

Neuroigin-1 is required for normal expression of LTP and associative fear memory in the amygdala of adult animals

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Neuroigin-1 is a potent trigger for the *de novo* formation of synaptic connections, and it has recently been suggested that it is required for the maturation of functionally competent excitatory synapses. Despite evidence for the role of neuroigin-1 in specifying excitatory synapses, the underlying molecular mechanisms and physiological consequences that neuroigin-1 may have at mature synapses of normal adult animals remain unknown. By silencing endogenous neuroigin-1 acutely in the amygdala of live behaving animals, we have found that neuroigin-1 is required for the storage of associative fear memory. Subsequent cellular physiological studies showed that suppression of neuroigin-1 reduces NMDA receptor-mediated currents and prevents the expression of long-term potentiation without affecting basal synaptic connectivity at the thalamo-amygdala pathway. These results indicate that persistent expression of neuroigin-1 is required for the maintenance of NMDAR-mediated synaptic transmission, which enables normal development of synaptic plasticity and long-term memory in the amygdala of adult animals.

synaptic plasticity | neuroigin | autism

Several studies have found that synaptically localized cell adhesion molecules not only trigger synapse formation but also play a major role in regulating both basal synaptic transmission and synaptic plasticity (1, 2). Among them, neurexins and neuroigins (NLs), which undergo a heterophilic interaction with each other, have emerged as important organizers of *de novo* synapse formation (3). Moreover, modifying the interaction of neuroigin-1 and PSD-95 alters the balance of neuronal excitation and inhibition required for normal brain function (4). The indispensable role of neuroigins for proper neuronal connectivity is further supported by the genetic linkage of neuroigin mutations with autism, a disease that is thought to be a disorder in social cognition that critically involves the amygdala (5, 6).

Because neuroigins are present both during development and throughout adulthood (7, 8), it is likely that neuroigins play roles other than that of an inducer of synaptogenesis in the adult brain. Indeed, a recent study of knockout (KO) mice deficient in neuroigin-1 demonstrated that neuroigin-1 regulates excitatory synaptic responses (9). Although neuroigin-1 has been suggested to be essential for maintaining normal *N*-methyl-D-aspartate (NMDA)-type glutamate receptor-mediated currents (9), the underlying mechanism and its physiological consequence remain to be identified. Furthermore, because the regulation of NMDA receptor (NMDAR) is critical for long-term synaptic modification (10), alterations of NMDAR-dependent currents regulated by neuroigin-1 are likely to have effects on synaptic plasticity and long-term memory in adult animals.

To address the functional role of neuroigin-1 at existing mature synapses, we used virus-mediated RNA interference to deplete endogenous neuroigin-1 in the lateral nucleus of the amygdala

(LA) of adult animals. We investigated the actions of neuroigin-1 in NMDAR-mediated synaptic transmission at the thalamo-amygdala synapse in brain slices and explored the physiological role of neuroigin-1 in intact behaving animals. In this fashion, we found that the continued presence of neuroigin-1 is required for the normal development of synaptic plasticity and memory retention.

Results

Expression of Endogenous Neuroigins and Acute Suppression of Neuroigin-1 in the Amygdala. Western blot analysis with a pan-neuroigin antibody revealed that the neuroigins were expressed in the amygdala of rat brain in both perinatal and postnatal stages and that their expression is slightly up-regulated during postnatal development (Fig. 1A). We attempted to suppress endogenous neuroigin-1 in neurons of the adult brain, using viral vectors to minimize the possibility of compensatory adaptations that can occur in genetically modified animals. We used a dual promoter lentiviral vector containing the small hairpin RNA (shRNA) targeted to neuroigin-1 and eGFP under control of U6 promoter and the CMV promoter, respectively (shNL1) (11). This viral knock-down system allowed us to suppress the expression of endogenous neuroigin-1 in a spatially and temporally controlled manner and to analyze the effects of neuroigin-1 on synaptic transmission, synaptic plasticity, and behavioral modification.

To validate that the viral vector was effective and selective in the intact brain, we infused shNL1 or the control eGFP-expressing virus with stereotactic microsurgery into the amygdala. Both shNL1 and control virus infected a substantial percentage of cells [$\approx 50\%$ cells; **supporting information (SI) Fig. S1**] only in the targeted area of the brain with minimum diffusion (< 1 mm from the injection sites). The virus infusion into the LA area led to infection of $18.3 \pm 1.4\%$ ($n = 9$) and $15.6 \pm 1.3\%$ ($n = 11$) (shNL1, and control virus, respectively) of total LA neurons identified with staining of NeuN (Fig. 1B). To confirm that the expression of neuroigin-1 in the LA was successfully suppressed *in vivo* by shNL1, we carried out serial

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Conflict of interest statement: E.R.K. is one of four founders of Memory Pharmaceuticals and is Chairman of its Scientific Advisory Board. Memory Pharmaceuticals is concerned with developing drugs for age-related memory loss. Some of these drugs are also potentially useful in depression and schizophrenia. E.R.K.'s laboratory is not involved in developing these drugs. E.R.K. is also a consultant for BrainCells, Inc., which works on neurogenesis, an area in which he is not directly involved.

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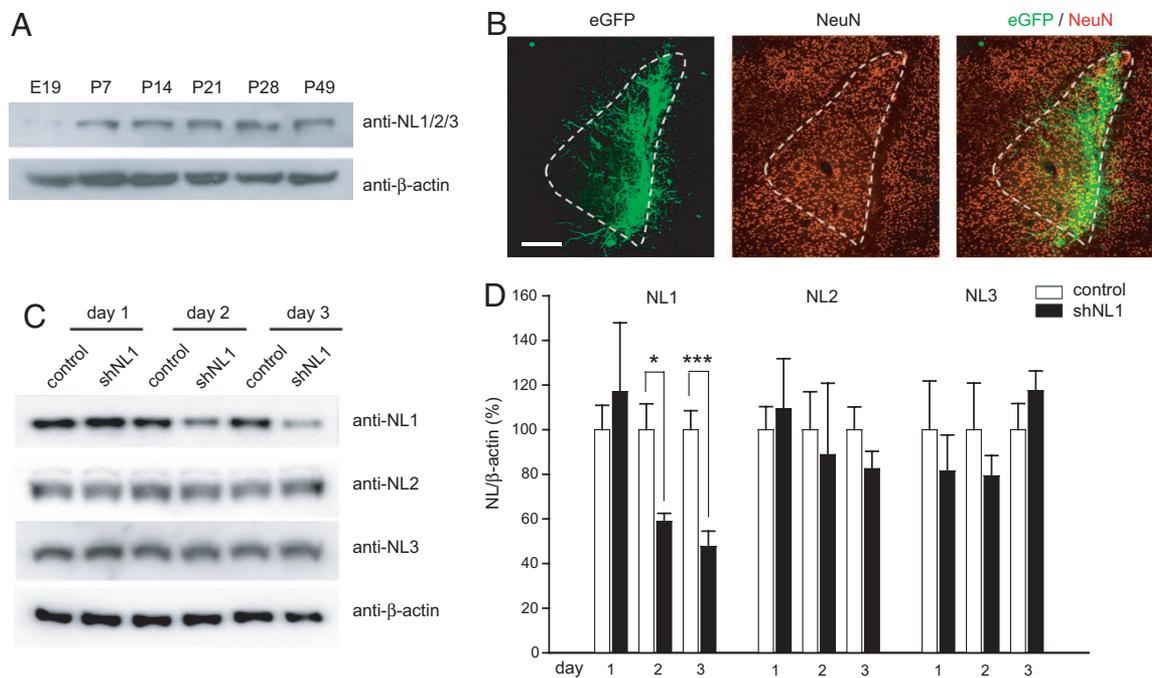


Fig. 1. Neuroigin expression at developmental stages and RNAi-mediated depletion of endogenous neuroigin-1 in the LA. (A) Immunoblots with pan-neuroigin antibody show that neuroigins are persistently expressed in the amygdala of rat brain at various developmental stages. (B) Immunohistochemistry images of the amygdala section (30 μ m thick) 3 days after the infusion of shNL1. The outline of the LA is depicted as a dotted line. Virus-infected neurons of the LA were identified by immuno-staining for eGFP (green) and a neuronal marker NeuN (red). More than 18% of the LA neurons were successfully infected by shNL1. The proportion of infected neurons from the total LA neurons was calculated as a percentage of GFP⁺ NeuN⁺/NeuN⁺ cells in the LA. (Scale bar: 300 μ m.) (C) Immunoblots exhibiting the time course of shNL1 effect after virus infusion. The level of neuroigin-1 in the shNL1-infected areas of LA started to decrease from 2 days after the virus infusion. Control or shNL1-infused animals ($n > 6$ per group; $n > 4$ per each time point) were killed at the indicated time after virus infusion. (D) Summary histograms of neuroigin-1, -2, and -3 immunoreactivity normalized to β -actin levels. The neuroigin-1 protein levels measured 2 days after virus infusion; control group, $100 \pm 11.6\%$ vs. shNL1 group, $58.8 \pm 3.7\%$ (*, $P < 0.05$), and 3 days after virus infusion; control group, $100 \pm 8.5\%$ vs. shNL1 group, $47.7 \pm 6.8\%$ (***, $P < 0.001$). The protein levels of neuroigin-2 and -3 were not significantly different between control and shNL1-infused groups at any time points ($P > 0.2$ for all comparisons).

Western blot analyses at multiple time points after virus infusion. Only the highly infected area of LA tissues but not the whole LA tissues were used because the effect of shNL1 occurring in a small proportion of cells could be probably masked by normal level of neuroigin-1 of the nearby uninfected cells of whole LA (see *SI Methods* for the detailed procedure). The analysis, using antibodies that selectively recognize either neuroigin-1 (7), neuroigin-2 (12), or neuroigin-3 (12) (Fig. S2), revealed that the amount of neuroigin-1 was decreased by $41.2 \pm 3.7\%$ (day 2 after infusion) and by $52.3 \pm 6.4\%$ (day 3 after infusion) of control levels (Fig. 1 C and D). In contrast with that of neuroigin-1, expression levels of neuroigin-2 and -3 were unaltered by infusion of shNL1 when probed with the specific antibodies, indicating that the suppression of neuroigin-1 elicited no compensatory up-regulation of other neuroigins (Fig. 1 C and D). In addition, to rule out possible diffusion of shNL1 to adjacent structures, we also assessed the expression level of neuroigin-1 in the central amygdala, which exhibited no difference between the groups (Fig. S3). Collectively, these quantitative measures verified that the *in vivo* RNA interference for neuroigin-1 was effective and selective in acutely suppressing endogenous neuroigin-1 in the LA. Considering the time course of RNA interference, we prepared brain slices for electrophysiological assays and started behavioral tests for fear memory 3 days after virus infusion.

Selective Reduction of NMDAR-Mediated Currents by *in Vivo* Depletion of Neuroigin-1. The principal neurons in dorsolateral division of the LA were identified visually and confirmed as showing spike frequency adaptation that was not observed in interneurons (13) (data not shown). We examined the electrophysiological properties of eGFP-expressing principal neurons in the LA slices of either

shNL1- or vehicle virus-infused rats. We were not able to detect any difference in the intrinsic electrophysiological properties between the two groups (Table S1). This analysis revealed that neuroigin-1 is not required for maintaining the intrinsic characteristics of principal neurons. The electrical properties and synaptic responses from uninfected principal neurons were indistinguishable from those of vehicle-virus infected neurons. Therefore, we pooled these data to form a control group.

Two major auditory afferents of the LA (cortico-amygdala and thalamo-amygdala) form two distinct types of synapses on the same target principal neurons (14). Synaptic regulation in the thalamo-amygdala pathway depends mostly on postsynaptic modifications (15, 16). To examine the role of neuroigin-1 localized to postsynaptic sites (7), we focused on the thalamo-amygdala synapses. We evoked excitatory postsynaptic currents (EPSCs) by stimulating the internal capsule with a bipolar stimulation electrode (Fig. 2A). To examine the efficacy of thalamic input to LA principal neurons, we obtained input-output curves (Fig. S4) and found no difference between shNL1-infected and control neurons, indicating that the postsynaptic suppression of neuroigin-1 did not cause any significant change in the ability of thalamic input to elicit EPSCs.

To assess and compare synaptic strength, we measured the ratio of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type glutamate receptor (AMPA)- and NMDAR-dependent EPSCs (NMDAR/AMPA). The NMDAR/AMPA ratio has been used reliably to compare synaptic strengths in different brain slices because it is independent of experimental conditions of slice recording such as the positioning of the electrodes and differences in slice preparation (17). We observed a $\approx 50\%$ decrease in the NMDAR/AMPA ratio in shNL1-infected neurons compared with that in control neurons (Fig. 2B). To test whether this

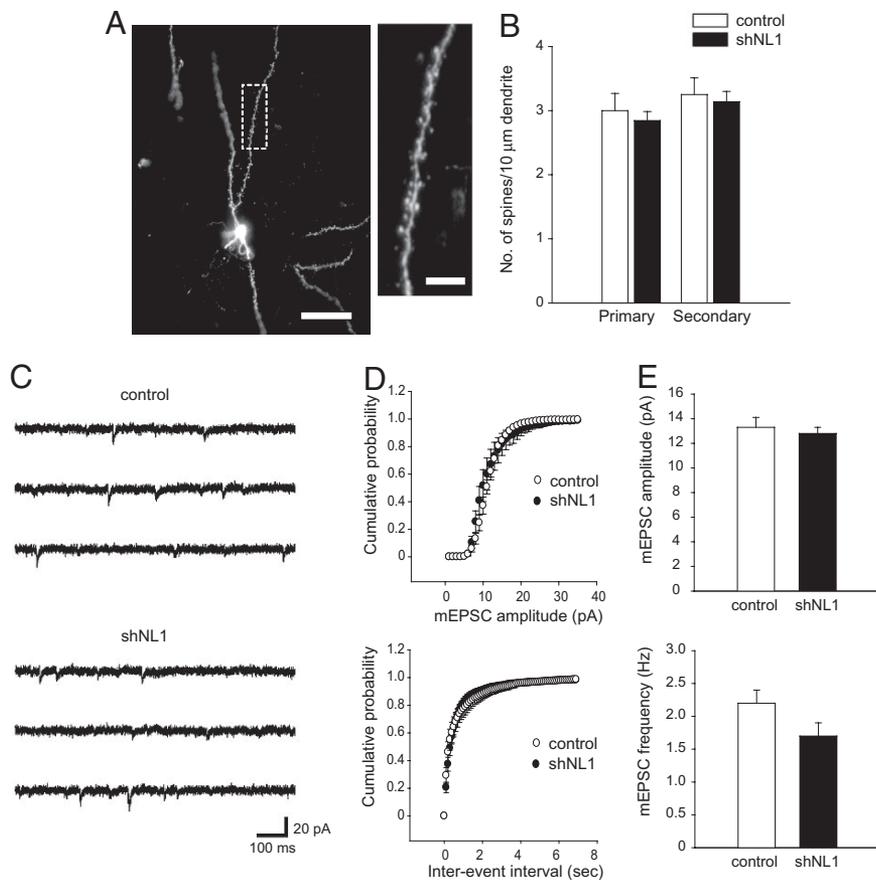


Fig. 3. Unaffected synaptic connections and basal synaptic transmission at thalamo-amygdala synapses. (A) Representative images of a principal neuron expressing shNL1 (Left). The dendritic region outlined with a dotted line was magnified to delineate spines (Right). (Scale bars: Left, 50 μm; Right, 10 μm.) (B) Summary histogram of spine densities (number of spines per 10 μm dendrite segment) for control and shNL1-infected neurons ($P > 0.3$ for both). (C) Sample traces of mEPSCs recorded from control and shNL1-infected neurons. (D) Cumulative amplitude (Upper) and inter-event interval (Lower) plots of mEPSCs for control ($n = 17$) versus shNL1- ($n = 20$) infected neurons. Kolmogorov–Smirnov test was used for the comparison ($P > 0.1$ for both). (E) Summary histograms of mEPSC parameters. The mean peak amplitudes ($P > 0.3$) and the mean frequencies ($P > 0.2$) were not significantly different between two groups.

Requirement of Neuroligin-1 for Storage of Associative Fear Memory. Various *in vivo* and *in vitro* results support the hypothesis that both LTP at the amygdala synapses and fear conditioning-induced neuronal plasticity share common molecular mechanisms (16, 21, 22). Therefore, we turned to a standard fear conditioning task to

examine the behavioral consequences of the depletion of neuroligin-1 at the intact animal level. Rats that were infused with viruses exhibited similar baseline behaviors to contextual and repeated auditory cues before the conditioning electrical shocks (repeated measures ANOVA, Fig. 5B). Indeed, freezing behaviors to the cues

Fig. 4. LTP impaired by depletion of neuroligin-1 or partial blockade of NMDARs at thalamo-amygdala pathway. (A) Mean EPSC amplitudes before and after the pairing (an arrow) were indicated after the normalization to preparing levels. LTP was abolished in shNL1-infected neurons (solid circles), whereas the pairing protocol induced LTP in control neurons (open circles). (Inset) Representative traces of EPSCs from control (Left) and shNL1- (Right) infected neurons were indicated before (black) and 30 min after (red) the pairing. Stimulus artifacts were omitted for clarity. (B) A summary histogram of normalized EPSC amplitudes (30 min after the pairing) was depicted (***, $P < 0.001$); control group, $195.1 \pm 53.9\%$ for ($n = 9$) vs. shNL1 group, $97.0 \pm 18.9\%$ ($n = 13$). (C) Mean EPSC amplitudes before and after the pairing (an arrow) in untreated control slices (open circles) and slices treated with 8 μM AP-5 (solid circles). LTP was also abolished in principal neurons from the LA slices that were pre-treated with AP-5 whereas the same pairing induced LTP in control neurons from untreated slices. (Inset) Representative EPSC traces from control (Left) and AP-5- (Right) treated neurons were indicated before (black) and 30 min after (red) the pairing. Stimulus artifacts were omitted for clarity. (D) A summary histogram of normalized EPSC amplitudes (30 min after the pairing) was presented for control and AP-5-treated neurons (**, $P < 0.01$); control group, $185.6 \pm 14.9\%$ ($n = 6$) vs. AP-5-treated group, $89.9 \pm 16.5\%$ ($n = 7$).

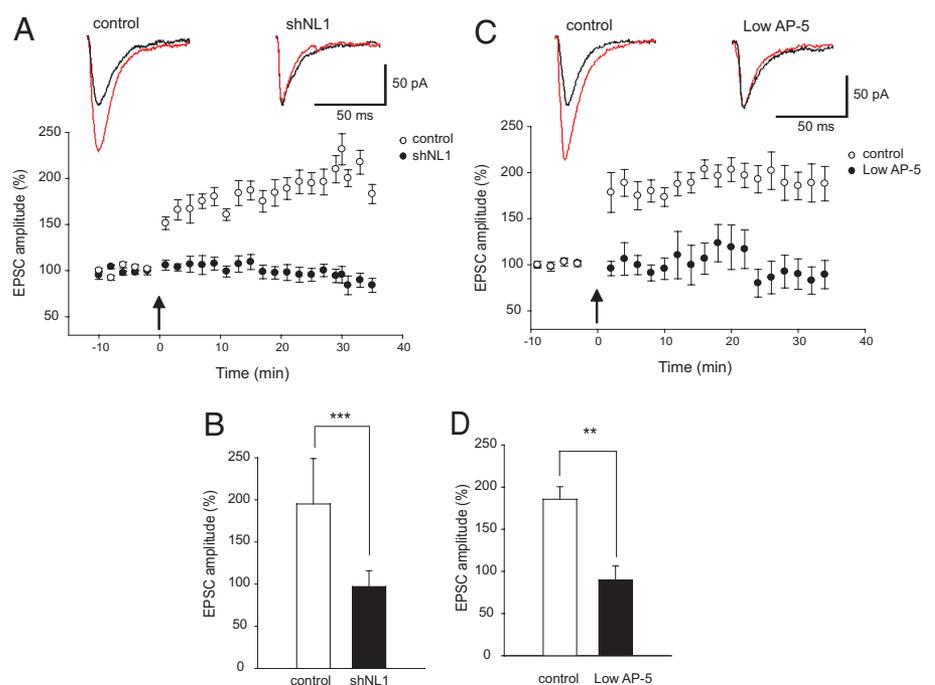
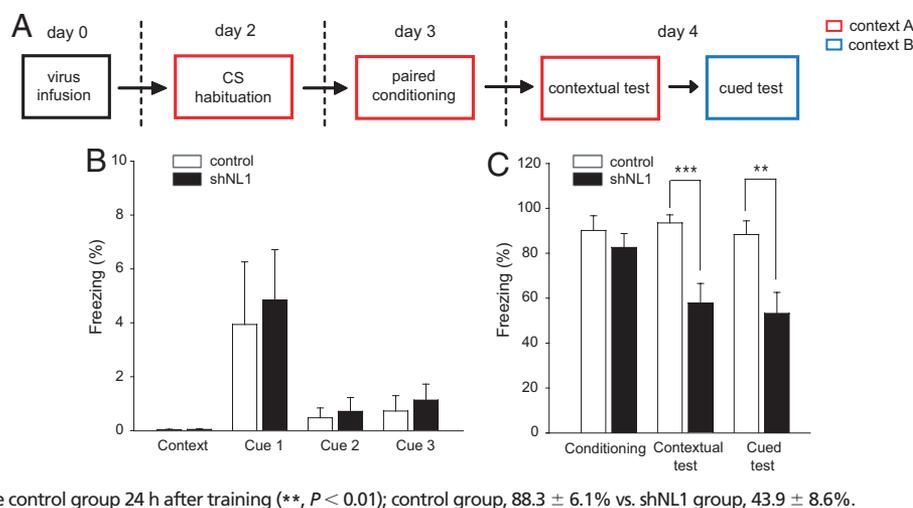


Fig. 5. Requirement of neuroligin-1 for storage of associative fear memory. (A) Schematized experimental procedures to test effects of virus infusion on fear conditioning. (B) No statistical difference in freezing levels appeared between control and shNL1 groups during the habituation period ($n = 11$ rats for control group vs. $n = 9$ rats for shNL1 group). Before the first auditory cue was given, animals of both groups showed almost no freezing in a recording chamber (context) and they exhibited similar baseline freezing responses to repeated auditory cues during habituation ($P > 0.6$). (C) There was no statistical difference in freezing level to the cues between the two groups ($P > 0.2$) during the cue-shock paired fear conditioning. In the contextual fear conditioning test, shNL1 group exhibited significantly less freezing in the conditioned context compared with the control group 24 h after training (***, $P < 0.001$); control group, $93.5 \pm 3.6\%$ vs. shNL1 group, $49.0 \pm 7.8\%$. In the cued fear conditioning test, shNL1 group also exhibited significant reduction of freezing behavior to the shock-associated cues compared with the control group 24 h after training (**, $P < 0.01$); control group, $88.3 \pm 6.1\%$ vs. shNL1 group, $43.9 \pm 8.6\%$.



of second and third sessions of paired-conditioning were similarly increased in both groups (Fig. 5C), which indicated that there was no difference in fear conditioning between control and shNL1-infused animals. After 24 h, however, shNL1-infused animals showed less freezing in the training chamber (contextual memory) than the control virus-infused animals (repeated measures ANOVA, Fig. 5C). We also tested cued fear memory in a novel context to a neutral tone that had been associated with electric shocks. The shNL1-infused animals again displayed a significant decrease in freezing response at the onset of the tone (repeated measures ANOVA, Fig. 5C). These results indicate that shNL1-infused animals have certain deficits for both contextual- and cued-fear memory.

To examine whether the suppression of neuroligin-1 in the LA compromised the anxiety states of subject animals, we assessed the anxiety levels with the open field test. No statistical difference was detected in all of the measurements reflecting the unaltered anxiety levels (Fig. S6). We also measured pain sensitivity by monitoring behavioral responses with increasing levels of electric shock, which revealed no difference between the groups (data not shown). Our data demonstrate that the presence of neuroligin-1 in the LA is essential for the storage of associative fear memory.

Discussion

Although neuroligins have been known to play a role in maturation and specification of synapses (8, 9), their physiological consequences in more complex neuronal circuitry—especially that found in normal adult brains—remain largely unknown. We have investigated the functions of neuroligin-1 in the context of synaptic transmission, synaptic plasticity and memory storage in adult animals.

We have found a specific effect of neuroligin-1 on NMDAR-mediated currents. Our method of acutely silencing neuroligin-1, using RNA interference delivered by virus, allowed us to avoid the possible developmental defects often associated with KO mouse models. Therefore, this study confirmed that the selective effect of neuroligin-1 on NMDAR-mediated transmission is physiological and not derived from potential developmental defects of the KO mice. In addition to this confirmation, the transient suppression of neuroligin-1 (≈ 2 days, Fig. 1) caused a decrease in NMDAR-mediated currents, providing a new insight into the normal function of excitatory synapses by showing that their normal maintenance requires the persistent presence of neuroligin-1.

The decrease in NMDAR-mediated currents upon suppression of neuroligin-1 could be attributed to reduced insertion of NMDARs to surface membrane or less effective lateral trafficking of NMDARs from extrasynaptic to synaptic sites, which reduced the number of NMDARs residing in postsynaptic membrane. Alter-

natively, it is also possible that neuroligin-1 modulates channel properties of individual NMDARs as EphB receptors (23). Future molecular studies of precisely how neuroligin-1 regulates NMDAR-mediated synaptic transmission and which proteins are involved in this process will ultimately lead to a better understanding of the physiological roles of neuroligin-1 in the mature neuronal circuitry of the adult brain.

We found that neuroligin-1 is not required for maintenance of synaptic connections and basal synaptic transmission in the mature neural network. The dispensability of neuroligin-1 is inconsistent with the previous report obtained in cultured neurons (11) but is supported well by recent *in vivo* data (8, 9). A possible explanation for the discrepancy of the results from cultured neurons and intact brain is that neuroligin-1 plays functional roles in a context-specific manner. When new synapses are produced intensively, such as in cultured neurons, neuroligin-1 is likely to control where new synapses are formed, whereas it would be more involved in regulation and specification of synaptic transmission after the establishment of mature synaptic connections.

One may argue that the reduction of NMDAR-mediated currents by RNA interference could be due to unspecific effects of shRNA. However, the off-target effects of shNL1 do not seem to account for the decrease in NMDAR-mediated currents and consequential effects for several reasons: (i) shNL1 did not affect the evoked AMPAR-mediated currents and basal synaptic transmission; (ii) the same sequence of shNL1 had been demonstrated to have no off-target effect (11); and (iii) typical off-target effects of shRNA, such as changes in spine morphology and intrinsic electrical properties, were absent (24). Thus, the shNL1-induced functional alterations are likely to arise from the depletion of endogenous neuroligin-1.

Our study has addressed a physiological consequence that a decreased availability of neuroligin-1 abolished LTP at the circuit level. A significant decrease in NMDAR-dependent EPSCs and the ensuing impairment of LTP expression are consistent with the results of a study demonstrating that activation of NMDARs in the amygdala is required for normal expression of synaptic plasticity (25). Furthermore, LTP was also abolished when NMDAR-mediated currents were partially attenuated to a similar extent as in shNL1-infected neurons, suggesting that the impairment of LTP expression appears to be an immediate consequence of the decrease in NMDAR-mediated currents that is induced by the depletion of neuroligin-1 and is less likely to result from the subsequent perturbation of biochemical signaling pathways downstream to NMDARs.

We also demonstrated that the depletion of neuroligin-1 in the LA caused a deficit in storage of associative fear memory in intact

behaving animals. Studies have shown that fear conditioning induces LTP in the amygdala, whereas inhibition of NMDARs blocks fear conditioning (21, 26). Although decreased NMDAR-dependent EPSCs and impaired LTP could account for the deficit in fear memory retention, one alternative possibility is that the depletion of neuroligin-1 might also affect inhibitory circuitry, which can influence both animal behavior and long-term memory. If the inhibitory drive is enhanced in the LA, fear memory could be impaired, as demonstrated with infusion of muscimol to the amygdala (27). Whether the depletion of neuroligin-1 enhances inhibitory circuitry could not be directly addressed in our electrophysiological analyses, which were carried out in the absence of inhibitory transmission. However, it was demonstrated that the suppression of neuroligins reduces inhibitory postsynaptic currents in cultured neurons (11), and the deletion of neuroligin-1 does not alter inhibitory transmission at all (9). Thus, the possible enhancement of inhibitory drive is unlikely to be the cause of the impairment in fear memory observed in this study.

Although predisposing genetic defects leading to autism are largely unknown, mutations in the human neuroligin genes have been reported to be linked to the disease (5, 6, 28). Because autism is thought to affect remodeling, maturation, and stabilization of mature synaptic connections (29), it is critical to study the physiological role of the autism-linked proteins in mature neuronal circuitry. A new knockin mouse model with a mutation in neuroligin-3 has recently been reported to exhibit autism-related behavioral abnormalities (30). It is interesting that this mice model exhibits an increase in inhibitory synaptic transmission without any alteration in excitatory transmission, which is not normally observed in autism patients (31). Therefore, it would appear to be necessary to investigate other neuroligins, especially those that regulate excitatory transmission. It is also essential to garner additional physiological and molecular insights in the brain regions highly involved in its etiology. We have focused on the amygdala, one of the brain regions consistently implicated in the pathophysiology of autism (32), to illustrate the physiological functions of neuroligin-1 on excitatory synaptic transmission. Thus, our findings are particularly relevant to autism research not only because they illustrate the *in vivo* physiological consequences of neuroligin-1 on synaptic plasticity and memory retention in the mature neural networks but because they provide insights into the regulation of

synaptic transmission in the amygdala, a structure thought to be important for autism, by a gene family shown to be a contributor to autism in certain patients.

Methods

For detailed experimental procedures, see *SI Methods*.

Biochemical and Animal Experiments. shNL1 lentiviral constructs were described in ref. 11. Anti-pan neuroligins (Synaptic System), anti-neuroligin-1 [from N. Brose (Max Planck Institute of Experimental Medicine, Gottingen, Germany)], and anti-neuroligin-2, -3 isoform-specific antibodies (12) were used for Western blot analyses. Male Sprague–Dawley rats (Orient Co.) were anesthetized with pentobarbital sodium, and then guide cannulae (Plastics One) were bilaterally implanted targeting the lateral amygdala, using coordinates from Paxinos and Watson (33). Lentivirus was infused through injection cannulae, and fear memory was tested according to experimental schemes as depicted in Fig. 5A. All procedures for animal experiments were performed in accordance with Pohang University of Science and Technology guidelines on animal care and use.

Electrophysiology. Animals were anesthetized with Ketamine/Medetomidine and decapitated, and the brains were quickly removed and chilled in ice-cold dissection buffer. Amygdala slices (300 μ m) were prepared with a vibratome (Leica) from control eGFP vehicle-virus and shNL1-injected rats. Whole-cell recording was made from principal neurons in the dorsolateral division of the LA under an upright microscope with DIC/infrared/fluorescence optics (Leica) with an Axopatch 200A amplifier and Clampex software, Version 9.2 (Molecular Devices). Cell excitability and intrinsic membrane properties were obtained in the current-clamp configuration, whereas evoked EPSCs and mEPSCs were recorded in the voltage-clamp mode. EPSCs were elicited by stimulating the internal capsule (thalamic input) as described in ref. 20. EPSCs were evoked at a holding potential of -60 mV unless otherwise specified.

Statistical Analysis. Results were denoted as means \pm SEM, and the statistical significance was expressed as *, $P < 0.05$; **, $P < 0.01$; or ***, $P < 0.001$. The Mann–Whitney test was used for comparison between two groups. Repeated measures ANOVA test was used to analyze a main effect for treatment (eGFP and shNL1) among sessions in habituation and between test types in conditioning test. The Kolmogorov–Smirnov test was used for the statistic analysis of cumulative histograms of mEPSCs.

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