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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.

eLife assessment

The manuscript looks at how dysregulated purine metabolism impacts the behavior, neural circuits and survival of the fruit fly with the possibility of generating a model for Lesch-Nyhan Disease. The study is **valuable** but the strength of evidence is currently **incomplete** regarding the use of this model for LND.

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

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

To date, the causes of the neurobehavioral troubles in LND are still not elucidated and it remains challenging to understand and cure this dramatic disease (Jinnah et al. 2010, Bell et al. 2016). The most favored hypothesis is a dysfunction of the basal ganglia, and particularly of its dopaminergic pathways (Baumeister and Frye 1985; Visser 2000; Nyhan 2000; Saito and Takashima, 2000; Egami et al. 2007). Indeed, analyses of LND patient brains revealed a

marked loss of dopamine (DA) levels (Lloyd et al. 1981; Ernst et al. 1996) and DA transporters (Wong et al. 1996) specifically in the basal ganglia, but the mechanisms linking HGPRT activity and the DA system are still under investigation. DA deficits have been reported in HGPRT knockout rodents, but these animal models do not exhibit the motor or neurobehavioral defects of human patients (Finger et al. 1988; Dunnett et al. 1989; Jinnah et al. 1993; Jinnah et al. 1994; Meek et al. 2016). Recent studies reported that HGPRT deficiency disrupts the proliferation and migration of developing midbrain dopamine (mDA) neurons in mouse embryos, arguing for a neurodevelopmental syndrome (Witteveen et al. 2022). This could result from ATP depletion and impaired energy metabolism (Bell et al., 2021) or an overactivation of *de novo* purine synthesis leading to the accumulation of potentially toxic intermediates of this pathway (López, 2008; López et al., 2020). The reason why dopaminergic neurons would be primarily affected in this disease is not currently known. Pharmacological models have also been developed by injecting the dopaminergic neurotoxin 6-hydroxydopamine into neonatally rat brains, which induced a self-mutilation behavior in response to DA agonist administration in adulthood. However, these models are of limited utility as they do not reproduce the basic genetic impairment of LND (Breese et al. 1990; Knapp et al. 2016; Bell et al. 2016).

New animal models are therefore critically needed to study LND pathogenesis and find efficient therapeutic molecules. The fruit fly *Drosophila melanogaster* presents many advantages for translational studies and drug discovery (Fernández-Hernández et al. 2016, Perrimon et al. 2016; Papanikolopoulou et al. 2019). It has been widely used to model a variety of human diseases, including neurodegenerative disorders such as Alzheimer, Huntington or Parkinson diseases (Jaiswal et al. 2012; McGurk et al 2015; Dung and Thao 2018; Nagoshi 2018), as well as heritable neurodevelopmental disorders such as Angelman, Rett or Fragile X syndromes (Gatto and Broadie 2011). Although the importance of this invertebrate model for studying rare human genetic diseases is now recognized (Oriel and Lasko 2018), a *Drosophila* LND model has 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 only recycling enzyme of the purine salvage pathway in this organism (Figure S1A and B, *step 12*). The $Aprt^5$ mutant was originally generated by chemical mutagenesis followed by selection of flies resistant to purine toxicity (Johnson and Friedman, 1981; Johnson and Friedman, 1983). Enzymatic assays confirmed a strong reduction in Aprt activity in extracts of heterozygous $Aprt^5$/+ mutants and its absence in homozygous and hemizygous ($Aprt^5$/*Df(3L)ED4284*) mutants (Figure S2 and Table S1). Sequencing of the $Aprt^5$ cDNA (Figure S3A) indicated that the $Aprt^5$ mRNA codes for a protein with several amino acid changes compared to *Drosophila melanogaster* wild-type Aprt, modifying in particular 3 amino acid residues that have been conserved in the Aprt sequences from *Drosophila* to humans (Figure S3B). These mutations are likely to be responsible for the loss of enzymatic activity. The homozygous $Aprt^5$ mutants are considered viable because they develop and reproduce normally. However, we observed that these mutants have a significantly reduced longevity, their median lifespan being only 38 days against 50 days for wild-type flies ($p < 0.001$) (**Figure 1A**).

Uric acid levels are increased in $Aprt^5$ mutants and normalized by allopurinol

In humans, one of the consequences of HGPRT deficiency on metabolism is the overproduction of uric acid (Harkness et al. 1988; Fu et al. 2015). We assayed the levels of purine metabolites by HPLC and found that the level of uric acid was substantially increased by 37.7% on average in *Drosophila* $Aprt^5$ mutant heads ($p < 0.01$) (**Figure 1B**). We then tried to normalize uric acid content in flies by providing allopurinol in the diet, as is usually done for LND patients. Allopurinol is a hypoxanthine analog and a competitive inhibitor of xanthine oxidase, an enzyme that catalyzes the oxidation of xanthine into uric acid (Figure S1A and B, *step 19*). Remarkably, the administration of 100 µg/ml allopurinol during 5 days decreased uric acid levels to a normal concentration range in $Aprt^5$ mutant heads (**Figure 1B**). Therefore, quite similarly to HGPRT deficit in humans, the lack of Aprt activity in flies increases uric acid levels and this metabolic disturbance can be prevented by allopurinol.

Aprt deficiency decreases motricity in young flies

LND patients present dramatic motor disorders that prevent them for walking at an early age. To examine whether a deficiency in the purine salvage pathway can induce motor disturbance in young flies, we monitored the performance of *Aprt*-null mutants in startle-induced negative geotaxis (SING), a widely used paradigm to assess climbing performance and locomotor reactivity (Feany and Bender, 2000; Friggi-Grelin et al. 2003; Riemensperger et al. 2013; Sun et al. 2018). Strikingly, $Aprt^5$ mutant flies showed a very early SING defect starting from 1 day after eclosion (d a.E.) (performance index (PI) = 0.73 vs. 0.98 for wild-type flies, $p < 0.001$) that was more pronounced at 8 d a.E. (PI = 0.51 vs. 0.96 for the wild-type flies, $p < 0.001$). The fly locomotor performance did not further decline afterwards until 30 d a.E. (**Figure 1C**). *Df(3L)ED4284* and *Df(3L)BSC365* are two partially overlapping small genomic deficiencies that covers *Aprt* and several neighbor genes. Hemizygous $Aprt^5$/*Df(3L)ED4284* or $Aprt^5$/*Df(3L)BSC365* flies also displayed SING defects at 10 d a.E. (PI = 0.71 and 0.68, respectively, compared to 0.97 for wild-type flies, $p < 0.01$) (Figure S4). In contrast to its beneficial effect on uric acid levels, we observed that allopurinol treatment did not improve the locomotor ability of $Aprt^5$ mutant flies, either administered 5 days before the test or throughout the development (Figure S5A, B). This is comparable to the lack of effect of this drug against neurological defects in LND patients.

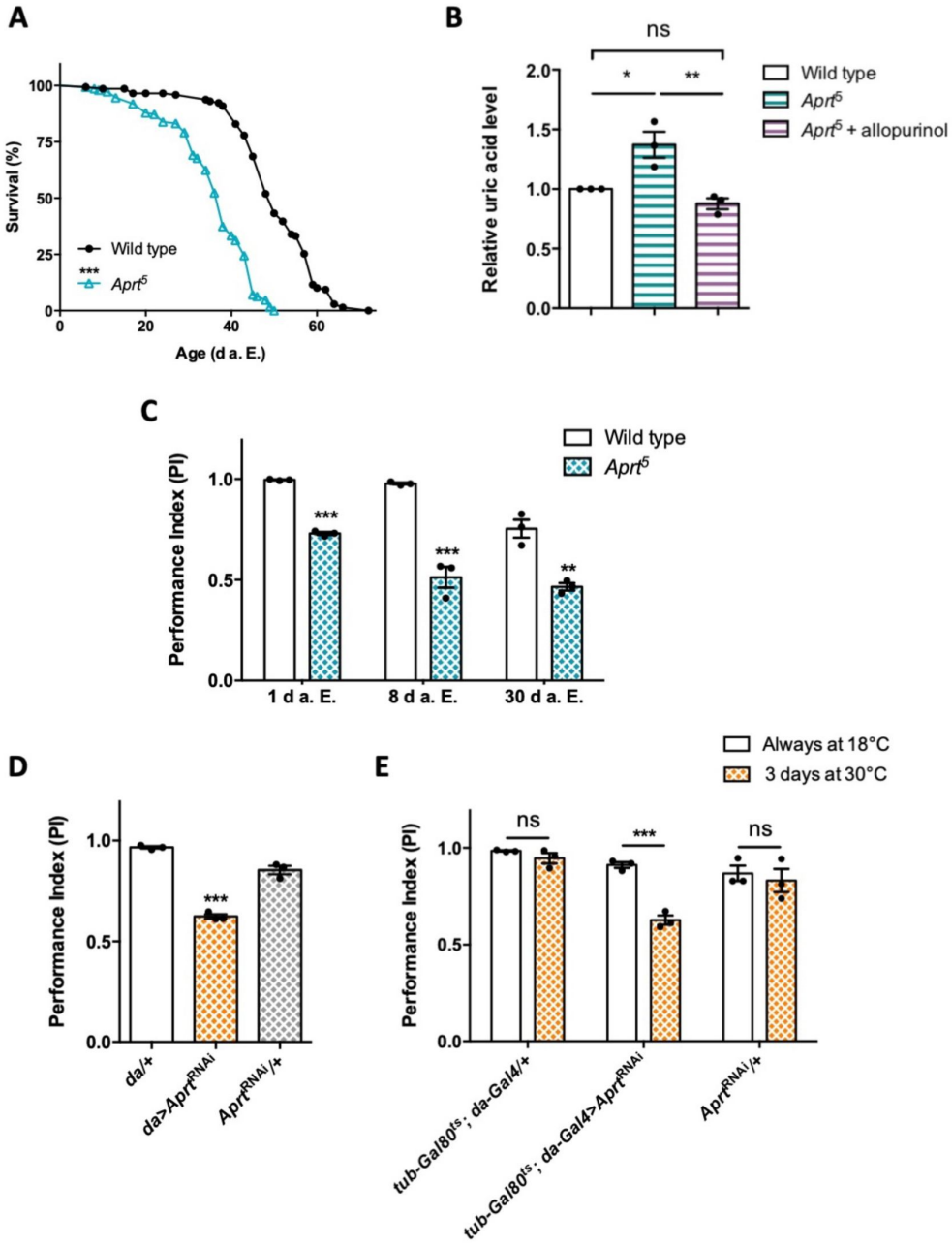

Figure 1.

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

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

Cell specificity of Aprt requirement for startle-induced locomotion

We then tried to identify the neural cells in which Aprt is required to ensure a normal locomotor reactivity in young flies by expressing *Aprt*^{RNAi} with selective Gal4 drivers. Expression in all neurons with *elav-Gal4* led to decreased locomotor performance in the SING test (PI = 0.68 at 10 d a.E., vs. 0.90 and 0.86 for the driver and RNAi controls, respectively, $p < 0.05$) (**Figure 2A**), which was comparable to the effects observed with the *Aprt*^{5} mutant or after ubiquitous expression of the RNAi. To confirm the role of neuronal Aprt in locomotor control, we generated a *UAS-Aprt* line which allowed for a substantial increase in Aprt expression and activity (Figure S6). We then found that re-expressing *Drosophila* Aprt selectively in neurons in *Aprt*^{5} background partially but significantly rescued the SING phenotype of the null mutant (PI = 0.70 vs. 0.52 for *driver* and *UAS-Aprt* controls in *Aprt*^{5} background, $p < 0.05$) (**Figure 2B**).

Furthermore, *Aprt* knockdown in all glial cells with *repo-Gal4*, or in sub-populations of glial cells that express the glutamate transporter Eaat1 with *Eaat1-Gal4*, which includes astrocyte-like glia, cortex glia and some subperineurial glia (Rival et al. 2004; Mazaud et al. 2019), also led to a lower SING performance of 10-day-old flies (PI = 0.72 for *repo-Gal4* vs. 0.91 for both controls, $p < 0.05$, and PI = 0.56 for *Eaat1-Gal4* vs. 0.77, $p < 0.05$, and 0.88, $p < 0.01$, for the driver and RNAi controls, respectively (**Figure 2C, D**). In contrast, *MZ0709-Gal4* (Doherty et al. 2009) and *NP6520-Gal4* (Awasaki et al. 2008) that selectively target the ensheathing glia, did not induce any significant locomotor defects when used to express the *Aprt* RNAi (Figure S7). Noticeably, re-expressing Aprt with *Eaat1-Gal4* in the *Aprt*^{5} background did not rescue the SING phenotype (**Figure 2E**), in contrast to the positive effect of neuronal re-expression (**Figure 2B**). Overall this suggests that Aprt is required both in neurons and glia subsets to ensure a normal SING performance in young flies, and that neuronal but not glial *Aprt* re-expression is sufficient to ensure a partially functional locomotor behavior.

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

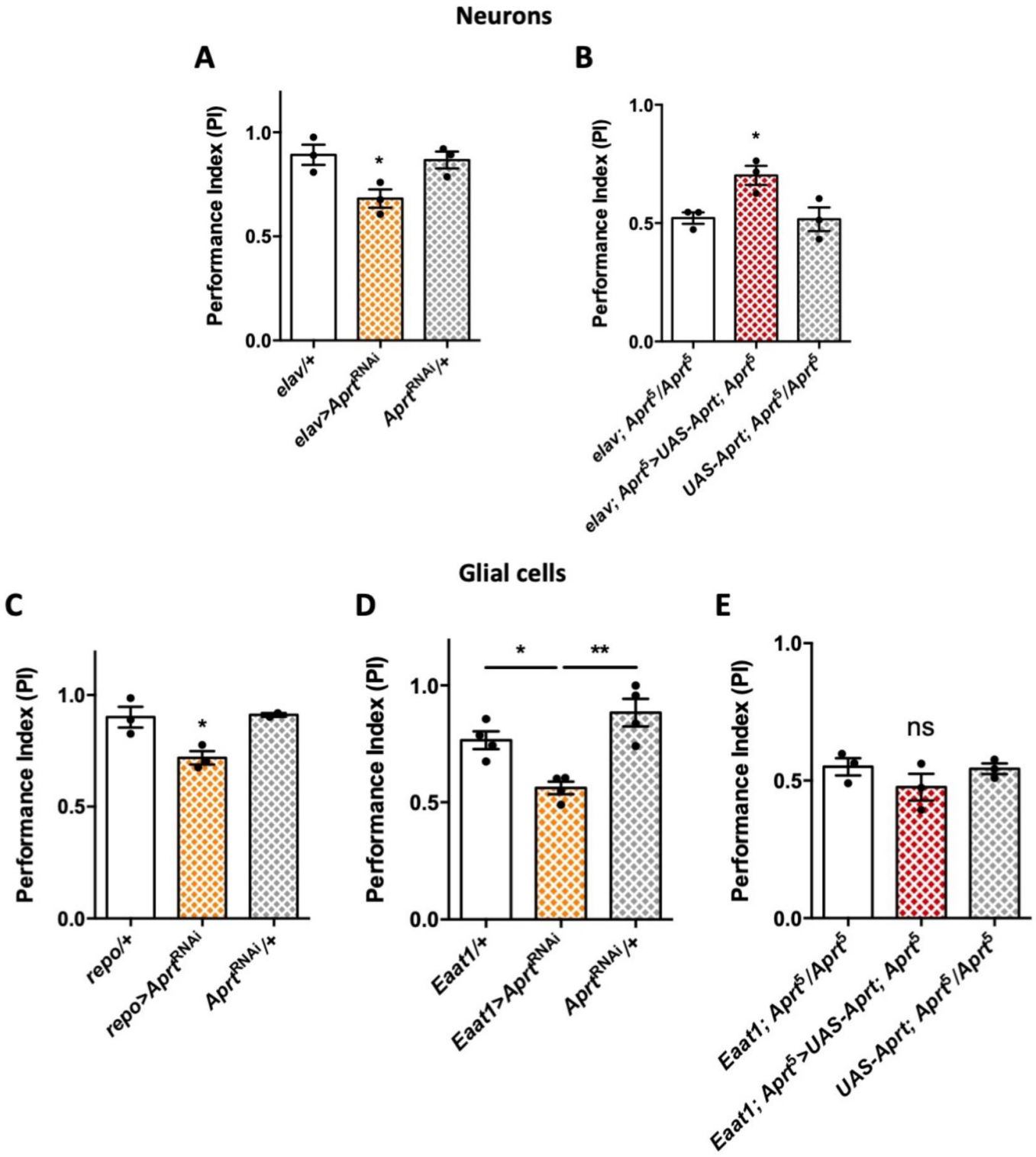

Figure 2.

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

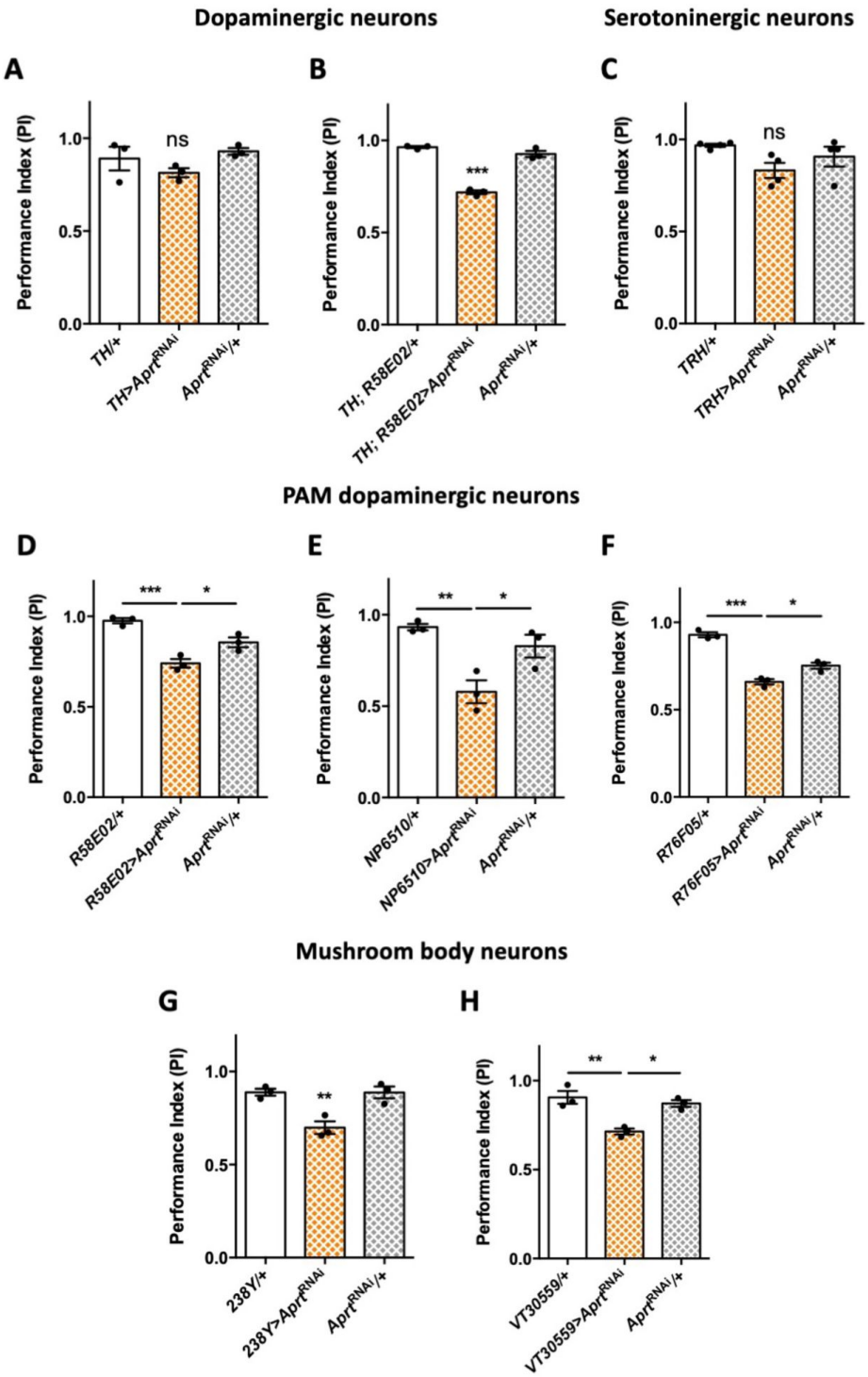

Figure 3.

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

These results suggest that some DA neurons in the PAM cluster are specifically involved in the locomotor impairments induced by *Aprt* deficiency. It has been previously shown in our laboratory that inhibiting DA synthesis in a subset of 15 PAM DA neurons cluster was able to alter markedly SING performance in aging flies (Riemensperger et al. 2013). We and others also reported that the degeneration or loss of PAM DA neurons is involved in the SING defects observed in several *Drosophila* models of Parkinson disease (Riemensperger et al. 2013; Bou Dib et al. 2014; Tas et al. 2018; Pütz et al. 2021). Here we found that expressing $Aprt^{RNAi}$ in the PAM cluster with *R58E02-Gal4* reproduced the same motor disturbance as pan-neuronal expression (PI = 0.74 vs. 0.96, $p < 0.001$, and 0.85, $p < 0.05$, for the driver and RNAi controls, respectively) (**Figure 3D**), and this result was confirmed by using two other PAM drivers (*NP6510-Gal4* – 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 al. 2018), we also inactivated *Aprt* in MB neurons with *238Y-Gal4* that strongly expresses in all the MB cells (Aso et al. 2009). Interestingly, this driver did induce a locomotor reactivity impairment (PI = 0.70 vs. 0.89 for both controls, $p < 0.01$) (**Figure 3G**), and the same result was observed with *VT30559-Gal4*, which is a very specific driver for all the MB intrinsic neurons (Plaçais et al. 2017) (**Figure 3H**). Overall, these results show that normal expression of the SING behavior depends on *Aprt* expression in the PAM and MB neurons in *Drosophila*.

Sleep disturbances induced by Aprt deficiency

Both the mushroom body and subpopulations of PAM DA neurons are known to be regulators of sleep in *Drosophila* (Nall et al. 2016; Artiushin and Sehgal 2017). The fact that *Aprt* deficiency in some of these cells impaired locomotor regulation prompted us to monitor the spontaneous locomotion and the sleep pattern of *Aprt* mutants. Compared to controls, *Aprt*-deficient flies did not have an altered circadian activity profile (Figure S8), nor any difference in total spontaneous locomotor activity during the day, as quantified by the number of infra-red beam cuts (*events*) per 30 min, but they showed a 26.2% increase in total activity during the night ($p < 0.001$) (**Figure 4A**). As usual, a sleep bout was defined as 5 min or more of fly immobility (Huber et al. 2004). We found that $Aprt^{5}$ mutants slept significantly less than wild-type flies and that it was the case both during day and night (**Figure 4B, C**). These mutants indeed showed a reduced walking speed (**Figure 4D**) and a smaller average sleep bout duration (**Figure 4E**), indicating a difficulty to maintain sleep. The reduced speed does not seem to be caused by a decreased energetic metabolism, since we could not detect different ATP levels in head and thorax of $Aprt^{5}$ mutants compared to wild-type flies (Figure S9).

Interestingly, RNAi-mediated downregulation of Aprt selectively in neurons (*elav*>$Aprt^{RNAi}$ flies) also significantly decreased sleep duration during day and night (**Figure 4F**), whereas glial-only expression (*repo*>$Aprt^{RNAi}$) had no effect (Figure S10A). Expressing the RNAi in both neurons and glia (*elav; repo*>$Aprt^{RNAi}$) had similar effects as in neurons alone (Figure S10B). Quantification of total sleep in these experiments, and the total amount of day and night sleep, are shown in **Figure 4G** and Figure S10C, D, respectively. Overall, these results suggest that *Aprt* expression is selectively needed in neurons for a normal sleep pattern in *Drosophila*.

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

Since we found that induced locomotion and sleep, two behaviors controlled by DA neurons in *Drosophila*, were altered in *Aprt*-deficient flies, we logically expected brain DA levels to be reduced in these mutants, as is the case in the basal ganglia of LND patients. To check for that, we performed comparative immunostaining for tyrosine hydroxylase (TH), the specific enzyme of DA

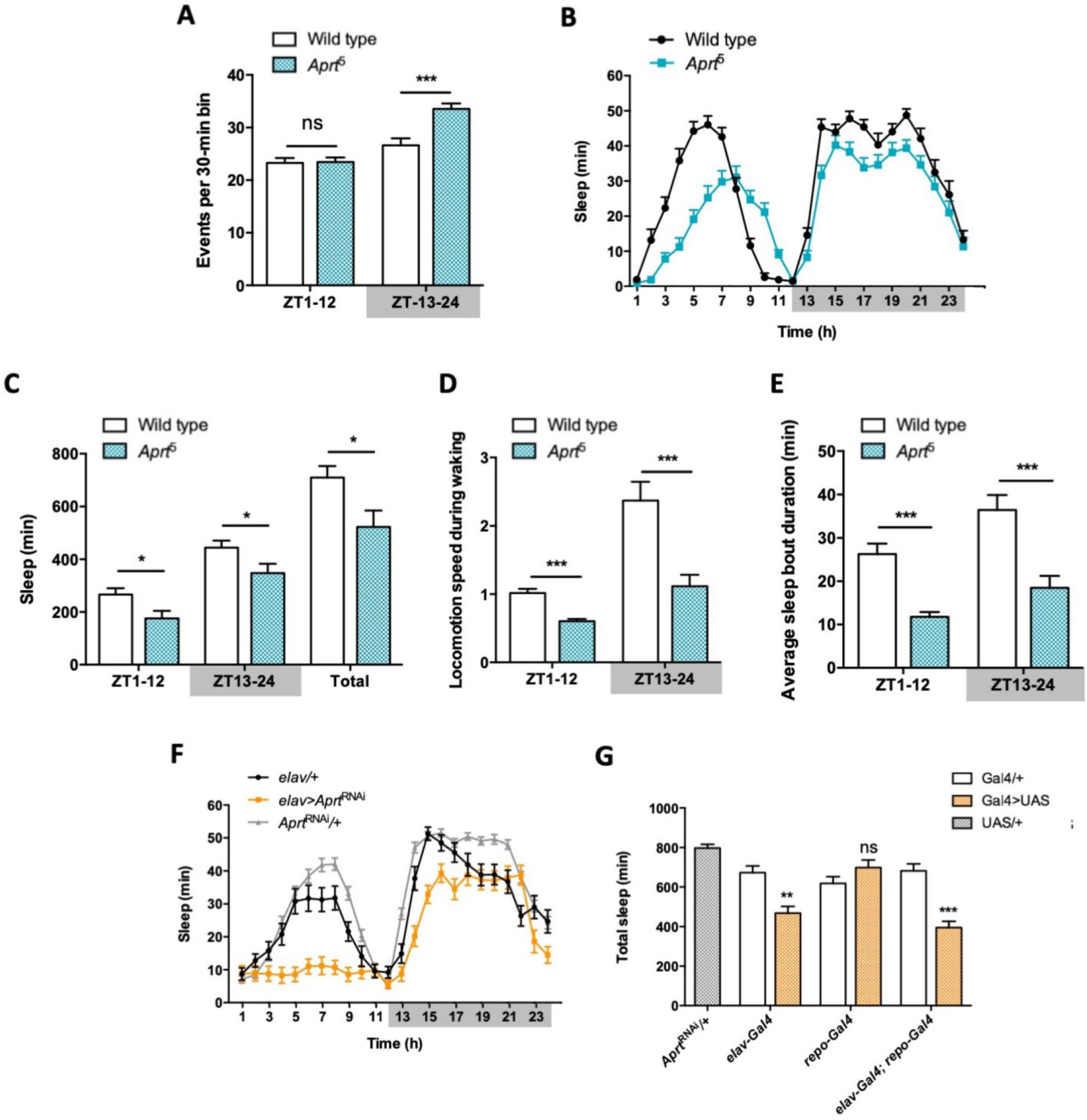

Figure 4.

Aprt-deficient flies sleep less and walk slower than wild-type flies. (**A**) Quantification of total spontaneous locomotor activity during day and night over 5 LD cycles. *Aprt*^{5} mutants show no difference in spontaneous locomotion with wild-type flies during the day but a higher activity at night. 3 independent experiments were performed on 32 flies per genotype and mean ± SEM was plotted. ***$p < 0.001$, ns: not significant. (**B**) Sleep pattern during a typical 24h LD cycle showing that the total amount of sleep is smaller during day and night in *Aprt*^{5} mutants compared to wild-type flies. (**C**) Quantification of day (ZT1-12), night (ZT13-24) and total sleep in *Aprt*^{5} mutants. ZT is for zeitgeber. (**D**) Locomotion speed during waking is reduced in *Aprt*^{5} mutants. (**E**) The average sleep bout duration is also decreased, indicating that *Aprt*^{5} mutants have a difficulty to maintain sleep. (**F**) Sleep pattern of *elav>Aprt*^{RNAi} flies, showing that knockdown of Aprt in all neurons led to sleep reduction during the night, similarly to the mutant condition, and an even more profound sleep defect during the day. (**G**) Quantification of total amount of sleep when *Aprt* was downregulated in all neurons (*elav-Gal4*), all glial cells (*repo-Gal4*) and both neurons and glial cells (*elav-Gal4*; *repo-Gal4*). Except for glia only, the resulting effect was a significant sleep reduction. For sleep and locomotion speed, means ± SEM were plotted. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns: not significant.

synthesis (Friggi-Grelin et al. 2003; Riemensperger et al. 2011), on dissected adult brains from wild-type and $Aprt^5$ mutant flies. Surprisingly, the global TH protein level appeared not to be decreased, but relatively increased by 17.5% in the $Aprt^5$ mutant brain ($p < 0.01$), in particular around the mushroom bodies, a structure that receives dense dopaminergic projections (**Figure 5A, B**). Moreover, DA immunostaining carried out on whole-mount dissected brains revealed a similarly increased level of this neuromodulator in $Aprt^5$ flies, by 17.0% on average in the entire brain ($p < 0.01$), but not specifically in the mushroom bodies or another part of the brain (**Figure 5C, D**). This indicates that disruption of the purine salvage pathway does not impede DA synthesis and levels in the *Drosophila* brain, which are instead slightly increased, perhaps as a compensatory mechanism. The behavior defects suggest that the lack of Aprt activity could interfere with correct functioning of the DA system in an indirect way. We therefore searched for another neurotransmitter system that could be affected by *Aprt* deficiency.

Interactions between Aprt and the adenosinergic system

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

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 flies. We found that *Aprt* transcripts were decreased by 29.5% on average ($p < 0.001$) in $AdoR^{KGex}$ mutant heads (**Figure 6C**, *left panel*), while Aprt activity was even more decreased by 78.4% ($p < 0.001$) compared to wild-type flies (**Figure 6C**, *right panel*). This effect likely results from the much increased level of adenosine uptake in the *AdoR* mutants, which would downregulate *Aprt* activity by a feedback mechanism. Overall, these data indicate that extracellular adenosine levels must be strongly decreased in Aprt mutant flies, both from the general reduction in adenosine levels and the increased expression of the Ent2 transporter. Although AdoR expression is not affected, AdoR signaling is therefore expected to be significantly reduced in *Aprt* mutants.

Aprt mutants show epilepsy-like seizure behavior

A number of *Drosophila* mutants with disrupted metabolism or neural function show increased susceptibility to seizure and paralysis following strong mechanical or electrophysiological stimulation (Fergestad et al. 2006, Parker et al. 2011, Kroll et al. 2015). These mutants are called bang-sensitive (BS) and commonly used as models of epileptic seizure (Song and Tanouye

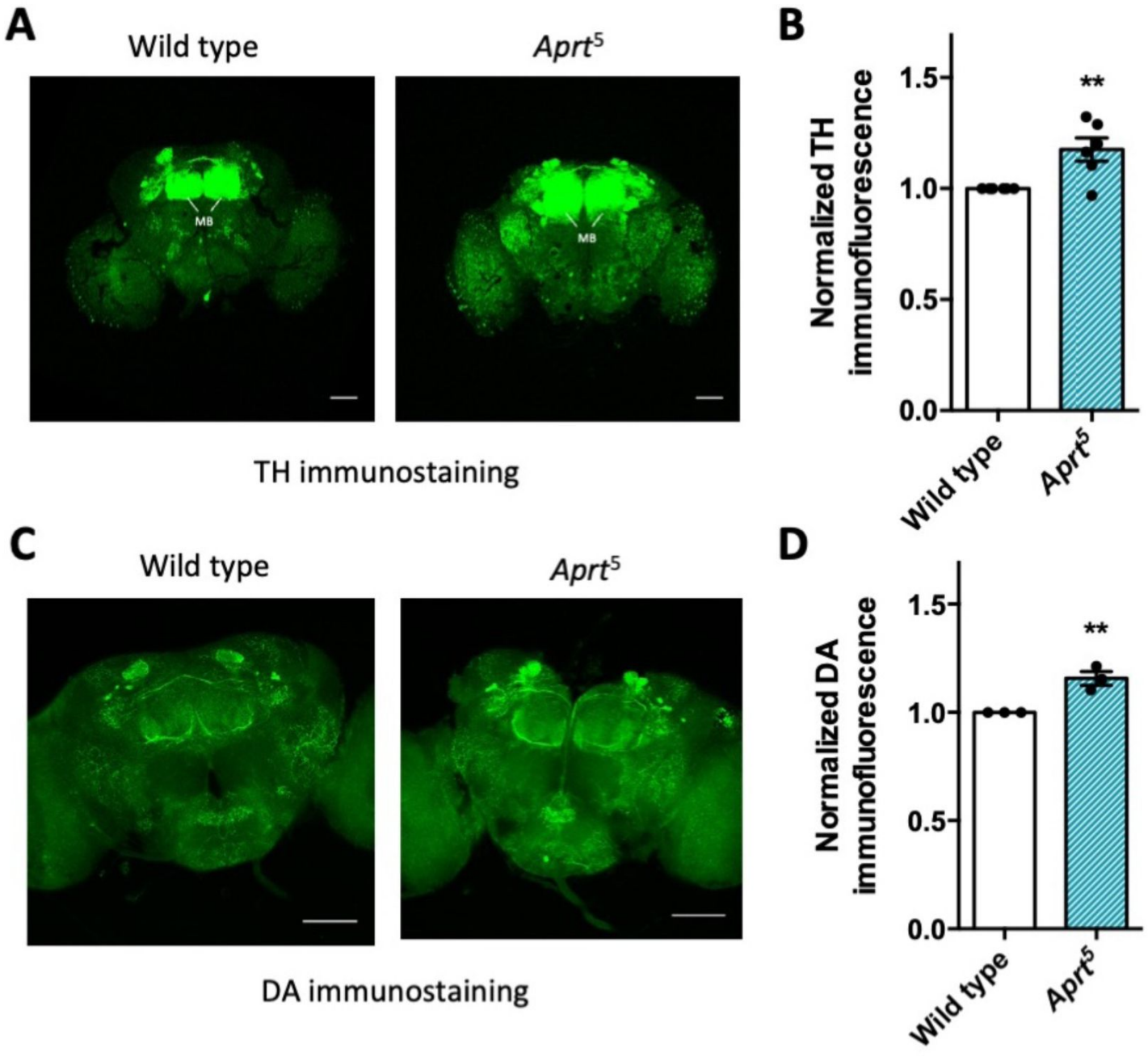

Figure 5.

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. $^{**}p < 0.01$.

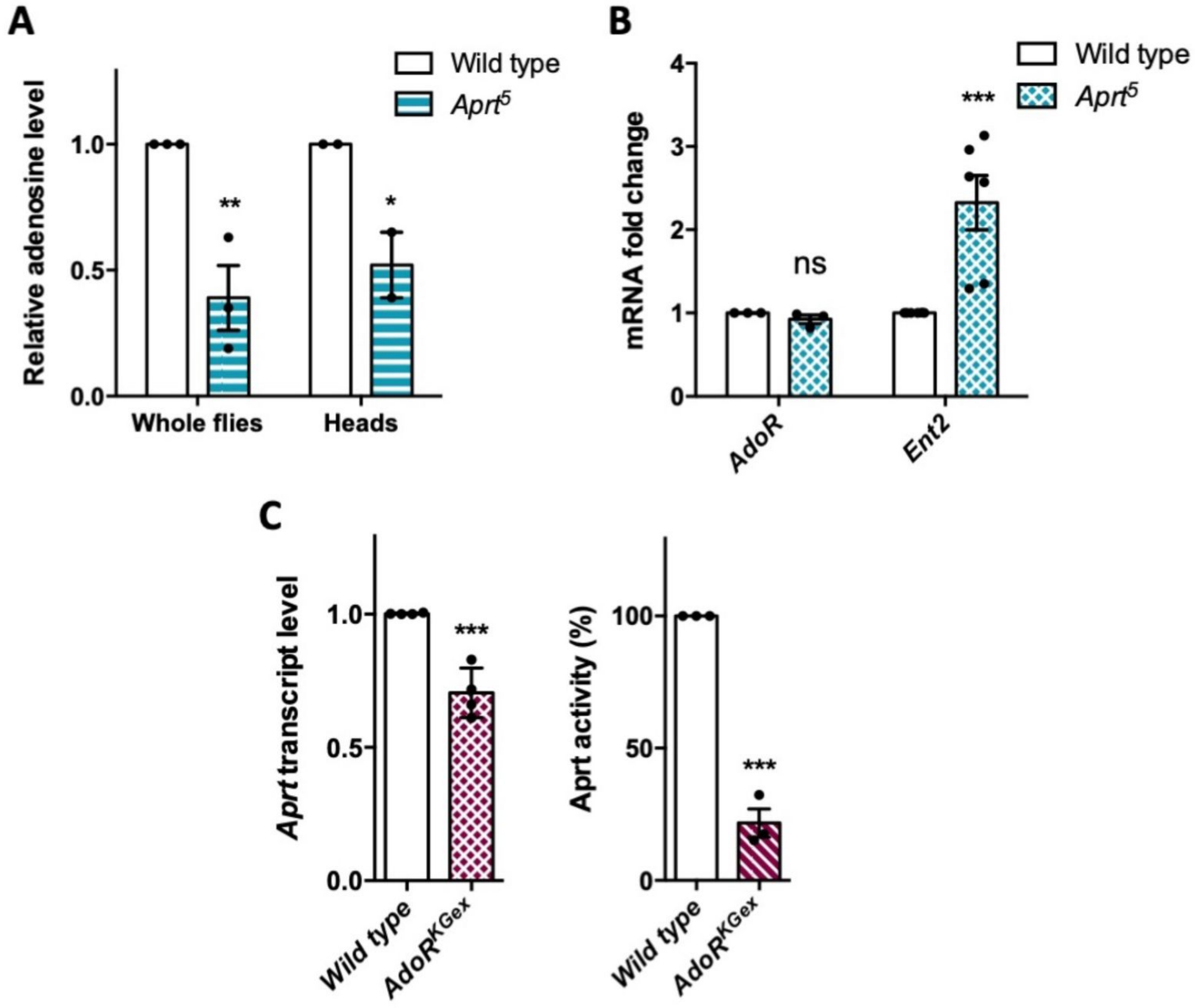

Figure 6.

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. ***$p < 0.001$.

2008). Here we checked whether Aprt deficiency could trigger a BS phenotype. We observed that aged *Aprt* mutants (at 30 d a.E.) recovered slowly after a strong mechanical stimulation applied by vortexing the vial for 10 s at high speed. These flies took on average 17.3 s to recover and get back on their legs, compared to 2.5 s for wild-type flies of the same age ($p < 0.01$) (**Figure 7A** and Movie S1). Some of the mutant flies appeared more deeply paralyzed as they did not spontaneously recover unless the vial was stirred, so their recovery time could not be scored. Hemizygous flies of the same age containing the $Aprt^5$ mutation over two partially overlapping genomic deficiencies covering *Aprt* also showed a BS behavior, with an average longer recovery time of 28.9 s and 33.2 s, respectively ($p < 0.001$) (**Figure 7B**).

We then downregulated *Aprt* by RNAi in all cells with the *da-Gal4* driver to check if this could also induce BS behavior. As shown in **Figure 7C**, *da>*$Aprt^{RNAi}$ flies at 30 d a.E. indeed displayed seizure after mechanical shock, quite similar to that of the $Aprt^5$ mutants (22.7 s recovery time on average compared to 1.55 s and 0.72 s for the driver and RNAi controls, respectively, $p < 0.05$). In contrast, inactivating *Aprt* selectively in neurons *(elav-Gal4)*, glial cells *(repo-Gal4)* or muscles *(24B-Gal4)* did not induce a BS phenotype (Figure S11). This suggests that the BS phenotype requires *Aprt* knockdown in other cells or in several of these cell types. Finally, in contrast to the SING defect, we observed that adult-specific *Aprt* knockdown in all cells with *da-Gal4* for 3 days did not trigger a BS behavior (**Figure 7D**), indicating that the bang sensitivity requires a longer downregulation of the gene or might be the consequence of a developmental defect.

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

Drosophila disease models are advantageously tractable for drug screening *in vivo* (Fernández-Hernández et al. 2016, Perrimon et al. 2016). We thus administered several compounds related to purine metabolism to $Aprt^5$ flies to check if they can rescue neurobehavioral impairments (locomotor defects and seizure). Feeding allopurinol at the same concentration used for uric acid normalization (100 µg/ml, **Figure 1B**), either in adults 5 days before the test or throughout all developmental stages, did not alter the BS phenotype (Figure S12), as was the case for the SING assay (Figure S5). Similarly in humans, it has been shown that the daily intake of allopurinol, even from infancy, does not mitigate the neurobehavioral impairments in LND patients (Marks et al. 1968; Torres et al. 2007; Jinnah et al. 2010; Madeo et al. 2019).

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

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

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

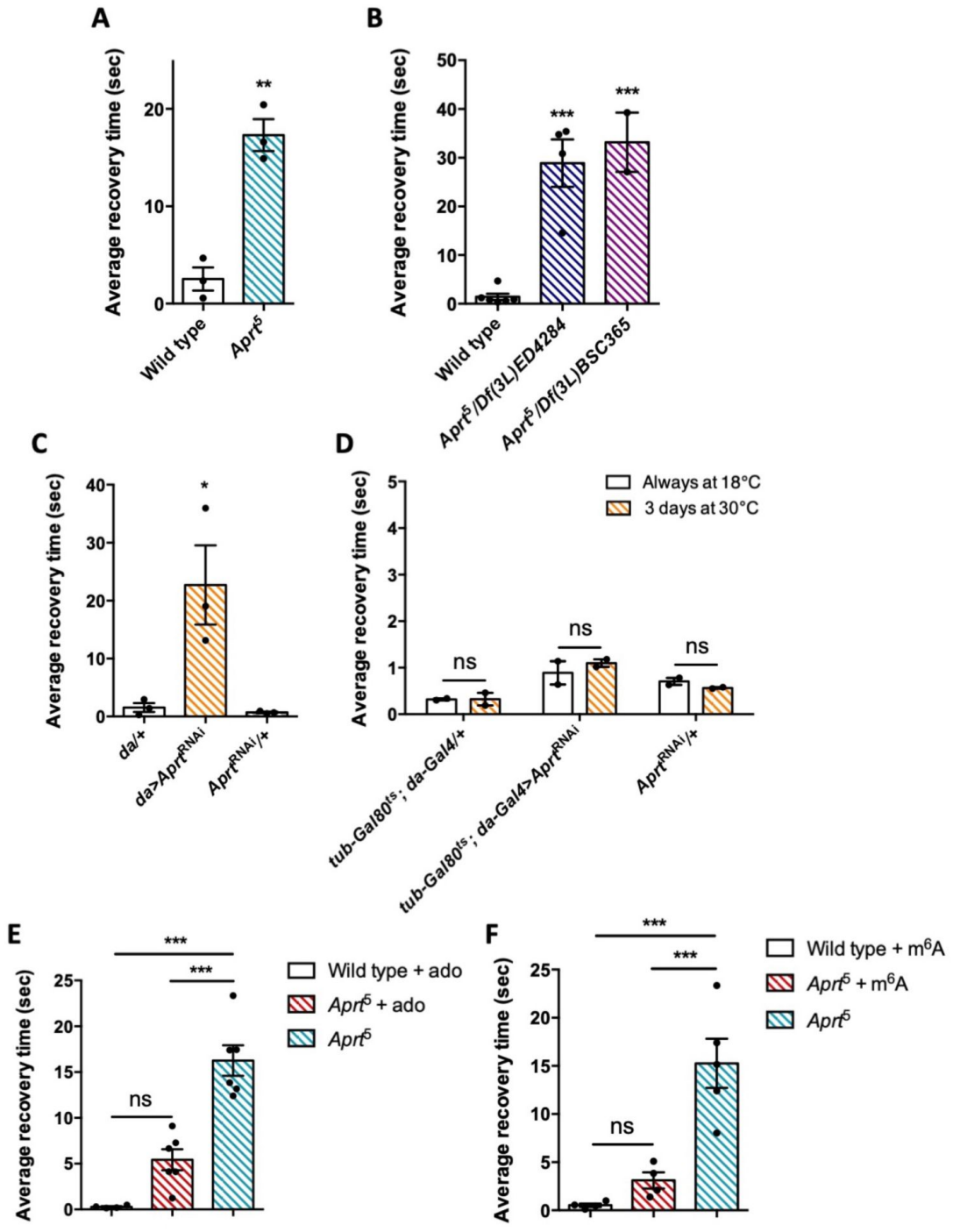

Figure 7.

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

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).

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

Discussion

Over the past 35 years or so, several animal models of LND have been developed in rodents based on HGPRT mutation in order to better understand the cause of the disease and test potential therapeutic treatments (Finger et al. 1988; Dunnett et al. 1989; Jinnah et al. 1993; Engle et al 1996; Meek et al. 2016; Witteveen et al. 2022). However, none of these models recapitulated the full LND syndrome and, particularly, motor or neurobehavioral symptoms as a consequence of HGPRT deficiency. To date, the causes of the neurobehavioral impairments in LND are not yet clearly elucidated and the disease is still incurable (Fu et al. 2014; Bell et al. 2016; López et al., 2020; Bell et al., 2021; Witteveen et al. 2022). Here we have tried to develop new models of this disease in *Drosophila*, a useful organism to conduct genetic and pharmacological studies. An apparent drawback of this project was that no homolog of the mammalian gene encoding HGPRT is conserved in the fly genome, which may explain why no LND models have been developed to date in this organism. To try to overcome that, we used two different strategies. First, because Aprt seems to be the only enzyme of the purine salvage pathway in *Drosophila*, we hypothesized that it could have analogous functions, both for purine homeostasis and behavior control, as HGPRT in humans. Indeed, we show here that *Aprt* deficiency induces both metabolic and neurobehavioral disturbances in *Drosophila*, similar to the loss of HGPRT, but not APRT, activity in humans. Second, we have expressed the wild-type human HGPRT and a LND-associated mutant form of this protein in *Drosophila* neurons to analyze their respective effects in living flies. Here we provide evidence that the fruit fly can be used to study the consequences of defective purine recycling pathway and HGPRT mutation in the nervous system, and perform pharmacological screens to find new therapy for LND.

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

Flies that carry a homozygous null-mutation in *Aprt* develop normally and live until the adult stage (Johnson and Friedman, 1983). Furthermore, a previous study reported that heterozygous *Aprt*/+ flies have an extended lifespan (Stenesen et al. 2013). We observed that homozygous $Aprt^{5}$ mutants have in contrast a significantly reduced longevity compared to wild-type flies. It can be noted that the lack of HGPRT activity in LND also causes reduced lifespan expectancy, which is generally less than 40 years of age for properly cared patients. Stenesen et al. (2013) have demonstrated that dietary supplementation with adenine, the Aprt substrate, prevented the longevity extension conferred either by dietary restriction or heterozygous mutations of AMP biosynthetic enzymes. This suggests that longevity depends on an accurate regulation of adenine

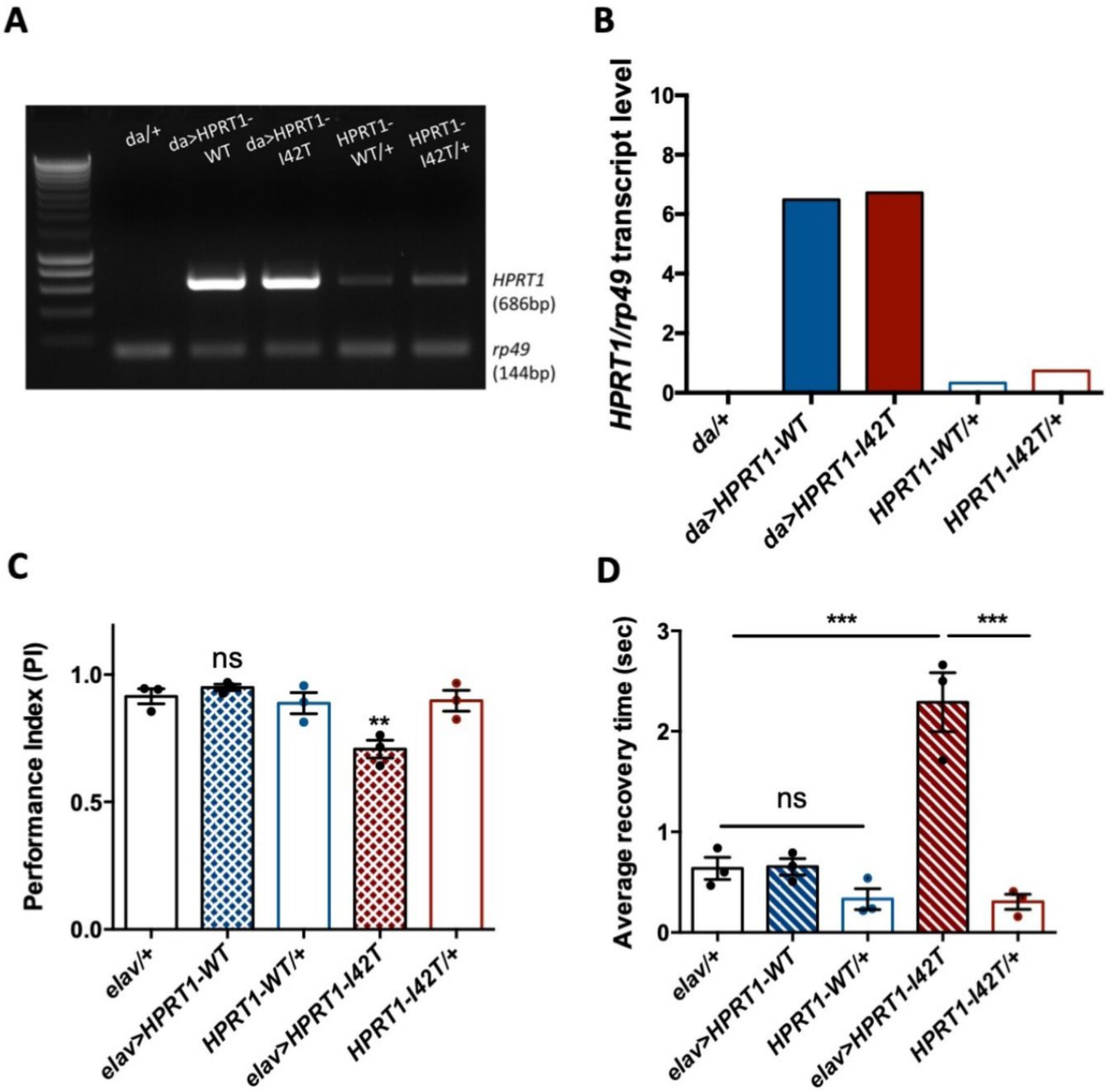

Figure 8.

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.

levels. It is possible that adenine could accumulate to toxic levels during aging in homozygous *Aprt* mutants, explaining their shorter lifespan. Alternatively, we could speculate that AMP, the Aprt product, will be decreased in the absence of a functional Aprt, and as a consequence, AMP-activated protein kinase (AMPK), an enzyme that protects cells from stresses inducing ATP depletion, would be less activated. Multiple publications explored the role of AMPK in lifespan regulation (Sinnett and Brenman 2016) and it has been demonstrated that downregulating AMPK by RNAi in *Drosophila* adult fat body or muscles reduces lifespan (Stenesen et al. 2013), as well as its ubiquitous inactivation under starvation (Johnson et al. 2010).

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

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

We observed that *Aprt*-null adult flies have a strikingly reduced performance in the SING test, which monitors locomotor reactivity to startle and climbing ability. This phenotype was already obvious at an early age, starting from 1 d a.E. The performance continued to decrease until 8 d a.E. and then appeared to stabilize up to 30 d a.E. Remarkably, this phenotype is clearly different from the defects usually found in α-synuclein-induced *Drosophila* Parkinson disease models, in which the locomotion starts declining at around 25 d a.E. and then decreases steadily with aging (Feany and Bender 2000, Riemensperger et al. 2013, Rahmani et al. 2022). Such a locomotor defect affecting newly eclosed *Aprt*-deficient flies could be reminiscent of the early onset of motor symptoms in LND patients, which most often appear from 3 to 6 months of age (Jinnah et al. 2006). Interestingly, we were also able to induce an impairment in startle-induced locomotion by knocking down *Aprt* during 3 days only in adult flies, which suggests that the motricity phenotype does not necessarily require a developmental flaw. Although *Aprt* mutants walked slower than wild-type flies, they were no less active and their ATP levels were not different than in controls, excluding that reduced SING performance resulted from a major failure in energetic metabolism.

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

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

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

Neuronal Aprt regulates spontaneous activity and sleep

Because sleep is regulated by the mushroom body as well as DA neurons in flies, (Artiushin and Sehgal 2017), we have also monitored the spontaneous locomotor activity and sleep pattern of $Aprt^{5}$ mutants. Their activity profile appeared normal, with unaltered morning and evening anticipation, indicating that the circadian rhythms are maintained in light-dark conditions in the absence of a functional purine recycling pathway (Figure S8). This experiment also highlighted that *Aprt*-deficient flies are hyperactive during the night, which suggests that they sleep less than wild-type flies. This could be confirmed by measuring their sleep pattern on video recordings. *Aprt* mutants show a reduced walking speed, sleep less during both day and night, and have a difficulty to maintain sleep. Downregulating *Aprt* selectively in neurons reproduced the sleep defect, whereas doing it in glial cells only had no effect. We have not attempted here to identify further the neuronal cells involved in this phenotype. Like for the SING behavior defect, it could involve the PAM DA neurons, as subpopulations of this cluster have been shown to regulate sleep in *Drosophila* (Nall et al. 2016). Therefore, the lack of purine recycling markedly disrupts sleep in *Aprt*-deficient flies, making them more active at night. This is strikingly comparable to young LND patients who have a much disturbed sleep-time during the night (Mizuno et al. 1979).

Lack of Aprt reduces adenosine signaling leading to DA neuron overactivation

Here we found that disruption of the purine salvage pathway induced by *Aprt* deficiency in *Drosophila* did not decrease the level of DA in brain, although the mutant flies showed reduction in induced locomotion and sleep. This prompted us to study another neuromodulator, adenosine, whose level was likely to be altered in *Aprt* mutants, since this nucleoside is indirectly a product of Aprt enzymatic activity. The purine nucleoside adenosine is one of the building blocks of RNA and the precursor of essential molecules such as ATP and cAMP, but it is also the endogenous ligand of adenosine receptors that modulate a wide range of physiological functions. In the brain, adenosine regulates many motor and cognitive processes, such as the sleep-wake cycle, anxiety, depression, epilepsy, memory and drug addiction (Soliman et al. 2018). In normal metabolic conditions, adenosine is present at low concentrations in the extracellular space and its level is highly regulated, either taken up by cells and incorporated into ATP stores or deaminated by adenosine deaminase into inosine. In mammals, several nucleoside transporters mediate the uptake of

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

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

Adenosine and dopamine receptors are known to interact closely in mammals (Franco et al. 2000; Kim and Palmiter 2008), whereas the function of AdoR has not been extensively studied in *Drosophila*, in particular with regard to its potential interactions with the DA system. However, a previous study performed in fly larvae showed that an increase in astrocytic Ca^{2+} signaling can silence DA neuron activity through AdoR stimulation on DA neurons, by a mechanism potentially involving the breakdown of released astrocytic ATP into adenosine (Ma et al. 2016). Conversely, the AdoR antagonist caffeine was shown to increase DA neuron signaling in the PAM cluster, although it is not certain if caffeine acts through AdoR or another receptor to activate these neurons (Nall et al. 2016). This suggests that a reduction in AdoR signaling, as seems to be the case in Aprt-deficient flies, will lead to DA neuron overactivation. We have previously shown that thermogenetic or optogenetic activation of the DA neurons strongly reduces fly performance in the SING test (Sun et al. 2018). As described here, this is precisely the locomotor phenotype of young Aprt-deficient flies. Furthermore, it has been shown in *Drosophila* that nocturnal hyperactivity is caused by an increase in dopamine signaling (Kumar et al. 2012; Lee et al. 2013). This is fully in accordance with our present observation that Aprt-deficient flies sleep less and are more active during the night. Therefore, both the locomotor and sleep defects induced by the lack of Aprt activity can be explained by an overactivation of DA neurons that would be a direct consequence of reduced adenosinergic signaling.

At first view, these observations do not seem to be in favor of the idea that the Aprt deficient fly could represent a useful model to study LND, since a constant feature of patients with this disease is a marked reduction in DA level in the basal ganglia. However, it has been previously proposed that adenosine could be a mediator of some of the neurological consequences of LND pathogenesis (Visser et al., 2000). Indeed, some studies showed that adenosine transport is decreased in peripheral blood lymphocytes of LND patients (Torres et al. 2004), as well as A_{2A} adenosine receptor mRNA and protein levels (García et al. 2009, García et al. 2011). In a murine HPRT knockout model of LND, expression of the adenosine A_1 receptor was found to be strongly increased and that of A_{2A} slightly decreased, while the expression of A_{2B} was not affected (Bertelli et al. 2006). Chronic administration of high doses of caffeine, which also acts as an antagonist of adenosine receptors in the mammalian brain, caused a self-injurious behavior in rats (Miñana et al. 1984; Jinnah et al. 1990). Moreover, central injection of an adenosine agonist is sufficient to prevent self-mutilation induced by dopamine agonist administration in neonatally 6-OHDA lesioned rats (Criswell et al. 1988). These striking observations encouraged us to study further the potential involvement of adenosine signaling in the neurobehavioral effects induced by *Aprt* deficiency in *Drosophila*.

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

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

Interestingly, inactivating *Aprt* by RNAi in all cells during development reproduced the same seizure-like behavior, whereas inactivation of *Aprt* for 3 days only in adult flies failed to induce bang sensitivity, in contrast to the SING phenotype. These results indicate that the absence of Aprt during development somehow alters a mechanism that controls bang sensitivity, but only has a repercussion with age. The role of Aprt being to recycle adenine into AMP, it was conceivable that *Drosophila Aprt* mutants lack nucleotides in the brain during development, possibly inducing neuronal defects that could trigger later the BS behavior. We have tried, therefore, to feed the mutants with a diet supplemented with various compounds involved in purine metabolism, such as allopurinol, adenine, hypoxanthine, adenosine or its analog N6-methyladenosine (m^6A), either throughout larval development or only in adult stage. Only adenosine and m^6A, ingested during development, were able to prevent the BS behavior. This suggests that the absence of Aprt induces a lack of adenosine in the developing nervous system, as we demonstrated, which has a profound impact on the formation or function of neural circuits controlling the BS behavior in adults. Adenosine is methylated at the nitrogen-6 position to form m^6A in RNA molecules by a large methyltransferase complex (Batista 2017). Once released during RNA turnover or degradation, m^6A cannot be incorporated back into nucleic acids and so it is excreted in urine (Schram 1998). The rescuing effect we observed with m^6A on the BS phenotype indicates thereby that both this compound and adenosine rather act as adenosine receptor agonists or allosteric regulators, than as nucleotide precursors, to prevent the nervous system disturbance induced by Aprt deficiency.

Adenosine has been shown to strongly inhibit brain electrical activity, and its role as an endogenous anticonvulsant and seizure terminator is well-established in humans (Boison 2005; Masino et al. 2014; Weltha et al. 2019). Accordingly, deficiencies in the adenosine-based neuromodulatory system can contribute to epileptogenesis. For instance, increased expression of astroglial adenosine kinase (ADK), which converts adenosine into AMP, leads to a reduction in brain adenosine level that plays a major role in epileptogenesis (Weltha et al. 2019). Hence, therapeutic adenosine increase is a rational approach for seizure control. One explanation for how adenosine release is responsible for seizure arrest implies the attenuation of the $GABA_A$ receptor-mediated depolarization (Ilie et al. 2012). Overall, such data could help explain why our observation that feeding adenosine or its derivative m^6A rescued the seizure-like phenotype of *Aprt* mutant flies, and further suggests that adenosinergic signaling has similar functions in the fly and mammalian brains. In addition, we did observe that adenosine level was decreased in *Aprt* mutant flies, perhaps because of an elevated ADK activity to compensate the absence of AMP production by Aprt. Although the reason why adenosine administration only in adult stage could not rescue the BS behavior remains to be elucidated, it is consistent with the fact that downregulating Aprt for three days in aged adult flies was not sufficient to give rise to the BS phenotype.

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

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

Why HGPRT deficit causes such dramatic neurobehavioral troubles in LND patients still remains a crucial question. To date, cellular (Smith & Friedman 2000; Torres et al. 2004; Ceballos-Picot et al. 2009; Cristini et al. 2010; Guibinga et al. 2012; Sutcliffe et al. 2021) and rodent (Finger et al. 1988; Dunnett et al. 1989; Jinnah et al. 1993; Meek et al. 2016; Witteveen et al. 2022) models only focused on the consequences of HGPRT deficiency to phenocopy the disease. Such an approach was justified by the fact that the lower the residual activity of mutant HGPRT, the more severe the symptoms are in patients (Fu and Jinnah 2012. Fu et al. 2014). However, it would be conceivable that part of the symptoms observed in the disease result from compensatory physiological mechanisms or a deleterious gain-of-function conferred to the HGPRT protein by the pathogenic mutations. Here we observed that neuronal expression of mutant human HGPRT-I42T, which expresses a low enzymatic activity, but not the wild-type protein, induced early locomotor defects in young flies and bang-sensitivity in older flies, very similarly to the defects induced by Aprt deficiency. This suggests a potential neurotoxicity of the pathological mutant form of HGPRT, which could be related to disturbances in purine metabolism or other signaling pathways. The human mutant form might not be properly degraded and accumulate as aggregates, potentially exerting neuronal toxicity. Such an approach opens interesting perspectives to better understand the endogenous function of HGPRT and its pathogenic forms. Indeed, the identification of a potential inherent neurotoxicity of defective forms of human HGPRT is a new element, which could be explored in further work in the fly and also in rodent models.

A new model of LND in an invertebrate organism

LND, a rare X-linked metabolic disorder due to mutations of the *HPRT1* gene, has dramatic neurological consequences for affected children. To date, no treatment is available to abrogate these troubles, and no fully satisfactory *in vivo* models exist to progress in the understanding and cure of this disease. HGPRT knockout rodents do not show comparable motor and behavioral troubles, which makes these models problematic for testing new therapeutic treatments. In *Drosophila*, no HGPRT homologue is expressed, indicating that Aprt is the only enzyme of the purine salvage pathway in this organism. Here we find that *Aprt* mutant flies show symptoms partly comparable to the lack of HGPRT in humans, including increase in uric acid levels, reduced longevity, and various neurobehavioral defects such as early locomotor decline, sleep disorders and epilepsy behavior. This animal model therefore recapitulates both salvage pathway disruption and motor symptoms, as observed in LND patients. Moreover, these results highlighted that *Aprt* deficiency in *Drosophila* has more similarities with HGPRT than APRT deficiency in humans. *Aprt* mutant flies also show a disruption of adenosine signaling and we found that adenosine itself or a derivative compound can relieve the epileptic symptoms. Moreover, we observed that neuronal expression of a mutant form of human HGPRT that causes LND also triggers abnormalities in fly locomotion and seizure-like behavior, which has not been documented to date in other models. The use of *Drosophila* to study LND should therefore prove valuable to better understand the link between purine recycling deficiency and brain functioning and carry out drug screening in a living organism, paving the way towards new improvements in the cure of this dramatic disease.

Materials and Methods

Drosophila culture and strains

Flies were raised at 25°C on standard cornmeal-yeast-agar medium supplemented with methylhydroxybenzoate as an antifungal under a 12h:12h light-dark (LD) cycle. The *Drosophila* mutant lines were obtained either from the Bloomington *Drosophila* Stock Center (BDSC), the Vienna *Drosophila* Resource Center (VDRC) or our own collection: $Aprt^{5}$ (Johnson and Friedman, 1983) (BDSC #6882), *Df(3L)ED4284* (BDSC #8056), *Df(3L)BSC365* (BDSC #24389), *UAS-*$Aprt^{RNAi}$ (VDRC #106836), $AdoR^{KG03964ex}$ (Wu et al. 2009) here named $AdoR^{KGex}$ (BDSC #30868), and the following Gal4 drivers: *238Y-Gal4*, *24B-Gal4*, *da-Gal4*, *EAAT1-Gal4* (Rival et al. 2004), *elav-Gal4* ($elav^{C155}$), *MZ0709-Gal4*, *NP6510-Gal4*, *NP6520-Gal4*, *R58E02-Gal4* (Liu et al. 2012), *R76F05-Gal4*, *repo-Gal4*, *TH-Gal4* (Friggi-Grelin et al. 2003), *TRH-Gal4* (Cassar et al. 2015), *tub-*$Gal80^{ts}$ and *VT30559-Gal4*. The Canton-S line was used as wild-type control. The simplified driver>effector convention was used to indicate genotypes in figures, e.g. *elav>*$Aprt^{RNAi}$ for *elav-Gal4*; *UAS-*$Aprt^{RNAi}$. In some experiments, to restrict RNAi-mediated *Aprt* inactivation at the adult stage, we have used the TARGET system (McGuire et al. 2003). Flies were raised at 18°C (permissive temperature) where Gal4 transcriptional activity was inhibited by *tub-*$Gal80^{ts}$, and shifted to 30°C (restrictive temperature) for 3 days before the test to enable Gal4-driven $Aprt^{RNAi}$ expression.

APRT and HPRT transgenic lines

To generate a *UAS-Aprt* strain, a 549-bp *Aprt* insert containing the coding sequence was PCR amplified from the ORF clone BS15201 (*Drosophila* Genomics Resource Center, Bloomington, IN, USA) using primers with added restriction sites (Table S3). After digestion with EcoRI and XbaI, the *Aprt* cDNA was subcloned into the *pUASTattB* vector (Bischof et al. 2007) using the In-Fusion HD Cloning Kit (Takara Bio, Kyoto, Japan) according to the manufacturer's instructions, and the insertion verified by sequencing (Eurofins Genomics, Ebersberg, Germany). The construction was sent to BestGene (Chino Hills, CA, USA) for *Drosophila* germline transformation into the attP14 docking site on the 2d chromosome. The *UAS-Aprt* line yielding the strongest expression was selected and used in the experiments.

A clone containing the human wild-type *HPRT1* cDNA was kindly provided to us by Prof. Hyder A. Jinnah (Emory University, GA, USA). We constructed the *HPRT1-I42T* cDNA from this clone by site-directed mutagenesis using the Quick Change II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA). Primers sequences used to create the mutation are shown in Table S3. The cDNA obtained was verified by sequencing. Then, the 657-bp *HPRT1-WT* and *HPRT1-I42T* inserts were PCR amplified using primers with added restriction sites and *Drosophila* translation start consensus sequence (Table S3). They were subcloned into *pUASTattB* and verified by sequencing. The transgenes were sent to BestGene for *Drosophila* transformation and inserted into the attP40 docking site on the 2d chromosome.

Sequencing of $Aprt^{5}$

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

Lifespan assay

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

Startle-induced negative geotaxis (SING)

SING assays were performed as previously described (Rival et al. 2004; Riemensperger et al. 2011; Sun et al. 2018). For each genotype, 50 adult males divided into 5 groups of 10 flies were placed in a vertical column (25-cm long, 1.5-cm diameter) with a conic bottom end and left for about 30 min for habituation. Then, columns were tested individually by gently tapping them down (startle), to which flies normally responded by climbing up. After 1 min, flies having reached at least once the top of the column (above 22 cm) and flies that never left the bottom (below 4 cm) were counted. Each fly group was assayed three times at 15 min intervals. The performance index (PI) for each column is defined as $½[1 + (n_{top}-n_{bot})/n_{tot}]$, where n_{tot} is the total number of flies in the column, n_{top} the number of flies at the top and n_{bot} the number of flies at the bottom after 1 min. SING was performed at 1, 8, 10 and 30 days after adult eclosion (d a.E.).

Spontaneous locomotion and sleep monitoring

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

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

Immunohistochemistry

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

Nikon A1R confocal microscope with identical laser, filter and gain settings for all samples in each experiment. Immunofluorescence intensity levels were quantified using the Fiji software. Experiments were repeated independently at least 3 times on 4 to 6 brains per genotype.

Bang-sensitivity test

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

Drug administration

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

Uric acid quantification

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

ATP assay

ATP level was measured by bioluminescence using the ATP Determination Kit (A22066, ThermoFisher Scientific, Waltham, MA, USA) on a TriStar 2 Spectrum LB942S microplate reader (Berthold Technologies, Bad Wildbad, Germany), according to the manufacturer's instructions. Samples were prepared as described previously (Fergestad et al. 2006). Briefly, 30 heads or 5 thoraces from 8-day-old flies were homogenized in 200 µL of 6 M guanidine-HCl to inhibit ATPases, boiled directly 5 min at 95°C and then centrifuged for 5 min at 13,000 g and 4°C. Total protein content of each supernatant was measured with the BCA Protein Assay Kit. Each supernatant was then diluted at 1:500 in TE buffer pH 8 and 10 µl were placed in a 96-well white-bottom plate. The luminescent reaction solution (containing D-luciferine, recombinant firefly luciferase, dithiothreitol and reaction buffer) was added to each well with an injector and high-gain 1 sec exposure glow reads were obtained at 15 min after reaction initiation. Results were compared to a standard curve generated with known ATP concentrations and final values were normalized to the protein content.

Enzyme activity assay

HGPRT and APRT activities were assessed in *Drosophila* by adapting methods previously established for human cells (Cartier and Hamet 1968, Ceballos-Picot et al. 2009). 20 whole male flies were homogenized in 250 µl of 110 mM Tris, 10 mM $MgCl_2$ pH 7.4 (Tris-$MgCl_2$ buffer), immediately frozen and kept at least one night at -80°C before the assay. After 5 min of centrifugation at 13,000 g, total protein content of each supernatant was measured with the BCA Protein Assay Kit to normalize activity level. Kinetics of [^{14}C]-hypoxanthine conversion to IMP and [^{14}C]-adenine conversion to AMP were assessed for HGPRT and Aprt assays, respectively. Compositions of reaction mediums were: 25 µl of a radioactive solution made with 38 µl of [^{14}C]-hypoxanthine (25 µCi/ml) diluted in 1 ml of 1.2 mM cold hypoxanthine, for HGPRT assay, or 25 µl of a radioactive solution made with 75 µl of [^{14}C]-adenine (50 µCi/ml) diluted in 1 ml of 1.2 mM cold adenine for Aprt assay, and a volume of fly extract equivalent to 200 µg protein diluted in Tris-$MgCl_2$ buffer to 150 µl. Reactions were monitored at 37°C and started by adding 25 µl of the co-factor 5-phosphoribosyl-1-pyrophosphate (PRPP) at 10 mM. After 6, 12, 24 and 36 min, 40 µL of each pool was placed in a tube containing either 25 µl of HIE (3 mM hypoxanthine, 6 mM IMP, 200 mM EDTA) or AAE (3 mM adenine, 6 mM AMP, 200 mM EDTA) solutions and incubated for 3 min at 95°C to stop the reaction. The different radioactive compounds were separated by paper chromatography on Whatman 3MM strips using 28% NH_4OH, 50 mM EDTA as solvent for about 1h30. Then, the substrates and products were visualized under a UV lamp at 254 nm and placed separately in vials in 2 ml of Scintran (VWR, Radnor, PA, USA). The radioactivity in disintegrations per minute (dpm) was measured in a Packard Tri-Carb 1600 TR liquid scintillation analyzer (PerkinElmer, Waltham, MA, USA). The percentage of substrate transformation as a function of time was converted in nmol/min/mg protein and finally normalized to wild-type control values.

Reverse transcription-PCR

Total RNA was isolated by standard procedure from 20-30 heads of 8-day-old males collected on ice and lysed in 600 µl QIAzol Reagent (Qiagen, Venlo, Netherlands). 1 µg of total RNA was treated by DNase (DNase I, RNase-free, ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. 5 µL of treated RNA was reverse transcribed using oligo d(T) primers (PrimeScript RT Reagent Kit – Takara Bio, Kyoto Japan). Then, at least 750 ng of the first strand cDNA was amplified in 20 µl of reaction mixture using PrimeStar Max DNA polymerase (Takara Bio, Kyoto Japan) with a Techne Prime Thermal Cycler apparatus (Bibby Scientific, Burlington, NJ, USA). The program cycles included 10 s denaturation at 98°C, 10 s annealing at 55°C and 10 s elongation at 72°C, repeated 30 times. PCR product levels were measured after electrophoresis by densitometry analysis with the Fiji software. Data were normalized to amplification level of the ribosomal protein *rp49/RpL32* transcripts as internal control. Sequences of the primers used were: sense *HPRT*, 5'-GAGATACAAAATGGCGACCCGCAGCCCT; antisense *HPRT*, 5'-GCTCGGATCCTTATCATTACTAGGCTTTG (amplicon 686bp); sense *rp49*, 5'-GACGCTTCAAGGGACAGTATC; antisense *rp49*, 5'- AAACGCGGTTCTGCATGAG (amplicon 144bp).

Quantitative reverse transcription PCR

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

Adenosine assay

Adenosine was determined by Ultra Performance Liquid Chromatography (UPLC). 5 whole flies or 30 heads were homogenized in 120 µl 0.9% (w/v) NaCl using a Minilys apparatus (Bertin Instruments, Montigny-le-Bretonneux, France) and frozen at -80°C. After unfreezing and 5 min of microcentrifugation, 20 µl of 10% perchloric acid were added to 70 µl of the supernatant and the mixture was left for 5 min on ice. After a new centrifugation, 20 µl of a neutralization solution (made by mixing 3 volumes of 3 M K_2CO_3 with 1 volume of 6.4 mM NaOH containing 0.4 mg/ml bromothymol blue) were added and the mixture was centrifuged again before injection (5 µl). Samples were analyzed with a UV diode-array detector on an Acquity UPLC HSS T3 column (1,8 µm, 2.1 × 150mm) (Waters Corporation, Milford, MA, USA). The mobile phases consisted in Buffer A (30 mM ammonium acetate, pH 4.0 with 1:10,000 heptafluorobutyric acid (HPFA)) and Buffer B (acetonitrile with 1:10,000 HPFA), using a flow rate of 0.3 ml/min. Chromatographic conditions were: 3.5 min 100% Buffer A, 16.5 min up to 6.3% Buffer B, 2 min up to 100% Buffer B, and 1 min 100% Buffer B. The gradient was then returned over 5 min to 100% Buffer A, restoring the initial conditions. Results were normalized to protein levels for each sample.

Statistics

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

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Authors contributions

Conceptualization, C.P., L.S., S.M., I.C.P. and S.B.; investigation, C.P., L.S., A.D., R.F., 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, 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., I.C.P. and S.B.; writing, review & editing, C.P., I.C.P and S.B.; funding acquisition and supervision, I.C.P and S.B.; project administration, S.B.

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Reviewer #1 (Public Review):

The current manuscript focuses on the adenine phosphoribosyltransferase (Aprt) and how the lack of its function affects nervous system function. It puts it into the context of Lesch-Nyhan disease, a rare hereditary disease linked to hypoxanthine-guanine phosphoribosyltransferase (HGPRT). Since HGPRT appears absent in Drosophila, the study focuses initially on Aprt and shows that aprt mutants have a decreased life-span and altered uric acid levels (the latter can be attenuated by allopurinol treatment). Moreover, aprt mutants show defects in locomotor reactivity behaviors. A comparable phenotype can be observed when specifically knocking down aprt in dopaminergic cells. Interestingly, also glia-specific knock-down caused a similar behavioral defect, which could not be restored when re-expressing UAS-aprt, while neuronal re-expression did restore the mutant phenotype. Moreover, mutants, pan-neuronal and pan-neuronal plus glia RNAi for aprt caused sleep-defects. Based on immunostainings Dopamine levels are increased; UPLC shows that adenosine levels are reduced and PCR showed in increase of Ent2 levels are increased (but not AdoR). Moreover, aprt mutants display seizure-like behaviros, which can be partly restored by purine feeding (adenosine and N6-methyladenosine). Finally, expression of the human HGPRT also causes locomotor defects.

The authors provide a wide range of genetic experimental data to assess behavior and some molecular assessment on how the defects may emerge. It is clearly written, and the arguments follow the experimental evidence that is provided.

The findings provide a new example of how manipulating specific genes in the fruit fly allows the study of fundamental molecular processes that are linked to a human disease.

Reviewer #2 (Public Review):

The manuscript by Petitgas et al demonstrates that loss of function for the only enzyme responsible for the purine salvage pathway in fruit-flies reproduces the metabolic and neurologic phenotypes of human patients with Lesch-Nyhan disease (LND). LND is caused by mutations in the enzyme HGPRT, but this enzyme does not exist in fruit-flies, which instead only have Aprt for purine recycling. They demonstrate that mutants lacking the Aprt enzyme accumulate uric acid, which like in humans can be rescued by feeding flies allopurinol, and have decreased longevity, locomotion and sleep impairments and seizures, with striking resemblance to HGPRT loss of function in humans. They demonstrate that both loss of function throughout development or specifically in the adult ubiquitously or in all neurons, or dopaminergic neurons, mushroom body neurons or glia, can reproduce the phenotypes (although knock-down in glia does not affect sleep). They show that the phenotypes can be rescued by over-expressing a wild-type form of the Aprt gene in neurons. They identify a decrease in adenosine levels as the cause underlying these phenotypes, as adenosine is a neurotransmitter functioning via the purinergic adenosine receptor in neurons. In fact, feeding flies throughout development and in the adult with either adenosine or m6A could

prevent seizures. They also demonstrate that loss of adenosine caused a secondary up-regulation of ENT nucleoside transporters and of dopamine levels, that could explain the phenotypes of decreased sleep and hyperactivity and night. Finally, they provide the remarkable finding that over-expression of the human mutant HGPRT gene but not its wild-type form in neurons impaired locomotion and induced seizures. This means that the human mutant enzyme does not simply lack enzymatic activity, but it is toxic to neurons in some gain-of-function form. Altogether, these are very important and fundamental findings that convincingly demonstrate the establishment of a Drosophila model for the scientific community to investigate LND, to carry out drug testing screens and find cures.

The experiments are conducted with great rigour, using appropriate and exhaustive controls, and on the whole the evidence does convincingly or compellingly support the claims. The exception is an instance when authors mention 'data not shown' and here data should either be provided, or claims removed: "feeding flies with adenosine or m6A did not rescue the SING phenotype of Aprt mutants (data not shown)". It is important to show these data (see below).

Sleep is used to refer to lack of movement of flies to cross a beam for more than 5 minutes. However, lack of movement does not necessarily mean the flies are asleep, as they could be un-motivated to move (which could reflect abnormal dopamine levels) or engaged in incessant grooming instead. These differences are important for future investigation into the neural circuits affect by LND.

The authors claim that based on BLAST genome searchers, there are no HPRTI (encoding HGPRT) homologues in Drosophila. However, such a claim would require instead structure-based searches that take into account structural conservation despite high sequence divergence, as this may not be detected by regular BLAST.

This work raises important questions that still need resolving. For example, the link between uric acid accumulation, reduced adenosine levels, increased dopamine and behavioural neurologic consequences remain unresolved. It is important that they show that restoring uric acid levels does not rescue locomotion nor seizure phenotypes, as this means that this is not the cause of the neurologic phenotypes. Instead, their data indicate adenosine deficiency is the cause. However, one weakness is that for the manipulations they test some behaviours but not all. The authors could attempt to improve the link between mechanism and behaviour by testing whether over-expression of Aprt in neurons or glia, throughout development or in the adult, and feeding with adenosine and m6A can rescue each of the behavioural phenotypes handled: lifespan, SING, sleep and seizures. The authors could also attempt to knock-down dopamine levels concomitantly with feeding with adenosine or m6A to see if this rescues the phenotypes of SING and sleep. Visualising the neural circuits that express the adenosine receptor could reveal why the deficit in adenosine can affect distinct behaviours differentially, and which neurologic phenotypes are primary and which secondary consequences of the mutations. This would allow them to carry out epistasis analysis by knocking-down AdoR in specific circuits, whilst at the same time feeding Aprt mutants with Adenosine.

The revelation that the mutant form of human HGPRT has toxic effects is very intriguing and important and it invites the community to investigate this further into the future.

To conclude, this is a fundamental piece of work that opens the opportunity for the broader scientific community to use Drosophila to investigate LND.

Reviewer #3 (Public Review):

The study attempts to develop a Drosophila model for the human disease of LND. The issue here, and the main weakness of this study, is that Drosophila does not express the enzyme,

HGPRT, which when mutated causes LND. The authors, instead, mutate the functionally-related Drosophila Aprt enzyme. However, it is unknown whether Aprt is also a structural homologue. Because of this, it will likely not be possible to identify pharmacological compounds that rescue HGPRT activity via a direct interaction (unless modelling predicts high conservation of substrate binding pocket between the two enzymes, etc). An additional weakness is that the study does not identify a molecule that may act as a lead compound for further development for treating LND. Rather, the various rescues reported are selective for only a subset of the disease-associated phenotypes. Thus, whilst informative, this first section of the study does not meet the study ambitions.

The second approach adopted is to express a 'humanised mutated' form of HGPRT in Drosophila, which holds more promise for the development of a pharmacological screen. In particular, the locomotor defect is recapitulated but the seizure-like activity, whilst reported as being recapitulated, is debatable. A recovery time of 2.3 seconds is very much less than timings for typical seizure mutants. Nevertheless, the SING behaviour could be sufficient to screen against. However, this is not explored.

In summary, this is a largely descriptive study reporting the behavioural effects of an Aprt loss-of-function mutation. RNAi KD and rescue expression studies suggest that a mix of neuronal (particularly dopaminergic and possibly adenosinergic signalling pathways) and glia are involved in the behavioural phenotypes affecting locomotion, sleep and seizure. There is insufficient evidence to have confidence that the Arpt fly model will prove valuable for understanding / treating LND.

Author Response:

We are very grateful to the Editors and the three Reviewers for their valuable reviews of our submission. We will take into account all the comments and provide a revised manuscript with our point-by-point responses as soon as possible. In the meantime, we would like to respond provisionally to the reservation expressed in the eLife editorial assessment and by Reviewer #3 about the validity of our models to study of the neurobehavioral consequences of purine deficiency and the pathogenesis of Lesch-Nyhan disease (LND) in Drosophila.

Two enzymes are responsible for purine recycling in mammals: APRT and HGPRT. Only HGPRT deficiency causes neurobehavioral disturbances and LND in humans, while APRT deficiency leads to metabolic deficits without neurological or behavioral symptoms. In contrast, as we have been able to confirm, Drosophila expresses a single purine recycling enzyme, Aprt, and no HGPRT or HGPRT-like activity. Here we propose different ways to model LND in Drosophila, based either on Aprt deficiency or the expression of mutant HGPRT.

Although it may be difficult to accept that the inactivation of a different gene in a distant organism could be a good model for LND, we have found that, in contrast to humans, Aprt deficiency has both metabolic and neurobehavioral consequences in Drosophila. This suggested that Aprt, being the unique fly purine recycling enzyme, might share the enzymatic function of human APRT and the neurodevelopmental function of human HGPRT, because its inactivation should recapitulate all pathological consequences of a lack of purine recycling in this organism, and in particular in the brain.

The statement by Reviewer #3 that “it is unknown whether Aprt is also a structural homologue [of HGPRT]” is not accurate. APRT and HGPRT are known to be functionally and structurally related. Both human APRT and HGPRT belong to the type I PRTases family identified by a conserved phosphoribosyl pyrophosphate (PRPP) binding motif, which is used as a substrate to transfer phosphoribosyl to purines. This binding motif is only found in PRTases from the nucleotide synthesis and salvage pathways (see: Sinha and Smith (2001)

Curr Opin Struct Biol 11(6):733-9. PMID: 11751055). The purine substrates adenine, hypoxanthine and guanine share the same chemical skeleton and APRT can bind hypoxanthine, indicating that APRT and HGPRT also share similarities in their substrate binding sites (Ozeir et al. (2019) J Biol Chem. 294(32):11980-11991. PMID: 31160323). Moreover, Drosophila Aprt and Human APRT are closely related as the amino acid sequences of APRTs have been highly conserved throughout evolution (shown in Fig. S3B of our paper). We apologize for not providing this information in our original submission. This point will be made clearer in the revised article.

Here we report a set of evidence that Drosophila can be used as a model to study LND. A strong argument, as we believe, is that the same drugs have been found effective in rescuing the seizure-like phenotype in Aprt-deficient flies (Figure 7 in our manuscript) and the viability of fibroblasts and neural stem cells derived from iPSCs of LND patients, in which de novo purine synthesis was prevented (as discussed on page 37). This is a good sign that Drosophila could be used to identify new genetic targets and pharmacological compounds capable to rescue HGPRT mutations in humans.

Finally, we would like to emphasize that Reviewer #1 and Reviewer #2 expressed confidence in the potential usefulness of our work to better understand and treat LND in their public reviews. Reviewer #1 indeed stated that: “The findings provide a new example of how manipulating specific genes in the fruit fly allows the study of fundamental molecular processes that are linked to a human disease”, and Reviewer #2 further wrote: "Altogether, these are very important and fundamental findings that convincingly demonstrate the establishment of a Drosophila model for the scientific community to investigate LND, to carry out drug testing screens and find cures”, and added: “To conclude, this is a fundamental piece of work that opens the opportunity for the broader scientific community to use Drosophila to investigate LND”.