Neuroligins Mediate Excitatory and Inhibitory Synapse Formation

INVolvement of PSD-95 and Neurexin-1β in Neuroligin-Induced Synaptic Specificity*§

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The balance between excitatory and inhibitory synapses is a tightly regulated process that requires differential recruitment of proteins that dictate the specificity of newly formed contacts. However, factors that control this process remain unidentified. Here we show that members of the neuroligin (NLG) family, including NLG1, NLG2, and NLG3, drive the formation of both excitatory and inhibitory presynaptic contacts. The enrichment of endogenous NLG1 at excitatory contacts and NLG2 at inhibitory synapses supports an important in vivo role for these proteins in the development of both types of contacts. Immunocytochemical and electrophysiological analysis showed that the effects on excitatory and inhibitory synapses can be blocked by treatment with a fusion protein containing the extracellular domain of neurexin-1α. We also found that overexpression of PSD-95, a postsynaptic binding partner of NLGs, resulted in a shift in the distribution of NLG2 from inhibitory to excitatory synapses. These findings reveal a critical role for NLGs and their synaptic partners in controlling the number of inhibitory and excitatory synapses. Furthermore, relative levels of PSD-95 alter the ratio of excitatory to inhibitory synaptic contacts by sequestering members of the NLG family to excitatory synapses.

Synapse formation is a tightly regulated process that involves the recruitment of specific cell adhesion molecules and scaffolding proteins to newly formed contacts between an axon and a dendrite (1–3). In the brain, excitatory and inhibitory synaptic transmission is mainly mediated by two neurotransmitters: glutamate, which is released at excitatory glutamatergic synaptic contacts, and GABA, which is released at inhibitory GABAergic synapses. Initial transformation of a contact to either an excitatory or inhibitory synapse is thought to be controlled by spatial and temporal changes in protein content. This process is critical because an appropriate balance between excitatory and inhibitory synapses is required for proper neuronal excitability and function (2, 4–6). However, molecular events that control differentiation of a contact into either an excitatory or inhibitory synapse remain unknown.

The postsynaptic density protein, PSD-95, is a molecule that is exclusively localized to glutamatergic synapses and regulates clustering of AMPA receptors through association with stargazin (7, 8). Through its third PDZ (PSD-95/Dlg/ZO-1) homology domain, PSD-95 also recruits neurexin-1β (NLG1), a cell adhesion molecule involved in synapse formation (9–11). These findings indicate that association of PSD-95 with NLG1 is involved in excitatory synapse development. Recent work by Prange et al. (12) showed that NLG1 can drive both excitatory and inhibitory presynaptic contact formation. These results suggested that NLGs are involved in inhibitory synapse formation. Our work also showed that the effects of NLG1 on postsynaptic differentiation were less dramatic. Overexpression of NLG1 modestly increased the total number of excitatory postsynaptic sites but did not enhance clustering of PSD-95 or AMPA receptors (12). In contrast, coexpression of PSD-95 with NLG1 coordinated the maturation of pre- and postsynaptic elements and the recruitment of AMPA receptors (12). Moreover, enhanced expression of PSD-95 induced changes in the number of excitatory versus inhibitory synapses and resulted in an overall increase in the ratio of excitatory/inhibitory (E/I) synaptic currents (12). These findings provided the first evidence that assembly of specific postsynaptic elements can regulate a balance between excitatory and inhibitory synapses. Thus, abnormalities in the expression of and/or interactions between these molecules may result in aberrant synapse formation and a change in E/I ratio, which underlie complex psychiatric disorders (13). The detection of mutations in NLG3 and NLG4 in autistic patients suggests that synaptic imbalance may underlie autism (14).

NLG1 has additional homologs in rat including NLG2 and NLG3, which also contain a PDZ-binding site (10). Thus, PSD-95 may similarly control the action of NLG2 and NLG3 in...
neurons. Binding of NLGs to presynaptic neurexin-1β, a specific isoform of β-neurexin, is required for synaptic contact formation through trans-synaptic heterophilic protein interactions (9, 11, 15–17). However, it remains unclear whether association of NLGs with β-neurexin is involved in the formation of both excitatory and inhibitory presynaptic contacts. Also, it remains unclear whether NLGs are localized to both excitatory and inhibitory synapses and whether PSD-95 modulates the E/I ratio through regulated distribution of these proteins.

To elucidate the events that regulate synaptic specificity, we have analyzed the role of NLGs and PSD-95 in this process. We show that NLG1, NLG2, and NLG3 are capable of inducing both excitatory and inhibitory presynaptic contact formation. The effect on inhibitory synapses was blocked by a soluble form of neurexin-1β. Moreover, enhanced expression of PSD-95 induced clustering of NLG2 and NLG3 and shifted endogenous NLG2 from inhibitory to excitatory synapses. These results demonstrate that members of the NLG family are involved in establishing excitatory and inhibitory synapses; however, association with postsynaptic scaffolding proteins regulates the distribution of NLGs and controls the type of synapses formed.

MATERIALS AND METHODS

cDNA Cloning and Mutagenesis—The hemagglutinin (HA)-tagged wild type NLG1 (lab splice variant) amplified from mouse cerebellum was a gift from Dr. Peter Scheiffele (Columbia University). The generation of GW1 PSD-95 fused to GFP was previously described (18, 19). The generation of NLG2 and NLG3 constructs was carried out by PCR using oligonucleotides containing BglIII and HindIII restriction sites (NLG2, GGGCCCAGATCTGGGAGGAGGGGTCCCGCC-CAAGCTTCTATACCCGAGTGGTGGA; NLG3, GGGCCCAGATCTGCGTATACCCGAGGGCCCG and GGGCCCAGAAGCTTATACCCGAGGGCCCG-CAAGCTTCTATACCCGAGTGGTGGA) and subcloning the resulting fragments into pEFP-C1 constructs. The final HA-tagged versions were made by removing GFP (using AgeI and BglII) and inserting an HA tag.

Neuronal Cell Culture and Transfections—Dissociated primary neuronal cultures were prepared from hippocampi of embryonic day 18 or 19 Wistar rats. The hippocampi were dissociated by enzymatic papain digestion followed by brief mechanical trituration. The cells were plated on poly-γ-lysine (Sigma)-treated glass coverslips. The cultures were maintained in neurobasal medium (Invitrogen) supplemented with B27, penicillin, streptomycin, and 1-glutamine as described by Brewer et al. (37). Hippocampal cultures were transfected by lipid-mediated gene transfer using the Lipofectamine 2000 agent (Invitrogen) or by the electroporation method (Clontech) at least 2 days prior to immunocytochemistry.

Immunocytochemistry—The coverslips were removed from culture wells and fixed in −20 °C methanol. The cells were washed three times with phosphate-buffered saline containing 0.3% Triton-X-100 before each antibody incubation. The following primary antibodies were used: goat (1:100; Becton Dickinson), mouse (1:1000; Synaptic Systems), guinea pig (1:100; Amersham Biosciences; anti-goat, 1:5000, Santa Cruz Biotechnology). The blots were visualized by use of ECL (Pierce). Purification of soluble neurexin-1β fusion protein and the control FC-IgG protein was carried out as described by Ushkaryov et al. (38). For treatment using the purified proteins, the neurons were transfected with the appropriate construct. Transfection medium was then replaced with neurobasal medium containing mCherry (HeNe laser-damaged salt solution), neurexin-1β fusion protein, or FC-IgG (for each well of neurons, ~8 μl of each was added to 500 μl of neurobasal medium).

Imaging and Analysis—The images were acquired on a Zeiss Axiovert M200 motorized microscope by using a monochrome 14-bit Zeiss Axiocam HR charge-coupled device camera. In some experiments, there were times individually adjusted keyord intensity values to create a binary image, and dendrites of the cell of interest were outlined by using fluorescence signal. Only clusters with average pixel values two times greater than corresponding background pixel values were used for analysis. The number of stained clusters was measured as a function of dendritic length. For analysis of changes in NLG2 localization, puncta from the NLG2 channel were manually outlined, and the intensity of each punctum was individually displayed keyord intensity values and multiplied by punctum area to obtain an integrated intensity. In the case of cells transfected with PSD-95 GFP, each punctum on the NLG2 channel was then scored for colocalization with either PSD-95 GFP or VGAT puncta. Mean intensity of NLG2 puncta colocalizing with PSD-95 GFP was then compared with that of NLG2 puncta colocalizing with VGAT, and a ratio was obtained. In the case of untransfected controls, the same process was conducted for VGLUT-positive or -negative NLG2 puncta. The ratios for PSD-95 GFP-transfected cells were then compared with those for untransfected controls. For statistical analyses, Mann-Whitney U or Wilcoxin signed ranks tests were used.

Electrophysiology—Recording of miniature excitatory postsynaptic currents (mEPSCs) and miniature inhibitory postsynaptic currents (mIPSCs) were performed at least 2 days post-transfection. Hippocampal neurons on coverslips were transferred to a recording chamber that was continuously perfused with extracellular solution (pH 7.4; 320–330 mOsm) containing 140 mM NaCl, 5.4 mM KCl, 1 mM MgCl2, 1.3 mM CaCl2, 25 mM HEPES, 33 mM glucose, and 0.0005 mM tetrodotoxin (Alomone Labs). Transfected cells were identified from their GFP signal under a fluorescence upright microscope. Patch pipettes were pulled from borosilicate glass capillary tubes (Sutter Instruments) with a positive pipette potential for GABAA receptor-mediated mIPSCs (10 mV) to isolate mIPSCs. The recorded spontaneous mIPSCs or at the reversal potential for ionotropic glutamate receptor-mediated mEPSCs or at the reversal potential for GABAA receptor-mediated mIPSCs (−60 mV) to isolate mEPSCs or at the reversal potential for ionotropic glutamate receptor-mediated mEPSCs (+10 mV) to isolate mIPSCs. The recorded spontaneous mIPSCs and mEPSCs were blocked completely by the GABA receptor antagonist bicuculline (Sigma) and by the ionotropic glutamate receptor antagonist cyanotoxin-7-nitroquinoxaline-2,3-dione (Sigma), respectively (data not shown). Recordings were low pass filtered at 2 kHz, sampled at 10 kHz, and stored in a PC using Clampex 8.2 (Axon). The t test was used for statistical analysis.

RESULTS AND DISCUSSION

Neuroligins Drive Excitatory and Inhibitory Presynaptic Contact Formation—Previous work showed that NLG1 drives excitatory synapse formation (17). However, recently we have revealed the surprising finding that NLG1 overexpression can also induce inhibitory presynaptic contact formation (12). These results suggest that NLG1 may be involved in vivo in
establishing both excitatory and inhibitory synapses. NLG2 and NLG3 are two other known members of the NLG family expressed in the brain (20). To explore whether the effect on inhibitory synapses is unique to NLG1, we analyzed the effects of HA-tagged NLG2 (HA-NLG2) and NLG3 (HA-NLG3) on both excitatory and inhibitory synapse formation. For this analysis, days in vitro (DIV) 5 hippocampal neurons were transfected with HA-NLG2 and HA-NLG3, fixed at DIV 8, and stained for either the vesicular GABA transporter (VGAT), a marker for inhibitory synapses, or VGLUT, a marker for excitatory synapses. Remarkably, HA-NLG2 and HA-NLG3 overexpression significantly enhanced the average number of contacting presynaptic boutons, both excitatory (1.6 ± 0.1-fold for HA-NLG2 and 1.5 ± 0.2-fold for HA-NLG3) and inhibitory (3.0 ± 0.9-fold for HA-NLG2 and 2.7 ± 0.4-fold for HA-NLG3), when compared with GFP-transfected cells (Fig. 1). Other changes observed include enhanced numbers of dendritic filopodia (data not shown). These results demonstrate that various members of the NLG family exert similar effects on establishing new synaptic contacts, regardless of type.

NLG-induced Inhibitory Synapse Formation Is Mediated by Neurexin-1β—The differential recruitment of proteins to their respective synaptic compartments is likely to be mediated by heterotypic trans-synaptic signaling. NLGs have been shown to associate with neurexin-1β, and this interaction leads to the recruitment of elements required for the structural reorganization of presynaptic compartments (10, 17, 20, 21). Work performed by Dean et al. (22) demonstrated that synapse formation involves direct interaction between NLG1 and neurexin-1β and that the effects of NLG1 on excitatory synapses are blocked using an FC fusion protein containing the extracellular domain of neurexin-1β (17). However, it remains unclear how NLGs influence the maturation of GABA presynaptic termi-
nals. Previous studies showed that β-neurexins are also expressed by inhibitory neurons (23). Moreover, recent work by Graf et al. (24) showed that expression of neurexin-1β can drive clustering of NLGs and several other excitatory and inhibitory postsynaptic proteins in neurons. To examine whether NLG interaction with neurexin-1β is re-

FIG. 3. Blocking neurexin-1β function diminishes NLG1-mediated excitatory and inhibitory synaptic function. Shown are electrophysiological recordings from cultured rat hippocampal neurons coexpressing HA-NLG1 and GFP, or GFP alone, and incubated with medium containing either 30 μg/ml FC-IgG or 30 μg/ml neurexin-1β-FC peptide (NXN-FC). Spontaneous mEPSCs and mIPSCs were recorded in voltage clamp mode at holding potentials of −60 mV and +10 mV, respectively. A, representative traces of mEPSCs (left) and mIPSCs (right) recorded from these neurons. B and C, both the mEPSC and mIPSC frequencies were enhanced in neurons expressing HA-NLG1 when compared with neurons transfected with GFP alone. In contrast, treatment of HA-NLG1 expressing cells with NXN-FC resulted in a significant reduction in both mEPSC and mIPSC frequency when compared with FC-IgG treated controls. Moreover, NXN-FC treatment reduced both basal mEPSCs and mIPSCs frequency in neurons expressing GFP alone. *, p < 0.05; **, p < 0.01.
quired for inhibitory synapse formation in vivo, DIV 6 neurons were transfected with either HA-NLG1 or HA-NLG2 and then incubated in a medium containing either vehicle solution, 30 μg/ml FC-IgG (control), or 30 μg/ml of a soluble form of neurexin-1β lacking splice site 4 fused to FC-IgG (NXN-FC; Supplemental Fig. 1). Two days post-transfection, the neurons were fixed and analyzed for induction of inhibitory synapses. Remarkably, inhibitory synapse formation mediated by either NLG1 or NLG2 was dramatically diminished upon incubation with NXN-FC (Fig. 2 and Supplemental Fig. 1). This was manifested by a decrease in the number of VGAT-positive puncta contacting dendrites of cells transfected with either construct as compared with vehicle-treated controls (Fig. 2, A and B; 34 ± 7% of control for NLG1 and 39 ± 9% of control for NLG2). Moreover, no significant effect of FC-IgG treatment was observed (Fig. 2B). These results show that a neurexin-1β-dependent interaction regulates NLG-induced inhibitory synapse formation. Significantly, treatment of GFP-transfected cells with NXN-FC resulted in a decrease in the number of inhibitory synapses (51 ± 9% of control) when compared with GFP cells treated with vehicle only (Fig. 2C and Supplemental Fig. 1C). This is further evidence that neurexin-1β is involved in the formation of inhibitory synapses in vivo.

Treatment with NXN-FC also induced aggregation of HA-NLG1 and HA-NLG2 (Supplemental Fig. 2). This phenomenon is consistent with that observed in the case of the integrin transmembrane cell-adhesion receptors, where ligand binding induces aggregation of the receptor (25). Interestingly, NLG clusters were present at sites positive for PSD-95 but lacking synaptophysin staining, indicating nonsynaptic localization (Supplemental Fig. 2, B and C). Similar coclustering of NLGs and PSD-95 by neurexin-1β was recently reported (24). The aggregation of NLGs and PSD-95 upon treatment with NXN-FC is intriguing because NLG expression alone was not sufficient to enhance PSD-95 clustering (Supplemental Fig. 2A). Further work is required to clarify these differences.

Functional Effects of Neurexin-1β on Excitatory and Inhibitory Synapses—To examine functional correlates of the immunocytochemical changes induced by application of NXN-FC fusion protein, an electrophysiological approach was taken (Fig. 3). DIV 8–9 hippocampal neurons were transfected with either GFP alone or HA-NLG1 and incubated with either NXN-FC or FC-IgG for 2–3 days. Changes in mEPSCs and mIPSCs were compared by using whole cell voltage clamp recordings (Fig. 3A). Ectopic expression of HA-NLG1 in cells treated with control peptide (FC-IgG) significantly increases the frequency of mEPSCs and mIPSCs compared with GFP transfected cells (Fig. 3, B and C, upper panels). Strikingly, the effects on both mEPSCs and mIPSCs were blocked by treatment with NXN-FC. In addition, NXN-FC treatment reduced basal frequency of mEPSCs and mIPSCs in cells transfected with GFP alone. These results parallel our immunocytochemical data and support a novel role for β-neurexins in the induction of inhibitory synapses. Importantly, treatment of HA-NLG1 transfected cells with NXN-FC increases the ratio of E/I synaptic currents (~4-fold), suggesting that β-neurexins play a more critical role in inhibitory synapse formation.

It should also be noted that neither HA-NLG1 expression nor NXN-FC treatment had a significant effect on mEPSC amplitude, and the effect on mIPSC amplitude was only moderate (Fig. 3, B and C, lower panels). The lack of effect on the amplitude of excitatory currents, which mainly reflects postsynaptic changes, is consistent with the lack of change in clustering of postsynaptic proteins such as PSD-95 (Supplemental Fig. 2A). These findings indicate that NLG1 is mainly involved in presynaptic rather than postsynaptic maturation.
proteins in building both excitatory and inhibitory synapses. Manipulation of PSD-95 Levels Regulates Clustering of NLGs and Shifts the Distribution of NLG2 from Inhibitory to Excitatory Synapses—We have previously shown that enhanced expression of PSD-95 accelerates maturation of excitatory synapses and that this process involves recruitment of NLG1 to clusters containing PSD-95 (7). We have also shown that PSD-95 overexpression results in a decrease in the total number of inhibitory synapses (53 ± 5%) and an overall increase in the E/I synaptic ratio (12). These effects are most likely due to sequestration of synaptogenic factors to specifically build excitatory synapses. Here we explored whether PSD-95 manipulates the balance between excitatory and inhibitory synapses by sequestering various members of the NLG family to excitatory synapses. We first evaluated whether PSD-95 manipulates the balance between excitatory and inhibitory synapses by sequestering various members of the NLG family to excitatory synapses. We first explored whether PSD-95 can enhance clustering of overexpressed NLG2 and NLG3. For this analysis, neurons were transfected at DIV 6 with GFP-tagged PSD-95 (PSD-95 GFP) and either HA-NLG2 or HA-NLG3 and then fixed at DIV 9. Similar to HA-NLG1 (12), PSD-95 GFP induced clustering of both HA-NLG2 and HA-NLG3 (Fig. 5). These results demonstrate that PSD-95 can accelerate clustering of members of the NLG family.

The presence of NLG2 at inhibitory contacts suggested that the decreased number of inhibitory synapses previously observed upon overexpression of PSD-95 may have resulted from manipulation of NLG2 accumulation at inhibitory sites, whereby it was sequestered to excitatory synapses. To assess this, hippocampal neurons were transfected with PSD-95 GFP, then fixed, and immunostained with antibodies specific to VGAT and NLG2 (at DIV 10). Strikingly, the relative amount of NLG2 at excitatory synapses in cells overexpressing PSD-95 GFP was significantly enhanced when compared with controls (Figs. 4, B and C, right panels, and 6, A and B). The ratio of intensity of staining of NLG2 at VGLUT-positive (excitatory) to VGLUT-negative (inhibitory) sites in untransfected cells was 0.30 ± 0.03. In contrast, the ratio of intensity of staining of NLG2 at excitatory (PSD-95 GFP-positive) to inhibitory (VGAT-positive) sites in PSD-95 GFP-overexpressing cells was 1.7 ± 0.2. The enhanced accumulation of NLG2 at excitatory synapses correlates with a reduced number of inhibitory contacts in neurons expressing PSD-95 GFP. This strongly suggests that enhanced levels of PSD-95 manipulate the E/I synapse ratio by depleting NLG2 from inhibitory synapses and directing it to excitatory postsynaptic sites. Fig. 6C depicts a model that illustrates this phenomenon.

Several lines of evidence indicate that appropriate stoichiometry of PSD-95 and NLGs controls the number and type of synapses formed (Fig. 6C). First, when transfected alone, NLGs increase the number of both excitatory and inhibitory presynaptic terminals; however, as previously shown by our lab, coexpression of NLG1 with PSD-95 restricts NLG-induced presynaptic effects to excitatory synaptic contacts (12). Second, clustering of both endogenous and transfected NLGs at postsynaptic excitatory sites was enhanced by PSD-95. Third, overexpression of PSD-95 resulted in a shift in the distribution of NLG2 from inhibitory sites to excitatory synapses, and this shift correlates with the previously observed decrease in the number and activity of inhibitory synapses in neurons overexpressing PSD-95 (12). Taken together, these observations indicate that the relative amount of endogenous PSD-95 controls the E/I ratio through modulation of the localization and/or retention of NLGs.

It is striking that PSD-95 can manipulate the localization of multiple members of the NLG family and that it can redistribute NLG2 from inhibitory to excitatory synapses. However, it remains unclear how endogenous NLG2 preferentially clusters at inhibitory synapses. In addition to PDZ-dependent interactions, the C-terminal domain of each of these proteins may

FIG. 5. Regulation of NLG2 and NLG3 clustering by PSD-95. Hippocampal cells were transfected with HA-NLG2 or HA-NLG3, either alone or with PSD-95 GFP. The cells were fixed and immunostained for HA and GFP. A, HA-NLG2 expression alone (left panel) results in a diffuse staining pattern. When PSD-95 GFP is coexpressed (right panels), clustering of HA-NLG2 is strongly enhanced. B, similar effects were observed in the case of HA-NLG3. The boxed areas in A and B are shown enlarged below. Scale bars, 10 μm (full view images) and 1 μm (enlarged panels).
associate with other specific elements that cooperate or compete with PSD-95 for targeting members of the NLG family to excitatory or inhibitory synapses. Further work is required to determine whether this process involves competition between PDZ-containing proteins and molecules such as gephrin or GABA receptor-interacting proteins to regulate NLG2 accumulation at inhibitory synapses (28, 29).

By manipulating the localization of cell adhesion molecules involved in building excitatory and inhibitory synapses, PSD-95 can alter the E/I synapse ratio and hence neuronal excitability. These findings are critical in light of the new observation that the balance in E/I ratio is affected in many psychiatric disorders, including autism and mental retardation (13). The implication of NLG genes, as well as PSD-95, in autism suggest that an imbalance in the E/I ratio may result in the manifestation of abnormalities in patients affected with this disease (14, 30–32). In addition to the NLGs examined in this study, a fourth member (NLG4) has recently been shown to be affected in autism (14). It will be important to determine the endogenous localization of the additional NLG proteins and their role in the process of synaptic development. The involvement of neurexin-1/β-neurexin in NLG-mediated inhibitory presynaptic contacts is also intriguing and provides a novel mechanism for the development of inhibitory synapses.

Of particular interest, physiological and pathological paradigms have recently been shown to alter the levels of PSD-95. For example, PSD-95 association with the PSD is dynamic and is regulated by synaptic activity and palmitate cycling on PSD-95 (33). Synaptic activity also up-regulates PSD-95 expression through a neuregulin-mediated pathway (34). In contrast, administration of cocaine, a drug known to cause hyperexcitability, results in down-regulation of PSD-95 in the striatum, a region mainly composed of inhibitory neurons (35). Moreover, mutation of FMRP, a gene associated with fragile X mental retardation, results in a loss of regulation of PSD-95 expression (36). Thus, changes in PSD-95 levels under these conditions may also induce profound effects on the localization of NLGs at excitatory and inhibitory synapses and thus a change in the E/I ratio.

It appears then that localization of NLGs rather than their intrinsic characteristics determine which members are involved in formation of a particular type of synapse. The following question therefore arises: How does sequestering particular NLGs to either excitatory or inhibitory postsynaptic sites result in induction of a particular synapse type with respect to the presynaptic side? It may be the case that NLGs are the cue for recruitment of presynaptic machinery that is common to both types of synapses. Additional postsynaptic cues would then be required for specification of synaptic type. Alternatively, NLGs may only be required for synaptic stabilization. Indeed, we observed that both NLG1 and NLG2 are only weakly clustered in young neurons, at an age when PSD-95 and presynaptic clusters have already been well established. Thus, the action of NLGs may come into play later in development, with different members stabilizing synapse formation at their respective synapse type.

We conclude that members of the NLG family exert similar effects on both excitatory and inhibitory synapses. These re-
sults reveal a novel role for NLGs in building inhibitory synapses through interaction with β-neurexin. Another critical finding is that synaptogenic activity of NLGs is dictated by interactions with scaffolding proteins, and these interactions regulate the E/I synaptic ratio.

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