

# Protein Kinase C $\beta$ : a New Target Therapy to Prevent the Long-Term Atypical Antipsychotic-Induced Weight Gain

**Alessandro Rimessi<sup>1,6</sup>, Chiara Pavan<sup>2,6</sup>, Elli Ioannidi<sup>1</sup>, Federica Nigro<sup>1</sup>, Claudia Morganti<sup>1</sup>, Alberto Brugnoli<sup>3</sup>, Francesco Longo<sup>3</sup>, Chiara Gardin<sup>4</sup>, Letizia Ferroni<sup>4</sup>, Michele Morari<sup>3</sup>, Vincenzo Vindigni<sup>5</sup>, Barbara Zavan<sup>4,7</sup> and Paolo Pinton<sup>\*,1,7</sup>**

<sup>1</sup>Department of Morphology, Surgery and Experimental Medicine, Section of Pathology, Oncology and Experimental Biology, Laboratory for Technologies of Advanced Therapies (LTTA), University of Ferrara, Ferrara, Italy; <sup>2</sup>Unit of Psychiatry, Department of Neurosciences NPSRR, University of Padua, Padua, Italy; <sup>3</sup>Department of Medical Sciences, Section of Pharmacology, Neuroscience Center and National Institute of Neuroscience, University of Ferrara, Ferrara, Italy; <sup>4</sup>Department of Biomedical Sciences, University of Padua, Padua, Italy; <sup>5</sup>Unit of Plastic Surgery, Department of Neurosciences NPSRR, University of Padua, Padua, Italy

Antipsychotic drugs are currently used in clinical practice for a variety of mental disorders. Among them, clozapine is the most effective medication for treatment-resistant schizophrenia and is most helpful in controlling aggression and the suicidal behavior in schizophrenia and schizoaffective disorder. Although clozapine is associated with a low likelihood of extrapyramidal symptoms and other neurological side effects, it is well known for the weight gain and metabolic side effects, which expose the patient to a greater risk of cardiovascular disorders and premature death, as well as psychosocial issues, leading to non-adherence to therapy. The mechanisms underlying these iatrogenic metabolic disorders are still controversial. We have therefore investigated the *in vivo* effects of the selective PKC $\beta$  inhibitor, ruboxistaurin (LY-333531), in a preclinical model of long-term clozapine-induced weight gain. Cell biology, biochemistry, and behavioral tests have been performed in wild-type and PKC $\beta$  knockout mice to investigate the contribution of endogenous PKC $\beta$  and its pharmacological inhibition to the psychomotor effects of clozapine. Finally, we also shed light on a novel aspect of the mechanism underlying the clozapine-induced weight gain, demonstrating that the clozapine-dependent PKC $\beta$  activation promotes the inhibition of the lipid droplet-selective autophagy process. This paves the way to new therapeutic approaches to this serious complication of clozapine therapy.

*Neuropsychopharmacology* (2017) **42**, 1491–1501; doi:10.1038/npp.2017.20; published online 15 February 2017

## INTRODUCTION

Antipsychotic drugs (APDs) are a class of compounds widely used for a variety of psychiatric conditions, including schizophrenia, mood disorders, bipolar disorder, autism, and many other conditions characterized by agitation and altered mental status (Deng, 2013). Although the majority of patients show some therapeutic benefit and symptomatic relief with first-line APD medications, a significant proportion, ie, 20–30%, of all patients with schizophrenia are resistant to standard APD treatment (Sinclair and Adams, 2014). For those patients who do not respond to standard therapy, treatment with clozapine may be warranted (Meltzer, 2012). Clozapine is the most effective medication

for treatment-resistant schizophrenia and is most helpful in controlling aggression and suicidal behavior in schizophrenia and schizoaffective disorder (Froegley *et al*, 2012; Mamo, 2007).

Although clozapine is associated with a low likelihood of extrapyramidal symptoms and other neurological side effects (Leucht *et al*, 2009), it presents a highest risk to induce body weight gain and general metabolic dysregulation, such as obesity, hyperlipidemia, insulin resistance, and diabetes (Foley and Morley, 2011). A recent meta-analysis reports that 51.9% of people on clozapine have metabolic syndrome compared with 28.2% for olanzapine and 27.9% for risperidone. In a 10-year follow-up on people treated with clozapine in the United States of America, 43% of people have developed diabetes with a mean weight gain of over 13.5 kg (Siskind *et al*, 2016). The clozapine has the propensity to induce weight gain, which has been demonstrated over both short- and long-term treatment (Henderson *et al*, 2010), exposing the patient to a greater risk of cardiovascular disorders, and premature death, as well as psychosocial issues, leading to non-adherence to therapy (Citrome and Vreeland, 2008; Stahl *et al*, 2009). Approximately 40% of patients discontinued clozapine during the first 2 years of therapy, causing a rapid clinic deterioration,

\*Correspondence: Professor P Pinton, Department of Morphology, Surgery and Experimental Medicine, Section of Pathology, Oncology and Experimental Biology, Laboratory for Technologies of Advanced Therapies (LTTA), University of Ferrara, Via Fossato di Mortara 70 (c/o CUBO), Ferrara 44121, Italy, Tel: +0039 0532455802, Fax: +0039 0532455351, E-mail: pnp@unife.it

<sup>6</sup>These authors contributed equally to this work.

<sup>7</sup>These authors share senior co-authorship.

Received 8 October 2016; revised 3 January 2017; accepted 21 January 2017; accepted article preview online 27 January 2017

onset of chronic disability, re-hospitalization, and poorer functioning (Legge *et al*, 2016).

The physiological mechanisms underlying clozapine-induced weight gain and metabolic syndrome remain unclear. Clozapine was shown to enhance the differentiation of adipose tissue precursor cells to mature adipocytes (Hemmrich *et al*, 2006). In our previous *in vitro* study, the effects of different APDs (clozapine, olanzapine, quetiapine, risperidone, and aripiprazole) on adipogenic events in cultured human adipocyte-derived stem cells (ADSCs) and rat muscle-derived stem cells (MDSCs) were analyzed. This study showed that APDs influenced the differentiation of uncommitted mesenchymal precursors and the transdifferentiation of MDSCs through protein kinase C isoform  $\beta$  (PKC $\beta$ ) activation (Pavan *et al*, 2010). Reactive oxygen species and activated PKC $\beta$  drove stem cells from both adipose and skeletal muscle tissues toward an adipogenic potential (Aguiari *et al*, 2008; Giorgi *et al*, 2010). The APD-induced weight gain involved not only preadipocytes derived from adipose tissue but also the commitment of stem cells derived from muscle tissue in adipogenic feature, providing some insight into the signals that underlie this process (Aguiari *et al*, 2008). Multiple mechanisms may be involved in clozapine-induced weight gain, thus preventive and therapeutic approaches can be undertaken to counteract the different targets. Among them, PKC $\beta$  resulted to be of particular importance in order to prevent weight gain during the APD treatment *in vitro* (Pavan *et al*, 2010). The crucial role of kinase was proven by small interfering RNA (siRNA) and pharmacological inhibition, leading to strong reduction of the neo-formation of adipose cells (Aguiari *et al*, 2008). This finding suggested that combining a PKC $\beta$  inhibitor with APDs could tackle the related weight gain *in vivo*.

Considering the extensive evidence that weight gain is a major issue in clozapine therapy and the proportional lack of published data regarding the treatment options for clozapine-induced weight gain, we have characterized the *in vivo* effects of the selective PKC $\beta$  inhibitor, ruboxistaurin (LY-333531, RBX), in a preclinical model of long-term clozapine-induced weight gain. In addition, we have explored through a combined behavioral and biochemical approach, the contribution of endogenous PKC $\beta$  and its pharmacological inhibition to the effects of clozapine. Finally, we also shed light on a novel aspect of the mechanism underlying the clozapine-induced weight gain, demonstrating that the ADP-dependent PKC $\beta$  activation promote the inhibition of the lipid droplet-selective autophagy process.

## MATERIAL AND METHODS

### Subjects and Housing

Animals employed in all procedures were 4-week-old male C57BL/6J wild-type (WT) and PKC $\beta$  null (PKC $\beta$ <sup>-/-</sup>) mice. All mice were individually housed in plastic 'tub' cages (26.5 × 17 × 12 cm<sup>3</sup>) with a stainless steel grid lid and wood shavings scattered on the floor. The vivarium was maintained at 23 °C on a 12-h light/12-h dark cycle with lights off at 0700 hours. The mice had access to pelleted Teklad Rodent Diet with 60% of calories from fat (cod. MV2 Envigo RMS S. R.L.). According to the manufacturer's specifications, by

weight; metabolizable energy content, 5.1 kcal/g. Deionized water and food were available *ad libitum*.

### Drug Administration, Food and Water Intake, and Body Weight Evaluations

The study was conducted on 12 mice for each experimental condition:

- Group A: male C57BL/6J WT, vehicle
- Group B: male C57BL/6J WT, clozapine (Haxal AG)
- Group C: male C57BL/6J WT, RBX (Axon Medchem)
- Group D: male C57BL/6J WT, RBX and clozapine
- Group E: male C57BL/6J PKC $\beta$ <sup>-/-</sup>, vehicle
- Group F: male C57BL/6J PKC $\beta$ <sup>-/-</sup>, clozapine

Mice were acclimated to individual cages for at least 6 days. After acclimation, food and water intakes were measured weekly for 15 weeks. Food intake was determined by weighing the metal cage top, including the food. During the test, deionized water was available through bottle choice where clozapine was added into water-beverage at 250 mg/l. The beverage containing the antipsychotic was replaced twice a week. Intakes were measured daily in the middle of the light period. Extensive experience has shown that fluid spillage and evaporation from drinking tubes rarely exceeds 0.2 ml over 48 h, and so these were ignored. The body weight was measured weekly, always between 0800 and 1000 hours.

The selective PKC $\beta$  inhibitor, RBX, was administered (0.5 mg/Kg) by oral gavage three times a week for 15 weeks, following the standard operating procedure. We have chosen this dose and route of administration based on the literature (Danis *et al*, 1998; Ishii *et al*, 1996; Lei *et al*, 2013; Nagareddy *et al*, 2009) and in-house experience gained in these years, through pilot experiments.

After 5 months, three animals from each group were sampled to obtain skeletal muscle and visceral white adipose tissue for RT-PCR and immunoblotting analysis; the tissues were processed and frozen for the analyses.

### MDSCs and Adipocyte Differentiation Detection

Primary cultures of MDSCs were prepared from newborn C57BL/6J WT and PKC $\beta$ <sup>-/-</sup> mice (3–5 days) as described in (Brini *et al*, 1997). Adipogenic conversion was observed both in the first preplating (2 h) and in the re-plating of non-rapidly adherent cells (24 h). The second pool of adherent cells was used for all experiments. The viable cells obtained were counted using the Trypan blue exclusion assay and seeded at a density of 1 × 10<sup>6</sup> cells per square centimeter for *in vitro* expansion in DMEM supplemented with glucose 25 mM after 5 days of expansion. At day 1, clozapine (1  $\mu$ M, 50  $\mu$ M, 100  $\mu$ M, 250  $\mu$ M, 500  $\mu$ M, and 1 mM) was added for the following 3 days (Supplementary Figure S2). For the differentiation experiments *in vitro*, we have chosen the concentration of 250  $\mu$ M because this concentration is able to induce adipose differentiation without causing cytotoxicity. DMEM in standard glucose was used as a negative control. Oil Red (Sigma-Aldrich) staining of the cytoplasmic droplets of neutral lipids was performed according to the standard procedure. Monolayer cultures were washed with PBS and then Oil Red working solution (0.5 g in 100 ml isopropanol) was added to well for 2 h at room temperature. After

washing, stained cells were kept in 10% paraformaldehyde and examined by light microscopy.

### Cell Proliferation Determination (3-4,5-Dimethylthiazol-2-yl-2,5-Diphenyltetrazolium Bromide (MTT) Assay)

Cell proliferation rates were determined by the MTT cytotoxicity test using the Denizot and Lang method with minor modifications (Denizot and Lang, 1986).

### DNA Content

DNA content was determined using a DNeasy Kit (Qiagen) to isolate total DNA from cell cultures according to the manufacturer's protocol, after overnight incubation in proteinase K (Qiagen). The concentration of DNA was detected by measuring the absorbance at 260 nm in a spectrophotometer.

### Behavioral Studies

The psychotropic properties of clozapine were investigated using a battery of behavioral tests (Marti *et al*, 2007; Viaro *et al*, 2008) encompassing the bar, drag, and accelerod tests, as well as the open field. To reduce the number of animals used, mice were tested at the end of the chronic clozapine therapy. All experiments were performed between 0830 and 1400 hours. Experiments were conducted blind by trained observers working in pairs. The behavior of mice was videotaped and analyzed and scored off-line by a different trained operator.

The bar test measures the ability of the mouse to respond to an externally imposed static posture. Also known as the catalepsy test, it can be used to quantify akinesia (ie, time to initiate a movement) also under conditions that are not characterized by increased muscle tone (ie, rigidity) as in the cataleptic/catatonic state. The time spent by each forelimb on bars of different heights (1.3, 3, and 6 cm) was measured (immobility cutoff: 20 s), and the akinesia was calculated as the total time spent on the bars (total maximal time of catalepsy: 60 s).

The drag test is a modification of the 'wheelbarrow test' (Schallert *et al*, 1979), and measures the ability of the animal to balance its body posture with forelimbs in response to an externally imposed dynamic stimulus. It gives information regarding both the time to initiate a movement and to execute it (bradykinesia). In the drag test, the mouse was lifted by the tail, leaving the front paws on the table and dragged backward at a constant speed of about 20 cm/s for a fixed distance (100 cm). The number of steps performed by each paw was recorded by two different observers. For each animal, five to seven measurements were collected.

The rotarod test measures different motor parameters such as motor coordination, gait ability, balance, muscle tone, and motivation to run. Animals were placed on a rotating cylinder whose speed was increased stepwise from 5 to 60 r.p.m. (5 r.p.m. every 3 min). The time spent on the cylinder was measured.

Spontaneous locomotor activity was measured by using the ANY-maze video-tracking system (Ugo Basile, application version 4.99 g Beta). The mouse was placed in a square

plastic cage (60  $\times$  60 cm<sup>2</sup>) located in a sound- and light-attenuated room and motor activity was monitored for the first 30 min. Four mice were monitored at the same time in each experiment. Total distance travelled (meters) was recorded every 10 min for a maximum of 30 min. To avoid mice olfactory cues, cages were carefully cleaned with a dilute (5%) ethanol solution and washed with water between animal trials.

### Real-time PCR

Total RNA was extracted by using the RNeasy Mini Kit (Qiagen), including DNase digestion with the RNase-Free DNase Set (Qiagen). Five-hundred nanograms of total RNA of each sample were reverse transcribed with SensiFAST cDNA Synthesis Kit (Bioline Reagents Ltd), following the manufacturer's instructions. Real-time PCR was performed using 300 nM primers and the SensiFAST SYBR No-ROX Kit (Bioline). Thermal cycling and fluorescence detection were performed using the Rotor-Gene 3000 (Corbett Research). The thermal cycling conditions were as follows: 2 min denaturation at 95 °C; 45 cycles of 5 s denaturation at 95 °C, annealing for 10 s at 60 °C, and 20 s elongation at 72 °C.

All reactions were performed twice. Results were normalized by GAPDH mRNA and expressed in terms of percentage variation with respect to vehicle.

### Fluorescence Microscopy

MDSCs were plated onto 24  $\times$  24-mm<sup>2</sup> coverslips at a density of 3  $\times$  10<sup>5</sup> cells/coverslip. Lipid droplets (LDs) were stained by incubating cells with BODIPY 493/503 (Invitrogen) for 30 min while lysosomes were highlighted with LysoTracker (Invitrogen). Mounting medium contained DAPI stain to highlight the cell nucleus. Images were acquired with an Axiovert 200 fluorescence microscope (Carl Zeiss Ltd), maintained at 37 °C in a temperature-controlled stage, with a  $\times$  100 objective. Quantification was performed in individual frames after deconvolution with the manufacturer's software and thresholding using the ImageJ software (NIH). Particle number was quantified with the 'analyze particles' function in thresholded single sections with size (pixel<sup>2</sup>) settings from 0.1 to 10 and circularity from 0 to 1. Co-localization was calculated by JACoP plugin in single Z-stack sections of deconvoluted images.

### Immunoblotting Assay

In brief, to obtain whole-cell extracts, cells were washed, harvested, and lysed in RIPA buffer supplemented with 2 mM Na<sub>3</sub>VO<sub>4</sub>, 2 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and complete protease inhibitor cocktail (Roche Diagnostics Corp.). Thereafter, protein extracts were separated on precast 4–12% SDS-PAGE gels (Life Technologies), electrotransferred onto PVDF membranes (Bio-Rad), and probed with the specific antibodies anti-LC3 (L7543 Sigma Aldrich) and anti-actin (A-3853 Sigma Aldrich). Finally, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (Southern Biotech), followed by chemiluminescence detection using West Pico reagent (Thermo Scientific–Pierce). The

immunoreactive bands were acquired using the ImageQuant LAS-4000 system (GE Healthcare).

### Statistical Analysis

All the data are given as mean  $\pm$  SEM. Unless otherwise indicated, all assays were performed independently and in triplicate, yielding comparable results. Data were analyzed using repeated-measure (RM) ANOVA. Results from treatments showing significant overall changes were subjected to *post hoc* Tukey tests with significance for  $p < 0.05$ . Student's *t*-test was used to determine statistical significance ( $p < 0.01$  and  $p < 0.05$ ) between two groups. Statistical analysis was performed with the Prism (GraphPad Prism, USA) and Microsoft Excel (Microsoft Co) softwares.

### Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Italian Ministry of Health. The experimental protocol has been approved by the Italian Ministry of Health (protocol number: 19184).

## RESULTS

### Clozapine Potentiates Weight Gain on High-Fat Diet

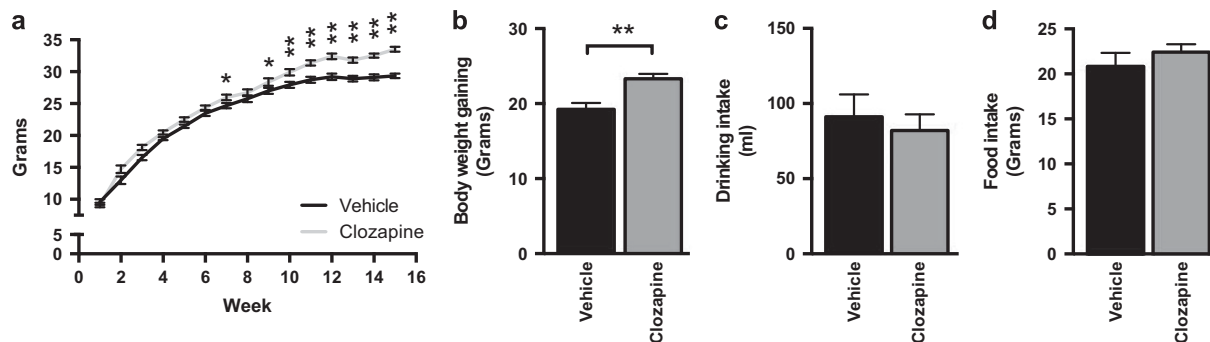
In order to develop a preclinical model for clozapine-induced weight gain in the mouse, we surveyed C57BL/6J WT inbred strain, which is the founder strain of PKC $\beta$ <sup>-/-</sup> mice (Bansode et al, 2008). Twelve males were randomized to receive either clozapine (dissolved into water-beverage at 250 mg/l) or vehicle and were fed a high-fat diet *ad libitum*. RM ANOVA revealed a highly significant increase in the weekly mean cumulative weight gain of the clozapine-treated group starting from week 7 (Figure 1a), with an average gain of 23 g over initial body weight (Figure 1b). Such difference in body weight between vehicle- and clozapine-treated mice was not due to a different drinking intake (Figure 1c) but likely to the about 3 mg/day of clozapine taken by each mice with access to the antipsychotic. Examining the tissues isolated from mice, the visceral white fat weight was higher in clozapine- than vehicle-treated mice, whereas no

differences in liver weight emerged (Supplementary Figure S1A). To verify the possibility that clozapine-dependent body weight increase was also due to changes in food consumption, food intake was monitored (Figure 1d, Supplementary Figure S1B and C). Clozapine increased food intake by about 8% per week. Although this increase was not statistically significant on a weekly basis (clozapine vs vehicle group), it contributes a greater cumulative food intake over time, which might sustain the clozapine-induced body weight increase, in line with some reports of clozapine-dependent hyperphagic behavior (Hartfield et al, 2003; Kluge et al, 2007).

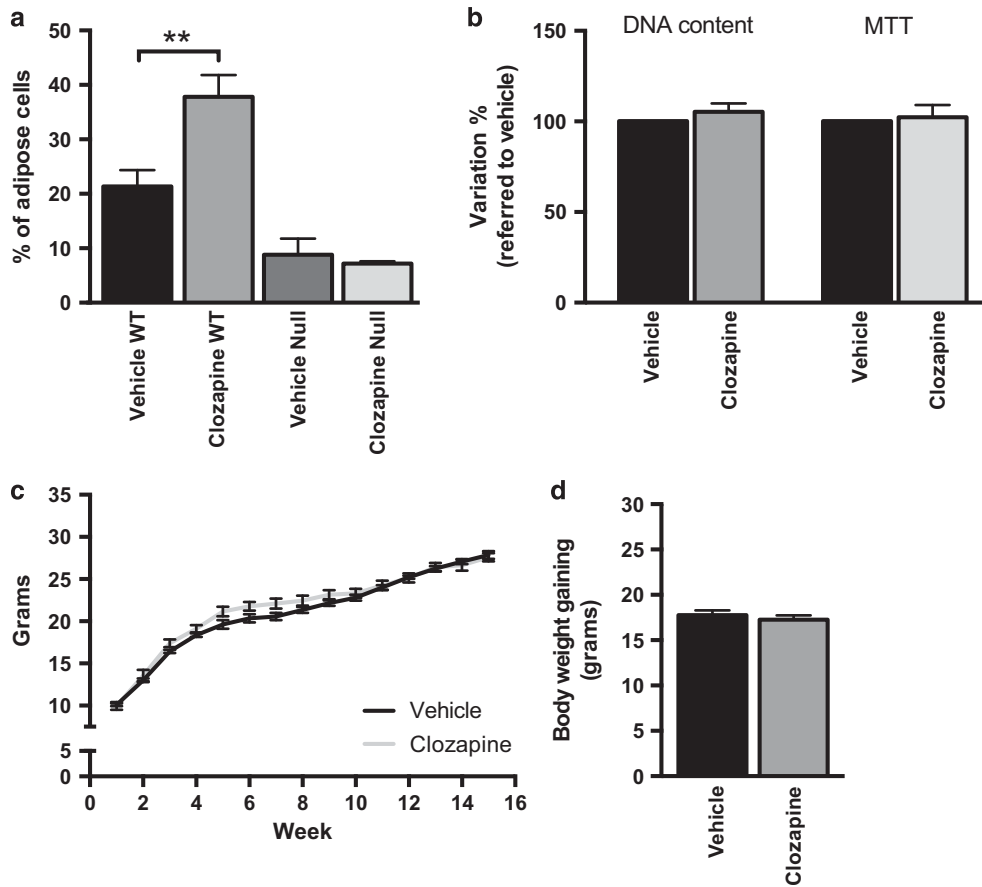
### Effect of PKC $\beta$ Deletion on Clozapine-Promoting Adipogenesis *In Vitro* and *In Vivo*

As reported in our previous work, pharmacological inhibition (hispidin) or genetically deletion of PKC $\beta$  (siRNA) reduced the adipogenic potential of clozapine in human-derived ADSCs and rat-derived MDSCs (Aguari et al, 2008).

The ability of clozapine to increase total lipid production in mouse-derived MDSC was first analyzed (Supplementary Figure 2). To this end, MDSCs derived from WT and PKC $\beta$ <sup>-/-</sup> mice were cultured in high glucose and then treated for 3 days with clozapine (see Material and Methods section). As shown in Figure 2a, a clear increase of lipid production was found in WT-derived MDSCs treated with clozapine. This increase was dependent on PKC $\beta$  as it was not observed in PKC $\beta$ <sup>-/-</sup>-derived MDSCs. These differences in lipid production were appreciated through the quantification of Oil Red-positive cells and reported as percentage in histograms. We then evaluated whether the increased lipid production observed in WT cells was due to an increase of MDSC proliferation. DNA content analysis and MTT vitality test did not reveal any major stimulatory effects of clozapine in WT and PKC $\beta$ <sup>-/-</sup> cells, indicating that the lipid accumulation in WT cells was not related to a proportional increase in cell number (Figure 2b). These *in vitro* results suggest that the lack of PKC $\beta$  is a determinant factor to prevent the differentiation of MDSCs to adipocytes. In order to confirm this finding *in vivo*, the same experimental procedure was performed in PKC $\beta$ <sup>-/-</sup> mice. As expected, we did not record any significant differences in body weight (Figures 2c and d) and food and drinking intake (data not



**Figure 1** (a, b) Long-term treatment with clozapine (250 mg/l for 15 weeks) induced body weight gain in C57BL/6J WT mice. The kinetics indicate that the weight gain became significant after the seventh week of treatment, gaining a mean of 23 g over initial body weight; \* $p < 0.05$ , \*\* $p < 0.01$ . (c) No changes in drinking intake between clozapine- and vehicle-treated mice were found. The histograms show the average weekly beverage supply for mice. (d) Comparison of food intake per week between clozapine- and vehicle-treated mice during the 15 weeks of treatment.



**Figure 2** (a) Percentage of adipocyte differentiation *in vitro* of WT and PKC $\beta^{-/-}$  muscle-derived stem cells (MDSCs) treated with clozapine 250  $\mu$ M. (b) Cells have been evaluated in terms of DNA content and MTT. Results are reported in terms of percentage variation compared with vehicle. (c, d) Body weight gain during long-term treatment with clozapine 250 mg/l for 15 weeks in C57BL/6J PKC $\beta^{-/-}$  mice. No variation in body weight gain over initial body weight between clozapine and vehicle groups was found. \*\* $p < 0.01$ .

shown) between the clozapine and the vehicle groups during the whole study (5 months), confirming once again that the weight gain linked to clozapine is dependent on PKC $\beta$ .

### Behavioral Effects of Clozapine in PKC $\beta$ WT and PKC $\beta^{-/-}$ mice

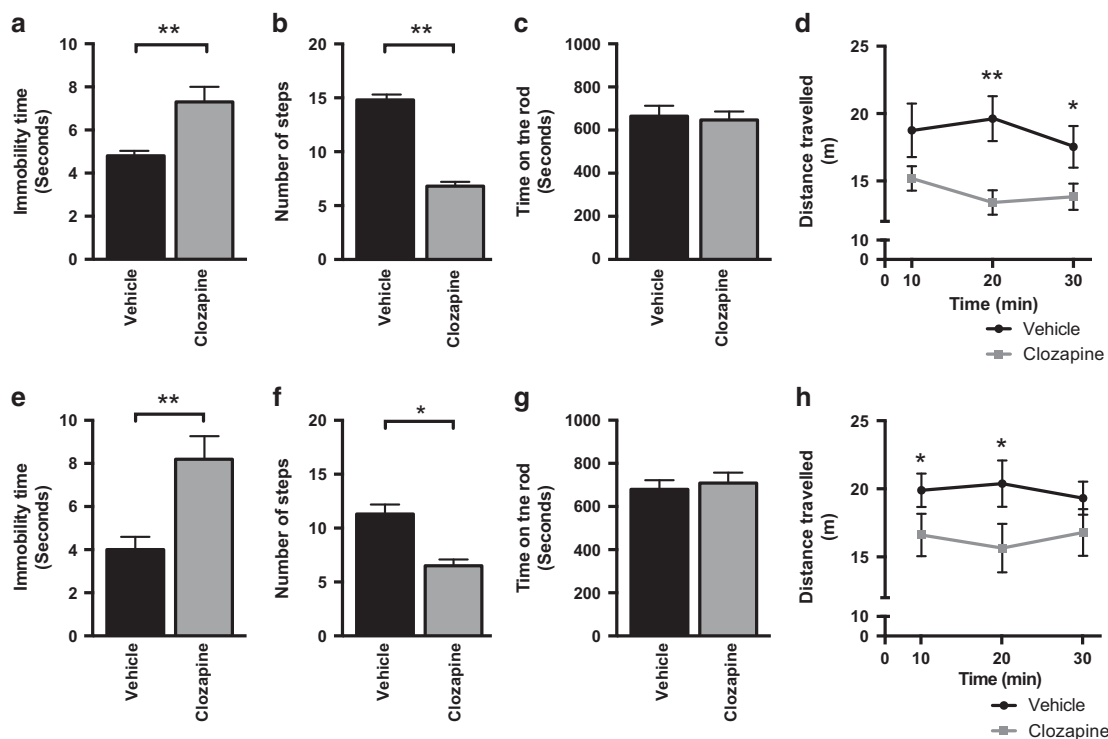
To investigate whether endogenous PKC $\beta$  contributes to the clozapine ability to modulate psychomotor functions, WT and PKC $\beta^{-/-}$  mice were subjected to a battery of behavioral tests (the bar, drag, rotarod, and open-field tests; Marti *et al*, 2007; Viaro *et al*, 2008) at the end of chronic clozapine treatment.

Supplementary Figure S3 shows that, under basal conditions, C57BL/6J WT and PKC $\beta^{-/-}$  mice had comparable motor performances and correlated behaviors. In C57BL/6J WT mice, the immobility time (bar test) was  $4.8 \pm 0.3$  s, the number of steps (drag test) was  $14.8 \pm 0.5$ , the time on rod (rotarod test) was  $664.9 \pm 47.7$  s and the mean distance travelled in 10 min (open-field test) was  $18.7 \pm 1.73$  m. Clozapine significantly impaired psychomotor activity, increasing by about 54% the immobility time ( $7.3 \pm 0.7$  s), reducing by about 50% the number of steps ( $6.8 \pm 0.4$ ) and by about 25% the distance travelled ( $14.1 \pm 0.93$  m), and leaving unaffected the time on rod (Figure 3a–d;

$647.4 \pm 38.9$  s). This indicates that clozapine inhibits psychomotor activity, inducing a state of apathy, lack of initiative, and limited range of emotion. Moreover, the same phenotype persisted in PKC $\beta^{-/-}$  mice after chronic treatment with clozapine (Figure 3e–h), suggesting that psychomotor effects were not affected by genetic removal of PKC $\beta$ .

### The Pharmacological Inhibition of PKC $\beta$ Prevents Antipsychotic-Induced Weight Gain Leaving Psychomotor Properties of Clozapine Unaffected

So far, these data have demonstrated that PKC $\beta$  is a key component of the clozapine-induced weight gain machinery, with hardly any implications in the psychomotor effects of this antipsychotic. On this basis, we have performed a pilot preclinical study *in vivo* to test the hypothesis that the selective PKC $\beta$  inhibitor RBX could prevent the metabolic side effects of clozapine without affecting its psychomotor properties. Previous *in vitro* tests have been performed to define the concentrations of RBX able to prevent clozapine-dependent lipid accumulation in primary cultured MDSCs without inducing changes in cell proliferation or surviving (Supplementary Figure S4A and B). These experiments provided some information for *in vivo* dosage.



**Figure 3** (a–d) Motor activity of C57BL/6J WT mice treated with clozapine. Effect of chronic administration of clozapine in the bar (a), drag (b), rotarod (c), and open-field (d) tests in mice. Data are expressed as immobility time on bar (in seconds) (a), number of steps (b), time rod (in seconds) (c), and travelled distance (in meters) (d). (e–h) Motor activity of C57BL/6J PKC $\beta$ <sup>-/-</sup> mice treated with clozapine in the bar (e), drag (f), rotarod (g), and open-field (h) tests. All data are means  $\pm$  SEM of 10–17 determinations per group; \* $p$  < 0.05, \*\* $p$  < 0.01.

C57BL/6J WT mice were randomized to clozapine (into water-beverage at 250 mg/l) and RBX (administered by oral gavage 0.5 mg/Kg three times a week) or RBX alone. They were fed a high-fat diet *ad libitum* for 5 months and then compared with the clozapine- and vehicle-treated groups. The results indicated that co-administration of RBX prevented the clozapine-induced weight gain. RM ANOVA revealed a similar increase of the weekly mean cumulative weight gain in RBX and clozapine co-treated mice with respect to vehicle-treated mice, which was also confirmed by body weight gains and visceral white fat accumulation (Figure 4a, b and Supplementary Figure S4C). No differences in weight gain, visceral white fat accumulation, and food and drinking intake emerged between RBX- and vehicle-treated mice, demonstrating that repeated RBX administration did not affect mice behavior and routine (data not shown).

Behavioral tests performed at the end of clozapine treatment corroborated genetic data showing that the PKC $\beta$  inhibitor did not interfere with the psychomotor effects of clozapine. In fact, mice co-administered with RBX and clozapine were as hypokinetic as clozapine-treated mice (Figure 4c–f).

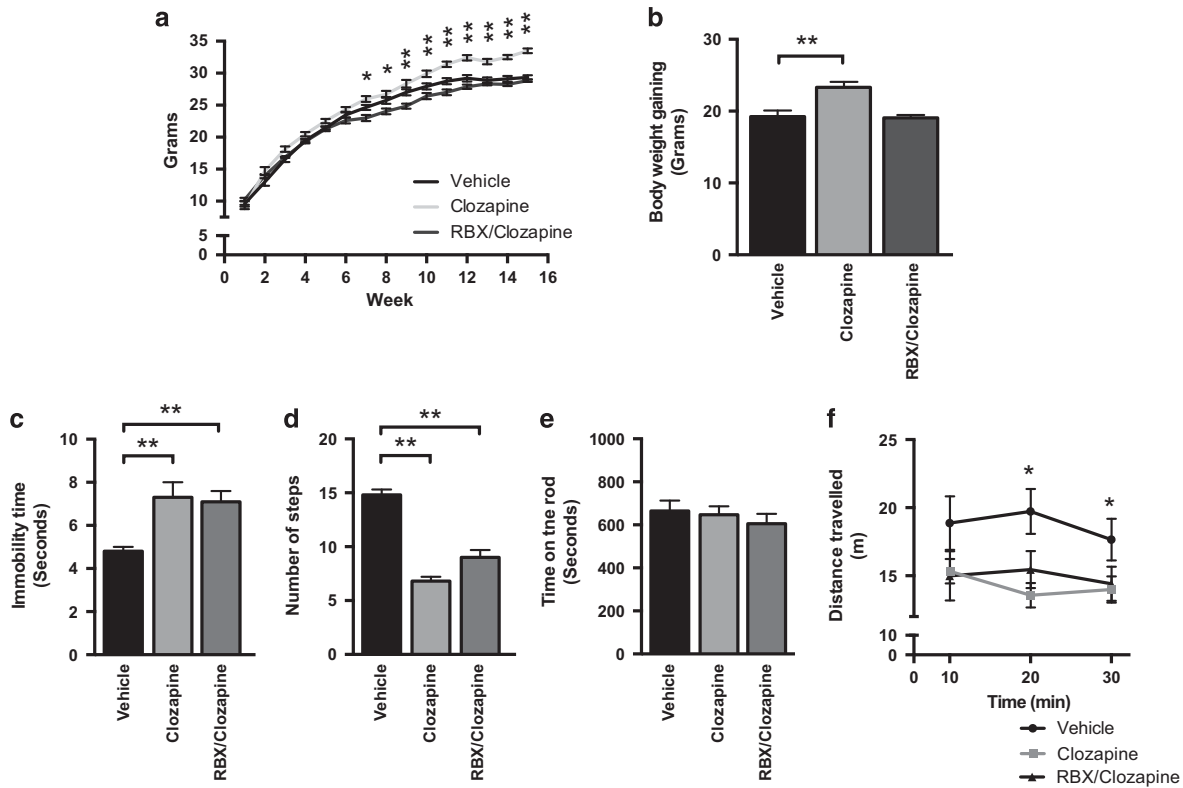
### Clozapine-Induced PKC $\beta$ Activation Modulates the Lipid Status by Inhibiting the Lipid-Selective Autophagy Process

Although the mechanisms underlying antipsychotic-induced weight gain remain unclear, our data highlight the pivotal role of PKC $\beta$ , indicating PKC $\beta$  blockade as a new strategy for

preventing lipid accumulation during long-term antipsychotic treatment without affecting the psychomotor properties of the drug. In order to better clarify the molecular pathway underlying PKC $\beta$  activity, a gene expression analysis related to lipid production (expression of adipose-specific markers) and lipid degradation (expression of lipophagic-specific markers) was performed in visceral white adipose and skeletal muscle tissues derived from mice administered with RBX/clozapine, clozapine, or vehicle. The results are expressed in terms of percentage variation with respect to vehicle (Figure 5a and b).

RT-PCR analysis of mRNA transcripts revealed that clozapine significantly upregulated adipocyte-specific proteins, such as adiponectin (adipoQ), leptin (LEP), and peroxisome proliferator activated receptor gamma (PPAR $\gamma$ ), and downregulated the lipophagic markers perilipin (PLIN1), TBC1D1, and transcription factor EB (TFEB). The differences in the adipose-specific markers were more pronounced in the skeletal muscle than in the adipose tissue, whereas the lipophagic marker expression was similar between tissues (Figure 5a and b). These data suggest that clozapine-induced weight gain is due to the overexpression of adipogenic molecular pathways, in particular, in the skeletal muscle. However, this effect is amplified by downregulation of the molecular pathways leading to LD clearance.

RBX counteracted the clozapine-induced lipid accumulation, especially upregulating the lipophagic-specific markers and downregulating the adipose-specific markers, as reported in Figure 5b. Indeed, the pro-lipophagic activity of



**Figure 4** (a) Effect of the pharmacological PKC $\beta$  inhibitor ruboxistaurin (RBX) 0.5  $\mu$ g/Kg on clozapine-induced weight gain and clozapine psychomotor properties in C57BL/6J WT mice. The differences in clozapine-induced body weight kinetic reported in the figure refers to the following experimental conditions: co-administration of RBX and clozapine vs the clozapine alone; \* $p < 0.05$ , \*\* $p < 0.01$ . (b) The motor activity of C57BL/6J WT mice co-treated with clozapine and RBX was compared with the vehicle and clozapine experimental groups. Motor performances in the bar (c), drag (d), rotarod (e), and open-field (f) tests have been reported. All data are means  $\pm$  SEM of 10–17 determinations per group; \* $p < 0.05$ , \*\*  $p < 0.01$ .

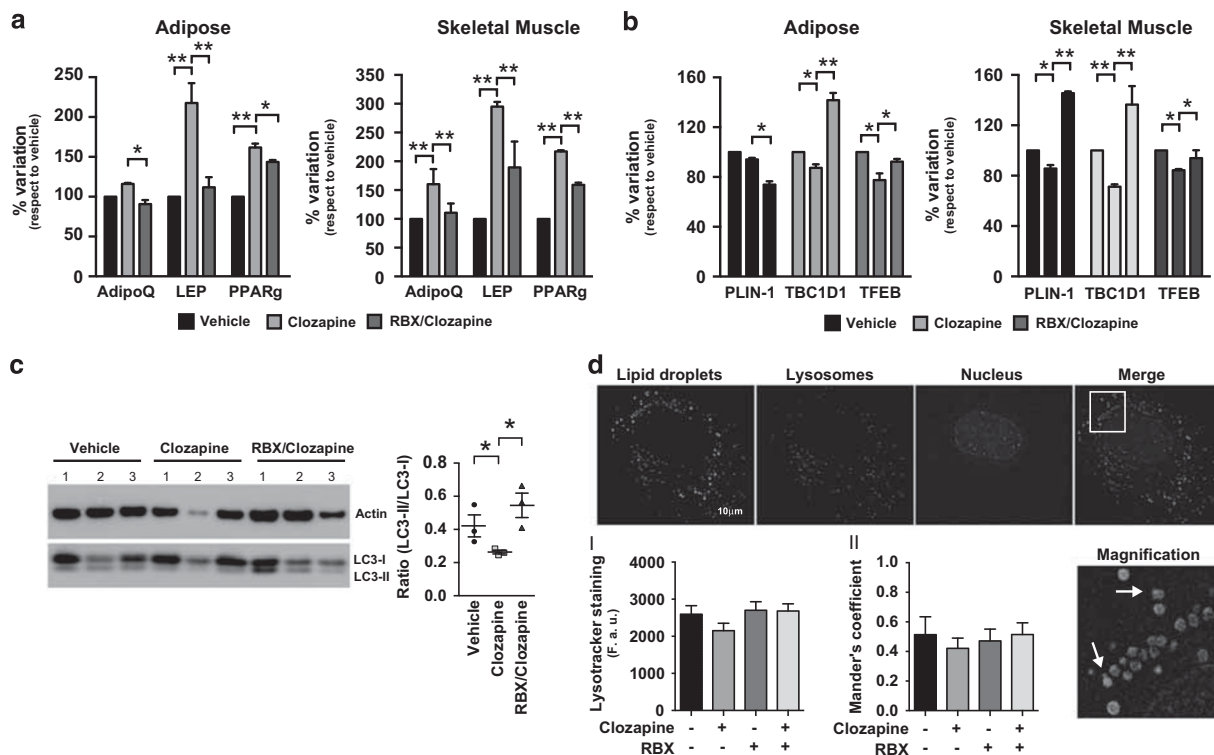
RBX was confirmed by immunoblot detection of the lipidation level of the autophagic effector microtubule-associated protein 1 light chain 3 (LC3) in visceral white adipose tissue at the fifteenth week of experiment *in vivo*. After translation, LC3 is processed to LC3-I, which localizes to the cytosol. Stimulatory signals modified LC3-I in a cleaved and lipidated membrane-bound form, LC3-II, which localizes to phagosomes. The level of autophagy was quantified as LC3-II/LC3-I ratio, as recommended by different guidelines for the study of autophagy (Klionsky *et al*, 2016). PKC $\beta$  inhibition, mediated by RBX, led to a significant activation of autophagy, as assessed by the increase in the endogenous LC3-II/LC3-I ratio (Figure 5c). This finding is supported by recovery *in vitro* of autophagic flow in RBX-treated WT MDSCs exposed to clozapine (Supplementary Figure 4D). However, the autophagic activation induced by RBX was not due to an enhanced intracellular lysosomal content or an enhanced colocalization with LDs, as suggested by double-staining studies in MDSCs (Figure 5d).

## DISCUSSION

One of the goals of recent antipsychotic research is the identification of drugs that lower and/or prevent the liability of antipsychotics to induce body weight gain and metabolic syndrome. Despite clozapine is the only treatment indicated

for resistant schizophrenia, its clinical use is limited mainly due to serious metabolic adverse side effects. Approximately 40% of patients discontinued clozapine treatment during the first 2 years of therapy, with severe implications for the health of patients who already have an average life expectancy of 16–25 years less than the general population (Saha *et al*, 2007). Overweight and, particularly, obesity are risk factors of cardiovascular disease, metabolic syndrome, diabetes, and cancer. Limited options are available that mitigate these metabolic problems; the antidiabetic drug Metformin and physical exercise are commonly prescribed options, providing partial benefits over clozapine-induced weight gain and metabolic abnormalities (Caemmerer *et al*, 2012; Maayan *et al*, 2010). Here we have demonstrated that a good candidate drug for preventing the clozapine-induced weight gain is RBX, an orally effective selective PKC $\beta$  inhibitor, which is used to treat diabetic retinopathy and macular edema (Deissler and Lang, 2016). It blocks the adenine triphosphate binding in the active site of PKC $\beta$ , thus preventing the phosphorylation of its substrates.

Our gene expression data indicate that the metabolic side effects of clozapine are not generalized but rather tissue specific as they were mainly observed in the skeletal muscle and less in visceral white adipose tissue. This difference in tissue response to clozapine is consistent with the changes in expression of PKC $\beta$  or PKC $\beta$  activator levels, such as Ca<sup>2+</sup> and/or diacylglycerol. It is of note that metabolic changes such as hyperglycemia and elevation of free fatty acids have



**Figure 5** Effect of the pharmacological PKC $\beta$  inhibitor ruxobistaurin (RBX) 0.5 mg/Kg on clozapine-induced (a) adipose-specific and (b) lipophagic-specific gene expression in C57BL/6J WT-derived visceral white adipose and skeletal muscle tissues. Data are expressed as percentage variation with respect to vehicle. (c) Representative immunoblotting of C57BL/6J WT-derived visceral white adipose tissues treated either with RBX plus clozapine or clozapine and vehicle. The level of autophagic lipolysis has been quantified and expressed as LC3-II/LC3-I ratio. (d) Representative fluorescent images of lipid droplets and lysosomes have been reported. MDSC WT and PKC $\beta^{-/-}$  cells were stained with BODIPY 493/503 (green), lysotracker RED (red), and Hoechst (blue). Bottom panel: magnification of merged images from both channels. (I) Quantification of intracellular lysosomes expressed as mean of lysotracker fluorescent intensity per single cells. (II) Co-localization analysis of lipid droplets and lysosomes calculated and expressed as the mean of Mander's coefficient.

been reported to increase diacylglycerol levels, which activates PKC (Inoguchi *et al*, 1992). PKC $\beta$  is a classic PKC isoform, which has an important role in the development of metabolic complications (Pavan *et al*, 2010; Pinton *et al*, 2011), becoming a target of great interest. In fact, previous studies suggested that antipsychotic-dependent PKC activation induces adipocyte differentiation under hyperglycemic and oxidative stress conditions (Aguari *et al*, 2008; Pavan *et al*, 2010). The intracellular redox state controls the selectivity of PKC isoform activation (Bononi *et al*, 2011; Giorgi *et al*, 2010), and therefore, it changes the sensitivity of the different isoforms to cell stimulation, explaining why stimuli that in principle could activate a broad number of PKC isoforms can become selective only for a specific isoform (Rimessi *et al*, 2007).

In our experimental model, the PPAR $\gamma$ /adiponectin axis is positively regulated by PKC $\beta$ , as evidenced by the high levels of expression of PPAR $\gamma$  and adiponectin genes in clozapine-treated WT-derived adipose and skeletal muscle tissue, which are normalized by the selective inhibitor RBX. This provides the first *in vivo* evidence that PKC $\beta$  positively regulates adipocyte differentiation, confirming previous *in vitro* findings (Aguari *et al*, 2008; Pavan *et al*, 2010). However, PKC $\beta$  has been shown to activate c-Jun N-terminal Kinase and, subsequently, to suppress PPAR $\gamma$ , the transcription factor promoting the expression of adiponectin (Liu and

Liu, 2010), suggesting that further studies are needed to elucidate the relationship between PKC $\beta$  and PPAR $\gamma$ .

Leptin mRNA levels are directly linked to adipocyte volume within each fat depot (Zhang *et al*, 2002). In obese people, leptin levels are higher (Seufert *et al*, 1999), and a significant number of studies found elevated leptin levels in patients taking atypical antipsychotics, suggesting a disruption of leptin homeostasis, probably caused by leptin resistance (Hagg *et al*, 2001). Leptin is widely considered an adipokine; however, cultured myocytes have also been found to release leptin, although with a pattern different from adipose tissue (Wolsk *et al*, 2012). Glucose and free fatty acids increase leptin expression and protein concentration in the skeletal muscle (Wang *et al*, 1998); unfortunately, the role that leptin has in skeletal muscle metabolism is unclear.

Unlike PKC $\beta^{-/-}$  mice, WT mice developed leptin resistance with high-fat diet, mimicking humans with diet-induced obesity (Huang *et al*, 2009). Two potential mechanisms by which PKC $\beta$  might influence leptin sensitivity in mice have been suggested: PKC $\beta$  might positively regulate leptin transcription, thus PKC $\beta$  deficiency or inhibition would reduce leptin expression. Alternatively, PKC $\beta$  might regulate leptin signal to the effector molecules. Our results demonstrate that the administration of RBX significantly reduces the leptin mRNA expression in visceral white adipose tissue from clozapine-treated WT mice, indicating



a tissue-specific role for PKC $\beta$  in the transcriptional control of leptin promoter.

In recent years, the metabolic significance of autophagy has attracted considerable attention. In addition to its well-characterized function in protein and organelle catabolism (Galluzzi *et al*, 2014; Rimessi *et al*, 2013), the role of autophagy in regulating lipid metabolism and adipocyte differentiation has also been explored (Singh *et al*, 2009). LD degradation was previously regarded to be solely dependent on the enzymatic activity of lipases. However, recent findings prove that LDs, or a portion of them, can also be degraded via autophagy, as underlined by the co-localization of phagosomal marker LC3-II LD surface (Singh *et al*, 2009). Lipophagy has been introduced to specify this selective autophagy-mediated lysosomal lipid degradation, an important pathway of LD clearance whose extent modulates the lipid content into the cells. Reduced lipophagy in a setting of chronic lipid loading promotes cellular lipid accumulation creating the basis for development of metabolic disease, such as obesity. On the contrary, an increased lipophagy facilitates basal lipolysis to counteract the adverse effects of lipid overload.

In this work, we have demonstrated that lipophagy represents an alternative pathway of lipid degradation and is targeted by chronic clozapine therapy. Our finding shows that PKC $\beta$  blockade by RBX ameliorates the metabolic syndrome induced by clozapine-dependent lipophagy-specific gene expression in WT mice. The discovery of lipophagy-specific genes and their modulation by PKC $\beta$  inhibition is an example of how regulation of autophagic function may have important implications for the development of alternative therapeutic strategies to counteract the metabolic side effects of clozapine, although further studies are needed to better elucidate the mechanisms behind LD recognition and sequestration by the autophagic machinery. It has been proposed that specific LD-associated proteins are required to initiate the interaction between LD membrane and the autophagic components.

The protein constituents of the LD surface are heterogeneous and made up of various combinations of members of the PLIN family, components that respond differently to the cellular metabolic state, making LD available to lipophagy. Among the PLINs, PLIN1 coats the LD surface and response to adrenergic beta 2 receptor stimulation, translocating from the endoplasmic reticulum to the LD surface during the maturation of the droplets (Skinner *et al*, 2013). Indeed, PLIN1 facilitates lipolysis by activating patatin like phospholipase domain containing 2 through the release of LD-binding protein CGI-58 and also by serving as the dock site for the activated hormone-sensitive lipase (Brasaemle *et al*, 2000).

Members of TBC domain-containing family have been studied for their ability to modulate the phagosome formation during the autophagic response. Among TBC1Ds, TBC1D1, TBC1D3, and weakly, TBC1D18, when overexpressed, strengthened the autophagic response (Longatti *et al*, 2012). TBC1D1 is a Rab-GTPase-activating protein involved also in glucose 4 transporter trafficking into the cell membrane, responding to insulin and muscle contraction (Szekeres *et al*, 2012). Genetic variation in *TBC1D1* gene has been linked to higher fat deposition in animals and to human obesity, although the precise roles of these mutations in

TBC1D1 function are not known (Stone *et al*, 2006). On the contrary, the deletion of TBC1D1 gene in mice showed normal glucose and insulin tolerance, with no difference in body weight compared with WT littermates (Stockli *et al*, 2015). Although the functional relevance of TBC1D family in lipophagy is not fully characterized, we have shown that RBX counteracted the clozapine-dependent reduction of TBC1D1 expression both in white adipose and skeletal muscle tissue, indicating its potential effect on lipophagy and glucose metabolism.

The lipophagy-inhibitory effect of clozapine has been confirmed also by the downregulation of the transcription factor TFEB. TFEB positively regulates lipophagy and promotes fatty acid  $\beta$ -oxidation, providing a regulatory link between different lipid degradation pathways (O'Rourke and Ruvkun, 2013). It is regulated by nutrients and growth factor levels and, in turn, regulates the expression levels of genes involved in energy metabolism and lipid catabolism. Consistently, genetic deletion of TFEB caused LD accumulation and defective peripheral fat mobilization after fasting, whereas overexpression of TFEB protected from diet-induced obesity and metabolic syndrome (Settembre *et al*, 2013), an effect possibly mediated through autophagy as suggested by a study reporting that TFEB overexpression in the liver of mice lacking the essential autophagy gene *ATG7* failed to induce lipid catabolism, resulting in accumulation of LDs in hepatocytes.

Interestingly, PKC $\beta$ <sup>-/-</sup> mice exhibit some similarities with a class of lean mice having a reduction in adipose tissue storage capacity or an enhanced lipolysis, which could be induced by the altered gene transcription (Abu-Elheiga *et al*, 2001; Huang *et al*, 2009). An enhancement of autophagy was observed both in cultured human and mouse PKC $\beta$ <sup>-/-</sup> cells (Patergnani *et al*, 2013). We thus favor the interpretation that protection from clozapine-induced weight gain with the PKC $\beta$  inhibitor RBX is mechanistically linked to reduced lipid storage as a consequence of increased autophagic lipolysis of LD. The way lipophagy is regulated and connected to other lipid catabolic pathways such as the intracellular uptake of lipids from the bloodstream and their  $\beta$ -oxidation in mitochondria is still unclear. These processes must be co-regulated to optimize lipid usage and prevent the lipotoxicity associated with the long-term effectiveness and potential side effects of strengthened lipophagy.

In conclusion, our *in vitro* and *in vivo* data point to the crucial role of PKC $\beta$  in the clozapine-induced weight gain. We therefore propose PKC $\beta$  blockade as a new therapeutic strategy able to prevent the metabolic side effects of chronic therapy with clozapine without interfering with its psychomotor properties, and possibly, neuroleptic effectiveness. Considering RBX is already in use in the clinic to treat diabetes-associated retinopathy, this study might foster clinical trials aimed at testing its efficacy in tackling metabolic complications of long-term therapy with atypical neuroleptics.

## FUNDING AND DISCLOSURE

AR is supported by local funds from the University of Ferrara and grants from the Italian Ministry of Health (GR-2011-02346964), the Italian Cystic Fibrosis Foundation (FFC no.

20/2015). PP is supported by grants from the Italian Association for Cancer Research (AIRC, IG-18624), Telethon (GGP15219B), local funds from the University of Ferrara, the Italian Ministry of Education, University and Research (COFIN: 20129JLHSY\_002, FIRB: RBAP11FXBC\_002, Futuro in Ricerca: RBFR10EGVP\_001), and the Italian Ministry of Health. PP is grateful to Camilla degli Scrovegni for her continuous support. The authors declare no conflict of interest.

## REFERENCES

- Abu-Elheiga L, Matzuk MM, Abo-Hashema KA, Wakil SJ (2001). Continuous fatty acid oxidation and reduced fat storage in mice lacking acetyl-CoA carboxylase 2. *Science* **291**: 2613–2616.
- Aguiari P, Leo S, Zavan B, Vindigni V, Rimessi A, Bianchi K et al (2008). High glucose induces adipogenic differentiation of muscle-derived stem cells. *Proc Natl Acad Sci USA* **105**: 1226–1231.
- Bansode RR, Huang W, Roy SK, Mehta M, Mehta KD (2008). Protein kinase C deficiency increases fatty acid oxidation and reduces fat storage. *J Biol Chem* **283**: 231–236.
- Bononi A, Agnoletto C, De Marchi E, Marchi S, Patergnani S, Bonora M et al (2011). Protein kinases and phosphatases in the control of cell fate. *Enzyme Res* **2011**: 329098.
- Brasaemle DL, Rubin B, Harten IA, Gruia-Gray J, Kimmel AR, Londos C (2000). Perilipin A increases triacylglycerol storage by decreasing the rate of triacylglycerol hydrolysis. *J Biol Chem* **275**: 38486–38493.
- Brini M, De Giorgi F, Murgia M, Marsault R, Massimino ML, Cantini M et al (1997). Subcellular analysis of Ca<sup>2+</sup> homeostasis in primary cultures of skeletal muscle myotubes. *Mol Biol Cell* **8**: 129–143.
- Caemmerer J, Correll CU, Maayan L (2012). Acute and maintenance effects of non-pharmacologic interventions for antipsychotic associated weight gain and metabolic abnormalities: a meta-analytic comparison of randomized controlled trials. *Schizophr Res* **140**: 159–168.
- Citrome L, Vreeland B (2008). Schizophrenia, obesity, and antipsychotic medications: what can we do? *Postgrad Med* **120**: 18–33.
- Danis RP, Bingaman DP, Jirousek M, Yang Y (1998). Inhibition of intraocular neovascularization caused by retinal ischemia in pigs by PKC $\beta$  inhibition with LY333531. *Invest Ophthalmol Vis Sci* **39**: 171–179.
- Deissler HL, Lang GE (2016). The protein kinase C inhibitor: ruboxistaurin. *Dev Ophthalmol* **55**: 295–301.
- Deng C (2013). Effects of antipsychotic medications on appetite, weight, and insulin resistance. *Endocrinol Metab Clin North Am* **42**: 545–563.
- Denizot F, Lang R (1986). Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J Immunol Methods* **89**: 271–277.
- Foley DL, Morley KI (2011). Systematic review of early cardiometabolic outcomes of the first treated episode of psychosis. *Arch Gen Psychiatry* **68**: 609–616.
- Frogley C, Taylor D, Dickens G, Picchioni M (2012). A systematic review of the evidence of clozapine's anti-aggressive effects. *Int J Neuropsychopharmacol* **15**: 1351–1371.
- Galluzzi L, Pietrocola F, Levine B, Kroemer G (2014). Metabolic control of autophagy. *Cell* **159**: 1263–1276.
- Giorgi C, Agnoletto C, Baldini C, Bononi A, Bonora M, Marchi S et al (2010). Redox control of protein kinase C: cell- and disease-specific aspects. *Antioxid Redox Signal* **13**: 1051–1085.
- Hagg S, Soderberg S, Ahren B, Olsson T, Mjorndal T (2001). Leptin concentrations are increased in subjects treated with clozapine or conventional antipsychotics. *J Clin Psychiatry* **62**: 843–848.
- Hartfield AW, Moore NA, Clifton PG (2003). Effects of clozapine, olanzapine and haloperidol on the microstructure of ingestive behaviour in the rat. *Psychopharmacology* **167**: 115–122.
- Hemmerich K, Gummersbach C, Pallua N, Luckhaus C, Fehsel K (2006). Clozapine enhances differentiation of adipocyte progenitor cells. *Mol Psychiatry* **11**: 980–981.
- Henderson DC, Sharma B, Fan X, Copeland PM, Borba CP, Freudenreich O et al (2010). Dietary saturated fat intake and glucose metabolism impairments in nondiabetic, nonobese patients with schizophrenia on clozapine or risperidone. *Ann Clin Psychiatry* **22**: 33–42.
- Huang W, Bansode R, Mehta M, Mehta KD (2009). Loss of protein kinase C $\beta$  function protects mice against diet-induced obesity and development of hepatic steatosis and insulin resistance. *Hepatology* **49**: 1525–1536.
- Inoguchi T, Battan R, Handler E, Sportsman JR, Heath W, King GL (1992). Preferential elevation of protein kinase C isoform  $\beta$  II and diacylglycerol levels in the aorta and heart of diabetic rats: differential reversibility to glycemic control by islet cell transplantation. *Proc Natl Acad Sci USA* **89**: 11059–11063.
- Ishii H, Jirousek MR, Koya D, Takagi C, Xia P, Clermont A et al (1996). Amelioration of vascular dysfunctions in diabetic rats by an oral PKC  $\beta$  inhibitor. *Science* **272**: 728–731.
- Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H (2016). Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy* **12**: 1–222.
- Kluge M, Schulz H, Himmerich H, Dalal M, Schacht A, Wehmeier PM et al (2007). Clozapine and olanzapine are associated with food craving and binge eating: results from a randomized double-blind study. *J Clin Psychopharmacol* **27**: 662–666.
- Legge SE, Hamshere M, Hayes RD, Downs J, O'Donovan MC, Owen MJ et al (2016). Reasons for discontinuing clozapine: a cohort study of patients commencing treatment. *Schizophr Res* **174**: 113–119.
- Lei S, Li H, Xu J, Liu Y, Gao X, Wang J et al (2013). Hyperglycemia-induced protein kinase C  $\beta$ 2 activation induces diastolic cardiac dysfunction in diabetic rats by impairing caveolin-3 expression and Akt/eNOS signaling. *Diabetes* **62**: 2318–2328.
- Leucht S, Corves C, Arbter D, Engel RR, Li C, Davis JM (2009). Second-generation versus first-generation antipsychotic drugs for schizophrenia: a meta-analysis. *Lancet* **373**: 31–41.
- Liu M, Liu F (2010). Transcriptional and post-translational regulation of adiponectin. *Biochem J* **425**: 41–52.
- Longatti A, Lamb CA, Razi M, Yoshimura S, Barr FA, Tooze SA (2012). TBC1D14 regulates autophagosome formation via Rab11- and ULK1-positive recycling endosomes. *J Cell Biol* **197**: 659–675.
- Maayan L, Vakhrusheva J, Correll CU (2010). Effectiveness of medications used to attenuate antipsychotic-related weight gain and metabolic abnormalities: a systematic review and meta-analysis. *Neuropsychopharmacology* **35**: 1520–1530.
- Mamo DC (2007). Managing suicidality in schizophrenia. *Can J Psychiatry* **52**(Suppl 1): 59S–70S.
- Marti M, Trapella C, Viaro R, Morari M (2007). The nociceptin/orphanin FQ receptor antagonist J-113397 and L-DOPA additively attenuate experimental parkinsonism through overinhibition of the nigrothalamic pathway. *J Neurosci* **27**: 1297–1307.
- Meltzer HY (2012). Clozapine: balancing safety with superior antipsychotic efficacy. *Clin Schizophr Relat Psychoses* **6**: 134–144.
- Nagareddy PR, Soliman H, Lin G, Rajput PS, Kumar U, McNeill JH et al (2009). Selective inhibition of protein kinase C  $\beta$ (2) attenuates inducible nitric oxide synthase-mediated cardiovascular abnormalities in streptozotocin-induced diabetic rats. *Diabetes* **58**: 2355–2364.
- O'Rourke EJ, Ruvkun G (2013). MXL-3 and HLH-30 transcriptionally link lipolysis and autophagy to nutrient availability. *Nat Cell Biol* **15**: 668–676.
- Patergnani S, Marchi S, Rimessi A, Bonora M, Giorgi C, Mehta KD et al (2013). PRKCB/protein kinase C,  $\beta$  and the mitochondrial axis as key regulators of autophagy. *Autophagy* **9**: 1367–1385.

- Pavan C, Vindigni V, Michelotto L, Rimessi A, Abatangelo G, Cortivo R *et al* (2010). Weight gain related to treatment with atypical antipsychotics is due to activation of PKC- $\beta$ . *Pharmacogenomics J* **10**: 408–417.
- Pinton P, Pavan C, Zavan B (2011). PKC-beta activation and pharmacologically induced weight gain during antipsychotic treatment. *Pharmacogenomics* **12**: 453–455.
- Rimessi A, Bonora M, Marchi S, Patergnani S, Marobbio CM, Lasorsa FM *et al* (2013). Perturbed mitochondrial Ca<sup>2+</sup> signals as causes or consequences of mitophagy induction. *Autophagy* **9**: 1677–1686.
- Rimessi A, Rizzuto R, Pinton P (2007). Differential recruitment of PKC isoforms in HeLa cells during redox stress. *Cell Stress Chaperones* **12**: 291–298.
- Saha S, Chant D, McGrath J (2007). A systematic review of mortality in schizophrenia: is the differential mortality gap worsening over time? *Arch Gen Psychiatry* **64**: 1123–1131.
- Schallert T, De Ryck M, Whishaw IQ, Ramirez VD, Teitelbaum P (1979). Excessive bracing reactions and their control by atropine and L-DOPA in an animal analog of Parkinsonism. *Exp Neurol* **64**: 33–43.
- Settembre C, De Cegli R, Mansueto G, Saha PK, Vetrini F, Visvikis O *et al* (2013). TFEB controls cellular lipid metabolism through a starvation-induced autoregulatory loop. *Nat Cell Biol* **15**: 647–658.
- Seufert J, Kieffer TJ, Leech CA, Holz GG, Moritz W, Ricordi C *et al* (1999). Leptin suppression of insulin secretion and gene expression in human pancreatic islets: implications for the development of adipogenic diabetes mellitus. *J Clin Endocrinol Metab* **84**: 670–676.
- Sinclair D, Adams CE (2014). Treatment resistant schizophrenia: a comprehensive survey of randomised controlled trials. *BMC Psychiatry* **14**: 253.
- Singh R, Kaushik S, Wang Y, Xiang Y, Novak I, Komatsu M *et al* (2009). Autophagy regulates lipid metabolism. *Nature* **458**: 1131–1135.
- Siskind DJ, Leung J, Russell AW, Wysoczanski D, Kisely S (2016). Metformin for clozapine associated obesity: a systematic review and meta-analysis. *PLoS ONE* **11**: e0156208.
- Skinner JR, Harris LA, Shew TM, Abumrad NA, Wolins NE (2013). Perilipin 1 moves between the fat droplet and the endoplasmic reticulum. *Adipocyte* **2**: 80–86.
- Stahl SM, Mignon L, Meyer JM (2009). Which comes first: atypical antipsychotic treatment or cardiometabolic risk? *Acta Psychiatr Scand* **119**: 171–179.
- Stockli J, Meoli CC, Hoffman NJ, Fazakerley DJ, Pant H, Cleasby ME *et al* (2015). The RabGAP TBC1D1 plays a central role in exercise-regulated glucose metabolism in skeletal muscle. *Diabetes* **64**: 1914–1922.
- Stone S, Abkevich V, Russell DL, Riley R, Timms K, Tran T *et al* (2006). TBC1D1 is a candidate for a severe obesity gene and evidence for a gene/gene interaction in obesity predisposition. *Hum Mol Genet* **15**: 2709–2720.
- Szekeres F, Chadt A, Tom RZ, Deshmukh AS, Chibalin AV, Bjornholm M *et al* (2012). The Rab-GTPase-activating protein TBC1D1 regulates skeletal muscle glucose metabolism. *Am J Physiol Endocrinol Metab* **303**: E524–E533.
- Viaro R, Sanchez-Pernaute R, Marti M, Trapella C, Isacson O, Morari M (2008). Nociceptin/orphanin FQ receptor blockade attenuates MPTP-induced parkinsonism. *Neurobiol Dis* **30**: 430–438.
- Wang J, Liu R, Hawkins M, Barzilai N, Rossetti L (1998). A nutrient-sensing pathway regulates leptin gene expression in muscle and fat. *Nature* **393**: 684–688.
- Wolsk E, Mygind H, Grondahl TS, Pedersen BK, van Hall G (2012). Human skeletal muscle releases leptin *in vivo*. *Cytokine* **60**: 667–673.
- Zhang Y, Guo KY, Diaz PA, Heo M, Leibel RL (2002). Determinants of leptin gene expression in fat depots of lean mice. *Am J Physiol Regul Integr Compar Physiol* **282**: R226–R234.

Supplementary Information accompanies the paper on the Neuropsychopharmacology website (<http://www.nature.com/npp>)