Brain tumor necrosis factor-α mediates anxiety-like behavior in a mouse model of severe obesity

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ABSTRACT

Although the high prevalence of anxiety in obesity increasingly emerges as significant risk factor for related severe health complications, the underlying pathophysiological mechanisms remain poorly understood. Considering that chronic inflammation is a key component of obesity and is well known to impact brain function and emotional behavior, we hypothesized that it may similarly contribute to the development of obesity-related anxiety. This hypothesis was experimentally tested by measuring whether chronic food restriction, a procedure known to reduce inflammation, or chronic anti-inflammatory treatment with ibuprofen improved anxiety-like behavior and concomitantly decreased peripheral and/or hippocampal inflammation characterizing a model of severe obesity, the *db/db* mice. In both experiments, reduced anxiety-like behaviors in the open-field and/or elevated plus-maze were selectively associated with decreased hippocampal tumor necrosis factor-α (TNF-α) mRNA expression. Highlighting the causality of both events, chronic central infusion of the TNF-α blocker etanercept was then shown to be sufficient to improve anxiety-like behavior in *db/db* mice. Lastly, by measuring the impact of *ex-vivo* etanercept on hippocampal synaptic processes underlying anxiety-like behaviors, we showed that the anxiolytic effect of central TNF-α blockade likely involved modulation of synaptic transmission within the ventral hippocampus. Altogether, these results uphold the role of brain TNF-α in mediating obesity-related anxiety and provide important clues about how it may modulate brain function and behavior. They may therefore help to introduce novel therapeutic strategies to reduce anxiety associated with inflammatory conditions.

Key words: TNF-α, Inflammation, Neuroinflammation, Anxiety, Obesity, Hippocampus

HIGHLIGHTS

- Decreasing inflammation reduces anxiety-like behavior in obese *db/db* mice
- Increased anxiety in obesity is associated with hippocampal inflammation
- Anti-inflammatory strategies improving anxiety target hippocampal TNF-α expression
- Blockade of central TNF-α expression reduces obesity-related anxiety-like behavior
- Blockade of TNF-α modulates synaptic transmission in the ventral hippocampus

1. INTRODUCTION

Obesity is a major public health concern with continuous and alarming growth worldwide. In addition, mounting evidence steadily reports a high prevalence of neuropsychiatric comorbidities, including both depressive and anxiety symptoms (Brumpton et al, 2013; Brunault et al. 2012; Gariepy et al, 2010; Gaysina et al, 2011). Although less extensively studied than obesity-related depressive comorbidities (Dawes et al, 2016; Luppino et al, 2010), available data suggest that anxiety and obesity are also tightly linked. Anxiety often promotes weight gain and obesity, which are reciprocally associated with a higher vulnerability for anxiety (Brumpton *et al*, 2013; Brunault *et al*, 2012; de Zwaan *et al*, 2009; Gariepy *et al*, 2010; Gaysina *et al*, 2011). To worsen the picture, both conditions represent significant risk factors for related severe comorbidities, including cardiovascular and metabolic diseases (Haslam and James, 2005; Roy-Byrne *et al*, 2008). Identifying the pathophysiological mechanisms underlying development of anxiety symptoms in the context of obesity may therefore prevent and/or reduce the development of multiple disorders in chain. Interestingly, mounting evidence suggests that obesity-related inflammatory processes may be key players in increasing anxiety associated with this condition (Almeida-Suhett *et al*, 2017; Pierce *et al*, 2017; van Reedt Dortland *et al*, 2013).

Chronic low-grade inflammation in the periphery and within the brain increasingly appears to be a key component in severe obesity, strongly connected with the metabolic dysregulations that classically characterize this condition (Capuron *et al*, 2017; Castanon *et al*, 2014; Castanon *et al*, 2015; Lasselin *et al*, 2014). Moreover, abundant data document a causal role for dysregulated activation of inflammatory processes, and associated brain function alterations, in the induction of anxiety-related symptoms in different chronic inflammatory conditions (Chen *et al*, 2015; Steptoe *et al*, 2013). Experimentally, peripherally-released inflammatory cytokines induce the production of brain cytokines and

influence pathways involved in emotional regulation, including neurotransmitter metabolism, neuroendocrine function and neural plasticity (Miller *et al*, 2013; Salim *et al*, 2012). Moreover, anti-inflammatory treatments seem to reduce anxiety-like behaviors in animal models of inflammatory diseases (Larrieu and Layé, 2018; Lee *et al*, 2016).

Converging evidence suggests that obesity-related inflammation may similarly be relevant to the onset of neuropsychiatric comorbidities (Ambrosio *et al*, 2018; Capuron *et al*, 2017; Castanon *et al*, 2014; Castanon *et al*, 2015), although the underlying mechanisms are still poorly understood. Firstly, peripheral inflammatory status correlates with symptoms of anxiety in patients with obesity or metabolic syndrome (Ambrosio *et al*, 2018; Pierce *et al*, 2017; Tayefi *et al*, 2017). Moreover, weight loss-induced reduction in inflammation is associated with decreased anxiety (Capuron *et al*, 2011). Supporting these clinical findings, obesity-driven inflammation in rodents is associated with impaired cognition and increased anxiety-like behaviors (de Cossio *et al*, 2017; Decarie-Spain *et al*, 2018; Dinel *et al*, 2011, 2014). However, the different behavioral alterations do not necessarily appear concomitantly in obese mice (André *et al*, 2014; Bocarsly *et al*, 2015; Kang *et al*, 2014), suggesting the potential involvement of distinct neurobiological mediators. Additionally, although increased inflammation has consistently been reported in the hypothalamus of obese subjects (Thaler and Schwartz, 2010; Thaler *et al*, 2012), mounting evidence shows that the hippocampus is another central site of obesity-related inflammation (de Cossio *et al*, 2017; Dinel *et al*, 2011, 2014; Erion *et al*, 2014). The key role this region plays in memory formation and emotional regulation (Bannerman *et al*, 2002) therefore suggests that increased inflammation in the hippocampus could play a pivotal role in both cognitive alterations and anxiety associated with obesity. Several studies point to increased hippocampal interleukin-1β (IL-1β) or IL-6 as strong mediators of obesity-related cognitive deficits (Erion *et al*, 2014; White *et al*, 2009), with intra-hippocampal administration of IL-1 receptor antagonist preventing synaptic

dysfunction and associated cognitive impairment in obese mice (Erion *et al*, 2014). Recent preclinical studies performed in rodent models of obesity also point to a role for inflammatory processes in the development of anxiety-like behaviors (Kurhe *et al*, 2014; Soto *et al*, 2018; Wang *et al*, 2018), but little is known in that case about the inflammatory mediators involved. Their identification is however an important issue given the high prevalence and impact of symptoms of anxiety on health and well-being in obese individuals, and the necessity of improving the management and treatment of these symptoms.

Leptin receptor-deficient *db/db* mice, which model leptin resistance and the consequent impairment of appetite and energy homeostasis often reported in severely obese patients (Pan and Myers, 2018), are particularly suitable to address this important issue. Their excessive food intake indeed promotes progressive development of severe obesity and related inflammation and metabolic alterations. More importantly for the present study, they also display enhanced anxiety-like behaviors associated with high levels of hippocampal IL-1β, IL-6 and tumor necrosis factor (TNF)-α (Dinel et al, 2011, 2014; Stranahan et al, 2016). We sought to determine whether caloric restriction or chronic anti-inflammatory treatment could decrease hippocampal inflammation, and concomitantly reduce anxiety-like behaviors in *db/db* mice. Indeed, caloric restriction is known to reduce obesity-related systemic inflammation, including in *db/db* mice (Bates *et al*, 2005; Kanda *et al*, 2015), and inflammation-related behavioral alterations in different inflammatory conditions (Kim *et al*, 2016; Kurhe *et al*, 2014; Lutter *et al*, 2008; MacDonald *et al*, 2014). As both strategies reduced anxiety-like behaviors and specifically decreased hippocampal TNF-α expression in *db/db* mice, we further studied for the first time the causality of both events. To this end, we measured the consequences of direct brain pharmacological TNF-α inhibition on obesity-related anxiety-like behavior and underlying hippocampal synaptic processes.

2. METHODS AND MATERIALS

2.1. Animals and treatments

All animal care and use were conducted according to the relevant French (Directive 87/148, Ministère de l'Agriculture et de la Pêche) and international (Directive 10/63, European Community) legislation and approved by the Institutional Animal Care and Use Committee (approval ID: 5012047-A). Every effort was made to minimize suffering and the number of animals used. Five-week-old male *db/db* (C57BLKS/J-*lepr*^{db}/*lepr*^{db}) and their control littermates *db/+* (C57BLKS/J-*lepr*$^{db/+}$) mice (Charles River Laboratories, France) were housed individually under standard housing conditions in a temperature (23±1 °C)- and humidity (40%)-controlled animal room with a 12-hour light/dark cycle (0700-1900) and with free access to food and water except otherwise specified. All mice were fed a standard diet (Lab-Diet 5K52), except for the ibuprofen experiment (see below). Independent sets of mice were used for each experiment.

Chronic food restriction: 24-hour food intake was monitored for the first 4 days in *ad libitum*-fed mice. They were then randomly allocated to control (fed *ad libitum*) or food-restricted groups (n=10/group) (Figure 1A). Within each genotype, food intake and body weight were similar when restriction begun. Restricted *db/db* mice were provided with the average amount of food consumed by control *db/+* mice daily for 7 weeks, leading to a restriction of about 30% compared to unrestricted *db/db* mice (Bates *et al*, 2005). A similar 30% restriction rate was applied to restricted *db/+* mice.

Ibuprofen: As described by Llorens-Martin *et al*, 2014, the non-steroidal anti-inflammatory drug ibuprofen (Sigma-Aldrich, MO, USA) was formulated into standard animal chow (Research-Diet D0933105, NJ, USA) at a final concentration of 375 ppm (about 2.8 mg/day). It was freely provided to ibuprofen-supplemented *db/db* mice (n=10) for 7 weeks, whereas control *db/db* (n=9) and *db/+* (n=10) mice consumed isocaloric standard

chow (Research-Diet D10001) (Figure 2A). This non-invasive treatment procedure was previously shown to reduce inflammation-induced increase of anxiety-like behavior without any detectable behavioral impact in saline-treated mice (Llorens-Martin *et al*, 2014). Based on these findings, and in order to minimize the number of animal used, the ibuprofen-supplemented diet was not provided to *db/+* mice.

Etanercept (ETN): The human recombinant TNF-α receptor fusion protein ETN (Enbrel; Wyeth Pharmaceuticals, France), which competitively binds murine TNF-α (Haji *et al*, 2012), was continuously infused into the third cerebral ventricle (28 days, 5 µg/kg/h) through a cannula connected to an osmotic mini-pump (Alzet, model 1004D) (Figure 3A). Control mice received artificial cerebrospinal fluid (aCSF). On the day of surgery, mice were anesthetized with isoflurane and placed in a stereotaxic apparatus. The cannula was implanted using the following coordinates: anteroposterior = 0.3mm from bregma; lateral = 1mm from midline; depth = 2.8mm from the skull. While still anesthetized, a subcutaneous incision was made upon the back of the mouse to insert the osmotic mini-pump between the skin and muscle. The dose, route and duration of treatment were chosen based on previously published data (Guggilam *et al*, 2011; Haji *et al*, 2012). The initial number of mice per group was 8 for *db/+* mice and 10 for *db/db* mice. However, 2 mice from each genotype died during surgery, reducing the numbers to 7 *db/+* mice per group (7 infused with aCSF and 7 with ETN), 8 *db/db* mice infused with aCSF (control mice) and 10 with ETN.

2.2. Behavioral measurements

Behavioral testing was always performed in the morning (0800-1200), under conditions of dim light and low noise, and started after 2 weeks of recovery post-surgery (ETN experiment) or 4 weeks of exposure to ibuprofen supplementation or food restriction. In order to reduce the potential effect of circadian influences on anxiety-like behavior, mice were tested in a

random order that was changed between each test. Anxiety-like behaviors were first assessed in the open-field (10-minute test) and a week later in the elevated plus-maze (5-min-test), by applying the experimental conditions previously used to highlight the "anxious" phenotype of *db/db* mice (Dinel *et al*, 2011; Dinel *et al*, 2014; Sharma *et al*, 2010). Behavior was automatically recorded and scored (Smart Videotrack System, Bioseb, France) as previously described (André *et al*, 2014; Dinel *et al*, 2011). All equipments were thoroughly cleaned between each session.

Open-field (OF): The OF apparatus was an unfamiliar, inescapable square area (40x40x16cm) without bedding litter. A central area, considered more anxiogenic compared to the periphery was defined as a virtual 10x10cm square in the middle. Mice were randomly placed in alternate corners and allowed to freely explore the OF for 10 minutes. Parameters measured to evaluate anxiety-like behavior were the number of entries and the percentage of time spent in the central area (Belzung and Griebel, 2001).

Elevated Plus-Maze (EPM): Mice were exposed to the EPM apparatus made of 4 arms (30x8cm), 2 of them closed with walls providing shelter as compared to 2 open-arms without walls, which are more anxiogenic since the EPM was placed 120cm above the floor (Dinel *et al*, 2011). Mice were placed in the center facing an open-arm and allowed to freely explore for 5 minutes. A reduction of the percent of time spent and number of entries into the open arms (scored as such when all four limbs were placed into an arm) is considered as an anxiety-like index, independent of locomotor activity (Belzung *et al*, 2001). When deeper analysis of anxiety-like behavior was needed, additional anxiety-like indexes, namely risk assessment behaviors known to be rarely displayed by anxious animals (Wall *et al*, 2003), were measured. This included reduced frequency of stretch-attend posture (when mouse stretched forward in an open-arm with forelegs only and then retracted back to its original

position) and unprotected head-dips (defined as peering over the edge of an open-arm with head, neck and shoulders).

2.3. Biochemical measurements

Five to seven days after completion of behavioral testing, mice were euthanized in a random order with terminal isoflurane anesthesia, within a few seconds after being picked up from their home cage. They were always euthanized in the morning (0800-1100) in order to reduce hormone level variability linked to circadian rhythmicity of release. Blood samples were immediately collected via cardiac puncture, centrifuged, and aliquots of plasma were stored for later analysis of cytokine and hormone content with mouse cytokine and metabolic hormone Milliplex kits respectively (Merk-Millipore, France) following the manufacturer's instructions (Dinel *et al*, 2011, 2014). Total plasma corticosterone was also measured with an in-house radioimmunoassay using a highly specific antibody provided by H. Vaudry (University of Rouen, France) as previously described (Richard *et al*, 2010). All samples were run in duplicate. Inter and intra-assay coefficients of variation and low limits of detectability for the Milliplex kits and the RIA are provided in Table S1.

After blood collection, mice were perfused with chilled PBS via the ascending aorta to remove all traces of blood from tissues. Brains were rapidly extracted from the skulls and carefully dissected. The hippocampus (HPC) and prefrontal cortex (PFC) were immediately collected into labeled-sterile tubes which were frozen with dry ice and stored at -80°C until assaying. Total RNA was extracted using TRIzol (Invitrogen) and RNA purity and concentration were determined using a Nanodrop spectrophotometer (Nanodrop technologies, Wilmington, DE). One microgram of RNA was reverse-transcribed to cDNA as previously described (Fourrier *et al*, 2017). Quantitative real-time RT-PCR was then performed using Taqman gene expression assays for sequence-specific primers (Applied Biosystems, Foster

City, CA), which are designed to span exon-exon junctions and therefore prevent amplification from contaminating genomic DNA. β2-microglobulin was used as the housekeeping gene. Reactions were performed in duplicate according to manufacturer instructions. The PCR program consisted of 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Fluorescence was measured using an AB 7500 Real-Time PCR system (Applied Biosystems, Foster city, CA). The calculation of relative expression levels was performed according to the methods of Schmittgen and Livak (2008) and plotted as fold change relative to the appropriate control condition.

2.4. Electrophysiology

Horizontal hippocampal slices (350µm) were prepared with a vibrating blade microtome (VT1000S, Leica Microsystems) in an oxygenated artificial cerebrospinal fluid (aCSF) containing (in mM): 23 $NaHCO_3$, 87 NaCl, 75 sucrose, 25 glucose, 4 $MgCl_2$, 2.5 KCl, 0.5 $CaCl_2$, 1.25 NaH_2PO_4. Slices were stored at room temperature and recordings started after at least 1hour of rest. aCSF solution for storage contained (in mM): 23 $NaHCO_3$, 130 NaCl, 11 Glucose, 2.4 $MgCl_2$, 2.5 KCl, 1.2 $CaCl_2$, 1.2 NaH_2PO_4. Electrophysiological consequences of TNF-α inhibition were assessed by adding ETN to the recording solution, at a final concentration of 10 µg/mL (Haji *et al*, 2012), for at least three hours. For whole-cell patch-clamp recordings, spontaneous excitatory postsynaptic currents (sEPSCs) were recorded in the CA1 stratum radiatum of the ventral hippocampus (vHPC) at the holding potential of -60mV (Figure 4A). Recording pipettes were filled with balanced intracellular solutions containing (in mM): 128 KGlu, 20 NaCl, 1 $MgCl_2$, 1 EGTA, 0.3 $CaCl_2$, 2 Na^{2+}-ATP, 0.3 Na^{+}-GTP, 10 glucose, buffered with 10 HEPES, pH 7.3, osmolarity 290mOsm (Thomazeau *et al*, 2016). GABAergic transmission was not blocked to preserve excitability of the network. However, GABAergic events were not detectable in our conditions (Le Roux *et al*,

2006). Whole-cell access resistances measured in voltage-clamp were in the range of 5-20mΩ. sEPSCs amplitude, rise time, decay time, and inter-event interval time were analyzed with Axograph X. One or two cells per animal and per condition (aCSF ± etanercept) were recorded, and at least four animals per group were employed.

2.5. Statistical analysis

Data was analyzed using the GraphPad Prism (version 5) software, using one-way (for the ibuprofen experiment: treatment) or two-way ANOVAs (genotype x treatment), with repeated measures for the time factor where appropriate. Between-group differences were determined using *post-hoc* Fisher's LSD test when interactions were significant. Pearson's correlation coefficients were calculated to determine potential relationships between treatment-induced behavioral and biochemical changes. Lastly, unpaired t-tests were used to compare sEPSCs between control aCSF- and etanercept-treated slices. As already mentioned, 4 mice died during surgery in the etanercept experiment. In addition, some values were identified as outliers using the extreme studentized deviate method, which allows for identification of a single outlier in a normal sample (Walfish, 2006). Lastly, some data are missing because of occasional experimental issues (e.g. videotrack recording failure, lack of enough plasma samples to perform all planned assays, wrong duplicates, low-quality RNA). Overall, data obtained from 7 to 10 mice/group was used to perform the statistical analyses depending on the experiments and measures considered.

3. RESULTS

3.1. Chronic food restriction (CFR) reduced anxiety-like behavior, plasma corticosterone levels and cytokine production in *db/db* mice

CFR significantly blunted body weight gain in both genotypes compared to their respective controls ($F_{(1,32)}$=30.6, P<0.001; Figure 1B), although *db/db* mice remained heavier than *db/+* mice by the end of the CFR period ($F_{(1,32)}$=129.2, P<0.001; Figure S1). By that time, plasma leptin levels also remained higher in *db/db* than *db/+* mice ($F_{(1,34)}$=33.3, P<0.001; Table S2) regardless of their feeding conditions (CFR: P>0.1). Plasma insulin levels were only affected by CFR in restricted-*db/+* mice (genotype x CFR: $F_{(1,35)}$=31.1, P< 0.001; Table S2) but not *db/db* mice (restricted *vs.* non-restricted: P>0.1).

We then assessed whether CFR may reduce enhanced anxiety-like behaviors displayed by *db/db* mice (Dinel *et al*, 2011, 2014). In the OF, percent of time spent exploring the anxiogenic central area was smaller in *db/db* than *db/+* mice ($F_{(1,32)}$=45.2, P<0.001; Figure 1C), but CFR attenuated this difference ($F_{(1,32)}$=4.5, P<0.05). Similarly, it reduced the latency of the first entry into this area in restricted-*db/db* mice compared to non-restricted ones (49.9±14.3 s *vs.* 126.6±23.7 s; genotype x CFR: $F_{(1,32)}$=7.4, P<0.01), whereas both *db/+* groups displayed the same latency (34.8±6.9 s and 35.2±5.9 s). Analyzing the time-course of central entries confirmed that CFR effect was only significant in *db/db* mice ($F_{(1,60)}$=11.7, P<0.01; CFR x time: $F_{(4,60)}$=7.0, P<0.001; Figure S2), which also displayed less entries ($F_{(1,33)}$=23.1, P<0.001; Figure 1D) and tended to spend less time ($F_{(1,31)}$=3.7, P=0.06; Figure 1E) in the open-arms of the EPM than *db/+* mice. CFR increased both measures ($F_{(1,33)}$=4.6, P<0.05 and $F_{(1,31)}$=5.3, P<0.05 respectively), this effect being particularly notable in restricted-*db/db* mice. Improvement of anxiety-like behavior was not due to locomotor changes since total distance travelled in the OF, although globally higher in *db/+* mice than *db/db* mice (17.85±0.68 m *vs.* 10.54±0.85 m; P<0.001), was not changed by feeding conditions regardless of the genotype (P>0.1). However, CFR significantly increased the distance travelled in the central area in restricted-*db/db* mice as compared to non-restricted ones (P<0.05). Similarly, CFR significantly increased the number of entries in the open-arms

of the EPM in *db/db* mice (Figure 1D) without changing the number of closed arms entries (9.56±1.42 *vs.* 12.90±2.15; P>0.1 for non-restricted *vs.* restricted *db/db* mice respectively). A question arose then of what biological correlates might underlie these behavioral improvements.

Among the main biological factors altered in *db/db* mice that are capable of acting centrally to modulate behavior, corticosterone and cytokines are likely candidates (Erion *et al*, 2014; Stranahan *et al*, 2008). We therefore determined whether CFR normalized hypothalamo-pituitary-adrenal (HPA) axis and/or inflammatory disturbances previously reported in *db/db* mice (Dinel *et al*, 2011, 2014; Stranahan *et al*, 2008). Plasma corticosterone levels were higher in *db/db* than *db/+* mice ($F_{(1,35)}$=15.2, P<0.001; Figure 1F), but selectively reduced in restricted-*db/db* mice (genotype x CFR: $F_{(1,35)}$=7.5, P<0.01; control- *vs.* restricted-*db/db* mice: P<0.05). Overall, these levels correlated with percent of time spent in the center of the OF (*r*=-0.38; P=0.05). Regarding plasma cytokines, all mice displayed similar plasma IL-1β and TNF-α levels (Table S2). In contrast, plasma IL-6 levels were increased in non-restricted-*db/db* mice compared to their *db/+* counterparts (P<0.05), but normalized after CFR (genotype x CFR: $F_{(1,30)}$=7.3, P<0.05; Figure 1G). *db/db* mice also displayed higher HPC mRNA expression of IL-1β ($F_{(1,32)}$=8.2, P<0.01), IL-6 ($F_{(1,33)}$=7.9, P<0.01), and TNF-α ($F_{(1,32)}$=24.3, P<0.001) than *db/+* mice (Figure 1H). CFR selectively reduced TNF-α expression in *db/db* mice ($F_{(1,32)}$=4.6, P<0.05; genotype x CFR: $F_{(1,32)}$=4.0, P=0.05), without impacting the other cytokines. In the PFC, *db/db* mice displayed higher mRNA expression of IL-1β ($F_{(1,33)}$=11.7, P<0.01), IL-6 ($F_{(1,34)}$=5.8, P<0.05), and TNF-α ($F_{(1,32)}$=4.5, P<0.05) than *db/+* mice (Figure 1I). A genotype x CFR interaction was found only for TNF-α ($F_{(1,32)}$=4.1, P=0.05), even if restricted- *vs.* non-restricted-*db/db* mice did not significantly differ (P>0.1). Importantly, hippocampal, but not cortical, TNF-α mRNA expression correlated with first

entry latency (r=0.75; P<0.001) and percent of time spent in the center of the OF (r=-0.53; P<0.01), and open-arm entries in the EPM (r=-0.41; P<0.05).

We then assessed the respective role of enhanced HPA axis activation and/or brain inflammation in mediating increased anxiety-like behaviors in *db/db* mice. Hippocampal glucocorticoid receptor (GR) activation is known to modulate HPA axis activity, inflammation and anxiety-like behaviors (Stranahan *et al*, 2008; Tronche *et al*, 1999). GR activation through its phosphorylation was higher in *db/db* than *db/+* mice (GR/GAPDH: $t_{(15)}$=0.06, p=0.95, Figure S3B; P-GR/GR: $t_{(13)}$=4.60; p<0.001; Figure S3C). Interestingly, chronic GR antagonism with mifepristone did not improve anxiety-like behaviors in *db/db* mice, despite selectively normalizing plasma IL-6 levels (genotype x treatment: $F_{(1,33)}$=4.9, P<0.05; Figure S3D-E). Conversely, it significantly increased anxiety-like behaviors in *db/+* mice compared to their placebo-treated counterparts (genotype x treatment: $F_{(1,36)}$=4.3, P<0.05; Figure S3F) and tended to concomitantly stimulate hippocampal TNF-α mRNA expression (Figure S3F). These results further support brain TNF-α as potential mediator of the enhanced anxiety-like behaviors in *db/db* mice.

3.2. Chronic anti-inflammatory treatment reduced anxiety-like behavior and hippocampal TNF-α mRNA expression in *db/db* mice

We then investigated whether directly targeting inflammation in *db/db* mice with ibuprofen may improve anxiety-like behaviors. In the OF, the percent of time spent in the center tended to be globally different between the 3 groups ($F_{(1,22)}$=3.41, P=0.06; Figure 2B). Further analysis revealed that control *db/db* mice spent significantly less time in the center than *db/+* mice (P<0.05), but this difference ceased after ibuprofen treatment. Importantly, both *db/db* groups displayed the same total distance travelled in the OF (9.89±0.70 *vs.* 8.65±0.68m; P>0.1), supporting an anxiolytic effect of chronic anti-inflammatory treatment

rather than non-specific locomotor alterations. The number of entries in the open-arms of the EPM was also globally different between groups ($F_{(1,23)}$=6.18, P<0.01; Figure 2C), with both *db/db* groups displaying less entries than control *db/+* mice (P<0.05). Similarly, *db/db* mice tended to display reduced percent of time in the open-arms of the EPM than *db/+* mice regardless of their treatment (P<0.05; Figure 2C), although global treatment effect did not reach significance. Considering the anxiolytic effect of ibuprofen in the OF, we decided to deeply analyze anxiety-like behaviors in the EPM by additionally measuring risk assessment behaviors known to be rarely displayed by anxious animals (Wall *et al*, 2003). The frequency of stretch-attend postures ($F_{(1,24)}$=5.38, P<0.05) and head dips ($F_{(1,20)}$=18.66, P<0.001) was different between the 3 groups. Increased anxiety-like behaviors in control *db/db* mice was confirmed by the significant reduced frequency of stretch-attend postures (P<0.01) and head dips (P<0.001) compared to *db/+* mice (Figure 2C). Conversely, ibuprofen displayed an anxiolytic effect in *db/db* mice by significantly increasing these frequencies as compared to untreated *db/db* mice (P<0.05).

Akin to the aforementioned data, ibuprofen improved anxiety-like behavior without normalizing plasma corticosterone levels in treated *db/db* mice. Regardless of their treatment, *db/db* mice also displayed similar plasma levels of IL-1β and TNF-α to *db/+* mice (Table S2), as well as higher body weight ($F_{(1,132)}$=110.72, P<0.001; Figure S1), plasma IL-6 levels ($F_{(1,20)}$=6.04, P<0.01; *db/db* vs. *db/+* mice: P<0.05; Figure 2D), and hippocampal IL-1β ($F_{(1,20)}$=4.11, P<0.05; *db/db* vs. *db/+* mice: P<0.05) and IL-6 ($F_{(1,22)}$=11.57, P<0.001; *db/db* vs. *db/+* mice: P<0.01) mRNA expression (Figure 2E). However, ibuprofen selectively reduced the increased hippocampal TNF-α mRNA expression displayed by untreated *db/db* mice ($F_{(1,21)}$=13.52, P<0.001; *db/db* vs. *db/+* mice: P<0.01 and untreated- *vs.* ibuprofen-treated *db/db* mice: P<0.05; Figure 2E). Together, this data suggests that increased TNF-α

expression specifically in the brain may contribute to increase anxiety-like behavior in *db/db* mice, as they are both impacted by ibuprofen.

3.3. Chronic brain TNF-α blockade improved anxiety-like behavior in *db/db* mice

To directly assess the role of brain TNF-α, the potent TNF-α blocker Etanercept (ETN) was chronically infused intracerebroventricularly (icv). By the time of behavioral assessment, all mice were considered to have totally recovered from surgery since their body weight (Figure S1), as well as general locomotor activity (total distance travelled in the OF: 15.08±1.15 m *vs.* 10.52±0.71 m for *db/+ vs. db/db* mice respectively; $P<0.01$) had returned to levels similar to those recorded in the previous experiments. Importantly, body weight and locomotor activity remained unchanged by ETN regardless of the genotype (Treatment: $p>0.1$ for both body weight and distance travelled). *db/db* mice displayed lower number of visits ($F_{(1,23)}=9.4$, $P<0.01$; Figure 3B) and distance travelled in the center of the OF ($F_{(1,23)}=8.6$, $P<0.01$; not shown) than *db/+* mice, both measures being increased by ETN ($F_{(1,23)}=4.4$, $P<0.05$ and $F_{(1,23)}=4.0$, $P=0.05$ respectively). Although the global effect of genotype and treatment did not reach significance for the total percent of time spent in this area, it appeared smaller in control *db/db* than *db/+* mice, but not anymore after ETN treatment (Figure 3C). Confirming these data, detailed time-course analysis of this measure showed the effect of ETN in *db/db* mice ($F_{(1,52)}=4.7$, $P<0.05$; Figure 3D). In the EPM, *db/db* mice also visited less often (genotype: $F_{(1,22)}=8.7$, $P<0.01$; Figure 3E) and spent proportionally less time (genotype x ETN: $F_{(1,22)}=5.7$, $P<0.05$; Figure 3F) in the open-arms than *db/+* mice. Interestingly, *db/db* mice, but not *db/+* mice, treated with ETN displayed increased time spent in the open-arms (aCSF- *vs.* ETN-*db/db* mice: $P<0.05$; Figure 3F). Of note, behavioral improvement was likely independent from changes in peripheral inflammatory alterations as brain ETN infusion did not change plasma cytokine levels (Table

S2). Together, these results uphold the specific involvement of brain TNF-α as an important player in inducing anxiety-like behavior in *db/db* mice.

3.4. Ex-vivo blockade of TNF-α modulated synaptic transmission in the vHPC of *db/db* mice

To precisely decipher the neurobiological mechanisms linking increased hippocampal TNF-α and anxiety-like behavior, we measured the impact of ETN on excitatory synaptic transmission in the vHPC, which has been related to anxiety-like behavior (Bannerman *et al*, 2002). TNF-α-induced increase of synaptic transmission, translated in increased sEPSCs size, was previously linked to increased anxiety (Haji *et al*, 2012). We therefore hypothesized that ETN may improve anxiety-like behavior in *db/db* mice by reducing these parameters. To test this hypothesis, whole-cell patch-clamp recordings were used to examine sEPSCs shape and frequency in vHPC CA1 pyramidal neurons (Figure 4B). Although basal transmission was similar in both genotypes (frequency: $t_{(7)}=1.07$, $p=0.83$; amplitude: $t_{(7)}=0.223$, $p=0.32$), their sEPSCs were differentially affected by ETN. Whereas no detectable effect was found in *db/+* mice, ETN in *db/db* mice reduced both sEPSCs frequency ($t_{(8)}=2.636$, $p<0.05$) and amplitude ($t_{(8)}=2.407$, $p<0.05$) (Figure 4C-D). ETN treatment of hippocampal slices also lowered sEPSCs decay time in *db/db* mice ($t_{(7)}=2.480$, $p<0.05$, Figure 4E), whereas sEPSC rise time was unchanged whatever the genotype (Figure 4F). Of note, ETN did not change local long-term potentiation (LTP) in *db/+* or *db/db* mice ($F_{(1,44)}=0.03$, $p=0.87$) (Figure S4). Collectively, these results suggest that inhibition of TNF-α signaling improved synaptic transmission in the vHPC of *db/db* mice.

4. DISCUSSION

By using complementary approaches ultimately reducing inflammation, we report here a causal link between obesity-related inflammation, particularly in the hippocampus, and increased anxiety. Importantly, our results highlight an anxiolytic effect of the central blockade of TNF-α in obese mice, and provide evidence that this effect likely involves changes in vHPC synaptic transmission.

In line with clinical studies (Capuron *et al*, 2011), CFR improved anxiety-like behavior in obese mice. In agreement with previously published data (Collins *et al*, 2008; Iio *et al*, 2015; Kirchner *et al*, 2012), this anxiolytic effect was not related to non-specific changes of locomotion since CFR, as well as ibuprofen and ETN treatment, did not alter global locomotor activity regardless of the genotype. Supporting this notion further, increased anxiety-like behavior reported in *db/db* mice was shown to be independent from motor or motivational disturbances, although they globally displayed reduced locomotion as compared to *db/+* mice (de Cossio *et al*, 2017; Dinel *et al*, 2011, 2014). Besides reducing body weight as expected, CFR normalized HPA axis activity and selectively targeted plasma IL-6 and hippocampal TNF-α. Although these factors are known to participate to the pathophysiology of anxiety, they do not similarly contribute to the present behavioral improvement. In line with findings recently reported in other conditions also associated with increased anxiety (Lee *et al*, 2016; Shang *et al*, 2015), ibuprofen reduced anxiety-like behaviors in *db/db* mice without decreasing body weight, neither normalizing circulating IL-6 nor corticosterone. Conversely, chronic GR blockade normalized circulating IL-6 without improving anxiety-like behavior. Moreover, we recently showed that reducing hippocampal IL-6 expression failed to improve anxiety-like behavior in *db/db* mice (de Cossio *et al*, 2017). This confirms the dissociation between IL-6, corticosterone, and anxiety-like behavior in diet-induced obesity (DIO) models (André *et al*, 2014). Of note, corticosterone and hippocampal IL-1β and IL-6 are known to contribute to obesity-related memory deficits (Erion *et al*, 2014; Stranahan *et al*,

2008; White *et al*, 2009; Wosiski-Kuhn *et al*, 2014). In addition, the onset of anxiety-like behaviors in DIO mice was delayed compared with memory deficits (André *et al*, 2014). Together, these findings strengthen the assumption of a mechanistic dissociation between obesity-related cognitive alterations and anxiety-like behaviors.

Altered circulating levels and signaling pathways of several metabolic factors, notably adipokines and insulin, are another trademark of severe obesity. Interestingly, they not only regulate energy homeostasis by targeting the hypothalamus, but also behavior and mood by acting in the hippocampus (McGregor *et al*, 2015). Considering that they can interact with inflammatory processes (Capuron *et al*, 2017), these metabolic factors may contribute to increase obesity-related anxiety, as suggested by findings reporting anxiolytic effects of leptin administration in leptin-deficient mice (Asakawa *et al*, 2003). Leptin resistance displayed by *db/db* mice may similarly contribute to the development of their behavioral alterations. This has not been directly tested here, but several lines of evidence suggest that its role is unlikely predominant regarding the anxiolytic effects of the different interventional strategies used in the present study. Indeed, previously published studies reported no impact of CFR on serum adiponectin or leptin levels in *db/db* mice (Kanda *et al*, 2015; Stranahan *et al*, 2009). Extending these findings, we found here that CFR, as well as ibuprofen and ETN treatment, improved anxiety-like behaviors in *db/db* mice without impacting circulating leptin levels and leptin resistance. Akin to these findings, a number of studies have suggested that defective leptin signaling in *db/db* mice is directly involved in the dysregulations reported in the hypothalamus (Bouret *et al*, 2012), but not the hippocampus (for review, see Stranahan, 2015). Instead, hippocampal dysfunction rather appears as an indirect consequence of other systems' or functions' alterations (Stranahan *et al*, 2008; Erion *et al*, 2014). This was previously studied regarding the mechanisms underlying cognitive impairments, but the present data suggests that it is also likely the case for those underlying

increased anxiety. More studies directly addressing the role of leptin signaling in that context are however still necessary to draw a definitive conclusion. In addition, the release of adipokines, including leptin, and their potential impact on behavioral reactivity fluctuating over the day (see for review Challet 2017), it would be important to determine the metabolic and behavioral effects of circadian rhythms in *db/db* mice by testing them during their wake phase. Of note, CFR also failed to normalize plasma insulin levels in *db/db* mice, supporting a previous study reporting improvement of anxiety-like behavior in *db/db* mice independent of any change of glucose levels (Zhao *et al*, 2012). Conversely, improving insulin resistance, hyperglycemia or circulating resistin concentrations in those mice has no impact on anxiety-like behaviors (de Cossio *et al*, 2017). Interestingly, these findings are consistent with pharmacological studies suggesting that the beneficial effect of several anti-diabetic drugs on neuropsychiatric symptoms may involve, beyond improvement of hyperglycemia, reduction of inflammation (Gupta *et al*, 2014; Pomytkin *et al*, 2015).

We showed that both CFR and ibuprofen improved anxiety-like behaviors in *db/db* mice and concomitantly reduced hippocampal TNF-α expression. Although we did not demonstrate that changes in mRNA expression correlated with protein levels, previous studies have reported this to be the case (van Dam *et al*, 1998), notably in *db/db* mice (Kumari *et al*, 2007). Interestingly, TNF-α mRNA levels in particular closely mirrored protein levels (Shebl *et al*, 2010). Moreover, we showed that modulating TNF-α mRNA expression in obese mice resulted in modulation of related signaling pathway activation and behavioral response alterations (André *et al*, 2014; Dinel *et al*, 2011, 2014). Several neuroactive compounds, including ibuprofen, have already been shown to improve stress-induced anxiety by similarly targeting hippocampal TNF-α (Todorovic and Filipovic, 2017). Moreover, blocking brain TNF-α with ETN reduced anxiety-like behaviors in *db/db* mice. It further supports that increased TNF-α mediates downstream actions on neurobiological

pathways that contribute to anxiety-like behaviors, including monoamine metabolism, neuroendocrine function, neuronal excitability and synaptic plasticity (Aguilar-Valles *et al*, 2018; Fleming *et al*, 2015; Himmerich *et al*, 2008; Kekow *et al*, 2011; Tyring *et al*, 2006). Conversely, mifepristone-treated *db/+* mice displayed both increased hippocampal TNF-α expression and anxiety-like behaviors compared to their controls. This result fits with the increased anxiety-like behavior reported following adenovirus-mediated enhancement of hippocampal TNF-α expression (Klaus *et al*, 2016). Interestingly, icv ETN was shown to prevent lipopolysaccharide-induced anxiety-like behaviors by specifically reversing the induction of TNF-α in the hippocampus (Camara *et al*, 2015), further emphasizing a pivotal role for hippocampal TNF-α in mood alterations.

Different, yet not exclusive, mechanisms have been proposed to explain the behavioral effects of TNF-α antagonism with ETN (Haji *et al*, 2012; Jia *et al*, 2007). While identifying the main pathways triggering the anxiolytic effect of TNF-α antagonism with ETN in the context of obesity remains a challenge, this research provides interesting clues supporting a role for modulation of synaptic transmission in the vHPC. Indeed, ETN decreased both anxiety-like behaviors and synaptic transmission in the vHPC of *db/db* mice, where TNF-α is over-expressed. In contrast, ETN has no effect in *db/+* mice, indicating that only supra-physiological concentrations of TNF-α may affect synaptic transmission, as previously suggested (Habbas *et al*, 2015). Supporting this assumption, TNF-α inhibition was shown to reduce anxiety-like behaviors in several models of chronic inflammatory diseases, by decreasing both EPSC frequency and amplitude in brain areas participating in mood regulation (Chen *et al*, 2013; Haji *et al*, 2012; Jia *et al*, 2007). Such electrophysiological changes are known to reflect modifications occurring at pre- and post-synaptic levels respectively, ultimately altering synaptic strength (Wierenga *et al*, 2006). Our results suggest similar mechanisms in obese *db/db* mice. Indeed, recorded synaptic events that were

modified by ETN treatment are likely glutamatergic, since acetylcholine and GABAergic events are too small to be recorded in our conditions (Le Roux *et al*, 2006). Moreover, glial TNF-α is known to mediate synaptic scaling, a homeostatic regulation of glutamatergic neuronal activity (Stellwagen *et al*, 2005). In basal conditions, TNF-α facilitates synaptic transmission by inhibiting astrocyte glutamatergic transporters at the synapse, or by inducing AMPA receptors exocytosis in hippocampal pyramidal cells (Stellwagen *et al*, 2005). Similarly, obesity-related inflammation and glial activation may increase glial TNF-α production, which may then alter homeostatic glutamatergic transmission (Jia *et al*, 2007), and in turn behavioral reactivity. Of note, ETN did not affect LTP in the vHPC of *db/db* mice. This further supports the hypothesis of a role of local synaptic scaling in the behavioral effect of TNF-α in *db/db* mice. As ETN was administered icv, we cannot exclude the contribution of other brain areas to the reported behavioral effect. Inflammation in the nucleus accumbens was for instance recently reported to contribute to anxiety-like behavior in DIO models (Decarie-Spain *et al*, 2018). Further investigation is required to fully address this question, but it is unlikely that the prefrontal cortex participates to the observed anxiety-like behaviors since they negatively correlated with hippocampal, but not prefrontal cortex, TNF-α expression. Moreover, the vHPC, which participates in the regulation of emotional behaviors (Bannerman *et al*, 2002) and in the development of high-fat diet-induced anxiety-like behavior (Krishna *et al*, 2015), is preferentially targeted by ETN (Camara *et al*, 2015). At the clinical level, it would now be important to evaluate the beneficial effect of TNF-α inhibition on anxiety disorders and brain activity in obese subjects, as already demonstrated in patients suffering from depression or autoimmune diseases (Fleming *et al*, 2015; Kekow *et al*, 2011; Tyring *et al*, 2006).

As mentioned earlier, a limitation of the current study is that behavioral tests have been only performed during the light phase. Although efforts were made to minimize circadian

influence on behavior, we cannot totally exclude that circadian disruption in *db/db* mice may have somehow influenced the results obtained. A second limitation is that this study only included males, while sex differences are prominent in anxiety disorders, with women being at greater risk (Maeng and Milad, 2015). This increased vulnerability is likely due to the combined effect of several factors, including environmental influences, sex differences in brain structure and function, and/or differential exposure to reproductive hormones (Altemus *et al*, 2014). These factors might therefore increase variability among data and highly complicate their interpretation when males and females are studied together. This issue was not addressed in the present study. Therefore, the potential effect of gender cannot be ruled out and any extrapolation of our results to females needs to be taken with caution.

In conclusion, this study points to brain TNF-α as an important mediator of obesity-related anxiety and provides important clues about how it may act to modulate brain function and ultimately induce anxiety symptoms. By extending our knowledge on the mechanistic dissociation between inflammation-related anxiety and cognitive alterations in the context of obesity, this study also highlights the necessity of considering the different dimensions of observable behaviors and neurobiological measures in psychiatry, as recommended by the NIH (Research Domain Criteria project, RDoC). Lastly, beyond obesity and related metabolic diseases, these findings should also be useful to identify potential new therapeutic strategies aiming to improve anxiety disorders associated with inflammatory conditions.

DECLARATION OF INTEREST

Declaration of interest: none.

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LEGENDS

Figure 1. Chronic food restriction (CFR) reduced anxiety-like behavior, plasma corticosterone levels and cytokine production in *db/db* mice. (A) Experimental timeline and design: half of the *db/+* and *db/db* mice were submitted for 7 weeks to a food restriction of about 30% compared to their unrestricted counterparts. Behavioral testing started after 4 weeks of exposure to this procedure. (B) Body weight gain (n=10/group). (C) Percent of time spent in the central area of the open-field (n=9-10/group). (D) Number of entries in the open-arms of the elevated plus-maze (n=9-10/group). (E) Percent of time spent in the open arms of the elevated plus maze (n=9-10/group). (F) Plasma corticosterone (n=9-10/group) and (G) IL-6 levels (n=7-10/group). Il-1β, Il-6 and Tnf-α mRNA expression (plotted as fold change relative to controls) measured in (H) the hippocampus and (I) the prefrontal cortex (n=8-10/group). $^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$ for genotype effect; $*p<0.05$; $**p<0.01$; $***p<0.001$ for treatment effect.

Figure 2. Chronic anti-inflammatory treatment with ibuprofen (375 ppm) reduced anxiety-like behavior and hippocampal Tnf-α mRNA expression in *db/db* mice. (A) Experimental timeline and design: a subgroup of *db/db* mice were fed with ibuprofen-supplemented standard chow (estimated intake 2.8 mg/day) for 7 weeks. Behavioral testing started after 4 weeks of exposure to this procedure. (B) Percent of time spent in the central area of the open-field (n=8-10/group). (C) Number of open-arms entries, percent of time spent in the open arms of the elevated plus maze and risk assessment behaviors (stretch-attend postures and head dips) measured in the elevated plus-maze (n=8-10 and 7-10/group respectively). (D) Plasma IL-6 levels (n=7-10/group). (E) Hippocampal expression of IL-1β, IL-6 and Tnf-α mRNA (plotted as fold change relative to controls) (n=7-9/group). $^{\#}p<0.05$; $^{\#\#}p<0.01$;

$^{###}$p<0.001 for comparison with *db/+* Standard chow; *p<0.05 for comparison with *db/db* Standard chow.

Figure 3. Chronic blockade of brain TNF-α with etanercept (ETN, 28 days, 5 µg/kg/h, intracerebroventricular (icv) infusion) improved anxiety-like behavior in *db/db* mice. (A) Experimental timeline and design: *db/+* and *db/db* mice were equipped with a cannula implanted into the right lateral cerebral ventricle (stereotaxic coordinates: 0.3mm posterior to bregma, 1.0mm lateral from midline, 2.8mm from the skull surface) under ketamine/xylazine anesthesia. The cannula was connected to an osmotic mini-pump implanted subcutaneously in the back of the mouse in order to continuously deliver (0.11 µl/h, icv) etanercept or artificial cerebrospinal fluid (vehicle). Behavioral testing started after 2 weeks of recovery post-stereotaxic surgery. (B) Number of entries and (C) percent of time spent in the center of the open-field (n=7-10/group). (D) Time-course of the time spent in the center of the open-field (n=7-10/group). (E) Number of entries and (F) time spent in the open-arms of the elevated plus-maze. (n=7-10/group). $^{#}$p<0.05; $^{##}$p<0.01 for genotype effect; *p<0.05 for treatment effect.

Figure 4. Ex-vivo blockade of TNF-α modulated synaptic transmission in the ventral hippocampus (vHPC) of *db/db* mice. (A) Upper panel: Experimental timeline and design: horizontal brain slices (350µm) containing the vHPC were pre-incubated for 3h in a recording solution supplemented with etanercept (ETN, final concentration: 10 µg/mL); Lower panel: Position of the stimulation electrode in the CA3 and of the recording electrode in the CA1 region. (B) Examples of spontaneous excitatory postsynaptic currents (sEPSCs) traces. (C) sEPSCs frequency, (D) amplitude, (E) decay time and (F) rise time. Data obtained

from *db/+* and *db/db* mice are respectively represented in the left and right panels. n=4-6 cells/group. *$p<0.05$ for comparison with *db/db* Control.

APPENDICES

Supplemental information file (word document) contains:

- a "Supplementary Methods and Materials" providing details about electrophysiological measurement of long-term potentiation (LTP) and chronic treatment with mifepristone,

- a short description of the Supplementary Tables S1 and S2,

- the legends of Supplementary Figures S1-S4,

- a list of the references cited in the Supplementary Information.

Supplementary Table S1: This table shows inter and intra-assay coefficients of variation and low limits of detectability for the Milliplex kits and the RIA.

Supplementary Table S2: This table shows plasma levels of leptin and insulin (expressed as ng/ml) in *db/+* and *db/db* mice fed *ad libitum* or submitted for 7 weeks to chronic food restriction (restricted groups) (chronic food restriction experiment). It also shows plasma levels of interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) (expressed as ng/ml) measured using the mouse adipokines Milliplex kit (Merk-Millipore, France) in *db/+* and *db/db* mice fed *ad libitum* or submitted for 7 weeks to chronic food restriction (restricted groups) (chronic food restriction experiment); in *db/+* and *db/db* mice fed with the standard chow and *db/db* mice fed with the Ibuprofen-enriched diet (Ibuprofen experiment); and in *db/+* and *db/db* mice treated with the vehicle or with Etanercept (Etanercept experiment). n=7-10/group. $^{###}p<0.001$ for genotype effect; ***p<0.001 for treatment effect (chronic food restriction experiment); *p<0.05: *db/db* ibuprofen vs *db/db* standard chow (Ibuprofen experiment).

Supplementary Figure S1: This figure shows body weight changes over time for the different experiments. Chronic food restriction for 7 weeks significantly blunted body weight gain in both genotypes compared to their respective controls, although *db/db* mice remained heavier than *db/+* mice by the end of the CFR period. In the ibuprofen experiment, Ibuprofen treatment did not normalize higher body weight in *db/db* mice. Finally, chronic etanercept brain administration did not change body weight in both *db/+* and *db/db* mice.

Supplementary Figure S2: This figure shows that chronic food restriction altered the time course of central entries in *db/db* mice only.

Supplementary Figure S3: This figure shows that chronic blockade of glucocorticoid receptors with mifepristone (25 mg/kg/day, subcutaneous pellets, 21 days) did not improve anxiety-like behavior or hippocampal cytokine expression in *db/db* mice, but rather increased both anxiety-like behavior and hippocampal TNF-α mRNA expression in *db/+* mice compared to their placebo-treated counterparts.

Supplementary Figure S4: This figure shows that blocking TNF-α *ex-vivo* by incubating ventral hippocampus (vHPC) brain slices with etanercept (added to the recording solution at a final concentration of 10 µg/mL) did not affect the induction and maintenance of long-term potentiation (LTP) in *db/+* or *db/db* mice.

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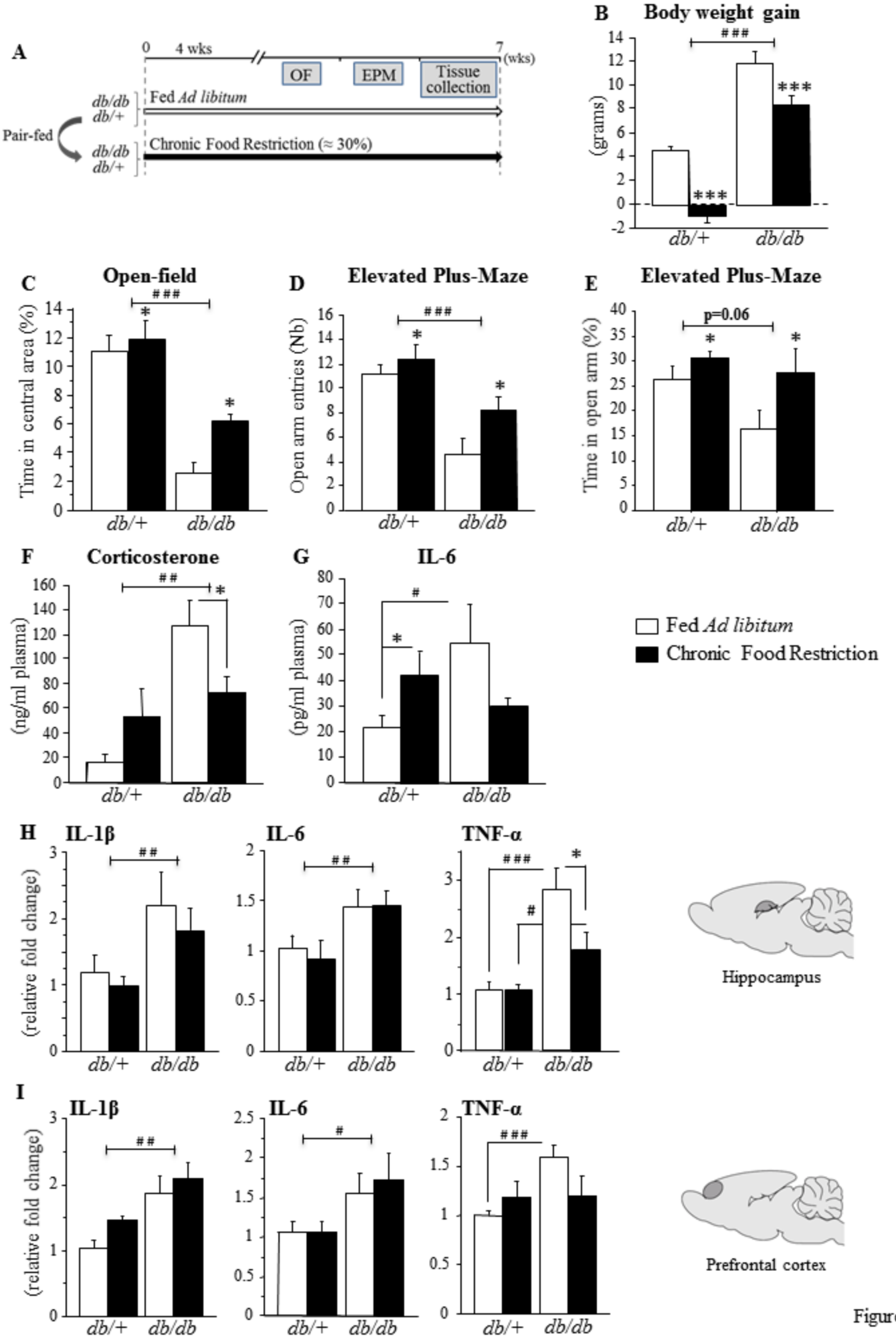
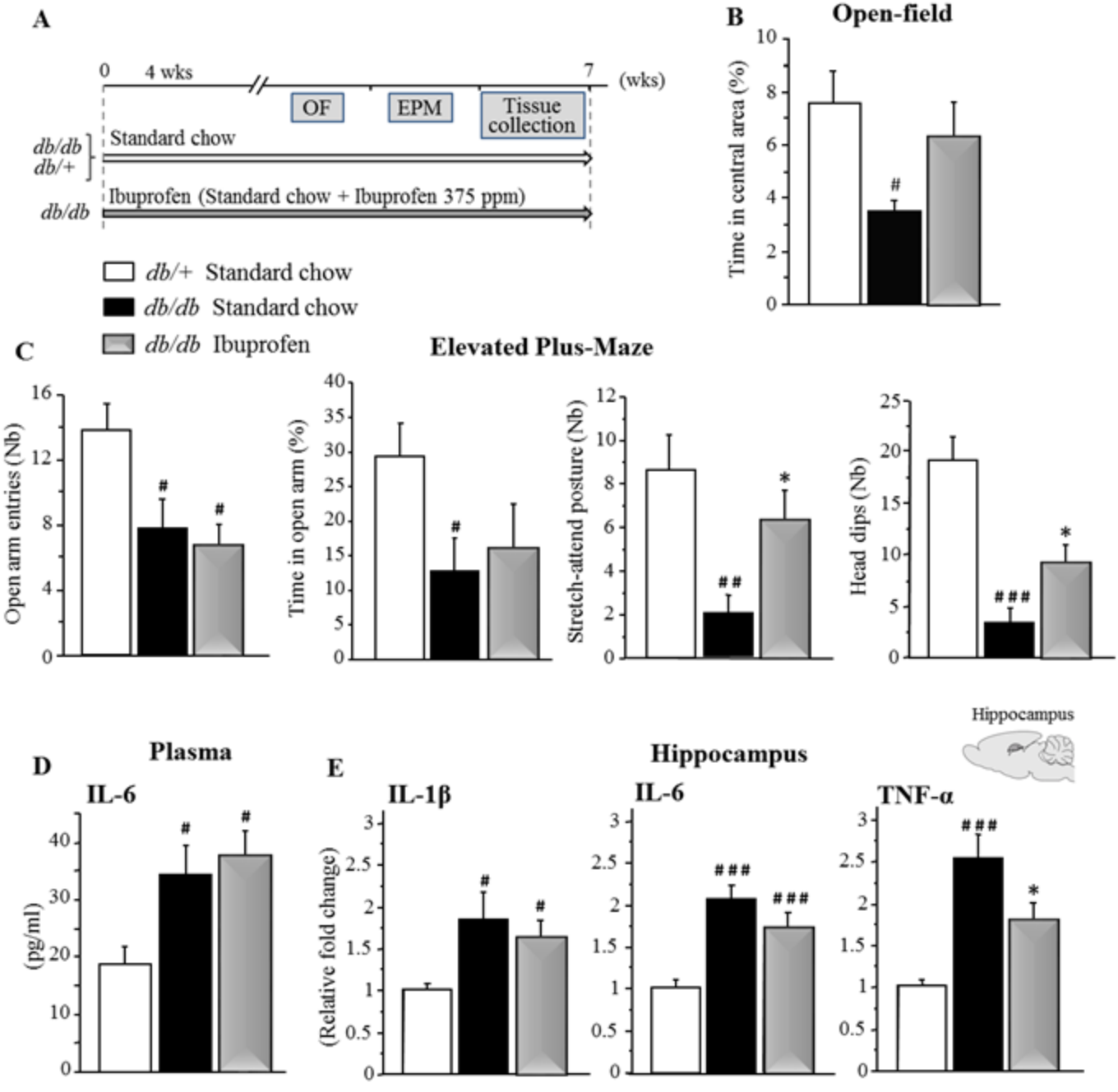

A
0
4 wks
7
(wks)
OF
EPM
Tissue collection
db/db
db/+
Fed Ad libitum
Pair-fed
Chronic Food Restriction (≈ 30%)
B
Body weight gain
(grams)
db/+
db/db
C
Open-field
Time in central area (%)
D
Elevated Plus-Maze
Open arm entries (Nb)
E
Elevated Plus-Maze
Time in open arm (%)
p=0.06
F
Corticosterone
(ng/ml plasma)
G
IL-6
(pg/ml plasma)
Fed Ad libitum
Chronic Food Restriction
H
IL-1β
IL-6
TNF-α
(relative fold change)
Hippocampus
I
IL-1β
IL-6
TNF-α
(relative fold change)
Prefrontal cortex

Figure 1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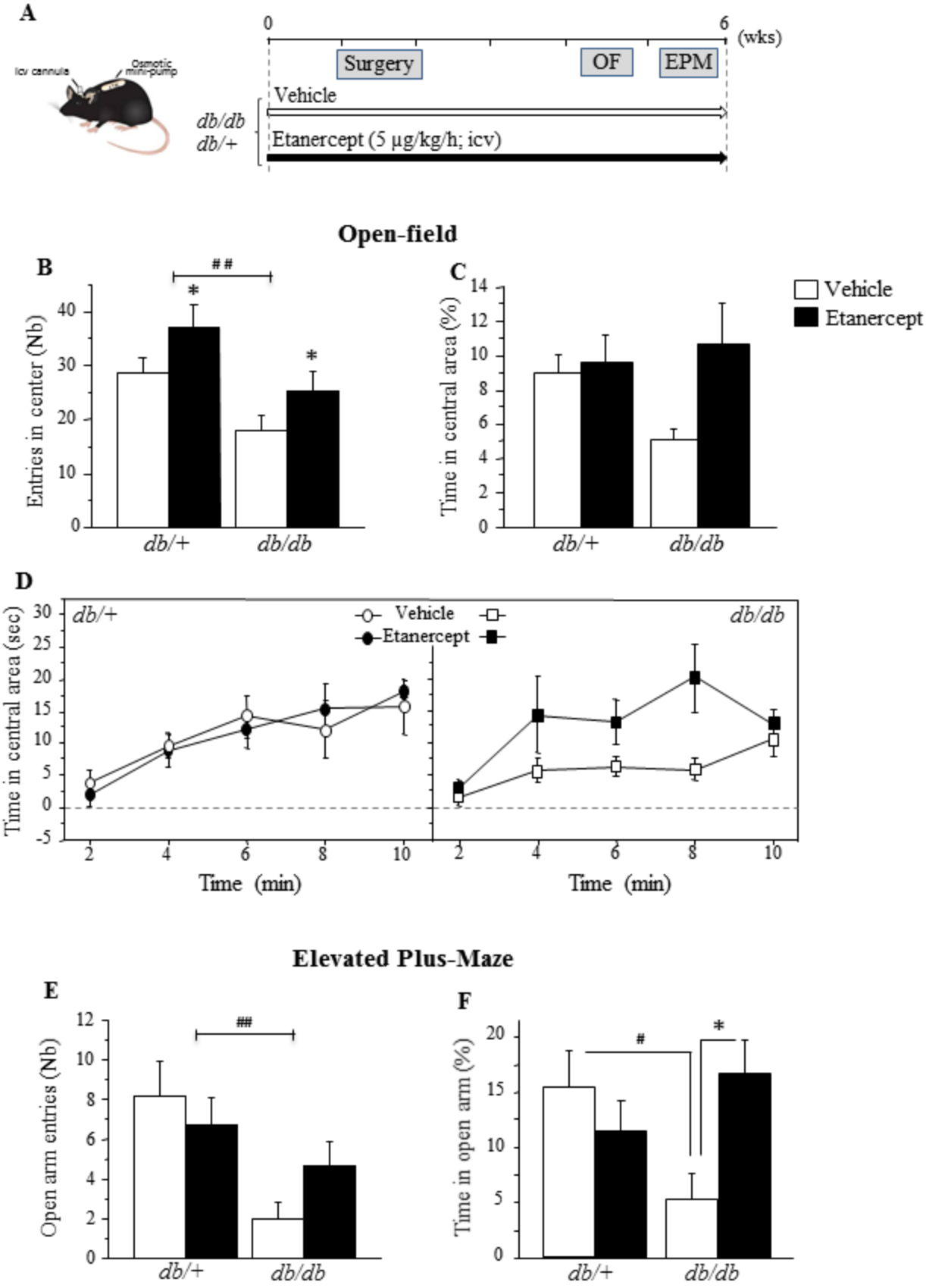
4 wks
7
(wks)
OF
EPM
Tissue collection
db/db
db/+
Standard chow
Ibuprofen (Standard chow + Ibuprofen 375 ppm)
db/db
db/+ Standard chow
db/db Standard chow
db/db Ibuprofen
B
Open-field
Time in central area (%)
C
Elevated Plus-Maze
Open arm entries (Nb)
Time in open arm (%)
Stretch-attend posture (Nb)
Head dips (Nb)
Hippocampus
D
Plasma
IL-6
(pg/ml)
E
Hippocampus
IL-1β
IL-6
TNF-α
(Relative fold change)

Figure 2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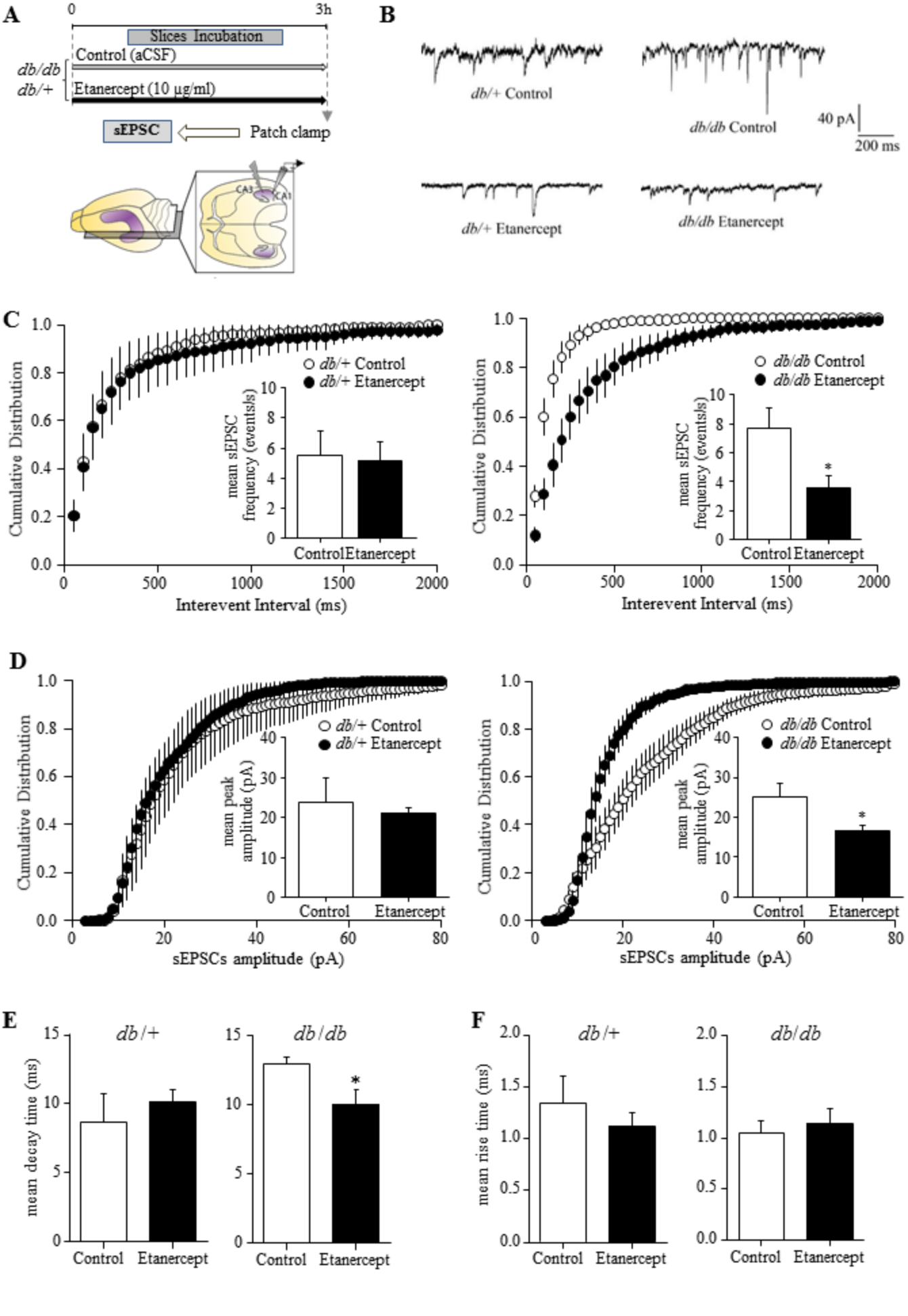
Icv cannula
Osmotic mini-pump
0
6
(wks)
Surgery
OF
EPM
Vehicle
db/db
db/+
Etanercept (5 µg/kg/h; icv)
Open-field
B
##
*
*
Entries in center (Nb)
db/+
db/db
C
Time in central area (%)
Vehicle
Etanercept
D
db/+
db/db
Vehicle
Etanercept
Time in central area (sec)
Time (min)
Elevated Plus-Maze
E
##
Open arm entries (Nb)
F
#
*
Time in open arm (%)

Figure 3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sEPSC
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CA1
B
db/+ Control
db/db Control
40 pA
200 ms
db/+ Etanercept
db/db Etanercept
C
Cumulative Distribution
db/+ Control
db/+ Etanercept
mean sEPSC frequency (events/s)
Control
Etanercept
Interevent Interval (ms)
db/db Control
db/db Etanercept
D
sEPSCs amplitude (pA)
mean peak amplitude (pA)
E
db/+
db/db
mean decay time (ms)
F
mean rise time (ms)

Figure 4