Published in final edited form as:

Neurosci Lett. 2014 March 20; 563: 96–100. doi:10.1016/j.neulet.2014.01.033.

Transgenic mice expressing S129 phosphorylation mutations in α -synuclein

Valerie Drews Escobar^{a,b}, Yien-Ming Kuo^{a,b}, Bonnie M. Orrison^c, Benoit I. Giasson^{d,1}, and Robert L. Nussbaum^{a,b,*}

^aDepartment of Medicine, University of California San Francisco, San Francisco, CA 94143, USA

^bInstitute for Human Genetics, University of California San Francisco, San Francisco, CA 94143, USA

^cGenetic Disease Research Branch, NHGRI, National Institutes of Health, Bethesda, MD 20892, USA

^dDepartment of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA

Abstract

Aggregated α -synuclein is a predominant constituent of Lewy bodies, the intracellular protein aggregates seen in Parkinson's disease. While most α -synuclein in the nervous system is unphosphorylated, the majority of α -synuclein in Lewy bodies is phosphorylated at serine 129 (S129). We developed transgenic mice expressing human *SNCA* with either a phosphomimic (S129D) or a non-phosphorylatable (S129A) mutation, on a mouse *Snca* knockout background. Transgenic lines with each mutation expressing the human α -synuclein protein at levels ranging from 0.3 to 1.9 fold of endogenous mouse protein were chosen to avoid toxic overexpression effects. We previously demonstrated an altered distribution of presynaptic vesicles in *Snca* knockout mice, as well as enhanced interaction between presynaptic cytoskeletal proteins and α -synuclein when phosphorylated at S129 or carrying an S129D mutation. We therefore examined α -synuclein's synaptic localization and the distribution of presynaptic vesicles in these mutants. In addition, we evaluated the transgenic lines for reduced colonic motility, an early marker of α -synuclein pathology, and α -synuclein aggregates. No abnormalities were detected in mice expressing either phosphorylation mutant protein as their only α -synuclein protein. These results suggest the S129A and S129D mutations have no obvious effect on α -synuclein function.

Keywords

α -synuclein; Parkinson's disease; Phosphorylation; Lewy bodies; Transgenic

1. Introduction

Several of lines of evidence implicate α -synuclein (α Syn) in the pathogenesis of Parkinson's disease (PD) including familial human mutations and genome wide association studies [15]. The pathognomonic Lewy bodies (LBs) found in PD brains consist predominantly of highly aggregated α Syn [23] that is phosphorylated at S129 [2,9]. The physiological function of α Syn phosphorylation and its role in the disease process remain unknown.

Attempts to study the effect of α Syn phosphorylation in animal models by overexpression, particularly with viral vectors, have produced conflicting results [3,6,8,12]. We studied the *in vivo* effect of S129 phosphorylation by analyzing transgenic mouse lines that express α Syn at approximately endogenous levels with mutations that either mimic (S129D) or prevent (S129A) phosphorylation at serine 129, on an endogenous murine *Snca* knockout background.

First we looked at the expression of the α Syn phosphomutant proteins using immunohistochemistry to detect aggregated LB-like α Syn deposits. Since α Syn phosphorylation has been shown to influence binding to synaptic proteins *in vitro* [16], we examined synaptic localization of α Syn and synapse formation using immunocytochemistry of neuronal cultures. Based on studies suggesting an effect of α Syn on presynaptic vesicle pools [5,18] we also analyzed vesicles using electron microscopy of neuronal cultures. Finally, we measured colonic motility in the transgenic mice, as enteric nervous system dysfunction is one of the earliest and most robust manifestations of α Syn pathology, preceding movement and cognitive dysfunction in both humans and mouse models of PD [4,13].

2. Material and methods

2.1. Transgenic mice

The 145 kb P1 artificial chromosome (PAC) RP1-27M07 (BAC/PAC Resource Center, Children's Hospital, Oakland, CA) containing wildtype human *SNCA* was described previously [24]. The S129A and S129D mutations were introduced by recombineering in the *E. coli* strain DY380 [14] by introducing GCA (S129A) or GAT (S129D).

We generated transgenic mouse lines carrying either the *SNCA*^{S129A} PAC or *SNCA*^{S129D} PAC in a FVB/N background using previously described methods [11]. Two independent lines for each construct were selected. Mice homozygous for each of the four S129 PAC transgenes in a *Snca*^{+/+} background grew, developed normally and bred, indicating the PAC transgenes did not cause insertion site mutations. Each of the PAC transgenic lines was crossed with a *Snca*^{-/-} knock-out (129S6//SvEvTac) [5] to generate homozygous PAC transgenics on a *Snca*^{-/-}, mixed FVB/N $\times$ 129S6 background. PCR and interphase FISH were used for genotyping and to determine transgene zygosity as reported previously [13]. The two S129A and two S129D homozygous transgenic lines on *Snca*^{-/-} were designated A1, A2, D1 and D2 respectively. Transgenic mice expressing wildtype human α Syn [13] and non-transgenic littermates with *Snca*^{+/+} or *Snca*^{-/-} genotypes were selected as controls with a similar mixed background. All animals were handled in accordance with the

requirements of NIH, the UCSF Animal Care and Use Committee, and EC Directive 86/609/EEC.

2.2. Quantitative RT-PCR

Brains were harvested from 6-week-old mice and stored in RNAlater (Qiagen). The mice expressed either endogenous mouse *Snca* (wildtype), a wildtype human *SNCA* transgene, or a phosphomutant human *SNCA* transgene (A1, A2, D1 and D2). The transgenic mice were homozygous for the transgenes on a *Snca*^{-/-} background. cDNA preparation and real-time PCR on 16–20 replicates were performed as previously described [13]. The difference (Ct) between the average cycle number at which threshold was reached (Ct) for each line and the average Ct for endogenous *Elavl4* or endogenous *Syp* was recorded. The difference of differences (Ct) and relative amount $2^{-(Ct)}$ between transgenic wildtype or phosphomutant human *SNCA* and endogenous mouse *Snca*, (both normalized to *Elavl4* or *Syp*), was calculated.

2.3. Western blotting

Brains from 8-week-old female mice from each transgenic line (A1, A2, D1 and D2), a *Snca*^{-/-} littermate, and two wildtype littermates were dissected. Western blotting with the brain protein lysates was performed as previously described [5,13] using anti- α Syn antibody (BD Transduction Laboratories) that recognizes both human and mouse α Syn. α Syn signals were normalized for total protein per lane using mouse anti- α -tubulin antibody (Calbiochem).

2.4. Immunohistochemistry

Immunohistochemistry was performed on the brains of one male 9-month-old A2, and one 12-month-old D2 mouse using anti- α Syn antibody Syn505 as described previously [13]. Anti- α Syn antibodies Syn505 and pSer129/81A were used on the brain of a 12-month-old mouse expressing A53T human α Syn (line M83) as a positive control [10]. Tissue sections were counterstained with hematoxylin.

2.5. Immunocytochemistry of cultured neurons

Hippocampal and cortical neurons from E15.5 mouse embryos were isolated and plated in Neurobasal medium (Invitrogen) with B-27 supplement (Invitrogen) following manufacturer's protocol. At 17 days post-plating, neurons were fixed and stained with mouse anti- α Syn antibody (BD Transduction Laboratories) and rabbit anti-synapsin antibody (Stressgen). They were then stained with Alexa Fluor 488 conjugated goat anti-mouse and Alexa Fluor 594 conjugated goat anti-rabbit secondary antibodies (Invitrogen) and mounted on slides using Vectashield containing DAPI nuclear stain (Vector Laboratories). Images were taken using a SPOT microscopy camera and software (SPOT imaging). Final images were corrected for brightness and contrast at a similar level for each genotype using the Photoshop Elements program (Adobe).

2.6. Electron microscopy

Two eight-week-old mice from each genotype (A2, D2, wildtype and knockout) were intracardially perfused with 4% paraformaldehyde/2% glutaraldehyde fixative. The brains were post-fixed in fresh fixative. Coronal brain sections of 70 nm were cut at the level of the caudate/putamen using a vibratome and fixed overnight. Vesicles were counted in fifteen synapses from each mouse in the center of the CA1 stratum radiatum as previously described [5].

2.7. Colonic motility

Colonic motility was measured in 6-month-old male and female A2 and D2 mice by the bead expulsion test as previously described [13]. Six-month-old mice expressing the wildtype human *SNCA* PAC on a mouse *Snca*^{-/-} background [13] were included as a control.

2.8. Statistical analysis

Standard deviations of Cts and Cts were calculated from the standard deviations (SD) as $[(SD_1)^2 + (SD_2)^2]$. Student's *t* tests were used for pair-wise comparison between wildtype and mutant lines in the electron microscopy vesicle counts. The Mann-Whitney *U* test was used in the colonic motility comparisons. The significance level was $p < 0.05$ for both tests. All data were analyzed by GraphPad Prism 4.0 statistical software (GraphPad Software).

3. Results

3.1. Expression of mutant human α Syn in transgenic mice

Human *SNCA* or mouse *Snca* mRNA expression was measured in brains from each of the two S129D and S129A transgenic lines and wildtype mice using qRT-PCR. The transgenic lines expressed human *SNCA* mRNA at 0.7-fold–39-fold higher levels in brain relative to expression of mouse *Snca* in wildtype mice, in all cases relative to *Elavl4* mRNA as a reference (Fig. 1a). Similar results were obtained when endogenous synaptophysin expression was used as a reference (data not shown). Western blot analysis of whole brain (Fig. 1b) revealed a modest increase in the amount of protein (0.3–1.9 fold) with lines A1 and D2 expressing at levels almost equal to endogenous mouse α Syn (0.7 fold). This discrepancy between steady state *SNCA* mRNA levels and α Syn protein has been observed in other PAC transgenic animals [13].

3.2. Brain histology

To verify the expression of the transgene and detect potential LB inclusions, one mouse from each of the highest expressing transgenic lines (A2 and D2) was histologically evaluated for α Syn expression and localization. Typical α Syn neuropil staining was observed in the A2 and D2 lines. No pathological inclusions were found in either transgenic line (Fig. 2a), as compared to a positive control mouse expressing a pathogenic A53T human α Syn transgene. In addition, there were no gross morphological abnormalities of the brain that would suggest significant neuronal toxicity (data not shown).

3.3. Phenotypic evaluation

If phosphorylation at S129 had an important physiological role in α Syn function, then mice expressing the phosphomimic or non-phosphorylatable α Syn at S129 might show a dominant negative or toxic gain-of-function effect in *Snca*^{+/+} and *Snca*^{-/-} backgrounds respectively, resulting in an enhancement of the modest phenotype seen with loss-of-function mutations of *Snca* in a 129S6 inbred strain [1,5]. We examined the mice cytologically for abnormalities in the ability of α Syn to localize to synapses and in the fraction of docked synaptic vesicles, as well as more grossly in overall growth, development, and enteric nervous system abnormalities seen in a mouse model of early PD.

3.4. Immunocytochemistry of neurons

Hippocampal neurons from mouse embryos were examined after 17 days in culture. The cells from all four phosphomutant lines were viable and had the same shape and size as those from a wildtype littermate (Fig. 2b). Staining with an anti-synapsin antibody showed normal location, size and density of synapses in all transgenic lines. Double labeling with anti-synapsin and anti- α Syn antibodies revealed α Syn staining overlapping with synapsin in wildtype, A1 and D2 transgenic animals (Fig. 2b) as well as in A2 and D1 transgenic animals (data not shown) but not in the *Snca*^{-/-} control, where it was absent as expected. Cortical embryonic neurons were also examined with similar results (data not shown).

3.5. Electron microscopy

We examined the stratum radiatum region of the hippocampus in two animals from each of the highest expressing transgenic lines (A2 and D2). Wildtype and knockout littermates were used as controls. The numbers of vesicles in the docked position and reserve pool, as well as the total number of vesicles, were the same across all genotypes (Fig. 3).

3.6. Gross development and breeding

S129A and S129D transgenic animals developed and bred normally, regardless of whether they were on an endogenous mouse *Snca*^{+/+} or *Snca*^{-/-} background.

3.7. Colon motility

We examined colonic motility, a sensitive measure of murine synucleinopathy, in the highest expressing transgenic mutants (A2 and D2) and in animals carrying the wildtype human α Syn transgene (*SNCA*^{WT}). Although in each group the males had a slower expulsion time than the females, a known difference trait between the sexes, there was no consistent difference between the *SNCA*^{WT} and the A2 or D2 animals (Fig. 4).

4. Discussion

Determining the normal physiological role for phosphorylation of α Syn as well as the role of phosphorylation in LB aggregates is important for the design of therapeutic interventions based on manipulating phosphorylation state. We created transgenic mouse lines that express human α Syn with either the S129D or S129A mutation as their only α Syn at approximately physiological levels in order to explore the physiological and pathological impact of phosphorylation. We then tested these transgenic S129D and S129A animals for

an abnormal phenotype above what was seen in animals that either expressed endogenous mouse α Syn, human wildtype α Syn or were ablated for α Syn expression.

Because we observed differential interactions *in vitro* between presynaptic cytoskeletal proteins and phosphorylated (and S129D) versus unphosphorylated C-terminal peptides of α Syn [16], we looked at synaptic localization of S129D and S129A α Syn. In hippocampal and cortical primary neurons, α Syn expression showed no difference between wildtype and the S129 phosphorylation mutants in their overlap with the synaptic marker, synapsin. Next, we examined the S129A and S129D mutations for a toxic gain of function on synaptic vesicle pools because previous studies using α Syn knock-down in cultured cells and inbred 129S6 *Snca*^{-/-} mice [5,18] showed decreased levels of undocked vesicles in the hippocampus. We examined similar neuronal cultures and found no effect of S129D or S129A α Syn on vesicle localization. As previously demonstrated in *Snca*^{-/-} mice on a mixed genetic background [1], there was no alteration in the fraction of undocked vesicles in the control *Snca*^{-/-} animals.

We chose transgenic mice that expressed the α Syn transgene protein at levels comparable to wildtype mouse α Syn, in order to avoid the effect of overexpression. The protein was expressed in the correct locations, as seen in the whole brain histology and in the neuronal immunocytochemistry. We did not see behavioral or motor defects that would suggest toxicity and did not see α Syn aggregates or neurodegeneration, in contrast to what has been reported in studies that expressed S129A or S129D α Syn in flies [6,7] or via viral injections into rat brains [8,12,17].

One early sign of Parkinson's disease, preceding movement and cognitive dysfunction, is enteric nervous system dysfunction. Mice carrying A53T or A30P mutations that cause autosomal dominant PD show abnormalities in enteric nervous system function by 6 months [13]. We measured colonic motility in S129D and S129A mice and found no consistent difference between the mutants and control mice expressing wildtype human α Syn, suggesting phosphorylation state does not cause enteric nervous system dysfunction.

However, there are significant limitations of this study. The first is only one phosphorylation site, S129, was examined and phosphorylation at S87 may also be involved in α Syn membrane binding, fibrillation, and aggregate formation [19]. Second, S129D and S129A mutations may not fully mimic the behavior of phosphorylated and unphosphorylated α Syn. The ability of α Syn to inhibit PLD2 *in vitro* is abolished by either phosphorylation or S129D [21,22]. In addition, S129A and S129D peptides mimic the binding of the S129 unphosphorylated and phosphorylated peptides in pull down experiments ([16]; and data not shown.) On the other hand, S129D did not recapitulate the inhibition of α Syn fibrillation *in vitro* [20] by S129PO₄. Finally, there may be subtle behavioral or motor effects that were not detected in this analysis.

In summary, we demonstrated transgenic mice expressing human S129D or S129A α Syn as their only α Syn show normal growth and development, localization of α Syn at synapses in culture, normal synaptic vesicle localization, no histological signs of α Syn aggregation in the brain, and no enteric nervous system dysfunction. Thus, within the limitations of this

study, there is no evidence that S129D or S129A interferes with the normal function of α Syn or causes a toxic gain of function as seen with mutations associated with PD in humans.

Acknowledgments

Supported by the Division of Intramural Research, NHGRI, the Michael J. Fox Foundation, and the UCSF Department of Medicine and Institute of Human Genetics (RLN), and NIH grant number NS053488 (BG). V.D.E. participated in design of experiments, carried out the majority of experiments, and was the primary author of the manuscript, B.M.O. constructed the engineered PACs, Y.M.K. performed real time PCR and colonic motility experiments, B.G. performed immunohistochemistry on the brain tissue, and R.L.N. contributed to design of experiments, review of data, and preparation of the manuscript.

We thank Dr. Deborah Cabin for helpful discussions and Mr. Larry Ackerman for expert technical assistance.

Abbreviations

PD	Parkinson's disease
αSyn	α -synuclein
LB	Lewy bodies
PAC	P1 artificial chromosome.

References

1. Abeliovich A, Schmitz Y, Farinas I, Choi-Lundberg D, Ho WH, Castillo PE, Shinsky N, Verdugo JM, Armanini M, Ryan A, Hynes M, Phillips H, Sulzer D, Rosenthal A. Mice lacking alpha-synuclein display functional deficits in the nigrostriatal dopamine system. *Neuron*. 2000; 25:239–252. [PubMed: 10707987]
2. Anderson JP, Walker DE, Goldstein JM, de Laat R, Banducci K, Caccavello RJ, Barbour R, Huang JP, Kling K, Lee M, Diep L, Keim PS, Shen XF, Chataway T, Schlossmacher MG, Seubert P, Schenk D, Sinha S, Gai WP, Chilcote TJ. Phosphorylation of Ser-129 is the dominant pathological modification of alpha-synuclein in familial and sporadic Lewy body disease. *J Biol Chem*. 2006; 281:29739–29752. [PubMed: 16847063]
3. Azeredo da Silveira S, Schneider BL, Cifuentes-Diaz C, Sage D, Abbas-Terki T, Iwatsubo T, Unser M, Aebischer P. Phosphorylation does not prompt, nor prevent, the formation of alpha-synuclein toxic species in a rat model of Parkinson's disease. *Hum Mol Genet*. 2009; 18:872–887. [PubMed: 19074459]
4. Braak H, Del Tredici K. Neuroanatomy and pathology of sporadic Parkinson's disease. *Adv Anat Embryol Cell Biol*. 2009; 201:1–119. [PubMed: 19230552]
5. Cabin DE, Shimazu K, Murphy D, Cole NB, Gottschalk W, McIlwain KL, Orrison B, Chen A, Ellis CE, Paylor R, Lu B, Nussbaum RL. Synaptic vesicle depletion correlates with attenuated synaptic responses to prolonged repetitive stimulation in mice lacking alpha-synuclein. *J Neurosci*. 2002; 22:8797–8807. [PubMed: 12388586]
6. Chen L, Feany MB. Alpha-synuclein phosphorylation controls neurotoxicity and inclusion formation in a Drosophila model of Parkinson disease. *Nat Neurosci*. 2005; 8:657–663. [PubMed: 15834418]
7. Feany MB, Bender WW. A Drosophila model of Parkinson's disease. *Nature*. 2000; 404:394–398. [PubMed: 10746727]
8. Febbraro F, Sahin G, Farran A, Soares S, Jensen PH, Kirik D, Romero-Ramos M. Ser129D mutant alpha-synuclein induces earlier motor dysfunction while S129A results in distinctive pathology in a rat model of Parkinson's disease. *Neurobiol Dis*. 2013; 56:47–58. [PubMed: 23567651]

9. Fujiwara H, Hasegawa M, Dohmae N, Kawashima A, Masliah E, Goldberg MS, Shen J, Takio K, Iwatsubo T. alpha-Synuclein is phosphorylated in synucleinopathy lesions. *Nat Cell Biol.* 2002; 4:160–164. [PubMed: 11813001]
10. Giasson BI, Duda JE, Quinn SM, Zhang B, Trojanowski JQ, Lee VM. Neuronal alpha-synucleinopathy with severe movement disorder in mice expressing A53T human alpha-synuclein. *Neuron.* 2002; 34:521–533. [PubMed: 12062037]
11. Gisbert S, Del Turco D, Garrett L, Chen A, Bernard DJ, Hamm-Clement J, Korf HW, Deller T, Braak H, Auburger G, Nussbaum RL. Transgenic mice expressing mutant A53T human alpha-synuclein show neuronal dysfunction in the absence of aggregate formation. *Mol Cell Neurosci.* 2003; 24:419–429. [PubMed: 14572463]
12. Gorbatyuk OS, Li S, Sullivan LF, Chen W, Kondrikova G, Manfredsson FP, Mandel RJ, Muzyczka N. The phosphorylation state of Ser-129 in human alpha-synuclein determines neurodegeneration in a rat model of Parkinson disease. *Proc Natl Acad Sci U S A.* 2008; 105:763–768. [PubMed: 18178617]
13. Kuo YM, Li Z, Jiao Y, Gaborit N, Pani AK, Orrison BM, Bruneau BG, Giasson BI, Smeyne RJ, Gershon MD, Nussbaum RL. Extensive enteric nervous system abnormalities in mice transgenic for artificial chromosomes containing Parkinson disease-associated alpha-synuclein gene mutations precede central nervous system changes. *Hum Mol Genet.* 2010; 19:1633–1650. [PubMed: 20106867]
14. Liu P, Jenkins NA, Copeland NG. A highly efficient recombineering-based method for generating conditional knockout mutations. *Genome Res.* 2003; 13:476–484. [PubMed: 12618378]
15. Martin I, Dawson VL, Dawson TM. Recent advances in the genetics of Parkinson's disease. *Annu Rev Genomics Hum Genet.* 2011; 12:301–325. [PubMed: 21639795]
16. McFarland MA, Ellis CE, Markey SP, Nussbaum RL. Proteomics analysis identifies phosphorylation-dependent alpha-synuclein protein interactions. *Mol Cell Proteomics.* 2008; 7:2123–2137. [PubMed: 18614564]
17. McFarland NR, Fan Z, Xu K, Schwarzschild MA, Feany MB, Hyman BT, McLean PJ. Alpha-synuclein S129 phosphorylation mutants do not alter nigrostriatal toxicity in a rat model of Parkinson disease. *J Neuropathol Exp Neurol.* 2009; 68:515–524. [PubMed: 19525899]
18. Murphy DD, Rueter SM, Trojanowski JQ, Lee VM. Synucleins are developmentally expressed, and alpha-synuclein regulates the size of the presynaptic vesicular pool in primary hippocampal neurons. *J Neurosci.* 2000; 20:3214–3220. [PubMed: 10777786]
19. Paleologou KE, Oueslati A, Shakked G, Rospigliosi CC, Kim HY, Lamberto GR, Fernandez CO, Schmid A, Chagini F, Gai WP, Chiappe D, Moniatte M, Schneider BL, Aebischer P, Eliezer D, Zweckstetter M, Masliah E, Lashuel HA. Phosphorylation at S87 is enhanced in synucleinopathies, inhibits alpha-synuclein oligomerization, and influences synuclein-membrane interactions. *J Neurosci.* 2010; 30:3184–3198. [PubMed: 20203178]
20. Paleologou KE, Schmid AW, Rospigliosi CC, Kim HY, Lamberto GR, Fredenburg RA, Lansbury PT, Fernandez CO Jr, Eliezer D, Zweckstetter M, Lashuel HA. Phosphorylation at Ser-129 but not the phosphomimics S129E/D inhibits the fibrillation of alpha-synuclein. *J Biol Chem.* 2008; 283:16895–16905. [PubMed: 18343814]
21. Payton JE, Perrin RJ, Woods WS, George JM. Structural determinants of PLD2 inhibition by alpha-synuclein. *J Mol Biol.* 2004; 337:1001–1009. [PubMed: 15033366]
22. Pronin AN, Morris AJ, Surguchov A, Benovic JL. Synucleins are a novel class of substrates for G protein-coupled receptor kinases. *J Biol Chem.* 2000; 275:26515–26522. [PubMed: 10852916]
23. Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M. α -Synuclein in Lewy bodies. *Nature.* 1997; 388:839–840. [PubMed: 9278044]
24. Touchman JW, Dehejia A, Chiba-Falek O, Cabin DE, Schwartz JR, Orrison BM, Polymeropoulos MH, Nussbaum RL. Human and mouse alpha-synuclein genes: comparative genomic sequence analysis and identification of a novel gene regulatory element. *Genome Res.* 2001; 11:78–86. [PubMed: 11156617]

HIGHLIGHTS

- We developed transgenic mice expressing only S129D or S129A human α -synuclein.
- Transgene expression and localization were normal and at endogenous levels.
- No gross abnormalities in behavior or motor defects indicating toxicity were seen.
- Mice had normal gut motility and no neurodegeneration or aggregation in the brain.
- Presynaptic vesicle localization was normal in phosphomutant mice.

Fig. 1.

SNCA transgenes are expressed in brain RNA and protein. (A) qPCR of 6-week-old mouse brain. Transgenic *SNCA* RNA levels relative to endogenous mouse *Snca* mRNA are expressed as $\log_{10}$ of 2^{-Ct} , each referenced against a mouse neuronal gene, *Elavl4*. The S129 transgenic lines A1, A2, D1, D2 and the human wildtype transgenic line express human *SNCA* at 0.7–39 fold higher levels than endogenous mouse *Snca* expression (wt). (B) Western blot of 8-week-old mouse brain. Protein levels were detected using an anti- α Syn antibody that recognizes both human and mouse α Syn. Anti- α -tubulin antibody was used to control for protein loading. An *Snca* knockout mouse is included as a negative control (-/-). The transgenic lines A1, A2, D1 and D2 on a mouse *Snca* knockout background express human α Syn from the transgene at 0.3–1.9 fold level of endogenous mouse α Syn shown as wt. Rel., relative.

Fig. 2.

Normal expression of the αSyn transgene and lack of pathophysiology in brains and neurons of phosphomutants. (A) Normal αSyn staining in brains of A2 and D2 transgenic mice. Immunohistochemistry with anti-αSyn antibody Syn 505 (A–D) in a 12-month-old D2 transgenic mouse (A, C) and a 9-month-old A2 transgenic mouse (B, D) in the hippocampus (A, B) and the pons/cerebellum (C, D). Typical αSyn neuropil staining was observed. In comparison, robust αSyn inclusion pathology was observed using antibodies Syn505 (E) or pSer129/81A (F) in the pons of a 12-month-old symptomatic transgenic mouse expressing A53T human αSyn (line M83). Tissue sections were counterstained with hematoxylin. Bar —100 μm. (B) Phosphomutant transgenics have normal hippocampal neuronal morphology, αSyn localization and synapse formation in culture. Labeling with anti-synapsin (red) and anti-αSyn (green) antibodies and DAPI nuclear stain (blue). Neurons from A1 and D2 transgenic lines were viable and had the same shape and size as those from a wildtype (wt) littermate. Anti-synapsin antibody staining showed normal location of synapsin and normal size and density of synapses in all lines. Diffuse αSyn staining was seen with intense staining overlapping with the synapsin stain in transgenic and wildtype neurons. αSyn staining was absent in *Snca* knockout neurons (–/–), as expected. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

Vesicle docking in neurons is unaffected by phosphomutations. (A) Electron microscopy of the stratum radiatum region of the hippocampus shows normal synapse morphology. (B) No significant differences were found in the number of total, docked or reserve pool vesicles between wildtype and each of three mutants. The Student's *t* tests were used for pair-wise comparison with a significance level of $p < 0.05$. wt, wildtype. $-/-$, *Snca* knockout.

Fig. 4.

Phosphomutations do not reduce colonic motility. Colonic motility was measured in male and female mice from the two highest expressing mutant transgenic lines A2 and D2, and a line carrying the human wildtype *SNCA* transgene. The time required for expulsion of a glass bead from the colon was recorded. There was no consistent difference between the human wildtype *SNCA* transgenic and the mutants, with a significant increase for S129A only in females ($p < 0.0052$ by Mann–Whitney *U*-test) and a significant decrease for S129D mice in males ($p < 0.0115$) compared to a human wildtype *SNCA* transgenic line.