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Arginine is a disease modifier for polyQ disease models that stabilizes polyQ protein conformation

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The polyglutamine (polyQ) diseases are a group of inherited neurodegenerative diseases that include Huntington's disease, various spinocerebellar ataxias, spinal and bulbar muscular atrophy, and dentatorubral pallidoluysian atrophy. They are caused by the abnormal expansion of a CAG repeat coding for the polyQ stretch in the causative gene of each disease. The expanded polyQ stretches trigger abnormal β-sheet conformational transition and oligomerization followed by aggregation of the polyQ proteins in the affected neurons, leading to neuronal toxicity and neurodegeneration. Disease-modifying therapies that attenuate both symptoms and molecular pathogenesis of polyQ diseases remain an unmet clinical need. Here we identified arginine, a chemical chaperone that facilitates proper protein folding, as a novel compound that targets the upstream processes of polyQ protein aggregation by stabilizing the polyQ protein conformation. We first screened representative chemical chaperones using an *in vitro* polyQ aggregation assay, and identified arginine as a potent polyQ aggregation inhibitor. Our *in vitro* and cellular assays revealed that arginine exerts its anti-aggregation property by inhibiting the toxic β-sheet conformational transition and oligomerization of polyQ proteins before the formation of insoluble aggregates. Arginine exhibited therapeutic effects on neurological symptoms and protein aggregation pathology in *Caenorhabditis elegans*, *Drosophila*, and two different mouse models of polyQ diseases. Arginine was also effective in a polyQ mouse model when administered after symptom onset. As arginine has been safely used for urea cycle defects and for mitochondrial myopathy, encephalopathy, lactic acid and stroke syndrome patients, and efficiently crosses the blood–brain barrier, a drug-repositioning approach for arginine would enable prompt clinical application as a promising disease-modifier drug for the polyQ diseases.

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Abbreviations: FCS = fluorescence correlation spectroscopy; FRET = fluorescence resonance energy transfer; polyQ = polyglutamine; SBMA = spinal and bulbar muscular atrophy; SCA = spinocerebellar ataxia

Introduction

The polyglutamine (polyQ) diseases are a group of inherited neurodegenerative diseases that include Huntington's disease, various spinocerebellar ataxias (SCAs), spinal and bulbar muscular atrophy (SBMA), and dentatorubral pallidoluysian atrophy (Orr and Zoghbi, 2007; Nagai and Popiel, 2008; Bauer and Nukina, 2009). These diseases are caused by the abnormal expansion of a CAG repeat (>35–40 repeats), which codes for a polyQ stretch in the causative gene of each disease. PolyQ diseases are characterized by various symptoms, including motor, cognitive and psychiatric impairments, which are different for each disease and depend on the regions affected in the nervous system. The treatments currently available for these diseases are symptomatic therapies, and thus disease-modifying therapies that attenuate both symptoms and molecular pathogenesis remains an unmet clinical need.

In the polyQ diseases, the expansion of the polyQ stretch within the disease-causing proteins triggers misfolding followed by their oligomerization and aggregation in affected neurons. These misfolded protein species can induce a wide range of downstream pathogenic events that eventually lead to neurodegeneration (Williams and Paulson, 2008; Takeuchi and Nagai, 2017). Various studies show that suppressing this misfolding or inhibiting the aggregation of the expanded polyQ protein exerts therapeutic effects on polyQ disease models. We and others showed that expression of molecular chaperones, such as Hsp70 and Hsp40, which facilitate proper protein folding, decreases polyQ protein aggregation and ameliorates the neurological phenotypes of various polyQ disease models (Warrick *et al.*, 1999; Cummings *et al.*, 2001; Popiel *et al.*, 2012). We also identified polyQ binding peptide, QBP1, and showed that QBP1 inhibits polyQ aggregation *in vitro* and ameliorates the neurodegenerative phenotypes of various polyQ disease models (Nagai *et al.*, 2000, 2003; Popiel *et al.*, 2013). Furthermore, various small chemical compounds, such as Congo red (Heiser *et al.*, 2000), benzothiazole derivatives (Heiser *et al.*, 2002; Hockly *et al.*, 2006), C2-8 (Zhang *et al.*, 2005; Chopra *et al.*, 2007), and (–)-epigallocatechin-3-gallate (Ehrnhoefer *et al.*, 2006, 2008) have been shown to inhibit polyQ aggregation *in vitro* and *in vivo*. However, most of these molecules have limited clinical application because of toxicity, poor blood–brain barrier permeability, or metabolic instability (Hockly *et al.*, 2006; Frid *et al.*, 2007).

Chemical chaperones are a group of low molecular weight molecules that facilitate protein folding and suppress protein aggregation, many of which are natural compounds and thus have a high safety profile when administered *in vivo* (Cortez and Sim, 2014). Here we identified the chemical chaperone, arginine, as a potent polyQ aggregation inhibitor that targets the upstream processes of polyQ protein aggregation such as β-sheet conformational transition and oligomer formation, both of which exert cellular toxicity (Nagai *et al.*, 2007; Takahashi *et al.*, 2008). Importantly, we demonstrate the therapeutic effects of arginine in mouse models of two different polyQ diseases, and even when administered after symptom onset. Arginine efficiently crosses the blood–brain barrier (Pardridge, 1983) and is in clinical use for other diseases in humans (Koga *et al.*, 2002; Boenzi *et al.*, 2012; Koenig *et al.*, 2016). Arginine would be a promising candidate therapeutic molecule for polyQ diseases that could achieve prompt clinical application.

Materials and methods

Protein expression and purification

Thioredoxin-polyQ fusion protein with 62 glutamines (thio-Q62) was expressed and purified as previously described (Nagai *et al.*, 2007). The concentration of purified thio-Q62 was determined using a DC protein assay kit (Bio-Rad Laboratories).

Protein aggregation assay

Aggregation of thio-Q62 or thio-Q19 was assessed by a turbidity assay as described previously (Nagai *et al.*, 2000). To assess the effect of arginine on turbidity, turbidity of the thio-Q62 solution that reached the aggregation plateau was measured before and immediately after the addition of arginine or phosphate-buffered saline (PBS). For seeding experiments, preformed thio-Q62 (10 μM) fibrils, prepared by incubation at 37°C for 6 days, were sonicated on ice for six pulses of 30 s with a 1-min interval between each pulse. The sonicated fibril solution (20 μl) was added as seeds to 200 μl of freshly purified thio-Q62 solution.

Circular dichroism

Circular dichroism spectra of thio-Q62 (8.2 μM) in PBS (pH 7.5) incubated at 37°C were measured using a spectropolarimeter Model J-820 (Jasco) as previously described (Nagai *et al.*, 2007).

Native PAGE

Thio-Q62 (8.2 μM) incubated with or without arginine-HCl (600 mM) at 37°C was separated by 10% (w/v) non-denaturing polyacrylamide gel electrophoresis (PAGE) and stained with Coomassie brilliant blue as previously described (Nagai *et al.*, 2007).

Fluorescence resonance energy transfer analysis

Cell culture and fluorescence resonance energy transfer (FRET) analysis was performed as previously described (Takahashi *et al.*, 2008). COS-7 cells co-expressing mCFP/mYFP-trD-polyQ fusion proteins with 56 glutamines (mCFP/mYFP-trD-Q56) were cultured with or without arginine (5–50 mM) and subjected to FRET analysis. The mean FRET signal intensity of 153–353 cells per experimental group was analysed. Cells showing FRET signal intensities above the cut-off value of 0.20 were considered to be FRET-positive, and the percentage of FRET-positive cells was calculated.

Fluorescence correlation spectroscopy analysis

Cell culture and fluorescence correlation spectroscopy (FCS) analysis was performed as previously described (Takahashi *et al.*, 2007). COS-7 cells expressing polyQ-GFP fusion protein with 45 glutamines (Q45-GFP) were cultured with or without arginine-HCl (25 mM) and subjected to FCS measurement.

Drosophila culture and experiments

MJDtr-Q78(S) transgenic flies expressing the haemagglutinin (HA)-tagged truncated form of the MJD (Machado-Joseph disease) protein with 78 glutamines (MJDtr-Q78) under the *gmr*-GAL4 driver (Warrick *et al.*, 1998) were cultured at 25°C in instant *Drosophila* medium (Carolina Biological Supply Company) with or without arginine-HCl (1–100 mM). To detect the MJDtr-Q78 protein, larval eye discs were immunostained with an anti-HA antibody (clone 3F10, Roche) as previously described (Nagai *et al.*, 2003). Quantitative analyses for eye pigmentation were performed as previously described (Saitoh *et al.*, 2015). In brief, to calculate the pigmentation score that reflects the area of remaining normal pigment in the fly eye, first, the total area of each fly eye was determined by obtaining a greyscale image of the compound eye, and was set as the region of interest. Then, the remaining normal pigment within the region of interest was measured by converting the greyscale image to a binary image and adjusting the threshold of the binary image to determine the area with normal pigment.

Caenorhabditis elegans culture and experiments

Caenorhabditis elegans lines expressing the polyQ protein were generated by injecting plasmids encoding Q40-GFP into wild-type strain N2 and were cultured with or without 10 mM arginine in the medium. Tail movement analysis was performed at 20°C on nematodes synchronized to 4 days old by counting body bends for 1 min as previously described (Brignull *et al.*, 2006). Nematodes without any movement after vigorous stimulation were considered dead. For aggregation analysis by semi-denaturing detergent agarose gel electrophoresis (SDD-AGE), 4-day-old nematodes were lysed in buffer containing 0.5% SDS (sodium dodecyl sulphate), 0.5% NP-40, 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA and a protease inhibitor cocktail. The samples were electrophoresed on a 1% agarose gel with 0.01% SDS in a running buffer of Tris-glycine containing 0.01% SDS, and were then transferred onto a nitrocellulose membrane. The membrane was then incubated with an anti-GFP antibody (1:10 000; MBL Life Sciences) at 4°C overnight and then with an HRP-conjugated anti-rabbit IgG antibody (1:20 000; Dako). For SDS-PAGE and western blot, 4-day-old nematodes were lysed in buffer A (0.5% SDS, 0.5% NP-40, 50 mM Tris-HCl , 150 mM NaCl, 5 mM EDTA, protease inhibitor cocktail) and sonicated. The extracted proteins were separated by SDS-PAGE using standard techniques and electroblotted onto PVDF (polyvinylidene difluoride) membranes. The membranes were immunoblotted with anti-GFP antibody (MBL Life Sciences) or anti-GAPDH antibody (MBL Life Sciences).

Mice and arginine treatment

All animal experiments were performed in accordance with the guidelines of the Animal Ethics Committees of the National Institute of Neuroscience, National Center of Neurology and Psychiatry and Osaka University Graduate School of Medicine, Japan. The *Sca1*$^{154Q/2Q}$ mouse model (Watase *et al.*, 2002) was provided by Dr Kei Watase (Tokyo Medical and Dental University, Japan), and the SBMA mouse model (Katsuno *et al.*, 2002) by Dr Gen Sobue (Nagoya University, Japan). Mice were housed with a 12-h light/dark cycle and with food and water provided *ad libitum*. Female SCA1 mice, male SBMA mice and their wild-type littermate controls were weaned at either 3 or 5 weeks of age and maintained in cages with water bottles containing either water, 2% or 6% L-arginine-HCl. The daily liquid intake was calculated by dividing the difference of the bottle weight measured before and after usage by the number of mice in each cage, days the bottle was placed on the cage, and the specific gravity of the liquid in the bottle. All male SCA1 mice were subjected to breeding and were not included in the study. The mice were randomly assigned to the different experimental groups.

Amino acid analyses of mouse tissues

Wild-type mice were orally administered 200 μl of water, 5% or 15% L-arginine-HCl using a sonde once an hour for 9 h. The total amount of L-arginine administered during this period was almost equal to the total daily intake of L-arginine of mice administered 2% or 6% L-arginine *ad libitum*, respectively (data

not shown). One hour after the final administration, the mice were anaesthetized with pentobarbital and euthanized by exsanguination from the abdominal aorta. Sera and the dissected brains were flash-frozen and stored at –80°C until use. L-arginine concentration in the samples was measured by Shiseido Inc. using high performance liquid chromatography (HPLC), as previously described (Hamase *et al.*, 2010).

Mouse phenotype analyses

Rotarod performance and spontaneous motor activity were assessed as described previously (Popiel *et al.*, 2012). Balance beam analysis was performed by assessing the time for mice to cross a 150-cm long beam of 10 mm in diameter. The beam was placed 50 cm above the ground with a bright light at the starting end and a dark box at the far end. From 4 weeks of age, mice were trained for three consecutive days, with two trials on each day, once every 2 weeks, to cross the beam without falling or turning around. The time for the mice to cross the beam on the two trials of the third day of the training was recorded from 6 weeks of age, and the faster time was used for analysis. If the mice took longer than 60 s to cross, the score was counted as 60 s. The analyses were performed with the raters blinded to treatment status but not to the genotype because the genotypes of SCA1 and SBMA mice can be identified by visual inspection.

Immunohistochemistry analyses

For SCA1 mice, 10-μm thick frozen sections of mouse brains were prepared as described previously (Popiel *et al.*, 2012). As for the analyses of inclusion bodies, sections were blocked in PBS containing 5% goat serum and 0.1% Triton™ X-100 for 1 h at room temperature, then incubated with a mouse anti-ubiquitin antibody (clone FK2, 1:500) at 4°C overnight, followed by an Alexa Fluor® 488-conjugated goat anti-mouse IgG antibody (1:1000, Life Technologies) for 1 h at room temperature. Sections were mounted with SlowFade® Gold Antifade reagent containing DAPI (Life Technologies) and examined using a confocal laser-scanning microscope (FV 1000; Olympus). Images obtained from three or four mice per group were analysed for quantification of the percentage of neuronal cells that contains inclusion bodies. In each mouse, the cell count was averaged from three sections (four or two images per section obtained in the cortex or hippocampus, respectively). Over 130 or 70 cells were counted in the cortex and hippocampus, respectively. As for the analyses of dendritic arborization of the Purkinje cells, brains were obtained from 30-week-old wild-type or SCA1 mice with or without 6% arginine administration from 3 weeks of age ($n = 5$ per group). Midsagittal frozen sections (10 μm) of mouse cerebellum were antigen retrieved by autoclaving at 121°C for 20 min, incubated with rabbit anti-calbindin antibody (1:16 000, Swant Swiss antibodies), then incubated with Histofine Simple Stain MAX PO MULTI (Nichirei Biosciences) for 30 min at room temperature, colourized using 3,3′-diaminobenzidine tetrahydrochloride (BioGenex Laboratories), and counterstained with haematoxylin. Two sections per mouse were stained and examined with NanoZoomer-XR (Hamamatsu Photonics).

For SBMA mice, spinal cords were dissected from 14-week-old animals, post-fixed with 10% neutral buffered formalin (Sigma-Aldrich) at 4°C overnight, embedded in paraffin, and coronally sectioned at the level of L1 at a thickness of 5 μm. Sections were then deparaffinized, antigen retrieved with 98% formic acid for 5 min at room temperature, blocked with 5% horse serum in PBS containing 0.1% Triton X-100, incubated with 1C2 antibody (Merck Millipore) at 4°C overnight, and incubated with biotinized horse anti-mouse antibody. Sections were then incubated with streptavidin-horseradish peroxidase (Dako), colourized using 3,3-diaminobenzidine tetrahydrochloride (BioGenex Laboratories), and counterstained with haematoxylin. Images obtained from five mice per group, 10 sections per mouse, were analysed for quantification of the percentage of neuronal cells that contains inclusion bodies.

Biochemical analyses of mice tissues

Immunoblotting of mutant ataxin-1 in the SCA1 mice brain using 1C2 antibody (Merck Millipore), which detects polyglutamine expansion, was performed as previously described (Watase *et al.*, 2002). For immunoblotting of androgen receptor (AR) in the SBMA mice tissue, spinal cords were homogenized in lysis buffer (50 mM Tris-HCl, pH 8.0, 2% SDS) containing protease inhibitor cocktail (Nakalai Tesque), incubated at 99°C for 5 min, centrifuged at 2500*g* for 15 min at 4°C, and the supernatants were collected. Proteins were separated by 4–20% polyacrylamide gels, transferred to PVDF membranes, and incubated overnight at 4°C with anti-AR antibody (H-280; Santa Cruz Biotechnology) or anti-α-tubulin antibody (Cell Signaling Technology). After incubation with HRP-conjugated secondary antibodies, the signals were visualized with ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Science), and captured by ImageQuant LAS 4000 (Fujifilm). Signal intensities were quantified by densitometry using MultiGauge (Fujifilm).

Statistical analyses

Turbidity, FRET, pigmentation score of the MJDtr-Q78(S) flies, and arginine concentration were analysed by one-way ANOVA followed by Tukey's multiple comparison test. FCS measurements, tail movements and the relative amounts of monomers and oligomers in *C. elegans*, and the percentage of cells with polyQ inclusions in mouse brains were analysed by Student's *t*-test. Data for daily liquid intake and motor phenotype assessment of SCA1 mice were analysed by two-way repeated-measures ANOVA followed by Tukey's multiple comparison test. Motor phenotype assessment of SBMA mice was analysed by mixed-effects analysis followed by multiple comparison by Holm-Sidak test. Survival data were analysed using the log-rank test. For all analyses, GraphPad Prism (GraphPad Software Inc.) was used. $P < 0.05$ was considered to indicate a statistically significant difference.

Data availability

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Results

Identification of arginine as a polyQ protein aggregation inhibitor *in vitro*

To identify chemical compounds that inhibit aggregation of polyQ proteins, we screened nine representative chemical chaperones using a previously established *in vitro* polyQ aggregation assay (Nagai *et al.*, 2000). In this assay, aggregation of thioredoxin-fused expanded polyQ proteins (thio-polyQ) can be quantified by turbidity. We incubated thio-Q62 on its own or with various chemical chaperones, and evaluated its aggregation on Day 4. While co-incubation of most chemical chaperones with thio-Q62 did not affect its aggregation, arginine significantly decreased the aggregation of thio-Q62 by >80% (Fig. 1A). Although glycerol also moderately inhibited thio-Q62 aggregation (Fig. 1A), we chose arginine as a therapeutic candidate for polyQ diseases because it is known to penetrate the blood–brain barrier (Pardridge, 1983) and has an established safety record in clinical trials for other human diseases (Koga *et al.*, 2002; Boenzi *et al.*, 2012; Koenig *et al.*, 2016).

We next characterized the inhibitory effect of arginine on the time course of polyQ aggregation. The turbidity of thio-Q62 solution increases in a time-dependent manner with a short lag phase followed by rapid elevation during the aggregation phase that eventually reaches a plateau (Nagai *et al.*, 2007) (Fig. 1B). Co-incubation of thio-Q62 with arginine remarkably elongated the lag phase and decelerated the aggregation process (Fig. 1B). Arginine itself did not interfere with turbidity measurements (Fig. 1B and Supplementary Fig. 1).

We then characterized dose-dependency of arginine at a neutral pH on the inhibition of polyQ aggregation. Arginine significantly inhibited thio-Q62 aggregation in a dose-dependent manner while proline, used as a negative control, did not exert any anti-aggregation effect on thio-Q62 (Fig. 1C). These results confirmed the inhibitory effect of arginine on polyQ aggregation in the initial screening and excluded the possibility that the high pH of free arginine used in the initial screening was the cause of aggregation inhibition.

We further tested the effect of arginine on seed-induced polyQ aggregation. In amyloidogenic proteins such as amyloid-β, α-synuclein and polyQ, preformed protein aggregates act as seeds to trigger aggregation of soluble protein monomers with a shortened lag phase, which is called seeding effects (Jarrett and Lansbury, 1993). Seeding is now believed to contribute to the spread of pathology in most neurodegenerative diseases (Goedert, 2015). Indeed, addition of a small amount of preformed thio-Q62 aggregate to soluble thio-Q62 eliminated the lag phase and triggered an immediate increase in turbidity (Fig. 1D), which is consistent with a previous study (Chen *et al.*, 2001). Arginine remarkably decelerated the increase of seed-induced turbidity of thio-Q62 (Fig. 1D). These results indicate that arginine alleviated seed-induced polyQ aggregation and that arginine has the potential to decelerate further aggregation of polyQ protein after initiation of the aggregation process.

Arginine inhibits the toxic conformational transition and oligomer formation of the polyQ protein

Recent studies have indicated that aggregation-prone proteins exert cellular toxicity before the formation of insoluble aggregates. At this stage, such proteins undergo conformation transition to a β-sheet rich structure and assemble into soluble oligomers (Williams and Paulson, 2008). Because elongation of the lag phase of thio-Q62 aggregation by arginine (Fig. 1B) suggested that arginine affects the initial misfolding processes before insoluble aggregate formation during the aggregation process, we investigated whether arginine alters the formation of these soluble toxic protein species.

We first tested the effect of arginine on the β-sheet conformational transition of the polyQ protein, which exerts cellular toxicity (Nagai *et al.*, 2007). Consistent with our previous report (Nagai *et al.*, 2007), native PAGE analysis of thio-Q62 revealed that the fast-migrating band corresponding to the β-sheet-rich monomer of thio-Q62 gradually appeared upon incubation, while the slow-migrating band corresponding to the α-helix-rich monomer gradually disappeared (Fig. 2A). This conformational transition of thio-Q62 from α-helix to β-sheet was confirmed by circular dichroism analysis (Fig. 2B), where two negative peaks at 208 and 222 nm at Day 0, which are characteristics of α-helical structure, shifted to a single negative peak at 210 nm, which are characteristic of β-sheet structure (Greenfield, 2006). Co-incubation of thio-Q62 with arginine inhibited the appearance of the fast-migrating β-sheet band (Fig. 2A), indicating that arginine inhibited the β-sheet conformational transition of thio-Q62.

We then investigated the effect of arginine on polyQ oligomer formation in cultured cells using FRET and FCS (Takahashi *et al.*, 2007, 2008). FRET is a sensitive technique to analyse protein-protein interactions of fluorescent molecules. We previously applied this method to detect interactions among polyQ proteins fused with CFP or YFP, which assemble into soluble oligomers in cultured cells (Takahashi *et al.*, 2008). We transfected COS-7 cells with mCFP/mYFP-trD-Q56, incubated them in the medium without or with various concentrations of arginine, and examined the percentage of FRET-positive cells after 48 h. Incubation with arginine (20–50 mM) significantly decreased the percentage of cells with a positive FRET signal (Fig. 2C and D). Arginine treatment at these concentrations did not affect the viability of COS-7 cells with mCFP/mYFP-trD-Q56 (data not shown). These results demonstrate that arginine inhibits the interactions among

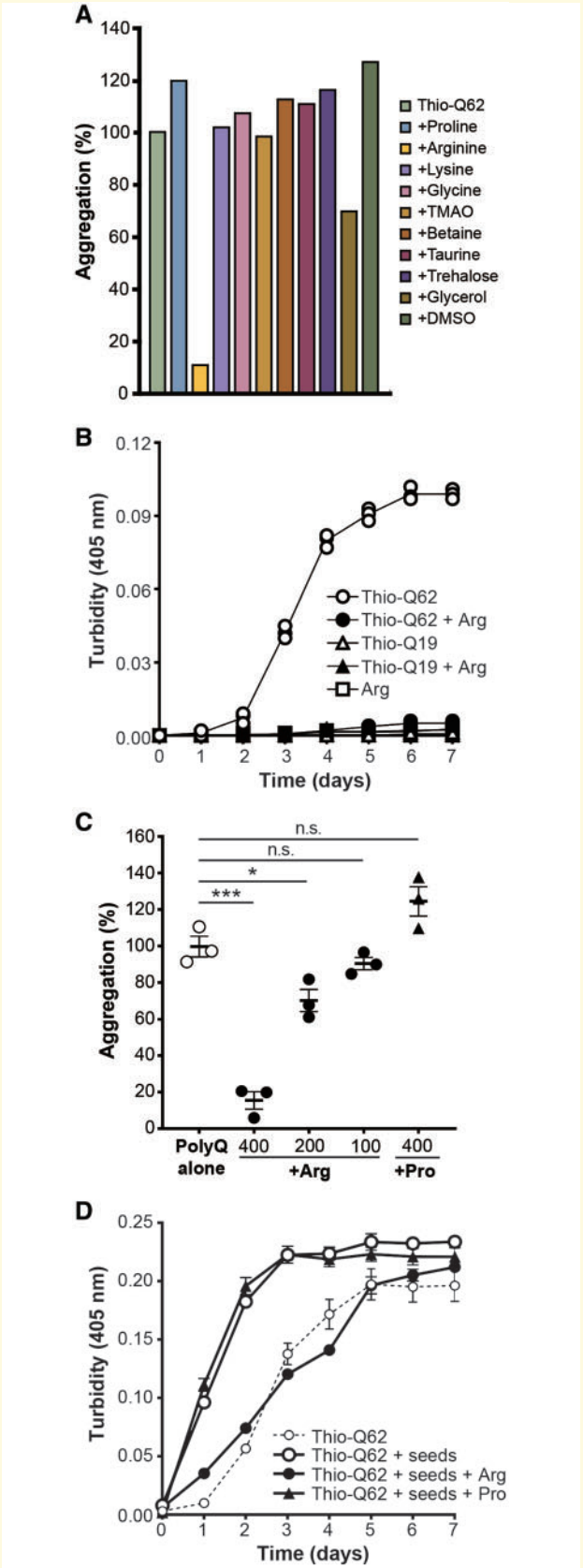

Figure 1 Arginine inhibits polyQ protein aggregation *in vitro*. (**A**) Screening of chemical chaperones for polyQ aggregation inhibitors using an *in vitro* aggregation assay. Thio-Q62 (10 μM) was incubated on its own or with various chemical chaperones (100 mM), and aggregation was evaluated on Day 4 by turbidity measurements. (**B**) Effect of arginine on the time course of thio-Q62 aggregation. Thio-Q62 or thio-Q19 (10 μM) with or without arginine (400 mM) or arginine alone (400 mM) was incubated at 37°C and was subjected to turbidity measurement every 24 h. Values represent means ± standard error of the mean (SEM), $n = 3$. (**C**) Dose dependency of the anti-aggregation effect of arginine. Arginine or proline was co-incubated with thio-Q62 (10 μM), and the aggregation was evaluated on Day 5. The turbidity of polyQ alone was set to 100%. Values represent means ± SEM, $n = 3$. *$P < 0.05$, ***$P < 0.001$; n.s. = not significant. (**D**) Inhibition of seed-induced polyQ aggregation by arginine. Soluble thio-Q62 (10 μM) was seeded by preformed thio-Q62 aggregates and co-incubated with arginine or proline (600 mM). Values represent means ± SEM, $n = 4$.

polyQ proteins that lead to oligomer assembly in cultured cells in a dose-dependent manner.

To confirm the FRET analysis results, we quantitatively evaluated the effect of arginine on polyQ oligomer formation by FCS. FCS is a sensitive technique for analysing the dynamic properties of fluorescent molecules. We previously utilized two indices obtained from FCS measurement to assess polyQ oligomers; counts per particle (CPP) and the diffusion time of the fast components (DT_1) (Takahashi *et al.*, 2007). CPP is the average fluorescence intensity of polyQ oligomeric particles and hence directly reflects the number of polyQ monomers per oligomer. DT_1 reflects the size of the polyQ oligomers. We transfected COS-7 cells with a polyQ-GFP fusion protein of 45 glutamines (Q45-GFP). Cells were then incubated with or without arginine for 48 h and then subjected to FCS. Arginine treatment significantly reduced the CPP and the DT_1 compared with the untreated cells (Fig. 2D and E). These results also indicate that arginine inhibits oligomer formation of polyQ proteins within cells.

Taken altogether, these findings indicate that arginine inhibits the β-sheet conformational transition and oligomer formation of the polyQ protein. We conclude that arginine interferes with these initial processes of polyQ protein aggregation during which misfolded polyQ proteins exert neuronal toxicity.

Arginine exerts therapeutic effects in invertebrate models of polyQ diseases

We next investigated the therapeutic effects of arginine *in vivo* using two well-established invertebrate models of polyQ diseases, *Drosophila melanogaster* (Warrick *et al.*, 1998) and *Caenorhabditis elegans* (Satyal *et al.*, 2000), to validate the effect of arginine in a time- and cost-effective manner.

Spinocerebellar ataxia type 3 (SCA3/MJD) model flies that express a truncated form of the MJD protein with an expanded repeat of 78 glutamines (MJDtr-Q78) in the compound eye (Warrick *et al.*, 1998) show compound eye

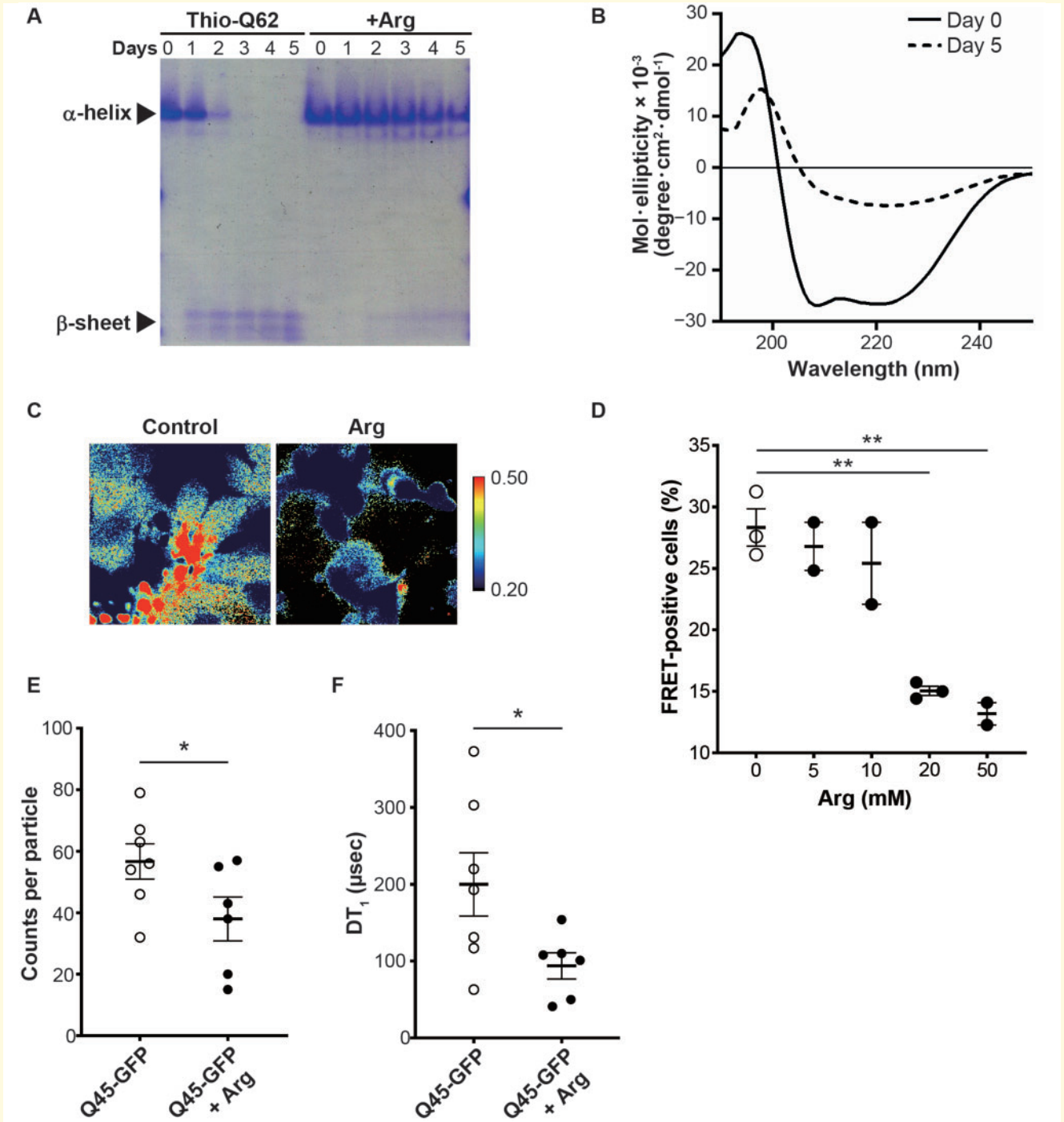

Figure 2 Arginine inhibits the toxic conformational transition and oligomer formation of a polyQ protein. (**A** and **B**) Inhibition of the conformational transition of a polyQ protein by arginine. Thio-Q62 (8.2 μM) was incubated at 37°C for 5 days with or without arginine (600 mM) and was then subjected to native PAGE (**A**). Circular dichroism analysis of thio-Q62 alone on Day 5 (**B**). (**C**–**F**) Inhibition of the oligomerization of polyQ protein by arginine detected by FRET (**C** and **D**) and FCS (**E** and **F**) analysis. COS-7 cells expressing Q56-mCFP and Q56-mYFP were incubated in medium containing arginine (0–50 mM) and were subjected to FRET analysis (**C** and **D**). Representative FRET images are shown in (**C**). COS-7 cells expressing Q45-GFP were incubated in medium with or without arginine (25 mM), then lysed and subjected to FCS analysis (**E** and **F**). In **D**–**F**, values represent the mean ± SEM, $n = 3$. *$P < 0.05$, **$P < 0.01$.

degeneration, which can be detected under the light microscope (Warrick *et al.*, 1998). We administered arginine by culturing the flies with food containing arginine (1–100 mM) and found that arginine significantly ameliorated the compound eye degeneration at 100 mM, and also showed a tendency of amelioration at 10 mM (Fig. 3A). We then

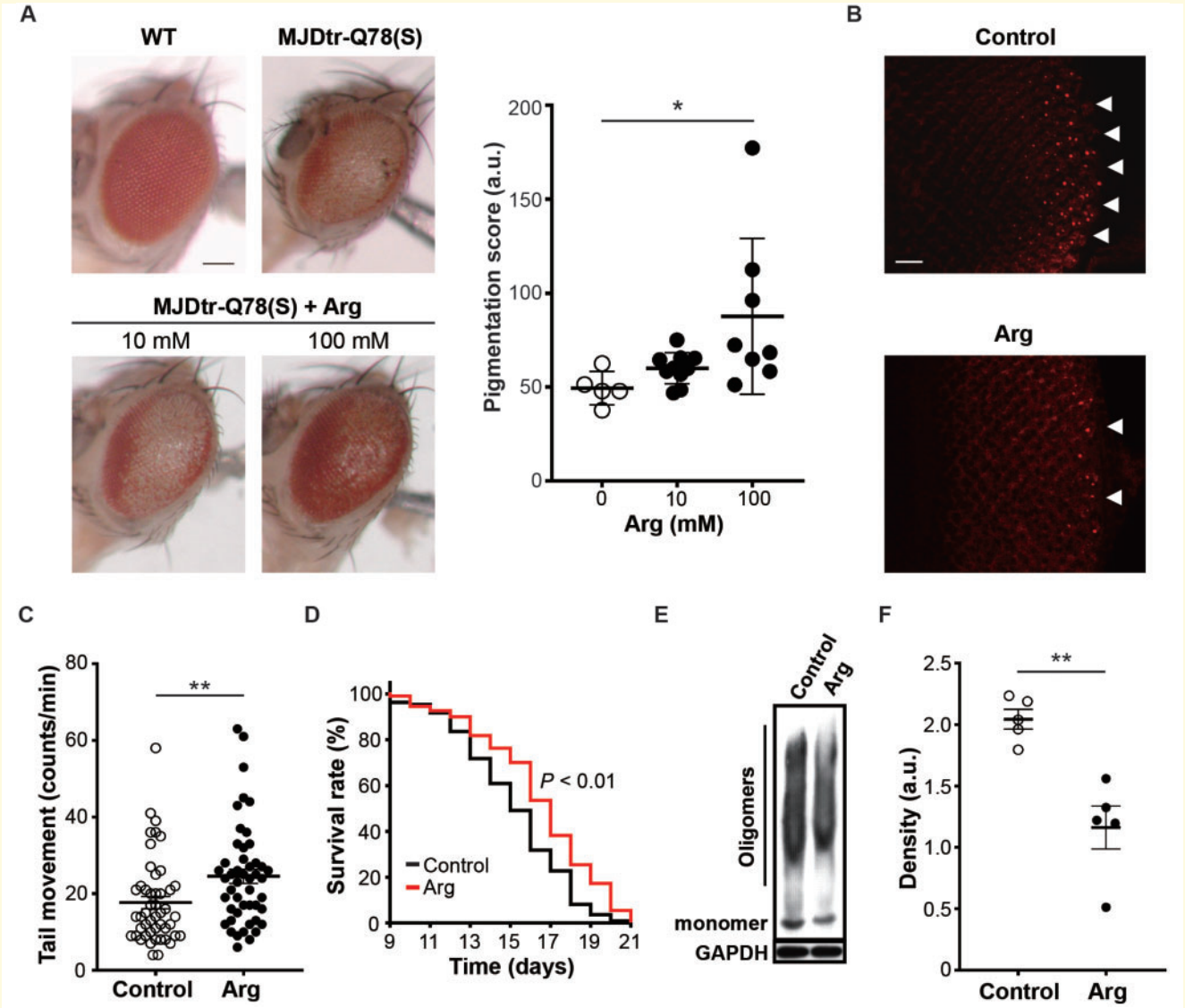

Figure 3 Arginine suppresses polyQ-induced phenotypes in invertebrate models of polyQ diseases. (**A** and **B**) Effect of arginine on the phenotypes of a *Drosophila* model of polyQ disease. (**A**) Light microscopy images and relative pigmentation scores of the external compound eyes of wild-type (WT) and MJDtr-Q78(S) flies cultured with or without arginine (10 or 100 mM). Scale bar = 100 μm. Values represent means ± SEM, n = 5, 10, 8 in 0, 10, 100 mM, respectively. (**B**) Confocal microscopy images of inclusion body formation in the eye discs of MJDtr-Q78(S) fly larvae cultured with or without arginine (100 mM) detected with an anti-haemagglutinin antibody. Scale bar = 10 μm. (**C**–**F**) Effect of arginine on the phenotypes of a *C. elegans* model of polyQ disease. Tail movements (**C**), survival rate (**D**), and SDD-AGE analysis (**E** and **F**) of the disease model worms cultured with or without arginine (10 mM). The relative amounts of oligomers in the polyQ worms were quantified (**F**) from the smear intensities of the SDD-AGE images (**E**). In **C** and **F**, values represent means ± SEM, n = 48 in **D**, n = 5 in **F**. $^{**}P < 0.01$. In **D**, $P < 0.01$, log-rank test.

analysed inclusion body formation of the MJDtr-Q78 protein in the eye imaginal discs of third instar larvae by immunohistochemistry. Larvae cultured with food containing arginine had remarkably fewer inclusion bodies compared with those cultured with standard food (Fig. 3B), indicating potent suppression of polyQ inclusion body formation by arginine administration.

We also tested the effect of arginine in a *C. elegans* model of polyQ disease, which expresses Q40-GFP and exhibits motor dysfunction and shortened lifespan. Addition of arginine to the culture medium significantly improved the motor dysfunction (Fig. 3C) and the survival rate (Fig. 3D) of the polyQ disease worms. We also tested the effect of arginine on polyQ oligomer formation in worms by SDD-AGE. While the untreated control polyQ disease worms exhibited a smear of Q40-GFP oligomers, the maximal vertical width of the smear band of the arginine-treated sample was less broad in the arginine-treated polyQ disease worms

(Fig. 3E and F). We confirmed that arginine treatment did not affect the expression level of Q40-GFP monomer proteins (Supplementary Fig. 2).

Taken together, we conclude that arginine exerts therapeutic effects *in vivo* in invertebrate models of polyQ diseases via the inhibition of polyQ protein oligomerization and aggregation.

Arginine ameliorates the neurological phenotypes of polyQ disease mouse models

To confirm the therapeutic effects of arginine, we extended our study to mammalian models, using a polyQ disease mouse model of SCA, the *Sca1*$^{154Q/2Q}$ knock-in model (Watase *et al.*, 2002) (hereafter referred to as SCA1 mice). This model expresses an expanded polyQ SCA1 protein at endogenous levels and with accurate temporal and spatial distribution. Furthermore, human SCA disease features are well replicated, including progressive motor deficit (Watase *et al.*, 2002).

To confirm the blood–brain barrier permeability of arginine, we orally administered water or arginine solution to wild-type mice and subjected sera and brains to HPLC (Hamase *et al.*, 2010). Oral administration of arginine led to a dose-dependent increase in the arginine concentration in serum and brain (Fig. 4A), which is consistent with a previous report (Pardridge, 1983).

We then examined the therapeutic effects of arginine by administering 6% arginine in drinking water to SCA1 mice from 3 weeks of age. In the rotarod test, SCA1 mice showed a clear deficit at 4 weeks of age, which steadily progressed until 36 weeks of age (Watase *et al.*, 2002) when the analysis ended (Fig. 4B). SCA1 mice treated with 6% arginine performed significantly better than the untreated mice and were comparable with the wild-type control mice from 4–12 weeks of age, while arginine administration to wild-type mice did not exert any obvious effects (Fig. 4B). The therapeutic effect of arginine remained significant until 24 weeks of age (Fig. 4B).

The motor coordination of SCA1 mice was also examined using the balance beam test. The motor phenotype of SCA1 mice, as an increased time to cross the balance beam, was consistently detected from 20 weeks of age (Fig. 4C), which was later than the rotarod deficit. SCA1 mice treated with 6% arginine were able to cross the balance beam in significantly less time compared with untreated SCA1 mice from 26 weeks of age, demonstrating the therapeutic effect of arginine (Fig. 4C). In addition, at 20 and 22 weeks of age, while the difference between untreated SCA1 mice and untreated wild-type mice was statistically significant, that between arginine-treated SCA1 mice and untreated wild-type mice was not significant, which suggests a therapeutic effect of arginine at these earlier ages.

We then analysed the effect of arginine on inclusion body formation in the SCA1 mouse brain. SCA1 mice develop polyQ inclusion bodies in their neurons from ~4 weeks of age, which are evident in the cerebral cortex and hippocampus (Watase *et al.*, 2002). Immunohistochemical analyses of 12-week-old arginine-treated SCA1 mouse brains exhibited significantly fewer inclusion bodies in these regions compared with untreated mice (Fig. 4D and E). In addition, 30-week-old arginine-treated SCA1 mice showed a tendency of better dendritic arborization of the Purkinje cells in the cerebellum (Supplementary Fig. 3). Consistent with the previous study (Watase *et al.*, 2002), no inclusion bodies were observed in the cerebellum (data not shown). We confirmed that arginine administration did not affect the expression level of monomeric mutant ataxin-1 in the brains of SCA1 mice (Fig. 4F).

We confirmed that all mouse groups had a comparable amount of liquid intake (Supplementary Fig. 4), showing that drinking water containing 6% arginine did not affect the *ad libitum* drinking activity of wild-type or SCA1 mice. Arginine administration did not affect the bodyweight of wild-type or SCA1 mice (Fig. 4G). All mice survived until 39 weeks of age, when all the behavioural analyses were ended (data not shown).

These results indicate that arginine exerts a therapeutic effect on the motor function and brain pathology of SCA1 mice.

Arginine is effective in two different polyQ disease mouse models

We next questioned whether arginine is effective in a mouse model of another polyQ disease based on the *in vitro* effect of arginine on the expanded polyQ stretch itself.

We used a mouse model of SBMA (Katsuno *et al.*, 2002), which expresses full-length human AR with an expanded polyQ stretch and exhibits progressive motor impairment that is reversible by drug treatment (Katsuno *et al.*, 2003). The motor impairment of these mice can be monitored as a deficit in spontaneous motor activity by measuring their horizontal activity and rearing behaviour in a cage (Katsuno *et al.*, 2002). At 12 weeks of age, untreated SBMA mice started to show a decrease in spontaneous activity compared with untreated wild-type mice (Fig. 5A and B). Arginine treatment significantly improved this motor impairment of SBMA mice to levels that were comparable to that of the wild-type mice until 14 weeks of age (Fig. 5A and B). Treatment of wild-type mice with arginine did not have a significant effect (Fig. 5A and B). Arginine did not affect the survival of SBMA mice (data not shown). Arginine treatment significantly improved the motor impairment of SBMA mice when assessed with rotarod test at 13 weeks of age (Fig. 5C), when the deficit of SBMA mice in an open-field test showed significant improvement by arginine administration. In contrast, no significant difference in the rotarod test performance was observed between SBMA mice with or without arginine treatment at 9 weeks of age (Fig. 5C), when SBMA mice did not show a deficit in the open-field test (Fig. 5A and B).

We then analysed the effect of arginine on inclusion body formation in the spinal cords of SBMA mice. Immunohistochemical analyses of 14-week-old arginine-treated SBMA mouse spinal cords exhibited a tendency of

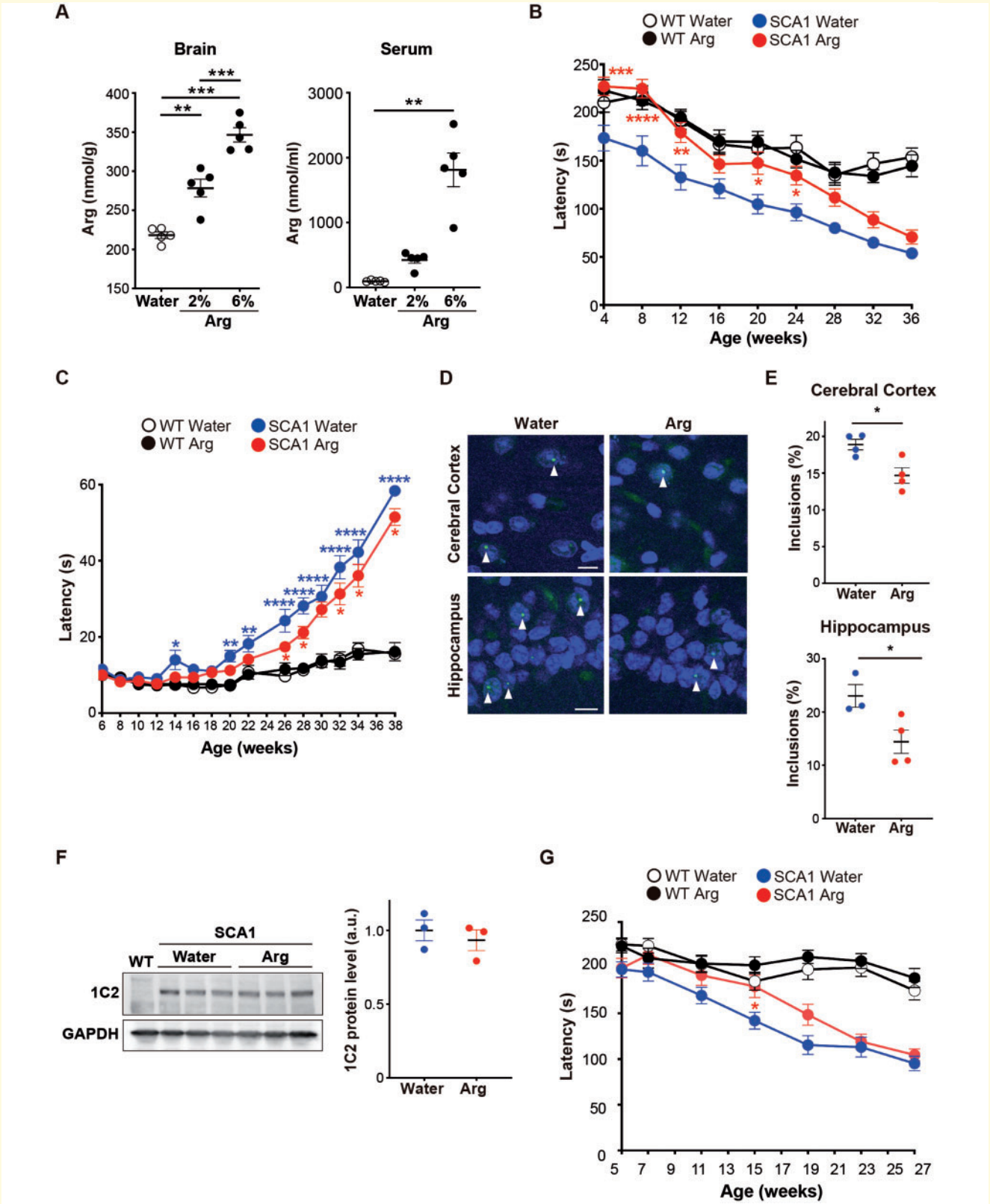

Figure 4 Arginine administration ameliorates the neurological and pathological phenotypes of SCA1 mice. (**A**) Arginine concentration per gram of brain (*left*) and per ml of serum (*right*) measured by HPLC in wild-type (WT) mice orally administered with the equivalent amount of arginine to the daily *ad libitum* intake of 2% or 6% arginine-containing water, or water as a control. Data represent the mean ± SEM, $n = 3$. $^{**}P < 0.01$, $^{***}P < 0.001$. (**B**) Rotarod activity of wild-type and SCA1 mice with or without arginine administration. The latency to fall from the accelerating rotarod is shown. (**C**) The latency to cross the balance beam of wild-type and SCA1 mice with or without arginine administration. (**D** and **E**) Inclusion body formation in the brains of SCA1 mice with or without 6% arginine treatment. The brains were obtained at 12

(continued)

fewer inclusion bodies compared with untreated mice, although the difference did not reach statistical significance (Fig. 5D). We confirmed that arginine administration did not affect the expression level of monomeric AR proteins in the spinal cords of SBMA mice (Fig. 5E). We confirmed that all mouse groups had a comparable amount of liquid intake and bodyweight (Supplementary Fig. 5), showing that drinking water containing 6% arginine did not affect the *ad libitum* drinking activity or the bodyweight of SBMA mice.

These results show that arginine is effective in two different mouse models of polyQ diseases, strengthening its potential as a therapeutic molecule for polyQ diseases.

Arginine administration after disease onset is effective in a polyQ disease mouse model

An important issue in the development of candidate therapeutic compounds for neurodegenerative diseases is whether the candidates exert a therapeutic effect at the symptomatic stage, when the pathological process is already well progressed (Katsuno *et al.*, 2012). Most preclinical studies reported so far administer therapeutic candidates to disease model mice prophylactically at the pre-symptomatic stage. To test whether arginine administration is also effective after symptom onset, we administered arginine to SCA1 mice from 5 weeks of age, when these mice already show neurological deficits. In addition, we administered the mice with 2% arginine, which is less than the mouse-equivalent dose (White and Seymour, 2005) of the maximal oral dose of arginine administered to patients with urea cycle deficits (0.5 g/kg/day as arginine-HCl), as approved by the Pharmaceutical and Medical Devices Agency (PMDA) of Japan. Untreated SCA1 mice demonstrated a deficit in rotarod performance at 5 weeks of age compared with untreated wild-type mice, which progressively worsened (Fig. 4G). Arginine-treated SCA1 mice demonstrated better performance than untreated mice from 7–19 weeks of age, with statistically significant differences at 15 weeks of age (Fig. 4G), although the effect of arginine was more evident in the presymptomatic administration (Fig. 4B). These results demonstrate that a PMDA-approved dose of arginine exerts therapeutic effects on the neurological phenotypes of a polyQ disease even when administration is started after the onset of motor symptoms. Arginine is, therefore, a promising therapeutic candidate for clinical application.

Discussion

In this study, we identified arginine as a potent polyQ aggregation inhibitor that acts by inhibiting the formation of misfolded and oligomeric toxic protein species before the formation of insoluble aggregates. We also confirmed its therapeutic effects on neurological symptoms and protein aggregation pathology using two different animal models of polyQ diseases. In addition, we showed that arginine may exert a therapeutic effect on the dendritic arborization of the Purkinje cell in the cerebellum.

Application of arginine as a novel therapy for polyQ diseases fits the recent concept of 'drug repositioning' (Corbett *et al.*, 2012) because arginine has been safely used for patients with urea cycle defects (Boenzi *et al.*, 2012) and for those with mitochondrial myopathy, encephalopathy, lactic acid and stroke (MELAS) syndrome (Koga *et al.*, 2002; Koenig *et al.*, 2016). The advantage of this approach is that the safety and blood–brain barrier permeability (Pardridge, 1983) of arginine is already established, greatly reducing the time and cost required for validation and optimization before clinical trial.

Another advantage of arginine as a therapeutic molecule is that arginine targets the upstream processes of insoluble aggregate formation. Aggregation-prone proteins undergo multiple processes before the formation of insoluble aggregates, such as misfolding and β-sheet conformation transition of the protein monomer, and assembly of the monomers into oligomers. The formation of insoluble aggregates is further accelerated in the presence of preformed aggregates, which act as seeds (Nagai *et al.*, 2007; Cheng *et al.*, 2013).

Figure 4 Continued

weeks of age and examined by immunohistochemistry. (**D**) Confocal microscopy images of the cerebral cortex (*top*) and hippocampus (*bottom*) of SCA1 mice. Green = ubiquitin staining (inclusion bodies); blue = nuclei visualized by DAPI staining. In each panel, nuclear inclusion bodies are indicated by white arrowheads. Scale bars = 10 μm. (**E**) The percentage of neuronal cells with inclusion bodies in the cortex (*left*) and hippocampus (*right*). Data are shown as the mean ± SEM. *$P < 0.05$. (**F**) Western blot analysis (*left*) and relative mutant ataxin-1 levels (*right*) in the brains of SCA1 mice with or without 6% arginine treatment. The brains were obtained at 12 weeks of age. Lines represent the mean ± SEM. In (**B**–**F**), SCA1 mice were administered drinking water containing 6% arginine from 3 weeks of age. Wild-type mice administered water or 6% arginine are included as controls. In **B** and **C**, and **F** and **G**, values represent the mean ± SEM, $n = 16$–18 per group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. In **B**, DFn = 1, DFd = 33, F = 4.915, $P = 0.0336$ for treatment; DFn = 17, DFd = 534, F = 53.92, $P < 0.0001$ for time/genotype; DFn = 17, DFd = 534, F = 3.840, $P < 0.0001$ for treatment × time/genotype. In **C**, DFn = 1, DFd = 27, F = 0.7713, $P = 0.0371$ for treatment; DFn = 29, DFd = 753, F = 115.3, $P < 0.0001$ for time/genotype; DFn = 29, DFd = 753, F = 1.593, $P = 0.0273$ for treatment × time/genotype. Blue asterisks in **C** indicate statistically significant differences between SCA1 and wild-type mice administered water, while red asterisks in **B**, **C** and **F** indicate statistically significant differences between SCA1 mice with or without arginine administration. (**G**) Rotarod activity of wild-type and SCA1 mice with or without 2% arginine administration from 5 weeks of age. Mice ($n = 23$–25) were analysed for each group. Data are shown as the mean ± SEM for each group. *$P < 0.05$, **$P < 0.01$. DFn = 1, DFd = 48, F = 1.304, $P = 0.2591$ for treatment; DFn = 13, DFd = 596, F = 54.70, $P < 0.0001$ for time/genotype; DFn = 13, DFd = 596, F = 1.751, $P = 0.0474$ for treatment × time/genotype.

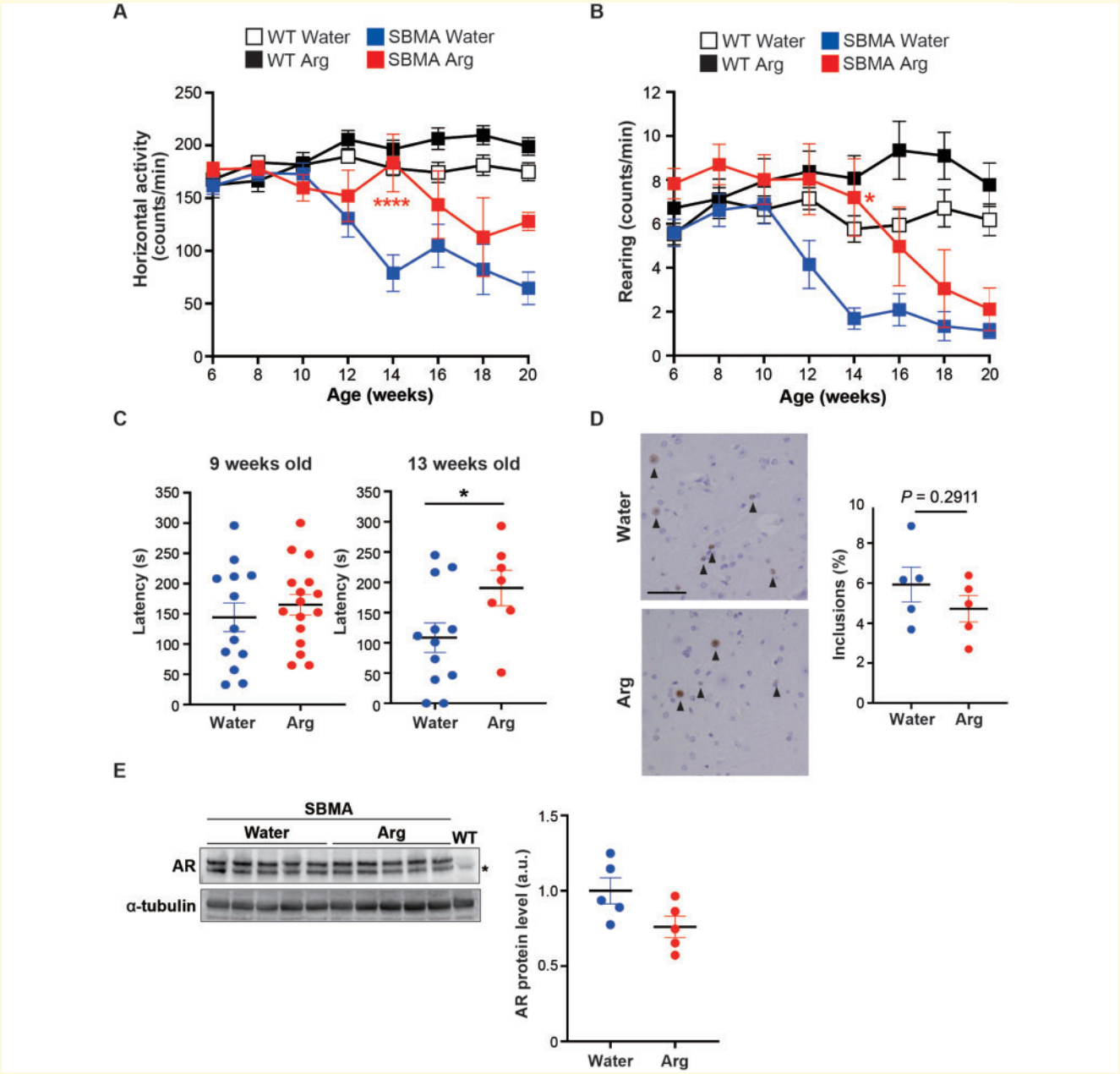

Figure 5 Arginine administration exerts therapeutic effects in SBMA mice, a model of another polyQ disease. (**A** and **B**) Spontaneous motor activity of SBMA mice administered 6% arginine water. Horizontal activity (**A**) and rearing behaviour (**B**) were measured. n = 12–16 mice for each group. Data are shown as the mean ± SEM. *$P < 0.05$, ****$P < 0.0001$. In **A**, DFn = 15, DFd = 301, F = 2.727, $P = 0.0006$ for treatment and time/genotype. In **B**, DFn = 1, DFd = 28, F = 6.248, $P = 0.0186$ for treatment; DFn = 15, DFd = 301, F = 8.359, $P < 0.0001$ for time/genotype; DFn = 15, DFd = 301, F = 1.328, $P = 0.1838$ for treatment × time/genotype. (**C**) Rotarod activity of wild-type and SBMA mice with or without arginine administration. The latency to fall from the accelerating rotarod is shown. Data are shown as the mean ± SEM. *$P < 0.05$. (**D**) Light microscopy images (*left*) and the percentage of neuronal cells with inclusion bodies (*right*) in the spinal cord of SBMA mice. Scale bar = 100 μm. Lines represent the mean ± SEM. (**E**) Western blot analysis (*top*) and relative mutant AR protein levels (*bottom*) in the spinal cords of SBMA mice with or without 6% arginine treatment. The brains were obtained at 12 weeks of age. Lines represent the mean ± SEM. The lower band in the AR blot (asterisk) corresponds to the endogenous AR protein.

Previous structural and functional studies of aggregation-prone proteins have suggested that it is the misfolded and oligomeric soluble protein species rather than the insoluble aggregates that exhibit toxicity (Williams and Paulson, 2008). We have shown in various *in vitro* and *in vivo* polyQ disease model systems that arginine inhibits β-sheet conformation transition (Fig. 2A), oligomer formation (Fig. 2C and D), aggregate formation (Fig. 1A–C) and seed-induced aggregation (Fig. 1D) of expanded polyQ proteins. These data indicate that arginine interferes with the early step of

aggregate formation, during which proteins exhibit neuronal toxicity.

We observed a tendency of improved dendritic arborization of the Purkinje cells in the cerebellum of SCA1 mice with arginine administration (Supplementary Fig. 3), although no neuronal inclusions were detected in the cerebellum (data not shown), consistent with the previous study (Watase *et al.*, 2002). The effect of arginine on dendritic arborization was not drastic and not quantifiable by standard methods, possibly because SCA1 mice show only a mild reduction in the dendritic arborization at baseline (Watase *et al.*, 2002). The mechanism underlying the improvement of dendritic arborization in the Purkinje cells without detectable inclusions may be the reduction of the soluble but toxic polyQ protein species in the Purkinje cells by arginine, or through effects of arginine or its metabolites on various pathways discussed below (Koga *et al.*, 2002; Morris, 2016; Xu *et al.*, 2018).

We also showed in this study that arginine is effective when administered either at the pre-symptomatic (Figs 4B, D–F, 5A and B) or the symptomatic stage (Fig. 5C). One of the critical issues in translational research is that interventions in preclinical studies are often initiated prophylactically during the pre-symptomatic stage, leading to insufficient analyses of their therapeutic efficacies after symptom onset (Katsuno *et al.*, 2012). Our data show that arginine would benefit patients at both the pre-symptomatic and symptomatic stages. Arginine administration at the symptomatic stage resembles the actual clinical setting where most patients seek medical support after the onset of neurological symptoms. However, we cannot exclude the possibility that arginine exhibited symptomatic relief instead of disease modification when administered after symptom onset (Fig. 5C). On the other hand, prophylactic treatment with arginine would benefit patients who underwent pre-symptomatic diagnosis. Arginine would also increase the benefit of predictive genetic testing for polyQ diseases because it enables early intervention.

We identified arginine through a screen for low molecular chemical compounds that inhibit aggregation of expanded polyQ proteins. This has been an attractive approach for the development of disease-modifying therapies for polyQ diseases and indeed led to the identification of various compounds, including benzothiazole derivatives (Heiser *et al.*, 2002; Hockly *et al.*, 2006), C2-8 (Zhang *et al.*, 2005; Chopra *et al.*, 2007), and (–)-epigallocatechin-3-gallate (Ehrnhoefer *et al.*, 2006, 2008) as anti-aggregation compounds. Most of these compounds have limited direct clinical application (Hockly *et al.*, 2006; Frid *et al.*, 2007); therefore, we focused on chemical chaperones, a subset of low molecular weight compounds, for the initial screening.

Among the anti-aggregation compounds, chemical chaperones exhibit anti-aggregation properties by stabilizing proteins in their native conformation and influencing the rate or fidelity of the protein-folding reaction (Welch and Brown, 1996). Trehalose (Tanaka *et al.*, 2004), proline (Ignatova and Gierasch, 2006) and cyclohexanol (McLaurin *et al.*, 2006) are chemical chaperones that suppress protein aggregation, including for polyQ proteins. Arginine is a chemical chaperone and is one of the most commonly used additives to prevent aggregation and increase the solubility of various recombinant proteins expressed in *Escherichia coli* (Arakawa and Tsumoto, 2003). This non-specific anti-aggregation effect of arginine indicates the possibility that arginine could be utilized for the treatment of other protein misfolding diseases, including Alzheimer's disease and Parkinson's disease. Indeed, recent studies have shown that arginine prevents aggregation of amyloid-$\beta_{1\text{-}42}$ or α-synuclein *in vitro* (Das *et al.*, 2007), which are major components of amyloid plaques in Alzheimer's disease or Lewy bodies in Parkinson's disease, and shown to exert neurotoxicity via oligomer formation (Benilova *et al.*, 2012). Furthermore, arginine may exert additional neuroprotective effects through an increased microcirculation in the cerebral blood flow via production of nitric oxide (Koga *et al.*, 2002) or other mechanisms including neuroinflammation, oxidative stress, neurotransmission, or neuromodulation via various metabolites of arginine (Morris, 2016; Xu *et al.*, 2018).

While these effects of arginine were sufficient to achieve significant improvement of behavioural and pathological disease phenotypes in polyQ disease models, arginine did not completely suppress the progression of disease phenotypes. This may be because a small amount of aggregated polyQ proteins could gradually accumulate in polyQ disease mice during the disease course even under arginine administration, considering that the effect of arginine in suppressing polyQ protein aggregation is not complete and that polyQ proteins are continuously produced *in vivo* during the entire disease course. Another possibility is that arginine did not affect pathogenic pathways other than polyQ protein aggregation, which could underlie polyQ disease progression. For example, the physiological function of causative genes/proteins of each polyQ disease could be altered by abnormal CAG/glutamine repeat expansion (Ashizawa *et al.*, 2018). RNA transcripts from the expanded CAG repeat might also exhibit neurotoxicity (Li *et al.*, 2008). In addition, protein products translated by repeat-associated non-ATG translation from expanded CAG repeat exhibit neurotoxicity in polyQ diseases (Cleary and Ranum, 2017). Various pathogenic pathways in addition to polyQ protein aggregation, including those described above, may need to be targeted to achieve a complete inhibition of polyQ disease progression.

Although numerous candidate compounds for disease-modifying therapy of neurodegenerative diseases have been identified through *in vitro* and *in vivo* studies, most compounds have failed to demonstrate their effects in clinical trials. One of the critical issues in the translational research of these therapeutic candidates is the lack of clinical and biological outcome measures that are sensitive enough to detect alteration of the disease course during the relatively short period of clinical trials and that are resistant to inter- and intra-rater variability and placebo effects (Katsuno *et al.*, 2012). We recently

developed a novel quantification method for sensitive and accurate assessment of ataxia with low inter- and intra-rater variability (Tada *et al.*, 2014; Nagai *et al.*, 2017). Using this and other well-established ataxia rating scales, we are currently planning a clinical trial to evaluate the efficacy and safety of long-term oral arginine administration in polyQ disease patients in Japan, which we hope will pave the way for arginine administration as a disease-modifying therapy for polyQ disease patients worldwide.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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