

1 **Spread of pathological α -synuclein from urogenital nerves initiates multiple**
2 **system atrophy-like symptoms**

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34

35 **Abstract**

36 Multiple system atrophy (MSA) is a fatal adult-onset movement disorder with autonomic failures,
37 especially urogenital dysfunction. The neuropathological feature of MSA is the accumulation of
38 misfolded α -synuclein (α -Syn) in the nervous system. Here, we show that misfolded α -Syn exist
39 in nerve terminals in detrusor (DET) and external urethral sphincter (EUS) of patients with MSA.
40 Moreover, α -Syn preformed fibrils inoculated into the EUS or DET in TgM83^{+/-} mice initiated the
41 transmission of misfolded α -Syn from the lower urinary tract to brain, and these mice developed
42 α -Syn inclusion pathology through micturition reflex pathways along with urinary dysfunction and
43 motor impairments. These findings indicate that spreading of misfolded α -Syn from the autonomic
44 control of the lower urinary tract to the brain via micturition reflex pathways induces autonomic
45 failure and motor impairments. These results provide important new insights into the pathogenesis
46 of MSA as well as highlight potential targets for early detection and therapeutics.

47

48 **Introduction**

49 Multiple system atrophy (MSA) is a fatal, multisystem, neurodegenerative disorder characterized
50 by a variable combination of rapidly progressive autonomic failures, ataxia, and parkinsonism.
51 According to the most recent guidelines, autonomic failures featuring urogenital dysfunction,
52 orthostatic hypotension, and respiratory disorder are premonitory symptoms and necessary for
53 the diagnosis of MSA (Gilman et al., 2008). Retrospective data indicate that among autonomic
54 failures, urological symptoms occur several years prior to the neurological symptoms in the
55 majority of MSA patients (Beck, Betts, & Fowler, 1994; Jecmenica-Lukic, Poewe, Tolosa, &

56 Wenning, 2012; Sakakibara et al., 2000). Urogenital dysfunction in patients with extrapyramidal
57 symptoms is thought to help differentiate between Parkinson's disease (PD) and MSA in early
58 disease stages (Wenning et al., 1999). Consistently, neuropathological studies reveal that
59 widespread pathological lesions of micturition reflex pathways, including the periaqueductal gray
60 (PAG), Barrington's nucleus (BN), intermediolateral columns (IML), Onuf's nucleus of the spinal
61 cord and so on, is present in central nervous system (CNS) of MSA patients (Stemberger, Poewe,
62 Wenning, & Stefanova, 2010; VanderHorst et al., 2015). So far, few animal models of MSA have
63 been established to display MSA-like urinary dysfunction and denervation-reinnervation of EAS
64 simultaneously. It has been acknowledged that the cellular hallmark lesion of MSA is misfolded
65 α -synuclein (α -Syn) accumulation within glial cytoplasmic inclusions along with neuronal
66 inclusions (NIs) in central nervous system (CNS). Moreover, Watts et. al. reported that brain
67 homogenates from MSA cases induced widespread deposits of phosphorylated α -Syn in the brains
68 of MSA-inoculated mice, suggesting that α -Syn aggregates in the brains of MSA are transmissible
69 (Watts et al., 2013).

70 In this study we show that misfolded α -Syn exist in nerve terminals in detrusor (DET) and
71 external urethral sphincter (EUS) of patients with MSA. Also, we injected α -Syn preformed fibrils
72 (PFFs) to the lower urinary tract of hemizygous TgM83^{+/-} mice, we observed the widespread α -
73 Syn inclusion pathology from the autonomic control of the lower urinary tract to the brain along
74 with urinary dysfunction and motor impairments

75

76 **Results**

77 **Clinical characteristics of patients**

78 Forty-five patients were diagnosed as MSA, PD, or PSP according to the consensus criteria
79 (Gilman et al., 2008; Kalia & Lang, 2015; Litvan et al., 1996). Informed consent was obtained for
80 each subject or their authorized surrogates on behalf of patients who lack decision-making ability.
81 Clinical descriptions for each type of disease are summarized in Table 1. Among 32 patients (12
82 males and 20 females) with MSA, 13 patients were MSA with predominant parkinsonism (MSA-
83 P) while 19 patients were MSA with predominant cerebellar ataxia (MSA-C). The mean \pm SD of
84 patients' ages for these two types of MSA at the time of clinically diagnosed were 62.1 ± 7.1 y and
85 57.8 ± 6.6 y, respectively. The UMSARS scores were utilized for the evaluation of patients'
86 urological function. The score values (mean \pm SD) of MSA-P and MSA-C were 44.4 ± 25.7 and

87 26.7 ± 15.3, respectively. In addition, these patients all had autonomic symptoms, including
88 urological dysfunction, orthostatic dysregulation or chronic constipation (Low et al., 2015;
89 Stefanova, Bucke, Duerr, & Wenning, 2009; Wenning et al., 2004). Furthermore, the positive rates
90 of urodynamic examination and perianal electromyography in MSA were 88.1% and 81.0%,
91 respectively. Altogether, these data indicate that urological dysfunction is specific and common in
92 patients with MSA.

93

94 **Detection of misfolded α -Syn in patients' samples**

95 We then investigated the deposits of misfolded α -Syn in MSA patients' DET or EUS, using anti-
96 α -Syn filament, anti-phosphorylated α -Syn (p α -Syn), and anti-aggregated- α -Syn (5G4) antibodies
97 (Fig. 1A-H). Among 32 patients with MSA, 23 MSA cases exhibited deposits of misfolded α -Syn
98 in biopsy tissues (Fig. 1A-F and Table 1). Moreover, the portion of misfolded α -Syn in the triangle
99 region, right wall, and left wall of bladder is similar ($P > 0.05$) and has no significant difference
100 between MSA-P and MSA-C ($P > 0.05$). Among the patients examined, one had a urinary
101 incontinence for 6 years before presence of movement deficits, and we made diagnosis for him as
102 MSA after 8 months of movement deficits. A large amount of misfolded α -Syn was found in his
103 DET and EUS. Most remarkably, no PD cases and PSP cases tested show misfolded α -Syn in
104 bladder (Fig. 1G, H, I). Twenty control subjects didn't show misfolded α -Syn in bladders either
105 (Fig. 1I). Taken together, these results show that misfolded α -Syn exists in DET or EUS from 71.9%
106 of the collected patients with MSA, while PD, PSP, and control subjects exhibit no detectable
107 misfolded α -Syn in their bladders from this study.

108

109 **Identification of the micturition reflex pathways controlling EUS or DET using** 110 **Fluoro-Gold (FG)**

111 After we found that misfolded α -Syn proteins exist in DET and EUS of the detected patients, we
112 then used Fluoro-Gold (FG) injection to trace the micturition reflex pathways controlling EUS or
113 DET in mice. FG was injected into both sides of EUS or DET in TgM83^{+/-} and C57BL/6 mice; the
114 sections from different parts of nervous system were detected at 14-day post-injection. FG-labeled
115 neurons were detected in pelvic ganglia, spinal cord, pons, and midbrain bilaterally in both mouse
116 models (Fig. 2). In spinal cord, we found that FG-labeled neurons also existed in T2 level, which
117 has not been previously reported. At T2 level, the FG-labeled neurons mostly appeared in ventral

118 horn and IML, which are closely associated with motor and autonomic functions. Among other
119 levels of spinal cord, FG-labeled neurons gathered in the EUS motoneurons of lamina IX (ExU9)
120 and sacral parasympathetic nucleus (SPSy) at S1 level, in EAS motoneurons of lamina IX (ExA9),
121 ExU9, gluteal motoneurons of lamina IX (Gl9), lamina VII of the spinal gray (7Sp), and lateral
122 spinal nucleus (LSp) at L6 level, in psoas motoneurons of lamina IX (Ps9), quadriceps
123 motoneurons of lamina IX (Q9), intercalated nucleus (ICL), IML, and lumbar dorsal commissural
124 nucleus (LDCom) at L2 level. In brain, FG-labeled neurons appeared in BN, PAG, and locus
125 coeruleus (LC), and these nuclei have been reported to participate in micturition reflex pathways
126 (Fowler, Griffiths, & de Groat, 2008). In addition, FG-labeled neurons were found to be in
127 parvocellular reticular nucleus alpha (PCRtA), mesencephalic trigeminal nucleus (Me5), and red
128 nucleus (RN), which are involved in the general locomotion, postural control, and modulation of
129 certain sensory and autonomic functions. Taken together, these results suggest that the micturition
130 reflex pathways controlling EUS and DET are connected not only with the autonomic nervous
131 system but also with the central motor pathways.

132

133 **Spreading of phosphorylated α -Syn from the lower urinary tract to the brain in** 134 **TgM83^{+/-} mice via micturition reflex pathways**

135 Our above-mentioned retrograde tracing study with FG has identified the pathways controlling
136 EUS or DET. To demonstrate whether spreading of misfolded α -Syn via the same pathways
137 induces the MSA-like neuropathology, we next injected α -Syn preformed fibrils (PFFs) (Figure 3-
138 figure supplement 1) to EUS or DET (Figure 3-figure supplement 1) in TgM83^{+/-} mice and
139 evaluated phosphorylated α -Syn in different sections at different time points.

140 Immunohistochemical results show that p α -Syn, immunostained with the anti-p α -Syn antibody,
141 were detected at 5-month post-injection in both EUS- and DET- α -Syn PFFs TgM83^{+/-} mice (Fig.
142 3). In contrast, p α -Syn has not been detected in EUS-PBS, DET-PBS TgM83^{+/-}, and C57BL/6
143 mice post-injection (Figure 3-figure supplement 2). In EUS- α -Syn PFFs TgM83^{+/-} mice, small
144 numbers of p α -Syn were detected in EUS and pelvic ganglia (Fig. 3A, B). Furthermore, p α -Syn
145 was detected at the S1, L6, L2, and T2 levels of spinal cord; and they mostly existed in laminae
146 V-VII and IX of these levels (Fig. 3C-F). In brain, p α -Syn existed in pons and midbrain (Fig. 3H,
147 D). These observations are consistent with the results of the above-mentioned FG study.
148 Additionally, using immunohistochemical approach, p α -Syn was also found in the cerebellar

149 nuclei (Fig. 3G). The neuropathological findings in DET- α -Syn PFFs TgM83^{+/-} mice were similar
150 to those in EUS- α -Syn PFFs TgM83^{+/-} mice. In conclusion, transmission of pathological α -Syn in
151 these mice invades not only the autonomic nervous system associated with urinary function, but
152 also the extrapyramidal system via the micturition reflex pathways, and these findings are
153 consistent with MSA pathology found in patient autopsy (Cykowski et al., 2015; Stemberger et al.,
154 2010; VanderHorst et al., 2015; Yoshida, 2007). Thus, these findings suggest that pathological α -
155 Syn spreads from the autonomic innervation of the lower urinary tract to extrapyramidal system
156 via the micturition reflex pathways, leading to widespread α -Syn inclusion pathology.

157 To further characterize the nature of α -Syn-positive deposits in diseased EUS- or DET- α -Syn
158 PFFs TgM83^{+/-} mice and the distribution of pathological α -Syn in various cells, double
159 immunofluorescence staining was then employed. First, we revealed that deposits of p α -Syn
160 colocalized with ubiquitin in spinal cord, cerebellum, and BN of diseased EUS- or DET- α -Syn
161 PFFs TgM83^{+/-} mice (Fig. 4A, C, G). Then, we found that p α -Syn colocalized with Iba-1
162 (microglia marker) in spinal cord, ventral pons, and midbrain (Fig. 4B, J, K), which indicates that
163 microglial activation is involved in the pathological process. Additionally, we observed a small
164 amount of p α -Syn in LC (Fig. 4F) and a large number of p α -Syn in PAG and RN (Fig. 4H). No
165 p α -Syn was detected in substantia nigra pars compacta (SNc) (Fig. 4H). We also noticed high level
166 of ubiquitin protein in dopamine (DA) neurons of SNc (Fig. 4I). Finally, we identified that
167 transmission of α -Syn PFFs through micturition reflex pathways resulted in axonal pathology and
168 demyelination in diseased EUS- α -Syn PFFs TgM83^{+/-} mice with a distinct loss of myelin basic
169 protein (MBP) and neurofilament (Fig. 4D, E). Taken together, these results suggest that injection
170 of α -Syn PFFs into EUS or DET of TgM83^{+/-} mice initiates pathological α -Syn transmission,
171 including microglia activation, axonal pathology, and demyelination.

172 An immunoblot of spinal cord, pons, and PAG homogenate probed with α -Syn (Ser129P) and
173 aggregated α -Syn (clone 5G4) antibodies was conducted to confirm that injection of α -Syn PFFs
174 into EUS or DET of TgM83^{+/-} mice initiates MSA-like neuropathology (Fig. 5). In the insoluble
175 fractions, immunostaining with α -Syn (Ser129P) reveals that bands around 15 kDa were detected
176 in all examined diseased EUS- α -Syn TgM83^{+/-} mice, while it was faintly detected in EUS- α -PBS
177 TgM83^{+/-} mice (Fig. 5A, D, F). Statistical analysis shows that p α -Syn is significantly increased in
178 diseased EUS- α -Syn TgM83^{+/-} mice (Fig. 5B, E, G). Immunoblotting of the insoluble fractions of
179 spinal cord homogenates using the aggregated α -Syn (clone 5G4) antibodies exhibits the bands

180 around 35 kDa in diseased EUS- α -Syn TgM83^{+/-} mice but not in age-matched EUS- α -PBS
181 TgM83^{+/-} mice (Fig. 5A). Statistical data indicate that the level of α -Syn aggregates is significantly
182 elevated in EUS- α -Syn TgM83^{+/-} mice (Fig. 5C). These data indicate that the injection of α -Syn
183 PFFs into EUS or DET can induce the spreading of pathological α -Syn in CNS.

184

185 **Early-onset denervation-reinnervation of EAS in EUS- or DET- α -Syn PFFs TgM83^{+/-}** 186 **and C57BL/6 mice**

187 Forty age-matched healthy TgM83^{+/-} and C57BL/6 male mice were used; no abnormal EAS
188 electromyogram (EMG) in these mice was detected. Based on the EMGs of the previous literature
189 (Daube & Rubin, 2009; Palace, Chandiramani, & Fowler, 1997; Schwarz, Kornhuber, Bischoff,
190 & Straube, 1997), abnormal EAS EMG would be defined if the EMG findings satisfied any one
191 of the following six conditions: (1) fibrillation potentials; (2) positive sharp waves; (3) CRD; (4)
192 fasciculation potentials; (5) myokymic discharges; and (6) satellite potential.

193 EUS- and DET- α -Syn PFFs TgM83^{+/-} mice show abnormal EAS EMGs at 2-month post-
194 injection ($P < 0.05$), while no abnormality of EAS EMG was detected in PBS groups.
195 Representative abnormal and normal EAS EMGs are shown in Fig. 6A-E and F, respectively. The
196 prevalence of abnormal EAS EMGs in EUS- α -Syn PFFs TgM83^{+/-} mice was 55%, 84%, and 90%
197 at 2-month, 4-month, and 6-month post-injection, respectively, versus 51%, 83%, and 91% in
198 DET- α -Syn PFFs TgM83^{+/-} mice, respectively (Fig. 6G, H). In C57BL/6 mice, the data of
199 abnormal EAS EMGs show no significant difference between EUS- or DET- α -Syn PFFs groups
200 and PBS groups (Fig. 6I, J). The results suggest prevalence of abnormal EAS EMGs increases
201 along with the progression of neural lesions caused by α -Syn PFFs. We also injected α -Syn PFFs
202 into the intestine wall of stomach and duodenum of TgM83^{+/-} mice. However, the TgM83^{+/-} mice
203 with intestine- α -Syn PFFs didn't develop abnormal EAS EMG while TgM83^{+/-} mice did. Taken
204 together, the denervation-reinnervation of EAS occurs in the early stage of neuropathological
205 process in a time dependent manner, and may be caused by spreading of α -Syn PFFs from the
206 lower urinary tract through micturition reflex pathways.

207

208 **Urinary dysfunction in EUS- or DET- α -Syn PFFs TgM83^{+/-} mice**

209 The urodynamic baseline is determined by cystometry results of 2-month-old male TgM83^{+/-} and
210 C57BL/6 mice prior to treatments. Urinary dysfunction was observed in EUS- and DET- α -Syn

211 PFFs TgM83^{+/-} mice between 3 and 4 months post-injection and persisted to the last stage
212 examined. At 4-month post-injection, both EUS- and DET- α -Syn PFFs TgM83^{+/-} mice exhibited
213 a significant increase in amplitude, PVR, and NVCs during the filling phase compared to PBS
214 groups ($P < 0.05$). Meanwhile, VV and ICI in EUS- or DET- α -Syn PFFs TgM83^{+/-} mice were
215 found less and shorter, respectively (Fig. 7). The body mass of EUS- or DET- α -Syn PFFs TgM83^{+/-}
216 mice was mostly lighter than EUS- or DET-PBS TgM83^{+/-} mice; however, the bladder of EUS- or
217 DET- α -Syn PFFs TgM83^{+/-} mice exhibited overtly greater size compared to EUS- or DET-PBS
218 mice (Figure 7-figure supplement 1), which was probably due to progressive urothelium and DET
219 hyperplasia in α -Syn PFFs TgM83^{+/-} mice. By the time of the 14th month post-injection, EUS- or
220 DET- α -Syn PFFs C57BL/6 mice did not show any urinary dysfunction. The intestine- α -Syn PFFs
221 TgM83^{+/-} mice didn't show any urinary dysfunction at 3.5-month post-injection neither when
222 EUS- and DET- α -Syn PFFs TgM83^{+/-} mice did already. All results mentioned above suggest that
223 urodynamic assessment in EUS- or DET- α -Syn PFFs TgM83^{+/-} mice was characterized by an
224 overactive, less stable, and inefficient bladder. In addition, α -Syn PFFs injection into EUS or DET
225 of TgM83^{+/-} mice caused potential dyssynergia between DET and EUS, leading to hyperactive
226 bladder and DET hyperreflexia (Boudes et al., 2013; Hamill et al., 2012), which resembles urinary
227 dysfunction in patients with MSA. Thus, we developed an animal model to replicate MSA-like
228 urinary disorders and abnormal EAS EMGs, which has not been previously reported.

229

230 **Motor impairments in EUS- or DET- α -Syn PFFs TgM83^{+/-} mice**

231 Both EUS- and DET- α -Syn PFFs TgM83^{+/-} mice began to exhibit motor impairments from 5-
232 month post-injection. Most diseased mice presented an arched back initially and then progressed
233 with weight loss, ataxia, paralysis, and a moribund state requiring euthanasia within 3 weeks (Fig.
234 8A). Compared with DET- α -Syn PFFs TgM83^{+/-} mice, the behavioral deficiency was more
235 obvious in EUS- α -Syn PFFs TgM83^{+/-} mice. At 5-month post-injection, α -Syn PFFs TgM83^{+/-}
236 mice showed significantly increased motor behavioral scale (MBS) score compared with EUS- or
237 DET-PBS TgM83^{+/-} mice, which is considered as a semi-quantitative assessment for MBS rating
238 (Fig. 8B and Figure 8-figure supplement 1). The rotarod test was carried out to assess coordination
239 capability. The performance on the rotating rod was significantly impaired in EUS- and DET- α -
240 Syn PFFs TgM83^{+/-} mice compared to PBS controls, as their latency to fall was markedly reduced
241 (Fig. 8C). In an open field test, EUS- and DET- α -Syn PFFs TgM83^{+/-} mice showed significantly

242 reduced spontaneous activities in comparison with PBS-injected mice (Fig. 8J, K). Footprint
243 analysis indicates that EUS- and DET- α -Syn PFFs TgM83^{+/-} mice have shorter stride length and
244 wider base width compared to PBS-injected mice (Fig. 8D, E and Figure 8-figure supplement 1).
245 Moreover, EUS- and DET- α -Syn PFFs TgM83^{+/-} mice also showed significantly motor
246 dysfunction in the beam walking test (Fig. 8F, G) and pole test (Fig. 8H, I). EUS- or DET-PBS
247 TgM83^{+/-} mice didn't show any phenotype until they were 22 months old, consistent with our
248 spontaneously sick TgM83^{+/-} mice in timeline. As the previous study reported, spontaneously sick
249 TgM83^{+/-} mice develop series of phenotypes between 22-28 months of age (Giasson et al., 2002).
250 Nevertheless, EUS- and DET- α -Syn PFFs C57BL/6 mice failed to exhibit behavioral
251 abnormalities up to 420-day post-injection (Figure 8-figure supplement 2).
252 Taken together, EUS- and DET- α -Syn PFFs TgM83^{+/-} mice developed distinct motor signs
253 including weight loss, bradykinesia, ataxia, and paralysis at 5-month post-injection. We conclude
254 that injection with α -Syn PFFs into EUS or DET in TgM83^{+/-} mice initiates MSA-like motor
255 deficits.

256

257 **Discussion**

258 We broadened the landscape of the pathogenesis of synucleinopathies. Prior to this study, we had
259 some understanding of the pathogenesis of PD, while we knew less about that of MSA. In MSA,
260 autonomic dysfunctions, especially urinary dysfunction (Kirby, Fowler, Gosling, & Bannister,
261 1986), are severe and common which are different from the other main synucleinopathy, e.g., PD.
262 Based our long-term observations of clinical subjects with these diseases, we reckoned that
263 pathological α -Syn might exist in the lower urinary tract at the early stage of MSA instead of gut
264 as shown in PD (Holmqvist et al., 2014). To test this hypothesis, we first performed bladder biopsy
265 in participants. The findings from this study show that misfolded α -Syn aggregates indeed exist in
266 DET or EUS in 71.9% of the included MSA patients. The subsequent results from
267 immunohistochemistry studies in experimental mice show that α -Syn aggregates invade the
268 micturition reflex pathways. In addition, we found widely positive staining of p α -Syn in ventral
269 white matter of spinal cord, possibly due to nerve tracts from brain and comprehensively
270 longitudinal connections by synapses of numerous nerve fibers. Moreover, we detected overt α -
271 Syn aggregates in cerebellar nucleus which indicate that α -Syn aggregates transmit to cerebellum
272 via rubro-cerebello-rubrospinal circuit (Larson-Prior & Cruce, 1992). α -Syn pathology in EUS- or

273 DET- α -Syn PFFs C57BL/6 mice was not detected at 14-month post-injection. The results of
274 double immunofluorescence analysis further demonstrate the pathological lesions in CNS of the
275 two mouse models. There was apparent microglial activation and demyelination in CNS of EUS-
276 α -Syn PFFs TgM83^{+/-} mice, which is a major pathological feature of MSA (Ettle et al., 2016). The
277 immunostaining results validate the hypothesis that pathological α -Syn transmits initially from
278 urogenital autonomic nerves to extrapyramidal system, inducing α -Syn inclusion pathology. EAS
279 EMG has been previously proposed as a diagnostic method for MSA (E. A. Lee, Kim, & Lee,
280 2002). Abnormalities of EAS EMG in MSA indicate the denervation-reinnervation of EAS caused
281 by neuronal loss of Onuf's nucleus in the anterior horn of the spinal cord (E. A. Lee et al., 2002;
282 Libelius & Johansson, 2000). In this study, we conducted EAS EMG in mouse models to assess
283 denervation-reinnervation of EAS. Remarkably, abnormal EAS EMGs emerged at 2-month post-
284 injection in EUS- or DET- α -Syn PFFs TgM83^{+/-} mice. Further, overall prevalence reached 90% in
285 EUS- α -Syn PFFs TgM83^{+/-} mice versus 91% in DET- α -Syn PFFs TgM83^{+/-} mice at 6-month post-
286 injection, while no abnormality was detected in PBS-injected TgM83^{+/-} mice. We demonstrate that
287 electromyography experimental results in the mouse models are similar to EAS EMG feature of
288 MSA patients preceding urinary dysfunction and movement disorders. Previous studies
289 (Yamamoto et al., 2005) presented a view that selective neuronal loss of Onuf's nucleus, which
290 innervates EAS, results in abnormal EAS EMGs in patients with MSA. We found that α -Syn
291 aggregates are present in Onuf's nucleus in both EUS- and DET- α -Syn PFFs TgM83^{+/-} mice.

292 In this study, we implemented urodynamic assessment in different time points to evaluate
293 urinary function in experimental mice. Consequently, EUS- or DET- α -Syn PFFs TgM83^{+/-} mice
294 exhibited urodynamic changes after the 3rd month post-injection, prior to motor impairments,
295 versus no changes in PBS groups. Here, we identified that urinary dysfunction, characterized by
296 urinary bladder hyperreflexia of α -Syn PFFs TgM83^{+/-} mice, replicates the altered bladder function
297 in MSA patients including urinary incontinence, frequency, urgency, and retention (Fowler,
298 Dalton, & Panicker, 2010; Ragab & Mohammed, 2011). Previous study (Libelius & Johansson,
299 2000) showed that the spontaneous TgM83^{+/-} mice developed urinary bladder dysfunction prior to
300 motor dysfunction due to A53T mutant α -Syn. In our study, EUS- or DET- α -Syn PFFs TgM83^{+/-}
301 mice started to perform urinary dysfunction at 3.5-month post-injection whereas EUS- or DET-
302 PBS and non-inoculated TgM83^{+/-} mice didn't show any urinary dysfunction until they were 22
303 months old. The occurrence of urinary dysfunction in EUS- or DET- α -Syn PFFs TgM83^{+/-} mice

304 is earlier than PBS control groups and non-inoculated TgM83^{+/-} mice. In our α -Syn PFFs TgM83^{+/-}
305 mice, the micturition reflex pathways, including EUS and DET, pelvic ganglia, Onuf's nucleus,
306 IML, PAG, BN, and LC, exhibited misfolded α -Syn aggregates, revealing pathological
307 mechanisms of the urinary dysfunction. However, we did not observe appreciable levels of
308 misfolded α -Syn deposition in oligodendrocytes within the TgM83^{+/-} mice. This observation could
309 be explained by misfolded α -Syn originating from different parts of PNS to CNS via neuronal
310 projections transsynaptically. In spontaneously ill TgM83^{+/-} mice, α -Syn aggregates have not been
311 detected in ExU9, ICL, ExA9, Gl9, sacral dorsal commissural nucleus, IML, LDCom, BN, and
312 PAG, which is different from the diseased EUS- α -Syn PFFs TgM83^{+/-} mice. As these spared areas
313 are involved in controlling the urinary bladder (Fowler et al., 2008), these data support that the
314 preceding autonomic dysfunction of EUS- or DET- α -Syn PFFs TgM83^{+/-} mice results from
315 exogenously injected α -Syn PFFs instead of A53T mutant α -Syn. Therefore, our results suggest
316 that misfolded α -Syn spreading through the micturition reflex pathways retrogradely may lead to
317 urinary dysfunction.

318 Previous studies indicate that pathological α -Syn spread from peripheral nervous system (PNS)
319 to CNS through retrograde axonal transport, in a stereotypical and topographical pattern (Bernis
320 et al., 2015; Braak et al., 2003; Holmqvist et al., 2014; Luk, Kehm, Carroll, et al., 2012).
321 Experimental studies suggest that PD pathology may originate in the vagal nerves from the gut
322 and gradually propagate to the brain (Holmqvist et al., 2014). These findings support the
323 hypothesis that different synucleinopathies may originate from different part of PNS and gradually
324 propagate to CNS. Hence, we speculate that pathological α -Syn originate from the autonomic
325 innervation of the lower urinary tract has the potential to propagate to CNS and induce MSA Our
326 data demonstrate that misfolded α -Syn can induce α -Syn inclusion pathology along with
327 autonomic failure and motor impairments by transmitting from the autonomic control of the lower
328 urinary tract to the brain via micturition reflex pathways.

329 As previously reported by others, peripheral injection of α -Syn PFFs into multiple sites could
330 promote the development of α -Syn pathology in the CNS of TgM83^{+/-} mice (Ayers et al., 2017;
331 Breid et al., 2016; Holmqvist et al., 2014; Luk, Kehm, Carroll, et al., 2012; Sacino, Brooks,
332 Thomas, McKinney, Lee, et al., 2014; Sacino, Brooks, Thomas, McKinney, McGarvey, et al.,
333 2014; Watts et al., 2013). We injected α -Syn PFFs into the striatum of TgM83^{+/-} mice in initial
334 studies. The results show that the lower urinary tract pathology can't be obtained at 6th month

335 after intracerebral α -Syn PFFs injection. We also injected α -Syn PFFs into the intestine wall of
336 stomach and duodenum of TgM83^{+/-} mice. However, the intestine- α -Syn PFFs TgM83^{+/-} mice
337 didn't develop abnormal EAS EMG and urinary dysfunction at 5-month post-injection when they
338 had α -Syn pathology in CNS and motor impairments already. Thus, α -Syn injection into intestine
339 wall alone couldn't induce the denervation-reinnervation of EAS and urinary dysfunction prior to
340 motor impairments in TgM83^{+/-} mice. Again, this further indicates that α -Syn of PD and MSA may
341 start in different places.

342 Here we further demonstrate that injection with α -Syn PFFs into EUS or DET induces a rapid
343 progression of motor dysfunctions. Our study shows that injection with α -Syn PFFs into EUS or
344 DET in TgM83^{+/-} mice causes not only seeding of α -Syn aggregation in the CNS, but also rapid
345 progressive motor dysfunctions evaluated using a spectrum of behavioral tests. From our findings,
346 the occurrence of motor impairments in our EUS- or DET- α -Syn PFFs TgM83^{+/-} mice was much
347 earlier than spontaneously ill TgM83^{+/-} mice (Giasson et al., 2002). According to previous studies
348 (Breid et al., 2016; Holmqvist et al., 2014; S. B. Prusiner et al., 2015; Sacino, Brooks, Thomas,
349 McKinney, Lee, et al., 2014; Sacino, Brooks, Thomas, McKinney, McGarvey, et al., 2014), the
350 animal models of synucleinopathy induced by exogenous inoculation involve different inocula and
351 inoculation positions, developing variable α -Syn pathology and motor impairments without
352 autonomic dysfunction. Furthermore, pathology of α -Syn inclusions observed in motor neuron of
353 ventral horn, cerebellum, and RN in α -Syn PFFs TgM83^{+/-} mice provides compelling
354 neuropathological evidence for the motor impairments.

355 In summary, this study suggests one possible pathogenic mechanism of MSA, which is the
356 spreading of α -Syn inclusion pathology from the autonomic control of the lower urinary tract to
357 the brain. Also, our data support the view that pathological α -Syn may originate from different
358 parts of PNS among different disorders of synucleinopathies. However, the pathogenic
359 mechanisms of MSA are not fully understood, other possibilities may exist. Thus, we and others
360 will need to investigate further.

361

362 **Materials and methods**

363 **Patients**

364 Forty-five patients (18 men, 27 women; age 60.6 ± 7.2 years) were enrolled consecutively from
365 2016 to 2018 with MSA (32 patients), PD (7 patients), or progressive supranuclear palsy (PSP) (6

366 patients) according to consensus criteria (Kalia & Lang, 2015; Litvan et al., 1996; Stefanova et al.,
367 2009), respectively. In MSA, the phenotype was characterized by prevalently cerebellar signs in
368 19 patients and by parkinsonian signs in the remaining 13 patients. Disease severity was evaluated
369 using Unified Multiple System Atrophy Rating Scale (UMSARS) (Low et al., 2015; Wenning et
370 al., 2004). UMSARS Total is a sum of UMSARS I and UMSARS II. Demographic and clinical
371 data are summarized in Table 1. At the time of enrollment, all subjects underwent clinical and
372 electrophysiological evaluation as well as EUS and bladder biopsies at 3 sites: left wall, right wall,
373 and triangle region. Twenty subjects were also included in the study as controls (7 men, 13 women;
374 age 58.5 ± 7.0 years). All biopsies were performed according to the outpatient procedures by
375 experienced urologists in a prescriptive exam room. Cystoscopy was performed using standard
376 cystoscope according to previously published procedures under local anesthesia with 1%
377 xylocaine (Butros, McCarthy, Karaosmanoglu, Shenoy-Bhangle, & Arellano, 2015). The
378 procedure was repeated until the EUS, left wall, right wall, and triangle region of bladder tissues
379 were obtained. Samples were immediately fixed in 4% paraformaldehyde and kept at 4 °C for at
380 least 2 days. The study was executed with the approval of the Institutional Ethics Committees of
381 the Zhengzhou University.

382

383 **Animals**

384 TgM83^{+/-} and C57BL/6 mice were purchased from Nanjing Biomedical Research Institute of
385 Nanjing University (Nanjing, China), and evaluated at the age of six to eight weeks. The
386 hemizygous TgM83 mice expressed the human A53T α -Syn driven by the prion gene promoter
387 (Giasson et al., 2002). C57BL/6 mice were chosen as the control mice because TgM83^{+/-} mice
388 were maintained on a mixed C57/C3H genetic background. Mice were kept in a near pathogen-
389 free environment under standard conditions with food and water (21 °C, 12h/12h light-dark cycle).
390 All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory
391 Animals. The protocols were approved by the Institutional Ethics Committees of the Zhengzhou
392 University.

393

394 **α -Synuclein preformed fibrils (PFFs) preparation**

395 α -Syn (S-1001, rPeptide) was resuspended in assembly buffer (20 mM Tris-HCl, 100 mM NaCl,
396 pH 7.4) at concentration of 1 mg/ml. To obtain PFFs, the samples were placed in 2 ml sterile

397 polypropylene tubes, sealed with parafilm, and agitated in a beaker with a magnetic stirrer (MS-
398 H-Pro+, Scilogex, China) at 350 rpm for 7 days at 37 °C. After 7 days of incubation, the α -Syn
399 fibrils were sonicated for 45 seconds using an ultrasonic cell disruptor at 10% of its peak amplitude
400 (Scientz-IID, Ningbo, China). α -Syn fibrils were stored at -80°C until use.

401

402 **Modeling surgery**

403 All injections were performed using a manual microinjector under an operating microscope. Mice
404 were anesthetized with isoflurane inhalation and fixed in a supine position. After disinfection, mice
405 were inoculated in the EUS with 15 μ l α -Syn PFFs (1 mg/ml) or phosphate buffered saline (PBS,
406 Solarbio), or DET with 20 μ l (1 mg/ml) α -Syn PFFs or PBS. Following the injection, the exposed
407 wound was sewn closed.

408

409 **EAS EMG**

410 EAS EMG was carried out in all animals before injection for control groups and at correspondingly
411 post-injection times to determine EAS denervation-reinnervation. Animals in each group
412 underwent EAS EMG as follows (Aghae-Afshar et al., 2009; Buffini, O'Halloran, O'Herlihy,
413 O'Connell, & Jones, 2012; Healy, O'Herlihy, O'Brien, O'Connell, & Jones, 2008; Lane et al., 2013):
414 anesthesia was induced using isoflurane inhalation. Limb withdrawal to paw pinch and corneal
415 reflexes of animals were observed to assess the level of anesthesia. After placing the animal supine,
416 shaving the thigh, and establishing a ground connection, a disposable concentric 30-gauge needle
417 electrode (Technomed Europe), which has a 25-mm length, 0.30-mm diameter, and 0.021-mm²
418 recording area, was inserted at the 3 or 9 o'clock position of the anal orifice perpendicularly into
419 the EAS from the perianal skin close to the mucocutaneous junction to a depth of approximately
420 1 to 2 mm. The point of the electrode insertion was adjusted under audio guidance until a
421 permanent tonic activity was recorded, in order to ensure that the electrode has entered EAS. If the
422 mouse discharged a fecal pellet during the recording process, a pair of forceps were used to gently
423 clip it out. EMG was performed with an EMG monitoring machine (MEB-2306C, NIHON
424 KOHDEN CORPORATION, Tokyo, Japan) at a sweep speed of 10 ms/div and a gain of 100
425 μ v/div. Abnormal EAS EMGs were simultaneously visualized and recorded. EMG activity was
426 quantified by prevalence of abnormal EAS EMGs (fibrillation potentials, positive sharp waves,

427 complex repetitive discharges (CRD), fasciculation potentials, myokymic discharges, and satellite
428 potential) in each group (Daube & Rubin, 2009; Palace et al., 1997; Schwarz et al., 1997).

429

430 **Cystometry evaluations and calculations**

431 The following urodynamic parameters (Boude et al., 2013; Fandel et al., 2016; Girard, Tompkins,
432 Parsons, May, & Vizzard, 2012; Y. S. Lee et al., 2013; Silva et al., 2015) were used for the current
433 study: (1) Maximum voiding pressure (P_{\max} ; cmH₂O); (2) Basal bladder pressure (BBP; cmH₂O):
434 the lowest bladder pressure during filling phase; (3) Amplitude (cmH₂O): P_{\max} - BBP; (4) Bladder
435 leak point pressure (BLPP; cmH₂O): intravesical pressure recorded at the first leaking/micturition
436 point; (5) Threshold pressure (Pt; cmH₂O); (6) Nonvoiding contractions during filling phase
437 (NVCs): rhythmic intravesical pressure rises (> 5 cmH₂O from baseline pressure) without any
438 fluid leakage from the urethra; (7) Postvoid residual volume (PVR; ml): the remaining saline in
439 the bladder collected and measured after stopping the infusion at the end of the final micturition
440 cycle; (8) Maximum bladder capacity (MBC; ml): volume between the start of infusion and the
441 BLPP; (9) Voided volume (VV; ml): MBC - PVR; (10) Intercontraction interval (ICI; s).

442

443 **Statistical analysis**

444 All statistical analyses were performed using SPSS 21.0 (IBM, Armonk, New York, USA).
445 Characteristics of patients presented as mean \pm SD, statistical differences among groups of subjects
446 were assessed using χ^2 test. Additionally, behavioral data and cystometry parameters of mice were
447 presented as mean \pm SD, employing Student's t test for comparison between two groups while
448 one-way ANOVA for three when these data were distributed normally ($P > 0.05$ by Shapiro-Wilk
449 test). Otherwise, the Mann-Whitney test was used for two groups versus Kruskal-Wallis test for
450 three. Comparative analysis for prevalence of abnormal EAS EMGs among groups was performed
451 by means of χ^2 test. Pvalues < 0.05 were considered to be statistically significant.

452

453 **Acknowledgements**

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455 81671267, 81471307, 81873791, 81301086, and 81430023). We thank Dr. Richard M. Niles for
456 his thoughtful editing of the manuscript.

457

458 **Figure legends**

459 **Figure 1. Immunohistochemical results of the sample tissues from subjects stained with anti-**
460 **α -Syn filament antibody (MJFR14).** (A-H) Representative images displayed misfolded α -Syn in
461 DET of MSA-P (A, C-E) and MSA-C (B), and in EUS of MSA-P (F), but not in PSP (G) and PD
462 (H). (C-E) Representative images displayed the right wall of MSA-P (C), the left wall of MSA-P
463 (D) and the triangle region of MSA-P (E). (I) Histogram shows the percentage of cases who had α -
464 Syn-positive inclusions in sample tissues in different groups. (J) Schematic displayed the anatomy
465 of the lower urinary tract and sampling positions (DET and EUS). Statistical significance was
466 analyzed employing the χ^2 test. *P < 0.05. [Scale bar, 400 μ m (A, B); 100 μ m (C-H).]

467 **Figure 2. Representative images of FG-labeled neurons in DET- and EUS-FG C57BL/6 mice.**
468 All FG-labeled neurons appeared bilaterally while displayed one side. Schematics in the upper panel
469 displayed the map of retrograde tracing areas (shaded areas) at different levels. Labeling appeared
470 in DET-FG C57BL/6 mice (middle panel) and EUS-FG C57BL/6 mice (lower panel). Insets show
471 a higher magnification relative to the main image. [Scale bars, 200 μ m (DET, EUS, pons); 100 μ m
472 (pelvic ganglia); 500 μ m (S1, L6, L2, T2, midbrain).] Abbreviation: 5SpL: lamina V of the spinal
473 gray, lateral part; 5SpM: lamina V of the spinal gray, medial part; 6SpL: lamina VI of the spinal
474 gray, lateral part; 6SpM: lamina VI of the spinal gray, medial part; 7Sp: lamina VII of the spinal
475 gray; 8Sp: lamina VIII of the spinal gray; 10Sp: lamina X of the spinal gray; Ad9: adductor
476 motoneurons of lamina IX; Ax9: axial muscle motoneurons of lamina IX; BN: Barrington's nucleus;
477 CC: central canal; cp: cerebral peduncle, basal part; Cr9: cremaster motoneurons of lamina IX; csc:
478 commissure of the superior colliculus; DET: detrusor; df: dorsal funiculus; Dk: nucleus of
479 Darkschewitsch; dl: dorsolateral fasciculus (Lissauer); ExA9: external anal sphincter motoneurons
480 of lamina IX; ExU9: external urethral sphincter motoneurons of lamina IX; EUS: external urethral
481 sphincter; FG: Fluoro-Gold; G19: gluteal motoneurons of lamina IX; gr: gracile fasciculus; Hm9:
482 hamstring motoneurons of lamina IX; ICL: intercalated nucleus; ICo9: intercostal muscle
483 motoneurons of lamina IX; IML: intermediolateral columns; IMM: intermediomedial column; InCG:
484 interstitial nucleus of Cajal, greater part; InC: interstitial nucleus of Cajal; LC: locus coeruleus;
485 LDCom: lumbar dorsal commissural nucleus; LPrCb: lumbar precerebellar nucleus; LSp: lateral
486 spinal nucleus; Me5: mesencephalic trigeminal nucleus; ml: medial lemniscus; PAG: periaqueductal
487 gray; PCRtA: parvocellular reticular nucleus alpha; Pes9: pes motoneurons of lamina IX; Ps9: psoas
488 motoneurons of lamina IX; Q9: quadriceps motoneurons of lamina IX; RN: red nucleus; rs:

489 rubrospinal tract; SDCom: sacral dorsal commissural nucleus; SMV: superior medullary velum;
490 SNC: substantia nigra pars compacta; SNL: substantia nigra, lateral part; SNR: substantia nigra,
491 reticular part; SPrcb: sacral precerebellar nucleus; SPSy: sacral parasympathetic nucleus; vf: ventral
492 funiculus.

493 **Figure 3. Representative immunohistochemical results of different segments from diseased**
494 **EUS- α -Syn PFFs TgM83^{+/-} mice.** (A-I) Pathological α -Syn stained with anti-phospho- α -Syn (Ser
495 129) antibody. Representative images displayed the distribution of p α -Syn in EUS (A1), pelvic
496 ganglia (B1), S1 (C1), L6 (D1), L2 (E1), T2 (F1), cerebellum (G1), pons (H1), midbrain (I1). (A2-
497 I2, C3-I3, H4) High-magnification views relative to the main image. [Scale bars, 500 μ m (A1, G1,
498 H2); 100 μ m (A2, B1); 50 μ m (B2, G2, I2, G3-I3, H4); 250 μ m (C1-F1); 25 μ m (C2-F2, C3-F3); 1
499 mm (H1, I1).] Abbreviation: DLPAG: dorsolateral periaqueductal gray; DMPAG: dorsomedial
500 periaqueductal gray; DpG: deep gray layer of the superior colliculus; DpMe: deep mesencephalic
501 nucleus; DpWh: deep white layer of the superior colliculus; EW: Edinger-Westphal nucleus; GiA:
502 gigantocellular reticular nucleus; IntA: interposed cerebellar nucleus, anterior part; IPC:
503 interpeduncular nucleus, caudal subnucleus; IPL: internal plexiform layer of the olfactory bulb; IPR:
504 interpeduncular nucleus, rostral subnucleus; IRt: intermediate reticular nucleus; Lat: lateral (dentate)
505 cerebellar nucleus; LDTg: tegmental nucleus; LPAG: lateral periaqueductal gray; LVe: lateral
506 vestibular nucleus; Med: medial (fastigial) cerebellar nucleus; MVePC: medial vestibular nucleus;
507 parvocellular part; MVe: medial vestibular nucleus; PnC: pontine reticular nucleus, caudal part;
508 Pr5DM: principal sensory trigeminal nucleus, dorsomedial part; Pr5VL: principal sensory
509 trigeminal nucleus, ventrolateral part; RLi: rostral linear nucleus of the raphe; RMg: raphe magnus
510 nucleus; SuVe: superior vestibular nucleus.

511 **Figure 4. Double immunofluorescence analysis of different segments from diseased EUS- α -**
512 **Syn PFFs TgM83^{+/-} mice and age-matched EUS-PBS TgM83^{+/-} mice.** (A, B) Double
513 immunofluorescence analysis of L6 from EUS- α -Syn PFFs TgM83^{+/-} mice for p α -Syn (red) and
514 ubiquitin (green, A1-A2), Iba-1 (green, B1-B2). (C) Double immunofluorescence analysis of
515 cerebellum from EUS- α -Syn PFFs TgM83^{+/-} mice for p α -Syn (red) and ubiquitin (green). (D, E)
516 Double immunolabeling for MBP (green) and neurofilament (red) in L2 of EUS-PBS TgM83^{+/-}
517 mice (D) and EUS- α -Syn PFFs TgM83^{+/-} mice (E). (F, G) Double immunofluorescence analysis of
518 pons from diseased EUS- α -Syn PFFs TgM83^{+/-} mice for p α -Syn (red) and TH (green, F), ubiquitin
519 (green, G1-G3). (H-J) Double immunofluorescence analysis of p α -Syn (red, H) and TH (green, H),

520 ubiquitin (red, I) and TH (green, I), p α -Syn (red, J) and Iba-1 (green, J) in midbrain of diseased
521 EUS- α -Syn PFFs TgM83^{+/-} mice. (K) Double immunolabeling for p α -Syn (red, K1) and Iba-1
522 (green, K2) in pons of EUS- α -Syn PFFs TgM83^{+/-} mice. Co-immunolabeling is represented by
523 signal in yellow. Cell nuclei were counter stained with Hoechst33258 (blue). [Scale bars, 500 μ m
524 (A1, C1, H); 200 μ m (B1, F, G1, I); 100 μ m (G2-G3, K1-K4); 50 μ m (A2, B2, C2, D, E, J).]

525 **Figure 5. Western blot analysis of diseased EUS- α -Syn PFFs TgM83^{+/-} mice and age-matched**
526 **EUS-PBS TgM83^{+/-} mice.** (A, D, F) Representative immunoblots of α -Syn in the soluble and
527 insoluble fractions of spinal cord, pons and PAG using the α -Syn (Ser129P) and aggregated α -Syn
528 (clone 5G4) antibodies. Blots were probed for GAPDH as a loading control (Bottom). Molecular
529 weight markers of migrated protein standards are expressed in kDa. (B, C, E, G) Quantification of
530 soluble and insoluble α -Syn levels in the spinal cord, pons and PAG (n = 3 per group). Data are the
531 means \pm SD. Statistical significance was analyzed by using the Student's t test and Mann-Whitney
532 test, *P < 0.05; n.s., non-significant.

533 **Figure 6. EAS EMG analysis of TgM83^{+/-} mice and C57BL/6 mice.** (A-E) Representative
534 abnormal EAS EMGs from diseased EUS- or DET- α -Syn PFFs TgM83^{+/-} mice. Abnormal EAS
535 EMGs show as fibrillation potential (A), positive sharp waves (B), CRD (C), satellite potential (D),
536 and myokymic discharges (E). (F) Representative normal EAS EMG referring to resting potential
537 from EUS-PBS TgM83^{+/-} mice at 5-month post-injection. (G-J) Time-dependent distribution of
538 abnormal EAS EMGs in different groups of TgM83^{+/-} mice (G, H) and C57BL/6 mice (I, J). EUS-
539 α -Syn PFFs mice n = 20, DET- α -Syn PFFs mice n = 18, EUS-PBS mice n = 18, DET-PBS mice n
540 = 16, control group n = 20. Statistics was analyzed employing the χ^2 test. *P < 0.05 relative to the
541 corresponding PBS groups and control groups.

542 **Figure 7. Urinary function analysis of TgM83^{+/-} mice.** (A-E) Representative cystometry traces in
543 DET- α -Syn PFFs (A), DET-PBS (B), EUS- α -Syn PFFs (C), EUS-PBS (D) TgM83^{+/-} mice at 5-
544 month post-injection and baseline group (E). Arrows indicate void events and asterisks indicate
545 NVCs. (F-J) Summary bar graphs from urodynamic evaluation for EUS and DET TgM83^{+/-} mice
546 including amplitude (F), #NVCs/Cycle (G), PVR (H), VV (I) and ICI (J). EUS- α -Syn PFFs
547 TgM83^{+/-} mice n = 18, DET- α -Syn PFFs TgM83^{+/-} mice n = 16, EUS-PBS TgM83^{+/-} mice n = 22,
548 DET-PBS TgM83^{+/-} mice n = 20. Data are the means \pm SD. Statistics was analyzed employing the
549 Student's t test and Mann-Whitney test. *P < 0.05 indicates a significant difference between EUS-
550 or DET- α -Syn PFFs groups and EUS- or DET-PBS groups.

551 **Figure 8. Behavioral analysis of TgM83^{+/-} mice.** (A) Kaplan-Meier survival plot shows decreased
552 survival time (due to death or euthanasia because of paralysis) for α -Syn PFFs TgM83^{+/-} mice
553 compared with age-matched PBS TgM83^{+/-} mice. (B) The mean score of MBS. (C) Latency to fall
554 from the rotarod. (D and E) Footprint analysis of the hindlimb stride length (D) and the hind-base
555 width (E). (F and G) The average time to cross the beam (F) and the average number of side slip
556 errors (G) on the beam. (H and I) T turn (H) and T total (I) of the pole test. (J and K) Average speed
557 (J) and total distance (K) traveled during 15 minutes in the open field test. EUS- α -Syn PFFs
558 TgM83^{+/-} mice n = 12, DET- α -Syn PFFs TgM83^{+/-} mice n = 20, EUS-PBS TgM83^{+/-} mice n = 14,
559 DET-PBS TgM83^{+/-} mice n = 14. Data are the means \pm SD. Statistical analysis was done by using
560 the Student's t test and Mann-Whitney test, *P < 0.05.

561 **Supplementary figure legends**

562 **Figure 3- figure supplement 1 (A).** Negatively stained transmission electron micrographs of α -Syn
563 PFFs (upper), α -Syn PFFs in a high magnification (lower left), and α -Syn PFFs which were
564 sonicated and used for modeling (lower right). (B) Schematic displayed the positions where were
565 inoculated by α -Syn PFFs, including DET and EUS. [Scale bars, 200 nm (a, upper); 100 nm (a,
566 lower left and right).]

567 **Figure 3-figure supplement 2.** Representative immunohistochemical results of different segments
568 from EUS-PBS TgM83^{+/-} mice and spontaneously ill TgM83^{+/-} mice. Sections were stained with
569 anti-phospho- α -Syn (Ser 129) antibody. Representative images displayed the distribution of p α -Syn
570 aggregates in S1, L6, L2, T2, pons, midbrain, and cerebellum. The locations, indicated by red dots
571 in the upper panel, represent nuclei where p α -Syn aggregates were only observed in diseased EUS-
572 α -Syn PFFs TgM83^{+/-} mice, but not in spontaneously ill TgM83^{+/-} mice.

573 **Figure 7-figure supplement 1.** Urinary function analysis. (A) Representative cystometric curve of
574 normal C57BL/6 mice. (B and C) Summary bar graphs from urodynamic evaluation about BBP (B)
575 and Pt (C) for EUS and DET TgM83^{+/-} mice. EUS- α -Syn PFFs TgM83^{+/-} mice n = 18, DET- α -Syn
576 PFFs TgM83^{+/-} mice n = 16, EUS-PBS TgM83^{+/-} mice n = 22, DET-PBS TgM83^{+/-} mice n = 20.
577 Data are the means \pm SD. Statistics was performed employing the Student's t test and Mann-
578 Whitney test. (D) Bladder size of EUS- α -Syn PFFs (right) and EUS-PBS (left) TgM83^{+/-} mice at 6-
579 month post-injection.

580 **Figure 8-figure supplement 1.** Motor and postural abnormalities of EUS- α -Syn PFFs TgM83^{+/-}
581 mice at 5-month postinjection. (A) Hindlimb claspings (arrow). (B) Truncal dystonia (arrow). (C)

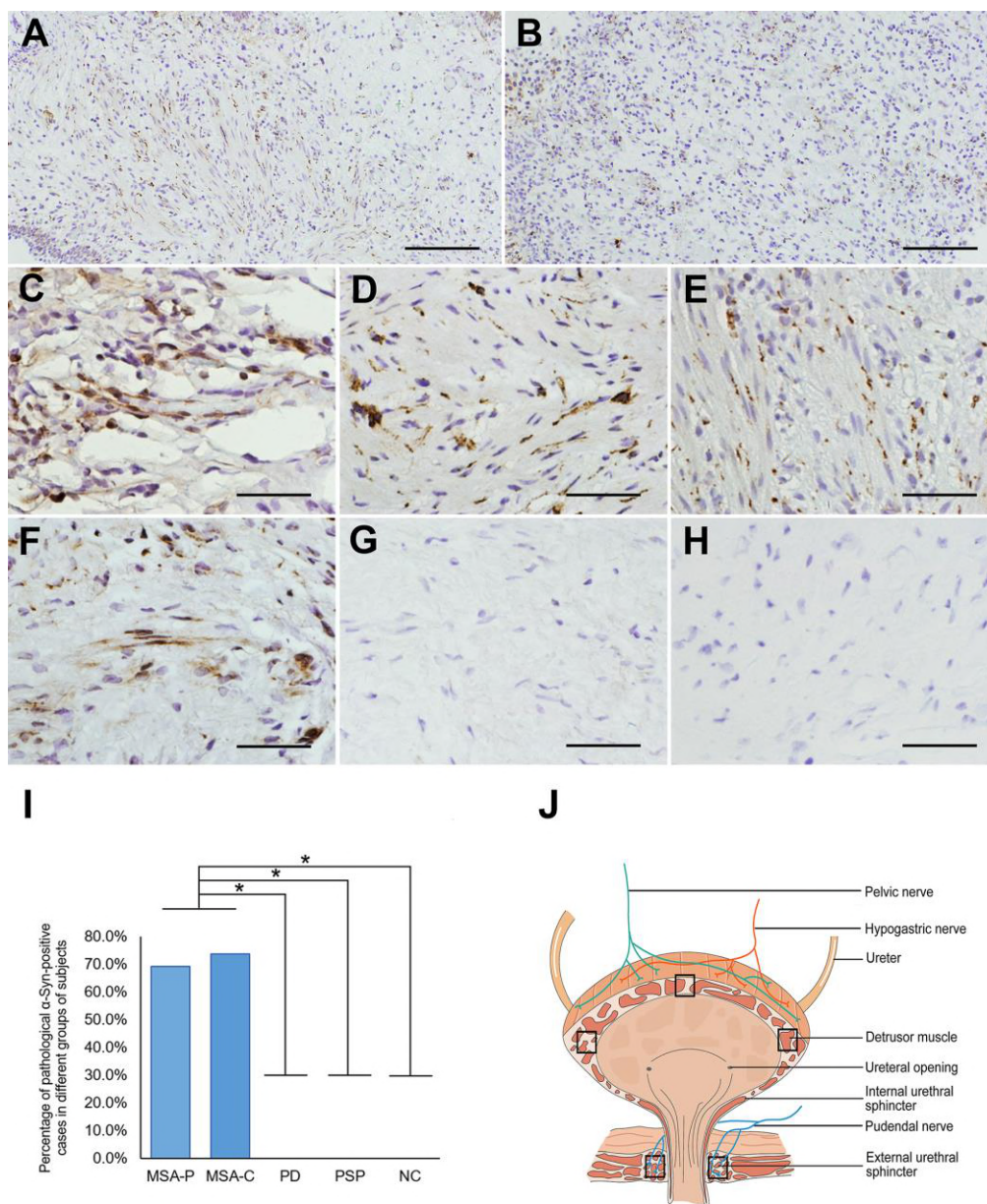
582 Hindlimb dystonia (arrow). **(D)** Impaired postural adjustments. **(E and F)** Representative footprints
583 of EUS-PBS TgM83^{+/-} mice **(E)** and diseased EUS- α -Syn PFFs TgM83^{+/-} mice **(F)**.

584 **Figure 8-figure supplement 2.** Behavioral analysis of C57BL/6 mice. **(A)** The mean score of MBS.
585 **(B)** Latency to fall from the rotarod. **(C and D)** Average speed **(C)** and total distance **(D)** traveled
586 during 15 minutes in the open field test. **(E and F)** Footprint analysis of the hindlimb stride length
587 **(E)** and the hind-base width **(F)**. EUS- α -Syn PFFs C57BL/6 mice n = 8, DET- α -Syn PFFs C57BL/6
588 mice n = 12, EUS-PBS C57BL/6 mice n = 7, DET-PBS C57BL/6 mice n = 8. Data are the mean \pm
589 SD. Statistical analysis was performed by using the Student's t test and Mann-Whitney test, n.s.,
590 non-significant.

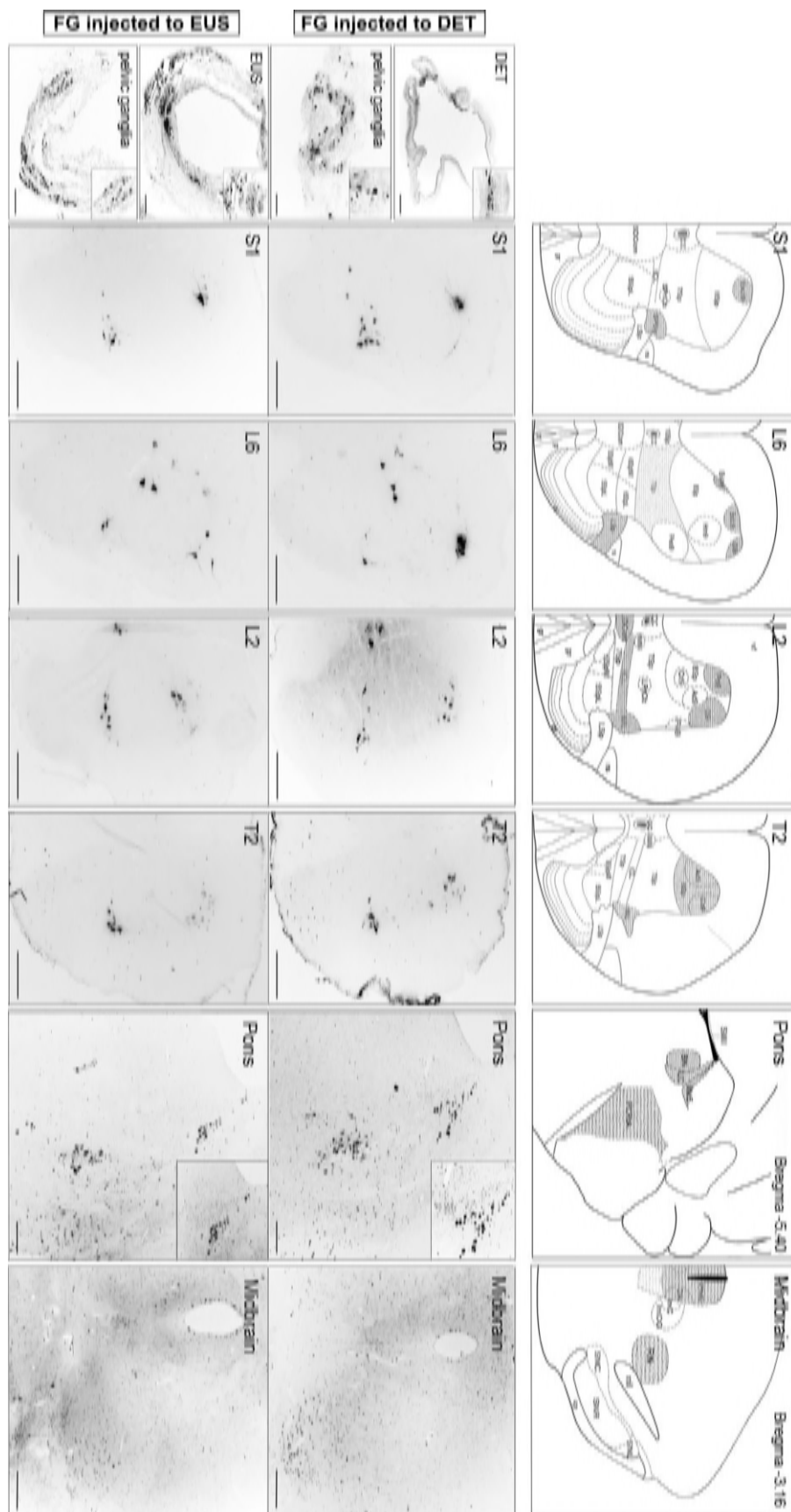
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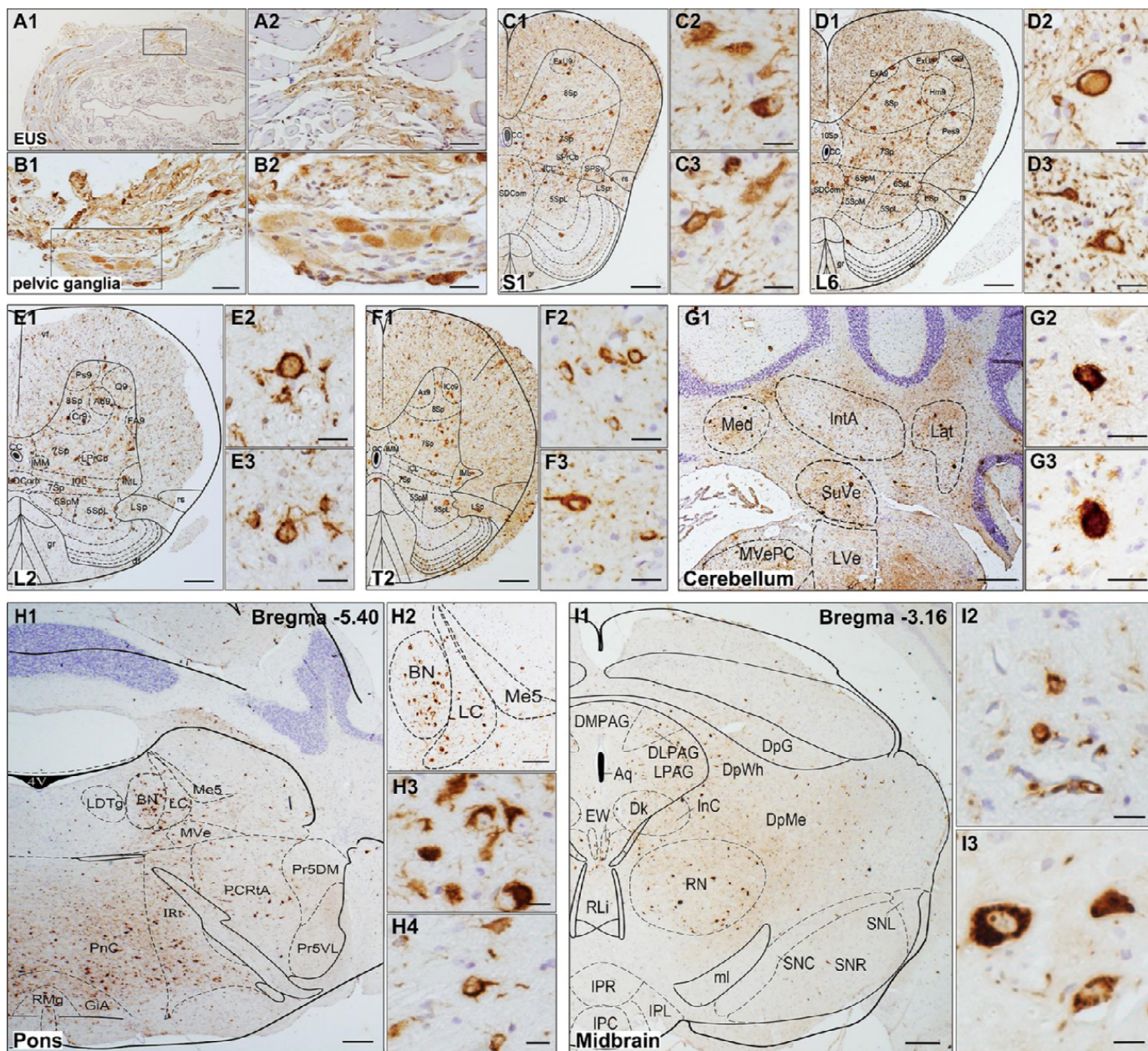
593 **Figures**



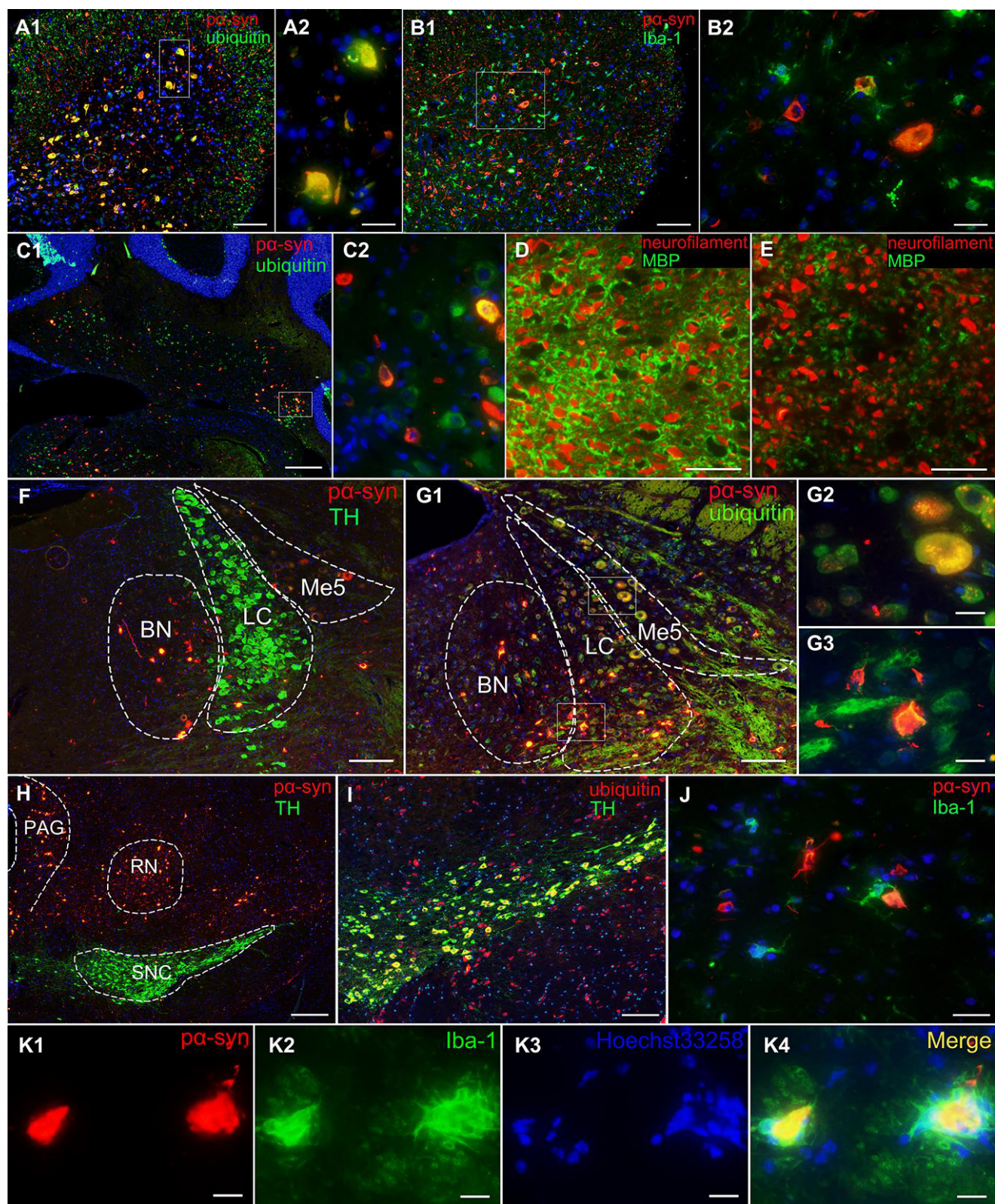
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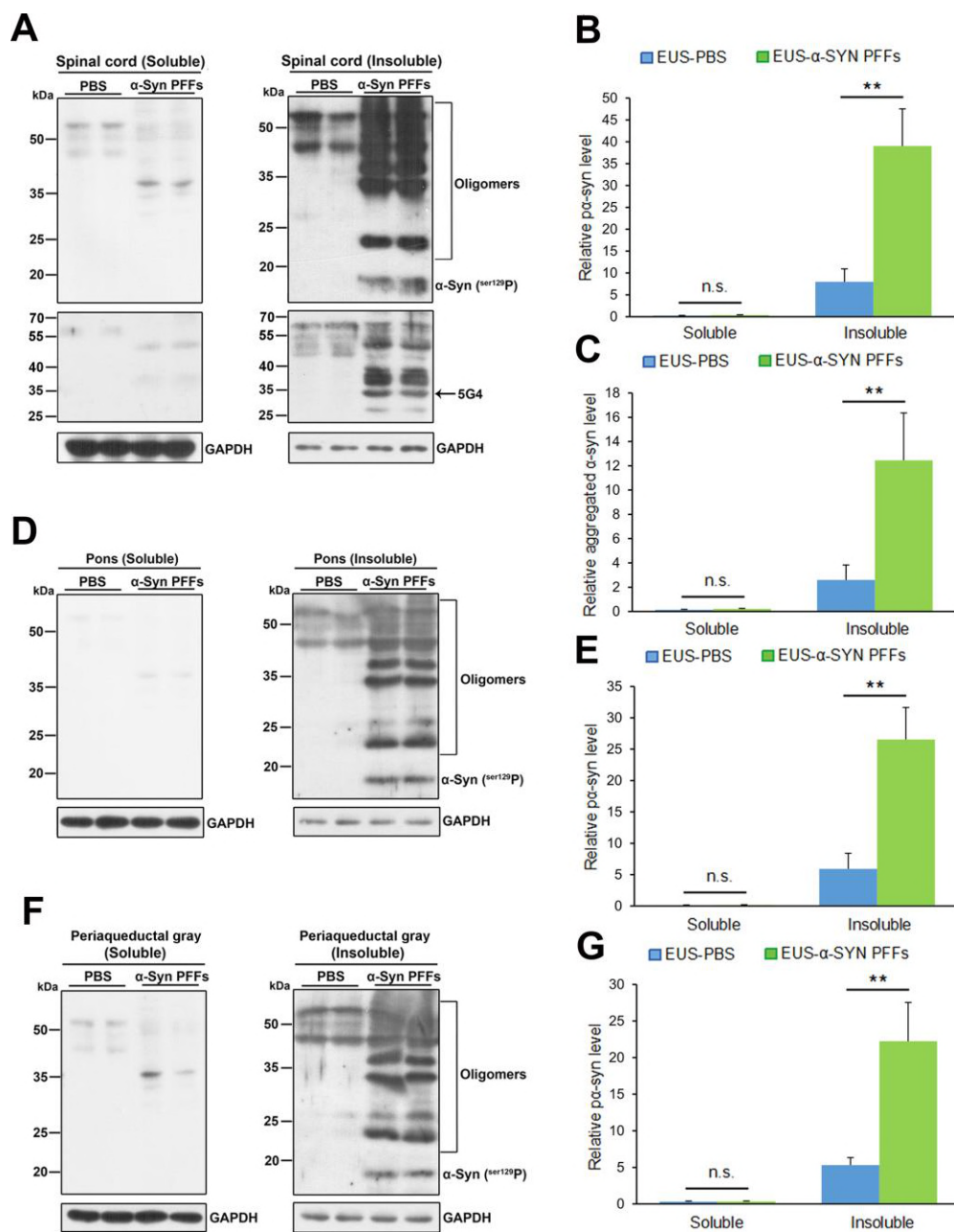


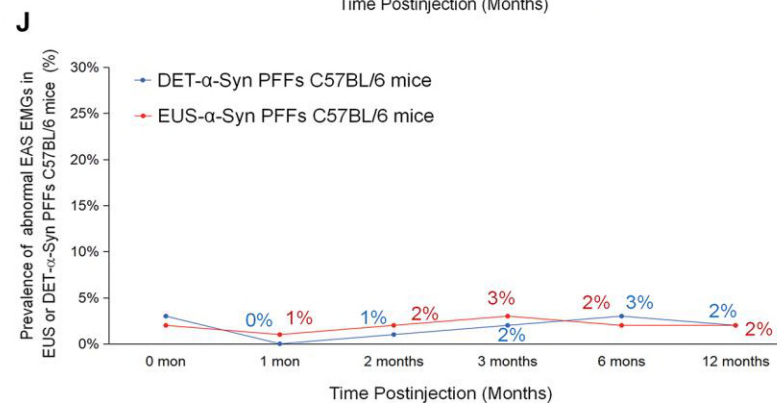
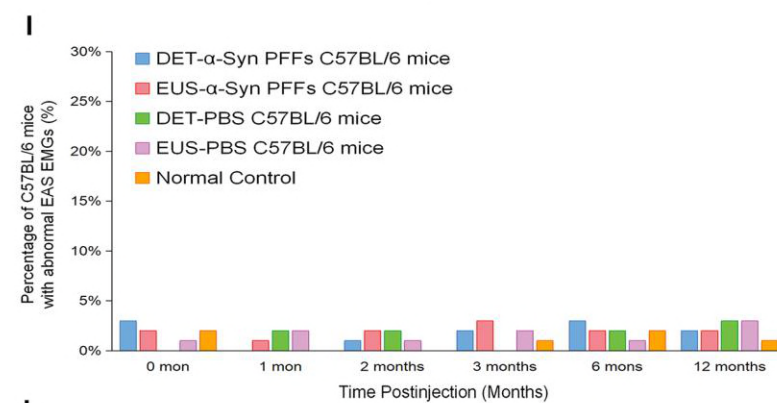
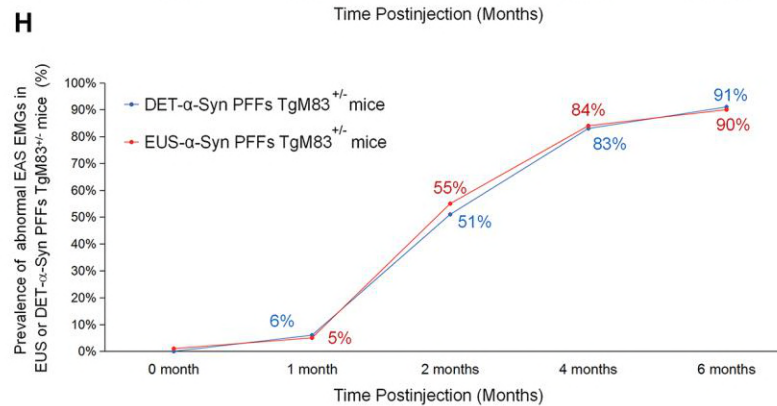
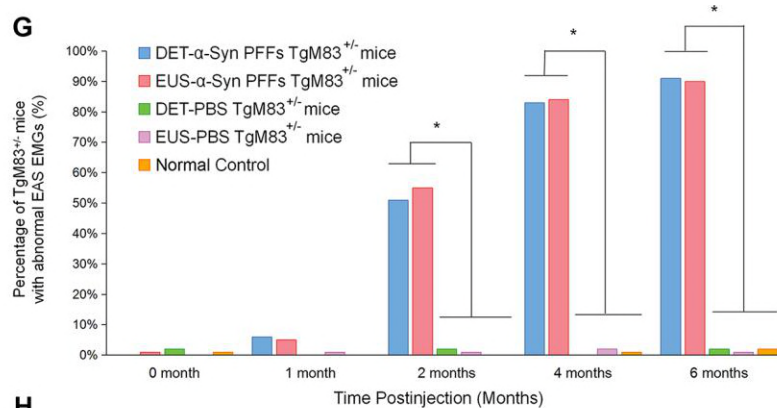
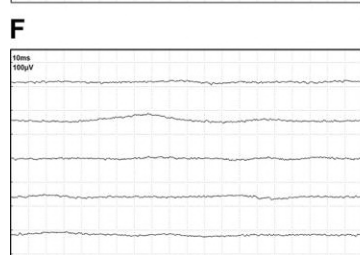
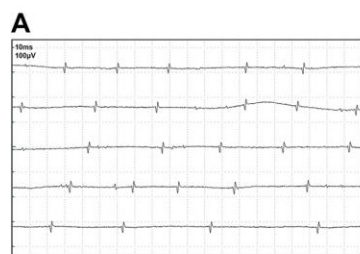
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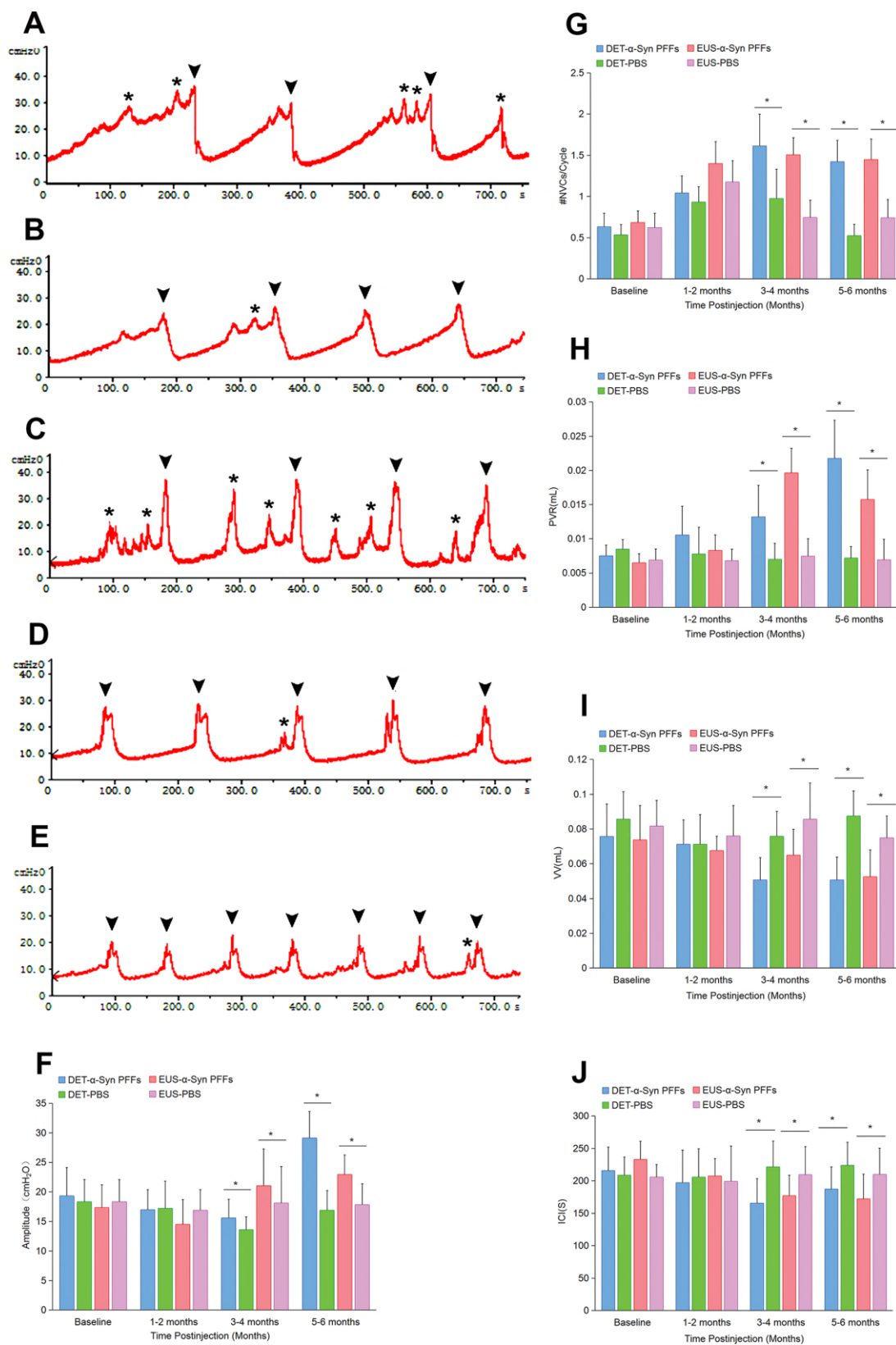
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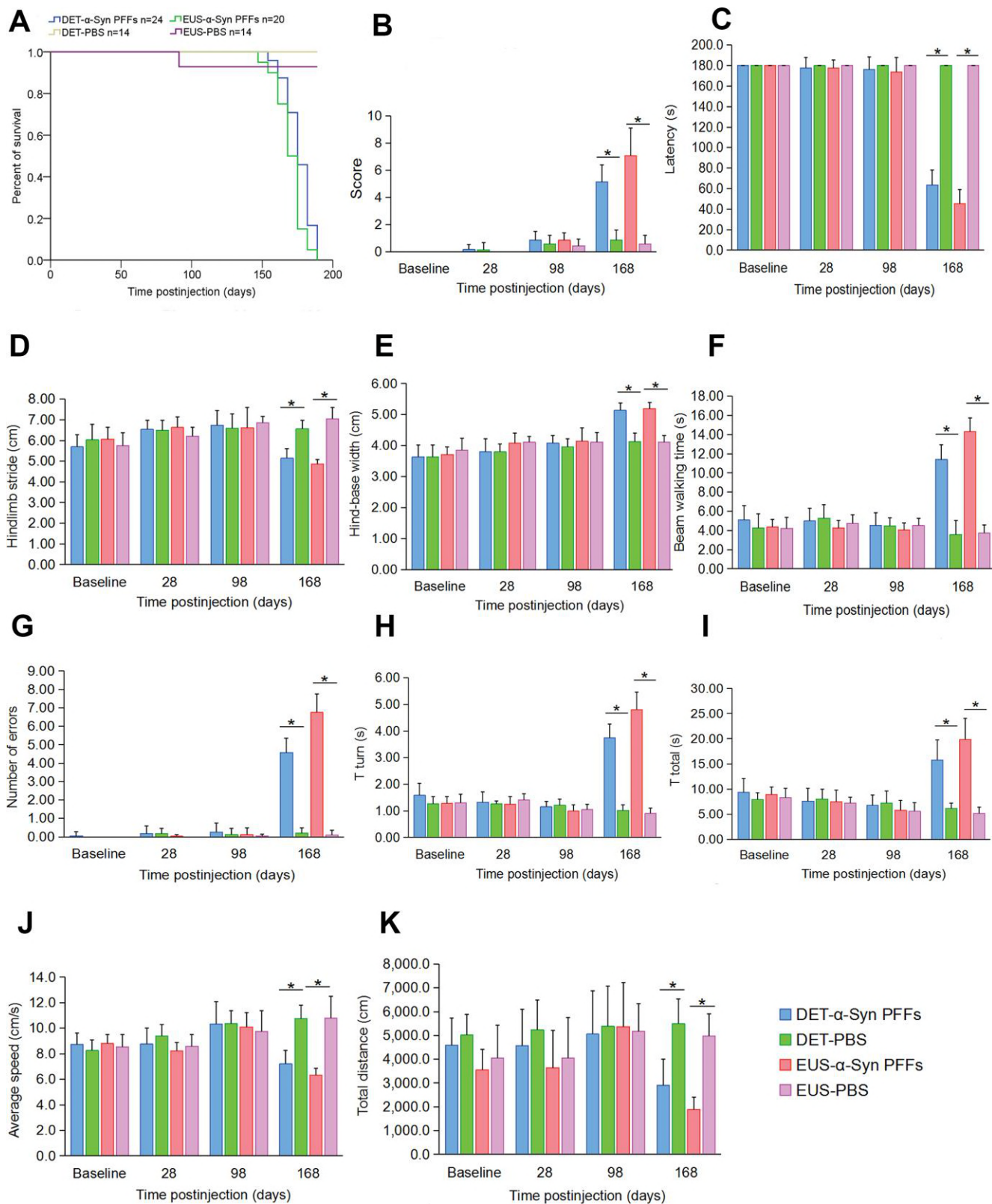






601





602

603

604 **Table 1. Characteristics and the exam findings of patients**

Age	Sex	Diagnosis	Duration (year)	MRI	UMSARS				Urodynamic examination†	Perianal electromyography†	α-synuclein filament in the bladder DET‡	α-synuclein filament in EUS§
					UMSARS I	UMSARS II	UMSARS III	UMSARS IV				
54	M	MSA-P	1.5	+	2	5	+	1	+	+	-	0
57	F	MSA-P	8	+	32	50	+	4	+	+	+	0
63	F	MSA-P	2	+	14	18	+	2	-	+	-	0
59	F	MSA-P	4	+	21	18	+	3	-	+	-	0
46	M	MSA-P	2	+	9	14	+	2	+	+	-	0
68	M	MSA-P	7	+	20	31	+	3	+	+	+	0
66	F	MSA-P	<1	+	7	12	+	1	-	+	+	0
70	F	MSA-P	3	+	32	46	+	4	+	+	+	0
66	F	MSA-P	3	+	18	24	+	3	+	+	-	+
72	F	MSA-P	5	+	34	42	+	4	+	+	+	+
63	M	MSA-P	2	+	7	13	+	2	+	-	-	+
64	F	MSA-P	7	+	33	42	+	5	+	+	+	+
59	F	MSA-P	4	+	18	15	+	4	+	-	+	+
59	M	MSA-C	2	+	10	13	+	2	+	+	+	0
50	M	MSA-C	1.5	+	2	3	+	1	-	-	-	0
47	M	MSA-C	8	+	30	21	+	4	+	+	+	0
63	F	MSA-C	2	+	21	14	+	1	+	-	-	0
50	M	MSA-C	2	+	5	6	+	1	+	-	-	0
61	M	MSA-C	3	+	20	18	+	4	+	+	+	0
61	M	MSA-C	5	+	8	9	+	2	+	+	+	0
51	F	MSA-C	3	+	8	6	+	1	-	+	-	0
58	F	MSA-C	3	+	4	5	+	2	+	+	-	0
50	F	MSA-C	2	+	12	14	+	2	+	+	+	0
64	F	MSA-C	2	+	14	24	+	4	+	+	+	0
62	F	MSA-C	3	+	14	18	+	3	+	+	+	0
64	M	MSA-C	3	+	10	11	+	2	+	+	+	0
64	F	MSA-C	3	+	30	12	+	4	+	-	-	+
52	F	MSA-C	8	+	33	22	+	5	+	+	+	+
53	F	MSA-C*	2	+	9	15	+	1	+	+	-	+
62	F	MSA-C	2	+	7	9	+	2	+	-	+	+
57	F	MSA-C	4	+	20	25	+	5	+	-	+	+
71	M	MSA-C	1	+	2	4	+	1	+	+	+	-
54	F	PD	10	-	NA				-	+	-	0
58	F	PD	3	-	NA				-	-	-	0
69	F	PD	10	-	NA				+	-	-	0
61	M	PD	6	-	NA				-	-	-	0
65	F	PD	10	-	NA				-	-	-	0
58	F	PD	4	-	NA				-	-	-	-
64	M	PD	8	-	NA				-	+	-	-
47	M	PSP	8	+	NA				-	-	-	0
69	M	PSP	3	+	NA				-	-	-	0
70	M	PSP	3	+	NA				-	-	-	0
69	F	PSP	3	+	NA				+	-	-	0
70	F	PSP-C	10	+	NA				-	-	-	0
67	M	PSP	4	+	NA				-	-	-	-

605 F, female; M, male; MSA-P, multiple system atrophy with predominant parkinsonism; MSA-C,
606 multiple system atrophy with predominant cerebellar ataxia; PD, Parkinson's disease; PSP,
607 progressive supranuclear palsy; PSP-C, progressive supranuclear palsy with predominant cerebellar
608 ataxia; MRI, magnetic resonance imaging, including atrophy on MRI of putamen, middle cerebellar
609 peduncle, pons, or cerebellum, -, absent; +, present; UMSARS, Unified Multiple System Atrophy
610 Rating Scale (I. historical review of disease-related impairments; II. motor examination; III.
611 autonomic examination, -, absent; +, present; IV. global disability scale); NA, not applicable.

612 *possible MSA-C.

613 †-, normal findings; +, abnormal findings.

614 ‡ DET, detrusor; -, absent; +, present.

615 § EUS, external urethral sphincter; -, absent; +, present; 0: not examined.

616

617 **Supplementary Materials**

618

619 **Urodynamic examination (UE) and external anal sphincter electromyography (EAS EMG)** 620 **of patients**

621 All human subjects underwent clinical and electrophysiological evaluation, including UE and EAS
622 EMG, as Yamamoto et al. previously described (Yamamoto et al., 2014), (Yamamoto et al., 2005).

623

624 **Surgery and retrograde tracing**

625 To retrogradely label micturition reflex pathways, C57BL/6 and TgM83^{+/-} mice were anesthetized
626 with isoflurane inhalation (S. Prusiner et al., 2015), and 15 µl Fluoro-Gold (FG) (Fluorochrome,
627 LLC, Denver, CO) was injected slowly into EUS or DET. Following the injections, the skin was
628 sutured. After 14 days, mice were perfused as described before (Bacsikai, Rusznak, Paxinos, &
629 Watson, 2014). The EUS or DET, pelvic ganglia, spinal cord and brain were removed and
630 postfixed at 4 °C in a 30% sucrose solution containing 4% paraformaldehyde for at least 2 days.
631 Serial transverse sections were cut at 20 µm using a freezing microtome (Leica CM1860 UV, Leica,
632 Nussloch, Germany). Consecutive sections were collected, mounted, and cover-slipped with
633 glycerol. Slides were then examined using an Olympus IX51 microscope equipped with
634 epifluorescence.

635

636 **Transmission electron microscopy (TEM) imaging**

637 The nature of the fibrillar α -Syn forms was assessed using Jeol 1400 (Jeol Ltd. Tokyo, Japan) TEM.
638 First, a drop of fibrillar solution was transferred onto a carbon-coated 200-mesh grid and then
639 negatively stained with 1% uranyl acetate. The images were recorded with Gatan Orius CCD
640 camera (Gatan, Pleasanton, CA).

641

642 **Immunohistochemical and double immunofluorescence staining**

643 Immunohistochemistry and double-labeling immunofluorescence analysis were conducted as
644 previously described by Luk et al. (Luk, Kehm, Zhang, et al., 2012) using the following antibodies:
645 phospho- α -synuclein (Ser 129) (mouse, Millipore, 1:600 or rabbit, Abcam, 1:400), α -synuclein
646 filament (MJFR14) (rabbit, Abcam, 1:500), Ubiquitin (rabbit, Cell Signaling Technology, 1:400),
647 Iba-1 (rabbit, Wako, 1:400), Myelin basic protein (rabbit, Abcam, 1:900), neurofilament heavy
648 polypeptide (mouse, Abcam, 1:400), anti-tyrosine hydroxylase (rabbit, Abcam, 1:400),
649 Rhodamine RedTM-X (RRX) AffiniPure donkey anti-mouse IgG (H+L) (donkey, Jackson
650 ImmunoResearch, 1:400), CyTM2 AffiniPure donkey anti-rabbit IgG (H+L) (donkey, Jackson
651 ImmunoResearch, 1:400). Cell nuclei were stained using Hoechst33258 (1:1000, Solarbio). Slides
652 were coverslipped with glycerol. Digital images were captured using Olympus IX51 microscope
653 mounted with DP71 Olympus digital camera. Photoshop CS6 (Adobe Systems) was used to
654 assemble montages.

655

656 **Western blotting analysis**

657 After mice were anesthetized and decapitated, the spinal cord and several brain regions such as
658 PAG, RN, pons, and cerebellum were separated on a cold stage. The isolated mice tissues were
659 then stored in liquid nitrogen for further treatment. The transferred polyvinylidene fluoride (PVDF)
660 membranes for the Western blotting as Kohl et al. previously described (Kohl et al., 2016) were
661 incubated with following primary antibodies: phospho- α -synuclein (Ser 129) (mouse, Millipore,
662 1:600 or rabbit, Abcam, 1:800), α -synuclein filament (MJFR14) (rabbit, Abcam, 1:500),
663 aggregated α -synuclein (5G4) (mouse, Millipore, 1:500). Forty-eight hours later, the membranes
664 were washed in TBST (TBS with 0.1% Tween-20) and incubated with HRP-conjugated goat anti-
665 mouse or goat anti-rabbit secondary antibodies for 2 hours at room temperature and visualized
666 with enhanced chemiluminescence (Thermo Fisher Scientific). Proteins' densities on the blots

667 were normalized against those of GAPDH. All immunoreactive bands from Western blotting
668 analysis were quantified by pixel intensity using FluorChem 8900 software (Alpha Innotech, San
669 Leandro, CA, USA).

670

671 **Cystometry surgery**

672 All animals were subjected to cystometric experiment to evaluate their urinary function before
673 injection and at corresponding time points of post-injection following the methods reported
674 previously (Boudes et al., 2013; Fandel et al., 2016; Girard et al., 2012; Silva et al., 2015). The
675 animal was put supine and the bladder was exposed via a lower midline abdominal incision under
676 isoflurane anesthesia. A polyethylene catheter-50 (Clay-Adams, Parsippany, New Jersey, USA)
677 was implanted into the apical bladder dome and secured in place with a 6/0 purse-string sutures
678 (Ethicon, Norderstedt, Germany). We flushed the catheter with saline to ensure no leakage and then
679 threaded it from neck to the lower back through the subcutaneous tunnel anchored to the neck skin,
680 finally closed the abdominal wall and skin. Through a three-way tap, the bladder catheter was
681 connected to an infusion pump (B. Braun Sharing Expertise, Germany) and a pressure transducer
682 (AD Instruments, Castle Hill, New South Wales, Australia) coupled to a computerized BL-420S
683 data acquisition and analysis system (Techman Soft, Chengdu, China) which amplified and
684 recorded intravesical pressure from the pressure transducer. We applied a heating lamp and room-
685 temperature saline to maintain the body temperature of mice. Bladders were given a continuous
686 infusion of 0.9% NaCl at a constant rate (20 μ l/min) and after an equilibration period of 20-30
687 minutes, the intravesical pressure was recorded and voiding events were observed and noted for
688 30 minutes.

689

690 **Behavioral test**

691 To evaluate α -Syn PFFs-induced behavioral deficits, mice were assessed by the following tests.
692 TgM83^{+/-} and C57BL/6 mice were tested every 7 days starting from the second month's post-
693 injection. Blinded experiments were performed to treatment group for all behavioral tests.

694

695 ***The motor behavioral scale (MBS)***

696 MBS was used as Fernagut et al. previously reported (Fernagut et al., 2002). Higher score indicated
697 higher disability and the maximum total score was 10. The total score was determined and used
698 for the statistical analysis.

699

700 ***Rotarod test***

701 Motor coordination was assessed following the method previously reported by Duclot et al.
702 (Duclot et al., 2012) with modifications. In brief, a rotating rod (Rotarod YLS-4C; YiYan Science
703 and Technology Development Co., Ltd. Shandong, China) was used. At each time point, mice
704 were placed on the rod rotating at 30 rpm. The latency to fall off the rotarod within the maximum
705 time (180 seconds) was recorded, if a mouse stayed on the rod until the end of the 3 minutes, a
706 time of 180 seconds was recorded. Mice received three trials per day with a 15-minute inter-trial
707 interval. The mean latency to fall off the rotarod was statistically analyzed.

708

709 ***Open field test***

710 To assess general activity, locomotion, and anxiety of the mice, the open field test system (Wuhan
711 YiHong Sci. & Tech.Co., Ltd) was applied. Mice were placed in the center of the open field (37.5
712 × 37.5 × 34.8 cm) and tested for 15 minutes at the same time of the day (6:00 p.m. to 9:00 p.m.).
713 Activity was analyzed by the Anilab software version 5.10, registered version (Anilab Software &
714 Instruments Co., Ltd., China). At the end of testing, the arena was cleaned with 75% alcohol to
715 remove olfactory cues. The tests were performed in a dark room that was isolated from external
716 noises and light during the test period. Total distance (cm), average speed (cm/s), and zone crossing
717 were statistically analyzed.

718

719 ***Footprint test***

720 The footprint test was performed to examine the gait of the mice. Paws of the mice were painted
721 with water-soluble non-toxic paint of different colors (fore-paws in red and hind-paws in green).
722 The animals were then allowed to walk along a restricted cardboard tunnel (50 cm long, 5 cm wide,
723 10 cm high) into an enclosed box and a sheet of white paper (42 cm long, 4.5 cm wide) was placed
724 on the floor of the tunnel, and one set of footprints was collected for each animal. Three steps from
725 the middle portion of each run were measured for four parameters (cm): (1) stride length (front

726 and hind legs). (2) The front- and hind-base width. The mean of each set of values was statistically
727 analyzed (Stefanova et al., 2005).

728

729 ***Beam walking test***

730 Balance and bradykinesia were assessed with the method described before with modifications
731 (Schafferer et al., 2016). The beams consisted of two different types of wood (each measuring 80
732 cm long, one was 1.6 cm, and the other 0.9 cm wide) placed horizontally 50 cm above the floor,
733 respectively. Two daily sessions of three trials were performed using the 1.6 cm width large beam
734 during training. Mice were then tested using the 0.9 cm width beam. Mice were allowed to perform
735 in three consecutive trials. The time for traversing 50 cm as well as the number of sideslip errors
736 were recorded on each trial. The average traverse duration and average number of sideslip errors
737 of the three trials were statistically analyzed.

738

739 ***Pole test***

740 The pole test was performed to assess motor coordination and balance. A vertical gauze-taped pole
741 (1 cm diameter, 50 cm height) with a small cork ball (3 cm diameter) at the top was applied. Mice
742 were placed with their head upward right below the ball. The time taken to turn completely
743 downward (T turn) and total time taken to reach the base of the pole with four paws (T total) were
744 recorded. The maximum cutoff of total time to stop this test was 120 seconds. This test was
745 performed three times for each mouse, while the average time was statistically analyzed (Zhou et
746 al., 2016).

747

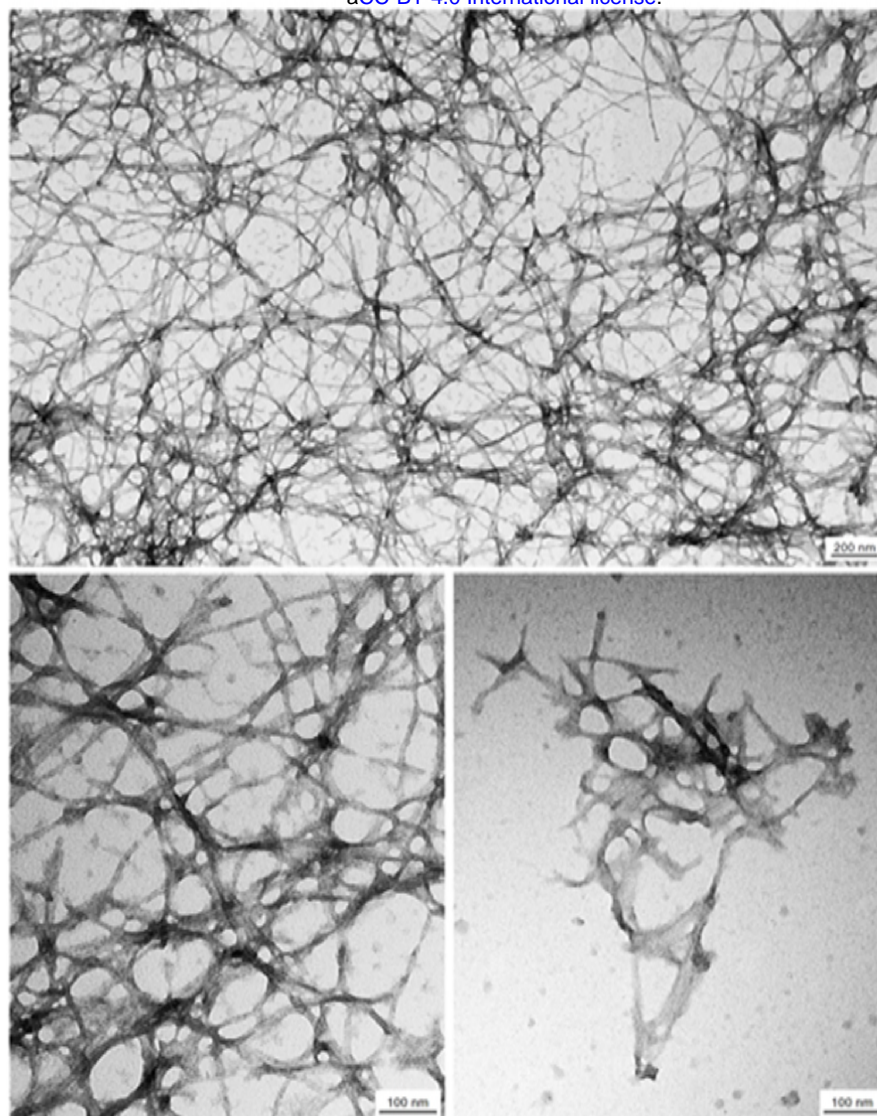
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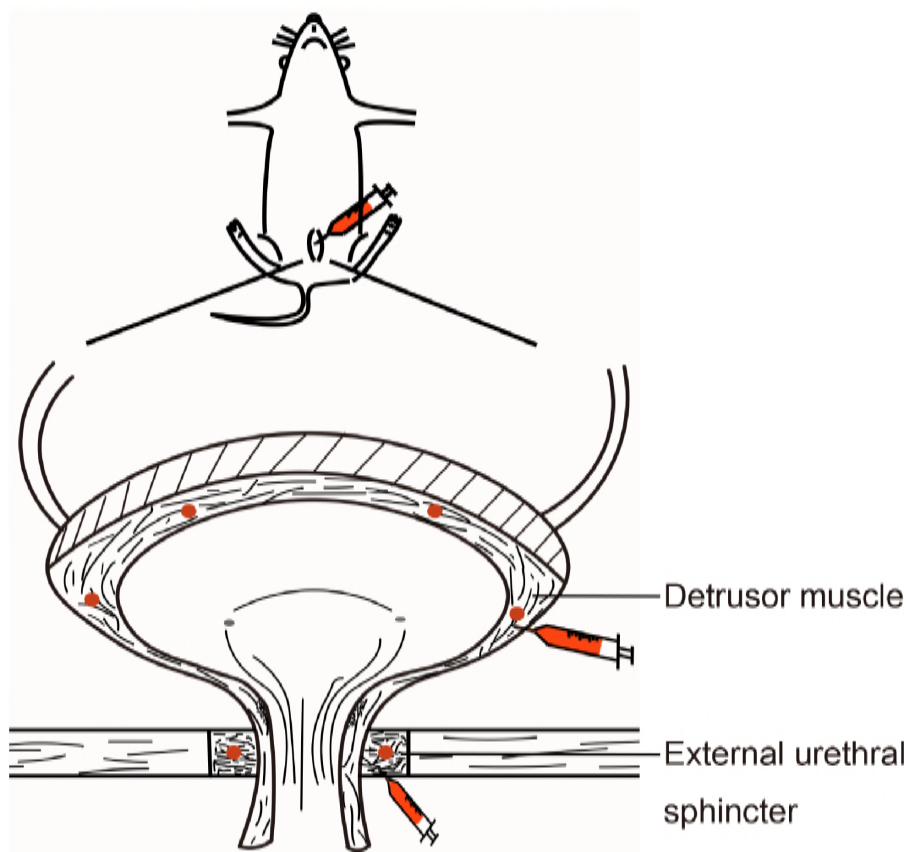
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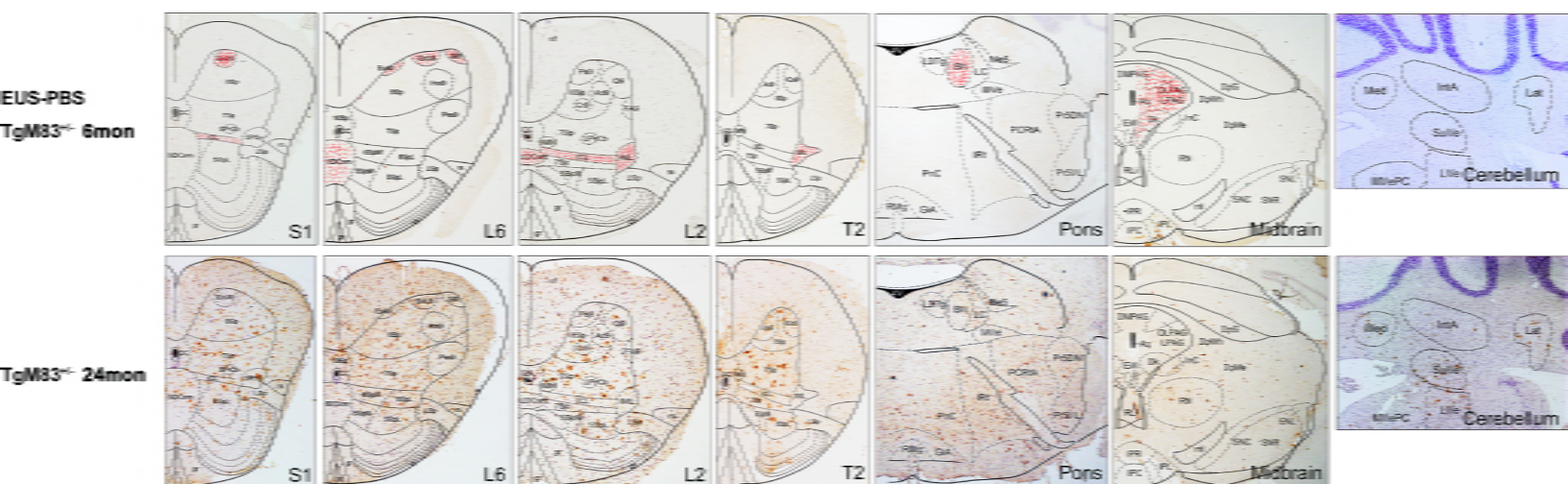
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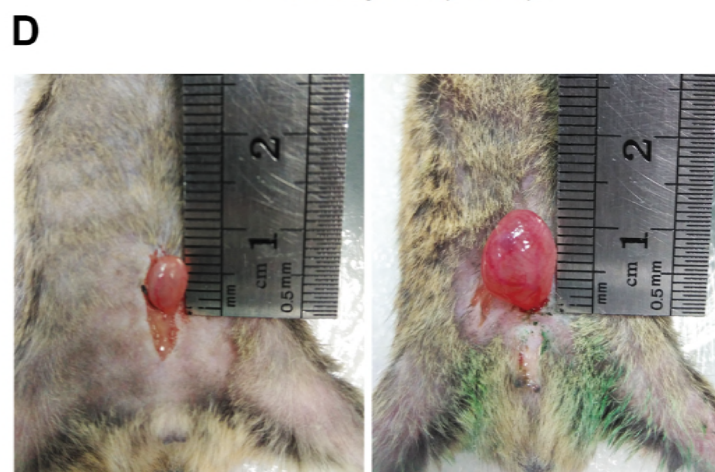
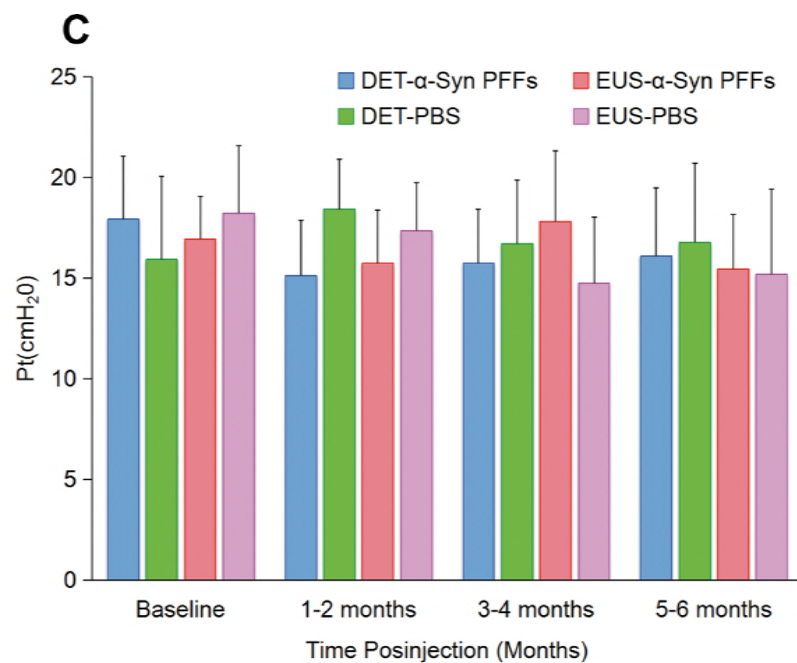
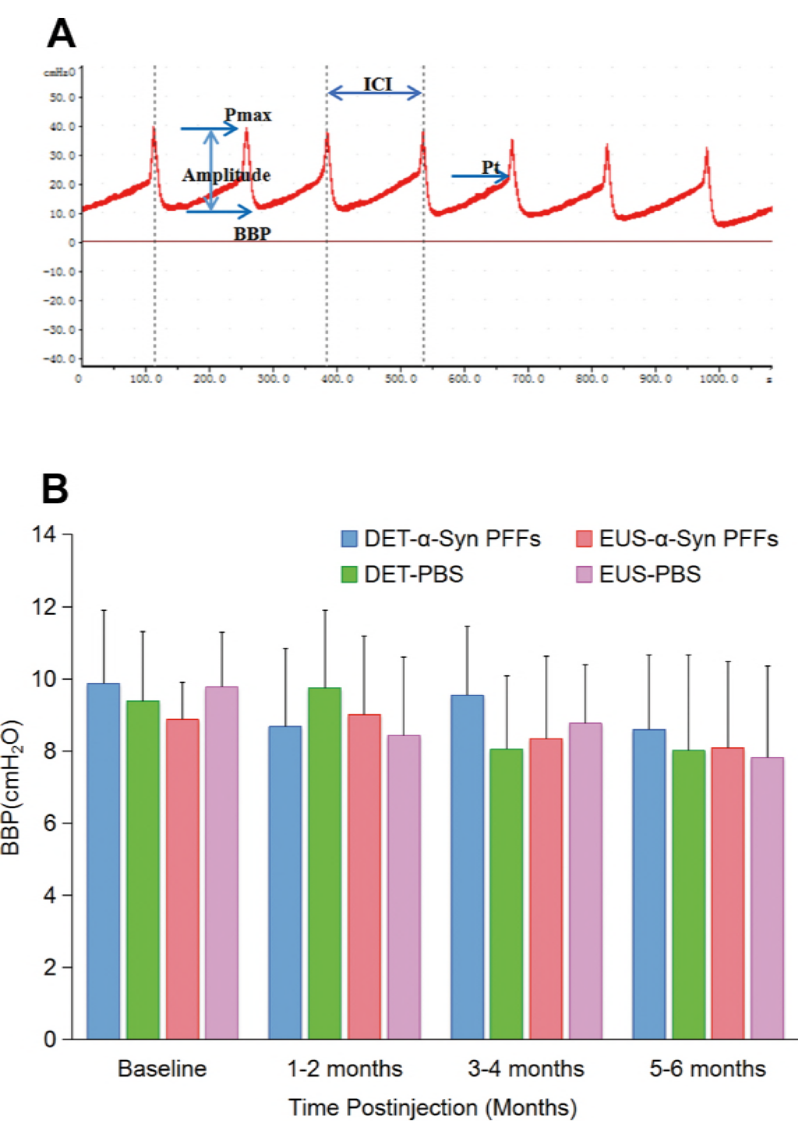
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B







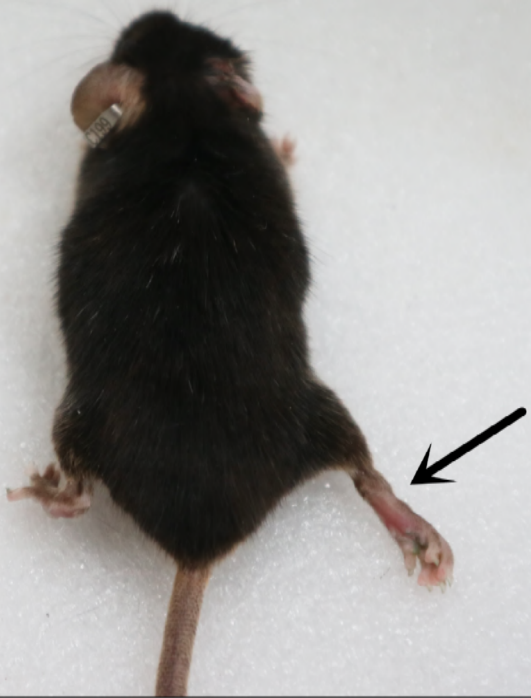
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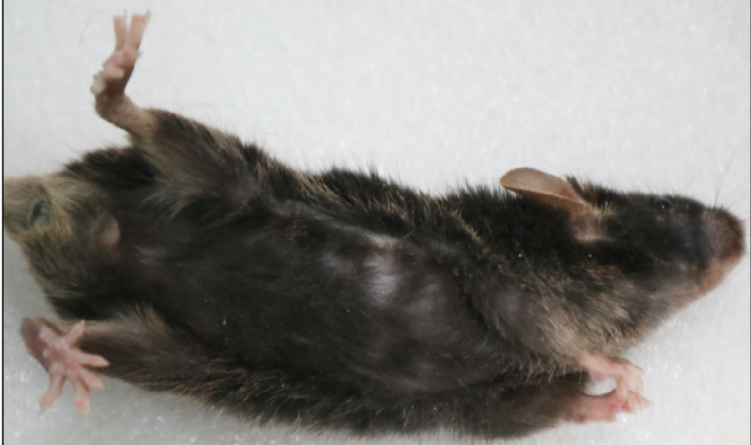
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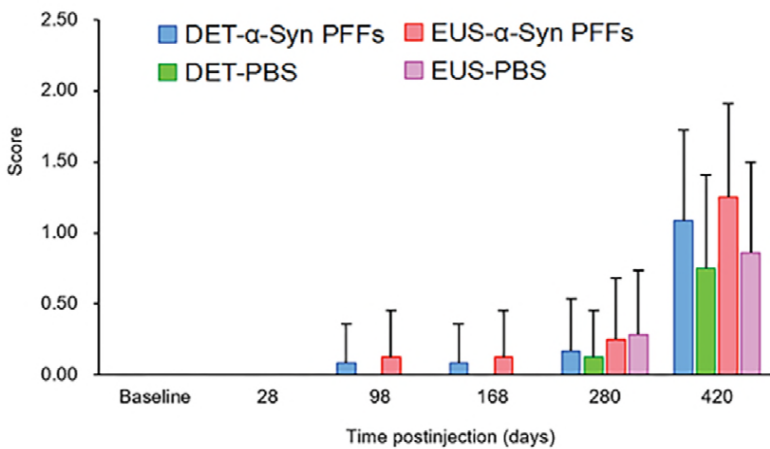
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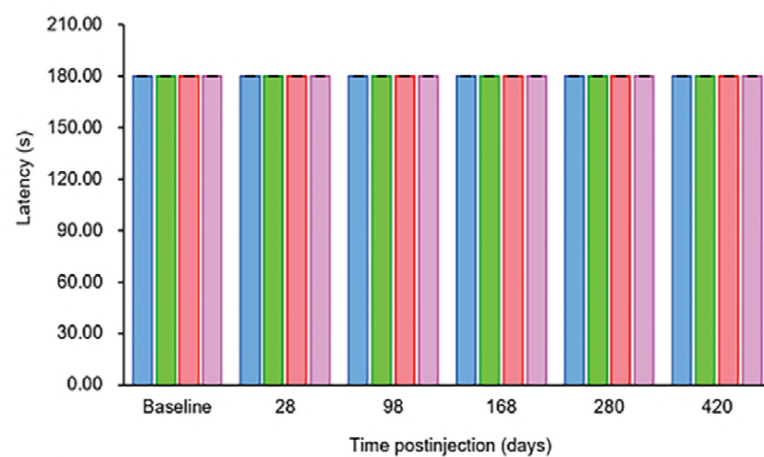
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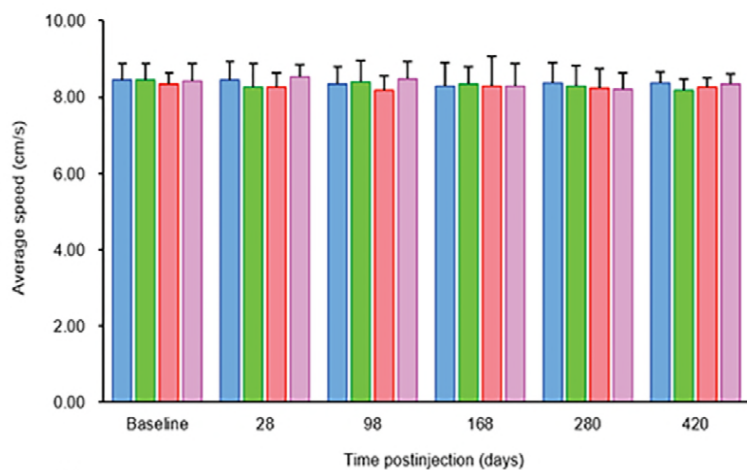
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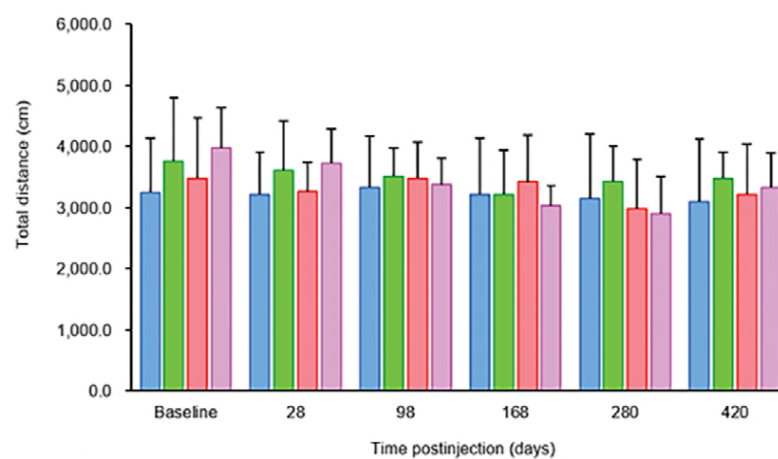
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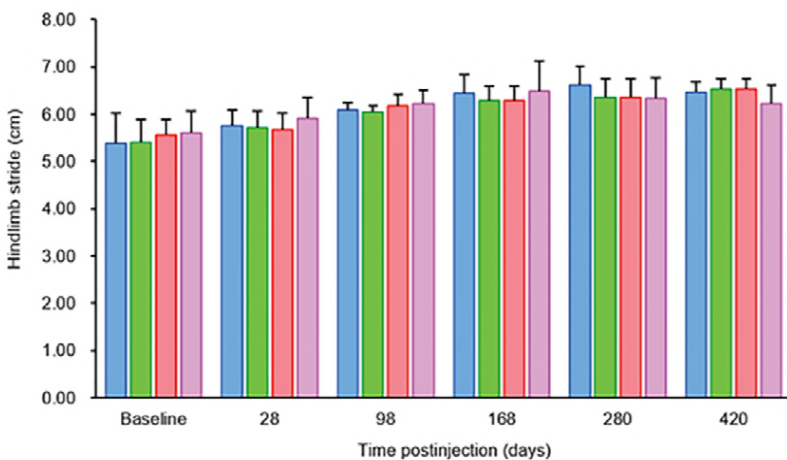
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