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Apelin controls emotional behavior in age- and metabolic state-dependent manner

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ABSTRACT

Apelin is a small peptide secreted by the adipose tissue notably in conditions of obesity-induced hyper-insulinemia. Apelin exerts a range of physiological functions at the periphery including the improvement of insulin sensitivity and the increase of muscle strength or cardiac contractibility. Interestingly, the brain is endowed with a high density of APJ, the single target of apelin, and growing evidence suggests various central actions of this adipokine. Interestingly, recent studies reported that the intracerebroventricular infusion of apelin modulates emotional states in middle age stressed animals. However, results are so far been mixed and have not allowed for definitive conclusions about the impact of apelin on anxio-depressive-like phenotype. This study aims 1) to evaluate whether serum apelin levels are associated with mood in older adults and 2) to determine the impact of the genetic apelin inactivation in 12-month old mice fed a standard diet (STD) or in 6-month old mice fed a high fat diet (HFD). A higher plasma apelin level was associated with higher depressive symptoms in older adults. In line with these clinical findings, 12-month old apelin knock-out ($Ap^{-/-}$) mice displayed a spontaneous antidepressant-like phenotype. In a marked contrast, 6-month old $Ap^{-/-}$ mice harbored a higher degree of peripheral insulin resistance than wild-types in response to HFD and were more prone to develop anxiety while the depressive-like state was not modified. We also provided evidence that such anxious behavior was associated with an impairment of central serotonergic and dopaminergic neuronal activities. Finally, although the insulin sensitizing drug metformin failed to reverse HFD-induced insulin resistance in 6-month old $Ap^{-/-}$ mice, it reversed their anxious phenotype.

These results emphasize a complex contribution of apelin in the regulation of emotional state that might depend on the age and the metabolic status of the animals. Further investigations are warranted to highlight the therapeutic potential of manipulating the apelinergic system in mood-related disorders.

INTRODUCTION

As reported by the World Health Organization, diabetes and depression each affect around 350 million people worldwide. These pathologies represent important healthcare burdens as well as critical economic issues due to their effects on disability, productivity and quality of life (Egede and Ellis, 2010). Interestingly, epidemiological studies revealed a bidirectional link between diabetes and depression pointing out a higher rate of depression in patients suffering from diabetes and vice-versa. In a meta-analysis, Anderson and collaborators showed that the prevalence of depression is at least doubled among diabetic patients compared to the general population (Anderson et al., 2001). This increase appears to be common in diabetes subtypes since such a relationship was reported not only in type 1 (Buchberger et al., 2016) but also in type 2 diabetic patients (Wang et al., 2019). In a more advanced way, Phillips and Perry proposed that impairment of insulin signaling might represent a metabolic predictor of depressive symptoms, notably in obese patients (Phillips and Perry, 2015). This hypothesis is congruent with animal studies conducted in mice and rats. Indeed, evidence indicates that mouse models of type 1 and type 2 diabetes, in response to streptozotocin (STZ) or high-fat diet (HFD) respectively, harbor anxio/depressive-like behaviors (Gupta et al., 2014; Zemdegs et al., 2016). Remarkably, it has been shown that intraperitoneal insulin injection reverses STZ-induced depressive-like behaviors (Gupta et al., 2014) whereas long-term treatments with the insulin-sensitizing drug metformin produced similar beneficial effects in mice fed an HFD for 16 weeks (Zemdegs et al., 2019).

Type 2 diabetes is the most prevalent subtype of diabetes and is characterized by an inability to respond adequately to endogenous insulin. Although the mechanisms underpinning insulin resistance are not fully understood, it is well known that the adipose tissue (AT) plays a major role through the release of a large variety of factors in plasma called adipokines (Rosen and Spiegelman, 2006). These hormones do not similarly influence insulin signaling. Adiponectin and leptin, for example, have been shown to improve insulin sensitivity whereas resistin and Tumor Necrosis Factor (TNF)-alpha would impair this physiological process (Dyck et al., 2006). Consequently, the contribution of various adipokines to the development of insulin resistance is complex and their putative role in the regulation of emotional state draws a particular attention (Nicolas et al., 2015; Cordeiro et al., 2019).

Apelin, a small peptide discovered by Fujinos' group in 1998 (Tatemoto et al., 1998) and secreted by the AT, has been recently characterized as an insulin sensitizer in both mice

(Boucher et al. 2005; Dray et al., 2008) and humans (Gourdy et al. 2017). Its biological action is mediated by a high affinity G protein-coupled receptor named apelin receptor (APJ). Both apelin and APJ are found in several peripheral tissues such as kidney, skeletal muscles, liver and heart (O'Carroll et al., 2000; Medhurst et al., 2003) where they exert positive effects on peripheral energy metabolism and insulin sensitivity by improving lipid oxidation, mitochondrial oxidative capacity and glucose utilization in muscles of HFD fed mice (Dray et al., 2008; Attané et al., 2012). Evidence demonstrates that apelin and APJ are also widely present within the brain (O'Carroll et al., 2000). Although less documented, the effects of apelin on neurobehavior have been addressed and its beneficial effects on emotionality have been proven. In particular, it has been shown that the acute intracerebroventricular injection of apelin reverses the depressive-like behaviors of rats induced by chronic stress procedures such as the social defeat or unpredictable chronic mild stress models (Tian et al., 2018; Zhang et al., 2019). Although the mechanisms by which apelin could improve behavioral outcome has yet to be solved, several studies reported that it exerts similar effects as antidepressant drugs including the improvement of hypothalamic-pituitary-adrenal axis (HPA axis) reactivity (Dai et al., 2018; Shen et al., 2019). However, the latter results have been challenged since it was also reported that apelin mimetic peptide produced depressive-like effects (Lv et al., 2012). Moreover, an upregulation of apelin and APJ expression has also been detected in the hippocampus of stressed rats (Xiao et al., 2018), indicating that the influence of this system on mood-related behaviors remains somewhat equivocal.

On this backdrop, this study aims at determining 1) the existence of a putative association between plasma apelin levels on depressive symptoms using the MAPT cohort composed of older subjects (Vellas et al., 2014) and 2) the impact of the genetic inactivation of the apelinergic system on different symptoms of depression in 12-month old mice or younger (6-month old) high fat fed animals. To do so, a comprehensive analysis of the metabolic and neurobehavioral status of $Ap^{-/-}$ and $APJ^{+/-}$ mice were conducted. The impact of the impairment of apelin signaling on monoaminergic neuronal activities (i.e., serotonergic and dopaminergic systems) was also determined to gain insight into the putative neurobiological circuits impacted by the genetic inactivation of the apelinergic system.

METHODS

Study Population

Participants in this study came from the randomized controlled trial Multidomain Alzheimer's Preventive Trial (MAPT, ClinicalTrials.gov [NCT00672685]), which included 1679 dementia-free older adults (aged 70+ years) for multidomain interventions (physical activity and nutritional counselling and cognitive training) with/without omega-3 supplementation, and placebo group over a 3-year period (Vellas et al., 2014). Older adults were recruited if any of the following criteria were met: 1) expressing spontaneous memory complaint, 2) having limitation in at least one instrumental activity of daily living, 3) demonstrating slow gait speed (i.e. lower than 0.8 m/s). Exclusion criteria were: 1) MMSE score \leq 24, 2) diagnosed dementia, 3) having difficulties in basic activities of daily living, 4) taking polyunsaturated fatty acid supplementation (see supplemental Table 1, for the consort flow diagram). The MAPT was approved by the ethics committee in Toulouse (CPP SOOM II) following the Declaration of Helsinki. Written consent forms were obtained from all participants. 839 participants (median age 75 years) in the MAPT has usable blood tests for apelin in the first year, and the current study analyzed 830 participants who had complete data of both the blood test and the geriatric depression scale (GDS).

Animals

All animals and experimental procedures complied with the ARRIVE guidelines and were conducted with respect to the European directive 2010/63/UE and approved by the French Ministry of Research (#12342-2017082111489451 v6). Apelin knock-out male mice ($Ap^{-/-}$) were generated by homologous recombination in embryonic stem cells (Genoway, Lyon, France). Briefly, C57Bl/6J blastocysts injected with recombined embryonic stem cells were implanted into surrogate mothers to generate chimeric mice. PCR and Southern blot analysis were then used to identify homozygous male mice of interest. APJ heterozygous mice ($APJ^{+/-}$) were generated by mating of C57Bl6/J WT littermates and heterozygous mice for the APJ mutation (Deltagen, San Carlos, CA, USA). $APJ^{+/-}$ were used because more than half of $APJ^{-/-}$ embryos die in utero due to cardiovascular impairments (Kang et al., 2013). Twelve-week-old male $Ap^{-/-}$, $APJ^{+/-}$ and C57/Bl6J mice (Charles River Lab) were used in this study. They were housed in standard conditions at the CRCA animal facility (12:12h light-dark cycle, 22°C, 60% relative humidity with ad libitum access to water and food).

Diet composition to induce metabolic disorders

Mutants and wild-type mice were fed a Standard diet (STD A04; SAFE diets, Augy, France) during the first weeks of their life and then maintained on this regimen or exposed to an HFD for 6 weeks (U8954P/251HF; SAFE diets, Augy, France) when they attained the age of 3 months. Diet was composed of proteins (20%), carbohydrates (35%) and lipids (45%) and we verified that tryptophan amounts– the precursor for 5-HT synthesis – were equivalent between STD and HFD to avoid putative bias for the interpretation of electrophysiological recordings of serotonergic neurons. Overall, the HFD was twice as high in term of Kcal compared to the STD (Zemdegs et al., 2016). Details of the experimental protocols are depicted in figures 1 and 2.

Metformin treatment

The insulin sensitizing agent metformin, was dissolved in tap water and delivered at the active dose of 300 mg/kg (Sigma-Aldrich, France) in the bottles for 4 weeks (Zemdegs et al., 2019). This pharmacological agent was maintained during the metabolic and neurobehavioral assessments, i.e. for 2 supplemental weeks. The route of administration allowed a constant amount of this compound during the exposition and daily water consumption was monitored in order to adjust metformin concentration. Details of the experimental protocol are depicted in figure 4.

Apelin assay

Plasma samples were collected at each visit of the participant and stored at -80 °C. Serum was then collected on EDTA, centrifuged at 1800 g, 4°C for 10 min and stored at -80°C. Serum apelin concentrations was measured using a nonselective apelin-12 EIA kit (Phoenix Pharmaceuticals; Belmont, CA) following manufacturer's instructions. Each sample was tested in duplicate and the manufacturer indicates that the inter-assay variation is <15%.

Metabolic parameters

Intraperitoneal glucose test tolerance. Animals were fasted for 5 hours with free access to water from 9 am to 2 pm. Blood glucose levels were monitored from tail sampling (Accu-check Performa glucometer; Roche, Boulogne-Billancourt, France) at 0, 15, 30, 45, 60, 90 and 120 min after the intraperitoneal glucose injection (2 g/kg).

Plasma insulin dosage. Blood samples were collected from the tip of the tail in EDTA coated tubes (Microcuvette CB300 EDTA K2, Sarstedt). Plasma was extracted after a 4°C centrifugation (13 500 rpm for 15min) and stored at -80°C. Insulin quantification was performed using an ELISA Kit (Mouse insulin ELISA #1247, Mercodia). The sensitivity of the kit mentioned by the supplier is 0.2 to 6.5 µg/L, which is perfectly compatible with the expected order of magnitude for plasma insulin levels. Importantly, due to the volume required for the analysis (10 µl per well), only one measure per animal was obtained.

HOMA-IR. The homeostasis model assessment of insulin resistance was calculated using the following equation: fasting blood glucose (mg/dL) x fasting insulin (ng/mL)/405. This value reflects the individual level of insulin resistance.

Behavioral tests

All behavioral experiments were assessed in the morning (between 9 am and 12 am) in order to avoid the impact of circadian rhythm on locomotor activity and metabolic parameters. During this behavioral phenotyping, mice were exposed once a day to a test from the less to the most stressful as follows: open field (OF), elevated plus maze (EPM), dark-light (DL), social interaction (SI), and tail suspension test (TST) or forced swim test (FST). All of them, except the DL were conducted in the presence of a weak light source in the experimental room (halogen lamp of 15-W 100 lx). The experiments presented in this study were conducted separately. Indeed, given the behavioral variability at different time of the year notably in term of aggressivity in the cages which then might impact behavioral performance (Theil et al., 2020), it was not relevant to plot the data.

The OF was assessed in an opaque circular Plexiglas arena (ø:40cm). Mice were placed the arena and were free to move for 10 minutes. They were then video-tracked with Noldus software (Ethovision, Wageningen, The Netherlands) in order to measure the number of entries and time spent in the center as well as the distance travelled. The latter parameter was used in all experiments to control putative bias related to hyper- or hypo-locomotion.

The EPM was performed in an apparatus composed of four 5 cm wide and 35 cm long arms. Two of them are opened (anxiogenic part) and the others are closed by 15 cm high walls. During 5 minutes, each mouse was free to explore the apparatus and the number of entries as well as the time spent in each the opened and closed arms were measured.

The DL was achieved in a two-chamber apparatus composed of a dark chamber and an enlightened one (White compartment: 250 (D) x 250 (W) x 240 (H) mm; Black compartment: 160 (D) x 250 (W) x 240 (H) mm; Door: 70 x 70 mm). During 10 minutes the mouse was free to explore the apparatus and the time spent in each compartment was measured. The dark compartment was illuminated by a dim red light (60 W; 4 lx), whereas the enlightened compartment was brightly illuminated with a 60-W (400 lx) light source as recommended (Bourin and Hascoët, 2003).

The SI test was realized in a three-chamber apparatus. During a first 10-minutes session, mice were allowed to explore the apparatus and two empty cages, one of them being used to contain an exhibited mouse. Indeed, during the second session, an exhibited familiar mouse was placed in one of the two empty cage and the tested mouse was free to explore and interact with its littermate or the empty cage. Social interaction was calculated as the percentage of interaction time with the exhibited mouse: $\text{time spent interacting with the mouse} / (\text{time spent interacting with the mouse} + \text{the empty cage}) \times 100$.

For the FST, mice were individually placed in a beaker (height: 23 cm; diameter: 20 cm) containing 15 cm of water (23 ± 2 °C) for 6 min. Floating or only slight movement to maintain balance was considered immobility. For the scoring, we followed the recommendations described in the original article, i.e., that the immobility time was considered only on the last 4 minutes of the test (Porsolt et al., 1977).

For the TST probing the same behavior than the FST (i.e., resignation, mice were hanged by their tails with a tape to a hook for 6 minutes. Immobility time was manually scored for the whole duration of the test as initially described (Steru et al., 1985). This test was specifically applied in mice fed an HFD because a strong impairment of swimming (inability to keep their head above water) was detected in these animals as previously observed in mice under this diet (Lloyd and Reyes, 2020).

Anxiety and depressive Z-scores

Z-normalization was applied for anxiety (EPM and DL) and depressive-like state (SI and TST/FST) in order to obtain an integrative score reflecting these specific endophenotypes. For each mouse in a specific behavioral test, normalized z-value was obtained by subtracting the

average of the control population by the value of the individual and then divided by the standard derivation of the control population as described previously (Guilloux et al., 2011). The anxiety z-score included the time spent in the center in the EPM, the time spent in the dark in the DL (and the center time/distance moved ratio of the OF for the last figure). The same weight was attributed to each test for the calculation of the z-score. With respect to the depressive phenotype, the z-score included the percentage of time interacting with the familiar mouse in the SI and the time of immobility in the FST or TST.

In vivo Electrophysiology

After behavioral and metabolic experiments mice were anesthetized with chloral hydrate (400mg/kg, ip) and then placed in a stereotaxic apparatus. Single-unit recordings of serotonergic neurons located in the dorsal raphe (DR) were performed using a glass micropipette (Stoelting, Wood Dale, IL, USA) prepared from a gravitational puller (Narishige, London, United-Kingdom) and filled with a 2M NaCl solution to reach an impedance ranging between 2.5 to 5 M Ω . Recording micropipettes were dipped into the DR using the following coordinates: 0.2-0.5 mm posterior to the interaural line on the midline and lowered at 3.5 mm from the brain surface. Serotonergic neurons were characterized using two main criteria: a regular firing rate (0,5 to 2,5 Hz) and a long-duration positive action potential. Several tracks were performed to measure the average discharge frequency of DR 5-HT neurons and the number of neurons recorded per track. We also characterized bursting 5-HT neurons with the peculiarity of an interspike interval <12ms. Their burst frequency and the mean of spike/burst were determined for this neuronal subpopulation.

Regarding the recording of dopaminergic neurons, micropipettes were dipped into the ventral tegmental area (VTA) using the following coordinates: -3 to -3.3 mm posterior, \pm 0.3 to 0.6 mm lateral and lowered at 4.1 to 4.6 mm from brain surface. Dopaminergic neurons were identified according to the criteria previously described by (Grace and Bunney, 1983). Regarding VTA DA neurons displaying a bursting mode, the onset of a burst was defined as two spikes occurring <80 ms apart, with the termination of the spike being defined as two spikes occurring >160 ms apart. The burst frequency and the mean spike/burst were also calculated using Spike2 software (Cambridge Electronic Design).

Statistical analyses

Clinical data

The data were presented as mean (standard deviation) or median (interquartile range) as appropriate. General linear models (GLMs) were performed with the first year GDS score (i.e., at the same time-point as plasma apelin assessment) as the dependent variable and the apelin value as the independent variable. To better understand the association between apelin and GDS, three different adjustments were included in the model, i.e., unadjusted (with only apelin data), age and sex adjusted, and full covariates (age, sex, first year BMI, MAPT group, education, vascular risk and baseline GDS) adjusted. Descriptive data among stratified GDS groups were compared by chi-square test for categorical variables and Wilcoxon rank-sum test for continuous variables with Mann-Whitney U test as post-hoc test. Ordinal logistic regression model was performed with stratified GDS groups as the dependent variable and the apelin value as the independent variable. The logistic regression also included three adjustments. Data analysis was performed by SAS 9.4 with a two-tailed significance of 0.05.

Preclinical data

Data were analyzed using Prism8 software (Graphpad, San Diego, USA). Results were represented as mean \pm SEM. Statistical analysis relied on two-tailed unpaired student's t-test. The threshold of significance was set at $p < 0.05$.

RESULTS

High apelin plasma levels of apelin is associated with the severity of depression in elderly patients

We first questioned the putative relationship between circulating apelin levels and the severity of depressive symptoms in patients from the MAPT cohort (Vellas et al., 2014) (supplemental Table 2). Results of GLMs showed that although plasma apelin levels were not found to be significantly associated with GDS in both the unadjusted and age- and sex-adjusted models, a significant association was found in the fully-adjusted model. Specifically, a higher plasma apelin level was associated with a higher GDS score ($p=0.022$, table 1). The latter results led us to envision that apelin might be critical in controlling emotional behavior.

The genetic inactivation of apelin in 12-month old mice produces antidepressant-like effects

To address this issue and to gain insight into the role of the apelinergic system in emotionality, we characterized the metabolic and neurobehavioral phenotypes of apelin knock-out ($Ap^{-/-}$) mice (Figure 1A). As a first step and in order to match the MAPT cohort performed in elderly subjects, we analyzed 12-month old mice for which biological markers of aging such as an accumulation of senescent cells in different tissues has been previously demonstrated (Hall et al., 2016).

Our results showed that 12-month old $Ap^{-/-}$ mice display the same metabolic status than wild-types (figures 1B-G). On the same animals, we first conducted two behavioral tests that interrogate core symptoms of depression ie, sociability and resignation using the SI and FST, respectively. In the SI test, a non-familiar mouse was introduced in one compartment and time spent interacting with this animal over the empty compartment was used as an index of sociability. As shown in figure 1H, $Ap^{-/-}$ mice showed a trend toward a higher time spent interacting with the familiar mouse compared to $Ap^{+/+}$ mice, albeit not statistically significant ($p=0.06$). Of note, during the habituation session for which there is no mouse in the device, wild-types and mutants spent the same time in the compartments, thus excluding any bias for one of the two compartments (data not shown). In the FST, which monitored the escape behavior of mice placed in an inescapable tank filled with water, our result indicated that the time of immobility was significantly lower in $Ap^{-/-}$ mice compared to wild-types. Consequently, it appeared that the genetic deletion of apelin decreased resignation (figure 1I)

without any bias related to change in locomotor activity (supplemental table 3). Collectively, these results provide evidence that the loss of apelin promote antidepressant-like effects as illustrated by a low value of depression z-score in $Ap^{-/-}$ mice relative to wild-types (figure 1J). Since anxiety is often associated with depression, we also assessed this behavior using two relevant paradigms namely the EPM and DL. In the EPM, a cross-shaped platform that consists of two open and two closed arms elevated from the floor, anxiety-like behavior is reflected by avoidance of the open (anxiogenic) arms. The frequencies of entry (not shown) and time spent in the open arms of apelin deficient mice were found indistinguishable from $Ap^{+/+}$ mice (figure 1K). In the DL, in which anxiety-like behavior is reflected by avoidance of an anxiogenic illuminated compartment, $Ap^{-/-}$ mice spent more time in the dark compartment relative to $Ap^{+/+}$ mice indicating an anxious behavior (figure 1L). However, when calculating the anxiety z-score, level of anxiety of $Ap^{-/-}$ mice was found in a normal range (figure 1M).

The genetic inactivation of apelin or its preferred APJ receptor in 6-month old mice fed an HFD impairs glucose metabolism and neurobehavior

Because a large body of evidence indicates that HFD can serve as a pathological trigger to promote signs of aging (Spencer et al., 2017), we then sought to determine to what extent the exposure of young 6-month old $Ap^{-/-}$ mice to an HFD for 6 weeks reverberate on metabolic and emotional parameters (figure 2A).

Our results showed that high fat fed $Ap^{-/-}$ mice displayed a significant higher fasting insulinemia and HOMA-IR without any alteration of glucose tolerance relative to wild-types under the same regimen (figures 2E-G). These metabolic impairments reminiscent of higher insulin resistance in young $Ap^{-/-}$ mice were accompanied by neurobehavioral changes. Indeed, although, depressive-like behavior remained unchanged in mutants (figures 2H-J), $Ap^{-/-}$ mice harbored a more pronounced anxiogenic-like profile compared to wild-types in the EPM and DL (figures 2K-L). These findings were further confirmed by the anxiety z-score (figure 2M).

In order to determine whether this behavioral phenotype involved the apelin receptor (APJ), we conducted the same metabolic and behavioral experiments in young $APJ^{+/-}$ mice harboring a partial loss of the apelin receptor. In these mice fed an HFD for 6 weeks, although fasting glycemia was not impacted relative to wild-types exposed to the same diet, signs of insulin resistance (ie, significant higher insulinemia and HOMA-IR index associated with a significant impairment of glucose tolerance) were noticed (supplemental figure 1). At the behavioral level and as observed in $Ap^{-/-}$ mice, $APJ^{+/-}$ mice did not show sign of depressive-

like state (supplemental figure 2A-C) but were more anxious compared to the corresponding group of wild-types (supplemental figure 2D-F).

Together, these results strongly suggested that the impairment of apelin signaling in 6-month old mice fed an HFD favored anxiety without impacting depressive-like phenotype.

The genetic inactivation of apelin in 6-month old mice fed an HFD impairs decreases serotonergic and dopaminergic neuronal activities

In attempting to identify the neurotransmitter that might underpin the association between the impairment of apelin signaling in 6-month old mice fed an HFD and their high level of anxiety, we then conducted electrophysiological experiments aimed at recording the firing activities of DR 5-HT and VTA DA neurons. Our results indicated that the genetic inactivation of apelin in young mice fed an HFD resulted in a significant decrease in monoaminergic neurons discharges (figure 3). In the DR the firing rate of non-bursting 5-HT neurons was decreased by 25% (figure 3B). Moreover, given that a proportion of DR 5-HT neurons discharge with a bursting activity, we also studied the firing rate of this neuronal sub-population. Interestingly, 5-HT neurons with this mode of discharge were not impacted by the mutation in term of firing rate, burst frequency and number of spikes/burst in $Ap^{-/-}$ mice (figures 3C-E). Regarding the VTA, the analysis of electrophysiological properties of DA neurons also indicated that a lower firing frequency in young $Ap^{-/-}$ mice fed an HFD relative to wild-types fed with the same diet. Such a decrease was not related to a lower burst frequency and/or spikes/burst (figures 3G-J).

The improvement of glucose metabolism is dispensable for the beneficial effects of the insulin sensitizer metformin on neurobehavior in 6-month old $Ap^{-/-}$ mice fed an HFD

A last series of experiments was conducted to highlight the putative causal relationship between insulin resistance and the higher level of anxiety. To this end, we evaluated the metabolic and behavioral effects of the insulin sensitizing drug metformin. Young $Ap^{-/-}$ mice were fed an HFD for 6 weeks while being administered with metformin or its vehicle (figure 4A). Our results clearly indicated that metformin did not improve metabolic parameters in these mutants (figures 4B-G). This lack of effect cannot be attributed to the use of a subactive dose since metformin significantly improved glucose tolerance in young $Ap^{+/+}$ mice fed an HFD (supplemental figure 3A-B). At the behavioral level, metformin attenuated anxiety in $Ap^{-/-}$ mice fed an HFD (figures 5A-C) compared to the corresponding group of mice injected

with the vehicle and this behavioral feature is confirmed by the lower anxiety z-score (figure 5D).

DISCUSSION

The present study shows that the severity of depressive symptoms in older patients is positively correlated to plasma apelin levels. The higher the plasma apelin levels, the greater the severity of depression. In line with this observation, we reported that 12-month old $Ap^{-/-}$ mice display an antidepressant-like phenotype, raising the possibility that apelin mediates pro-depressive effects. However, the role of this hormone in the regulation of emotional states is more complex than it appears since different results were obtained in 6-month old $Ap^{-/-}$ mice fed an HFD. Indeed, in these mice, the genetic inactivation of apelin combined with an obesogenic environment is associated with a higher insulin resistance, an exacerbated anxiety and a reduction of DR 5-HT and VTA DA neuronal activities relative to wild-types. Remarkably, although metformin fails to improve insulin sensitivity, it attenuates anxiety in these 6-month old $Ap^{-/-}$ mice fed an HFD. Collectively, our results lead us to envision that the enhancement of apelin signaling might exert differential effects on emotionality according to the age of the mice and, likely, their metabolic state.

One of the most remarkable results reported herein, is the fact that high plasma apelin levels are associated with increased depressive symptoms in older adults. Although such a relationship has been poorly investigated, a recent study shows that depression also positively correlates with plasma apelin levels in peritoneal dialysis patients. In particular, the percentage of depressive patients was 7% in the low group, 23% in the medium group and 92% in the high group (Gok Oguz et al., 2016). These findings echo an earlier preclinical study reporting a pro-depressive-like effect in response to the administration of apelin-13 in the mouse central nervous system (CNS) (Lv et al., 2012). They are also consistent with the observation that the intracerebroventricular (ICV) administration of apelin increased plasma ACTH and corticosterone (Taheri et al., 2020) thereby highlighting an upregulation of the HPA axis. However, conflicting results have been yielded and several lines of evidence suggest, on the contrary, that the ICV administration of apelin reverses the depressive-like behaviors of rats subjected to chronic stress procedures such as the social defeat (Tian et al., 2018; Xiao et al., 2018), the repeated forced swim stress (Dai et al., 2018) or the unpredictable chronic mild stress models (Zhang et al., 2019). In an attempt to gain insight into the role of apelin in emotional states, we sought to determine whether $Ap^{-/-}$ mice show

neurobehavioral changes. Our results indicated that 12-month old mutant mice displayed an antidepressant-like behavior in the SI and FST tests whereas anxiety was not changed. The latter results support a pro-depressive role for apelin and confirm our predictions based on our clinical data. Although a direct pathogenetic link cannot be established between apelin and affective disorders, the presence of this hormone and its receptor in the brain (O'Carroll et al., 2000) raises the possibility of a central action of apelin. In particular, given that brain inflammation or oxidative stress may precipitate MDD, one would expect that such mechanisms are activated by apelin. Nevertheless, the apelinergic system has been shown to promote neuroprotective effects through anti-inflammatory and antioxidants properties in the central nervous system (Zeng et al., 2014; Xin et al., 2016). Regarding possible indirect effects, one of the main effects of apelin at the peripheral level is to improve insulin sensitivity (Dray et al., 2008) and glyceamic control in diabetic patients (Habchi et al., 2014). Again, it is thus difficult to assign the putative pro-depressive effect of this hormone in 12-month old mice through its action on insulin signaling since emotional disorders often occur in response to insulin resistance (André et al., 2014; Gupta et al., 2014; Zemdegs et al., 2016; Chireh et al., 2019; Zemdegs et al., 2019).

To further address the relationship between the apelinergic system and emotional behavior, we decided to test younger $Ap^{+/+}$ and $Ap^{-/-}$ mice (i.e., 6-month old) challenged with an HFD for 6 weeks. Since $Ap^{-/-}$ mice exhibit markers of senescence (Rai et al., 2017) and a short-period of HFD is sufficient to induce age-related impairments (Adler et al., 2014; Al-Kuraishy et al., 2019; Santos et al., 2019), we consider these animals suitable to model an accelerated aging and therefore to compare their metabolic and behavioral phenotypes to those of 12-month old $Ap^{-/-}$ mice fed an STD diet. Regarding metabolic parameters, we showed that both $Ap^{-/-}$ and $APJ^{+/-}$ mice exhibited more pronounced signs of insulin resistance than wild-types as evidenced by a higher HOMA-IR or a lower ability to face a glucose challenge. These findings were consistent with data demonstrating that the APJ receptor is expressed in the pancreas and that the specific deletion of APJ in beta-cells produced an impairment of glucose tolerance and an attenuation of glucose-stimulated insulin secretion (Han et al., 2015). At the behavioral level, we provided evidence that 6-month old $Ap^{-/-}$ and $APJ^{+/-}$ mice fed an HFD displayed a higher level of emotional disturbance. Indeed, using the z-score method normalizing and integrating emotional behaviors across complementary measures, we showed that depressive symptoms remained unchanged in $Ap^{-/-}$ and $APJ^{+/-}$ mice fed an HFD compared to the corresponding groups of wild-types under the same diet.

However, these mutants displayed a higher level of anxiety. It has recently been demonstrated that long-term HFD negatively reverberates on both depressive-like symptoms and anxiety (Zemdegs et al., 2016; Zemdegs et al., 2019). Consequently, it seems that a short HFD exposure promotes only anxiety and this is consistent with the fact that such behavioral impairment often precedes the onset of depression (Malhi and Mann, 2018). It is important to note that apelin is released under HFD, especially under the influence of TNF-alpha (Daviaud et al., 2006). This could be considered as an adaptive mechanism aimed at limiting insulin resistance in wild-type mice fed an HFD whereas this process would be abolished or limited respectively in $Ap^{-/-}$ and $APJ^{+/-}$ mutant mice thereby explaining their metabolic and behavioral anomalies. In any case, it is clearly demonstrated that apelin expression is positively correlated to body mass index (Heinonen et al., 2005) whereas hypocaloric diet associated with weight loss reduces apelin expression and secretion (Castan-Laurell et al., 2008). This is a further argument to support the idea that apelin is secreted under HFD to counteract pathological insulin-resistance.

To gain insight into the mechanisms underlying the anxiogenic phenotype of 6-month old $Ap^{-/-}$ mice fed an HFD, we conducted *in vivo* electrophysiological recordings of monoaminergic neurons (i.e., serotonergic and dopaminergic neurons). These systems are commonly associated with anxio/depressive-like phenotype and their activity can be modulated by changes in peripheral glucose and insulin homeostasis (Kleinridders et al., 2015). In this study, we found that the firing rate of dorsal raphe (DR) 5-HT neurons was decreased in $Ap^{-/-}$ mice. Specifically, our results indicated that the firing rate of DR 5-HT neurons was decreased in the subpopulation of neurons discharging in a single spike manner. Consequently, one might assume that the extracellular levels of 5-HT could be decreased at the nerve terminals such as the amygdala, the frontal cortex or the ventral hippocampus. To the best of our knowledge, the influence of apelin on the serotonergic system has been poorly investigated although the DR is endowed with a high density of APJ (De Mota et al., 2000). With respect to VTA DA neurons, we also found a decrease in VTA DA neuronal activity. Hence, the attenuation of brain apelin signaling in $Ap^{-/-}$ or $APJ^{+/-}$ mice might cause an attenuation of monoaminergic tone in discrete brain areas at the origin of the anxiety phenotype. This hypothesis is compatible with the observation that the ICV injection of apelin-13 causes anxiolytic effects which is reversed by the 5-HT_{2A} receptor antagonist methysergide or the D₂ receptor antagonist haloperidol (Telegdy and Jaszberenyi, 2014).

Although our results strongly suggest a role of central apelin signaling in the regulation of anxiety in young animals, we next questioned the putative causal link between peripheral

metabolism and neurobehavior. To do so, we fed these 6-month old $AP^{-/-}$ mice an HFD with metformin. This pharmacological agent is an insulin sensitizing drug whose beneficial effects on glucose metabolism have been attributed to several processes, including the activation of AMPK pathway, reduction in hepatic gluconeogenesis, and stimulation of glucose uptake in skeletal muscle (Rena et al. 2017). Our results showed that $Ap^{-/-}$ mice fed an HFD were resistant to metformin since this pharmacological agent failed to improve peripheral insulin sensitivity in these mutants. This property could result from the inability of the metformin to increase plasma apelin levels since clinical evidence indicates that metformin increases plasma levels of apelin in patients with T2D (Fan et al., 2015; Al-Kuraishy et al., 2018). Despite this lack of beneficial effects on metabolic parameters, metformin produced anxiolytic effects in $Ap^{-/-}$ mice as previously reported in animal models of comorbid diabetes and anxiodepressive-like phenotype (Ji et al., 2019; Zemdegs et al., 2019). This lack of effect of metformin on metabolism may appear surprising in light of previous data showing that compounds such as rosuvastatin reversed short-term HFD induced-metabolic impairments in mice (Al-Kuraishy et al., 2019). Such a discrepancy might depend on the ability of drugs to impact body weight since rosuvastatin decreases this parameter (Al-Kuraishy et al., 2019) while metformin fails to do so. It is also conceivable that the combination of metformin with another insulin sensitizing drug would have positively reverberate on metabolism (Al-Kuraishy et al., 2020). Nevertheless, our results are remarkable as they suggest that the behavioral properties of metformin on anxiety in 6-month old $Ap^{-/-}$ mice fed an HFD do not require an improvement of peripheral metabolism. This agrees with recent observations (Milnstein and Ferris, 2021) and reinforces the idea that the regulation of emotional state by the apelinergic system recruits central mechanisms. In particular, one would expect that metformin promotes anxiolytic effects in $Ap^{-/-}$ mice fed an HFD by improving brain insulin sensitivity.

Overall, our findings obtained in $Ap^{-/-}$ mice strongly suggest a differential role of the apelinergic system in the regulation of emotionality. Indeed, it is believed that in young animals, the stimulation of apelin signaling exerts a beneficial effect on emotionality and more particularly on anxiety by stimulating monoaminergic neurotransmissions. On the contrary, in older animal, the activation of the apelin system would exert a pro-depressive effect. It is possible that such negative effects result from a progressive depletion, with age, of apelin in the plasma and the brain (Rai et al., 2017) thereby limiting the ability of this adipokine to counteract emotional disorders. In light of these results, future experiments are

required to test the influence of the acute or chronic pharmacological modulation of the apelinergic system in animal models of anxio-depressive disorders (with or without metabolic comorbidity). This will likely shed some light on the finely-tuned regulation of emotional responses by the central and/or peripheral apelinergic system. Another issue that should be addressed, in addition to age and metabolic status, is the question of the sex-dependent vulnerability. It was demonstrated that diabetes is a stronger risk factor for depression in women than in men (Deischinger et al., 2020) suggesting that our study would have brought more robust conclusions if conducted in female mice. However, a recent study reported that males were more prone than females to develop anxiety in response to HFD (Bridgewater et al., 2017) likely due to a higher vulnerability to HFD-induced metabolic impairments (Shin et al., 2020). This question remains open and deserves our attention for future investigations.

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Table 1

Adjustment	Variables	Sample size	Coefficient	p value	95% CI
Unadjusted		830			
	Apelin		1.36E-04	0.3473	-1.48E-04, 4.21E-04
Age and sex adjusted		830			
	Apelin		7.00E-05	0.6280	-2.14E-04, 3.54E-04
	Age		0.10	<.0001**	0.06, 0.13
	Sex (Male)		-0.32	0.0883	-0.68, 0.05
Full adjusted		812			
	Apelin		2.48E-04	0.0220*	3.58E-05, 4.60E-04
	Age		0.05	0.0010**	0.02, 0.08
	Sex (Male)		-0.06	0.6896	-0.33, 0.22

*: p < 0.05, **: p < 0.01

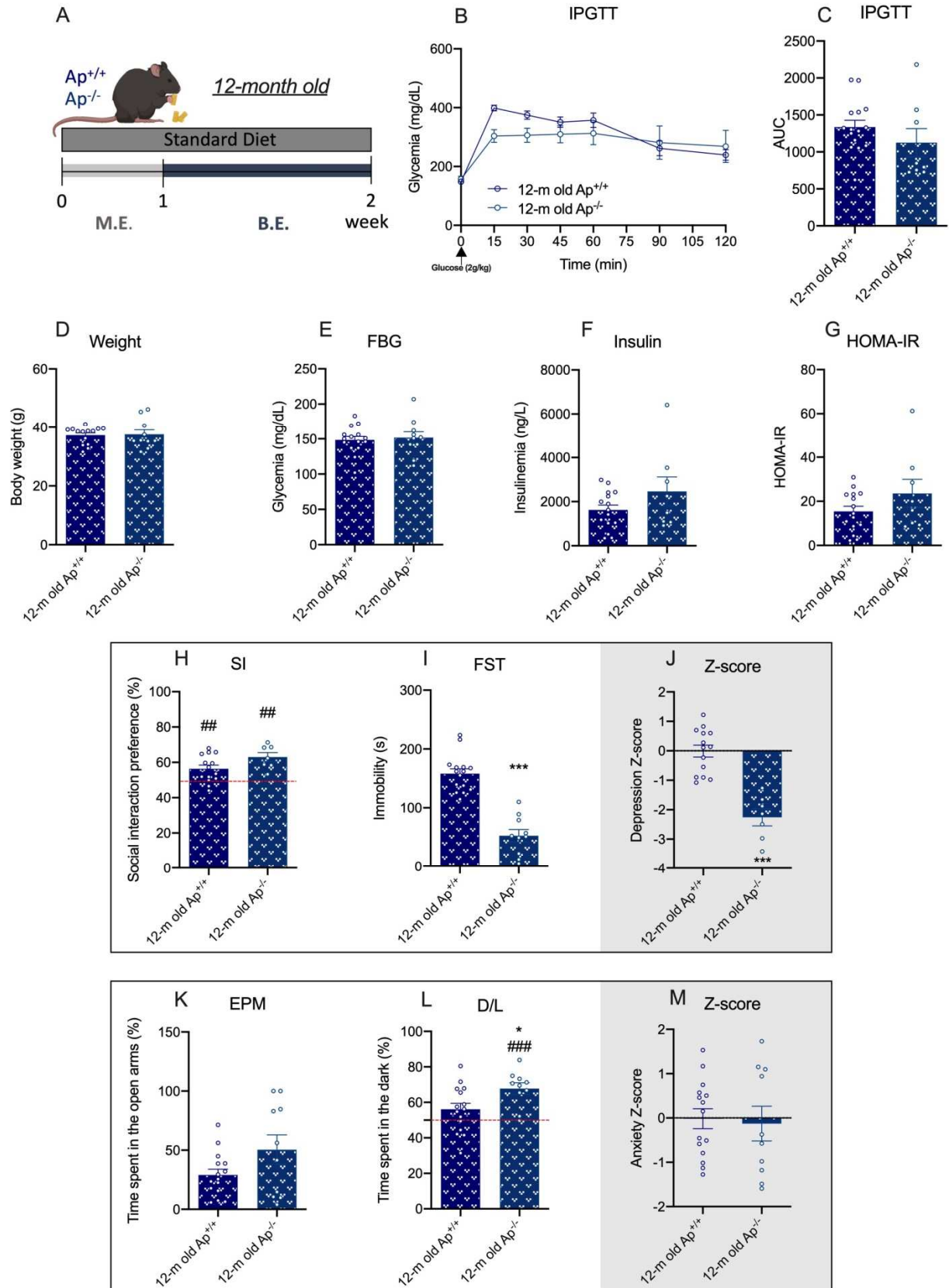


Figure 1: Peripheral metabolism and anxio/depressive-like phenotype in 12-month old $Ap^{-/-}$ mice fed a ND. (A) Experimental protocol. M.E.: Metabolic Experiments, B.E.:

Behavioral Experiments. All data are mean \pm SEM. **(B)** Time course of fasting glycemia (mg/dL) during an intraperitoneal glucose tolerance test (IPGTT). **(C)** Area Under the Curve (AUC) of IPGTT. T-test $p=0.04$. **(D)** Mouse body weight (g). T-test $p=0.86$. **(E-F)** Fasting blood glucose (mg/dL) and insulinemia (ng/L). T-tests: $p=0.731$ and $p=0.149$; respectively. **(G)** Homeostasis model assessment of insulin resistance (HOMA-IR). T-test $p=0.159$. **(H)** Social interaction time (%) in the social interaction (SI) test. T-test $p=0.066$. One sample T-test compared to 50% p-values: $p=0.008$ ($Ap^{+/+}$) and $p=0.002$ ($Ap^{-/-}$). **(I)** Time of immobility (sec) in the forced swim test (FST). T-test $p<0.001$. **(J)** Depression Z-score including social interaction preference (SI) and immobility time (FST). T-test $p<0.001$. **(K)** Time spent in open arms (%) in the elevated plus-maze experiment (EPM). T-test $p=0.080$. **(L)** Time spent in the dark compartment (%) in the dark-light (DL) test. T-test $p=0.026$. One sample T-test compared to 50% p-values: $p=0.093$ ($Ap^{+/+}$) and $p<0.001$ ($Ap^{-/-}$). **(M)** Anxiety Z-score including time spent in the open arms (EPM) and time spent in the dark compartment (D/L). T-test $p=0.797$. * $p<0.05$ and *** $p<0.001$: significantly different from wild-type $Ap^{+/+}$ mice. ## $p<0.01$ and ### $p<0.001$ significantly different from chance level (50%).

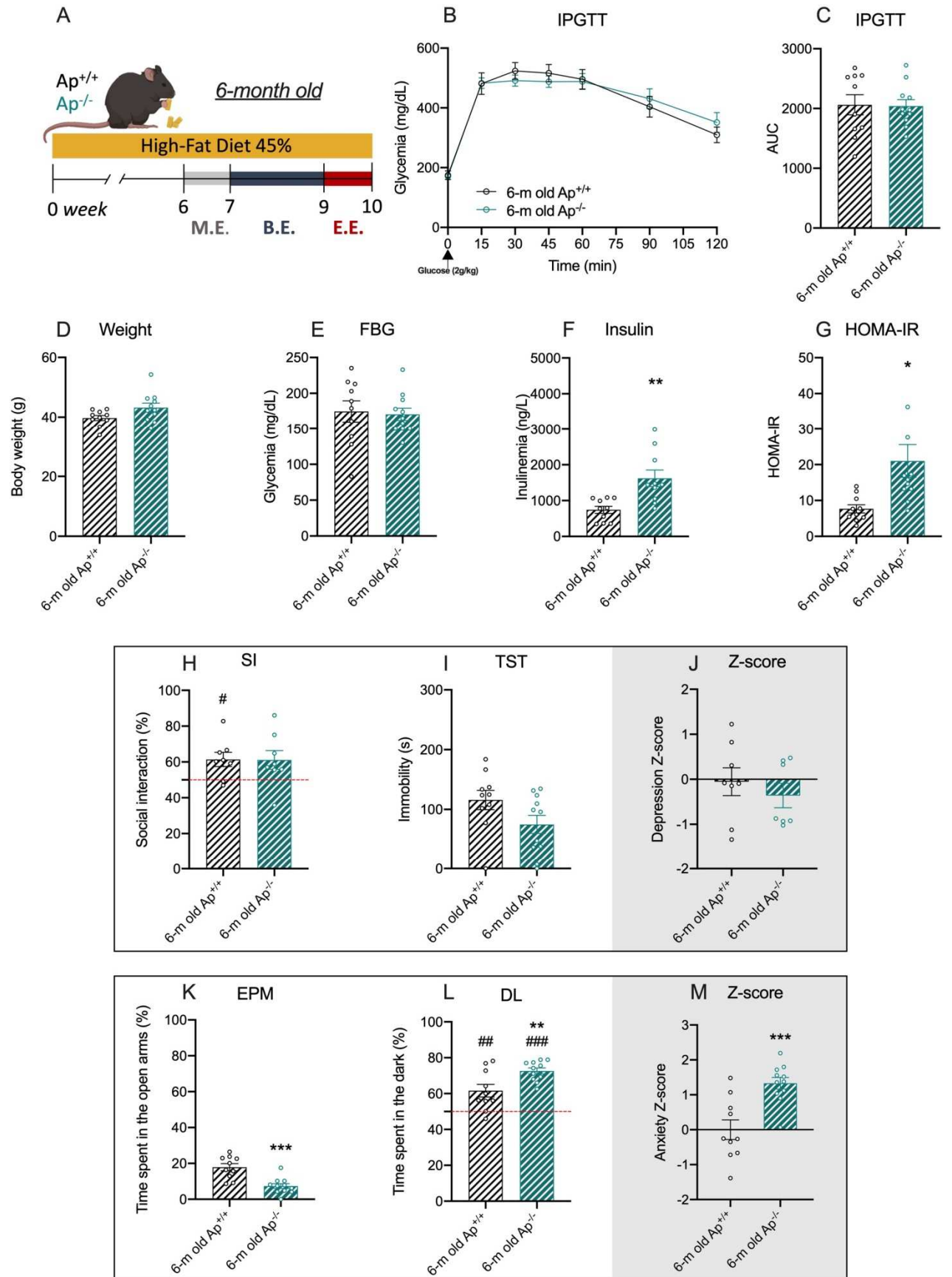


Figure 2: Peripheral metabolism and anxio/depressive-like phenotype in 6-month old $Ap^{-/-}$ mice fed an HFD for 6 weeks. (A) Experimental protocol. M.E.: Metabolic Experiments, B.E.: Behavioral Experiments, E.E.: Electrophysiology Experiments. All data are mean \pm SEM. (B) Time course of fasting glycemia (mg/dL) during an intraperitoneal glucose test tolerance test (IPGTT). (C) Area Under the Curve (AUC) of IPGTT. T-test $p=0.918$. (D) Mouse body weight (g). T-test $p=0.063$. (E-F) Fasting blood glucose (mg/dL) and insulinemia (ng/L). T-tests: $p=0.816$ and 0.002 ; respectively. (G) Homeostasis model assessment of insulin resistance (HOMA-IR). T-test $p=0.013$. (H) Social interaction time (%) in the social interaction (SI) test. T-test $p=0.969$. One sample T-test compared to 50% p-values: $p=0.022$ ($Ap^{+/+}$) and $p=0.071$ ($Ap^{-/-}$). (I) Time of immobility (sec) in the tail suspension test (TST). T-test $p=0.079$. (J) Depression Z-score including social interaction preference (SI) and immobility time (TST). T-test $p=0.473$. (K) Time spent in open arms (%) in the elevated plus-maze experiment (EPM). T-test $p<0.001$. (L) Time spent in the dark compartment (%) in the dark-light (DL) test. T-test $p=0.008$. One sample T-test compared to 50% p-values: $p=0.009$ ($Ap^{+/+}$) and $p<0.001$ ($Ap^{-/-}$) (M) Anxiety Z-score including time spent in the open arms (EPM) and time spent in the dark compartment (D/L). T-test $p<0,001$. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$: significantly different from wild-type $Ap^{+/+}$ mice. #, $p<0.05$, ## $p<0.01$ and ### $p<0.001$ significantly different from chance level (50%).

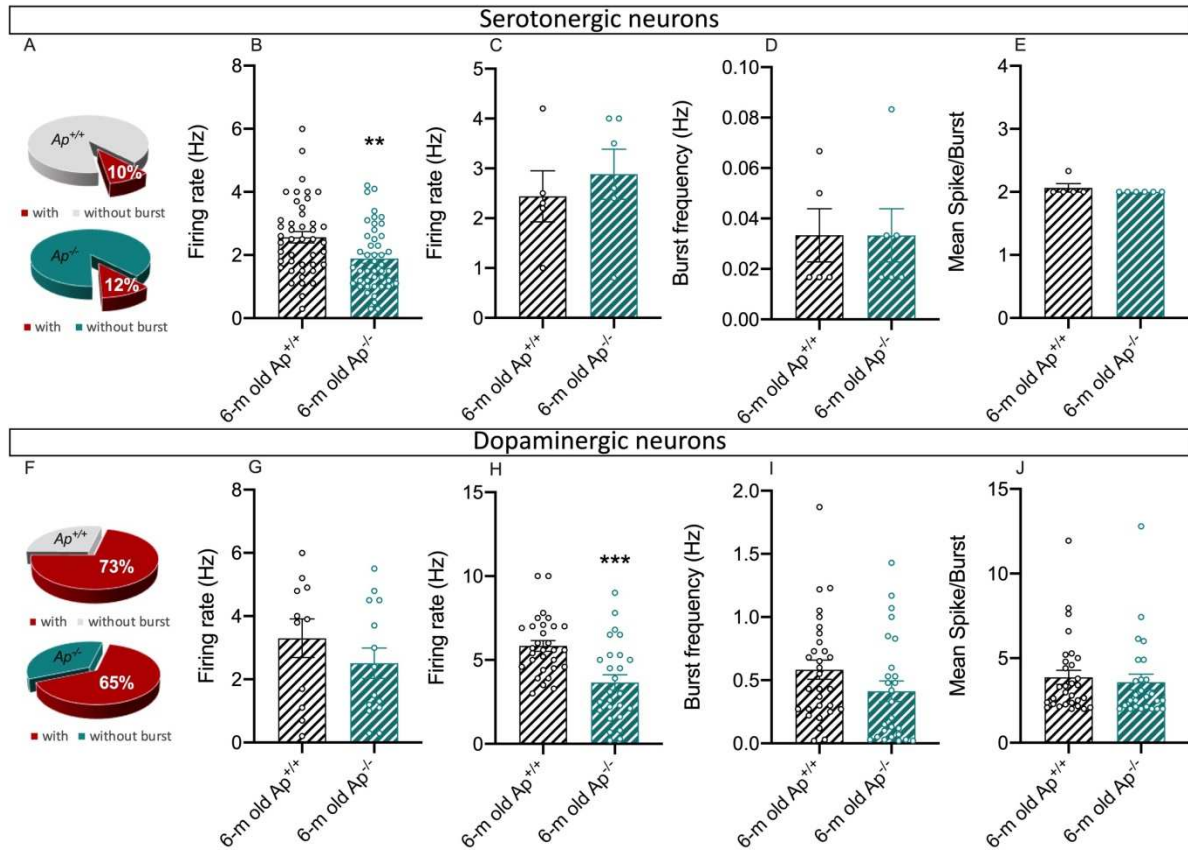


Figure 3: Decreased serotonergic and dopaminergic firing rates in 6-month old $Ap^{-/-}$ mice fed an HFD for 6 weeks. *In vivo* electrophysiology in anesthetized mice. (A) Percentage of DRN 5-HT non-bursting and bursting neurons in $Ap^{+/+}$ and $Ap^{-/-}$ mice. All data are mean \pm SEM. (B) Firing rate of non-bursting 5-HT neurons, T-test $p=0.005$; (C) Firing rate, T-test $p=0.554$, (D) burst frequency, T-test $p=0.999$ and (E) spike per burst, T-test $p=0.967$ of DRN 5-HT bursting neurons. (F) Percentage of VTA DA non-bursting and bursting neurons in $Ap^{+/+}$ and $Ap^{-/-}$ mice (G) Firing rate of non-bursting DA neurons, T-test $p=0.005$; (H) Firing rate of bursting DA neurons, T-test $p=0.554$, (I) burst frequency, T-test $p=0.131$ and (J) mean spike per burst, T-test $p=0.649$ of VTA DA bursting neurons. ** $p<0.01$ and *** $p<0.001$: significantly different from wild-type $Ap^{+/+}$ mice.

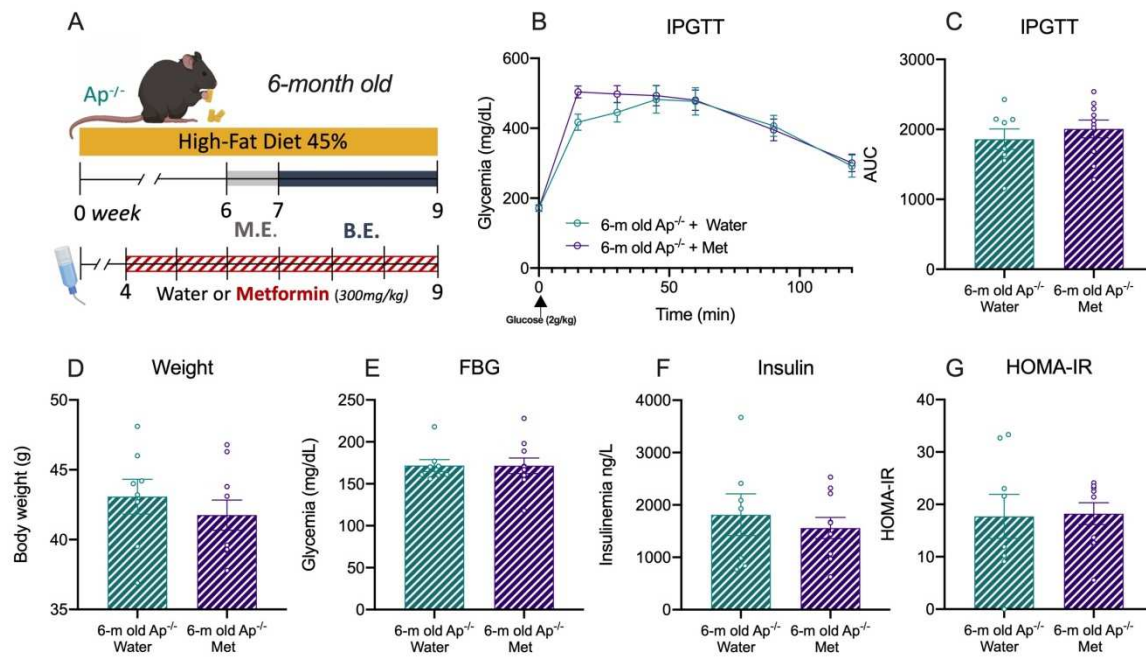


Figure 4: Effects of metformin on peripheral metabolism in 6-month old $Ap^{-/-}$ mice fed an HFD for 6 weeks. (A) Experimental protocol. M.E.: Metabolic Experiments, B.E.: Behavioral Experiments. All data are mean \pm SEM. (B) Time course of fasting glycemia (mg/dL) during an intraperitoneal glucose test tolerance test (IPGTT). (C) Area Under the Curve (AUC) of IPGTT. T-test $p=0.4599$. (D) Mouse body weight (g). T-test $p=0.4316$. (E-F) Fasting blood glucose (mg/dL) and insulinemia (ng/L). T-test $p=0.9838$ and $p=0.6094$. (G) Homeostasis model assessment of insulin resistance (HOMA-IR). T-test $p=0.9108$.

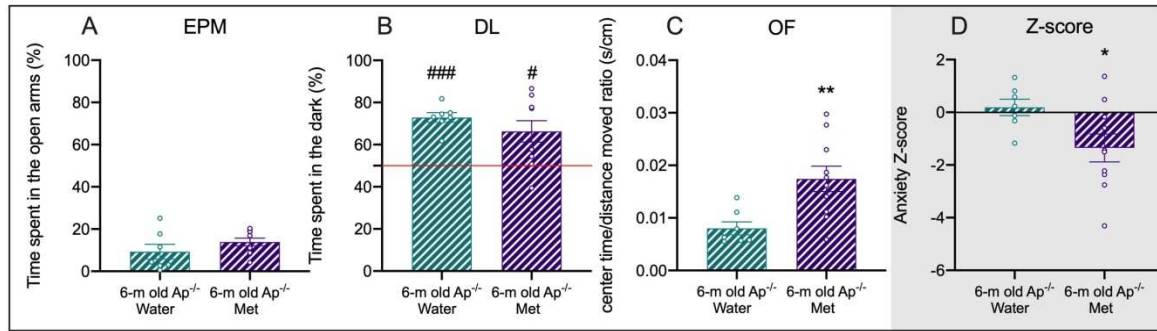


Figure 5: Effects of metformin on anxious behavior in $Ap^{-/-}$ mice. All data are mean \pm SEM. **(A)** Time spent in open arms (%) during the elevated plus-maze (EPM). T-test $p=0.2244$. **(B)** Time spent in the dark compartment (%) during the dark-light (DL) test. T-test $p=0.3209$; One sample T-test compared to 50% p -values: $p<0.001$ ($Ap^{+/+}$) and $p=0.0109$ ($Ap^{-/-}$). **(C)** Ratio of time spent in center (sec) / distance travelled (cm) in the open field (OF). T-test $p=0.0081$. **(D)** Anxiety Z-score including time spent in the open arms (EPM), time spent in the dark compartment (D/L) and center time/distance moved ratio (OF). T-test $p=0.0405$. * $p<0.05$ and ** $p<0.01$: significantly different from $Ap^{+/+}$ mice. # $p<0,05$ and ### $p<0.001$: significantly different from chance level (50%).

GRAPHICAL ABSTRACT

