Dopamine synapse is a neuroligin-2–mediated contact between dopaminergic presynaptic and GABAergic postsynaptic structures

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Midbrain dopamine neurons project densely to the striatum and form so-called dopamine synapses on medium spiny neurons (MSNs), principal neurons in the striatum. Because dopamine receptors are widely expressed away from dopamine synapses, it remains unclear how dopamine synapses are involved in dopaminergic transmission. Here we demonstrate that dopamine synapses are contacts formed between dopaminergic presynaptic and GABAergic postsynaptic structures. The presynaptic structure expressed tyrosine hydroxylase, vesicular monoamine transporter-2, and plasmalemmal dopamine transporter, which are essential for dopamine synthesis, vesicular filling, and recycling, but was below the detection threshold for molecules involving GABA synthesis and vesicular filling or for GABA itself. In contrast, the postsynaptic structure of dopamine synapses expressed GABAergic molecules, including postsynaptic adhesion molecule neuroligin-2, postsynaptic scaffolding molecule gephyrin, and $GABA_A$ receptor α1, without any specific clustering of dopamine receptors. Of these, neuroligin-2 promoted presynaptic differentiation in axons of midbrain dopamine neurons and striatal GABAergic neurons in culture. After neuroligin-2 knockdown in the striatum, a significant decrease of dopamine synapses coupled with a reciprocal increase of GABAergic synapses was observed on MSN dendrites. This finding suggests that neuroligin-2 controls striatal synapse formation by giving competitive advantage to heterologous dopamine synapses over conventional GABAergic synapses. Considering that MSN dendrites are preferential targets of dopamine synapses and express high levels of dopamine receptors, dopamine synapse formation may serve to increase the specificity and potency of dopaminergic modulation of striatal outputs by anchoring dopamine release sites to dopamine-sensing targets.

dopamine synapse | neuroligin-2 | medium spiny neuron | striatum

Chemical synapses comprise presynaptic machinery for transmitter release and postsynaptic machinery for receptor-mediated signal transduction in a neurochemically matched manner. They are classified into Gray type-I and type-II synapses by asymmetric or symmetric membrane density, respectively, of the pre- and postsynaptic structures (1). Most, if not all, asymmetric and symmetric synapses are excitatory and inhibitory, respectively, as they selectively express ionotropic glutamate or GABA/glycine receptors together with their specific scaffolding proteins (2). Neurochemical matching of chemical synapses is controlled by activity-dependent mechanisms (3, 4), and mediated by transmembrane adhesion proteins and secreted molecules (5). The neuroligin (NL) family comprises postsynaptic adhesion molecules that form transsynaptic contacts with presynaptic neurexins (Nrxn) (6). Of note, NL1 and NL2 are selectively expressed at glutamatergic and GABAergic synapses, respectively (7, 8), and are required for activity-dependent specification of the corresponding synapses (9).

Midbrain dopamine neurons project densely to the striatum and are strongly involved in motor and cognitive functions (10, 11). Anatomically, dopamine synapses are frequently found on dendritic shafts and spines of GABAergic medium spiny neurons (MSNs), and exhibit ultrastructural features common to symmetric synapses (12–14). MSNs constitute around 90% of all striatal neurons. They are divided equally into direct and indirect pathway MSNs (d-MSN and i-MSN), differing in their connectivity with output nuclei of the basal ganglia and in the expression of dopamine receptors (i.e., D_1R in d-MSNs and D_2R in i-MSNs) (10). Considering the broad extrasynaptic expression of D_1R and D_2R (15–17), the mode of dopaminergic transmission at dopamine synapses (i.e., whether it is mediated by wired transmission like that at conventional glutamatergic and GABAergic synapses) remains unclear. It is also unknown which types of molecules comprise pre- and postsynaptic membrane specializations at dopamine synapses.

In the present study, we show that the presynaptic side of dopamine synapses is indeed dopaminergic, whereas the postsynaptic side is exclusively GABAergic, expressing $GABA_A$ receptor α1 ($GABA_AR\alpha1$), gephyrin, and NL2. In vivo knockdown of NL2 in the striatum reduced the density of dopamine synapses on MSN dendrites, but reciprocally increased that of GABAergic synapses. Our findings suggest that NL2 regulates striatal synapse formation by giving competitive advantage to dopamine synapses over GABAergic synapses, and that the formation of dopamine synapses may serve to provide dopamine-sensing MSNs with structural anchorage of dopamine release sites.

Significance

Nigrostriatal dopaminergic projections form a number of dopamine synapses onto medium spiny neurons in the striatum, and have strong influence on emotion, motivation, voluntary movement, and cognition. Despite the functional importance, the molecular composition at dopamine synapses remains unknown. Here we demonstrate that dopamine synapses are neurochemically mismatched contacts formed between dopaminergic presynaptic and GABAergic postsynaptic structures. Intriguingly, neuroligin-2 expressed at the GABAergic postsynaptic structure controls striatal synapse formation by giving competitive advantage to heterologous dopamine synapses over conventional GABAergic synapses. Therefore, our findings suggest that neuroligin-mediated formation of such neurochemically mismatched synapses could be a novel strategy to increase the target selectivity and potency of modulation by anchoring release sites of neuromodulators to their receptive targets.

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Results

Presynaptic Phenotypes at Dopamine Synapses. To characterize the molecular–anatomical organization of the so-called dopamine synapse, we studied the dorsolateral striatum in the adult mouse. We first examined the neurochemical properties of dopaminergic terminals, which were identified by immunolabeling for plasmalemmal dopamine transporter (DAT) or tyrosine hydroxylase (TH) (Fig. 1 *A* and *B*). Triple immunofluorescence revealed that DAT-labeled dopaminergic terminals overlapped extensively with TH and vesicular monoamine transporter-2 (VMAT2) (Fig. 1*C*). DAT-labeled dopaminergic terminals had no immunodetectable signals for glutamatergic terminal markers, vesicular glutamate transporters VGluT1, VGluT2, and VGluT3, or for GABAergic terminal markers, 65/67-kDa glutamic acid decarboxylases (GAD) or vesicular inhibitory amino acid transporter (VIAAT) (Fig. S1 *A–E*). However, plasmalemmal GABA transporter GAT1 was expressed in dopaminergic terminals, albeit at a much lower intensity than in VIAAT-labeled GABAergic terminals (Fig. S1 *F* and *G*). We then compared the GABA content in dopaminergic, GABAergic, and glutamatergic terminals using immunoelectron microscopy for GABA and TH or VIAAT. According to previous studies (12, 13, 18), TH- or VIAAT-labeled terminals forming symmetric synapses were judged to be dopaminergic or GABAergic, respectively. Terminals that were unlabeled for either TH or VIAAT and formed asymmetric synapses were judged to be glutamatergic, although a few asymmetric synapses are serotonergic (19). GABAergic terminals were intensely labeled for GABA (Fig. 1*D*, red), whereas dopaminergic (Fig. 1*E*, green) and glutamatergic (Fig. 1*D*, blue) terminals were scarcely labeled for GABA. Density quantification revealed a significantly higher level of immunogold labeling for GABA in GABAergic terminals than in dopaminergic and glutamatergic terminals (Fig. 1*F*). Therefore, dopaminergic terminals contain very little, if any, GABA and GABAergic proteins, except for GAT1.

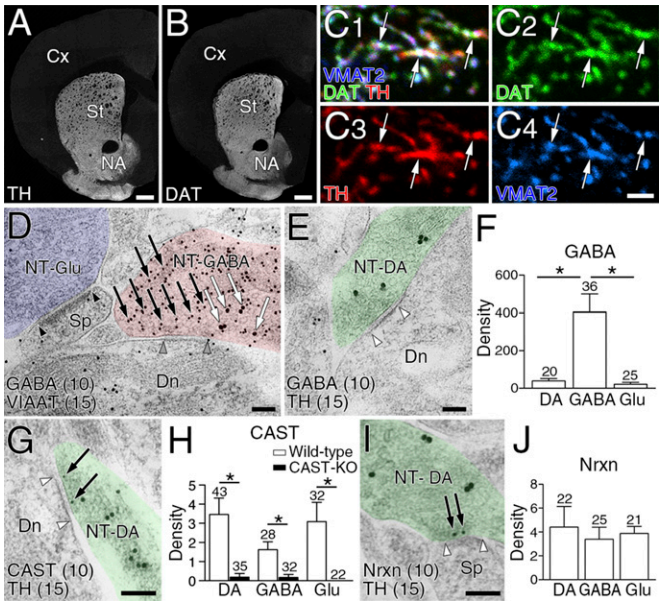

Fig. 1. Dopaminergic presynaptic phenotype at striatal dopamine synapses. (*A* and *B*) Immunofluorescence labeling for TH (*A*) and DAT (*B*) in the striatum. Cx, cortex; NA, nucleus accumbens; St, striatum. (*C*) Triple immunofluorescence for TH (red), DAT (green), and VMAT2 (blue) in ultrathin (100 nm) cryosections showing their extensive overlap (arrows). (*D* and *E*) Double-label postembedding immunoelectron microscopy for GABA [Ø (diameter) = 10-nm colloidal gold particles] and VIAAT (*D*, Ø = 15 nm) or TH (*E*, Ø = 15 nm). GABA (arrows) is concentrated on VIAAT$^+$ GABAergic terminals forming symmetric synapses (NT-GABA, red terminal), but not detected in VIAAT$^-$ glutamatergic terminals forming asymmetric synapses (NT-Glu, blue) or TH$^+$ dopaminergic terminals (NT-DA, green) forming symmetric synapses. Arrowhead pairs indicate the synaptic membrane. Dn, dendrite; NT, nerve terminal; Sp, spine. (*F*) The density of immunogold labeling for GABA (particles per 1 μm^2) in dopaminergic (DA), GABAergic (GABA), and glutamatergic (Glu) terminals. (*G* and *I*) Double-label postembedding immunogold microscopy for TH (Ø = 15 nm) and CAST (*G*, Ø = 10 nm) or Nrxn (*I*, Ø = 10 nm). Immunogold labeling (arrows) for CAST and Nrxn is observed beneath the presynaptic membrane of TH$^+$ dopaminergic terminals. (*H* and *J*) The mean density of immunogold particles per 1 μm of synaptic membrane for CAST (*H*) and Nrxn (*J*) at dopaminergic, GABAergic, and glutamatergic synapses in wild-type (*H* and *J*, open columns) and CAST-KO (*H*, filled columns) mice. In *F*, *H*, and *J*, numbers of terminals analyzed are indicated above each column, and error bars on columns represent SEM. *$P < 0.05$ (unpaired *t* test). [Scale bars: 1 mm (*A* and *B*), 2 μm (*C*), and 100 nm (*D*, *E*, *G*, and *I*).]

Presynaptic differentiation of dopaminergic terminals was tested by expression of the active zone protein CAST and the presynaptic adhesion molecule Nrxn. Immunofluorescence showed punctate immunolabeling for CAST in DAT$^+$ dopaminergic terminals (Fig. S1*H*). Immunoelectron microscopy revealed CAST expression beneath the presynaptic membrane of dopaminergic (Fig. 1*G*), GABAergic (Fig. S1*I*), and glutamatergic (Fig. S1*J*) terminals. The density of immunogold labeling for CAST on the presynaptic membrane was comparable across the three types of terminals (Fig. 1*H*, open columns), and specificity was confirmed by significantly low densities of immunogold labeling in the corresponding terminals of CAST-knockout (KO) mice (Fig. 1*H*, filled columns). This was also true for Nrxn, which was detected at comparable levels on the presynaptic membrane of the three types of terminals (Fig. 1 *I* and *J* and Fig. S1 *K* and *L*). Therefore, presynaptic molecules are recruited to the contact sites at dopaminergic terminals, like those at GABAergic and glutamatergic terminals. Taking these data together, this finding indicates that the presynaptic neurochemical phenotype at dopamine synapses is exclusively dopaminergic, and their contact sites exhibit presynaptic differentiation.

Dopamine Receptor Expression. Before investigating postsynaptic neurochemical phenotypes, we examined the basic expression profile of dopamine receptors in the striatum. Expression of D_1R and D_2R was mutually exclusive at both the transcription and protein levels (Fig. S2 *A* and *B*), and detected predominantly along dendrites of MSNs labeled for 32-kDa dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP32) (Fig. S2*C*). In comparison, D_1R and D_2R expression was low or undetectable in dendrites of GABAergic and cholinergic interneurons expressing their neuronal markers parvalbumin (PV), neuronal nitric oxide synthase (nNOS), and choline transporter (CHT) (Fig. S2 *D–F*). In MSNs, the density of cell membrane-attached metal particles for D_1R and D_2R was comparably high in the soma, dendritic shaft, and dendritic spines (Fig. 2 *A–C*). In the neuropil, weak immunolabeling for D_1R and D_2R was also observed in terminals forming symmetric—but not asymmetric—synapses (Fig. 2*C*). Triple immunofluorescence revealed low to moderate axonal immunolabeling for D_1R in DARPP32$^+$ MSN axons, and D_2R in CHT-labeled cholinergic and DAT-labeled dopaminergic axons (Fig. S2 *G–I*).

Next, we examined the distribution of D_1R and D_2R in relation to dopamine synapses using double-label pre-embedding immunoelectron microscopy. Metal particles for D_1R and D_2R were densely distributed on the extrasynaptic surface of spiny dendrites, whereas the postsynaptic membrane in contact with TH-labeled dopaminergic terminals (diaminobenzidine precipitates) was rarely labeled (Fig. 2 *D* and *E*). We quantified this by measuring the density of metal particles on the synaptic, perisynaptic (<100 nm from the edge of the dopamine synapse), and extrasynaptic (>100 nm) membranes (Fig. 2*F*). The mean labeling density for D_1R and D_2R was significantly lower in the synaptic membrane than in the extrasynaptic membrane (Fig. 2*G*). To test the possibility of hindered antibody penetration into dopamine synapses, we performed postembedding immunoelectron

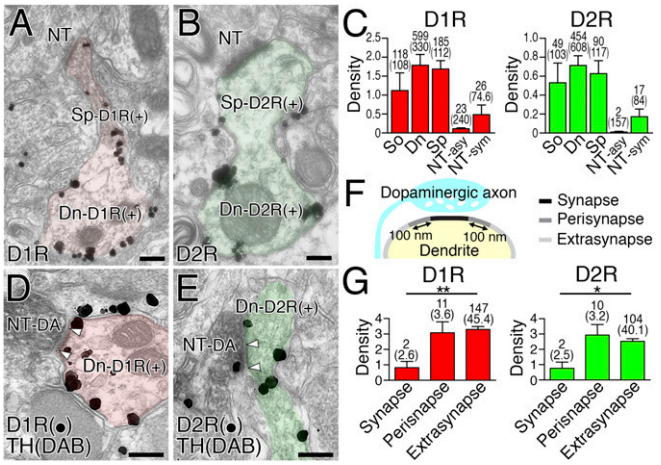

Fig. 2. Expression profiles of dopamine receptors in the striatum. (*A* and *B*) Pre-embedding immunoelectron microscopy for D_1R (*A*) and D_2R (*B*). D_1R- and D_2R-labeled spiny dendrites are colored red and green, respectively. (*C*) Labeling densities for D_1R (*Left*) and D_2R (*Right*) per 1 μm of the plasma membrane in somata (So), dendritic shafts (Dn), dendritic spines (Sp), and nerve terminals forming asymmetric (NT-asy) or symmetric (NT-sym) synapses. (*D* and *E*) Double-label pre-embedding immunoelectron microscopy for TH (DAB) and D_1R (*D*, particles) or D_2R (*E*, particles). Arrowhead pairs indicate dopamine synapses formed by TH-labeled dopaminergic terminals (NT-DA). (*F*) The synaptic (black), perisynaptic (dark gray), and extrasynaptic (light gray) membranes of dendrites around dopamine synapses. (*G*) Densities for D_1R (*Left*, 18 synapses) and D_2R (*Right*, 16 synapses) labelings per 1 μm of the synaptic, perisynaptic, and extrasynaptic membranes. The length of the plasma membrane (μm) and the number of metal particles analyzed are indicated in parentheses or above each column, respectively. Error bars represent SEM. $*P < 0.05$ and $**P < 0.01$ (unpaired *t* test). (Scale bars, 200 nm.)

microscopy using tissue specimens fixed mildly with 0.2% picric acid/2% paraformaldehyde fixative (Fig. S2 *J* and *K*). Again here, the synaptic membrane of TH-labeled dopamine synapses was significantly low for D_1R or D_2R labeling than the extrasynaptic membrane (Fig. S2 *L* and *M*). Thus, dopamine receptors are widely expressed on the extrasynaptic somatodendritic surface of MSNs, with no particular accumulation at—or gradient toward—dopamine synapses.

Postsynaptic Phenotypes at Dopamine Synapses. We then examined which types of molecules construct postsynaptic membrane specializations at dopamine synapses. From their symmetric nature, we examined expression levels of the following GABAergic postsynaptic proteins: $GABA_AR\alpha1$, gephyrin (a scaffolding protein interacting with $GABA_A$ and glycine receptors) and NL2 (a synaptic adhesion protein interacting with $GABA_A$ receptors and gephyrin) (20, 21). All three proteins were clustered in the neuropil, and tightly apposed to VIAAT-labeled GABAergic (Fig. 3 *A*–*C*, arrowheads) and DAT-labeled dopaminergic (Fig. 3 *A*–*C*, arrows) terminals. Postsynaptic localization of these proteins was further tested by postembedding double-label immunoelectron microscopy (Fig. 3 *D*–*F* and Fig. S3 *A*–*C*). The density of immunogold labeling for $GABA_AR\alpha1$, gephyrin, and NL2 was almost comparable between dopamine and GABAergic synapses, whereas the density at glutamatergic synapses was not different from background (Fig. 3 *G*–*I*). In contrast, glutamatergic postsynaptic proteins, PSD-95 and AMPA receptors, were enriched at glutamatergic synapses, but hardly detected at dopamine or GABAergic synapses (Fig. S3 *D*–*G*). Therefore, the postsynaptic phenotype at dopamine synapses is exclusively GABAergic.

Postsynaptic Target of Dopamine Synapses. Dopamine synapses are frequently found in dendritic shafts and spines of MSNs (12, 13). In the present study, we quantitatively assessed target striatal neurons by lentivirus-mediated single neuronal labeling with GFP (Fig. S4 *A* and *B*). GFP-labeled dendrites (green) were further examined by immunofluorescence for TH (blue), NL2 (red), and neuronal markers (white) (Fig. 4 *B* and *C* and Fig. S4 *C*–*E*). The adenosine receptor $A_{2A}R$ was used as a marker for i-MSNs instead of D_2R, because of its exclusive somatodendritic expression (22). The location of dopamine synapses was identified by the presence of NL2 clusters apposing TH^+ dopaminergic terminals on GFP-labeled dendrites (Fig. 4*A*). These NL2-clustered dopamine synapses were richly expressed on spiny dendrites of both D_1R^+ d-MSNs and $A_{2A}R^+$ i-MSNs (Fig. 4 *B* and *C*, arrows), whereas they were rare on aspiny dendrites of GABAergic and cholinergic interneurons labeled for PV, nNOS, and CHT (Fig. S4 *C*–*E*). Instead, most NL2 clusters on these interneuron dendrites were judged to be non-dopamine synapses because they did not appose TH^+ dopaminergic terminals (Fig. S4 *C*–*E*, arrowheads).

We measured the density of NL2-clustered dopamine and non-dopamine synapses on MSNs. The density of dopamine synapses on dendritic shafts (0.75- to 1.5-μm diameter) in d-MSNs and i-MSNs was significantly (three- to fourfold) higher than that in interneurons (Fig. 4*D*, open columns), whereas that of non-dopamine synapses showed no significant differences among striatal neurons (Fig. 4*E*). The density of dopamine synapses on dendritic spines was also comparable between the two types of MSNs (Fig. 4*D*, filled columns). Therefore, dendrites of d-MSNs and i-MSNs are targeted equally as a substrate for dopamine synapse formation.

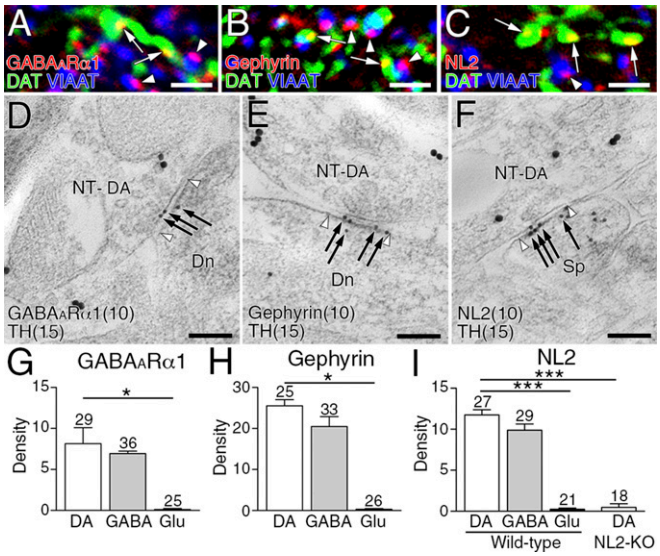

Fig. 3. GABAergic postsynaptic phenotype at striatal dopamine synapses. (*A*–*C*) Triple immunofluorescence for DAT (green) and VIAAT (blue), and for $GABA_AR\alpha1$ (*A*, red), gephyrin (*B*, red), or NL2 (*C*, red). Note close apposition of $GABA_AR\alpha1$, gephyrin, and NL2 clusters to both $VIAAT^+$ GABAergic terminals (arrowheads) and DAT^+ dopaminergic terminals (arrows). (*D*–*F*) Double-label postembedding immunoelectron microscopy for TH [Ø (diameter) = 15 nm] and for $GABA_AR\alpha1$ (*D*, Ø = 10-nm colloidal gold particles), gephyrin (*E*, Ø = 10 nm), or NL2 (*F*, Ø = 10 nm). Immunogold particles (arrows) are concentrated at symmetric synapses formed by TH-labeled dopaminergic terminals (NT-DA, arrowhead pairs). (*G*–*I*) The density of immunogold labeling for $GABA_AR\alpha1$ (*G*), gephyrin (*H*), or NL2 (*I*) per 1 μm of synaptic membrane at dopamine (DA), GABAergic (GABA), and glutamatergic (Glu) synapses. The specificity of NL2 labeling is confirmed by almost blank labeling in NL2-KO mice (*I*). Representative images of GABAergic synapses are shown in Fig. S3. Numbers of synapses analyzed are indicated above each column. Error bars represent SEM. $*P < 0.05$ and $***P < 0.001$ (unpaired *t* test). [Scale bars: 2 μm (*A*–*C*) and 100 nm (*D*–*F*).]

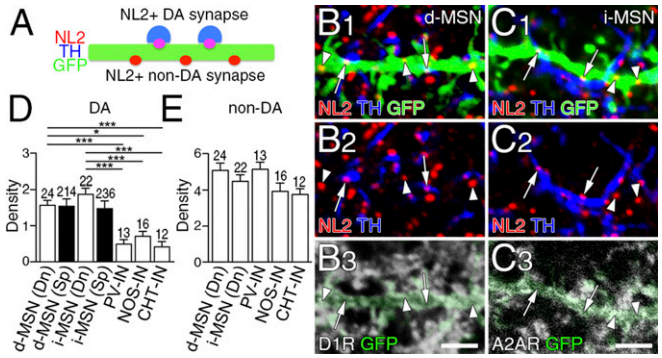

Fig. 4. Dopamine synapses are preferentially formed on to dendrites of two types of MSNs. (*A*) Schematic to distinguish NL2-clustered dopamine and non-dopamine synapses on GFP-labeled dendrites of striatal neurons. (*B* and *C*) Quadruple immunofluorescence for NL2 (red), GFP (green), and TH (blue), and for D_1R (*B*, white) or A_{2A}R (*C*, white). NL2-clustered dopamine synapses (arrows) are preferentially distributed on dendrites of D_1R-labeled d-MSNs (*B*) and A_{2A}R-labeled i-MSNs (*C*). (*D* and *E*) The density of NL2-clustered dopamine (*D*) and non-dopamine (*E*) synapses per 10 μm of dendritic shafts (Dn) and spines (Sp) in d-MSNs and i-MSNs, and dendrites of striatal interneurons. Immunofluorescence images for striatal interneurons are shown in Fig. S4. The number of dendrites analyzed is indicated above each column. Error bars represent SEM. $*P < 0.05$ and $***P < 0.001$ (one-way ANOVA with Tukey's post hoc test). (Scale bars, 2 μm.)

NL2-Mediated Presynaptic Differentiation in Vitro. We hypothesized that GABAergic postsynaptic molecules mediated dopamine synapse formation, as they do for GABAergic synapse formation (21). To pursue this possibility, we cocultured primary midbrain or striatal neurons at 9–11 d in vitro with HEK293T cells expressing $GABA_AR\alpha1$, NL2, or GFP, and examined whether presynaptic molecules were recruited to their contact sites (Fig. 5*A* and Fig. S5*A*). In this coculture assay, CAST, VMAT2, and Nrxn were robustly and significantly recruited to contact sites between DAT-labeled dopaminergic axons and HEK293T cells expressing NL2 (Fig. 5 *B* and *E*–*G*), but not $GABA_AR\alpha1$ or GFP (Fig. 5 *C* and *D*). Similar presynaptic differentiation was observed in cocultures of striatal GABAergic neurons with HEK293T cells expressing NL2 (Fig. S5 *B*–*E*). Therefore, NL2 induces presynaptic differentiation in axons of midbrain dopamine neurons and striatal GABAergic neurons in vitro.

NL3 Up-Regulates at Dopamine Synapses in NL2-KO Mice. To explore this role in vivo, changes in the density of dopamine synapses and molecular expression were investigated in NL2-KO mice. We assessed the density of DAT-labeled dopamine synapses by measuring their nearest-neighbor distances. No significant differences in dopamine synapse density were found between wild-type and NL2-KO mice (Fig. S5 *F*–*H*). Immunofluorescence and immunogold labelings for presynaptic VMAT2 (Fig. S5 *I*–*M*) and postsynaptic gephyrin (Fig. S5 *N*–*R*) were comparable at TH-labeled dopamine synapses between wild-type and NL2-KO mice, whereas significant up-regulation of NL3 occurred at dopamine synapses in NL2-KO mice (Fig. S5 *S*–*W*). In cocultures with HEK293T cells expressing NL3, NL3 also induced presynaptic differentiation in dopaminergic axons in vitro (Fig. S5*X*). Therefore, we concluded that the role of NL2 in dopamine synapse formation in vivo could not be addressed using NL2-KO mice, because of compensatory up-regulation of NL3.

Reciprocal Changes of Dopamine and GABAergic Synapses by NL2 Knockdown. To overcome this problem, we used a sparse knockdown (KD) of NL2, which has been used to uncover the role of NL1 in cortical synaptogenesis (23). We prepared control-microRNA (miR), NL2-miR#1, and NL2-miR#2. Both NL2-miRs effectively and selectively reduced NL2 expression in HEK293T cells (Fig. S6*A*). We injected lentivirus vectors carrying GFP and one of the three miRs into the striatum of newborn pups (Fig. S6*B*). Two months after injection, a few MSNs expressed GFP (Fig. S6*C*) (12 of 765 $DARPP32^+$ MSNs). We first checked NL2 expression at gephyrin clusters on GFP-labeled spiny dendrites. No significant changes were observed in the density or fluorescent intensity of gephyrin clusters between control-miR-infected (control) and NL2-miR–infected (NL2-KD) neurons (Fig. S6 *D*–*F*, red, and *G* and *H*). At gephyrin clusters, fluorescent intensity for NL2 was markedly and significantly lower in NL2-KD neurons (white arrowheads in Fig. S6 *D*–*F* and *I*), but not in neighboring non-infected neurons (Fig. S6 *D*–*F*, yellow arrowheads). Notably, the fluorescence intensity for NL3 at gephyrin clusters showed no significant differences between control and NL2-KD neurons (Fig. S6 *J*–*L*, white arrowheads, and *M*). Therefore, injection of NL2-miRs effectively reduces NL2 expression in MSNs without affecting the total number and intensity of gephyrin clusters or NL3 expression at gephyrin clusters.

Next, we analyzed the density of dopamine and GABAergic synapses by counting the number of gephyrin clusters on GFP-labeled spiny dendrites that apposed TH^+ dopaminergic terminals (Fig. 6 *A*–*C*, arrows) or $VIAAT^+$ GABAergic terminals (Fig. 6 *A*–*C*, arrowheads), respectively. In NL2-KD neurons, the density of dopamine synapses was significantly lower than in control neurons (Fig. 6*D*), whereas that of GABAergic synapses was significantly greater (Fig. 6*E*).

Discussion

In the present study, we have shown that striatal dopamine synapses are neurochemically mismatched contacts, and NL2 is involved in their formation.

Neurochemically Mismatched Contact at Dopamine Synapses. Dopaminergic terminals were extensively colabeled for TH, DAT, and VMAT2, which are essential for the synthesis, recycling, and vesicular filling of dopamine (24). Moreover, CAST and Nrxn, which are involved in active zone formation and synaptic adhesion, respectively (6, 25), accumulated on the presynaptic membrane at dopamine synapses and were expressed at levels comparable to

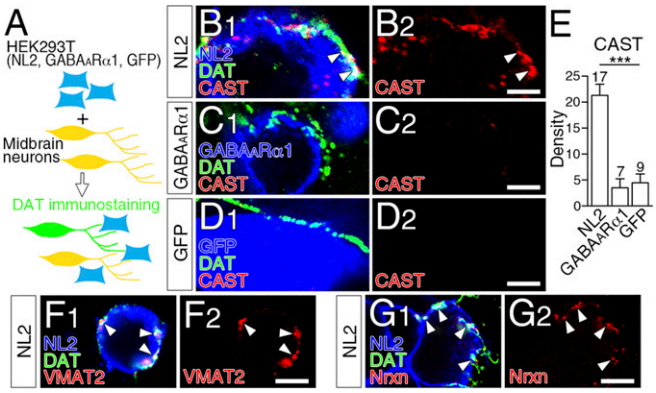

Fig. 5. NL2-mediated presynaptic differentiation of dopaminergic axons in vitro. (*A*) Schematic of coculture assay of midbrain neurons with HEK293T cells expressing NL2, $GABA_AR\alpha1$, or GFP. Axons of midbrain dopamine neurons were identified by DAT immunofluorescence. (*B*–*D*) Triple immunofluorescence for CAST (red) and DAT (green), and for NL2 (*B*, blue), $GABA_AR\alpha1$ (*C*, blue) or GFP (*D*, blue). CAST clusters are recruited to contact sites of dopaminergic axons with HEK293T cells expressing NL2 (*B*, arrowheads), but not $GABA_AR\alpha1$ (*C*) or GFP (*D*). (*E*) The density of CAST clusters per 100 μm of dopaminergic axon in contact with HEK293T cells. The number of HEK293T cells contacted by DAT-labeled dopaminergic axons is indicated above each column. Error bars represent SEM. $***P < 0.001$ (Mann–Whitney *u* test). (*F* and *G*) Triple immunofluorescence for VMAT2 (*F*, red) or Nrxn (*G*, red), DAT (green), and NL2 (blue) in cocultures of midbrain dopamine neurons and HEK293T cells expressing NL2. (Scale bars, 2 μm.)

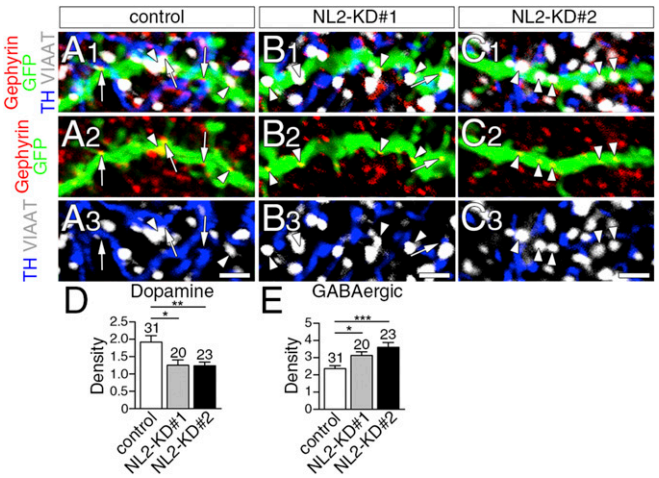

Fig. 6. Decrease of dopamine synapses and reciprocal increase of GABAergic synapses after sparse NL2 knockdown in striatal MSNs. (*A–C*) Quadruple immunofluorescence for gephyrin (red), GFP (green), TH (blue), and VIAAT (gray) in spiny dendrites of control (*A*), NL2-KD#1 (*B*), or NL2-KD#2 (*C*) neurons. Arrows and arrowheads indicate dopamine and GABAergic synapses, respectively, on GFP-labeled dendrites. (*D* and *E*) The density of dopamine (*D*) and GABAergic (*E*) synapses per 10 μm of control and KD dendrites. The total number of dendrites analyzed is indicated above each column. Error bars represent SEM. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ (Mann–Whitney *u* test). (Scale bars, 2 μm.)

conventional glutamatergic and GABAergic synapses. This orchestrated molecular architecture consolidates the dopaminergic phenotype in the presynaptic side of dopamine synapses. Surprisingly, GABAergic proteins $GABA_AR\alpha1$, gephyrin, and NL2 were expressed in the postsynaptic side at densities comparable to those at conventional GABAergic synapses. Therefore, dopamine synapses are contacts between dopaminergic presynaptic and GABAergic postsynaptic structures. It has been reported that 40.7% of symmetric synapses expressing $GABA_A$ receptors in the striatum are formed by terminals in which GABA is low or undetectable (26). We assume that they represent most, if not all, dopamine synapses.

An increasing number of reports is emerging of cases in which two or more classic transmitters are coreleased at single synapses (27), and dopamine has been shown to be coreleased with glutamate or GABA (27, 28). However, we found no significant immunoreactivity for GABA, GAD, or VIAAT at dopaminergic terminals. The scarcity of GABA in dopaminergic terminals is consistent with previous studies reporting that no or few (11–13%) dopaminergic terminals are significantly labeled for GABA (18, 29). This finding suggests that GABA is coreleased little, if at all, at most dopamine synapses. Nevertheless, optogenetic stimulation of dopaminergic axons evokes $GABA_A$ receptor-mediated postsynaptic currents in MSNs through GAT1-mediated uptake and VMAT2-dependent vesicular filling of GABA (28, 30). In the present study, we observed weak expression of GAT1, as well as intense expression of VMAT2 in dopaminergic terminals. Taken together, this evidence indicates that the phenotypes of the major transmitters and receptors are neurochemically mismatched at dopamine synapses, but transporter-mediated GABA release could be conducted from dopaminergic terminals whose GABA content is undetectable by conventional immunohistochemistry or from a small subpopulation of dopaminergic terminals expressing high content of GABA.

NL-Mediated Dopamine Synapse Formation. Synapse-type–specific transsynaptic interaction between NLs and Nrxns plays a key role in bidirectional synaptic differentiation (6). NL2 is selectively expressed at inhibitory synapses (8), binds gephyrin, and promotes its membrane targeting through collybistin activation (31). NL2 also interacts with $GABA_A$ receptors (20). These interactions underlie NL2-mediated specification of inhibitory synapses (9). Expression of NL2 at dopamine synapses, together with that of $GABA_AR\alpha1$ and gephyrin, is thus consistent with the framework of synapse-type–dependent expression of the NL family. Our finding that significant amounts of CAST, VMAT2, and Nrxn were recruited to contact sites between dopaminergic axons and NL2-expressing HEK293T cells indicates that NL2 functions as a presynaptic organizer for dopaminergic axons in vitro, similarly to GABAergic axons (20, 32).

In vitro experiments clearly demonstrate that manipulations that up- or down-regulate NL expression alter synapse formation positively or negatively, respectively, indicating that NLs are intrinsically synaptogenic (9, 33). However, there is little or no change in the number and structure of synapses between global NL-KO and wild-type mice (23, 34). Indeed, we found no significant changes in the density of dopamine synapses in the striatum of NL2-KO mice. These conflicting results between the in vitro and in vivo conditions seem to be ascribed to the redundancy in the molecular form of NLs and Nrxns and their interactions (6). In support of this notion, NL3 was up-regulated at dopamine synapses in NL2-KO mice, which would mask synaptogenic actions by NL2. To overcome this problem, we adopted the sparse NL2-KD strategy to minimize compensatory up-regulation of NL3. Under this condition, the density of dopamine and GABAergic synapses on MSN dendrites was significantly decreased and increased, respectively, without a change in their total density. This finding suggests that NL2 mediates striatal synapse formation by giving competitive advantage to heterologous dopamine synapses over conventional GABAergic synapses. Therefore, NL2 appears to regulate the formation of striatal synapses in a competitive and input-dependent manner in vivo rather than in a simple synaptogenic manner in vitro. Such NL-dependent competitive synaptogenesis has also been reported for NL1 in the cortex, where cortical synapse formation is sensitive to sparse NL1-KD, causing transcellular differences in the relative amount of NL1 (23). Thus, different relative amounts of NL2 caused by sparse KD may also affect competitive synaptogenic processes between NL2-KD and control MSNs.

Postulated Role of Dopamine Synapses. D_1R and D_2R are widely expressed on the extrasynaptic surface of MSN dendrites (15–17). The present immunohistochemistry confirmed this, and further clarified the lack of dopamine receptor accumulation at dopamine synapses. The mismatching between dopamine release and reception sites indicates that dopamine synapses are neither the neural device for the wired transmission that is conducted for fast and point-to-point signaling typical to conventional glutamatergic and GABAergic synapses, nor that for the volume transmission for slow and global modulation, such as muscarinic M1-mediated cholinergic transmission in the hippocampus (35, 36). Then, what is the role of neurochemically mismatched dopamine synapses?

Glutamatergic transmission to MSNs by cortical and thalamic inputs, and its modulation by dopaminergic, cholinergic, and GABAergic inputs, are the basis of functional regulation in the basal ganglia (10, 14). The diffusion model of quantal dopamine release postulates an effective radius of ∼2 and 7 μm for the activation of D_1R and D_2R, respectively (37). A recent study using a D_2R biosensor points out that the spatiotemporal extent of dopaminergic transmission is more limited and D_2R on MSNs functionally behaves as low-affinity receptors for fast dopaminergic transmission (38). Considering that MSN dendrites are both the preferential postsynaptic target of dopamine synapse formation and the neuronal element enriched with dopamine receptors, we suggest that such heterologous contacts function as a device to increase the target specificity and potency of dopaminergic modulation by anchoring dopamine release sites to dopamine-sensing neurons. In this regard, it should be noted that synaptic contacts are also formed by other neuromodulatory neurons (39–41).

Of these, NL2 is expressed at symmetric synapses formed by cholinergic neurons in the forebrain (42). The possibility that NL2-dependent formation of neurochemically mismatched contacts could be the general strategy to attract neuromodulatory inputs to specific targets is an intriguing issue to be explored in future studies.

Materials and Methods

All animal experiments were approved by the Hokkaido Univeristy Animal Care and Use Committee. In the present study, we performed immunoblot, immunohistochemistry, lentiviral experiments, and coculture assay. More information about the experimental procedures, the specificity and combination of antibodies, and the labeling density of synaptic molecules in individual mice is available in *SI Materials and Methods*, Fig. S7, and Tables S1–S3.

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