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**Impaired fast network oscillations and mitochondrial dysfunction in a mouse model of
alpha-synucleinopathy (A30P)**

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Highlights

Age-dependent impairment in hippocampal gamma activity in a mouse model of alpha-synucleinopathy.

Hippocampal slices from mice over-expressing alpha-synuclein show greater sensitivity of gamma oscillations to inhibition of mitochondrial function.

Age-dependent impairment in mitochondrial function in a mouse model of alpha-synucleinopathy.

Keywords

Hippocampus

Gamma oscillations

Alpha-synuclein

Mitochondria

Rotenone

Abbreviations

α -syn	alpha-synuclein
ACSF	artificial cerebrospinal fluid
AD	Alzheimer's disease
ANOVA	analysis of variance
CA1/CA3	cornu amonis 1/3
COX	cytochrome c oxidase
DG	dentate gyrus
DLB	dementia with Lewy bodies
DAB	3,3'-diaminobenzidine
EEG	electroencephalogram
EPSCs	excitatory post-synaptic potentials
Hz	Hertz
IQR	interquartile range
KCN	potassium cyanide
LTP	long-term potentiation
mtDNA	mitochondrial DNA
NBT	nitroblue tetrazolium
NMDA	N-methyl-D-aspartate
PBS	phosphate buffered saline
PD	Parkinson's disease
PDD	Parkinson's disease dementia
PMS	phenazine methosulfate
PV	parvalbumin
RGB	Red, green, blue
r.m.p.	resting membrane potential
SDH	succinate dehydrogenase
s.e.m.	standard error of the mean

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Abstract

Intracellular accumulation of alpha-synuclein (α -syn) is a key pathological process evident in Lewy body dementias (LBD), including Parkinson's disease dementia (PDD) and dementia with Lewy bodies (DLB). LBD results in marked cognitive impairments and changes in cortical networks. To assess the impact of abnormal α -syn expression on cortical network oscillations relevant to cognitive function, we studied changes in fast beta/gamma network oscillations in the hippocampus in a mouse line that over-expresses human mutant α -syn (A30P). We found an age-dependent reduction in the power of the gamma (20-80 Hz) frequency oscillations in slices taken from mice aged 9-16 months (9+), that was not present in either young 2-6 months old (2+) A30P mice, or in control mice at either age. The mitochondrial blockers potassium cyanide and rotenone both reduced network oscillations in a concentration-dependent manner in aged A30P mice and aged control mice but slices from A30P mice showed a greater reduction in the oscillations. Histochemical analysis showed an age-dependent reduction in cytochrome c oxidase (COX) activity, suggesting a mitochondrial dysfunction in the 9+A30P group. A deficit in COX IV expression was confirmed by immunohistochemistry. Overall, our data demonstrate an age-dependent impairment in mitochondrial function and gamma frequency activity associated with the abnormal expression of α -syn. These findings provide mechanistic insights into the consequences of over-expression of α -syn which might contribute to cognitive decline.

Introduction

Abnormal intraneuronal deposition of the synaptic protein alpha-synuclein (α -syn) occurs in several neurodegenerative diseases, collectively referred to as the synucleinopathies, which include conditions such as Parkinson's disease (PD), Parkinson's disease dementia (PDD), dementia with Lewy bodies (DLB), and a subgroup of patients with Alzheimer's disease (AD). α -syn is a small, 140 amino-acid, soluble protein enriched in the presynaptic terminal of neurons where it plays a role in vesicular trafficking, neurotransmitter release and synaptic maintenance (Cabin et al., 2002; Nemani et al., 2010; Scott et al., 2010). Excess misfolded α -syn forms deposits called Lewy bodies, the pathological hallmark of the synucleinopathies, which are present throughout the brain including within the hippocampus and neocortex (Spillantini et al., 1998; Lassen et al., 2016).

Cognitive dysfunction is a key feature of both PD dementia (PDD) and DLB, which together, are the second most common cause of dementia in the elderly after AD. Both PDD and DLB are associated with progressive cognitive impairment including memory loss, Parkinsonism, attention deficits and complex visual hallucinations (McKeith et al., 2005). The link between abnormal α -syn expression and cognitive impairment is still unclear, but changes in network oscillations may underlie many of the cognitive deficits seen in neurodegenerative diseases (Nimmrich et al., 2015). Both AD and DLB patients have a general slowing of the electroencephalogram (EEG) activity, particularly from alpha- (8-12 Hz) frequency oscillations to slower theta- (4-8 Hz), and delta- (1-4 Hz) oscillations (Bonnani et al., 2008; Morris et al., 2015; Bonnani et al., 2016). The majority of the clinical EEG studies in patients have focused on lower frequency oscillations in the theta 4-8 Hz and alpha 8-12 Hz bands (e.g. Bonnani et al., 2016). However, fast network oscillations in the beta (20-30 Hz) and gamma (30-80 Hz) frequency ranges are also important for cognitive tasks including attention (Clayton et al., 2015), and memory functions (Watrous et al., 2015; Zheng et al., 2016). Several studies have

reported impaired gamma activity in patients with AD (Herrmann and Demiralp 2005; Basar et al., 2016).

Missense mutations, including (A30P) and (A53T), in the human α -syn (SNCA) gene have been identified that are linked to early onset familial Parkinson's disease (Polymeropoulos et al., 1997; Kruger et al., 1998). Several transgenic mouse lines are available that over-express either human or mutant α -syn (Kahle et al., 2008; Hatami and Chesselet 2015). To assess the impact of abnormal α -syn on cortical network oscillations we have used the A30P mouse line that over-expresses human mutant α -syn under the control of the CNS promoter Thy-1 (Khale et al., 2000; Neumann et al., 2002). Previous studies have confirmed widespread expression of human mutant α -syn throughout the mouse brain, including the hippocampus and neocortex, with cognitive deficits evident at ~12 months of age and motor deficits from ~14 months (Kahle et al., 2000; Freishel et al., 2007; Schell et al., 2009). One recent study, using an alternative A53T α -syn transgenic mouse line, reported a general slowing of the EEG in the theta and alpha bands (Morris et al., 2015), consistent with patient data, but the impact of excess α -syn expression on the generation of beta and gamma frequency network oscillations is unknown.

Mitochondrial dysfunction is also a key feature of many neurodegenerative diseases such as PD and AD (Schon and Przedborski 2011; Spano et al., 2015). Previous studies have shown that acute blockade of complex I of mitochondria in slices from normal animals impairs the generation of fast network oscillations (Kann et al., 2011; Whittaker et al., 2011). Excess α -syn can translocate to the mitochondrial inner membranes and inhibit function (Nakamura et al., 2011), but whether there is a link between excess α -syn, impaired mitochondrial function, and network oscillations remains to be determined.

In this study we wanted to determine whether α -syn over-expression was associated with changes in gamma network oscillations and mitochondrial function. We recorded hippocampal gamma frequency activity *ex vivo* in A30P mice aged between 2 and 16 months and compared results with age-matched control C57BL6 mice. We found significant impairments in the power of the gamma frequency activity *ex vivo* in A30P mice aged >9+ months, but not at 2-6 months of age, or in age-matched control mice. Application of the known inhibitors of mitochondrial function (KCN and rotenone) reduced the power of network oscillations in older C57BL6 mice and A30P mice but there was a trend for a greater reduction in the A30P mice. The deficits in network oscillations were associated with a reduced mitochondrial function as indicated by changes in COX/SDH activity in slices only in the older 9+A30P mice. Our data suggest that excess human mutant α -syn expression in mice causes abnormal hippocampal gamma frequency oscillations and impaired mitochondrial function that could contribute to cognitive decline.

Methods

Transgenic mice

The A30P mice (male and female) were bred in house from breeding pairs supplied by Dr. P Kahle, University of Tübingen. Transgenic mice of C57BL6 background expressed human mutant A30P α -syn under the control of the CNS specific promoter Thy-1 (Kahle et al., 2000; Neumann et al., 2002). The transgenic A30P α -syn were maintained as a homozygous colony and aged (2-16 months) in the animal facility. Control mice (male and female) were age-matched C57BL6 supplied from Charles River and also aged in house. Mice were genotyped after transport and breeding to ensure homozygous expression of A30P α -syn in the mouse colony. Animals were housed according to ARRIVE guidelines and were maintained on a 12-hour dark/light cycle with lights on at 7.00 am. For most analyses A30P mice were divided into two age groups referred to as 2+A30P (2-6 months) and 9+ A30P (9-16 months) but see Results.

Slice preparation and solutions

All mice were anaesthetised with inhaled isoflurane prior to intramuscular injection of ketamine ($\geq 100 \text{ mg kg}^{-1}$) and xylazine ($\geq 10 \text{ mg kg}^{-1}$) as described previously (Driver et al., 2007). When all response to noxious stimuli, such as pedal withdrawal reflex, had terminated the animals were intracardially perfused with ~25 mls of modified artificial cerebrospinal fluid (ACSF) that was composed of (mM) 252 sucrose, 3.0 KCl, 1.25 NaH_2PO_4 , 24 NaHCO_3 , 2.0 MgSO_4 , 2.0 CaCl_2 and 10 glucose. This procedure was in accordance with the UK Animals (Scientific Procedures) Act 1986 and European Union directive 2010/63EU. Following brain removal, 450 μm thick horizontal slices were cut using a Leica VT1000S vibratome. Slices were then trimmed and transferred to a holding chamber at room temperature for approximately 1 hour before being placed in the recording chamber where they were

maintained at 32-34°C at the interface between normal ACSF (where sucrose was replaced with 126 mM NaCl) and humidified 95% O₂/5% CO₂.

Drugs:

Carbachol (10 µM); nitroblue tetrazolium (NBT), phenazine methosulfate (PMS); phosphate buffered saline (PBS); potassium cyanide (KCN 50-100 µM); rotenone (250-1000 nM); (Sigma-Aldrich, Gillingham, Dorset, UK); HistoClear (Fischer Scientific Ltd., Leicester, UK). Rotenone was dissolved in DMSO and stored at -20 °C.

Recording and data acquisition

Extracellular recording electrodes were filled with normal ACSF (resistance 2-5 MΩ). Gamma frequency oscillations were evoked using the cholinergic agonist carbachol (10 µM) and field traces were recorded from the border between *stratum radiatum* and *stratum lacunosum moleculare* in CA3. Data were recorded with an Axoclamp-2B amplifier (Axon Instruments Inc., Union City, CA, USA). Extracellular data were filtered at 0.001-0.4 kHz and intracellular signals were low pass filtered at 2 kHz using Bessel filters. Mains noise was subtracted from the signal with a Humbug (Digitimer, Welwyn Garden City, Herts, UK). Data were redigitised at 10 kHz using an ITC-16 interface (Digitimer, Welwyn Garden City, UK). Data were recorded and analysed using Axograph 4.6 software (Axon Instruments Inc., Union City, CA, USA). Further offline analysis was performed using MATLAB (Mathworks, Natick, USA).

Data analysis

Power spectra were generated using Axograph by Fourier analysis of 60 sec epochs of recorded activity to give the peak frequency of the oscillations. Gamma frequency oscillations in the mouse have previously been shown to have a slower frequency compared to rat ~20-35 Hz (Driver et al., 2007), therefore, a value for area power was determined as the

area under the peak in the power spectra between 15-80 Hz. Control oscillations were considered “stable” when area power measurements were within ± 10 -15% for three consecutive recordings at 10-15 min intervals. Rhythmicity was measured from the amplitude of the first side peak of the auto-correlations performed over 1 sec traces. The rhythmicity index (RI) was on a scale of 0-1. As multiple slices can be obtained from each animal, in all the data reported below two n values N/n represent animal/slice respectively.

α -synuclein and COX IV immunohistochemistry

Upon termination of electrophysiological recordings, slices were immediately fixed in 4% buffered paraformaldehyde solution and stored in phosphate buffered saline (PBS) at 4 °C. Following cryoprotection in 30% sucrose in PBS slices were resectioned at 40 μ m with a freezing microtome and immunoperoxidase- stained free-floating for either human α -syn using anti-human α -syn (Abcam, Cambridge, UK) 1:5000 dilution, or COX IV antibody (Abcam, Cambridge, UK) 1:100 dilution using standard histological procedures (secondary reagents supplied by Vector Laboratories, Peterborough, UK). Sections were photographed using an Axiovision camera and software and Olympus BX-50 microscope.

COX/SDH activity

Mice were anaesthetised with inhaled isoflurane before an intramuscular injection of ketamine and xylazine. Once eye blink and pedal withdrawal reflexes ceased, the brain was removed and snap frozen using isopentane immersed in liquid nitrogen. Brains were then stored in a -80°C freezer prior to cryosectioning. Snap frozen whole brains were sectioned at 10 μ m using a cryostat at -20°C and collected on to slides. Three sections were collected per slide, with each containing at least one hippocampus. All slides were stored at -80°C until ready to use. Slides were removed from the freezer and left to dry at room temperature for 1 hour before use. Wherever possible individual slides were processed in large batches to maintain consistency and avoid day-to-day variability in staining. The first section of every

slide was used as a control to ensure consistency of COX and SDH media. Half of the control slices received COX incubation media only, and the other half received SDH incubation media only. All other sections received both COX and SDH media. The COX incubation media was prepared by adding 100 μ M cytochrome c to 5 mM 3,3'-diaminobenzidine (DAB) and vortexing with a few crystals of catalase. COX media (50 μ L) was applied to each section, and all slides were incubated for 40 minutes at 37°C. COX media was then removed and 2 x five minute washes with 0.1 M phosphate buffered saline (PBS) were performed. After the final wash, excess PBS was removed from the slides.

The SDH incubation media was prepared by vortexing together 1.5 mM nitroblue tetrazolium (NBT), 130 mM sodium succinate, 0.2 mM phenazine methosulfate (PMS), and 1.0 mM sodium azide. Care was taken to protect the sensitive PMS (and resultant final media) from light. SDH media (50 μ L) was applied to each section, and all sections were incubated for 40 minutes at 37°C. SDH media was then removed and 2 x five minute washes with 0.1M PBS were carried out. Slides were dehydrated through an ethanol gradient (70%, 95%, 100%, 100%) with 5 minutes in each ethanol solution and 10 minutes in the final 100% ethanol solution. Slides were then transferred to two consecutive pots of HistoClear for five minutes of clearing in each and then mounted with DPX.

Quantification of COX/SDH staining and anti-COX IV immunohistochemical staining

Using an Olympus BX51 stereology microscope with an Olympus U-TV1X-2 T2 camera, colour images of COX/SDH staining were taken at x10 magnification of the CA3 region of the hippocampus. All slides were assigned a random numerical value to blind the imaging and quantification processes. In the presence of normal COX activity, the brown DAB precipitate which forms inhibits the formation of the blue NBT precipitate with SDH activity (Trifunovic et al., 2004). Therefore, normal COX activity would appear as brown, while a complete absence of COX activity would appear as blue. Using ImageJ's histogram tool (<http://rsb.info.nih.gov/ij/>), a freehand selected area of the CA3 pyramidal layer could be

analysed for its mean RGB (red, green, blue) constitution. All values were noted, but the mean blue value (no blue = 0, maximum blue = 255) was compared as a measure of mitochondrial dysfunction. Values obtained from sections were compared to control sections stained for either COX (brown) or SDH (blue) alone. Multiple sections (2-7) per mouse were averaged to give an average per animal, and then animals were compared on average for the young 2+A30P and older 9+A30P groups using an unpaired *t* test.

Low power black and white Images of COX IV immunoperoxidase staining were analysed using ImageJ. The *stratum pyramidale* of the CA3 region was outlined and the area and mean optical density (white = 0, black = 255) of the immunostaining within this region of interest was measured and normalized against background staining by subtracting the mean optical density of a nearby area of white matter. Six sections were analysed for each animal, and for each section the total optical density was calculated by multiplying area by the mean optical density. The mean optical density for each animal was calculated by dividing total density by total area from the six sections analysed. Results from 2+A30P (N=3) mice were compared with 9+A30-P (N=3) mice.

Statistical Analysis

Data were statistically analysed using Sigmaplot (v12.5) to determine whether it corresponded to a normal (Gaussian) distribution. Normally distributed data were plotted as mean $\pm$ s.e.m and compared using a Student's *t*-test. Due to the normal variability, especially in gamma frequency area power, all electrophysiological data were analysed using non-parametric statistics on the raw data and described as median and interquartile ranges (IQR) or percentage change. When comparing two different groups we used a Mann Whitney rank sum test. A Wilcoxon signed rank test were used for comparing before and after conditions in the same sample. Repeated measures on the same sample were analysed using a Friedman's repeated measures ANOVA. Multiple groups were compared using Kruskal-Wallis one-way ANOVA on ranks. If the *p* value for the ANOVA tests showed a significant difference

a Dunn's or Tukey's (all pairwise multiple comparison) test was performed to determine which parameters were significantly different. p values are reported as significant <0.05 and <0.001 .

Results

Alpha-syn expression in A30P mice

Immunohistochemical analysis using the human-specific α -syn antibody (see Methods) in slices from the 2+A30P group, confirmed widespread expression of human α -syn throughout the brain including *stratum pyramidale* in the hippocampus (Fig. 1A). There was no staining for human α -syn in the hippocampal pyramidal cell layer in age-matched control C57BL6 mice (Fig. B). A similar staining pattern was observed for both 9+A30P and the 9+C57 mice (data not shown).

Over-expression of α -syn results in an age-dependent decline in gamma frequency activity.

Cognitive impairments in the A30P mice have been reported at 12 months of age (Kahle et al., 2000; Freichel et al., 2007), therefore, to assess network oscillations we initially divided A30P mice into four age groups referred to as 2+ (2-6 months n = 21 mice/45 slices); 9-11 months n = 7 mice/18 slices, 12-13 months n = 7 mice/18 slices, and 14+ (14-16 months, n = 3 mice/8 slices). Gamma frequency oscillations can be readily evoked in the CA3 region of the hippocampus following activation of cholinergic receptors with carbachol (Fisahn et al., 1998; Mann et al., 2005). After application of carbachol (10 μ M) the area power of the gamma frequency oscillations increased gradually for 2-4 hours as previously reported (Lu et al., 2012; Haggerty et al., 2013), and measurements were made once oscillations had stabilised (see Methods).

Our data demonstrated an age-dependent decrease in the area power of the gamma frequency oscillations in the A30P mice (Fig. 2A-E, Table 1). Area power of the gamma activity was significantly different between the 2+A30P, 9-11A30P, 12-13A30P and 14+A30P groups (Kruskal-Wallis one-way ANOVA on ranks, $p < 0.001$). A pairwise multiple comparisons test (Dunn's method) showed that slices from the 2+A30P group had significantly larger oscillations than the 9-11A30P, 12-13A30P, 14+A30P groups, and versus all 9+A30P data

combined. However, there was no significant difference between any of the older age groups (Fig. 2E). Table 1 shows the median and IQR values for area power in each age group.

In contrast, the frequency of the gamma oscillation was not significantly different (Fig. 1F) between any of the different age groups of A30P mice (Kruskal-Wallis one-way ANOVA on ranks, $p>0.001$; Table 1). In addition, there was a significant difference in RI values (Fig. 1G, Table 1) overall (Kruskal-Wallis one-way ANOVA on ranks, $p>0.001$; Table 1); however, a pairwise multiple comparisons test (Dunn's method) did not reveal any significant differences between specific groups. As slices from the 9-11A30P, 12-13A30P or 14+A30P and all 9+A30P combined groups all showed reduced gamma frequency area power compared to the 2+A30P group, these older age groups were combined into the so-called 9+A30P group for all subsequent analysis (Table 1).

No gender differences in the power of network oscillations in the A30P mice

Both the 2+ and 9+A30P groups contained data obtained from male and female mice. Therefore, we also assessed these groups separately, but found no significant difference in the stable area power between slices taken from male and female mice (Fig. 3A). Stable area gamma frequency power in 2+A30P male mice was 2066 (IQR 1115 - 4966; $n = 5$ mice/11 slices) μV^2 compared with 3325 (IQR 2252 - 6216; $n = 16$ mice/34 slices) μV^2 in the female 2+A30P mice (Kruskal-Wallis one-way ANOVA on ranks $p>0.05$). In the 9+A30P group there was also no significant difference between slices taken from male and female mice. Stable area gamma frequency power in 9+A30P male group was 306 (IQR 124 - 1355; $n = 11$ mice/30 slices) μV^2 compared with 1484 (IQR 265 - 2254; $n = 5$ mice/14 slices) μV^2 in the female 2+A30P group (Kruskal-Wallis one-way ANOVA on ranks $p>0.05$). Importantly, slices taken from male and female mice both showed an age-dependent reductions in the area power of the gamma frequency activity between 2+A30P and 9+A30P age groups ($p<0.05$, Kruskal-Wallis one-way ANOVA on ranks, Fig. 3A).

The frequency of the gamma oscillation (Fig. 3B) for the 2+A30P male mice was 25.6 (IQR 23.9 - 27.0; n = 5 mice/11 slices) Hz compared with 25.1 (IQR 23.4 - 26.8; n = 16 mice/34 slices) Hz in the female 2+A30P mice ($p>0.05$). In the 9+A30P group there was a median frequency of 23.6 (IQR 21.6 - 25.7; n = 11 mice/30 slices) Hz in male mice compared with 26.7 (IQR 23.7 - 27.7; n = 5 mice/14 slices) Hz in the female 2+A30P group. There were no significant differences between the groups (Kruskal-Wallis one-way ANOVA on ranks, $p>0.05$). RI values were also assessed separately for slices from male and female mice (Fig. 3C). For the 2+A30P male mice RI was 0.59 (IQR 0.16 - 0.75) compared to 0.67 (IQR 0.48 - 0.74) for female. In the 9+A30P group RI in the male group was 0.29 (IQR 0.13 - 0.59) compared to 0.48 (IQR 0.24 - 0.75) in the female group. Again, as above for RI values, there was a significant difference overall (Kruskal-Wallis one-way ANOVA on ranks, $p<0.05$) but a multiple comparison procedure did not reveal specific group differences.

No age-dependent change in stable gamma frequency activity in control C57 mice

Previous *in vitro* studies have shown an age-dependent decrease in hippocampal gamma oscillations in control mice, but only after approximately >16 months (Vreugdenhil et al., 2005; Driver et al., 2007). In order to confirm the age-dependent deficit observed in the A30P mice was a consequence of human mutant α -syn expression, and not due to normal ageing, we compared a similar age-matched group of control C57BL6 (male only) mice divided into similar 2+ and 9+ age groups.

In contrast to the data reported above for the A30P mice, we found no age-dependent changes in gamma frequency activity between 2+ and 9+C57 mice (Fig. 4A-G). Stable gamma frequency area power in slices taken from 2+C57 mice was 4779 (IQR 1876 - 9309; n = 6 mice/19 slices) μV^2 compared with 2190 (IQR 415 - 7928; n = 6 mice/15 slices) μV^2 in slices taken from 9+C57 mice (Mann Whitney rank sum test $p>0.05$). There was also no difference in the frequency of the gamma oscillations between 2+C57 and 9+C57 control mice (Fig. 5F). The median frequencies in 2+C57 and 9+C57 were 23.3 (IQR 22 - 24.7; n = 6

mice/19 slices) Hz and 25.2 (IQR 22.3 – 26.4; n = 6 mice/15 slices) Hz, respectively (Mann Whitney rank sum test $p > 0.05$). We also assessed possible changes in rhythmicity between the 2+ and 9+C57 mice but there was no significant difference in RI values between the two groups. Median RI in the 2+C57 mice was 0.57 (IQR 0.5 – 0.65) and 0.49 (IQR 0.2 – 0.6) in the 9+C57 mice (Mann Whitney rank sum test $p > 0.05$).

A one way-ANOVA comparing all four groups (2+A20P, 9+A30P, 2+C57 and 9+C57) showed a significance difference ($p < 0.001$; Kruskal-Wallis one-way ANOVA on ranks). Post-hoc analysis confirmed that the gamma frequency area power was significantly smaller only in the 9+A30P (Dunn's pairwise multiple comparison). The above data, therefore, demonstrate an age-dependent reduction in gamma frequency power in the older A30P mice that occurs prior to any normal age-related decline in gamma activity in control mice.

Effects of mitochondrial inhibitors on network gamma frequency oscillations

Previous studies have shown that hippocampal gamma frequency oscillations evoked *in vitro* are sensitive to blockade of mitochondrial complex I and complex IV, with rotenone and KCN respectively (Kann et al., 2011; Whittaker et al., 2011). If the impaired gamma frequency activity observed in the 9+A30P mice reflected an age-dependent reduction in mitochondrial function, we predicted we might observe a greater sensitivity of the oscillatory activity to inhibition of mitochondrial function in slices from the older A30P mice. Thus we compared the concentration-dependent effects of potassium cyanide (KCN 25, 50 and 100 μ M), and rotenone (250, 500, and 1000 nM), on stable carbachol-evoked gamma frequency oscillations from older C57 and A30P mice (Fig. 5).

Once oscillations were stable KCN was bath applied incrementally every 30 mins with readings taken at 10 min intervals (Fig. 5A-B). There was a rapid reduction in gamma oscillatory power within 10 mins in slices from both 9+C57 (n = 4 mice/10 slices) and 9+A30P C57 (n = 3 mice/6 slices) mice. Compared to control stable values statistically significant

reductions were evident at 50 μ M and 100 μ M in both 9+C57 and 9+A30P groups (Freidman's repeated measures ANOVA on ranks followed by Tukey post-hoc multiple comparisons test $p < 0.05$). At the final concentration of KCN tested the power of the gamma activity was reduced by $-77 \pm 7.6\%$ in 9+C57 group and $-82.6 \pm 8.3\%$ in the 9A30P group.

In a further set of experiments, we also tested rotenone and this appeared less effective than KCN. In the control 9+C57 group ($n = 7$ mice/21 slices) there was a clear trend towards a concentration dependent reduction in gamma power (Fig. 5C) but this was not statistically different at any point when compared to stable control levels (Freidman's repeated measures ANOVA on ranks followed by Tukey post-hoc multiple comparisons test $p > 0.05$). However, in the 9+A30P group ($n = 6$ mice/18 slices) at the higher concentrations rotenone did cause a statistically significant decrease compared to stable gamma power (Freidman's repeated measures ANOVA on ranks followed by Tukey post-hoc multiple comparisons test $p < 0.05$). At the final concentration of rotenone tested the power of the gamma activity was reduced by $-29.6 \pm 9.8\%$ in 9+C57 group and $-41.9 \pm 8.8\%$ in the 9A30P group. The greater decrease in gamma frequency oscillations with rotenone suggests slices from the 9+A30P mice are more sensitive to this mitochondrial inhibitor.

Age-dependent mitochondrial dysfunction in the A30P mice

The above data suggested that the age-dependent impairment in the power of the gamma frequency oscillation seen in older A30P mice might reflect a mitochondrial dysfunction in these animals. COX/SDH histochemistry (see Methods) was performed on sections from 2+A30P ($N = 5$ mice) and 9+A30P ($N = 5$ mice) groups. While we did not detect COX deficiency within individual neurons, a general reduction in COX reactivity was seen across the hippocampus. In order to ascertain if there was any difference in the activity of mitochondria in these sections we measured the changes in RGB balance in the CA3 *stratum pyramidale*, with increased blueness indicating a loss of COX activity (see Methods). The

mean blue values for COX alone control was 39.5 ± 2.8 compared to 120.4 ± 4.1 for SDH alone control (Fig. 6A-B) and these control values were highly significantly different ($p < 0.001$). Overall in the 2+A30P the mean blue level was 45.17 ± 2.9 compared to 52.59 ± 1.73 in the 9+A30P group, which was a statistically significant change in COX/SDH histochemistry in the CA3 *stratum pyramidale* (Fig. 6E-F) in the older 9+A30P (Un-paired t-test $p < 0.05$). The increased mean blue level suggested an age-dependent decline in mitochondrial COX activity and expression and, therefore, a decrease in mitochondrial function in the 9+A30P group. Of note, there was considerable variability between individual mice in the younger 2+A30P group (Fig. 6E). In a further set of experiments immunohistochemistry for COX IV immunoperoxidase in the *stratum pyramidale* of the CA3 region also showed a significant 23.3% reduction in optical density in the 9+A30P group compared to slices from 2+A30P mice (136.7 ± 5.0 to 104.9 ± 5.0 , $N = 3$, $p < 0.05$, Fig. 6I). Combined, these data suggest an age-dependent impairment in COX activity and expression indicating a mitochondrial dysfunction was present in the older 9+A30P group.

Discussion

In this study we have demonstrated an age-dependent reduction in hippocampal gamma frequency oscillations in slices from older 9+A30P mice, compared with younger 2+A30P mice, or age-matched control animals. Oscillations in both control C57BL6 mice and A30P mice were sensitive to inhibitors of mitochondrial function but a greater reduction in area power was seen in the older A30P group. COX/SDH staining and anti-COX IV immunohistochemistry revealed a significant age-related decline in mitochondrial function in A30P mice.

Although human α -syn expression was clearly evident in young animals (2+A30P), the deficits in gamma activity were only seen in the older 9+A30P mice. One possible explanation for the age-dependence of the change is that reductions in gamma frequency oscillations might be a consequence of a progressive increase in pathology. α -syn is expressed in the A30P mice from one month of age (Kahle et al., 2000). However, posttranslational modifications of α -syn, such as phosphorylation or ubiquitination, are associated with Lewy-like pathology in both humans and in mouse α -synucleinopathy models (Lim et al., 2011). In the A30P mice an age-dependent increase in Serine-129 phosphorylation has been reported, that correlated with the cognitive deficits observed in these animals at 12 months of age (Schell et al., 2009). In this study the deficits in gamma activity were evident at 9-11 months of age in the A30P mice, with no significant further changes observed up to 14-16 months of age. This suggests the reductions in gamma frequency activity occur just prior to the onset of quantifiable cognitive dysfunction.

It is not yet known how abnormal α -syn expression might affect synaptic transmission, and the generation of gamma frequency oscillations in the hippocampus. In human post-mortem tissue from patients with a confirmed diagnosis of DLB, α -syn aggregates were found to localize at the synaptic terminal (Kramer and Schultz-Schaffer 2007). α -syn is known to

undergo misfolding and oligomerisation forming dimers, oligomers and fibrils (Ingelsson 2016). It is now generally accepted that the oligomeric form is the most toxic, rather than the larger intracellular inclusions. These oligomeric structures may also be transmitted in a prion-like manner through brain structures (Ingelsson 2016), and thus could impair synaptic transmission in subtle ways long before major cognitive decline is evident in the A30P mice. Gamma oscillations in the hippocampus depend upon reciprocal interactions between pyramidal cells and fast-spiking GABAergic interneurons (Whittington et al., 2011) that contain the calcium binding protein parvalbumin (PV). The age-dependent reduction in gamma activity observed in the 9+A30P mice could reflect changes in either pyramidal cells, interneurons or both and further studies are required to see which cell types are affected or might be selectively vulnerable to α -syn toxicity. However, evidence suggests PV interneurons are particularly vulnerable to metabolic stress due to the high energy demand of their fast firing rates (Kann et al., 2014). Data from AD transgenic mice have implicated loss of PV interneuron function as a key contributor to deficits in network oscillations (Palop and Mucke 2016). In addition, reductions in PV-positive interneuron staining have been reported in human post-mortem studies in both AD (Brady and Mufson 1997) and DLB (Bernstein et al. 2011). Further studies are needed to determine if the deficits in gamma frequency activity observed in aged A30P also reflect dysfunction of PV interneurons.

Abnormal α -syn expression in transgenic mice has been associated with both network hyperexcitability (Morris et al., 2015) and mitochondrial dysfunction (Chinta et al., 2010; Rothman et al., 2014; Martin et al., 2014; Chen et al., 2015). α -syn is found in the mitochondria and recently it was shown that α -syn associated with the mitochondria modulates mitochondrial transport and morphology (Pozo Deveto et al., 2017). In transgenic mice that over-express human mutant A53T α -syn both mitochondrial motility (Li et al., 2013) and function (Martin et al., 2014) are impaired. The study by Pozo Deveto et al., (2017) also showed, however, that the effects of over-expression of A53T α -syn in human and mouse

derived neuronal cultures were significantly greater than those of over-expression of A30P α -syn. This is in alignment with the observation that the A30P mutation in humans is associated with later onset and less severe clinical symptoms in patients than those seen with the A53T mutation (Kruger et al., 2001; Puschman et al., 2009). Our data showed a significant age-dependent impairment of both gamma frequency activity, and mitochondrial function, which would be consistent with a slow progression of neurodegenerative changes in the A30P transgenic mice.

Our data using the mitochondrial inhibitors KCN and rotenone showed a concentration dependent reduction in the power of the gamma frequency activity in both aged wild type C57 mice and A30P mice. Rotenone blocks mitochondrial complex I while KCN blocks complex IV. Previously, high concentrations of both KCN (100 μ M) and rotenone (1 μ M) have been shown to cause a rapid collapse of the gamma oscillations (Kann et al., 2011; Whittaker et al., 2011). We had predicted that if A30P mice developed compromised mitochondrial function with age then slices taken from the older A30P mice may be more sensitive to lower concentrations of the inhibitors. While we saw no difference between 9+C57 and 9+A30P groups with KCN, the results with rotenone did suggest a slightly greater sensitivity of the oscillations in the 9+A30P group.

A previous study (Whittaker et al., 2011) showed that KCN (100 μ M) had no effect on pyramidal cell properties or excitatory post-synaptic potentials (EPSP) onto the pyramidal cells, suggesting the changes in oscillations were not mediated by reductions in glutamatergic neurotransmission. In contrast, these authors found a reduction in inhibitory post-synaptic potential (IPSPs) on the pyramidal cells, while fast-spiking interneurons were depolarized and spiking activity in the interneurons was abolished (Whittaker et al., 2011). This loss of action potential firing in fast spiking interneurons, and the subsequent decrease in IPSP amplitude could account for the reduction in gamma frequency oscillations observed both in their study

and in the current experiments. As outlined above the higher sensitivity of interneurons to inhibition of mitochondrial function is consistent with their high metabolic requirements (Kann et al., 2016). Rotenone block of complex I can cause either an increase in reactive oxygen species (ROS) or ATP depletion. It would be interesting in further studies to assess whether interventions that prevent oxidative stress and energy failure have any impact on the impaired gamma frequency activity in the older A30P mice.

COX/SDH histochemistry detects alterations in the activity of complex IV and II respectively, and we found a significant deficit in COX activity and presumably expression in the 9+A30P group, although SDH expression in complex II was retained. An age-dependent deficit in COX IV expression was confirmed by immunohistochemistry in the 9+A30P group. This result is suggestive of mitochondrial dysfunction, which may be caused by mutations in key mitochondrial genes either within mitochondrial DNA (mtDNA) or the nucleus. In ageing human neurons, a reduction in COX activity is predominantly associated with deletions within mtDNA, which is vulnerable to oxidative damage, whereas the preservation of SDH activity in these situations is because it is entirely nuclear encoded, meaning that its activity is not affected by mutations in mtDNA (Bender et al., 2006; Kraytsberg et al., 2006; Krishnan et al., 2012). The variability in COX staining seen between different mice in the 2+A30P group may also indicate early changes, which while not significant as a group, suggest changes in mitochondrial function may already be starting to occur in some animals. Although from the current study we cannot ascertain whether the mitochondrial dysfunction is cell type specific, impairments in pyramidal cells and/or fast-spiking interneurons could result in the reduction in gamma frequency activity observed in the older 9+A30P mice.

Conclusions

Several studies have shown that α -syn oligomers impair hippocampal LTP, which is thought to represent the cellular mechanism of learning and memory. However, to our knowledge, this is the first study to report deficits in cognitively relevant, beta/gamma

frequency network oscillations in the hippocampus in mice that express abnormal human mutant α -syn. We have shown a consistent deficit in oscillations in aged mice overexpressing α -syn that precedes reported behavioural deficits and correlates temporally with observed changes in mitochondrial function.

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Figure Legends

Figure 1 – Alpha synuclein expression in the hippocampus.

A) Immunohistochemistry shows strong human α -syn immunostaining in the hippocampus, especially in the stratum pyramidale, of 2+A30P mice. (B) 2+C57 mice do not express human α -syn, although some non-specific staining is observed in erythrocytes and around the section edges. Hippocampal regions *Cornu Ammonis* 1 (CA1), *Cornu Ammonis* 3 (CA3) and dentate gyrus (DG) are indicated. Scale bar = 100 μ M.

Figure 2 - Age-dependent reduction in gamma frequency activity in A30P mice.

A) Representative 1 sec extracellular field trace in 2+A30P (blue) following 30 min bath application of carbachol (10 μ M) and the corresponding power spectra. Inset shows auto-correlation. (B) Representative 1 sec extracellular field trace when stable gamma activity was reached and corresponding power spectra. Inset shows auto-correlation. (C) Representative 1 sec extracellular field trace (maroon) in 9+A30P following 30 min bath application of carbachol (10 μ M) and corresponding power spectra. Inset shows auto-correlation. (D) Representative 1 sec extracellular field trace when stable gamma activity was reached and corresponding power spectra. Inset shows auto-correlation. Note different scales on power spectra for 2+ and 9+A30P mice. E) Boxplot shows the median (whiskers max to min) area power of the gamma frequency activity in all age groups (1 outlier removed from plot). Asterix (*) indicates power in the 2+A30P group was significantly different from all other groups ($p < 0.05$; Kruskal-Wallis One-way ANOVA on ranks) and post-hoc all pairwise multiple comparison (Dunn's method). F) Boxplot shows the median (whiskers max to min) frequency of the gamma frequency activity in all age groups. There was no significant difference between any group ($p > 0.05$; Kruskal-Wallis One-way ANOVA on ranks). G) Boxplot shows median (whiskers max to min) RI index of the gamma frequency activity in all age groups. There was a significant difference between the groups ($p < 0.05$; Kruskal-Wallis One-way ANOVA on ranks but post

hoc all pairwise multiple comparison (Dunn's method) did not reveal specific group differences. Scale bars for auto-correlation 0.5/100 ms.

Figure 3 – Area power and frequency in slices from male versus female A30P mice.

A) Boxplot shows the median (whiskers max - min) area power of the gamma frequency activity for slices from male versus female 2+A30P and 9+A30P mice. Power in the male 2+A30P group was significantly different from male 9+A30P group ($p < 0.05$; Mann Whitney rank sum test). Power in the female 2+A30P group was significantly different from female 9+A30P group ($p < 0.05$; Mann Whitney rank sum test). B) Boxplot shows the median (whiskers max - min) frequency of the gamma frequency activity for slices from male versus female 2+A30P and 9+A30P mice. There was no significant difference in frequency between male and female mice in the 2+A30P or 9+A30P groups ($p > 0.05$; Mann Whitney rank sum test). C) Boxplot shows the median (whiskers max - min) RI of the gamma frequency activity for slices from male versus female 2+A30P and 9+A30P mice. There was a significant difference between the groups ($p < 0.05$; Kruskal-Wallis One-way ANOVA on ranks but post-hoc all pairwise multiple comparison (Dunn's method) did not reveal specific group differences.

Figure 4 – No age-dependent changes in gamma frequency activity in control C57BL6 mice.

A) Representative 1 sec extracellular field trace in 2+C57 (green) following 30 min bath application of carbachol ($10 \mu\text{M}$) and the corresponding power spectra. Inset shows auto-correlation. (B) Representative 1 sec extracellular field trace when stable gamma activity was reached and corresponding power spectra. Inset shows auto-correlation. C) Representative 1 sec extracellular field trace in 9+C57 (brown) following 30 min bath application of carbachol ($10 \mu\text{M}$) and corresponding power spectra. Inset shows auto-correlation. (D) Representative 1 sec extracellular field trace when stable gamma activity was reached and corresponding power spectra. Inset shows auto-correlation. E) Boxplot shows the median (whiskers max –

min) area power of the gamma frequency activity in both age groups. Power in the 2+C57 group was not significantly different from the 9+C57 group ($p>0.05$; Mann Whitney rank sum test). F) Boxplot shows the median (whiskers max - min) frequency of the gamma frequency activity in both age groups. There was no significant difference between any group ($p>0.05$; Mann Whitney rank sum test). G) Boxplot shows the median (whiskers max - min) RI of the gamma frequency activity in both age groups. There was no significant difference between any group ($p>0.05$; Mann Whitney rank sum test). Scale bars for auto-correlation 0.5/100 ms.

Figure 5 – Effects of mitochondrial inhibitors on network oscillations in the hippocampus.

A) Histogram shows percentage change in area power at 10, 20 and 30 mins after application at each concentration of KCN (25 μ M, 50 μ M and 100 μ M) in the 9+C57 group. B) Histogram shows percentage change in area power at 10, 20 and 30 mins after application at each concentration of KCN (25 μ M, 50 μ M and 100 μ M) in the 9+A30P group. C) Histogram shows percentage change in area power at 10, 20 and 30 mins after application at each concentration of rotenone (250 nM, 500 nM and 1000 nM) in the 9+C57 group. D) Histogram shows percentage change in area power at 10, 20 and 30 mins after application at each concentration of rotenone (250 nM, 500 nM and 1000 nM) in the 9+A30P group. * indicates significantly different from stable control area power (Freidman's repeated measures ANOVA on ranks and post hoc Tukey test $p<0.05$).

Figure 6 – Age-dependent reduction in mitochondrial function in A30P mice.

A) Representative sections from the hippocampal CA3 region showing COX activity and (B) SDH activity only in sections from the 2+A30P group. Inset histograms show the individual blue stain intensity in the RGB spectra using ImageJ for each section shown. C) Representative sections from the hippocampal CA3 region and inset histograms of blue stain intensity in the RGB spectra using ImageJ values from 2+A30P animals and (D) 9+A30P

where the mean blue intensity level is significantly increased, indicating a reduction in COX activity. Scale bars = 100 μ M. E) Mean blue intensity levels for each mouse in 2+A30P and (F) 9+A30P groups. There was significant variability between values for individual mice in the 2+A30P group. G) Shows examples of anti-COX immunostaining in 2+A30P and (H) 9+A30P mice. I) Mean optical density measurements from the CA3 *stratum pyramidale* revealed a significant decrease in immunoreactivity in the 9+A30P group.

Robson et al Figure 2 revised

Robson et al Figure 3 revised

Robson et al Figure 4 revised

Robson et al Figure 5 revised

Robson et al Figure 6 revised