Metabolic and Neurobehavioral Disturbances Induced by Purine Recycling Deficiency in *Drosophila*

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Abstract

Lesch-Nyhan disease (LND) is a rare genetic disorder induced by deficiency in hypoxanthine-guanine phosphoribosyltransferase (HGPRT), an enzyme of the purine salvage pathway. This leads in early age to hyperuricemia and severe neurobehavioral disturbances, including dystonia, spasticity and compulsive self-injury. To date, no treatment is available for these neurological symptoms and no animal model recapitulates all the defects observed in LND patients. Here we studied LND-related mechanisms in the fruit fly *Drosophila melanogaster*. We confirmed that no HGPRT activity is expressed in this organism, where the only purine-recycling enzyme is adenine phosphoribosyltransferase (Aprt). This enzyme is also present in humans but its deficiency does not trigger neurological defects. In contrast, we observed that *Drosophila Aprt* mutants showed both metabolic and neurobehavioral disturbances, including increased uric acid levels, locomotor reactivity impairments, sleep alterations, seizure-like behavior, reduced lifespan, and reduction of adenosine signaling and content. Locomotor defects could be rescued by neuronal *Aprt* re-expression in mutant context and reproduced by knocking down *Aprt* selectively in the protocerebral anterior medial (PAM) clusters of dopaminergic neurons, the mushroom bodies and glia subsets. Ingestion of allopurinol normalized uric acid levels in *Aprt* mutants but not their neurological defects, as is the case in LND patients, whereas feeding adenosine or N^6-methyladenosine 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, this shows that *Drosophila* can be used as a new model 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 cells and brain neurons, 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 deficiency 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

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

81 Noticeably, the clinical severity in LND appears to be inversely correlated to the
82 enzymatic activity of HGPRT (Fu and Jinnah 2012; Fu et al. 2014). The full LND phenotype
83 which is the most severe corresponds to mutations that leave less than 2% of residual activity,
84 whereas 2-10% of activity leads to an intermediate phenotype with various neurological
85 abnormalities but without self-injury, and more than 10% protects from the neurobehavioral
86 symptoms and only results in hyperuricemia-related disorders. These two last groups are named
87 Lesch-Nyhan variants (Jinnah et al. 2010).

88 To date, the causes of the neurobehavioral troubles in LND are still not elucidated and it
89 remains challenging to understand and cure this dramatic disease (Jinnah et al. 2010, Bell et al.
90 2016). The most favored hypothesis is a dysfunction of the basal ganglia, and particularly of its
91 dopaminergic pathways (Baumeister and Frye 1985; Visser 2000; Nyhan 2000; Saito and
92 Takashima, 2000; Egami et al. 2007). Indeed, analyses of LND patient brains revealed a marked
93 loss of dopamine (DA) levels (Lloyd et al. 1981; Ernst et al. 1996) and DA transporters (Wong et
94 al. 1996) specifically in the basal ganglia, but the mechanisms linking HGPRT activity and the

95 DA system are still under investigation. DA deficits have been reported in HGPRT knockout
96 rodents, but these animal models do not exhibit the motor or neurobehavioral defects of human
97 patients (Finger et al. 1988; Dunnett et al. 1989; Jinnah et al. 1993; Jinnah et al. 1994; Meek et al.
98 2016). Recent studies reported that HGPRT deficiency disrupts the proliferation and migration of
99 developing midbrain dopamine (mDA) neurons in mouse embryos, arguing for a
100 neurodevelopmental syndrome (Witteveen et al. 2022). This could result from ATP depletion and
101 impaired energy metabolism (Bell et al., 2021) or an overactivation of *de novo* purine synthesis
102 leading to the accumulation of potentially toxic intermediates of this pathway (López, 2008;
103 López et al., 2020). The reason why dopaminergic neurons would be primarily affected in this
104 disease is not currently known. Pharmacological models have also been developed by injecting
105 the dopaminergic neurotoxin 6-hydroxydopamine into neonatally rat brains, which induced a
106 self-mutilation behavior in response to DA agonist administration in adulthood. However, these
107 models are of limited utility as they do not reproduce the basic genetic impairment of LND
108 (Breese et al. 1990; Knapp et al. 2016; Bell et al. 2016).

109 New animal models are therefore critically needed to study LND pathogenesis and find
110 efficient therapeutic molecules. The fruit fly *Drosophila melanogaster* presents many advantages
111 for translational studies and drug discovery (Fernández-Hernández et al. 2016, Perrimon et al.
112 2016; Papanikolopoulou et al. 2019). It has been widely used to model a variety of human
113 diseases, including neurodegenerative disorders such as Alzheimer, Huntington or Parkinson
114 diseases (Jaiswal et al. 2012; McGurk et al 2015; Dung and Thao 2018; Nagoshi 2018), as well
115 as heritable neurodevelopmental disorders such as Angelman, Rett or Fragile X syndromes
116 (Gatto and Broadie 2011). Although the importance of this invertebrate model for studying rare
117 human genetic diseases is now recognized (Oriel and Lasko 2018), a *Drosophila* LND model has
118 not yet been developed to our knowledge. This is probably due to the fact that no HGPRT

activity has been detected in this organism (Miller and Collins 1973; Becker 1974a; Becker 1974b). However, an ortholog of APRT is expressed in *Drosophila* (Johnson et al. 1987), encoded by the *Adenine phosphoribosyltransferase* (*Aprt*) gene, which seems to be the only enzyme of the purine salvage pathway in this insect. It is therefore possible that part of the functions of human HGPRT, and in particular those essential for nervous system development and physiology, were endorsed by Aprt in *Drosophila*.

Here, we tested whether purine recycling disruption in *Drosophila* can have similar consequences as lack of HGPRT activity in humans, by studying the effects of *Aprt* deficiency on purine metabolism, lifespan and various behaviors, including spontaneous and startle-induced locomotion, sleep and seizure-like bang-sensitivity. We provide evidence that Aprt prolongs lifespan and prevents seizure, and is required during development and in adult stage for many aspects of *Drosophila* life. Its activity seems to be of particular importance in subpopulations of dopaminergic neurons and glial cells in the brain. To our knowledge, this is the first LND model that recapitulates both purine recycling disruption and neurobehavioral symptoms. We also find that the expression of a LND-associated mutant form of HGPRT, but not the wild-type enzyme, in *Drosophila* neurons, induced neurobehavioral impairments similar to those of *Aprt*-deficient flies. Such a potential toxic gain-of-function effect of mutated HGPRT has not been demonstrated in an animal model before.

Results

Drosophila lacking Aprt activity have a shortened lifespan

To assess the importance of purine recycling in brain development and activity in *Drosophila*, we analyzed the phenotypes induced by a deficiency in Aprt, which is potentially the

142 only recycling enzyme of the purine salvage pathway in this organism (Figure S1A and B, *step*
143 *12*). The *Aprt*^{5} mutant was originally generated by chemical mutagenesis followed by selection of
144 flies resistant to purine toxicity (Johnson and Friedman, 1981; Johnson and Friedman, 1983).
145 Enzymatic assays confirmed a strong reduction in Aprt activity in extracts of heterozygous
146 *Aprt*^{5}/+ mutants and its absence in homozygous and hemizygous (*Aprt*^{5}/*Df(3L)ED4284*) mutants
147 (Figure S2 and Table S1). Sequencing of the *Aprt*^{5} cDNA (Figure S3A) indicated that the *Aprt*^{5}
148 mRNA codes for a protein with several amino acid changes compared to *Drosophila*
149 *melanogaster* wild-type Aprt, modifying in particular 3 amino acid residues that have been
150 conserved in the Aprt sequences from *Drosophila* to humans (Figure S3B). These mutations are
151 likely to be responsible for the loss of enzymatic activity. The homozygous *Aprt*^{5} mutants are
152 considered viable because they develop and reproduce normally. However, we observed that
153 these mutants have a significantly reduced longevity, their median lifespan being only 38 days
154 against 50 days for wild-type flies ($p < 0.001$) (Figure 1A).
155
156 **Uric acid levels are increased in *Aprt*^{5} mutants and normalized by allopurinol**
157 In humans, one of the consequences of HGPRT deficiency on metabolism is the
158 overproduction of uric acid (Harkness et al. 1988; Fu et al. 2015). We assayed the levels of
159 purine metabolites by HPLC and found that the level of uric acid was substantially increased by
160 37.7% on average in *Drosophila Aprt*^{5} mutant heads ($p < 0.01$) (Figure 1B). We then tried to
161 normalize uric acid content in flies by providing allopurinol in the diet, as is usually done for
162 LND patients. Allopurinol is a hypoxanthine analog and a competitive inhibitor of xanthine
163 oxidase, an enzyme that catalyzes the oxidation of xanthine into uric acid (Figure S1A and B,
164 *step 19*). Remarkably, the administration of 100 µg/ml allopurinol during 5 days decreased uric
165 acid levels to a normal concentration range in *Aprt*^{5} mutant heads (Figure 1B). Therefore, quite

166 similarly to HGPRT deficit in humans, the lack of Aprt activity in flies increases uric acid levels

167 and this metabolic disturbance can be prevented by allopurinol.

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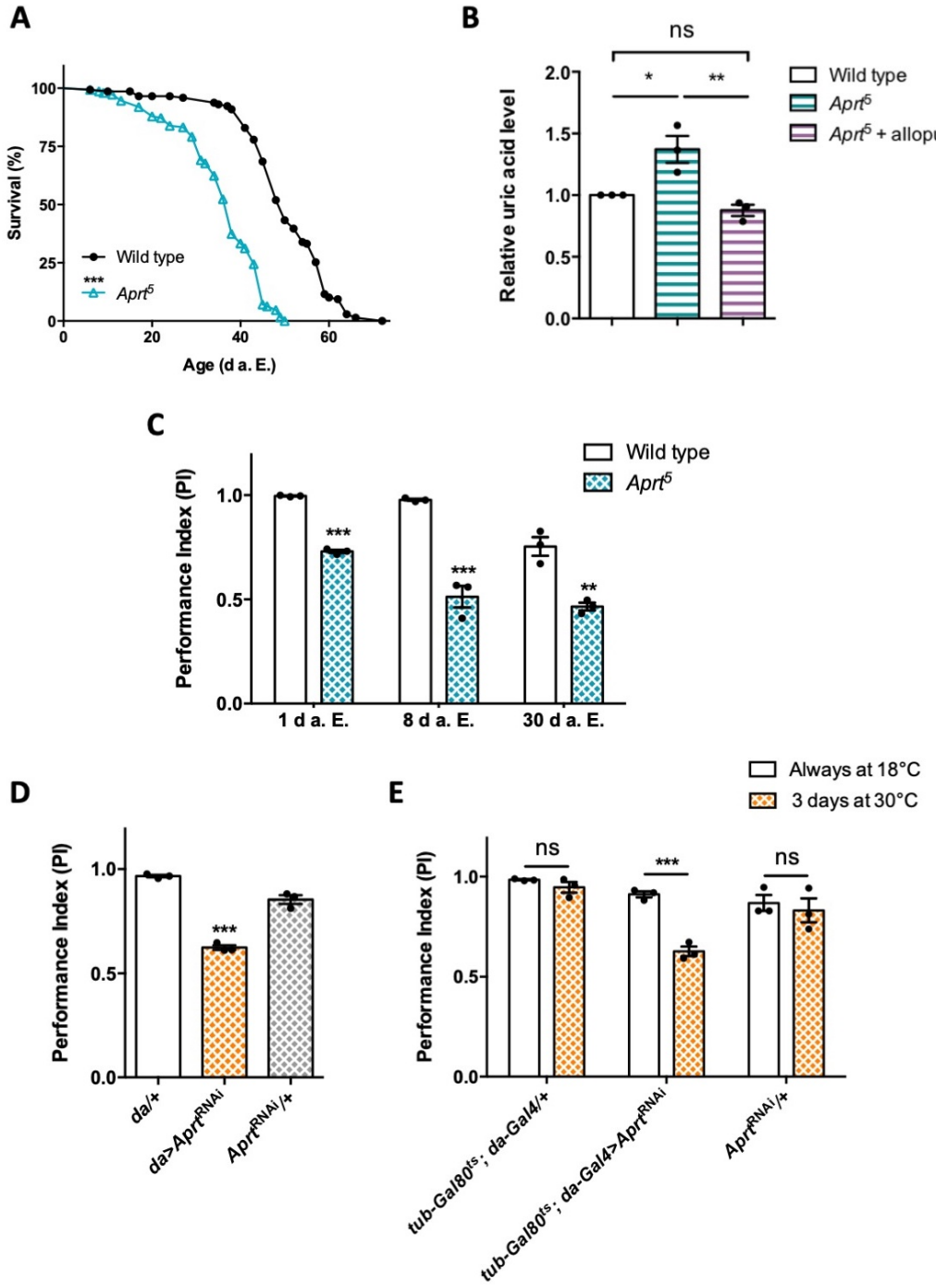


169

170 **Figure 1.** Aprt deficiency shortens lifespan and induces metabolic and neurobehavioral defects.
171 (**A**) *Aprt*^{5} mutant flies have a reduced lifespan compared to wild-type flies (median lifespan: 38
172 and 50 days, respectively). Three independent experiments were performed on 150 males per
173 genotype with similar results and a representative experiment is shown. ***$p < 0.001$. (**B**) HPLC
174 profiles on head extracts revealed an increase in uric acid levels in *Aprt*^{5} mutant flies.
175 Administration of 100 μg/ml allopurinol for 5 days before the test normalized uric acid levels.
176 Mean of 3 independent experiments performed on 40 flies per genotype. *$p < 0.05$, **$p < 0.01$,
177 ns: not significant. (**C-E**) Effect on climbing ability. (**C**) *Aprt*^{5} mutants show early defects in the
178 SING paradigm that monitors locomotor reactivity and climbing performance. This deficit was
179 already obvious at 1 d a.E. (days after eclosion) and further decreased up to 8 d a.E., after which
180 it did not change significantly up to 30 d a.E. Mean of 3 independent experiments performed on
181 50 flies per genotype. **$p < 0.01$, ***$p < 0.001$. (**D**) Downregulating *Aprt* by RNAi in all cells
182 (*da>Aprt*^{RNAi}) also led to an early impairment in climbing responses in the SING assay at 10 d
183 a.E., as compared to the driver (*da/+*) and effector (*Aprt*^{RNAi}*/+*) only controls. Mean of 3
184 independent experiments performed on 50 flies per genotype. ***$p < 0.001$. (**E**) Adult-specific
185 inactivation of *Aprt* (*tub-Gal80*^{ts}*; da-Gal4>Aprt*^{RNAi}) decreased startle-induced climbing abilities
186 in the SING paradigm, suggesting that the locomotor impairment induced by Aprt deficiency is
187 not caused by a developmental effect. Flies were raised at permissive temperature (18°C) in
188 which Gal80^{ts} suppressed Gal4-controlled *Aprt*^{RNAi} expression, and were shifted from 18 to 30°C
189 for 3 days before the test (between 7 and 10 d a.E.) to activate transgene expression. Mean of 3
190 independent experiments performed on 50 flies per genotype. ***$p < 0.001$, ns: not significant.
191

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

193 LND patients present dramatic motor disorders that prevent them for walking at an early
194 age. To examine whether a deficiency in the purine salvage pathway can induce motor
195 disturbance in young flies, we monitored the performance of *Aprt*-null mutants in startle-induced
196 negative geotaxis (SING), a widely used paradigm to assess climbing performance and locomotor
197 reactivity (Feany and Bender, 2000; Friggi-Grelin et al. 2003; Riemensperger et al. 2013; Sun et
198 al. 2018). Strikingly, *Aprt*^{5} mutant flies showed a very early SING defect starting from 1 day
199 after eclosion (d a.E.) (performance index (PI) = 0.73 vs. 0.98 for wild-type flies, $p < 0.001$) that
200 was more pronounced at 8 d a.E. (PI = 0.51 vs. 0.96 for the wild-type flies, $p < 0.001$). The fly
201 locomotor performance did not further decline afterwards until 30 d a.E. (Figure 1C).
202 *Df(3L)ED4284* and *Df(3L)BSC365* are two partially overlapping small genomic deficiencies that
203 covers *Aprt* and several neighbor genes. Hemizygous *Aprt*^{5}*/Df(3L)ED4284* or

204 *Aprt*^{5}/*Df(3L)BSC365* flies also displayed SING defects at 10 d a.E. (PI = 0.71 and 0.68,
205 respectively, compared to 0.97 for wild-type flies, $p < 0.01$) (Figure S4). In contrast to its
206 beneficial effect on uric acid levels, we observed that allopurinol treatment did not improve the
207 locomotor ability of *Aprt*^{5} mutant flies, either administered 5 days before the test or throughout
208 the development (Figure S5A, B). This is comparable to the lack of effect of this drug against
209 neurological defects in LND patients.

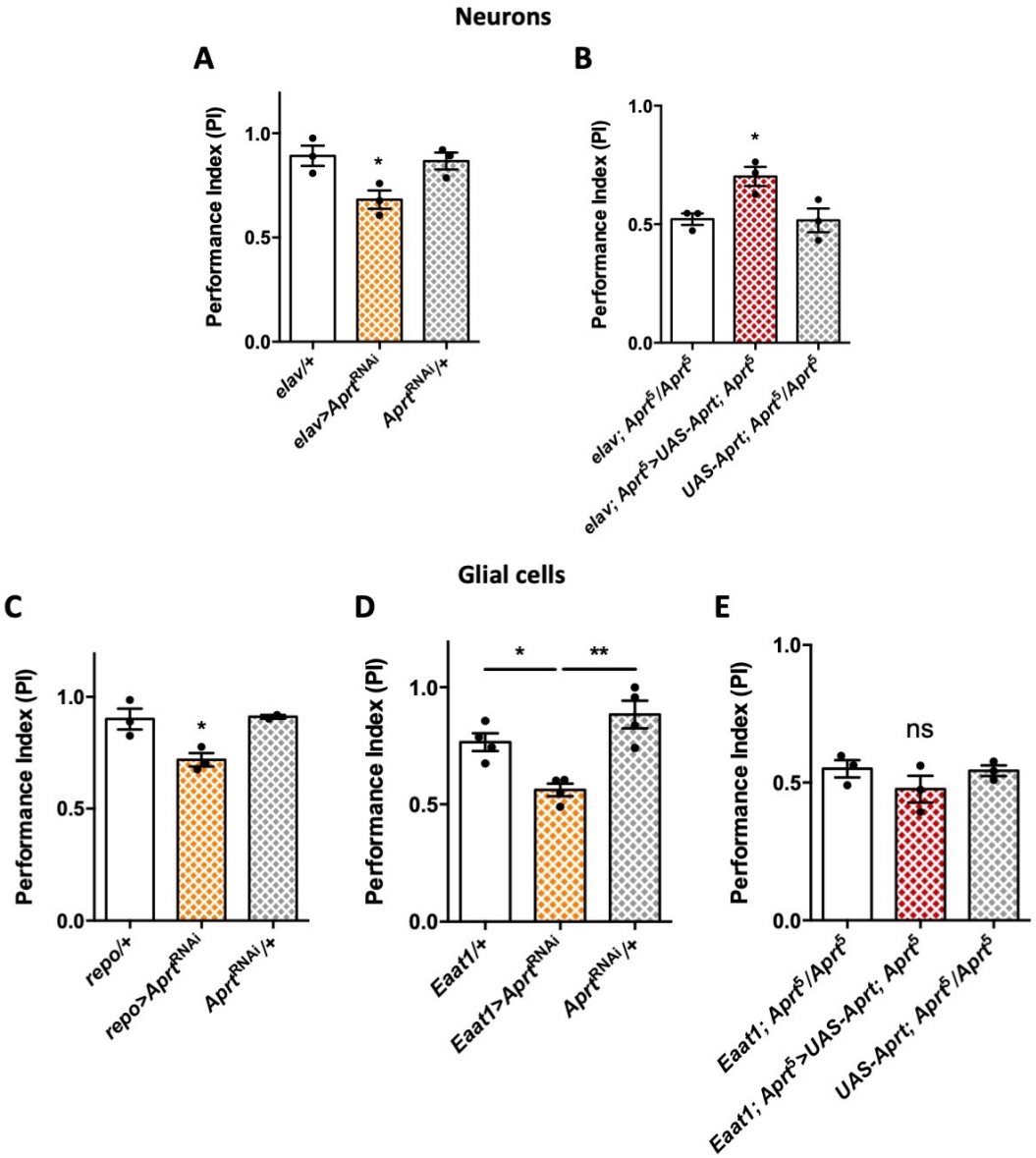
210 To confirm the effect of *Aprt* deficiency on the SING behavior, we used an *UAS-Aprt*^{RNAi}
211 line that reduced Aprt activity by more than 95% when expressed in all cells with the *da-Gal4*
212 driver (Table S1). These *Aprt* knocked down flies also showed a strong SING defect at 10 d a.E.
213 (PI = 0.62 against 0.97 and 0.85 for the driver and *UAS-Aprt*^{RNAi} alone controls, respectively, $p <$
214 0.001), like the *Aprt*^{5} mutant (Figure 1D). Next, we used *tub-Gal80*^{ts}, which inhibits Gal4 activity
215 at permissive temperature (McGuire et al. 2003), to prevent Aprt knockdown before the adult
216 stage. *tub-Gal80*^{ts}*; da-Gal4>Aprt*^{RNAi} flies raised at permissive temperature (18°C) did not show
217 any locomotor impairment (Figure 1E, *black bars*). However, after being transferred for 3 days
218 (between 7 and 10 d a.E.) at a restrictive temperature (30°C) that inactivates Gal80, the same flies
219 demonstrated a similar SING defect as *Aprt*^{5} mutants (PI = 0.63 compared to 0.94 and 0.83 for
220 the driver and RNAi alone controls, respectively, $p < 0.001$) (Figure 1E, *yellow bars with dots*).
221 This demonstrates that *Aprt* inactivation at the adult stage only is sufficient to alter SING
222 performance in *Drosophila*, strongly suggesting that this genotype does not result from
223 developmental defects.

224

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

226 We then tried to identify the neural cells in which Aprt is required to ensure a normal
227 locomotor reactivity in young flies by expressing *Aprt*^{RNAi} with selective Gal4 drivers.

228 Expression in all neurons with *elav-Gal4* led to decreased locomotor performance in the SING
229 test (PI = 0.68 at 10 d a.E., vs. 0.90 and 0.86 for the driver and RNAi controls, respectively, $p <$
230 0.05) (Figure 2A), which was comparable to the effects observed with the *Aprt*^{5} mutant or after
231 ubiquitous expression of the RNAi. To confirm the role of neuronal Aprt in locomotor control,
232 we generated a *UAS-Aprt* line which allowed for a substantial increase in Aprt expression and
233 activity (Figure S6). We then found that re-expressing *Drosophila* Aprt selectively in neurons in
234 *Aprt*^{5} background partially but significantly rescued the SING phenotype of the null mutant (PI =
235 0.70 vs. 0.52 for *driver* and *UAS-Aprt* controls in *Aprt*^{5} background, $p < 0.05$) (Figure 2B).
236 Furthermore, *Aprt* knockdown in all glial cells with *repo-Gal4*, or in sub-populations of
237 glial cells that express the glutamate transporter Eaat1 with *Eaat1-Gal4*, which includes
238 astrocyte-like glia, cortex glia and some subperineurial glia (Rival et al. 2004; Mazaud et al.
239 2019), also led to a lower SING performance of 10-day-old flies (PI = 0.72 for *repo-Gal4* vs.
240 0.91 for both controls, $p < 0.05$, and PI = 0.56 for *Eaat1-Gal4* vs. 0.77, $p < 0.05$, and 0.88, $p <$
241 0.01, for the driver and RNAi controls, respectively (Figure 2C, D). In contrast, *MZ0709-Gal4*
242 (Doherty et al. 2009) and *NP6520-Gal4* (Awasaki et al. 2008) that selectively target the
243 ensheathing glia, did not induce any significant locomotor defects when used to express the *Aprt*
244 RNAi (Figure S7). Noticeably, re-expressing Aprt with *Eaat1-Gal4* in the *Aprt*^{5} background did
245 not rescue the SING phenotype (Figure 2E), in contrast to the positive effect of neuronal re-
246 expression (Figure 2B). Overall this suggests that Aprt is required both in neurons and glia
247 subsets to ensure a normal SING performance in young flies, and that neuronal but not glial *Aprt*
248 re-expression is sufficient to ensure a partially functional locomotor behavior.
249



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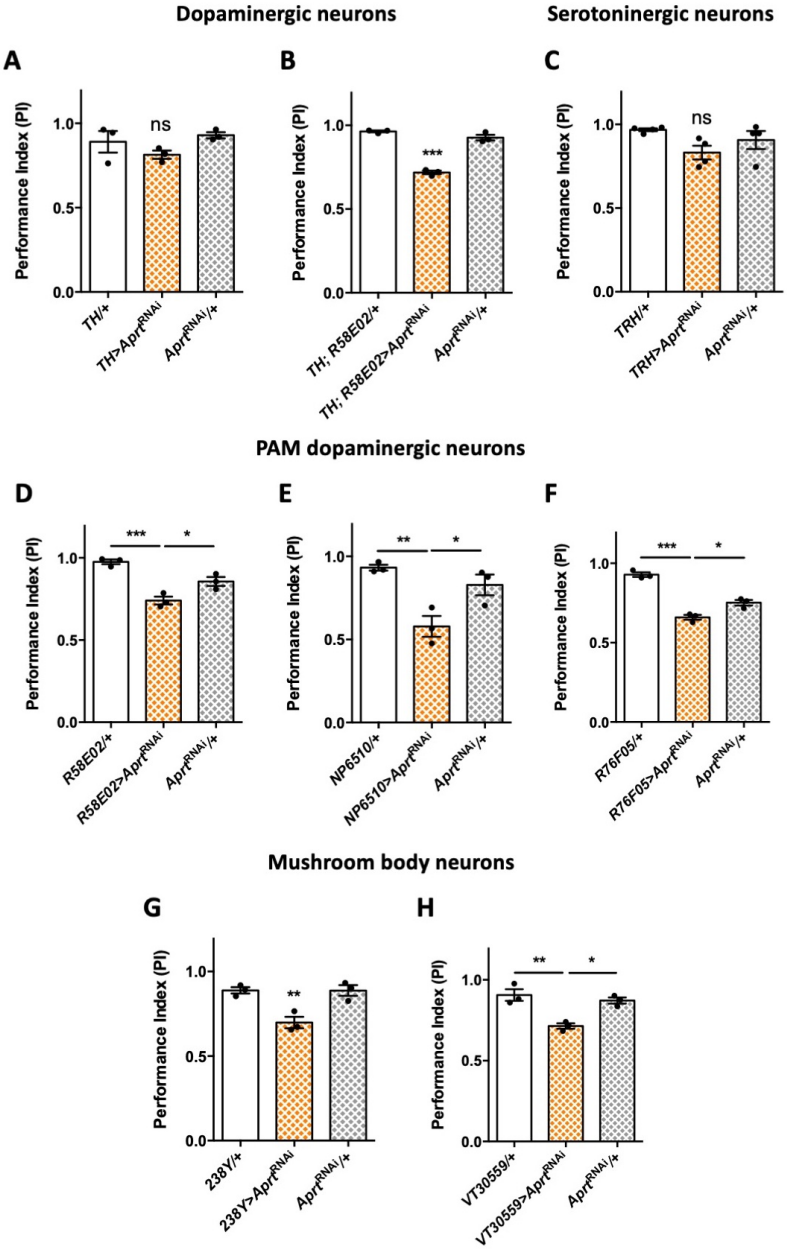
251 **Figure 2.** *Aprt* knockdown in neurons or glial cells disrupts startle-induced locomotion in
252 *Drosophila*. (**A**) $Aprt^{RNAi}$ expression in all neurons with *elav-Gal4* decreased SING performance
253 in 10-day-old flies. (**B**) Pan-neuronal expression of *Drosophila Aprt* with the UAS-Gal4 system
254 partially rescued the locomotor response of $Aprt^5$ mutants. (**C-D**) Downregulation of *Aprt*
255 expression in all glia with *repo-Gal4* (**C**) or in glial cell that express the glutamate transporter
256 Eaat1 with *Eaat1-Gal4* (**D**) also altered SING performances. (**E**) *Aprt* re-expression in glial cells
257 with *Eaat1-Gal4* driver did not rescue the climbing response of $Aprt^5$ mutants. Results of 3 or 4
258 independent experiments performed on 50 flies per genotype at 10 d a. E. $*p < 0.05$, $**p < 0.01$,
259 ns: not significant.
260

261 To identify the neuronal subpopulations in which Aprt is required to ensure proper
262 locomotor responses in young flies, we first tested dopaminergic drivers since we previously
263 showed that DA neurons play an important role in the control of the SING behavior
264 (Riemensperger et al. 2011; Riemensperger et al. 2013; Sun et al. 2018). *Aprt* knockdown
265 targeted to these neurons with *TH-Gal4* did not induce significant impairments (Figure 3A). This
266 driver expresses in all brain DA neurons except a large part of the protocerebral anterior medial
267 (PAM) cluster (Friggi-Grelin et al. 2003; Mao and Davis 2009; Pech et al. 2013). In contrast,
268 downregulating *Aprt* with the *TH-Gal4, R58E02-Gal4* double driver, which labels all DA
269 neurons including the PAM cluster (Sun et al. 2018), induced a quite similar locomotor defect as
270 did pan-neuronal *Aprt* knockdown (PI = 0.72 vs. 0.96 and 0.93 for the driver and RNAi controls,
271 respectively, $p < 0.001$) (Figure 3B). Besides, downregulating *Aprt* in a majority of the
272 serotoninergic neurons with *TRH-Gal4* (Cassar et al. 2015) did not induce a SING defect (Figure
273 3C).

274 These results suggest that some DA neurons in the PAM cluster are specifically involved
275 in the locomotor impairments induced by *Aprt* deficiency. It has been previously shown in our
276 laboratory that inhibiting DA synthesis in a subset of 15 PAM DA neurons cluster was able to
277 alter markedly SING performance in aging flies (Riemensperger et al. 2013). We and others also
278 reported that the degeneration or loss of PAM DA neurons is involved in the SING defects
279 observed in several *Drosophila* models of Parkinson disease (Riemensperger et al. 2013; Bou Dib
280 et al. 2014; Tas et al. 2018; Pütz et al. 2021). Here we found that expressing $Aprt^{RNAi}$ in the PAM
281 cluster with *R58E02-Gal4* reproduced the same motor disturbance as pan-neuronal expression (PI
282 = 0.74 vs. 0.96, $p < 0.001$, and 0.85, $p < 0.05$, for the driver and RNAi controls, respectively)
283 (Figure 3D), and this result was confirmed by using two other PAM drivers (*NP6510-Gal4* –
284 expressing only in 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



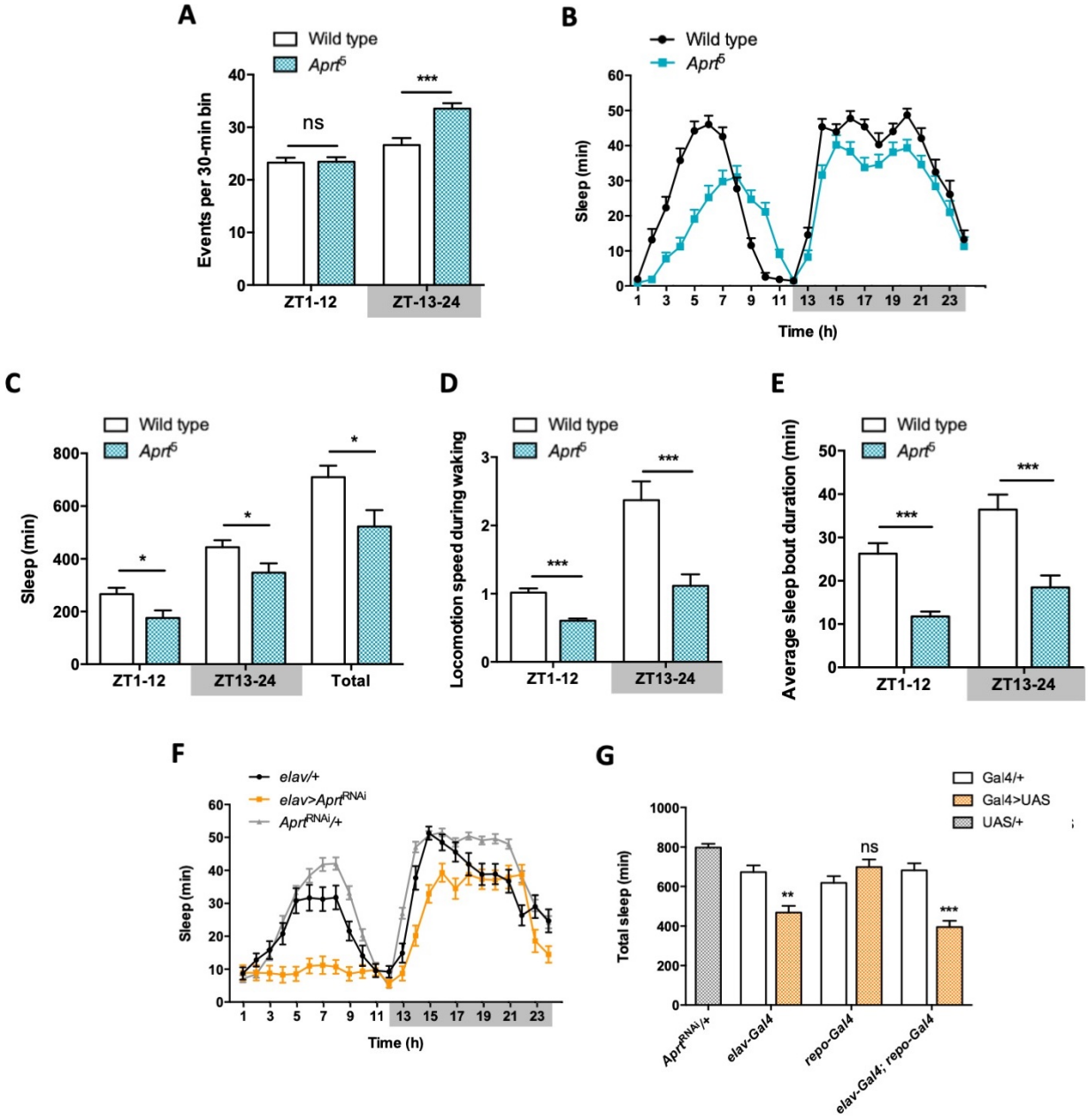
291 **Figure 3.** *Aprt* downregulation in DA neurons of the PAM cluster and in mushroom body
292 neurons impairs startle-induced locomotion. (**A**) RNAi-mediated *Aprt* inactivation in brain DA
293 neurons except a large part of the PAM cluster with the *TH-Gal4* driver did not lead to locomotor
294 defects in the SING assay. (**B**) In contrast, *Aprt* knockdown in all dopaminergic neurons
295 including the PAM cluster with the *TH-Gal4, R58E02-Gal4* double-driver led to a decrease in
296 SING performance. (**C**) *Aprt* downregulation in serotoninergic neurons with *TRH-Gal4* did not
297 alter startle-induced climbing response of the flies. (**D-F**) *Aprt* knockdown selectively in DA
298 neurons of the PAM cluster using either *R58E02-Gal4* (**D**), *NP6510-Gal4* (**E**) or *R76F05-Gal4*
299 (**F**) significantly decreased climbing performance. (**G-H**) *Aprt* knockdown in all the mushroom
300 body intrinsic neurons with *238Y-Gal4* (**G**) or *VT30559-Gal4* (**H**) also led to a decrease in SING
301 performance. Results of 3 or 4 independent experiments performed on 50 flies per genotype at 10
302 d a. E. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns: not significant.
303

304 al. 2018), we also inactivated *Aprt* in MB neurons with *238Y-Gal4* that strongly expresses in all
305 the MB cells (Aso et al. 2009). Interestingly, this driver did induce a locomotor reactivity
306 impairment (PI = 0.70 vs. 0.89 for both controls, $p < 0.01$) (Figure 3G), and the same result was
307 observed with *VT30559-Gal4*, which is a very specific driver for all the MB intrinsic neurons
308 (Plaçais et al. 2017) (Figure 3H). Overall, these results show that normal expression of the SING
309 behavior depends on *Aprt* expression in the PAM and MB neurons in *Drosophila*.
310

311 **Sleep disturbances induced by Aprt deficiency**

312 Both the mushroom body and subpopulations of PAM DA neurons are known to be
313 regulators of sleep in *Drosophila* (Nall et al. 2016; Artiushin and Sehgal 2017). The fact that *Aprt*
314 deficiency in some of these cells impaired locomotor regulation prompted us to monitor the
315 spontaneous locomotion and the sleep pattern of *Aprt* mutants. Compared to controls, *Aprt*-
316 deficient flies did not have an altered circadian activity profile (Figure S8), nor any difference in
317 total spontaneous locomotor activity during the day, as quantified by the number of infra-red
318 beam cuts (*events*) per 30 min, but they showed a 26.2% increase in total activity during the night
319 ($p < 0.001$) (Figure 4A). As usual, a sleep bout was defined as 5 min or more of fly immobility
320 (Huber et al. 2004). We found that $Aprt^5$ mutants slept significantly less than wild-type flies and

321 that it was the case both during day and night (Figure 4B, C). These mutants indeed showed a
322 reduced walking speed (Figure 4D) and a smaller average sleep bout duration (Figure 4E),
323 indicating a difficulty to maintain sleep. The reduced speed does not seem to be caused by a
324 decreased energetic metabolism, since we could not detect different ATP levels in head and
325 thorax of *Aprt*^{5} mutants compared to wild-type flies (Figure S9).



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327 **Figure 4.** *Aprt*-deficient flies sleep less and walk slower than wild-type flies. (**A**) Quantification
328 of total spontaneous locomotor activity during day and night over 5 LD cycles. $Aprt^5$ mutants
329 show no difference in spontaneous locomotion with wild-type flies during the day but a higher
330 activity at night. 3 independent experiments were performed on 32 flies per genotype and mean ±
331 SEM was plotted. ***$p < 0.001$, ns: not significant. (**B**) Sleep pattern during a typical 24h LD
332 cycle showing that the total amount of sleep is smaller during day and night in $Aprt^5$ mutants
333 compared to wild-type flies. (**C**) Quantification of day (ZT1-12), night (ZT13-24) and total sleep
334 in $Aprt^5$ mutants. ZT is for zeitgeber. (**D**) Locomotion speed during waking is reduced in $Aprt^5$
335 mutants. (**E**) The average sleep bout duration is also decreased, indicating that $Aprt^5$ mutants
336 have a difficulty to maintain sleep. (**F**) Sleep pattern of *elav>*$Aprt^{RNAi}$ flies, showing that
337 knockdown of Aprt in all neurons led to sleep reduction during the night, similarly to the mutant
338 condition, and an even more profound sleep defect during the day. (**G**) Quantification of total
339 amount of sleep when *Aprt* was downregulated in all neurons (*elav-Gal4*), all glial cells (*repo-*
340 *Gal4*) and both neurons and glial cells (*elav-Gal4*; *repo-Gal4*). Except for glia only, the resulting
341 effect was a significant sleep reduction. For sleep and locomotion speed, means ± SEM were
342 plotted. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns: not significant.
343
344 Interestingly, RNAi-mediated downregulation of Aprt selectively in neurons
345 (*elav>*$Aprt^{RNAi}$ flies) also significantly decreased sleep duration during day and night (Figure 4F),
346 whereas glial-only expression (*repo>*$Aprt^{RNAi}$) had no effect (Figure S10A). Expressing the
347 RNAi in both neurons and glia (*elav; repo>*$Aprt^{RNAi}$) had similar effects as in neurons alone
348 (Figure S10B). Quantification of total sleep in these experiments, and the total amount of day and
349 night sleep, are shown in Figure 4G and Figure S10C, D, respectively. Overall, these results
350 suggest that *Aprt* expression is selectively needed in neurons for a normal sleep pattern in
351 *Drosophila*.
352
353 **Brain DA synthesis and levels are increased in *Aprt*-deficient flies**
354 Since we found that induced locomotion and sleep, two behaviors controlled by DA
355 neurons in *Drosophila*, were altered in *Aprt*-deficient flies, we logically expected brain DA levels
356 to be reduced in these mutants, as is the case in the basal ganglia of LND patients. To check for
357 that, we performed comparative immunostaining for tyrosine hydroxylase (TH), the specific
358 enzyme of DA synthesis (Friggi-Grelin et al. 2003; Riemensperger et al. 2011), on dissected

359 adult brains from wild-type and *Aprt*^{5} mutant flies. Surprisingly, the global TH protein level
360 appeared not to be decreased, but relatively increased by 17.5% in the *Aprt*^{5} mutant brain ($p <$
361 0.01), in particular around the mushroom bodies, a structure that receives dense dopaminergic
362 projections (Figure 5A, B). Moreover, DA immunostaining carried out on whole-mount dissected
363 brains revealed a similarly increased level of this neuromodulator in *Aprt*^{5} flies, by 17.0% on
364 average in the entire brain ($p < 0.01$), but not specifically in the mushroom bodies or another part
365 of the brain (Figure 5C, D). This indicates that disruption of the purine salvage pathway does not
366 impede DA synthesis and levels in the *Drosophila* brain, which are instead slightly increased,
367 perhaps as a compensatory mechanism. The behavior defects suggest that the lack of Aprt
368 activity could interfere with correct functioning of the DA system in an indirect way. We
369 therefore searched for another neurotransmitter system that could be affected by *Aprt* deficiency.

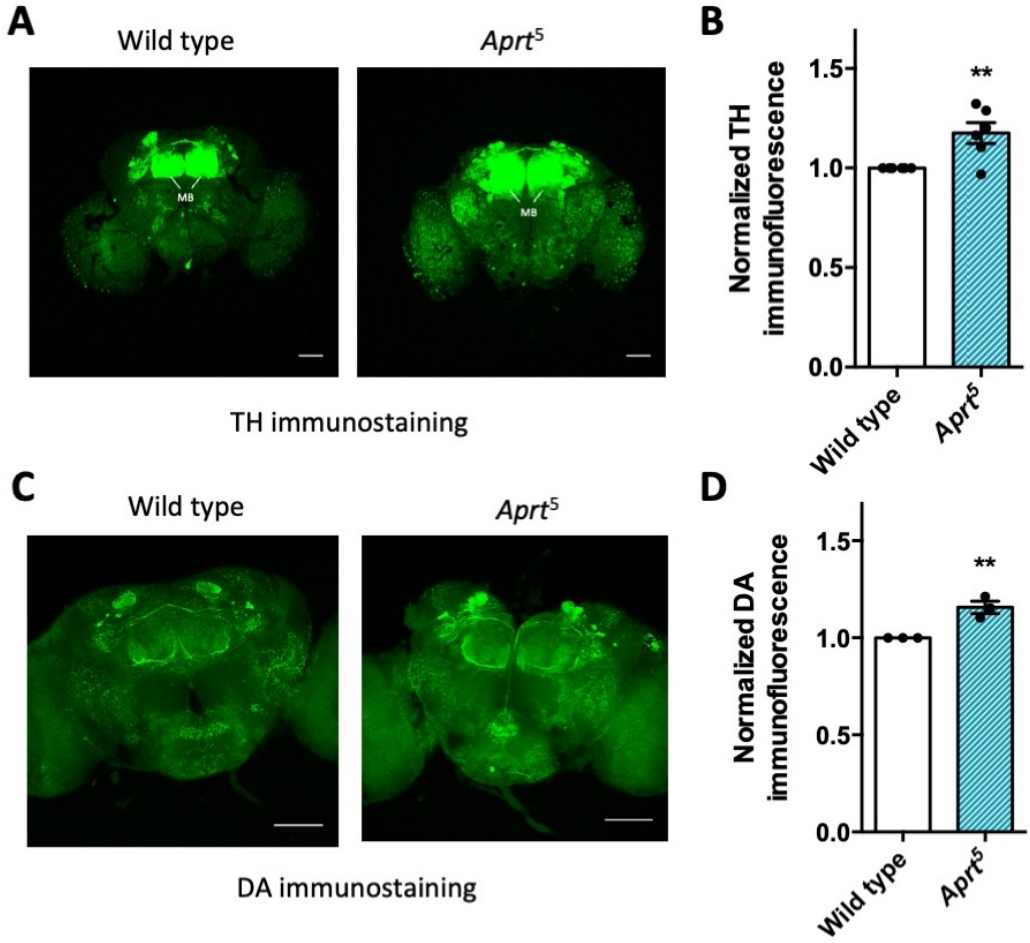

Figure 5. *Aprt* deficiency increases DA synthesis and content in the *Drosophila* brain. (**A**) Representative confocal projections of TH-immunostained whole-mount adult brains from wild-type flies and *Aprt*^{5} mutants. MB: mushroom body. Scale bars: 100 µm. (**B**) Quantification of TH immunofluorescence intensity normalized to the controls in the entire brain. 4 to 6 brains were dissected per experiment and genotype, and 6 independent experiments were performed. $^{**}p < 0.01$. (**C**) Representative confocal projections of DA immunostaining in whole-mount adult brains of wild type and *Aprt*^{5} mutants. Scale bars: 100 µm. (**D**) Quantification of DA immunofluorescence intensity over the entire brain showed a slight increase in DA content in *Aprt*^{5} mutants compared to wild-type controls. 6 brains were dissected per experiment and genotype, and 3 independent experiments were performed. $^{**}p < 0.01$.

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

386 Aprt catalyzes the conversion of adenine into AMP, and AMP breakdown by the enzyme
387 5'-nucleotidase produces adenosine, primarily in the extracellular space. Adenosine then acts as a
388 widespread neuromodulator in the nervous system by binding to adenosine receptors. We
389 therefore suspected that adenosine level could be reduced in the absence of Aprt activity, leading
390 to alterations in adenosinergic neurotransmission. Indeed, we found a significant decrease in
391 adenosine level either in whole flies (by 61.0% on average, $p < 0.01$) or in heads (by 48.0%, $p <$
392 0.05) in *Aprt*^{5} mutants (Figure 6A). We then examined the consequences of this reduction on
393 molecular components of the adenosinergic system, namely G protein-coupled adenosine
394 receptors and equilibrative nucleoside transporters (ENTs), which carry out nucleobase and
395 nucleoside uptake of into cells. Only one seven-transmembrane-domain adenosine receptor,
396 AdoR, is present in *Drosophila*, that is very similar to mammalian adenosine receptors
397 (Dolezelova et al. 2007; Brody and Cravchik, 2000), and three putative equilibrative nucleoside
398 transporters (Ent1-3) have been identified (Sankar et al. 2002) but only one, Ent2, showed
399 nucleoside transport activity (Machado et al. 2007). Interestingly, Ent2 has been shown to be
400 enriched in the mushroom bodies and its transcript level to be highly elevated in *AdoR* mutant
401 flies (Knight et al. 2010). In *Aprt*^{5} background, we also observed a strong increase in *Ent2*
402 mRNAs (2.3-fold higher than wild-type flies, $p < 0.001$), but no noticeable effect on *AdoR*
403 transcript level (Figure 6B). The increased expression of *Ent2* could correspond to a
404 compensatory response to adenosine shortage in *Aprt*^{5} mutants.

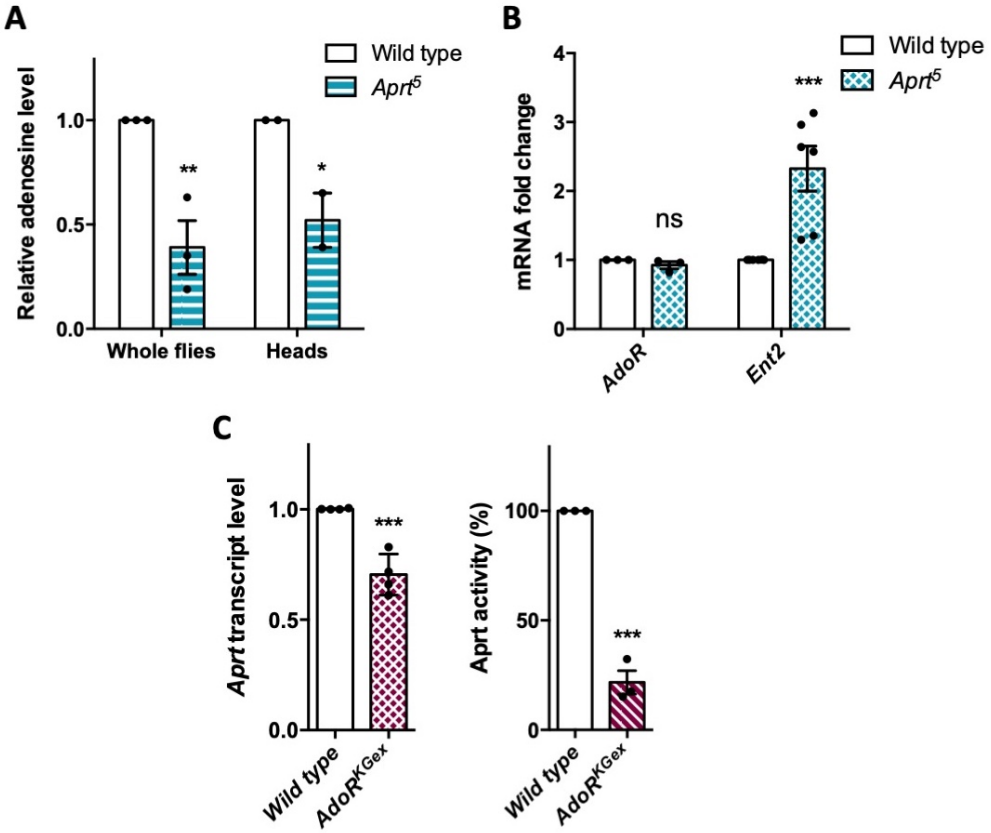

Figure 6. Relations between Aprt and molecular components of adenosinergic signaling. (**A-B**) Impacts of the lack of Aprt activity on the adenosinergic system. (**A**) Adenosine level was measured in whole flies or heads of *Aprt*^{5} flies by UPLC. Compared to wild-type flies, adenosine level was significantly reduced in the mutants. Results of 3 independent experiments performed with 5 males per genotype in triplicates, and 2 independent experiments with 30 heads per genotype in triplicates. *$p < 0.05$, **$p < 0.01$. (**B**) *Aprt*^{5} mutation did not affect *AdoR* expression but induced a marked increase in adenosine transporter *Ent2* mRNA abundance. 3-6 different RNA extractions were performed on 20-30 male heads. Results of 3 to 6 independent experiments. ***$p < 0.001$, ns: not significant. (**C**) Null *AdoR*^{KGex} mutants showed decreased *Aprt* expression (*left panel*) and a stronger decrease in Aprt activity (*right panel*). 4 independent RNA extractions were carried out on 20-30 male heads and 4 independent real-time PCR determinations were done per RNA sample. For Aprt activity, 3 independent determinations were performed on 20 whole flies per genotype. ***$p < 0.001$.

The AdoR receptor is highly expressed in adult fly heads and its ectopic overexpression leads to early larval lethality (Dolezelova et al. 2007). In contrast, a null mutant of this receptor, *AdoR*^{KGex}, in which the entire coding sequence is deleted, is fully viable (Wu et al. 2009). This enabled us to examine the consequences of a complete lack of AdoR on purine recycling in adult

424 flies. We found that *Aprt* transcripts were decreased by 29.5% on average ($p < 0.001$) in
425 *AdoR*^{KGex} mutant heads (Figure 6C, *left panel*), while Aprt activity was even more decreased by
426 78.4% ($p < 0.001$) compared to wild-type flies (Figure 6C, *right panel*). This effect likely results
427 from the much increased level of adenosine uptake in the *AdoR* mutants, which would
428 downregulate *Aprt* activity by a feedback mechanism. Overall, these data indicate that
429 extracellular adenosine levels must be strongly decreased in Aprt mutant flies, both from the
430 general reduction in adenosine levels and the increased expression of the Ent2 transporter.
431 Although AdoR expression is not affected, AdoR signaling is therefore expected to be
432 significantly reduced in *Aprt* mutants.
433
434 ***Aprt* mutants show epilepsy-like seizure behavior**
435 A number of *Drosophila* mutants with disrupted metabolism or neural function show
436 increased susceptibility to seizure and paralysis following strong mechanical or
437 electrophysiological stimulation (Fergestad et al. 2006, Parker et al. 2011, Kroll et al. 2015).
438 These mutants are called bang-sensitive (BS) and commonly used as models of epileptic seizure
439 (Song and Tanouye 2008). Here we checked whether Aprt deficiency could trigger a BS
440 phenotype. We observed that aged *Aprt* mutants (at 30 d a.E.) recovered slowly after a strong
441 mechanical stimulation applied by vortexing the vial for 10 s at high speed. These flies took on
442 average 17.3 s to recover and get back on their legs, compared to 2.5 s for wild-type flies of the
443 same age ($p < 0.01$) (Figure 7A and Movie S1). Some of the mutant flies appeared more deeply
444 paralyzed as they did not spontaneously recover unless the vial was stirred, so their recovery time
445 could not be scored. Hemizygous flies of the same age containing the *Aprt*^{5} mutation over two
446 partially overlapping genomic deficiencies covering *Aprt* also showed a BS behavior, with an
447 average longer recovery time of 28.9 s and 33.2 s, respectively ($p < 0.001$) (Figure 7B).

448 We then downregulated *Aprt* by RNAi in all cells with the *da-Gal4* driver to check if this
449 could also induce BS behavior. As shown in Figure 7C, *da>Aprt*^{RNAi} flies at 30 d a.E. indeed
450 displayed seizure after mechanical shock, quite similar to that of the *Aprt*^{5} mutants (22.7 s
451 recovery time on average compared to 1.55 s and 0.72 s for the driver and RNAi controls,
452 respectively, $p < 0.05$). In contrast, inactivating *Aprt* selectively in neurons *(elav-Gal4)*, glial
453 cells *(repo-Gal4)* or muscles *(24B-Gal4)* did not induce a BS phenotype (Figure S11). This
454 suggests that the BS phenotype requires *Aprt* knockdown in other cells or in several of these cell
455 types. Finally, in contrast to the SING defect, we observed that adult-specific *Aprt* knockdown in
456 all cells with *da-Gal4* for 3 days did not trigger a BS behavior (Figure 7D), indicating that the
457 bang sensitivity requires a longer downregulation of the gene or might be the consequence of a
458 developmental defect.
459
460 **Administration of Adenosine or *N*^{6}-methyladenosine to *Aprt*$^{-}$deficient flies prevents seizure**
461 *Drosophila* disease models are advantageously tractable for drug screening *in vivo*
462 (Fernández-Hernández et al. 2016, Perrimon et al. 2016). We thus administered several
463 compounds related to purine metabolism to *Aprt*^{5} flies to check if they can rescue
464 neurobehavioral impairments (locomotor defects and seizure). Feeding allopurinol at the same
465 concentration used for uric acid normalization (100 μg/ml, Figure 1B), either in adults 5 days
466 before the test or throughout all developmental stages, did not alter the BS phenotype (Figure
467 S12), as was the case for the SING assay (Figure S5). Similarly in humans, it has been shown
468 that the daily intake of allopurinol, even from infancy, does not mitigate the neurobehavioral
469 impairments in LND patients (Marks et al. 1968; Torres et al. 2007; Jinnah et al. 2010; Madeo et
470 al. 2019).

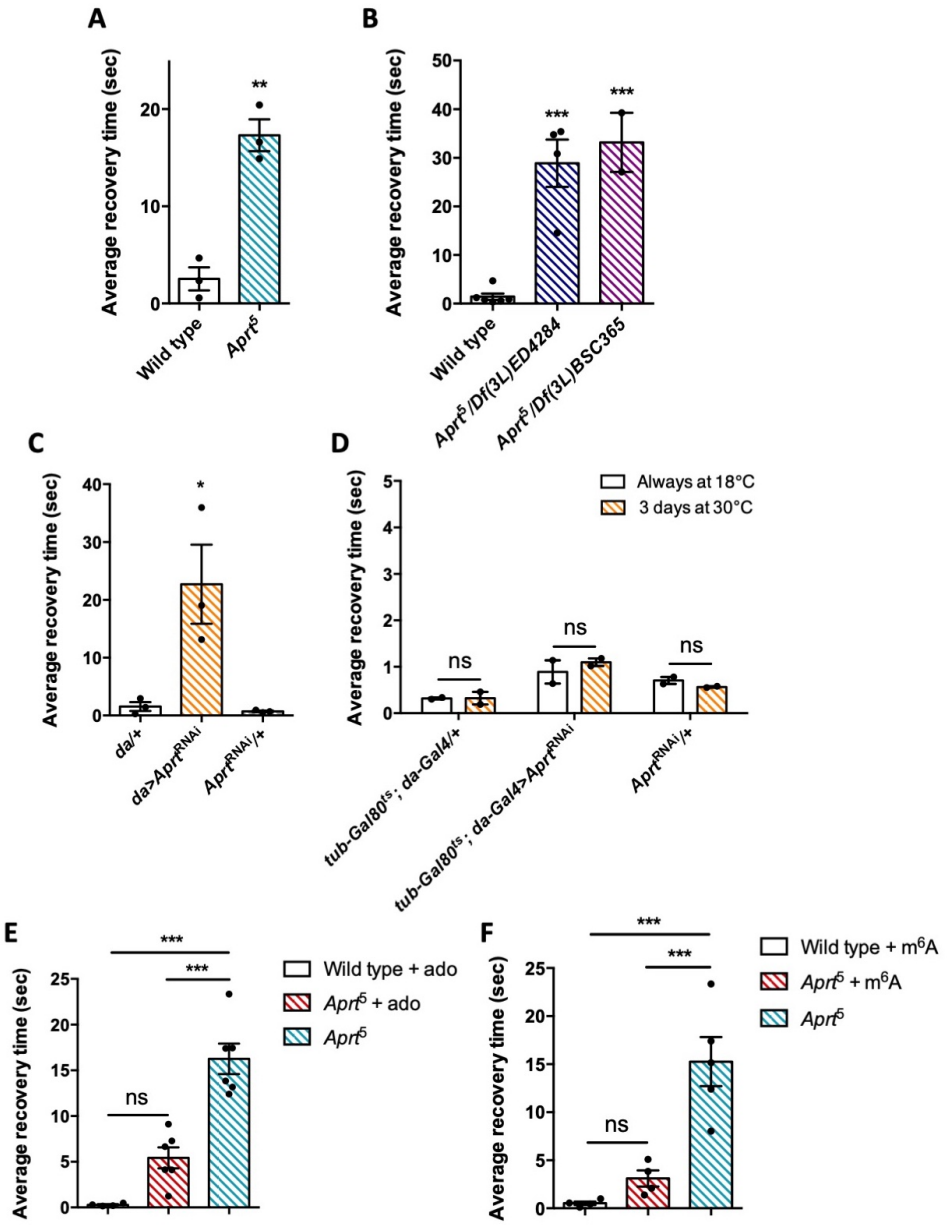

Figure 7. *Aprt* deficiency triggers a seizure-like phenotype. (**A**) At 30 d a.E., *Aprt*^{5} mutants need a much longer time than wild-type flies to recover from a strong mechanical shock, showing a bang-sensitive (BS) paralysis comparable to tonic-clonic seizure. Results of 3 independent experiments performed on 50 flies per genotype. $^{**}p < 0.01$. (**B**) At 30 d a. E., hemizygous *Aprt*^{5} mutants also showed a marked BS phenotype. Results of 2-4 independent experiments performed on 50 flies per genotype. $^{***}p < 0.001$. (**C**) RNAi-mediated downregulation of *Aprt* in all cells (*da>Aprt*^{RNAi}) also led to BS phenotype in adults at 30 d a.E., but not with the driver and effector controls. Results of 3 independent experiments performed on 50 flies per genotype. $^{*}p < 0.05$. (**D**) *Aprt* knockdown by RNAi during the adult stage for 3 days before the test was not sufficient to induce bang-sensitivity, suggesting that this phenotype could be caused by a developmental defect or a longer downregulation of *Aprt*. Results of 2 independent experiments performed on 50

483 flies per genotype. ns: not significant. (**E, F**) The BS phenotype of 30-day-old *Aprt*^{5} mutants was
484 rescued by feeding either 500 µM adenosine (ado) (**E**) or 500 µM N^6-methyladenosine (m^6A) (**F**)
485 during all developmental stages plus 5 days before the test. Results of 4 or 6 independent
486 experiments performed on 50 flies per genotype. ***$p < 0.001$, ns: not significant.
487

488 Then, we tried to supplement *Aprt* mutants with various purine compounds, including
489 adenine, hypoxanthine, adenosine and N^6-methyladenosine (m^6A), at 100 or 500 µM, either in
490 adult stage 5 days before the test or throughout larval development plus 5 days before the test.
491 None of these drugs was able to rescue the SING defect (data not shown). In contrast and
492 interestingly, administration of 500 µM adenosine or m^6A during development rescued the BS
493 phenotype of *Aprt*^{5} mutants (Figure 7E-F). This further indicates that different mechanisms
494 underpin SING alteration and BS behavior in *Aprt* mutants and provide another evidence that the
495 bang sensitivity may be caused by a developmental defect. The results also suggest that the lower
496 adenosine levels of *Aprt* mutant flies could be at the origin of their bang sensitivity.
497

498 **Neuronal expression of mutant HGPRT induces early locomotor defects and seizure**
499 **behavior**

500 In concordance with reports from about 50 years ago (Miller and Collins 1973; Becker
501 1974a; 1974b), we could confirm that no HGPRT enzymatic activity can be detected in wild-type
502 *Drosophila* extracts (Table S2). Furthermore, searching the *Drosophila* genome with BLAST did
503 not retrieve any *Drosophila* homologue of *HPRT1*, the human gene encoding HGPRT. In order to
504 potentially develop another *Drosophila* model mimicking LND conditions, we generated new
505 transgenic UAS lines to express in living flies either the human wild-type HGPRT (HGPRT-WT)
506 or a pathogenic LND-associated mutant form of this protein (HGPRT I42T), both isoforms being
507 inserted at the same genomic docking site. These lines were validated by showing that they are

transcribed at similar levels (Figure 8A, B). Enzymatic assays on adult extracts of flies expressing the wild-type form HGPRT-WT in all cells showed significant HGPRT enzyme activity, while an 80.5% lower activity was detected in *Drosophila* expressing the mutant form HGPRT-I42T (Table S2).

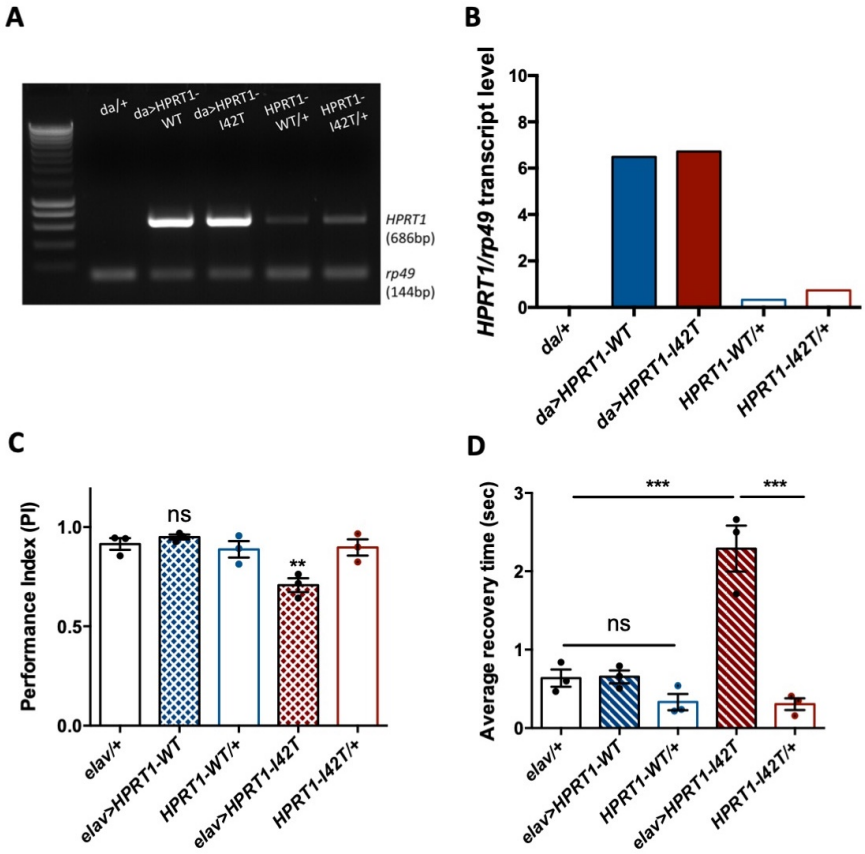

Figure 8. Expression of a pathogenic mutant isoform of human HGPRT induces neurobehavioral defects in flies. (**A-B**) Ubiquitous expression of human *HPRT1* with *da-Gal4*. (**A**) Amplification of human *HPRT1* transcripts detected by RT-PCR in head extracts of *da>HPRT1-WT* and *da>HPRT1-I42T* flies. 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. $^{**}p < 0.01$, $^{***}p < 0.001$, ns: not significant.

526 We next analyzed the consequences of human HGPRT expression on the SING and bang
527 sensitivity behaviors. Interestingly, the pan-neuronal expression of mutant I42T isoform
528 specifically induced a significant early locomotor defect at 15 d a.E. (PI = 0.71 vs. 0.92 and 0.90
529 for the driver and effector controls, respectively, $p < 0.01$) (Figure 8C) and a relatively small but
530 robust bang-sensitivity behavior at 30 d a.E. (2.3 s average recovery time vs. 0.64 s and 0.31 s for
531 the driver and effector only controls, $p < 0.001$) (Figure 8D). These defective phenotypes could
532 not be seen when HGPRT-WT was expressed. Therefore, and remarkably, whereas wild-type
533 HGPRT expression appears to be innocuous in *Drosophila*, we observed that the neuronal
534 expression of a pathogenic LND-associated isoform triggered neurobehavioral impairments quite
535 similar to those of *Aprt*-deficient flies.
536

537 **Discussion**

538 Over the past 35 years or so, several animal models of LND have been developed in
539 rodents based on HGPRT mutation in order to better understand the cause of the disease and test
540 potential therapeutic treatments (Finger et al. 1988; Dunnett et al. 1989; Jinnah et al. 1993; Engle
541 et al 1996; Meek et al. 2016; Witteveen et al. 2022). However, none of these models recapitulated
542 the full LND syndrome and, particularly, motor or neurobehavioral symptoms as a consequence
543 of HGPRT deficiency. To date, the causes of the neurobehavioral impairments in LND are not
544 yet clearly elucidated and the disease is still incurable (Fu et al. 2014; Bell et al. 2016; López et
545 al., 2020; Bell et al., 2021; Witteveen et al. 2022). Here we have tried to develop new models of
546 this disease in *Drosophila*, a useful organism to conduct genetic and pharmacological studies. An
547 apparent drawback of this project was that no homolog of the mammalian gene encoding HGPRT
548 is conserved in the fly genome, which may explain why no LND models have been developed to

549 date in this organism. To try to overcome that, we used two different strategies. First, because
550 Aprt seems to be the only enzyme of the purine salvage pathway in *Drosophila*, we hypothesized
551 that it could have analogous functions, both for purine homeostasis and behavior control, as
552 HGPRT in humans. Indeed, we show here that *Aprt* deficiency induces both metabolic and
553 neurobehavioral disturbances in *Drosophila*, similar to the loss of HGPRT, but not APRT,
554 activity in humans. Second, we have expressed the wild-type human HGPRT and a LND-
555 associated mutant form of this protein in *Drosophila* neurons to analyze their respective effects in
556 living flies. Here we provide evidence that the fruit fly can be used to study the consequences of
557 defective purine recycling pathway and HGPRT mutation in the nervous system, and perform
558 pharmacological screens to find new therapy for LND.
559

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

561 Flies that carry a homozygous null-mutation in *Aprt* develop normally and live until the
562 adult stage (Johnson and Friedman, 1983). Furthermore, a previous study reported that
563 heterozygous *Aprt*/+ flies have an extended lifespan (Stenesen et al. 2013). We observed that
564 homozygous $Aprt^5$ mutants have in contrast a significantly reduced longevity compared to wild-
565 type flies. It can be noted that the lack of HGPRT activity in LND also causes reduced lifespan
566 expectancy, which is generally less than 40 years of age for properly cared patients. Stenesen et
567 al. (2013) have demonstrated that dietary supplementation with adenine, the Aprt substrate,
568 prevented the longevity extension conferred either by dietary restriction or heterozygous
569 mutations of AMP biosynthetic enzymes. This suggests that longevity depends on an accurate
570 regulation of adenine levels. It is possible that adenine could accumulate to toxic levels during
571 aging in homozygous *Aprt* mutants, explaining their shorter lifespan. Alternatively, we could
572 speculate that AMP, the Aprt product, will be decreased in the absence of a functional Aprt, and

573 as a consequence, AMP-activated protein kinase (AMPK), an enzyme that protects cells from
574 stresses inducing ATP depletion, would be less activated. Multiple publications explored the role
575 of AMPK in lifespan regulation (Sinnett and Brenman 2016) and it has been demonstrated that
576 downregulating AMPK by RNAi in *Drosophila* adult fat body or muscles reduces lifespan
577 (Stenesen et al. 2013), as well as its ubiquitous inactivation under starvation (Johnson et al.
578 2010).

579 In humans, HGPRT deficiency induces an accumulation of hypoxanthine and guanine,
580 resulting from the lack of recycling and an increased *de novo* purine synthesis (Harkness et al.
581 1988, Fu et al. 2015; Ceballos-Picot et al. 2015) leading to an overproduction of uric acid which
582 increases the risk for nephrolithiasis, renal failure and gouty arthritis if not properly treated. In
583 insects, the end-product of purine catabolism is not uric acid, as in humans, but allantoin.
584 However, urate oxidase, the enzyme which converts uric acid into allantoin, is expressed
585 selectively in the Malpighi tubules, an excretory organ that forms the pre-urine in flies (Wallrath
586 et al. 1990). Hence, an accumulation of uric acid could occur in the hemolymph if the purine
587 salvage pathway is impaired. Here, we have indeed observed an increase in uric acid level in
588 *Drosophila Aprt* mutant heads. Interestingly, we were able to reduce uric acid to normal levels in
589 *Aprt* mutant flies by feeding them allopurinol, a xanthine oxidase inhibitor that is used to
590 decrease uric acid levels and protect against renal failure in LND patients.

591

592 **Aprt is required in dopamine and mushroom body neurons for young fly motricity**

593 We observed that *Aprt*-null adult flies have a strikingly reduced performance in the SING
594 test, which monitors locomotor reactivity to startle and climbing ability. This phenotype was
595 already obvious at an early age, starting from 1 d a.E. The performance continued to decrease
596 until 8 d a.E. and then appeared to stabilize up to 30 d a.E. Remarkably, this phenotype is clearly

597 different from the defects usually found in α-synuclein-induced *Drosophila* Parkinson disease
598 models, in which the locomotion starts declining at around 25 d a.E. and then decreases steadily
599 with aging (Feany and Bender 2000, Riemensperger et al. 2013, Rahmani et al. 2022). Such a
600 locomotor defect affecting newly eclosed *Aprt*-deficient flies could be reminiscent of the early
601 onset of motor symptoms in LND patients, which most often appear from 3 to 6 months of age
602 (Jinnah et al. 2006). Interestingly, we were also able to induce an impairment in startle-induced
603 locomotion by knocking down *Aprt* during 3 days only in adult flies, which suggests that the
604 motricity phenotype does not necessarily require a developmental flaw. Although *Aprt* mutants
605 walked slower than wild-type flies, they were no less active and their ATP levels were not
606 different than in controls, excluding that reduced SING performance resulted from a major failure
607 in energetic metabolism.

608 Since the complete absence of *Aprt* induced a strong and early SING defect, we tried to
609 identify in which cells *Aprt* is required to ensure a normal locomotor behavior. Downregulating
610 *Aprt* in all neurons by RNAi reproduced the locomotor defect of the $Aprt^5$ mutant, and *Aprt*
611 mutant locomotion could be partially rescued by neuronal Aprt re-expression. The fact that
612 rescue was not complete was possibly due to a dominant negative effect of the $Aprt^5$ mutation on
613 Aprt activity, as suggested by enzymatic assays on extracts of heterozygous mutant flies (Figure
614 S2). We then considered that DA neurons could be involved in this motor phenotype. Previous
615 studies from our and other laboratories have indeed demonstrated the role of DA neurons in the
616 control of SING in *Drosophila* (Feany and Bender 2000; Friggi-Grelin et al. 2003;
617 Riemensperger et al. 2011; Vaccaro et al. 2017; Sun et al. 2018), and that the degeneration or loss
618 of PAM DA neurons underpin the SING defects observed in different *Drosophila* Parkinson
619 disease models (Riemensperger et al. 2013; Bou Dib et al. 2014; Tas et al. 2018; Pütz et al.
620 2021). Here, we showed that knocking down *Aprt* in all DA neurons or only in the PAM DA

621 neurons was sufficient to induce early SING defects. Moreover, inactivation of Aprt with *TH-*
622 *Gal4* that labels all DA neurons except for a large part of the PAM cluster did not affect the
623 SING, suggesting that Aprt expression in subsets of PAM neurons is critical for this locomotor
624 behavior.

625 Here we found that inactivating *Aprt* in all MB neurons also induced a lower performance
626 in the SING assay. This important brain structure receives connections from DA neurons,
627 including the PAM, and is enriched in DA receptors (Waddell 2010). We have recently shown
628 that activation of MB-afferent DA neurons decreased the SING response, an effect that requires
629 signalization by the DA receptor dDA1/Dop1R1 in MB neurons (Sun et al. 2018). We also
630 observed a SING defect after knocking down *Aprt* either in all glial cells, or more selectively in
631 glial subpopulations expressing the glutamate transporter Eaat1, but not in the ensheathing glia.
632 We reported before that some astrocyte-like glial cells that express *Eaat1* extend processes
633 forming a thick mesh-like network around and inside the entire MB neuropil (Sinakevitch et al.
634 2010). So it could be hypothesized that these MB-associated astrocytes also require Aprt
635 expression to ensure a full SING behavior. However, re-expressing *Aprt* with *Eaat1-Gal4* did not
636 lead to SING rescue in Aprt mutant background, in contrast to the positive effect observed with
637 *elav-Gal4*. This suggests that the presence of Aprt in neurons can somehow compensate for *Aprt*
638 deficiency in glia, but the reverse is not true. In conclusion, proper startle-induced locomotion in
639 young flies depends on Aprt activity in PAM DA and MB neurons, and in *EAAT1*-expressing
640 astrocytes.

641

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

643 Because sleep is regulated by the mushroom body as well as DA neurons in flies,
644 (Artiushin and Sehgal 2017), we have also monitored the spontaneous locomotor activity and

645 sleep pattern of *Aprt*^{5} mutants. Their activity profile appeared normal, with unaltered morning
646 and evening anticipation, indicating that the circadian rhythms are maintained in light-dark
647 conditions in the absence of a functional purine recycling pathway (Figure S8). This experiment
648 also highlighted that *Aprt*-deficient flies are hyperactive during the night, which suggests that
649 they sleep less than wild-type flies. This could be confirmed by measuring their sleep pattern on
650 video recordings. *Aprt* mutants show a reduced walking speed, sleep less during both day and
651 night, and have a difficulty to maintain sleep. Downregulating *Aprt* selectively in neurons
652 reproduced the sleep defect, whereas doing it in glial cells only had no effect. We have not
653 attempted here to identify further the neuronal cells involved in this phenotype. Like for the
654 SING behavior defect, it could involve the PAM DA neurons, as subpopulations of this cluster
655 have been shown to regulate sleep in *Drosophila* (Nall et al. 2016). Therefore, the lack of purine
656 recycling markedly disrupts sleep in *Aprt*-deficient flies, making them more active at night. This
657 is strikingly comparable to young LND patients who have a much disturbed sleep-time during the
658 night (Mizuno et al. 1979).
659
660 **Lack of Aprt reduces adenosine signaling leading to DA neuron overactivation**
661 Here we found that disruption of the purine salvage pathway induced by *Aprt* deficiency
662 in *Drosophila* did not decrease the level of DA in brain, although the mutant flies showed
663 reduction in induced locomotion and sleep. This prompted us to study another neuromodulator,
664 adenosine, whose level was likely to be altered in *Aprt* mutants, since this nucleoside is indirectly
665 a product of Aprt enzymatic activity. The purine nucleoside adenosine is one of the building
666 blocks of RNA and the precursor of essential molecules such as ATP and cAMP, but it is also the
667 endogenous ligand of adenosine receptors that modulate a wide range of physiological functions.
668 In the brain, adenosine regulates many motor and cognitive processes, such as the sleep-wake

669 cycle, anxiety, depression, epilepsy, memory and drug addiction (Soliman et al. 2018). In normal
670 metabolic conditions, adenosine is present at low concentrations in the extracellular space and its
671 level is highly regulated, either taken up by cells and incorporated into ATP stores or deaminated
672 by adenosine deaminase into inosine. In mammals, several nucleoside transporters mediate the
673 uptake of adenosine and other nucleosides into cells, named equilibrative and concentrative
674 nucleoside transporters (ENTs and CNTs), respectively (Gray et al. 2004; Boswell-Casteel and
675 Hays 2017; Pastor-Anglada and Pérez-Torras 2018).

676 As expected, we observed a marked reduction in adenosine levels in *Aprt* mutant flies.
677 While we did not observe any alteration in the transcript levels of *AdoR*, the gene coding for the
678 only adenosine receptor in flies, transcript levels of the known adenosine transporter, Ent2, were
679 increased more than 2-fold in *Aprt* mutants. Interestingly, one paper reported a similar strong
680 increase in *Ent2* transporter expression in *AdoR* mutant flies (Knight et al. 2010). This suggests
681 that AdoR signaling is less activated in Aprt-deficient flies compared to controls, and the
682 decrease in adenosine we observed in *Aprt* mutants also supports this hypothesis. The fact that
683 AdoR expression was not affected in these conditions suggests that a role of this receptor is to
684 inform the cells about the external adenosine concentration and accordingly regulate Ent2
685 expression level. We also observed that the lack of AdoR decreased Aprt expression and activity
686 in *Drosophila*, probably as a consequence of the increase in Ent2 expression, which would lead
687 to higher adenosine influx and then Aprt downregulation by a feedback mechanism.

688 Adenosine and dopamine receptors are known to interact closely in mammals (Franco et
689 al. 2000; Kim and Palmiter 2008), whereas the function of AdoR has not been extensively
690 studied in *Drosophila*, in particular with regard to its potential interactions with the DA system.
691 However, a previous study performed in fly larvae showed that an increase in astrocytic Ca^{2+}
692 signaling can silence DA neuron activity through AdoR stimulation on DA neurons, by a

693 mechanism potentially involving the breakdown of released astrocytic ATP into adenosine (Ma et
694 al. 2016). Conversely, the AdoR antagonist caffeine was shown to increase DA neuron signaling
695 in the PAM cluster, although it is not certain if caffeine acts through AdoR or another receptor to
696 activate these neurons (Nall et al. 2016). This suggests that a reduction in AdoR signaling, as
697 seems to be the case in Aprt-deficient flies, will lead to DA neuron overactivation. We have
698 previously shown that thermogenetic or optogenetic activation of the DA neurons strongly
699 reduces fly performance in the SING test (Sun et al. 2018). As described here, this is precisely
700 the locomotor phenotype of young Aprt-deficient flies. Furthermore, it has been shown in
701 *Drosophila* that nocturnal hyperactivity is caused by an increase in dopamine signaling (Kumar
702 et al. 2012; Lee et al. 2013). This is fully in accordance with our present observation that Aprt-
703 deficient flies sleep less and are more active during the night. Therefore, both the locomotor and
704 sleep defects induced by the lack of Aprt activity can be explained by an overactivation of DA
705 neurons that would be a direct consequence of reduced adenosinergic signaling.

706 At first view, these observations do not seem to be in favor of the idea that the Aprt
707 deficient fly could represent a useful model to study LND, since a constant feature of patients
708 with this disease is a marked reduction in DA level in the basal ganglia. However, it has been
709 previously proposed that adenosine could be a mediator of some of the neurological
710 consequences of LND pathogenesis (Visser et al., 2000). Indeed, some studies showed that
711 adenosine transport is decreased in peripheral blood lymphocytes of LND patients (Torres et al.
712 2004), as well as A_{2A} adenosine receptor mRNA and protein levels (García et al. 2009, García et
713 al. 2011). In a murine HPRT knockout model of LND, expression of the adenosine A_1 receptor
714 was found to be strongly increased and that of A_{2A} slightly decreased, while the expression of
715 A_{2B} was not affected (Bertelli et al. 2006). Chronic administration of high doses of caffeine,
716 which also acts as an antagonist of adenosine receptors in the mammalian brain, caused a self-

717 injurious behavior in rats (Miñana et al. 1984; Jinnah et al. 1990). Moreover, central injection of
718 an adenosine agonist is sufficient to prevent self-mutilation induced by dopamine agonist
719 administration in neonatally 6-OHDA lesioned rats (Criswell et al. 1988). These striking
720 observations encouraged us to study further the potential involvement of adenosine signaling in
721 the neurobehavioral effects induced by *Aprt* deficiency in *Drosophila*.
722

723 **Adenosine or N6-methyladenosine supplementation rescues the epilepsy behavior of *Aprt***
724 **mutants**

725 LND is characterized by several behavioral troubles including dystonia, spasticity and
726 choreoathetosis. This mainly corresponds to uncontrolled, sustained or repetitive muscular
727 contractions, and stiffness or involuntary twitching and writhing that can affect the posture,
728 walking ability and normal movements. Some patients can also show an epileptic disorder
729 (Jinnah et al. 2006; Madeo et al. 2019). In flies, the bang-sensitivity test is often used to model
730 epileptic seizures (Song et al. 2008; Parker et al. 2011). Here, we have found that 30-day-old *Aprt*
731 mutant flies show a typical BS phenotype after a strong mechanical shock. Previous works have
732 shown that bang sensitivity is often linked to neuronal dysfunction in flies (Parker et al. 2011;
733 Kroll and Tanouye 2013; Kroll et al. 2015; Saras and Tanouye 2016). The fact that we could not
734 reproduce the BS phenotype by knocking down *Aprt* in specific cells such as neurons, glia, or
735 muscles, indicates that Aprt has to be inactivated in several or all cell types to induce this defect.
736 This may suggest that the crucial component missing in the absence of Aprt activity is diffusible
737 and can be provided to neurons from the extracellular space or the hemolymph.

738 Interestingly, inactivating *Aprt* by RNAi in all cells during development reproduced the
739 same seizure-like behavior, whereas inactivation of *Aprt* for 3 days only in adult flies failed to
740 induce bang sensitivity, in contrast to the SING phenotype. These results indicate that the

741 absence of Aprt during development somehow alters a mechanism that controls bang sensitivity,
742 but only has a repercussion with age. The role of Aprt being to recycle adenine into AMP, it was
743 conceivable that *Drosophila Aprt* mutants lack nucleotides in the brain during development,
744 possibly inducing neuronal defects that could trigger later the BS behavior. We have tried,
745 therefore, to feed the mutants with a diet supplemented with various compounds involved in
746 purine metabolism, such as allopurinol, adenine, hypoxanthine, adenosine or its analog N6-
747 methyladenosine (m^6A), either throughout larval development or only in adult stage. Only
748 adenosine and m^6A, ingested during development, were able to prevent the BS behavior. This
749 suggests that the absence of Aprt induces a lack of adenosine in the developing nervous system,
750 as we demonstrated, which has a profound impact on the formation or function of neural circuits
751 controlling the BS behavior in adults. Adenosine is methylated at the nitrogen-6 position to form
752 m^6A in RNA molecules by a large methyltransferase complex (Batista 2017). Once released
753 during RNA turnover or degradation, m^6A cannot be incorporated back into nucleic acids and so
754 it is excreted in urine (Schram 1998). The rescuing effect we observed with m^6A on the BS
755 phenotype indicates thereby that both this compound and adenosine rather act as adenosine
756 receptor agonists or allosteric regulators, than as nucleotide precursors, to prevent the nervous
757 system disturbance induced by Aprt deficiency.

758 Adenosine has been shown to strongly inhibit brain electrical activity, and its role as an
759 endogenous anticonvulsant and seizure terminator is well-established in humans (Boison 2005;
760 Masino et al. 2014; Weltha et al. 2019). Accordingly, deficiencies in the adenosine-based
761 neuromodulatory system can contribute to epileptogenesis. For instance, increased expression of
762 astroglial adenosine kinase (ADK), which converts adenosine into AMP, leads to a reduction in
763 brain adenosine level that plays a major role in epileptogenesis (Weltha et al. 2019). Hence,
764 therapeutic adenosine increase is a rational approach for seizure control. One explanation for how

765 adenosine release is responsible for seizure arrest implies the attenuation of the $GABA_A$ receptor-
766 mediated depolarization (Ilie et al. 2012). Overall, such data could help explain why our
767 observation that feeding adenosine or its derivative m^6A rescued the seizure-like phenotype of
768 *Aprt* mutant flies, and further suggests that adenosinergic signaling has similar functions in the
769 fly and mammalian brains. In addition, we did observe that adenosine level was decreased in *Aprt*
770 mutant flies, perhaps because of an elevated ADK activity to compensate the absence of AMP
771 production by Aprt. Although the reason why adenosine administration only in adult stage could
772 not rescue the BS behavior remains to be elucidated, it is consistent with the fact that
773 downregulating Aprt for three days in aged adult flies was not sufficient to give rise to the BS
774 phenotype.

775 We and others recently identified m^6A, and other compounds sharing an adenosine
776 moiety, as able to rescue the viability of LND fibroblasts and neural stem cells derived from
777 induced pluripotent stem cells (iPSCs) of LND patients, when cultured in the presence of
778 azaserine, a potent blocker of *de novo* purine synthesis (Petitgas and Ceballos-Picot, unpublished
779 results; Ruillier et al. 2020). Like for flies again, allopurinol was not capable of rescuing the cell
780 viability in this *in vitro* model. The similarity of these results provides further evidence
781 suggesting that the *Aprt*-deficient *Drosophila* could be used as a model to study LND.

782

783 **Expression of mutant HGPRT triggers locomotor and seizure phenotypes similar to *Aprt***
784 **deficiency**

785 Why HGPRT deficit causes such dramatic neurobehavioral troubles in LND patients still
786 remains a crucial question. To date, cellular (Smith & Friedman 2000; Torres et al. 2004;
787 Ceballos-Picot et al. 2009; Cristini et al. 2010; Guibinga et al. 2012; Sutcliffe et al. 2021) and
788 rodent (Finger et al. 1988; Dunnett et al. 1989; Jinnah et al. 1993; Meek et al. 2016; Witteveen et

789 al. 2022) models only focused on the consequences of HGPRT deficiency to phenocopy the
790 disease. Such an approach was justified by the fact that the lower the residual activity of mutant
791 HGPRT, the more severe the symptoms are in patients (Fu and Jinnah 2012. Fu et al. 2014).
792 However, it would be conceivable that part of the symptoms observed in the disease result from
793 compensatory physiological mechanisms or a deleterious gain-of-function conferred to the
794 HGPRT protein by the pathogenic mutations. Here we observed that neuronal expression of
795 mutant human HGPRT-I42T, which expresses a low enzymatic activity, but not the wild-type
796 protein, induced early locomotor defects in young flies and bang-sensitivity in older flies, very
797 similarly to the defects induced by Aprt deficiency. This suggests a potential neurotoxicity of the
798 pathological mutant form of HGPRT, which could be related to disturbances in purine
799 metabolism or other signaling pathways. The human mutant form might not be properly degraded
800 and accumulate as aggregates, potentially exerting neuronal toxicity. Such an approach opens
801 interesting perspectives to better understand the endogenous function of HGPRT and its
802 pathogenic forms. Indeed, the identification of a potential inherent neurotoxicity of defective
803 forms of human HGPRT is a new element, which could be explored in further work in the fly and
804 also in rodent models.
805
806 **A new model of LND in an invertebrate organism**
807 LND, a rare X-linked metabolic disorder due to mutations of the *HPRT1* gene, has
808 dramatic neurological consequences for affected children. To date, no treatment is available to
809 abrogate these troubles, and no fully satisfactory *in vivo* models exist to progress in the
810 understanding and cure of this disease. HGPRT knockout rodents do not show comparable motor
811 and behavioral troubles, which makes these models problematic for testing new therapeutic
812 treatments. In *Drosophila*, no HGPRT homologue is expressed, indicating that Aprt is the only

813 enzyme of the purine salvage pathway in this organism. Here we find that *Aprt* mutant flies show
814 symptoms partly comparable to the lack of HGPRT in humans, including increase in uric acid
815 levels, reduced longevity, and various neurobehavioral defects such as early locomotor decline,
816 sleep disorders and epilepsy behavior. This animal model therefore recapitulates both salvage
817 pathway disruption and motor symptoms, as observed in LND patients. Moreover, these results
818 highlighted that *Aprt* deficiency in *Drosophila* has more similarities with HGPRT than APRT
819 deficiency in humans. *Aprt* mutant flies also show a disruption of adenosine signaling and we
820 found that adenosine itself or a derivative compound can relieve the epileptic symptoms.
821 Moreover, we observed that neuronal expression of a mutant form of human HGPRT that causes
822 LND also triggers abnormalities in fly locomotion and seizure-like behavior, which has not been
823 documented to date in other models. The use of *Drosophila* to study LND should therefore prove
824 valuable to better understand the link between purine recycling deficiency and brain functioning
825 and carry out drug screening in a living organism, paving the way towards new improvements in
826 the cure of this dramatic disease.
827

828 # Materials and Methods

829 ***Drosophila* culture and strains**.

830 Flies were raised at 25°C on standard cornmeal-yeast-agar medium supplemented with
831 methylhydroxybenzoate as an antifungal under a 12h:12h light-dark (LD) cycle. The *Drosophila*
832 mutant lines were obtained either from the Bloomington *Drosophila* Stock Center (BDSC), the
833 Vienna *Drosophila* Resource Center (VDRC) or our own collection: $Aprt^{5}$ (Johnson and
834 Friedman, 1983) (BDSC #6882), *Df(3L)ED4284* (BDSC #8056), *Df(3L)BSC365* (BDSC
835 #24389), $UAS\text{-}Aprt^{RNAi}$ (VDRC #106836), $AdoR^{KG03964ex}$ (Wu et al. 2009) here named $AdoR^{KGex}$

836 (BDSC #30868), and the following Gal4 drivers: *238Y-Gal4*, *24B-Gal4*, *da-Gal4*, *EAAT1-Gal4*
837 (Rival et al. 2004), *elav-Gal4* (*elav*^{C155}), *MZ0709-Gal4*, *NP6510-Gal4*, *NP6520-Gal4*, *R58E02-*
838 *Gal4* (Liu et al. 2012), *R76F05-Gal4*, *repo-Gal4*, *TH-Gal4* (Friggi-Grelin et al. 2003), *TRH-Gal4*
839 (Cassar et al. 2015), *tub-Gal80*^{ts} and *VT30559-Gal4*. The Canton-S line was used as wild-type
840 control. The simplified driver>effector convention was used to indicate genotypes in figures, e.g.
841 *elav>Aprt*^{RNAi} for *elav-Gal4*; *UAS-Aprt*^{RNAi}. In some experiments, to restrict RNAi-mediated *Aprt*
842 inactivation at the adult stage, we have used the TARGET system (McGuire et al. 2003). Flies
843 were raised at 18°C (permissive temperature) where Gal4 transcriptional activity was inhibited by
844 *tub-Gal80*^{ts}, and shifted to 30°C (restrictive temperature) for 3 days before the test to enable
845 Gal4-driven *Aprt*^{RNAi} expression.
846
847 ***APRT and HPRT* transgenic lines**
848 To generate a *UAS-Aprt* strain, a 549-bp *Aprt* insert containing the coding sequence was
849 PCR amplified from the ORF clone BS15201 (*Drosophila* Genomics Resource Center,
850 Bloomington, IN, USA) using primers with added restriction sites (Table S3). After digestion with
851 EcoRI and XbaI, the *Aprt* cDNA was subcloned into the *pUASTattB* vector (Bischof et al. 2007)
852 using the In-Fusion HD Cloning Kit (Takara Bio, Kyoto, Japan) according to the manufacturer's
853 instructions, and the insertion verified by sequencing (Eurofins Genomics, Ebersberg, Germany).
854 The construction was sent to BestGene (Chino Hills, CA, USA) for *Drosophila* germline
855 transformation into the attP14 docking site on the 2d chromosome. The *UAS-Aprt* line yielding the
856 strongest expression was selected and used in the experiments.
857 A clone containing the human wild-type *HPRT1* cDNA was kindly provided to us by Prof.
858 Hyder A. Jinnah (Emory University, GA, USA). We constructed the *HPRT1-I42T* cDNA from this
859 clone by site-directed mutagenesis using the Quick Change II Site-Directed Mutagenesis Kit

860 (Agilent Technologies, Santa Clara, CA, USA). Primers sequences used to create the mutation
861 are shown in Table S3. The cDNA obtained was verified by sequencing. Then, the 657-bp
862 *HPRT1-WT* and *HPRT1-I42T* inserts were PCR amplified using primers with added restriction sites
863 and *Drosophila* translation start consensus sequence (Table S3). They were subcloned into
864 *pUASTattB* and verified by sequencing. The transgenes were sent to BestGene for *Drosophila*
865 transformation and inserted into the attP40 docking site on the 2d chromosome.
866

867 **Sequencing of *Aprt*^{5}**

868 For sequencing of the *Aprt*^{5} cDNA, total RNA was isolated by standard procedure from 20-30
869 heads of homozygous *Aprt*^{5} flies and reverse transcribed using oligo d(T) primers (PrimeScript RT
870 Reagent Kit, Takara Bio, Kyoto, Japan). At least 750 ng of the first strand cDNA was amplified by
871 PCR in 20 µl of reaction mixture using PrimeStar Max DNA polymerase (Takara Bio, Kyoto
872 Japan) with a Techne Prime Thermal Cycler apparatus (Bibby Scientific, Burlington, NJ, USA).
873 The program cycles included 10 s denaturation at 98°C, 10 s annealing at 55°C and 10 s elongation
874 at 72°C, repeated 35 times. 1µL of the PCR product was amplified again with the same program, in
875 30 µl of reaction mixture. After elution on 1% agarose gel, DNA were purified using the
876 Wizard® SV Gel and PCR Clean-Up System protocol (Promega, Madison, Wisconsin, USA)
877 according to the manufacturer's instructions. Finally, 7.5 µL of purified DNA were sent with 2.5
878 µL of primers (sense and antisense in separate tubes) for sequencing (Eurofins Genomics,
879 Ebersberg, Germany).

880 **Lifespan assay**

881 Longevity study was performed as previously described (Riemensperger et al. 2011).
882 *Drosophila* adult males were collected within 24h of emergence and maintained on standard

883 medium at 25°C under a 12:12h LD cycle. They were transferred into fresh bottles every 2-3
884 days and the number or surviving flies was scored. 50 flies per bottle in triplicate were tested for
885 each genotype and the experiment was done 3 times.
886

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

888 SING assays were performed as previously described (Rival et al. 2004; Riemensperger et
889 al. 2011; Sun et al. 2018). For each genotype, 50 adult males divided into 5 groups of 10 flies
890 were placed in a vertical column (25-cm long, 1.5-cm diameter) with a conic bottom end and left
891 for about 30 min for habituation. Then, columns were tested individually by gently tapping them
892 down (startle), to which flies normally responded by climbing up. After 1 min, flies having
893 reached at least once the top of the column (above 22 cm) and flies that never left the bottom
894 (below 4 cm) were counted. Each fly group was assayed three times at 15 min intervals. The
895 performance index (PI) for each column is defined as $\frac{1}{2}[1 + (n_{top}-n_{bot})/n_{tot}]$, where n_{tot} is the total
896 number of flies in the column, n_{top} the number of flies at the top and n_{bot} the number of flies at the
897 bottom after 1 min. SING was performed at 1, 8, 10 and 30 days after adult eclosion (d a.E.).
898

899 **Spontaneous locomotion and sleep monitoring**

900 Spontaneous locomotor activity was recorded as previously described (Vaccaro et al.
901 2017), using *Drosophila* activity infra-red beam monitors (DAM, TriKinetics Inc, Waltham, MA
902 USA) placed in incubators at 25°C equipped with standard white light. 8-day-old male flies were
903 maintained individually for 5-6 days under 12:12h LD cycle in 5 x 65 mm glass tubes containing
904 5% sucrose, 1.5% agar medium. Data analysis was performed with the FaasX software (Klarsfeld

905 et al. 2003). Histograms represent the distribution of the activity through 24h in 30-min bins,
906 averaged for 32 flies per genotype over 4-5 cycles.

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

918

919 **Immunohistochemistry**

920 Brains of 8 to 10-day-old adult flies were dissected in ice-cold Ca^{2+}-free *Drosophila*
921 Ringer's solution and processed for whole-mount anti-TH or anti-DA immunostaining as
922 previously described (Riemensperger et al. 2011, Cichewicz et al. 2017). The primary antibodies
923 used were mouse monoclonal anti-TH (1:1000, cat. n° 22941, ImmunoStar, Hudson, WI, USA)
924 and mouse monoclonal anti-DA (1:100, cat. n° AM001, GemacBio, Saint-Jean-d'Illac, France).
925 The secondary antibody was goat anti-mouse conjugated to Alexa Fluor 488 (1:1000,
926 ThermoFisher Scientific, Waltham, MA, USA). Brains were mounted with antifade reagent,
927 either Prolong Gold (ThermoFisher Scientific, Waltham, MA, USA) or, alternatively, 65% 2,2'-

928 thiodiethanol (Sigma-Aldrich, St. Louis, MI, USA) (Cichewicz et al. 2017) for DA staining.
929 Images were acquired on a Nikon A1R confocal microscope with identical laser, filter and gain
930 settings for all samples in each experiment. Immunofluorescence intensity levels were quantified
931 using the Fiji software. Experiments were repeated independently at least 3 times on 4 to 6 brains
932 per genotype.
933

934 **Bang-sensitivity test**

935 Bang-sensitivity assays were performed as previously described (Howlett et al. 2013). 30-
936 day-old males were divided into 5 groups of 10 flies under CO_2 and allowed to recover overnight.
937 The following day, each group was placed in a vial without food and after 20 min of habituation,
938 the vials were stimulated vigorously with a vortex mixer for 10 s at 2,500 rpm. The recovery time
939 was measured for each fly, from the end of the stimulation until they reached a normal standing
940 position. Results are the mean of the recovery time for at least 50 flies per genotype.
941

942 **Drug administration**

943 Allopurinol (A8003, Sigma Aldrich, St. Louis, MI, USA) was diluted in standard medium
944 at 100 μg/ml and flies were placed for 5 days on this medium before metabolite extraction.
945 Adenine, adenosine and hypoxanthine (A2786, A9251 and H9377, Sigma Aldrich, St. Louis, MI,
946 USA) or N^6-methyladenosine (m^6A) (QB-1055, Combi-Blocks, San Diego, CA, USA) were
947 diluted in fly food medium at 500 μM. Parents were allowed to lay eggs on this medium in order
948 to have exposition to the drug throughout all larval development of the progeny. Adults were
949 collected and placed in normal medium until 5 days before the test, when they were placed again
950 in food supplemented with adenosine or m^6A at the same concentrations.

951

952 **Uric acid quantification**

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

965

966 **ATP assay**

967 ATP level was measured by bioluminescence using the ATP Determination Kit (A22066,
968 ThermoFisher Scientific, Waltham, MA, USA) on a TriStar 2 Spectrum LB942S microplate
969 reader (Berthold Technologies, Bad Wildbad, Germany), according to the manufacturer's
970 instructions. Samples were prepared as described previously (Fergestad et al. 2006). Briefly, 30
971 heads or 5 thoraces from 8-day-old flies were homogenized in 200 µL of 6 M guanidine-HCl to
972 inhibit ATPases, boiled directly 5 min at 95°C and then centrifuged for 5 min at 13,000 g and
973 4°C. Total protein content of each supernatant was measured with the BCA Protein Assay Kit.

974 Each supernatant was then diluted at 1:500 in TE buffer pH 8 and 10 µl were placed in a 96-well
975 white-bottom plate. The luminescent reaction solution (containing D-luciferine, recombinant
976 firefly luciferase, dithiothreitol and reaction buffer) was added to each well with an injector and
977 high-gain 1 sec exposure glow reads were obtained at 15 min after reaction initiation. Results
978 were compared to a standard curve generated with known ATP concentrations and final values
979 were normalized to the protein content.
980

981 **Enzyme activity assay**

982 HGPRT and APRT activities were assessed in *Drosophila* by adapting methods
983 previously established for human cells (Cartier and Hamet 1968, Ceballos-Picot et al. 2009). 20
984 whole male flies were homogenized in 250 µl of 110 mM Tris, 10 mM $MgCl_2$ pH 7.4 (Tris-
985 $MgCl_2$ buffer), immediately frozen and kept at least one night at -80°C before the assay. After 5
986 min of centrifugation at 13,000 g, total protein content of each supernatant was measured with the
987 BCA Protein Assay Kit to normalize activity level. Kinetics of [^{14}C]-hypoxanthine conversion to
988 IMP and [^{14}C]-adenine conversion to AMP were assessed for HGPRT and Aprt assays,
989 respectively. Compositions of reaction mediums were: 25 µl of a radioactive solution made with
990 38 µl of [^{14}C]-hypoxanthine (25 µCi/ml) diluted in 1 ml of 1.2 mM cold hypoxanthine, for
991 HGPRT assay, or 25 µl of a radioactive solution made with 75 µl of [^{14}C]-adenine (50 µCi/ml)
992 diluted in 1 ml of 1.2 mM cold adenine for Aprt assay, and a volume of fly extract equivalent to
993 200 µg protein diluted in Tris-$MgCl_2$ buffer to 150 µl. Reactions were monitored at 37°C and
994 started by adding 25 µl of the co-factor 5-phosphoribosyl-1-pyrophosphate (PRPP) at 10 mM.
995 After 6, 12, 24 and 36 min, 40 µL of each pool was placed in a tube containing either 25 µl of
996 HIE (3 mM hypoxanthine, 6 mM IMP, 200 mM EDTA) or AAE (3 mM adenine, 6 mM AMP,

997 200 mM EDTA) solutions and incubated for 3 min at 95°C to stop the reaction. The different
998 radioactive compounds were separated by paper chromatography on Whatman 3MM strips using
999 28% NH_4OH, 50 mM EDTA as solvent for about 1h30. Then, the substrates and products were
1000 visualized under a UV lamp at 254 nm and placed separately in vials in 2 ml of Scintran (VWR,
1001 Radnor, PA, USA). The radioactivity in disintegrations per minute (dpm) was measured in a
1002 Packard Tri-Carb 1600 TR liquid scintillation analyzer (PerkinElmer, Waltham, MA, USA). The
1003 percentage of substrate transformation as a function of time was converted in nmol/min/mg
1004 protein and finally normalized to wild-type control values.
1005

1006 **Reverse transcription-PCR**

1007 Total RNA was isolated by standard procedure from 20-30 heads of 8-day-old males
1008 collected on ice and lysed in 600 µl QIAzol Reagent (Qiagen, Venlo, Netherlands). 1 µg of total
1009 RNA was treated by DNase (DNase I, RNase-free, ThermoFisher Scientific, Waltham, MA,
1010 USA) according to the manufacturer's instructions. 5 µL of treated RNA was reverse transcribed
1011 using oligo d(T) primers (PrimeScript RT Reagent Kit – Takara Bio, Kyoto Japan). Then, at least
1012 750 ng of the first strand cDNA was amplified in 20 µl of reaction mixture using PrimeStar Max
1013 DNA polymerase (Takara Bio, Kyoto Japan) with a Techne Prime Thermal Cycler apparatus
1014 (Bibby Scientific, Burlington, NJ, USA). The program cycles included 10 s denaturation at 98°C,
1015 10 s annealing at 55°C and 10 s elongation at 72°C, repeated 30 times. PCR product levels were
1016 measured after electrophoresis by densitometry analysis with the Fiji software. Data were
1017 normalized to amplification level of the ribosomal protein *rp49/RpL32* transcripts as internal
1018 control. Sequences of the primers used were: sense *HPRT*, 5'-
1019 GAGATACAAAATGGCGACCCGCAGCCCT; antisense *HPRT*, 5'-

1020 GCTCGGATCCTTATCATTACTAGGCTTTG (amplicon 686bp); sense *rp49*, 5'-
1021 GACGCTTCAAGGGACAGTATC; antisense *rp49*, 5'- AAACGCGGTTCTGCATGAG
1022 (amplicon 144bp).
1023

1024 **Quantitative reverse transcription PCR**

1025 For RT-qPCR, total RNA was isolated by standard procedure from 20-30 heads of 8-day-
1026 old males collected on ice and lysed in 600 µl QIAzol Lysis Reagent (Qiagen, Venlo,
1027 Netherlands). 1 µg total RNA was reverse transcribed using oligo d(T) primers (PrimeScript RT
1028 Reagent Kit, Takara Bio, Kyoto, Japan). Approximately 40 ng of the first strand cDNA was
1029 amplified in 10 µl of reaction mixture using the LightCycler 480 SYBR Green I Master reaction
1030 mix (Roche Applied Science, Mannheim, Germany) and a LightCycler 480 Instrument (Roche
1031 Applied Science, Mannheim, Germany). The program cycles included a 10-min preincubation
1032 step at 95°C, 40 cycles of amplification (10 s denaturation at 95°C, 10 s annealing at 55°C, 20 s
1033 elongation at 72°C), followed by a melting curves analysis for PCR product identification. Data
1034 were normalized to amplification level of the ribosomal protein *rp49*/*RpL32* transcripts as
1035 internal control. The genes analyzed and primer sequences used for qPCR are indicated in Table
1036 S4.
1037

1038 **Adenosine assay**

1039 Adenosine was determined by Ultra Performance Liquid Chromatography (UPLC). 5
1040 whole flies or 30 heads were homogenized in 120 µl 0.9% (w/v) NaCl using a Minilys apparatus
1041 (Bertin Instruments, Montigny-le-Bretonneux, France) and frozen at -80°C. After unfreezing and
1042 5 min of microcentrifugation, 20 µl of 10% perchloric acid were added to 70 µl of the

1043 supernatant and the mixture was left for 5 min on ice. After a new centrifugation, 20 µl of a
1044 neutralization solution (made by mixing 3 volumes of 3 M K_2CO_3 with 1 volume of 6.4 mM
1045 NaOH containing 0.4 mg/ml bromothymol blue) were added and the mixture was centrifuged
1046 again before injection (5 µl). Samples were analyzed with a UV diode-array detector on an
1047 Acquity UPLC HSS T3 column (1,8 µm, 2.1 x 150mm) (Waters Corporation, Milford, MA,
1048 USA). The mobile phases consisted in Buffer A (30 mM ammonium acetate, pH 4.0 with
1049 1:10,000 heptafluorobutyric acid (HPFA)) and Buffer B (acetonitrile with 1:10,000 HPFA), using
1050 a flow rate of 0.3 ml/min. Chromatographic conditions were: 3.5 min 100% Buffer A, 16.5 min
1051 up to 6.3% Buffer B, 2 min up to 100% Buffer B, and 1 min 100% Buffer B. The gradient was
1052 then returned over 5 min to 100% Buffer A, restoring the initial conditions. Results were
1053 normalized to protein levels for each sample.

1054

1055 **Statistics**

1056 Statistical significance was determined with the Prism 6 software (GraphPad Software, La
1057 Jolla, CA, USA). Survival curves for longevity experiments were analyzed using the log-rank
1058 test. The Student's *t*-test was used to compare two genotypes or conditions, and one-way or two-
1059 way ANOVA with Tukey's or Sidak's *post-hoc* multiple comparison tests for three or more
1060 conditions. Results are presented as mean ± SEM. Probability values in all figures: *$p < 0.05$, **p
1061 < 0.01, ***$p < 0.001$.

1062

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1069

1070 ## Authors contributions

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1072 M.S., J.D. and S.M.; formal analysis, C.P., L.S., A.D., R.F., M.S., J.D. and S.M.; methodology,
1073 C.P., L.S., A.M., B.C.Z and S.M.; validation, C.P., I.C.P and S.B.; writing, original draft, C.P.,
1074 I.C.P. and S.B.; writing, review & editing, C.P., I.C.P and S.B.; funding acquisition and
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