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GABAergic-like dopamine synapses in the brain

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Summary

Dopaminergic axons originate in the midbrain and establish widely spread synapses throughout the brain¹. Synaptic transmission at these synapses plays a crucial role for volitional movement and reward-related behaviors^{2,3}, while dysfunction of dopamine (DA) synapses causes various psychiatric and neurological disorders. Despite this significance, the true nature of brain-wide spatial and functional features of DA synapses remains poorly understood due to difficulties defining functional DA synapses at the molecular and physiological levels. Here we show that DA synapses are structured and function like GABAergic synapses in the mouse brain with marked regional heterogeneity. DA transmission is strongly correlated with GABA co-transmission at DA synapses across the brain areas. In addition, functional DA synapses possess GABAergic postsynaptic markers and are unevenly distributed throughout the brain with distinct spatial clustering. In the dorsal striatum, GABAergic-like DA synapses are uniquely clustered on the dendrites and GABA transmission at DA synapses has disparate physiological properties. Remarkably, the attenuation of GABA co-transmission precedes deficits in dopaminergic transmission in animal model of Parkinsonism. Our findings unravel distinct spatial and functional nature of GABAergic-like DA synapses in health and disease. Furthermore, the broader implication of our results is that GABAergic-like features of DA synapses can be utilized to better understand the real complexity of synaptic actions at DA synapses in regulating neural circuits.

Modulatory neurotransmitter dopamine (DA) critically orchestrates essential brain functions including voluntary movement, action selection, and reward-related behaviors via volume transmission. Due to its spatiotemporal release properties, synaptic transmission through DA synapses has been considered as slow and diffuse one in space, possibly causing non-synapse-specific transfer of information to postsynaptic neurons. In addition, DA acts mainly through extrasynaptic G protein-coupled receptors (GPCRs)^{4,5}. Thus, the early idea that DA transmission employing volume transmission is slow, inaccurate, and non-synapse-specific has been prevalent for a long time. However, recent studies highlighted the possibility that synaptic action via DA synapses can be rapid, synapse-specific, and far more complicated than we think. It appears that there exist active zone-like release sites in some populations of DA boutons, which can potentially support spatially and temporally precise coding⁶. More interestingly, co-transmission of classical, fast-acting neurotransmitters such as glutamate and γ -aminobutyric acid (GABA) at DA synapses, along with DA, has been found in the striatum⁷⁻⁹. Given that DA release and its propagation to neighboring synapses via volume transmission are not strictly limited in space, such co-transmission of fast-acting neurotransmitters at DA synapses and its synaptic effects would be spatially more restricted and probably localized to individual synapses, rendering the physiological actions of DA synapses more synapse-specific. Although potentially critical for realistic synaptic actions at DA synapses, the central question of whether this co-transmission of DA, glutamate, and GABA at DA synapses is ubiquitous throughout the brain or is just one of the distinctive features of some specific DA projections remains not fully explored.

DA transmission is strongly correlated with GABA transmission at DA synapses in the brain

To determine brain-wide heterogeneity of synaptic actions at DA synapses caused by co-transmission of multiple neurotransmitters, we first investigated regional variations of DA axon fibers by carrying out double-immunostaining for tyrosine hydroxylase (TH) and dopamine transporter (DAT) that are key molecular markers for DA neurons and axon fibers. Two DA axonal projections (nigrostriatal and mesocorticolimbic) from midbrain DA neurons in the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA) heavily innervate striatum, which is the input region of basal ganglia (Fig. 1a). However, they also project to cortex and other nuclei of basal ganglia, including globus pallidus (GPe and GPi) and subthalamic nucleus (STN)¹⁰. As

many of the previous studies reported, most of areas in the striatum were occupied by highly dense arborization of DA axons (Fig. 1a, b) and the areas of TH-positive axon fibers were comparable among striatal regions (Fig. 1b, c). The amount of axonal projections to cortex, however, was significantly less than DA projections in the striatum (Fig. 1b, c, e). Extrastriatal basal ganglia nuclei, such as GPe, GPi, and STN, showed much sparser axonal fibers than the striatum, but TH-positive DA axons in these regions were still denser than other cortical areas (Fig. 1b, c, e). The regional pattern of DAT-positive axons projecting to different brain regions was largely similar to the one of TH-positive axons (Fig. 1b, d, f). Notably, although small, regional heterogeneity and differences were found within the striatum in DAT-positive axons (Fig. 1d, f).

We next questioned whether neurotransmitters co-transmission at DA synapses would follow the quantitative pattern of axon fibers across the brain. We selectively expressed channelrhodopsin 2 (ChR2) in DA neurons by crossing DAT-IRES-Cre mice (in which Cre recombinase is selectively expressed in DA transporter-expressing neurons) with transgenic mice containing a conditional floxed allele of ChR2 in the Rosa26 locus (Ai32 mice)⁸. We first performed fast-scanning cyclic voltammetry (FSCV) to measure and compare brain-wide DA transmission among each DA projection region (Fig. 1g). As documented before^{11,12}, light-evoked (450 nm) DA transmission was the highest in the dorsal striatum and ventral domains of striatum exhibited relatively smaller DA release than dorsal striatum. Photo-stimulation of DA axons elicited very low DA release in the cortex as well as extrastriatal basal ganglia nuclei (Fig. 1h-j).

We then sought to determine the regional heterogeneity in the co-transmission of GABA and glutamate at DA synapses. To measure GABAergic inhibitory postsynaptic current (IPSC) and glutamatergic excitatory postsynaptic current (EPSC), we performed whole-cell voltage clamp recording in various postsynaptic neurons of DA target areas and recorded optogenetically induced IPSC (oIPSC) and EPSC (oEPSC) from the same postsynaptic neuron. The strongest GABAergic responses were observed from the spiny projection neurons (SPNs) in the dorsal striatum, while the SPNs in the ventral striatum only displayed GABAergic responses less than half of the mean amplitude in the dorsal striatum. GABA co-transmission at DA synapses in both cortex and extracellular basal ganglia nuclei was minimal, if not non-existent, and very few neurons only produced small amplitude of oIPSC (Fig. 1k-m, Extended Data Fig. 1a, b). Unlike GABA co-transmission, the highest responses by glutamatergic transmission at DA synapses came from the

nucleus accumbens (NAc) shell in the ventral striatum (Fig. 1n-p, Extended Data Fig. 1c, d). Glutamatergic oEPSCs in other striatal domains were much smaller than that of NAc shell and there were almost no EPSC responses from the recorded neurons in the cortex and extrastriatal basal ganglia nuclei. Of particular note, when this regional transmission of DA, GABA, and glutamate at DA synapses was compared with each other, we found a strong correlation between DA and GABA release, whereas no pronounced correlations existed between glutamate and other neurotransmitters (Fig. 1q). In addition, when the area of DA axon fibers and neurotransmitters co-transmission were compared throughout DA target areas, significant correlations were found between DA axonal areas (TH- and DAT-positive) and GABA (or DA) transmission, while there was no meaningful correlation between DA fibers and glutamate transmission (Fig. 1r, Extended Data Fig. 2). Interestingly, the correlation between DAT-positive axonal area and GABA (or DA) transmission was stronger than the one by TH-positive axons both in the whole brain and in the striatal regions. Together, it is very likely that DA transmission reliably accompanies GABA transmission at DA synapses in the brain. Furthermore, regional heterogeneity of DAT-positive axons in the brain, although small, mirrors regional differences in DA and GABA transmissions better than TH-positive axonal areas.

Spatial distribution and characteristics of GABAergic-like DA synapses in the brain

DA axon terminals make synaptic contacts with neurons in numerous brain areas, although the majority of these synapses reside within the striatum. These multiple brain regions are implicated in diverse neural functions, which are critically mediated by synaptic transmission via DA synapses. It is important to note that not all TH-positive bouton-like structures are able to release DA⁶ and postsynaptic DA receptors are typically at extrasynaptic sites^{4,5,13}. Thus, unlike glutamatergic and GABAergic synapses, this mismatch between presynaptic and postsynaptic sites, and our lack of knowledge about presynaptic and postsynaptic molecular markers defining functional DA synapses, have hampered so far the comprehensive identification of spatial and functional features of DA synapses throughout the brain. Recently, neuroligin-2 (NL2), a common postsynaptic cell adhesion molecule (CAM) that has been thought to be exclusively localized to inhibitory synapses¹⁴, was found in the postsynaptic membrane specializations at striatal DA synapses⁵. Despite this finding, however, our understanding of molecular components defining DA

synapses in the brain is still far from complete.

We investigated brain-wide distribution and spatial characteristics of DA synapses by labeling molecular markers for both presynaptic and postsynaptic sites. We utilized NL2 as a postsynaptic marker for DA synapse. We also considered the co-localization of TH and vesicular monoamine transporter 2 (VMAT2) immunofluorescences as a putative marker for dopaminergic presynaptic boutons that are able to co-transmit DA and GABA⁷. In multi-color immunostaining and enhanced confocal microscopy by airyscan, TH- and VMAT2-positive presynaptic boutons, unlike TH-positive DA axons, showed marked regional heterogeneity in their distribution across the nigrostriatal and mesocorticolimbic pathways (Fig. 2a-d, Extended Data Fig. 3a, c-e). Dorsal striatum largely contained more TH- and VMAT2-positive boutons than ventral striatum. As expected from our results (Fig. 1b-f), the number of TH- and VMAT2-positive boutons in cortical areas and extrastriatal basal ganglia was much smaller than that of striatal regions (Fig. 2b, Extended Data Fig. 3c). Notably, the number of NL2-positive postsynaptic sites also exhibited regional differences across the brain, although the relative difference among brain areas was smaller than that of dopaminergic presynaptic boutons (Fig. 2c, Extended Data Fig. 3d). We then regarded the co-localization of a TH- and VMAT2-positive presynaptic bouton and a NL2-positive postsynaptic site as a potential GABAergic-like DA synapse. When this triple co-localization of TH, VMAT2, and NL2 was compared, the regional heterogeneity in dopaminergic presynaptic boutons was accentuated by variation in NL2-positive postsynaptic sites, leading to more prominent differences in the number of GABAergic-like DA synapses across the brain (Fig. 2d, Extended Data Fig. 3e).

It is likely that TH- and VMAT2-positive presynaptic boutons may not represent exclusively functional DA terminals capable of releasing neurotransmitters as VMAT2 can also exist outside synaptic boutons. To confirm this, we examined the spatial properties of GABAergic-like DA synapses by labeling presynaptic protein Bassoon as a functional presynaptic marker at DA synapses⁶. When VMAT2 was replaced by Bassoon, relative regional differences in the numbers of both presynaptic boutons and GABAergic-like DA synapses were largely maintained (Fig. 2g-j), while the estimated numbers of dopaminergic presynaptic boutons and GABAergic-like DA synapses were considerably reduced (Extended Data Fig. 3b, f-h). In addition, when Bassoon was used as a presynaptic marker, the difference in the number of GABAergic-like DA

synapses among brain regions, especially between dorsal and ventral striatum, became more pronounced (Fig. 2j, Extended Data Fig. 3h). We further validated these findings and analyzed GABAergic-like DA synapses by using Gephyrin as a postsynaptic marker for DA synapses. Gephyrin is an assembly protein which anchors inhibitory neurotransmitter receptors to the postsynaptic membrane and has been determined as a major postsynaptic protein of GABAergic synapses^{15,16}. Spatial distribution and regional disparity of GABAergic-like DA synapses throughout the brain, revealed by Gephyrin, were also comparable to the pattern identified by NL2, suggesting that regional properties of GABAergic-like DA synapses exist independent of the molecular markers used. Moreover, compared to the co-localization of VMAT2 and Gephyrin, the identification of GABAergic-like DA synapses by the co-localization of Bassoon and Gephyrin made the regional difference between dorsal and ventral striatum more obvious (Extended Data Fig. 4, 5). The proportion of GABAergic-like DA synapses out of TH- and VMAT2-positive presynaptic boutons ranged on average from 27.9 to 42.7 percent in the striatum. The percentage of GABAergic-like DA synapses in the cortex was comparable to the striatum, while this percentage was much lower (7.8 to 12.3 percent) in the extrastriatal basal ganglia (Fig. 2e). Meanwhile, the percentage of GABAergic-like DA synapses out of all the NL2-positive postsynaptic sites varied between 13 to 25.4 percent in the striatal areas and this ratio was relatively low in the cortical areas and extrastriatal basal ganglia (Fig. 2f). Noticeably, when Bassoon was used in place of VMAT2 as a presynaptic marker, the proportion of GABAergic-like DA synapses out of all the presynaptic boutons rose, whereas the ratio of GABAergic-like DA synapses out of all the postsynaptic sites significantly decreased (Fig. 2k, l, Extended Data Fig. 4k, l).

We then examined the correlation and found that the correlation between GABAergic-like DA synapses and dopaminergic presynaptic boutons was remarkably higher than the one between GABAergic-like DA synapses and GABAergic postsynaptic sites throughout the brain regions (Fig. 2m, n, Extended Data Fig. 6). Of particular note, Bassoon-NL2 combination exhibited the strongest correlation between GABAergic-like DA synapses and dopaminergic presynaptic boutons and the weakest correlation between GABAergic-like DA synapses and GABAergic postsynaptic sites when the correlations were separately calculated and compared from each brain area (Fig. 2o). More importantly, the correlation between GABAergic-like DA synapses and GABA (or DA) transmission was the highest and highly significant in the whole brain as well as

in the striatum, when both Bassoon and NL2 were simultaneously used as molecular markers for GABAergic-like DA synapses (Fig. 2p, q, Extended Data Fig. 7). No significant correlations were found between GABAergic-like DA synapses and glutamate transmission both in the whole brain and in the striatum, regardless of the combinations between presynaptic and postsynaptic markers. We further performed nearest neighbor analysis on the distribution of GABAergic-like DA synapses across the brain, measuring inter-synaptic distances in the population of GABAergic-like DA synapses. Among the different combinations of presynaptic and postsynaptic markers, the Bassoon-NL2 combination showed a clear distinction in the distribution of GABAergic-like DA synapses between dorsal and ventral striatum (Fig. 2r, Extended Data Fig. 7g). This Bassoon-NL2 combination also displayed a significant separation of inter-synaptic distances between the striatum and other brain areas, including cortex and extrastriatal basal ganglia (Fig. 2r). Together, these results indicate that unlike just the area of DA axons, GABAergic-like DA synapses are distributed throughout the brain with significant regional and spatial heterogeneity, irrespective of synaptic markers used for analysis. Furthermore, GABAergic-like DA synapses determined by Bassoon-NL2 combination do correlate best with GABA-DA transmission at DA synapses.

Spatial clustering of GABAergic-like DA synapses in the striatum

Spatial arrangement and clustering of synapses can exert complex effects on the computation of neural circuits^{17,18}. To delve into the potential clustering of GABAergic-like DA synapses throughout the brain, we performed multi-color immunostaining and enhanced confocal imaging via airyscan. We used triple co-localization of TH, Bassoon, and NL2 as a marker for GABAergic-like DA synapse. To better capture region-specific spatial clustering of GABAergic-like DA synapses, image area per each brain region was expanded (Fig. 3a). For spatial clustering analysis, we utilized Ripley's H function that is particularly sensitive to point pattern clustering^{19,20}. Owing to the limited number of GABAergic-like DA synapses in both cortical and extrastriatal basal ganglia areas, we analyzed spatial clustering for only striatal regions that contains sufficient number of GABAergic-like DA synapses to perform Ripley's H function. In the mean NND (nearest neighbor distance), dorsomedial striatum (DMS) alone showed significantly shorter inter-synaptic distances than the null model (random distribution) (Fig. 3b). However, according to Ripley's H function, all the striatal regions exhibited marked differences in the clustering of

GABAergic-like DA synapses when compared to the simulated random arrangement of synapses from the null models (Fig. 3c, d), suggesting that significant clustering exists in each striatal region.

To further compare the relative clustering of GABAergic-like DA synapses between striatal regions, we analyzed the same Ripley's H function with one of the striatal regions pooled as a null model. We found that a significantly strong differences were observed between dorsolateral striatum (DLS) and all the striatal regions when DLS was used as the null model (Fig. 3e, f). Both dorsal and ventral striatum showed more than 60% of rejection rates calculated as the number of samples for which the null model rejected on the Diggle-Cressie-Loosmore-Ford (DCLF) test²¹. Especially, olfactory tubercle (OT) displayed 100% rejection rate, implicating that in terms of synapse clustering pattern, OT appears to be the most different from DLS. Moreover, when OT was used as the null model, these regional differences in the clustering of GABAergic-like DA synapses were preserved (Fig. 3g, h). It is important to note that GABAergic-like DA synapses in the DLS seem to be the most dispersed among the striatal regions even though DLS has the greatest number of GABAergic-like DA synapses. Interestingly, despite the similar number of GABAergic-like DA synapses, DMS showed more clustered patterns of synapses than DLS. NAc shell and OT also exhibited strongly clustered patterns of GABAergic-like DA synapses compared to other regions, which might stem from the specific structures of neural circuit in NAc shell and OT, including cell layers.

Physiological differences between GABAergic-like DA synapses and conventional GABAergic synapses in the dorsal striatum

Spatial and functional properties of GABAergic-like DA synapses have not been explored previously in the brain and many fundamental questions remain, such as to what extent spatial and functional differences exist between GABAergic-like DA synapses and classical GABAergic synapses. To address this question, we chose striatal collateral inhibition from indirect SPNs (iSPNs) to other SPNs as a case of conventional GABAergic projection and first compared the spatial clustering of GABAergic-like DA synapses and conventional GABAergic synapses in the dorsal striatum. As both DA and local GABAergic inputs form synapses on the dendrites of SPNs, we sparsely labeled SPNs in the dorsal striatum of both DAT-Cre and A2A-Cre mice (in which Cre recombinase is selectively expressed in iSPNs) by expressing GFP via virus injection. For

selectively labeling conventional GABAergic axons from iSPNs to other SPNs, we also injected AAV virus expressing RFP upon exposure to Cre recombinase, into the dorsal striatum of A2A-Cre mice (Extended Data Fig. 8a). Then we conducted multi-color immunostaining and enhanced confocal imaging to quantify and compare the distributions of GABAergic-like DA synapses and conventional GABAergic synapses. Triple co-localization of TH, Bassoon, and NL2 on the GFP-positive postsynaptic dendrite was considered as a GABAergic-like DA synapse, as in figure 3. In addition, we regarded the triple co-localization of RFP, VGAT (vesicular GABA transporter), and NL2 as a conventional GABAergic synapse (Fig. 4a). We found that the density of GABAergic-like DA synapses on the single dendrite of SPNs was markedly higher than that of conventional GABAergic synapses made by local iSPNs. Furthermore, this difference in the density of synapses was maintained irrespective of the specific location on the dendrite (distal vs. proximal) (Fig. 4b). Inter-synaptic distances of GABAergic-like DA synapses (DAT-Cre mice) were also shorter than that of GABAergic synapses (A2A-Cre mice) (Fig. 4c). Spatial clustering of synapses on a single dendrite matters since it can critically affect functional computation of dendrites^{22,23}. Hence, we employed Ripley's H function to characterize the spatial clustering of GABAergic-like DA synapses and GABAergic synapses within a single dendrite. Importantly, when considering both Ripley's H function analysis and DCLF test, conventional GABAergic synapses made by iSPNs appeared to be more clustered than GABAergic-like DA synapses, only at the distal portion of dendrites (Fig. 4d, e). Although conventional GABAergic synapses observed from the whole and proximal dendrites also exhibited more than 50% rejection rate in DCLF test, this seemed to be caused by the fact that the clustering patterns of GABAergic synapses are more variable than that of GABAergic-like DA synapses on the entire and proximal dendrites of SPNs.

Meanwhile, the functional properties of synaptic transmission by DA-containing synaptic vesicles have never been investigated in detail by whole-cell patch clamp recording, which have been mostly studied either by extracellular DA recording through FSCV or by overexpression of G protein-coupled inwardly-rectifying potassium channel (GIRK) to SPNs. Taking advantage of GABA co-transmission at DA synapses, we first examined whether GABA co-transmission at DA synapses and GABA transmission at conventional GABAergic synapses rely on distinct types of voltage-dependent Ca^{2+} channels for vesicular release. We recorded GABA co-transmission at DA synapses by stimulating Chr2-expressing DA axons from DAT-Cre;Ai32 mice and evaluated the

effects of 3 different types of Ca^{2+} channels blockers on the amplitude of oIPSC in SPNs. We found that GABA release at DA terminals is dependent on N-type as well as P/Q type Ca^{2+} channels in the dorsal striatum, whereas T-type Ca^{2+} channels were not implicated in vesicular release (Fig. 4f, g, Extended Data Fig. 8b). On the other hand, conventional GABAergic transmission that were examined at GABAergic synapses between iSPN and dSPN by using A2A-Cre;Ai32 mice, was mediated by not only N- and P/Q-type Ca^{2+} channels but also T-type channel (Fig. 4h, i, Extended Data Fig. 8c).

Next, we questioned whether GABA co-transmission at DA synapses and conventional GABA transmission share similar or distinct quantal properties in the striatum. To analyze and compare the quantal properties of oIPSC between dopaminergic GABA co-transmission and conventional GABA transmission in the dorsal striatum, Ca^{2+} was replaced with Sr^{2+} in the bath-solution to promote asynchronous release (Fig. 4j). Sr^{2+} facilitated asynchronous vesicle release both in DAT-Cre;Ai32 and A2A-Cre;Ai32 mice, while GABA co-transmission at DA synapses was less prone to evoke asynchronous release than conventional GABAergic transmission (Fig. 4j, k). Furthermore, the quantal amplitude of GABA co-transmission at DA synapses was markedly smaller than that of conventional GABA transmission between the SPNs (Fig. 4k, l). To search for the presynaptic molecular mechanisms behind the differential susceptibility to asynchronous release by Sr^{2+} , we checked the individual isoforms of synaptotagmin enriched at DA and GABAergic terminals, respectively (Fig. 4m, n). We found that DA terminals were preferentially co-localized with synaptotagmin 1, followed by synaptotagmin 7 and 5/9 in the dorsal striatum. In addition, the relative expression levels of each synaptotagmin isoform at conventional GABAergic terminals were similar to DA terminals (Fig. 4m-o). However, the co-localization of synaptotagmin 1 that is critical for synchronous vesicular release at various synaptic terminals²⁴⁻²⁶, was considerably higher at DA terminals than at GABAergic terminals (Fig. 4p), possibly accounting for the observed differential susceptibility to asynchronous release by Sr^{2+} at DA and GABAergic terminals.

Weakening of GABA transmission precedes the deficit of DA transmission at DA synapses in an animal model of Parkinsonism

Physiological changes such as altered neuronal firing in DA neurons and the attenuation of DA

transmission have been reported to precede the degeneration of DA neurons in the course of Parkinson's disease^{12,27}. We questioned whether the co-transmission of multiple neurotransmitters at DA synapses is equally susceptible to the progression of Parkinsonism. To unravel the potential deficits of synaptic transmission at DA synapses, we induced the animal model of Parkinsonism by infusing 6-OHDA (6-hydroxydopamine) unilaterally into the medial forebrain bundle (MFB) of DAT-Cre mice (Fig. 5a). We first checked the degeneration of GABAergic-like DA synapses in the dorsal striatum by simultaneously labeling TH, Bassoon, and NL2 (Fig. 5b). We found that the number of GABAergic-like DA synapses in the ipsilateral striatum was largely intact one day after 6-OHDA injection (Fig. 5c). However, when compared with the contralateral striatum, GABAergic-like DA synapses have begun to decrease substantially 3 days after 6-OHDA injection. The number of GABAergic-like DA synapses was significantly reduced 7 days after 6-OHDA injection (Fig. 5c). In addition, the spatial distribution of GABAergic-like DA synapses became sparser as 6-OHDA-induced Parkinsonism progressed (Fig. 5d).

We then examined the temporal alteration of DA transmission 1, 3, and 7 days after 6-OHDA injection. To identify DA synapse-specific physiological changes, we unilaterally injected 6-OHDA into the MFB of DAT-Cre;Ai32 mice (Fig. 5e). As with the number of GABAergic-like DA synapses over time, DA transmission in the ipsilateral striatum was not affected one day after 6-OHDA injection, while the release of DA was dramatically reduced 3 and 7 days after 6-OHDA injection (Fig. 5f, g). Most interestingly, however, GABA transmission at DA synapses in the ipsilateral striatum began to significantly diminish even one day after 6-OHDA injection (Fig. 5h, i, Extended Data Fig. 9a). Like DA transmission, GABA transmission measured 3 and 7 days after 6-OHDA injection was also considerably weakened when compared with the contralateral striatum. In case of glutamate transmission at DA synapses, no attenuation of oEPSC was observed one day after injection at the ipsilateral side of striatum. Since the magnitude of glutamate co-transmission at DA synapses was relatively small when compared with DA or GABA co-transmission, glutamate transmission measured by oEPSC from SPNs was almost abolished 3 and 7 days after 6-OHDA injection (Fig. 5j, k, Extended Data Fig. 9b). These results strongly suggest that although GABA transmission is highly correlated with DA transmission at DA synapses in the normal brain, DA and GABA transmission may change over the course of Parkinsonism with differential temporal susceptibility.

Discussion

Electron microscopy (EM) studies have shown that DA terminals immunoreactive for TH make symmetrical contacts with striatal SPNs^{28,29}. NL2 and Gephyrin, major postsynaptic proteins exclusively found at inhibitory synapses in the brain, were recently reported to exist at postsynaptic sites of striatal DA synapses⁵. In addition, it appears that GABA along with DA is co-transmitted at striatal DA synapses from DA terminals^{7,8} and we previously showed that dopaminergic GABA can be synthesized in midbrain DA neurons via non-canonical GABA synthesis pathway⁸. Here, we for the first time demonstrate that there exist structurally and functionally GABAergic-like DA synapses in the brain, establishing the comprehensive landscape of GABAergic-like DA synapses throughout the brain. Our results first show the strong correlation between DA and GABA transmission at DA synapses in both nigrostriatal and mesocorticolimbic pathways. GABA in DA neurons can be loaded into dopaminergic synaptic vesicles through VMAT2, a vesicular membrane protein for transporting DA from cytosol into synaptic vesicles^{7,30}. As such, it is reasonable to think that DA and GABA can be co-transmitted together from a considerable population of DA synapses (26.30% to 50.96% by Bassoon-NL2 combination) in the brain. Our results are also roughly consistent with the previous observation that about 30% of DA varicosities, which contain Bassoon, can release DA in the striatum⁶. Notably, both DA and GABA transmission at DA synapses exhibit much higher correlation with the number of GABAergic-like DA synapses identified by Bassoon than with that of GABAergic-like DA synapses by VMAT2. These results indicate that Bassoon is a more reliable presynaptic marker for GABAergic-like DA synapses that can co-transmit DA and GABA. However, it bears mentioning that there still remain many portions of dopaminergic presynaptic boutons that are not co-localized with inhibitory postsynaptic markers such as NL2 and Gephyrin. Those DA boutons may (or may not) release DA, glutamate, or both²⁹.

Unlike DA axons immunoreactive for TH and DAT, these GABAergic-like DA synapses exhibit marked regional and spatial heterogeneity in the brain, especially within the striatum. GABAergic-like DA synapses also show region-specific clustering in the striatal subregions. GABAergic-like DA synapses are more dispersed in DLS and NAc core, whereas DMS, NAc shell, and OT seem to exhibit more clustered pattern of these synapses. This spatial heterogeneity is well suited to provide regionally distinct functions of DA synapses throughout the brain.

Importantly, the density of GABAergic-like DA synapses is the highest in the DLS within the striatum, while OT and DMS display a relatively higher degree of clustering of these synapses than DLS. Although the region-specific structure of neural circuits may partly underlie the high degree of clustering of GABAergic-like DA synapses in OT, the relative difference of spatial clustering of GABAergic-like DA synapses between DLS and DMS might have physiological implications in diverse functions mediated by DLS and DMS. Furthermore, GABAergic-like DA synapses in DLS show relatively even distribution on the dendrite of SPN and exist in higher density than conventional GABAergic synapses made by local iSPNs. On the other hand, GABAergic inputs projected from neighboring iSPNs are likely to be more clustered at the distal part of dendrites. Given the distinct physiological features of GABA co-transmission at DA synapses, spatial distribution of GABAergic-like DA synapses on the dendrite might further contribute to functional specificity of these synapses in regulating postsynaptic neurons.

Our data suggest that DA transmission in the brain, if not always, may accompany GABA transmission with high fidelity at GABAergic-like DA synapses. Although DA transmission under certain conditions can also provide spatially and temporally precise coding^{6,31}, the co-transmission of GABA at DA synapses, in conjunction with glutamate, can reliably convey not only fast, but also synapse-specific and more precise signals to postsynaptic neurons. GABA co-transmission can be utilized to mark salient events with high temporal resolution³². On the other hand, depending on the intracellular concentration of Cl⁻ in postsynaptic neurons, GABA co-transmission may also confer on DA synapses the ability to subtly modulate the excitation of subcellular regions of a postsynaptic neuron. Therefore, distinct combination of DA and GABA transmission, and region-specific spatial distribution of GABAergic-like DA synapses may provide critical repertoire to finely tune various types of target neurons for the appropriate movements and reward-related behaviors. In 6-OHDA-lesioned mice, the attenuation of GABA transmission precedes the impairment of DA and glutamate transmission at DA synapses. In addition, GABA transmission begins to alter when GABAergic-like DA synapses are intact by 6-OHDA injection. These results strongly suggest that GABA co-transmission at DA synapses may temporally undergo different pathological processes in the degeneration of DA system and might be more susceptible to Parkinsonism. Considered together, our findings that a significant population of DA synapses are structurally and functionally GABAergic in the brain open

numerous doors to a deeper mechanistic understanding of synaptic actions at DA synapses in health and disease.

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Fig. 1 | DA transmission is strongly correlated with GABA transmission at DA synapses in the brain. **a**, Schematic illustration and fluorescent image of dopaminergic pathways in the brain. **b**, Representative confocal images of TH⁺ and DAT⁺ DA fibers in the brain of DAT-Cre mice. **c-f**, Summary statistics of TH⁺ and DAT⁺ axonal areas in the brain. **g**, Schematic illustration depicting electrophysiological recording of synaptic transmission at DA synapses in the multiple brain regions. **h**, Representative recording traces of DA transmission in the brain, evoked by optogenetic stimulation. **i, j**, Summary statistics of DA transmission in the brain. **k**, Representative recording traces of GABA transmission at DA synapses. **l, m**, Summary statistics of brain-wide GABA transmission at DA synapses. **n**, Representative recording traces of glutamate transmission at DA synapses. **o, p**, Summary statistics of brain-wide glutamate transmission at DA synapses. **q**, Relationships between DA, GABA, and glutamate transmission. **r**, Relationships between DAT⁺ area and neurotransmitters release. Blue bar indicates optogenetic light stimulation. Abbreviations (DLS: dorsolateral striatum, DMS: dorsomedial striatum, NAc c: nucleus accumbens core, NAc sh: nucleus accumbens shell, OT: olfactory tubercle, ACC: anterior cingulate cortex, M1: primary motor cortex, GPe: external globus pallidus, GPi: internal globus pallidus or entopeduncular nucleus, STN: subthalamic nucleus). Statistical significance is determined by one-way ANOVA with post hoc Sidak's multiple comparison test, one-way ANOVA with post hoc Sidak's multiple comparison test (DLS as a control group), and unpaired t-test. Data are presented as box and whisker plot or mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

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Fig. 2 | Spatial distribution and characteristics of GABAergic-like DA synapses in the brain.

a, Enhanced confocal (airyscan) fluorescence images of brain-wide DA terminals and synapses immunostained by TH, VMAT2, and NL2 antibodies (white arrow: triple co-localization). **b-f**, Summary statistics of brain-wide GABAergic-like DA synapses labeled by TH, VMAT2, and NL2. **g**, Enhanced confocal fluorescence images of brain-wide DA terminals and synapses immunostained by TH, Bassoon, and NL2 antibodies. **h-l**, Summary statistics of brain-wide GABAergic-like DA synapses labeled by TH, Bassoon, and NL2. **m-o**, Relationships between GABAergic-like DA synapses and DA presynaptic boutons (or GABAergic postsynaptic sites) in the brain. **p, q**, Relationships between GABAergic-like DA synapses and neurotransmitters release at DA synapses. **r**, Brain-wide cumulative frequency for nearest neighbor distance among GABAergic-like DA synapses. Statistical significance is determined by one-way ANOVA with post hoc Sidak's multiple comparison test (DLS as a control group). Data are presented as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

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Fig. 3 | Spatial clustering of GABAergic-like DA synapses in the striatum. **a**, Representative enhanced confocal images of GABAergic-like DA synapses in the striatum, analyzed by custom MATLAB codes (Bassoon and NL2 fluorescence signals are not shown for clarity). **b**, Cumulative distribution for mean NND of GABAergic-like DA synapses and null model (generated from 1000 simulated images). **c**, Edge-corrected Ripley's H function analysis of GABAergic-like DA synapses and null model (generated from 1000 simulated images, CE: confidence envelope). **d**, Rejection rates of striatal regions from null models. **e**, Edge-corrected Ripley's H function analysis of GABAergic-like DA synapses when DLS is set as a null model. **f**, Rejection rates of striatal regions from DLS (null model). **g**, Edge-corrected Ripley's H function analysis of GABAergic-like DA synapses when OT is set as a null model. **h**, Rejection rates of striatal regions from OT (null model). Kolmogorov-Smirnov test is used to determine statistical differences between two groups in cumulative probability graphs. Rejection rates are evaluated by DCLF test. * $P < 0.05$.

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Fig. 4 | Physiological differences between GABAergic-like DA synapses and conventional GABAergic synapses in the dorsal striatum. **a**, Enhanced confocal images of GABAergic-like DA (or conventional GABAergic) synapses and sparsely labeled SPN dendrites in the dorsal striatum of DAT-Cre (immunostained with TH, Bassoon, NL2, and GFP antibodies) and A2A-Cre (immunostained with RFP, VGAT, NL2, and GFP antibodies) mice, analyzed by custom MATLAB codes (only GFP signal and synapses are shown for clarity). **b**, Summary statistics of synapse density on SPN dendrites. **c**, Cumulative frequency of nearest neighbor distance for GABAergic-like DA synapses (DAT-Cre) or GABAergic synapses (A2A-Cre) in DLS. **d**, Edge-corrected Ripley's H function analysis of GABAergic synapses (A2A-Cre) and GABAergic-like DA synapses (DAT-Cre) (null model, CE: confidence envelope). **e**, Rejection rate of conventional GABAergic synapses from GABAergic-like DA synapses (null model) evaluated by DCLF test. **f**, **g**, Representative recording traces and summary statistics of GABA transmission at GABAergic-like DA synapses, showing the effects of calcium channel blockers on GABA release. **h**, **i**, Representative recording traces and summary statistics of GABA transmission at conventional GABAergic synapses (iSPN to dSPN). **j-l**, Representative recording traces and summary statistics (quantal properties) of optogenetically evoked asynchronous GABA release in DAT-Cre;Ai32 and A2A-Cre;Ai32 mice during the bath-application of strontium. **m**, Enhanced confocal images of TH, Bassoon, and Synaptotagmins (1, 7, 5/9) immunofluorescence from DLS in DAT-Cre mice (white arrow: presynaptic boutons co-localized with Synaptotagmins). **n**, Enhanced confocal images of GFP, VGAT, and Synaptotagmins (1, 7, 5/9) immunofluorescence from DLS in A2A-Cre;Ai32 mice. **o**, **p**, Proportion of DA (or GABAergic) presynaptic boutons colocalized with Synaptotagmin 1, 7, and 5/9 in DAT-Cre and A2A-Cre;Ai32 mice. Blue bar indicates optogenetic light stimulation. Statistical significance is determined by unpaired t-test, one-way ANOVA with post-hoc Sidak's multiple comparison test, one-way ANOVA with post hoc Sidak's multiple comparison test (vehicle as a control group), two-way repeated measures ANOVA with post-hoc Sidak's multiple comparison test, and Kolmogorov-Smirnov test. Data are presented as box and whisker plot or mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

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Fig. 5 | Weakening of GABA co-transmission precedes the deficit of DA transmission at DA synapses in an animal model of Parkinsonism. **a**, Schematic illustration describing the injection of 6-OHDA into MFB (left hemisphere) of DAT-Cre mice. **b**, Enhanced confocal fluorescence images of GABAergic-like DA synapses (immunostained with TH, Bassoon, and NL2 antibodies) from DLS in DAT-Cre mice injected with 6-OHDA (1-, 3-, and 7-day incubation). **c**, Summary statistics for the number of GABAergic-like DA synapses after 6-OHDA lesion. **d**, Cumulative frequency for the nearest neighbor distance of GABAergic-like DA synapses in 6-OHDA-lesioned mice. **e**, Schematics illustrating the injection of 6-OHDA into MFB (left hemisphere) of DAT-Cre;Ai32. **f, g**, Representative recording traces and summary statistics of DA transmission at DA synapses in 6-OHDA-injected mice. **h, i**, Representative recording traces and summary statistics of GABA transmission at DA synapses in 6-OHDA-injected mice. **j, k**, Representative recording traces and summary statistics of glutamate transmission at DA synapses in 6-OHDA-injected mice. Blue bar indicates optogenetic light stimulation. Statistical significance is determined by unpaired t-test and Kolmogorov-Smirnov test. Data are presented as box and whisker plot or mean $\pm$ SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

Methods

Animals

Adult (8 – 16 weeks, male and female) mice were used for this study. To generate dopaminergic neuron-specific ChR2 expressing mice, DAT-Cre (B6.SJL-*Slc6a3*^{tm1.1(*cre*)*Bkmn*}/J, Jackson stock number: 006660) mice were crossed with Ai32 (B6.Cg-*Gt(ROSA)26Sor*^{tm32(CAG-COP4**H134R/EYFP*)*Hze*}/J, Jackson stock number: 024109) mice, producing DAT-Cre;Ai32. These mice were used for optogenetic stimulation of dopaminergic terminals in whole-cell patch clamp recording and fast-scanning cyclic voltammetry (FSCV) experiments. DAT-Cre mice were used in immunohistochemistry experiments. To generate indirect pathway spiny projection neuron (iSPN)-specific ChR2 expressing mice, A2A-Cre (B6.FVB(Cg)-Tg(*Adora2a-cre*)KG139Gsat/Mmucd, MMRRC stock number: 036158-UCD) mice were crossed with Ai32 mice, producing A2A-Cre;Ai32. These mice were used in whole-cell patch clamp recording and immunohistochemistry experiments. A2A-Cre mice were also used for immunohistochemistry experiments. All mice were maintained in C57BL/6J background. The experiment was not blind to the genotypes of mice. Mice were group-housed (up to 5 mice per cage) and bred under standard pathogen-free housing conditions in the animal facility of Ulsan National Institute of Science and Technology (UNIST) on 12-hour light/dark cycle (lights on from 7 AM to 7 PM). Food and water were available ad libitum. All experimental procedures were conducted in accordance with protocols approved by Institutional Animal Care and Utilization Committee of UNIST.

Immunohistochemistry and confocal imaging

Immunohistochemistry experiments were performed similarly as previously described³³. For immunofluorescence staining, mice were anesthetized by intraperitoneal injection of zoletil (60 mg/kg, Virbac Korea) and rompun (15 mg/kg, Bayer Korea) mixture solution (zoletil : rompun : saline = 4 : 1 : 20) and perfused transcardially with phosphate buffer (PB), followed by 4% paraformaldehyde (PFA). Brains were rapidly removed and maintained in 4% PFA at 4°C for overnight. Fixed brains were transferred to 30% sucrose in 0.01 M PB for cryoprotection. Brain sections containing striatum, cortex, and extrastriatal basal ganglia were made using frozen section technique (Microtome SM2010, Leica). Obtained brain sections were washed with PBS, PBST (0.5% Triton X-100) and blocked with PBST containing 10% normal goat serum (NGS) and 2%

bovine serum albumin (BSA). After blocking, brain sections were incubated with anti-tyrosine hydroxylase antibody (rabbit polyclonal, 1:1000, ab112), anti-tyrosine hydroxylase antibody (chicken polyclonal, 1:1000, ab76442), anti-dopamine transporter antibody (rat monoclonal, 1:500, ab5990), anti-vmat2 antibody (rabbit polyclonal, 1:1000, VMAT2-Rb-Af720), anti-gephyrin antibody (mouse monoclonal, 1:500, 147 111), anti-RFP antibody (mouse monoclonal, 1:1000, MA5-15257), anti-GFP antibody (chicken polyclonal, 1:1000, GFP-1010), anti-bassoon antibody (guinea pig polyclonal, 1:500, 147 004), anti-bassoon antibody (mouse monoclonal, 1:500, ADI-VAM-PS003-D), anti-neurologin2 antibody (guinea pig polyclonal, 1:1000, Nlgn2-GP-Af760), anti-vgat antibody (rabbit polyclonal, 1:500, 131 003), anti-synaptotagmin1 antibody (mouse monoclonal, 1:500, 105 011), anti-synaptotagmin7 antibody (rabbit polyclonal, 1:200, 105 173), anti-synaptotagmin5/9 antibody (rabbit polyclonal, 1:500, 105 053) in blocking solution at 4°C overnight, followed by secondary antibodies (goat anti-rabbit, goat anti-rat, goat anti-mouse, goat anti-chicken, goat anti-guinea pig, Invitrogen, 1:1000) conjugated to Alexa 405, Alexa 488, Alexa 594, and Alexa 647 fluorophores in blocking solution at room temperature for 2 hours. After washing with PBST and PBS, brain slices were mounted to slides using mounting medium (P36934 or P36935, Invitrogen).

For quantitative analysis, images were captured by FV1000 confocal laser scanning microscope (Olympus) using a 60x/1.35 NA oil immersion objective (image size: 512 x 512 pixels), LSM780N multi-photon confocal laser scanning microscope with airyscan (Carl Zeiss) using a 63x/1.46 NA oil immersion objective (zoom factor: 3, image size: 912 x 912 pixels), and LSM880 multi-photon confocal laser scanning microscope with airyscan (Carl Zeiss) using a 63x/1.4 NA oil immersion objective (zoom factor: 3, image size: 6972 x 6972 pixels) and a 40x/1.2 NA water immersion objective (zoom factor: 3, image size: 1248 x 1248 pixels). Imaging areas were randomly selected and then images were captured from each target brain region. Acquired images were further analyzed by Zen software (Carl Zeiss), ImageJ program (NIH, measure function), and MATLAB (MathWorks, custom codes). For dendritic synapse clustering analysis, the whole dendrite was divided into two compartments (proximal and distal). The half of the dendritic compartment close to the soma was analyzed as proximal dendrite and the other half of the dendrite away from the soma was considered as distal dendrite.

Brain slice preparation for electrophysiology

Acute brain slices were similarly prepared as previously described³³. Coronal brain slices containing striatal regions (DLS, DMS, NAc core, NAc shell, OT), cortex (ACC, M1), and extrastriatal basal ganglia (GPe, GPi, STN) were obtained (300 μ m thick) for whole-cell patch clamp recording and FSCV. Mice were anesthetized with isoflurane (Piramal Critical Care), decapitated, and the brain was taken out, briefly exposed to ice-chilled artificial cerebrospinal fluid (ACSF) containing 125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 25 mM NaHCO₃, 1 mM MgCl₂, 2mM CaCl₂ and 15 mM glucose oxygenated with 95% O₂ and 5% CO₂. Acute brain slices were prepared using a tissue vibratome (Leica, VT 1200S) in ice-cold ACSF. Brain slices were first maintained in ACSF for 30 min at 34°C and then another 30 min at room temperature. After recovery, slices were transferred to a submerged recording chamber perfused with ACSF at a rate of 2 – 3 ml/min at 30 – 31°C. Brain slices were used for electrophysiological recording within 4 hours after recovery.

Electrophysiology, optogenetic stimulation, and pharmacology

Electrophysiological recording was performed similarly as previously described³³. Neurons in striatal regions, cortex, and extrastriatal basal ganglia were visually identified by conventional IR-DIC optics (BX51WI, Olympus). Whole-cell voltage clamp recordings were made with borosilicate glass pipettes (2.5 – 3.5 M Ω) filled with Cs⁺ based low Cl⁻ internal solution containing 135 mM CsMeSO₃, 10 mM HEPES, 1 mM EGTA, 3.3 mM QX-314, 0.1 mM CaCl₂, 4 mM Mg-ATP, 0.3 mM Na₃-GTP, 8 mM Na₂-phosphocreatine (290 – 300 mOsm, pH 7.3 with CsOH). To measure inhibitory and excitatory synaptic currents from the same neuron, membrane potential was first held at +0 mV (reversal potential of ionotropic glutamate receptors) to measure inhibitory synaptic currents and then continuously held at -70 mV (reversal potential of chloride) to measure excitatory synaptic currents under the voltage clamp mode (liquid junction potential corrected). Access resistance was 10 – 20 M Ω and only cells with a change in access resistance < 20% were included in the analysis. Whole-cell patch clamp recordings were performed using Multiclamp 700B (Molecular Devices) and signals were filtered at 2 kHz and digitized at 10 kHz (NI PCIe-6259, National Instruments). Recording data were monitored and acquired by WinWCP (Strathclyde software, http://spider.science.strath.ac.uk/sipbs/software_ses.htm), further analyzed

offline using Clampfit 10.7 software (Molecular Devices) and OriginPro 2017 (OriginLab).

To stimulate ChR2-expressing dopaminergic or intra-striatal collateral axons, blue laser light (450 nm, 1 msec pulses with 60 sec intervals, 50% of saturation power under the objective less than 10 mW) from diode laser (MDL-III-450, Opto Engine LLC) was focused on the back focal plane of the objective to generate wide-field illumination. GABA and glutamate co-transmission from DA axon terminals were recorded from striatal regions (DLS, DMS, NAc core, NAc shell, OT), cortex (ACC, M1), and extrastriatal basal ganglia (GPe, GPi, STN) by optogenetically activating DA terminals. ChR2 stimulation of DA terminals induced fast, monosynaptic, optogenetically-evoked excitatory postsynaptic currents (oEPSCs) and inhibitory postsynaptic currents (oIPSCs) in all neurons. To test the physiological effects of acute inhibition of calcium channels on GABAergic transmission from DA and GABAergic neurons, we used selective T-type calcium channel blocker (NNC 55-0396, 1 μ M, alomone labs, N-205), N-type calcium channel blocker (ω -conotoxin, 100 nM, alomone labs, C-300), P/Q-type calcium channel blocker (ω -agatoxin, 200 nM, alomone labs, STA-500) and these blockers were bath-applied during the recording session. For the experiment measuring strontium-induced asynchronous GABA release from DA and GABAergic terminals, another ACSF which contained strontium chloride hexahydrate (4 mM, Sigma-Aldrich, Cat. No. 255521) in place of calcium was made and after recording oIPSC with normal ACSF, this calcium-containing ACSF was replaced by strontium-containing ACSF. Asynchronous GABA release at DA and GABAergic (iSPN to dSPN) synapses were recorded from SPNs in DLS by optogenetically activating DA and GABAergic axon terminals. The amplitude and frequency of strontium-induced asynchronous IPSC were analyzed by using Mini Analysis software (Synaptosoft).

Fast-scanning cyclic voltammetry (FSCV)

FSCV was performed similarly as previously described³³. Extracellular DA release was recorded by FSCV using carbon-fiber microelectrodes (7 μ m diameter carbon fiber sealed by glass, 50 – 100 μ m exposed tip length beyond the tapered glass seal) from striatal regions (DLS, DMS, NAc core, NAc shell, OT), cortex (ACC, M1), and extrastriatal basal ganglia (GPe, GPi, STN). To detect DA release, a triangular scanning waveform was applied to carbon fiber electrode from -0.4 V (vs. AgCl reference electrode) to +1.3 V and back (8.5 msec waveform width) at a scan rate of 400

V/sec (repeated at 10 Hz). Carbon fiber electrode was held at -0.4 V between scans. Cyclic voltammograms were background-subtracted by averaging 10 background scans. To evoke DA release from DA synapses, ChR2-expressing DA axon terminals were stimulated by blue laser light (450 nm, 2 ms pulse width). Oxidation current by optogenetically evoked DA release was detected and monitored using TarHeel CV system. Changes in dopamine concentration were quantified by plotting the peak oxidation current of the voltammogram over time. Carbon fiber electrode was calibrated at the end of each experiment to convert oxidation current to DA concentration using DA (10 μ M, Tocris, Cat. No. 3548) in ACSF.

Virus production

AAV-hSyn-GFP (AV5075E, UNC) and AAV-hSyn-DIO-mCherry (50459-AAV5, Addgene) were purchased from UNC vector core and Addgene, respectively. The production titer was 5.6×10^{12} virus molecules/ml for AAV-hSyn-GFP and $> 7 \times 10^{12}$ viral genomes/ml for AAV-hSyn-DIO-mCherry.

Stereotaxic viral injection for sparse labeling

Stereotaxic injection was performed similarly as previously described³³. Stereotaxic virus injection was conducted on over 8 – 10 weeks old male and female mice (DAT-Cre, A2A-Cre) and performed using a stereotaxic system (51730, Stoelting). Before surgery, mice were deeply anesthetized by intraperitoneal injection of zoletil (60 mg/kg, Virbac Korea) and rompun (15 mg/kg, Bayer Korea) mixture solution (zoletil : rompun : saline = 4 : 1 : 20). A total volume of 300 nl virus solution (1:50 dilution of AAV-hSyn-GFP for DAT-Cre, 1:50 dilution of AAV-hSyn-GFP mixed with 1:2 dilution of AAV-hSyn-DIO-mCherry for A2A-Cre) was injected into DLS (coordinates used, AP: +1.3 mm, ML: ± 2.25 mm from bregma, DV: -2.2 mm from exposed dura mater) to achieve sparse labeling of neurons. A glass micropipette with a long narrow tip (size: 10 – 20 μ m) was made using a micropipette puller (P-1000, Sutter Instrument) to deliver the virus. The glass pipette was slowly reached to target area and left for 5 min before virus injection. Virus solution (300 nl) was injected at an infusion rate of 100 nl/min and withdrawn 10 min after the end of injection. After injection, the scalp was sutured and mice were returned to their home cages. Virus-injected mice were used for immunohistochemistry experiment 3 weeks after injection.

Stereotaxic 6-OHDA injection

Stereotaxic 6-OHDA injection was conducted on over 8 – 14 weeks old male and female mice (DAT-Cre, DAT-Cre; Ai32) and performed using a stereotaxic system (51730, Stoelting). Before surgery, mice were deeply anesthetized by intraperitoneal injection of zoletil (60 mg/kg, Virbac Korea) and rompun (15 mg/kg, Bayer Korea) mixture solution (zoletil : rompun : saline = 4 : 1 : 20). A total volume of 300 nl 6-OHDA solution was injected unilaterally into left MFB (coordinates used, AP: -1.2 mm, ML: +1.2 mm from bregma, DV: -4.75 mm from exposed dura mater). A glass micropipette with a long narrow tip (size: 10 – 20 μ m) was made using a micropipette puller (P-1000, Sutter Instrument) to deliver 6-OHDA. The glass pipette was slowly reached to target area and left for 5 min before 6-OHDA injection. 6-OHDA solution (300 nl) was injected at an infusion rate of 100 nl/min and withdrawn 10 min after the end of injection. After injection, the scalp was sutured and mice were returned to their home cages. 6-OHDA-injected mice were used for experiment 1, 3, 7 days after injection.

Spot detection and synapse extraction analysis

To extract synapses from the acquired image, we implemented a general method for spot detection³⁴⁻³⁶. The method exploits mathematical morphological processing based on set theory. By considering objects in the acquired images as sets, signal enhancement and spot detection can be accomplished exploiting combinations of logical operators in set theory. This method is divided into three parts: denoising, signal enhancement, and spot extraction. At the denoising step, the acquired images are processed by Gaussian filter with $\sigma = 0.5$. For signal enhancement, three combined thresholding algorithms with top-hat transform, hard thresholding, and Otsu thresholding are applied to the resulting images. Top-hat transform of an image $I(m)$ is represented as:

$$T(I(m)) = I(m) - I(m) \circ s(m)$$

where $\circ$ denotes the opening operator in morphological processing and $s(m)$ denotes the structuring element. Our structuring element $s(m)$ for applying top-hat transform is circular shape with size of 10 X 10. Hard thresholding is expressed as:

$$H(I(m)) = \begin{cases} I(m) & \text{if } I(m) > \text{threshold} \\ 0 & \text{if } I(m) < \text{threshold} \end{cases}$$

Threshold value of hard thresholding is 40000. After applying top-hat transform and hard thresholding to the acquired images, Otsu's thresholding method is exploited to the processed images to enhance fluorescent signal from the actual object and suppress background signal. From the signal-enhanced images, spots are extracted using combined morphological filters, consisting of two morphological operations: filling and opening. Filling is employed to fill out holes in fluorescent spots, and opening is performed to smooth contour of spots and remove irrelevant fluorescent signal to synaptic structure. The extracted spots are clustered within the set of images (presynaptic and postsynaptic) corresponding to distance among the spots, which allows the images to delineate presynaptic and postsynaptic structure. By calculating the distance between presynaptic and postsynaptic clusters, the clusters with less than 2 pixels in distance (80 nm) were combined to constitute synapses.

Point pattern analysis

The spatial analysis of GABAergic-like DA synapses was performed using Ripley's analysis^{37,38} on both single group and group vs. group scale. Ripley's function describes spatial characteristics of the sample (clustering or dispersion) over a range of distances. The edge corrected Ripley's function is defined as:

$$K(r) = \lambda^{-1} \sum_{i \neq j} k_{i,j} I(d_{i,j} \leq r) / N$$

where λ is the density of a point pattern; $k_{i,j}$ is the inverse of a portion of circumference with center at point i and radius r , that lies within the boundary and passes through point j ; function I is an indicator function; d is the Euclidean distance between points i and j ; N is the number of points in the pattern. A variance stabilized version of the Ripley's K function³⁹ was used in this study:

$$H_r = \sqrt{\frac{K(r)}{\pi} - r}$$

Ripley's function was computed for each observed point pattern. Group vs. group analysis was also performed to establish whether point patterns from one group shared similar spatial characteristics with patterns from another (null) group. We assume that all m (the total number of generated patterns) patterns in the null group are generated by the same point process.

Diggle-Cressie-Loosmore-Ford (DCLF) test

Diggle-Cressie-Loosmore-Ford (DCLF) test is based on the quantification of the difference between the H function of the observed pattern and the H function for the null group at the given spatial scale. We computed $H(r)$ for the test pattern $H_{obs}(r)$ and each pattern in the null group $H_i(r)$, then we estimated the summary function $\hat{H}(r)$ for the null group as follows:

$$\hat{H}(r) = \frac{1}{m+1} (H_1(r) + H_2(r) + \dots + H_m(r) + H_{obs}(r))$$

Then, the maximum vertical separation between $H(r)$ and $\hat{H}(r)$ is:

$$T_{obs} = \int_0^R (H_{obs}(r) - \hat{H}(r))^2 dr$$

where R is maximum value of the interaction distance. To compute p-value, the following formula can be used: $p = \frac{\sum_1^m I(T_{obs} > T_i)}{m+1}$, where $I(T_{obs} > T_i) = \begin{cases} 1, & T_{obs} > T_i \\ 0, & otherwise \end{cases}$ and T_i is computed between the observed pattern and each pattern in the test group ($i = \overline{1, m}$).

Statistics

All data were analyzed using Clampfit 10.7 (Molecular Devices), Mini Analysis (version 6.0.7, Synaptosoft), OriginPro 2017 (OriginLab), Zen software (version 2.5, Carl Zeiss), ImageJ (version 1.53c, NIH), and MATLAB (version R2017a, MathWorks). Statistical analysis was performed using GraphPad Prism software (version 7, GraphPad software). Summary statistics were all shown with box and whisker plot or mean $\pm$ SEM. Unpaired and paired student t-tests, one-way ANOVA with post-hoc Sidak's multiple comparison test, two-way repeated measures ANOVA with post-hoc Sidak's multiple comparison test, linear regression, Kolmogorov-Smirnov test were used to determine statistical differences (two-sided) between the groups. $P < 0.05$ was considered as statistically significant. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. Measured values and the details of statistical analysis were presented in the supplementary table.

Data availability

All data generated and analyzed in the current study are available from the corresponding author upon reasonable request.

Code availability

Custom code used for performing synapse extraction and analyzing spatial distributions of synapses is available from the corresponding author upon reasonable request.

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922 J.-H.B., J.-H.P., and J.-I.K.; Investigation, H.-J.K., M.R., J.L., B.E.L., Y.L., and J.-I.K.;
923 Interpretation of Data; H.-J.K., M.R., J.-H.B., and J.-I.K.; Writing - Original Draft, H.-J.K. and J.-
924 I.K.; Writing - Review and Editing, H.-J.K., M.R., J.-H.B., and J.-I.K.; Funding Acquisition, K.M.,
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926

927 **Competing interest**

928 The authors report no competing interests.

Figures

Figure 1

DA transmission is strongly correlated with GABA transmission at DA synapses in the brain. a, Schematic illustration and fluorescent image of dopaminergic pathways in the brain. b, Representative confocal images of TH+ and DAT+ DA fibers in the brain of DAT-Cre mice. c-f, Summary statistics of TH+ and

DAT+ axonal areas in the brain. g, Schematic illustration depicting electrophysiological recording of synaptic transmission at DA synapses in the multiple brain regions. h, Representative recording traces of DA transmission in the brain, evoked by optogenetic stimulation. i, j, Summary statistics of DA transmission in the brain. k, Representative recording traces of GABA transmission at DA synapses. l, m, Summary statistics of brain-wide GABA transmission at DA synapses. n, Representative recording traces of glutamate transmission at DA synapses. o, p, Summary statistics of brain-wide glutamate transmission at DA synapses. q, Relationships between DA, GABA, and glutamate transmission. r, Relationships between DAT+ area and neurotransmitters release. Blue bar indicates optogenetic light stimulation. Abbreviations (DLS: dorsolateral striatum, DMS: dorsomedial striatum, NAc c: nucleus accumbens core, NAc sh: nucleus accumbens shell, OT: olfactory tubercle, ACC: anterior cingulate cortex, M1: primary motor cortex, GPe: external globus pallidus, GPi: internal globus pallidus or entopeduncular nucleus, STN: subthalamic nucleus). Statistical significance is determined by one-way ANOVA with post hoc Sidak's multiple comparison test, one-way ANOVA with post hoc Sidak's multiple comparison test (DLS as a control group), and unpaired t-test. Data are presented as box and whisker plot or mean $\pm$ SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure 2

Spatial distribution and characteristics of GABAergic-like DA synapses in the brain. a, Enhanced confocal (airyscan) fluorescence images of brain-wide DA terminals and synapses immunostained by TH, VMAT2, and NL2 antibodies (white arrow: triple co-localization). b-f, Summary statistics of brain-wide GABAergic-like DA synapses labeled by TH, VMAT2, and NL2. g, Enhanced confocal fluorescence images of brain-wide DA terminals and synapses immunostained by TH, Bassoon, and NL2 antibodies. h-l, Summary

statistics of brain-wide GABAergic-like DA synapses labeled by TH, Bassoon, and NL2. m-o, Relationships between GABAergic-like DA synapses and DA presynaptic boutons (or GABAergic postsynaptic sites) in the brain. p, q, Relationships between GABAergic-like DA synapses and neurotransmitters release at DA synapses. r, Brain-wide cumulative frequency for nearest neighbor distance among GABAergic-like DA synapses. Statistical significance is determined by one-way ANOVA with post hoc Sidak's multiple comparison test (DLS as a control group). Data are presented as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Figure 3

Spatial clustering of GABAergic-like DA synapses in the striatum. a, Representative enhanced confocal images of GABAergic-like DA synapses in the striatum, analyzed by custom MATLAB codes (Bassoon

and NL2 fluorescence signals are not shown for clarity). b, Cumulative distribution for mean NND of GABAergic-like DA synapses and null model (generated from 1000 simulated images). c, Edge-corrected Ripley's H function analysis of GABAergic-like DA synapses and null model (generated from 1000 simulated images, CE: confidence envelope). d, Rejection rates of striatal regions from null models. e, Edge-corrected Ripley's H function analysis of GABAergic-like DA synapses when DLS is set as a null model. f, Rejection rates of striatal regions from DLS (null model). g, Edge-corrected Ripley's H function analysis of GABAergic-like DA synapses when OT is set as a null model. h, Rejection rates of striatal regions from OT (null model). Kolmogorov-Smirnov test is used to determine statistical differences between two groups in cumulative probability graphs. Rejection rates are evaluated by DCLF test. *P < 0.05.

Figure 4

Physiological differences between GABAergic-like DA synapses and conventional GABAergic synapses in the dorsal striatum. **a**, Enhanced confocal images of GABAergic-like DA (or conventional GABAergic) synapses and sparsely labeled SPN dendrites in the dorsal striatum of DAT-Cre (immunostained with TH, Bassoon, NL2, and GFP antibodies) and A2A-Cre (immunostained with RFP, VGAT, NL2, and GFP antibodies) mice, analyzed by custom MATLAB codes (only GFP signal and synapses are shown for clarity). **b**, Summary statistics of synapse density on SPN dendrites. **c**, Cumulative frequency of nearest

neighbor distance for GABAergic-like DA synapses (DAT-Cre) or GABAergic synapses (A2A-Cre) in DLS. d, Edge-corrected Ripley's H function analysis of GABAergic synapses (A2A-Cre) and GABAergic-like DA synapses (DAT-Cre) (null model, CE: confidence envelope). e, Rejection rate of conventional GABAergic synapses from GABAergic-like DA synapses (null model) evaluated by DCLF test. f, g, Representative recording traces and summary statistics of GABA transmission at GABAergic-like DA synapses, showing the effects of calcium channel blockers on GABA release. h, i, Representative recording traces and summary statistics of GABA transmission at conventional GABAergic synapses (iSPN to dSPN). j-l, Representative recording traces and summary statistics (quantal properties) of optogenetically evoked asynchronous GABA release in DAT-Cre;Ai32 and A2A-Cre;Ai32 mice during the bath-application of strontium. m, Enhanced confocal images of TH, Bassoon, and Synaptotagmins (1, 7, 5/9) immunofluorescence from DLS in DAT-Cre mice (white arrow: presynaptic boutons co-localized with Synaptotagmins). n, Enhanced confocal images of GFP, VGAT, and Synaptotagmins (1, 7, 5/9) immunofluorescence from DLS in A2A-Cre;Ai32 mice. o, p, Proportion of DA (or GABAergic) presynaptic boutons colocalized with Synaptotagmin 1, 7, and 5/9 in DAT-Cre and A2A-Cre;Ai32 mice. Blue bar indicates optogenetic light stimulation. Statistical significance is determined by unpaired t-test, one-way ANOVA with post-hoc Sidak's multiple comparison test, one-way ANOVA with post hoc Sidak's multiple comparison test (vehicle as a control group), two-way repeated measures ANOVA with post-hoc Sidak's multiple comparison test, and Kolmogorov-Smirnov test. Data are presented as box and whisker plot or mean $\pm$ SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure 5

Weakening of GABA co-transmission precedes the deficit of DA transmission at DA synapses in an animal model of Parkinsonism. **a**, Schematic illustration describing the injection of 6-OHDA into MFB (left hemisphere) of DAT-Cre mice. **b**, Enhanced confocal fluorescence images of GABAergic-like DA synapses (immunostained with TH, Bassoon, and NL2 antibodies) from DLS in DAT-Cre mice injected with 6-OHDA (1-, 3-, and 7-day incubation). **c**, Summary statistics for the number of GABAergic-like DA synapses after 6-OHDA lesion. **d**, Cumulative frequency for the nearest neighbor distance of GABAergic-like DA synapses in 6-OHDA-lesioned mice. **e**, Schematic illustrating the injection of 6-OHDA into MFB (left hemisphere) of DAT-Cre;Ai32. **f**, **g**, Representative recording traces and summary statistics of DA transmission at DA

synapses in 6-OHDA-injected mice. h, i, Representative recording traces and summary statistics of GABA transmission at DA synapses in 6-OHDA-injected mice. j, k, Representative recording traces and summary statistics of glutamate transmission at DA synapses in 6-OHDA-injected mice. Blue bar indicates optogenetic light stimulation. Statistical significance is determined by unpaired t-test and Kolmogorov-Smirnov test. Data are presented as box and whisker plot or mean $\pm$ SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Supplementary Files

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