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Brief communication

BEHAVIOURAL DEFICITS IN TRANSGENIC MICE EXPRESSING HUMAN TRUNCATED (1-120 AMINO ACID) ALPHA-SYNUCLEIN

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ABSTRACT

Accumulation and aggregation of alpha-synuclein in cortical and hippocampal areas is a pathological sign for dementia with Lewy bodies (DLB) and Parkinson's disease with dementia. However the mechanisms of alpha-synuclein triggered cellular dysfunction leading to the development of memory impairment is not clear. We have created a mouse model of DLB, where aggregation-prone human truncated (120 amino acid) alpha-synuclein is expressed in forebrain areas under the calcium/calmodulin-dependent protein kinase II alpha (CamKII-alpha) promoter. We have observed the presence of the transgenic protein in target forebrain areas, with small granular cytoplasmic accumulation of aggregated alpha-synuclein. This was associated with a progressive deficit in cortical-hippocampal memory tests including the Barnes Maze and Novel Object Recognition. This data suggests that low levels of aggregation prone alpha-synuclein are sufficient to induce memory deficits in mice and that forebrain regions associated with cognitive function may have an increased sensitivity to the truncated toxic form of alpha-synuclein.

INTRODUCTION

Dementia is an increasing burden in society as the proportion of elderly individuals is becoming larger, affecting both patients and their caregivers with an estimated cost of up to £23 billion per year in the UK alone (Dementia 2010 report, Alzheimer Research UK). It is a chronic syndrome generally characterised by a progressive decline in cognitive function and leads to a disruption of daily activities and quality of life.

The second most frequent dementia after Alzheimer disease (AD) is dementia with Lewy bodies (DLB), which accounts for 15-30% of all dementias. DLB occurs at the same age as AD, which sets it apart from Parkinson's disease with dementia (PDD). The onset of motor parkinsonian syndrome aids in clinical differentiation between these two dementias – with the occurrence of dementia at least one year earlier than motor syndrome in DLB (McKeith *et al.* 2005). Similarly, PDD is very common – it is considered that up to 80% of patients with advanced PD will suffer from various degrees of dementia (Hely *et al.* 2008).

Neuropathologically, there is an accumulation of aggregated alpha-synuclein as Lewy bodies (LB) in the frontal and parietal cortex, parahippocampal gyrus, cingulate gyrus and insula (Irwin *et al.* 2012), in addition to the aggregation of alpha-synuclein in the basal nucleus of Meynert and in the diencephalon. Alpha-synuclein aggregation as Lewy bodies (LB) or neurites in cortical areas is a pathological manifestation for dementia in both PD and DLB, however the time-course of the protein aggregation and corresponding neurochemical correlates are not clearly understood.

Alpha-synuclein is a ubiquitous presynaptic protein of 140 amino acid residues. While point-mutations or multiplication of the alpha-synuclein gene result in autosomal-dominant PD (Singleton *et al.* 2013), recent genome wide association studies showed clear association

between alpha-synuclein and idiopathic PD (Satake *et al.* 2009; Simon-Sanchez *et al.* 2009). The function of native alpha-synuclein protein is not exactly clear, however it has been shown that alpha-synuclein regulates neurotransmitter-filled vesicle transport to the release zone and assembly of presynaptic protein complexes (Kahle *et al.* 2000; Burre *et al.* 2010). Alpha-synuclein can aggregate to form insoluble intracellular filamentous inclusions – LB or Lewy neurites (Spillantini *et al.* 1997), which has become the defining pathological signs of PD and DLB. Events leading to the aggregation of alpha-synuclein are not well understood, however modification and truncation of the C-terminus of alpha-synuclein was shown to induce its aggregation in several models. Moreover, carboxyl terminus truncation of human alpha-synuclein was identified in the human DLB brain, and this truncation increases propensity of alpha-synuclein to aggregate *in vitro* (Tofaris *et al.* 2003; Crowther *et al.* 1998).

We have previously reported that overexpression of 1-120 amino acid truncated alpha-synuclein within catecholaminergic cells produces aggregates of protein within the olfactory bulbs and striatum, accompanied with stimulated dopamine release deficit, presynaptic proteins re-distribution (Garcia-Reitbock *et al.* 2010) and a LTP deficit in the hippocampus (Costa *et al.* 2012). We have asked now whether presence of human truncated alpha-synuclein within forebrain structures will be sufficient to induce deficits in mouse behaviour, and whether this deficit will be associated with alpha-synuclein aggregation. We report here the generation of a novel mouse transgenic line (CamS), where expression of human truncated 1-120 amino acid alpha-synuclein is driven by the calcium/calmodulin-dependent protein kinase II alpha (CamKII-alpha) promoter, which resulted in alpha-synuclein accumulation in forebrain neurons leading to measurable alteration in memory-related tests.

MATERIALS AND METHODS

Animals

Wild type mice used in this study were purchased from Jackson Laboratories, USA (B6CBAF1/J), Harlan Laboratories, UK (C57Bl/6 HsdOla) and Charles River Laboratories, UK (C57Bl/6J). Transgenic mice were generated in the Centre of Biological Sciences Transgenic Unit of the University of Cambridge (UK). All animals were kept in a temperature and humidity-controlled (22 °C) room on a 12-hour light/dark cycle with water and food *ad libitum*. All the experimental procedures were performed in strict accordance with appropriate Home Office project and personal licences and approved by the ethical committee of the University of Cambridge (UK).

Generation of CamS transgenic mouse line

pcDNA4/HisMax-TOPO plasmid containing CamKII 1.3 alpha promoter was kindly provided by Dr. Lynn Bedford (University of Nottingham, United Kingdom). Human truncated (120-amino acid) alpha-synuclein cDNA with flanking NotI restriction sites and Kozak consensus sequence was generated by PCR and subcloned at the end of the promoter sequence. The nucleotide sequence of the promoter and subcloned transgene was verified by the direct sequencing of the construct. Approximately 4.7 kb of DNA containing CamKII-alpha promoter, 1-120 truncated alpha-synuclein and SV40 poly-A sequence were excised from the plasmid using Bst11071 and XhoI restriction enzymes and 30 ng/μl of DNA was injected into the pronucleus of oocytes in of B6CBAF1/J mice (Jackson Laboratories, USA).

Six founders were identified using conventional PCR genotyping and were crossed with C57Bl/6HsdOla mice (C57Bl/6S, Harlan Laboratories, UK) which have a spontaneous chromosomal deletion of the alpha-synuclein locus (Specht and Schoepfer 2004; Specht and

Schoepfer 2001), to detect the transmission of the transgene protein to the progeny by Western Blot. At least 7 subsequent crosses of CamS mice with C57Bl/6S mice were performed to generate a line of CamS mice expressing human transgenic alpha-synuclein on the endogenous alpha-synuclein free background.

PCR and RT-PCR

For the routine genotyping, total genomic DNA was isolated by the incubation of mouse ear biopsies with 0.4 mg/ml Protease K (Sigma Aldrich, UK) in a buffer containing 0.05M KCl, 0.01M Tris-HCl pH 9.0, 0.1% Triton X100 at +55 ^{0}C for 2 h, followed by +95 ^{0}C for 30 min. The sample was then centrifuged at 13,000 rpm for 1 min at room temperature (RT), and 1 µl of DNA was used for a conventional PCR with a forward primer covering a part of the CamKII alpha promoter sequence (5'-GGC ACA CTG CAA ACT GAA AC-3') and the reverse primer targeting exon 4-5 border of human alpha-synuclein cDNA (5'-GGC TCC TTC TTC ATT CTT GCC C-3').

To semi-quantitatively evaluate the levels of transgenic alpha-synuclein mRNA, brain areas of transgene expression (olfactory bulbs, hippocampus, frontal cortex) were dissected from fresh mouse brains at 1, 3, 6 and 11 months of age. RNA was isolated using an RNeasy mini kit (Qiagen, UK) and the levels of the transgenic alpha-synuclein mRNA were assessed using a OneStep RT-PCR kit (Qiagen, UK) with the forward primer 5'-TGG ATG TGT TCA TGA AAG GAC T-3' and the reverse primer targeting exon 4-5 border of human alpha-synuclein cDNA 5'-GGC TCC TTC TTC ATT CTT GCC C-3'. The resulting reaction was run on 0.8% agarose gel with ethidium bromide in TBE buffer and visualised in UV light.

Immunohistochemistry

Mice aged 1, 3, 6 and 11 months were deeply anaesthetized and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4. Brains were removed and post-fixed overnight, followed by immersion in 30% sucrose in PBS containing 0.02% sodium azide. Serial 30 μm sections were cut on a freezing microtome (Leica) and stored at +4 ^{0}C in PBS/sodium azide until processed further. Free-floating sections were quenched in 3% hydrogen peroxide, 20% methanol in PBS for at least 60 min and blocked in 5% normal goat serum PBS with 0.03% Triton X100 (PBS-T) for 1 hour at room temperature. Sections were then incubated with the primary antibody in PBS-T overnight at 4°C. This was followed by incubation with biotinylated secondary antibodies and the staining was visualised using the ABC Elite Kit, with 3,3'-diaminobenzidine (all from Vector Laboratories, USA) as the chromogen. Sections were then dehydrated and analysed using a Leitz DMRB microscope.

The following antibodies were used: mouse anti-human specific Syn204 (Abcam, Cambridge, UK) 1:250, mouse anti-CamKII alpha (Novus Biologicals, Cambridge, UK) 1:1000, mouse anti-alpha-synuclein Syn-1 (BD Transduction laboratories, USA) 1:1000, and mouse anti-beta actin (Sigma Aldrich, UK) 1:7000.

Western blotting

For routine PAGE, tissues were dissected and homogenised in RIPA buffer (Sigma-Aldrich) containing protease inhibitors (Roche). Protein concentrations were determined using the BCA Protein Assay Kit (Pierce). Equal amounts of protein were run on 14% SDS-PAGE and transferred onto Immobilion P (Millipore). Membranes were briefly washed with Tris-buffered saline with 0.02% Tween 20 (TTBS), blocked in 5% milk TTBS and then incubated with the primary antibody overnight at 4°C. They were subsequently incubated with HRP-

conjugated polyclonal goat anti-mouse or goat anti-rabbit antibody (GE Healthcare, at 1:10,000) in 1% milk TTBS. Samples were developed using ECL western blotting substrate (Pierce).

Buried food olfactory test

We have used the protocol described by Yang and Crawley (Yang and Crawley 2009) with minor modifications. Mice were examined at 1 and 4 months of age (n=5 per group). Mice were habituated to individual cages for 2 consecutive days, where they were allowed to explore the cage for 30 min before being returned to their home cages. Food access was removed from their home cages the evening before the test, whereas water was provided *ad libitum*. For the test, mice were placed into their individual habituation cages and left to settle for 30 min. After this, a piece of commercially available Coco-Pop pellet was hidden approximately 1 cm below the bedding in random locations on the opposite side of the cage, and the time spent to find the food pellet was recorded. The timer was stopped when the food pellet was held by both their front paws and/or when the mouse started eating it.

Spontaneous Novel Object Recognition test

We have followed the protocol described earlier (Winters *et al.* 2004) with minor modifications for mice. Mice were examined at 1 month (n=8 per group), 3 months and 6 months of age (CamS n=5, wt n=6). Briefly, animals were split from their home cage into single cages approximately 30 min prior to the habituation or test. For habituation, mice were left to spontaneously explore an empty Y-maze for 5 min for two days prior to the test. The novel object recognition test consisted of two phases: a sample and choice phase. During the sample phase mice were placed carefully into the long arm of the Y-maze and left to explore two identical objects placed into the short arms of the Y-maze for 5 min, after

which they were returned to their single cages for 1 hour. After the 1 hour delay, during the choice phase, mice were returned to the Y-maze and left to explore a copy of one previously seen (familiar) object and one novel object. A video recording of both phases was made. A discrimination index (D.I.) was calculated according to the equation $(Tn-Tf)/(Tn+Tf)$; where Tn was the time spent exploring the novel object and Tf was the time spent exploring the familiar object, both during the 5 min choice phase. In addition, the total time examining left and right identical objects during the sample phase was calculated to examine any side bias. We have considered sniffing, biting, touching with front paws but not climbing on the top of the object as explorative behaviour.

Barnes maze

We followed an adapted Barnes Maze protocol (Barnes 1979) based on that published by Sunyer *et al.* 2007 to examine hippocampal-dependant spatial reference learning and memory. Mice were examined at 9 months of age (n=4 mice per group). Prior to daily testing, mice were placed into individual cages 30 minutes prior to the start of the experiment and remained in these cages between trials. Mice received 5 consecutive days of testing consisting of 6 trials per day, with an inter-trial interval (ITI) of approximately 15 minutes. Prior to the very first testing trial, mice received 3 training trials whereby they were placed in the centre of the maze in a start tube and released after 10 seconds. After 2 hole-explorations, mice were gently guided to the escape hole and left in the recessed chamber for 90 seconds. During each test trial, mice were placed in the centre of the maze and left to spontaneously explore for a maximum of 3 minutes (or until they found the escape hole). Mice were guided to the escape hole if not located within the 3 minutes. Mice then remained in the recessed chamber for 1 minute. Over the course of trials, mice use the

extra-maze environment to cue the target location and navigate themselves to the target hole. The number of errors (the number of visits to any of the 19 non-escape holes) and the latency (time taken to locate the single target escape hole) was measured for each trial. Following the test, mice received a single probe trial 24 hours after the last test trial to assess memory retention of the escape hole. During the probe trial, mice received one 90-second trial which was identical to training trials except the target hole was now closed, appearing identical to the other 19 holes of the maze. After 90 seconds had elapsed, mice were removed from the maze. The latency to visit the escape hole location was recorded.

A number of experimental factors were counterbalanced including the running order of groups, the direction of release from the start tube (allocated randomly) and the location of the target hole. The target hole location and extra-maze spatial cues remained consistent for each mouse throughout testing. Between trials, the maze (including all holes and the escape chamber) were wiped down with water-soaked paper towel and dried to ensure no odour cues would assist performance.

RESULTS

Pronuclear injection of linearized transgene construct, consisting of CamKII alpha promoter and 120 amino acid truncated human alpha-synuclein cDNA, resulted in six positive founders. Three of which were shown to transmit transgene to the progeny, detected by PCR and western blotting (data not shown). These three founders were consecutively crossed with endogenous alpha-synuclein free C57Bl/6S mice for at least 7 generations, and the lack of endogenous alpha-synuclein was verified by both PCR and western blotting.

Line 3 showed lowest levels of transgene mRNA, detected by RT-PCR (Figure 1A), and was not used for experiments. Line 2 was bred to homozygous status, verified by test breeding

with wild type C57Bl/6S mice. RT-PCR evaluation of mRNA levels did not show age-related changes in the homozygous line (Figure 1B). However, levels of monomeric alpha-synuclein were highest at 11 months of age, showing additional higher molecular weight bands of aggregated protein already at 1 month (Figure 1C) in the olfactory bulbs of line 2 mice. As CamS mice do not have endogenous murine alpha-synuclein after consecutive breeding into C57Bl/6S mice, we have used a corresponding age of wild type C57Bl/6J (Charles River, UK) mouse brain to assess the levels of transgene protein. Our transgenic mice have expressed lower levels of transgenic protein then endogenous alpha-synuclein in the brain of age-matched wild type mouse (Figure 1F).

Transgenic protein was distributed in areas corresponding to the sites of CamKII promoter activity; with hippocampus, olfactory bulbs, striatum and prefrontal areas stained most abundantly (Figure 1D-E). In the hippocampus, the CA3-2 area displayed prominent staining, where cells showed small granular accumulation of the transgenic protein (Figure 1G) without obvious cellular degeneration (data not shown). A similar degree and pattern of staining was observed in frontal and parietal cortical areas, where the transgenic protein was present in layer 2-3 of the cortex and cells displayed granular accumulation of alpha-synuclein (perirhinal cortex in Figure 1H).

As transgenic protein was expressed in olfactory bulbs, detected by both mRNA and protein levels, we have asked if this will manifest in the alteration of olfactory sensitivity in CamS mice. The buried food test showed a slight but significant delay in the time spent to locate the food pellet at 1 month of age, however this was not present at 3 or 4 months of age (Figure 1I).

Pathologically, DLB is manifested with accumulation and aggregation of alpha-synuclein in hippocampal and cortical areas, however it is not clear whether this accumulation will be a triggering event for the development of memory impairment and dementia. We have observed the accumulation of the transgenic protein in similar areas of the CamS brain and have asked if this will result in behavioural and cognitive deficits in our mice. The novel object recognition (NOR) test assesses memory formation and retention related to perirhinal cortex function (Winters *et al.* 2004). We did not observe any difference between the control and transgenic mice at 1 month of age, where both groups spent more time exploring the novel object. At 3 months of age however, the transgenic mice spent significantly less time exploring the novel object, which was even more pronounced at 6 months of age. In contrast, the ability for control mice to discriminate between novel and familiar objects remained intact across all time points (Figure 1J).

We explored the memory deficit in our mice further, employing a test sensitive in detecting deficits in hippocampal-related learning and memory (the Barnes maze). At 9 months of age, we did not observe any difference between the control and transgenic mice in their latency to find the target hole across the 5 days of testing, however the transgenic mice showed an increased number of errors at days 3 and 4 of the experiment suggesting a spatial learning impairment. In addition to this, transgenic mice showed a significant deficit in their memory for the correct location of the target escape hole in the probe trial of the experiment (Figure 1K-L).

DISCUSSION

DLB is a second most prevalent dementia disorder, and there is an increasing need in developing a simple suitable model to represent crucial pathological pathways of this disease. While aggregation of alpha-synuclein is significant and pathologically relevant in post-mortem human tissues, it is not clear whether it is a trigger ensuring development of memory impairment and neurodegeneration, or a secondary mechanism.

Several animal models have been suggested to recapitulate aspects of human alpha-synuclein accumulation present in human disease. Pan-neuronal murine Thy-1 promoter driven expression of full length alpha-synuclein on C57Bl/6 x DBA2 background has produced accumulation of alpha-synuclein in various regions, including the substantia nigra and locus coeruleus, which resulted in a decrease of cortical norepinephrine and acetylcholine, as well as some deficits in NOR and reversal learning (Rockenstein *et al.* 2002;Magen and Chesselet 2010). Two other models employed targeted inducible overexpression of full length alpha-synuclein within forebrain tissues using CamKII alpha promoter, where accumulation and aggregation of the transgenic protein caused a deficit in Morris water maze performance (Nuber *et al.* 2008) and a decrease in fear-related memory (Lim *et al.* 2011).

Our mouse model demonstrates that forebrain-specific expression of lower than endogenous levels of human truncated alpha-synuclein is sufficient to induce clear morphological signs of protein aggregation and memory deficits recapitulating hippocampal and cortical pathology observed in human DLB. This suggests that accumulation and aggregation of even small amounts of human truncated alpha-synuclein could be detrimental for the pathways ensuring acquisition and retention of memory, further

strengthening the notion that early aggregation of alpha-synuclein is crucial in pathogenesis of alpha-synucleinopathies.

An accumulation of human truncated alpha-synuclein within forebrain areas resulted in an early and transient deficit in the buried food olfactory test, suggesting altered olfactory sensitivity in CamS mice. A deficit in odour identification is common in early dementia (McLaughlin and Westervelt 2008) and PD (Damholdt *et al.* 2011), and has been positively linked with a conversion from mild cognitive impairment to dementia in AD (Conti *et al.* 2013). Changes in olfactory sensitivity has also been reported in rodent models of AD (Wu *et al.* 2013) and PD (Nuber *et al.* 2013). Although the results indicate an olfactory deficit consistent with the literature, we cannot rule out the possible contribution of a motor problem or motivation changes to find the food pellet in this task. Further examination is therefore required to fully interpret these results, and to understand the transient nature of this deficit.

An ability to recall and recognise a familiar visual stimulus is central in animal models of dementia (reviewed in (Antunes and Biala 2012). We have used a modified approach to test spontaneous novel object recognition with a Y-shaped maze to limit the influence of spatial factors (Winters *et al.* 2004). It has been shown this modification of the NOR test relies primarily on an intact perirhinal cortex, but not hippocampus, at least in rats (Winters *et al.* 2004; Forwood *et al.* 2005). When assessing CamS mice in the NOR test, a clear progressive decrease in spontaneous novel object recognition in transgenic mice starting at 3 months of age was detected, which together with an accumulation of alpha-synuclein within the target areas (temporal cortex, perirhinal cortex and hippocampus), indicate that low amounts of

aggregation-prone alpha-synuclein in these regions can result in alterations of memory processes.

These findings were further verified in an additional test exploring hippocampal spatial memory acquisition and retention – the Barnes maze. It is conceptually similar to the Morris water maze, as both require finding a hidden goal on the basis of distal visual stimuli. While they differ with respect to motivational and sensorimotor factors, effective learning of either task requires an intact hippocampus (Sutherland *et al.* 1982; McNaughton *et al.* 1989). The Barnes maze does not involve swimming and is therefore considered to be less anxiogenic than the Morris water maze (Pompl *et al.* 1999) and it should be less affected by motoric deficits, which can both bias the results. Our results indicated that while CamS transgenic mice perform as well as control mice by day 5 of the test, they tend to make more errors during the process of learning. More importantly, the spatial memory retention seems to be impaired in CamS mice, as they make more errors in trying to find the escape box during the probe trial, 24 hours after the last training session.

The behavioural test data together with morphological signs for alpha-synuclein aggregation in this novel mouse model (CamS mice) for forebrain alpha-synuclein aggregation, confirms that even small amounts of aggregation-prone alpha-synuclein present within memory-related areas of the brain is sufficient to induce memory impairment. This further strengthens the idea that alpha-synuclein dis-aggregation strategies may be beneficial in slowing down the pathological cascade in human alpha-synucleinopathies and suggests that memory-related forebrain areas of the brain may have an increased sensitivity to toxic forms of alpha-synuclein.

Figure legend

(A) Levels of the transgenic alpha-synuclein mRNA in the olfactory bulbs of three lines of CamS mice as detected by RT-PCR. M – size marker, +ve – mRNA from the olfactory bulb of α-syn (1-120) transgenic mouse (Tofaris *et al.* 2006). (B) Levels of the transgenic alpha-synuclein mRNA in olfactory bulbs of the CamS 2nd line at 1, 2, 6 and 11 months of age. M – size marker, +ve – mRNA from the olfactory bulb of α-syn (1-120) transgenic mouse. (C) Transgenic alpha-synuclein shows accumulation of monomeric protein at 11 months of age (arrow), as well as the presence of higher molecular weight bands indicative of an aggregation of the protein, as detected by human specific alpha-synuclein Syn204 antibodies. (F) Levels of alpha-synuclein in the olfactory bulbs (OB) and hippocampus (hip) of a 6 month old CamS mouse and wild type C57Bl/6J mouse (C/R). Transgenic alpha-synuclein, detected by alpha-synuclein Syn1 antibody, is present in the brain of a 6 month old mouse (E) in the areas corresponding to the CamKII-alpha protein distribution (D). It forms small cytoplasmic granules (arrowed) in the CA2-3 region of the hippocampus (G) and in the perirhinal cortex (H) of a 6 month old CamS brain. (I) The buried food olfactory test reveals a transient deficit in CamS mice at 1 month of age. * = 0.05 by the Student t-test, n=5 per group. (J) Spontaneous novel object recognition test shows a decrease in the Discrimination Index (D.I., as specified in Materials and methods) in CamS mice at 3 and 6

months of age. ** = 0.01 the Student t-test, n=5-8 per group. (K) The Barnes maze test shows an increased number of errors made by 9 month old CamS mice on day 3 and 4. * = 0.05, ** = 0.01 by two-way ANOVA, n=4 per group. (L) The Barnes Maze probe trial results show a decrease in the memory retention of 9 month old CamS mice 24 hours after the end of testing. ** = 0.01 by two-way ANOVA, n=4 per group.

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