Research article

Level of plasma neuregulin-1 SMDF is reduced in patients with idiopathic Parkinson's disease

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HIGHLIGHTS

- We detected and measured NRG1 SMDF protein in plasma.
- Levels of plasma NRG1 SMDF were correlated with cerebral spinal fluid levels.
- Levels of plasma NRG1 SMDF were significantly reduced in sporadic PD.

ARTICLE INFO

ABSTRACT

Parkinson's disease (PD) is characterised by the progressive loss of dopaminergic neurons, neurons that are regulated by the development, protection and function of neuregulin-1 (NRG1)-ErbB4 signals, in the substantia nigra (SN). NRG1 is a neurotrophic differentiation factor and one of its isoforms is a sensory and motor neuron-derived factor (SMDF), mostly expressed in neurons. To examine the relationship between NRG1 SMDF and PD, we tested whether NRG1 SMDF can be detected and measured in plasma and whether their level in plasma correlates with the clinical severity of PD. We detected NRG1 SMDF to be immunoreactive in plasma. Using an ELISA method specific for NRG1 SMDF, we found that NRG1 SMDF levels were significantly reduced in sporadic PD as compared to controls. However, levels of plasma NRG1 SMDF showed no correlation with the clinical severity of PD. Additionally, we found that there was a correlation of NRG1 SMDF levels in CSF with that in plasma where levels in plasma were significantly higher, at approximately ten times that in CSF. Finally, we also examined the expression of NRG1 SMDF in the post-mortem brain using immunohistochemistry and showed that Lewy bodies in the SN of patients with PD were immunoreactive for NRG1 SMDF. In summary, we found that the reduction of plasma NRG1 SMDF is specifically associated with PD, but has no correlation with the clinical severity of PD. These findings of NRG1 SMDF may provide important complementary information for diagnosing the onset of PD.

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1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder of adulthood, especially in the elderly population. The loss of dopaminergic neurons and Lewy body formation in the substantia nigra pars compacta (SNpc) is the hallmark pathology of this disorder. Apart from some hereditary PD, the majority of PD is sporadic and the cause of this disorder is not known. Furthermore, several neurodegenerative disorders manifest clinical features similar to those of PD, also known as 'Parkinsonism' [1,2].

Neuregulin-1 (NRG1) is a neurotrophic factor that contains an epidermal growth factor (EGF)-like domain, and can be classified into subgroups through different N-amino termini [3]. NRG1 type III, sensory and motor neuron-derived factor (SMDF), is the most dominant type of NRG1 in the human adult brain, accounting for ~73% of total NRG1 [4]. In addition, it is selectively expressed in neurons and plays important roles in the differentiation of oligodendrocytes from neural precursor cells, control of neuronal

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nicotinic acetylcholine receptor expression in the central nervous system (CNS), and differentiation, proliferation, regeneration of Schwann cells in the peripheral nervous system (PNS) [5–8].

NRG1 signals act through ErbB receptors and ErbB4, one of the receptors, is an NRG1-specific ErbB. It is highly expressed in dopaminergic neurons of the SN in human adults and regulates their development and function. NRG1–ErbB signalling plays important roles in neurons, astrocytes, and oligodendrocytes, regulating NMDA, GABA, and ACh receptor expression, and is involved in dopaminergic neurotransmission as well as the survival of dopaminergic neurons [9–12].

Taken together, NRG1 SMDF acting through ErbB4 is an important protein for the dopaminergic neurons of SN. In this study, we focused on NRG1 SMDF and the possible link it has with neurodegenerative disorders including PD.

2. Material and methods

2.1. Participants and samples

A total of 45 patients with sporadic PD (mean age 70.6 ± 6.4 years), 51 patients with MSA (63.0 ± 7.7), 36 patients with ALS (65.9 ± 9.4) and 30 healthy adult controls (61.7 ± 7.1) were analyzed. In our bank of samples, there were 9 sets of plasma and CSF samples from 6 patients with MSA, 2 with ALS, and one with iNPH. In each case, peripheral blood was obtained immediately after CSF sampling by lumbar puncture. The clinical severity of PD was rated according to the Hoehn and Yahr Scale ($n = 45$) [13] and the Unified Parkinson's Disease Rating Scale (UPDRS) Parts I and III ($n = 34$) [14]. In patients with PD, 11 were Yahr II, 23 were Yahr III, 8 were Yahr IV, and 3 were at Yahr V. The score on UPDRS Part I was 2.82 ± 2.44 (mean $\pm$ SD), and Part III was 29.09 ± 17.59.

Plasma and CSF samples were centrifuged at 3,000 rpm for 30 min at 4 rpm for 30 min at 4 °C. The aliquots were collected and frozen at −80 °C until measured. This study was approved by the Institutional Ethics Committee of Hokkaido University Graduate School of Medicine and written informed consents were obtained from all participants or their families when patients were unable to sign themselves.

2.2. Western blot methods

Plasma samples (100 μl) were concentrated with a Microcon YM-100 centrifugal filter (Millipore, Billerica, MA, USA) at 14,000 g for 12 min. The concentrated plasma samples (per 10 μl of non-concentrated plasma) were separated by SDS-PAGE and transferred to a PVDF membrane (Bio-Rad Hercules, CA, USA). The membranes were incubated with anti-human NRG1 isoform SMDF antibody (R&D systems, Minneapolis, MN) (0.1 μg/ml) at room temperature (RT) for 3 h. After washing, they were incubated with peroxidase-conjugated anti-goat IgG (H + L) (Jackson ImmunoResearch, West grove, PA) (1:5000) at RT for 1 h, followed by detection with ECL Plus Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK). As a positive control for NRG1 SMDF, recombinant human NRG1 isoform SMDF (R&D systems, Minneapolis, MN) was used.

2.3. ELISA methods

In brief, wells of Maxisorp black ELISA plates (Nunc, Roskilde, Denmark) were coated with 70 ng anti-human NRG1 isoform SMDF antibody (R&D systems, Minneapolis, MN) at 4 °C overnight. After washing and blocking, plasma (1:5) or standard, 100 μl recombinant human NRG1 isoform SMDF (R&D Systems, Minneapolis, MN) (0.125–10 ng/ml) was applied in duplicate into each well and incubated at 4 °C overnight. After washing the wells, 15 ng

of human NRG1β1/HRGβ1 extracellular domain biotinylated antibody (R&D systems, Minneapolis, MN) was added to each well where it indicated a soluble mature form of NRG1 type 1HRGβ1. Biotinylated antibodies bound to wells were incubated with 100 μl avidin-β-galactosidase (1:10000; Rockland, Gilbertsville, PA) and followed by the fluorogenic substrate, 200 μM MUG (Sigma, St Louis, MO). The fluorescence was measured using a Varioskan Flash microplate reader (Thermo Scientific; Waltham, MA) with excitation and emission at 365 and 450 nm. To test the cross-reactivity of NRG1 SMDF in ELISA, we used recombinant proteins of the EGF family; EGF, betacellulin, heparin-binding EGF-like growth factor (HB-EGF), transforming growth factor-α (TGF-α), NRG1α/HRG1α EGF domain and human NRG1β/HRG1β extracellular domain (R&D systems, Minneapolis, MN).

2.4. Immunohistochemistry

Immunohistochemical analysis was carried out using formalin-fixed, paraffin-embedded sections from the midbrain and upper pons of patients with PD ($n = 5$) and normal controls ($n = 5$). The primary antibody used was a rabbit polyclonal antibody against human NRG1 SMDF (1:100; Novus Biologicals, Littleton, Co., USA).

2.5. Statistical Analysis

Group comparisons were conducted using two-tailed Wilcoxon rank-sum tests and comparisons of age were conducted using the two-tailed Student's t-test. The relationship between plasma NRG1 SMDF levels and clinical severity scales were evaluated using Pearson's correlation coefficient. Data was analyzed with JMP® statistical software, version 11.0 (SAS Inc., Cary, North Carolina) and p-values < 0.05 were considered significant.

3. Results

3.1. Detection of NRG1 SMDF in peripheral blood

The NRG1 SMDF protein was found to be immunoreactive in plasma samples. They were found to be around 50 kDa in size, presumably as a soluble form of a C-terminal processed product (Fig. 1A). The MW of recombinant human NRG1 SMDF protein, as a positive control, was slightly lower than NRG1 SMDF in human plasma. This was thought to be because the recombinant protein is a baculovirus-derived NRG1 type III (SMDF) β3 protein whereas those in plasma are NRG1 SMDF proteins that are released, glycosylated, and have undergone proteolytic processing in vivo. NRG1 SMDF proteins expressed in mammalian tissue have a slightly different MW than those of cell lines [15].

3.2. Measurement of NRG1 SMDF by ELISA

In this especially designed ELISA system, the linearity of NRG1 SMDF detection was obtained using concentrations ranging from 0.125 ng/ml to 100 ng/ml recombinant human NRG1 SMDF (Fig. 1B). No cross-reactivity or interference with other NRG1-like proteins (~100 ng/ml) was seen when using EGF, HB-EGF, TGF-α, betacellulin, NRG1αEGF domain, or a type I NRG1β extracellular domain (Table S1), indicating that this ELISA system was able to detect a domain specific to NRG1 SMDF.

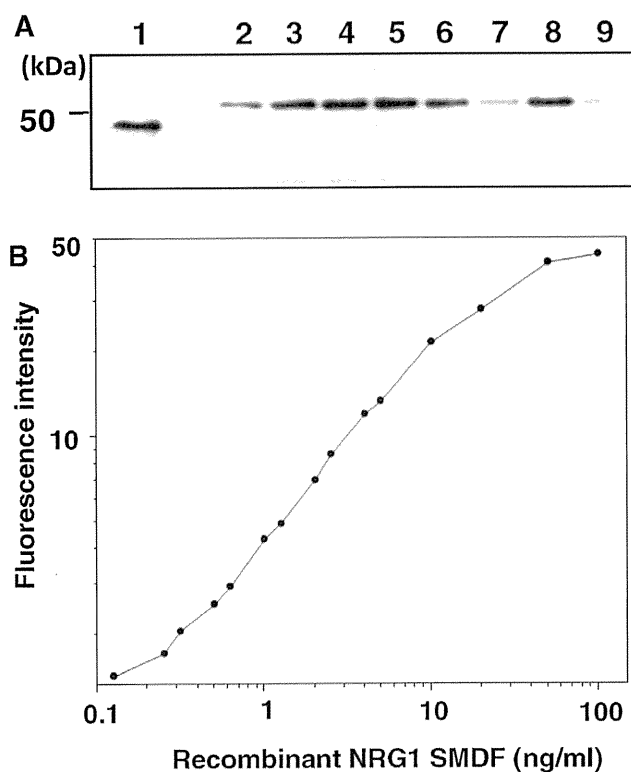Supplementry material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2014.12.024.

A

B

Fig. 1. Characterization of plasma neuregulin 1 (NRG1) SMDF by W.B and ELISA.
A. Representative immunoblot analysis of plasma NRG1 SMDF from PD and normal controls. Concentrated plasma samples (per 10 μl of non-concentrated plasma) of controls (lane 2–5) and PD patients (lane 6–9). Positive control of recombinant NRG1 SMDF protein used (lane 1). Signals showed presence of NRG1 SMDF protein only. The variability of individuals was not observed by this W.B.
B. A standard curve of ELISA with control NRG1 SMDF protein.
Various concentrations of human recombinant NRG1 SMDFβ3 (0.125–100 ng/ml) applied to ELISA as a standard.

3.3. Level of NRG1 SMDF in samples

The mean NRG1 SMDF level of the 30 healthy normal controls was 9.5 ± 2.2 (mean $\pm$ SE; ng/ml); 9.8 ± 3.5 in 16 females and 9.0 ± 2.8 in 14 males. There was no significant difference between genders ($p = 0.866$) or associated with age ($r = 0.098$, $P = 0.605$).

There were no statistical significant differences between the levels of plasma NRG1 SMDF in controls (9.5 ± 2.2) and ALS (9.3 ± 2.7, $p = 0.315$) or controls and MSA (12.7 ± 2.6, $p = 0.837$), but plasma NRG1 SMDF levels were significantly lower in PD (4.4 ± 0.7, $p = 0.025$) as compared to controls (Fig. 2A). Additionally, when plasma NRG1 SMDF levels between parkinsonism dominant type MSA (MSA-P) and ataxia dominant type MSA (MSA-C) were compared, we found the level in the MSA-P group ($n = 22$, 8.7 ± 2.7) to be slightly lower than that in the MSA-C group ($n = 29$, 15.8 ± 4.1), but the difference was not statistically significant (Fig. 2B).

We also assessed plasma NRG1 SMDF levels and PD clinical severity and found that they did not correlate with Yahr stage ($r = 0.174$, $p = 0.254$), UPDRS Part I ($r = -0.116$, $p = 0.514$), UPDRS Part III ($r = 0.150$, $p = 0.398$).

Finally, we analyzed the potential effectiveness of PD medications (levodopa, amantadine, anticholinergic agents, catechol-O-methyl transferase inhibitors and monoamine oxidase type B inhibitors) with NRG1 SMDF, but found that there was no correlation (Fig. S1).

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3.4. NRG1 SMDF is present in human CSF and plasma

In order to examine NRG1 SMDF levels in CSF and plasma (sampled at the same time), we used 9 sample sets comprised of 6 MSA patients, 2 with ALS and one with idiopathic normal pressure hydrocephalus (iNPH). We found there was a significant correlation of NRG1 SMDF levels in CSF with that in plasma ($r = 0.667$, $p = 0.049$) (Fig. 2C): levels in plasma were significantly higher, at approximately 10 times that in CSF (10.2 ± 3.9 in plasma; 1.1 ± 0.5 in CSF; $p = 0.034$).

3.5. Immunohistochemical analysis

In PD and normal controls, the neuronal cytoplasm and processes were weakly immunolabeled with the anti-NRG1 SMDF antibody (Fig. 3). No significant difference was noted in the NRG1 SMDF immunoreactivity of the neurons between PD and controls (data not shown) and there was also no difference in the staining intensity between the substantia nigra pars compacta (dopaminergic neurons) and pars reticularis (GABAergic neurons) or pedunculopontine tegmental nucleus (cholinergic neurons). In PD, Lewy bodies present inside and outside the SN were homogeneously stained in all the cases examined.

4. Discussion

This is the first report using samples of patients and controls that makes evident several findings of NRG1 SMDF. It can be detected and measured in the peripheral blood and confirmed by ELISA. Its levels in plasma and CSF are positively correlated, and the levels in plasma are significantly lower in PD than in controls though this reduction is not seen in other neurodegenerative disorders including ALS and MSA. Levels of NRG1 SMDF are not correlated with clinical severity or the stage of PD, although interestingly, some normal control subjects showed lower values similar to those seen in the PD group. Lastly, Lewy bodies in the SN of PD are immunoreactive for the anti-human NRG1 SMDF antibody.

PD results from the progressive loss of dopaminergic neurons in the SN [1], where NRG1 regulates dopamine levels and is an indicator of neuroprotectivity [16,12] and ErbB4, a receptor of NRG1, is highly expressed in these neurons [10,11]. We examined the levels of NRG1β1/HRG1β1, a soluble form protein, in plasma, but found that the concentrations did not differ between controls, MSA, and PD (Fig. S2). We initially chose to analyze NRG1 (type III) SMDF since it has been reported that it is selectively expressed in neurons [5]. In addition, it has generally been thought that the mature protein of NRG1 SMDF is expressed on the cell-surface as a membrane-anchored molecule. However, there are also some reports that have shown that NRG1 SMDF is released and detected as a soluble form by proteolytic processing [17,18]. To test the latter idea, we examined the presence of NRG1 SMDF as a soluble form by proteolytic processing in peripheral blood, and were able to detect and measure it (Fig. 1A).

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In this study, we showed that NRG1 SMDF levels positively correlated with plasma and CSF, and levels in PD patients were significantly lower than in healthy controls, and the levels were slightly lower in MSA-P patients than MSA-C patients. These results suggest that the loss of dopaminergic neurons relates to the downregulation of the NRG1-ErbB4 signal because NRG1 SMDF is a member of the EGF family and contains an EGF-like domain. We consider it to coincide with previous studies that have shown levels of EGF in plasma and brain decrease with PD [19,20]. Additionally, it is widely known that the prevalence of PD increases with age.
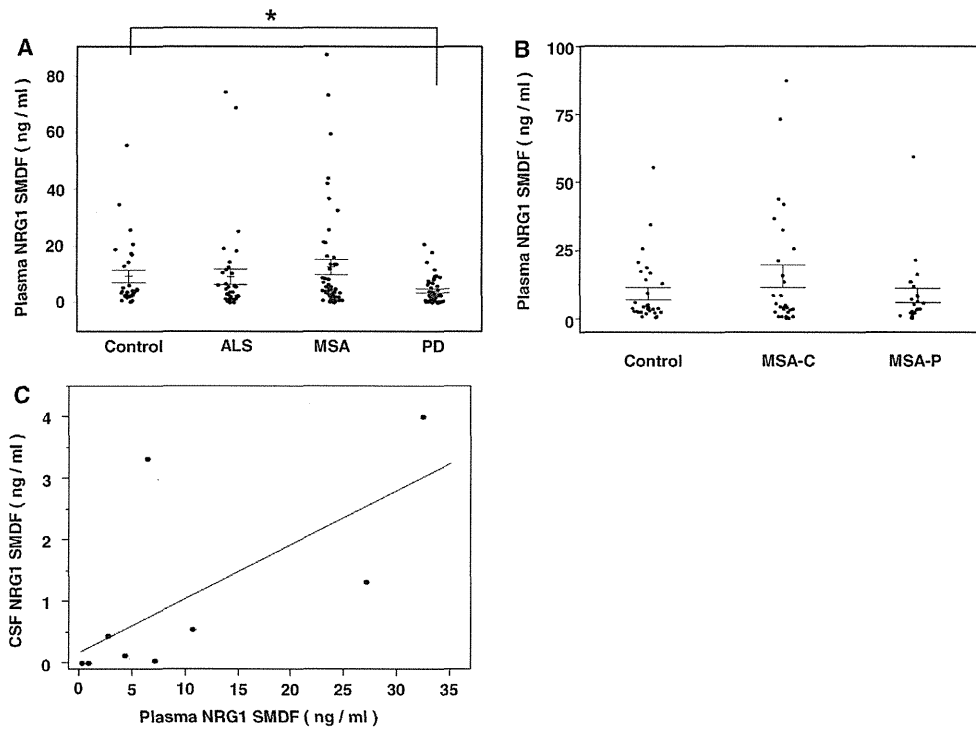
Fig. 2. NRG1 SMDF levels of plasma and CSF in samples by ELISA.
A. Plasma NRG1 SMDF levels in patients with ALS, MSA, PD, and normal controls.
Plasma NRG1 SMDF levels with PD were significantly lower than controls ($p = 0.025$).
B. Plasma NRG1 SMDF levels in MSA-C, MSA-P and normal controls.
MSA-P was slightly lower than MSA-C ($p = 0.470$).
C. Correlation of NRG1 SMDF levels in plasma and CSF obtained at the same time.
Plasma and CSF NRG1 SMDF levels of 9 sets of samples were assayed. Plasma NRG1 SMDF levels were positively correlated with CSF levels ($r = 0.667$, $p = 0.049$).
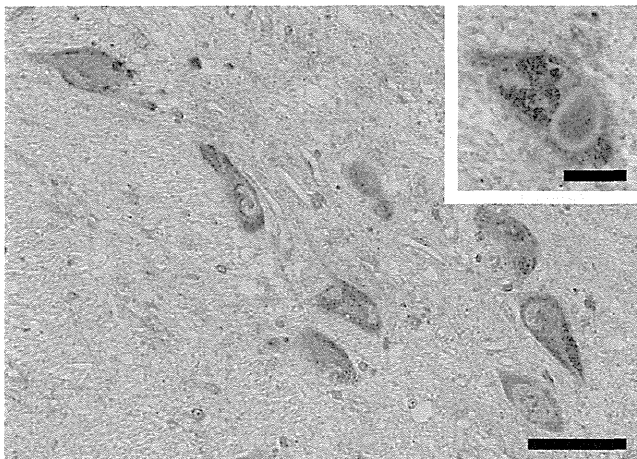

Fig. 3. Immunohistochemistry of NRG1 SMDF in PD.
NRG1 immunoreactivity in the substantia nigra pars compacta of patients with PD. The neuronal cytoplasm and processes are weakly immunolabeled. Lewy bodies in the pigmented neurons are intensely immunolabeled (inset). Bar = 20 μm.
S. Fahn, R.L. Elton,;1; Members of the UPDRS development committee. Unified Parkinson's disease rating scale, in: S. Fahn, C.D., Marsden, M., Goldstein, D.B. Calne, (Eds), Recent developments in Parkinson's disease. Volume 2. Macmillan Healthcare Information, Florham Park, New Jersey, 1987, pp. 153–163, 293–305.

Further, the appearance of new dopaminergic neurons by stimulation of a neurotrophic factor such as NRG1 and the expression of ErbB4 decreases with an increase in age [21,22]. Given the above, the idea of the reduction of NRG1 SMDF being associated with the progression of dopaminergic cell loss could be seen as pathology of PD. However, it is unclear whether the reduction of NRG1 SMDF is due to the reduction of dopaminergic cell or if dopaminergic cells decline because of NRG1 SMDF reduction.

One of the earliest signs of PD is olfactory dysfunction [23]. In mice experiments, ErbB4 is highly expressed in the olfactory bulb (OB) and NRG1 SMDF modulates neuroblast migration through ErbB4, and is expressed more than other NRGs in this area [24], indicating that NRG1 SMDF plays an important role in olfactory development. Our experiments showed that plasma NRG1 SMDF levels were lower in patients PD when compared with other neurodegenerative disorders, but the reduction levels were not correlated with the clinical severity or the stage of PD. The idea that the reduction of NRG1 SMDF may be considered as one of the causes of the onset of PD is worth investigating, and could be achieved by monitoring a large cohort of asymptomatic individuals with low plasma NRG1 SMDF levels along with elderly individuals having low levels of NRG1 SMDF but asymptomatic for PD.

When examining NRG1 SMDF, the question arises of where it originates. NRG1 SMDF is the most dominant NRG1 in the human adult brain and expression is reported to excitatory neurons, GABAergic interneurons, and astrocytes [4,5]. Further, the NRG1β1-extracellular domain is able to penetrate the blood–brain barrier [25,26]. We initially assumed that NRG1 SMDF originates in the brain and moves from CSF to plasma, but results of our experiment showed that the MW of NRG1 SMDF is approximately 50 kDa and the levels in plasma are 10 times higher than the levels in CSF. There was also no significant difference in the NRG1 immunoreactivity between PD and controls by immunohistochemistry. We think that NRG1 SMDF possibly originates in the PNS, not just from the CNS. It has been reported that NRG1 SMDF is expressed in the

PNS and is known to be a factor involving axonal growth and regulation of myelination [8,27]. Additionally, it has been reported that the number of sympathetic axons of the heart decreased in PD [28] and PD medication may help in the repair of myelin [29]. We think that a reduction of plasma NRG1 SMDF in PD is related to the PNS but the mechanism is not clear. Additional research is needed to confirm this idea.

Acknowledgements

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Self-Reported Adherence after Overnight Switching from Immediate- to Extended-Release Pramipexole in Parkinson's Disease

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Abstract

Background: Drug adherence decreased in patients with Parkinson' s disease (PD) because of taking many different types of drugs. We evaluated drug adherence after switching from immediate-release (IR) to once-daily extended-release (ER) pramipexole (PPX) in PD. Methods: This study included 35 PD patients (20 men, 15 women); 10 were taking oral PPX alone, and 25 were also using another anti-PD drug. PPX-IR was switched overnight to PPX-ER without gradual tapering. One month after switching, improvement in timing adherence and reduction in medication burden were evaluated by a questionnaire using a visual analog scale (VAS) (0: No change; 10: Better). Motor function was assessed using part III of the Unified Parkinson's Disease Rating Scale (UPDRS). Results: The VAS score for improvement in timing adherence was 8.1 ± 0.5 (mean ± standard error), and that for reduction in medication burden was 7.3 ± 0.6. There was a significant negative correlation (ρ = −0.43, p = 0.01) between the VAS score and number of types of medications. The UPDRS part III score improved significantly after switching (p < 0.01). Only one patient discontinued PPX-ER after switching. Conclusion: Switching from PPX-IR to once-daily PPX-ER is safe and improves motor function in patients by improving timing adherence and reducing medication burden.

Keywords

Parkinson's Disease, Timing Adherence, Pramipexole, Extended-Release, Immediate-Release, Motor Function

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1. Introduction

Maintaining good drug adherence requires a feasible treatment plan that a patient can follow and continue that regimen between each patient and their health care provider. Patients with Parkinson's disease (PD) take many different drugs even in the early stage, and as PD progresses, the doses and types of drugs increase, and the times for taking these medications become more complex. Research on drug adherence in PD has reported "not taking drugs on time", in other words, decreased timing adherence, as an issue [1] [2]. Poor adherence in PD is directly correlated with the complexity of drug regimen, with the depression, and with cognitive impairment [2]-[5]. PD patients prefer drugs that can be taken less often each day and in lower amounts [6] [7]. Moreover, better adherence with anti-PD drugs taken once daily, compared to drugs taken multiple times daily, has been shown [2] [8].

Once-daily extended-release formulations of non-ergot dopamine agonists (DAs) as anti-PD drugs are now available. However, studies on adherence when switching from multiple-daily to once-daily administration of these drugs have not been conducted. Pamipexole is a non-ergot dopamine agonist, and directly stimulates as a partial/full agonist at the D2, D3 and D4 receptors in the striatum. In this study, we investigated the effects on adherence of switching from the pramipexole immediate-release (PPX-IR) to pramipexole extended-release (PPX-ER) formulation.

2. Methods

2.1. Subjects

We conducted a hospital-based study involving 35 consecutive patients with PD in the Department of Neurology at the Tokyo Women's Medical University Hospital between July 2012 and November 2013. Subjects were included 35 PD patients (20 men, 15 women) ranging in age from 49 to 83 years (mean ± SD: 67.8 ± 9.1 years), with disease duration of 7 to 132 months (65.1 ± 31.1 months). PD severity was Hoehn and Yahr stage I in 5, II in 19, III in 10, and IV in 1 patient. Cognitive function was evaluated using the Mini-Mental State Examination (MMSE), and MMSE scores for the patients with PD ranged from 21 to 30 points (21 points in 1 patient, and another patients were above 25 points). The oral PPX dose before switching was 1.83 ± 0.5 mg, including 10 patients taking the PPX alone and 25 patients also taking other anti-PD drugs. These other drugs included levodopa in 22 (mean: 288 ± 120 mg), zonisamide in 11 (40.1 ± 23.1 mg), selegiline in 9 (5.3 ± 2.0 mg), amantadine in 6 (166 ± 40.8 mg), trihexyphenidyl in 5 (2.4 ± 0.9 mg), and entacapone in 2 (400.0 ± 141.4 mg) patients. The total number of drug types, including anti-PD drugs and other drugs, was 1 drug in 7, 2 drugs in 7, 3 drugs in 7, 4 drugs in 9, 5 drugs in 1, 6 drugs in 1, 7 drugs in 1, and 9 drugs in 2 patient. This study was performed in accordance with the guidelines of the Committee of Medical Ethics of Tokyo Women's Medical University. Informed consent was obtained from patients before study participation.

2.2. Switching from PPX-IR to PPX-ER

PPX-IR was switched overnight to PPX-ER without any gradual tapering. The dosage of the ER formulation was the same as the IR formulation in 27 patients; in 8 patients without a dosage corresponding to the IR formulation, the dose was increased (0.25-mg increase, 5 patients; 0.5-mg increase, 3 patients). The daily dose of PPX was 1.83 ± 0.5 mg before switching and 1.90 ± 0.6 mg after switching. The switched dose was significantly higher (p < 0.01).

2.3. Evaluation of Drug Adherence and Medication Burden

Patients were asked the following questions 4 - 6 weeks after switching to PPX-ER: Q1. Have you forgotten to take your anti-PD drug? Q2. Do you now take your medication on time with once-daily dosing? (timing adherence) and Q3. Has the burden of taking your medication decreased with once-daily dosing? Responses for Q 2 and Q3 were assessed using a visual analog scale (VAS): (0: No change; 10: Better) (Figure 1).

2.4. Patient Survey about Motor Function and Mood

Patients filled out a survey form with the following questions: Q1. How are your daily activities compared to previously? Q2. How are your evening activities compared to previously? Q3. How are your morning activities
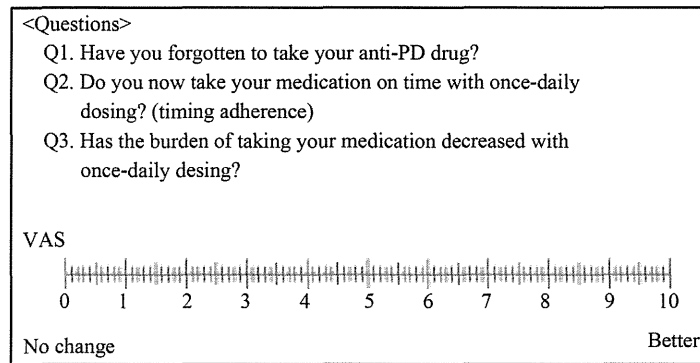
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<Questions>
Q1. Have you forgotten to take your anti-PD drug?
Q2. Do you now take your medication on time with once-daily
    dosing? (timing adherence)
Q3. Has the burden of taking your medication decreased with
    once-daily desing?

VAS

|++|++|++|++|++|++|++|++|++|++|++|++|++|++|++|++|++|++|++|++|
0   1   2   3   4   5   6   7   8   9   10

No change   Better

Figure 1. Questionnaire of drug adherence and medication burden Responses for Q2 and Q3 were assessed using a visual analog scale (VAS) (0: No change; 10: Better).

compared to previously? and Q4. How is your mood compared to previously? There were 7 grades of response (much worse, worse, somewhat worse, no change, somewhat better, better, and much better) (Figure 2).

2.5. Motor Function

Motor function was assessed before and 4 - 6 weeks after switching using part III of the Unified Parkinson's Disease Rating Scale (UPDRS) that is composed of 14 items (speech, facial expression, tremor at rest, action or postural tremor of hands, rigidity, finger taps, hand movements, rapid alternating movements of hands, leg agility, arising from chair, posture, gait, postural stability and body bradykinesia and hypokinesia).

2.6. Statistical Analysis

Spearman's rank correlation was used to examine the correlation between the VAS scores for questions 2 and 3 about taking medications with each of the following: number of types of medications, age, severity, disease duration, and MMSE score. One-way ANOVA was used to compare the single-drug and multiple-drug groups. The paired t-test was used to compare doses and analyze UPDRS changes before and after switching. The level of significance was $p < 0.05$.

3. Results

3.1. Adherence

The responses to Q1 (Have you forgotten to take your PPX-ER?) were 100 % "no" and 0 % "yes".

The VAS score for Q2 (Do you now take your medication on time with once-daily dosing?) was 10 in 48% of patients and ≥8 in 74% of patients (Figure 3, left). The VAS score (mean ± SE) for all patients was 8.1 ± 0.5, including 9.4 ± 0.8 in the single drug group and 7.6 ± 0.5 in the multiple drug group. The single-drug group took their medication more on time, but the difference was not significant (p = 0.07). Two patient using 6 and 9 concomitant drugs responded "no change".

There was a significant negative correlation between the VAS score and the number of types of medications ($\rho = -0.43$, p = 0.01). There was no significant correlation between the VAS score and age, PD severity, disease duration, or MMSE score.

3.2. Medication Burden

The VAS score for Q3 (Has the burden of taking your medication decreased with once-daily dosing?) was 10 in 49% of patients and ≥8 in 61% of patients (Figure 3, right).

The VAS score (mean ± SE) for all patients was 7.3 ± 0.6, including 8.6 ± 1.1 in the single-drug group and 6.9 ± 0.7 in the multiple-drug group. The single-drug group reported more of a reduction in medication burden, but the difference was not significant (p = 0.18). Four patients using concomitant drugs in the multiple drug group responded "no reduction in burden".

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<Questions>
Q1. How are your daily activities compared to previously?
Q2. How are your evening activities compared to previously?
Q3. How are your morning activities compared to previously?
Q4. How is your mood compared to previously?

| much worse | worse | somewhat worse | no change | somewhat better | better | much better |

Figure 2. Patient survey about motor function and mood. Patients filled out a survey form with the questions.
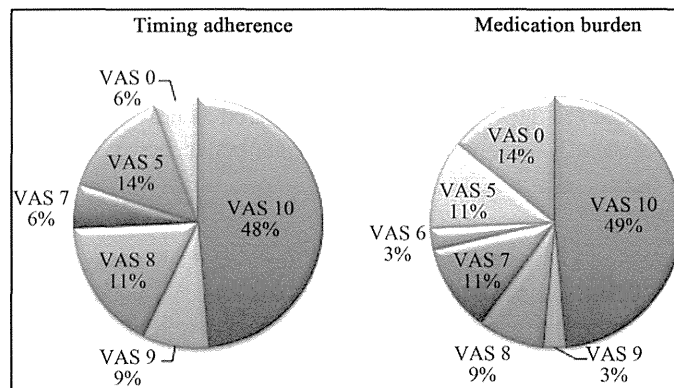

Figure 3. Survey results for timing adherence (left) and medication burden (right) after switching. VAS: visual analog scale; 0: No change; 10: Better.

There was no significant correlation between the VAS score for reduction in medication burden and age, PD severity, disease duration, MMSE score, or number of types of medications.

3.3. Patient Survey about Motor Function and Mood (Figure 4)

1) Daily activities

The responses in 27 patients switched to the same dose were: no change 59%, better 19%, somewhat better 11%, somewhat worse 7%, and worse 4%. On the other hand, the responses in the 8 patients with an increased dose were: no change 50%, much better 12%, better 13%, and somewhat better 25%; none reported subjective worsening.

2) Evening activities

The responses in the patients switched to the same dose were: no change 74%, better 4%, somewhat better 15%, and somewhat worse 7%. The responses in patients with an increased dose were: no change 75%, much better 12%, and better 13%, with none reporting subjective worsening.

3) Morning activities

The responses in the patients switched to the same dose were: no change 74%, somewhat better 19%, and somewhat worse 7%. The responses in patients with an increased dose were: no change 87% and somewhat better 13%, with none reporting subjective worsening.

4) Mood

The responses in the patients switched to the same dose were: no change 55%, much better 4%, somewhat better 22%, somewhat worse 11%, worse 4%, and much worse 4%. The responses in patients with an increased dose were: no change 50%, better 12%, and somewhat better 38%, with none reporting subjective worsening.

3.4. UPDRS Part III

In the group switched to the same dose, the UPDRS part III score ranged from 3 to 28 (10.4 ± 1.3) before
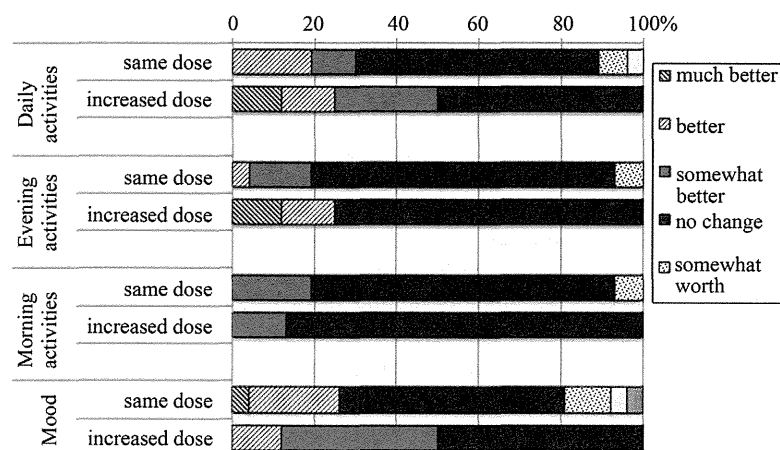
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Figure 4. Patient survey about motor function and mood in the group switched to the same dose and an increased dose.

switching and 3 to 26 (8.8 ± 1.0) after switching. In the group switched to an increased dose, the scores ranged from 2 to 22 (11.3 ± 4.1) before switching and from 1 to 22 (7.3 ± 2.3) after switching. After switching to the extended release formulation, the UPDRS part III scores improved significantly in both groups ($p < 0.01$).

3.5. Safety

Because of chest discomfort and worsening mood, one patient discontinued PPX-ER and was switched back to PPX-IR. In addition, there was mild somnolence in 5, constipation in 1, and mild worsening of visual hallucinations in 1 patient.

4. Discussion

4.1. Adherence by Switching to PPX-ER

Adherence includes the percentage of doses taken vs. the total number of doses prescribed (total adherence), the percentage of days a drug is actually taken as prescribed vs. the total number of days prescribed (days adherence), and the percentage of taking a drug at a correct time vs. the prescribed frequency of administration (timing adherence). In a multicenter collaborative study of drug adherence in PD, total adherence, days adherence, and timing adherence were significantly better in a group taking a DA once-daily compared to 3 times daily [2] [8]. In particular, timing adherence differed markedly between groups: ≥90% in the once-daily group, but it decreased to about 25% in the 3-times-daily group. Even the impact of twice-daily dosing was a marked decrease in adherence to 33% [2] [8]. Based on the above reports, the present study focused on and evaluated timing adherence using a VAS patient survey. After patients switched from multiple daily PPX-IR to once-daily PPX-ER, timing adherence improved, and medication burden was reduced. In addition, timing adherence was negatively correlated with the number of types of medications being taken. In patients using ≥6 types of medications, timing adherence did not improve even after switching to PPX-ER. Drug non-adherence reportedly increases 1.86 times with a 100-mg increase in levodopa dose, and there is a negative correlation with total number of drugs, with a 1.68-fold increase in non-adherence with each additional drug [5] [9]. Factors associated with decreased adherence besides multiple drugs and complicated dosing regimens include mood disturbance, decreased cognitive function, poor control, long disease duration, educational level, absence of a spouse, younger age, and low income [2]-[5]. Attention to these patient factors and therapeutic interventions are essential to maintain good adherence.

A limitation of this study is that we evaluated adherence by using the self-reported questionnaires. In previous studies on adherence, differences in adherence rates have been observed between evaluations using patient surveys and evaluations using electronic monitoring systems of the actual number of doses taken and times when taken, with a trend toward more over-estimation on patient surveys [1] [2] [10]-[12]. In this study, although almost patients showed normal MMSE scores, and none of the patients responded that they "forgot to take PPX-

ER", because of evaluation of timing adherence using a VAS patient survey, some discrepancy between prescribed times and actual times taken may have arisen. However, in every clinical practice, it was useful to have a simple questionnaire to assess the adherence to therapy in PD.

4.2. Changes in Motor Function by Switching to PPX-ER

In some patients switched to the same dose, there was worsening of daily, evening, and morning activities and of mood. In patients switched to an increased dose, there was no worsening of these parameters. However, the UPDRS part III scores increased significantly ($p < 0.01$) not only in the increased dose group, but also in patients switched to the same dose. Overnight switching from PPX-IR to PPX-ER can be safely accomplished without worsening of symptoms in about 85% of patients [7] [13]-[15]. Moreover, improvement in UPDRS part III scores and reduction in off-times in advanced PD patients have been reported [7] [14]. Because of some issues with corresponding dosage formulations in this study, the mean dose increased after switching, and this may have been a factor in the improved UPDRS part III scores. There was also significant improvement, however, in patients switched to the same dose, and taking into consideration the effect of drug adherence on therapeutic efficacy and QOL [16], the on-time dosing with better drug adherence and longer lasting drug delivery with the extended release formulation probably contributed to improved motor function.

5. Conclusion

This study showed that switching from PPX-IR to once-daily PPX-ER was safe and improved motor function in patients by improving timing adherence and reducing medication burden.

Disclosures of Conflicts of Interest

None of the authors report any disclosures.

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Differential Expression of Alpha-Synuclein in Hippocampal Neurons

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Abstract

α-Synuclein is the major pathological component of synucleinopathies including Parkinson's disease and dementia with Lewy bodies. Recent studies have demonstrated that α-synuclein also plays important roles in the release of synaptic vesicles and synaptic membrane recycling in healthy neurons. However, the precise relationship between the pathogenicity and physiological functions of α-synuclein remains to be elucidated. To address this issue, we investigated the subcellular localization of α-synuclein in normal and pathological conditions using primary mouse hippocampal neuronal cultures. While some neurons expressed high levels of α-synuclein in presynaptic boutons and cell bodies, other neurons either did not or only very weakly expressed the protein. These α-synuclein-negative cells were identified as inhibitory neurons by immunostaining with specific antibodies against glutamic acid decarboxylase (GAD), parvalbumin, and somatostatin. In contrast, α-synuclein-positive synapses were colocalized with the excitatory synapse marker vesicular glutamate transporter-1. This expression profile of α-synuclein was conserved in the hippocampus in vivo. In addition, we found that while presynaptic α-synuclein colocalizes with synapsin, a marker of presynaptic vesicles, it is not essential for activity-dependent membrane recycling induced by high potassium treatment. Exogenous supply of preformed fibrils generated by recombinant α-synuclein was shown to promote the formation of Lewy body (LB) -like intracellular aggregates involving endogenous α-synuclein. GAD-positive neurons did not form LB-like aggregates following treatment with preformed fibrils, however, exogenous expression of human α-synuclein allowed intracellular aggregate formation in these cells. These results suggest the presence of a different mechanism for regulation of the expression of α-synuclein between excitatory and inhibitory neurons. Furthermore, α-synuclein expression levels may determine the efficiency of intracellular aggregate formation in different neuronal subtypes.

Introduction

α-Synuclein is one of the major components of Lewy bodies (LBs) and Lewy neurites (LNs), which are pathological hallmarks of synucleinopathies including Parkinson's disease (PD) and dementia with Lewy bodies (DLB) [1–3]. Several mutations in the α-synuclein gene are responsible for familial PD [4–6]. Therefore, insights into the mechanisms underlying α-synuclein pathology are crucial for the understanding of these neurodegenerative disorders. To elucidate the mechanisms of neuronal loss in these diseases, it is also important to determine the physiological roles of α-synuclein in normal neurons.

α-Synuclein is localized at presynapses in vivo and in vitro [7–9]. It has been suggested that α-synuclein is involved in the generation and maintenance of synapses, because this protein appears earlier than synaptophysin during development of the central nervous system and is localized to presynaptic terminals throughout the adult mammalian brain [10,11]. α-Synuclein directly binds to synaptobrevin-2 in presynaptic regions and functions to sustain

soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-complex assembly in vivo and in vitro [12]. In addition, in a recent study using an overexpression and knockout model in vitro, it was reported that α-synuclein maintains the size of the vesicular recycling pool [13].

In studies of α-synuclein pathogenicity, overexpression of α-synuclein in neurons resulted in formation of inclusion bodies and neuronal loss [14], and decreased the survival and dendritic development of newborn neurons [15]. In humans, the number of α-synuclein-positive neurons in the hippocampus of DLB patients is significantly increased [15]. In addition, parvalbumin-containing neurons in the neocortex are reported to be free of LBs in DLB patients [16]. These results suggest that an increase in the intracellular amount of α-synuclein is a probable risk factor for neurodegeneration, and that the mechanisms underlying aggregate formation are distinct in different neuronal cell types.

In the present study, we investigated the precise expression level and subcellular localization of α-synuclein in hippocampal neuronal cell cultures, and verified that there is a difference in

the expression of α-synuclein between excitatory and inhibitory neurons. We also examined activity-dependent synaptic vesicular recycling following treatment with high potassium. A recent report demonstrated that preformed fibrils generated by recombinant α-synuclein can promote the formation of LB-like aggregates containing endogenous α-synuclein [17]. We also treated cells with these preformed fibrils, and explored whether aggregate formation depends on the expression level of endogenous α-synuclein. Finally, we examined the expression of α-synuclein in the mouse hippocampus *in vivo* and confirmed the observations of differential expression found *in vitro*.

Materials and Methods

Animals

C57BL/6N mice were used, and all experimental designs and procedures were approved by the Committee for Animal Research, Kyoto Prefectural University of Medicine (M23-241), Kyoto Japan, and followed the guidelines of the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Antibodies

For α-synuclein detection, mouse monoclonal antibody Syn-1 (BD Biosciences, San Diego, CA, USA) and rabbit polyclonal antibody C-20 (Santa Cruz Biotechnology, Dallas, TX, USA) were used. For specific detection of human α-synuclein, mouse monoclonal antibody Syn211 (Thermo Fisher Scientific, UK) was used, because Syn211 recognizes human α-synuclein, but not mouse α-synuclein [18]. Phosphorylated α-synuclein was detected by mouse monoclonal antibody pSyn#64 (Wako Pure Chemical Industries, Japan) or rabbit polyclonal antibody phospho-S129 (Abcam, UK). Glutamic acid decarboxylase (GAD) was detected by rabbit polyclonal antibodies purchased from Sigma-Aldrich (St Louis, MO, USA). Anti-synapsin antibody was produced as previously described [19]. The following antibodies were purchased from these manufacturers: anti-synaptotagmin (Developmental Studies Hybridoma Bank, Iowa city, IA, USA), anti-vesicular glutamate transporter-1 (vGluT-1; Millipore, Billerica, MA, USA), anti-parvalbumin (Sigma-Aldrich), anti-somatostatin (Millipore), and anti-NeuN (Millipore).

Cell culture and transfection

Dissociated cells prepared from hippocampi of mouse embryos (E16-18) were disseminated onto polyethylimine coated coverslips and cultured in Neurobasal medium (Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with B-27 (Gibco), L-glutamine (Nacalai Tesque, Kyoto, Japan) and penicillin/streptomycin (Nacalai Tesque). In the occasion of neuronal culture without glial cells, cells cultured for 2 days were treated with a medium containing cytosine arabinoside (AraC, 10 μM) (Sigma-Aldrich) for 12 h, followed by replacement with a complete medium. Half of the volume of medium was changed every 4 days. Unless specifically mentioned, cells were cultured without AraC treatment and most experiments were performed at 16–22 days *in vitro*.

To form intracellular aggregates containing human α-synuclein, transfection of plasmid DNA for the expression of human α-synuclein was performed using the electroporator CUY21Pro-Vitro (Nepagene, Chiba, Japan) in accordance with the manufacturer's protocol. After transfection, cells were disseminated onto coverslips. For depolarizing the neuronal cell membrane, cultured cells were treated with fresh medium containing 100 mM potassium chloride for 30 min. After depolarization, cells were treated with original complete medium for 10 min, to analyze the recovery of the cell membrane.

Immunocytochemistry

Cells were fixed with 2% paraformaldehyde (PFA) in culture medium for 10 min at room temperature. The fixed cells were permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS, pH 7.4) for 10 min, and blocked with 5% normal goat serum (NGS) in PBS for 30 min. Cells were then incubated with primary antibody in the blocking solution for 1–2 h. Cells were then washed with PBS and treated with a secondary antibody. For double staining, this staining procedure was repeated. The primary antibodies were detected with Alexa488- and Alexa594-conjugated secondary antibodies (Molecular Probes, Life Technologies, Carlsbad, CA, USA). For detection of the primary antibody produced in guinea pig, a fluorescein isothiocyanate-conjugated secondary antibody (Vector Laboratories, Burlingame, CA, USA) was used. Cells were washed with PBS and then with milliQ water (Millipore), and mounted with FluorSave (Millipore). Images were acquired as Z stacks (20–30 z-sections, 0.3–0.5 μm apart, 1024×1024 pixels) using a Plan-Apochromat 63x/1.40 Oil DIC objective (Carl Zeiss, Oberkochen, Germany) with an inverted laser-scanning confocal microscope, LSM510 (Carl Zeiss).

Formation of intracellular aggregates of α-synuclein

Neurons containing intracellular aggregates of α-synuclein were obtained using previously described methods [17]. Briefly, following sonication, preformed fibrils prepared from human or mouse α-synuclein were suspended in culture medium, and the final concentration was adjusted to 5 μg/ml. Cultured cells were treated with complete medium containing the fibrils for 7 days.

Fibril preparation

For preparation of recombinant human and mouse α-synuclein from bacterial culture, plasmid vectors were constructed as described previously (Tatebe et al., 2010; Watanabe et al., 2012). Briefly, PCR fragments of human and mouse α-synuclein were inserted into pTrc-His-TOPO vector (Invitrogen, Life Technologies, Carlsbad, CA, USA). Recombinant α-synuclein with a His-tag was purified by His-Accept column (Nacalai Tesque) [20,21]. Purified protein was electrophoresed and the protein band was stained by Coomassie Brilliant Blue and the purity confirmed by SDS-PAGE. Fibril forms of α-synuclein were prepared in accordance with a previous report [17] with slight modifications. Briefly, fibrils of α-synuclein were generated by incubating purified α-synuclein in PBS (final solution 2 mg/ml) at 37°C with constant agitation for 7 days. After agitation, the solution containing fibrils was ultracentrifuged at 200,000×g for 2 h. Fibrils were recovered as a precipitate at the bottom of the centrifuge tube. The resulting pellet was dissolved in the original volume of PBS and stored at −20°C until use. The supernatant was also stored. Each sample was then subjected to western blot analysis.

Construction of plasmid vector

For expression of human α-synuclein in cultured neurons, α-synuclein was amplified from a human cDNA library with the primers 5′-CGCCACCATGGATGTATTCATGAAA-3′ and 5′-GATATCTTAGGCTTCAGGTTCGTAG-3′. The PCR fragment was digested with *NotI* and then inserted into the *NotI-EcoRV* site of the pcDNA3.1/myc-HisB vector. The vector was then sequenced for confirmation.

SDS-PAGE and western blotting

Samples were denatured by heating with SDS-sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 0.003% Pyronin Y, and 10% glycerol) containing 1% 2-mercaptoethanol at 98°C for 3 min. Proteins were separated by 15% polyacrylamide gel, and transferred to polyvinylidenedifluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked with 5% skim milk in Tris-buffered saline (TBS, pH 7.4) containing 0.1% Tween-20 for 1 h at room temperature. After treatment with primary antibody, the membrane was incubated with alkaline phosphatase-conjugated secondary antibodies. Protein bands were detected using the NBT-BCIP system (NacalaiT-esque).

Image processing

The digital images obtained on the LSM510 were processed using Adobe Photoshop 7.0. Projections of Z stack images were processed with LSM Image Browser (Carl Zeiss).

Quantitative analysis

For quantification of the ratio of α-synuclein-expressing cells or aggregate-containing GAD-positive cells, two independent cell cultures were performed and three coverslips per culture were subjected to the immunocytochemical procedures. Endogenous α-synuclein and its aggregates were detected by Syn-1 and pSyn#64 antibodies, respectively. GAD-positive cell bodies (more than 75 cells per coverslip) were subjected to the cell counting procedure. For quantification of the ratio of GAD-positive cells having intracellular aggregates composed of transfected human α-synuclein, two coverslips per culture were subjected to the immunocytochemical procedures. Human α-synuclein was detected by the Syn211 antibody, followed by counting the number of GAD-positive neurons harboring human α-synuclein aggregates. These experiments were independently repeated twice. Data represent mean ± SEM. Statistical significance of the difference in the ratio of intracellular aggregate formation was analyzed using Graphpad Prism 5 with an unpaired, two-tailed Student's t test with or without Welch's correction.

After acquisition of the confocal images, quantitative colocalization analysis using Pearson's correlation test was performed (LSM Image Browser, Carl Zeiss).

Immunohistochemistry

Under deep anesthesia with pentobarbital, adult mice were intracardially perfused with PBS followed by 4% PFA in PBS. Brains were dissected into blocks including the cerebral cortex and hippocampus, and post-fixed with the same fixative for 12 h at 4°C. Coronal sections (40 μm) were obtained using a vibratome (DSK, Kyoto, Japan). Sections were permeabilized with 0.3% Triton X-100 in PBS (PBST) for 1 h, and blocked with 10% NGS in PBST for 6 h. Sections were then treated with primary and secondary antibodies following the immunocytochemical procedures described above. After these treatments, the sections were washed with PBST and then with 20 mM Tris-HCl buffer, and mounted with FluorSave. Images were acquired as Z stacks (10–20 z-sections, 1 μm apart, 1024×1024 pixels) using a Plan-Apochromat 63x/1.40 Oil DIC objective with an inverted laser-scanning confocal microscope, LSM510.

Results

Inhibitory neurons showed low expression of α-synuclein *in vitro*

Immunocytochemistry of cultured hippocampal neurons showed that α-synuclein was present in a punctate distribution, with an intense signal observed in the cell body and nucleus of some neurons (Fig. 1A). However, other neurons were unstained with either monoclonal or polyclonal antibodies against α-synuclein (Fig. 1 and Fig. S1A). There were clearly differential expression patterns of α-synuclein in cultured neurons. To characterize the neurons with low expression of α-synuclein, we performed immunostaining using a specific antibody against GAD, a well-known marker of inhibitory neurons. GAD-positive neurons were immunonegative for α-synuclein (Fig. 1A). Although GAD-positive puncta were juxtaposed with the α-synuclein-positive dendrites, most GAD signals were not colocalized with α-synuclein (Fig. 1A, merged images). 89±1.4% of GAD-positive neurons displayed no expression of α-synuclein (6 coverslips were analyzed). About 10% of the GAD-positive neurons, however, showed faint immunoreactivity for α-synuclein. Double immunofluorescence with antibodies against α-synuclein and parvalbumin or somatostatin, other marker proteins of inhibitory neurons, confirmed that α-synuclein was not detected in parvalbumin- or somatostatin-positive neurons (Fig. 1C and 1D). These results indicated that inhibitory neurons show very low expression of α-synuclein *in vitro*. Even after depletion of glial cells by AraC treatment, this differential expression pattern of α-synuclein was still observed (data not shown).

Cultured neurons exhibited differential expression of α-synuclein within 30 h after cell dissemination

To investigate the expression of α-synuclein during cell maturation, double immunostaining for NeuN, a neuron-specific marker, and α-synuclein was performed at various times after cell-disemination (Fig. 2A). Expression of α-synuclein gradually increased during cell maturation, and after 30 h cultured neurons already exhibited differential expression levels of α-synuclein. There were α-synuclein-negative neurons among the NeuN-positive cells. At this time point, GAD expression was not sufficient to distinguish inhibitory neurons (Fig. 2B).

α-Synuclein was localized at excitatory synapses

We examined the synaptic localization of α-synuclein by double immunostaining for α-synuclein and synaptotagmin, a presynaptic marker (Fig. 3A and 3B). Although α-synuclein was colocalized with synaptotagmin in some presynapses, a subset of the synaptotagmin-positive synapses lacked α-synuclein expression. In contrast, GAD immunoreactivity was colocalized with some of the signals of synapsin, another presynaptic marker (Fig. S1B). Because α-synuclein was not co-expressed with either GAD, parvalbumin, or somatostatin in nerve terminals (Fig. 1), α-synuclein-positive puncta are not likely to be presynapses of inhibitory neurons.

Further characterization by immunostaining for vGluT-1, an excitatory synaptic marker, revealed that almost all α-synuclein-positive synapses co-expressed vGluT-1 (Fig. 3C and 3D). We also confirmed that vGluT-1 was not colocalized with GAD (Fig. S1C). Furthermore, we performed quantitative colocalization analysis using Pearson's correlation test. The correlation of pixel intensities between α-synuclein and vGluT-1 was excellent (Fig. S2A; Pearson's correlation coefficient, R = 0.83). However, the correlations of pixel intensities between α-synuclein and inhibitory neuronal markers were weak (Fig. S2B, GAD R = 0.20;
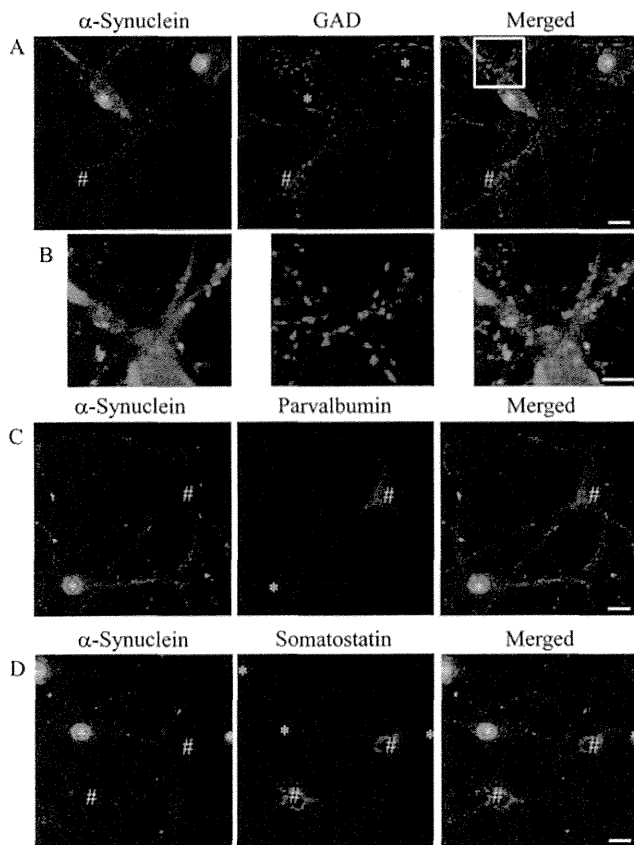
Figure 1. Low expression of α-synuclein in inhibitory neurons. Cells expressing the inhibitory neuronal marker proteins GAD (A), parvalbumin (C), and somatostatin (D) (indicated by #) showed low expression of α-synuclein. Cells with high expression of α-synuclein are labeled with asterisks. The region marked by a white square in A is magnified in B. Immunoreactivity of GAD was not colocalized with that of α-synuclein (B). Three independent cultures were performed and the differential expression pattern of α-synuclein was reproducibly confirmed. These images were further subjected to the quantitative colocalization analyses shown in Fig. S2. Scale bars: 10 μm in A, C, and D; 5 μm in B.
doi:10.1371/journal.pone.0089327.g001

Figure 2. Differential expression of α-synuclein during cell maturation. (A) Expression of α-synuclein was examined at the indicated times after cell dissemination. Immunoreactivity of α-synuclein increased during cell maturation. By 30 h after cell dissemination, some NeuN-positive cells expressed α-synuclein (asterisks), but other NeuN-positive cells did not. (B) Expression of GAD at 30 h was not sufficient to distinguish inhibitory neurons from other types of neurons. Three independent cultures were performed. Scale bars: 10 μm.
doi:10.1371/journal.pone.0089327.g002

Fig. S2C, parvalbumin R = 0.38; Fig. S2D, somatostatin R = 0.14). These results indicated that *in vitro* α-synuclein was predominantly expressed in excitatory neurons, but not in inhibitory neurons.

α-Synuclein was colocalized with synapsin during presynaptic membrane recycling

Previous reports have demonstrated that α-synuclein is involved in SNARE-complex assembly and the resulting release of synaptic vesicles [12]. However, in this study some neurons were immunonegative for α-synuclein (Fig. 1). Depolarization by treatment with culture medium containing high potassium altered the synaptic localization of synapsin from synaptic vesicles to the presynaptic plasma membrane during exocytosis (Fig. 4). α-Synuclein was colocalized with synapsin during the presynaptic membrane recycling. This change in synapsin localization at synapses during depolarization also occurred in the absence of α-synuclein. This differential expression pattern was still evident at synapses following recovery from the membrane depolarization (Fig. 4). Activity-dependent recycling of synaptic membrane therefore seems to occur independently of the existence of α-synuclein.
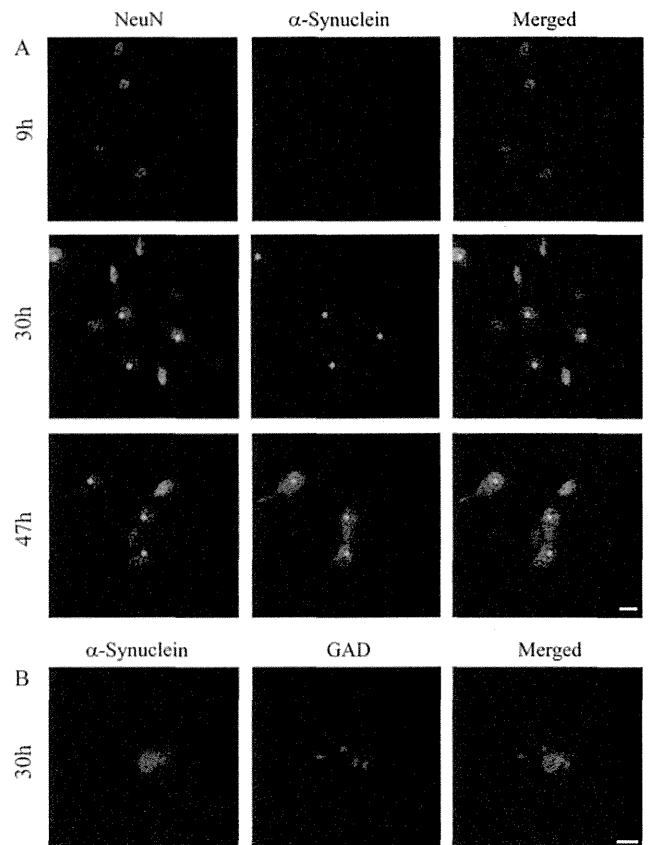
GAD-positive neurons were free of intracellular aggregates of α-synuclein

Intracellular aggregates such as LBs and LNs are mainly composed of α-synuclein. These aggregates are formed by recruitment of intrinsic soluble α-synuclein into the insoluble aggregate core. Endogenous expression of α-synuclein, therefore, should be required for the formation of intracellular aggregates of α-synuclein [17]. Preformed fibrils prepared from recombinant α-synuclein can induce the formation of LB-like intracellular aggregates [17]. LB-like aggregates contain α-synuclein with a phosphorylated serine 129 residue [22]. If inhibitory neurons have low expression levels of α-synuclein, they may not be able to form intracellular aggregates, because of an inability to recruit endogenous α-synuclein. After treatment with preformed fibrils prepared from recombinant α-synuclein (Fig. S3), intracellular fibrous aggregates or inclusion bodies were detected by a specific antibody against phosphorylated α-synuclein (Fig.5A and 5B). This antibody can exclusively detect intracellular aggregates of α-synuclein [22]. As expected, most GAD-positive neurons were free of intracellular aggregates of α-synuclein (Fig. 5A). We also performed quantitative colocalization analysis using Pearson's correlation test. The correlation of pixel intensities between phosphorylated α-synuclein and GAD was weak (Fig. S2E;
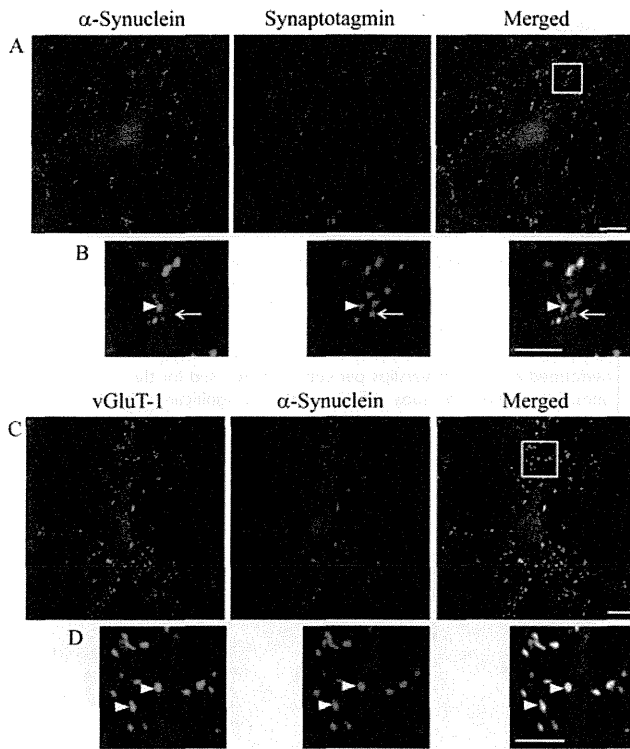
Figure 3. Presynaptic localization of α-synuclein in excitatory neurons. (A, B) Confocal images of double immunostaining for α-synuclein and synaptotagmin. The region marked by a white square in A is magnified in B. Arrowhead in B indicates the presynapse, expressing both α-synuclein and synaptotagmin. However, there are some synaptotagmin-positive synapses lacking α-synuclein (arrow). (C, D) Confocal images of double immunostaining for α-synuclein and vGluT-1, an excitatory neuronal marker protein. The region marked by a white square in C is magnified in D. α-Synuclein is colocalized with vGluT-1 in D (arrowheads). Three independent cultures were performed and colocalization between α-synuclein and vGluT-1 was reproducibly confirmed. Panel C was subjected to the quantitative colocalization analysis shown in Fig. S2. Scale bars: 10 μm in A and C; 5 μm in B and D. doi:10.1371/journal.pone.0089327.g003

R = 0.07). The proportion of aggregate-containing GAD-positive cells was significantly lower than that of NeuN-positive cells (see note "a" in Table 1). This result is probably due to the lower amount of endogenous α-synuclein expressed in the GAD-positive cells. Another possibility is that the cell entry efficiency of the seeds derived from the preformed fibrils was different between inhibitory neurons and other types of neurons. Next, we confirmed the dose-dependent effect of the intracellular amount of α-synuclein on aggregate formation. After transfection of human α-synuclein and further treatment with preformed fibrils, intracellular accumulation of exogenous α-synuclein was observed in GAD-positive neurons. We used the Syn211 monoclonal antibody to detect only exogenous expression of human α-synuclein without detecting endogenous mouse α-synuclein, and prepared preformed fibrils from mouse α-synuclein. Before fibril treatment, exogenous human α-synuclein was diffusely expressed in the cell body (Fig. 5C). After fibril treatment, accumulation of exogenous α-synuclein was observed in some of the transfected GAD-positive cells (Fig. 5C and Table 1). The efficiency of aggregate formation was significantly increased by exogenous expression of α-synuclein (see note "b" in Table 1). These results indicated that inhibitory neurons can form intracellular aggregates under conditions where there is sufficient intracellular α-synuclein.
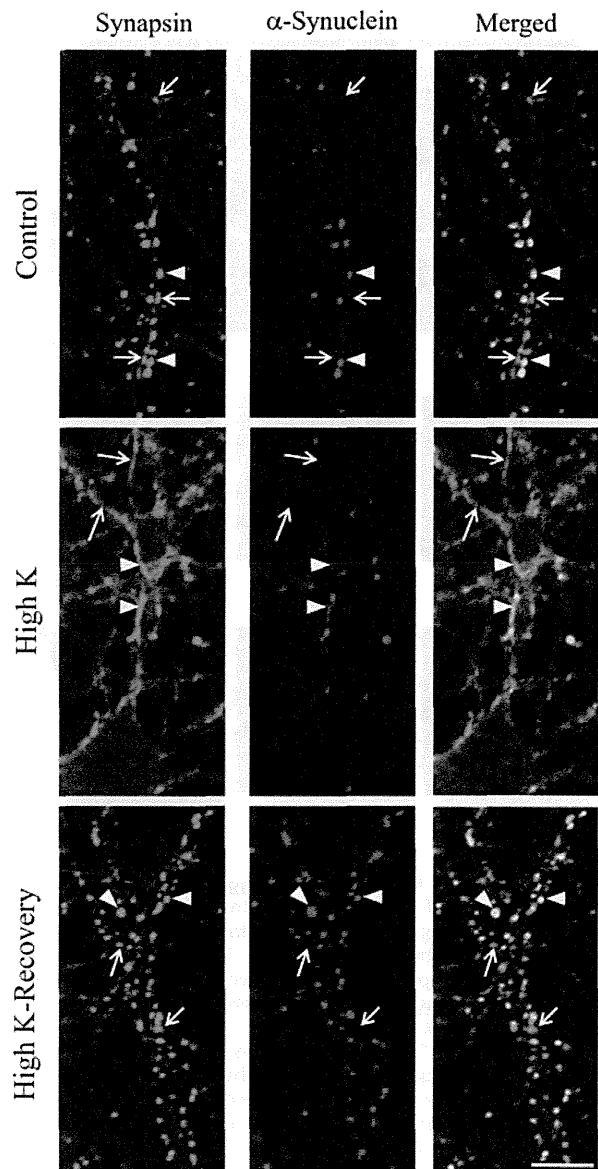
Figure 4. Colocalization of synapsin and α-synuclein during presynaptic membrane recycling. Synapsin immunoreactivity was observed as punctate signals before treatment (Control). After membrane depolarization by applying a medium containing high potassium, the punctate signals of synapsin changed shape and fused to the presynaptic plasma membrane (High K). The change in localization was reversed by treatment with a low-potassium medium (High K-Recovery). α-Synuclein was colocalized with synapsin during presynaptic membrane recycling (arrowheads). However, there are also instances of synapsin immunoreactivity without α-synuclein expression (arrows). Three independent cultures were performed, and these staining patterns were observed in all cultures. Scale bar: 5 μm. doi:10.1371/journal.pone.0089327.g004

Low expression of α-synuclein in GAD-positive cells was also conserved *in vivo*

In sections of mouse hippocampus, α-synuclein was detected as puncta in the periphery of neurons. Diffuse expression of α-synuclein was not observed in the cell body or nucleus. α-Synuclein was colocalized with vGluT-1 in the CA1 region (Fig. 6A and 6B). In contrast, α-synuclein was juxtaposed with the punctuate immunoreactivity of GAD and never colocalized with GAD signals (Fig. 6C and 6D). Furthermore, acquired confocal
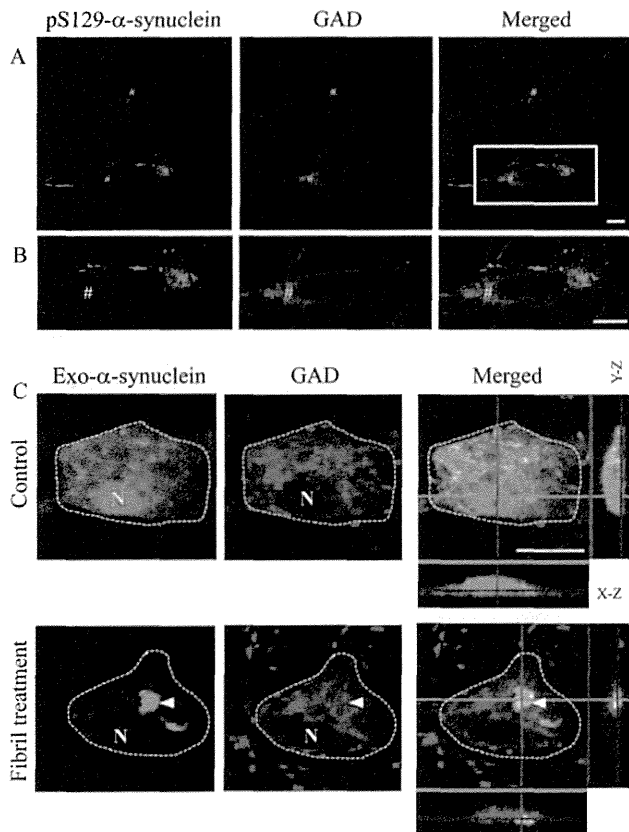
Figure 5. Formation of intracellular aggregates of α-synuclein. (A) Confocal images of double immunostaining for phosphorylated α-synuclein and GAD after treatment with preformed fibrils of α-synuclein. The region marked by a white square in A is magnified in B. Immunoreactivity of phosphorylated α-synuclein was observed as intracellular fibrous aggregates or inclusion bodies. GAD-positive neurons indicated by # were free of α-synuclein aggregate formation. GAD signals, including GAD-positive neurites, were not colocalized with phosphorylated α-synuclein. (C) In the absence of fibril treatment, exogenous human α-synuclein was diffusely distributed in the cell body of GAD neurons (Control). After fibril treatment, intracellular inclusions positive for α-synuclein were induced in the GAD-positive cells expressing exogenous α-synuclein. Cell bodies are shown surrounded by white dotted lines. 'N' indicates the location of the nucleus. Three independent cultures were performed and in all cases confirmed that intracellular inclusions were predominantly formed in GAD-negative neurons. Exogenous expression of human α-synuclein enhanced the aggregate formation in GAD-positive cells. These results were quantified and are described in Table 1. Scale bars: 10 μm.
doi:10.1371/journal.pone.0089327.g005

images were subjected to the quantitative colocalization analysis. The correlation of pixel intensities between the α-synuclein and vGluT-1 shown in Fig. 6A was excellent (Fig. S2F; R = 0.78). In contrast, the correlation of α-synuclein and GAD was weak (Fig. S2G; R = 0.11). Thus the expression pattern of α-synuclein observed in cultured inhibitory neurons was also conserved *in vivo*.

Discussion

α-Synuclein is localized at presynapses [7,8] and it was shown to regulate the size of the presynaptic vesicular pool in a study where antisense oligonucleotides were introduced in cultured hippocampal neurons [23]. More recently, using knockout and overexpressing neurons, Scott and Roy showed that α-synuclein plays a role in maintaining the overall size of the recycling pool of vesicles [13].

Table 1.

Ratio	%
pS129-α-Synuclein-positive cells/NeuN-positive cells	35±0.89[a]
pS129-α-Synuclein-positive cells/GAD-positive cells	2.7±0.64[a*]
Aggregate-positive cells/GAD- and syn211-positive cells	24±2.2[b*]

[a]Data represent mean ± SEM (6 coverslips). Two independent cultures were performed and three coverslips per culture were used for the immunocytochemical study. Statistical analysis showed significant difference with $p<0.0001$ by student's *t* test unpaired, two-tailed.
[b]Data represent mean ± SEM (4 coverslips). Two independent cultures were performed and two coverslips per culture were used for the immunocytochemical study. Asterisk indicates significant difference with $p<0.01$ by student's *t* test unpaired, two-tailed with Welch's correction.
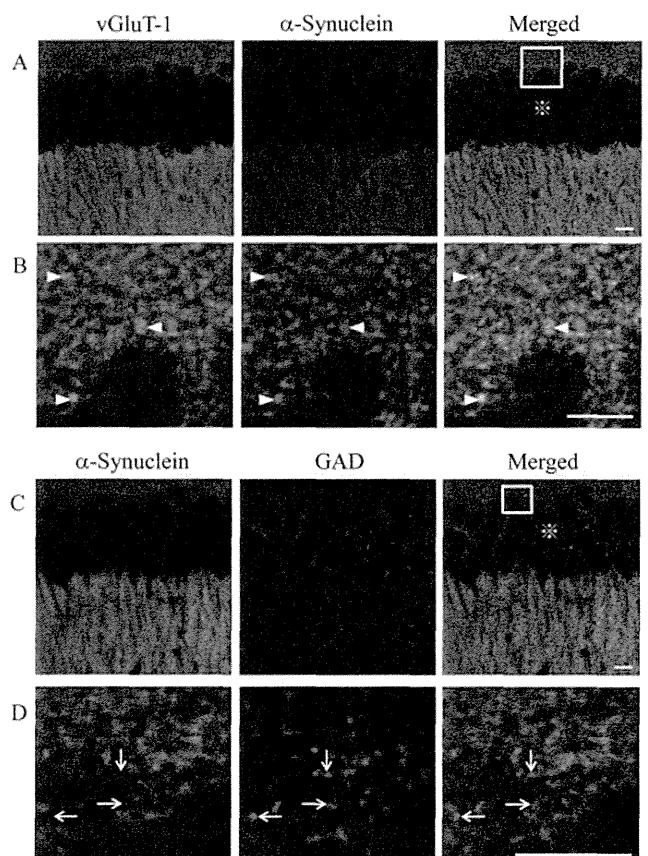doi:10.1371/journal.pone.0089327.t001

Figure 6. Localization of α-synuclein in the hippocampal CA1 region. (A, B) Confocal images of hippocampal neurons double immunostained for vGluT-1 and α-synuclein. The region marked by a white square in A is magnified in B. α-Synuclein is colocalized with vGluT-1 in B (arrowheads). (C, D) Confocal images double immunostained for α-synuclein and GAD. The region marked by a white square in C is magnified in D. As indicated by arrows in D, colocalization of α-synuclein and GAD was not detected. Mouse brains from two littermates were used for the immunohistochemical study. This experiment was repeated three times, and the differential expression pattern of α-synuclein was reproducibly confirmed. Panels A and C were used for the quantitative colocalization analyses shown in Fig. S2. Scale bars: 10 μm. ※ indicates stratum pyramidale.
doi:10.1371/journal.pone.0089327.g006

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We observed expression and synaptic localization of α-synuclein in excitatory neurons, but not in inhibitory neurons, although there were no obvious differences between the two types of neurons in expression of the synaptic markers synaptotagmin and synapsin. We also observed that activity-dependent presynaptic membrane recycling (induced by high potassium treatment) occurred independently of the presence of α-synuclein.

While there have been many studies investigating the properties of postsynaptic channels including kinetics [24–26] and the signaling molecules involved in synaptic transduction [27], less is known about the presynaptic differences between excitatory and inhibitory neurons. Recent work revealed that the sizes of both the recycling pool and total vesicular pool are more variable at glutamatergic synapses than gamma-aminobutyric acid (GABAergic) synapses [28]. This heterogeneity of the size of the recycling pool at glutamatergic synapses may provide a dynamic range of synaptic strength that is not present at GABAergic synapses [28,29]. α-Synuclein might act as a modulator of the size of the recycling pool at excitatory synapses. α-Synuclein is also suggested to be involved in mobilization of glutamate from the reserve pool using electrophysiology of hippocampal slices [30].

There is a possibility that the differential expression of α-synuclein is due to a difference in protein turnover between excitatory and inhibitory neurons. However, treatment with inhibitors of proteasomes or lysosomes did not alter the immunostaining patterns of α-synuclein (data not shown), suggesting that differential synthesis rather than degradation of α-synuclein is responsible for the distinct expression patterns in neurons.

Recently, it was reported that α-synuclein promotes early neurite outgrowth in cultured primary neurons [31]. It has also been suggested that α-synuclein plays important roles in the early development of synapses [10]. The expression ratio of α-synuclein/synaptophysin is higher during early development than in adult and aged rat brain [11]. We demonstrated that cultured neurons exhibit differential expression of α-synuclein by 30 h after cell dissemination. At this stage, there were no synaptic connections established between neurons. In addition, expression of GAD was very weak and not strong enough to distinguish inhibitory neurons. These results suggest that α-synuclein is involved in the differentiation of neurons.

Concerning the pathogenicity of α-synuclein, we observed that inhibitory neurons did not exhibit aggregate formation after treatment with preformed fibrils. This result was due to the low expression level of α-synuclein, because the expression of exogenous human α-synuclein in GAD neurons enabled them to form α-synuclein aggregates. A study in DLB patients showed that parvalbumin-containing cortical neurons are free of LBs and spared from degeneration, although the basal expression level of α-synuclein in these neurons was not determined [16]. In addition, it was found that overexpression of α-synuclein in a transgenic mice model caused inclusion body formation in hippocampal neurons, suggesting that high expression of α-synuclein is important for the intracellular accumulation and formation of LBs [14]. Recently, accumulation of α-synuclein was observed at the presynaptic terminals expressing vGluT-1in SNARE protein (SNAP-25) mutant mice [32].

These reports seem to be consistent with our present results. Intracellular aggregate formation composed of α-synuclein might be related to the endogenous expression level of α-synuclein, which is cell-type specific.

In conclusion, we have demonstrated differential expression patterns of α-synuclein between excitatory and inhibitory neurons *in vitro*. Importantly, these observations were also confirmed *in vivo*. Further studies will elucidate how α-synuclein works differently in the synaptic machinery of excitatory and inhibitory neurons, including in the regulation of the membrane recycling pool. Further analysis of the regulation of intracellular expression of α-synuclein will provide new insights for understanding the pathological conditions of neurodegenerative disorders including PD and DLB.

Supporting Information

Figure S1 Double staining for NeuN and α-synuclein. (A) Confocal images of cultured hippocampal neurons double immunostained for NeuN and α-synuclein. α-Synuclein was differentially expressed among the NeuN-positive cells. (B) GAD-immunoreactive puncta occupied a part of the synapsin-positive synapses. (C) Immunoreactivity of GAD was not colocalized with that of vGluT-1. Two independent cultures were performed and the reactivity of the antibodies was confirmed. Scale bars: 10 μm in A; 5 μm in B and C.
(TIF)

Figure S2 Quantification of colocalization between α-synuclein and marker proteins. (A–G) The degree of the colocalization shown in Figs. 3C, 1A, 1C, 1D, 5A, 6A, and 6C was determined using LSM colocalization analysis and quantified using Pearson's correlation coefficient (R).
(TIF)

Figure S3 Preparation of fibrils of α-synuclein. (A) Purity of the recombinant α-synuclein was confirmed by SDS-PAGE. Lane M indicates the molecular masses, given in kilodaltons. His-tagged α-synuclein was detected as a 20 kDa band (lane F). This fraction predominantly contained the induced protein. CBB: Coomassie Brilliant Blue staining. (B) Western blot analysis of α-synuclein. α-Synuclein was oligomerized by agitation and the insoluble aggregates with high molecular masses were recovered in the precipitant after ultracentrifugation (Lane P). Lane Or: original fraction before agitation; Lane S: supernatant after ultracentrifugation; IB: immunoblot.
(TIF)

Author Contributions

Conceived and designed the experiments: KT MT. Performed the experiments: KT YW AT HT. Analyzed the data: KT YW TT MT MT. Contributed reagents/materials/analysis tools: AT HT SM. Wrote the paper: KT MT.

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