

Intact Olfaction in a Mouse Model of Multiple System Atrophy

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Abstract

Background: Increasing evidence suggests that olfaction is largely preserved in multiple system atrophy while most patients with Parkinson's disease are hyposmic. Consistent with these observations, recent experimental studies demonstrated olfactory deficits in transgenic Parkinson's disease mouse models, but corresponding data are lacking for MSA models.

Methods: Olfactory function and underlying neuropathological changes were investigated in a transgenic multiple system atrophy mouse model based on targeted oligodendroglial overexpression of α -synuclein as well as wild-type controls. The study was divided into (1) a pilot study investigating olfactory preference testing and (2) a long-term study characterizing changes in the olfactory bulb of aging transgenic multiple system atrophy mice.

Results: In our pilot behavioral study, we observed no significant differences in investigation time in the olfactory preference test comparing transgenic with wild-type animals. These findings were accompanied by unaffected tyrosine hydroxylase-positive cell numbers in the olfactory bulb. Similarly, although a significant age-related increase in the amount of α -synuclein within the olfactory bulb was detected in the long-term study, progressive degeneration of the olfactory bulb could not be verified.

Conclusions: Our experimental data show preserved olfaction in a transgenic multiple system atrophy mouse model despite α -synucleinopathy in the olfactory bulb. These findings are in line with the human disorder supporting the concept of a primary oligodendroglial pathology with variable neuronal involvement.

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Introduction

Multiple system atrophy (MSA) is a rapidly progressive neurodegenerative disorder of unknown etiopathogenesis. It is characterized clinically by autonomic failure accompanied by parkinsonism and cerebellar ataxia [1]. The distinction of early stage MSA from related parkinsonian syndromes including Parkinson's disease (PD) can be challenging [2]. However, previous reports suggested that assessment of olfactory function is an important pointer in the differential diagnosis. MSA patients show intact or mildly impaired olfaction whereas most PD patients are hyposmic or sometimes anosmic [3–8]. Even more interestingly, olfactory disturbances may predate the onset of classic motor features in PD [9,10]. Deficits in PD patients include impairment of odor detection, discrimination and identification [10,11].

α -synuclein (α SYN) is a key protein in the pathogenesis of MSA and PD with the former being characterized by glial cytoplasmic inclusions (GCIs, Papp-Lantos bodies) and the latter by neuronal Lewy bodies as their subcellular hallmark feature. These α SYN-positive inclusions are also observed in the olfactory tract, predominantly affecting the anterior olfactory nucleus [12,13].

In preclinical research, α SYN pathology may be replicated by transgenic (tg) overexpression of α SYN under oligodendroglial [14–16] or neuronal promoters [17] mimicking MSA- or PD-like inclusion pathology, respectively.

Recently, olfactory disturbances have been studied in tg mouse models of PD. Behavioral alterations and olfactory bulb pathology in these models are reminiscent of the human disorder with age-related impairment in odor detection and discrimination [18–20] as well as extensive olfactory bulb pathology [21–23]. In contrast, smell disturbances in MSA models were only studied once in the context of glial derived neurotrophic factor (GDNF) replacement therapy [24]. This study reported olfactory impairment in tg versus wild-type (wt) animals in the saline-treated study arm; however, olfactory bulb pathology was not investigated [24].

In the present study, we investigated olfactory behavior and assessed neuropathological changes within the olfactory bulb (OB) and their age-related evolution in an established tg MSA mouse model featuring overexpression of α SYN in oligodendrocytes [14].

Methods

The study was split into two parts: (1) a pilot study determining behavioral olfactory deficits and immunohistochemical differences in 9-months old animals and (2) a confirmatory long-term study (LTS) focusing on the analysis of OB aging. In the LTS, mice with 2, 6 and 18 months of age were studied. Both subprotocols compared homozygous tg MSA mice to age- and strain-matched non-littermate wt controls of the inbred C57BL/6 strain.

Animals

The generation and characterization of tg mice with targeted overexpression of human α SYN (h α SYN) under the oligodendroglial proteolipid protein promoter (PLP-h α SYN) were described previously [14]. Tg and wt mice were originally obtained from P. Kahle (University of Tübingen, Tübingen, Germany) and Charles River Laboratories (Charles River Laboratories, Sulzfeld, Germany), respectively. Mice were bred and maintained in a temperature-controlled specific pathogen free room with a 12-h light/dark cycle and free access to food and water at the Animal Facility of Innsbruck Medical University. Genotyping was performed by tail clip polymerase chain reaction (PCR) using the following primers: Forward: 5'-ATG GAT GTA TTC ATG AAA GG-3'; reverse: 5'-TTA GGC TTC AGG TTC GTA G-3'.

This study was carried out in strict accordance with the Austrian guidelines for the care and use of laboratory animals and all in vivo protocols were approved by the Austrian Federal Ministry of Science and Research (do. Zi. 6001). All efforts were made to minimize the number of animals used and their suffering.

Behavioral testing

We performed olfactory preference testing in 9 month old mice according to a previously published protocol [25,26]. This test is designed to identify specific odor detection deficiencies, based on the inability to sense attractive scents [25,26]. Briefly, four home cages sized 26 cm \times 45 cm \times 20 cm (width \times length \times height) were lined up next to each other separated by opaque filter paper. Animals were habituated to the unknown surrounding of an empty cage for a period of 1 hour with transfer steps to the next cage every 15 minutes. Moreover, a video camera was placed such that the entire arena cage (= last habituation cage) was in focus. Thereafter, 5 cm \times 5 cm squares of scented filter paper were placed on the opposite end of the mice's current position. The animal's behavior was recorded on video for a period of 3 minutes. Different scents were randomly presented at an interval of at least one minute. The following odorants were used: distilled water (control, inherent smell of the filter paper), peanut butter (Skippy, Unilever) dissolved in mineral oil (Sigma-Aldrich, Vienna, Austria), vanilla (Oetker GmbH, Bielefeld, Germany) and cinnamon (Invero, Wiener Neudorf, Austria) dissolved in mineral oil. Test cages were extensively cleaned after each mouse. Finally, to avoid confounding of data owing to task learning, mice performed the test once only.

Tissue preparation and immunohistochemistry

Mice were sacrificed at the designated time points by transcardial perfusion with 10 ml of 0.1 M phosphate buffered saline (PBS; Sigma-Aldrich, Vienna, Austria) followed by ice-cold 4% paraformaldehyde (PFA; Merck, Darmstadt, Germany) in PBS under deep thiopental (Sandoz, Kundl, Austria) anesthesia (i.e. 120 mg/kg body-weight thiopental). Brains were quickly removed and post-fixed in 4% PFA dissolved in PBS at 4°C over night. After cryoprotection in 25% PBS-sucrose solution (Sigma-Aldrich, Vienna, Austria), brains were slowly frozen in 2-methylbutane

(Merck, Darmstadt, Germany) and stored at -80°C until further processing.

Serial coronal sections (40 μ m) were cut on a freezing microtome (Leica, Nussloch, Germany). One series of sections per animal was mounted on slides and underwent cresyl violet (CV) staining. Free floating sections were stained according to a standard immunoperoxidase protocol [27,28]. The following primary antibodies were used: mouse anti-tyrosine hydroxylase (TH, 1:1000; Sigma, St. Louis, Missouri, USA), rat anti-mouse CD11b (1:150; Serotec, Oxford, UK), rat anti-human- α -synuclein (15G7, 1:200; Enzo Life Sciences, Exeter, UK). For immunohistochemistry, secondary antibodies were biotinylated anti-mouse or anti-rat IgG (Vector Laboratories, Burlingame, California, USA) as appropriate. Following incubation with avidin-biotin complex (ABC) reagent (Vectastain ABC kit, Vector Laboratories, Burlingame, California, USA), immunohistochemical reactions were visualized by 3,3'-diamino-benzidine-tetrahydrochloride (DAB; Sigma, St. Louis, Missouri, USA). For immunofluorescence, Alexa 488- or Alexa 594-conjugated anti-rat or anti-mouse IgG (Molecular Probes, Life Technologies, Paisley, UK), as appropriate, were applied as secondary antibodies.

Image analysis

Image analyses were performed by a blinded investigator. Nikon E-800 (Nikon, Vienna, Austria) microscope equipped with a digital camera (Nikon DXM 1200, Nikon, Vienna, Austria) connected to a computer-assisted analysis system (Stereo Investigator Software, MicroBrightField Europe, Magdeburg, Germany) was used. Regions of interest were outlined manually according to the Paxinos and Franklin Mouse Brain Atlas (1997, Academic Press, San Diego). The optical fractionator workflow was exploited to generate an unbiased estimate of TH- and 15G7-immunoreactive cell numbers in the granular layer and glomerular layer of the OB. Microglial activation was determined by measuring optical density (OD) of CD11b immunoreactivity. Briefly, staining brightness was measured in the glomerular and granular layer of the OB (OD_{ROI}) and a blank area ($OD_{Background}$). Next, the OD ratio was calculated according to the following formula: OD ratio = $-\log(OD_{ROI}/OD_{Background})$ as previously described.[27] OB atrophy was evaluated by outlining the OB bilaterally on 3 adjacent CV stained sections and measuring the respective area using Stereo Investigator Software.

For immunofluorescence, imaging was performed using a DMI 4000B Leica microscope equipped with Digital Fire Wire Color Camera DFC300 FX and Application Suite V3.1 software by Leica (Leica, Nussloch, Germany).

Statistical analysis

Statistical analysis was performed using SPSS 20.0 (SPSS Inc., Chicago, Illinois, USA). If not stated otherwise, data are expressed as mean \pm standard error of mean (SEM). Group differences were analyzed by Student's T-test, Kruskal-Wallis-Test, one-way analysis of variance (ANOVA) or two-way ANOVA and Bonferroni correction of multiple comparisons as appropriate. The significance level was set at $p < 0.05$; all tests were two-sided.

Results

Pilot study: olfactory preference test in aged MSA mice

In the olfactory preference testing paradigm, there was no significant difference in investigation time between 9-month old tg and wt animals ($F_{1, 16} = 0.000$, $p = 0.989$, ANOVA). Although the different scents were a non-significant term in our ANOVA model ($F_{3, 48} = 2.189$, $p = 0.101$, ANOVA), pairwise comparisons re-

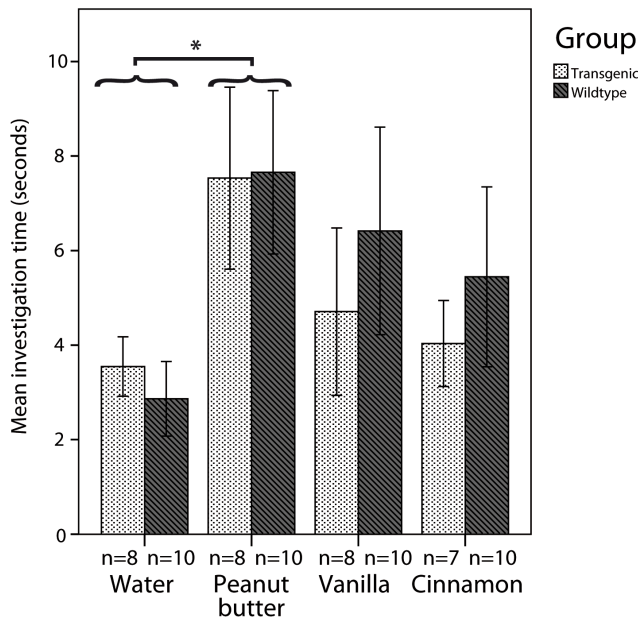


Figure 1. Olfactory behavior. Mean investigation time in seconds in olfactory preference testing of 9 months old mice. Data are expressed as mean; error bars indicate the standard error of mean. Sample sizes are reported below the X-axis. * P<0.05. doi:10.1371/journal.pone.0064625.g001

vealed that the inherent smell of the filter paper (water sample) was less attractive than peanut butter to the animals irrespective of the underlying genotype (p = 0.041, post-hoc analysis of ANOVA model with Bonferroni correction for multiple comparisons) (Figure 1).

Immunohistochemical analysis of TH Immunoreactive cells within the glomerular layer of the OB yielded no difference in the number of dopaminergic neurons in 9 months old mice (p = 0.129, Student’s T-test, Table 1). In addition, stereological analysis of 15G7-immunoreactive cells revealed αSYN pathology of the OB in 9 months old mice already (Table 1).

Long-term study: OB ageing

As indicated above, the LTS involved assessment of neuropathology in 2, 6 and 18 month-old animals to detect age- and αSYN-related neurodegeneration in the OB.

We observed an age-dependent accumulation of hαSYN immunoreactive inclusions in the OB of tg mice (p = 0.036, Kruskal-Wallis; Table 1). However, the highest number of MSA-like cytoplasmic inclusion pathology was observed in 9 months old animals (Table 1) with a statistically significant difference to 2 months (p = 0.048, Mann-Whitney-U-Test with Bonferroni correction for multiple comparisons) and 6 months (p = 0.024, Mann-Whitney-U-Test with Bonferroni correction for multiple comparisons) old animals, but not compared to 18 months old mice (p = 0.558, Mann-Whitney-U-Test with Bonferroni correction for multiple comparisons). Transgenic hαSYN driven by the PLP promoter was detected in CNPase-positive glial cells of the OB (Figure 2b) similar to other CNS regions [29,30]. The increase in αSYN load did not convert into OB degeneration, in particular, there were no significant differences in OB volume between the two genotypes (F_{1, 39} = 3.263, p = 0.079, ANOVA; Figure 2a). Likewise, the number of TH-ir cells remained stable over time (F_{3, 39} = 1.248, p = 0.305, ANOVA; Table 1) and genotype was a non-significant term in the ANOVA model (F_{1, 39} = 0.377, p = 0.543, ANOVA; Table 1).

In contrast, tg animals featured early OB microglial activation with significantly increased CD11b immunoreactivity in the glomerular layer at 2 months compared to wt animals (p<0.01, ANOVA model with Bonferroni correction). However, wt animals showed an age-related increase in microglial activation catching up the difference which was present at 2 months (Figure 2c). In the granular layer, we observed age-related enhancement of CD11b immunoreactivity in tg as well as wt animals (F_{3, 38} = 21.5, p<0.001, ANOVA) with differences between the two genotypes failing to reach statistical significance (Figure 2d).

Discussion

The presence of olfactory deficits is an important diagnostic pointer in patients presenting with parkinsonism. MSA patients show largely preserved olfactory function whereas most PD patients are hyposmic [3–8]. In preclinical research, olfaction has been extensively studied in PD αSYN mouse models revealing deficits of odor detection and discrimination [18–20]. In addition,

Table 1. Stereological cell counts of 15G7 and TH immunohistochemistry.

Age	Genotype	15G7, mean ± SEM (n)	TH, mean ± SEM (n)	p-value ¹
2 months	Transgenic	77532.1 ± 7258.3 (n = 5)	67893.9 ± 8327.7 (n = 5)	0.226
2 months	Wildtype	Not applicable	53583.1 ± 5948.7 (n = 4)	
6 months	Transgenic	108711.4 ± 11531.2 (n = 6)	73244.8 ± 10118.7 (n = 6)	0.619
6 months	Wildtype	Not applicable	82077.8 ± 13940.0 (n = 6)	
9 months	Transgenic	160520.2 ± 10012.3 (n = 5) ^{2,3}	69613.5 ± 17201.7 (n = 6)	0.129
9 months	Wildtype	Not applicable	111041.0 ± 18096.1 (n = 7)	
18 months	Transgenic	124320.8 ± 10888.0 (n = 8) ²	75983.0 ± 7448.5 (n = 8)	0.197
18 months	Wildtype	Not applicable	62436.0 ± 5622.8 (n = 6)	

π... Data from the pilot study;

¹... Comparing TG vs. WT animals in TH IHC by Student’s T Test;

²... p<0.05 compared to 2 month old mice (Mann-Whitney U Test with Bonferroni correction for multiple comparisons);

³... p<0.05 compared to 6 month old mice (Mann-Whitney U Test with Bonferroni correction for multiple comparisons). 15G7... human αSYN, TH... tyrosine hydroxylase, TG... transgenic, WT... wildtype, IHC... immunohistochemistry, SEM... standard error of mean.

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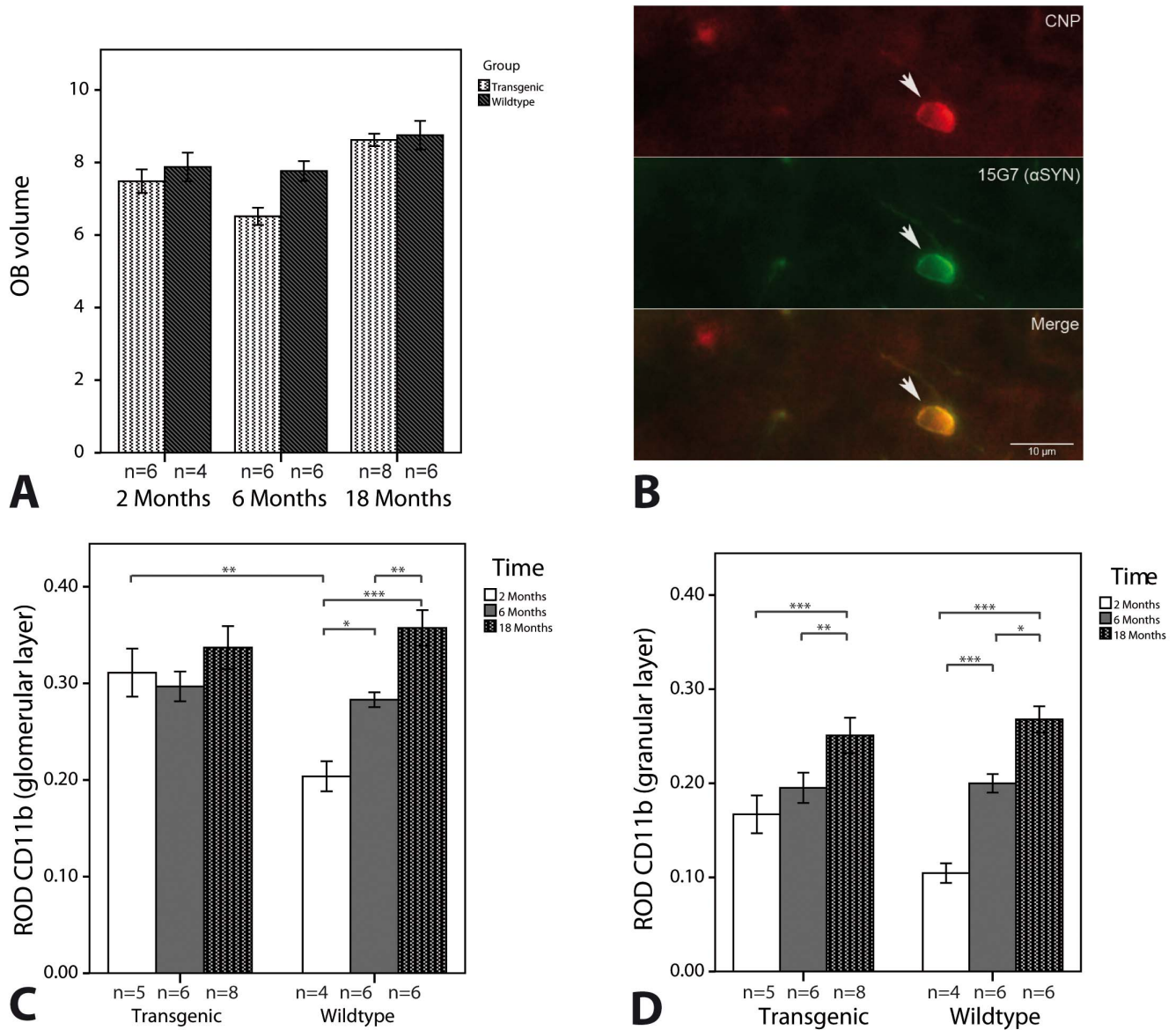


Figure 2. Immunohistochemistry of confirmatory long-term study. A...OB volume; B... Immunofluorescence for CNPase (red) and 15G7 (green) confirmed α SYN expression in glial cells (arrow), scale bar: 10 μ m; ROD of CD11b immunohistochemistry in the (C) glomerular layer and the (D) granular layer of the OB; All data are expressed as mean; error bars indicate the standard error of mean. Sample sizes are reported below the X-axis; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. doi:10.1371/journal.pone.0064625.g002

extensive olfactory bulb pathology has been reported in PD α SYN mouse models [21–23].

In the present study, we explored olfactory function and OB pathology in a transgenic mouse model with targeted oligodendroglial overexpression of h α SYN driven by the PLP promoter [14] featuring MSA-like inclusion pathology. Our data clearly show that olfactory preference testing, a widely used behavioral paradigm to identify specific olfactory deficiencies (i.e. the ability to sense attractive scents) [25,26], was not impaired in the PLP-h α SYN mouse model. This finding is in contrast to a previous study in a related mouse model overexpressing α SYN under the myelin basic protein (MBP) promoter which reported increased pellet retrieval latencies in tg compared to wt animals [24]. The discrepancy might be due to differences between the models used - including myelination with PLP-h α SYN mice lacking obvious

demyelination at ages up to 18 months [14] and MBP-h α SYN mice showing myelin damage at young ages already [15]. Other methodological issues (i.e. olfactory preference testing versus buried pellet test) might also have contributed to the observed differences. To verify that the absence of obvious smell deficits in PLP-h α SYN mice is a reliable observation, we performed neuropathological work-up demonstrating lack of genotype-specific OB atrophy as well as lack of accelerated neuronal loss in the transgenic OB, despite a trend towards a lower TH-immunoreactive cell number within the OB of 9 months old tg animals. The PLP-h α SYN transgenic model reproduces MSA-like selective vulnerability of the different subpopulations of dopaminergic neurons in SNc and OB. Putting these considerations into a clinical perspective, it has to be emphasized that previous clinical studies in human MSA found varying degrees of olfactory deficits

with impairment being less pronounced in MSA compared to PD [3,7,31–33]. In addition, α SYN inclusion pathology has been demonstrated in the human OB [12], however, a recent clinicopathological case series could not identify any smell deficit in MSA [5].

To exclude oversights due to age-related neurodegeneration, we subsequently conducted a long-term confirmatory study focusing on neuropathological read-outs. Age-dependent accumulation of α SYN immunoreactive inclusions with a significant increase at 9 and 18 months compared 2 months was observed in transgenic OB. This finding is partly in agreement with the human disorder showing GCIs in the OB [12], however, the temporal evolution of GCI pathology in the OB has not been studied in MSA patients so far. Surprisingly, the inclusion pathology did not convert into neurodegeneration. Neither olfactory bulb volume nor TH-ir cell numbers within the glomerular layer of the OB were significantly different between wt and tg animals. In the glomerular layer of the OB, microglial activation was more pronounced in tg compared to wt animals at 2 months of age. However, there was no age-related effect on CD11b immunoreactivity in tg animals, whereas microglial activation continuously increased in wt animals. In the granular layer, age-related enhancement of CD11b immunoreactivity was observed in both, tg and wt animals. These findings are in line with a previous study reporting early and sustained microglial activation affecting the striatum and the SNc of PLP- α SYN mice [34]. Finally, it has to be acknowledged that additional olfactory tests may be helpful to study independent functional domains associated with olfaction in 9 months old mice and further longitudinal studies are required to exclude late-onset

olfactory deficits paralleling the progressive OB α -synucleinopathy that has been observed in the present study. However, preserved olfaction at 9 months of age clearly separates the PLP- α SYN MSA mouse model from corresponding PD mouse models [18,19]. Semi-quantitative analysis of microglial activation by OD measurements of CD11b immunostainings may be affected by various factors; therefore, we applied counter-measures including (1) the calculation of relative OD values to account for different labeling intensities, and (2) the acquisition of all images during a single microscopy session at uniform microscopy settings to vigorously control confounding factors.

To the best of our knowledge this is the first analysis of olfactory behavior as well as candidate neuropathology in the context of a transgenic MSA mouse model. Our experimental data suggest preserved olfactory function providing further support to a recent notion claiming that olfactory deficits are unlikely in MSA [5] reflecting the unique oligodendroglial pathology.

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Author Contributions

Conceived and designed the experiments: FK GKW YL WP NS. Performed the experiments: FK YL NS. Analyzed the data: FK NS. Wrote the paper: FK NS. Reviewed/critiqued statistical analysis: GKW YL WP. Reviewed/critiqued the manuscript: GKW YL WP. Approved the final manuscript version: FK GKW YL WP NS.

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