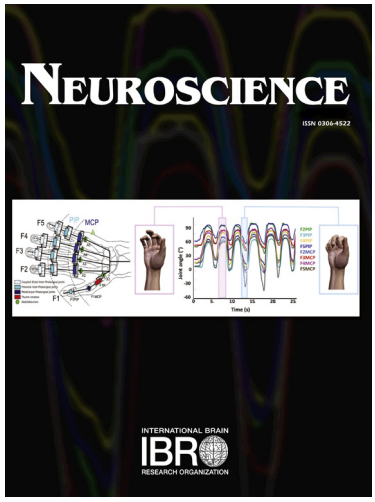

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Alterations in neuronal control of body weight and anxiety behavior by glutathione peroxidase 4 deficiency

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Abstract

Elevated levels of oxidative stress and neuronal inflammation in the hypothalamus or ventral midbrain, respectively, represent common denominators for obesity and Parkinson's Disease (PD). However, little is known about defense mechanisms that protect neurons in these regions from oxidative damage. Here, we aimed to assess whether murine Gpx4, a crucial antioxidant enzyme that protects neurons from membrane damage and ferroptosis, is critical for the protection from neuronal inflammation in two distinct pathophysiologic diseases, namely metabolic dysfunction in diet-induced obesity or PD. Gpx4 was deleted from either AgRP or POMC neurons in the hypothalamus, essential for metabolic homeostasis, or from dopaminergic neurons in the ventral midbrain, governing behaviors such as anxiety or voluntary movement. To induce a pro-inflammatory environment, AgRP and POMC neuron-specific Gpx4 knockout mice were subjected to high-fat high-sucrose (HFHS) diet. To exacerbate oxidative stress in dopaminergic neurons of the ventral midbrain, we systemically co-deleted the PD-related gene DJ-1. Gpx4 was dispensable for the maintenance of cellular health and function of POMC neurons, even in mice exposed to obesogenic conditions. In contrast, HFHS-fed mice with Gpx4 deletion from AgRP neurons displayed increased body adiposity. Gpx4 expression and activity were diminished in the hypothalamus of HFHS-fed mice compared to standard diet-fed controls. Gpx4 deletion from dopaminergic neurons induced anxiety behavior, and diminished spontaneous locomotor activity when DJ-1 was co-deleted. Overall, these data suggest a physiological role for Gpx4 in balancing metabolic control signals and inflammation in AgRP but not POMC neurons. Moreover, Gpx4 appears to constitute an important rheostat against neuronal dysfunction and PD-like symptoms in dopaminergic circuitry within the ventral midbrain.

Keywords: Obesity, Parkinson disease, DJ-1, hypothalamus, antioxidant, lipid peroxidation.

Abbreviations: Gpx4 - Glutathione Peroxidase 4; AgRP - Agouti Related Protein, POMC - Proopiomelanocortin, Glutathione – GSH; SN – Substantia Nigra; PD - Parkinson Disease.

Introduction

Metabolic diseases such as obesity and neurodegenerative diseases such as Parkinson's Disease (PD) share a prominent common feature: increased oxidative stress, manifested by a surge in reactive oxygen species (ROS) and inflammatory signaling in neurons within the hypothalamus or ventral midbrain, respectively. Obesity has been associated with increased circulating levels of inflammatory cytokines, which activate ROS-releasing pathogen defense enzymes such as superoxide ($O_2^{\bullet-}$)-producing NADP oxidase in multiple tissues (Erdos et al., 2009; Jaillard et al., 2009). Obesity was further linked with the excessive release of ROS from mitochondria due to nutritional substrate overloading, which results in electrons escaping from the electron transport chain to form $O_2^{\bullet-}$ (Brownlee, 2005). For PD, the role of inflammatory processes in its pathology is firmly established (Joers et al., 2016; Moehle and West, 2015). Mitochondrial dysfunction, high constitutive firing activity and dopamine metabolism itself, which all translate into a constant and high level of ROS, are thought to render dopaminergic neurons of the ventral midbrain particularly vulnerable to degeneration (Blesa et al., 2015; Surmeier et al., 2012).

ROS, such as $O_2^{\bullet-}$ or hydrogen peroxide (H_2O_2), induce cellular damage at their site of production, for instance mitochondrial DNA damage or protein oxidation which may trigger the unfolded protein response (Santos et al., 2009; Shokolenko et al., 2009). Moreover, H_2O_2 can form the highly reactive hydroxyl radicals ($OH^{\bullet}$) that attack polyunsaturated fatty acids thus forming alkoxyl/peroxyl radicals and phospholipid hydroperoxides. Lipid peroxidation was associated with severe pathophysiological consequences in multiple peripheral tissues and brain areas, including perturbed membrane structure and function, activated cellular stress signaling and ferroptosis, a non-apoptotic form of cell death (Benani et al., 2007; Dixon et al., 2012; Furukawa et al., 2004; Keaney, 2003; Yoo et al., 2010). There is no enzymatic defense mechanism against highly reactive hydroxyl radicals; solely nutrient-derived antioxidants such as vitamin E provide a certain protection (Pfluger et al., 2004). However, once lipid peroxides are formed, they can be efficiently detoxified by glutathione peroxidase 4 (Gpx4) at the expense of the highly abundant antioxidant glutathione (GSH; Fig. 1a).

Gpx4 is a selenocysteine-containing member of the glutathione peroxidase family with three isoforms transcribed from the same gene: a ubiquitously expressed cytosolic form (cGpx4), and the mitochondrial (mGpx4) and sperm nuclei (snGpx4) forms whose expression is largely restricted to testes. Global germline deletion of mGpx4 and snGpx4 yielded fully viable but

infertile mice that displayed perturbed sperm maturation (Conrad et al., 2005; Schneider et al., 2009). In contrast, global Gpx4 deletion evoked early embryonic lethality at day 7.5 (Yant et al., 2003). Mice with germline CAMKIIalpha-driven Gpx4 ablation from the forebrain, while born unconspicuously, developed an atactic gate and fatal hyperexcitable phenotype by postpartem day 13 (Seiler et al., 2008). The essential role of Gpx4 was also corroborated in inducible CAMKIIalpha-driven Gpx4 knockout (KO) mice, which displayed spinal motor neuron degeneration, paralysis and death within 8 days of tamoxifen injection due to excacerbated lipid peroxidation, mitochondrial dysfunction and ferroptosis (Chen et al., 2015).

Mice with Gpx4 haploinsufficiency ($Gpx4^{+/-}$) were viable and fertile when fed standard low fat diet (Katunga et al., 2015). However, when chronically exposed to high fat high sucrose diet (HFHS), $Gpx4^{+/-}$ mice displayed classical symptoms of the metabolic syndrome as well as cardiac hypertrophy and cardiac fibrosis. These metabolic perturbations were associated with increased levels of lipid peroxidation, mitochondrial dysfunction, increased ROS release and the upregulation of pro-inflammatory genes in livers and hearts of obese $Gpx4^{+/-}$ but not wildtype (WT) mice (Katunga et al., 2015). Putative detrimental effects of Gpx4 haploinsufficiency on the CNS were not assessed by Katunga et al. (Katunga et al., 2015). However, earlier reports showed increased lipid peroxidation in the CNS of $Gpx4^{+/-}$ mice and early signs of Alzheimer's Disease such as increased amyloidogenesis (Chen et al., 2008).

Here we aimed to assess the role of Gpx4 in hypothalamic proopiomelanocortin (POMC) and agouti-related protein (AgRP) neurons residing in the arcuate nucleus. These two neuronal subpopulations, which are characterized by neuropeptide production of either POMC or AGRP, sense peripheral nutrients and hormones and govern adaptive metabolic responses to environmental changes (Belgardt et al., 2009; Denis et al., 2014; Varela and Horvath, 2012). Our focus on Gpx4 in AgRP and POMC neurons was driven by the following rationale: a) Gpx4 is expressed in hypothalamic neurons (Cong et al., 2012); b) HFHS feeding (Y. Gao et al., 2014; Thaler et al., 2012) and fatty acid species such as monounsaturated fatty acids (Kleinridders et al., 2009; Obici et al., 2002) or ceramides (S. Gao et al., 2011) were shown to induce hypothalamic inflammation and metabolic dysfunction; c) AgRP and POMC neurons display profound baseline ROS production which can be elevated by hormonal factors, neurotransmitters and macronutrients such as glucose or lipids (Andrews et al., 2008; Drougard et al., 2015); d) POMC neurons display excacerbated firing rates, increased mitochondrial ROS production, abnormal

mitochondrial morphology and apoptosis upon chronic HFHS exposure (García-Cáceres et al., 2016; Schneeberger et al., 2013; Thaler et al., 2012); e) HFHS exposure modulates the neuronal activity of AgRP neurons by increasing mitochondrial ROS production (Andrews et al., 2008), translating into massively increased neuronal firing activities (Diano et al., 2011) and altered mitochondrial fusion dynamics (Dietrich et al., 2013) and f) buffered ROS production in AgRP/POMC neurons is essential to maintain energy and glucose homeostasis, but factors gatekeeping ROS-induced lipotoxicity remain unknown (Gyengesi et al., 2012).

Our focus on Gpx4 in ventral midbrain dopaminergic neurons was prompted by a) decreased Gpx4 mRNA levels and increased levels of oxidized lipid by-products in the brains of Alzheimer mouse models (Yoo et al., 2010), which suggest a protective role of Gpx4 in neurodegenerative diseases by removing lipid hydroperoxides, and b) a recent report on PD-associated protein deglycase DJ-1, which was shown to interact with Gpx4 mRNA, thereby increasing Gpx4 protein expression in a post-transcriptional fashion in brains of human PD patients (Blackinton et al., 2009). DJ-1 is expressed in brain regions affected by PD (Bandopadhyay et al., 2004; Olzmann et al., 2007) and appears to constitute a crucial anti-oxidant enzyme that protects dopaminergic neurons from oxidative stress.

In a first set of experiments, we hypothesized that Gpx4 represents an anti-lipotoxic defense mechanism to prevent AgRP and POMC neurons from metabolic damage incurred by dietary HFHS exposure. Specifically, we hypothesized perturbed metabolic adaptation to HFHS exposure in mice with AgRP- or POMC-neuron specific Gpx4 deficiency. In a second, independent set of experiments we hypothesized that the loss of Gpx4 will initiate cellular damage specifically in dopaminergic neurons of the ventral midbrain, thereby leading to PD-like symptoms in adult mice. To increase oxidative stress tailored for the respective disease pathologies “metabolic dysfunction” or “PD”, we used HFHS-feeding in our first set of experiments, and systemic co-deletion of DJ-1 to exaberate potential PD-like symptoms in our second set of experiments. This allowed us to test the precise roles of Gpx4 in specific neuronal populations that are driving the respective disease pathophysiologies. Ultimately, by studying the role of Gpx4 in two distinct disease models, both associated with neuroinflammation due to increased ROS-induced damage, we aimed to shed light on common mechanistic underpinnings for two diseases of highest relevance for public health.

Materials & Methods

Mouse husbandry

Conditional Gpx4 mutant mice (Seiler et al., 2008) were crossed to AgRP-Cre mice (Wang et al., 2010) and POMC-Cre mice (Postic et al., 1999), and group housed in positive individual ventilation cages in dedicated animal housing rooms with a 12-h light, 12-h dark cycle (6am-6pm) at 22°C. Mice were either maintained on a standard chow diet (Altromin 1314) with free access to food and water, or exposed to high fat high sucrose (HFHS) diet (Research Diets D12451, New Brunswick, NJ, USA) containing 45% of calories from fat and 35% of calories from carbohydrates. For the analysis of Gpx1 and Gpx4 expression and activity, male C57Bl/6J mice (Janvier, Saint-Berthevin, France) were subjected to chow or HFHS diet for a total of 17 weeks.

For the selective disruption of Gpx4 in midbrain dopaminergic neurons, conditional Gpx4 mice were crossed to Slc6a3-CreERT2 mice (Jackson Laboratory, Strain 016583). Intercross with DJ-1 deficient mice (Pham et al., 2010) resulted in mouse mutants ($Gpx4^{Slc6a3\text{-}CreERT2}$ KO x DJ-1 KO) deficient of Gpx4 in dopaminergic neurons on a DJ-1 deficient background after activation of the inducible Cre by repeated injection of tamoxifen. Behavioral experiments were conducted in littermates from intercrosses of $DJ\text{-}1^{+/-}/Gpx4^{flox/flox}$/Slc6a3-CreERT2 x $DJ\text{-}1^{+/-}/Gpx4^{flox/flox}$ mice. Mice of all resulting genotypes were tamoxifen injected. Tamoxifen administration was performed in week 27 (2 mg tamoxifen, Sigma-Aldrich: T5648) i.p. for 5 consecutive days dissolved in Miglyol® 812 (Caelo, Caesar & Loretz GmbH, Hilden, Germany). Behavior tests were carried out 18 days after the last injection. At 34 weeks of age, mice were sacrificed and processed for immunohistochemical detection of tyrosine-hydroxylase expressing neurons. All studies were approved by and performed according to the guidelines of the Institutional Animal Care and Use Committee of the State of Bavaria, Germany.

Genotyping, gene expression and gene array analyses

Genotyping to corroborate ablation of Gpx4 from the hypothalamus or midbrain was performed by using Q5 High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA) with primers 5´- GTG TAC CAC GTA GGT ACA GTG TCT GC -3´ and 5´- GGA TCT AAG GAT CAC AGA GCT GAG GCT GC-3´ to detect the deleted allele. RNA was isolated from tissues using a commercially available kit (NucleoSpin, Macherey-Nagel, Dueren, Germany). For qPCR

analyses, equal amounts of RNA were transcribed to cDNA using QuantiTect (Qiagen, Hilden, Germany). Gene expression was analyzed using TaqMan probes (Applied Biosystems, Carlsbad, CA, USA) and the TaqMan gene expression mastermix (Applied Biosystems). qPCRs were carried out using an Applied Biosystems ViiA7 Real-Time PCR System. Gene expression was evaluated using the Δ-Δ Ct method and HPRT was used as house keeping gene.

Enzymatic activity of Gpx4 and Gpx1

Tissues were homogenized in 9 volumes (w/vol) of sucrose buffer (20 mM tris/HCl, pH 7.4, 0.25 M sucrose, 1.1 mM EDTA and 0.1% peroxide-free Triton X 100) followed by centrifugation as previously described (Schriever et al., 2009). Gpx4 activity was measured by the coupled assay procedure using phosphatidyl choline hydroperoxide (PCOOH), a substrate specific for Gpx4. In the same samples, Gpx1 activity was measured using H_2O_2 as substrate. For both assays, 1 enzyme unit (EU) was defined as amount of enzyme that will oxidize 1 µmole GSH/min. Protein concentrations were determined using the BCA assay kit (Pierce, ThermoFisher Scientific, Schwerte, Germany).

Indirect calorimetry and measurement of body composition

Fat mass and lean mass were assessed by using NMR technology (EchoMRI, Houston, TX, USA). Food intake, energy expenditure, respiratory quotients and locomotor activity were measured by using a combined indirect calorimetry system (TSE Systems GmBH, Bad Homburg, Germany). Mice had free access to food and water and were adapted to the cages for a minimum of 48 hours. Oxygen consumption and carbon dioxide production were measured every 10 min to determine the respiratory quotient and energy expenditure. Home-cage locomotor activity was determined using a multidimensional infrared light beam system.

Glucose and insulin tolerance tests (GTT and ITT)

For the measurements of glucose tolerance and insulin sensitivity, mice were subjected to 6 hours of fasting and injected intraperitoneally with 1.5 g glucose / kg body weight (20% D-glucose (Sigma) in 0.9% saline) for the GTT, and 0.75 U insulin / kg body weight (Humolog, Lily, Indianapolis, USA) for the ITT, as indicated. Tail blood glucose levels [mg/dL] were measured

using a handheld glucometer (TheraSense Freestyle) before (0 min) and at 15, 30, 60 and 120 min after injection.

Immunohistochemical (IHC) stainings

For histological analyses, animals were sacrificed and intracardially perfused with paraformaldehyde (PFA, 4%, pH 7.5). After dissection, brains were post-fixed in PFA overnight and stored at 4°C in 25% sucrose solution until cutting in 40 µm sections. Histological methods using primary anti-tyrosine hydroxylase (TH) antibody (1:10,000; Pel-Freez Biologicals, USA) were performed as described (http://empress.har.mrc.ac.uk/). Subsequent DAB (3,3' Diaminobenzidine) IHC was conducted using biotin-labeled secondary antibody (1:500; Jackson ImmunoResearch, UK) in combination with the Vectastain ABC System (Vector Laboratories, USA) and DAB as chromogen. Images were acquired using an Axioplan2 microscope and an AxioCam MRc camera (Carl Zeiss AG, Germany). Images were processed with AxioVision 4.6 (Carl Zeiss AG, Germany) and Adobe Photoshop CS3 (Adobe Systems Inc., USA) software. Unbiased stereology has been used to determine the total numbers of dopaminergic neurons in blinded experiments using the optical fractionator method and analyzed with the StereoInvestigator software (MicroBrightField Inc., USA). Immunopositive cells in the substantia nigra pars compacta (SNc) were marked and quantified by systematic random sampling using a scan grid-size of 250x250 µm.

Immunofluorescence stainings for hypothalamic AgRP and POMC were done using anti-AgRP goat antibody (1:1000; LifeSpan BioSciences, Seattle, WA, USA) and anti-POMC rabbit antibody (1:1000; Phoenix Pharmaceuticals, Burlingame, CA, USA). Primary antibodies were amplified by biotin-conjugated horse anti-goat and biotin-conjugated horse anti-rabbit antibodies (1:400; Vector Laboratories, Peterborough, UK), respectively and then detected with streptavidin Alexa-555 bound biotin-conjugation and (1:200; Thermo Fisher Scientific, Darmstadt, Germany) was therefore used to detect secondary antibody. Imaging was performed with a Leica TCS SP5 microscope and images were processed with Fiji ImageJ software (www.imagej.net).

Behavior Testing

Behavioral tests were performed 18 days after tamoxifen application in 27-weeks-old animals. For Open Field tests, animals were placed in a square arena at the middle of the back wall, which

was illuminated at approximately 200 lux (Actimot, TSE, Bad Homburg, Germany). For 20 minutes the behavior was recorded via infra-red beam breaks as previously described (Hölter et al., 2015). Vertical Pole test were conduced by placing mice head upwards on a vertical taped pole of 50 cm length and 1 cm diameter, as previously described (Hölter and Glasl, 2011). The striatal function-specific time to orient themselves downwards and descend the length of the pole to the ground was subsequently recorded. Mice received two training trials and three to five test trials with inter-trial intervals of 5 to 10 minutes. Grip Strength tests and Ladder Walk tests were conducted as previously described (Hölter and Glasl, 2011).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc. La Jolla, CA, USA) or SPSS (IBM Corporation, Armonk, NY, USA). Differences between genotypes were assessed using unpaired students t-tests, or 1-way or 2-way Anova with Sidak's multiple comparison post-test, as indicated. Differences in energy expenditure were assessed by Ancova, with lean and fat mass as covariates. P values lower than 0.05 were considered significant. Results are presented as means ± SEM or as Box and Whisker Plots with 5-95% percentiles.

Results

Reduced Gpx4 expression and activity in the hypothalamus of male HFHS-fed mice

We here aimed to assess whether the expression of Gpx4, a key antioxidant enzyme involved in the protection of biological membranes from lipid peroxidation (**Fig. 1a**), is altered by chronic exposure to high-fat diet feeding. C57Bl/6J mice were fed for 17 weeks with HFHS diet, which increased body weight (55.08 ± 4.02 g) compared to chow-fed and age-matched lean control mice (body weight: 33.48 ± 0.46 g). HFHS-induced body adiposity was associated with decreased Gpx4 mRNA levels in the hypothalamus (**Fig. 1b**), and increased Gpx4 levels in white adipose tissue (WAT) (**Fig. 1c**). Expression levels of Gpx4 remained unaltered in the extensor digitorum longus (EDL) muscle, the soleus and the liver. To assess whether these expression changes are of functional relevance, we next applied the classical Gpx4-specific enzyme activity assay with PCOOH as lipid peroxide substrate. We did not observe alterations in enzymatic Gpx4 activity in all peripheral tissues tested (testis, quadriceps (QUAD), WAT), but found diminished activity in the hypothalamus (**Fig. 1d**). We next used gene expression data (GSE68177) from the publicly available GEO database to confirm the presence of Gpx4 in POMC neurons and AgRP neurons of the arcuate nucleus, a key hypothalamic area that governs energy and glucose homeostasis. FACS-based sorting and subsequent RNA sequencing of POMC- and AgRP neurons from mice that were either fed ad libitum or fasted for 24 hrs (Henry et al., 2015) revealed unchanged Gpx4 expression levels in AgRP neurons, but diminished Gpx4 expression levels in POMC neurons of food-deprived mice, compared to ad libitum fed controls (**Fig. 1e**). Last, to assess whether decreased Gpx4 activity is accompanied by a general decrease in antioxidant peroxidation capacity, we performed enzymatic activity assays with H_2O_2 as substrate (**Fig. 1f**). Enzymatic peroxidation of H_2O_2, which involves several enzymes such as catalase or Gpx1, was unchanged in all peripheral tissues tested. However, in hypothalamic tissue we found a trend for decreased H_2O_2 detoxification in mice fed with HFHS ($p=0.054$).

Gpx4 deletion from POMC neurons does not affect body weight, energy expenditure or glucose homeostasis

The diminished expression of Gpx4 in POMC neurons upon calorie deprivation (**Fig. 1e**, (Henry et al., 2015)) prompted us to hypothesize that Gpx4 may be an important antioxidant enzyme in POMC neurons that protects from HFHS-induced metabolic damage. To assess this potential role

of Gpx4, we crossed conditional Gpx4 mutants (Seiler et al., 2008) to a mouse with Cre expression restricted to POMC neurons (Balthasar et al., 2004). The resulting offspring of $Gpx4^{POMC\text{-}Cre}$ WT and KO mice were born healthy and viable with normal Mendelian ratio. We confirmed deletion of Gpx4 by PCR for the deleted allele, which is only present in $Gpx4^{POMC\text{-}Cre}$ KO mice (**Fig. 2a**). Body weights of male $Gpx4^{POMC\text{-}Cre}$ WT and KO mice were similar on chow diet (**Fig. 2b**). Upon exposure to HFHS for 12 weeks male $Gpx4^{POMC\text{-}Cre}$ WT and KO mice displayed similar propensities for weight gain (**Fig. 2c**). A subcohort of male $Gpx4^{POMC\text{-}Cre}$ WT and KO mice further displayed similar body weight, fat mass and lean mass when exposed to HFHS for 14 weeks (**Fig. 2d**). These male $Gpx4^{POMC\text{-}Cre}$ WT and KO mice were then monitored for metabolic perturbations by indirect calorimetry. First, we corroborated that $Gpx4^{POMC\text{-}Cre}$ WT and KO mice consume equal amounts of food (**Fig. 2e**). Moreover, we revealed similar energy expenditure (**Fig. 2f**) and locomotor activity (**Fig. 2g**) in both genotypes. Comparable respiratory quotients in $Gpx4^{POMC\text{-}Cre}$ WT and KO mice further suggest unperturbed nutrient partitioning (**Fig. 2h**). Overall, these data confirm a balanced energy metabolism in mice with Gpx4 deficiency in POMC neurons.

Ablation of Gpx4 from AgRP neurons increases diet-induced body weight gain and fat mass in male mice by altering locomotor activity and nutrient partitioning

Due to the presence of Gpx4 in AgRP neurons (Diano et al., 2011) we hypothesized that Gpx4 deficiency in AgRP neurons should hamper their antioxidant capacity, augment lipid oxidation and membrane damage, and ultimately impair AgRP neuronal function. In consequence, $Gpx4^{AgRP\text{-}Cre}$ KO mice should in theory reveal a phenotype similar to mice undergoing AgRP neuronal ablation, i.e. decreased hunger and starvation-induced weight loss (Gropp et al., 2005; Luquet et al., 2005).

Crossing conditional Gpx4 mutants to mice with Cre expression restricted to AgRP neurons resulted in viable offspring and the expected Mendelian ratio of $Gpx4^{AgRP\text{-}Cre}$ WT and KO mice. Genotyping for the deleted allele revealed efficient ablation of Gpx4 upon Cre expression (**Fig. 3a**). Body weights of male WT and $Gpx4^{AgRP\text{-}Cre}$ KO mice were similar on chow diet (**Fig. 3b**). However, after 8 weeks of HFHS exposure male $Gpx4^{AgRP\text{-}Cre}$ KO mice displayed a trend for increased body weight ($p=0.078$, **Fig. 3c**) and significantly induced weight gain compared to their respective WT counterparts (**Fig. 3d**). The increased propensity for weight gain was mirrored by

increased body adiposity in a subset of male HFHS-fed Gpx4$^{AgRP\text{-}Cre}$ KO, which showed higher body weights (**Fig. 3e left panel**) and fat mass (**Fig. 3e middle panel**) and a redistribution of fat vs. lean mass when displayed as percent of body weight (**Fig. 3e, right panel**), compared to male Gpx4$^{AgRP\text{-}Cre}$ WT mice.

We next subjected a subcohort of male Gpx4$^{AgRP\text{-}Cre}$ WT and KO mice to a combined indirect calorimetry system to assess the impact of AgRP neuronal Gpx4 deletion on metabolic physiology in more detail. After 17 weeks of HFHS feeding, male Gpx4$^{AgRP\text{-}Cre}$ WT and KO mice consumed similar amounts of HFHS in the 3-day monitoring period (**Fig. 3f**). Energy expenditure was similar for both Gpx4$^{AgRP\text{-}Cre}$ WT and KO mice, but KO mice tended to expend more energy in the dark phase (p=0.068, **Fig. 3g**). In contrast, we observed significantly decreased locomotor activity in Gpx4$^{AgRP\text{-}Cre}$ KO mice during the light phase (**Fig. 3h**), and a concomittant decrease in the respiratory quotient of Gpx4$^{AgRP\text{-}Cre}$ KO mice, compared to Gpx4$^{AgRP\text{-}Cre}$ WT mice (**Fig. 3i**). In summary, classical readouts for the measurement of energy balance, i.e. food intake vs. energy expenditure, were similar for Gpx4$^{AgRP\text{-}Cre}$ WT and KO mice, suggesting a balanced energy physiology. However, decreased physical activity and a change in nutrient partitioning in male Gpx4$^{AgRP\text{-}Cre}$ KO mice appear to contribute to the increased propensity for high-fat diet induced weight gain and body adiposity.

Female mice with ablation of Gpx4 from AgRP neurons show no metabolic perturbations

In contrast to male HFHS-fed Gpx4$^{AgRP\text{-}Cre}$ WT and KO mice, female mice showed no differences in body weight or body composition (**Fig. 4a,b**). Moreover, we found no evidence for changes in energy expenditure, locomotor activity and respiratory quotients when monitored for 2 days in the indirect calorimetry system (**Fig. 4c-e**). Overall, effects of Gpx4 ablation from AgRP neurons on metabolic physiology appear to be sexually bimorphic and restricted to males, with female mice being protected from HFHS-induced metabolic damage, likely due to elevated estradiol levels.

Ablation of Gpx4 from AgRP and POMC neurons does not affect glucose homeostasis or AgRP and POMC neuronal cell density

Independent from their role in controlling food intake and body weight, POMC neurons are also involved in the central regulation of glucose homeostasis (Parton et al., 2007). Accordingly, we

next assessed whether Gpx4 deletion from POMC neurons affects systemic glucose control in mice challenged with a high fat diet. After 9 weeks of exposure to HFHS, male $Gpx4^{POMC\text{-}Cre}$ WT and KO mice displayed nearly identical glucose excursions (**Fig. 5a, left panel**) or area under the curve (AUC) values (**Fig. 5a, right panel**) when challenged with a glucose bolus. The administration of insulin evoked similar decreases in glucose and AUC levels in HFHS-fed male $Gpx4^{POMC\text{-}Cre}$ WT and KO mice (**Fig. 5b**). Overall, these data clearly demonstrate that Gpx4 is dispensable for the glucoregulatory role of POMC neurons. Similarly, we performed glucose and insulin tolerance tests in male and female $Gpx4^{AgRP\text{-}Cre}$ WT and KO mice that were exposed to HFHS for 12-13 weeks to assess whether the absence of Gpx4 in AgRP neurons attenuates their physiological function as glucoregulatory neurons. Overlapping glucose excursions and similar AUC values in male $Gpx4^{AgRP\text{-}Cre}$ WT and KO mice, challenged with a single glucose bolus, did not support a glucoregulatory role for Gpx4 in AgRP neurons (**Fig. 5c**). A single intraperitoneal injection of insulin in an ITT was unable to decrease glucose levels in male HFHS-fed $Gpx4^{AgRP\text{-}Cre}$ WT and KO mice, thus suggesting severe but similar insulin resistance for both genotypes (**Fig. 5d**). Female HFHS-fed $Gpx4^{AgRP\text{-}Cre}$ WT and KO mice challenged with glucose or insulin (**Fig. 5e,f**) displayed similar glucose excursions and AUC values, further proving that Gpx4 is dispensable for the glucoregulatory role of AgRP neurons.

Moreover, we performed immunohistochemical stainings for AgRP or POMC to assess the cellular density of AgRP or POMC neurons after Gpx4 ablation in HFHS-fed $Gpx4^{AgRP\text{-}Cre}$ or $Gpx4^{POMC\text{-}Cre}$ WT and KO mice. POMC staining was localized within the cell soma and allow a direct quantification of POMC neuronal cell numbers, which were similar between $Gpx4^{POMC\text{-}Cre}$ WT and KO mice (**Fig. 5g**). AgRP stainings was found in neuronal processes and soma, which prohibited an accurate counting of neurons (**Fig. 5h**). Accordingly, we compared fluorescent intensities of AgRP staining between genotypes, and found no difference in cellular density between HFHS-fed $Gpx4^{AgRP\text{-}Cre}$ WT and KO mice.

Overall, these data indicate that Gpx4 ablation fom AgRP or POMC neurons does not affect systemic glucose homeostasis. Moreover, immunohistochemical stainings for POMC and AgRP reveal that Gpx4 ablation does not induce profound cellular damage with a loss of POMC and AgRP neurons.

Inducible dopamine transporter (Slc6a3) Cre driven ablation of Gpx4 in adult mice does not induce a loss of dopaminergic neurons

In a second set of studies unrelated to HFHS-induced metabolic dysfunction, we aimed to assess the role of Gpx4 for PD, focusing on Gpx4 in dopaminergic neurons based on multiple reports that unequivocally localize cellular damage within this large neuronal population (Joers et al. 2016, Moehle and West 2015). Specifically, due to the proposed interaction of Gpx4 with the PD-associated protein deglycase DJ-1 (Blackinton et al., 2009), we hypothesized that the loss of Gpx4 might initiate cellular damage in dopaminergic neurons of the ventral midbrain, thereby leading to PD-like symptoms in adult mice.

Inititially, we aimed to confirm successful ablation of Gpx4 from Slc6a3-Cre positive neurons after tamoxifen adminsitration in adult mice. Genotyping for the deleted allel confirmed the correct recombination in Slc6a3-Cre positive areas such as the midbrain, but not in Slc6a3-Cre negative brain areas such as the cerebellum (**Fig. 6a**). Next, we performed immunohistochemical stainings for tyrosine hydroxylase in littermate control mice ($Gpx4^{Slc6a3\text{-}CreERT2}$ WT x DJ-1 WT), mice lacking Gpx4 in dopaminergic neurons ($Gpx4^{Slc6a3\text{-}CreERT2}$ KO x DJ-1 WT) and mice lacking both DJ-1 and Gpx4 in dopaminergic neurons ($Gpx4^{Slc6a3\text{-}CreERT2}$ KO x DJ-1 KO), and assessed the number of TH-positive neurons in the substantia nigra (SN) (**Fig. 6b,c**). We revealed that even co-deletion of Gpx4 and DJ-1 was not sufficient to induce overt cellular damage with cell loss, as indicated by equal TH-positive cell numbers in $Gpx4^{Slc6a3\text{-}CreERT2}$ KO x DJ-1 WT and $Gpx4^{Slc6a3\text{-}CreERT2}$ KO x DJ-1 KO mice compared to WT controls **(Fig. 6c)**

Inducible ablation of Gpx4 in dopaminergic neurons leads to increased anxiety and in combination with DJ-1 deficiency to motoric impairments

Next, we assessed behavioural changes induced by the loss of Gpx4 in dopaminergic neurons and the additional systemic depletion of DJ-1. Specifically, we assessed spontaneous locomotor activity, exploration and anxiety-related behaviour in the Open Field, muscle strength in the Grip Strength test and alterations in motor abilities in the Ladder Walk and in the Vertical Pole test. Of note, the groups did not differ in body weight at the time point of testing **(Fig. 7a)**. No group differences were detected in Grip Strength and in the Ladder Walk test (data not shown). In the Open Field, double knockout ($Gpx4^{Slc6a3\text{-}CreERT2}$ KO x DJ-1 KO) mice displayed significantly reduced spontaneous locomotor activity in comparison to the control group, an effect that was not

evident in mice only lacking Gpx4 in dopaminergic neurons **(Fig. 7b,c)**. The only detectable genotype effect in the latter group was an increase in anxiety-related behaviour as measured by a reduction of the time spent in the anxiogenic centre of the Open Field, which was also evident in the double knockouts **(Fig. 7d)**. There were no genotype effects in the turning time of the Vertical Pole test **(Fig. 7e)**, which detects alterations in nigrostriatal function (Matsuura et al., 1997). These modest behavioral changes suggested – specifically in the $Gpx4^{Slc6a3\text{-}CreERT2}$ KO x DJ-1 KO mice, a disturbance of the dopaminergic system.

Discussion

We here present evidence for a novel role of Gpx4 in controlling body weight, locomotor activity and anxiety behavior. In mice lacking Gpx4 in dopaminergic neurons of the ventral midbrain, we observed increased anxiety, and decreased spontaneous activity when DJ-1 was co-deleted. We demonstrate that deletion of Gpx4 from AgRP neurons augments HFHS-induced weight gain in male mice. Altered locomotor activity and nutrient partitioning accompanied the increased propensity for diet-induced obesity in $Gpx4^{AgRP\text{-}Cre}$ KO mice. In contrast, we found no evidence for metabolic perturbations in $Gpx4^{POMC\text{-}Cre}$ KO mice. Gpx4 expression and activity were both diminished in the hypothalamus of HFHS-fed mice, suggesting an important physiological role for this membrane-protecting enzyme in the adaptive response to nutritional changes in our environment.

Global or forebrain-specific Gpx4 KO mice (Imai et al., 2003; Seiler et al., 2008) displayed embryonic or early postpartem mortality, respectively, and Gpx4 is described as key regulating enzyme in ferroptosis driven via lipid peroxidation and oxidative damage (Dixon et al., 2012; Friedmann Angeli et al., 2014). Indeed, global Gpx4 haploinsufficiency led to lipid peroxidation and protein carbonylation in peripheral tissues such as the liver or heart (Katunga et al., 2015), and renal Gpx4 ablation led to phospholipid peroxidation (Friedmann Angeli et al., 2014). Whether such oxidative damage can also be found in the respective brain areas of our KO cohorts remains to be determined. The targeted neuronal populations comprise only a fraction of neurons within the arcuate nucleus or ventral midbrain. Further, current analytical tools and molecular sensors do not allow the quantification of lowest levels of protein carbonylation and lipid peroxidation. In consequence, technical limitations and the high dilution of potential signal prevented us from the analytical detection of oxidation end products in our $Gpx4^{POMC\text{-}Cre}$, $Gpx4^{AgRP\text{-}Cre}$ or tamoxifen-induced $Gpx4^{Slc6a3\text{-}CreERT2}$ KO mice. Nevertheless, immunohistochemical stainings and cell counting in $Gpx4^{AgRP\text{-}Cre}$, $Gpx4^{POMC\text{-}Cre}$ KO and $Gpx4^{Slc6a3\text{-}CreERT2}$ KO mice revealed no signs of neuronal loss or cell damage, even when exposed to HFHS diet or co-deletion of DJ-1, respectively. Moreover, $Gpx4^{AgRP\text{-}Cre}$, $Gpx4^{POMC\text{-}Cre}$ KO and tamoxifen-induced $Gpx4^{Slc6a3\text{-}CreERT2}$ mice were viable and fertile. Previous mouse studies on diphteria toxin-induced cell death of POMC or AgRP neurons revealed profound metabolic perturbations, i.e. starvation with weight loss and death in case of AgRP neuronal loss, and

obesity in case of POMC neuronal loss. The paucity of a phenotype in our GPx4$^{POMC\text{-}Cre}$ KO mice, and slightly increased body weight in Gpx4$^{AgRP\text{-}Cre}$ KO mice, thus clearly suggests intact POMC and AgRP neurons. Also, the stereological analysis of dopaminergic neurons does not reveal a loss of neurons in in Gpx4 $^{Slc6a3\text{-}CreERT2}$ mice. Overall, our data does not support a crucial role for Gpx4 in the neurogenesis and maintenance of these neuronal subpopulations. Future studies should nevertheless aim to clarify the occurence of ROS-induced metabolic damage in AgRP, POMC or dopaminergic neurons, and their impact as contributing factors for metabolic dysfunction and PD.

The absence of metabolic perturbations and neuronal loss in HFHS-fed Gpx4$^{POMC\text{-}Cre}$ KO mice suggests that Gpx4 is not a necessary component of the antioxidant defense system of POMC neurons, which is surprising given previous reports of relatively high mitochondrial ROS production in POMC neurons of mice exposed to environmental lipids (Diano et al., 2011). Gpx4-independent mechanisms of antioxidant defense and membrane protection may include non-enzymatic protection via lipid-soluble antioxidants such as tocopherols or tocotrienols (Carlson et al., 2016; Pfluger et al., 2004), or the partial replacement of damage-susceptible polyunsaturated fatty acids (PUFA) with saturated fatty acids. Indeed, nutritional supplementation of mice with diets enriched in n-6 and/or n-3 PUFAs induced hypothalamic POMC expression and neurogenesis of POMC-expressing cells, which highlights the importance of essential fatty acid supply in POMC cells (Dziedzic et al., 2007; Nascimento et al., 2016). Antioxidant defense and membrane protection in POMC neurons may further depend on the action of mitochondrial uncoupling protein 2 (UCP2) (Andrews et al., 2008) or detoxifying enzymes such as superoxide dismutase (SOD) or Gpx1. However, exact molecular events that prevent POMC neurons from exceeding a yet-to-be-defined treshold ROS production with subsequent lipid peroxidation and cell damage remain to be determined.

Deletion of Gpx4 from AgRP neurons increased body weight in male mice fed HFHS diet. This finding points towards increased AgRP activity in HFHS-fed Gpx4$^{AgRP\text{-}Cre}$ KO males, as AgRP neurons promote a positive energy balance and weight gain upon activation via increased feeding, reduced energy expenditure and increased fat storage (Krashes et al., 2011). Recently, we further showed a sustained decrease in locomotor activity after rats were centrally treated with

AgRP {Pfluger:2011em}. Our finding of decreased locomotion in HFHS-fed Gpx4$^{AgRP\text{-}Cre}$ KO males is in line with auggmented AgRP neuronal activity, and suggests chronically decreased activity thermogenesis as potential reason for the positive energy balance and increase in weight gain of male HFHS-fed Gpx4$^{AgRP\text{-}Cre}$ KO male. Reasons for the increased susceptibility for weight gain remain elusive. ROS were previously shown to be crucial signaling moieties activating AgRP neurons (Gyengesi et al., 2012). Moreover, hypothalamic ROS production was revealed as prerequisite for central insulin action on food intake (Jaillard et al., 2009) or as trigger for the sensing of nutrients (Benani et al., 2007). AgRP neurons were shown to release elevated levels of ROS when mice were administered the orexigenic stomach hormone ghrelin (Diano et al., 2011), and ROS-scavenging by mitochondrial UCP2 enabled AgRP neurons to sustain their responsiveness to hormones such as ghrelin even under fasting conditions (Andrews et al., 2008). A potential link between Gpx4 activity and UCP2 was reported by Findeisen and colleagues, who showed a compensatory elevation of ROS-scavenging UCP2 levels in hypothalami of mice after chemical ablation of hypothalamic Gpx1 and Gpx4 activity (Findeisen et al., 2009). Our finding of weight gain in Gpx4$^{AgRP\text{-}Cre}$ KO mice is thus supporting a model whereby cellular function in AgRP neurons deficient for Gpx4 is not impaired due to oxidative damage, but rather increased. In this context, it is interesting to notice that mice undergoing chronic HFHS exposure diminished Gpx4 and in tendency also Gpx1 activity in the hypothalamus. Lower Gpx activity in hypothalamic neurons may facilitate adaptive metabolic processes by an increase in ROS-induced signaling. In cell culture, intracellular ROS levels remained unchanged upon partial knockdown of Gpx4, but the levels of oxidized lipid by-products increased profoundly (Yoo et al., 2010). Higher levels of lipid peroxidation products can subsequently act as lipophilic signaling molecules to aggravate stress response signaling cascades such as Jnk (Davis, 2000). Increased Jnk activity in AgRP neurons was recently linked to increased body adiposity in mice (Tsaousidou et al., 2014), but whether AgRP neuron specific Gpx4 deficiency elicits a similar response remains to be tested.

Mice with a loss of Gpx4 and DJ-1 from dopaminergic neurons displayed significant changes in the speed and overall distance of locomotion in an Open Field test compared to WT controls indicating disturbed motoric fuction. We furthermore observed a decrease in the time spent in the open field in Gpx4$^{Slc6a3\text{-}CreERT2}$ KO mice and Gpx4$^{Slc6a3\text{-}CreERT2}$ KO x DJ-1 double KO mice,

which indicates increased anxiety in the absence of Gpx4.. However, Gpx4 deletion from dopaminergic neurons, regardless of the presence or absence of DJ-1, had no effects on body weight or the number of dopaminergic neurons. Oxidized chinone metabolites of dopamine were shown to covalently bind Gpx4, leading to a loss in protein and reduced Gpx4 activity (Hauser et al., 2013). Recent reports showed, relative to cell density, increased levels of Gpx4 and selenoprotein P (Sepp1), a selenium transport protein necessary for the synthesis of selenocysteine-containing proteins, in dystrophic dopaminergic neurons of the SN (Bellinger et al., 2011; 2012). Gpx4 and Sepp1 levels were further enriched in axons and presynaptic terminals within the putamen of patients with PD (Bellinger et al., 2012; 2011). These reports point to a protective role of Gpx4 against the dysfunction of the nigrostriatal pathway. Although in our study overt neurodegeneration was not induced by the loss of GPx4 in dopaminergic neurons, the observed anxiety-related phenotype is indicative for the prodromal stage of PD (Kalia and Lang, 2015; Postuma and Berg, 2016), suggesting that the functionality of these neurons is sligthly impaired. Furthermore, an additional disturbance of the antioxidant defense system - in form of DJ-1 deficiency - induced slight motoric dysfunction. Thus, dopaminergic neurons appear to utilize antioxidant defense mechanisms that compensate for the loss of lipid peroxidase activity. The exact nature of these compensatory defense mechanisms remains elusive, but may include other antioxidant enzymes or the enrichment with dietary antioxidants such as omega-3 PUFAs (Bousquet et al., 2008). Compensatory mechanism(s) may further be overcome – according to the multifactorial nature of PD aetiology – by challenging the system with environmental toxins, genetic disturbances and/or ageing (Kalia and Lang, 2015).

In summary, we reveal that GPx4 deletion, and thus the loss of protection from membrane damage, can exacerbate pathologies in two independent experimental disease models for neuroinflammatory pathologies, namely HFHS-induced metabolic dysfunction or PD. Gpx4 deficiency in dopaminergic neurons incurred prodromal symptoms of PD like anxiety and decreased locomotion in the presence of a second hit. The protective function appears to go beyond the protection from immediate cellular death, but exact mechanisms remain elusive. Our data further indicates that Gpx4 is part of a molecular machinery in AgRP but not POMC neurons that is required for ROS buffering and metabolic homeostasis, at least under obesogenic conditions. We propose that Gpx4 ablation mediates AgRP activation, thereby stimulating a

positive energy balance and additional weight gain. The exact molecular nature of this response remains elusive, but may include a compensatory increase in anti-oxidant defense mechanisms or the selective activation of pro-inflammatory signaling cascades. Overall, our data suggest common pathophysiological mechanisms underlying the two distinct disease models, which may further potential future avenues of disease protection by specifically augmenting GPx4-mediated membrane protection.

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Figure legends:

Fig. 1. Diet-induced obesity reduces hypothalamic Gpx4 expression and activity in mice. A) Schematic representation of Gpx4-mediated membrane protection. Reactive oxygen species (ROS), especially hydroxyl radicals (OH*), induce the formation of phospholipid hydroperoxides (LH --> LOOH), which are detoxified by Gpx4 at the expense of glutathione (GSH). Expression levels of Gpx4 in the hypothalamus (**B**) and peripheral tissues (**C**) of male C57Bl/6J mice exposed to chow or HFHS for 17 weeks (Chow: n=4-6; HFHS: n=15-18). **D)** Enzymatic activity of Gpx4 in the hypothalamus and peripheral tissues of male C57Bl/6J mice exposed to chow or HFHS for 17 weeks (n=4). **E)** Logarithmic expression levels for Gpx4 in AgRP and POMC neurons of young male chow-fed C57Bl/6J mice exposed to ad lib feeding or 24 hours of fasting (n=5-6). Data were generated by Henry et al. (Henry et al., 2015) and deposited under GSE68177. **F**) Enzymatic peroxidation capacity of H_2O_2 in the hypothalamus and peripheral tissues of male C57Bl/6J mice exposed to chow or HFHS for 17 weeks (n=4). Means ± SEM (b-d) or Box and Whiskers with 5-95% percentile (e). * $p<0.05$, ** $p<0.01$.

Fig. 2: Unperturbed body adiposity, food intake and energy expenditure in male HFHS-fed $Gpx4^{POMC\text{-}Cre}$ WT and KO mice. A) Gpx4 locus before (upper line) and after (lower line) Cre-driven recombination, illustrating the PCR-based genotyping strategy. The agarose gel depicts the conditional as well as deleted alleles amplified from murine hypothalamic lysates ± Cre-driven ablation of Gpx4 exons 5-7 from POMC neurons. Male $Gpx4^{POMC\text{-}Cre}$ WT and KO mice (n=20-24) display **B**) similar body weight on chow at 8-10 weeks of age and **C**) near-identical propensities for diet induced body weight gain when exposed to HFHS for 12 weeks. **D**) After 14 weeks of HFHS exposure, male $Gpx4^{POMC\text{-}Cre}$ WT and KO mice (n=10-12) display similar body weight, fat mass and lean mass. Combined indirect calorimetry recordings of **E)** ad libitum food intake, **F)** energy expenditure, **G)** locomotor activity and **H)** respiratory quotients - interrupted by a short power failure at the end of day 3 - revealed unchanged metabolism in male $Gpx4^{POMC\text{-}Cre}$ WT and KO mice that were exposed to HFHS for 14 weeks (n=10). Box and Whisker Plots with 5-95% percentiles (B, D) or means ± SEM (C, E, F, G, H).

Fig. 3. Ablation of Gpx4 from AgRP neurons increases the susceptibility for HFHS induced weight gain, reduced locomotor activity and respiratory quotient. A) Agarose gel depicting the conditional and deleted alleles amplified from murine hypothalamic lysates ± Cre-driven ablation of Gpx4 exons 5-7 from AgRP neurons **B**) Male $Gpx4^{AgRP\text{-}Cre}$ WT and KO mice (n=11-12) display similar body weight on

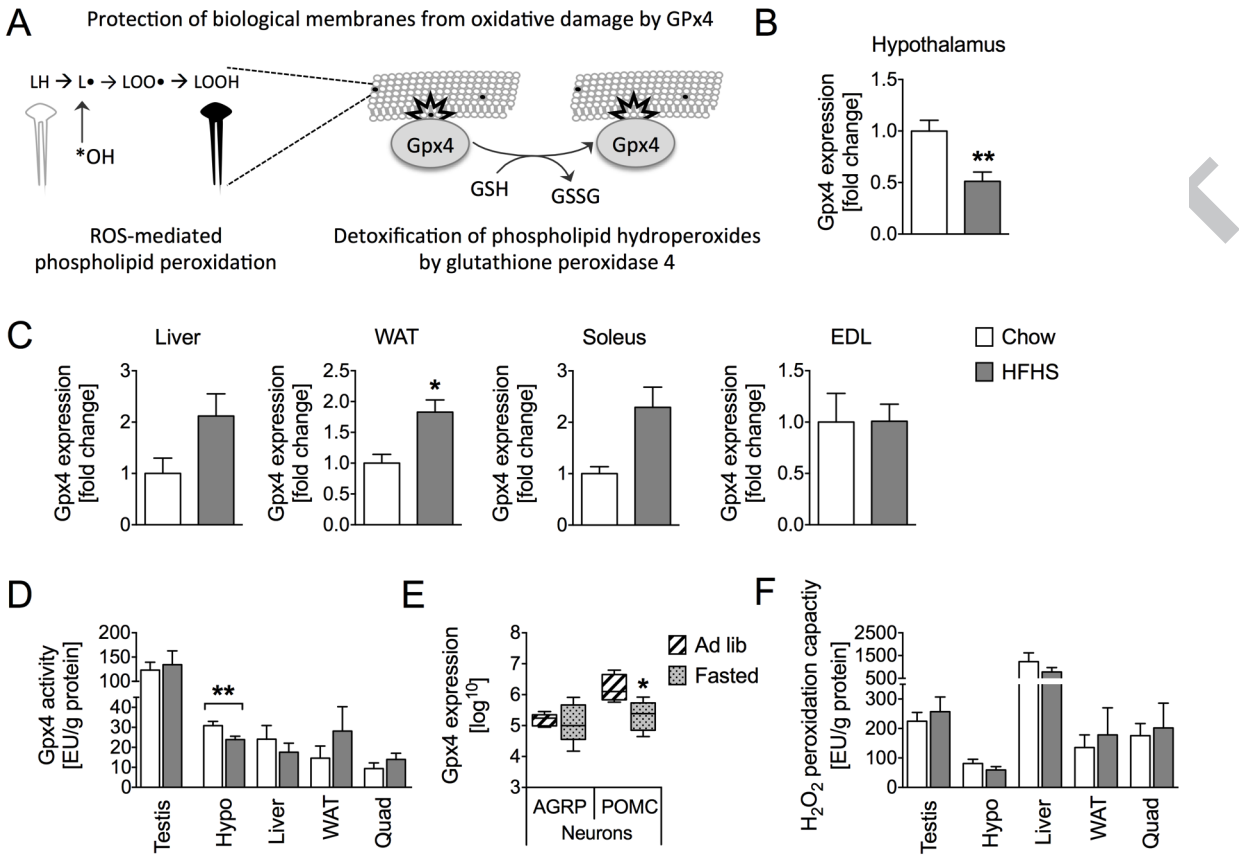
chow at 8-10 weeks of age. **C**) Body weight and **D**) body weight gain were recorded in male $Gpx4^{AgRP\text{-}Cre}$ WT and KO mice (n=11-12) that were exposed to HFHS for 12 weeks. **E)** Body weight and body composition in subsets of male $Gpx4^{AgRP\text{-}Cre}$ WT and KO mice (n=7-10) after 17 weeks of HFHS feeding. Combined indirect calorimetry recordings of **F)** ad libitum food intake, **G)** energy expenditure, **H)** locomotor activity and **I)** respiratory quotients in male $Gpx4^{AgRP\text{-}Cre}$ WT and KO mice that were exposed to HFHS for 17 weeks (n=7). Box and Whisker Plots with 5-95% percentiles (B,E) or means ± SEM (C,D,F,G,H,I). *p<0,05, **p<0.01.

Fig. 4. Unperturbed body adiposity, energy expenditure, locomotor activity and respiratory quotients in female HFHS-fed $Gpx4^{AgRP\text{-}Cre}$ WT and KO mice. A) Female $Gpx4^{AgRP\text{-}Cre}$ WT and KO mice (n=8-10) display near-identical propensities for diet induced body weight gain when exposed to HFHS for 12 weeks. **B**) After 17 weeks of HFHS exposure, female $Gpx4^{AgRP\text{-}Cre}$ WT and KO mice (n=4) display similar body weight, fat mass and lean mass. Combined indirect calorimetry recordings of **C)** energy expenditure, **D)** locomotor activity and **E)** respiratory quotients revealed unchanged metabolism in female $Gpx4^{AgRP\text{-}Cre}$ WT and KO mice that were exposed to HFHS for 17 weeks (n=4). Means ± SEM (A,C,D,E) or Box and Whisker Plots with 5-95% percentiles (B).

Fig. 5. Unperturbed glucose homeostases and neuronal cell densities in HFHS-fed $Gpx4^{AgRP\text{-}Cre}$ and $Gpx4^{POMC\text{-}Cre}$ KO mice. Male $Gpx4^{POMC\text{-}Cre}$ WT and KO mice (n=10-12) were exposed to **A)** glucose or **B)** insulin tolerance tests after 9 (GTT) or 10 (ITT) weeks of HFHS feeding, with intraperitoneal administrations of 1.5 g glucose (GTT) or 0.75 U insulin (ITT) per kg body weight, respectively. Male $Gpx4^{AgRP\text{-}Cre}$ WT and KO mice (n=11-12) were subjected to **C)** glucose or **D)** insulin tolerance tests after 12 (GTT) or 13 (ITT) weeks of HFHS feeding, with intraperitoneal administrations of 1.5 g glucose (GTT) or 0.75 U insulin (ITT) per kg body weight, respectively. Female $Gpx4^{AgRP\text{-}Cre}$ WT and KO mice were (n=8-10) subjected to a GTT **(E)** after 12 weeks of HFHS exposure (1.5 g/kg) and an ITT **(F)** after 13 weeks of HFHS exposure (0.75 U/kg). **G**) Immunohistochemical stainings for POMC-positive nuclei revealed no difference between male HFHS-fed $Gpx4^{POMC\text{-}Cre}$ WT and KO mice (n=4). **H**) Similar fluorescent densities of AgRP-positive neuronal projections in male HFHS-fed $Gpx4^{AgRP\text{-}Cre}$ WT and KO mice (n=4). Means ± SEM. Scale bars represent 100 µm.

Fig. 6. Inducible dopamine transporter (Slc6a3) Cre driven ablation of Gpx4 in adult WT and DJ-1 deficient mice does not induce a loss of dopaminergic neurons. A) Agarose gel depicting the conditional and deleted alleles amplified from dopaminergic midbrain and non-dopaminergic cerebellum lysates ± tamoxifen-driven ablation of Gpx4 exons 5-7. **B**) Horizontal brain sections stained with anti-tyrosine-hydroxylase antibody revealed no alterations in **C**) the number of dopaminergic neurons after unbiased stereology in littermate control mice ($Gpx4^{Slc6a3\text{-}CreERT2}$ WT x DJ-1 WT), mice lacking Gpx4 in dopaminergic neurons ($Gpx4^{Slc6a3\text{-}CreERT2}$ KO x DJ-1 WT) and double knockouts ($Gpx4^{Slc6a3\text{-}CreERT2}$ KO x DJ-1 KO). Means ± SEM. Scale bars represent 200 µm.

Fig. 7. In dopaminergic neurons, Gpx4 is only relevant for motor behavior with an additional hit. Littermate control mice ($Gpx4^{Slc6a3\text{-}CreERT2}$ WT x DJ-1 WT), mice lacking DJ-1 ($Gpx4^{Slc6a3\text{-}CreERT2}$ WT x DJ-1 KO), mice lacking Gpx4 in dopaminergic neurons ($Gpx4^{Slc6a3\text{-}CreERT2}$ KO x DJ-1 WT) and double knockouts ($Gpx4^{Slc6a3\text{-}CreERT2}$ KO x DJ-1 KO) were subjected to the Open Field test **(A-D)** and the Vertical Pole test **(E)**. Double knockouts for Gpx4 in dopaminergic neurons and DJ-1 show reduced spontaneous activity **(B,C)**, and both double and single Gpx4 knockouts are more anxious **(D)**. There were no differences in body weight **(A)** or nigrostriatal function-specific behavior **(E)**. Box and Whisker Plots with 5-95% percentiles. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. control group.


A
Protection of biological membranes from oxidative damage by GPx4
LH → L• → LOO• → LOOH
*OH
Gpx4
Gpx4
GSH
GSSG
ROS-mediated phospholipid peroxidation
Detoxification of phospholipid hydroperoxides by glutathione peroxidase 4
B
Hypothalamus
Gpx4 expression [fold change]
**
C
Liver
WAT
Soleus
EDL
Chow
HFHS
Gpx4 expression [fold change]
*
D
Gpx4 activity [EU/g protein]
**
Testis
Hypo
Liver
WAT
Quad
E
Gpx4 expression [log10]
Ad lib
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F
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Liver
WAT
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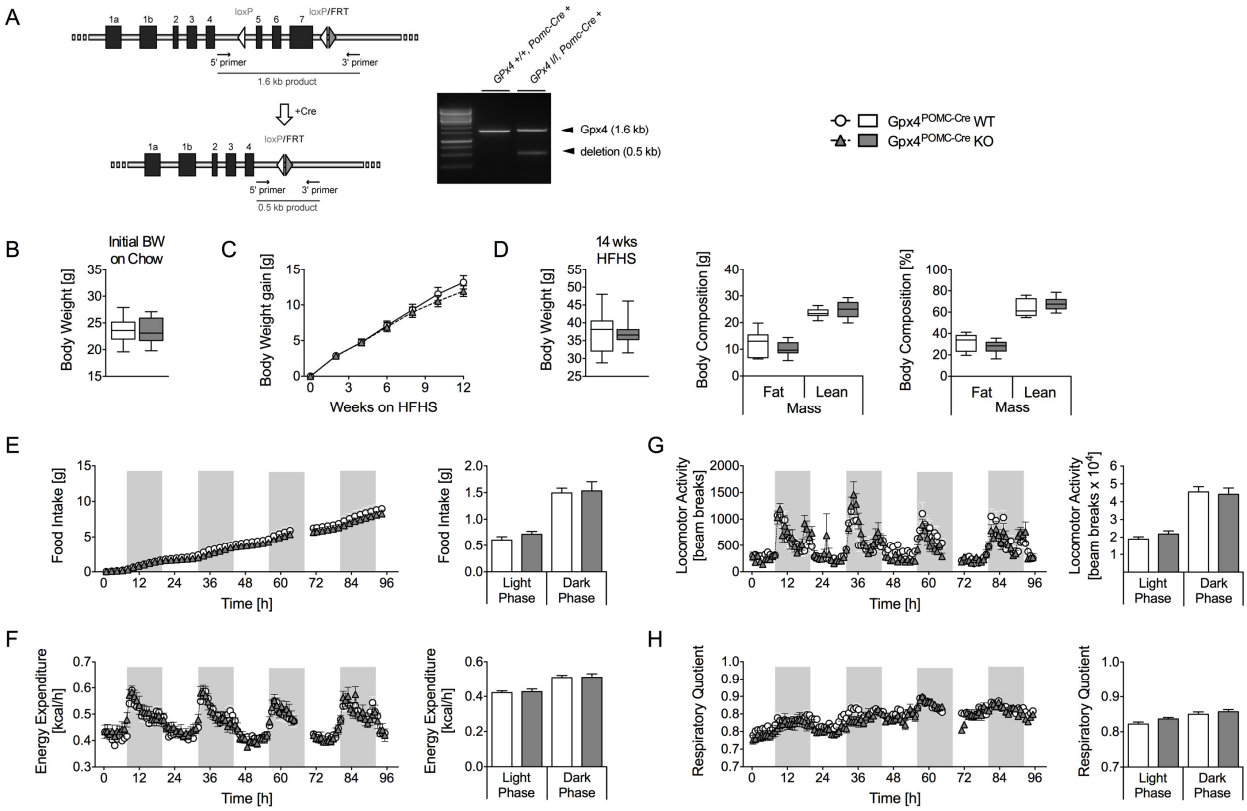

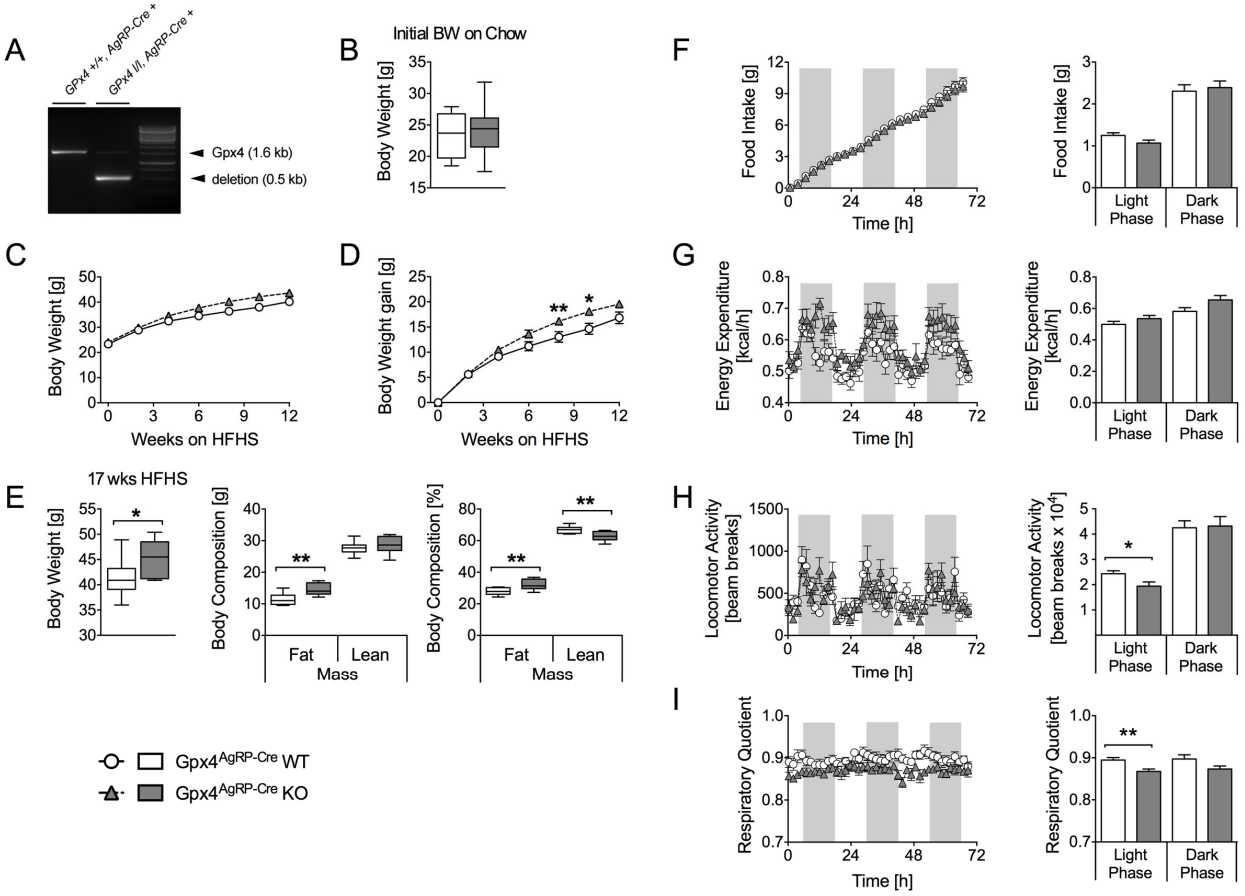

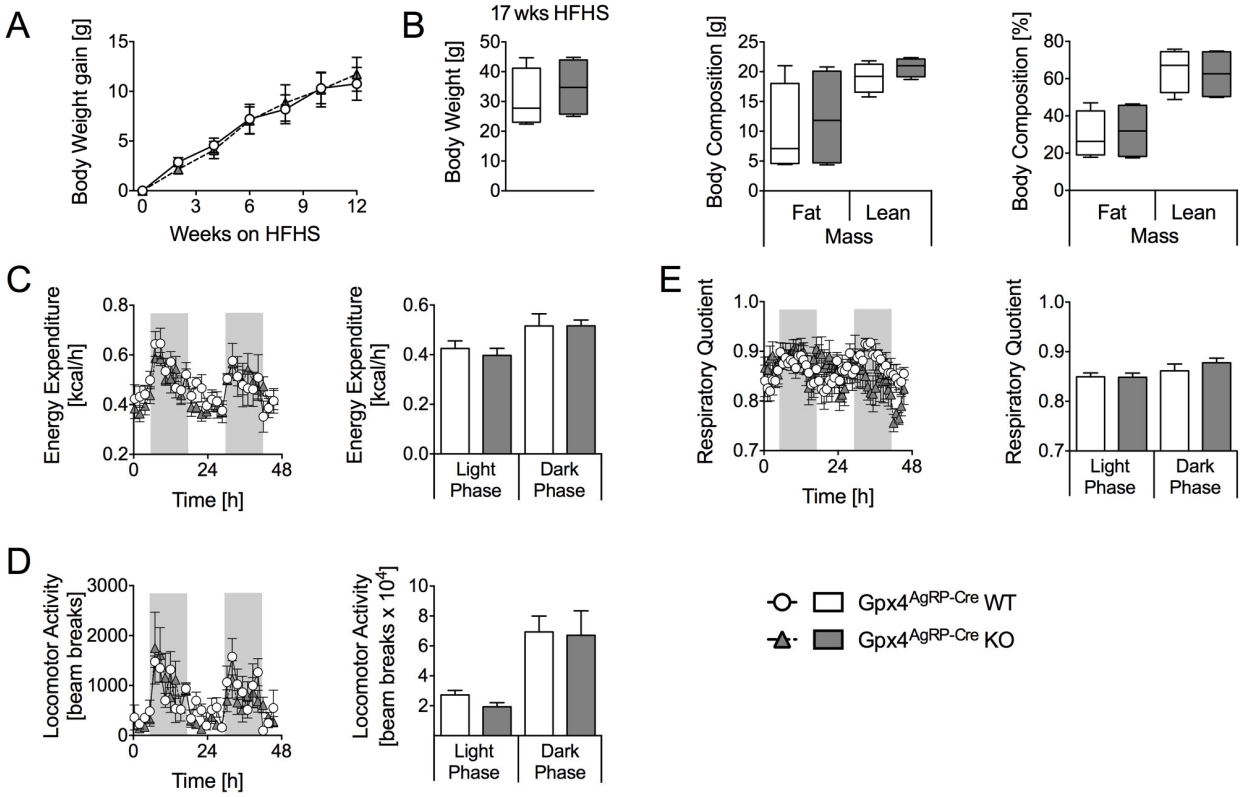

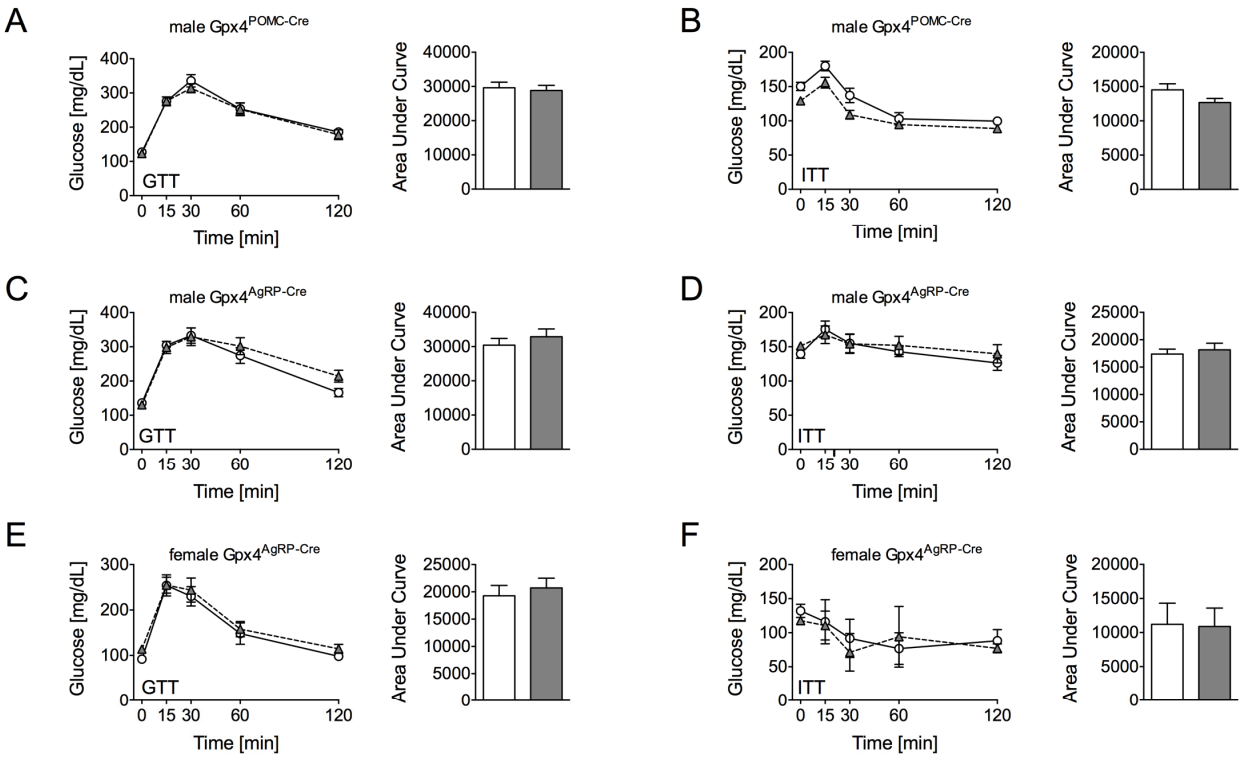

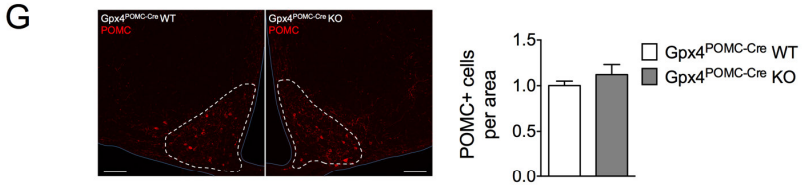

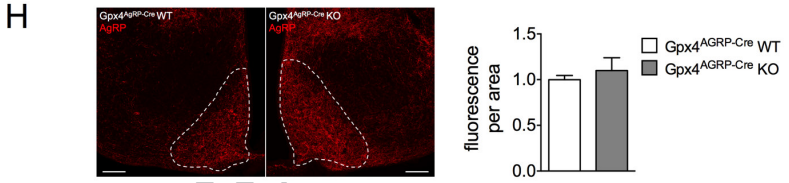

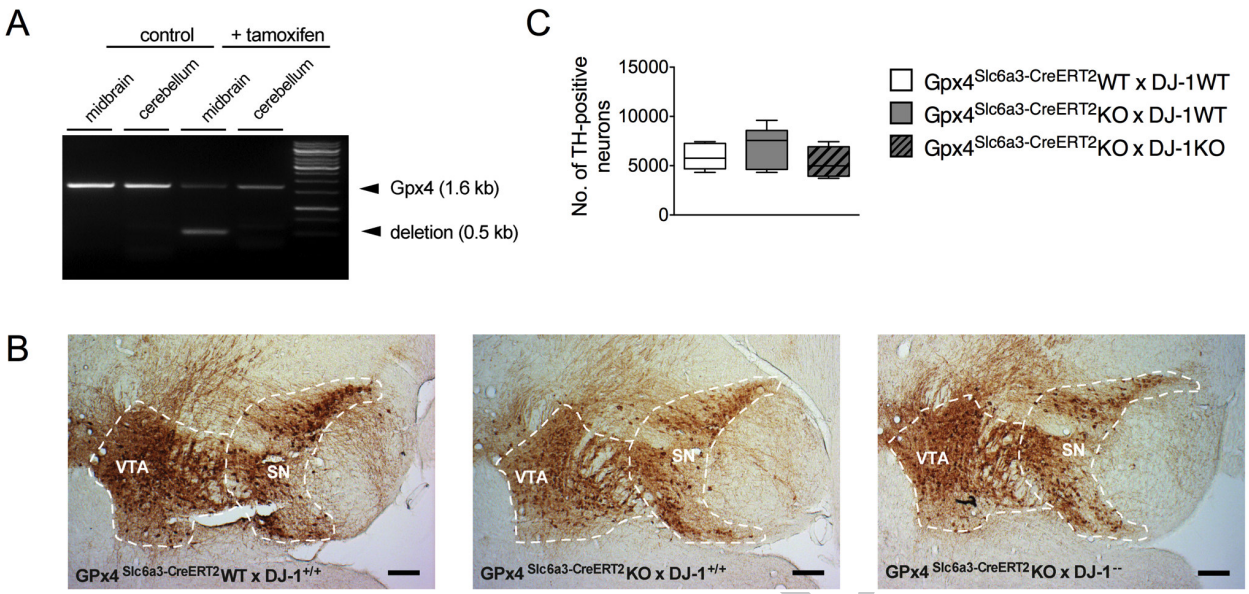

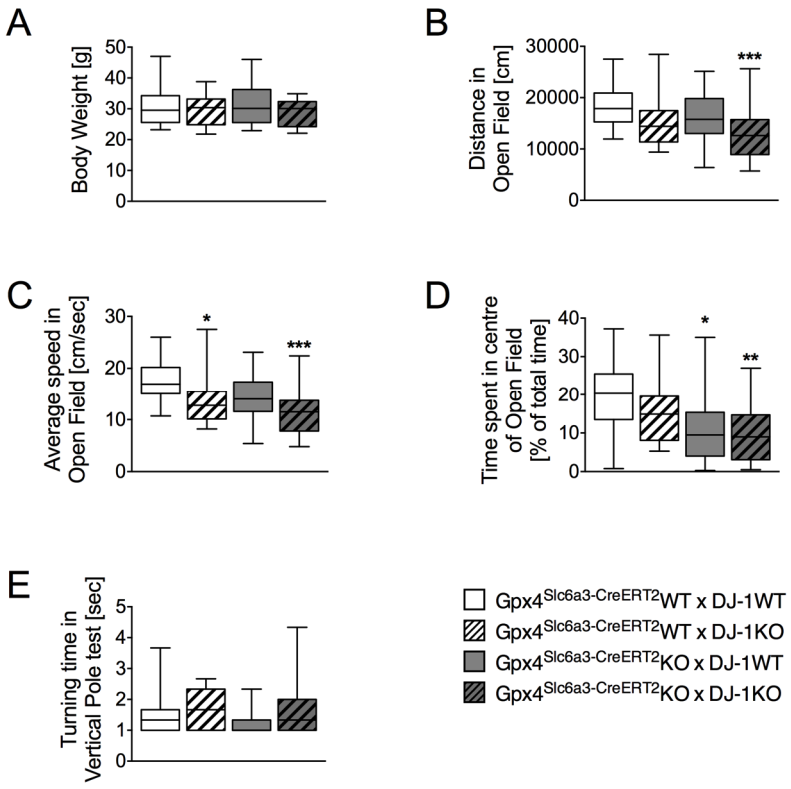

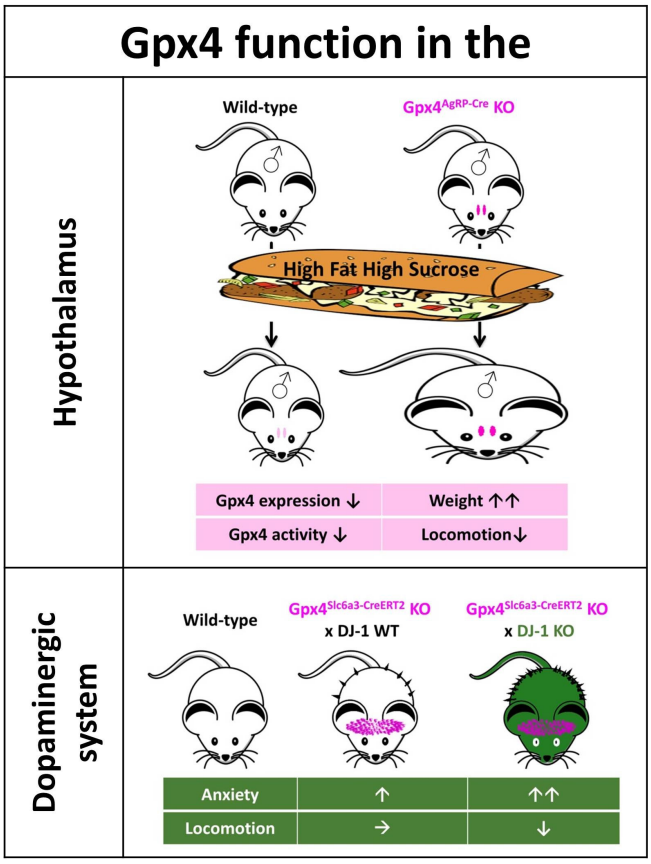

Highlights:

- Gpx4 deletion from dopaminergic neurons leads to symptoms of PD-like anxiety
- Gpx4 and DJ-1 co-deletion from dopaminergic neurons decreases spontaneous locomotion
- HFHS diet exposure attenuates hypothalamic Gpx4 expression and activity in mice
- HFHS-fed $Gpx4^{AgRP\text{-}Cre}$ KO males show increased weight gain and reduced locomotion