

Rim1 α and Neurexin3 Regulate Synaptosomal Dopamine Release

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Neurotransmitter release is regulated by various proteins of the active zone in the presynaptic nerve terminals. Dopamine (DA) is an essential neurotransmitter associated with the pathophysiology of diverse behavioral and mental illness such as schizophrenia and drug addiction. We measured synaptosomal DA release of knockout (KO) mice which lacked major genes related to neurotransmitter release. Synaptosomal DA uptake and release were performed and measured using [³H]-DA and superfusion experiments. 3 of the 17 KO mice exhibited altered DA release compared to their littermate controls. In Rim1 α KO, [³H]-DA release evoked by membrane depolarization significantly decreased. Both basal (physiological buffer-evoked) and membrane depolarization-evoked DA release significantly decreased in dopaminergic conditional KO of Rim1 α . Dopaminergic conditional KO of neurexin3 demonstrated a significant increase of membrane depolarization-evoked DA release. These data explain the similarities and distinctions between DA and other classical neurotransmitters such as glutamate and GABA (γ -aminobutyric acid) release. In conclusion, Rim1 α and neurexin3 may be important regulators of presynaptic DA release and related to disorders of the nervous system.

Key words : Dopamine release, neurotransmitter, Rim1 α , neurexin3

Introduction

Regulation of neurotransmitter release is crucial to the control of neuronal signaling. Presynaptic terminals are organized into junctions known as active zones where various proteins serve as the regulators of neurotransmitter release. Active zones are highly systemized structures consisting of protein networks [30]. The majority of proteins involved in neurotransmitter release at active zone include two types of protein complexes such as the fusion machinery and the cytomatrix of active zones [19]. SNARE-complexes, the fusion machinery, are constructed from the synaptic vesicle-transmembrane protein VAMP and the plasma membrane proteins syntaxin-1 and SNAP25 mediating exocytotic fusion of synaptic vesicles with the plasma membrane. The cytomatrix of active zones have several functions including construction of a scaffold for the recruitment of various proteins.

Neuronal dopaminergic system is considered to affect a wide range of behavioral or mental disorders including schizophrenia, autism, Parkinson's disease, and Attention Deficit Hyperactivity Disorder [25]. Molecular cloning of multiple dopamine (DA) receptor subtypes has ushered in many new insights of the molecular mechanisms of DA ac-

tions [29]. Neuronal dopamine release has also undergone extensive investigation due to its known involvement in various physiological and pathological conditions [9]. Unlike most classical neurotransmitters, monoamines such as DA, serotonin, and norepinephrine (NE) undergo release from somatodendritic region as well as axon terminals [20]. Somatodendritic DA release may engage either regulated exocytosis of vesicles containing transmitter or efflux by a nonvesicular mechanism. While most classical neurotransmitters involve regulated exocytosis using only synaptic vesicles, monoamines are stored in large dense core vesicles besides the synaptic vesicles [32]. Because of these complexities of DA release, we are still at the early stages in unraveling its molecular mechanism.

Advances in classic electrophysiological technique which postsynaptically measures the response evoked by the presynaptic release of neurotransmitters have contributed to the development for the study of neurotransmitter release. However, the vesicular release of catecholamines such as DA and NE does not evoke postsynaptic quantal responses [33]. Alternatively, *in vivo* electrochemical detection (voltammetry and amperometry) and microdialysis have been widely used to measure brain extracellular monoamine dynamics [23]. *In vivo* electrochemical detection has likewise disadvantages because it does not reflect synaptic events.

The aim of this study is to investigate the effect of various

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knockout (KO) in which the genes of fusion machineries and the cytomatrix of active zones were genetically turned off on DA release. We used superfused mice synaptosomes because the superfusion study is relatively easy for screening and synaptosomes constitute a useful model for the investigation of functional proteins at the presynaptic level [1].

Materials and Methods

Experimental animals

KO and/or knockin (KI) mice in this screening test listed below: Rim1 α KO (RAK) [27]; Rim1 α serine-413 Flipped KI [14]; β -neurexin KO [2]; synaptophysin1, 2/synaptogyrin1, 3 quadruple KO [12]; synaptotagmin2 KO [22]; synuclein $\alpha\beta\gamma$ KO [4]; syntaxin1A KO with open conformation KI of syntaxin1B [8]; Rim2 $\alpha\beta\gamma$ KO [28]; Cspa KO [5]; Rabphilin KO [6]; Rab3A/D double KO (DKO) [26]; synapsin1/2 DKO [31]; ERC2 (ELK2 or CAST) KO [13]; C-terminal GFP KI of syntaxin1B [8]; Piccolo KO [7]; dopaminergic conditional KO of Rim1 $\alpha\beta$ (DRABK) [15]; dopaminergic conditional KO of neurexin3 (DNK) [21] (Table 1). These mice in the experimental groups and the age (2.5-4 months)/sex (male)-matched littermates (control) were ob-

tained from Dr. Thomas C. Südhof (University of Texas, Southwestern Medical Center, Dallas, TX, USA) [30].

Preparation of synaptosomes

Synaptosomes from mouse striatum were prepared essentially as described previously [18]. All manipulations were performed at 0-4°C. The tissue was dissected and homogenized in a glass-Teflon homogenizer at 900 rpm in homogenizing solution (0.32 M sucrose, 5 mM HEPES-NaOH, pH 7.4, 0.1 mM EDTA). Homogenates were cleared by low-speed centrifugation (1,000 \times g for 5 min) and centrifuged at 14,500 \times g for 15 min to obtain the crude synaptosome fraction (P₂). The P₂ was resuspended in 3 ml ice-cold gassed (95% O₂/5% CO₂) Krebs-bicarbonate buffer (composition in mM: 118 NaCl, 3.5 KCl, 1.25 CaCl₂, 1.25 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, 5 HEPES-NaOH, pH 7.4, 11.5 glucose) and used within 2-3 hr. The buffers for release experiments additionally contained 0.6 mM ascorbic acid, 100 μ M EDTA and 10 μ M pargyline.

Superfusion experiments

These were carried out as described previously [17].

Table 1. The list of KO and/or KI mice in the screening test

KO and/or KI mice	Function	Ref.
Rim1 α KO	maintenance of normal probability of neurotransmitter release, regulation of release during short-term synaptic plasticity	[27]
Rim1 α serine-413 Flipped KI	presynaptic long-term potentiation	[14]
β -neurexin KO	cell-adhesion protein in synapse function	[2]
synaptophysin1, 2/ synaptogyrin1, 3 quadruple KO	short-term and long-term synaptic plasticity	[12]
synaptotagmin2 KO	Ca ²⁺ triggering of evoked release	[22]
synuclein $\alpha\beta\gamma$ KO	long-term regulation of presynaptic function.	[4]
syntaxin1A KO with open conformation KI of syntaxin1B	Closed conformation initiates the synaptic vesicle fusion reaction	[8]
Rim2 $\alpha\beta\gamma$ KO	Ca ²⁺ -triggering of exocytosis	[28]
Cspa KO	cochaperone for neuronal survival	[5]
Rabphilin KO	regulation of synaptic vesicular recovery from use-dependent depression	[6]
Rab3A/D DKO	Ca ²⁺ -triggered synaptic vesicle exocytosis	[26]
synapsin1/2 DKO	boost of the release probability during high-frequency stimulation	[31]
ERC2 (ELK2 or CAST) KO	functions in synaptic transmission of autonomic synapses	[13]
C-terminal GFP KI of syntaxin1B	Closed conformation initiates the synaptic vesicle fusion reaction	[8]
Piccolo KO	short-term synaptic plasticity during repetitive stimulation.	[7]
dopaminergic conditional KO of Rim1 $\alpha\beta$	synaptic vesicle priming, long-term presynaptic plasticity	[15]
dopaminergic conditional KO of neurexin3	coupling Ca ²⁺ channels to the presynaptic machinery	[21]

Synaptosomes were loaded with 600 nM [3 H]-DA (specific activity 10 Ci/mmol) or 150 nM [3 H]-NE (40 Ci/mmol) for 5 min. Labeled synaptosomes (0.1 ml) were trapped on glass fiber filters (GF/B, Whatman) and superfused at 0.8 ml/min with Krebs-bicarbonate buffer at 34°C under continuous aeration with a 95% O₂/5% CO₂ gas mixture. After 10 min of washing, two 1-min fractions were collected to determine baseline release, and neurotransmitter release was stimulated by application of 25 mM KCl for 30 sec and 5 μ M α -latrotoxin for 1 min. The amounts of neurotransmitters secreted into the superfusate and retained in the synaptosomes at the end of the experiment were quantified by liquid scintillation counting. The fractional release rate of neurotransmitters was calculated as the fraction of radioactivity released divided by the amount remaining on the filter at that particular time point. Total evoked transmitter secretion was calculated from the area under the peak and compared with basal neurotransmitter secretion.

Synaptosomal uptake assays

Synaptosomal uptake was performed using the same incubation condition and concentration of radiolabelled neurotransmitters as those of superfusion experiments. The assay was stopped by centrifuging the samples (1,000 \times g for 5 min) and washing the pellets twice with ice-cold buffer. The pellets were resuspended for scintillation counting. Synaptosomal protein concentrations were also determined using BSA as the standard. Uptake was calculated as pmol/mg protein.

Statistical analyses

Data are presented as mean \pm SEM. Statistical significance was evaluated with paired t- tests.

Results and Discussion

In the 17 groups of KO/KI mice screened, 3 of them showed different patterns of DA release compared to their littermate controls. These different patterns were found in the groups of RAK, DRABK, and DNK (Fig. 1-3). Others did not show any different patterns in the synaptosomal DA release compared to the control mice (data not shown).

In RAK, basal release of [3 H]-DA by normal Krebs-bicarbonate buffer from striatal synaptosomes did not change. [3 H]-DA release evoked by membrane depolarization (30-s pulse of 25 mM KCl) significantly decreased in RAK compared to wild-type mice ($p<0.001$) (Fig. 1A, B). However, 1-min pulse of 5 μ M α -latrotoxin did not affect [3 H]-dopamine release from synaptosome of RAK mice. The difference in DA release between RAK and control was not caused by a difference in the [3 H]-dopamine uptake into synaptosome (Fig. 1C).

In DRABK, basal release of [3 H]-DA significantly decreased compared to control ($p<0.001$). [3 H]-DA release evoked by membrane depolarization also significantly decreased in DRABK ($p<0.01$) (Fig. 2A, 2C). Additionally, basal release of [3 H]-NE significantly decreased in DRABK mice ($p<0.01$). There was no difference in KCl-evoked [3 H]-NE release between DRABK and control (Fig. 2B, 2D). There were no significant differences either in [3 H]-DA or [3 H]-NE

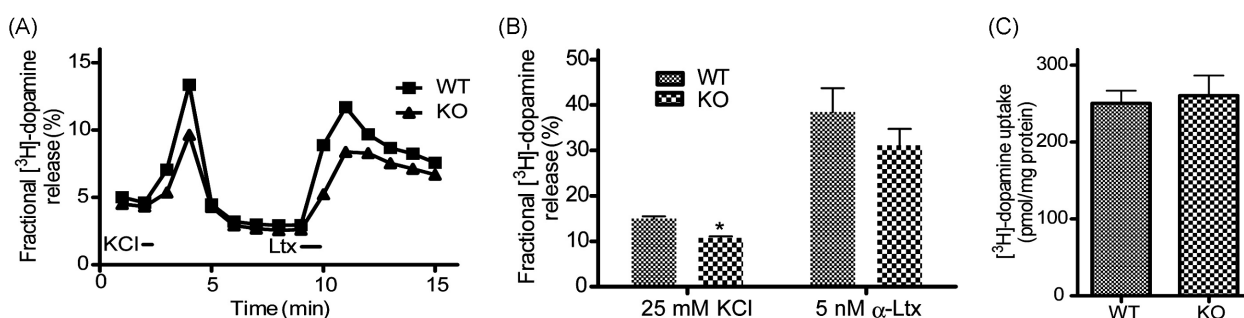


Fig. 1. Uptake and release of [3 H]-dopamine in striatal synaptosomes of Rim1a- knockout and wild-type mice. Striatal synaptosome were loaded with [3 H]-dopamine and superfused with normal Krebs-bicarbonate buffer. Release was triggered sequentially by membrane depolarization (30-s pulse of 25 mM KCl) and 1-min pulse of 5 μ M α -latrotoxin. Released [3 H]-dopamine was monitored continuously in the superfusate. (A) A representative experiment of superfusion. (B) [3 H]-dopamine release evoked by 25 mM KCl but not by α -latrotoxin significantly decreases in mice lacking Rim1a compared to wild-type mice (* $p<0.001$). (C) There is no significant difference in [3 H]-dopamine uptake between the knockout and the wild-type mice. The wild-type mice were age- (3-4 months) and sex- (male) matched littermates to the knockout group. Data are given as mean \pm SEM (n=9).

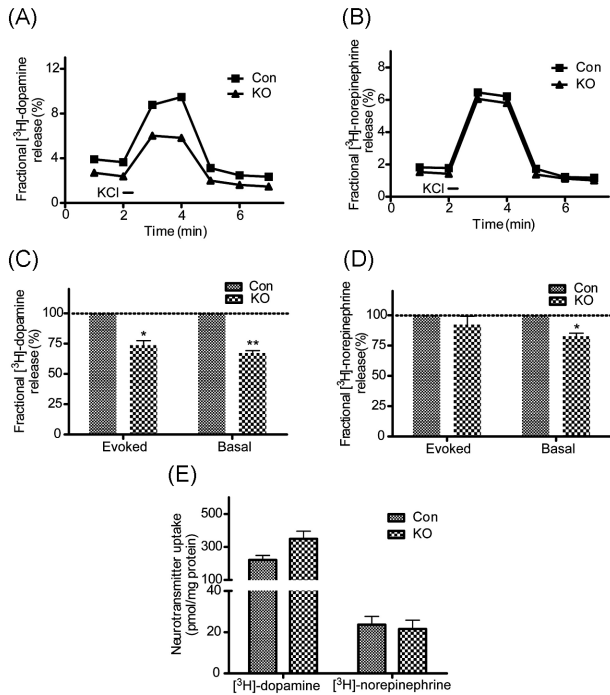


Fig. 2. Uptake and release of $[^3\text{H}]$ -dopamine and $[^3\text{H}]$ -norepinephrine in synaptosomes of conditional-knockout of Rim1 $\alpha\beta$ and control mice. Synaptosomes were loaded with $[^3\text{H}]$ -dopamine or $[^3\text{H}]$ -norepinephrine and superfused with normal Krebs-bicarbonate buffer. Release was triggered sequentially by membrane depolarization (30-s pulse of 25 mM KCl). Released $[^3\text{H}]$ -dopamine and $[^3\text{H}]$ -norepinephrine were monitored continuously in the superfusate. Representative experiments of superfusion are shown (A and B). Both KCl-evoking and basal release of $[^3\text{H}]$ -dopamine (C) and basal release of $[^3\text{H}]$ -norepinephrine (D) significantly decrease in mice conditionally lacking Rim1 $\alpha\beta$ compared to controls (* $p < 0.01$; ** $p < 0.001$). There are no significant differences either in $[^3\text{H}]$ -dopamine or $[^3\text{H}]$ -norepinephrine uptake between the knockout and the control mice (E). The control mice were age- (2.5-3 months) and sex- (male) matched littermates to the knockout group. Data are given as mean \pm SEM ($n=5$).

uptake between DRABK and control mice (Fig. 2E).

In DNK mice, basal release of $[^3\text{H}]$ -DA and $[^3\text{H}]$ -NE by normal Krebs-bicarbonate buffer did not change compared to littermate controls (Fig 3A, 3B). $[^3\text{H}]$ -dopamine but not $[^3\text{H}]$ -norepinephrine release by 30-s pulse of 25 mM KCl increased in DNK compared to controls (* $p < 0.05$) (Fig. 3A, 3B, 3C). There were no significant differences either in $[^3\text{H}]$ -DA or $[^3\text{H}]$ -NE uptake between DRABK and control mice (Fig. 3D).

RAK and DRABK showed decreased DA release evoked by membrane depolarization in our study. Rim1 α is a

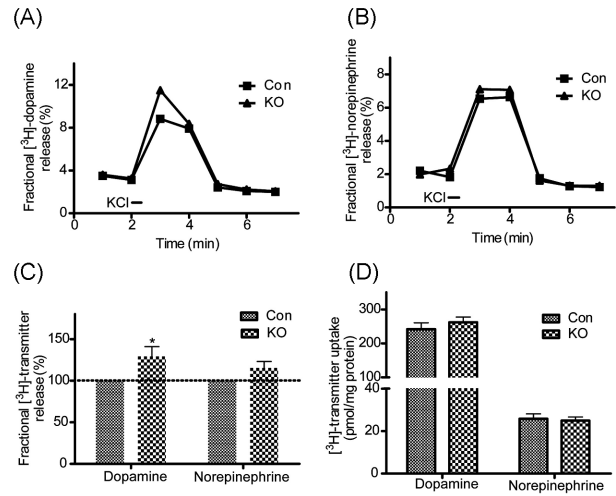


Fig. 3. Uptake and release of $[^3\text{H}]$ -dopamine and $[^3\text{H}]$ -norepinephrine in synaptosomes of conditional-knockout of Neurexin3 and control mice. Synaptosomes were loaded with $[^3\text{H}]$ -dopamine or $[^3\text{H}]$ -norepinephrine and superfused with normal Krebs-bicarbonate buffer. Release was triggered sequentially by membrane depolarization (30-s pulse of 25 mM KCl). Released $[^3\text{H}]$ -dopamine and $[^3\text{H}]$ -norepinephrine were monitored continuously in the superfusate. Representative experiments of superfusion are shown (A and B). $[^3\text{H}]$ -dopamine but not $[^3\text{H}]$ -norepinephrine release increases in mice conditionally lacking Neurexin3 compared to controls (* $p < 0.05$) (C). There are no significant differences either in $[^3\text{H}]$ -dopamine or $[^3\text{H}]$ -norepinephrine uptake between the knockout and the control mice (D). The control mice were age- (2.5-3 months) and sex- (male) matched littermates to the knockout group. Data are given as mean \pm SEM ($n=4-6$).

multidomain protein that constitutes an essential component of the active zone by binding to other active zone proteins such as Munc13s, α -liprins, and ELKS, and to synaptic vesicle proteins such as Rab3 and synaptotagmin1. In glutamatergic neurons of RAK mice, there is significant impairment of synaptic vesicle priming and presynaptic long-term plasticity, but these mice are not lethal [15]. Synaptic vesicle priming is defined as preparation of synaptic vesicles filled with neurotransmitters to make them competent for Ca^{2+} triggered fusion-pore opening [30]. So, impairment of priming causes dysregulation of depolarization-induced neurotransmitter release. Rim1 α may be related to synaptic vesicle priming not only in glutamatergic neurons but also in dopaminergic neuron according to the result of our study. This possibility becomes more likely as DNK mice also demonstrated decreased DA release evoked by membrane depolarization.

Even at rest, synapses have a finite but low probability of release, causing spontaneous events of exocytosis that are

reflected in electrophysiological recordings as miniature postsynaptic currents [30]. Conceptually, Basal neurotransmitter release in our study corresponds to miniature postsynaptic currents. In contrast to the result of DA release evoked by membrane depolarization, basal DA release by physiological buffer did not change in RAK and DNK compared to wild type. A previous article reported that miniature postsynaptic currents of RAK were not different from wild type in agreement with our data [27].

A matter which requires attention is that the results of basal DA release in RAK and DRABK were different. Conventional and conditional KO may reveal different effects. As in DRABK, intervention from neurons excepting dopaminergic neurons to dopaminergic neurons is not possible in RAK during the experiments because the experiments were done using synaptosomes. However, the effect of the intervention may be already reflected during the development via certain processes such as synaptic plasticity. The other consideration is that DA transport decreased up to 20% in wild type and experimental groups of DRABK (data not shown). This change came from the course of making the conditional KO and might be related to different phenotype of RAK and DRABK.

At the synapse, neurotransmitter release is triggered physiologically by Ca^{2+} influx through voltage-gated Ca^{2+} channels. Non-physiologically, release can be evoked by a potent neurotoxin from black widow spider venom, α -latrotoxin [18]. α -latrotoxin binds to a plasma membrane protein, neurexin which interacts with a synaptic vesicular protein synaptotagmin. Such an interaction has an important role in the docking and targeting of synaptic vesicles in the nerve terminal [10]. Thus, α -latrotoxin promotes neurotransmitter release via modulation of docking step distinguishable from priming step. Unlike Ca^{2+} influx through membrane depolarization, α -latrotoxin did not affect [^3H]-dopamine release in RAK mice. This means that Rim1 α plays an important role in the priming step rather than in the docking and supports earlier findings [15].

Depolarization-induced NE release did not change in the DRABK while basal NE release decreased in the present study. These patterns are very different compared to those of DA release in the DRABK. One thing postulated from these results is that Rim1 α 's crucial role in the docking could be limited to glutamate and DA not to NE. Rim1 α may regulate NE release with different way than others. A study using α -latrotoxin set a good example that different neuro-

transmitter release are modulated in different manners [16].

Neurexins are presynaptic cell surface proteins that may function as *trans*-synaptic cell adhesion molecules. Vertebrates have three large neurexin genes, each of which expresses longer α - and shorter β -neurexins, producing six major neurexins (neurexins 1 α , 2 α , 3 α and 1 β , 2 β , 3 β). As important adhesion molecules, interaction of neurexin and its binding partner neuroligin is highlighted by the finding that, in humans, mutations in X-chromosomal neuroligin genes are associated with autistic spectrum disorders and/or mental retardation [3]. Neurexin3, investigated protein here, is thought to be related to alcohol dependence, cocaine addiction, and illegal substance abuse [11]. The key neurotransmitter of both autistic disorder and addiction is DA [24]. Hence, changed DA release by depolarization in DNK in the study is not surprising.

In this short report, we pass by discussions about the reasons and meanings why the other molecules did not show significant results. Further investigation needs to be done to elucidate the more detailed roles and mechanisms of Rim1 α and neurexin3 in the DA release.

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초록 : Rim1 α 와 neurexin3의 시냅소좀 도파민 분비 조절

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신경전달물질의 분비는 시냅스전 신경말단의 active zone에 있는 다양한 단백질들에 의해 조절된다. 도파민은 정신분열병, 약물중독과 같은 여러 가지 행동, 정신질환의 병태생리와 연관된 필수적인 신경전달물질이다. 저자들은 본 연구에서 신경전달물질 분비와 관련된 주요 유전자가 결여된 knockout (KO) 생쥐의 시냅소좀(synaptosome) 도파민 분비를 측정하였다. 시냅소좀 도파민 흡수와 분비는 [³H]-도파민과 관류실험을 이용하여 시행, 측정하였다. 17 KO 생쥐 가운데 3 종류의 생쥐에서 그들의 littermate 대조군과 비교하였을 때 변화된 도파민 분비를 보였다. Rim1 α KO에서 세포막 탈분극에 의한 [³H]-도파민은 유의하게 감소되었으며, 또한 Rim1 α 의 도파민 신경에서의 조건 KO에서는 생리적 완충용액에 의한 기본적인 도파민 분비 및 세포막 탈분극에 의한 도파민 분비 모두가 유의하게 감소되어 있었다. neurexin3의 도파민 신경에서의 조건 KO에서는 세포막 탈분극에 의한 도파민 분비의 증가를 보였다. 이 데이터들은 도파민 분비와 글루타메이트, GABA와 같은 전통적 신경전달물질 분비의 유사성과 차별성을 설명한다. 결론적으로, Rim1 α 와 neurexin3는 시냅스전 도파민 분비의 중요한 조절자이며 신경계 질환과 연관될 가능성이 있다.