RNA G-quadruplexes forming scaffolds for α-synuclein aggregation lead to progressive neurodegeneration

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Abstract

Synucleinopathies, including Parkinson's disease, dementia with Lewy bodies, and multiple system atrophy, are primarily neurodegenerative diseases with progressive decline in motor function[1,2,3,4,5,6]. Aggregates composed of α-synuclein, which are known as Lewy bodies, are a neuropathological hallmark of synucleinopathies; their pathogenesis has been attributed to neuronal loss owing to intracellular α-synuclein accumulation [1,2,3,7,8]. However, the mechanism of α-synuclein aggregation remains unclear. Here we show that the RNA G-quadruplexes assembly forms scaffolds for α-synuclein aggregation, thereby contributing to neurodegeneration. RNA G-quadruplexes undergo phase separation and form scaffolds for co-aggregation with α-synuclein. Upon pathogenic α-synuclein seeds-induced cellular stress and an optogenetic assembly of RNA G-quadruplexes, phase-separated RNA G-quadruplexes served as scaffolds for α-synuclein phase transition, and the co-aggregates initiated synaptic dysfunction and Parkinsonism in mice. Treatment with 5-aminolevulinic acid and protoporphyrin IX, which prevents RNA G-quadruplexes phase separation[9], attenuates α-synuclein phase transition, neurodegeneration, and motor deficits in synucleinopathy model mice. Together, the RNA G-quadruplexes assembly accelerates α-synuclein phase transition and aggregation owing to intracellular Ca^{2+} homeostasis, thereby contributing to the pathogenesis of synucleinopathies.

57 **Main**

58 α-synuclein (αSyn) is an intrinsically disordered protein (IDP) of 140 amino
59 acids[10]. A relatively high concentration of αSyn is present in synaptic vesicle membranes;
60 it is also present as a natively unfolded monomer in the cytoplasm[11]. Although the
61 physiological role of αSyn remains elusive, it is presumably involved in synaptic vesicle
62 clustering, docking, fusion, and recycling[12]. In pathological conditions, monomeric αSyn
63 assembles into toxic oligomers, protofibrils, and amyloid-like fibrils in synucleinopathies,
64 such as Parkinson's disease (PD), dementia with Lewy bodies (DLB), and multiple
65 system atrophy (MSA)[1,2,3,4,5,6]. The aggregates composed of αSyn, which are known as
66 Lewy bodies (LBs), are a neuropathological hallmark of synucleinopathies, and their
67 pathogenesis has been attributed to neuronal loss[1,2,3,7,8]. However, the mechanism
68 underlying the transformation of native αSyn into pathological aggregates is not
69 completely understood.

70 Liquid–liquid phase separation (LLPS) represents a ubiquitous phenomenon in
71 which molecules transition from a homogeneous state into a dense phase with different
72 physiochemical properties (e.g., molecular crowding), inducing the formation of liquid
73 droplets, hydrogels, and aggregates[13,14,15,16,17]. Proteins and RNAs often undergo LLPS
74 due to multivalent macromolecular interactions with themselves and other
75 biomolecules[9,13,18]. LLPS results in the formation of membraneless ribonucleoprotein
76 (RNP) organelles enriched in RNA and RNA-binding proteins (RBPs), including nucleoli,
77 Cajal bodies, nuclear speckles, stress granules (SGs), processing bodies (P-bodies), and
78 neuronal RNA granules[19,20]. The liquid–solid phase transition via LLPS could be an
79 important intermediate step for aggregate formation in neurodegenerative disease-
80 associated IDPs, such as αSyn[21], tau [22], fused in sarcoma (FUS)[23], TAR DNA-binding
81 Protein of 43kDa (TDP-43)[24], and heterogeneous nuclear ribonucleoprotein A1 (hnRNP
82 A1)[25], whereas the intrinsic factor that facilitates the phase transition has not been
83 identified.

84 We found that RNA G-quadruplexes (rG4s), which are quadruple-stranded
85 structures formed by contiguous guanines[26], bind to αSyn and induce aggregation. rG4
86 itself undergoes LLPS in a Ca^{2+}-dependent manner, acting as a scaffold for αSyn
87 aggregation. In αSyn pre-formed fibrils (PFFs)-treated mouse neurons forming LB-like
88 inclusions similar to human synucleinopathies[27,28], αSyn co-aggregated with rG4
89 assembly consisting of synaptic mRNAs associated with intracellular Ca^{2+} overload,
90 thereby demonstrating synaptic dysfunction. In pharmacological experiments, oral
91 administration of 5-aminolevulinic acid (5-ALA), which intracellularly generates
92 protoporphyrin IX (PPIX) that inhibits rG4s LLPS[9,29], suppressed αSyn aggregation and

improved behavioral motor deficits in αSyn PFF-treated mice. These results provide evidence that rG4s assembly induces sol–gel phase transition of αSyn, eliciting progressive neurodegeneration.

αSyn binds to rG4s and undergoes sol–gel phase transition

First, we analyzed the temporal changes in the aggregation of endogenous αSyn using primary mouse cortical neurons treated with human αSyn PFF. In untreated controls, αSyn was found to be intracellularly diffuse assembling at synaptophysin-positive presynapses along microtubule-associated protein 2 (MAP2)-positive neurites (**Extended Data Fig. 1a**). However, after PFF treatment, αSyn showed significantly higher inhomogeneity in the cytoplasm, existing as granules (**Fig. 1a, b**). The immunoreactivity of αSyn phosphorylated at serine 129 (pS129), a central feature of the LB inclusion, increased in a time-dependent manner and was evident eight days after PFF treatment (**Fig. 1b** and **Extended Data Fig. 1b**). Of note, αSyn granules mainly co-localized with decapping mRNA 1A (Dcp1a)-positive P-bodies (43.0%) but rarely with fragile X mental retardation protein (FMRP)-positive RNA granules (3.2%) or p62-positive aggresomes (18.9%) four days after the treatment (**Fig. 1a, b** and **Extended Data Fig. 1c**). However, αSyn granules localized primarily in the aggresomes (43.9%) but rarely in the P-bodies (6.1%) or the RNA granules (3.1%) eight days after the treatment (**Fig. 1a, b** and **Extended Data Fig. 1c**). Since human antigen R (HuR)-positive SGs were not detected upon PFF treatment (**Extended Data Fig. 1c**), neurons were treated with sodium arsenite for SG formation. Although both HuR-positive SGs as well as αSyn granules were detected in the cytoplasm after the treatment, they did not co-localize (**Extended Data Fig. 1d**).

To assess the dynamic nature of αSyn with PFF treatment in cells, we utilized HEK293 cells overexpressing green fluorescent protein (GFP)-tagged αSyn in the present study. The GFP-tagged αSyn granules exhibited rapid recovery kinetics 24 h after PFF treatment in fluorescence recovery after photobleaching (FRAP) assay (**Fig. 1c**, **Extended Data Fig. 1e** and **Supplementary Video**), and the GFP-tagged αSyn granules were not co-localized with pS129 or p62 (**Extended Data Fig. 1f**). however, the fluorescence recovery was markedly reduced and αSyn granules co-localized with pS129 and p62 48 h after the treatment (**Fig. 1c**, **Extended Data Fig. 1e, f** and **Supplementary Video**). These results suggest that αSyn transiently forms granules with RNPs via LLPS, subsequently forming aggregates contributing to the pathogenesis of synucleinopathies. Thereafter, we employed clustered regularly interspaced short palindromic repeats/Cas9 technology in several human cell lines (Human embryonic kidney 293 (HEK293), Hela,

and Hutu-80 cells) for endogenous tagging with mNeonGreen at *SNCA* loci to reveal the dynamic nature of endogenous αSyn with PFF treatment; however, both the N- and C-terminal tagging was unsuccessful owing to unexplained cell death.

Since our data suggested that RNP granules containing RNAs and/or proteins provide scaffolds for initial αSyn aggregation, we subsequently examined whether the addition of cell-derived RNAs or proteins undergo sol–gel phase transition of αSyn *in vitro*. Purified αSyn (69 μM) underwent LLPS according to the concentration of polyethylene glycol (PEG), a molecular crowding agent, whereas no droplet formation was observed without PEG (**Extended Data Fig. 2a**). Thereafter, we examined whether RNAs impact αSyn LLPS by adding total RNAs extracted from Neuro-2a cells. RNA addition decreased the PEG concentration required for αSyn LLPS and elicited a transition from a droplet to a gel-like state (**Fig. 1d**). Fluorescence imaging studies using mCherry-tagged αSyn (mCh-αSyn) confirmed that these gel-like aggregates contain αSyn (**Fig. 1d**). In FRAP experiments in the presence of RNAs, the fluorescence recovery of αSyn was faster in the droplets but not in the gel-like states (**Fig. 1e**). Similar experiments were performed with nuclease-treated proteins: αSyn droplets formed in the presence of PEG; however, no transition to a gel-like state occurred (**Extended Data Fig. 2b**).

These observations motivated us to examine the nucleotide-binding capacity of αSyn and its sequence; therefore, we subsequently performed RNA Bind-n-Seq[30] using recombinant human αSyn and a pool of random 24-nt RNA oligonucleotides *in vitro* (**Fig. 1f**). The top 10 αSyn binding RNA motifs were significantly guanine-rich sequences (**Extended Data Fig. 3a–e** and **Supplementary Table 1**). Several runs at different concentrations of αSyn revealed that these motifs require guanines in a contiguous but not random sequence, thereby suggesting rG4s as a potential structure for αSyn binding (**Fig. 1f** and **Extended Data Fig. 3f, g**). To investigate whether αSyn preferentially interacts with rG4s, we performed an electrophoresis mobility shift assay (EMSA) using various RNA oligonucleotide structures: G4tr—a typical rG4, telomeric repeat-containing RNA (TERRA) $(UUAGGG)_4$ repeats, G4mt—a TERRA mutant that is unable to form rG4 $(UUACCG)_4$ repeats, a hairpin structure—$(CAG)_8$ repeats, and polyA—single-stranded and non-structured $(AAA)_8$ repeats (**Extended Data Fig. 4a**). αSyn demonstrated high affinity only for G4tr and little or no affinity for other RNA structures (**Fig. 1g** and **Extended Data Fig. 4b**). In quantitative surface plasmon resonance (SPR) binding analysis, αSyn demonstrated much higher selectivity for G4tr (K_D = 3.63 ± 0.63 μM) compared to that with G4mt (K_D = n.d.) (**Fig. 1h** and **Extended Data Fig. 4c, d**). αSyn is defined by three regions: 1) the N-terminal region (Nterm; residues 1–60), which is basic and composed of seven imperfect repeats of 11 amino acids that contain a 4-

residue core with the consensus sequence KTKEGV; 2) the non-amyloid-β component region (NAC; residues 61–95), which is hydrophobic and forms the core of the fiber during aggregation; and 3) the C-terminus (Cterm; residues 96–140), which is a highly negatively charged region that binds to metal ions[31]. To identify the domains responsible for rG4-binding capability, we constructed three mutant αSyns: ΔNterm, ΔNAC, and ΔCterm. SPR analysis demonstrated ΔNAC and ΔCterm but not ΔNterm segments showing binding affinity for G4tr, suggesting that the N-terminus of αSyn preferentially binds to rG4s (**Extended Data Fig. 4c, d**). We also obtained similar results using the Quartz Crystal Microbalance system.

As for αSyn LLPS *in vitro*, G4mt demonstrated no additional effects on αSyn droplet formation in the presence of 15% PEG, while G4tr evoked αSyn transition into gel-like states (**Fig. 1i**). Furthermore, αSyn ΔNterm mutant formed droplets but not gel-like aggregates with G4tr (**Extended Data Fig. 4e**). In FRAP studies, the fluorescence recovery of αSyn droplets was rapid when formed alone, with G4mt, and as ΔNterm with G4tr, whereas that of gel-like assemblies with G4tr was not recovered (**Fig. 1i** and **Extended Data Fig. 4e**). The intensity of Proteostat signals was significantly higher in gel-like assemblies of αSyn with G4tr compared to that in droplets of αSyn alone, αSyn with G4mt, and ΔNterm with G4tr (**Fig. 1j** and **Extended Data Fig. 4f**). Considered together, rG4s bind directly to the N-terminus of αSyn, accelerating the sol–gel phase transition *in vitro*.

Ca^{2+}-induced rG4 assembly is essential for αSyn aggregation

We examined the intracellular mechanism underlying the rG4s-induced αSyn aggregation on pathological conditions. rG4 itself has the propensity to undergo LLPS *in vitro*, which can be boosted electrostatically by Mg^{2+} divalent cation[16,18]. Indeed, G4tr underwent LLPS without PEG; however, G4mt did not (**Extended Data Fig. 5a**). Moreover, other divalent cation Ca^{2+} promoted G4tr LLPS droplets and enlarged the size of droplets (**Extended Data Fig. 5a,b**). Furthermore, the Ca^{2+}-induced increase in droplet size was markedly suppressed by the addition of EGTA, a Ca^{2+} chelator (**Extended Data Fig. 5b**). Notably, G4tr droplets co-aggregated with αSyn in the presence of Ca^{2+} (**Fig. 2a**). In the absence of G4tr, αSyn droplets or gel-like aggregates were not observed regardless of the presence of Ca^{2+} (**Fig. 2a**). Ca^{2+} transitioned G4tr-αSyn coacervates to a gel-like state thereby significantly reducing FRAP, whose effect was abolished by EGTA (**Fig. 2b**). As expected, Proteostat signals were detected only in the G4tr-αSyn complex in the presence of Ca^{2+} (**Fig. 2c**). Moreover, lower concentration of αSyn (34.5 μM) underwent sol–gel transition in the presence of G4tr and Ca^{2+} (**Extended Data Fig. 5c**).

These results suggested that Ca^{2+}-induced rG4 assembly is essential for the efficient initiation of αSyn aggregation.

The persistent and excessive Ca^{2+} entry in neurons by certain metabolic stress conditions could be a major factor in the development of neurodegeneration, including PD[32,33]. Indeed, in response to PFF-induced cellular stress, cultured neurons demonstrated an excessively elevated intracellular Ca^{2+} concentration (**Extended Data Fig. 5d**)[34]. Thus, we examined whether Ca^{2+}-induced rG4 assembly is observed in neurons by immunostaining with G4-specific antibody BG4[35]. PFF and Ca^{2+} ionophore (ionomycin) treatments significantly increased the number of cytosolic G4 granules of both small (0.05–0.2 μm^2) and large (> 0.2 μm^2) sizes compared to vehicle-treated controls (**Fig. 2d**). Furthermore, the number of G4 granules co-localized with αSyn significantly increased after PFF treatment (**Extended Data Fig. 6a**). Eight days after PFF treatment, pS129-positive aggregates were extensively co-localized with G4 granules in mouse cultured neurons (**Fig. 2e**). In the synucleinopathy model mice injected with PFF into the dorsal striatum, pS129-positive inclusions were observed in the tyrosine hydroxylase (TH)-positive dopaminergic (DA) neurons of the substantia nigra, which co-localized with large G4 granules (**Fig. 2f**). In the brainstems of postmortem human patients with PD, the majority (> 90%) of pS129-positive inclusions co-localized with large G4 granules (**Fig. 2g** and **Extended Data Fig. 6b**). The immunoreactivity of cytosolic G4 granules seen in asymptomatic controls was completely eliminated by RNase pretreatment, whereas immunoreactivity of cytosolic G4 granules co-localized with pS129-positive inclusions in PD brains was not (**Extended Data Fig. 6b**).

αSyn co-accumulated with rG4 containing synaptic mRNAs following Ca^{2+} overload

To identify the rG4s as scaffolds of αSyn aggregation in neurons, BG4-bound RNAs were analyzed in PFF-treated cultured mouse neurons using RNA immunoprecipitation coupled with sequencing (BG4 RIP-seq)[18].

We identified 254 and 524 gene transcripts enriched in vehicle- and PFF-treated neurons, respectively, of which 162 were common (cutoff: P-value < 0.05 and fold-change > 1.5) (**Fig. 3a** and **Supplementary Tables 2 and 3**). We compared fragments per kilobase of exon per million mapped reads (FPKM) obtained from BG4-enriched fractions with those from IgG-treated fractions to identify the rG4s. To predict quadruplex-forming guanine-rich sequences (QGRS) mapper[36], more than 95% of BG4-enriched mRNAs were found to have putative rG4-forming sequences with two or three G-tetrad layers (**Fig. 3b, Extended Data Fig. 7a** and **Supplementary Table 4,5**). In the gene ontology (GO) analysis, BG4-binding RNAs obtained from PFF-treated neurons

contributed to neurodegeneration (**Extended Data Fig. 7b** and **Supplementary Tables 6 and 7**), and most of these RNAs were linked to the synapse (**Fig. 3c** and **Supplementary Table 8**). Among the rG4 enriched in PFF-treated neurons, we focused on two mRNAs encoding synaptic proteins, *Camk2a* and *dlg4*, which have been validated to fold into G4 structures by biophysical assays[37] and conserved in both mice and humans (**Extended Data Fig. 7c**). Moreover, these synaptic proteins (i.e., CaMKIIα and PSD95) are essential for synaptic plasticity, an important cellular mechanism underlying learning and memory[38,39]. We also validated that both mRNAs contain rG4-forming sequences (G4ck and G4ps; **Fig. 3d** and **Extended Data Fig. 7d**). Both G4ck and G4ps co-aggregated with αSyn in the presence of Ca^{2+} *in vitro*, which were detected by Proteostat (**Fig. 3e** and **Extended Data Fig. 7e**). To obtain an insight into the interaction of αSyn with these mRNAs in the context of pathogenesis, we performed *in situ* hybridization together with immunostaining in mouse cultured cortical neurons eight days after PFF treatment. We observed high co-localization of these mRNAs with pS129-positive aggregates in the cytoplasm (**Fig. 3f** and **Extended Data Fig. 7f**).

Optogenetics-induced rG4 assembly evokes αSyn aggregation and neuronal dysfunction

To provide direct evidence that rG4 assembly induces αSyn aggregation, we developed an optogenetics-induced rG4 assembly approach, "optoG4 system" under spatiotemporal control of blue light (BL) stimulation. The optoG4 system consists of two plasmids: *G4tr-MS2* RNA—rG4-forming $(TTAGGG)_{23}$ repeats tagged 12×*MS2*-hairpin loops[40] and MCP-Cry2—*MS2*-coat protein tagged mCh-Cry2olig, which functions by co-expressing them in the cells (**Fig. 4a** and **Extended Data Fig. 8a**). We confirmed that MCP-Cry2 clusters were formed in the optoG4-expressing Neuro-2a cells in a BL exposed-time dependent manner (**Fig. 4b**). In the cells co-expressing optoG4 and αSyn, MCP-Cry2 clusters co-assembled with αSyn and *G4tr-MS2* RNA 3-h following BL stimulation (**Fig. 4c**). The BL-induced αSyn granules were p62-, pS129-, and BG4-positive (**Extended Data Fig. 8b, c**). Since Cry2olig possesses the property of reversible homo-oligomerization in response to BL stimulation[41], we examined whether BL-induced αSyn aggregation in the optoG4 system is irreversible. In the cells co-expressing optoG4 and αSyn, MCP-Cry2 formed irreversible complexes containing αSyn and *G4tr-MS2* RNA following 3-h BL stimulation and subsequent 3-h withdrawal (**Extended Data Fig. 8d, e**). However, MCP-Cry2 clusters were significantly more dispersed in cells expressing only optoG4 than in cells co-expressing optoG4 and αSyn under the same stimulatory condition (**Extended Data Fig. 8d, e**). We developed "optoG4mt system" using *G4mt-*

MS2 RNA consisting of rG4-unforming $(TTACCG)_{23}$ repeats tagged 12×*MS2*-hairpin loops as a negative control for rG4-forming structure (**Extended Data Fig. 8a, b**). In cells co-expressing optoG4mt and αSyn, MCP-Cry2 and *G4mt-MS2* RNA complexes formed 3-h after BL stimulation; however, they did not co-assemble with αSyn or G4s (**Extended Data Fig. 8f**).

In mouse cultured cortical neurons expressing the optoG4 system, endogenous αSyn formed pS129- and p62-positive aggregates following 3-h BL stimulation (**Fig. 4d** and **Extended Data Fig. 8g**), but did not in those expressing optoG4mt (**Fig. 4e**) or MCP-Cry2 alone (**Extended Data Fig. 8h**). Endogenous αSyn aggregation induced by optoG4 were retained even after 3-h BL stimulation and subsequent 3-h withdrawal (**Extended Data Fig. 8i**). We measured spontaneous excitatory postsynaptic currents (sEPSCs) to investigate the effect of optoG4-induced αSyn aggregation on neuronal activity. The sEPSC amplitude and frequency were significantly reduced in BL-stimulated optoG4 neurons compared to non-stimulated optoG4 neurons (**Fig. 4f**). Changes in the neuronal activities were not observed in the neurons expressing MCP-Cry2 alone or with optoG4mt, regardless of the BL conditions (**Fig. 4f** and **Extended Data Fig. 8j**). Impaired sEPSCs in the BL-stimulated optoG4 neurons were also sustained after 3-h BL withdrawal (**Extended Data Fig. 8k**).

Thereafter, we employed the optoG4 system in a series of *in vivo* experiments, injecting recombinant adeno-associated viruses (AAVs) into the nigral DA neurons of mice resulting in expression of improved Cre under the control of mouse TH promoter (AAV-mTH-iCre;[42]), iCre recombinase-dependent expression of MCP-Cry2 flanked by double-floxed inverse open reading frame (DIO) (AAV- MCP-Cry2), and *G4tr-MS2* RNA (AAV-*G4-MS2*). Fourteen days after AAV injection, mice were implanted with a wireless optogenetic device to enable BL stimulation in the substantia nigra. Two days after the implantation, the mice were subjected to BL stimulation for 1-h per day for four weeks (**Fig. 4g**).

Immunohistochemical analysis confirmed MCP-Cry2 expression in the nigral DA neurons (**Extended Data Fig. 9a**). In optoG4 mice, BL stimulation formed αSyn granules, which co-assembled with MCP-Cry2 and *G4-MS2* RNA (**Fig. 4h**). In optoG4 mice with BL, the number of TH-positive DA neurons was significantly reduced compared to that without BL, and pS129-positive inclusions were observed (**Fig. 4i**). No pathological changes were observed in the other groups (**Extended Data Fig. 9b, c**). Rotarod and beam-walking tests were performed to determine whether optoG4 system induces PD-like motor deficits. OptoG4 mice with BL demonstrated a progressive and significant decrease in fall latency in the rotarod test and increase in the number of footslips in the

309 beam-walking test compared to those without BL (**Fig. 4j**). The other groups did not show
310 motor deficits, regardless of BL stimulation (**Extended Data Fig. 9d**).
311
312 **Treatment with 5-ALA ameliorates motor dysfunctions observed in PFF-injected**
313 **mice**
314 Since PPIX binds to rG4s[9], we examined whether PPIX affects rG4s assembly *in*
315 *vitro*. Treatment with PPIX reduced the size of G4tr-containing liquid droplets (**Fig. 5a**).
316 Importantly, the formation of gel-like aggregates consisting of G4tr and αSyn was also
317 inhibited by PPIX (**Fig. 5b** and **Extended Data Fig. 10a**).
318 PPIX is not available *in vivo* owing to its cytotoxicity[43]; thus, we investigated
319 whether 5-ALA, an extremely low-toxic and blood–brain barrier-permeable prodrug of
320 PPIX[9,29], could inhibit αSyn aggregation in cells. The application of 5-ALA significantly
321 prevented increase in G4 granules and αSyn aggregation 24-h following PFF treatment in
322 Neuro-2a cells expressing mCh-αSyn (**Fig. 5c** and **Extended Data Fig. 10b**). Thereafter,
323 5-ALA was administered to primary mouse cultured PFF-treated neurons. 5-ALA
324 significantly restored the reduction in amplitude and frequency in sEPSC eight days after
325 co-treatment with PFF (**Fig. 5d**). 5-ALA also reduced the pS129-positive inclusions (**Fig.**
326 **5e**), and attenuated reduction of PSD95 and CaMKIIα protein levels without changes in
327 the mRNA levels of *Dlg4* and *Camk2a* in PFF-treated neurons (**Fig. 5f and Extended**
328 **Data Fig. 10c**).
329 Finally, we investigated the effects of 5-ALA on αSyn aggregation and
330 Parkinsonism *in vivo*. Mice were injected with PFF into the dorsal striatum followed by
331 daily oral administration of 5-ALA (1 or 3 mg/kg/day). Two months after PFF injection,
332 5-ALA (3 mg/kg) prevented motor dysfunctions in PFF-injected mice (**Fig. 5g**).
333 Importantly, 5-ALA also prevented the formation of pS129-positive inclusion and the
334 resultant degeneration of nigral DA neurons (**Fig. 5h**).
335
336

Discussion

In this study, we demonstrated that Ca^{2+}-induced rG4 assembly via LLPS served as scaffolds for αSyn aggregation, contributing to neurodegeneration. rG4s bind directly to the N-terminus of αSyn, accelerating the aggregation. In PFF-treated neurons, αSyn aggregated with rG4 assembly composed of synaptic mRNAs, such as *Camk2a* and *Dlg4,* due to Ca^{2+} excess influx, eliciting neuronal dysfunction. In addition, rG4 assembly by the optoG4 system caused endogenous αSyn aggregation and neuronal dysfunction, reproducing the pathology of synucleinopathies. 5-ALA and PPIX administration suppressed rG4 LLPS and inhibited the co-aggregation of rG4s and αSyn, thereby ameliorating neurodegeneration and motor deficits in PFF-injected synucleinopathy mice. Considered together, rG4 assembly attributed to aberrant intracellular Ca^{2+} influx promotes αSyn aggregation, which may be involved in the pathogenesis of synucleinopathies **(Extended data Fig. 10**).

Although the aggregation of many neurodegenerative disease-related IDPs has been attributed to LLPS-mediated sol–gel phase transitions[22,23,24,25,44], purified αSyn does not form liquid droplets nor aggregates *in vitro*, except under non-physiological conditions, such as high protein concentrations above 100 μM, addition of molecular crowding reagents, low pH, and agitation (**Fig. 1**)[21,45,46,47]. The difficulty of αSyn aggregation *in vitro* is presumably attributed to the monomeric state preserved by biased electrostatic and hydrophobic intramolecular interactions and intermolecular repulsion. αSyn has opposite charges at the N- and C-termini and has overall low hydrophobicity; the N-terminus with positively charged, the NAC region with high hydrophobicity, and the C-terminus with highly negatively charged that binds to bivalent metal ions, including Ca^{2+}[48,49]. The long-range electrostatic interactions through the N- and C-termini, as well as hydrophobic interactions between the C-terminus and NAC regions form a compact, autoinhibitory conformation that limits the exposure of the NAC region. In addition, intermolecular interactions of the negatively charged C-terminus produce electrostatic repulsion, inhibiting multimer formation[48,49].

Distinct from "αSyn LLPS", we here proposed the "rG4 LLPS" hypothesis that Ca^{2+}-induced association of rG4s via LLPS is responsible for the initiation of αSyn aggregation. The rG4-induced αSyn aggregation occurred under near physiological conditions (neutral pH, concentration at 34.5 μM) without both agitation and the presence of the molecular crowding reagent (**Extended Data Fig. 5**). The preferential binding of the N-terminus of αSyn to rG4s was required for αSyn aggregation **(Extended Data Fig. 4e, f**). Since the N-terminus of αSyn is involved in membrane anchoring[31], a critical period could exist at which the interaction factor with αSyn shifts from the membrane to

373 rG4s in the pathogenic process. Importantly, both rG4s and Ca^{2+} are necessary and
374 sufficient for αSyn aggregation. Since rG4 unfolding mutant G4mt did not bind to αSyn,
375 the binding of rG4s to αSyn was not through the electrostatic interaction of the negatively
376 charged phosphate backbone of the RNAs with the positively charged N-terminus of the
377 protein. Although how rG4 modulates αSyn conformation and the subsequent aggregation
378 is unclear, it is possible that rG4s and Ca^{2+} shield both the N-terminus and negatively
379 charged C-terminus, respectively, thereby exposing the NAC region upon release of the
380 compact conformation. This would result in αSyn bearing aggregation-prone states via
381 the hydrophobic core domain (NAC-NAC interaction). More structural analyses are
382 warranted in the rG4s binding to the N-terminus through non-covalent bonds, such as
383 hydrogen bonds and hydrophobic interactions using cryogenic electron microscopy and
384 X-ray crystallography techniques.

385 In mammals, rG4-forming potential sequences are abundant in transcripts, with
386 3,800 predicted in approximately 2,300 different genes[50]. Although the rG4 assembly
387 organizes the formation of SGs in neurons[18], the intracellular mechanism of the rG4
388 assembly attributed to stress stimulation remains unclear. We here proposed that persistent
389 and excessive intracellular Ca^{2+} influx by PFF treatment triggers rG4 assembly (**Fig. 2**).
390 Interestingly, mRNAs encoding synaptic proteins accounted for most of the RNAs
391 identified by BG4 RIP-seq in the PFF-treated neurons (**Fig. 3**). Consistent with this result,
392 a significant reduction in the synaptic activity was observed in neurons challenged with
393 optoG4 system and PFF (**Figs. 4 and 5**). Indeed, excessive Ca^{2+} influx has been identified
394 in the pathogenesis of various neurodegenerative diseases, including synucleinopathies,
395 Alzheimer's disease, amyotrophic lateral sclerosis, Huntington's disease, and
396 spinocerebellar ataxias[32]. Furthermore, rG4-induced sol–gel phase transition of tau has
397 been observed *in vitro* (under preparation). These results suggest that rG4 is a key factor
398 in the aggregation of prionoid proteins, including αSyn, tau, and FMRpolyG[9], and
399 excessive Ca^{2+} influx-induced rG4 assembly may be a main source of the pathogenesis in
400 neurodegenerative diseases. In the future, it is necessary to elucidate why neuronal rG4
401 assembly contains a majority of synaptic mRNAs and how rG4 assembly aggregation of
402 prionoid proteins contributes to the pathogenesis of neurodegeneration.

403 In conclusion, rG4 assembly by excessive Ca^{2+} influx could induce αSyn
404 aggregation and neuronal dysfunction. In addition, we found that PPIX can inhibit the
405 rG4 assembly, and its prodrug 5-ALA significantly ameliorated motor deficits in PFF-
406 injected mice. Importantly, 5-ALA can act on the brain through oral administration and
407 produce PPIX intracellularly without serious adverse effects[29]. 5-ALA can be
408 prophylactically administered before progressive motor dysfunction, and could be a

409 promising novel agent for neurodegenerative diseases, including synucleinopathies.
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Figures and Figure legends

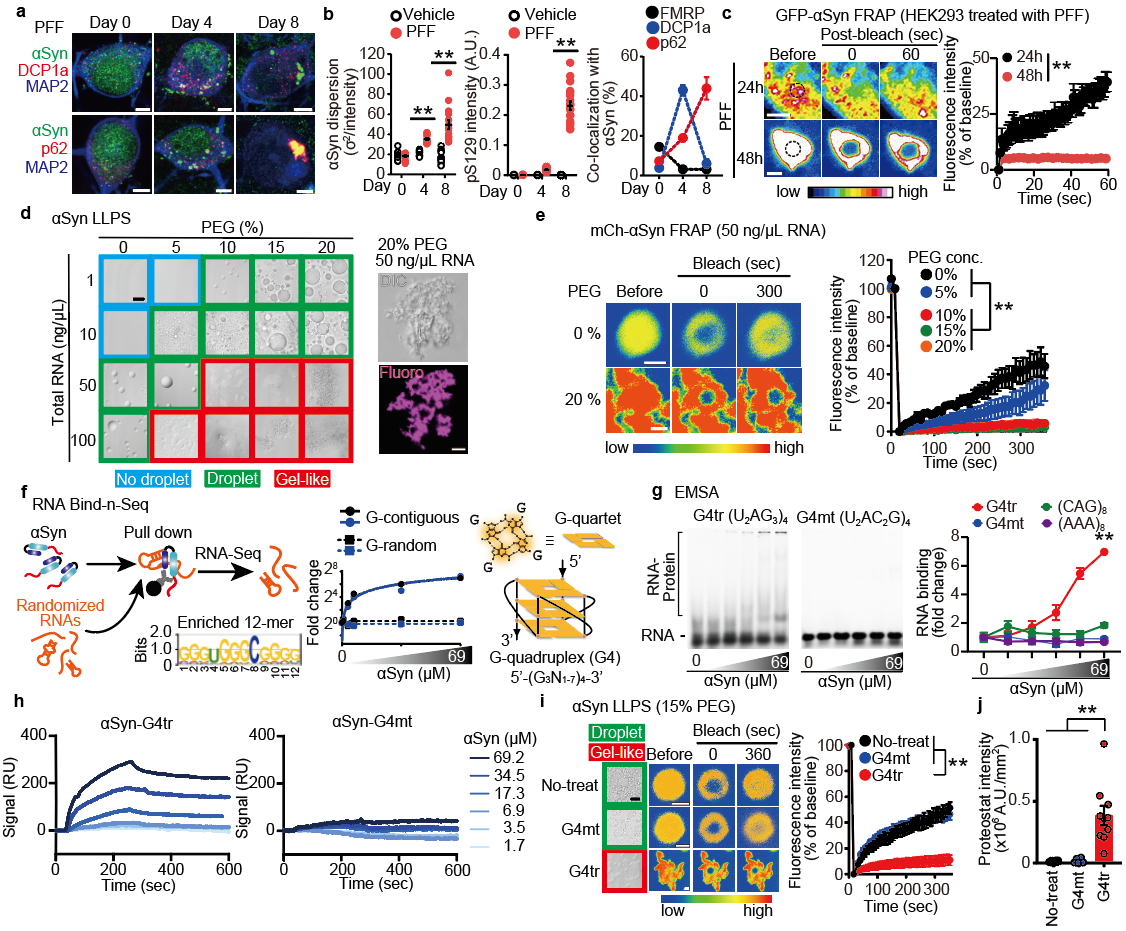

Fig. 1: RNA G-quadruplexes initiates α-synuclein (αSyn) sol–gel phase transition. **a**, Representative images of MAP2 (blue) and mouse αSyn (green) with DCP1a or p62 (red) in mouse cultured neurons following pre-formed fibril (PFF) treatment. Scale bars, 5 μm. **b**, Analyses of αSyn dispersion (left), pS129^{+} immunoreactivity relative to MAP2^{+} area (center), and co-localization of αSyn with markers of RNA granule and aggresome (right) in mouse cultured neurons after PFF treatment (images shown in **Extended Data Fig. 1b, c**). **c**, αSyn fluorescence recovery after photobleaching (FRAP) assay of PFF-treated HEK293T cells following transient expression of GFP-αSyn. Scale bars, 2 μm. **d**,**e**, *In vitro* αSyn phase separation (d) and FRAP assay (e) dependent on polyethylene glycol (PEG) and total RNA. Scale bars, 5 and 1 μm, respectively. DIC, differential interference contrast. **f**, Schematic of RNA Bind-n-Seq experiment for αSyn and the top enriched RNA motif (left). Fold enrichment of the top two 12-mers (circle marks) and two randomly chosen 12-mers (square marks) across αSyn concentrations (0, 0.69, 3.45, 6.9, 34.5, and 69 μM; center). Schematic illustration of intramolecular parallel G4 (right). **g**, electrophoresis mobility shift assay for the interaction of αSyn with RNA oligomers in the presence of 100 mM NaCl. αSyn concentrations were the same as those of RNA Bind-

n-Seq (**f**). **h**, surface plasmon resonance sensorgrams for the interaction of αSyn with G4tr or G4mt. RU, response unit. **i**, *In vitro* αSyn phase separation (left) and FRAP assay (right) with G4tr or G4mt in the presence of 15% PEG. Scale bars, 5 and 1 μm, respectively. **j**, Proteostat intensity within phase-separated RNA/αSyn in the presence of 15% PEG. Data are presented as mean ± standard error of mean. $^{**}P < 0.01$ by two-way (**b**,**c**,**e**,**g** and **i**) and one-way (**j**) Analysis of variance with Bonferroni's multiple comparisons test. Number of replicates is shown in Supplementary Table 9.

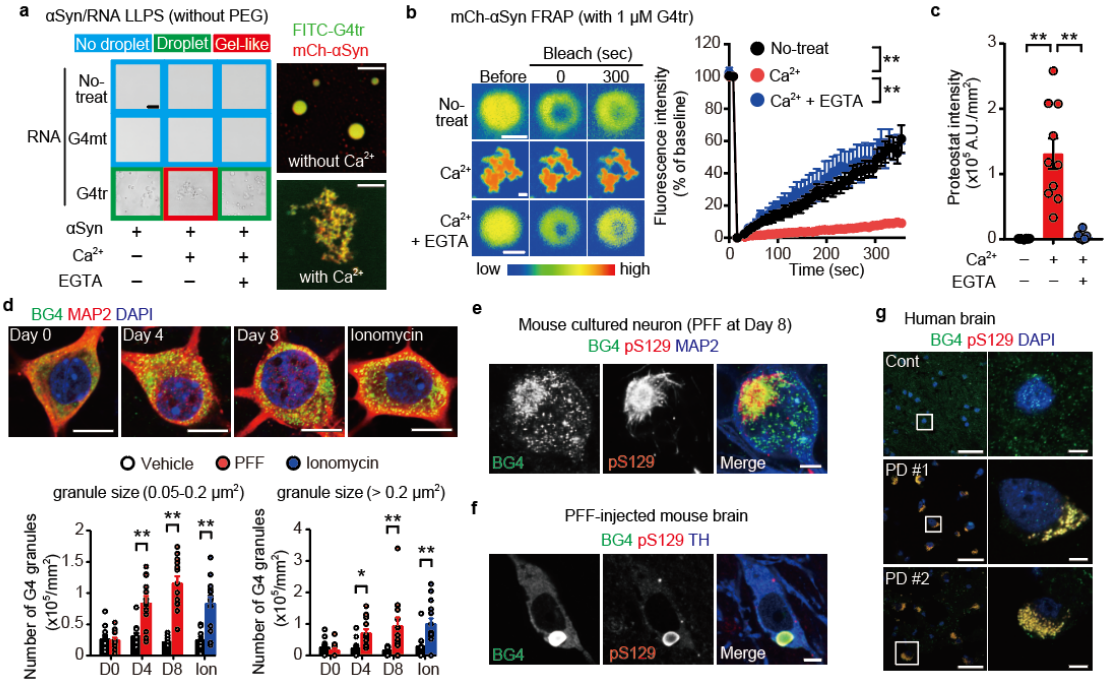

Fig. 2: Ca^{2+} triggers RNA G-quadruplexes -induced α-synuclein (αSyn) sol–gel phase transition.

a, Representative images of *in vitro* αSyn phase transition dependent on G4tr and Ca^{2+}. Scale bars, 10 (left) and 5 (right) μm, respectively. Veh., vehicle. **b**, *In vitro* αSyn fluorescence recovery after photobleaching assay dependent on G4tr and Ca^{2+}. Scale bars, 1 μm. **c**, Proteostat intensity within phase-transitioned RNA/αSyn dependent on Ca^{2+}. **d**, Representative images (top) and quantification of the number (bottom) of BG4 granules (green) in primary cultured mouse neurons treated with pre-formed fibrils (PFF) or Ionomycin (Ion; 30 min). Scale bars, 10 μm. **e–g**, Representative images of BG4 (green) and pS129 (red) with MAP2, TH, or 4',6-diamidino-2-phenylindole (blue) in PFF-treated mouse cultured neurons (**e**), PFF-injected mouse brain (**f**), and human normal control and postmortem brains of patients with Parkinson's disease (**g**). Low and high magnification scale bars, 20 and 5 μm, respectively. Data are presented as mean ± standard error of mean. $^{**}P < 0.01$ by two-way (**b**,**d** (vehicle vs. PFF)) and one-way (**c**) Analysis of variance with Bonferroni's multiple comparisons test and by two-sided, unpaired Student's *t*-test (**d** (vehicle vs. Ionomycin)). Number of replicates is shown in Supplementary Table 9.

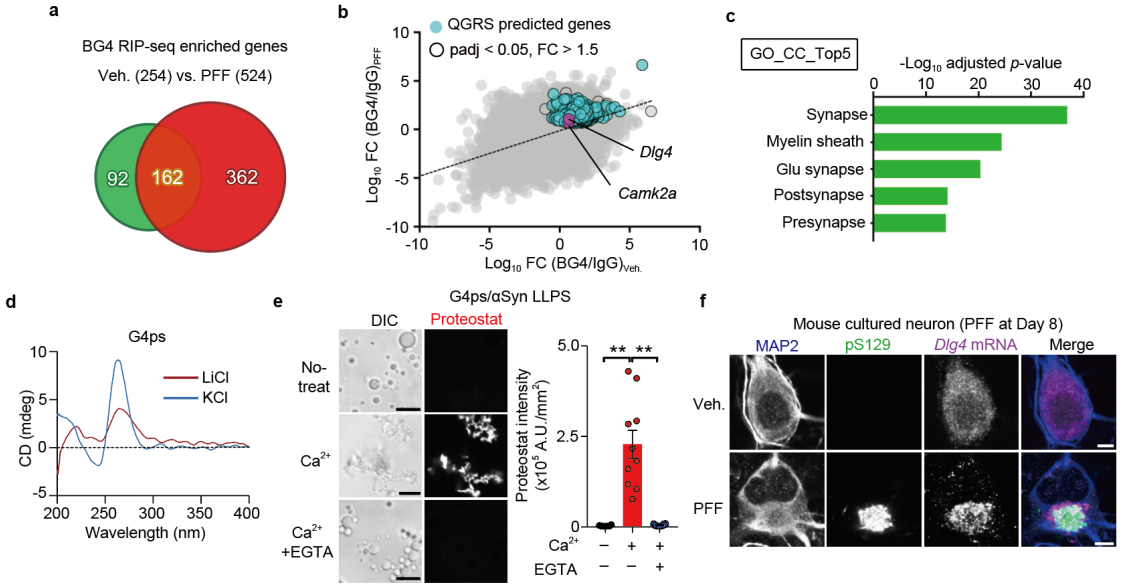

Fig. 3: RNA G-quadruplexes-containing synaptic mRNA are involved in α-synuclein (αSyn) aggregation.

a, Venn diagram of BG4-enriched RNAs in vehicle- and pre-formed fibrils (PFF)-treated mouse cultured neurons compared with immunoglobulin G (IgG) control. **b**, Scatter plots of BG4-enriched RNAs in vehicle versus PFF treatment. Open black dots show RNAs with $P < 0.05$ and fold-change (FC) > 1.5, and light blue dots exhibit predicted genes in the quadruplex-forming guanine-rich sequences (QGRS) mapper. **c**, Gene Ontology (GO) enrichment analysis by cellular component (CC) terms in BG4-enriched RNAs only detected in PFF-treated mouse cultured neurons. **d**, Circular dichroism (CD) spectra of mouse G4ps (sequence shown in **Extended Data Fig. 9c**) in the presence of KCl or LiCl. **e**, Representative images (left) and quantification (right) of Proteostat intensity within phase-transitioned G4ps/αSyn dependent on Ca^{2+}. Scale bars, 5 μm. **f**, Representative confocal images of MAP2 (blue), pS129 (green), and *Dlg4* RNA (magenta) in mouse cultured neurons following PFF treatment. Scale bars, 5 μm. Data are presented as mean ± standard error of mean. $^{**}P < 0.01$ by one-way analysis of variance with Bonferroni's multiple comparisons test. Number of replicates is shown in Supplementary Table 9.

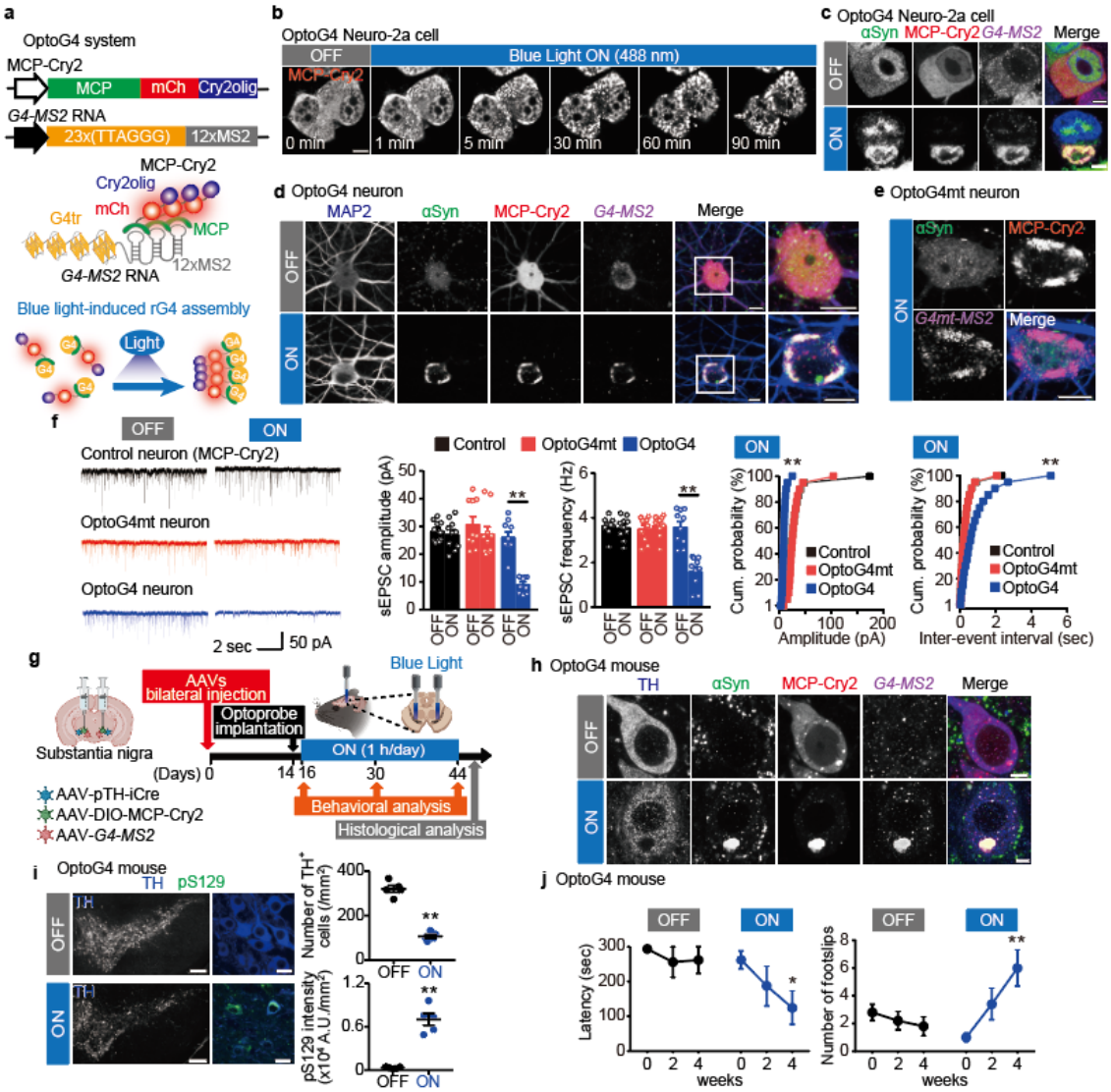

Fig. 4: OptoG4-induced α-synuclein (αSyn) aggregation triggers neurodegeneration. **a**, Schematic of the light-inducible RNA G-quadruplexes assembly approach using the Cry2olig photoreceptor, MCP protein, and 12×*MS2*-hairpin loops. **b**, Representative time-lapse images of Neuro-2a cells challenged with blue light (BL) for the indicated time under expression of optoG4. Scale bar, 20 μm. **c**, Representative images of αSyn (green), MCP-Cry2 (red), *G4-MS2* RNA (magenta), and 4',6-diamidino-2-phenylindole (blue) in optoG4 and αSyn co-expressing Neuro-2a cells with or without exposure to BL stimulation. Scale bars, 5 μm. **d**,**e**, Representative images of MAP2 (blue), αSyn (green), MCP-Cry2 (red), and *MS2* RNA fluorescent *in situ* hybridization (magenta) in optoG4 (**d**) and optoG4mt (**e**) mouse cultured neurons exposed to BL stimulation or darkness. Scale bars, 10 μm. **f**, Measurement of spontaneous excitatory postsynaptic currents (sEPSCs) in MCP-Cry2 (control)-, optoG4mt-, and optoG4-expressing mouse cultured neurons with BL stimulation. Cum., Cumulative. **g**, Schematic of viral constructs for *in*

652 *vivo* optoG4 modeling and experimental schedules. **h**, Representative images of tyrosine
653 hydroxylase (TH) (blue), αSyn (green), MCP-Cry2 (red), and *G4-MS2* RNA (magenta)
654 in the nigral dopaminergic neurons exposed to BL stimulation or darkness. Scale bars, 5
655 μm. **i,** Representative images (left) and quantification (right) of nigral TH^+ cells (blue)
656 and pS129 intensity (green) in the optoG4 mouse exposed to BL stimulation or darkness.
657 Low and high magnification scale bars, 200 and 20 μm, respectively. **j**, Motor function of
658 optoG4 mice exposed to BL stimulation or darkness in rotarod (left) and beam-walking
659 (right) tasks. Data are presented as mean ± standard error of mean. $*P < 0.05$ and $**P <$
660 0.01 by one-way (**f** sEPSC amplitude and frequency, and two-way (**f** (cumulative
661 probability of sEPSC amplitude and inter-event interval), and **j**) analysis of variance with
662 Bonferroni's multiple comparisons test and two-sided, unpaired Student's *t*-test (**i**).
663 Number of replicates is shown in Supplementary Table 9.
664

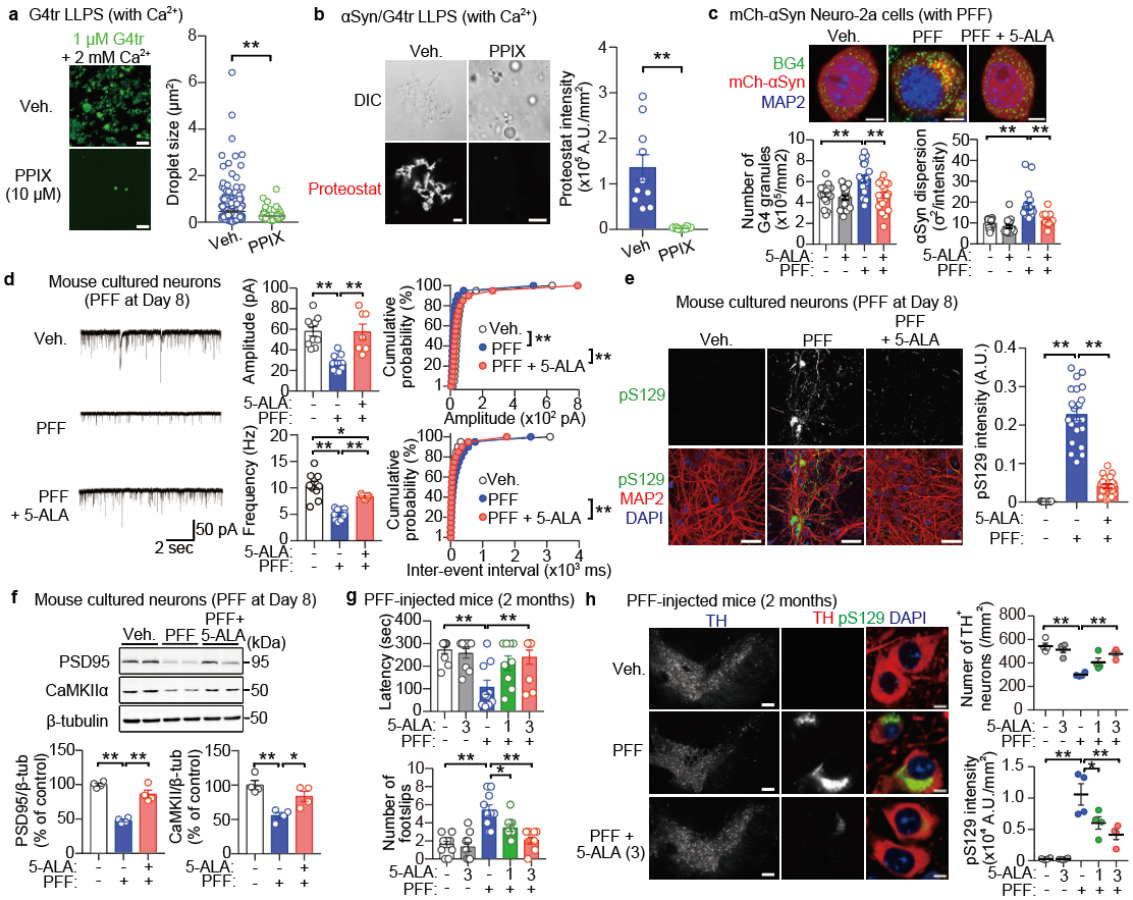

Fig. 5: G4 ligand ameliorates neuronal dysfunction by preventing α-synuclein (αSyn) aggregation.

a, Representative images (left) and quantification of droplet size (right) of *in vitro* phase-separated G4tr dependent on Ca^{2+} and the effect of protoporphyrin IX (PPIX). Scale bar, 5 μm. **b**, Proteostat intensity within *in vitro* phase-transitioned G4tr/αSyn dependent on Ca^{2+} and the effect of PPIX. Scale bar, 5 μm. **c**, Representative images (top) and quantification (bottom) of the number of G4 granules (green) and the dispersion of αSyn (red) in PFF-treated mCh-αSyn-expressing Neuro-2a cells and the effects of 3μM 5-aminolevulinic acid (5-ALA). Scale bars, 5 μm. **d**, Measurements of the spontaneous excitatory postsynaptic currents (sEPSCs) in PFF-treated mouse cultured neurons and the effects of 3 μM 5-ALA. **e**, Representative images (left) and quantification (right) of pS129 immunoreactivity (green) relative to $MAP2^{+}$ area (red) in pre-formed fibrils (PFF)-treated mouse cultured neurons and the effect of 3 μM 5-ALA. Scale bars, 20 μm. **f**, Representative images (top) and quantification (bottom) of immunoblots probed with the indicated antibodies in PFF-treated mouse cultured neurons and the effects of 5-ALA. **g**, Motor function of PFF-injected mice and the effects of 5-ALA in the rotarod (top) and beam-walking (bottom) tasks. **h**, Representative images (left) and quantification (right)

682 of the number of nigral tyrosine hydroxylase (TH^+) cells (blue) and pS129 intensity
683 (green) relative to the TH^+ area of PFF-injected mice and the effects of 5-ALA. Low and
684 high magnification scale bars, 200 and 20 μm, respectively. Data are presented as mean
685 ± standard error of mean. *P < 0.05 and **P < 0.01 by two-way (**d** (cumulative
686 probability of sEPSC amplitude and inter-event interval)) and one-way (**c**,**d**,**e** (sEPSC
687 amplitude and frequency),**f**,**g**) analysis of variance with Bonferroni's multiple
688 comparisons test and by unpaired two-sided, unpaired Student's *t*-test (**a**,**b**). Number of
689 replicates is shown in Supplementary Table 9.
690

691 **Methods**

692 **Animals**

693 C57BL/6J mice (Japan SLC) were used for all the experiments. Mice were
694 housed under climate-controlled conditions in a 12-hour light/12-hour dark cycle and
695 were provided standard food and water *ad libitum*. Animal studies were conducted in
696 accordance with the Kumamoto University institutional guidelines. Ethical approval was
697 obtained from the Institutional Animal Care and Use Committee of the Kumamoto
698 University Environmental and Safety Committee.

699

700 **Cell culture**

701 Neuro-2A and HEK293T cell lines were grown in Dulbecco's modified essential
702 medium (Sigma-Aldrich) containing 10% fetal bovine serum (FBS; Gibco), 100 units/mL
703 penicillin, and 100 μg/mL streptomycin at 5% CO_2 and 37°C. Transfection was performed
704 using the Lipofectamine 3,000 Reagent (Invitrogen) according to the manufacturer's
705 protocol. Primary cultures of mouse neurons were performed as previously described[9].
706 Briefly, cortical brain tissue was dissected from 18 days old embryonic mice, trypsin-
707 digested, and mechanically dispersed. Cells were seeded with minimum essential medium
708 (Gibco) supplemented with 10% FBS, 0.6% glucose (FUJIFILM), and 1 mM pyruvate
709 (Sigma-Aldrich). Cells were cultured in Neuron Culture Medium (FUJIFILM) with
710 medium changed every three days. Transfection was performed by electroporation
711 (NEPAGENE) or Magnetofection (OZ Biosciences) according to the manufacturer's
712 protocol.

713

714 **Plasmid constructs**

715 For protein purification, αSyn constructs with mouse coding sequence (CDS),
716 mutations, and CDS of fluorescent proteins were subcloned into pET-human αSyn[51] using
717 the KOD-Plus Mutagenesis kit (TOYOBO) and In-Fusion HD Cloning Kit (Takara Bio)
718 according to the manufacturers' protocol. For preparing the mammalian expression
719 plasmids, pCAG-GFP[9], pHR-FUSN-mCh-Cry2WT (Addgene #101223), and MCP-YFP
720 (Addgene #101160) were used as backbones. Repeat sequences of $(TTAGGG)_{23}$ and
721 $(TTACCG)_{23}$ were synthesized by GeneScript and subcloned into pHR-Tre3G-
722 29×GGGGCC-12×MS2 (Addgene #99149) using Ligation high (TOYOBO). The
723 fragments with MS2 were inserted in the 3'-untranslated region of pCAG-HaloTag vector[9].
724 For recombinant AAV production, pAAV-TH-iCre, pAAV-hSyn-DIO-MCP-Cry2, pAAV-
725 hSyn-G4tr-MS2, and pAAV-hSyn-G4mt-MS2 were synthesized by GeneScript, using
726 pAAV-hSyn-DIO{ChETA-mRuby2}on-W3SL (Addgene #111389) as a backbone.

727
728 **Antibodies**
729 The primary and secondary antibodies for immunoblotting and
730 immunohistochemical analyses utilized in the present study are shown in the
731 Supplementary Method.
732
733 **Preparation of recombinant proteins**
734 pET-αSyn plasmids were transformed into *Escherichia coli* BLR (DE3) cells and
735 purified as described previously[52]. Briefly, bacterial lysate by ultrasonication was heated
736 (75°C, 15 min) and precipitated by ammonium sulfate. The dialysate was fractionated by
737 ion exchange chromatography (Resource-Q or HiTrap SP HP; GE Healthcare) on an
738 AKTA-explorer system (GE Healthcare) at 4°C. Samples were desalted, lyophilized, and
739 stored at 4°C until the assay was conducted.
740
741 **Preparation of αSyn PFF**
742 αSyn PFF was prepared as previously described[51]. Briefly, recombinant αSyn (5
743 mg/mL in 1× phosphate buffer saline (PBS)) was centrifuged for an hour at 100,000g at
744 4°C and the supernatants were shaken in an orbital thermomixer at 1,000 rpm for seven
745 days. αSyn PFFs were sonicated by Bioruptor (Cosmo Bio) for eight cycles at high power
746 (on, 1-pulse every other second for 15 s in total; off, 15 s; at 23°C), and then stored at -
747 80°C until use.
748
749 **AAV preparation**
750 Recombinant AAV9 vectors were generated by co-transfection of AAVpro 293T
751 cells (Takara Bio) with three plasmids: pAAV, pHelper (Stratagene), and pAAV2/9 (a gift
752 from James M. Wilson). The viral particles were purified from transfected cells and
753 titrated using the AAVpro Purification Kit Maxi and AAVpro Titration Kit (Takara Bio)
754 according to the manufacturer's instructions.
755
756 **Treatment with αSyn PFF and other compounds in cultured cells**
757 Neuro-2a or HEK293T cells expressing mCh- or GFP-human αSyn were treated
758 with human αSyn PFF at 50 μg/mL (3.45 μM) for the indicated time. Mouse cultured
759 neurons at DIV10 were incubated with mouse or human αSyn PFF at 50 μg/mL for the
760 indicated time, some of which were further treated with 5 μM Calbryte 520AM (AAT
761 Bioquest) for 30 min. Several mouse cultured neurons were treated with 0.5 mM $NaAsO_2$
762 (Sigma-Aldrich) for 60 min or 1.0 μM ionomycin (AdipoGen Life Sciences) for 30 min

763 on DIV14.
764
765 **Intracellular Ca^{2+} imaging**
766 αSyn PFF- and Calbryte 520AM-treated mouse cultured neurons were subjected
767 to measurement of intracellular Ca^{2+} levels using LSM900 microscopy system (Carl
768 Zeiss) in an imaging chamber (KYODO INTERNATIONAL).
769
770 **Stereotaxic injection of αSyn PFF and AAV**
771 Mouse αSyn PFFs were injected into the bilateral dorsal striatum (2
772 μg/hemisphere) at the following coordinates (in mm): anterior, +0.7; lateral, ±2.5; and
773 depth, -2.8 relative to the bregma. Viral particles of 1-μL with the same titer (1.4×10^{10}
774 vector genomes/mL) were injected into the bilateral substantia nigra at the following
775 coordinates (in mm): anterior, -3.5; lateral, ±1.2; and depth, -4.0 relative to the bregma.
776 αSyn PFFs and AAV were injected at 0.2 μL/min using a Hamilton syringe under 1%
777 isoflurane anesthesia.
778
779 **FRAP assay in living cells**
780 GFP-αSyn-transfected and αSyn PFF-transduced HEK293T cells were subjected
781 to FRAP assay in the imaging chamber. Fluorescent images were obtained using the
782 LSM900 microscopy system. Photobleaching was performed with 50% laser power with
783 20 iterations. Time-lapse images were recorded every 250 ms at room temperature.
784
785 ***In vitro* LLPS, Proteostat, and FRAP assay**
786 Non-labeled or mCh-labeled human αSyn (34.5 or 69 μM) was prepared in the
787 LLPS assay buffer: 50 mM Tris-HCl buffer (pH 7.5) containing 140 mM KCl, 15 mM
788 NaCl, 10 mM $MgCl_2$, 0–20% PEG8000 (MP Biomedicals), and 1 unit/μL RNase inhibitor
789 (TOYOBO) in the presence or absence of 2 mM $CaCl_2$ and 2.5 mM EGTA. Total protein,
790 total RNA, or synthesized 24-mer RNA oligomer (1 μM; Hokkaido System Science) was
791 added and incubated at 37°C for three hours. Total protein was lysed by homogenization
792 in radioimmunoprecipitation (RIPA) buffer (FUJIFILM) containing protease inhibitor
793 cocktail (NACALAI TESQUE) and Cryonase Cold-active Nuclease (TAKARA), while
794 total RNA was extracted using an RNeasy Mini Kit (Qiagen), both from intact Neuro-2a
795 cells. The resultant solutions were mounted on glass slides using a 0.12 nm spacer
796 (Sigma-Aldrich) and a coverslip. In some conditions, 2% (v/v) Proteostat reagent (Enzo
797 Life Sciences) was added into the LLPS assay buffer to detect aggregates. Differential
798 interference contrast images were obtained using the TCS SP8 confocal microscopy

799 system (Leica Microsystems). Samples were photobleached with 50% laser power and
800 time-lapse images were recorded every 8-s using the LSM780 microscopy system.
801
802 **Circular dichroism (CD) spectroscopy**
803 G4tr $(UUAGGG)_4$ and G4mt $(UUACCG)_4$ were prepared using an *in vitro* the
804 HiScribe T7 Quick High Yield RNA Synthesis Kit (New England Biolabs) following the
805 manufacturers' instructions, respectively. Other RNA oligomers were synthesized by
806 Hokkaido System Science. Each RNA (2.5 μM) was prepared in 10 mM Tris-HCl buffer
807 (pH 7.5) containing 100 mM LiCl or KCl. The oligomers were then refolded by a
808 heating/cooling process (90°C for 3 min, followed by cooling down to 10°C for 1.5 hours)
809 before measurement. The CD spectra were recorded at room temperature over the range
810 of 200–350 nm using a spectrometer (J-805LST; JASCO) equipped with a 3 mm path-
811 length quartz cuvette.
812
813 **EMSA**
814 Fluorescein phosphoramidite (FAM)-labeled RNAs (20 nM) in 10 mM Tris-HCl
815 buffer (pH 7.5) containing 100 mM NaCl were refolded as described above. The folded
816 RNA samples were incubated with a human αSyn (0–138.4 μM) or BG4 (320 nM) with
817 1 unit/μL RNase inhibitor (TOYOBO) at room temperature for at least 30 min. The
818 resulting mixtures were analyzed by 6% native TBE polyacrylamide gel electrophoresis
819 and visualized using Typhoon Trio equipment (GE Healthcare).
820
821 **SPR-binding assays**
822 SPR experiments were performed on OpenSPR (Nicoya). After biotin-labeled
823 G4tr or G4mt was captured by a sensor chip using biotin-streptavidin sensor kit (Nicoya),
824 αSyn in running buffer (50 mM Tris-HCl pH 7.5 and 10 mM KCl) was streamed over the
825 sensor chip to allow interaction. The interaction time was at least 5 min to allow the ligand
826 signal to stabilize. The running buffer was then flowed at a rate of 20 μL/min for 5 min
827 to collect the dissociation data. Binding kinetic parameters were obtained by fitting the
828 curve to a One-to-One binding model using TraceDrawer software (Ridgeview
829 Instruments).
830
831 **RNA Bind-n-Seq**
832 RNA Bind-n-Seq was performed as previously described[30]. Briefly, the purified
833 αSyn (0–69 μM) and BG4 (0.32 μM) were equilibrated in binding buffer (25 mM Tris-
834 HCl pH 7.5, 150 mM KCl, 3 mM $MgCl_2$, 0.01% Tween 20, 1 mg/ml bovine serum

835 albumin (BSA), and 1 mM dithiothreitol) with 1 units/μL RNase inhibitor (TOYOBO)
836 for 30 min at room temperature. 24-mer random RNAs of 1 μM (Hokkaido System
837 Science) were added to the solutions and incubated for an hour at 4°C. During the
838 incubation, Dynabeads Protein G (Invitrogen) was washed with wash buffer (25 mM Tris-
839 HCl pH 7.5, 150 mM KCl, 60 μg/mL BSA, 0.5 mM EDTA, and 0.01% Tween 20) and
840 then equilibrated in binding buffer including 1 units/μL RNase inhibitor with anti-αSyn
841 or anti-DYKDDDDK tag antibody until use. The protein/RNA complex was pulled down
842 by the beads and incubated for an hour at room temperature. Supernatant was removed
843 and the beads were washed once. The beads were incubated at 70°C for 10 min in elution
844 buffer (10 mM Tris-HCl pH 7.0, 1% sodium dodecyl sulfate (SDS), and 1 mM EDTA).
845 Bound RNA was extracted and reverse-transcribed into cDNA using Superscript III
846 (Invitrogen) according to the manufacturer's instructions together with the following
847 primer: 5'-GCCTTGGCACCCGAGAATTCCA-3'. To control for any nucleotide biases
848 in the input random library, the input RNA pool (0.5 pmol) was also reverse-transcribed
849 and Illumina sequencing library prep was followed by 8–10 cycles of polymerase chain
850 reaction (PCR) using High Fidelity Phusion (New England Biolabs). All the libraries were
851 barcoded in the PCR step, pooled together, and sequenced on a NextSeq 500 sequencer.
852 Motif enrichment analyses were performed by the MERMADE program[53]. Base contents,
853 GC contents, and GC skew were calculated by the SeqKit program[54]. The motifs were
854 processed by enoLOGOS21 (http://www.benoslab.pitt.edu/cgi-
855 bin/enologos/enologos.cgi), and putative G4 sequences were predicted using QuadBase2
856 (https://quadbase.igib.res.in/).
857
858 **RIP-seq**
859 Immunoprecipitation of RNPs was performed using a RiboCluster Profiler RIP-
860 Assay kit for microRNA (MBL) according to the manufacturer's protocol. Cells were
861 harvested, lysed, and precleared on Dynabeads Protein G for an hour at 4°C. The
862 precleared lysates were incubated at 4°C for three hours in the beads immobilized with
863 normal rabbit IgG, anti-αSyn, or BG4 with anti-DYKDDDDK tag antibody. Each fraction
864 containing protein-bound RNA was purified and prepared for RNA-sequencing library
865 using NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (New England
866 Biolabs). All the libraries were sequenced on a NextSeq 500 sequencer (Illumina). Fastq
867 files were processed for quality check and adapter trimming with the Trimgalore! (v0.6.6).
868 Trimmed reads were used for mapping the mouse genome (mm10) using the STAR
869 (v2.7.9a). DESeq2(v1.38.3) was used for identifying BG4-enriched transcripts. Putative
870 rG4-forming motifs of mRNAs were predicted using the QGRS mapper

871 (http://bioinformatics.ramapo.edu/QGRS/index.php). GO analysis was performed using
872 DAVID Bioinformatics Resources (https://david.ncifcrf.gov/home.jsp).
873
874 **Photoactivation by exposure of BL stimulation**
875 For live cell imaging, OptoG4-expressing Neuro-2a cells were placed in the
876 imaging chamber and imaged typically with two laser wavelengths (488 nm for Cry2olig
877 photoactivation/560 nm for mCh imaging) using the LSM780 microscopy system.
878 Cry2olig photoactivation was induced at 5% power and time-lapse acquisitions were
879 performed every 20 s at room temperature. For electrophysiological and
880 immunohistochemical analyses, cells were photoactivated using 470 nm blue light-
881 emitting diode (LED) light (Bio Research Center) with an estimated light intensity of
882 1.0 mW/cm^2. For *in vivo* photoactivation, mice were implanted with dual-LED optic
883 cannulae at 470 nm (Bio Research Center) into the bilateral substantia nigra two weeks
884 following AAV injection. Daily photoactivation was performed using Teleopto, a wireless
885 optogenetic stimulation system (Bio Research Center).
886
887 **Drugs**
888 PPIX was purchased from Sigma-Aldrich. 5-ALA hydrochloride and sodium
889 ferrous citrate were provided by SBI Pharmaceuticals. PPIX (10 μM) was added to the
890 LLPS assay buffer. Cultured cells were co-treated with PFF and 5-ALA (3 μM with
891 sodium ferrous citrate [20:1 molar ratio] dissolved in sterilized distilled water) or vehicle
892 (sodium ferrous citrate). Animals were orally administered with vehicle or 5-ALA (1 or
893 3 mg/kg/day) with sodium ferrous citrate 24 hours following αSyn PFF injection daily
894 for two months.
895
896 **Electrophysiology**
897 sEPSC was recorded at room temperature for mouse cultured neurons on DIV14
898 (optoG4) or DIV18 (αSyn PFF) using an EPC10 amplifier (HEKA Instruments) as
899 previously described[9]. The following buffers were used in the present study: extracellular
900 buffer (143 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM glucose, and
901 10 mM HEPES at pH 7.4 adjusted with NaOH) and intracellular buffer (135 mM CsMeS,
902 5 mM CsCl, 0.5 mM EGTA, 1 mM $MgCl_2$, 4 mM Mg_2ATP, 0.4 mM NaGTP, 5 mM QX-
903 314 and 10mM HEPES at pH 7.4 adjusted with CsOH). Recording pipettes had a
904 resistance of 3.5–4.5 MΩ when filled with the intracellular buffer. The sEPSC was
905 recorded for 2 min at a holding potential of -70 mV in the presence of 10 μM bicuculline
906 in the extracellular buffer. Recordings were filtered at 2 kHz and digitized at 10 kHz. Data

907 were collected and initially analyzed using Patchmaster software (HEKA). Further
908 analysis was performed using SutterPatch v2.2 (Sutter Instrument).
909
910 **Immunohistochemical analysis**
911 Immunohistochemistry was performed as previously described[9]. Cells and
912 mouse brain slices (50 μm thickness) were fixed with 4% paraformaldehyde, washed with
913 1× PBS, permeabilized with 0.1–0.3% Triton X-100, and incubated with 1% BSA and
914 0.1–0.3% Triton X-100 in 1× PBS. Human frozen brain sections (5–10 μm thickness)
915 were obtained from BioChain Institute and a part of them were treated with RNase A. An
916 hour after blocking, samples were treated with primary antibodies for 1–3 days at 4°C.
917 After incubation with fluorophore-conjugated secondary antibodies, samples were
918 mounted in Vectashield (Vector Laboratories). Some samples were counterstained with
919 4',6-diamidino-2-phenylindole. Immunofluorescence images were obtained using
920 LSM900 or TCS SP8 confocal microscopy system.
921
922 **Fluorescent *in situ* hybridization (FISH)**
923 FISH was performed as previously described[9]. After permeabilization as
924 described above, the samples were prehybridized with hybridization solution (2× saline-
925 sodium citrate (SSC), 40% formamide, 10% dextran sulfate, 2 mM vanadyl sulfate, 0.5
926 mg/mL yeast transfer RNA) for 60 min at 37 (*MS2*) or 60°C (*Camk2a* and *Dlg4*), and
927 then incubated with 1 nM (*MS2*) or 0.3 μM (*Camk2a* and *Dlg4*) probe in a hybridization
928 solution at 37 or 60°C overnight. After hybridization, samples were washed and subjected
929 to immunofluorescence procedures and images were collected as described above. FISH
930 probe sequences are shown in the Supplemental Method.
931
932 **Western blotting analysis**
933 Western blot analyses were performed as previously described[9]. Briefly, primary
934 mouse cultured neurons were homogenized in RIPA buffer with a protein inhibitor
935 cocktail. The lysate was centrifuged and protein concentration of the supernatant was
936 determined by BCA method, and samples were then boiled for 3 min in Laemmli's sample
937 buffer. Equal amounts of protein were loaded onto SDS-polyacrylamide gels. After
938 transferring the separated proteins to polyvinylidene difluoride membranes, the
939 membranes were blocked with 5% non-fat skim milk and incubated with primary
940 antibody at 4°C overnight. Blots were developed using an ECL Western Blotting
941 Substrate (Thermo Scientific), and the signals were detected using LAS-4000mini (GE
942 Healthcare).

943

944 **Quantitative PCR with reverse transcription (RT-qPCR)**

945 Sample preparation for RT-qPCR from primary cultured neurons was performed
946 using the RNeasy Mini Kit and PrimeScript RT Master Mix (Takara Bio). RT-qPCR was
947 performed using KOD SYBR qPCR Mix (TOYOBO) on a CFX Connect Real-Time PCR
948 System (Bio-Rad Laboratories). Gene expression was assessed using the differences in
949 normalized Ct (cycle threshold) ($^{\Delta\Delta}$Ct) method after normalization to *Gapdh* expression.
950 Fold change was calculated by $2^{-\Delta\Delta Ct}$. PCR primers are shown in the Supplemental
951 Method.

952

953 **Behavioral analysis**

954 The rotarod and beam-walking tasks were performed according to a previous
955 report[51]. Briefly, mice were trained before the stereotaxic injection. In the rotarod task,
956 mice were placed on the rod rotating at 20 rpm and falling latency was measured for up
957 to 5 min. In the beam-walking task, the number of foot-slips from the end of beam to goal
958 box was recorded.

959

960 **Statistical analysis**

961 Data are presented as the mean ± standard error of the mean. Comparisons
962 between two experimental groups were made using unpaired Student's t-test. Statistical
963 significance for differences among groups was tested by one-way or two-way analysis of
964 variance with *post-hoc* Bonferroni's multiple comparison test. $P < 0.05$ represented a
965 statistically significant difference. All the statistical analyses were performed using
966 GraphPad Prism 7 (GraphPad Software). Source data are provided in Supplementary
967 Table 9.

968

969 **Methods references**

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983

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Author Contributions

N.S. and Y.Y. designed the study. K.Matsuo, S.A., K.Maeda, K.Kawakubo, G.K., K.Kudo, Y.S., K.H., S.I., S.U., S.F., and Y.Y. performed the experiments. Y.K. and T.M. provided critical advice for αSyn synthesis and analysis of the interaction between rG4 and αSyn. K.Matsuo, N.S., and Y.Y. wrote the manuscript. N.S. and Y.Y. supervised the project.

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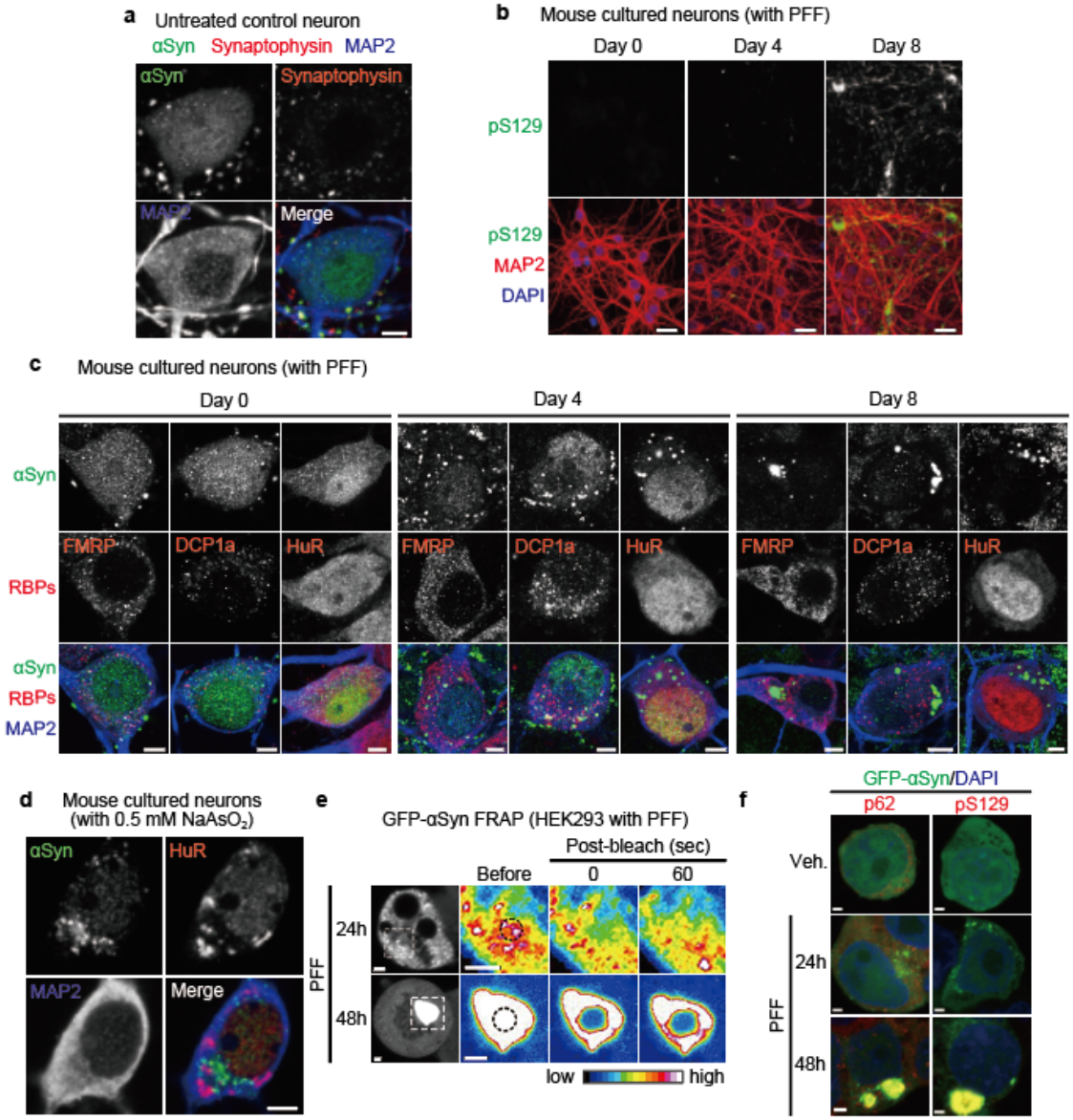
Competing interests

The authors declare that they have no competing interests.

Data Availability

The raw data of RNA-Bind-n-Seq and RIP-Seq analyses are available at Gene Expression Omnibus (accession number: GSE235418 and GSE234238, respectively). Additional data related to this paper may be requested from the corresponding author N.S. and Y.Y. upon reasonable request.

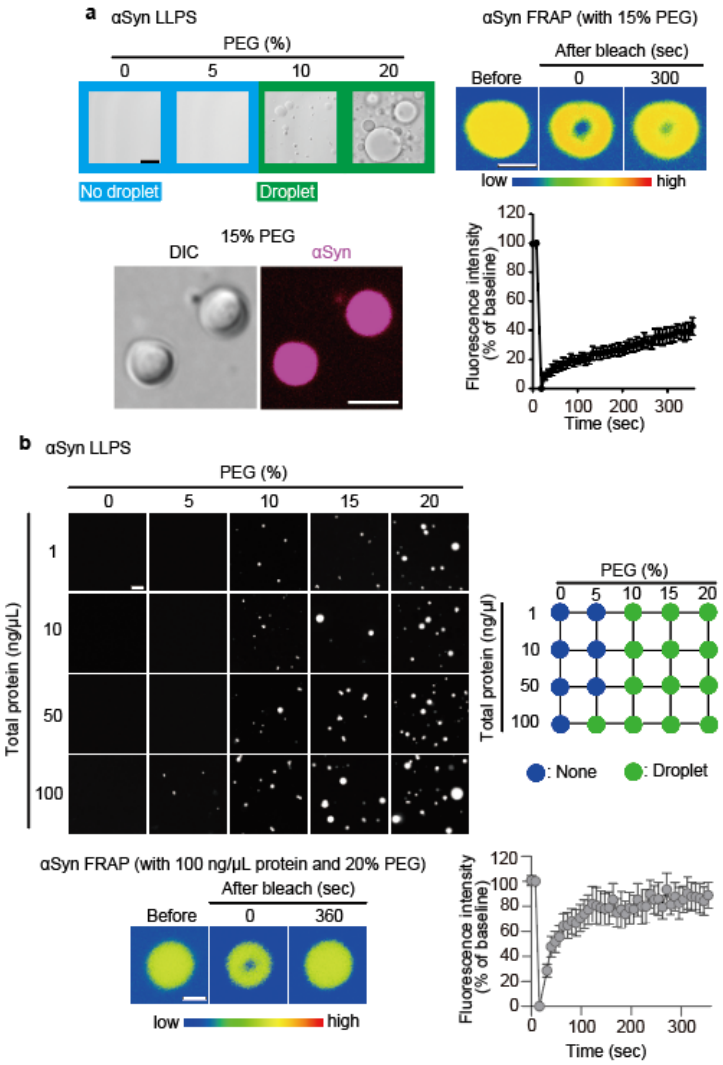
Supplementary Information is available for this paper



Extended Data Fig. 1: Localization and dynamics of α-synuclein (αSyn) under cellular stress.

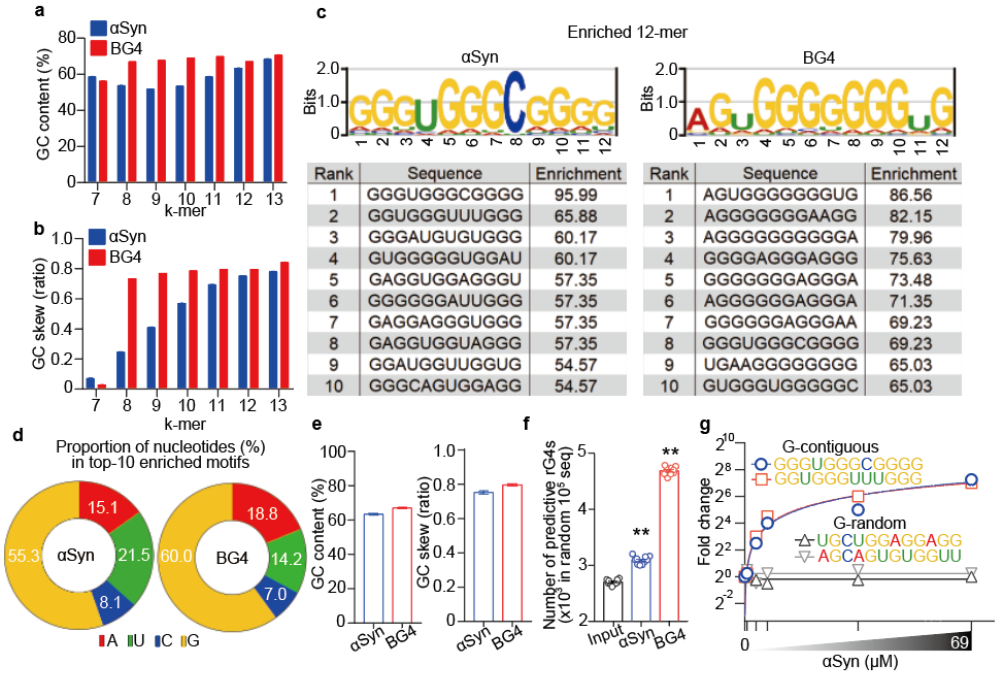
a, Representative images of αSyn (green), synaptophysin (red), and MAP2 (blue) in naïve mouse cultured neurons. Scale bar, 5 μm. **b**, Representative images of pS129 (green), MAP2 (red), and 4',6-diamidino-2-phenylindole (DAPI) (blue) in mouse cultured neurons following pre-formed fibrils (PFF) treatment for the indicated time. Scale bars, 20 μm. **c**, Representative images of αSyn (green), RNA binding proteins (red), and MAP2 (blue) in mouse cultured neurons after PFF treatment for the indicated time. Scale bars, 5 μm. **d**, Representative images of αSyn (green), HuR (red), and MAP2 (blue) in mouse cultured neurons after treatment with 0.5 mM $NaAsO_2$ for 60 min. Scale bar, 5 μm. **e,** Representative images of αSyn fluorescence recovery after photobleaching assay in PFF-

1051 treated GFP-αSyn expressing HEK293T cells. Scale bars, 2 μm. **(f)**, Representative
1052 images of p62 or pS129 (red) and DAPI (blue) in HEK293T cells treated with PFF
1053 following GFP-αSyn overexpression (green). Scale bars, 2 μm.
1054

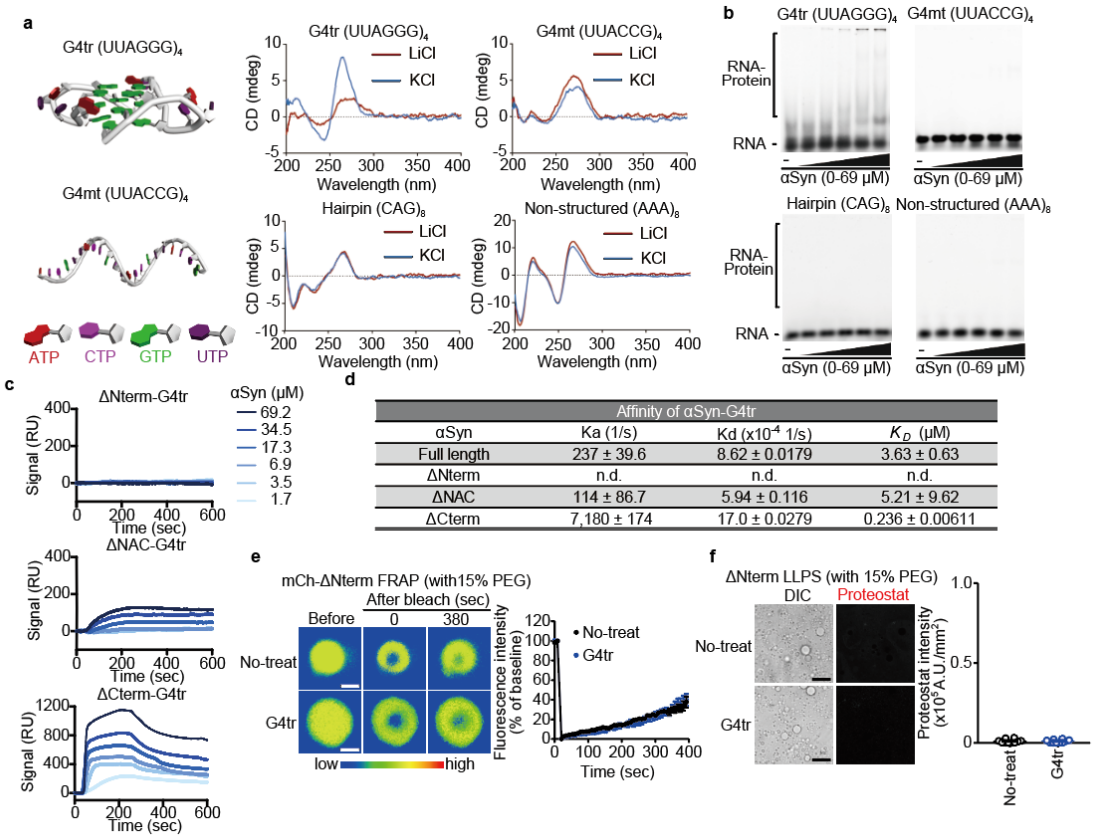


Extended Data Fig. 2: *In vitro* α-synuclein (αSyn) phase separation in the presence of a molecular crowding agent.

a, *In vitro* αSyn phase separation (left) and fluorescence recovery after photobleaching (FRAP) assay (right) dependent on polyethylene glycol (PEG). Scale bars, 5 (Differential interference contrast image) and 2 (Fluorescent images) μm, respectively. **b**, *In vitro* αSyn phase separation (top) and FRAP assay (bottom) dependent on PEG and total protein extracted from Neuro-2a cells. Data are presented as mean ± standard error of mean. n = 7 (**a**) and n = 7 (**b**).



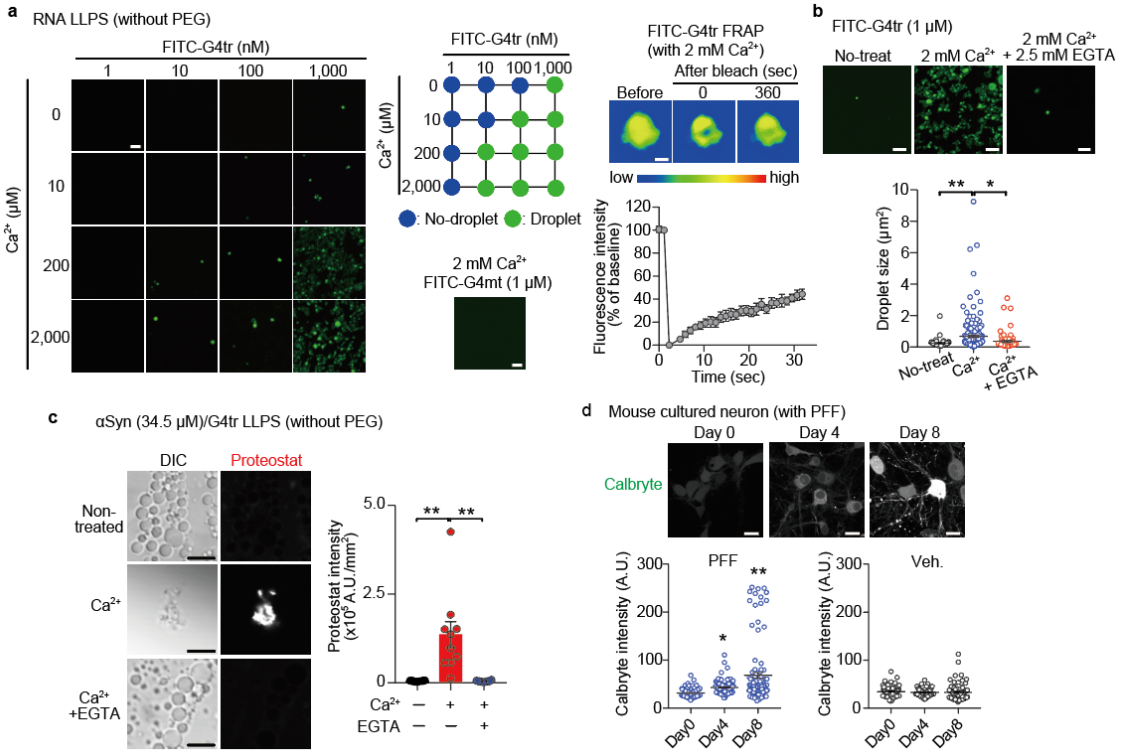
Extended Data Fig. 3: Analysis of RNA Bind-n-Seq for α-synuclein (αSyn). **a**,**b**, GC contents (**a**) and GC skew (**b**) in *k*-mer sequences in enriched motifs for αSyn and BG4. **c**–**e**, The top 10 motifs enriched in αSyn and BG4 (**c**), the proportion of nucleotides (**d**), and the GC contents and GC skew (**e**). **f**, Number of sequences with predicted rG4 structures enriched in αSyn and BG4. **g**, Fold enrichment of the top two 12-mers (circle and square marks) and two randomly chosen 12-mers (triangle marks) across αSyn concentrations (see legends in **Fig. 1f**). Data are presented as mean ± standard error of mean. $^{**}P < 0.01$ by one-way analysis of variance with Bonferroni's multiple comparisons test. Number of replicates is shown in Supplementary Table 9.



Affinity of αSyn-G4tr			
αSyn	Ka (1/s)	Kd (x10^{-4} 1/s)	K_D (μM)
Full length	237 ± 39.6	8.62 ± 0.0179	3.63 ± 0.63
ΔNterm	n.d.	n.d.	n.d.
ΔNAC	114 ± 86.7	5.94 ± 0.116	5.21 ± 9.62
ΔCterm	7,180 ± 174	17.0 ± 0.0279	0.236 ± 0.00611

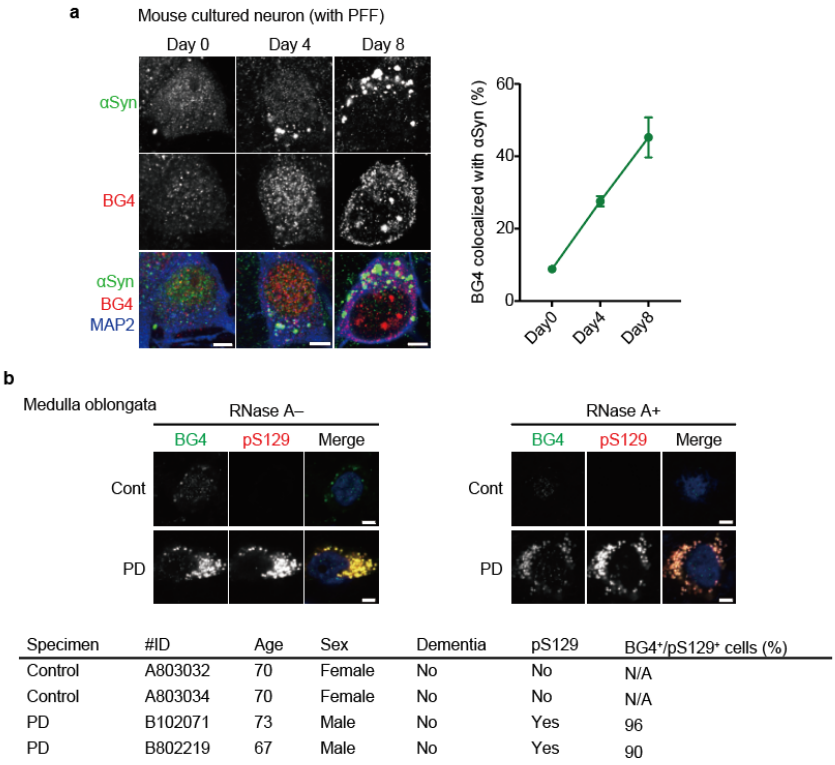
Extended Data Fig. 4: Physicochemical and biochemical analyses of RNA structures interacting to α-synuclein (αSyn).

a, Schematic illustration of G4tr and G4mt (left). Circular dichroism spectra of the indicated RNAs in the presence of 100 mM KCl or LiCl (right). **b**, Representative images of electrophoresis mobility shift assay for the interaction of αSyn (0, 0.69, 3.45, 6.9, 34.5, and 69 μM μM) with Fluorescein phosphoramidite-labeled G4tr, G4mt, $(CAG)_8$, or $(AAA)_8$ oligomers (20 nM) in the presence of 100 mM NaCl. **c**,**d**, Representative surface plasmon resonance binding curves (**c**) and the detailed parameters (**d**) related to the interaction of G4tr with αSyn ΔNterm, ΔNAC, and ΔCterm. K_D, dissociation constant; Ka, association rate constant; Kd, dissociation rate constant; n.d., not detected. **e**,**f**, *In vitro* G4tr/αSyn ΔNterm fluorescence recovery after photobleaching assay (**e**) and Proteostat intensity within the phase-separated droplets (**f**) dependent on 15% PEG. Scale bars, 1 (**e**) and 10 (**f**) μm, respectively. Data are presented as mean ± standard error of mean. Number of replicates is shown in Supplementary Table 9.



Extended Data Fig. 5: Ca^{2+}-dependent G4tr assembly promotes α-synuclein (αSyn) sol–gel phase transition.

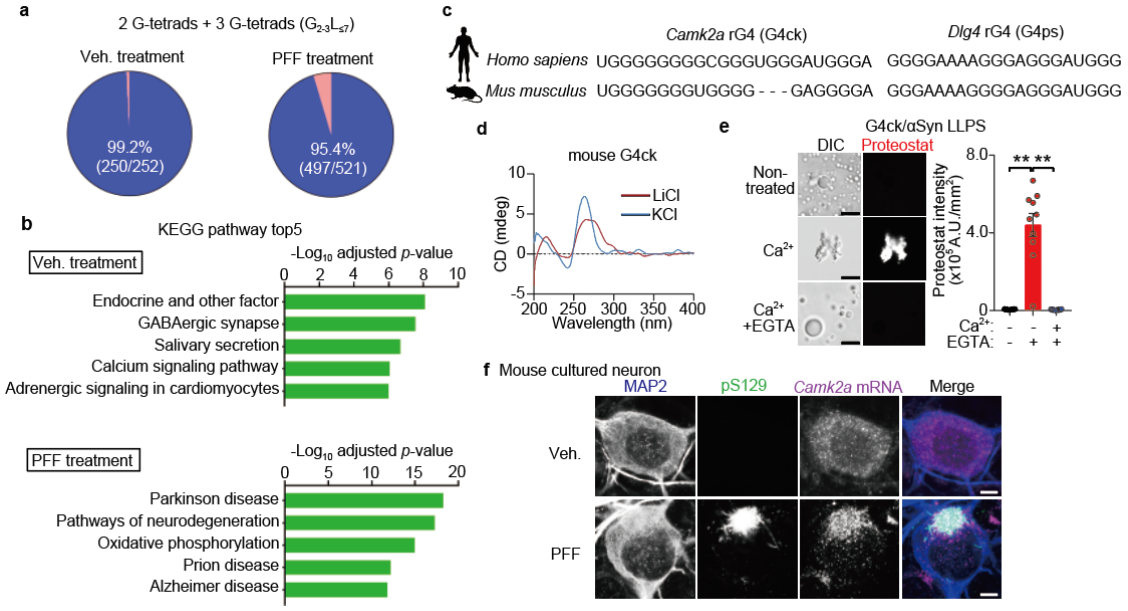
a, *In vitro* G4tr and G4mt phase separation (left, center) and fluorescence recovery after photobleaching (FRAP) assay (right) dependent on Ca^{2+}. Scale bars, 5 μm. n = 5 (FRAP). **b**, Representative images (top) and quantification of droplet size (bottom) of *in vitro* phase-separated G4tr dependent on Ca^{2+} and the effect of EGTA. Scale bars, 5 μm. **c**, Representative images (left) and quantification (right) of Proteostat intensity within phase-transitioned G4tr/αSyn dependent on Ca^{2+}. Scale bars, 5 μm. **d**, Representative images (top) and quantification (bottom) of Calbryte 520AM intensity in mouse cultured neurons after PFF treatment for the indicated time. Scale bars, 20 μm. Data are mean ± s.e.m. $*P < 0.05$ and $**P < 0.01$ by one-way analysis of variance with Bonferroni's multiple comparisons test. Number of replicates is shown in Supplementary Table 9.



Specimen	#ID	Age	Sex	Dementia	pS129	BG4⁺/pS129⁺ cells (%)
Control	A803032	70	Female	No	No	N/A
Control	A803034	70	Female	No	No	N/A
PD	B102071	73	Male	No	Yes	96
PD	B802219	67	Male	No	Yes	90

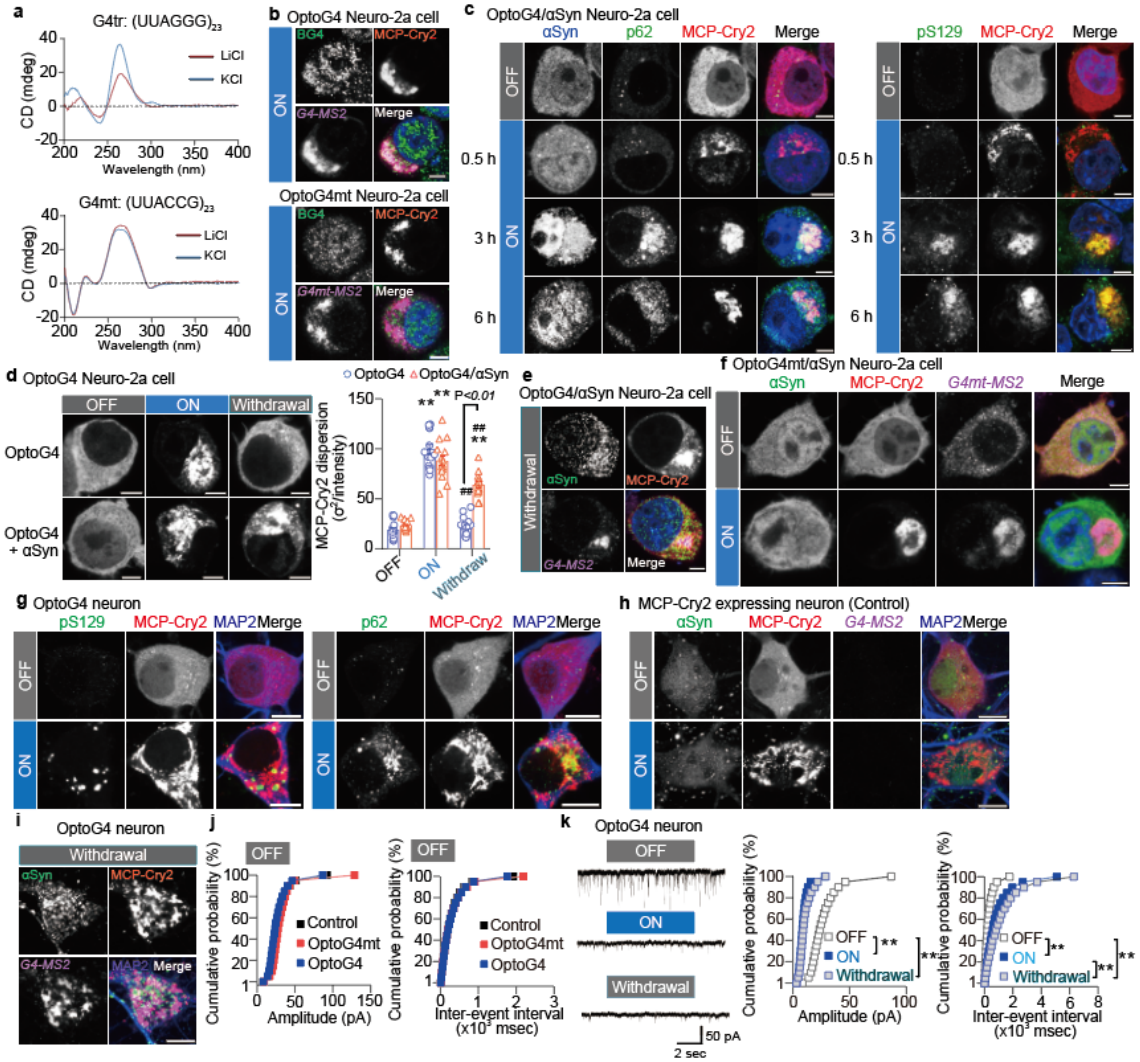
Extended Data Fig. 6: Co-aggregation of RNA G-quadruplexes and α-synuclein (αSyn) in pre-formed fibrils (PFF)-treated mouse cultured neurons and postmortem brain of patients with Parkinson's disease.

a, Representative images (left) and quantification (right) of co-localization of αSyn (green) and BG4 (red) granules in MAP2^{+} cells (blue) in mouse cultured neurons after PFF treatment. n = 12 (day 0), 15 (day 4), and 8 (day 8). Scale bars, 5 μm. **b**, Representative images of BG4 (green), pS129 (red), and 4',6-diamidino-2-phenylindole (blue) in the medulla oblongata of human normal control and patients with PD treated with or without RNase A (top). Scale bars, 5 μm. Information for human samples and the proportion of BG4^{+} cells among pS129^{+} cells (bottom). Data are presented as mean ± standard error of mean.



Extended Data Fig. 7: Bioinformatic and biophysical analyses of BG4-RIP-seq in pre-formed fibrils (PFF)-treated mouse cultured neurons.

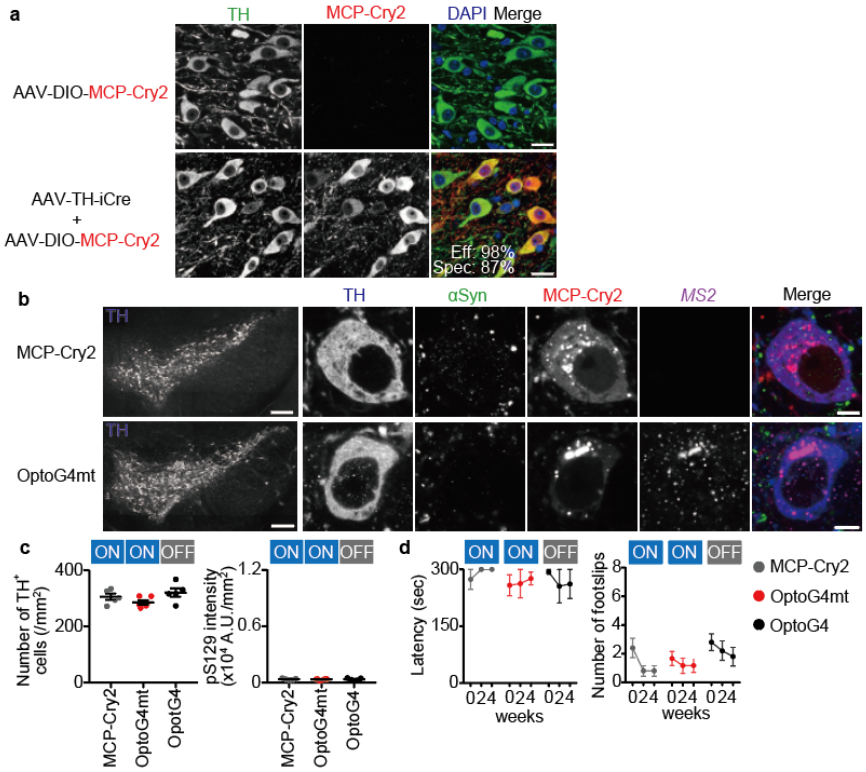
a, RNA G-quadruplexes (rG4s) in BG4-enriched RNAs containing two or three G-tetrads ($G_{2-3}L_{\leq 7}$) predicted by quadruplex-forming guanine-rich sequences (QGRS) mapper in mouse cultured neurons after PFF treatment. **b**, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis in BG4-enriched RNAs in mouse cultured neurons after PFF treatment. **c**,**d**, Predicted rG4 sequences in human/mouse G4ck and G4ps (**c**) and circular dichroism spectra of mouse G4ck in the presence of KCl or LiCl (**d**). **e**, Representative images (left) and quantification (right) of Proteostat intensity within phase-transitioned G4ck/αSyn dependent on Ca^{2+}. Scale bars, 5 μm. **f**, Representative images of MAP2 (blue), pS129 (green), and *Camk2a* RNA (magenta) in mouse cultured neurons after PFF treatment. Scale bars, 5 μm. Data are presented as mean ± standard error of mean. $^{**}P < 0.01$ by one-way analysis of variance with Bonferroni's multiple comparisons test. Number of replicates is shown in Supplementary Table 9.



Extended Data Fig. 8: Establishment of forced RNA assembly in cells using optogenetics.

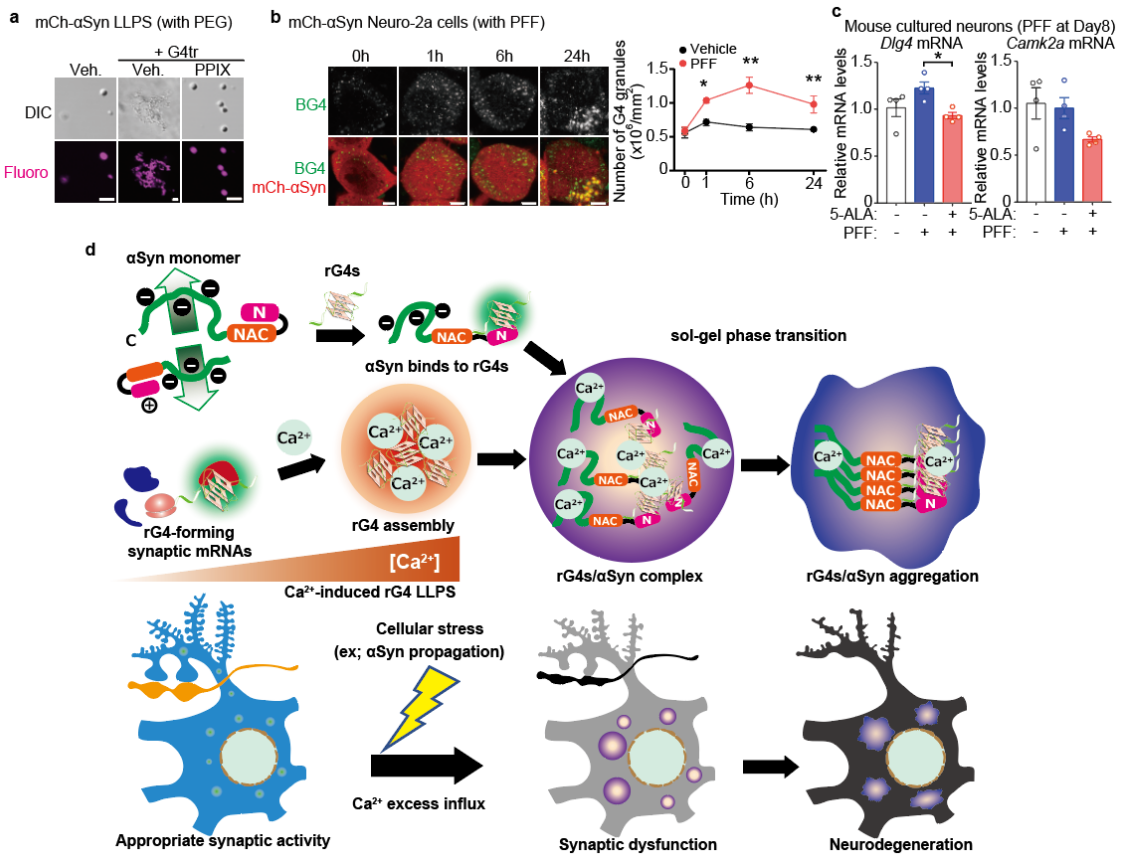
a, Circular dichroism spectra of G4tr $(UUAGGG)_{23}$ (left) and G4mt $(UUACCG)_{23}$ (right) in the presence of KCl or LiCl. **b**, Representative images of G4tr (top) or G4mt (bottom) assembly in OptoG4- (top) and OptoG4mt- (bottom) expressing Neuro-2a cells respectively, with 3-h BL stimulation. Scale bars, 5 μm. **c**, Representative images of p62 (left) and pS129 (right) in Neuro-2a cells co-expressing optoG4 and α-synuclein (αSyn) with blue light (BL) stimulation. Scale bars, 5 μm. **d**, Representative images (left) and quantification (right) of MCP-Cry2 dispersion in Neuro-2a cells co-expressing optoG4 and αSyn for 3 hours of BL stimulation followed by 3 hours of withdrawal. Scale bars, 5 μm. **e**, Representative images of co-assembly of αSyn (green) and *G4-MS2* RNA (magenta) in Neuro-2a cells co-expressing optoG4 and αSyn under the withdrawal condition. Scale bar, 5 μm. **f**, Representative images of Neuro-2a cells co-expressing optoG4mt and αSyn with 3-h BL stimulation. Scale bars, 5 μm. **g**, Representative images

of pS129 (left) and p62 (right) in optoG4-expressing mouse cultured neurons with 3-h BL stimulation. Scale bars, 5 μm. **h**, Representative images in MCP-Cry2-expressing mouse cultured neurons with BL stimulation for 3 h. Scale bars, 5 μm. **i**, Representative images of αSyn (green), MCP-Cry2 (red), *G4-MS2* RNA (magenta), and MAP2 (blue) in optoG4-expressing mouse cultured neurons under the withdrawal condition. Scale bar, 5 μm. **j**, Cumulative probability of sEPSCs related to **Fig. 4f** without BL stimulation. **k**, sEPSCs in OptoG4-expressing mouse cultured neurons for 3 hours of BL stimulation followed by 3 hours of withdrawal. Data are presented as mean ± standard error of mean. $^{**}P < 0.01$ vs. non-stimulated group and $^{\#\#}P < 0.01$ vs. stimulated group by one-way analysis of variance (ANOVA) with Bonferroni's multiple comparisons test (**d**). $^{**}P < 0.01$ by two-way ANOVA with Bonferroni's multiple comparisons test (**k**). Number of replicates is shown in Supplementary Table 9.



Extended Data Fig. 9: Forced RNA assembly in mouse nigral dopaminergic neurons by optogenetics.

a, Representative images of tyrosine hydroxylase (TH) (green), MCP-Cry2 (red), and 4',6-diamidino-2-phenylindole (blue) in the substantia nigra two weeks after the injection of AAV-DIO-MCP-Cry2 with AAV-pTH-iCre. Eff, efficiency; Spec, specificity. Scale bars, 20 µm. **b**, Representative images of TH (blue), αSyn (green), MCP-Cry2 (red), and *MS2* RNA (magenta) in the substantia nigra with blue light (BL) stimulation. Low and high magnification scale bars, 200 and 5 µm, respectively. **c**, Quantification of the number of TH-positive cells (left) and the fluorescent intensity of pS129 (right) in the substantia nigra with BL stimulation. **d**, Motor function in rotarod (left) and beam-walking (right) tasks with BL stimulation measured at the indicated time. Data are presented as mean ± standard error of mean. Number of replicates is shown in Supplementary Table 9.



Extended Data Fig. 10: Effects of G4 ligand on RNA G-quadruplexes (rG4)-induced α-synuclein (αSyn) aggregation.

a, Representative images of *in vitro* phase-transitioned G4tr/αSyn dependent on polyethylene glycol) and the effect of protoporphyrin IX. Scale bars, 5 μm. **b**, Representative images (left) and quantification (right) of BG4 granules (green) of Neuro-2a cells expressing mCh-αSyn (red) followed by pre-formed fibril (PFF) treatment for the indicated time. Scale bars, 5 μm. **c**, *Dlg4* and *Camk2a* mRNA levels in mouse cultured neurons following PFF treatment and the effects of 3 μM 5-aminolevulinic acid (5-ALA). **d**, Schematic showing rG4 assembly-induced αSyn aggregation and neurodegeneration: The monomeric state of αSyn is maintained by biased electrostatic and hydrophobic intramolecular interactions and intermolecular repulsion. rG4s bind to N-terminus of αSyn directly and then promote its aggregation. In the presence of Ca^{2+}, enhanced rG4 assembly via liquid-liquid phase separation may serve as scaffolds for αSyn aggregation contributing to neurodegeneration. When neurons suffer from cellular stress such as αSyn propagation, αSyn aggregates with rG4 assembly composed of G4-forming synaptic mRNAs by Ca^{2+} excess influx, inhibiting synaptic protein translation, eliciting synaptic dysfunction, and eventually resulting in neurodegeneration. *$P < 0.05$ and **$P < 0.01$ by two-way (**b**) and one-way (**c**) analysis of variance with Bonferroni's multiple

1187 comparisons test. Number of replicates is shown in Supplementary Table 9.

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Supplementary Table 1. Enriched 12-mer sequences in αSyn and BG4 by MERMADE program.

Supplementary Table 2. BG4-enriched mRNAs in vehicle-treated mouse cultured neurons.

Supplementary Table 3. BG4-enriched mRNAs in pre-formed fibrils-treated mouse cultured neurons.

Supplementary Table 4. Prediction of G4 structure in BG4-enriched mRNAs in vehicle-treated mouse cultured neurons by quadruplex-forming guanine-rich sequences mapper.

Supplementary Table 5. Prediction of G4 structure in BG4-enriched mRNAs in pre-formed fibrils-treated mouse cultured neurons by quadruplex-forming guanine-rich sequences mapper.

Supplementary Table 6. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis in BG4-enriched mRNAs in vehicle-treated neurons.

Supplementary Table 7. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis in BG4-enriched mRNAs in pre-formed fibrils-treated neurons.

Supplementary Table 8. Gene Ontology (GO) analysis in BG4-enriched mRNAs in pre-formed fibrils-treated neurons.

Supplementary Table 9. Statistical analysis and number of replicates.

Supplementary Method.

Supplementary Video. Live imaging of pre-formed fibrils (PFF)-treated cells following GFP-α-synuclein (αSyn) expression in a fluorescence recovery after photobleaching (FRAP) assay.

FRAP assay in HEK293T cells-expressing GFP-αSyn treated with PFF for the indicated time. Scale bars, 2 μm. Open circles in green indicate the regions of photobleaching.