreting these results, however, it should be noted that with their conditional deletion approach, synapse formation was already underway when the NL genes were lost. Chanda et al. (2017, their Fig. 1) show that synapse formation begins at 4 d *in vitro* (DIV), while cKO commenced at 3DIV. Given that NL2 and NL3 have half-lives of ~2.6 d in culture (Cohen et al., 2013), NLs were likely present in the early stages of synaptogenesis in this study. Furthermore, NL1 has been shown to recruit PSD-95, which promotes synapse formation and stability (El-Husseini et al., 2000), within 2–4 h of binding to NRX1β (Heine et al., 2008). Verifying the time course of NL protein and mRNA loss following recombination would clarify this issue. Furthermore, it would be informative to compare neurons with cKO commencing at 3DIV to an earlier KO, by preparing cultures from TKO pups (Varoqueaux et al., 2006), by expressing Cre *in utero* in cKO animals via a neuronal Cre driver, or by transfecting Cre on the day of culturing, so that cKO occurs at 0DIV.

Chanda et al. (2017) also observed decreased inhibitory synaptic puncta density when triple cKO cultures were left until 28DIV, suggesting that NLs also play a role in the long-term stability of synapses. Consistent with this, NL2 has been shown to couple with IGSF9B, a homophilic adhesion molecule thought to structurally stabilize inhibitory synapses (Woo et al., 2013). Furthermore, cKO of NL2 in the medial prefrontal cortex causes a delayed decrease in the number of inhibitory synapses that only becomes apparent 6–7 weeks after NL2 is knocked out (Liang et al., 2015). Thus, NL2, in addition to its role in regulating synapse maturation, may play a structural role in stabilizing mature synapses such that its ablation causes a slow, stochastic decay of inhibitory synapse numbers. A role for NL1 in stabilizing nascent synapses has also been shown in the developing *Xenopus* optic tectum (Chen et al., 2010). Alternately, neuronal activity itself can stabilize synapses (Trachtenberg et al., 2002; Keck et al., 2011), suggesting that NLs could play an indirect role here simply by promoting maturation of synapse strength. Moving forward, it will be important to tease apart these possibilities, by further investigating the role of NL2 in inhibitory synapse stability, and to assess whether NL1 and NL3 also play structural roles in the long-term stability of excitatory synapses.

The results of the study by Chanda et al. (2017) suggest that NLs do not contribute to the initial stages of synaptic development in neuronal culture but are necessary for postsynaptic maturation, and that there is only limited redundancy between NL1 and NL3. Importantly, many questions remain, and, as recent *in vivo* findings suggest, NLs play complex, cell type-specific roles in maintaining circuit function in different brain areas (Rothwell et al., 2014; Zhang et al., 2015). These will need to be understood to treat disorders caused by NL mutations, such as autism and schizophrenia.

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