

Research Article

Neurexin-Neuroigin Synaptic Complex Regulates Schizophrenia-Related DISC1/Kal-7/Rac1 “Signalosome”

Sylwia Owczarek,^{1,2} Marie Louise Bang,¹ and Vladimir Berezin¹

¹Laboratory of Neural Plasticity, Department of Neuroscience and Pharmacology, University of Copenhagen, 2200 Copenhagen, Denmark

²Research Laboratory for Stereology and Neuroscience, Bispebjerg Hospital, 2400 Copenhagen, Denmark

Correspondence should be addressed to Sylwia Owczarek; sylwia@sund.ku.dk

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Neurexins (NXs) and neuroligins (NLs) are cell adhesion molecules that are localized at opposite sites of synaptic membranes. They interact with each other to promote the assembly, maintenance, and function of synapses in the central nervous system. Both NX and NL are cleaved from a membrane-attached intracellular domain in an activity-dependent manner, generating the soluble ectodomain of NX or NL. Expression of the *NX1* and *NX3* genes in the brain appears to be regulated by a schizophrenia-related protein, DISC1. Here, we show that soluble ecto-NX1 β can regulate the expression of DISC1 and induce signaling downstream of DISC1. We also show that NL1 binds to a well-characterized DISC1 interaction partner, Kal-7, and this interaction can be compromised by DISC1. Our results indicate that the NX/NL synaptic complex is intrinsically involved in the regulation of DISC1 function, thus contributing to a better understanding of the pathology of schizophrenia.

1. Introduction

The development and maintenance of synaptic connections are a dynamic process that requires bidirectional interactions between pre- and postsynaptic components. Adhesion molecules are present at the synapse and organize synaptic specializations. The synaptic adhesion complex neurexin/neuroigin (NX/NL) mediates synapse formation and triggers pre- and postsynaptic signal transduction by recruiting components to developing synapses [1]. Depending on the composition of NX/NL complexes, they function as molecular switches that regulate excitatory-inhibitory (E/I) balance in the brain [1]. Mammals have three *NX* genes, each with two promoters. Each gene produces both longer α -NX and shorter β -NX transcripts. Additionally, each isoform can be alternatively spliced at five and two positions, respectively [1]. Humans express five *NL* genes, whereas other mammals express only three or four [1]. Each *NL* gene has a canonical splicing site (SS#A), and *NL1* additionally has the SS#B splicing site. Interestingly, the expression and function of both NXs and NLs are regulated

by neural activity. *NX* mRNA expression increases as synaptic activity increases [2], and both NX and NL can be cleaved from the pre- or postsynaptic membrane in an activity-dependent manner [3–6]. The expression of the *NX1* and *NX3* genes was recently shown to be dysregulated in the *Disc1* (disrupted in schizophrenia 1) mouse model of schizophrenia [7]. DISC1 is a scaffolding protein that plays several roles during many aspects of neural development [8]. For example, the inactivation of DISC1 during early development results in alterations in the density and morphology of dendritic spines [9]. Spines appear to be regulated by DISC1 in association with kalirin-7 (Kal-7) [10–12]. Kal-7 is an activator of a small guanosine triphosphatase (GTP), Rac1. DISC1, together with Kal-7 and Rac1, forms a multifunctional protein complex, called the DISC1/Kal-7/Rac1 signalosome, that is important for development of the nervous system [10]. Under baseline conditions, DISC1 binds Kal-7, blocking the access of Kal-7 to Rac1. The activation of ionotropic glutamate receptors (*N*-methyl-D-aspartate [NMDA] receptors) weakens this protein-protein interaction, allowing free access of Kal-7 to Rac1, which leads to the activation of Rac1 and eventually

spine maturation [10]. The aforementioned findings led us to hypothesize that NX/NL- and DISC1-induced signaling might be interconnected. Here, we show that NL1 binds to Kal-7, and alterations in NX-NL interactions lead to disturbances in the DISC1/Kal-7/Rac1 signalosome, thus allowing the activation of Rac1.

2. Materials and Methods

2.1. Materials. The recombinant ectodomain of human neurexin 1 beta Fc Chimera (ecto-NX1 β) was obtained from R&D Systems (catalog number 5268-NX-050; Abingdon, UK). An expression vector that encodes NL1-AB, pCAGNL1-AB was a gift from Peter Scheiffele (Addgene plasmid #15262) [13]. An expression vector that encodes Kal-7, pEAK10-His-Myc-Kal7 was a gift from Betty Eipper (Addgene plasmid #25454) [14]. An expression vector that encodes mouse FLAG-myc-tagged DISC1 was purchased from Origene (Rockville, MD, USA). The PKA inhibitor H89 was obtained from Invivogen (Toulouse, France). The following antibodies were used in this study: rabbit anti-S897-NR1, anti-S896-NR1 (1:500; Millipore, Billerica, MA, USA), phospho-Erk1/2, phospho-Akt (1:1000; Cell Signaling, Beverly, MA, USA), mouse anti- β -tubulin (1:50,000; Sigma-Aldrich, St. Louis, MO, USA), anti-HA tag (Abcam, Cambridge, UK), anti-myc-tag (Sigma-Aldrich), anti-Kal-7 (Abcam), and IRDye secondary antibodies (1:20,000; Odyssey, Lincoln, NE, USA).

2.2. Cell Cultures. Cortical neurons were obtained on embryonic day 18 from Wistar rat embryos (Charles River Laboratories, Kisslegg, Germany) as described previously [15]. For each single experiment, the cortices from all embryos were dissected, cleared from blood vessels and meninges, pooled together, mechanically chopped, and trypsinized. The cells were then washed in the presence of DNase and trypsin inhibitor, resuspended, and seeded at a density of 2×10^5 cells/cm² in poly-D-lysine coated (1 μ g/mL) 6 cm Petri dishes (Nunc, Roskilde, Denmark) in Neurobasal medium supplemented with 2% (v/v) B27, 1% (v/v) GlutaMax, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 2% (v/v) of 1M HEPES (all from Gibco, Waltham, MA, USA). At 3 DIV, cytosine β -D-arabinofuranoside (Ara-C) in concentration 2 μ M was added to inhibit the growth of nonneuronal cells. Cortical neurons were grown for 8 days. At this developmental stage the majority of synapses are immature. The advantage of using these young cultures is the high survival rate of neurons and general low background due to low level of spontaneous activity [16].

Human embryonic kidney 293 (HEK293) cells were obtained from the European Cell Culture Collection (Salisbury, UK) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal calf serum, 2 mm GlutaMax, 100 units/mL penicillin, and 100 μ g/mL streptomycin. All of the cell cultures were kept in a humidified incubator at 37°C in a 5% CO₂ atmosphere.

2.3. Immunoblotting. Cortical neurons were grown for 8 days before treatment. Thereafter, neurons were stimulated with

ecto-NX1 β (0.01, 0.05, and 0.2 nM) for 5 min. The cells were lysed, and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting using the antibody of interest. The bands were visualized using the Odyssey CLX Infrared Imaging System (Odyssey).

2.4. Rac1-GTP Assay. Cortical neurons were grown for 8 days and stimulated with ecto-NX1 β (0.01, 0.05, and 0.2 nM) for 20 min. The activation of Rac1 was measured with the Rac1 activity assay kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. A fusion protein that consisted of the p21-binding domain of Pak1, which specifically binds to the active form of Rac1 (Rac-GTP) but not to the inactive form of Rac1 (Rac-GDP), was used for Rac-GTP precipitation. Immunoprecipitated samples were analyzed by SDS-PAGE and immunoblotting using a primary mouse anti-Rac1 antibody (1:1000; Thermo Scientific) and goat anti-mouse RDye secondary antibodies (1:20,000; Odyssey).

2.5. Real-Time Quantitative Polymerase Chain Reaction. Cortical neurons were grown for 8 days and treated with ecto-NX1 β (0.01, 0.05, and 0.2 nM) for 2 h (*c-fos*), 24 h (DISC1), or 48 h (NR1 splice variants). Total RNA was prepared using the GeneJET RNA Purification Kit (Thermo Scientific) according to the manufacturer's instructions. RNA integrity and quantity were verified using an Agilent 2100 Bioanalyzer (Agilent Technologies). Only samples with RIN \geq 8 were used for further analyses. The RNA samples were stored at -80°C until use. Quantitative reverse-transcription polymerase chain reaction (PCR) and the data analyses were performed as described previously [17] using the primers that are shown in Table 1. All of the samples were run in duplicate. The relative levels of the PCR products in all of the samples were evaluated by the Pfaffl method [18].

2.6. Coimmunoprecipitation. HEK293 cells were transfected with cDNA expression vectors that encoded the proteins of interest. Forty-eight hours after transfection, cells were lysed in immunoprecipitation (IP) buffer (phosphate-buffered saline [PBS] and 1% NP40) supplemented with complete protease inhibitors (Roche), and cleared lysates were incubated with appropriate antibodies overnight. Immunoprecipitates were collected with Pierce protein A/G magnetic beads (Thermo Scientific), washed twice in IP buffer (once with PBS), and analyzed by SDS-PAGE and immunoblotting.

2.7. Data Analysis. The statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple-comparison *post hoc* test, Sidak's multiple-comparison *post hoc* test, or one-sample *t*-test when appropriate using Prism 6 software (GraphPad, San Diego, CA, USA).

TABLE 1: List of primers used in the study.

Gene	Accession number	Primer		Fragment size (bp)
		Sense	Antisense	
<i>c-fos</i>	NM_022197.2	5' GGA GGA GGG AGC TGA CAG ATA C 3'	5' GGT CAT TGG GGA TCT TGC AGG 3'	159
<i>Disc1</i>	NM_175596.2	5' TGC ACT CTG GGG TTC ATT CCG 3'	5' CCA TTC GAC GCC GGT GAT 3'	190
<i>NRI-N1</i>	L08228.1	5' ATC ATC CTG CTG GTC AGC GAC G 3'	5' GCA CCT TCT CTG CCT TGG GTC C 3'	160
<i>NRI-C1</i>	L08228.1	5' CGT GAA CGT GTG GAG GAA GAA C 3'	5' CTA CGT CTC TTG AAG CTG GAG G 3'	121
<i>NRI-C2</i>	L08228.1	5' CTC AAC CTC TCA GAT CCC TC 3'	5' GCA GCA GGA CTC ATC AGT GT 3'	102
<i>NRI-pan</i>	L08228.1	5' TGT ACG TCA AGC CCA CAA TGA G 3'	5' ACC AGG TGC ACC TCA TAG GTA 3'	211
<i>GAPDH</i>	NM_017008.3	5' AGC TGG TCA TCA ACG GGA AAC C 3'	5' CCT TCT CCA TGG TGG TGA AGA C 3'	126

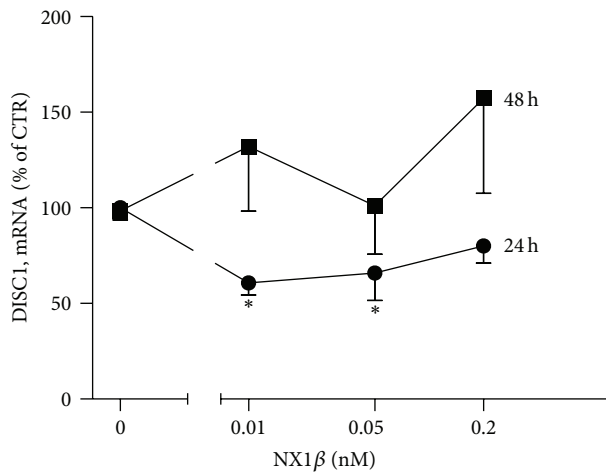


FIGURE 1: NX1 β decreases the mRNA level of DISC1. Cortical neurons were grown for 8 days and subsequently treated with different concentrations of recombinant NX1 β for (a) 24 h and (b) 48 h. Total RNA was isolated, and mRNA levels were assessed using RT-PCR. The results from $n = 3$ experiments are expressed as a percentage \pm SEM. * $P < 0.05$, compared with the untreated control set at 100%.

3. Results and Discussion

The expression of the *NX1* gene appears to be regulated by a DISC1-dependent mechanism [7], and this regulation is important for brain development and function. We investigated whether this regulation might be reciprocal and whether NX might also affect the expression of *Disc1*. Indeed, we found that 24 h treatment with ecto-NX1 β decreased the mRNA level of *Disc1* (Figure 1). This effect was inversely proportional to the dose of the protein. The highest concentration had no effect on *Disc1* expression. Notably, the longer 48 h treatment with ecto-NX1 β did not have a significant effect on *Disc1* expression (Figure 1).

DISC1 has many identified protein interaction partners, including synaptic proteins [8]. We hypothesized that either NX or NL might interact with DISC1 itself or might affect DISC1 interactions with other partners. Using LALIGN [18] to find the best local alignments between two sequences, we inspected the cytoplasmic domains of both NX1 (EAX00186.1) and NLI (ADB12633.1) for homology to

DISC1 (Q9NRI5.3). We found that the cytoplasmic region of NLI contains at least two sequence motifs that are homologous to DISC1 (Figure 2(a)). Moreover, these motifs are located in the DISC1 domain, which is crucial for binding with Kal-7 (amino acids 350–394 in DISC1) [10]. Hence, we examined whether NLI might directly interact with Kal-7. The interaction was confirmed by co-IP from heterologous cells (Figure 2(b)). Moreover, in the presence of DISC1, the binding of Kal-7 to NLI decreased substantially (Figure 2(c)), suggesting possible competition between DISC1 and NLI for binding to Kal-7. In contrast, the presence of NLI did not affect the DISC1-Kal-7 interaction, suggesting that DISC1 must first be released from Kal-7 to allow NLI-Kal-7 interactions.

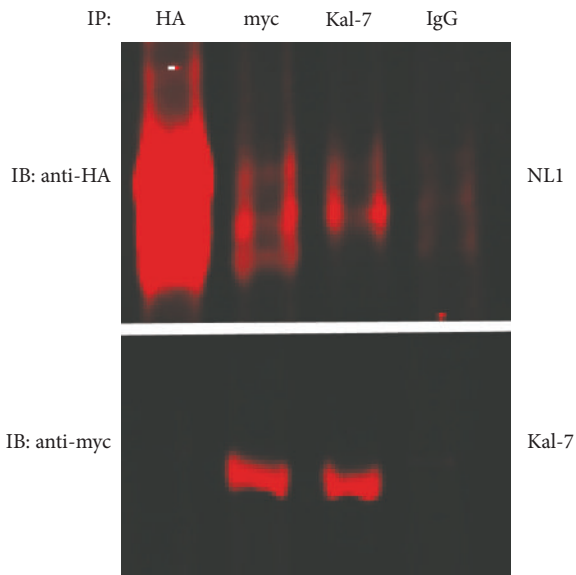
The interaction between Kal-7 and DISC1 depends on synaptic activity. Upon neuronal activation, DISC1 dissociates from Kal-7, which in turn leads to the activation of Rac1 [10]. Neuronal activity also leads to the destabilization and shedding of both NX1 and NLI from the synaptic membrane [3–6]. Additionally, treatment with recombinant ecto-NX1 β mimics some of the effects of neuronal activity associated with the cleavage of NLI from the postsynaptic membrane [6]. We observed that treatment with ecto-NX1 β resulted in an increase in the mRNA level of the immediate early gene *c-fos* (Figure 3), indirectly supporting the hypothesis that treatment with soluble NX1 augments neuronal activity. Thus, we tested whether ecto-NX1 β itself affects Rac1 activation. As shown in Figure 4, ecto-NX1 β increased the level of Rac1-GTP at a concentration as low as 0.01 nM. At higher concentrations, ecto-NX1 β did not have a statistically significant effect on Rac1-GTP.

The signaling of Kal-7 to Rac1 is coupled with neuronal activity via NMDA receptors [10]. Therefore, we examined whether NX can modulate the phosphorylation levels of an obligatory subunit of the NMDA receptor, NRI. NRI can be phosphorylated at three distinct serine residues (S897, S896, and S890) in the intracellular carboxyl tail region [19]. Active protein kinase C (PKC) phosphorylates the NRI subunit at S890 and S896 [20], whereas the activation of cyclic adenosine monophosphate- (cAMP-) dependent protein kinase A (PKA) leads to phosphorylation at S897 [21]. As shown in Figure 5(a), treatment with ecto-NX1 β led to phosphorylation at both S896 and S897. Interestingly, the level of Ser896 was markedly increased by the highest concentration of ecto-NX1 β (0.2 nM), whereas the level of

DISC1 342 LRNRRQMEVISLRK 356
 : . . . :
 Cyt NL1 762 LTHAQEEEEIMSLQMK 776
 Identities 5/15 33%
 Similarities 12/15 80%

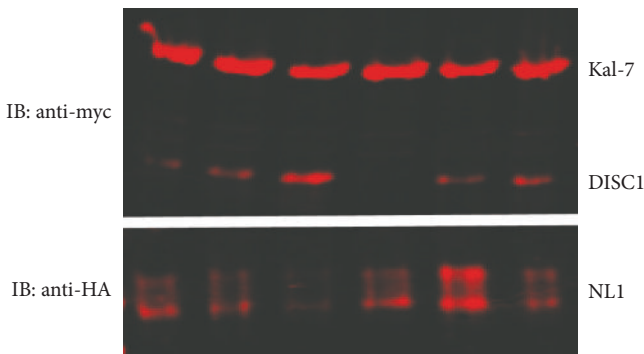
DISC1 375 TLQQRLEDLEQEKISLH 391
 : : . .
 Cyt NL1 772 SLQMKHTDLDECEESI 788
 Identities 7/17 41%
 Similarities 12/17 70%

(a)



(b)

Kal-7	+					
DISC1	+	++	+++	0	++	++
NL1	++	++	++	++	+++	+



(c)

FIGURE 2: Neuroligin 1 interacts with kalirin 7. (a) 293T cells were transfected with HA-tagged NLI-AB or myc-tagged Kal-7. Cell lysates were mixed and subsequently immunoprecipitated with anti-HA, anti-myc, or anti-Kal antibody, and immunoblots were probed with the indicated antibodies. Mouse IgG was used as a control. (b) HEK293 cells were transfected with HA-tagged NLI-AB or myc-tagged Kal-7 or FLAG-tagged DISC1. Cell lysates were mixed and subsequently immunoprecipitated. Immunoblots were probed with the indicated antibodies. Representative immunoblots of $n = 3$ experiments are shown.

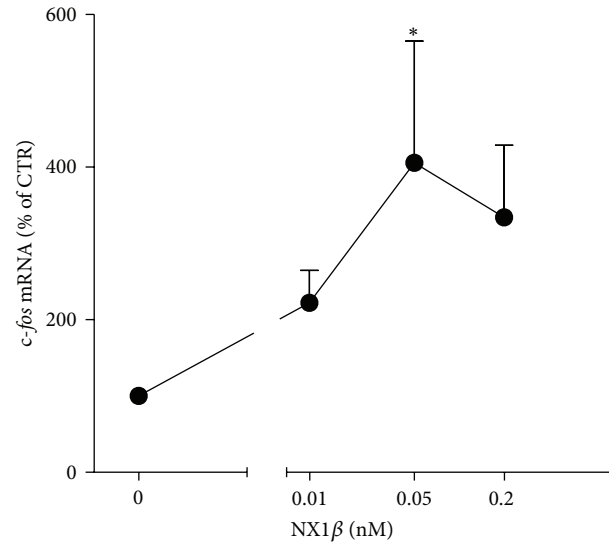


FIGURE 3: NX1β activates Rac1. Cortical neurons were grown for 8 days and subsequently treated with different concentrations of recombinant NX1β for 20 min, and the level of active Rac1 (Rac1-GTP) was analyzed using immunoblotting. The results from $n = 4$ experiments are expressed as a percentage \pm SEM. * $P < 0.05$, compared with the untreated control set at 100%.

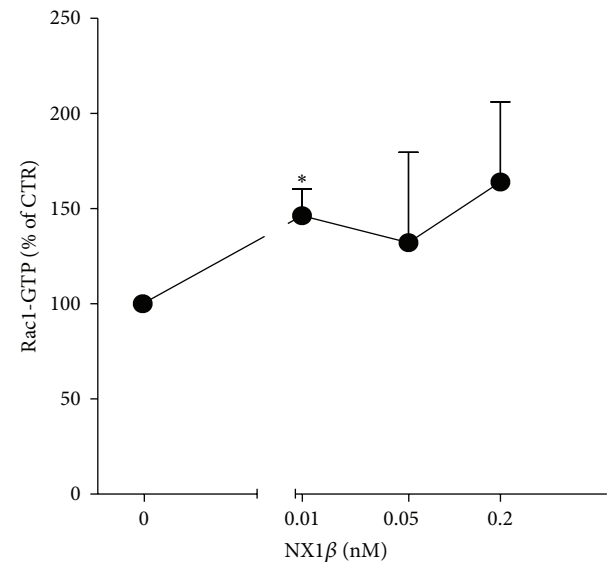


FIGURE 4: NX1β increases the mRNA level of the immediate early gene *c-fos*. Cortical neurons were grown for 8 days and treated with different concentrations of recombinant NRXN1b for 2 h. Total RNA was isolated, and mRNA levels were assessed using RT-PCR. The results from $n = 4$ experiments are expressed as a percentage \pm SEM. * $P < 0.05$, compared with the untreated control set at 100%.

Ser897 was significantly increased by 0.05 nM ecto-NX1β. In contrast to PKC, PKA responses are strong and brief. One of the targets that PKA phosphorylates and activates is phosphodiesterase, which rapidly lowers cAMP levels [22]. This might explain the differential effect of ecto-NX1β on PKA- and PKC-dependent phosphorylation sites in NRI.

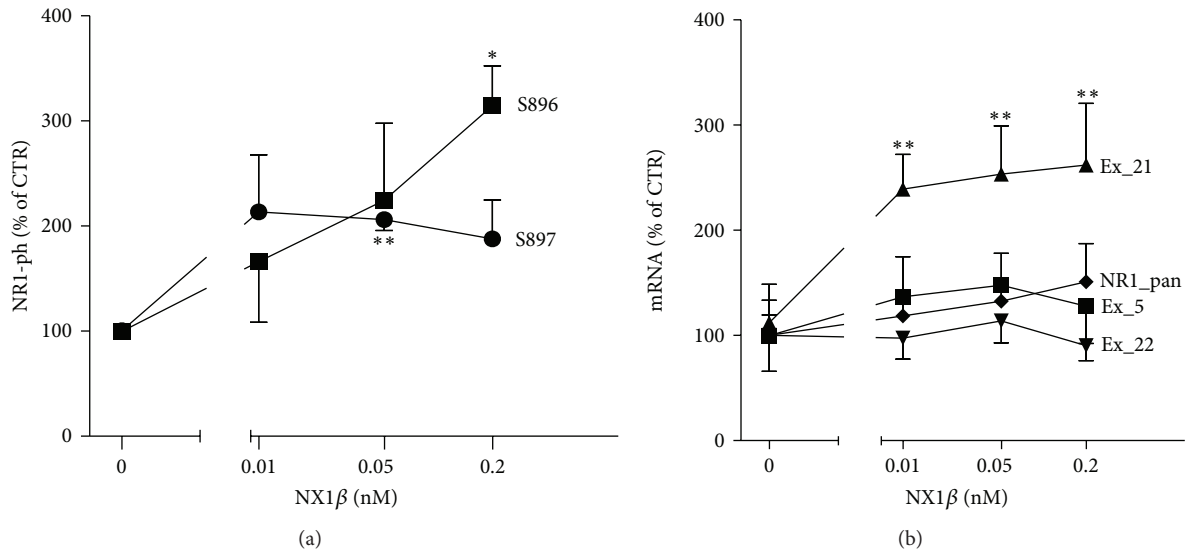


FIGURE 5: NX1 β differentially affects the phosphorylation and expression of NRI. (a) Cortical neurons were grown for 8 days, subsequently treated with different concentrations of recombinant NX1 β for 5 min, and further immunoblotted for the detection of NRI phosphorylation at S896 and S897. Representative immunoblots of three to four individual experiments are shown. The results from $n \geq 4$ experiments are expressed as a percentage \pm SEM. * $P < 0.05$, compared with the untreated control set at 100%. (b) Cortical neurons were grown for 8 days and treated with different concentrations of recombinant NX1 β for 48 h. Total RNA was isolated, and mRNA levels were assessed using RT-PCR. The results from $n = 5$ experiments are expressed as a percentage \pm SEM. * $P < 0.05$, compared with the untreated control set at 100%.

NRI exists in many isoforms through the alternative splicing of exons 5, 21, and 22. Exon 5, which encodes the extracellular N-terminal domain, is known to modulate the pharmacological properties of NMDA receptors [19]. Exon 21 and exon 22 (also known as the C1 and C2 cassettes, resp.) encode the intracellular C-terminal domain. mRNA splicing at these exons regulates protein-protein interactions, receptor trafficking, and NRI phosphorylation [19]. We found that long-term 48 h treatment but not 24 h treatment (data not shown) with ecto-NX1 β dose-dependently increased the relative expression levels of the *NRI-C1* splice variant (Figure 5(b)) but not other splice variants.

The C1 cassette contains two independent endoplasmic reticulum (ER) retention motifs and plays a unique role in the trafficking of NRI subunits to the cell surface. Thus, the effect of ecto-NX1 β on NMDA receptor trafficking appears to be time-dependent because short-term treatment increased NRI phosphorylation, which might promote the forward trafficking of NMDA receptors to the surface, but prolonged treatment led to the retention of NRI in the ER.

Finally, we investigated which signaling pathways are activated by ecto-NX1 β downstream of the NMDA receptor and Kal-7. Three kinases that represent three different signaling cascades, ERK1/2, p38 MAPK, and Akt (PKB), are known to mediate signal transduction that underlies neuronal activity in response to the activation of NMDA receptors [23, 24]. As shown in Figure 6, ecto-NX1 β had an effect on the phosphorylation levels of ERK1/2 (Figure 6(a)) and Akt (Figure 6(b)) but not p38 (Figure 6(c)). The effect on ERK1/2 and Akt was most pronounced at low concentrations and not seen at the highest concentration. To confirm the involvement of PKA in the NX-induced signaling

pathway, we concomitantly treated cultured cortical neurons with ecto-NX1 β and the specific PKA inhibitor H89. H89 abolished the effect of 10 μ M ecto-NX1 β on ERK1/2 phosphorylation and had no effect in control (nonstimulated) cultures (Figure 6(d)). We did not observe any effect of the PKC inhibitor Calphostin C on NX-mediated ERK1/2 phosphorylation (data not shown).

Our data indicate that the NX1:NLI complex is involved in the formation of the DISC1/Kal-7/Rac1 “signalosome,” which is an important determinant of the neuronal activity-dependent morphological and functional plasticity of dendritic spines [10]. The overexpression of either Kal-7 [14] or Rac1 led to an increase in spine size [10], and a similar effect was observed with DISC1 downregulation [10]. The NX-NL complex is also involved in remodeling dendritic spines. Importantly, NL can act by itself to increase the number of synapses, but NX-NL binding is essential for the increase in apparent synapse size [25]. Except NX, NLI also interacts extracellularly with NMDA receptor and this interaction is crucial for the synaptic recruitment and retention of NMDARs at glutamatergic synapses [26]. We suggest (see Figure 7) that, at the baseline condition, NLI forms a homodimer [1] interacting with NMDA receptor while DISC1 binds Kal-7, blocking the access of Kal-7 to Rac1. Upon synaptic activity and activation of NMDA receptors, NX1 β is cleaved from the presynaptic membrane what leads to the partial cleavage of NLI from the postsynaptic membrane followed by the release of NLI from NMDA receptor and DISC1 from Kal-7. After the shedding, NLI is no longer a dimer which probably changes the mobility of N-terminally truncated NLI, and it can diffuse allowing NLI to bind Kal-7 and in turn lead to the activation of Rac1. Additionally, binding of

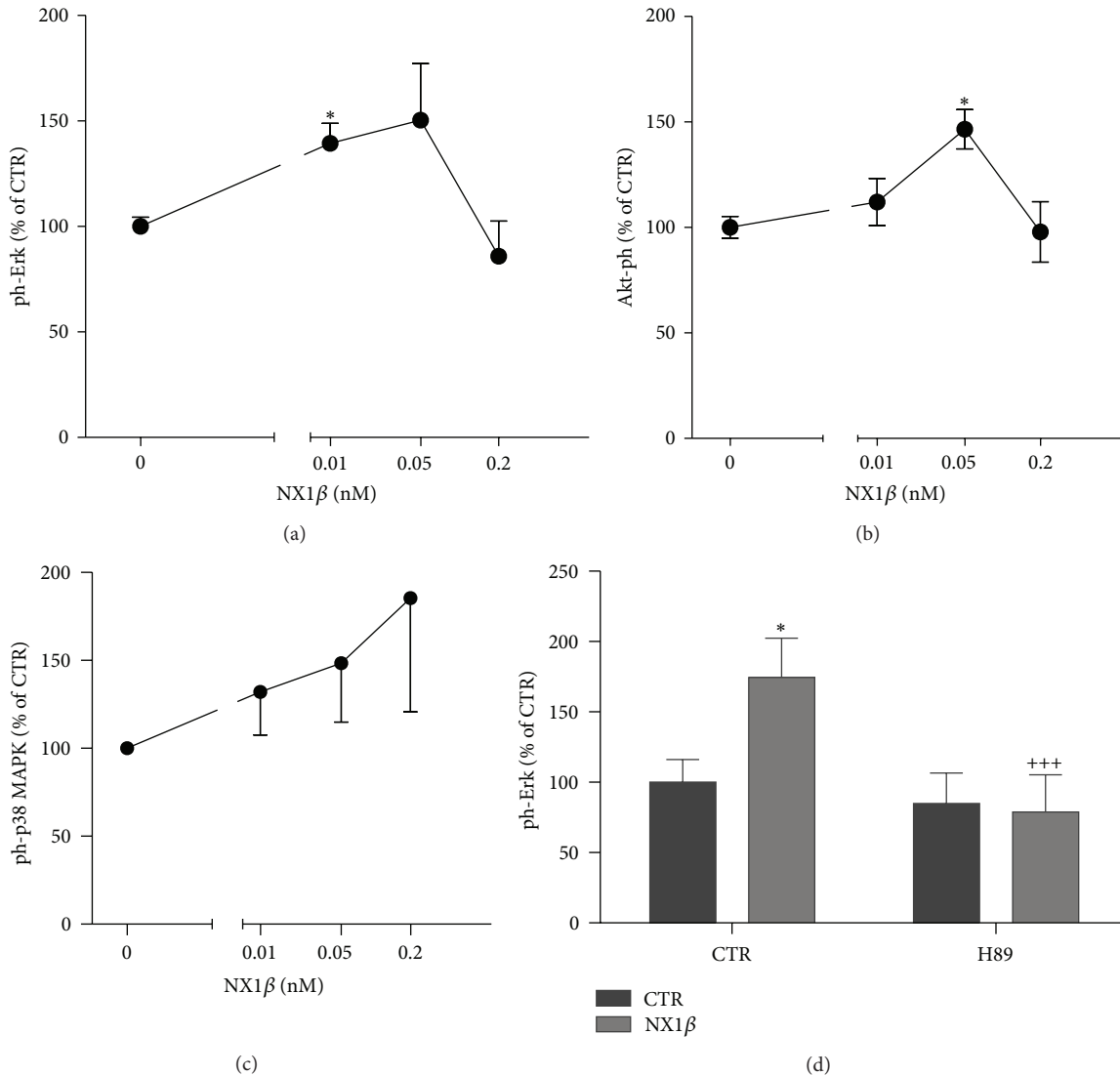


FIGURE 6: NX1 β activates ERK1/2 and Akt kinases. Cortical neurons were grown for 8 days, subsequently treated with different concentrations of recombinant NX1 β for 5 min, and further immunoblotted for the detection of (a) ERK1/2, (b) p38 MAPK, and (c) Akt. (d) Effect of the PKA inhibitor H89 on NX1 β -induced ERK1/2 phosphorylation. The results from $n \geq 4$ experiments are expressed as a percentage \pm SEM. * $P < 0.05$, compared with the untreated control set at 100%.

ecto-NX1 β to NLI results in NR1 subunit phosphorylation at S896 and S897 and phosphorylation of ERK and Akt. Interestingly, the 24 h exposure to ecto-NX1 β results in the diminished level of *DISC1* expression (Figure 1) and increased NLI shedding from the cell surface [6] while prolonged (48 h) treatment with ecto-NX1 β did not have any effect on mRNA level of *DISC1* suggesting that all these events are reversible, in a sort of equilibrium, because the dynamics of these events determines spine plasticity.

Some mutations and structural variations in both the *NX1* and *DISC1* genes have been associated with schizophrenia and autism spectrum disorders [26–28]. The common denominator for these diseases is most likely disturbances in neuronal connectivity in the brain [29, 30]. Interestingly, the abnormal expression of *NX1* in mice with mutated *DISC1* is most prominent during the stages of synapse formation,

neuronal maturation, and the general development of neuronal connectivity [7]. The DISC1/Kal-7/Rac1 signalosome also plays a role in neuronal development and may also contribute to the juvenile onset of mental diseases [31–33]. Our data support the functional connection between the DISC1/Kal-7/Rac1 signalosome and NX1 β -NLI complex. This interaction might play an important role in establishing proper neural connectivity, and any disturbance of which might underlie pathologies seen in schizophrenia and autism spectrum disorders.

4. Conclusion

We found physical and functional connections between the NX1 β -NLI complex and the DISC1/Kal-7/Rac1 signalosome. The NX1 β -NLI complex appears to regulate the signalosome,

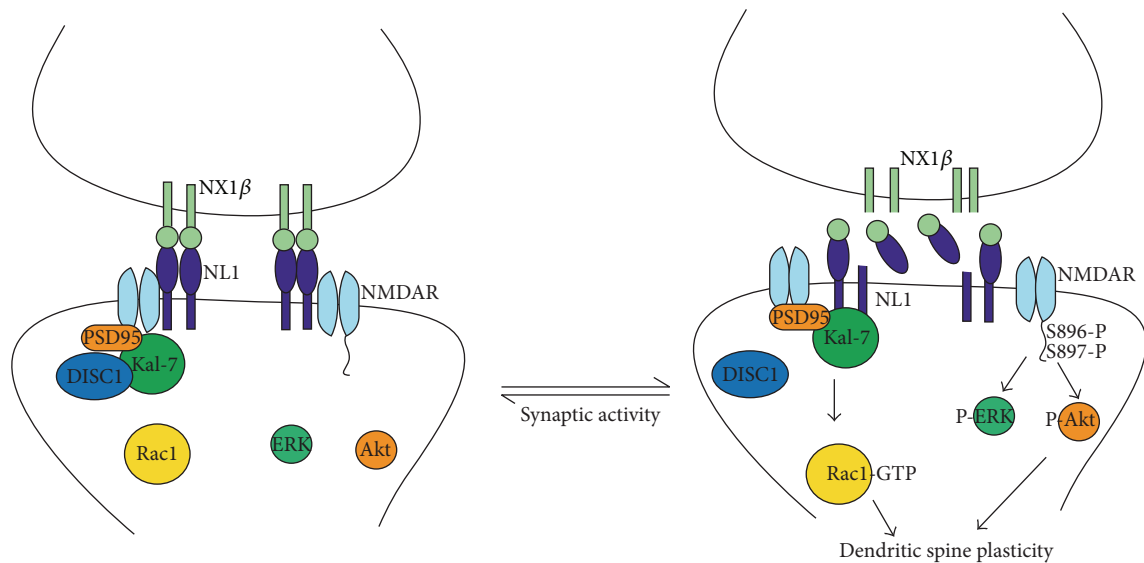


FIGURE 7: Schematic illustration of possible consequences of the NRI:NL1 interaction with the DISC1/Kal-7/Rac1 signalosome. At the baseline condition, NL1 forms homodimer interacting with NMDA receptor while DISC1 binds Kal-7, blocking the access of Kal-7 to Rac1. Upon synaptic activity and activation of NMDA receptors, NX1 β is cleaved from the presynaptic membrane leading to partial cleavage of NL1 from the postsynaptic membrane followed by the release of NL1 from NMDA receptor and DISC1 from Kal-7. After the shedding, NL1 is no longer a dimer which probably changes the mobility of N-terminally truncated NL1, and it can diffuse allowing NL1 to bind Kal-7 and in turn lead to the activation of Rac1. These events eventually allow NL1 to bind Kal-7 and in turn lead to the activation of Rac1. Additionally, binding of ecto-NX1 β to NL1 results in NR1 subunit phosphorylation at S896 and S897 and phosphorylation of ERK and Akt.

and this regulation is most likely closely coupled with synaptic activity and the activation of NMDA receptors.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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