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Behavioral deficits and brain α -synuclein and phosphorylated serine-129 α -synuclein in male and female mice overexpressing human α -synuclein

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Abstract

Background: Alpha-synuclein (α -syn) is a molecule involved in pathology of Parkinson's disease, and 90% of α -syn in Lewy bodies is phosphorylated at serine 129 (pS129 α -syn).

Objectives—To assess motor and non-motor behaviors in male and female mice overexpressing human α -syn under Thy1 promoter (Thy1- α -syn) and wild type (wt) littermates.

Methods: Motor and non-motor behaviors Brain human α -syn levels by ELISA, and mapped α -syn and pS129 α -syn in the brain by immunohistochemistry.

Results: Male and female wt littermates did not show differences in the behavioral tests. Male Thy1- α -syn mice displayed more severe impairments than female counterparts in cotton nesting, pole tests, adhesive removal, finding buried food and marble burying. Concentrations of human α -syn in the olfactory regions, cortex, nigrostriatal system and dorsal medulla were significantly increased in Thy1- α -syn mice, higher in males than females. Immunoreactivity of α -syn was not simply increased in Thy1- α -syn mice, but had altered localization in somas and fibers in a few brain areas. Abundant pS129 α -syn existed in many brain areas of Thy1- α -syn mice, while there was none or only a small amount in a few brain regions of wt mice. The substantia nigra, olfactory regions, amygdala, lateral parabrachial nucleus and dorsal vagal complex displayed different distribution patterns between wt and transgenic mice, but not between sexes.

Conclusions: The severer abnormal behaviors in male than female Thy1- α -syn mice may be related to higher brain levels of human α -syn, in the absence of sex differences in the altered brain immunoreactivity patterns of α -syn and pS129 α -syn.

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Conflict of Interest/Disclosure Statement

The authors have no conflicts of interest to report.

Keywords

α -synuclein; behavior; brain; mouse; phosphorylated serine-129 α -synuclein; synucleinopathy

INTRODUCTION

Alpha-synuclein (α -syn) is a key molecule involved in the pathology of Parkinson's disease (PD), and it is the primary component in Lewy bodies [1, 2]. α -syn pathology is not restricted to the substantia nigra (SN), but spreads to other brain regions [3], which is associated with non-motor symptoms [4]. Evidence indicates that α -syn accumulation is also observed in other neurodegenerative diseases [1, 5, 6]. Phosphorylation is one of the post-translational modifications of α -syn [7, 8]. Approximately 90% of α -syn in Lewy bodies is phosphorylated at serine 129 (pS129 α -syn) [9, 10], and pS129 α -syn promotes formation of α -syn fibrils and oligomers, which could be neurotoxic [9, 11]. pS129- α -syn was also shown to reduce α -syn membrane binding [12] and modulate α -syn toxicity [13–15]. pS129 α -syn levels in cerebrospinal fluid of PD patients were correlated with disease severity [16]. Therefore, pS129 α -syn may play a critical role in synucleinopathies [7].

Animal models based on α -syn overexpression display Parkinsonian motor and non-motor phenotypes and provide valuable tools used to comprehensively understand some of the pathogenic mechanisms of PD [17]. A mouse model over-expressing full-length human wild-type α -syn under the Thy-1 promoter (Thy1- α -syn mice, Line 61) has been most extensively characterized. The model recapitulates various features of PD [18], and shows progressive impairments [18, 19]. Thy1- α -syn mice display deficits in motor and sensorimotor functions [19, 20], cognition [21, 22], olfaction [23], social behavior [24, 25], gut transit [26] and metabolism [27], as well as stress and anxiety-like behaviors [28].

Two studies reported sex differences in motor functions of Thy1- α -syn mice [29, 30] potentially associated with lower α -syn mRNA expression in the SN of female transgenic (tg) mice [29]. Given that the transgene is located on the X chromosome in this mouse line, random inactivation of the X chromosome carrying the transgene may explain the less robust motor deficits in female mice [18]. Therefore, most studies in Thy1- α -syn mice have been performed in male mice. Prior studies on α -syn levels and immunoreactivity in Line 61 mouse brains used both male and female tg mice, but did not report sex differences [31, 32]. In this study, we assessed first motor and sensorimotor functions, as well as behaviors associated with olfactory function and anxiety in male and female Thy1- α -syn mice, then measured protein levels of human α -syn in brain areas involved in regulation of the altered behaviors. We further mapped distribution of α -syn immunoreactivity in the brain to potentially identify brain regions demonstrating sex differences. Since pS129 α -syn is known to play a role in neurotoxicity [9] and information about its brain distribution in mice is insufficient [33, 34], we also mapped pS129 α -syn immunoreactivity in male and female Thy1- α -syn mice and wild type (wt) littermates.

MATERIALS AND METHODS

Animals

Thy1- α -syn mice overexpressing human wild type α -syn under the membrane glycoprotein Thy1 promoter (line 61) were originally generated by the laboratory of Dr. E. Masliah (UCSD) as previously described [31]. The mice were crossed into a mixed C57BL/6-DBA2 (BDF1) background and maintained on this background by mating N5 females hemizygous for the transgene with wt male mice [35]. Five pairs of breeders were obtained from the colony maintained in the vivarium of the Department of Laboratory Animal Medicine at UCLA and reproduced 13 batches of offspring. The genotypes of all Thy1- α -syn and wt mice were verified by polymerase chain reaction (PCR) amplification analysis of tail DNA both after birth and at the end of the experiments. The experiments were performed in 11 male wt, 7 female wt, 17 male Thy1- α -syn, and 8 female Thy1- α -syn mice. Transgenic (tg) or wt mice were housed in groups of 4 or fewer per cage under controlled conditions of temperature (21–23°C) and illumination (6:00 AM to 6:00 PM) with free access to water and standard rodent diet (Prolab RMH 2500; LabDiet, PMI Nutrition, Brentwood, MO, USA) unless otherwise stated. Animal care was conducted in accordance with the United States Public Health Service Guide for the Care and Use of Laboratory Animals and animal experiments were approved by the UCLA and the West Los Angeles Veterans Administration Committees for Animal Use and Care (protocol number 06015–08).

Behavior tests

The behavioral tests were performed in hemizygous tg male and female mice and their wt littermates when they were 4–7 months of age. The mice were tested for pole and cotton nesting performance at 4 and 6–7 months old, and other behaviors were tested once when the mice were 5–7 months old. The mice were not interrupted for 1–2 weeks between the tests.

Cotton nesting: Nest-building is a natural mouse behavior related to thermoregulation, creation of a comfortable environment, and pup survival [36, 37]. Mice were housed individually and supplied with a piece of cotton pad weighed before being placed in the cage. All the pieces of the cotton pad were collected 24 h later, air-dried for one day, and weighed. The difference in cotton weight was calculated by subtracting the cotton weight after 24 h from the initial weight.

Pole test: The pole test has been used previously to assess basal ganglia-related movement disorders in mice. Briefly, animals were placed head-up on top of a vertical wooden pole 50 cm in length and 1 cm in diameter. The base of the pole was placed in the home cage. When placed on the pole, the animals oriented themselves downward and descended along the pole back into their home cage. The mice received training that consisted of five trials for each session. On the test day, the animals were tested in five trials, and time to orient downward and total time to descend were measured. The times recorded during the five trials were averaged for each mouse.

Adhesive removal: This is a fine movement test of sensorimotor function. The test was performed as described previously, with some modification [20, 37]. Mice were placed in a clean cage for 1 h for acclimation to the environment. One adhesive label (Avery adhesive-backed labels, round with diameter of one-quarter inch) was placed onto the snout and gently pressed down. We recorded the time from when the mouse was placed back in the cage until the label was removed. If the mouse did not remove the sticker within 60 sec, the trial was ended and the score was 60 sec. Each mouse was tested over 3 trials, with 2 min intervals between the trials. The times recorded for the three trials were averaged for each mouse.

Marble burying: The method was adapted from Angoa-Perez M [38]. This test is used to assess the effects of anti-depressant and/or anxiolytic drugs, and normal rodents actively bury glass marbles [39]. A clean cage (15 × 25 × 13 cm) with bedding of 5 cm deep containing 15 standard glass toy marbles in assorted colors and styles (15 mm diameter, 5.2 g in weight) was used. The marbles were placed gently on the surface of the bedding in 3 rows of 5. One mouse was carefully placed in a corner of the cage on the bedding as far as possible from the marbles, and then the cage was covered with a filter-top. There was no access to food or water during the test. The mouse was left undisturbed in the cage for 30 min. The number of buried marbles was recorded at the end of the test. A marble was scored as buried when two-thirds of its surface area was covered by bedding.

Olfaction: The buried pellet test was performed as described previously [23], with the exception that mice were tested only once to avoid compensatory strategies developed over repeated trials. Briefly, mice were food-restricted and maintained at ~90% body weight 5 days prior to and during testing. Food-restricted mice were given 3–4 g of chow per animal per day depending on weight (monitored each day during food restriction). A clean mouse cage (15 × 25 × 13 cm) was filled with 3 cm of clean bedding. One piece of sweetened cereal (~250 mg; Cap'n Crunch, Quaker, Chicago, IL) was buried in the front left corner of the cage approximately 0.5 cm below the bedding so that it was not visible. A mouse was then placed in the center of the cage and the latency to dig up and begin eating the cereal was timed using a stopwatch. After the trial, each animal was returned to its home cage. If a mouse did not locate the food pellet within 5 min, the animal was removed, returned to its home cage, and given a score of 5 min. The bedding was changed between mice, and gloves were replaced before touching fresh bedding to avoid possible contamination of the new bedding with traces of mouse smell. Extra care was taken to ensure that food pellets were fresh and could be smelled by the wt mice, and a ruler was used to ensure that the pellet was buried at approximately the same level below the bedding surface.

ELISA

An enzyme-linked immunosorbent assay (ELISA) was performed to measure human α -syn levels in selected brain areas. Brains of male and female Thy1- α -syn and wt mice of 7–8 months old were removed, frozen on dry ice, and stored at -80°C until use in the assay. Brain areas of interest were dissected in a cryostat with a scalpel, using a technique similar to the protocol published in Mo et al. [40]. The dissected brain regions included the olfactory bulb and olfactory cortex, cortex (cingulate and sensorimotor cortex), striatum,

SN and ventral tegmental area (VTA), and dorsal half of the medulla at the area postrema level. Brain samples were extracted in RIPA buffer (Thermo Fisher Scientific, Carlsbad, CA) with protease inhibitor cocktail (Roche 11697498001, Sigma-Aldrich, St. Louis, MO) using a homogenizer (Fastprep fp120, MP Biomedicals, Irvine, CA). The protein concentrations were measured using a Pierce™ BCA Protein Assay Kit (Cat# 23225, Thermo Fisher Scientific). Human α -syn concentration was measured using a human α -syn ELISA kit (KHB0061, Thermo Fisher Scientific) following the manufacturer's protocol.

Immunohistochemistry and imaging

The procedures were similar to those previously described [41]. Frozen brains from Thy1- α -syn and wt mice of 7–8 months old were sectioned at 25 μ m in a cryostat (Microm International GmbH, Walldorf, Germany) and divided into 4 sets. Avidin-biotin-peroxidase complex method was used. Endogenous peroxidase was blocked using 0.3% hydrogen peroxide (Sigma) in PBS. One set of sections each was incubated as follows: (1) a mouse monoclonal anti-rat synuclein-1 antibody against amino acids 15–123 recognizing rat, mouse and human α -syn (1:4,000, BD Transduction Laboratories, Clone 42, Cat# 610787, San Jose, CA, USA), (2) same antibody against α -syn used on sections pretreated with 5 μ g/ml proteinase K (Invitrogen/ThermoFisher, Cat# 25530–049) in 0.01 M PBS (pH 7.4) for 10 min at room temperature, and (3) a rabbit monoclonal anti-pS129 α -syn antibody (1:2,000, Abcam ab51253, clone # EP1536Y, Cambridge, MA) [42]. The secondary antibody was biotinylated goat anti-mouse or anti-rabbit IgG (1:1000, Jackson ImmunoResearch Laboratories Inc., Cat No. 111–067-003; West Grove, PA). Secondary antibody incubation was followed by incubation with the avidin–biotin–peroxidase complex (1:200; Vector, Burlingame, CA). Immunoreactivity was visualized by diaminobenzidine tetrachloride precipitation in the presence of hydrogen peroxide.

The specificity of the pS129 α -syn antibody was checked by pre-absorption with human pS129 α -syn. Diluted pS129 α -syn primary antibody (1:2,000) was incubated with 50 μ g/ml of synthetic human pS129 peptide (amino acids 116–131, Abcam ab49441) for 2 days prior to the immunohistochemical procedures described above.

Immunoreactivity in brain sections was examined by light microscopy (Axioscop II, Carl Zeiss, Germany), and images were acquired by a digital camera (AxioCam 506 Color, Carl Zeiss) using the image acquisition system ZEN 2 (Carl Zeiss).

To verify that the immunoreactivity of α -syn and pS129 α -syn was not different between male and female mice, representative areas were selected and mean gray values were measured using FIJI (ImageJ2, NIH).

The semi-quantitative analysis using “-” and “+” labels was scored based on the density and intensity of the immunoreactivity. “-” meant no specific immunoreactivity, as observed in wild type mice in Fig. 10A and B, and the AP and DVC in Fig. 10D; “+” indicated scarce and/or light staining, as in the somas and neuropil in the CeA in Fig. 8C and D; “++” indicated moderate immunoreactivity, as in the somas in the GP and the neuropil in the CPu of Thy1- α -syn mice in Fig. 8; “+++” indicated dense immunoreactivity, as in the somas in the Rt and the neuropil in the cortex of Thy1- α -syn mice in Fig. 8; “++++” indicated very

dense and intensive immunoreactivity, as in the somas in the BLA in Fig. 8 and the neuropil in the hippocampus CA1 and overlying cortex in Thy1- α -syn mice in Fig. 7D-F.

Statistics

Statistical analysis was performed using SigmaPlot 13 (Systat Software, Inc., San Jose, CA, USA). Data are presented as mean $\pm$ SEM. Comparisons between two groups were performed using Student's t-tests. Comparisons among multiple groups were made using one-way or two-way ANOVA followed by the Tukey test for post hoc multiple comparisons. P values <0.05 were considered significant. Outliers were excluded based on Grubbs' test (<https://www.graphpad.com/quickcalcs/Grubbs1.cfm>, Graph Pad).

RESULTS

Male Thy1- α -syn mice have more severe motor and olfactory deficits and anxiety than female mice

Body weights were monitored during the behavior tests. Male and female Thy1- α -syn mice had similar body weights to female wt mice (28.5 ± 1.1 , 27.0 ± 0.8 and 25.8 ± 1.7 g, $n = 13$, 8 , and 7 , respectively; $p > 0.05$), and were significantly lighter than wt male mice (35.0 ± 0.9 g, $n = 11$; $p < 0.001$) at 6–7 months of age. No sex differences were observed in the wt littermates in any of the behavior tests (Fig. 1).

Cotton nesting—Wt mice of both sexes used almost all of the cotton pad available to them for nesting in 24 h (98.2 ± 1.2 vs. $93.1 \pm 5.6\%$, $n = 11$ and 7 , respectively). Male Thy1- α -syn mice used significantly less of the cotton pad ($34.6 \pm 6.3\%$, $n = 15$, $p < 0.001$), whereas female Thy1- α -syn mice used similar amounts of cotton pad as their wt littermates ($84.7 \pm 9.9\%$, $n = 8$, $p > 0.05$; Fig. 1A). We observed significant effects of sex ($p = 0.002$, $F_{1,37} = 11.5$), genotype ($p < 0.001$, $F_{1,37} = 29.5$), and sex x genotype ($p < 0.001$, $F_{1,37} = 17.4$) by two-way ANOVA.

Pole test—Male Thy1- α -syn mice took more time to turn around on the top of the pole than mice in the other 3 groups (Fig. 1B), although this difference did not reach statistical significance ($p = 0.057$, one-way ANOVA). Similarly, male Thy1- α -syn mice took a longer time than female Thy1- α -syn and male and female wt mice to descend the pole (50.1 ± 4.1 vs. 27.0 ± 6.2 , 29.6 ± 4.6 , and 25.7 ± 4.9 sec; $n = 15$, 8 , 11 , and 7 , respectively; Fig. 1C). Two-way ANOVA revealed significant effects of sex ($p < 0.05$, $F_{1,35} = 7.2$) and genotype ($p < 0.05$, $F_{1,37} = 4.7$).

Adhesive removal—Male and female wt mice removed the sticker in similar time (1.0 ± 0.2 vs. 0.7 ± 0.1 min, $n = 6$ and 4 , respectively). Male Thy1- α -syn mice took a significantly longer time than female Thy1- α -syn (5.3 ± 0.9 vs. 1.6 ± 0.5 min, $p < 0.05$) and wt mice to remove the sticker. There was no significant difference between female Thy1- α -syn and wt mice (Fig. 1D). Two-way ANOVA revealed significant effects of sex ($p < 0.05$, $F_{1,21} = 6.4$), genotype ($p = 0.005$, $F_{1,21} = 10.0$), and sex x genotype ($p < 0.05$, $F_{1,21} = 4.5$).

Marble test—Wt mice of both sexes buried similar numbers of marbles (12.5 ± 0.8 vs. 12.4 ± 1.0 marbles/30 min, $n = 11$ and 7). Thy1- α -syn mice buried fewer marbles than the wt littermates (Fig. 1E), as female Thy1- α -syn buried 7.4 ± 1.2 marbles/30 min ($n = 8$, $p < 0.05$), while male Thy1- α -syn only buried 1.8 ± 0.7 marbles/30 min ($n = 13$, $p < 0.05$). During most of the testing period, the tg mice displayed freezing behavior in the corner of the cage. Significant effects of sex ($p < 0.01$, $F_{1,35} = 9.2$), genotype ($p < 0.001$, $F_{1,35} = 73.5$), and sex $\times$ genotype ($p < 0.01$, $F_{1,35} = 9.4$) were observed by two-way ANOVA.

Olfaction—Female Thy1- α -syn mice did not spend significantly more time to find the buried food than male or female wt mice (3.8 ± 0.8 vs. 2.5 ± 0.3 and 2.7 ± 0.5 sec, respectively, $n = 11$, 7 , and 8 ; Fig. 1F). Male Thy1- α -syn mice took more time (5.1 ± 0.8 sec, $n = 13$; $p < 0.05$) than male wt mice to find the buried food. There were no significant differences between wt and Thy1- α -syn female mice, or between male and female tg mice. Two-way ANOVA revealed a significant effect of genotype ($p < 0.01$, $F_{1,35} = 38.1$).

Brain levels of human α -synuclein were higher in male Thy1- α -syn mice than in female Thy1- α -syn mice

Using an ELISA with an antibody for human α -syn, we detected very low α -syn signal in the olfactory structures of male and female wt mice (Fig. 2), and very low or no detectable signals in the other brain areas that were tested (data not shown). The detected signals in wt brain could be non-specific immunoreactivity. Levels of human α -syn were higher in male tg mice than in female tg mice (0.7-fold in the olfactory structures, 3.2-fold in the cortex, 0.7-fold in the striatum, 1.7-fold in the SN and VTA, and 1.2-fold in the dorsal medulla; Fig. 2).

Immunoreactivity of α -synuclein in male and female Thy1- α -syn mice

It is important to note that unlike the antibody in the ELISA assay, the antibody used for immunohistochemistry cross-reacts with human and mouse α -syn. Semi-quantitative assessment of α -syn immunoreactivity did not reveal obvious differences between male and female mice of either genotype, as shown in the example in Fig. 3. Analysis of mean gray values in the substantia nigra, reticular part (SNr) confirmed that there was no difference between male and female mice (Fig. 4). As such, descriptions of α -syn immunoreactivity patterns and the representative photomicrographs in the figures are not repeated for both male and female mice.

Immunoreactivity of α -syn was observed in bead-like structures around neurons and in the neuropil, i.e. pre-synaptic structures, possibly in the extracellular space as an evenly immunostained brown matrix, and in neuronal somas. In general, the distributions of presynaptic and somatic α -syn immunoreactivity were similar to those described in a previous report in the same mouse model [31, 32] and in C57BL/6J mice [43]. No α -syn immunoreactivity existed in proteinase K-treated brain sections from Thy1- α -syn mice. In this study, we focused on differences in the distribution of α -syn immunostaining between wt and tg mice, and unique altered features not previously reported.

In wt littermates, dense presynaptic and extracellular α -syn immunoreactivity was found in the SNr, lateral parabrachial nucleus (LPB), area postrema and dorsal vagal complex (Figs. 3 and 5). In Thy1- α -syn mice, most of the brain areas had increased α -syn immunoreactivity, while the density of presynaptic and extracellular α -syn immunoreactivity was decreased in the SNr, LPB, area postrema and dorsal vagal complex, and α -syn-immunoreactive (ir) neurons were observed in the SNr (Figs. 3 and 5).

Neuronal somas immunostained with α -syn were located in a few brain areas of wt littermates, namely the SN, compact part (SNc), ventral tegmental area (VTA), and dorsal motor nucleus of the vagus (DMV). Interestingly, there were fewer or no α -syn-ir somas in those brain areas in Thy1- α -syn mice (Figs. 3 and 5). Instead, there were α -syn-ir somas in many other brain regions, consistent with Tables 1-3 in Delenclos' report [32].

Mapping of phosphor-Ser-129 α -synuclein immunoreactivity in male and female Thy1- α -syn mice

The pS129 α -syn antibody used cross-reacted with human and mouse pS129 α -syn. No sex differences in pS129 α -syn immunoreactivity were observed, as seen in representative images of the midbrain (Fig. 6). Measurements of mean gray values of pS129 α -syn immunoreactivity did not reveal sex differences in the SNr or hippocampus CA1 and CA3, areas that contained well-labeled neurons and/or fibers (Fig. 4). Semi-quantitative assessment of the brain regions did not reveal differences between male and female mice of either genotype (the semi-quantitation of some brain regions in individual mice are presented in supplementary Table S1 as examples). Therefore, the distribution of pS129 α -syn immunoreactivity (Table 1) was compared between wt and Thy1- α -syn mice in groups of both male and female mice.

Pre-absorption of the primary antibody used for pS129 α -syn immunoreactivity with human pS129 α -syn resulted in no signal in the brain sections of Thy1- α -syn mice. In the brains of wt littermates, pS129 α -syn-ir cells were found in the olfactory bulb and central and basolateral amygdala (Figs. 7A and 8C). Low to moderate pS129 α -syn immunoreactivity in presynaptic and extracellular structures were observed in the olfactory bulb, striatum, amygdala, SNr and interpeduncular nucleus in the wt mice (Table 1; Figs. 6–8).

In Thy1- α -syn mice, intensive pS129 α -syn immunostaining was observed in neuronal nuclei, cytoplasm, and processes in many brain areas. The staining was particularly dense and intensive in the olfactory bulb and cortex, neocortex, hippocampus, amygdala, SNr, medial geniculate nucleus, interpeduncular nucleus and LPB. There were also well-labeled somas scattered in the reticular formations of the thalamus and brainstem (Table 1). Some brain regions such as the cortex, amygdala, thalamus and SNr, displayed light nuclear pS129 α -syn immunoreactivity or negative staining in wt mice, but dense labeling of neuronal somas, dendrites and axons in Thy1- α -syn mice (Figs. 6–10).

Telencephalon: In the olfactory bulbs of male and female wt littermates, scattered pS129 α -syn-ir fibers were observed in the glomerular and external plexiform layers, and some ir neurons were noted in the mitral layer. In Thy1- α -syn mice, spherical and tree-like immunolabeled fibers were observed in the glomerular layer, dense and strongly stained

neurites were seen in the external plexiform layers, and many dark brown cells were lined up in the mitral cell layer (Fig. 7A and B, Table 1). There were also a few periglomerular cells and tufted cells that were pS129 α -syn-positive (Fig. 7B). In the anterior olfactory nuclei of the tg mice, many cells were deeply immunostained, while only a few cells had lightly stained nuclei. Strong pS129 α -syn-ir somas and neurites were abundant in the nucleus of the lateral olfactory tract (Fig. 8B).

Cortical regions of the Thy1- α -syn mice from the prefrontal to auditory and visual cortices bore many pS129 α -syn-ir neuronal somas, dark-stained nuclei, and neurites in layers II-VI. Dendritic trees and axons of pyramidal neurons showed intensive pS129 α -syn immunoreactivity in the cortical columns of the sensorimotor cortex (Fig. 7D and E).

In the hippocampus, pyramidal neurons were strongly pS129 α -syn-positive, and densely stained neurites were observed in the CA1 oriens and radiatum layers and dark brown somas in the pyramidal layers of CA1 and CA3 in Thy1- α -syn mice (Fig. 7D and F). Neurons in the polymorph layer of the dentate gyrus were lightly pS129 α -syn-ir (Fig. 7D and G).

In the basal ganglia, moderate numbers of pS129 α -syn-ir neurons were located in the globus pallidus and ventral pallidum. In the caudate and putamen, there was presynaptic pS129 α -syn immunoreactivity (Fig. 8B). The cortical and basolateral nuclei of the amygdala contained densely pS129 α -syn-ir neurons and neurites. As in the wt littermates, there was some light nuclear pS129 α -syn immunoreactivity in the central nucleus of the amygdala (Fig. 8C and 8D).

Diencephalon: Many thalamic cells were pS129 α -syn-positive in Thy1- α -syn mice. These cells were found in the anterior, paratenial, ventrolateral, ventroposterior and parafascicular nuclei in the reticular formation, and in the subthalamic nucleus (Fig. 9D). Less pS129 α -syn immunoreactivity was observed in the hypothalamus. Interestingly, the superior optic and paraventricular nuclei looked “pale” – no or only a few immunoreactive nuclei were observed in these regions (Fig. 9D).

Mesencephalon: The SNr, interpeduncular nucleus and medial geniculate nucleus displayed pS129 α -syn immunoreactivity in wt mice. In Thy1- α -syn mice, there were abundant pS129 α -syn-ir cells and dense pS129 α -syn staining in the neuropil in these regions. The red nucleus, superior and inferior colliculi, and periaqueductal gray contained moderate numbers of pS129 α -syn-ir neurons (Fig. 6).

Rhombencephalon—There was almost no pS129 α -syn immunoreactivity in the pons, medulla, or cerebellum in wt mice. In contrast, pS129 α -syn-ir neurons were distributed in many areas of these brain parts in Thy1- α -syn mice (Table 1, Fig. 9). Strikingly, there was no pS129 α -syn immunoreactivity in the area postrema, medial nucleus of the solitary tract, dorsal motor nucleus, or the midline raphe, and there were no or few lightly stained neuronal nuclei in these areas in Thy1- α -syn mice. Areas with strong pS129 α -syn immunoreactivity included the parabrachial nuclei, dorsal tegmental areas, ventrolateral area (A1/C1) and nucleus ambiguus. There were scattered pS129 α -syn-ir neurons in the reticular formation

(Fig. 10B, D). In the cerebellum of Thy1- α -syn mice, the molecular layer was deeply immunostained with few pS129 α -syn-ir neurons (Fig. 10B).

DISCUSSION

Male Thy1- α -syn mice displayed severer deficits than female Thy1- α -syn mice in all of the behavioral tasks assessed in this study. Human α -syn levels were higher in male than female tg mice in the SN/VTA, striatum, olfactory regions, cortex and dorsal medulla. These are brain centers regulating motor behavior, olfactory function and stress responses. Interestingly, the distribution and density of α -syn and pS129 α -syn immunoreactivity detected using antibodies with cross-reactivity to human and mouse α -syn were similar in the brains of male and female Thy1- α -syn and wt mice.

The mice used in this study were 4–7 months old. Compared to wt littermates, male Thy1- α -syn mice displayed reduced performance in cotton nesting by 65% and in marble-burying by 85%. They also spent up to 1.8-fold more time to descend the pole, took 5.2-fold longer to remove the sticker in the adhesive removal task, and searched 2.1-fold longer to find the buried food pellet. In contrast, female Thy1- α -syn mice did not perform differently from wt female mice in cotton nesting, the pole test, or the buried food pellet test. They did have deficits in adhesive removal and marble burying, although these deficits were significantly less severe than in male Thy1- α -syn mice. In general, the female Thy1- α -syn mice displayed less severe Parkinsonian phenotypes in the behavioral tests than their male counterparts. The data support previously reported sex differences in motor functions [29, 30]. In addition, it is known that male Thy1- α -syn mice weigh less than wt littermates [20], and our data showed that the tg mice weighed 16% less at 6 months of age. Importantly, we found no difference in weight between female tg and wt mice, consistent with the less severe and/or absent behavioral deficits.

About 50–77% of PD patients have Lewy body pathology that is not confined to the nigrostriatal pathways [44, 45]. Assessing the brain levels and distributions of α -syn and pS129 α -syn may unveil the mechanisms underlying some of the pathological features of mouse models of synucleinopathy. A previous study using male Thy1- α -syn mice reported robust human α -syn mRNA expression in the SN and 1.5–3.0-fold increases in total α -syn immunoreactivity in various brain regions [18]. A study on sex differences in motor deficits in Thy1- α -syn mice detected higher levels of human α -syn mRNA in the SN of male vs. female mice [29]. We found that concentrations of human α -syn in the SN-VTA region, striatum, olfactory regions, cortex and dorsal medulla were respectively 1.7, 0.8, 0.7, 2.3, and 1.3 times higher in male than female Thy1- α -syn mice. These data suggest that there may be more neurotoxicity in the brains of male than female Thy1- α -syn mice due to higher human α -syn levels, which may be associated with severer deficits in behavioral tasks. The lower brain levels of human α -syn in female tg mice likely result from X inactivation, as the transgene is located on the X chromosome [18].

Commercially available antibodies against α -syn and pS129 α -syn used for immunohistochemistry cross-react with human and mouse α -syn [42]. Therefore, we were unable to differentiate human α -syn and pS129 α -syn from the endogenous protein in the

brains of Thy1- α -syn mice. However, the immunolabeling in the wt mouse brains could be attributed to only mouse α -syn and pS129 α -syn. The low levels of pS129 α -syn immunoreactivity in the brains of wt mice suggest that there could be some phosphorylation of mouse α -syn. The different α -syn distributions in the brains of Thy1- α -syn mice when compared with those of wt mice indicate possible abnormal alterations in α -syn expression, conformation, and/or deposition. Analysis of the immunostaining patterns revealed interesting alterations in the distributions of α -syn-ir somas and presynaptic elements, and the widespread and abundant pS129 α -syn immunoreactivity in the Thy1- α -syn mice could be attributed to human α -syn overexpression. Interestingly, we did not find any obvious differences when comparing the immunoreactivity patterns in male and female Thy1- α -syn brains.

When immunostained using the same antibody as that used in this study, normal C57BL/6 mouse brains contain presynaptic α -syn in many regions. In contrast, immunoreactive neuronal somas are only observed in a few areas, namely the glomerular layer of the olfactory bulb, the mammalian nucleus of the hypothalamus, SNc, and DMV [43]. In addition to the above regions, α -syn-ir somas were observed in the VTA in the wt mice in this study. Moderate presynaptic α -syn immunoreactivity was observed in the olfactory bulb and hippocampus, and there was dense α -syn immunoreactivity in the SNr, lateral parabrachial nuclei, area postrema, nucleus tractus solitarii, and DMV. This characteristic distribution of α -syn immunoreactivity in wt mice indicates that α -syn conformation may be sensitive to pathological insults such as oxidative stress, inflammation, and neurotoxicity in some brain regions [44, 46, 47].

In the Thy1- α -syn mice, which overexpress human α -syn, immunoreactivity of α -syn (antibody cross-reacted with mouse and human α -syn) was increased throughout the brain in regions consistent with those reported previously when using an anti-human α -syn serum, although there was less immunoreactivity in the 6–7 month-old mice in this study when compared to the 12 month-old mice in the previous studies [31, 32]. No abnormal α -syn-ir structures were observed after proteinase K treatment in any of the brain regions tested. This result is different from those in previous publications reporting proteinase K-resistant α -syn immunostaining in the SN of 6 month-old Thy1- α -syn mice [18]. The discrepancy may result from the mice being raised in different colonies. There were interesting differences in the distribution patterns of α -syn between wt and tg mice. The α -syn-ir somas in the SNc, VTA, and DMV found in wt mice were not observed in Thy1- α -syn mice. The presynaptic α -syn immunoreactivity in the SNr and dorsal vagal complex was dense in wt mice, but was less strong in Thy1- α -syn mice; instead, many α -syn-ir somas were found in the SNr in the tg mice. These alterations may reflect potential changes in the conformation and subcellular localization of α -syn [48] in response to human α -syn overexpression, although the precise nature of the resulting α -syn forms and their functions are not known [49, 50].

α -syn has been reported to have both “good” and “bad” effects on neuronal activity and communication [51, 52]. It is a presynaptic molecule and involved in the maintenance of synaptic vesicle binding and trafficking, and different forms of α -syn may play various roles in normal cellular functions and disease pathogenesis [49, 53]. In addition to presynaptic areas, α -syn is found in neuronal nuclei, where it binds DNA [13, 54, 55]; and in the

extracellular space [56] in response to neuronal activity *in vitro* and *in vivo* in mice [57]. The presence of normal α -syn may counteract its fibrillation [58]. There are also complex interactions between α -syn and individual neurotransmitter systems. For instance, different species of α -syn have been reported to decrease, increase, or have no effect on DA release depending on the brain region studied [47]. Transgenic mice overexpressing human α -syn exhibit impaired synaptic vesicle exocytosis and reduced neurotransmitter release [51].

Phosphorylated α -syn has attracted attention as a pathological species, since pS129 α -syn was found to comprise the majority of α -syn in Lewy bodies. Although the distribution patterns of pS129 α -syn in human and mouse brain have been previously described, such data are limited and have been mainly reported on regions associated with α -syn-mediated pathogenesis, such as the SN, olfactory bulb, cortex, and amygdala [33, 59–62]. The rabbit monoclonal pS129 α -syn antibody used in this study was developed against a peptide with amino acid residues that are identical in human and rodent α -syn, and has high sensitivity and specificity. Pre-absorption of the primary antibody with human pS129 α -syn led to an absence of immunostaining in the Thy1- α -syn mouse brain. Immunoreactivity of pS129 α -syn was located in a few brain regions of the wt littermates, including the olfactory bulb, hippocampus, basolateral and central amygdala, and especially the SNr, where there was strong immunolabeling. Only lightly stained nuclei existed in the amygdala. These observations indicate that α -syn is phosphorylated in the above brain regions in normal mice. In the presence of human α -syn overexpression in the Thy1- α -syn mice, intensive pS129 α -syn immunostaining was widely distributed from the olfactory bulb to the medulla. Most of the densely stained regions were those bearing α -syn immunoreactivity, but the subcellular localization of the pS129 α -syn staining was different from that observed with the α -syn antibody. pS129 α -syn immunoreactivity was present in nuclei, perikarya, dendrites, and axons, particularly in the olfactory bulb, hippocampus, and cortex, where there were dense “bushes” of neurites. Brain pS129 α -syn -positive structures are much more abundant in Thy1- α -syn mice than in A30P α -syn transgenic mice [33], indicating that there is more widespread synucleinopathy caused by wild type α -syn than by the A30P familial mutant form of the protein. The absence of sex differences in the distribution of pS129 α -syn immunoreactivity in the brain indicates that this type of α -syn may not be associated with the observed sex differences in the behavioral deficits in Thy1- α -syn mice. However, it does not suggest that there are no differences in pS129 α -syn and α -syn formation, and the interactions between different α -syn species will need to be investigated in brain regions associated with phenotypes of the model. It is worth noting that the semi-quantitative analysis of the pS129 α -syn immunostaining used in this study may not be sufficiently sensitive to detect subtle differences in the levels of the protein in male vs. female mice. As such, more quantitative assays to measure pS129 α -syn, such as custom ELISAs or semi-quantitative immunoblot experiments are needed to confirm our findings.

Whether phosphorylation at serine-129 suppresses or enhances α -syn aggregation and toxicity *in vivo* also remains a subject of active debate [63]; thus, there is limited understanding of the role of specific forms of α -syn in the development of pathological alterations in PD [64]. The presence of pS129 α -syn immunoreactivity in wt mouse brains and normal human brains supports the hypothesis that pS129 α -syn may have some normal function [59, 60].

Human α -syn was originally isolated as the non-amyloid component of plaques from the brains of patients with Alzheimer's disease [65, 66]. Synucleinopathies encompass not only PD, but also other neurodegenerative diseases. In fact, α -syn may interact with other pathological proteins, such as tau and amyloid- β [1, 5, 67, 68]. α -syn may contribute to PD pathology by adoption of abnormal conformations, disturbing various cellular signaling pathways, and causing neuroinflammation [68]. Although α -syn has been investigated for its role in neurodegenerative diseases called synucleinopathies, much about its cellular functions and the mechanisms by which it causes pathogenesis remains unclear [67]. Mouse models with human α -syn overexpression in the brain can be useful tools to investigate pathogenic mechanisms associated with synucleinopathy in other neurodegenerative diseases [17].

Vulnerability of brain regions is recognized as an important factor in the development of neurodegenerative diseases, including synucleinopathies [44, 47]. The neuronal connectome could be involved in the spreading of synucleinopathy in the brain [47, 69]. Most of the studies on PD pathogenesis have focused on neurons in the nigrostriatal system, cortex, and hippocampus [46, 70–72]. For instance, in 6-OHDA models, DA and non-DA neurons in the mesencephalon of male mice have been shown to be more vulnerable to oxidative stress than those in female mice [73]. The nigrostriatal circuit may also be involved in non-motor disorders, e.g. through its connections to the cortex and thalamus [74]. Rats display cognitive deficits when DA neurons in the VTA are damaged following adeno-associated virus-induced α -syn overexpression [75].

Our data indicate that the olfactory pathway, amygdala, LPB, and dorsal vagal complex should be further investigated for their effects on α -syn-mediated neuronal activity and pathology. Of these regions, the LPB is the least studied. In the LPB, α -syn immunoreactivity was dense in wt mice, but was absent in Thy1- α -syn mice. In contrast, there was no pS129 α -syn immunoreactivity in the LPB in wt mice, but many intensely stained neurons in this region were observed in tg mice. This may indicate that the conformations of α -syn and its functions are altered in this region in the Thy1- α -syn model; the LPB may thus be one of the key regions for α -syn pathophysiology. The LPB responds to many visceral stimuli and plays roles in autonomic functions and nociceptive processing [76, 77]. In particular, the LPB has direct reciprocal connections with the vagal afferent receiving nucleus, the NTS, the subcortical autonomic regulation center, the hypothalamus, and one of the brain regions integrating responses to stress and autonomic dysfunction, namely the amygdala [78–81].

In conclusion, the differences in behavioral impairments between male and female Thy1- α -syn mice may be associated with the higher human α -syn expression in the brains of male mice. The lower levels of α -syn expression in female mice are due to random inactivation of the transgene, which is located on the X chromosome. The widespread α -syn pathology and altered cellular localization of α -syn in neurons in select brain regions of Thy1- α -syn mice when compared to those in wt mice provide evidence for the complexity of the functions of α -syn. Overexpression of human α -syn may lead to the loss of the normal forms of the protein in the SN, VTA, LPB, and dorsal vagal complex. The absence of differences between male and female Thy1- α -syn mice in the distributions of α -syn and pS129 α -syn

in the brain may suggest that even the lower levels of human α -syn overexpression in female Line 61 mice are sufficient to induce pathological alterations of brain α -syn. The specific conformations of α -synuclein, their brain region-specific distributions, and their relationships to the behavioral deficits observed in this mouse model warrant further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Comparison between male and female Thy1- α -syn mice (4–7 months old) versus wt littermates in motor/behavioral tests. Male Thy1- α -syn mice significantly nested less cotton (A), and buried fewer marbles (E) than female mutant mice, and took longer time to descend from a pole top (C), remove a sticker from the nose (D) and find the food pellets (F). The difference of turn time in pole test did not reach significance (B). Female Thy1- α -syn mice showed significant deficits in adhesive removal and marble burying, and performed significantly better than males (D, E). Data are mean $\pm$ SEM; mice/group indicated in each bar; *, $p < 0.05$ vs. wt of same sex; and #: $p < 0.05$ vs. male Thy1- α -syn.

Fig. 2.

Concentrations of human α -syn in selective brain regions measured by enzyme-linked immunosorbent assay. Male and female wt mice had no human α -syn (hasyn) immunoprecipitation except very low levels in olfactory structures. Concentrations of human α -syn in the substantia nigra and ventral tegmental area (SN-VTA), striatum, olfactory structures, cortex (cingulate, primary motor and sensory: cing, M&S) and dorsal medulla of male Thy1- α -syn were 1.7, 0.8, 0.7, 2.3, and 1.3 times significantly higher than female

Thy1- α -syn mice, respectively. Data are mean $\pm$ SEM; mice/group indicated in each bar; *: $p < 0.05$ vs. wt mice of the same sex.

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Fig. 3. Microphotographs of α -syn immunoreactivity in the substantia nigra (SN) of male and female wild type and Thy1- α -syn mice. Immunoreactivity of α -syn in some neuronal somas in SN compact part (SNc) and intensive presynaptic staining in SN reticular part (SNr) of wild type mice, and few somas in the SNc in Thy1- α -syn mice, but many in SNr where less presynaptic α -syn-ir. Immunoreactivity of α -syn was similar in male and female mice of both genotypes. Scale in A = 100 μ m for all panels.

Fig. 4.

Mean gray value of immunoreactivity of α-synuclein (α-syn) in the substantia nigra, reticular part (SNr) and phosphorylated serine 129 α-synuclein (pS129 α-syn) in SNr, hippocampus CA1 and CA3 of male and female Thy1-α-syn and wt mice, measured in FIJI (ImageJ). Data are mean ± SEM; mice/group indicated in each bar; *: p < 0.05 vs. WT mice of the same sex.

Fig. 5.

Microphotographs of α -syn immunoreactivity in the ventral tegmental area (VTA), lateral parabrachial nucleus (LPB) and medial-dorsal medulla of wt and Thy1- α -syn mice. The VTA of wild type mice contains α -syn positive somas (A) and few in Thy1- α -syn mice (B). The LPB shows dense presynaptic α -syn immunoreactivity in wt mice (C), but less in Thy1- α -syn mice (D). The area postrema (AP), nucleus tractus solitarius (NTS) and dorsal motor nucleus of the vagus (DMV) of wt mice display higher α -syn immunoreactivity than that in Thy1- α -syn mice (E, F). The insert in E: magnified DMN showing α -syn

positive neurons. fr: fasciculus retroflexus; MPB: medial parabrachial nucleus; scp: superior cerebellar peduncle. Scale in A = 100 μ m for panels A-F.

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Fig. 6. Microphotographs of pS129 α -syn immunoreactivity in midbrain of male and female wild type and Thy1- α -syn mice. IP: interpeduncular nucleus; MG: medial geniculate nucleus; PAG: periaqueductal gray; Red n: red nucleus; SC: superior colliculus; SNc: substantia nigra, compact part; SNr: substantia nigra, reticular part. Scale in A = 500 μ m for all panels.

Fig. 7. Microphotographs of pS129 α -syn immunoreactivity in the olfactory bulb (A, B), cortex and hippocampus (C, D) wt and Thy1- α -syn mice. pS129 α -syn immunoreactive neurons were found in the mitral layer (arrows) of both wild type (A) and transgenic mice (B). The cortex and hippocampus of wt mice contained almost no specific pS129 α -syn immunoreactivity (C), in contrast dense staining in Thy1- α -syn mice (D), and somas in the cortex and the pyramidal layer of CA1 and CA3 were intensively immunostained. Panels of E-G: magnification of the cortex, CA1 and dentate gyrus (DG) from panel D. EPI: external

plexiform layer; Gl: glomerular layer; Mi: mitral cell layer. Scale bar in A = 50 μm , same for B; scale bar in C = 500 μm , same for D; scale bar in E = 100 μm , same for F and G.

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Fig. 8. Microphotographs of pS129 α-syn immunoreactivity in the striatum (A, B) and amygdala (C, D) of wild type and Thy1-α-syn. BLA: basolateral nucleus of amygdala; CeA: central nucleus of the amygdala; CPu: caudate putamen; GP: globus pallidus; LOT: nucleus of the lateral olfactory tract; RT: reticular thalamic nucleus; VP: ventral pallidus. Scale bar = 500 μm for panels A and B; and scale bar = 100 μm for panels C and D.

Fig. 9. Microphotographs of pS129 α-syn immunoreactivity in the hypothalamus (A, B) and thalamus (C, D) of wild type and Thy1-α-syn mice. pS129 α-syn immunoreactivity was light in the paraventricular nucleus (PVN) of the hypothalamus of the transgenic mice (B) and many pS129 α-syn immunoreactive cells were located in the thalamus (D), including the ventral posteromedial thalamic nucleus (VPM) and subthalamic nucleus (STh, arrow). PF: parafascicular thalamic nucleus; Rt: reticular thalamic nucleus; VPL: ventral posterolateral thalamic nucleus. Scale bar in A = 100 μm, same for B; scale bar in C = 500 μm, same for D-F.

Fig. 10.

Microphotographs of pS129 α-syn immunoreactivity in pons (A, B) and medulla (C, D) of wild type and Thy1-α-syn mice. Arrows in panel B indicate pS129 α-syn immunoreactivity in the molecular layer of cerebellum. A1/C1: ventrolateral medullar (A1/C1) area; AP: area postrema; DMV: dorsal vagal complex; DTg: dorsal tegmental area; LC: locus ceruleus; LPB: lateral parabrachial nucleus; MPB: medial parabrachial nucleus; RF: reticular formation; Vsp: spinal nucleus of the trigeminal nerve; XII: hypoglossal nucleus. Scale bar = 500 μm, same for all panels.

Table 1.

Brain distribution of phosphor ser-129 α -synuclein in mice overexpressing human wide type α -synuclein driven by Thy-1 promoter (Thy1-asyn) and wild type (WT) littermates.

Brain structure	WT		Thy1-asyn	
	Somas	Neuropil	Somas	Neuropil
Olfactory bulb and cortex				
Glomerular layer	-	+	-	+
External plexiform layer	-	+	-	++
Mitral layer	++	+	+++	+
Internal plexiform layer	-	-	-	+
Anterior olfactory nu	+	-	+++	+
Anterior cingulate cortex	+	-	+++	-
Cortex (cingulate, M&S)	+	-	++++	++
Striatum				
Caudate putamen	-	-	±	+
Globus pallidum	-	+	++	+
Ventral pallidum	-	-	++	+
Amygdala				
Anterior cortical nu	-	-	+++	+
Central nu	+	-	+	+
Basolateral nu	++	-	++++	++
Hippocampus				
CA1	-	-	++++	++++
CA2	-	-	+	+
CA3	±	-	+++	++
Dentate gyrus	-	-	+	+
Thalamus				
Paratenial nu	-	-	+++	+
Anterodorsal nu	-	-	++	-
Anteromedial nu	-	-	+	-
Ventrolateral nu	-	-	++	+
Reticular formation	-	-	+++	+++
Hypothalamus				
medial preoptic area	-	-	++	+
Lateral preoptic area	-	-	++	+
Superior optic nu	-	-	-	-
Paraventricular nu	-	-	±	-
Lateral area	-	-	+	+
Dorsomedial area			++	-
Zona incerta	-	-	++	++

Brain structure	WT		Thy1-asyn	
	Somas	Neuropil	Somas	Neuropil
Subthalamus	-	+	+++	+
Brainstem reticular formation	-	-	+++	++
Midbrain				
Substantia nigra, compact part	-	-	+	+
Substantia nigra, reticular part	-	++	+++	+++
Substantia nigra, lateral part	-	-	+	+
Ventral tegmental area	±	-	++	+
Medial geniculate nu	-	-	++	++++
Red nucleus	-	-	+++	++
Periaqueductal gray	-	-	++	+
Interpeduncular nu	-	+	+++	++
Superior colliculus	-	-	++	++
Inferior colliculus	-	-	++	++
Pons				
Medial parabrachial nu	-	-	++	+
Lateral parabrachial nu	-	-	+++	+
Locus coeruleus	-	-	±	±
Dorsomedial tegmental area	-	-	++	+
Dorsolateral tegmental area	-	-	++	+
Raphe nu	-	-	+	+
Medulla oblongata				
Nu of solitary tract	-	-	-	-
Lateral nu of solitary tract	-	-	+	+
Dorsal motor nu of vagus	±	-	+	-
Nu ambiguous	-	-	+++	++
Spinal nu of V	-	-	++	+
Hypoglossal nu	-	-	+	+
Raphe nu	-	-	±	-
Ventrolateral area	-	-	+++	++
Gigantocellular reticular nu	-	-	++	+
Gracile and cuneate nu	-	-	++	+