TITLE

Mice lacking PDZD8 exhibit lipid accumulation in the SNr and dysregulation of basal ganglia circuits.

Running title

Dysregulation of basal ganglia circuits due to SNr lipid accumulation

Authors

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Keywords: Brain, Basal ganglia, Dopamine, SNr, Lipid, PDZD8, ADHD

ABSTRACT

The basal ganglia integrate cortical inputs to regulate motor, cognitive, and emotional behaviors through precisely balanced inhibitory and excitatory circuits. The substantia nigra pars reticulata (SNr) serves as a major output nucleus exerting tonic inhibition over thalamic and midbrain targets. However, how lipid metabolic disturbances affect SNr circuitry and dopaminergic regulation remains unclear. Here, we identify a crucial role for the lipid transport protein PDZD8 in maintaining basal ganglia circuit integrity. PDZD8 deficiency leads to marked lipid and lipofuscin accumulation selectively in the SNr, accompanied by enhanced striatal inhibitory inputs and reduced SNr projections to thalamus and midbrain dopaminergic nuclei. This circuit reorganization results in functional disinhibition and hyperactivation of dopaminergic neurons, producing maladaptive reinforcement of striatal inhibition. Our previous findings revealed that PDZD8$^{-/-}$ mice exhibit hyperactivity, reduced anxiety, and impaired fear memory, behavioral phenotypes reminiscent of attention-deficit/hyperactivity disorder (ADHD). These findings demonstrate that lipid accumulation in the SNr disrupts inhibitory output and dopaminergic regulation, forming a maladaptive basal ganglia–thalamocortical loop. Our study provides the first mechanistic link between lipid metabolic dysfunction, dopaminergic disinhibition, and ADHD-like behavioral phenotypes, highlighting PDZD8 as a key regulator of metabolic–circuit coupling in the basal ganglia.

INTRODUCTION

Basal ganglia and striatonigral pathway

The basal ganglia constitute a key component of the volitional motor system and contribute to essential aspects of cognition, sensorimotor integration, emotion, and learning. Dysfunction of basal ganglia circuits is implicated in a wide range of neurological and neuropsychiatric disorders(Park et al., 2020; Foster et al., 2021; McElvain et al., 2021). Current models describe three partially segregated channels of information flow through the basal ganglia—associative, limbic, and sensorimotor—which are further refined into multiple parallel subnetworks(Alexander et al., 1986; Grillner and Robertson, 2016). The cortico–basal ganglia–thalamic network (the basal ganglia–thalamocortical circuitry) represents one of the fundamental network motifs of the brain, consisting of precisely organized and reciprocally connected nodes(Foster et al., 2021; McElvain et al., 2021). Within this loop, the striatum, pallidum, and substantia nigra integrate widespread cortical inputs. The striatonigral (direct) pathway exhibits greater convergence than the striatopallidal (indirect) pathway, yet both ultimately converge onto the same output neurons within the substantia nigra pars reticulata (SNr), emphasizing the integrative nature of SNr-mediated control (Foster et al., 2021).

SNr as a major output nucleus of basal ganglia

SNr is the principal output nucleus of the basal ganglia, composed predominantly of inhibitory GABAergic neurons that project to specific thalamic nuclei and brainstem regions(Foster et al., 2021; McElvain et al., 2021; Falasconi et al., 2025). SNr neurons exhibit high tonic firing rates that maintain downstream targets under constant inhibition, whereas transient decreases in firing rate permit movement initiation via disinhibition. Recent studies have revealed that SNr neurons display multiphasic, bidirectional firing dynamics—both decreases and increases—allowing temporally precise modulation of downstream circuits during sequential motor acts(Falasconi et al., 2025). Notably, the caudal and lateral subregions of the SNr, which receive inputs from defined striatal territories, exhibit highly organized topographic projections(Lee et al., 2020; McElvain et al., 2021). These findings suggest that SNr subcircuits play specialized roles in coordinating movement and behavioral control.

SNr dysfunction and neuropsychiatric disorders

Attention-deficit/hyperactivity disorder (ADHD) is characterized by inattention, hyperactivity, and impulsivity, and is linked to altered dopaminergic and noradrenergic signaling(Biederman, 2005; Thapar and Cooper, 2016). Reduced cortical and subcortical gamma-amino butyric acid (GABA) levels have also been reported in ADHD. Among inhibitory neurons, parvalbumin-positive (PV^+) neurons play a key role in attentional

processes and motor control, and their dysfunction has been implicated in multiple psychiatric conditions, including schizophrenia, autism spectrum disorder, and ADHD(Lewis et al., 2005; Sohal et al., 2009). PV^+ neurons in the SNr provide critical feedforward inhibition and disinhibition within basal ganglia circuits. Recent work demonstrated that loss of histamine H_2 receptor (H_2R) expression in SNr PV^+ neurons leads to diminished neuronal activity and behavioral phenotypes resembling ADHD—hyperactivity, impulsivity, and inattention(Kim et al., 2016; Rizzi and Tan, 2019; An et al., 2025). Notably, this dysfunction in SNr PV^+ neurons, but not in medial prefrontal PV^+ neurons, was associated with elevated striatal dopamine levels, implicating the SNr as a key node in the dopaminergic dysregulation underlying ADHD(An et al., 2025).

Lipid metabolism and neuronal function

Lipid accumulation has long been associated with neurodegenerative conditions such as Alzheimer's disease (AD)(Dahm, 2006; Foley, 2010). The discovery of apolipoprotein E4 (ApoE4) as the strongest genetic risk factor for AD highlighted the importance of lipid metabolism in brain health(Lambert et al., 2013). Abnormal accumulation of cholesteryl esters (CEs)—neutral lipids involved in cholesterol transport—has been observed in brains of AD patients and AD model animals(van der Kant et al., 2019; Nugent et al., 2020; Blanchard et al., 2022; Litvinchuk et al., 2024). ApoE4-containing lipoproteins tend to aggregate within lysosomes, impairing degradation and promoting lipid peroxidation and inflammation. In neurons and glia carrying ApoE4, CEs accumulate in lysosomes and transform into lipofuscin, an autofluorescent granule composed of peroxidized lipids and damaged proteins that is highly resistant to degradation(Guo et al., 2025). Excessive lipofuscin accumulation leads to lysosomal stress and chronic neuroinflammation, processes increasingly recognized as drivers of neurodegeneration(Terman and Brunk, 2004; Gray and Woulfe, 2005; Moreno-Garcia et al., 2018).

PDZD8 and lipid homeostasis in the brain

We previously identified PDZD8 as a tethering protein at intracellular membrane contact sites (MCSs) between the endoplasmic reticulum (ER) and endosomes through proteomic analysis of the protrudin complex(Shirane and Nakayama, 2006; Shirane et al., 2020). PDZD8 belongs to the tubular lipid-binding (TULIP) superfamily and contains a synaptotagmin-like mitochondrial-lipid-binding (SMP) domain that mediates lipid transfer. We demonstrated that PDZD8 facilitates cholesterol transport(Shirane et al., 2020), and that PDZD8-deficient ($PDZD8^{-/-}$) mice exhibit brain-specific accumulation of CEs due to impaired lipophagy—a form of microautophagy in which lipid droplets directly fuse with lysosomes for degradation by lysosomal lipase(Morita et al., 2022). Behavioral battery analysis revealed that $PDZD8^{-/-}$ mice display pronounced

hyperactivity, reduced anxiety and fear, and deficits in fear-conditioned memory and working memory(Kurihara et al., 2023), collectively resembling ADHD-like phenotypes. Moreover, multiple PDZD8 mutations has been linked to human intellectual developmental disorder with autism and dysmorphic facies (IDDADF), a syndromic intellectual disability (ID) accompanied by regional brain hypertrophy and atrophy(Al-Amri et al., 2022; Pantiru et al., 2025).

Present study

Despite these findings, the specific brain regions and neural circuits affected by PDZD8 deficiency remained unclear. Here, we demonstrate that PDZD8$^{-/-}$ mice exhibit marked lipid accumulation in the SNr, a major output nucleus of the basal ganglia. This lipid deposition disrupts the striato–nigro–thalamo–cortical circuitry, leading to disinhibition of dopaminergic neurons and maladaptive reinforcement of striatal inhibitory feedback. These results provide the first evidence that abnormal lipid metabolism in the SNr can remodel basal ganglia circuits, offering new insights into the mechanistic links between lipid dysregulation, dopaminergic imbalance, and ADHD-like behavioral phenotypes.

RESULTS

PDZD8 expression in midbrain neurons and astrocytes

We performed immunostaining using the PDZD8 antibody in PDZD8$^{+/+}$ and PDZD8$^{-/-}$ mice to comprehensively analyze the distribution of PDZD8 expression in the brain. Though the specificity of the PDZD8 antibody has been demonstrated in a previous paper (Morita et al., 2022), we compared PDZD8$^{+/+}$ and PDZD8$^{-/-}$ signals in each region and only considered cells with specific signals as PDZD8-expressing cells (Supplementary figure 1A). The results showed that PDZD8 is expressed in multiple regions throughout the brain, but the intensity of expression varied considerably depending on the region and cell type. Brain regions showing notably high expression are shown in Supplementary figure 1B.

To determine the cellular distribution of PDZD8, we examined its expression in the midbrain of wild-type mice. PDZD8 was abundantly expressed in SNc, VTA, and SNr, regions defined by dopamine transporter (DAT) expression (Figure 1A). In the SNc and VTA, PDZD8 was localized in DAT- and tyrosine hydroxylase (TH)-positive dopaminergic neurons, respectively (Figure 1B, C). In contrast, PDZD8 was expressed in PV-positive GABAergic neurons and glial fibrillary acidic protein (GFAP)-positive astrocytes within the SNr (Figure 1D, E). Though the SNr is a large region with distinct cellular distributions according to rostral, caudal, medial, and lateral areas, we selected regions for high concordance between PDZD8 and each cell marker. These findings

150 indicate that PDZD8 is highly distributed across neuronal and glial populations around
151 the substantia nigra in midbrain, suggesting its potential role in modulating dopaminergic
152 circuitry.

153 **Marked lipid accumulation in SNr in PDZD8$^{-/-}$ mice**

154 Given that PDZD8$^{-/-}$ mice exhibit abnormal CE accumulation in the brain, we next
155 identified the affected brain regions. LipiDye II staining revealed significant lipid
156 accumulation in several areas, including SNr, dorsal raphe (DR), basolateral amygdala
157 (BLA), and central amygdala (CeA), with the SNr showing the most prominent signal
158 (Figure 2A). These results were confirmed by LipiDye II and LipidTOX staining, both of
159 them specifically labels neutral lipids in the hydrophobic core of LDs with a high signal-
160 to-noise ratio (Figure 2B, C). High-resolution Z-stack imaging demonstrated dense
161 intracellular lipid droplets, likely representing lipofuscin, within SNr cells (Figure 2D).
162 Thus, the SNr represents the principal site of lipid deposition in PDZD8$^{-/-}$ mice.

163 **Lipofuscin deposits are enriched in SNr in PDZD8$^{-/-}$ mice**

164 To characterize the lipid deposits in greater detail, serial midbrain sections were stained
165 with LipiDye II and anti-DAT. Large lipid deposits were concentrated in the caudal SNr,
166 while smaller aggregates appeared rostrally (Figure 3A). Ectopic anti-DAT signals were
167 detected in PDZD8$^{-/-}$ SNr, especially in caudal regions, likely overlapping with LipiDye
168 II fluorescence (Figure 3B). Then triple labeling with LipiDye II, DAT, and GFAP
169 confirmed complete overlap of these signals, consistent with autofluorescent lipofuscin
170 (Figure 3C). These results demonstrate that most large lipid deposits in the caudal SNr of
171 PDZD8$^{-/-}$ brain are lipofuscin.

172 **Extracellular and intracellular lipid deposits in SNr in PDZD8$^{-/-}$ mice**

173 Z-stack 3D imaging combined with GFAP staining revealed two types of lipid deposits
174 in the SNr of PDZD8-/- mice: small extracellular deposits, likely lipoproteins, and large
175 intracellular deposits, likely lipofuscin (Figure 4A–C). Extracellular deposits were more
176 abundant medially, while intracellular deposits predominated laterally. 3D
177 reconstructions further distinguished intra- versus extracellular localization
178 (Supplementary movies A, B). Notably, intracellular lipofuscin overlapped with GFAP
179 signals, suggesting autofluorescent properties of astrocytic lipofuscin.

180 **Increased striatal projection to SNr in PDZD8$^{-/-}$ mice**

181 To test whether lipid accumulation alters SNr connectivity, we performed retrograde
182 neuronal tracing by injecting Alexa594-CTb into the SNr of PDZD8$^{+/+}$ and PDZD8$^{-/-}$ mice
183 (Figure 5A, B). In both genotypes, injection sites were equivalent. However, corpus
184 striatal (CP) neurons, including pallidum (GP) neurons, projecting to SNr were markedly
185 increased and spatially expanded in PDZD8$^{-/-}$ mice (Figure 5C–E). In addition, we also

observed the similar results with a different mouse pair using the alternative tracer Alexa488-CTb (Supplementary figure 2A–E). These changes indicate enhanced striatal inputs to the SNr (Figure 5F). PDZD8 expression was also detected in dopamine- and cAMP-regulated phosphoprotein (DARPP-32)-positive medium spiny neurons (MSNs) in the striatum (Figure 5G), and DARPP-32 immunoreactivity was significantly elevated in the SNr of PDZD8$^{-/-}$ mice (Figure 5H). Together, these findings suggest strengthened GABAergic striatal inputs to SNr in PDZD8 deficiency.

Decreased projection from SNr in PDZD8$^{-/-}$ mice.

Given that SNr serves as a major output nucleus of the basal ganglia that regulates both thalamic and midbrain dopaminergic neurons, we examined downstream projections by injection of anterograde neuronal tracer palmitoylated GFP (palGFP) into the SNr of PDZD8$^{+/+}$ and PDZD8$^{-/-}$ mice. Injection sites were comparable between groups (Figure 6A, B). In PDZD8$^{-/-}$ mice, projections from the SNr to the VAL/VM and nst (Figure 6C), as well as to the VTA and SNc (Figure 6D), were markedly reduced compared to controls (Figure 6E), indicating disrupted SNr output (Figure 6F). Although GPi is also a major output nucleus from SNr, palGFP signal in GPi was too faint to determine whether there was a difference between PDZD8$^{+/+}$ and PDZD8$^{-/-}$.

Abnormal dopaminergic circuits in PDZD8$^{-/-}$ mice

Based on these observations, we propose that lipid accumulation in the SNr induces functional disinhibition within the basal ganglia–thalamocortical circuitry (Figure 7). Specifically, lipid deposition in SNr neurons leads to reduced inhibitory output from the SNr to both VAL/VM and the nst, as well as to the VTA and SNc. The consequent disinhibition of thalamic and dopaminergic targets results in hyperactivation of dopaminergic neurons, which in turn enhances inhibitory feedback from striatal GABAergic neurons to SNr, thereby reinforcing the suppression of SNr output. This establishes a maladaptive feedback loop—reduced SNr–thalamic output, dopaminergic disinhibition, and strengthened striatal inhibition—that possibly destabilizes the functional balance of the basal ganglia–thalamocortical circuitry. Such large-scale circuit dysregulation likely underlies the abnormal behavioral phenotypes observed in PDZD8$^{-/-}$ mice, including hyperactivity, reduced anxiety and fear, and impaired fear-memory regulation.

DISCUSSION

Summary of the findings

In this study, we identified a crucial role of the lipid transport protein PDZD8 in maintaining basal ganglia circuit homeostasis. PDZD8 deficiency led to prominent lipid

accumulation in the SNr, accompanied by structural and functional remodeling of basal ganglia output pathways. Neuronal tracing analyses revealed reduced SNr projections to both thalamic (VAL/VM, nst) and midbrain dopaminergic targets (VTA, SNc), together with enhanced inhibitory input from striatal GABAergic neurons. These anatomical changes were consistent with functional disinhibition of dopaminergic circuits, resulting in hyperactivation of dopaminergic neurons and maladaptive reinforcement of striatal inhibition. Given that PDZD8$^{-/-}$ mice exhibit hyperactivity and reduced anxiety and fear at the behavioral level—features reminiscent of ADHD-like phenotypes—these findings establish a direct mechanistic link between lipid metabolic disruption in the SNr, basal ganglia circuit disinhibition, and behavioral dysregulation.

SNr lipid accumulation and circuit disinhibition

The SNr serves as a tonic inhibitory gate in the cortico–basal ganglia–thalamo–cortical loop (basal ganglia–thalamocortical circuit), controlling both motor and cognitive outputs. Our data suggest that lipid accumulation within SNr neurons compromises this inhibitory control, likely by affecting cellular excitability and synaptic output. Lipofuscin-like deposits, enriched in cholesteryl esters and peroxidized lipids, are known to induce lysosomal stress, oxidative imbalance, and altered membrane conductance. These effects may collectively reduce the firing capacity or GABA release efficacy of SNr neurons, thereby reducing inhibitory output to their downstream targets. The observed reduction of SNr projections to VAL/VM and nst implies a loss of inhibitory tone at the thalamic level, which would increase cortical excitability and contribute to hyperactivity. Similarly, decreased inhibition of VTA and SNc dopaminergic neurons produces dopaminergic disinhibition, leading to excessive dopamine release in the striatum. The resulting dopaminergic hyperactivation enhances corticostriatal drive, which strengthens striatal GABAergic feedback to the SNr. This closed-loop maladaptive feedback—SNr hypoactivity, dopaminergic hyperactivation, and enhanced striatal inhibition—creates a self-reinforcing circuit instability. Such loop-level dysregulation likely underlies the behavioral abnormalities observed in PDZD8$^{-/-}$ mice.

Disinhibition-driven distortion of RPE signaling in the VTA–BLA circuit

Beyond basal ganglia output pathways, the disinhibition of dopaminergic neurons observed in PDZD8$^{-/-}$ mice likely extends its impact to broader reward-related networks, particularly the VTA–BLA circuit, which encodes reward prediction error (RPE) signals during associative learning. The VTA conveys phasic dopamine release to the BLA during both appetitive and aversive conditioning, where dopaminergic modulation shapes the associative strength between conditioned and unconditioned stimuli. Disruption of inhibitory control from the SNr to midbrain dopaminergic neurons may bias RPE

computation—amplifying positive prediction signals while diminishing aversive or omission-related responses. Such imbalance could account for the hyperactivity and impaired fear memory observed in PDZD8$^{-/-}$ mice, reflecting aberrant valuation of outcomes and a failure to encode negative prediction errors. In this framework, lipid accumulation–induced disinhibition of the SNr may distort dopaminergic gain control within the VTA–BLA network, leading to maladaptive reinforcement learning and emotional dysregulation. This suggests that PDZD8-dependent lipid homeostasis in the SNr is essential for maintaining the dynamic balance between positive and negative RPE signaling across cortico–amygdalo–dopaminergic loops.

Comparison with prior studies on basal ganglia and ADHD

Our findings extend previous evidence implicating SNr dysfunction in the pathophysiology of ADHD. Earlier studies demonstrated that H_2R deficiency in PV^+ neurons of the SNr reduces inhibitory output and induces hyperactivity and impulsivity. Similarly, reduced GABAergic tone and enhanced dopaminergic transmission have been reported in ADHD patients and model animals. The present work suggests that lipid metabolic stress may represent a previously unrecognized upstream mechanism producing a similar disinhibitory outcome. PDZD8 is an ER–endosome tethering protein required for lipid transport and membrane contact site integrity. Loss of PDZD8 impairs lipophagy, leading to intracellular lipid droplet accumulation and lipofuscin formation. These pathological deposits are not inert: they alter calcium handling, disrupt mitochondrial dynamics, and perturb synaptic vesicle turnover. The convergence of lysosomal and GABAergic dysfunction in SNr neurons likely explains the observed circuit-level changes. Therefore, disinhibition of SNr neurons emerges as a new paradigm linking lipid homeostasis to dopaminergic regulation and behavioral control. However, areas beyond those shown in this study may also be involved in ADHD-like behavior of PDZD8$^{-/-}$. While lipid accumulation in PDZD8$^{-/-}$ is most pronounced in SNr, significant lipid accumulation is also observed in DR, BLA, and CeA (Figure 2A). Therefore, future studies should investigate abnormalities in regions beyond SNr. ADHD is primarily considered a dysfunction of circuits connecting PFC and basal ganglia, which govern attention and executive function, with dopamine and noradrenaline involved in controlling these circuits. Furthermore, amygdala, which regulates emotions, and raphe nuclei, which control impulsivity, are also implicated in ADHD, with serotonin involved in regulation of these circuits. It is necessary to investigate abnormalities in these circuits and neurotransmitters in PDZD8$^{-/-}$ mice.

Regulation of inhibitory circuits by lipid metabolism

Although direct evidence for lipid deposition within neurons in PDZD8$^{-/-}$ has not been

294 obtained, the morphology and size of lipid-accumulating cells in PDZD8$^{-/-}$ mice suggest
295 they must be neurons. For example, in Figure 4C (LipiDyeII+GFAP), the two central
296 white cells exhibit the characteristic pyramidal shape of neurons, with a cell body
297 diameter exceeding 10 μm, significantly larger than the size of astrocyte cell bodies,
298 which have a diameter of 5 μm or less. Similarly, in Figure 2D (LipiDyeII+DIC), lipid-
299 accumulating cells in PDZD8$^{-/-}$ also exhibit the morphology and size of pyramidal cells
300 thought to be neurons. Lipid homeostasis is increasingly recognized as a determinant of
301 neuronal function. Our findings highlight that inhibitory GABAergic circuits are
302 particularly sensitive to metabolic stress. The SNr, characterized by high tonic firing rates,
303 may be especially vulnerable to disruptions in lipid trafficking and degradation.
304 Accumulation of peroxidized lipids and lipofuscin can impair GABA release machinery
305 and ion channel stability, thereby compromising tonic inhibition. Astrocytic PDZD8
306 expression observed in our study suggests that glia–neuron lipid coupling may be
307 essential for maintaining SNr function. Disruption of such glial lipid buffering could
308 amplify local oxidative stress and propagate dysfunction across the basal ganglia network.

309 **Implications for neuropsychiatric and neurodegenerative disorders**

310 The link between lipid accumulation and circuit disinhibition identified here may have
311 broader implications beyond ADHD. Lipofuscin accumulation and lysosomal
312 dysfunction are hallmarks of Alzheimer's disease and aging-related cognitive decline.
313 The present findings suggest that even in the absence of overt neurodegeneration, lipid
314 metabolic perturbations can remodel inhibitory circuits and alter behavioral state. Given
315 the prominent dopaminergic involvement, PDZD8 dysfunction may represent a shared
316 vulnerability factor bridging metabolic stress, dopamine dysregulation, and cognitive-
317 behavioral disorders related to basal ganglia or SNr.

318 **Limitation of the study**

319 Because lipofuscin exhibits strong autofluorescence, it remains unproven whether the
320 lipid-accumulating cells observed in PDZD8$^{-/-}$ mice are neurons. Although
321 immunostaining using wavelength-specific filters to visualize lipofuscin has been
322 reported(Guo et al., 2025), we were unable to establish this method in the present study.
323 While quantitative analysis of the entire SNr region was performed (Figure 2A),
324 quantitative analyses across serial sections (Figure 3) and three-dimensional
325 reconstructions (Figure 4) will be important in future studies.
326 The causal relationship and mechanisms linking lipid accumulation to circuit
327 reorganization in PDZD8$^{-/-}$ mice remain unresolved. However, previous studies have
328 shown that excessive lipofuscin accumulation induces lipid peroxidation–mediated
329 reactive oxygen species production, leading to lysosomal stress and chronic

330 neuroinflammation that promote neurodegeneration(Terman and Brunk, 2004; Gray and
331 Woulfe, 2005; Moreno-Garcia et al., 2018; Guo et al., 2025). Future studies should
332 directly quantify intracellular lipid peroxides and reactive oxygen species to test this
333 hypothesis.
334 Direct measurements of dopamine and its metabolites would provide important insight
335 but are technically challenging and were not feasible in the present study.
336 Although PDZD8 is broadly expressed throughout the brain, lipid accumulation was
337 selectively prominent in the SNr of PDZD8$^{-/-}$ mice. The basis of this regional
338 vulnerability remains unclear and warrants further investigation, including potential
339 contributions of PDZD8-interacting complex components. Future studies combining in
340 vivo dopamine imaging, electrophysiological recordings of SNr neurons, and cell-type–
341 specific PDZD8 rescue approaches will be required to define the relative contributions of
342 neuronal and glial populations. In addition, pharmacological or genetic restoration of lipid
343 homeostasis may help determine whether normalization of lipid metabolism can rescue
344 basal ganglia circuit stability and associated behavioral deficits.
345 **Conclusion**
346 Together, our findings reveal that PDZD8-dependent lipid metabolism is indispensable
347 for preserving inhibitory control within the basal ganglia and ensuring accurate
348 dopaminergic prediction error signaling, providing a unifying mechanistic framework
349 linking metabolic stress, circuit disinhibition, and behavioral dysregulation.
350
351 **METHODS**
352 **Animals**
353 Mice were housed under 12-h light/dark cycle with food and water ad libitum. All
354 mouse experiments were approved by the animal ethics committee of Nagoya City
355 University. All experiments were performed using at least two sets of paired mice
356 (PDZD8$^{+/+}$ and PDZD8$^{-/-}$). Mouse sex and age were matched across experiments, and
357 both mice in each pair were analyzed simultaneously. In all experiments, mice were
358 aged 4–9 months and were either male or female. Figures show representative images
359 from one of the multiple experiments performed. A summary table listing these mouse
360 details is shown in Supplementary table 1.
361 **Mutant mice**
362 Generation of PDZD8-KO mice was described previously(Morita et al., 2022). In brief,
363 Cas9 nickase mRNA, Generic tracrRNA, and target-specific crRNA were mixed and
364 injected into C57BL/6J mouse zygotes. The resultant mutant mice were backcrossed
365 with C57BL/6J mice before experiments. Animals were genotyped by PCR analysis.

366 **Antibodies and reagents**
367 Rabbit polyclonal antibodies to PDZD8 were from Sigma-Aldrich; mouse monoclonal
368 antibodies to TH were from Sigma-Aldrich; mouse monoclonal antibodies to GFAP
369 were from Proteintech; guinea pig polyclonal antibodies to DAT, PV, and DARPP were
370 from Frontier institute; Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 555 goat anti-
371 mouse IgG, Alexa Fluor 555 goat anti-guinea pig IgG, LipidTox, and Alexa Fluor 594
372 cholera toxin B were from Thermo Fisher Scientific; LipiDye II was from Funakoshi.
373 **Immunohistofluorescence analysis**
374 Mouse brain was fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS,
375 Wako), exposed consecutively to 15% and 30% sucrose in PBS for 2 days, and
376 embedded in tissue-Tek OCT compound (SAKURA). Thin sections were prepared with
377 a cryostat microtome (CM1850 UV, Leica) and stained consecutively with primary
378 antibodies and Alexa Fluor–labeled secondary antibodies in PBS containing 0.5% Triton
379 X-100, or stained with LipiDye II or LipidTox in PBS. The sections were observed with
380 an LSM800 confocal microscope (Zeiss), and the images were processed for calculation
381 of fluorescence intensity with ZEN imaging software (Zeiss). Brain regions were
382 referenced from Allen Brain Atlas (http://atlas.brain-map.org).
383 **Stereotaxic injection**
384 Mice were anesthetized intraperitoneally with a mixture of midazolam (4 mg kg^{-1},
385 SANDOZ, Tokyo, Japan), medetomidine (0.75 mg kg^{-1}, Kyoritsu Seiyaku, Tokyo,
386 Japan) and butorphanol (5 mg kg^{-1}, Meiji Seika Pharma, Tokyo, Japan) and placed in a
387 stereotaxic frame (NARISHIGE, Tokyo, Japan). Body temperature was maintained at
388 37 °C. The scalp was sterilized and incised, and a small craniotomy was made above the
389 target. Alexa Fluor 594– or 488–conjugated cholera toxin subunit B (CTb, Thermo
390 Fisher Scientific, MA, USA) was prepared at 2 μg μL^{-1} in sterile PBS. AAV2-CMV-
391 palGFP virus particles were from Dr. Nakamura(Yahiro et al., 2023). 200 nl of Alexa
392 Fluor 594-CTb was injected into the SNr (coordinates from Bregma: AP: -3.2 mm, ML:
393 ±1.4 mm, DV: -4.4 mm). 150 nl of Alexa Fluor 488-CTb was injected into the SNr
394 (coordinates from Bregma: AP: -3.7 mm, ML: ±1.8 mm, DV: -4.5 mm). 100 nl of AAV
395 solution was injected into the SNr (coordinates from Bregma: AP: -3.7 mm, ML: ±1.8
396 mm, DV: -4.5 mm). Tracer (200 nL per site) was delivered using a Hamilton
397 microsyringe (1701RN Neuros Syringe, 33G; Hamilton Company, NV, USA) connected
398 to a motorized stereotaxic microinjector (NARISHIGE, Tokyo, Japan ; 100 nL min^{-1}).
399 After infusion, the needle was left in place for 10–15 min to minimize reflux before
400 being withdrawn slowly. For each mouse, injections were made at the following
401 stereotaxic coordinates relative to bregma: [AP -3.2], [ML ±1.4], [DV -4.4] (mm). The

402 scalp was closed with sutures, and mice were monitored until full recovery. After
403 surgery, mice received postoperative care including an intraperitoneal injection of
404 antibiotic (amoxicillin, Kyoritsu Seiyaku, Tokyo, Japan; 50 mg kg^{-1}, i.p.). Information
405 of tracer injection was summarized in Supplementary table 2.

406 **Histological analysis of neural tracing**

407 Mouse brain was fixed with 2% paraformaldehyde in PBS, exposed consecutively to 15%
408 and 30% sucrose in PBS for 2 days, and embedded in OCT compound. Thin sections (50
409 μm) were prepared with a cryostat microtome. The sections were observed with an
410 LSM800 confocal microscope. The distribution of the tracer at the injection site was
411 verified, and only sections without substantial displacement of the injection site were
412 included in the analysis. The sections were imaged using a LSM800 to acquire
413 fluorescence images. The images were processed for calculation of fluorescence intensity
414 with ZEN imaging software. Regions of interest (ROIs) were defined on the acquired
415 images, and fluorescence intensity values were extracted. Background fluorescence was
416 subtracted to obtain tracer-specific signal intensities. Tracer signals were quantified for
417 both the injection sites and the target regions, and values from the target regions were
418 normalized to those of the corresponding injection sites.

419 **Statistical analysis**

420 For lipid imaging, statistical analysis was performed with the use of BellCurve for
421 Excel software (Social Survey Research Information, Tokyo, Japan). Normality of data
422 was assessed with the Shapiro-Wilk test. If the normality was not met, the Mann-
423 Whitney U test was applied (Fig. 2A, SNr; DR; BLA). If data were normally distributed
424 and variance was homogeneous between genotypes, comparisons were performed with
425 Student's *t* test. If homogeneity of variance was not assumed, Welch's *t* test was applied
426 instead of Student's *t* test (Fig. 2A, CeA). For neuronal tracing, statistical analysis was
427 performed using Microsoft Excel. Variance homogeneity was assessed using an F-test,
428 and based on the results, appropriate t-tests, Welch's t test (Fig. 6E, VAL/VM; SNc) or
429 Student's t test (Fig. 5E, Str; Fig. 6E, VTA; SNc) were applied to evaluate statistical
430 significance. Quantitative data are presented as mean ± SE. Statistical significance of a
431 *P* value is indicated as $p < 0.05$ (*) and $p < 0.001$ (***). Sample size (*n*) are in the figure
432 legends.
433

434 **Acknowledgements**

435 We thank K. Nakamura for AAV2-CMV-palGFP virus particles, I. Yamahata for
436 Immunofluorescent analysis, N. Takahashi for discussion, and the Research Equipment
437 Sharing Center at Nagoya City University for assistance.

Author contributions statement

M.S. conceptualized and supervised the study, acquired funding, designed experiments, and wrote the manuscript; T.O., Y.M., M.K., and H.T. performed experiments; M.I. and H.M. prepared resources.

Funding information

This work was supported in part by KAKENHI grants from Japan Society for the Promotion of Science (JSPS) to M.S. (24K02122).

Ethics

The animal study was approved by Nagoya City University, Animal Research Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Data availability and sources

The original contributions presented in the study are included in the article/ supplementary material, further inquiries can be directed to the corresponding author.

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559 **FIGURE LEGENDS**

560 **Figure 1. PDZD8 is highly expressed in neurons and astrocytes in the midbrain.**
561 Immunofluorescence analysis of wild-type mouse brain.
562 (A) Dopamine transporter (DAT, magenta) and PDZD8 (green) in the midbrain. Regions
563 corresponding to substantia nigra pars compacta (SNc), ventral tegmental area (VTA),
564 and substantia nigra pars reticulata (SNr) are indicated in the DAT image. The merged
565 image is also shown. Scale bars, 200 μm.
566 (B) PDZD8 (green) and DAT (magenta) in SNc.
567 (C) PDZD8 (green) and tyrosine hydroxylase (TH, magenta) in VTA.
568 (D) PDZD8 (green) and parvalbumin (PV, magenta) in SNr.
569 (E) PDZD8 (green) and GFAP (magenta) in SNr. Scale bars, 20 μm (B–E).
570

571 **Figure 2. Lipofuscin deposits are markedly increased in SNr in PDZD8$^{-/-}$ mice.**
572 Lipid aggregate analysis of PDZD8$^{+/+}$ and PDZD8$^{-/-}$ mouse brains.
573 (A) Dot plots showing mean fluorescence intensity of LipiDye II in SNr, dorsal raphe
574 (DR), basolateral amygdala (BLA), and central amygdala (CeA). Schematic brain
575 sections indicate the regions analyzed. Red lines in sagittal sections (upper) indicate the
576 positions of coronal sections (lower), and red boxes mark the analyzed regions. Sample
577 size (*n*) denotes the number of images.
578 (B, C) Confocal images of LipiDye II (B) and LipidTox (C) in SNr. Boxed regions (left)
579 are shown at higher magnification (right). Scale bars, 50 μm (left) and 20 μm (right).
580 (D) High-resolution images of LipiDye II in SNr of PDZD8$^{-/-}$ mice. Boxed regions (left)
581 are shown enlarged (right). A differential interference contrast (DIC) image is also
582 shown. The dotted line outlines a cell periphery densely packed with lipofuscin (right).
583 Scale bars, 5 μm.
584

585 **Figure 3. Lipofuscin deposits are enriched in SNr in PDZD8$^{-/-}$ mice.**
586 Lipid aggregate analysis of SNr in PDZD8$^{+/+}$ and PDZD8$^{-/-}$ mouse brains.
587 (A) Confocal images of DAT (magenta) and LipiDye II (green) in serial sections of SNr
588 from rostral (left) to caudal (right) regions at 240 μm intervals. Scale bars, 100 μm. For
589 PDZD8-/-, enlarged images of LipiDye II are also shown. Scale bars, 20 μm.
590 (B) The merged images of DAT and DIC for the images outlined in (A) in PDZD8$^{+/+}$
591 and PDZD8$^{-/-}$. The merged images of LipiDye II and DIC in PDZD8$^{-/-}$ are also shown.
592 The region of ectopic DAT signal is outlined with a dotted line. Scale bars, 100 μm.
593 (C) Confocal images of LipiDye II (green), DAT (blue), and GFAP (magenta) in SNr in
594 PDZD8$^{-/-}$. The merged image is also shown. Scale bars, 20 μm.

595

596 **Figure 4. Extracellular and intracellular lipid deposits in SNr in PDZD8-/- mice.**
597 (A) Scheme of coronal section containing SNr from mouse brain atlas (left), in which
598 SNr is shown enlarged (right) with the medial (M) and lateral (L) regions indicated.
599 (B, C) 3D images of LipiDye II (green) and GFAP (magenta) in medial (B) and lateral
600 (C) regions of SNr in PDZD8$^{-/-}$ mice, which are reconstructed from z-stack confocal
601 images using Imaris software. Scale bars, 10 μm.
602

603 **Figure 5. Abnormal striatal projection to SNr in PDZD8$^{-/-}$ mice.**
604 (A, B) Confocal images of Alexa594-conjugated cholera toxin subunit B (Alexa594-
605 CTb) injected to SNr merged with DIC images. Tiling images of cerebral hemisphere
606 (A) and serial sections from rostral (left) to caudal (right) regions at 50 μm intervals
607 focusing on SNr (B). Scale bars, 500 μm (A) and 200 μm (B).
608 (C, D) Confocal images of Alexa594-CTb merged with DIC images of brain region
609 containing striatum, indicating projection sources. Tiling images of cerebral hemisphere
610 (C) and serial sections from rostral (left) to caudal (right) regions at 50 μm intervals
611 focusing on striatum (D). Scale bars, 500 μm (C) and 200 μm (D).
612 (E) Dot plots showing normalized ratio of mean fluorescence intensity of Alexa594-
613 CTb at Str (striatum). Sample size (*n*) denotes the number of images.
614 (F) Schematic representation of striatal projections to SNr, with showing increase in
615 PDZD8$^{-/-}$ mice compared to PDZD8$^{+/+}$ mice. Injection sites of Alexa594-CTb to SNr are
616 also indicated.
617 (G) Immunofluorescence images of PDZD8 (green) and dopamine- and cAMP-
618 regulated phosphoprotein (DARPP-32, magenta) in striatum of wild-type mice, with
619 merged image. Scale bars, 20 μm.
620 (H) Immunofluorescence images of DARPP and merged with DIC in SNr of PDZD8$^{+/+}$
621 and PDZD8$^{-/-}$. Scale bars, 100 μm.
622

623 **Figure 6. Abnormal projection from SNr in PDZD8$^{-/-}$ mice.**
624 (A, B) Confocal images showing palmitoylated GFP (palGFP) injection sites in SNr
625 merged with DIC images. Tiling images of cerebral hemisphere (A) and serial sections
626 from rostral (left) to caudal (right) regions at 100 μm intervals focusing on SNr (B).
627 Scale bars, 500 μm (A); 200 μm (B).
628 (C) Confocal and merged DIC images of thalamic regions showing SNr projection
629 targets. PalGFP signals indicate SNr projections to the ventral anterior-lateral complex

of the thalamus (VAL), ventromedial thalamus (VM), and the nigrostriatal tract (nst). Scale bars, 200 μm.

(D) Confocal and merged DIC images of midbrain regions showing SNr projections to the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc). Scale bars, 200 μm.

(E) Dot plots showing normalized ratio of mean fluorescence intensity of palGFP at VAL/VM, nst, VTA, and SNc. Sample size (*n*) denotes the number of images.

(F) Schematic illustration summarizing the anterograde tracing results. Projections from the SNr to both VM/nst and VTA/SNc are markedly reduced in PDZD8$^{-/-}$ mice compared with PDZD8$^{+/+}$ mice. Injection sites of palGFP in the SNr are indicated.

Figure 7. Abnormal dopaminergic circuits in PDZD8$^{-/-}$ mice.

Proposed circuit model depicting the consequences of lipid accumulation in the SNr of PDZD8$^{-/-}$ mice. In PDZD8$^{+/+}$ mice (left), striatal GABAergic inputs transiently inhibit SNr neurons, which tonically suppress activity in thalamic (VM/nst) and midbrain dopaminergic (VTA/SNc) targets, maintaining balanced inhibitory control within the basal ganglia–thalamocortical circuit. In PDZD8$^{-/-}$ mice (right), lipid accumulation in SNr neurons reduces their inhibitory output to VM/nst and VTA/SNc, resulting in functional disinhibition of thalamic and dopaminergic pathways. The consequent hyperactivation of dopaminergic neurons increases dopamine release in the striatum, thereby enhancing inhibitory feedback from striatal neurons to the SNr. This establishes a maladaptive feedback loop—reduced SNr–thalamic output, dopaminergic hyperactivation, and strengthened striatal inhibition—that destabilizes the functional balance of the basal ganglia–thalamocortical circuit and underlies the ADHD-like behavioral abnormalities observed in PDZD8$^{-/-}$ mice.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary figure 1. Specificity of anti-PDZD8 antibody.

(A) Immunofluorescence analysis of PDZD8$^{+/+}$ and PDZD8$^{-/-}$ mice. PDZD8 (green) and the merged image of PDZD8 (green), DAT (red), and Hoechst (blue) in the VTA region are shown. Scale bars, 10 μm.

(B) The summary of brain regions and cell types whether they were colocalized or not with PDZD8. Immunostaining with the anti-PDZD8 antibody in PDZD8$^{+/+}$ and PDZD8$^{-/-}$ mice were comprehensively analyzed. (+) in the yellow columns indicates "colocalization was observed between the cell marker and PDZD8". (-) in the white

665 columns indicates "colocalization was not observed between the cell marker and
666 PDZD8". Vacant column indicates "not tested."
667
668 **Supplementary figure 2. Increased striatal projection to SNr in PDZD8$^{-/-}$ mice.**
669 (A, B) Confocal images of Alexa488-CTb injected to SNr merged with DIC images.
670 Tiling images of cerebral hemisphere (A) and images focused on SNr (B). Scale bars,
671 500 μm (A) and 200 μm (B).
672 (C, D) Confocal images of Alexa488-CTb merged with DIC images of brain region
673 containing Str (striatum), indicating projection sources. Tiling images of cerebral
674 hemisphere (C) and images focused on striatum (D). Scale bars, 500 μm (C) and 200
675 μm (D).
676 (E) Dot plots showing normalized ratio of mean fluorescence intensity of Alexa488-
677 CTb at Str (striatum). Sample size (*n*) denotes the number of images.
678
679 **Supplementary figure 3. Decreased SNr projection to thalamus in PDZD8$^{-/-}$ mice.**
680 Confocal and merged DIC images of thalamic regions showing SNr projection targets.
681 PalGFP signals in thalamus indicate SNr projections to the ventral anterior-lateral
682 complex of the thalamus (VAL) and ventromedial thalamus (VM). Scale bars, 200 μm.
683
684 **Supplementary table 1. Information of mouse age and sex.**
685 The age and sex of mice used in this study were summarized.
686
687 **Supplementary table 2. Information of neuronal tracer injection.**
688 Concentration, volume, target region, and coordinates from Bregma for Alexa594-CTb,
689 Alexa488-CTb, and AAV2/1-CMV-palGFP were summarized.
690
691 **Supplementary movie. 3D movies of lipid deposits in SNr in PDZD8$^{-/-}$ mice.**
692 (A, B) 3D movies of LipiDye II (green) and GFAP (magenta) in medial (A) and lateral
693 (B) regions of SNr in PDZD8$^{-/-}$ mice, which are corresponding to Figure 4B and C,
694 respectively. Scale bars, 10 μm.
695

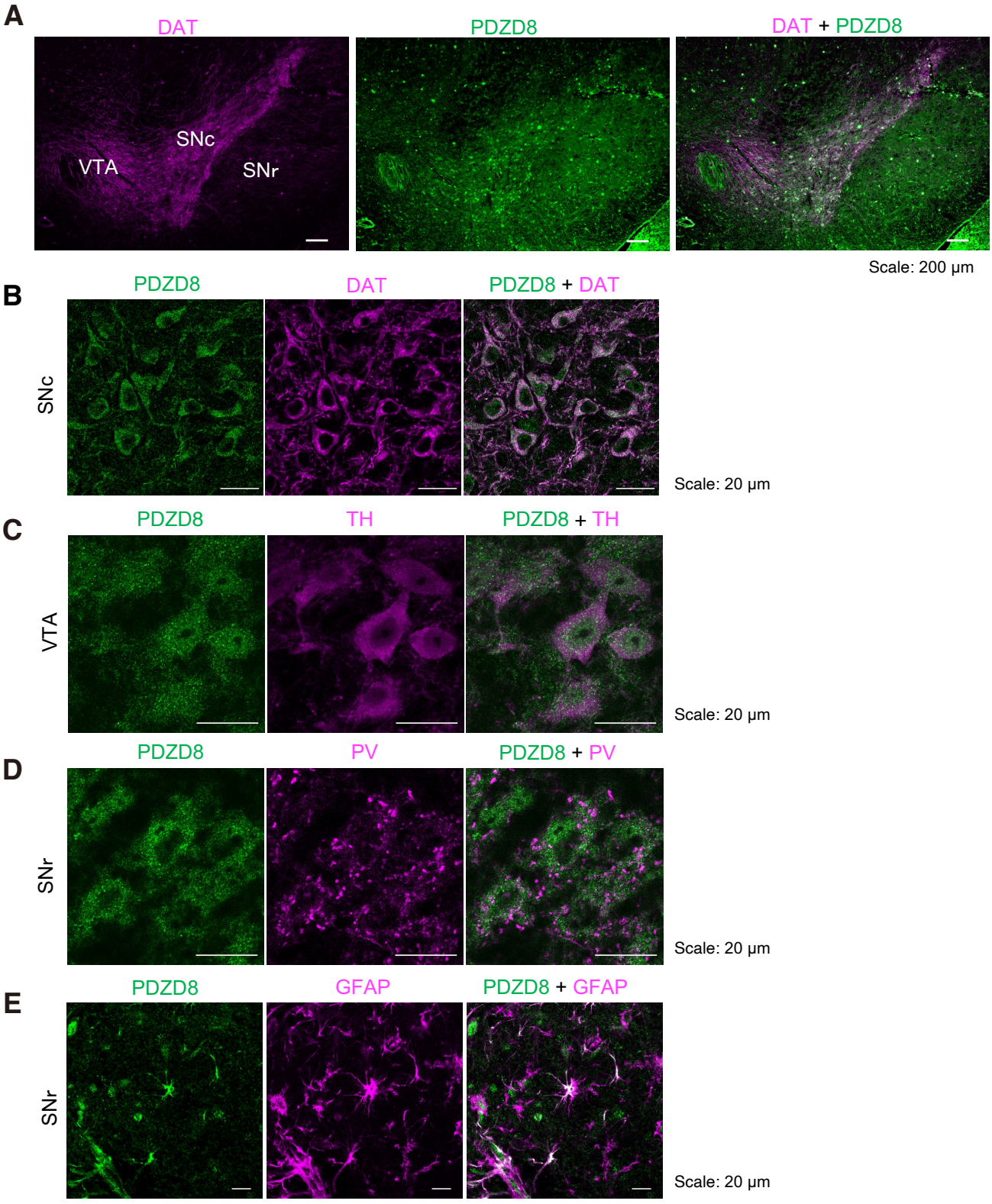

A
DAT
PDZD8
DAT + PDZD8
SNc
VTA
SNr
Scale: 200 μm
B
PDZD8
DAT
PDZD8 + DAT
SNc
Scale: 20 μm
C
PDZD8
TH
PDZD8 + TH
VTA
Scale: 20 μm
D
PDZD8
PV
PDZD8 + PV
SNr
Scale: 20 μm
E
PDZD8
GFAP
PDZD8 + GFAP
SNr
Scale: 20 μm

Figure 1

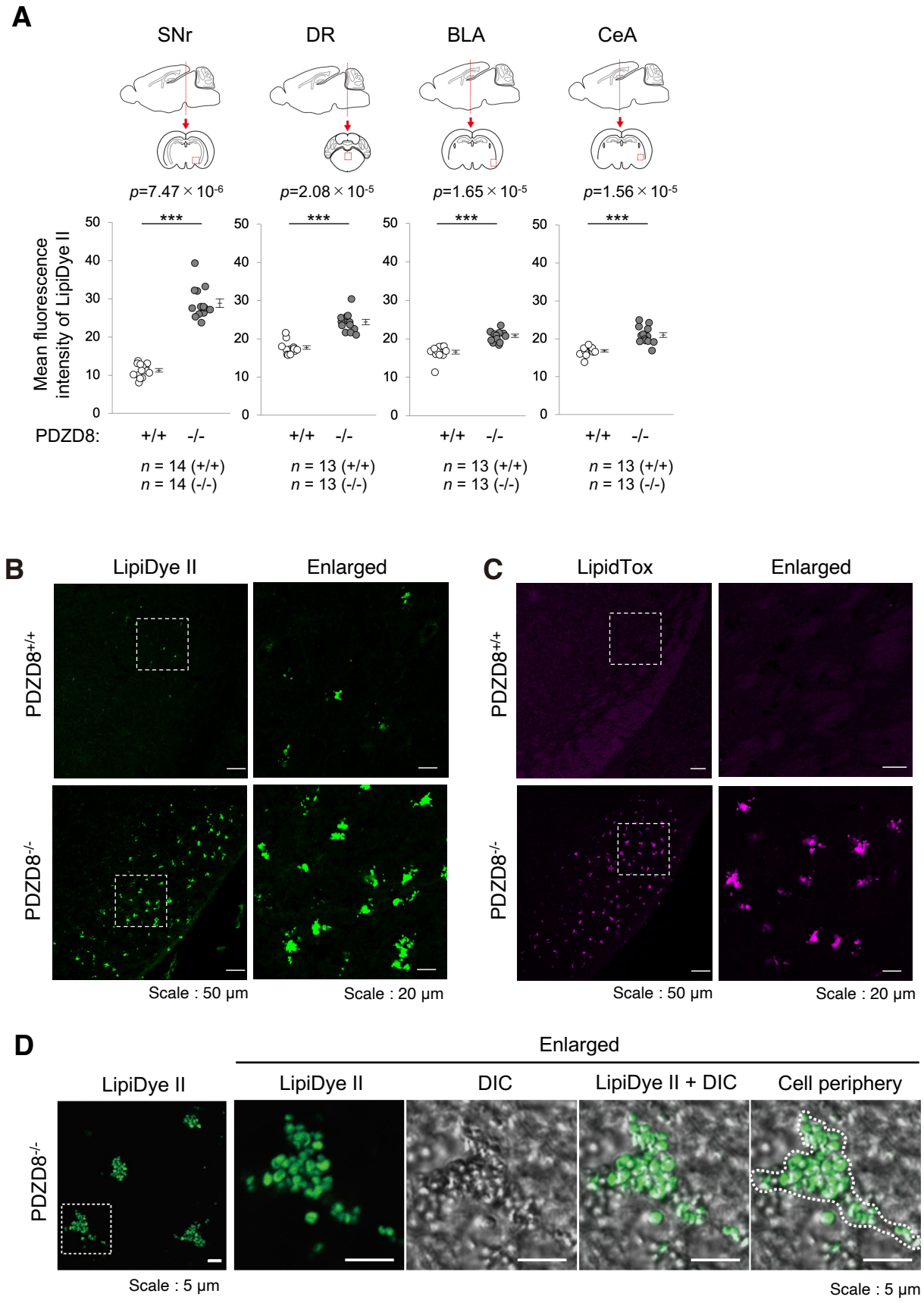
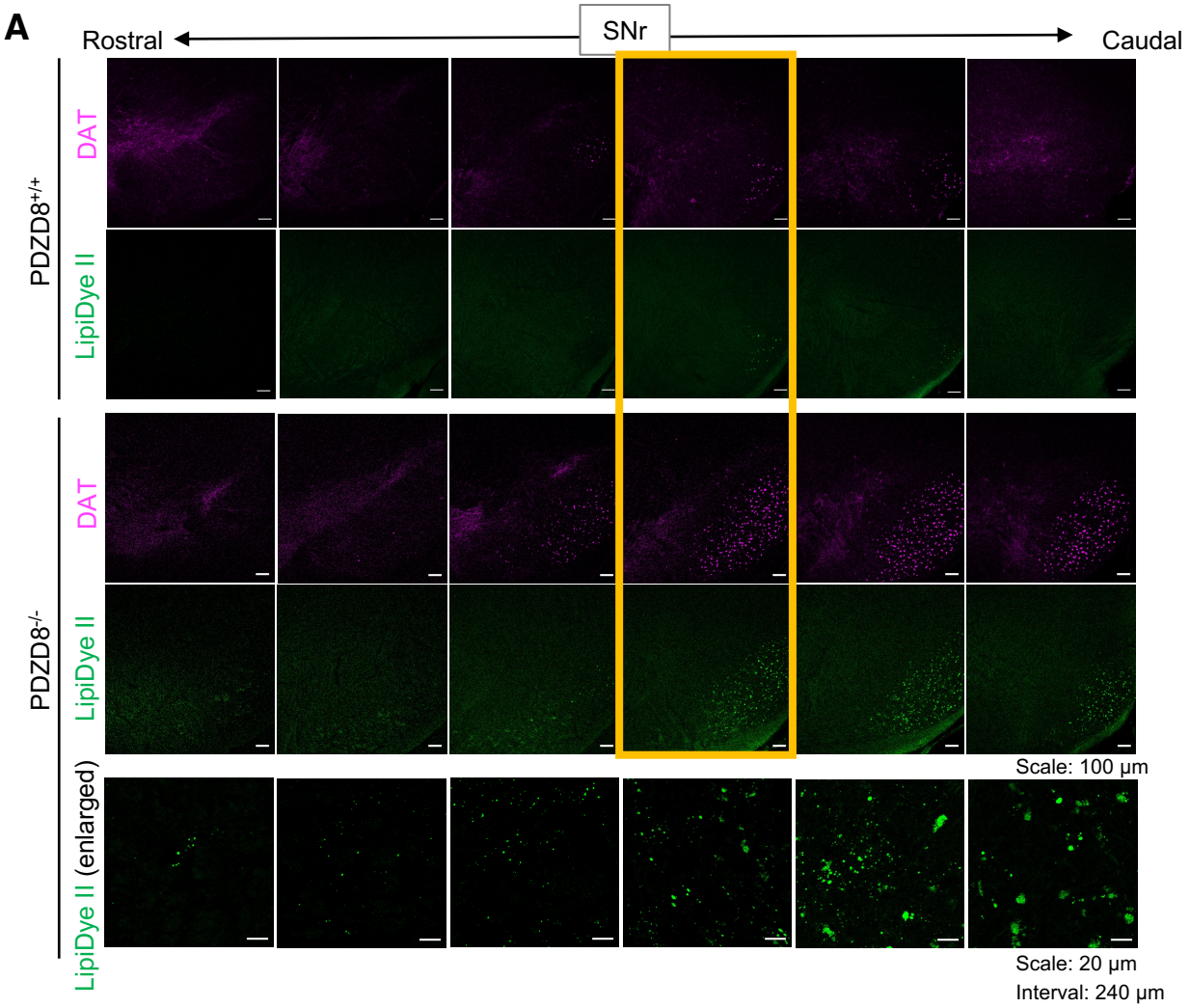
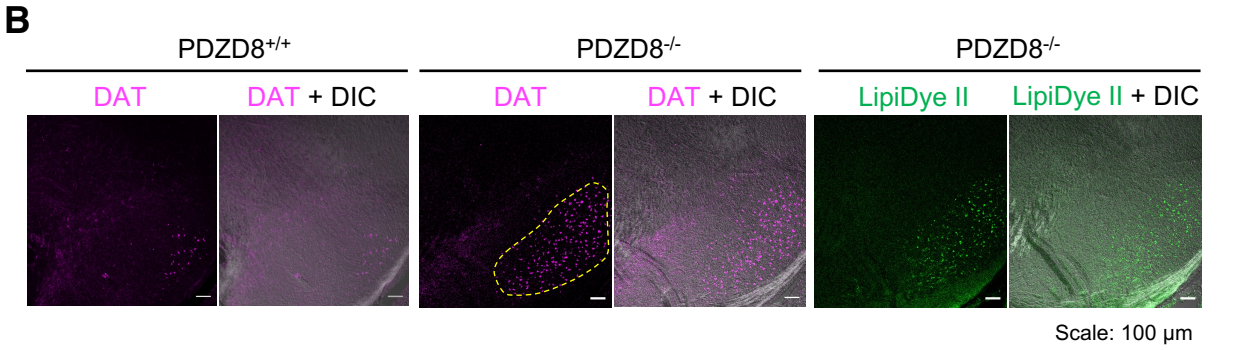

A
SNr
DR
BLA
CeA
p=7.47 × 10-6
p=2.08 × 10-5
p=1.65 × 10-5
p=1.56 × 10-5
***
Mean fluorescence intensity of LipiDye II
PDZD8:
+/+
-/-
n = 14 (+/+)
n = 14 (-/-)
n = 13 (+/+)
n = 13 (-/-)
B
LipiDye II
Enlarged
PDZD8+/+
PDZD8-/-
Scale : 50 μm
Scale : 20 μm
C
LipidTox
Enlarged
PDZD8+/+
PDZD8-/-
Scale : 50 μm
Scale : 20 μm
D
Enlarged
LipiDye II
LipiDye II
DIC
LipiDye II + DIC
Cell periphery
PDZD8-/-
Scale : 5 μm
Scale : 5 μm

Figure 2


A
Rostral
SNr
Caudal
PDZD8+/+
DAT
LipiDye II
PDZD8-/-
DAT
LipiDye II
LipiDye II (enlarged)
Scale: 100 μm
Scale: 20 μm
Interval: 240 μm


B
PDZD8+/+
PDZD8-/-
PDZD8-/-
DAT
DAT + DIC
DAT
DAT + DIC
LipiDye II
LipiDye II + DIC
Scale: 100 μm

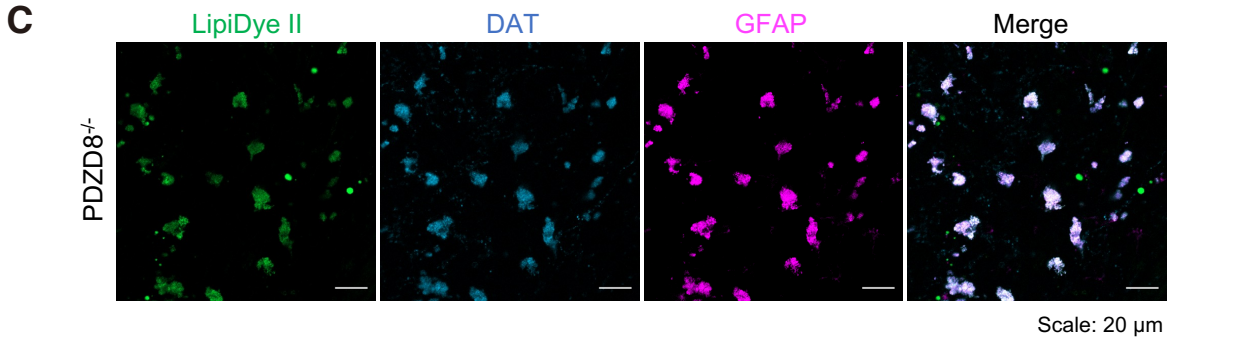
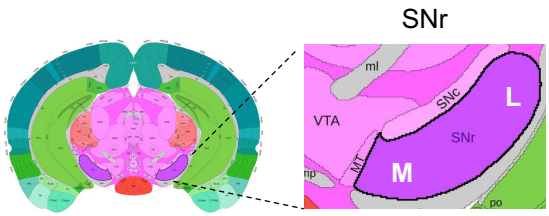
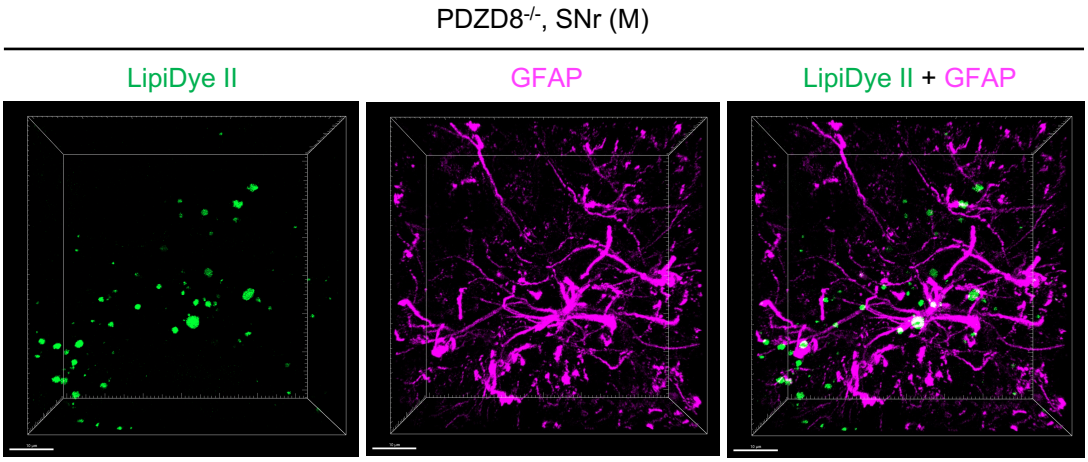

C
LipiDye II
DAT
GFAP
Merge
PDZD8-/-
Scale: 20 μm

Figure 3

A

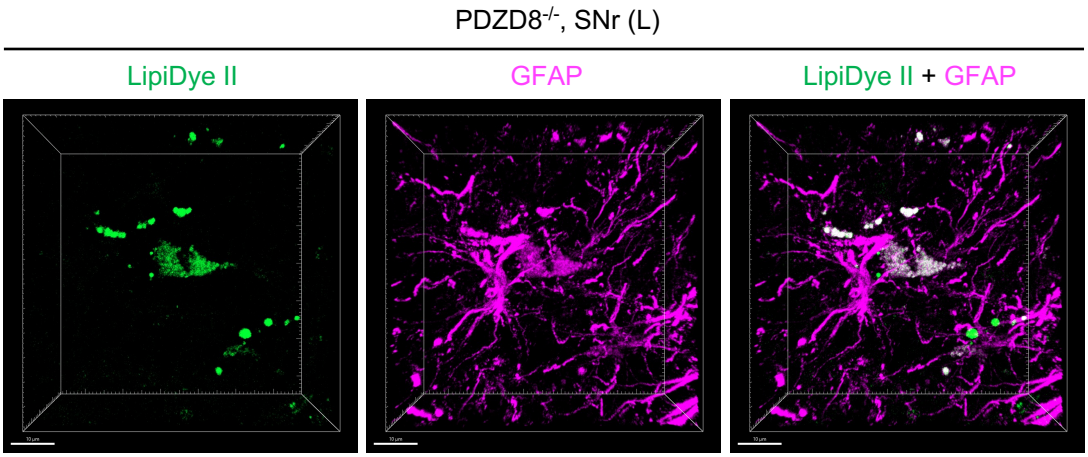


B



Scale: 10 μm

C



Scale: 10 μm

Figure 4

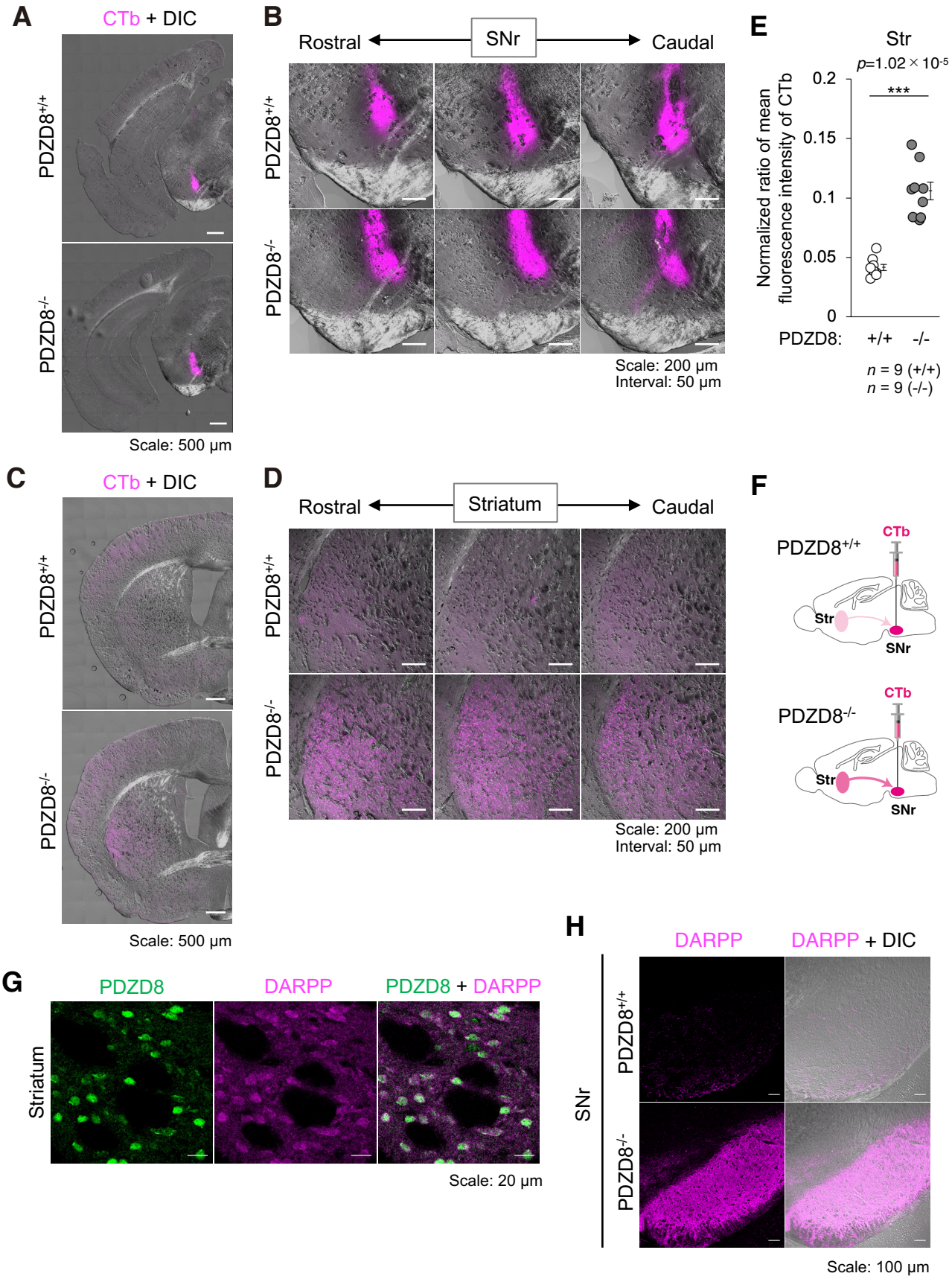

A
CTb + DIC
PDZD8+/+
PDZD8-/-
Scale: 500 μm
B
Rostral
SNr
Caudal
PDZD8+/+
PDZD8-/-
Scale: 200 μm
Interval: 50 μm
E
Str
p=1.02 × 10-5
***
Normalized ratio of mean fluorescence intensity of CTb
0.2
0.15
0.1
0.05
0
PDZD8:
+/+
-/-
n = 9 (+/+)
n = 9 (-/-)
C
CTb + DIC
PDZD8+/+
PDZD8-/-
Scale: 500 μm
D
Rostral
Striatum
Caudal
PDZD8+/+
PDZD8-/-
Scale: 200 μm
Interval: 50 μm
F
PDZD8+/+
CTb
Str
SNr
PDZD8-/-
CTb
Str
SNr
G
PDZD8
DARPP
PDZD8 + DARPP
Striatum
Scale: 20 μm
H
DARPP
DARPP + DIC
SNr
PDZD8+/+
PDZD8-/-
Scale: 100 μm

Figure 5

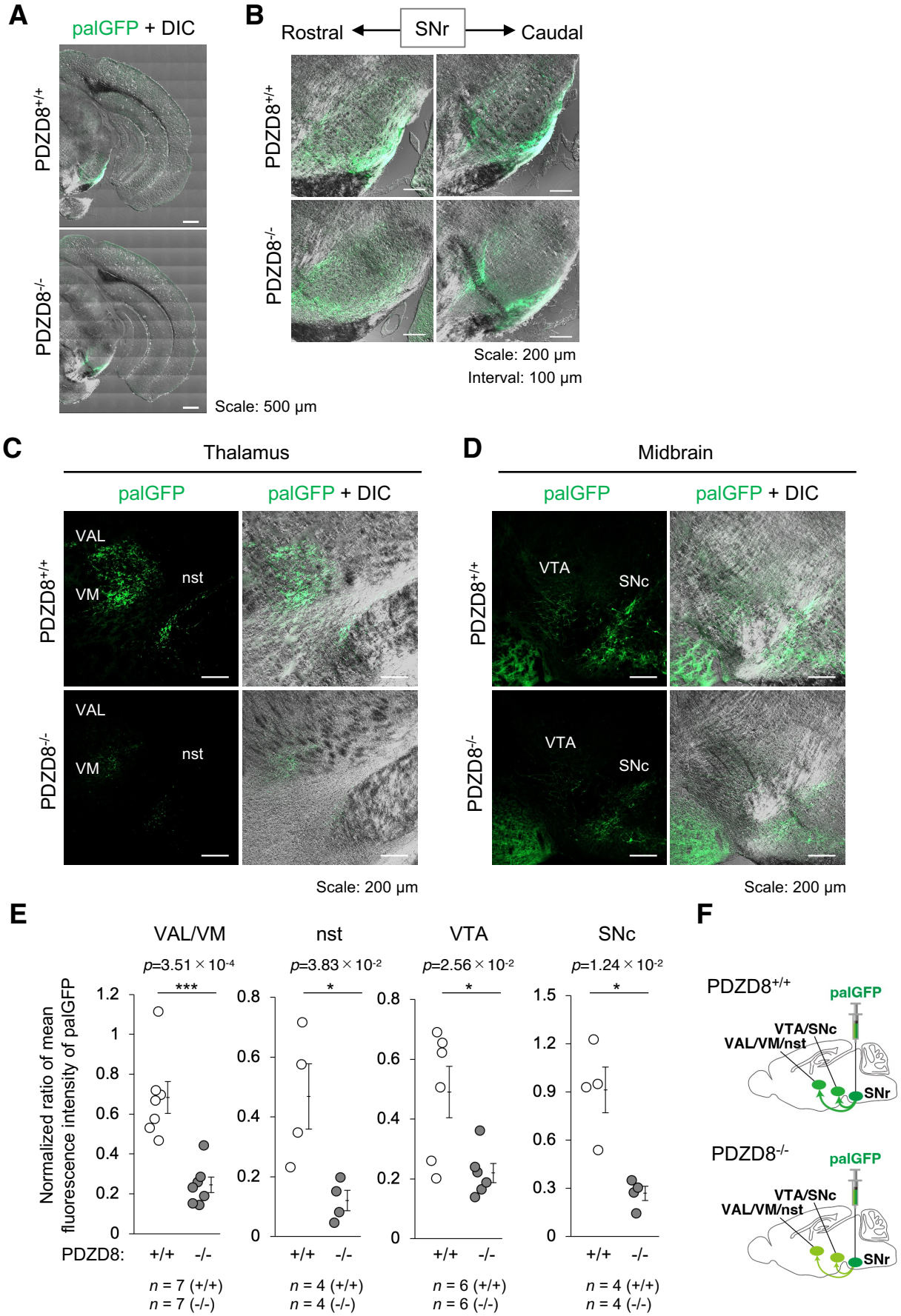

A
palGFP + DIC
PDZD8+/+
PDZD8-/-
Scale: 500 μm
B
Rostral
SNr
Caudal
PDZD8+/+
PDZD8-/-
Scale: 200 μm
Interval: 100 μm
C
Thalamus
palGFP
palGFP + DIC
VAL
VM
nst
PDZD8+/+
PDZD8-/-
Scale: 200 μm
D
Midbrain
palGFP
palGFP + DIC
VTA
SNc
PDZD8+/+
PDZD8-/-
Scale: 200 μm
E
VAL/VM
nst
VTA
SNc
p=3.51 × 10-4
p=3.83 × 10-2
p=2.56 × 10-2
p=1.24 × 10-2
***
*
*
*
Normalized ratio of mean fluorescence intensity of palGFP
PDZD8:
+/+
-/-
n = 7 (+/+)
n = 7 (-/-)
n = 4 (+/+)
n = 4 (-/-)
n = 6 (+/+)
n = 6 (-/-)
n = 4 (+/+)
n = 4 (-/-)
F
PDZD8+/+
palGFP
VTA/SNc
VAL/VM/nst
SNr
PDZD8-/-
palGFP
VTA/SNc
VAL/VM/nst
SNr

Figure 6

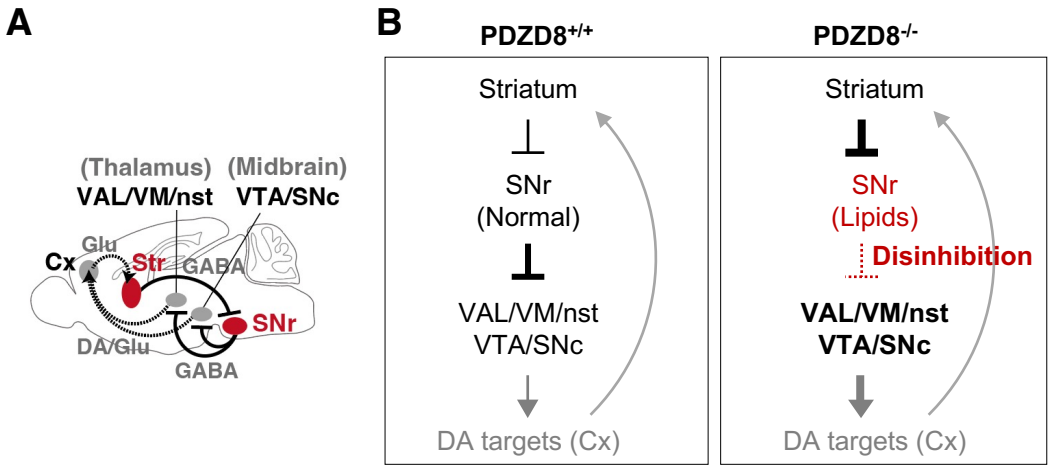

A
(Thalamus)
VAL/VM/nst
(Midbrain)
VTA/SNc
Glu
Cx
Str
GABA
SNr
DA/Glu
GABA
B
PDZD8+/+
Striatum
SNr
(Normal)
VAL/VM/nst
VTA/SNc
DA targets (Cx)
PDZD8-/-
Striatum
SNr
(Lipids)
Disinhibition
VAL/VM/nst
VTA/SNc
DA targets (Cx)

Figure 7