# Metabolic and neurobehavioral disturbances induced by purine recycling deficiency in *Drosophila*

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## Abstract

Adenine phosphoribosyltransferase (APRT) and hypoxanthine-guanine phosphoribosyltransferase (HGPRT) are two structurally related enzymes involved in purine recycling in humans. Inherited mutations that suppress HGPRT activity are associated with Lesch-Nyhan disease (LND), a rare X-linked metabolic and neurological disorder in children, characterized by hyperuricemia, dystonia and compulsive self-injury. To date, no treatment is available for these neurological defects and no animal model recapitulates all symptoms of LND patients. Here we studied LND-related mechanisms in the fruit fly. By combining enzymatic assays and phylogenetic analysis, we confirm that no HGPRT activity is expressed in *Drosophila melanogaster*, making the APRT homologue (Aprt) the only purine-recycling enzyme in this organism. Whereas APRT deficiency does not trigger neurological defects in humans, we observed that *Drosophila Aprt* mutants show both metabolic and neurobehavioral disturbances, including increased uric acid levels, locomotor impairments, sleep alterations, seizure-like behavior, reduced lifespan, and reduction of adenosine signaling and content. Locomotor defects could be rescued by Aprt re-expression in neurons and reproduced by knocking down *Aprt* selectively in the protocerebral anterior medial (PAM) dopaminergic neurons, the mushroom bodies or glia subsets. Ingestion of allopurinol rescued uric acid levels in *Aprt*-deficient mutants but not neurological defects, as is the case in LND patients, while feeding adenosine or $N^6$-methyladenosine ($m^6A$) during development fully rescued the epileptic behavior. Intriguingly, pan-neuronal expression of an LND-associated mutant form of human HGPRT (I42T), but not the wild-type enzyme, resulted in early locomotor defects and seizure in flies, similar to *Aprt* deficiency. Overall, our results suggest that *Drosophila* could be used in different ways to better understand LND and seek a cure for this dramatic disease.

## Keywords

Adenine phosphoribosyltransferase (APRT) – Adenosine signaling – *Drosophila melanogaster* – Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) – Lesch-Nyhan disease – Mushroom bodies – PAM dopamine neurons – Purine salvage pathway – Seizure-like behavior – Startle-induced locomotion – Sleep – Uric acid level

## Introduction

The purine salvage pathway is an essential component of cellular metabolism that allows the recovery of free purine bases derived from the diet or from the degradation of nucleic acids and nucleotides, thus avoiding the energy cost of *de novo* purine biosynthesis (Nyhan et al. 2014). Energy-intensive tissues, such as cardiac muscle and brain cells, extensively use this pathway to maintain their purine levels (Ipata 2011, Johnson et al. 2019). The two main recycling enzymes involved in the salvage pathway in mammals are hypoxanthine-guanine phosphoribosyltransferase (HGPRT), which converts hypoxanthine and guanine into IMP and GMP, respectively, and adenine phosphoribosyltransferase (APRT), which converts adenine into AMP.

APRT and HGPRT deficiencies induce very different disorders in humans. Loss of APRT seems to have only metabolic consequences, leading to the formation of 2,8-dihydroxyadenine crystals in kidney, which can be fatal but is readily prevented by allopurinol treatment (Bollée et al. 2012; Harambat et al. 2012). In contrast, highly inactivating mutations in HGPRT trigger Lesch-Nyhan disease (LND), a rare neurometabolic X-linked recessive disorder with dramatic consequences for child neurodevelopment (Lesch and Nyhan 1964, Seegmiller et al. 1967). The metabolic consequence of HGPRT deficiency is an overproduction of uric acid in the blood

72 (hyperuricemia) that can lead to gout and tophi, or nephrolithiasis (Sass et al. 1965; Kelley et al.
73 1967). Affected children also develop severe neurological impairments, such as dystonia,
74 choreoathetosis, spasticity, and a dramatic compulsive self-injurious behavior (Nyhan 1997; Jinnah
75 et al. 2006; Torres and Puig 2007; Schretlen et al. 2005; Madeo et al. 2019). They have a
76 developmental delay from 3 to 6 months after birth and most of them never walk or even sit without
77 support. Xanthine oxidase inhibitors, such as febuxostat or allopurinol, are given to patients after
78 diagnosis to decrease their uric acid levels and prevent the formation of urate crystals in kidney,
79 which can lead to renal failure (Kelley et al. 1967; Torres et al. 2007; Lahaye et al. 2014). However,
80 there is as yet no treatment to alleviate the neurobehavioral symptoms of LND (Torres et al. 2007;
81 Jinnah et al. 2010; Madeo et al. 2019).

82 To date, the causes of neurobehavioral troubles in LND are still not elucidated (Jinnah et
83 al. 2010, Bell et al. 2016). The most favored hypothesis is a dysfunction of the brain's basal ganglia,
84 and particularly of its dopaminergic pathways (Baumeister and Frye 1985; Visser 2000; Nyhan
85 2000; Saito and Takashima, 2000; Egami et al. 2007). Indeed, analyses revealed a marked loss of
86 dopamine (DA) (Lloyd et al. 1981; Ernst et al. 1996) and DA transporters (Wong et al. 1996) in
87 the basal ganglia of LND patients. DA deficits have also been reported in HGPRT knockout
88 rodents, but without motor or neurobehavioral defects (Finger et al. 1988; Dunnett et al. 1989;
89 Jinnah et al. 1993; Jinnah et al. 1994; Meek et al. 2016). Recent studies reported that HGPRT
90 deficiency disrupts proliferation and migration of developing midbrain DA neurons in mouse
91 embryos, arguing for a neurodevelopmental syndrome (Witteveen et al. 2022). This could result
92 from ATP depletion and impaired energy metabolism (Bell et al. 2021) or an overactivation of *de*
93 *novo* purine synthesis leading to the accumulation of potentially toxic intermediates of this pathway
94 (López, 2008; López et al. 2020). Pharmacological models have also been developed by injecting
95 the neurotoxin 6-hydroxydopamine into neonatally rat brains, which induced a self-mutilation

96 behavior in response to DA agonist administration in adulthood. However, these models are of
97 limited utility as they do not reproduce the basic genetic impairment of LND (Breese et al. 1990;
98 Knapp et al. 2016; Bell et al. 2016).

99 New animal models are therefore needed to study LND pathogenesis and find efficient
100 therapeutic molecules. The fruit fly *Drosophila melanogaster* presents many advantages for
101 translational studies and drug discovery (Fernández-Hernández et al. 2016, Perrimon et al. 2016;
102 Papanikolopoulou et al. 2019). Although the importance of this invertebrate model for studying
103 rare human genetic diseases is now recognized (Oriel and Lasko 2018), a *Drosophila* LND model
104 has not yet been developed to our knowledge. This is probably due to the fact that no HGPRT
105 activity has been detected in this organism (Miller and Collins 1973; Becker 1974a; Becker 1974b).
106 However, an ortholog of APRT is expressed in *Drosophila* (Johnson et al. 1987), encoded by the
107 *Aprt* gene. It is therefore possible that part of the functions of human HGPRT, and in particular
108 those essential for nervous system development and physiology, are endorsed by Aprt in
109 *Drosophila*.

110 Here, we studied the effects of *Aprt* deficiency on purine metabolism, lifespan and various
111 behaviors, including spontaneous and startle-induced locomotion, sleep and seizure-like bang-
112 sensitivity. We show that Aprt is required during development and in adult stage for many aspects
113 of *Drosophila* life, and that its activity is of particular importance in subpopulations of brain
114 dopaminergic neurons and glial cells. Lack of Aprt appears to decrease adenosinergic signaling
115 and induces both metabolic and neurobehavioral symptoms in flies, as is the case with HGPRT in
116 humans. We also find that expression of an LND-associated mutant form of HGPRT, but not the
117 wild-type enzyme, in *Drosophila* neurons, induced neurobehavioral impairments similar to those
118 of *Aprt*-deficient flies. Such a potential toxic gain-of-function effect of mutated HGPRT had not
119 yet been demonstrated in an animal model.

120

121 # Results

122 **Evolution of HGPRT proteins**

123 As shown in Figure S1, the pathways of purine anabolism/catabolism and recycling have been
124 closely conserved between *Drosophila* and humans: all genes related to purine metabolism have
125 homologs in both species, except for the human *HPRT1* gene encoding HGPRT (*step 13* in Figure
126 S1) which has no orthologue in flies (see below), and the lack of urate oxidase in humans (*step 20*).
127 In accordance with reports from about 50 years ago (Miller and Collins 1973; Becker 1974a;
128 1974b), we confirmed that no HGPRT enzymatic activity can be detected in extracts of wild-type
129 *Drosophila melanogaster* (shown in Table S2), using either hypoxanthine or guanine as substrate
130 in the reaction medium. This intriguing observation prompted us to carry out a more precise
131 analysis of the evolution of HGPRT.

132 HGPRT proteins are ancient, for they are present in both Bacteria and Archaea. However, the
133 analysis of the phyletic distribution of HGPRT proteins revealed their striking rareness in Insecta.
134 This conclusion is based on PSI-Blast sequence similarity searches on the NCBI Insecta database
135 (taxid: 6960, 50557). The phylogenetic analysis showed that all the 11 HGPRT proteins found in
136 Insecta cluster mainly with Bacteria, but also with Fungi, Apicomplexa, and Acari (see Figure S2,
137 *red font*, see legend for details). These and further evidence support the acquisition of HGPRT in
138 a few Insecta species by horizontal gene transfer (G. Matassi, unpublished observations). In
139 particular, HGPRT has no homologue in Drosophilidae, with the potential exception of a single
140 species, *Drosophila immigrans*, in which our most recent PSI-BLAST analysis detected one hit
141 (accession KAH8256851.1, annotated as hypothetical protein). Yet this sequence is 100% identical
142 to the HGPRT in the Gammaproteobacterium *Serratia marcescens*. A phylogenetic analysis

143 showed that the *D. immigrans* HGPRT clusters with the *Serratia* genus (Figure S3), suggesting
144 either a contamination of the sequenced sample or a very recent horizontal gene transfer event. The
145 second scenario is more likely for the corresponding nucleotide sequences differ by 5 synonymous
146 substitutions (out of 534 positions). We also carried out structural similarity searches against the
147 RCSB Protein Data Bank repository, using the human HGPRT structure as query (PDB identifiers:
148 5HIA or 1Z7G). This analysis did not identify any protein with a divergent sequence and relevant
149 similarity with HGPRT 3D structure in *Drosophila melanogaster*, consistent with the lack of
150 HGPRT enzymatic activity in this organism.
151

152 ***Drosophila* lacking Aprt activity have a shortened lifespan**

153 Both the phylogeny and enzymatic assays therefore strongly suggest that Aprt (Figure S1,
154 *step 12*) is the only recycling enzyme of the purine salvage pathway in *Drosophila*. To assess the
155 importance of purine recycling in brain development and function in this organism, we analyzed
156 the phenotypes induced by a deficiency in Aprt. The $Aprt^5$ mutant was originally generated by
157 chemical mutagenesis followed by selection of flies resistant to purine toxicity (Johnson and
158 Friedman, 1981; Johnson and Friedman, 1983). Enzymatic assays confirmed a strong reduction in
159 Aprt activity in extracts of heterozygous $Aprt^5$/+ mutants and its absence in homozygous and
160 hemizygous ($Aprt^5$/*Df(3L)ED4284*) mutants (Figure S4 and Table S1). Sequencing of the $Aprt^5$
161 cDNA (Figure S5A) indicated that the $Aprt^5$ mRNA codes for a protein with several amino acid
162 changes compared to *Drosophila melanogaster* wild-type Aprt, modifying in particular 3 amino
163 acid residues that have been conserved in the Aprt sequences from *Drosophila* to humans (Figure
164 S5B). These mutations are likely to be responsible for the loss of enzymatic activity. The
165 homozygous $Aprt^5$ mutants are considered viable because they develop and reproduce normally.

166 However, we observed that these mutants have a significantly reduced longevity, their median
167 lifespan being only 38 days against 50 days for wild-type flies ($p < 0.001$) (Figure 1A).
168
169 **Uric acid levels are increased in *Aprt*$^5$ mutants and rescued by allopurinol**
170 In humans, one of the consequences of HGPRT deficiency on metabolism is the
171 overproduction of uric acid (Harkness et al. 1988; Fu et al. 2015). We assayed the levels of purine
172 metabolites by HPLC and found that the level of uric acid was substantially increased by 37.7%
173 on average in *Drosophila Aprt*$^5$ mutant heads ($p < 0.01$) (Figure 1B). We then tried to rescue uric
174 acid content in flies by providing allopurinol in the diet, as is usually done for LND patients.
175 Allopurinol is a hypoxanthine analog and a competitive inhibitor of xanthine oxidase, an enzyme
176 that catalyzes the oxidation of xanthine into uric acid (Figure S1, *step 19*). Remarkably, the
177 administration of 100 μg/ml allopurinol during 5 days decreased uric acid levels to a normal
178 concentration range in *Aprt*$^5$ mutant heads (Figure 1B). Therefore, quite similarly to HGPRT deficit
179 in humans, the lack of Aprt activity in flies increases uric acid levels and this metabolic disturbance
180 can be prevented by allopurinol.

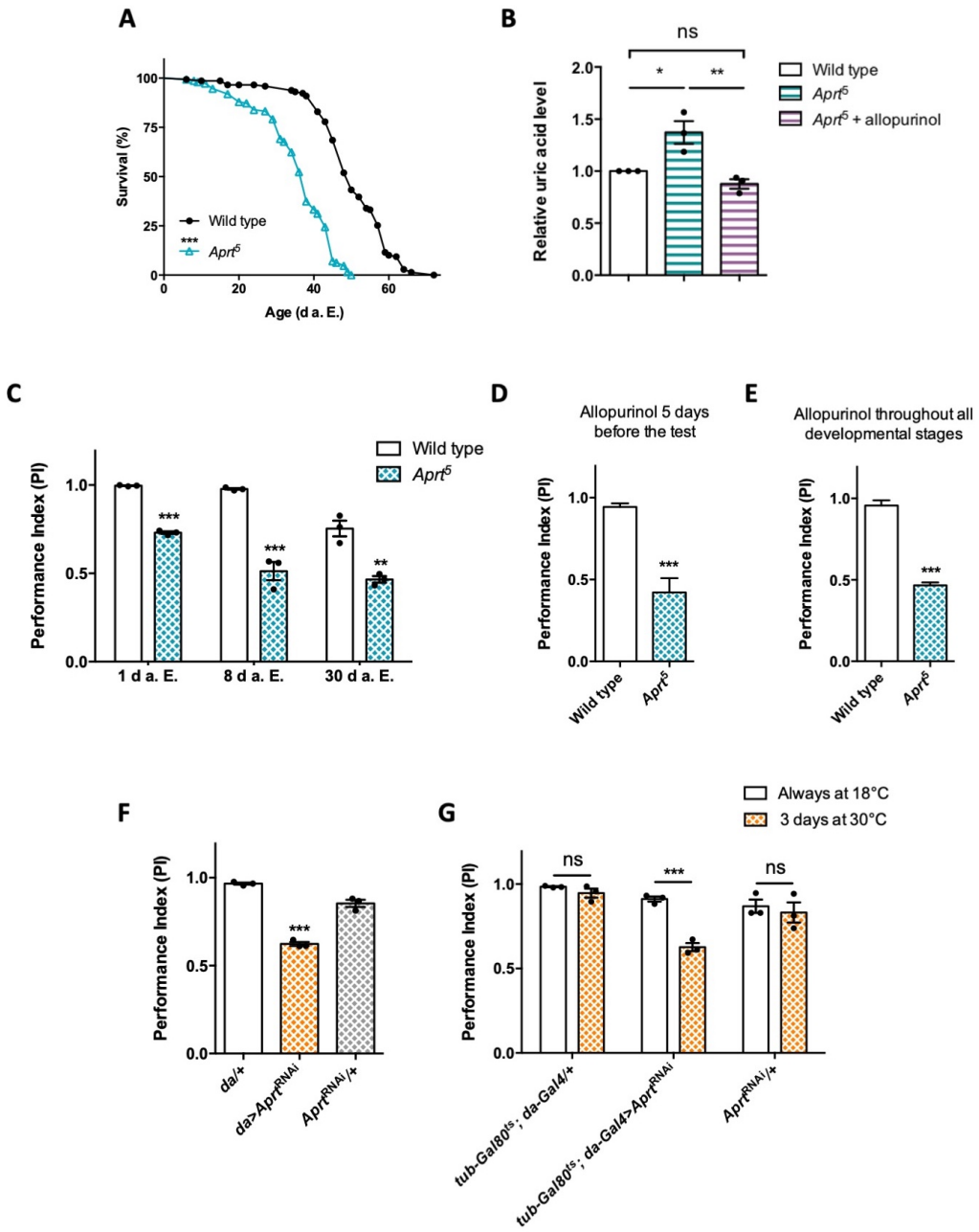



**Figure 1.** Aprt deficiency shortens lifespan and induces metabolic and neurobehavioral defects. (**A**) *Aprt*$^5$ mutant flies have a reduced lifespan compared to wild-type flies (median lifespan: 38 and 50 days, respectively). Three independent experiments were performed on 150 males per genotype with similar results and a representative experiment is shown. Log-rank test (***$p < 0.001$). (**B**) HPLC profiles on head extracts revealed an increase in uric acid levels in *Aprt*$^5$ mutant flies. Administration of 100 µg/ml allopurinol for 5 days before the test rescued uric acid levels. Mean of 3 independent experiments performed on 40 flies per genotype. One-way ANOVA with Tukey's *post-hoc* test for multiple comparisons (*$p < 0.05$; **$p < 0.01$; ns: not significant). (**C-E**) Effect on climbing ability. (**C**) *Aprt*$^5$ mutants show early defects in the SING paradigm that monitors locomotor reactivity and climbing performance. This deficit was already obvious at 1 d

192 a.E. (days after eclosion) and further decreased up to 8 d a.E., after which it did not change
193 significantly up to 30 d a.E. Mean of 3 independent experiments performed on 50 flies per
194 genotype. Unpaired Student's *t* test (**$p < 0.01$; ***$p < 0.001$). (**D, E**) Administration of
195 allopurinol does not rescue the motricity defects of *Aprt*-deficient mutants. Feeding the *Aprt*5
196 mutants with allopurinol (100 µg/ml) either in adults 5 days before the test (**D**) or throughout all
197 developmental stages (**E**) did not alter the defects observed in SING behavior. Results of one
198 experiment performed on 50 flies per genotype at 10 d a. E. Unpaired Student's *t* test (***$p <$
199 $0.001$). (**F**) Downregulating *Aprt* by RNAi in all cells (*da>Aprt*$^{RNAi}$) also led to an early impairment
200 in climbing responses in the SING assay at 10 d a.E., as compared to the driver (*da/+*) and effector
201 (*Aprt*$^{RNAi}$*/+*) only controls. Mean of 3 independent experiments performed on 50 flies per genotype.
202 One-way ANOVA with Tukey's *post-hoc* test for multiple comparisons; (***$p < 0.001$). (**G**) Adult-
203 specific inactivation of *Aprt* (*tub-Gal80*$^{ts}$*; da-Gal4>Aprt*$^{RNAi}$) decreased startle-induced climbing
204 abilities in the SING paradigm, suggesting that the locomotor impairment induced by Aprt
205 deficiency is not caused by a developmental effect. Flies were raised at permissive temperature
206 (18°C) in which Gal80$^{ts}$ suppressed Gal4-controlled *Aprt*$^{RNAi}$ expression, and were shifted from 18
207 to 30°C for 3 days before the test (between 7 and 10 d a.E.) to activate transgene expression. Mean
208 of 3 independent experiments performed on 50 flies per genotype. Two-way ANOVA with Sidak's
209 *post-hoc* test for multiple comparisons (***$p < 0.001$; ns: not significant).

210

211 **Aprt deficiency decreases motricity in young flies**

212 LND patients present dramatic motor disorders that prevent them for walking at an early
213 age. To examine whether a deficiency in the purine salvage pathway can induce motor disturbance
214 in young flies, we monitored the performance of *Aprt*-null mutants in startle-induced negative
215 geotaxis (SING), a widely used paradigm to assess climbing performance and locomotor reactivity
216 (Feany and Bender, 2000; Friggi-Grelin et al. 2003; Riemensperger et al. 2013; Sun et al. 2018).
217 Strikingly, *Aprt*$^{5}$ mutant flies showed a very early SING defect starting from 1 day after eclosion
218 (d a.E.) (performance index (PI) = 0.73 vs. 0.98 for wild-type flies, $p < 0.001$) that was more
219 pronounced at 8 d a.E. (PI = 0.51 vs. 0.96 for the wild-type flies, $p < 0.001$). The fly locomotor
220 performance did not further decline afterwards until 30 d a.E. (Figure 1C). *Df(3L)ED4284* and
221 *Df(3L)BSC365* are two partially overlapping small genomic deficiencies that uncovers *Aprt* and
222 several neighbor genes. Hemizygous *Aprt*$^{5}$*/Df(3L)ED4284* or *Aprt*$^{5}$*/Df(3L)BSC365* flies also
223 displayed SING defects at 10 d a.E. (PI = 0.71 and 0.68, respectively, compared to 0.97 for wild-

224 type flies, $p < 0.01$) (Figure S6). In contrast to its beneficial effect on uric acid levels, we observed
225 that allopurinol treatment did not improve the locomotor ability of *Aprt*$^{5}$ mutant flies, either
226 administered 5 days before the test or throughout the development (Figure 1D, E). This is
227 comparable to the lack of effect of this drug against neurological defects in LND patients.

228 To confirm the effect of *Aprt* deficiency on the SING behavior, we used an *UAS-Aprt*$^{RNAi}$
229 line that reduced Aprt activity by more than 95% when expressed in all cells with the *da-Gal4*
230 driver (Table S1). These *Aprt* knocked down flies also showed a strong SING defect at 10 d a.E.
231 (PI = 0.62 against 0.97 and 0.85 for the driver and *UAS-Aprt*$^{RNAi}$ alone controls, respectively, $p <$
232 $0.001$), like the *Aprt*$^{5}$ mutant (Figure 1F). Next, we used *tub-Gal80*$^{ts}$, which inhibits Gal4 activity
233 at permissive temperature (McGuire et al. 2003), to prevent Aprt knockdown before the adult stage.
234 *tub-Gal80*$^{ts}$*; da-Gal4>Aprt*$^{RNAi}$ flies raised at permissive temperature (18°C) did not show any
235 locomotor impairment (Figure 1G, *white bars*). However, after being transferred for 3 days
236 (between 7 and 10 d a.E.) at a restrictive temperature (30°C) that inactivates Gal80, the same flies
237 demonstrated a similar SING defect as *Aprt*$^{5}$ mutants (PI = 0.63 compared to 0.94 and 0.83 for the
238 driver and RNAi alone controls, respectively, $p < 0.001$) (Figure 1G, *yellow bars with dots*). This
239 demonstrates that *Aprt* inactivation at the adult stage only is sufficient to alter SING performance
240 in *Drosophila*, strongly suggesting that this genotype does not result from developmental defects.
241

242 **Cell specificity of Aprt requirement for startle-induced locomotion**

243 We then tried to identify the neural cells in which Aprt is required to ensure a normal
244 locomotor reactivity in young flies by expressing *Aprt*$^{RNAi}$ with selective Gal4 drivers. Expression
245 in all neurons with *elav-Gal4* led to decreased locomotor performance in the SING test (PI = 0.68
246 at 10 d a.E., vs. 0.90 and 0.86 for the driver and RNAi controls, respectively, $p < 0.05$) (Figure
247 2A), which was comparable to the effects observed with the *Aprt*$^{5}$ mutant or after ubiquitous

248 expression of the RNAi. To confirm the role of neuronal Aprt in locomotor control, we generated
249 a *UAS-Aprt* line which allowed for a substantial increase in Aprt expression and activity (Figure
250 S7). We then found that re-expressing *Drosophila* Aprt selectively in neurons in $Aprt^5$ background
251 partially but significantly rescued the SING phenotype of the null mutant (PI = 0.70 vs. 0.52 for
252 *driver* and *UAS-Aprt* controls in $Aprt^5$ background, $p < 0.05$) (Figure 2B).

253 Furthermore, *Aprt* knockdown in all glial cells with *repo-Gal4*, or in sub-populations of
254 glial cells that express the glutamate transporter Eaat1 with *Eaat1-Gal4*, which includes astrocyte-
255 like glia, cortex glia and some subperineurial glia (Rival et al. 2004; Mazaud et al. 2019), also led
256 to a lower SING performance of 10-day-old flies (PI = 0.72 for *repo-Gal4* vs. 0.91 for both
257 controls, $p < 0.05$, and PI = 0.56 for *Eaat1-Gal4* vs. 0.77, $p < 0.05$, and 0.88, $p < 0.01$, for the driver
258 and RNAi controls, respectively (Figure 2C, D). In contrast, *MZ0709-Gal4* (Doherty et al. 2009)
259 and *NP6520-Gal4* (Awasaki et al. 2008) that selectively target the ensheathing glia, did not induce
260 any significant locomotor defects when used to express the *Aprt* RNAi (Figure S8). Noticeably, re-
261 expressing Aprt with *Eaat1-Gal4* in the $Aprt^5$ background did not rescue the SING phenotype
262 (Figure 2E), in contrast to the positive effect of neuronal re-expression (Figure 2B). Overall this
263 suggests that Aprt is required both in neurons and glia subsets to ensure a normal SING
264 performance in young flies, and that neuronal but not glial *Aprt* re-expression is sufficient to ensure
265 a partially functional locomotor behavior.
266

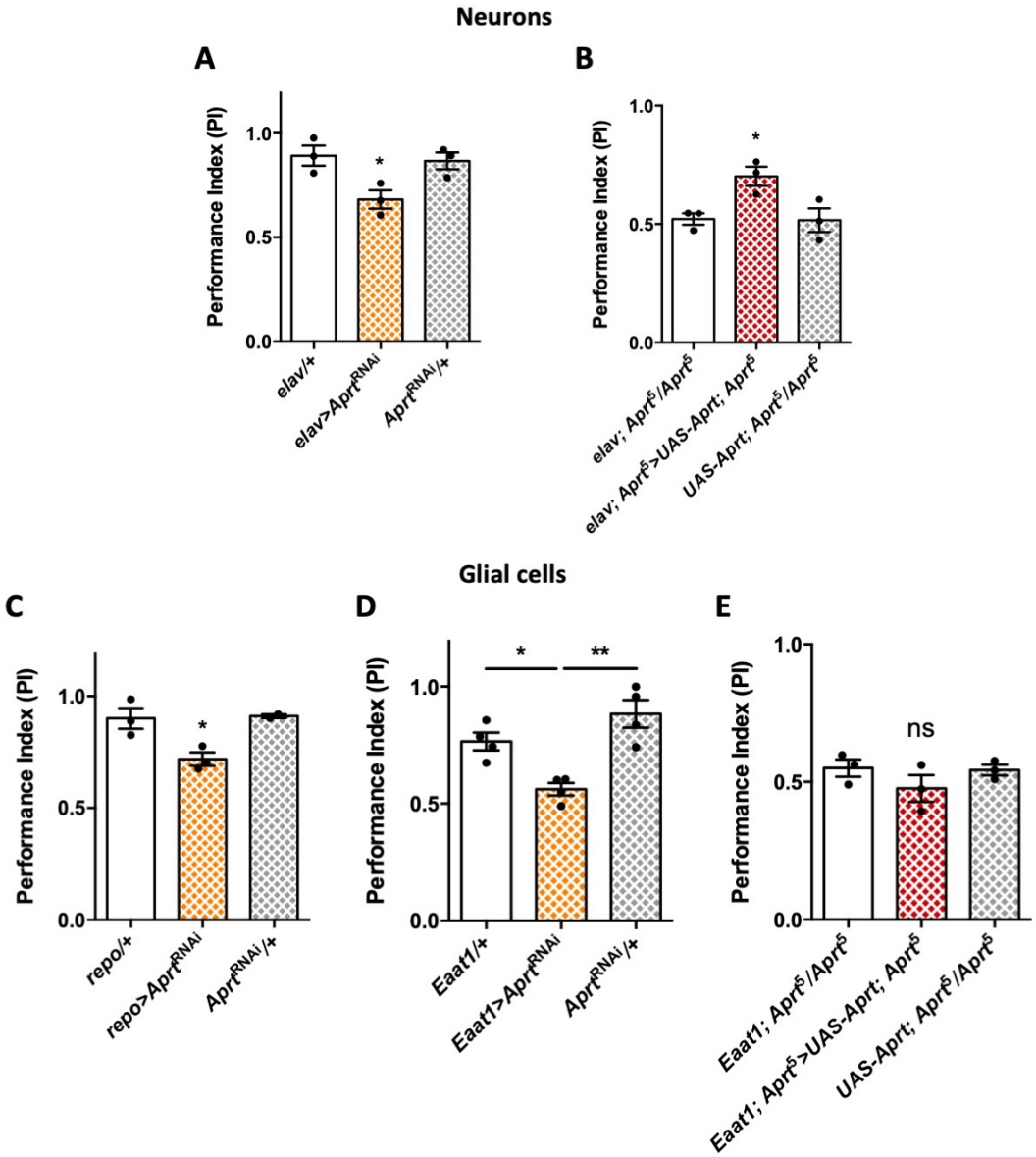


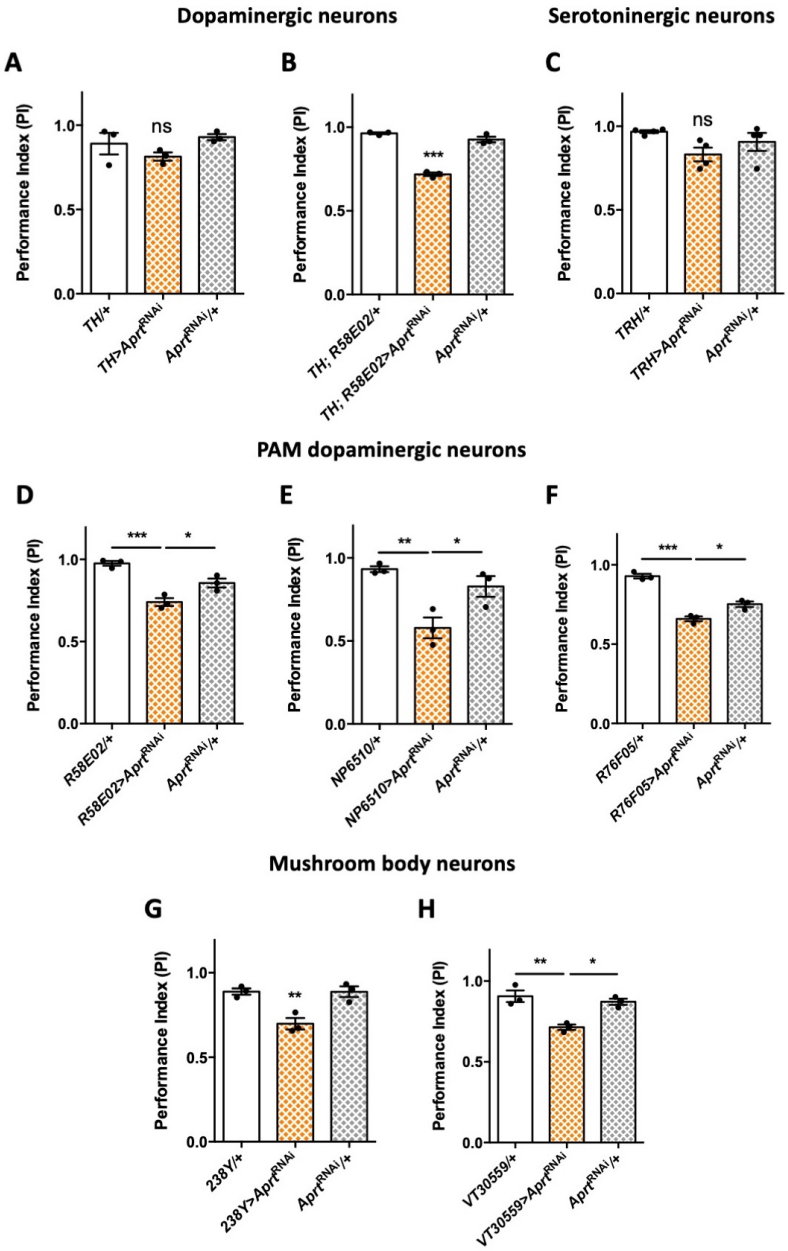

**Figure 2.** *Aprt* knockdown in neurons or glial cells disrupts startle-induced locomotion in *Drosophila*. (**A**) $Aprt^{RNAi}$ expression in all neurons with *elav-Gal4* decreased SING performance in 10-day-old flies. (**B**) Pan-neuronal expression of *Drosophila Aprt* with the UAS-Gal4 system partially rescued the locomotor response of $Aprt^5$ mutants. (**C-D**) Downregulation of *Aprt* expression in all glia with *repo-Gal4* (**C**) or in glial cell that express the glutamate transporter Eaat1 with *Eaat1-Gal4* (**D**) also altered SING performances. (**E**) *Aprt* re-expression in glial cells with *Eaat1-Gal4* driver did not rescue the climbing response of $Aprt^5$ mutants. Results of 3 or 4 independent experiments performed on 50 flies per genotype at 10 d a. E. One-way ANOVA with Tukey's *post-hoc* test for multiple comparisons (*$p < 0.05$; **$p < 0.01$; ns: not significant).

278 To identify the neuronal subpopulations in which Aprt is required to ensure proper
279 locomotor responses in young flies, we first tested dopaminergic drivers since we previously
280 showed that DA neurons play an important role in the control of the SING behavior
281 (Riemensperger et al. 2011; Riemensperger et al. 2013; Sun et al. 2018). *Aprt* knockdown targeted
282 to these neurons with *TH-Gal4* did not induce significant impairments (Figure 3A). This driver
283 expresses in all brain DA neurons except a large part of the protocerebral anterior medial (PAM)
284 cluster (Friggi-Grelin et al. 2003; Mao and Davis 2009; Pech et al. 2013). In contrast,
285 downregulating *Aprt* with the *TH-Gal4, R58E02-Gal4* double driver, which labels all DA neurons
286 including the PAM cluster (Sun et al. 2018), induced a quite similar locomotor defect as did pan-
287 neuronal *Aprt* knockdown (PI = 0.72 vs. 0.96 and 0.93 for the driver and RNAi controls,
288 respectively, $p < 0.001$) (Figure 3B). Besides, downregulating *Aprt* in a majority of the
289 serotoninergic neurons with *TRH-Gal4* (Cassar et al. 2015) did not induce a SING defect (Figure
290 3C).

291 These results suggest that some DA neurons in the PAM cluster are specifically involved
292 in the locomotor impairments induced by *Aprt* deficiency. It has been previously shown in our
293 laboratory that inhibiting DA synthesis in a subset of 15 PAM DA neurons cluster was able to alter
294 markedly SING performance in aging flies (Riemensperger et al. 2013). We and others also
295 reported that the degeneration or loss of PAM DA neurons is involved in the SING defects observed
296 in several *Drosophila* models of Parkinson disease (Riemensperger et al. 2013; Bou Dib et al. 2014;
297 Tas et al. 2018; Pütz et al. 2021). Here we found that expressing $Aprt^{RNAi}$ in the PAM cluster with
298 *R58E02-Gal4* reproduced the same motor disturbance as pan-neuronal expression (PI = 0.74 vs.
299 0.96, $p < 0.001$, and 0.85, $p < 0.05$, for the driver and RNAi controls, respectively) (Figure 3D),
300 and this result was confirmed by using two other PAM drivers (*NP6510-Gal4* – expressing only in
301 15 neurons - *and R76F05-Gal4*) (Figure 3E, F). This strongly suggests that purine recycling

deficiency compromises the correct functioning of these neurons, leading to a defective startle-induced locomotion.

Because PAM DA neurons innervate the horizontal lobes of the mushroom bodies (Liu et al. 2012; Riemensperger et al. 2013), and because this structure has been shown to be involved in the control of startle-induced climbing (Riemensperger et al. 2013; Bou Dib et al. 2014; Sun et



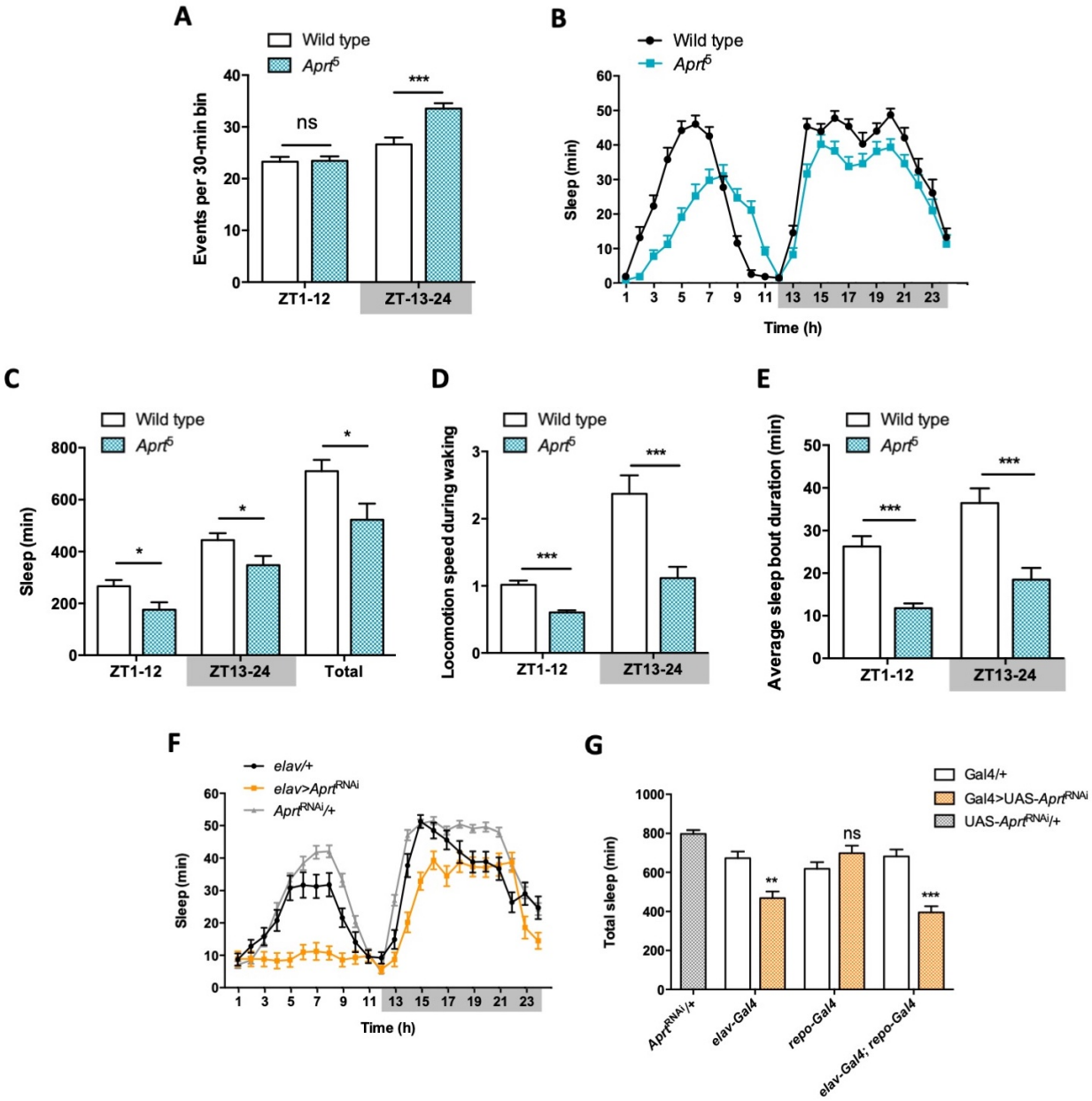
308 **Figure 3.** *Aprt* downregulation in DA neurons of the PAM cluster and in mushroom body neurons
309 impairs startle-induced locomotion. (**A**) RNAi-mediated *Aprt* inactivation in brain DA neurons
310 except a large part of the PAM cluster with the *TH-Gal4* driver did not lead to locomotor defects
311 in the SING assay. (**B**) In contrast, *Aprt* knockdown in all dopaminergic neurons including the
312 PAM cluster with the *TH-Gal4, R58E02-Gal4* double-driver led to a decrease in SING
313 performance. (**C**) *Aprt* downregulation in serotoninergic neurons with *TRH-Gal4* did not alter
314 startle-induced climbing response of the flies. (**D-F**) *Aprt* knockdown selectively in DA neurons of
315 the PAM cluster using either *R58E02-Gal4* (**D**), *NP6510-Gal4* (**E**) or *R76F05-Gal4* (**F**)
316 significantly decreased climbing performance. (**G-H**) *Aprt* knockdown in all the mushroom body
317 intrinsic neurons with *238Y-Gal4* (**G**) or *VT30559-Gal4* (**H**) also led to a decrease in SING
318 performance. Results of 3 or 4 independent experiments performed on 50 flies per genotype at 10
319 d a. E. One-way ANOVA with Tukey's *post-hoc* test for multiple comparisons (*$p < 0.05$; **$p <$
320 $0.01$; ***$p < 0.001$; ns: not significant).
321

322 al. 2018), we also inactivated *Aprt* in MB neurons with *238Y-Gal4* that strongly expresses in all
323 the MB cells (Aso et al. 2009). Interestingly, this driver did induce a locomotor reactivity
324 impairment (PI = 0.70 vs. 0.89 for both controls, $p < 0.01$) (Figure 3G), and the same result was
325 observed with *VT30559-Gal4*, which is a very specific driver for all the MB intrinsic neurons
326 (Plaçais et al. 2017) (Figure 3H). Overall, these results show that normal expression of the SING
327 behavior depends on *Aprt* expression in the PAM and MB neurons in *Drosophila*.
328

329 **Sleep disturbances induced by Aprt deficiency**

330 Both the mushroom body and subpopulations of PAM DA neurons are known to be
331 regulators of sleep in *Drosophila* (Nall et al. 2016; Artiushin and Sehgal 2017). The fact that *Aprt*
332 deficiency in some of these cells impaired locomotor regulation prompted us to monitor the
333 spontaneous locomotion and the sleep pattern of *Aprt* mutants. Compared to controls, *Aprt*-
334 deficient flies did not have an altered circadian activity profile (Figure S9), nor any difference in
335 total spontaneous locomotor activity during the day, as quantified by the number of infra-red beam
336 cuts (*events*) per 30 min, but they showed a 26.2% increase in total activity during the night ($p <$
337 $0.001$) (Figure 4A). As usual, a sleep bout was defined as 5 min or more of fly immobility (Huber

338 et al. 2004) and we checked that wild-type and *Aprt* mutant flies that did not move for 5 min were
339 indeed asleep because they were less sensitive to mild mechanical stimulation (Figure S10). We
340 found that $Aprt^5$ mutants slept significantly less than wild-type flies and that it was the case both
341 during day and night (Figure 4B, C). These mutants indeed showed a reduced walking speed
342 (Figure 4D) and a smaller average sleep bout duration (Figure 4E), indicating a difficulty to
343 maintain sleep. The reduced speed does not seem to be caused by a decreased energetic
344 metabolism, since we could not detect different ATP levels in head and thorax of $Aprt^5$ mutants
345 compared to wild-type flies (Figure S11).




346

347 **Figure 4.** *Aprt*-deficient flies sleep less and walk slower than wild-type flies. (**A**) Quantification of
348 total spontaneous locomotor activity during day and night over 5 LD cycles. *Aprt*$^5$ mutants show
349 no difference in spontaneous locomotion with wild-type flies during the day but a higher activity
350 at night. 3 independent experiments were performed on 32 flies per genotype and mean ± SEM
351 was plotted. Unpaired Student's *t* test (***$p < 0.001$; ns: not significant). (**B**) Sleep pattern during
352 a typical 24h LD cycle showing that the total amount of sleep is smaller during day and night in
353 *Aprt*$^5$ mutants compared to wild-type flies. (**C**) Quantification of day (ZT1-12), night (ZT13-24)
354 and total sleep in *Aprt*$^5$ mutants. ZT is for zeitgeber. (**D**) Locomotion speed during waking is
355 reduced in *Aprt*$^5$ mutants. (**E**) The average sleep bout duration is also decreased, indicating that
356 *Aprt*$^5$ mutants have a difficulty to maintain sleep. (**F**) Sleep pattern of *elav*>*Aprt*$^{RNAi}$ flies, showing
357 that knockdown of Aprt in all neurons led to sleep reduction during the night, similarly to the

358 mutant condition, and an even more profound sleep defect during the day. (**G**) Quantification of
359 total amount of sleep when *Aprt* was downregulated in all neurons (*elav-Gal4*), all glial cells (*repo-*
360 *Gal4*) and both neurons and glial cells (*elav-Gal4*; *repo-Gal4*). Except for glia only, the resulting
361 effect was a significant sleep reduction. For sleep and locomotion speed, means ± SEM were
362 plotted. Unpaired Student's *t* test (C-E) and one-way ANOVA with Tukeys's *post-hoc* test for
363 multiple comparisons (G) (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns: not significant).

364

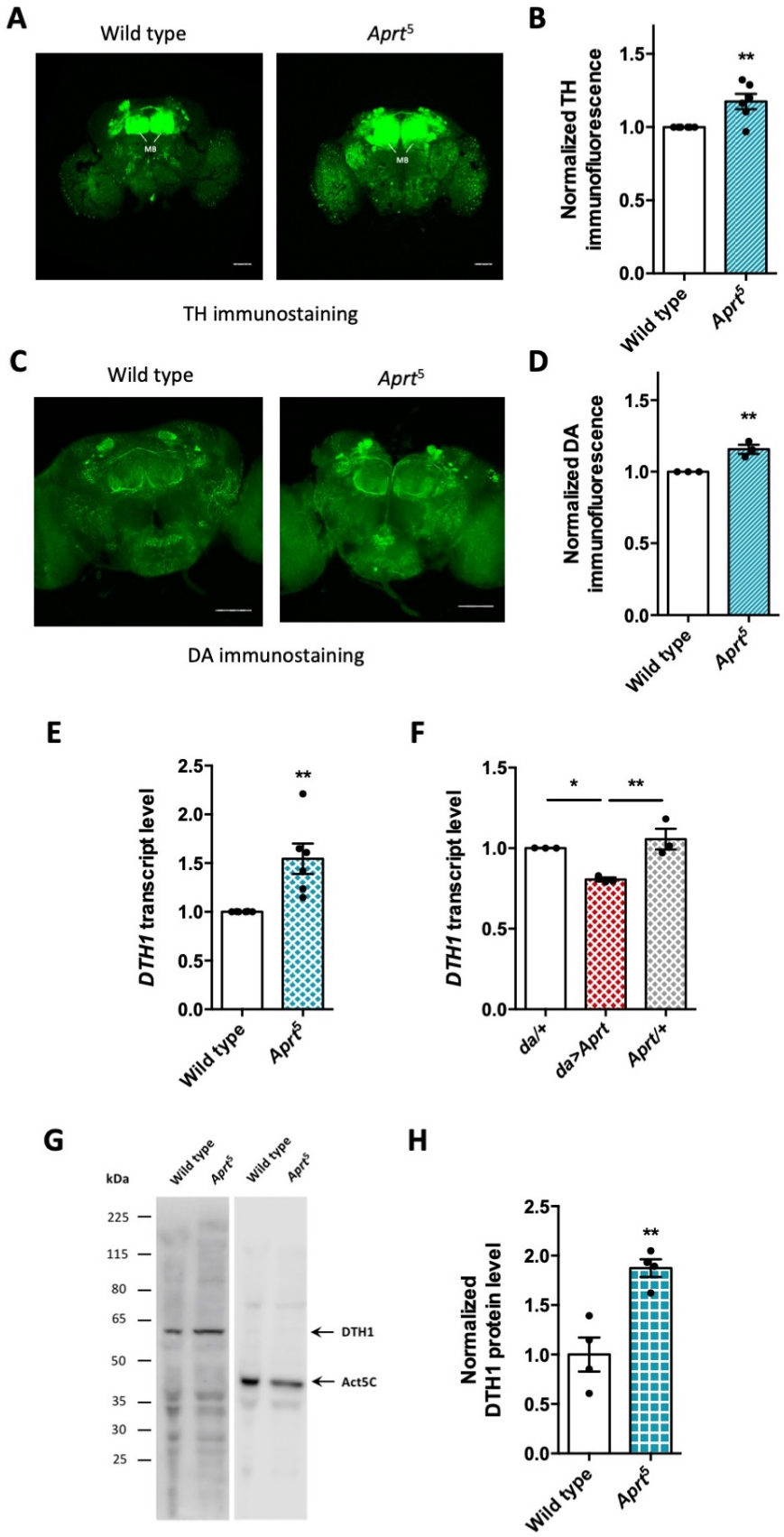
365 Interestingly, RNAi-mediated downregulation of Aprt selectively in neurons
366 (*elav>Aprt*$^{RNAi}$ flies) also significantly decreased sleep duration during day and night (Figure 4F),
367 whereas glial-only expression (*repo>Aprt*$^{RNAi}$) had no effect (Figure S12A). Expressing the RNAi
368 in both neurons and glia (*elav; repo>Aprt*$^{RNAi}$) had similar effects as in neurons alone (Figure
369 S12B). Quantification of total sleep in these experiments, and the total amount of day and night
370 sleep, are shown in Figure 4G and Figure S12C, D, respectively. Overall, these results suggest that
371 *Aprt* expression is selectively needed in neurons for a normal sleep pattern in *Drosophila*.

372

373 **Brain DA synthesis and levels are increased in *Aprt*-deficient flies**

374 Since we found that induced locomotion and sleep, two behaviors controlled by DA neurons
375 in *Drosophila*, were altered in *Aprt*-deficient flies, we logically expected brain DA levels to be
376 reduced in these mutants, as is the case in the basal ganglia of LND patients. We therefore carried
377 out comparative immunostaining for tyrosine hydroxylase (TH), the specific enzyme for DA
378 synthesis (Friggi-Grelin et al. 2003; Riemensperger et al. 2011), on dissected adult brains from
379 wild-type and *Aprt*$^{5}$ mutant flies. However, the global TH protein level appeared not to be
380 decreased, but relatively increased by 17.5% in the *Aprt*$^{5}$ mutant brain ($p < 0.01$), in particular
381 around the mushroom bodies, a structure that receives dense dopaminergic projections (Figure 5A,
382 B). Moreover, DA immunostaining carried out on whole-mount dissected brains revealed a
383 similarly increased level of this neuromodulator in *Aprt*$^{5}$ flies, by 17.0% on average in the entire
384 brain ($p < 0.01$), but not specifically in the mushroom bodies or another part of the brain (Figure

385 5C, D). We also found that the transcript levels of *DTH1*, encoding the TH neuronal isoform in
386 *Drosophila*, were increased in *Aprt*$^{5}$ mutants compared to wild-type flies (Figure 5E), and,
387 conversely, decreased when Aprt was overexpressed ubiquitously (Figure 5F). Western blot
388 experiments further indicated that DTH1 protein levels are increased in *Aprt*$^{5}$ compared to controls
389 (Figure 5G-H). This indicates that disruption of the purine salvage pathway does not impede DA
390 synthesis and levels in the *Drosophila* brain, which are instead slightly increased. We therefore
391 searched for another neurotransmitter system that could be affected by *Aprt* deficiency.


A
Wild type
Aprt5
MB
MB
TH immunostaining
B
Normalized TH immunofluorescence
1.5
1.0
0.5
0.0
**
Wild type
Aprt5
C
Wild type
Aprt5
DA immunostaining
D
Normalized DA immunofluorescence
1.5
1.0
0.5
0.0
**
Wild type
Aprt5
E
DTH1 transcript level
2.5
2.0
1.5
1.0
0.5
0.0
**
Wild type
Aprt5
F
DTH1 transcript level
1.5
1.0
0.5
0.0
*
**
da/+
da>Aprt
Aprt/+
G
kDa
Wild type
Aprt5
Wild type
Aprt5
225
115
80
65
50
35
30
25
DTH1
Act5C
H
Normalized DTH1 protein level
2.5
2.0
1.5
1.0
0.5
0.0
**
Wild type
Aprt5

392

393 **Figure 5.** *Aprt* deficiency increases DA synthesis and content in the *Drosophila* brain. (**A**)
394 Representative confocal projections of TH-immunostained whole-mount adult brains from wild-
395 type flies and *Aprt*$^5$ mutants. MB: mushroom body. Scale bars: 100 µm. (**B**) Quantification of TH
396 immunofluorescence intensity normalized to the controls in the entire brain. 4 to 6 brains were
397 dissected per experiment and genotype, and 6 independent experiments were performed (**$p <$
398 $0.01$). (**C**) Representative confocal projections of DA immunostaining in whole-mount adult brains
399 of wild type and *Aprt*$^5$ mutants. Scale bars: 100 µm. (**D**) Quantification of DA immunofluorescence
400 intensity over the entire brain showed a slight increase in DA content in *Aprt*$^5$ mutants compared
401 to wild-type controls. 6 brains were dissected per experiment and genotype, and 3 independent
402 experiments were performed. Unpaired Student's *t* test (**$p < 0.01$). **(E)** mRNA levels of *tyrosine*
403 *hydroxylase* neuronal form *DTH1* are markedly increased in *Aprt*$^5$ mutant heads compared to wild-
404 type flies. Results of 6 independent RT-qPCR experiments carried out on 3 to 4 different RNA
405 extractions from 20-30 male heads per genotype. Unpaired Student's *t* test (**$p < 0.01$). (**F**)
406 Conversely, overexpressing Aprt in all cells with the *da-Gal4* driver (*da>Aprt*) reduced mRNA
407 level of *DTH1* in heads, as compared to the driver (*da*/+) and effector (*Aprt*/+) controls. Mean of 3
408 independent experiments performed on 3 different RNA extractions from 20-30 male fly heads.
409 One-way ANOVA with Tukey's *post-hoc* test for multiple comparisons (*$p < 0.05$, **$p < 0.01$).
410 (**G**) Representative western blot of wild-type and *Aprt*$^5$ mutant head extracts probed with anti-TH
411 and anti-actin beta antibodies. (**H**) Quantification of DTH1 protein levels in adult wild-type and
412 *Aprt*$^5$ mutant heads by western blots showed an increase in DTH1 protein level (60 kDA) in *Aprt*$^5$
413 mutants. Actin (Act5C, 42kDa) was used as a loading control. Results are the mean of four
414 determinations in two independent experiments. Unpaired Student's *t* test (**$p < 0.01$).

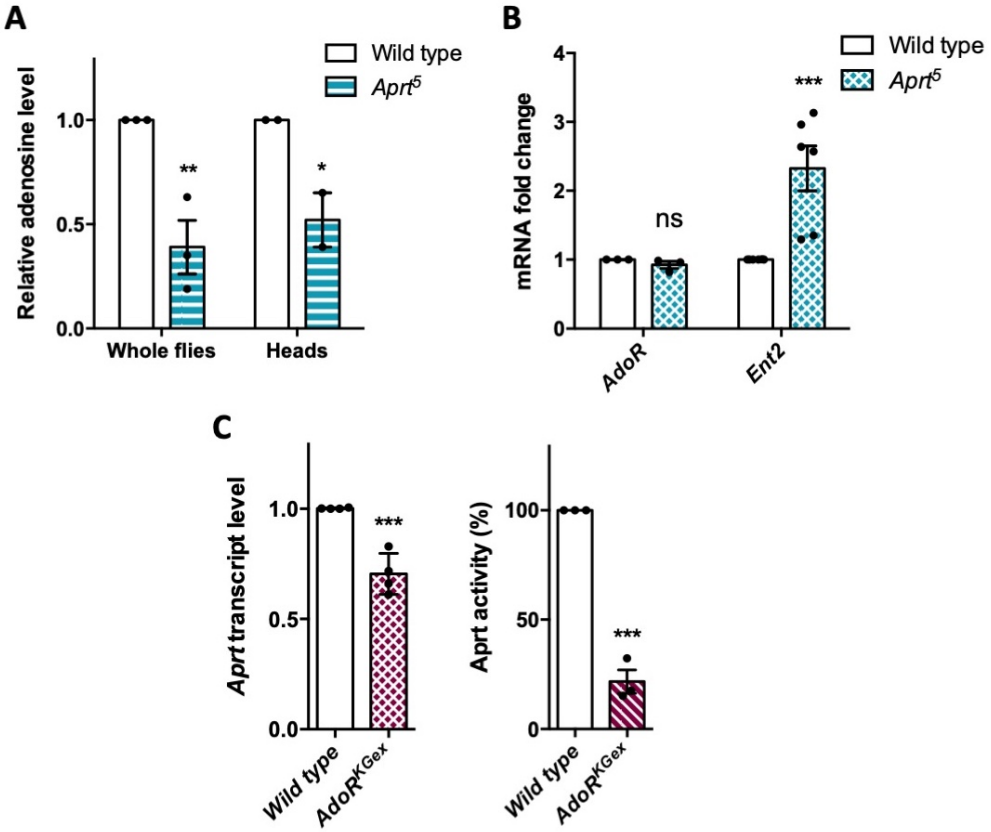
415

416

417 **Interactions between Aprt and the adenosinergic system**

418 Aprt catalyzes the conversion of adenine into AMP, and AMP breakdown by the enzyme
419 5'-nucleotidase produces adenosine, primarily in the extracellular space. Adenosine then acts as a
420 widespread neuromodulator in the nervous system by binding to adenosine receptors. We therefore
421 suspected that adenosine level could be reduced in the absence of Aprt activity, leading to
422 alterations in adenosinergic neurotransmission. Indeed, we found a significant decrease in
423 adenosine level either in whole flies (by 61.0% on average, $p < 0.01$) or in heads (by 48.0%, $p <$
424 $0.05$) in *Aprt*$^5$ mutants (Figure 6A). We then examined the consequences of this reduction on
425 molecular components of the adenosinergic system, namely G protein-coupled adenosine receptors
426 and equilibrative nucleoside transporters (ENTs), which carry out nucleobase and nucleoside

427 uptake of into cells. Only one seven-transmembrane-domain adenosine receptor, AdoR, is present
428 in *Drosophila*, that is very similar to mammalian adenosine receptors (Dolezelova et al. 2007;
429 Brody and Cravchik, 2000), and three putative equilibrative nucleoside transporters (Ent1-3) have
430 been identified (Sankar et al. 2002) but only one, Ent2, showed nucleoside transport activity
431 (Machado et al. 2007). Interestingly, Ent2 has been shown to be enriched in the mushroom bodies
432 and its transcript level to be highly elevated in *AdoR* mutant flies (Knight et al. 2010). In $Aprt^5$
433 background, we also observed a strong increase in *Ent2* mRNAs (2.3-fold higher than wild-type
434 flies, $p < 0.001$), but no noticeable effect on *AdoR* transcript level (Figure 6B). The increased
435 expression of *Ent2* could correspond to a compensatory response to adenosine shortage in $Aprt^5$
436 mutants.




437

438 **Figure 6.** Relations between Aprt and molecular components of adenosinergic signaling. (**A-B**)
439 Impacts of the lack of Aprt activity on the adenosinergic system. (**A**) Adenosine level was measured

440 in whole flies or heads of *Aprt*$^5$ flies by UPLC. Compared to wild-type flies, adenosine level was
441 significantly reduced in the mutants. Results of 3 independent experiments performed with 5 males
442 per genotype in triplicates, and 2 independent experiments with 30 heads per genotype in
443 triplicates. Two-way ANOVA with Sidak's *post-hoc* test for multiple comparisons (*$p < 0.05$; **$p$
444 $< 0.01$). (**B**) *Aprt*$^5$ mutation did not affect *AdoR* expression but induced a marked increase in
445 adenosine transporter *Ent2* mRNA abundance. 3-6 different RNA extractions were performed on
446 20-30 male heads. Results of 3 to 6 independent experiments. Two-way ANOVA with Sidak's
447 *post-hoc* test for multiple comparisons (***$p < 0.001$; ns: not significant). (**C**) Null *AdoR*$^{KGex}$
448 mutants showed decreased *Aprt* expression (*left panel*) and a stronger decrease in Aprt activity
449 (*right panel*). 4 independent RNA extractions were carried out on 20-30 male heads and 4
450 independent real-time PCR determinations were done per RNA sample. For Aprt activity, 3
451 independent determinations were performed on 20 whole flies per genotype. Unpaired Student's *t*
452 test (***$p < 0.001$).
453

454 The AdoR receptor is highly expressed in adult fly heads and its ectopic overexpression
455 leads to early larval lethality (Dolezelova et al. 2007). In contrast, a null mutant of this receptor,
456 *AdoR*$^{KGex}$, in which the entire coding sequence is deleted, is fully viable (Wu et al. 2009). This
457 enabled us to examine the consequences of a complete lack of AdoR on purine recycling in adult
458 flies. We found that *Aprt* transcripts were decreased by 29.5% on average ($p < 0.001$) in *AdoR*$^{KGex}$
459 mutant heads (Figure 6C, *left panel*), while Aprt activity was even more decreased by 78.4% ($p <$
460 $0.001$) compared to wild-type flies (Figure 6C, *right panel*). This effect likely results from the
461 much increased level of adenosine uptake in the *AdoR* mutants, which would downregulate *Aprt*
462 activity by a feedback mechanism. Overall, these data indicate that extracellular adenosine levels
463 must be strongly decreased in Aprt mutant flies, both from the general reduction in adenosine levels
464 and the increased expression of the Ent2 transporter. Although AdoR expression is not affected,
465 AdoR signaling is therefore expected to be significantly reduced in *Aprt* mutants.
466

467 ***Aprt* mutants show epilepsy-like seizure behavior**

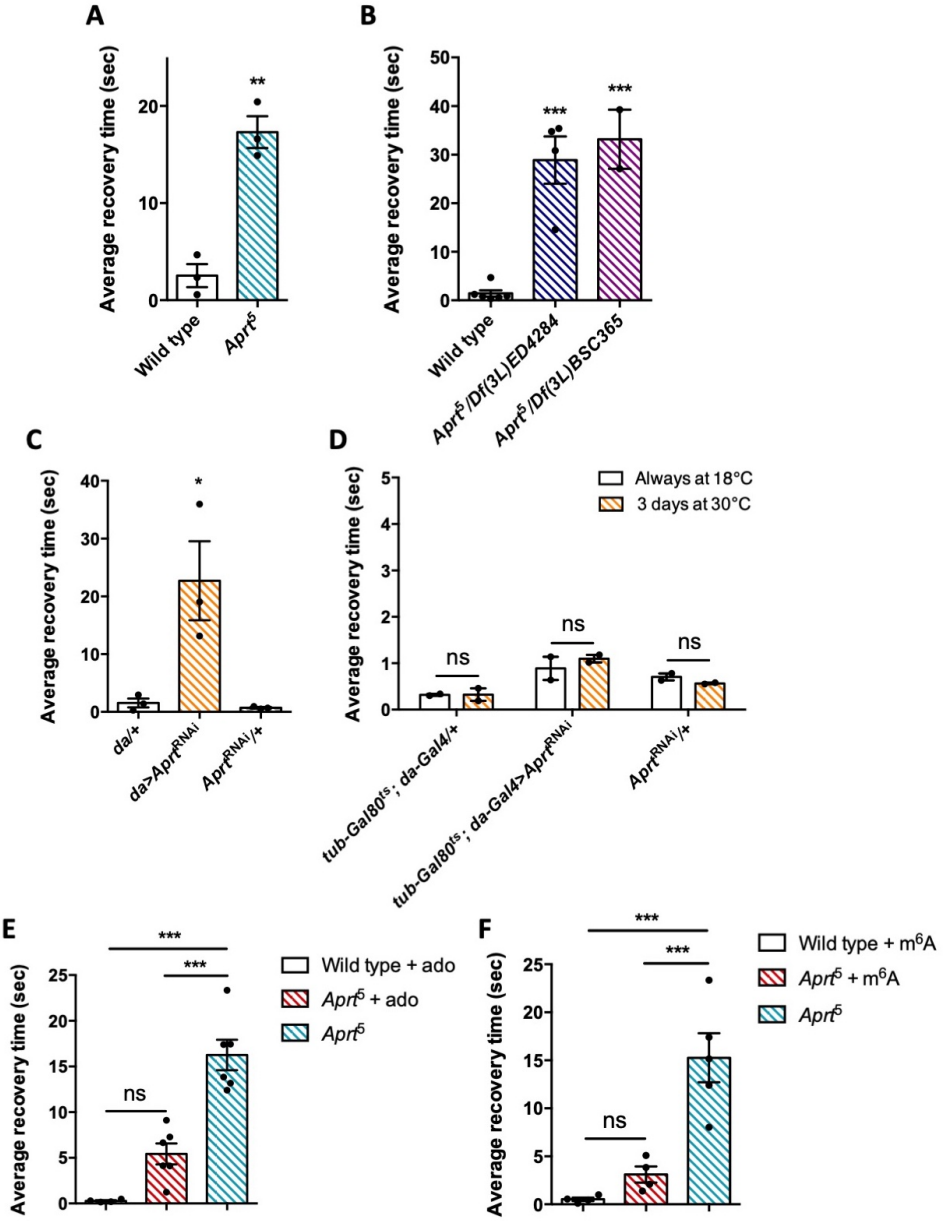
468 A number of *Drosophila* mutants with disrupted metabolism or neural function show
469 increased susceptibility to seizure and paralysis following strong mechanical or

470 electrophysiological stimulation (Fergestad et al. 2006, Parker et al. 2011, Kroll et al. 2015). These
471 mutants are called bang-sensitive (BS) and commonly used as models of epileptic seizure (Song
472 and Tanouye 2008). Here we checked whether Aprt deficiency could trigger a BS phenotype. We
473 observed that aged *Aprt* mutants (at 30 d a.E.) recovered slowly after a strong mechanical
474 stimulation applied by vortexing the vial for 10 s at high speed. These flies took on average 17.3 s
475 to recover and get back on their legs, compared to 2.5 s for wild-type flies of the same age ($p <$
476 $0.01$) (Figure 7A and Movie S1). Some of the mutant flies appeared more deeply paralyzed as they
477 did not spontaneously recover unless the vial was stirred, so their recovery time could not be scored.
478 Hemizygous flies of the same age containing the $Aprt^5$ mutation over two partially overlapping
479 genomic deficiencies covering *Aprt* also showed a BS behavior, with an average longer recovery
480 time of 28.9 s and 33.2 s, respectively ($p < 0.001$) (Figure 7B).

481 We then downregulated *Aprt* by RNAi in all cells with the *da-Gal4* driver to check if this
482 could also induce BS behavior. As shown in Figure 7C, $da>Aprt^{RNAi}$ flies at 30 d a.E. indeed
483 displayed seizure after mechanical shock, quite similar to that of the $Aprt^5$ mutants (22.7 s recovery
484 time on average compared to 1.55 s and 0.72 s for the driver and RNAi controls, respectively, $p <$
485 $0.05$). In contrast, inactivating *Aprt* selectively in neurons *(elav-Gal4)*, glial cells *(repo-Gal4)* or
486 muscles *(24B-Gal4)* did not induce a BS phenotype (Figure S13). This suggests that the BS
487 phenotype requires *Aprt* knockdown in other cells or in several of these cell types. Finally, in
488 contrast to the SING defect, we observed that adult-specific *Aprt* knockdown in all cells with *da-*
489 *Gal4* for 3 days did not trigger a BS behavior (Figure 7D), indicating that the bang sensitivity
490 requires a longer downregulation of the gene or might be the consequence of a developmental
491 defect.

492

493




494

495 **Figure 7.** *Aprt* deficiency triggers a seizure-like phenotype. (**A**) At 30 d a.E., *Aprt*$^5$ mutants need a
496 much longer time than wild-type flies to recover from a strong mechanical shock, showing a bang-
497 sensitive (BS) paralysis comparable to tonic-clonic seizure. Results of 3 independent experiments
498 performed on 50 flies per genotype. Unpaired Student's *t* test; $^{**}p < 0.01$. (**B**) At 30 d a. E.,
499 hemizygous *Aprt*$^5$ mutants also showed a marked BS phenotype. Results of 2-4 independent
500 experiments performed on 50 flies per genotype. One-way ANOVA with Dunnett's *post-hoc* test
501 for multiple comparisons ($^{***}p < 0.001$). (**C**) RNAi-mediated downregulation of *Aprt* in all cells
502 (*da>Aprt*$^{RNAi}$) also led to BS phenotype in adults at 30 d a.E., but not with the driver and effector
503 controls. Results of 3 independent experiments performed on 50 flies per genotype. One-way
504 ANOVA with Tukeys's *post-hoc* test for multiple comparisons ($^{*}p < 0.05$). (**D**) *Aprt* knockdown

505 by RNAi during the adult stage for 3 days before the test was not sufficient to induce bang-
506 sensitivity, suggesting that this phenotype could be caused by a developmental defect or a longer
507 downregulation of *Aprt*. Results of 2 independent experiments performed on 50 flies per genotype.
508 Two-way ANOVA with Sidak's *post-hoc* test for multiple comparisons; ns: not significant. (**E, F**)
509 The BS phenotype of 30-day-old $Aprt^5$ mutants was rescued by feeding either 500 µM adenosine
510 (ado) (**E**) or 500 µM $N^6$-methyladenosine ($m^6A$) (**F**) during all developmental stages plus 5 days
511 before the test. Results of 4 or 6 independent experiments performed on 50 flies per genotype. One-
512 way ANOVA with Tukeys's *post-hoc* test for multiple comparisons (***$p < 0.001$, ns: not
513 significant).

514 **Administration of Adenosine or $N^6$-methyladenosine to *Aprt*-deficient flies prevents seizure**

515 *Drosophila* disease models are advantageously tractable for drug screening *in vivo*
516 (Fernández-Hernández et al. 2016, Perrimon et al. 2016). We thus administered several compounds
517 related to purine metabolism to $Aprt^5$ flies to check if they can rescue neurobehavioral impairments
518 (locomotor defects and seizure). Feeding allopurinol at the same concentration used for uric acid
519 normalization (100 µg/ml, Figure 1B), either in adults 5 days before the test or throughout all
520 developmental stages, did not alter the BS phenotype (Figure S14), as was the case for the SING
521 assay (Figure 1D, E). Similarly in humans, it has been shown that the daily intake of allopurinol,
522 even from infancy, does not mitigate the neurobehavioral impairments in LND patients (Marks et
523 al. 1968; Torres et al. 2007; Jinnah et al. 2010; Madeo et al. 2019).

524 Then, we tried to supplement *Aprt* mutants with various purine compounds, including
525 adenine, hypoxanthine, adenosine and $N^6$-methyladenosine ($m^6A$), at 100 or 500 µM, either in adult
526 stage 5 days before the test or throughout larval development plus 5 days before the test. None of
527 these drugs was able to rescue the SING defect (Figure S15). In contrast and interestingly,
528 administration of 500 µM adenosine or $m^6A$ during development rescued the BS phenotype of
529 $Aprt^5$ mutants (Figure 7E-F). This further indicates that different mechanisms underpin SING
530 alteration and BS behavior in *Aprt* mutants and provide another evidence that the bang sensitivity

531 may be caused by a developmental defect. The results also suggest that the lower adenosine levels
532 of *Aprt* mutant flies could be at the origin of their bang sensitivity.
533
534 **Neuronal expression of mutant HGPRT induces early locomotor defects and seizure behavior**
535 In order to potentially develop another *Drosophila* model mimicking LND conditions, we
536 generated new transgenic UAS lines to express in living flies either the human wild-type HGPRT
537 (HGPRT-WT) or a pathogenic LND-associated mutant form of this protein (HGPRT I42T), both
538 isoforms being inserted at the same genomic docking site. These lines were validated by showing
539 that they are transcribed at similar levels (Figure 8A, B). Enzymatic assays on adult extracts of
540 flies expressing the wild-type form HGPRT-WT in all cells showed significant HGPRT enzyme
541 activity, while an 80.5% lower activity was detected in *Drosophila* expressing the mutant form
542 HGPRT-I42T (Table S2).

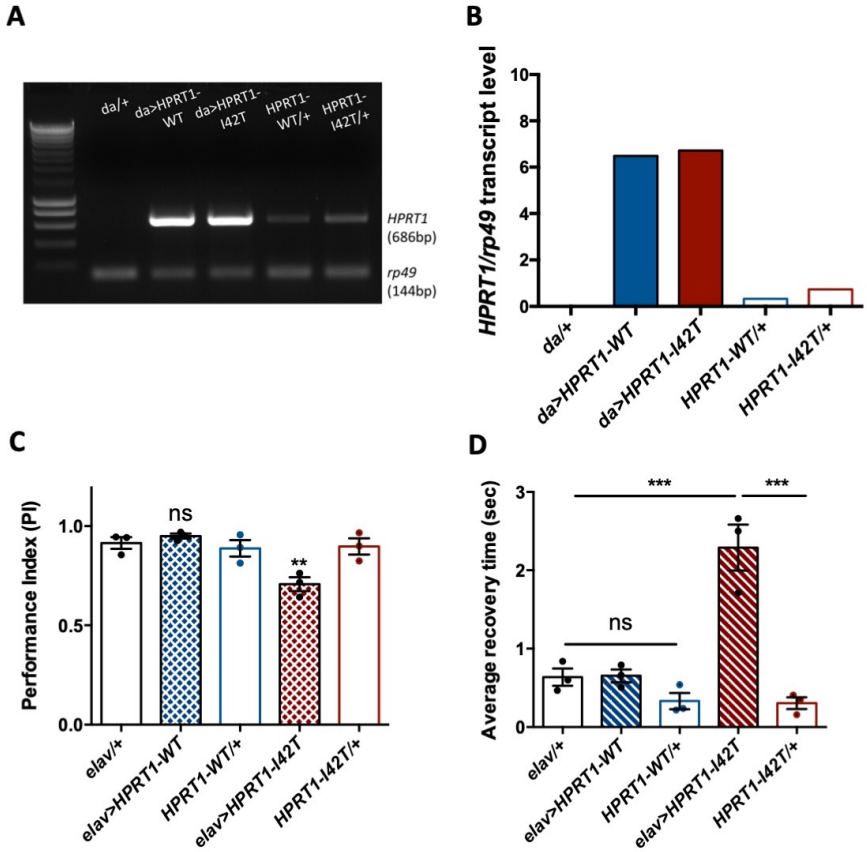



**Figure 8.** Expression of a pathogenic mutant isoform of human HGPRT induces neurobehavioral defects in flies. (**A-B**) Ubiquitous expression of human *HPRT1* with *da-Gal4*. (**A**) Amplification of human *HPRT1* transcripts detected by RT-PCR in head extracts of *da>HPRT1-WT* and *da>HPRT1-I42T* flies. A band with lower intensity was also detected in the effector controls (*UAS-HPRT1-WT/+ and UAS-HPRT1-I42T/+*), and not in the driver control (*da/+*), which indicates a small amount of driver-independent transgene expression. (**B**) Quantification of the previous experiment. (**C, D**) Expression of the LND-associated I42T isoform in all neurons (*elav>HPRT1-I42T*), but not of the wild-type form (*elav>HPRT1-WT*), induced an early locomotor SING defect at 15 d a.E. (**C**) and a prominent bang-sensitivity phenotype at 30 d a.E. (**D**), as compared to the driver (*elav/+*) and effector (*UAS-HPRT1 I42T/+*) only controls. Results of 3 independent experiments performed on 50 flies per genotype. One-way ANOVA with Tukeys's *post-hoc* test for multiple comparisons (**$p < 0.01$; ***$p < 0.001$; ns: not significant).

We next analyzed the consequences of human HGPRT expression on the SING and bang sensitivity behaviors. Interestingly, the pan-neuronal expression of mutant I42T isoform specifically induced a significant early locomotor defect at 15 d a.E. (PI = 0.71 vs. 0.92 and 0.90 for the driver and effector controls, respectively, $p < 0.01$) (Figure 8C) and a relatively small but

561 robust bang-sensitivity behavior at 30 d a.E. (2.3 s average recovery time vs. 0.64 s and 0.31 s for
562 the driver and effector only controls, $p < 0.001$) (Figure 8D). These defective phenotypes could not
563 be seen when HGPRT-WT was expressed. Therefore, and remarkably, whereas wild-type HGPRT
564 expression appears to be innocuous in *Drosophila*, we observed that the neuronal expression of a
565 pathogenic LND-associated isoform triggered neurobehavioral impairments quite similar to those
566 of *Aprt*-deficient flies.
567

568 **Discussion**

569 Over the past 35 years, several animal models of LND have been developed in rodents
570 based on HGPRT mutation in order to better understand the cause of the disease and test potential
571 therapeutic treatments (Finger et al. 1988; Dunnett et al. 1989; Jinnah et al. 1993; Engle et al 1996;
572 Meek et al. 2016; Witteveen et al. 2022). However, none of these models recapitulated the full
573 LND syndrome and, particularly, motor or neurobehavioral symptoms as a consequence of HGPRT
574 deficiency. To date, the causes of the neurobehavioral impairments in LND are not yet clearly
575 elucidated and the disease is still incurable (Fu et al. 2014; Bell et al. 2016; López et al. 2020; Bell
576 et al. 2021; Witteveen et al. 2022). Here we used two different strategies to develop new models
577 of this disease in *Drosophila*, a useful organism to conduct genetic and pharmacological studies..
578 First, we show that *Aprt* deficiency induces both metabolic and neurobehavioral disturbances in
579 *Drosophila*, similar to the loss of HGPRT, but not APRT, activity in humans. Secondly, we
580 expressed an LND-associated mutant form of human HGPRT in *Drosophila* neurons, which also
581 yielded behavioral symptoms. Our results suggest that the fruit fly can be used to study the
582 consequences of defective purine recycling pathway and HGPRT mutation in the nervous system.
583

584 ***Aprt*-deficient flies replicate lifespan and metabolic defects caused by HGPRT deficiency**

585 Flies that carry a homozygous null-mutation in *Aprt* develop normally and live until the
586 adult stage (Johnson and Friedman, 1983). While a previous study reported that heterozygous
587 *Aprt*/+ flies have an extended lifespan (Stenesen et al. 2013), we observed that homozygous $Aprt^5$
588 mutants have in contrast a significantly reduced longevity. The lack of HGPRT activity in LND
589 also reduces lifespan expectancy, generally under 40 years of age for properly cared patients.
590 Stenesen et al. (2013) showed that dietary supplementation with adenine, the Aprt substrate,
591 prevented the longevity extension conferred either by dietary restriction or heterozygous mutations
592 of AMP biosynthetic enzymes. This suggests that lifespan depends on accurate adenine level
593 regulation. It is possible that adenine could accumulate to toxic levels during aging in homozygous
594 *Aprt* mutants, explaining their shorter lifespan. Alternatively, since AMP is the Aprt product, AMP-
595 activated protein kinase (AMPK), an enzyme that protects cells from stresses inducing ATP
596 depletion, could be less activated in Aprt mutants. Multiple publications explored the role of
597 AMPK in lifespan regulation (Sinnett and Brenman 2016) and downregulating AMPK by RNAi in
598 adult fat body or muscles (Stenesen et al. 2013), as well as its ubiquitous inactivation under
599 starvation (Johnson et al. 2010), both reduce fly lifespan.

600 In humans, HGPRT deficiency induces hypoxanthine and guanine accumulation, resulting
601 from lack of recycling, and increased *de novo* purine synthesis (Harkness et al. 1988, Fu et al. 2015;
602 Ceballos-Picot et al. 2015). This in turn leads to uric acid overproduction that increases the risk for
603 nephrolithiasis, renal failure and gouty arthritis if not properly treated. In insects, the end-product
604 of purine catabolism is not uric acid but allantoin (Figure S1, *step 20*). However, urate oxidase, the
605 enzyme which converts uric acid into allantoin, is specifically expressed in the Malpighi tubules,
606 an excretory organ producing pre-urine (Wallrath et al. 1990). Uric acid could therefore accumulate
607 in fly tissues and hemolymph if purine salvage pathway is impaired. Accordingly, we observed an

608 increase in uric acid levels in *Drosophila Aprt* mutant heads, which could be reduced to normal
609 levels by providing allopurinol, a xanthine oxidase inhibitor used to protect against renal failure in
610 LND patients.
611
612 **Aprt is required in dopamine and mushroom body neurons for young fly motricity**
613 We found that *Aprt*-null adult flies show reduced performance in the SING test, which
614 monitors locomotor reactivity to startle and climbing ability. This phenotype appears at an early
615 age, starting from 1 d a.E. The performance continued to decrease until 8 d a.E. and then appeared
616 to stabilize up to 30 d a.E. This defect is quite different from the locomotor impairments described
617 in *Drosophila* Parkinson's disease models, in which SING performance starts declining at around
618 25 d a.E. (Feany and Bender 2000, Riemensperger et al. 2013, Rahmani et al. 2022). This
619 phenotype of *Aprt*-deficient flies could be reminiscent of the early onset of motor symptoms in
620 LND patients, which appear most often between 3 to 6 months of age (Jinnah et al. 2006).
621 Interestingly, knocking down *Aprt* during 3 days only in adult flies also induced SING impairment,
622 which argues against a developmental flaw. Although *Aprt* mutants walked slower than wild-type
623 flies, they were no less active and their ATP levels were not different compared to controls,
624 excluding a major failure in energy metabolism.
625 Downregulating *Aprt* in all neurons reproduced the locomotor defect of the $Aprt^5$ mutant,
626 and *Aprt* mutant locomotion could be partially rescued by neuronal Aprt re-expression. The fact
627 that rescue was not complete could be due to a dominant negative effect of the mutation, as
628 suggested by enzymatic assays on extracts of heterozygous $Aprt^5$ mutants (Figure S4). Previous
629 work from our and other laboratories showed that DA neurons control the SING behavior in
630 *Drosophila* (Feany and Bender 2000; Friggi-Grelin et al. 2003; Riemensperger et al. 2011; Vaccaro
631 et al. 2017; Sun et al. 2018) and that PAM DA neuron degeneration induce SING defects in various

632 Parkinson's disease models (Riemensperger et al. 2013; Bou Dib et al. 2014; Tas et al. 2018; Pütz
633 et al. 2021). Here, we found that knocking down *Aprt* either in all DA neurons or only in the PAM
634 DA neurons was sufficient to induce early SING defects. In contrast, knocking down Aprt with
635 *TH-Gal4* that labels all DA neurons except for a major part of the PAM cluster did not induce
636 SING defects, indicating that Aprt expression in subsets of PAM neurons is critical for this
637 locomotor behavior.

638 Inactivating *Aprt* in all MB neurons also induced a lower performance in the SING assay.
639 This important brain structure receives connections from DA neurons, including the PAM, and is
640 enriched in DA receptors (Waddell 2010). We recently reported that activation of MB-afferent DA
641 neurons decreased the SING response, an effect that requires signalization by the DA receptor
642 dDA1/Dop1R1 in MB neurons (Sun et al. 2018). We also observed a SING defect after knocking
643 down *Aprt* either in all glial cells, or more selectively in glial subpopulations expressing the
644 glutamate transporter Eaat1, but not in the ensheathing glia. Astrocyte-like glial cells expressing
645 *Eaat1* extend processes forming a thick mesh-like network around and inside the entire MB
646 neuropil (Sinakevitch et al. 2010). It could be hypothesized that SING also requires Aprt expression
647 in these MB-associated astrocytes. However, re-expressing *Aprt* with *Eaat1-Gal4* did not lead to
648 SING rescue in Aprt mutant background. This suggests that the presence of Aprt in neurons can
649 somehow compensate for *Aprt* deficiency in glia, but the reverse is not true. In conclusion, proper
650 startle-induced locomotion in young flies depends on Aprt activity in PAM DA and MB neurons,
651 and in *Eaat1*-expressing glial cells.

652

653 **Neuronal Aprt regulates spontaneous activity and sleep**

654 Because sleep is regulated by the mushroom body as well as DA neurons in flies, (Artiushin
655 and Sehgal 2017), we monitored spontaneous locomotor activity and sleep pattern of $Aprt^5$ mutants.

656 Their activity profile appeared normal, with unaltered morning and evening anticipation, indicating
657 that the circadian rhythms are surprisingly maintained in light-dark conditions in the absence of a
658 functional purine recycling pathway (Figure S9). This experiment also highlighted that *Aprt*-
659 deficient flies are hyperactive during the night, which suggests that they sleep less than wild-type
660 flies. This could be confirmed by measuring their sleep pattern on video recordings. *Aprt* mutants
661 show a reduced walking speed, sleep less during both day and night, and have a difficulty to
662 maintain sleep. Downregulating *Aprt* selectively in neurons reproduced the sleep defect, whereas
663 doing it in glial cells only had no effect. We have not attempted here to identify further the neuronal
664 cells involved in this phenotype. Like for the SING behavior defect, it could involve PAM DA
665 neurons, as subpopulations of this cluster have been shown to regulate sleep in *Drosophila* (Nall
666 et al. 2016). Therefore, the lack of purine recycling markedly disrupts sleep in *Aprt*-deficient flies,
667 making them more active at night. This is strikingly comparable to young LND patients who have
668 a much disturbed sleep-time during the night (Mizuno et al. 1979).
669
670 **Lack of Aprt reduces adenosine signaling leading to DA neuron overactivation**
671 We observed that *Aprt* deficiency did not decrease DA levels in the *Drosophila* brain. This
672 prompted us to study another neuromodulator, adenosine, which is indirectly a product of Aprt
673 enzymatic activity. The purine nucleoside adenosine is one of the building blocks of RNA and the
674 precursor of ATP and cAMP, but is also the endogenous ligand of adenosine receptors that
675 modulate a wide range of physiological functions. In brain, adenosine regulates motor and
676 cognitive processes, such as the sleep-wake cycle, anxiety, depression, epilepsy, memory and drug
677 addiction (Soliman et al. 2018). In normal metabolic conditions, adenosine is present at low
678 concentrations in the extracellular space and its level is highly regulated, either taken up by cells
679 and incorporated into ATP stores or deaminated by adenosine deaminase into inosine. In mammals,

680 several nucleoside transporters mediate the uptake of adenosine and other nucleosides into cells,
681 named equilibrative and concentrative nucleoside transporters (ENTs and CNTs), respectively
682 (Gray et al. 2004; Boswell-Casteel and Hays 2017; Pastor-Anglada and Pérez-Torras 2018).

683 We observed a marked reduction in adenosine levels in *Aprt* mutant flies. While we did not
684 observe any alteration in the transcript levels of *AdoR*, the gene coding for the only adenosine
685 receptor in *Drosophila*, transcript levels of an adenosine transporter, Ent2, were increased more
686 than 2-fold in *Aprt* mutants. Interestingly, one paper reported a similar strong increase in *Ent2*
687 expression in *AdoR* mutant flies (Knight et al. 2010). These results suggest that AdoR signaling is
688 less activated in *Aprt*-deficient flies compared to controls. We also observed that the lack of AdoR
689 decreased Aprt expression and activity in *Drosophila*, possibly from increased *Ent2* expression and
690 so higher adenosine influx which could downregulate Aprt by a feedback mechanism.

691 Adenosine and dopamine receptors are known to interact closely in mammals (Franco et al.
692 2000; Kim and Palmiter 2008). A previous study performed in fly larvae showed that an increase
693 in astrocytic $Ca^{2+}$ signaling can silence DA neurons through AdoR stimulation, by a mechanism
694 potentially involving the breakdown of released astrocytic ATP into adenosine (Ma et al. 2016).
695 We previously showed that DA neuron activation can decrease fly performance in the SING test
696 (Sun et al. 2018). Fly nocturnal hyperactivity can also be caused by an increase in dopamine
697 signaling (Kumar et al. 2012; Lee et al. 2013), in accordance with our observation that Aprt-
698 deficient flies sleep less and are more active during the night. Therefore, both the locomotor and
699 sleep defects induced by the lack of Aprt activity could be explained by DA neuron overactivation
700 that would result from reduced adenosinergic signaling.

701 Adenosine has been proposed before to be involved in neurological consequences of LND
702 (Visser et al. 2000). Adenosine transport is decreased in peripheral blood lymphocytes of LND
703 patients (Torres et al. 2004), as well as $A_{2A}$ adenosine receptor mRNA and protein levels (García

704 et al. 2009, García et al. 2011). In a murine LND model, adenosine $A_1$ receptor expression was
705 found to be strongly increased and that of $A_{2A}$ slightly decreased, while $A_{2B}$ expression was not
706 affected (Bertelli et al. 2006). Chronic administration of high doses of caffeine, an adenosine
707 receptor antagonist, caused self-injurious behavior in rats (Miñana et al. 1984; Jinnah et al. 1990).
708 Moreover, central injection of an adenosine agonist is sufficient to prevent self-mutilation induced
709 by dopaminergic agonist administration in neonatally 6-OHDA lesioned rats (Criswell et al. 1988).
710

711 **Adenosine or N6-methyladenosine supplementation rescues the epilepsy behavior of *Aprt***
712 **mutants**

713 LND is characterized by severe behavioral troubles including dystonia, spasticity and
714 involuntary movements (choreoathetosis). Some patients can also show an epileptic disorder
715 (Jinnah et al. 2006; Madeo et al. 2019). In flies, the bang-sensitivity (BS) test is often used to model
716 epileptic seizures (Song et al. 2008; Parker et al. 2011). Here, we observed that 30-day-old *Aprt*
717 mutant flies show a transient seizure-like phenotype after a strong mechanical shock. Although
718 seizure duration appeared shorter in $Aprt^5$ than in typical BS mutants such as *bss*, at least one other
719 BS mutant, *porin*, was reported to have similar short recovery times as *Aprt*-deficient flies (Graham
720 et al, 2010). Previous works demonstrated that bang sensitivity is linked to neuronal dysfunction
721 in *Drosophila* (Parker et al. 2011; Kroll and Tanouye 2013; Kroll et al. 2015; Saras and Tanouye
722 2016). Knocking down *Aprt* in specific cells such as neurons, glia, or muscles, did not trigger this
723 phenotype, suggesting that Aprt must be inactivated in several cell types to induce seizure.

724 Interestingly, knocking down *Aprt* by RNAi in all cells during development also induced
725 the BS behavior, but not for 3 days only in adult flies, at variance with the SING phenotype. We
726 have fed the mutants with a diet supplemented with various compounds involved in purine
727 metabolism, including allopurinol, adenine, hypoxanthine, adenosine or its analog N6-

728 methyladenosine ($m^6A$), either throughout larval development or in adult stage. Only adenosine
729 and $m^6A$, ingested during development, rescued the BS behavior. This suggests that loss of Aprt
730 induces a lack of adenosine in the developing nervous system, as we observed in adult flies (Figure
731 6), which may alter neural circuits controlling BS behavior in adults. The adenosine analogue $m^6A$
732 cannot be incorporated into nucleic acids and is excreted in the urine (Schram 1998; Batista 2017).
733 The rescuing effect we observed with $m^6A$ suggests thereby that both this compound and adenosine
734 are required as adenosine receptor agonists or allosteric regulators during development, rather than
735 nucleotide precursors.

736 Adenosine can strongly inhibit cerebral activity and its role as endogenous anticonvulsant
737 and seizure terminator is well-established in humans (Boison 2005; Masino et al. 2014; Weltha et
738 al. 2019). Conversely, deficiencies in the adenosine-based neuromodulatory system can contribute
739 to epileptogenesis. For instance, increased expression of astroglial adenosine kinase (ADK), which
740 converts adenosine into AMP, leads to a reduction in brain adenosine level that plays a major role
741 in epileptogenesis (Weltha et al. 2019). Hence, therapeutic adenosine increase is a rational
742 approach for seizure control. Our observation that feeding adenosine or its derivative $m^6A$ rescued
743 the seizure-like phenotype of *Aprt* mutant flies further suggests that adenosinergic signaling has
744 partly similar functions in the fly and mammalian brains. In addition, the decrease in adenosine
745 levels we observed in *Aprt* mutants could result from enhanced ADK activity that would
746 compensate for the lack of Aprt-produced AMP.

747 We and others recently observed that $m^6A$, and related compounds sharing an adenosine
748 moiety, are able to rescue the viability of LND fibroblasts and neural stem cells derived from
749 induced pluripotent stem cells (iPSCs) of LND patients cultured in the presence of azaserine, a
750 potent blocker of *de novo* purine synthesis (Petitgas and Ceballos-Picot, unpublished results;
751 Ruillier et al. 2020). Like in flies again, allopurinol was not capable of rescuing the cell viability

752 in this *in vitro* model. The similarity of these results does increase confidence that *Aprt*-deficient
753 *Drosophila* could be used as an animal model of LND.
754
755 **Expression of mutant HGPRT triggers locomotor and seizure phenotypes as does *Aprt***
756 **deficiency**
757 How HGPRT deficiency can cause such dramatic neurobehavioral troubles in LND patients
758 still remains a crucial question. To date, cellular (Smith & Friedman 2000; Torres et al. 2004;
759 Ceballos-Picot et al. 2009; Cristini et al. 2010; Guibinga et al. 2012; Sutcliffe et al. 2021) and
760 rodent (Finger et al. 1988; Dunnett et al. 1989; Jinnah et al. 1993; Meek et al. 2016; Witteveen et
761 al. 2022) models only focused on the consequences of HGPRT deficiency to phenocopy the
762 disease. Such an approach was justified by the fact that the lower the residual activity of mutant
763 HGPRT, the more severe the symptoms are in patients (Fu and Jinnah 2012. Fu et al. 2014).
764 However, it could be conceivable that part of these symptoms result from compensatory
765 physiological mechanisms or a deleterious gain-of-function conferred to the HGPRT protein by
766 the pathogenic mutations. Here we observed that neuronal expression of mutant human HGPRT-
767 I42T, which expresses a low enzymatic activity, but not the wild-type HGPRT protein, induced
768 early locomotor defects in young flies and bang-sensitivity in older flies, similarly to the defects
769 induced by Aprt deficiency. This suggests a potential neurotoxicity of the pathological mutant form
770 of HGPRT, which could be related to disturbances in purine metabolism or other signaling
771 pathways. The human mutant form might not be properly degraded and accumulate as aggregates,
772 potentially exerting neuronal toxicity. Such an approach opens interesting perspectives to better
773 understand the endogenous function of HGPRT and its pathogenic forms. Indeed, the identification
774 of a potential inherent neurotoxicity of defective forms of human HGPRT is a new element, which
775 could be explored in further work in the fly and also in rodent models.

776

777 **A new model of LND in an invertebrate organism?**

778 LND, a rare X-linked metabolic disorder due to mutations of the *HPRT1* gene, has dramatic
779 neurological consequences for affected children. To date, no treatment is available to abrogate
780 these troubles, and no fully satisfactory *in vivo* models exist to progress in the understanding and
781 cure of this disease. HGPRT knockout rodents do not show comparable motor and behavioral
782 troubles, which makes these models problematic for testing new therapeutic treatments. *Drosophila*
783 does not express HGPRT-like activity and our phylogenetic analysis established that no HGPRT
784 homologue is present in *Drosophila melanogaster* (Figure S2 and S3), confirming that Aprt is the
785 only enzyme of the purine salvage pathway in this organism. APRT and HGPRT are known to be
786 functionally and structurally related. Both human APRT and HGPRT belong to the type I PRTases
787 family identified by a conserved phosphoribosyl pyrophosphate (PRPP) binding motif, which is
788 used as a substrate to transfer phosphoribosyl to purines. This binding motif is only found in
789 PRTases from the nucleotide synthesis and salvage pathways (Sinha and Smith 2001). Moreover,
790 the purine substrates adenine, hypoxanthine and guanine share the same chemical skeleton and
791 APRT can bind hypoxanthine, indicating that APRT and HGPRT also share similarities in their
792 substrate binding sites (Ozeir et al. 2019).

793 Here we find that *Aprt* mutant flies show symptoms partly comparable to the lack of
794 HGPRT in humans, including increase in uric acid levels, reduced longevity, and various
795 neurobehavioral defects such as early locomotor decline, sleep disorders and epilepsy behavior.
796 This animal model therefore recapitulates both salvage pathway disruption and motor symptoms,
797 as observed in LND patients. Moreover, these results highlighted that *Aprt* deficiency in
798 *Drosophila* has more similarities with HGPRT than APRT deficiency in humans. *Aprt* mutant flies
799 also show a disruption of adenosine signaling and we found that adenosine itself or a derivative

800 compound can relieve the epileptic symptoms. Finally, neuronal expression of a mutant form of
801 human HGPRT that causes LND also triggers abnormalities in fly locomotion and seizure-like
802 behavior, which has not been documented to date in other models. The use of *Drosophila* to study
803 LND could therefore prove valuable to better understand the link between purine recycling
804 deficiency and brain functioning and carry out drug screening in a living organism, paving the way
805 towards new improvements in the cure of this dramatic disease.
806

807 # Materials and Methods

808 ***Drosophila* culture and strains**.

809 Flies were raised at 25°C on standard cornmeal-yeast-agar medium supplemented with
810 methylhydroxybenzoate as an antifungal under a 12h:12h light-dark (LD) cycle. The *Drosophila*
811 mutant lines were obtained either from the Bloomington *Drosophila* Stock Center (BDSC), the
812 Vienna *Drosophila* Resource Center (VDRC) or our own collection: $Aprt^{5}$ (Johnson and Friedman,
813 1983) (BDSC #6882), *Df(3L)ED4284* (BDSC #8056), *Df(3L)BSC365* (BDSC #24389), *UAS-*
814 $Aprt^{RNAi}$ (VDRC #106836), $AdoR^{KG03964ex}$ (Wu et al. 2009) here named $AdoR^{KGex}$ (BDSC #30868),
815 and the following Gal4 drivers: *238Y-Gal4*, *24B-Gal4*, *da-Gal4*, *Eaat1-Gal4* (Rival et al. 2004),
816 *elav-Gal4* ($elav^{C155}$), *MZ0709-Gal4*, *NP6510-Gal4*, *NP6520-Gal4*, *R58E02-Gal4* (Liu et al. 2012),
817 *R76F05-Gal4*, *repo-Gal4*, *TH-Gal4* (Friggi-Grelin et al. 2003), *TRH-Gal4* (Cassar et al. 2015), *tub-*
818 $Gal80^{ts}$ and *VT30559-Gal4*. The Canton-S line was used as wild-type control. The simplified
819 driver>effector convention was used to indicate genotypes in figures, e.g. $elav>Aprt^{RNAi}$ for *elav-*
820 *Gal4*; $UAS\text{-}Aprt^{RNAi}$. In some experiments, to restrict RNAi-mediated *Aprt* inactivation at the adult
821 stage, we have used the TARGET system (McGuire et al. 2003). Flies were raised at 18°C
822 (permissive temperature) where Gal4 transcriptional activity was inhibited by $tub\text{-}Gal80^{ts}$, and

823 shifted to 30°C (restrictive temperature) for 3 days before the test to enable Gal4-driven *Aprt*$^{RNAi}$
824 expression.
825
826 **Construction of transgenic lines**
827 To generate a *UAS-Aprt* strain, a 549-bp *Aprt* insert containing the coding sequence was PCR
828 amplified from the ORF clone BS15201 (*Drosophila* Genomics Resource Center, Bloomington, IN,
829 USA) using primers with added restriction sites (Table S3). After digestion with EcoRI and XbaI,
830 the *Aprt* cDNA was subcloned into the *pUASTattB* vector (Bischof et al. 2007) using the In-Fusion
831 HD Cloning Kit (Takara Bio, Kyoto, Japan) according to the manufacturer's instructions, and the
832 insertion verified by sequencing (Eurofins Genomics, Ebersberg, Germany). The construction was
833 sent to BestGene (Chino Hills, CA, USA) for *Drosophila* germline transformation into the attP14
834 docking site on the 2d chromosome. The *UAS-Aprt* line yielding the strongest expression was
835 selected and used in the experiments.
836 A clone containing the human wild-type *HPRT1* cDNA was kindly provided to us by Prof.
837 Hyder A. Jinnah (Emory University, GA, USA). We constructed the *HPRT1-I42T* cDNA from this
838 clone by site-directed mutagenesis using the Quick Change II Site-Directed Mutagenesis Kit
839 (Agilent Technologies, Santa Clara, CA, USA). Primers sequences used to create the mutation are
840 shown in Table S3. The cDNA obtained was verified by sequencing. Then, the 657-bp *HPRT1-WT*
841 and *HPRT1-I42T* inserts were PCR amplified using primers with added restriction sites and
842 *Drosophila* translation start consensus sequence (Table S3). They were subcloned into *pUASTattB*
843 and verified by sequencing. The transgenes were sent to BestGene for *Drosophila* transformation
844 and inserted into the attP40 docking site on the 2d chromosome.
845

846 **Sequencing of *Aprt*$^5$**

847 For sequencing of the *Aprt*$^5$ cDNA, total RNA was isolated by standard procedure from 20-30 heads
848 of homozygous *Aprt*$^5$ flies and reverse transcribed using oligo d(T) primers (PrimeScript RT Reagent
849 Kit, Takara Bio, Kyoto, Japan). At least 750 ng of the first strand cDNA was amplified by PCR in
850 20 µl of reaction mixture using PrimeStar Max DNA polymerase (Takara Bio, Kyoto Japan) with a
851 Techne Prime Thermal Cycler apparatus (Bibby Scientific, Burlington, NJ, USA). The program
852 cycles included 10 s denaturation at 98°C, 10 s annealing at 55°C and 10 s elongation at 72°C,
853 repeated 35 times. 1µL of the PCR product was amplified again with the same program, in 30 µl of
854 reaction mixture. After elution on 1% agarose gel, DNA were purified using the Wizard® SV Gel
855 and PCR Clean-Up System protocol (Promega, Madison, Wisconsin, USA) according to the
856 manufacturer's instructions. Finally, 7.5 µL of purified DNA were sent with 2.5 µL of primers (sense
857 and antisense in separate tubes) for sequencing (Eurofins Genomics, Ebersberg, Germany).

858

859 **Phylogenetic analyses**

860 HGPRT homologs were identified by BlastP searching the NCBI GenBank non-redundant
861 protein database (last October 2019 version). A subset of interest was selected for phylogenetic
862 analyses. For Figure S2, multiple sequence alignment was performed using MAFFT (-ensi) (Katoh
863 and Standley 2013). The confidence of aligned residues was assessed using the TCS index (Chang
864 et al. 2014); only columns with TCS index 7-9 (on a 0–9 scale) were retained. ProtTest v3.2
865 (Darriba et al. 2011) was used to assess the best model fitting of the data. Maximum Likelihood
866 tree was inferred in IQ-TREE (Trifinopoulos et al. 2016), under the LG + R6 model. Bayesian
867 inference was carried out in PhyloBayes v. 3.3 (Lartillot et al. 2009), under the LG + Γ4 model.
868 For Figure S3, the whole analysis was performed in SeaView 5.0.4 (Gouy et al. 2010). Multiple

869 sequence alignment was performed using MUSCLE (Edgar 2004). Maximum likelihood tree was
870 inferred using PhyML 3.0 (Guindon et al. 2010) (under the LG + Γ4 model; best of NNI & SPR
871 tree searching option; invariable sites optimized).

872

873 **Lifespan assay**

874 Longevity study was performed as previously described (Riemensperger et al. 2011).
875 *Drosophila* adult males were collected within 24h of emergence and maintained on standard
876 medium at 25°C under a 12:12h LD cycle. They were transferred into fresh bottles every 2-3 days
877 and the number or surviving flies was scored. 50 flies per bottle in triplicate were tested for each
878 genotype and the experiment was done 3 times.

879

880 **Startle-induced negative geotaxis (SING)**

881 SING assays were performed as previously described (Rival et al. 2004; Riemensperger et
882 al. 2011; Sun et al. 2018). For each genotype, 50 adult males divided into 5 groups of 10 flies were
883 placed in a vertical column (25-cm long, 1.5-cm diameter) with a conic bottom end and left for
884 about 30 min for habituation. Then, columns were tested individually by gently tapping them down
885 (startle), to which flies normally responded by climbing up. After 1 min, flies having reached at
886 least once the top of the column (above 22 cm) and flies that never left the bottom (below 4 cm)
887 were counted. Each fly group was assayed three times at 15 min intervals. The performance index
888 (PI) for each column is defined as $\frac{1}{2}[1 + (n_{top}-n_{bot})/n_{tot}]$, where $n_{tot}$ is the total number of flies in the
889 column, $n_{top}$ the number of flies at the top and $n_{bot}$ the number of flies at the bottom after 1 min.
890 SING was performed at 1, 8, 10 and 30 days after adult eclosion (d a.E.).

891

892 **Spontaneous locomotion and sleep monitoring**

893 Spontaneous locomotor activity was recorded as previously described (Vaccaro et al. 2017),
894 using *Drosophila* activity infra-red beam monitors (DAM, TriKinetics Inc, Waltham, MA USA)
895 placed in incubators at 25°C equipped with standard white light. 8-day-old male flies were
896 maintained individually for 5-6 days under 12:12h LD cycle in 5 x 65 mm glass tubes containing
897 5% sucrose, 1.5% agar medium. Data analysis was performed with the FaasX software (Klarsfeld
898 et al. 2003). Histograms represent the distribution of the activity through 24h in 30-min bins,
899 averaged for 32 flies per genotype over 4-5 cycles.

900 For sleep monitoring, 2-4-day-old virgin female flies were transferred individually into 5 x
901 65 mm glass tubes containing standard food and their movements were recorded for up to 5 days
902 using DAM infra-red beam monitors (TriKinetics Inc, Waltham, MA USA) or the *Drosophila*
903 ARousal Tracking (DART) video system (Faville et al. 2015), in a 12h:12h LD cycle with 50-60%
904 humidity. Control and experimental flies were recorded simultaneously. Each experiment included
905 at least 14 flies for each condition and was repeated 2-3 times with independent groups of flies. Fly
906 sleep, defined as periods of immobility lasting more than 5 min (Huber et al. 2004), was computed
907 with a Microsoft Excel macro for the infra-red beam data and with the DART software for the
908 video data. Distribution and homogeneity as well as statistical group comparisons were tested using
909 Microsoft Excel plugin software StatEL. The *p* value shown is the highest obtained among *post-*
910 *hoc* comparisons and means ± SEM were plotted.

911

912 **Immunohistochemistry**

913 Brains of 8 to 10-day-old adult flies were dissected in ice-cold $Ca^{2+}$-free *Drosophila*
914 Ringer's solution and processed for whole-mount anti-TH or anti-DA immunostaining as

915 previously described (Riemensperger et al. 2011, Cichewicz et al. 2017). The primary antibodies
916 used were mouse monoclonal anti-TH (1:1000, cat. n° 22941, ImmunoStar, Hudson, WI, USA)
917 and mouse monoclonal anti-DA (1:100, cat. n° AM001, GemacBio, Saint-Jean-d'Illac, France).
918 The secondary antibody was goat anti-mouse conjugated to Alexa Fluor 488 (1:1000,
919 ThermoFisher Scientific, Waltham, MA, USA). Brains were mounted with antifade reagent, either
920 Prolong Gold (ThermoFisher Scientific, Waltham, MA, USA) or, alternatively, 65% 2,2'-
921 thiodiethanol (Sigma-Aldrich, St. Louis, MI, USA) (Cichewicz et al. 2017) for DA staining. Images
922 were acquired on a Nikon A1R confocal microscope with identical laser, filter and gain settings
923 for all samples in each experiment. Immunofluorescence intensity levels were quantified using the
924 Fiji software. Experiments were repeated independently at least 3 times on 4 to 6 brains per
925 genotype.
926

927 **Bang-sensitivity test**

928 Bang-sensitivity assays were performed as previously described (Howlett et al. 2013). 30-
929 day-old males were divided into 5 groups of 10 flies under $CO_2$ and allowed to recover overnight.
930 The following day, each group was placed in a vial without food and after 20 min of habituation,
931 the vials were stimulated vigorously with a vortex mixer for 10 s at 2,500 rpm. The recovery time
932 was measured for each fly, from the end of the stimulation until they reached a normal standing
933 position. Results are the mean of the recovery time for at least 50 flies per genotype.
934

935 **Drug administration**

936 Allopurinol (A8003, Sigma Aldrich, St. Louis, MI, USA) was diluted in standard medium
937 at 100 μg/ml and flies were placed for 5 days on this medium before metabolite extraction.

938 Adenine, adenosine and hypoxanthine (A2786, A9251 and H9377, Sigma Aldrich, St. Louis, MI,
939 USA) or $N^6$-methyladenosine ($m^6A$) (QB-1055, Combi-Blocks, San Diego, CA, USA) were diluted
940 in fly food medium at 500 µM. Parents were allowed to lay eggs on this medium in order to have
941 exposition to the drug throughout all larval development of the progeny. Adults were collected and
942 placed in normal medium until 5 days before the test, when they were placed again in food
943 supplemented with adenosine or $m^6A$ at the same concentrations.
944

945 **Uric acid quantification**

946 For purine metabolite extraction, 40 heads from 8-day-old flies were ground in 80%
947 ethanol, heated for 3 min at 80°C and evaporated in a Speedvac apparatus. Dried residues were
948 resuspended in MilliQ water and total protein content of each homogenate was measured with the
949 Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA, USA). 20 µl of each
950 sample was injected into a 25 cm x 4.6 mm C18 Nucleosil column with 5-µm particles (Interchim,
951 Montluçon, France). Purine metabolites were detected by an Agilent 1290 Infinity HPLC system
952 coupled with a diode array detector as recommended by the ERDNIM advisory document. 7
953 wavelengths were used for detection (230, 240, 250, 260, 266, 270 and 280 nm) in order to have
954 the spectrum of each compound for identification in addition to the retention time. The mobile
955 phases contained 0.05 M monopotassium phosphate pH 5 and 65% (v/v) acetonitrile. The flux was
956 fixed at 1 ml/min. For analysis, the maximum height of the compound was normalized to protein
957 content and compared to the control genotype.

958 **ATP assay**

959 ATP level was measured by bioluminescence using the ATP Determination Kit (A22066,
960 ThermoFisher Scientific, Waltham, MA, USA) on a TriStar 2 Spectrum LB942S microplate reader

961 (Berthold Technologies, Bad Wildbad, Germany), according to the manufacturer's instructions.
962 Samples were prepared as described previously (Fergestad et al. 2006). Briefly, 30 heads or 5
963 thoraces from 8-day-old flies were homogenized in 200 µL of 6 M guanidine-HCl to inhibit
964 ATPases, boiled directly 5 min at 95°C and then centrifuged for 5 min at 13,000 g and 4°C. Total
965 protein content of each supernatant was measured with the BCA Protein Assay Kit. Each
966 supernatant was then diluted at 1:500 in TE buffer pH 8 and 10 µl were placed in a 96-well white-
967 bottom plate. The luminescent reaction solution (containing D-luciferine, recombinant firefly
968 luciferase, dithiothreitol and reaction buffer) was added to each well with an injector and high-gain
969 1 sec exposure glow reads were obtained at 15 min after reaction initiation. Results were compared
970 to a standard curve generated with known ATP concentrations and final values were normalized to
971 the protein content.
972

973 **Enzyme activity assay**

974 HGPRT and APRT activities were assessed in *Drosophila* by adapting methods previously
975 established for human cells (Cartier and Hamet 1968, Ceballos-Picot et al. 2009). 20 whole male
976 flies were homogenized in 250 µl of 110 mM Tris, 10 mM $MgCl_2$ pH 7.4 (Tris-$MgCl_2$ buffer),
977 immediately frozen and kept at least one night at -80°C before the assay. After 5 min of
978 centrifugation at 13,000 g, total protein content of each supernatant was measured with the BCA
979 Protein Assay Kit to normalize activity level. Kinetics of [$^{14}$C]-hypoxanthine conversion to IMP
980 (or in some cases [$^{14}$C]-guanine conversion to GMP), and [$^{14}$C]-adenine conversion to AMP were
981 assessed for HGPRT and Aprt assays, respectively. Compositions of reaction mediums were: 25
982 µl of a radioactive solution made with 38 µl of [$^{14}$C]-hypoxanthine (25 µCi/ml) diluted in 1 ml of
983 1.2 mM cold hypoxanthine (or in some cases 38 µl of [$^{14}$C]-guanine (25 µCi/ml) diluted in 1 ml of

984 1.2 mM cold guanine), for HGPRT assay, or 25 µl of a radioactive solution made with 75 µl of
985 [$^{14}$C]-adenine (50 µCi/ml) diluted in 1 ml of 1.2 mM cold adenine for Aprt assay, and a volume of
986 fly extract equivalent to 200 µg protein diluted in Tris-$MgCl_2$ buffer to 150 µl. Reactions were
987 monitored at 37°C and started by adding 25 µl of the co-factor 5-phosphoribosyl-1-pyrophosphate
988 (PRPP) at 10 mM. After 6, 12, 24 and 36 min, 40 µL of each pool was placed in a tube containing
989 either 25 µl of HIE (3 mM hypoxanthine, 6 mM IMP, 200 mM EDTA) or AAE (3 mM adenine, 6
990 mM AMP, 200 mM EDTA) solutions and incubated for 3 min at 95°C to stop the reaction. The
991 different radioactive compounds were separated by paper chromatography on Whatman 3MM
992 strips using 28% $NH_4OH$, 50 mM EDTA as solvent for about 1h30. Then, the substrates and
993 products were visualized under a UV lamp at 254 nm and placed separately in vials in 2 ml of
994 Scintran (VWR, Radnor, PA, USA). The radioactivity in disintegrations per minute (dpm) was
995 measured in a Packard Tri-Carb 1600 TR liquid scintillation analyzer (PerkinElmer, Waltham, MA,
996 USA). The percentage of substrate transformation as a function of time was converted in
997 nmol/min/mg protein and finally normalized to wild-type control values.
998

999 **Protein extraction and western blots**

1000 30 heads of 8-10 day-old-males per genotype were homogenized in 30 µL Laemmli buffer
1001 containing protease inhibitor (Complete mini Protease Inhibitor Cocktail, Roche Diagnostics),
1002 using a Minilys apparatus (Bertin Instruments, Montigny-le-Bretonneux, France). The lysates were
1003 incubated on ice for 30 min and centrifuged at 8,000 g for 10 min at 4°C. The extracted proteins
1004 were heated at 95°C for 10 min. Western blots were performed as previously described (Issa et al.
1005 2018). Briefly, proteins were separated in 4–12% Novex NuPAGE Bis-Tris precast polyacrylamide
1006 gels (Life Technologies) following the manufacturer's protocol in a MOPS-SDS running buffer. A

1007 semi-dry transfer was done onto polyvinylidene difluoride membranes (Amersham Hybond P 0.45
1008 μm) using a Hoefer TE77 apparatus. Membranes were probed overnight at 4°C with mouse
1009 monoclonal anti-TH (1:5000, cat. n° 22941, ImmunoStar, Hudson, WI, USA) and mouse
1010 monoclonal anti-actin beta (1:5000, cat. n° ab20272, Abcam, Cambridge, UK) that cross-reacts
1011 with *Drosophila* Actin 5C (Act5C). After incubation with horseradish peroxidase (HRP)-
1012 conjugated anti-mouse (1:5000, cat. n° 115–035–146, Jackson ImmunoResearch) as secondary
1013 antibody, immunolabeled bands were revealed by chemiluminescence staining using ECL
1014 RevelBlOt Intense (Ozyme, Saint-Cyr-l'École, France, cat. n° OZYB002-1000) and then digitally
1015 acquired with the ImageQuant TL software (GE Healthcare Life Science). Densitometry measures
1016 were made with the Fiji software and normalized to Act5C values as internal controls.
1017

1018 **Reverse transcription-PCR and quantitative PCR**

1019 Total RNA was isolated by standard procedure from 20-30 heads of 8-day-old males
1020 collected on ice and lysed in 600 μl QIAzol Reagent (Qiagen, Venlo, Netherlands). 1 μg of total
1021 RNA was treated by DNase (DNase I, RNase-free, ThermoFisher Scientific, Waltham, MA, USA)
1022 according to the manufacturer's instructions. 5 μL of treated RNA was reverse transcribed using
1023 oligo d(T) primers (PrimeScript RT Reagent Kit, Takara Bio, Kyoto Japan). Then, at least 750 ng
1024 of the first strand cDNA was amplified in 20 μl of reaction mixture using PrimeStar Max DNA
1025 polymerase (Takara Bio, Kyoto Japan) with a Techne Prime Thermal Cycler apparatus (Bibby
1026 Scientific, Burlington, NJ, USA). The program cycles included 10 s denaturation at 98°C, 10 s
1027 annealing at 55°C and 10 s elongation at 72°C, repeated 30 times. PCR product levels were
1028 measured after electrophoresis by densitometry analysis with the Fiji software. Data were
1029 normalized to amplification level of the ribosomal protein *rp49/RpL32* transcripts as internal

1030 control. Sequences of the primers used were: sense *HPRT*, 5'-
1031 GAGATACAAAATGGCGACCCGCAGCCCT; antisense *HPRT*, 5'-
1032 GCTCGGATCCTTATCATTACTAGGCTTTG (amplicon 686bp); sense *rp49*, 5'-
1033 GACGCTTCAAGGGACAGTATC; antisense *rp49*, 5'- AAACGCGGTTCTGCATGAG
1034 (amplicon 144bp).

1035 For RT-qPCR, approximately 40 ng of the first strand cDNA was amplified in 10 µl of
1036 reaction mixture using the LightCycler 480 SYBR Green I Master reaction mix (Roche Applied
1037 Science, Mannheim, Germany) and a LightCycler 480 Instrument (Roche Applied Science,
1038 Mannheim, Germany). The program cycles included a 10-min preincubation step at 95°C, 40
1039 cycles of amplification (10 s denaturation at 95°C, 10 s annealing at 55°C, 20 s elongation at 72°C),
1040 followed by a melting curves analysis for PCR product identification. Data were normalized to
1041 amplification level of the ribosomal protein *rp49*/*RpL32* transcripts as internal control. The genes
1042 analyzed and primer sequences used for qPCR are indicated in Table S4.

1043

1044 **Adenosine assay**

1045 Adenosine was determined by Ultra Performance Liquid Chromatography (UPLC). 5 whole
1046 flies or 30 heads were homogenized in 120 µl 0.9% (w/v) NaCl using a Minilys apparatus (Bertin
1047 Instruments, Montigny-le-Bretonneux, France) and frozen at -80°C. After unfreezing and 5 min of
1048 microcentrifugation, 20 µl of 10% perchloric acid were added to 70 µl of the supernatant and the
1049 mixture was left for 5 min on ice. After a new centrifugation, 20 µl of a neutralization solution
1050 (made by mixing 3 volumes of 3 M $K_2CO_3$ with 1 volume of 6.4 mM NaOH containing 0.4 mg/ml
1051 bromothymol blue) were added and the mixture was centrifuged again before injection (5 µl).
1052 Samples were analyzed with a UV diode-array detector on an Acquity UPLC HSS T3 column (1,8

1053 µm, 2.1 x 150mm) (Waters Corporation, Milford, MA, USA). The mobile phases consisted in
1054 Buffer A (30 mM ammonium acetate, pH 4.0 with 1:10,000 heptafluorobutyric acid (HPFA)) and
1055 Buffer B (acetonitrile with 1:10,000 HPFA), using a flow rate of 0.3 ml/min. Chromatographic
1056 conditions were: 3.5 min 100% Buffer A, 16.5 min up to 6.3% Buffer B, 2 min up to 100% Buffer
1057 B, and 1 min 100% Buffer B. The gradient was then returned over 5 min to 100% Buffer A,
1058 restoring the initial conditions. Results were normalized to protein levels for each sample.

1059

1060 **Statistics**

1061 Statistical significance was determined with the Prism 6 software (GraphPad Software, La
1062 Jolla, CA, USA). Survival curves for longevity experiments were analyzed using the log-rank test.
1063 The Student's *t*-test was used to compare two genotypes or conditions, and one-way or two-way
1064 ANOVA with Tukey's, Dunnett's or Sidak's *post-hoc* multiple comparison tests for three or more
1065 conditions. Results are presented as mean ± SEM. Probability values in all figures: $*p < 0.05$, $**p$
1066 $< 0.01$, $***p < 0.001$.

1067

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1076

1077 ## Authors contributions

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1079 R.F., M.S., J.D. and S.M.; formal analysis, C.P., L.S., A.D., G.M., R.F., M.S., J.D. and S.M.;
1080 methodology, C.P., L.S., A.M., G.M., B.C.Z and S.M.; validation, C.P., I.C.P and S.B.; writing,
1081 original draft, C.P., I.C.P. and S.B.; writing, review & editing, C.P., I.C.P and S.B.; funding
1082 acquisition and supervision, I.C.P and S.B.; project administration, S.B.

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