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Investigating the neuroprotective effect of AAV-mediated β-synuclein overexpression in

a transgenic model of synucleinopathy

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22 **ABSTRACT**

23 Parkinson's disease (PD) and multiple system atrophy (MSA) are neurodegenerative diseases

24 characterized by inclusions mainly composed of α-synuclein (α-syn) aggregates. The

25 objective of this study was to investigate if β-synuclein (β-syn) overexpression could have

26 beneficial effects by inhibiting the aggregation of α-syn. The M83 transgenic mouse is a

27 model of synucleinopathy, which develops severe motor symptoms associated with

28 aggregation of α-syn. M83 neonate or adult mice were injected with adeno-associated virus

29 vectors carrying the human β-syn gene (AAVβ-syn) or green fluorescent protein gene

30 (AAVGFP) using different injection sites. One or two months later, M83 disease was

31 accelerated or not using brain M83 extracts from mouse (M83) or human (MSA) origins.

32 AAV mediated β-syn overexpression detected by ELISA did not delay the disease onset,

33 regardless of the AAV injection route and of the inoculation of brain extracts. Accordingly,

34 phosphorylated α-syn levels detected by ELISA in sick mice were similar after injecting

35 AAVβ-syn or AAVGFP. Instead, immunohistochemistry analysis of β-syn indicated the

36 presence of proteinase-K resistant β-syn staining specifically in sick M83 mice inoculated

37 with AAVβ-syn. This study indicated for the first time that β-syn could form aggregates in a

38 model of synucleinopathy when it is expressed by a viral vector.

39 **Introduction**

40   Parkinson's disease (PD), dementia with Lewy bodies (DLB) and multiple system

41 atrophy (MSA) are synucleinopathies, characterized by inclusions mainly composed of an

42 aggregated form of α-synuclein (α-syn) in the central nervous system (CNS). As for prions,

43 aggregated forms of α-syn propagate within the CNS during the associated neuro-

44 degenerative diseases. This characteristic was initially suggested in humans by Braak's

45 description of PD stages, and was confirmed later in experimental models of

46 synucleinopathies, in particular in the M83 transgenic mouse model [1]. M83 mice express the

47 human A53T mutated α-syn found in some familial PD forms, under the control of the mouse

48 prion promoter [1]. These mice spontaneously develop severe motor impairment at 8-16 months

49 of age at the homozygous state. The symptomatology is associated with the accumulation in

50 the CNS, of a pathological form of α-syn (α-syn^P), heavily phosphorylated at serine 129

51 residue. We previously showed that, in M83 mice, disease onset can be accelerated by the

52 intracerebral inoculation of brain homogenates from sick M83 mice [2]. This model was further

53 characterized by development of an original ELISA that specifically detects, and allows to

54 easily quantify, the pathological α-syn in sick M83 mice [3-5]. This *in vivo* model may be useful

55 for testing novel therapeutic strategies, particularly targeting progression of the α-syn

56 aggregation.

57 β-synuclein (β-syn) is another member of the synuclein family, lacking a part of the non-

58 amyloid component, a specific region suggested to be amyloidogenic in α-syn. According to

59 *in vitro* studies, unlike α-syn, β-syn alone is not able to form aggregates, but could instead

60 interact with α-syn and reduce its capacity to aggregate [6]. These anti-aggregative features

61 have been investigated i*n vivo*, and some of these studies indeed reported a neuroprotective

62 effect and reduction of α-syn inclusions after overexpression of the human β-syn mediated by

63 DNA microinjection or lentiviral vectors in the transgenic D mouse model, overexpressing

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64   human wild-type α-syn [7,8]. Another study also suggested that crossing mice overexpressing

65   human β-syn with M83 mice delayed the M83 disease onset and reduced the α-syn

66   aggregation [9]. However, two recent studies by Taschenberger *et al* and Landeck *et al* which

67   analyzed the impact of human β-syn overexpression mediated by adeno-associated viral

68   vectors (AAV) on nigral dopaminergic neurons in rats, described a neurodegeneration with β-

69   syn aggregates [10,11].

70   Here, in order to assess the effects of β-syn overexpression in the context of these recent and

71   unexpected data suggesting that β-syn produced using AAV may be able to aggregate, we

72   investigated the impact of human β-syn overexpression mediated by AAV on the onset of the

73   synucleinopathy of M83 mice. Two strategies of inoculation of the AAV vector were

74   sequentially tested: (i) an intracerebroventricular (ICV) inoculation at birth, in order to

75   generate a widespread overexpression of the vector in the central nervous system [12], and (ii)

76   an inoculation of the AAV vector into the ventral tegmental area (VTA) [13], a region of the

77   mesencephalon in which prominent α-syn aggregation occurs in sick M83 mice [1]. Our results

78   indicate that, regardless of the AAV inoculation strategy, continuous AAV-mediated β-syn

79   overexpression in neurons did not delay the onset of the disease and did not modify the α-syn^P

80   levels measured by ELISA, strongly suggesting that β-syn may not protect against α-syn

81   aggregation and propagation.

82

83   **Results**

84   To overexpress β-syn in the M83 CNS, we used self-complementary (sc)AAV9 vectors

85   expressing human β-syn (AAVβ-syn) gene or, as a control, the green fluorescent protein

86   (AAVGFP) gene under the control of the human synapsin1 promoter and with a post-

87   transcriptional WPRE regulatory sequence (Supplementary figure 1A).

88   To functionally evaluate the AAV vectors, we first inoculated AAVβ-syn in the right and left

89   cerebral ventricles of wild-type B6C3H newborn mice (genetic background of M83 transgenic

90   mice) to obtain a widespread expression of the transgene in the CNS [12]. One month later,

91   using viral specific primers, vector mRNA was detected in all regions of the brain and in the

92   spinal cord (Supplementary figure 1B), as described [14], with higher levels in rostral regions of

93   the brain (olfactory bulbs, cortex, hippocampus, striatum) and mesencephalon.

94   AAV vectors were first tested in M83 mice challenged by inoculation of brain extracts from

95   sick M83 mice, an experimental design which is characterized by a quicker onset of the

96   disease with a lower intragroup variability as compared to unchallenged M83 mice [2]. AAV

97   vectors were ICV inoculated at birth and two months later challenged with different brain

98   extracts of sick M83 mice, one derived from a second passage of a sick M83 brain extract in

99   M83 mice (M83/M83 inoculum), and the other from a second passage of a human brain

100   extract from a MSA patient in M83 mice (MSA/M83 inoculum) (Figure 1A) [5]. M83 mice

101   were euthanized after the detection of the first symptoms of disease, *i.e* balance disorders or

102   hind limb paralysis, identified by two independent observers.

103   In both experiments, no delay in the onset of the disease was observed in AAVβ-syn injected

104   mice as compared to control AAVGFP mice, regardless of the type of inoculum (M83/M83 or

105   MSA/M83). Instead, AAVβ-syn injection significantly accelerated the onset of the disease in

106   animals challenged with the M83/M83 extract as compared to control mice (log rank test

107   p<0,05) (Figure 1B). In contrast, no difference was observed in animals challenged with the

108   MSA/M83 extract (Figure 1E). After dissection of the brains, biochemical analyses were

109   realized. As the vector expression was higher in the rostral regions of the brain than in other

110   CNS regions after ICV injections, we first checked the vector expression by detecting total β-

111   syn in the hippocampus. An ELISA test adapted from Krassnig *et al.* allowed to detect a

112   significant overexpression of β-syn (both murine β-syn and human β-syn produced by the

113   vector) in the hippocampus, confirming the continuous expression of the AAVβ-syn until the

114   disease onset (Figure 1C, and F). We next focused on the mesencephalon, the brainstem and

115   the spinal cord because these CNS regions are known to be strongly positive in α-syn^P in sick

116   M83 mice [3-5]. A significant overexpression of β-syn was only detected in the mesencephalon

117   and in the spinal cord of sick M83 mice injected with AAVβ-syn challenged with M83/M83

118   inoculum, but not in mice challenged with the MSA/M83 inoculum (Figure 1C and F). Since

119   the M83 disease is associated with moderate or high α-syn^P levels into different brain regions

120   and in the spinal cord [5], we quantified α-syn^P using ELISA in order to detect a potential effect

121   of β-syn on this biochemical marker of the disease. Our analyses did not show any significant

122   difference in the α-syn^P levels in any of the examined CNS regions after AAVβ-syn

123   inoculation, regardless of the nature of the inoculum used to accelerate the disease (Figure

124   1D, and G).

125   To further study the levels of β-syn protein after AAV injections in M83 mice throughout the

126   CNS, we used Western blot and ELISA which allowed to detect total β-syn in specific brain

127   regions. Comparing total β-syn levels of 5 to 7 months old sick M83 mice inoculated at birth

128   with AAVβ-syn or AAV GFP then challenged with the MSA/M83 inoculum (same mice as in

129   Figure 1E), an overexpression of β-syn protein was detected by ELISA in the olfactory bulbs,

130   cortex, striatum, hippocampus, but not in the other regions (Figure 2A), consistent with the

131   results obtained by qRT-PCR at one month after AAV inoculation (Supplementary figure 1B).

132   Western blot analyses confirmed an overexpression of β-syn protein in the hippocampus of

133   the same mice after AAVβ-syn inoculation (Figure 2B and C). Most of the animals inoculated

134   with AAVβ-syn showed a β-syn overexpression detectable until the disease onset (Figure 1C,

135   F). Only a few animals did not show significant overexpression of β-syn detected by ELISA

136   or by immunohistochemistry (5/48 animals, in the entire study); these animals were excluded

137   for the study analyzing the onset of disease.

138

139   We next examined the possible effects of AAVβ-syn ICV injection on the spontaneous

140   development of the M83 disease during aging, without any challenge to accelerate the disease

141   (Figure 3A). Here again, AAVβ-syn injection neither delayed the disease onset of M83 mice

142   nor modified the α-syn^P levels (Figure 3B and D), despite a sustained expression of the

143   transgene, at least in the hippocampus, up to the disease onset (Figure 3C) (ELISA data

144   correspond to 2 mice for the treated group and 3 mice for the control group; consequently, no

145   statistical analysis was done here).

146   Altogether, these results indicated that AAVβ-syn injection in the ventricles resulted in a

147   widespread expression of the transgene in the CNS, but at a moderate level, especially in the

148   structures that are the most heavily affected by the synucleinopathy lesions. We thus

149   considered another strategy by inoculating the AAV vectors in the ventral tegmental area

150   (VTA), which is located into the mesencephalon, where major brain lesions and accumulation

151   of α-syn^P are detected in sick M83 mice and which is also connected to multiple brain areas

152   [13].

153

154   In order to validate this injection protocol of AAV vectors into the VTA, two months old

155   wild-type mice were first inoculated with AAVβ-syn in the VTA and sacrificed one month

156   later. After dissection of the brains, vector mRNA was mostly detected in the mesencephalon,

157   but also in the cortex, striatum, hippocampus and brainstem; only traces of vector mRNA

158   were identified in the other regions of the brain (except the olfactory bulbs) and in the spinal

159   cord, as compared to mesencephalon (Supplementary figure 1C).

160   The same protocol was then applied to M83 mice. After the inoculation of AAV in the VTA

161   in two months old M83 mice, the disease was accelerated by intracerebrally inoculating the

162   mice one month later with the M83/M83 or the MSA/M83 inoculum (Figure 4A). No

163   significant difference in the survival was observed between the AAVβ-syn and AAVGFP

164   injected mice, between 3 and 8 months (Figure 4B, E). Importantly, analyses by ELISA

165   confirmed the strong overexpression of β-syn in the mesencephalon although no difference

166   was detected in the levels of pathological α-syn^P at the disease stage (Figure 4C, D, F, and G).

167   By immunohistochemistry, using an antibody targeting total β-syn (human and murine β-syn),

168   a specific β-syn staining punctate pattern was detected in the inoculated mesencephalon, as

169   well as in the striatum and brain stem which represent connected regions [13], of all the sick

170   M83 mice inoculated with AAVβ-syn (5/5), but not in sick M83 mice inoculated with

171   AAVGFP (0/2) (Figure 4H). Further analysis indicated that these β-syn immunoreactive dots,

172   which are specifically detected in mice inoculated with AAVβ-syn, mostly co-localize with

173   the presynaptic protein synaptophysin (Supplementary figure 2A). Co-localization of GFP

174   with neuronal marker β-tubulin type 3 in sick M83 mice inoculated with AAVGFP also

175   confirmed that these vectors allowed a neuronal specific expression of the transgene

176   (Supplementary figure 2B). We further biochemically characterized vectors expression after

177   injection in the VTA in M83 mice euthanized 4 months later. Total β-syn levels were

178   quantified by ELISA and showed a significant increase of the protein in the mesencephalon of

179   mice injected with AAVβ-syn as well as in the hippocampus and brain stem compared to

180   control mice injected with the AAVGFP (Figure 5A). β-syn protein overexpression was also

181   readily detected by Western blot in the mesencephalon of most of these mice (Figure 5B).

182

183   We finally asked whether the absence of any significant effect of AAVβ-syn could be due to

184   an insufficient dose of AAV vector. We thus injected 4-times more vector in the VTA one

185   month before the challenge by intra-cerebral inoculation of the M83/M83 inoculum. This

186   AAV dose allowed to significantly increase the overexpression of β-syn at the disease stage

187   (p<0,05) (Figure 5C, D). However, the M83 disease was still not delayed by β-syn

188   overexpression, but instead, as previously observed after ICV injection of AAV (Figure 1B),

189   the disease appeared significantly earlier in the AAVβ-syn injected mice as compared to

190   control AAVGFP animals (p<0,001) (Figure 5E). As in previous experiments, no impact was

191   found on the α-syn^P levels even if β-syn was significantly increased at the stage of clinical

192   signs in all the CNS regions (Figure 5D, F).

193   By immunohistochemistry, as with the low dose of AAV, a specific β-syn staining punctate

194   pattern was detected in the inoculated mesencephalon, as well as in the striatum and brain

195   stem in two sick M83 mice inoculated with AAVβ-syn, but not in one mouse inoculated with

196   AAVGFP (data not shown).

197

198   Proteinase K (PK)-resistant β-syn aggregates have been previously described after inoculation

199   of AAV vectors expressing human β-syn in the *substantia nigra* of rats [10,11]. We thus analyzed

200   by immunohistochemistry the brain of several sick M83 mice after inoculation of AAVs in

201   the VTA (Figure 6). After PK digestion, total β-syn antibody revealed a punctate pattern

202   specifically detected after the inoculation of AAVβ-syn, in the mesencephalon of 7/7 sick

203   M83 mice (5 mice injected with low dose of AAV, of which 2 were challenged with

204   M83/M83 inoculum and 3 with MSA/M83 inoculum, as well as 2 mice injected with high

205   dose of AAV and challenged with M83/M83 inoculum). This punctate pattern was also

206   detected in the striatum and the brain stem where the AAV is also expressed, but not in the

207   cerebellum where the AAV is less expressed (Supplementary figure 3). A few cell bodies

208   were also stained with total β-syn antibody after PK digestion, particularly in the

209   mesencephalon of these mice (data not shown). Even if diffuse, PK resistant staining was also

210   detected in the hippocampal region of two sick M83 mice injected with AAVβ-syn and not

211   challenged with brain extract inoculation, but not in one sick M83 control mouse (data not

212   shown). These results confirm that overexpression of β-syn resulted in PK-resistant β-syn

213   staining which could be detected independently of the vector dose, the inoculation site or the

214   age of inoculation.

215

Discussion

217   β-syn was previously described as a neuroprotective protein, able to inhibit the aggregation

218   process of α-syn *in vitro* and having benefic effects on synucleinopathies *in vivo* [6-9,15]. Here,

219   we tried to slow down a synucleinopathy in a transgenic mouse model with dramatic clinical

220   symptoms linked to pathological α-syn aggregation. In particular, we analyzed the effects of

221   the overexpression of β-syn mediated by an AAV vector on α-syn aggregation. We show here

222   that   the   delivery   of   the   AAVβ-syn   vector   *via*   two   different   injection   routes

223   (intracerebroventricular or in the ventral tegmental area) in transgenic M83 mice, neither

224   delayed the disease onset nor reduced the levels of pathological α-syn at the disease stage,

225   despite a sustained overexpression of β-syn. A previous study suggested that constitutive

226   overexpression of β-syn in transgenic M83 mice by generating double transgenic mice delays

227   the M83 disease onset by several months [9]. In our study, interestingly, M83 mice

228   overexpressing β-syn showed a higher spontaneous activity in experiments in which open

229   field was performed (Supplementary figure 4). These results suggest that β-syn expression

230   might have an impact on mice behavior without affecting the ultimate development of the

231   disease. Importantly, in this study of Fan and colleagues, human β-syn was expressed from

232   the pan-neuronal mouse prion promoter, which is the same promoter used to express human

233   mutated α-syn in the M83 model. It is possible that the strategies tested here did not provide

234   sufficient expression of β-syn, in the CNS regions targeted by the disease. Indeed, as

235   expected, after the injection of AAV in the VTA, we obtained a very high overexpression of

236   β-syn in the mesencephalon in which the aggregation of α-syn is high at the disease stage, but

237   this region may not be the region where the aggregation process of α-syn begins [16]. It should

238   be emphasized that the major signs of the clinical disease of M83 mice are in relation with

239   some - still poorly explained- disorders in the spinal cord [17]. In an additional series of

240   experiments, we were unable to identify a degeneration of motor neurons in the lumbar spinal

241   cord after the injection of brain extract from sick M83 mouse or preformed fibrils of human α-

242   syn in M83 mice, as previously reported after intra-muscular injections [17], further

243   interrogating the cause of the appearance of symptoms (Supplementary figure 5). The ICV

244   injection of AAV9 vector in SMNΔ7 mice, a severe model of spinal muscular atrophy,

245   allowed to express a codon-optimized human SMN1 coding sequence in the spinal cord,

246   which as a result, has improved significantly their survival [18]. After the ICV inoculation of

247   AAV β-syn, the overexpression of β-syn was diffuse and lower in caudal regions of the brain

248   and spinal cord, as described [14], but it may have been insufficient to slow down the

249   synucleinopathy process. It must however be noted that an *in vitro* study using AAV vectors

250   suggests that β-syn overexpression is benefic on α-syn linked toxicity only at a low ratio of β-

251   syn/α-syn expression [10]. Even if it was not evaluated here, another study has described

252   transgene expression in the neuromuscular junction after AAV injection into the ventricles at

253   birth; this may be important since the degeneration of the neuromuscular junction was also

254   described as a possible explanation of the appearance of symptoms in M83 mice [14,17]. Another

255   study suggested that the benefic effect of β-syn is the result of the down regulation of the

256   expression of α-syn, but we did not detect any decrease of α-syn expression, even in the brain

257   regions overexpressing β-syn (data not shown). In another transgenic mouse model (D line)

258   overexpressing human wild type α-syn, two studies reported the reduction of the

259   synucleinopathy by either generating bigenic mice that also overexpress β-syn, or

260   vehiculating the human β-syn transgene, under the control of cytomegalovirus (CMV)

261   promoter, in a lentiviral vector [7,8]. As some reports indicate a downregulation of the CMV

262   promoter in the CNS over time [19], we chose here to use the human synapsin 1 promoter to

263   obtain a long term expression of β-syn, which was confirmed in the present study, even in old

264   mice. Importantly, as previously described [20], the synapsin 1 promoter also allowed to express

265   human β-syn specifically in neurons, where α-syn aggregates are found in the M83 model [1].

266   According to the study by Hashimoto *et al.*, the neuroprotective effect of β-syn was associated

267   to the activation of Akt, a neuroprotective protein which could be indirectly down-regulated

268   by α-syn during the synucleinopathy process [21]. However, we did not detect any significant

269   increase of Akt activation by Western blot in 6 months-old M83 mice inoculated with AAVβ-

270   syn in the VTA at 2 months, and challenged one month later with the M83/M83 inoculum

271   (Supplementary figure 6). It should be noticed that such Akt changes were not consistently

272   observed, as shown in a study with a lentiviral vector used for β-syn overexpression [22].

273   Surprisingly, in the present study, two experiments in mice challenged with a M83/M83

274   inoculum rather showed a significant acceleration of the disease after AAV β-syn injection

275   (Fig 1B and 5E). Interestingly, several studies reported that β-syn could be implicated in the

276   pathological process of synucleinopathies. Indeed, an axonal pathology with β-syn

277   accumulation in axons was reported in patients with Parkinson's disease, DLB or

278   Hallervorden-Spatz syndrome [23,24]. In addition, two mutations of β-syn, identified in DLB

279   cases, were suggested to be responsible for α-syn aggregation and DLB [25]. Generating

280   transgenic mice co-expressing P123H mutated β-syn with human α-syn resulted in an

281   enhanced pathology, suggesting that mutated β-syn could potentiate α-syn aggregation [26].

282   Variable results were also observed after overexpression of β-syn using a lentiral vector after

283   inoculation into the hippocampus of transgenic mice overexpressing a mutated form of the

284   human β-amyloid precursor protein (APP) involved in Alzheimer's disease, as assessed by

285   examining plaque load, memory deficits, and anxiety [22]. However, it should be noted that the

286   expression level of β-syn was relatively low in this latter study (not detectable at the protein

287   level). Also, it is interesting to notice that a truncated form of human recombinant α-syn

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288   lacking the 71-82 residues not able to form aggregates *in vitro*, like β-syn [27], accelerated the

289   disease of a few M83 mice, after injection in the muscle or in the peritoneal cavity [17,28]. These

290   results raise the possibility that β-syn might be able to accelerate the aggregation of α-syn in

291   M83 mice, although this has not been reported so far.

292   Further analyzing the effects of β-syn expression in sick M83 mice inoculated with the AAV

293   vector in the VTA allowed detecting a punctate pattern of β-syn staining in the targeted brain

294   region and some connected regions, only in mice inoculated with AAVβ-syn. This staining

295   was more resistant to PK digestion than that endogenous β-syn in control mice. This result

296   suggests that overexpressed β-syn formed aggregates which could explain why β-syn did not

297   act as expected, *i.e* as an α-syn aggregation inhibitor. Landeck *et al.* have described the same

298   staining of β-syn resistant to PK digestion referred as "dark punctae", two months after the

299   inoculation of AAV5 carrying human β-syn under the control of chicken beta-actin promoter

300   including a CMV enhancer element (CBA) in the *substantia nigra* of rats [11]. Before this study,

301   Taschenberger *et al.* have also described the detection of PK-resistant β-syn aggregates *in*

302   *vivo*, as early as two weeks following the inoculation of AAV2 carrying human β-syn gene in

303   the *substantia nigra* of rats [10]. This time, the vector design was closer to ours, using the same

304   promoter (human synapsin) to express human β-syn, with a WPRE sequence to enhance

305   transgene expression. It is interesting to notice that, even if the AAV serotype used as well as

306   the vector design can differ between studies, all these studies that used AAV to carry β-syn

307   also reported the detection of β-syn aggregates. These observations suggest that the

308   neuroprotective effect of β-syn may vary according to the strategy followed to overexpress it

309   and the animal model. Also, it is important to point out that even if most of neurons express

310   the transgene in the cerebral region injected, there is an important variability of the expression

311   of the transgene in transduced cells after AAV vectors injection (Supplementary figure 7). It

312   might explain at least partly the difficulty to detect a benefic effect of β-syn overexpression as

313   it was suggested to depend on a specific α-syn/β-syn ratio in cell culture [10].

314   The present study suggest for the first time that β-syn overexpression could not be benefic in a

315   synucleinopathy model. This may be due at least partly to the formation of a PK-resistant β-

316   syn species after AAVβ-syn inoculation. According to a very recent study, a mildly acidic pH

317   environment, found in several organelles, could induce β-syn aggregation, highlighting the

318   complexity of β-syn fibrillation mechanisms occurring *in vivo* [29]. Further studies are needed to

319   better understand how and to what extent β-syn could play a role in synucleinopathies.

320   However, even though we could not detect any protective effects of β-syn, our study confirms

321   that an AAV vector is suitable for long-term overexpression of proteins into the CNS.

322   Notably, after ICV inoculations of AAV in neonates, we confirmed a widespread expression

323   of the protein, as this was also recently described using an α-syn AAV vector [30]. The injection

324   of AAV in the VTA also allowed to express human β-syn in various connected regions, as

325   suggested in a previous study using serotype 9 AAV vector carrying a lysosomal enzyme

326   gene [13]. Our data also illustrate the robustness of the experimental model of M83 mice

327   intracerebrally challenged by inocula containing aggregated α-syn, showing rather short and

328   relatively uniform survival periods before the appearance of characteristics clinical signs,

329   which can be used for the assessment of future therapeutic strategies.

330

331   **Methods**

332   *Animals:* M83 transgenic mice were used in this study (B6;C3H-Tg[SNCA]83Vle/J,

333   RRID:MGI:3603036, The Jackson Laboratory, Bar Harbor, ME, USA). These mice express

334   A53T mutated human α-syn protein and spontaneously develop severe motor impairment

335   leading to early death [1]. Homozygous M83 mice develop characteristic motor symptoms

336   between 8 and 16 months of life, beginning with reduced ambulation, balance disorders,

337   partial paralysis of a hind leg and then progressing to prostration, difficulty in feeding, weight

338   loss, hunched back and general paralysis [1]. The animals were housed per group in enriched

339   cages in a temperature-controlled room on a 12h light/dark cycle, and received water and food

340   *ad libitum,* in our approved facilities (No. C69 387 0801) for breeding and experimental

341   studies, in accordance with EEC Directive 86/609/EEC and French decree No. 2013-118. The

342   experimental studies described in this article were performed in containment level 3 facilities

343   and authorized by the « Comité d'éthique » CE2A – 16, ComEth ANSES/ENVA/UPEC and

344   by the « Ministère de l'enseignement supérieur, de la recherche et de l'innovation » (ref 16-

345   006).

346

347   *AAV vectors:* Recombinant self-complementary AAV9 vectors (scAAV9) encoding human β-

348   syn or enhanced GFP (eGFP) were produced by calcium phosphate transfection of HEK-293

349   cells [31]. Three plasmids were transfected simultaneously: (i) a vector plasmid containing the

350   human gene of β-syn or eGFP under control of the neuron-specific synapsin 1 gene promoter

351   (Figure 1A) (ii) a helper plasmid pXX6 [32] and (iii) a plasmid carrying rep2 and cap9 genes [33].

352   Vector particles were extracted and purified on an iodixanol step gradient. Titers were

353   determined by quantitative polymerase chain reaction (qPCR) and expressed as viral genomes

354   per milliliter (vg/mL).

355

356   *Inoculations:* AAV vectors were injected at birth or at adulthood (two months of age), at

357   different sites of inoculation. Neonates were inoculated using 5 μL Hamilton syringe, with

358    $9.38*10^8$ vg of AAVβ-syn or $2.73*10^8$ vg of AAVGFP vectors per lateral ventricle of the

359   viral solution with 0.05% Trypan blue [12]. Adults mice were inoculated with $3.75*10^8$ vg of

360   AAVβ-syn or $1.09*10^8$ vg of AAVGFP (or with high dose: $1.5*10^9$ vg of AAVβ-syn or

361    $4.36*10^8$ vg of AAVGFP) of viral solution in the ventral tegmental area located in the

362   mesencephalon, using stereotaxic coordinates (anteroposterior: -3.16 mediolateral: +0.25

363   dorsoventral: -4.50). Two months after the inoculation of the viral solution in neonates or one

364   month after the inoculation of the viral solution in adults, M83 mice were inoculated with

365   brain homogenates from a second passage of a sick M83 brain sample in M83 mice

366   (M83/M83 inoculum) or a second passage of a patient with multiple system atrophy (MSA)

367   brain sample in M83 mice (MSA/M83 inoculum) (same sample used in a previous publication

368   [5]). These homogenates were injected in the left striatum, using stereotaxic coordinates (AP:

369   +0.14 ML: +2 DV: -2.75). Before each stereotaxic surgery (AAV or brain extract

370   inoculation), mice have been anesthetized with a xylazine (10 mg/kg) and ketamine (100

371   mg/kg) mixture.

372

373   *qRT-PCR:* B6C3H brains were dissected and RNA was extracted from brain regions using

374   RNeasy lipid tissue Minikit (ref 74804, Qiagen, Courtaboeuf, France). RNA samples were

375   tittered using Nanodrop (Nanodrop 1000, Thermo Fisher scientific, Villebon sur Yvette,

376   France). 500 ng of RNA were reverse-transcribed with Quanta qScript cDNA Super Mix (ref

377   95048-100, VWR international, Fontenay-sous-Bois, France) on a Biorad iCycler. After a

378   1:10 dilution, complementary DNA samples were analyzed by qPCR using primers specific

379   for the WPRE region of viral mRNA, and primers specific for GAPDH mRNA. We used the

380   LC 480 SYBR Green 1 Master kit (ref 04887 352 001, Roche, Boulogne-Billancourt, France)

381   on a Roche Light Cycler 480 to perform the qPCR and analyzed the results on the LC 480

382   software.

383

384   *ELISA:* Brains were dissected as described [4] and proteins were extracted in high salt buffer

385   (50 mM Tris-HCl, pH 7.5, 750 mM NaCl, 5 mM EDTA, 1 mM DTT, 1% phosphatase and

386   protease inhibitor cocktails), using a mechanical homogenizer (grinding balls, Precellys 24,

387   Bertin Technologies, Montigny-le-Bretonneux, France) to obtain a 10% homogenate (w/v).

388   Each sample was titered using the DC^{TM} protein assay kit (ref 5000111, Biorad, Marnes-la-

389   Coquette, France).

390   As already described for α-syn^P detection [3,5], plates were saturated with Superblock T20

391   (Thermo Scientific, Rockford, IL, USA) for 1 h at 25°C, under agitation (150 rpm). After 5

392   washes in PBST, 10 µg of protein (for α-syn^P detection) diluted in PBST were incubated for 2

393   h at 25°C, under agitation (150 rpm). α-syn^P was detected with a rabbit polyclonal antibody

394   against PSer129 α-syn (ref ab59264, Cambridge, UK) diluted to 1:3,000 in PBST with 1%

395   bovine serum albumin (BSA); plates were incubated for 1 h at 25°C under agitation. After 5

396   washes, anti-rabbit IgG HRP conjugate (ref 4010-05, SouthernBiotech, Birmingham, AL,

397   USA) was added at 1:2,000 (for α-syn^P detection). After washing, 100 µL of 3,3′,5,5′-

398   tetramethylbenzidine solution (ref. T0440, Sigma, Saint-Quentin-Fallavier, France) were

399   added to each well and plates were incubated for 15 min with shaking. The reaction was

400   stopped with 100 µL of 1 N HCl, and the absorbance was measured at 450 nm with the

401   microplate reader Model 680 (Clariostar, BMG Labtech, Champigny sur Marne, France).

402   ELISA allowing total β-syn quantification was adapted based on an ELISA already published

403   [22]. Briefly, 2 µg or 0.2 µg (for Supplementary figure 3) of brain homogenate was diluted in

404   PBST BSA 1% and incubated at 4°C overnight. After 5 washes in PBST, plates were

405   saturated with Superblock T20 for 1 h at 25°C, under agitation (150 rpm). After 5 washes in

406   PBST, plates were incubated with 1:2,000 of an anti-β-syn antibody ab76111 (ref EP1537Y,

407   Abcam, Cambridge, UK). After 5 washes in PBST, plates were incubated with an anti-rabbit

408   IgG HRP conjugate (ref 4010-05, SouthernBiotech, Birmingham, AL, USA) at 1:4,000. After

409   washing, immunoreactivity was revealed with the same protocol as for α-syn ELISA.

410

411 *Western blot:* 50 µg (for phosphorylated Akt detection) or 10 µg of proteins (for total Akt

412 detection) or 2 µg of proteins (for GFP and β-syn detection) were separated in 12% SDS-

413 polyacrylamide gels and electroblotted onto polyvinylidene fluoride (PvF) membranes 0,45

414 µm (Bio-Rad). The membranes were washed 3 times in Tris-Buffered Saline (TBS) for 5 min

415 at room temperature (RT) under agitation and were saturated 1 h with 5% BSA in TBS 0.1%

416 Tween20 (TBST). Membranes were incubated with rabbit antibody against phosphorylated

417 Akt at Ser473 (ref 9271S, Ozyme, Montigny-le-Bretonneux, France) at 1:1,000 or with rabbit

418 antibody against total Akt (ref 9272, Ozyme, Montigny-le-Bretonneux, France) at 1:1,000 or

419 with anti-β-syn antibody ab76111 (ref EP1537Y, Abcam, Cambridge, UK) at 1:5,000 or with

420 anti-GFP antibody (ref ab290, Abcam, Cambridge, UK) at 1:1,000 or with anti-β-actin

421 antibody (ref ab8226, Abcam, Cambridge, UK) at 1:2000 overnight at 4°C. After 3 washes,

422 the membranes were incubated for 1 h at RT with anti-rabbit HRP-linked antibody (ref

423 7074P2, Ozyme, Montigny-le-Bretonneux, France) for detection of total Akt and

424 phosphorylated Akt at 1:2,000, or with anti-rabbit HRP-linked antibody (ref 4010-05,

425 SouthernBiotech, Birmingham, AL, USA). The immunocomplexes were revealed with

426 chemiluminescent reagents (Supersignal WestDura, ref 34076, Pierce, Interchim, MontLuçon,

427 France), and analyzed using the ChemiDoc system (Bio-Rad) and Image Lab software (Bio-

428 Rad) which allowed to quantify the intensity of the bands.

429

430 *Immunohistochemistry/Immunofluorescence:* After dissection, brain samples were fixed in 4%

431 paraformaldehyde and paraffin embedded to be cut into serial 6 µm sections. After

432 deparaffinization, endogeneous peroxidase activity was directly blocked with oxygenated

433 water 3% during 5 minutes at RT. For immunofluorescence, brain sections were pretreated

434 with a citrate solution (ref C9999, Sigma, Saint-Quentin-Fallavier, France) with heat antigen

435 retrieval. After washing, sections were saturated using a blocking reagent (ref 11096176001,

436   Roche), 1 h at RT and incubated with antibody against β-syn ab76111 in PBST (ref EP1537Y,

437   Abcam, Cambridge, UK) diluted at 1:500, anti-synaptophysin antibody SY38 (ref ab8049,

438   Abcam, Cambridge, UK) diluted at 1:10 or anti-GFP antibody (ref ab290, Abcam,

439   Cambridge, UK) diluted at 1:500 with anti-tubulin β-3 antibody (ref MMS-435P, Biolegend,

440   San diego, USA) diluted at 1:100 at 4°C overnight. After another blocking step 30 minutes at

441   RT, sections were incubated 1 h at RT with anti-rabbit IgG HRP conjugate (ref 4010-05,

442   SouthernBiotech, Birmingham, AL, USA) diluted at 1:250 in PBST. Antibody binding was

443   detected using DAB peroxidase substrate (ref SK-4100, Vector Laboratories, Burlingame, CA

444   USA) intensified with nickel chloride. For immunofluorescence, sections were incubated 1 h

445   at RT with anti-rabbit IgG AlexaFluor 488 or 555 (ref A-11008 and ref A21428, respectively,

446   Thermofischer scientific, Villebon sur Yvette, France) or with anti-mouse IgG AlexaFluor

447   488 or 555 (ref A11001 and A21127 respectively, Thermofischer scientific, Villebon sur

448   Yvette, France) diluted at 1:1000. After washings in PBST then in PBS, sections were treated

449   with an autofluorescence eliminator reagent (ref 2160, Millipore, Temecula, USA), before

450   mounting.

451

452   *PK digestion:* After being deparaffinized, sections were incubated with a 10 µg/mL solution

453   of proteinase K (ref EU0090-B, Euromedex, Souffelweyersheim, France) diluted in PBS, 10

454   minutes at RT (protocol adapted from [10]). Sections were washed three times in water before

455   blocking the endogenous peroxidase activity and pursuing the immunohistochemistry staining

456   protocol.

457

458   *Statistical analysis:* Survival time was defined as the time from birth until death (Figure 3A,

459   exclusively) or as the time from the inoculation of the brain extract until the appearance of the

460   first specific M83 symptoms and euthanasia of the mouse. We right-censored mice found

461 dead without M83 disease identification. Survival times were compared using log-rank test.

462 Concerning statistical analysis of ELISA tests results, means were compared using Student

463 test, when at least 6 animals were included in all groups of animals which were compared or

464 using Wilcoxon test, when at least one group of mice was composed of less than 6 animals.

465 The difference was significant when $p<0,05$ (*), $p<0,01$(**), $p<0,001$(***) .

466

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474

475 **Author contributions**

476 DS, DB, MD, DG, performed experiments. JV, JNA contributed to biochemical and

477 immunohistochemistry assays respectively. LL supervised animal experiments in containment

478 level 3 facilities. AS supervised all the construction and the production of viral vectors. TB,

479 DB designed experiments and supervised the study. DS, TB, DB, AS wrote the paper. All

480 authors read and approved the final manuscript.

481

482 **Conflicts of interest**

483 The authors declare that they have no competing interests.

484

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589

590

591   **LEGENDS**

592

593   **Figure 1. Impact of the intracerebroventricular inoculation of AAV on the accelerated**

594   **disease of M83 mice.** (A) M83 mice were injected at birth (Week 0) with AAVβ-syn or

595   AAVGFP vectors per lateral ventricle and challenged two months later (Week 8) by the

596   injection of M83/M83 (B-D) or MSA/M83 (E-G) inoculum in the striatum to accelerate the

597   disease. After the appearance of the first specific symptoms of M83 disease (from 3 months to

598   8 months after challenge), mice were sacrificed and dissected in order to perform biochemical

599   analysis. (B) M83 disease-associated survival after the inoculation of the M83/M83 brain

600   extract is represented (significant difference according to log rank test, $p<0.05$). (C, D)

601   Quantification of total β-syn (mouse β-syn and human β-syn produced by the vector) (C) or of

602   pathological Ser129-phosphorylated form of α-syn (α-syn^{P}) (D) by ELISA in CNS regions of

603   the same sick M83 mice groups (n=6 for the treated and the control group). (E) M83 disease-

604   associated survival after the inoculation of the MSA/M83 brain extract (no significant

605   difference according to log-rank test). (F, G) Quantification of total β-syn (F) or α-syn^{P} (G) by

606   ELISA in CNS regions of the same sick M83 mice groups (n=6 for the treated group and n=5

607   for the control group). Hi: hippocampus, Mes: mesencephalon, BS: brain stem, CSC: cervical

608   spinal cord, TSC: thoracic spinal cord, LSC: lumbar spinal cord. Data are shown as mean ±

609   sd. *$p<0,05$; **$p<0,01$; ***$p<0,001$ according to Student test (B, C) or Wilcoxon test (E, F).

610

611   **Figure   2.   Characterization   of   the   AAV   vectors   expression   after   their**

612   **intracerebroventricular inoculation in M83 mice.** M83 mice were injected with AAV

23

613   vectors in the ventricles at birth and challenged with MSA/M83 inoculum (same mice than in

614   Figure 1 E-G). (A) Detection of total β-syn synthesis by ELISA in cerebral regions and spinal

615   cord of sick M83 mice 5 to 7 months after ICV injection of AAV. (B) Detection of total β-syn

616   or GFP protein in the hippocampus of the same M83 mice by Western blot. Recombinant β-

617   syn was loaded as a positive control. Numbers refer to each mouse (n=6 in the treated group

618   and 5 in the control group). (C) Quantification of total β-syn levels detected by WB. OB:

619   olfactory bulbs, Cx: cerebral cortex, Str: striatum, Hi: hippocampus, Mes: mesencephalon,

620   BS: brain stem, CSC: cervical spinal cord, TSC: thoracic spinal cord, LSC: lumbar spinal

621   cord. Data are shown as means ± sd. *p<0,05; **p<0,01 according to Wilcoxon test.

622

623   **Figure 3. Impact of the intracerebroventricular inoculation of AAVβ-syn or AAVGFP**

624   **on the spontaneous M83 disease.** (A) M83 mice were injected at birth (Week 0) with

625   AAVβ-syn or AAVGFP vectors per lateral ventricle. After the appearance of the first specific

626   symptoms of M83 disease (from 11 months to 20 months of age), mice were sacrificed and

627   dissected in order to perform biochemical analysis. (B) M83 disease-associated survival after

628   the intracerebroventricular (ICV) inoculation of AAV vectors at birth (no significant

629   difference according to log-rank test). (C, D) Quantification of total β-syn (B) or α-syn[P] (C)

630   by ELISA in CNS regions of the same sick M83 mice. No statistical analysis was done for

631   ELISA results because the number of mice analyzed was too small (2 mice for the treated

632   group and 3 mice for the control group). Hi: hippocampus, Mes: mesencephalon, BS: brain

633   stem, CSC: cervical spinal cord, TSC: thoracic spinal cord, LSC: lumbar spinal cord. Data are

634   shown as means ± sd (n=2 and 3).

635

636   **Figure 4. Impact of the inoculation of AAV in the ventral tegmental area on M83**

637   **disease.** (A) Two months old M83 mice (Week 8) were injected with AAVβ-syn or AAVGFP

638   vectors in the ventral tegmental area (VTA) and challenged one month later (Week 12) by the

639   injection of M83/M83 (B-D) or MSA/M83 (E-G) inoculum in the striatum to accelerate the

640   disease. As before, after the appearance of the first specific symptoms of M83 disease (from 3

641   months to 8 months after challenge), mice were sacrificed and the CNS was dissected in order

642   to realize biochemical analysis and immunohistochemistry /immunofluorescence studies

643   (Figure 4H, Supplementary figure 2, 3). (B) M83 disease-associated survival after the

644   inoculation of the brain extract (no significant difference according to log-rank test). (C, D)

645   Quantification of total β-syn (C) or pathological Ser129-phosphorylated form of α-syn (α-

646   syn^P) (D) by ELISA in CNS regions of the same sick M83 mice groups (n=5 for the treated

647   group, n=5 for the control group). (E) The M83 disease-associated survival after the

648   inoculation of the MSA/M83 inoculum (no significant difference according to log-rank test).

649   (F, G) Quantification of total β-syn (F) or α-syn^P (G) by ELISA in CNS regions of the same

650   mice groups (n=5 for the treated group, n=5 for the control group). (H)

651   Immunohistochemistry pictures showing total β-syn staining (mouse β-syn and human β-syn

652   produced by the vector) in the mesencephalon of 7 months old sick M83 mice inoculated with

653   AAV β-syn or AAV GFP and challenged with MSA/M83 inoculum. Mes: mesencephalon,

654   BS: brain stem, CSC: cervical spinal cord, TSC: thoracic spinal cord, LSC: lumbar spinal

655   cord. Data are shown as means ± sd. *p<0,05; **p<0,01 according to Wilcoxon test. Scale bar

656   100µm.

657

658   **Figure 5. Characterization and impact of inoculation of AAV in the ventral tegmental**

659   **area of M83 mice.** (A-B) Two months old M83 mice were injected with AAVβ-syn or

660   AAVGFP vectors in the VTA and challenged one month later by the injection of M83/M83

661   inoculum in the striatum to accelerate the disease. In this experiment, all mice were

662   euthanized 3 months after the challenge for biochemical analysis. (A) Detection of β-syn and

25

663 GFP proteins by ELISA in the brain and the spinal cord of M83 mice after inoculation of

664 AAVβ-syn or AAVGFP in the VTA (n=4 and 6, respectively). (B) Detection of the total β-

665 syn protein by Western blot in the mesencephalon of the same sick M83 mice. Recombinant

666 β-syn was loaded as a positive control. Numbers refer to mice. (C) Total β-syn protein

667 quantification by ELISA test using 0,2 µg (instead of 2 µg) of mesencephalon homogenates

668 extracted from sick M83 mice inoculated with $1,5*10^9$ vg (high dose, 4-times more than the

669 low dose) or $3,75*10^8$ vg (low dose) of AAVβ-syn or AAVGFP. These results were obtained

670 with the same samples used in Figure 4C and 5D, but 10 times diluted. (D-F) Two months old

671 M83 mice (Week 8) were injected with high dose of AAV vectors (4-times higher than in

672 Figure 4 for each vector) in the VTA and challenged one month later (Week 12) by injecting

673 M83/M83 inoculum in the striatum. (D, F) Quantification of total β-syn (D) or α-syn[P] (F) by

674 ELISA in CNS regions of the same sick M83 mice groups (n=3 and 7 for the treated and

675 control group). (E) M83 disease-associated survival after the inoculation of the brain extract

676 (significant difference according to log-rank test, p<0,001). Mes: mesencephalon, BS: brain

677 stem, CSC: cervical spinal cord, TSC: thoracic spinal cord, LSC: lumbar spinal cord. Data are

678 shown as means ± sd. *p<0,05, **p<0,01, according to Wilcoxon test. Scale bar 100µm.

679

680 **Figure 6. Proteinase K (PK)-resistant β-syn staining in sick M83 mice inoculated with**

681 **AAV β-syn.** (A-D) Immunoreactivity to total β-syn antibody in mesencephalon sections of

682 two sick M83 mice inoculated with low dose of AAVβ-syn or AAVGFP in the VTA and

683 challenged by MSA/M83 inoculum (mice from the study Figure 4E-G) is shown without PK

684 digestion (A, B) or after PK digestion (C, D). After PK digestion, these β-syn immunoreactive

685 dots were detected in all mice analyzed by immunohistochemistry inoculated with the AAVβ-

686 syn in the VTA (7/7 mice, comprising 2 mice challenged with M83/M83 inoculum, 3 mice

687 challenged with MSA/M83 inoculum, and also in 2 mice inoculated with high dose of AAV

688   and challenged with M83/M83 inoculum), but not in sick M83 mice inoculated with

689   AAVGFP (3/3, comprising 2 mice challenged with MSA/M83 inoculum and 1 mouse

690   challenged with M83/M83 inoculum). Scale bars 100µm (low magnification) and 25µm (high
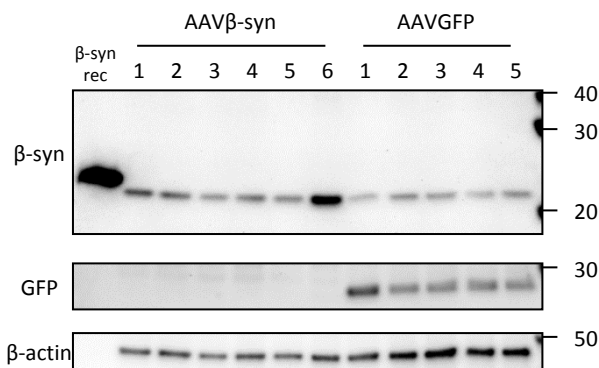
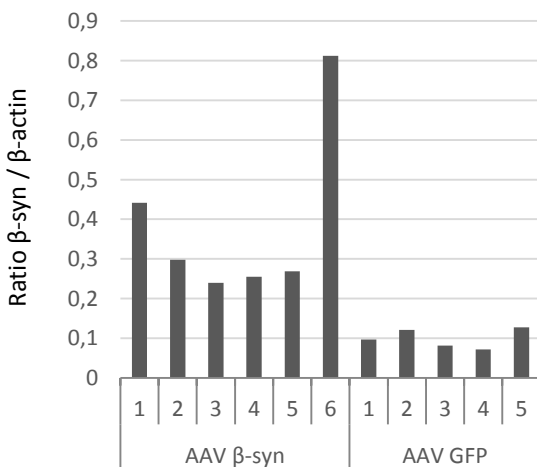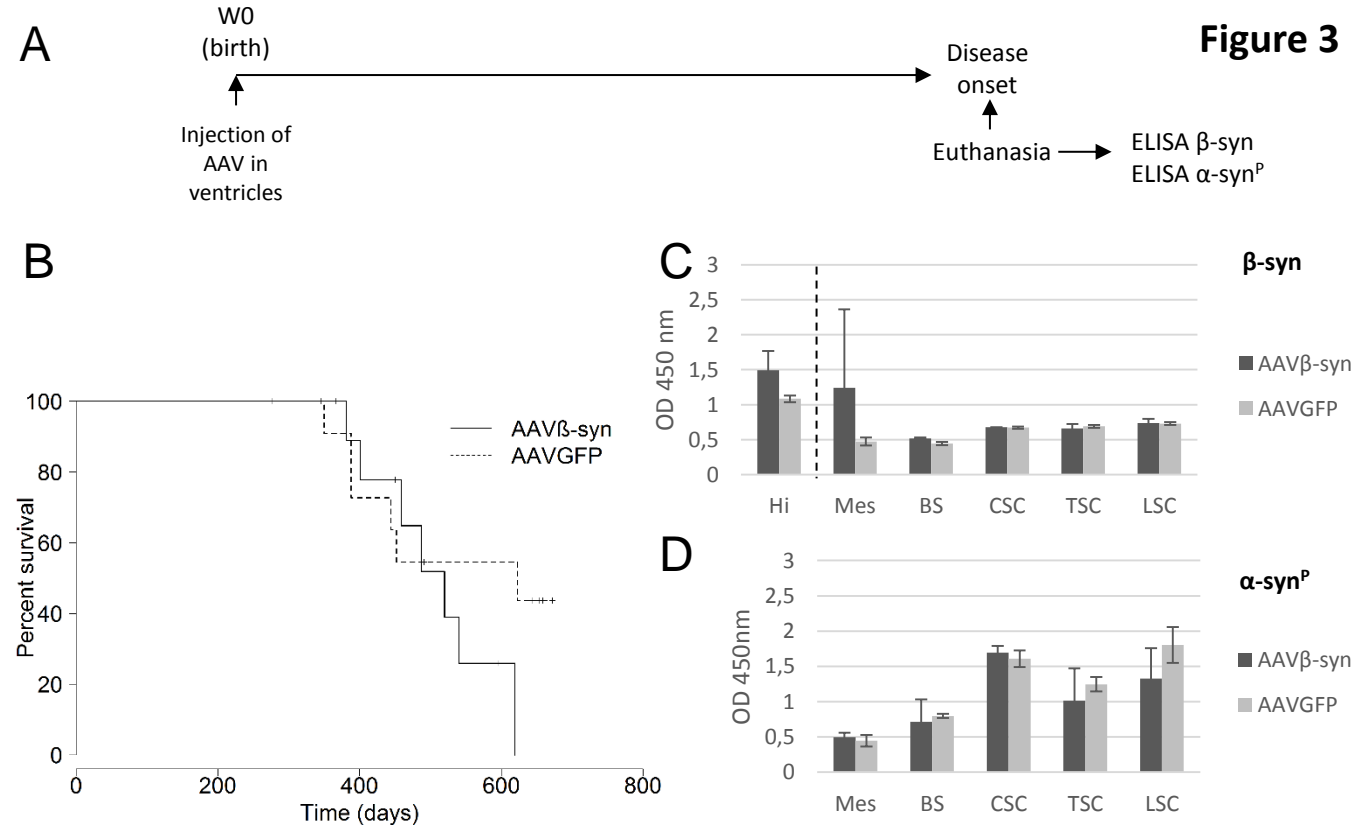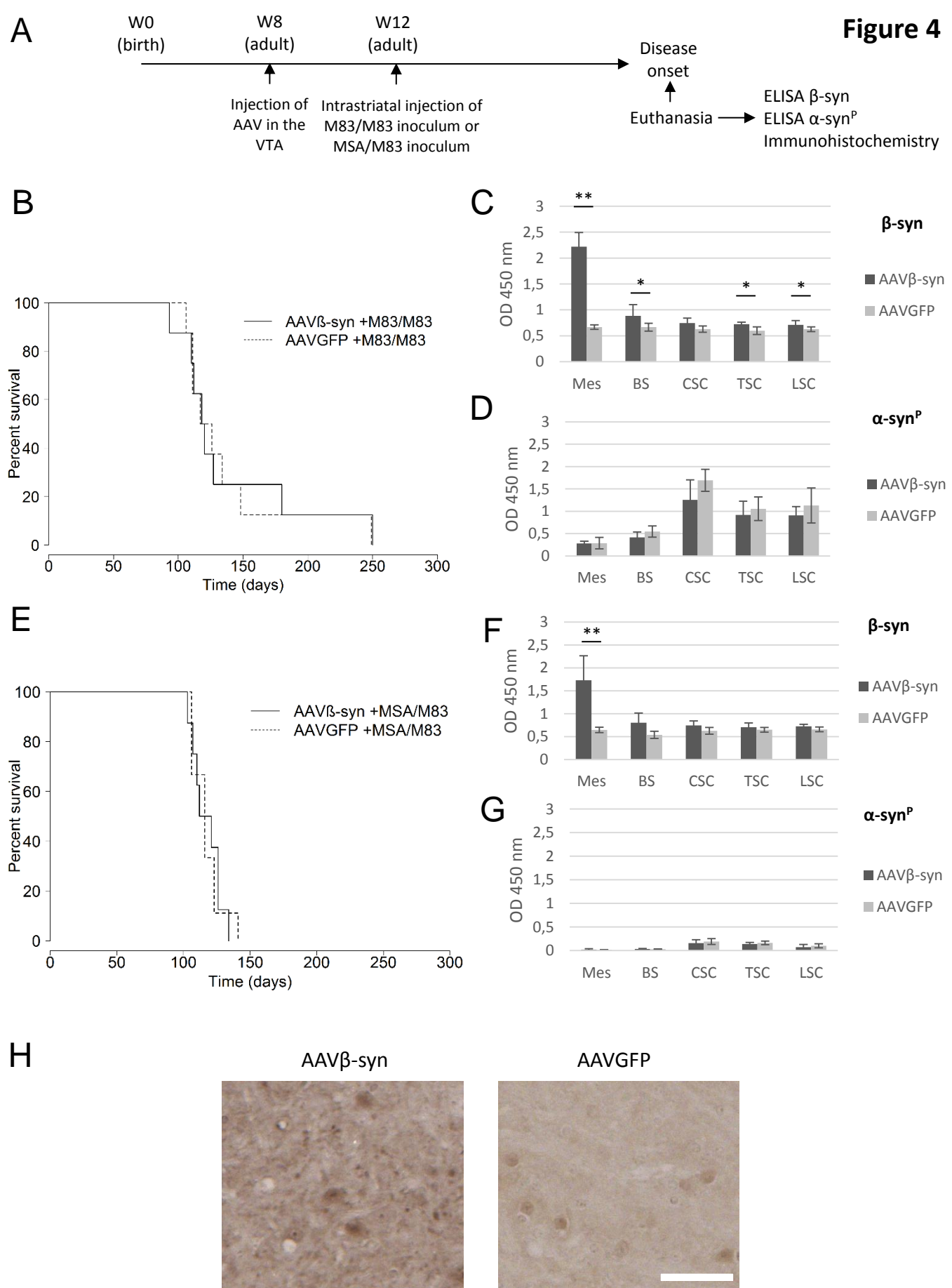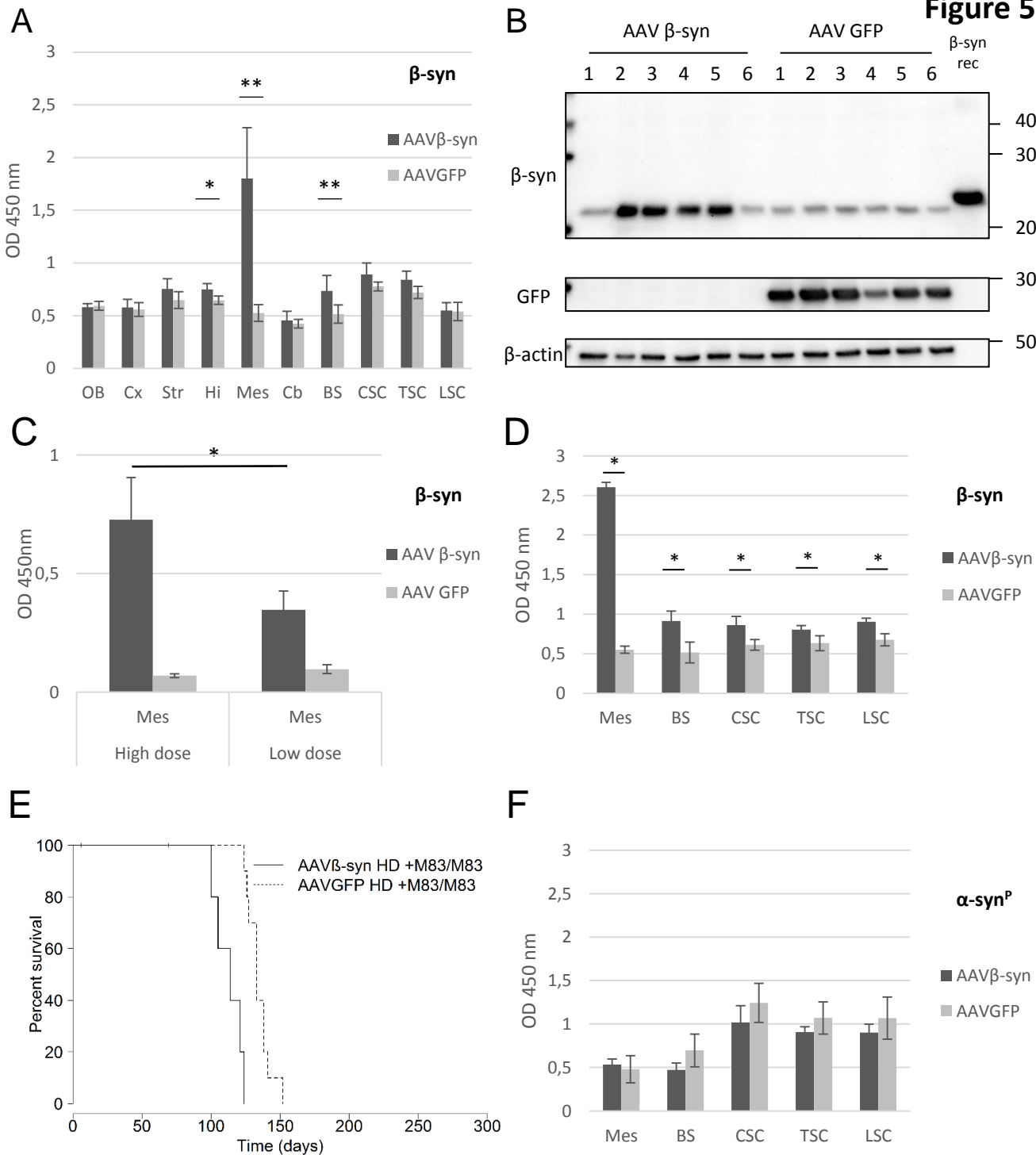691   magnification).

Figure 1
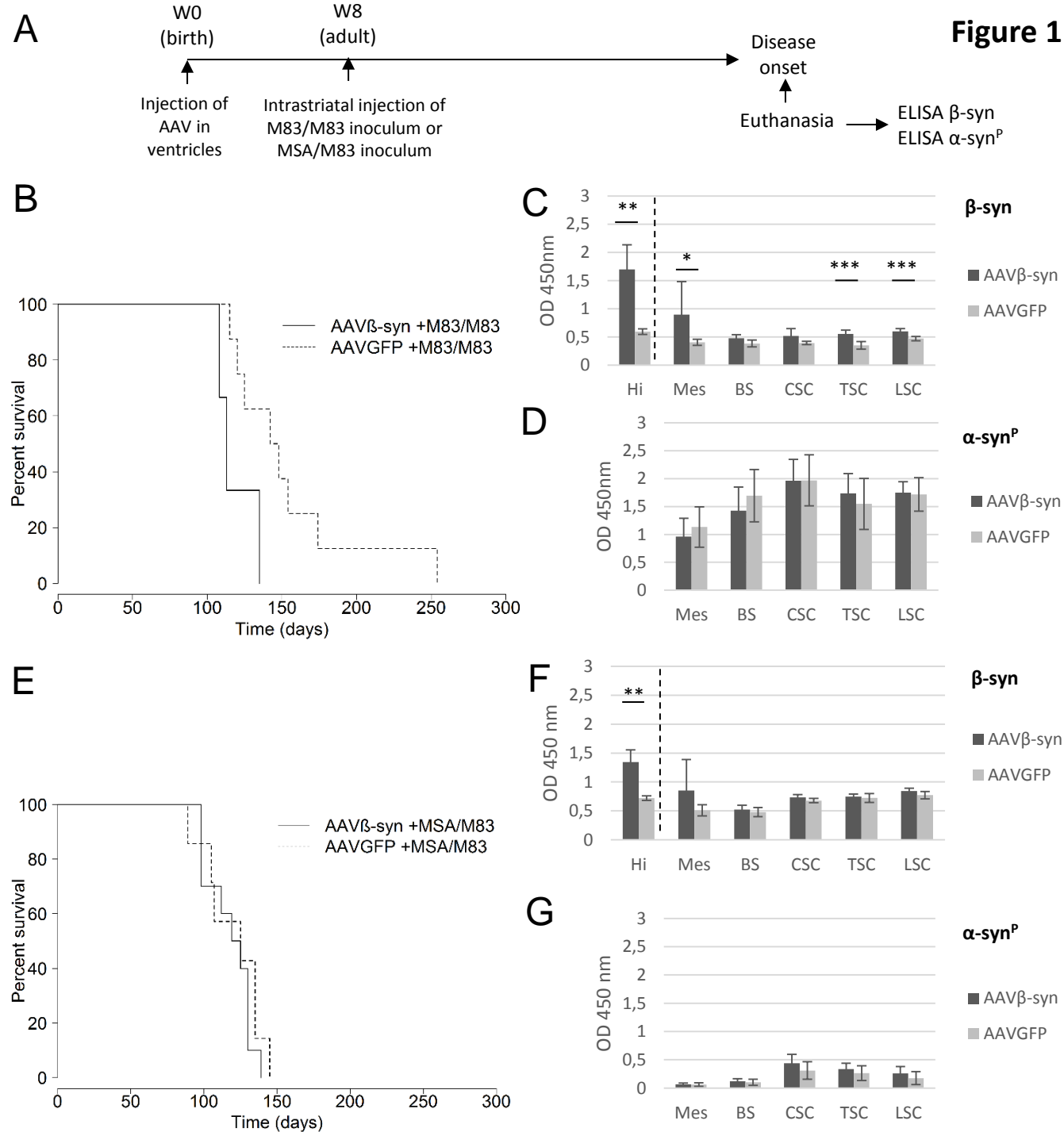
Figure 2

A
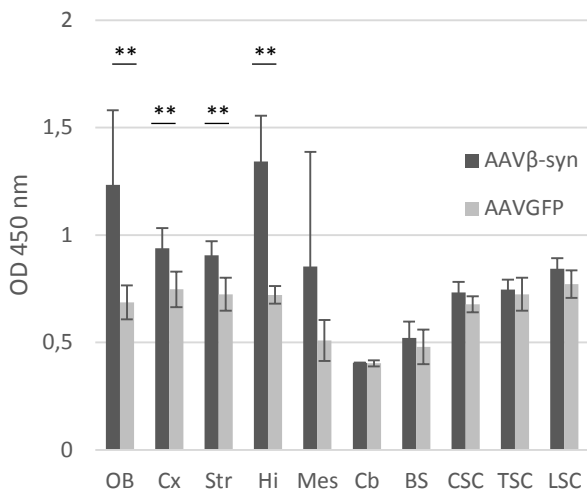
B

C

Figure 3

Figure 4

Figure 5

Figure 6

AAV β-syn

AAV GFP

PK −

PK +