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Biochemical and Biophysical Research Communications 352 (2007) 226–230
BBRC
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Ca^{2+}-dependent splicing of neurexin IIα

G. Rozic-Kotliroff, N. Zisapel *

Department of Neurobiochemistry, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel

Received 17 October 2006
Available online 10 November 2006

Abstract

Neurexins are synaptic adhesion proteins encoded by 3 genes (NRXN1, NRXN2, and NRXN3) each transcribed from 2 promoters to yield longer (α) and shorter (β) forms. The primary gene transcripts undergo extensive alternative splicing leading to products that may differ in synaptic coupling properties. Here we show that depolarization of neurons modulates splicing of NRXN2α, particularly at splice sites 1 and 3. Furthermore, we demonstrate that exclusion of exon 11 at splice site 3 is calcium-dependent. These data indicate neuronal activity-dependent splicing of NRXN2α. This dynamic process may be important for maintenance of mature neuronal circuits.

Keywords: Neurexin; Calcium; Depolarization; Splicing; mRNA

Neurexins are neuron-specific, cell-surface proteins that may function as synaptic adhesion molecules [1–5]. Mammalian neurexins are encoded by three genes (*NRXN1, NRXN2*, and *NRXN3*), each controlled by two promoters [1,6] resulting in a longer α-neurexin and a shorter β-neurexin. α-Neurexins are composed of large extracellular sequences containing six laminin/neurexin/sex-hormone-binding globulin (LNS) domains with three interspersed epidermal growth factor (EGF)-like regions, whereas β-neurexins include only the sixth LNS domain of α-neurexins, and have no EGF-like sequence. Five canonical sites of differential splicing, referred to as SS#1–#5, are known in α-neurexins, the last two of which are also present in β-neurexins. Many of the alternatively spliced sequences exhibit multiple variants, resulting in potentially over 1000 forms of neurexins [2].

Neurexin immunoreactivity has been localized mainly to pre-synaptic nerve terminals [1,3] while their known ligands are post-synaptic membranal proteins (neuroligins), external membrane proteins (dystroglycans), and diffusible factors released by inhibitory interneurons (neurexophilins) [7–10]. Recent findings indicate that neuroligin 1 splice variants differentially interact with NRXN1α- and β SS#4 splice variants; this observation suggested that expression of different neuroligin and neurexin forms specifies a trans-synaptic signaling code [11–13]. If so, it is important to know whether neurexin splicing patterns are affected by neuronal activity, as such processes may be key in synaptic refinement and memory.

In the present study, we have focused on the effects of depolarization on splicing of NRXN2; the shortest of the three NRXN genes [14,15]. We show that depolarization of primary culture of cortical neurons, previously shown to induce calcium-dependent neurotransmitter release [16,17], specifically affects NRXN2 splicing at SS#1 and SS#3 (exon 11 exclusion). Because the prime effect of depolarization in neurons is to provoke calcium influx, the involvement of calcium in exclusion of exon 11 from NRXN2 SS#3 transcripts was further investigated.

Materials and methods

Materials. Minimal essential medium (MEM), Dulbecco's modification of Earle's medium (DMEM), heat-inactivated horse serum (IHS), L-glutamine, penicillin–streptomycin, and EZ- First Strand cDNA Synthesis RNA kit were purchased from Biological Industries (Beit Haemek, Israel), B-27 supplement from Invitrogen (Carlsbad, CA), *Taq* DNA polymerase from Bioline (Luckenwalde, Germany), actinomycin,

* Corresponding author. Fax: +972 3 6407643.
E-mail address: navazis@post.tau.ac.il (N. Zisapel).

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doi:10.1016/j.bbrc.2006.11.008

cycloheximide, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), $CdCl_2$ and poly-L-lysine from Sigma (Sigma–Aldrich, St. Louis, MO).

Cell culture and treatment. Cultures were prepared from postnatal day 1 Sprague–Dawley rats. Cortical tissue was digested with papain (100 U; 20 min) triturated to a single-cell suspension. Cells were plated onto 6-well plates (2×10^6 cells/plate) pre-coated with 100 μg/ml poly-L-lysine in MEM containing 5% IHS, 2% B-27 neuronal supplement, 100 IU/ml of penicillin, 100 g/ml of streptomycin, 2 mM glutamine, and 6 mg/ml D-glucose. Cultures were maintained at 37 °C in humidified air containing 5% CO_2.

Pharmacological treatments. Cells grown for 11–13 days in culture were incubated with 50 (“high K”) or 5 mM (“low K”) KCl in growth medium at 37 °C in humidified atmosphere with 5% CO_2 for 6 h (found in optimization studies to ensure a fully developed response). RNA was extracted using EZ-First Strand cDNA Synthesis RNA kit. In some experiments, actinomycin D (20 μg/ml), cycloheximide (20 μg/ml), BAPTA (100 μM), $CdCl_2$ (100 μM) or equivalent amount of respective vehicle was added to culture medium 40 min before and during the high and low K treatments.

Cell viability. Cell viability was assessed by the MTT method [18]. Cells were incubated for 6 h with low or high K medium as described above. MTT was then added to a final concentration of 0.1 mg/ml and incubation resumed for 40 min at 37 °C. The insoluble formazan was dissolved in 200 μl dimethyl sulfoxide and quantified at 570 nm with a reference wavelength at 630 nm in a microtiter plate reader. In other experiments, prior to viability assessment the high/low K medium was replaced with fresh medium and cells were allowed to recover for 6 additional hours.

RNA extraction and reverse transcription. Total RNA (1 μg) was denatured in the presence of a specific primer for 10 min at 70 °C. cDNA was then prepared (1 h at 42 °C) using EZ- First Strand cDNA Synthesis RNA kit. Reactions were blocked by 15-min incubation at 70 °C and samples were stored at −20 °C.

PCR amplification and DNA sequencing. cDNA sample aliquots were added to reaction mixtures containing 1.5 mm $MgCl_2$, 200 μm dNTP, 1 μm of each primer, and 1 U *Taq* DNA polymerase. Amplification reactions (PTC-200 thermal cycler) started with denaturation phase (3 min at 94 °C) followed by repeated cycles (25 for NRXN2, 19 for β-actin, and 20 for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) found within the linear amplification range) of incubations (30 s at 94 °C, 30 s at 62 °C, and 30 s at 72 °C). PCR products were subjected to electrophoresis on 2.5% agarose gel stained with ethidium bromide [10 μg/ml]. Gels were photographed on top of a 280-nm UV light box and images densitometrically quantified with the PC Image J. RT-PCR values are presented as a ratio of the neurexin splice variant's signal in the selected linear amplification cycle divided by the β-actin signal. To compare between experiments, these values were calculated as % of the respective value for the exon-included splice variant in control cells treated with low K.

At SS#1, NRXN2α exons 2, 3, and 4 may be alternatively spliced in 8 possible ways depending on which exons are included and excluded. At SS#2, exon 7, which contains two alternative 5′ splice sites (exons 6a and 6 b), is alternatively spliced to generate three possible isoforms. At SS#3 exon 11 is alternatively spliced in and out of the mRNA. For the exon that follows exon 11, there are two possible splice acceptor sites (12a and 12b). Finally, at SS#4, exon 20, which can be included in the NRXNα and β transcripts, can be alternatively spliced to generate two different isoforms for each transcript [2,14].

RT-PCR analysis was performed with primers designed to amplify across splice sites SS# 1, 2, 3, and 4 in NRXN2α, and SS# 4 in NRXN2βessentially as described [19] except for SS# 2 reverse (5′- GGT GTAATCCTCCTGCGTGT) and SS# 3 reverse (5′-TTGTGGAACTC CAGCCGCGT). For β-actin, 381-bp fragment was amplified using the primers forward, 5′-GCCCTAGACTTCGAGCAAGAGA and reverse, 5′-CCAGGATAGAGCCACCAATC. For GAPDH, a 300-bp fragment was amplified using the primers forward 5-′ GTCATCCCAGAGCTG AACGG and reverse 5′-GTCCACCACCCTGTTGCTGT.

Real-time PCR analysis. Amplification reactions were performed in ABI PRISM® 7000 (Applied Biosystems, Foster City, CA, USA) with 1× SYBR Green I PCR Mastermix (Applied Biosystems). The protocol consisted of two initial steps (50 °C 2 min, followed by 95 °C 10 min), 40 cycles of DNA amplification (95 °C 15 s, 60 °C 1 min) and a PCR-product dissociation stage for quality control. Each experiment was repeated three times in triplicate. Single band PCR products were verified by 2.5% agarose gel electrophoresis. Expression of NRXN2 splice variants normalized to β-actin levels is presented relative to respective value for the exon-included splice variant in control cells at low K. The primers used for NRXN2 exon 10 were forward 5′-GAGGGCTTATGGACTC ATGATGG, reverse 5′-GCAGGCAGTCGAGGTTGACT, exon 12a reverse 5′-CTGCAGCACCGAGGTTGACT, exon 12b reverse 5-GGCC TTACCGAGGTTGACTG, and β-actin forward 5′-CTACAATGAGCT GCGTGTGG, reverse 5′-GACAACACAGCCTGGATGGC.

Statistical evaluations. All data presented in the figures are representative of 3–4 experiments. The semi-quantitative evaluations are expressed as means ± SD and compared by analysis of variance (ANOVA) followed by Tukey's post hoc tests. Differences between treatment groups were judged to be statistically significant at $p < 0.05$.

Results

The effects of depolarization of the cortical neurons on Neurexin 2α splicing at SS#1, SS#2, SS#3, and SS#4 and of Neurexin 2β SS#4 were explored. The cortical neurons expressed neurexin 2α and β isoforms and all expected splice variants were detected. Depolarization significantly affected Neurexin 2α splicing at #SS1 and #SS3 (Supplement 1) and did not significantly affect splicing at #SS2 and #SS4. Total NRXN2α mRNA expression (sum of all transcripts at each splice site) was not significantly affected by depolarization. Expression of β-actin gene relative to the housekeeping gene GAPDH (see Fig. 2 for example) or total RNA was not affected by depolarization and other pharmacological treatments. Means ± SD β-actin/GAPDH expression values under low vs. high K were 0.99 ± 0.08 vs 0.94 ± 0.02, respectively; $p > 0.2$. Respective values under BAPTA, $CdCl_2$, and their combinations were $1.04 + 0.05$, $0.96 + 0.01$, and $1.12 + 0.04$; $p > 0.1$ for all, compared to controls.

Under basal conditions 2 main transcripts of approximately 320 and 280 base pairs were seen for SS#1. Depolarization (6 h) reduced the amount of transcripts corresponding to the included exons 2, 3, and 4 at SS#1 and enhanced the transcripts corresponding to the excluded exons. For SS#3, we observed two of the 4 possible transcripts and identified them by sequencing to the exon 11 included and excluded variants. Depolarization reduced the amount of transcript corresponding to the included and enhanced that corresponding to the excluded exon 11 (Supplement 1). At SS#2 and SS#4, the amount of the variants lacking the alternative exon is already high at low K and not affected by high K. High K treatment reduced NRXN2β mRNA expression as evidenced from the decrease in levels of both NRXN2β SS #4 transcripts.

We have further focused on the depolarization-induced change in NRXN2 SS#3 splicing as that involved the exclusion of the single miniexon 11. Real-time PCR experiments confirmed that expression of the exon-11 included variant (E10–E11) decreased, whereas that of the two

transcripts corresponding to excluded exon-11 (E10–E12a and E10–E12b which contain the respective exons 12a and 12b) increased upon depolarization (Table 1).

Cell viability following 6-h depolarization or the 6-h recovery period was equal to that in control cells. Furthermore, the depolarization-induced changes in NRXN2α exon 11 excluded and included variants were reversible and values returned to baseline levels within the recovery period (Fig. 1).

The mechanism of the depolarization-induced change in NRXN2α SS#3 splicing was further explored. The effects of the mRNA synthesis inhibitor actinomycin D on the depolarization-induced changes in neurexin 2α expression and splicing were studied to determine the need in newly synthesized mRNA for this response. In the control low K treated cells actinomycin D slightly enhanced the level of transcript corresponding to the included exon 11 and reduced the level of transcript corresponding to the excluded exon 11 variant. However, actinomycin D completely abolished the depolarization-induced exclusion of exon 11 (Supplement 2). We further tested the requirement for protein synthesis for this response and found that cycloheximide did not affect the amount of transcripts (Supplement 2).

The role of Ca^{2+} influx in the depolarization-induced exclusion of exon 11 was explored using Cd^{2+} to block the influx of extracellular Ca^{2+} through voltage-dependent Ca^{2+} channels (VDCCs). As can be seen in Fig. 2, Cd^{2+} completely abolished the depolarization-induced changes in SS#3 splicing; in the presence of Cd^{2+} the expression of the SS#3 splice variants was not significantly different in the high vs. low K treated cells. The role of intracellular Ca^{2+} in NRXN2 expression and splicing was studied using the cell-permeable Ca^{2+} chelator BAPTA. BAPTA completely abolished the effects of depolarization on SS#3 splicing; in its presence the expression of the SS#3 splice variants was not significantly different in the high K vs. low K treated cells. BAPTA/Cd^{2+} combination significantly reduced the transcript corresponding to the excluded exon 11 in the control low K treated cells and abolished the effect of depolarization.

Real-time PCR confirmed that intracellular Ca^{2+} inhibition with BAPTA abolished the depolarization induced NRXN2 SS#3 splicing (Table 1).

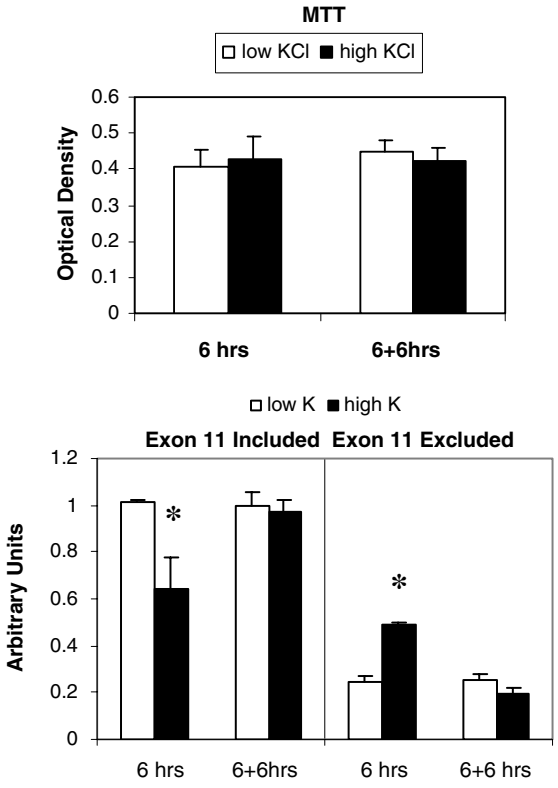

Fig. 1. Effects of depolarization of cultured cortical neurons on viability and NRXN2α SS#3 splice variants expression. Neurons were incubated with low or high K for 6 h. Viability (MTT) and expression of exon 11 included and excluded NRXN2α variants (RT-PCR) were measured immediately after this period (6 h) or follwing an additional recovery period of 6 h in culture medium (6 + 6 h). Expression (means + SD from at least 4 experiments) is relative to the expression of exon-included variant/β-actin in control cells treated with low KCl. *Significant high K effect ($p < 0.05$ *t*-test).

Discussion

The importance of neurexin splice variants in synapse formation and maintenance is only beginning to unravel. Most studies show a role for splicing at SS#4 in the β-neurexin–neuroligin link [20–24]. The results of the present study show for the first time external stimulus evoked changes in NRXN2α splicing at SS#1 and SS#3 rather

Table 1
Real-time PCR analysis of NRXN2α SS#3 splice variants after depolarization in the presence and absence of BAPTA

Transcript	Treatment conditions	RQ[a]	RQ std. err.	Significance[b]
E10–E11	High K	0.5	±0.11	0.04
E10–E12a	High K	1.9	±0.34	0.03
E10–E12b	High K	2.1	±0.34	0.03
E10–E11	BAPTA/Low K	1.18	±0.29	—
	BAPTA/High K	1.30	±0.15	>0.05
E10–E12a	BAPTA/Low K	1.18	±0.22	—
	BAPTA/High K	1.13	±0.30	>0.05
E10–E12b	BAPTA/Low K	1.67	±0.35	—
	BAPTA/High K	1.75	±0.24	>0.05

[a] Means + SE ratio to the corresponding expression under low K; $N = 3$.
[b] Compared to low K control (Student's *t*-tests).

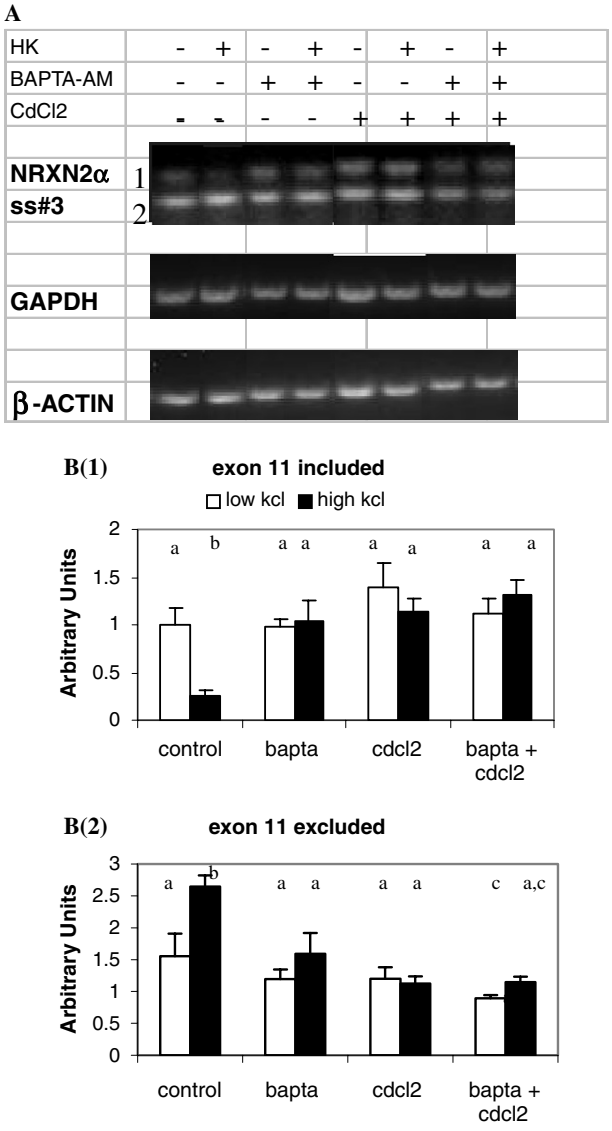

Fig. 2. Involvement of Ca^{2+} in NRXN2α SS#3 splice variants expression. (A) RT-PCR products generated from RNA extracted from cells treated with low and high K for 6 h in the absence (control) and presence of $CdCl_2$, BAPTA or their combination. Quantification of expression (means + SD from at least 4 experiments) is presented in B(1) and B(2) relative to the expression of variant 1 in control cells treated with low KCl. Bars sharing a common letter do not differ significantly (ANOVA; Tukey's post hoc tests).

than SS# 2 and #4 which may preferentially increase NRXN2α forms lacking exons 2,3,4,11 and their combinations.

One plausible outcome of exon 11 exclusion is a change in properties or specificity of NRXN2α binding with trans-synaptic or diffusible ligands. NRXNα SS #2, SS#3, and SS#4 are located within LNS domains which function as autonomous ligand-binding sites with a high degree of specificity [25]. Crystal structure of the second LNS/LG domain of NRXN1α has demonstrated the importance of splice inserts at SS#2 in regulation of the Ca^{2+}-binding properties of this domain [26]. SS#2 or SS#4 inserts at the second and sixth NRXNα LNS domains prevent interaction with dystroglycan [9], whereas the presence of the SS#4 insert modulates the binding affinity and specificity of NRXN1α and β to neuroligins [11–13]. In analogy to SS#2 and SS#4, the change in NRXN2α splicing at SS#3 may result in structural modification of the fourth LNS domain and consequently alter NRXN2α-binding properties.

Results on NRXNα knockout mice, demonstrate that deletions of α-neurexins cause loss of Ca^{2+}-channel postsynaptic *N*-methyl-D-aspartate receptor function, decrease in spontaneous release and decline in short-term plasticity [27,28]. NRXN2α was postulated as a plasticity-related gene but may also participate in the response of the neuron to external insults and ischemia [19,23,27,28]. Unlike response to insult [19,29], the depolarization did not cause changes in NRXN2α mRNA levels and SS#4 splicing. Furthermore, in agreement with previous studies showing enhanced cortical neuron survival following extended (24 h) depolarization [30], depolarization of the cortical neurons for 6 h did not induce cell death within or after this period.

The depolarization-induced changes in SS#3 of NRXN2α splicing are Ca^{2+}-dependent as they can be completely blocked by BAPTA, a chelating agent that lowers intracellular Ca^{2+}, and $CdCl_2$, a VDCC blocker. The dependence of these changes (at least those at SS#3) on Ca^{2+} influx supports the notion that they are neuron-activity driven. Although the depolarization-induced change in NRXN2α splicing depends on RNA but not protein synthesis, it takes several hours to develop as also reported for other depolarization-induced Ca^{2+}-regulated splicing, e.g. the mammalian large-conductance, Ca^{2+}-sensitive K^+ channel [31]. Importantly, the change in NRXN2α splicing was reversible and the pattern reverted to baseline following recovery at low K. Hence, the change in NRXN2α splicing does not represent a stress response or effect on neuron survival but rather depends on the presence of sustained (and presumably repeated) depolarization.

Altogether these findings indicate a role for Ca^{2+} not only in regulation of α-neurexins, ligand-binding affinity but also splicing patterns thereby presumably affecting NRXN2α-binding properties and specificity. The activity related modifications in NRXN2α splicing thus potentially represent a novel route of synaptic or maintenance in mature networks.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.11.008

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