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PKC BETA AS A NEW THERAPY TARGET ON  
PREVENTION OF WEIGHT GAIN DURING LONG-  
TERM ATYPICAL ANTIPSYCHOTICS TREATMENT

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# 1. INTRODUCTION

## 1.1 Antipsychotic drugs

Antipsychotics medications are a class of drugs mainly used for the symptomatic treatment of psychosis including schizophrenia, mania, bipolar disorder and many other conditions characterized by agitation and altered mental status. A common feature of these drugs is the antagonistic activity at dopamine D2 receptors in the central nervous system but other receptors are involved in their mechanism of action including D1 and D4 in addition to serotonin (5HT),  $\alpha$ -adrenergic, cholinergic and histaminic receptors (1). The first generation of antipsychotic agents, also designated as 'neuroleptic' or 'typical', are potent dopaminergic D2 antagonists that have a strong propensity towards producing extrapyramidal side effects (EPSEs) arising from activity in the striatum. In contrast, the so-called newer generation of antipsychotics exhibits much less marked EPSEs and for this reason are named 'atypical antipsychotics' (APDs).

### 1.1.2 *The History*

Antipsychotic medications were pioneered by the development of phenothiazine compounds as industrial dyes (i.e. methylene blue) at the end of 19th century. Subsequently, in the 1930s, one of these compounds, promethazine, was found to have antihistaminic and sedative properties and, following tests on rodents, it was found to prolong sleep induced by barbiturates. It was introduced by Laborit in the clinic for its ability to prolong and stabilize anaesthesia for surgery (2).

A few years later, another significant phenothiazine derivative was synthesized by Charpenter and later tested by Laborit. This compound, named chlorpromazine, was found to reduce anxiety and induce mild sedation without causing loss of consciousness, which led to its use in the treatment of psychosis in France by Delay and Deniker and later, chlorpromazine was licensed in the USA in 1955.

The antidepressant and antipsychotic properties of promethazine were recognized in individuals using promethazine to treat topical infections (Hood et al, 2007). Chlorpromazine, was developed in 1951, providing anticholinergic and antiemetic effects (3). Then, the antipsychotic effects of chlorpromazine were appreciated. Haloperidol was developed in 1958, in a continued effort to refine the newly developed antipsychotic medications (3).

### ***1.1.3 First Generation Antipsychotics (Typical)***

Antipsychotics have been classified into two separate classes: typical and atypical. This distinction was originally made by the pharmaceutical companies while they sought to promote sales of the newly developed atypical drugs.

The term “classic antipsychotic” is synonymous with “first generation antipsychotic” and “typical antipsychotic.” Typical antipsychotics are known for extreme sedation and causing severe extrapyramidal symptoms (3).

Side effects can include:

- acute and chronic movement disorders
- increased prolactin levels
- neuroleptic malignant syndrome

- anticholinergic symptoms
- sedation
- postural hypotension
- reduced seizure threshold”

Due to increases in prolactin levels, EPSE and worsening of negative symptoms, it became evident that the typical antipsychotics effects were limited to treating the positive symptoms of schizophrenia (Hood et al, 2007).

Typical drugs cause dyskinesias, including tardive dyskinesia. These are thought to arise from disruption to dopamine transmission in the basal ganglia. Atypical drugs have metabolic side effects. Although the exact consequence varies in degree from drug to drug, for example olanzapine may cause excessive weight gain-but not with all patients, while many atypicals cause diabetogenesis.

The typical antipsychotics are commonly divided into three main categories that reflect different classes of chemical structure: (a) phenothiazine, (b) thioxanthines and (c) butyrophenones.

First generation antipsychotics are also classified on the basis of their relative potency as *low, medium and high potency*, according to the dosage necessary to cause an antipsychotic effect. Aliphatic phenothiazines such as chlorpromazine are designated as ‘lowpotency’, while piperazine phenothiazines such as fluphenazine and trifluoperazine, thioxanthines and butyrophenones are referred to as ‘high potency’ antipsychotics.

All the first generation antipsychotics antagonize the D2-subtype of dopamine receptors in the CNS. Although these drugs possess different affinities at D1, D4, serotonin 5HT2, histamine H1, adrenergic and cholinergic

receptors, it appears that D2 antagonism represents a major component of their antipsychotic activity (4).

While dopamine is ubiquitous in the central nervous system, particularly in the nucleus caudate (nigrostriatal system), nucleus accumbens (mesocortical system), the mesolimbic system and the tuberoinfundibular pathway, dopamine D2 receptors localized in the limbic and striatal areas are implicated in antipsychotic efficacy and EPSEs.

Due to the blockade of the dopamine D2 innervations in the striatum, the conventional antipsychotics generate a range of neurological adverse effects that are much less marked or even absent in the case of the newer generation drugs. While *akathisia*, *dystonia*, *parkinsonism* and *neuroleptic malignant syndrome* represent acute reactions to these medications, *tardive dyskinesia* can occur after chronic treatment.

Typical antipsychotics have long been recognized to elevate blood levels of the hormone prolactin. In fact, since the stimulation of the D2 receptors in the tuberoinfundibular area suppresses prolactin release, all antipsychotics, particularly those acting in that brain area, cause some degree of hyperprolactinaemia *via* D2 antagonism. As a consequence, hyperprolactin-related side effects such as gynaecomastia, galactorrhoea and erectile dysfunction may occur.

Allied to the high levels of prolactin induced by antipsychotics, these drugs are also subject of investigation with regard to their potential contribution to the development of bone mineral density loss, which has been reported in several studies (Liu-Seifert et al., 2004; Meaney et al., 2004; Meaney and O'Keane, 2007; Kishimoto et al., 2008) and breast cancer (Harvey et al., 2008).

Conventional first generation antipsychotics are not the current first choice for the treatment of schizophrenia and although they are effective in first-episode schizophrenia, especially in improving the positive symptoms, they are not superior to atypical agents such as clozapine (Lieberman et al., 2003). Although chlorpromazine and haloperidol reduce the risk of relapse (Thornley et al., 2003; Joy et al., 2001), they are not effective in refractory schizophrenia compared to second-generation antipsychotics (Kane et al., 1988).

At present, treatment with typical agents is only recommended for patients showing a good clinical response with minimal side effects (Sharif, 1998).

#### ***1.1.4 Second Generation Antipsychotics (Atypical)***

The history of atypical antipsychotics began with the discovery of clozapine and its antipsychotic properties.

In an effort to find a medication with less severe side-effects, clozapine was developed. This was the first “atypical” antipsychotic or “second generation antipsychotic”, and was found to be highly effective for treatment resistant schizophrenia. However, it posed the risk of agranulocytosis (3). Agranulocytosis is a granulocyte count less than  $500/\text{mm}^3$  in which the individual presents with signs and symptoms of infection (4).

Three features of SGA (Second Generation Antipsychotics) include:

- efficacy in treating positive symptoms
- low incidence of EPSE and 5-HT<sub>2</sub> serotonin receptor antagonism
- D<sub>2</sub> dopamine receptor antagonism



The clinical features of SGA including improvement in positive symptoms and negative symptoms, low incidence of EPSE after acute dosing, no elevation in prolactin, improved cognitive symptoms and improved mood, have shown benefit in treatment resistant patients.

It is now known that Atypical drugs act on the 5HT<sub>2A</sub> receptor. They are not antagonists but inverse agonists, in effect causing a reversed effect. They also act upon a range of other receptors, but the respective affinities vary from drug to drug. In sharp contrast to haloperidol, drugs like risperidone have a weak potential to cause injury to cells. These atypical drugs also cause neurogenesis (5).

Studies with clozapine have shown that pretreatment and post treatment reverse the effects of haloperidol toxicity (5). In addition, the drug can increase NGF plasma levels in medication naïve schizophrenia patients. The level of NGF is increased to approximately normal levels. Although this presents a possibility of using neurotrophins to treat schizophrenia, it is important to remember that while we can stimulate neurogenesis, we cannot ensure that the proper connections are made.

Atypical drugs have other effects that are in opposition to haloperidol, and these actions promote cells survival. Atypical drugs reduce caspase activation (6), block glutamate toxicity and ameliorate rotenone induced toxicity.

Clozapine was introduced to the market in the 1960s and while the chemical structure first suggested that it might possess a potential effect as an antidepressant, it was soon discovered that it actually had neuroleptic properties. However, there was an anomaly reported about this finding because clozapine yielded antipsychotic activity without causing EPSEs and this stimulated interest in the nature of this drug. In fact, at that time, the

onset of EPSEs was considered an inseparable pre-requirement for antipsychotic activity (7).

Nevertheless, clozapine was marketed in several European countries until a study conducted in Finland in 1975 reported several cases of agranulocytosis resulting from treatment (8). Clozapine was then withdrawn from the clinical use, but in the following years its unique properties were extensively investigated until the validation of its efficacy in treating resistant-schizophrenia was established, leading to the reappearance on the market in 1990.

Soon after clozapine returned to the market, a new generation of antipsychotic drugs, called 'atypical', was developed and marketed. Amongst these olanzapine, quetiapine, risperidone, ziprasidone and aripiprazole were introduced into clinical usage.

Side Effects of Second Generation Antipsychotics SGA have been noted to improve adherence due to more tolerable side effects, in comparison to the side effects associated with FGA (9). Although SGA are a new addition to the available treatments for bipolar and schizophrenia, their side effect profile has been appreciated. It is possible that the side effects of SGA may not be less serious than those of FGA. Issues that present with SGA use include metabolic disturbance, weight gain and QT prolongation (9).

**Table 1: side effects of typical and atypical antipsychotics**

	Extrapyramidal	Sedation	Weight gain	Hyperglycaemia	Anticholinergic	Orthostatic hypotension
<b>Atypical antipsychotics</b>						
Risperidone	●●	●● initially	●●	●●	●	●● initially
Quetiapine	●*	●●●	●●	●●●	●●	●●
Olanzapine	●	●●●	●●●	●●●	●●●	●
Clozapine	●	●●●	●●●	●●●	●●●	●●
Amisulpride	●●*	●	●	●	●	●
Aripiprazole	●	●	●	●	●	●
Ziprasidone	●	●●	●	●	●	●●
<b>Typical antipsychotics</b>						
Haloperidol	●●●	●	●●	●●	●	●
Chlorpromazine	●●	●●●	●●●	●●●	●●●	●●●

Approximate frequency of adverse effects: ● (<2%) = negligible or absent; ● (>2%) = infrequent; ●● (>10%) = moderately frequent; ●●● (>30%) = frequent. \* rarely a problem at usual therapeutic doses

➤ Metabolic Disturbance and Weight Gain:

With the use of SGA, weight gain and increased appetite are noted as prominent side effects (Stahl, 2008). As a result, obesity and an increase in BMI can result; thus, increasing an individual’s cardiometabolic risk, predisposing one to a premature death (Stahl, 2008). The H1 histamine receptor and the 5HT2C serotonin receptor in the brain are associated with weight gain in the use of SGA. When blocked, especially synchronously, individuals taking SGA can experience weight gain (10). The hypothalamic centers are also partially responsible for enhancing appetite (10).The risks to cardiovascular health as a result of SGA treatment are equivalent to the risks of cigarette smoking (Bell et al., 2009).

Patients treated with SGA have an increased risk of diabetes mellitus, high blood pressure, coronary heart disease and related conditions (11). In addition, variables including patient’s age, family history, lifestyle, smoking

and exercise play an important role in the evaluation of metabolic syndrome (Bell et al., 2009, p. 142).

SGA weight gain varies according to baseline BMI. If the patient is obese, thus having a higher BMI, prior to the initiation of SGA treatment, the patient will not gain as much weight as their counterpart with a lower BMI(12). Among the SGA, clozapine and olanzapine have the highest association with metabolic disturbance (13).

Second generation antipsychotics have also been found to induce a preference for carbohydrate foods (14). Following an initial weight gain during the first few months of therapy, an individual taking SGA can continue to gain weight even after one year of discontinuation of treatment (14). Patients on olanzapine who gain two to three kilograms within the first few weeks of treatment present a significant risk for long term weight gain (14). Clozapine, olanzapine and risperidone are three SGA that have been associated with the greatest weight gain.

### ***1.1.5 Metabolic adverse effects***

Metabolic adverse effects associated with antipsychotics are of particular concern because of the increase in cardiovascular morbidity and mortality.<sup>4</sup> Olanzapine and clozapine are particularly associated with substantial weight gain, dyslipidaemia and hyperglycaemia.<sup>4</sup> People taking these medicines often report that they always feel hungry. Weight gain can be substantial – a gain of 2 kg in two weeks should prompt a medicine review. All patients prescribed antipsychotics should be given appropriate advice on diet and lifestyle interventions and monitored carefully for diabetes.

### ***1.1.6 Extrapyramidal effects***

Atypical antipsychotics are generally considered to cause fewer extrapyramidal adverse effects than typical antipsychotics. A meta-analysis showed clozapine, olanzapine and risperidone to be significantly less commonly associated with extrapyramidal symptoms than low potency typical antipsychotics (i.e. chlorpromazine 600 mg daily or equivalent).<sup>8</sup> The majority of studies have found no differences within the atypical group in terms of extrapyramidal effects.

### ***1.1.7 Tardive dyskinesia***

Rates of new-onset tardive dyskinesia (orofacial and trunk movements) have been estimated at 3% with risperidone and 1% to 2% for other atypical antipsychotics. In comparison, tardive dyskinesia develops in around 20% of people receiving typical antipsychotics. Tardive dyskinesia is of particular concern as it may not be evident immediately, is often resistant to treatment, may be persistent and may worsen on treatment withdrawal.

## **1.2 Schizophrenia**

In the general population, approximately 1% suffers from schizophrenia. This chronic disorder often manifests in youth, and are characterized by a number of symptoms, all of which can be classified as either positive (paranoid delusions, auditory hallucinations, thought disorders etc.), negative (flattened affect, apathy, social withdrawal etc.) or cognitive symptoms (reduced sustained attention and executive functions). Even though all of these symptoms are evident in different cases of schizophrenia, none of the symptoms is pathognomonic for schizophrenia.

### ***1.2.1 The etiology of schizophrenia***

Genetics play a key role in schizophrenia, but the exact etiology of schizophrenia remains largely unknown. Through the last decades, different Original article causes of schizophrenia have been investigated, including changes in neurotransmitter systems and neurodevelopment abnormalities. The dopamine hypothesis was first described in the 1970's,. Evidence of correlation between blockade of dopamine D2 receptors (DRD2), and response to antipsychotic drugs lead to a theory of dopamine hyperfunction in subcortical regions (nucleus accumbens) in the brain (7). Subcortical regions are dense in DRD2, and the number of DRD2 have been shown further increased in these areas in schizophrenia<sup>8</sup>. The dopamine hyperfunction in the limbic system are considered responsible for the positive symptoms of schizophrenia (7). Since the 1970's, the hypothesis has been refined to include a dopaminergic hypofunction in the prefrontal cortex (9). The prefrontal cortex is an area dense in dopamine D1 receptors, in which abnormalities may account for the negative symptoms as well as the cognitive impairment of schizophrenia. More recently, studies suggests that dopamine activity can be influenced by abnormalities in glutamate transmission in areas such as substantia nigra and the ventral tegmental area. Deficiency of Nmethyl aspartate (NMDA) transmission, a receptor of glutamate, can lead to a hypofunction of dopamine receptors in the prefrontal cortex, subsequently leading to a decrease in mesocortical dopamine transmission. Over time, the deficiency of the meso-limbic dopamine transmission might cause the positive symptoms of schizophrenia. N-desmethylclozapine (NDMC) Clozapine undergoes hepatic metabolization by several P450 CYP enzymes, the most significant being CYP1A2, CYP2D6 and CYP3A410. The high first pass metabolism reduces the bioavailability of

clozapine to 50-60%. The majority of clozapines metabolites are physiological inactive, but N-desmethylozapine (NDMC) is active. Only a few studies have investigated the effects of NDMC, but studies suggest different properties of NDMC than clozapine (14-16). NDMC acts as a partial agonist to DRD2 (14), with a binding affinity ( $K_i$ ) of 115, (17). Clozapine on the other hand, acts as an inverse agonist, or antagonist (15) with a  $K_i$  of 431 (17). An animal study evaluating Conditioned avoidance responding and Amphetamine-induced locomotion, models of antipsychotic efficacy, has revealed differences between clozapine and NDMC. Clozapine inhibited effects in both models, the inhibition were only present at high dosages of NDMC (16). A model of regional activation of the central nervous system (Fos expression), showed both clozapine and NDMC ability to induce Fos in nucleus accumbens but not in the dorsolateral striatum. Again, NDMC required high dosages to do the same (16). No differences between clozapine and NDMC in studies investigating catalepsy (measure of motor-side effects) or prolactin measurement (measure of side-effects) were found (16). Treatment of schizophrenia In most cases of schizophrenia, antipsychotic drugs are the treatment of choice (18). However, discontinuation of treatment is a common obstacle in this patient group, due to side-effects or lack of efficacy, the latter one also leaving a large proportion of patients symptomatic despite adequate antipsychotic treatment (19). Up to 30% of patients suffering from schizophrenia, are classified as treatment resistant schizophrenia (TRS) (20). TRS are often defined as inadequate response to two, or more first generation antipsychotics (FGA) and second generation antipsychotics (SGA).

Clozapine is the most efficient antipsychotic drugs, proven efficient for alleviating both positive and negative symptoms (21), as well as having

antisuicidal properties. On the other hand, studies fail to find any substantial superiority of clozapine compared to other dopamine antagonists in first episode schizophrenia, suggesting a biological homogeneity of patients responding to clozapine. Clozapine is not considered a first line antipsychotic drug in non-resistant schizophrenia, due to the risk of agranulocytosis and myocarditis (26). The side-effects warrant close monitoring of patients (26). Nevertheless, clozapine possesses the unique property of being efficient in 3 Original article approximately 50% of patient with TRS, making it the golden standard for treating TRS.

The mechanism of action of clozapine is unknown, but several theories exist; I) Dysregulation of the immune system have been observed in schizophrenia (28), and preclinical studies suggest that improvements by clozapine in factors influencing the immune systems, correlates with symptoms relieve. Thus, a theory of an antiinflammatory effect of clozapine was proposed as being the reason for the unique effect. II) Evidence of clozapine affinity towards a large number of receptors has been acknowledged for a long time (30). The high affinity of clozapine to muscarinic receptors, a property mainly clozapine possess, lead to a theory that the receptor might be an important factor in clozapine response (32). III) Blockage of DRD2 is a shared feature of all present antipsychotic drugs (7).

In addition, a DRD2 occupation of at least 70% is considered optimum for antipsychotic response. Interestingly, the high occupancy is not the case for clozapine, which rarely reaches occupancy of 70% even at high dosages. Clozapine superior effectiveness might be caused by regional selectivity, since clozapine specifically target DRD2 in limbic and cortical regions. In addition, clozapine display a fast dissociation rate and are more loosely bound to DRD2, unlike other antipsychotic drugs (5).



### 1.3 PKC Structure, Classification, and Regulation

Protein Kinase C is  $\text{Ca}^{2+}$  activated, defined by a structurally related family of serine/threonine protein kinases that are involved in several biological processes including proliferation, differentiation, apoptosis, adhesion and migration. All members contain a highly conserved kinase domain and regulatory domain. The kinase domain resides in the C-terminal half of the protein and consists of motifs that are required for ATP/substrate binding and catalysis. The regulatory domain is contained within the amino terminal half of the protein and is defined by an auto-inhibitory pseudo-substrate domain and one or two discrete membrane targeting domains; a C1 domain that binds diacylglycerol (DAG) or phorbol ester, and a C2 domain that binds  $\text{Ca}^{2+}$  (15). This family is sub-classified into three groups of isoforms based on their cofactor requirements for activation and corresponding structural differences in their regulatory domain (16).

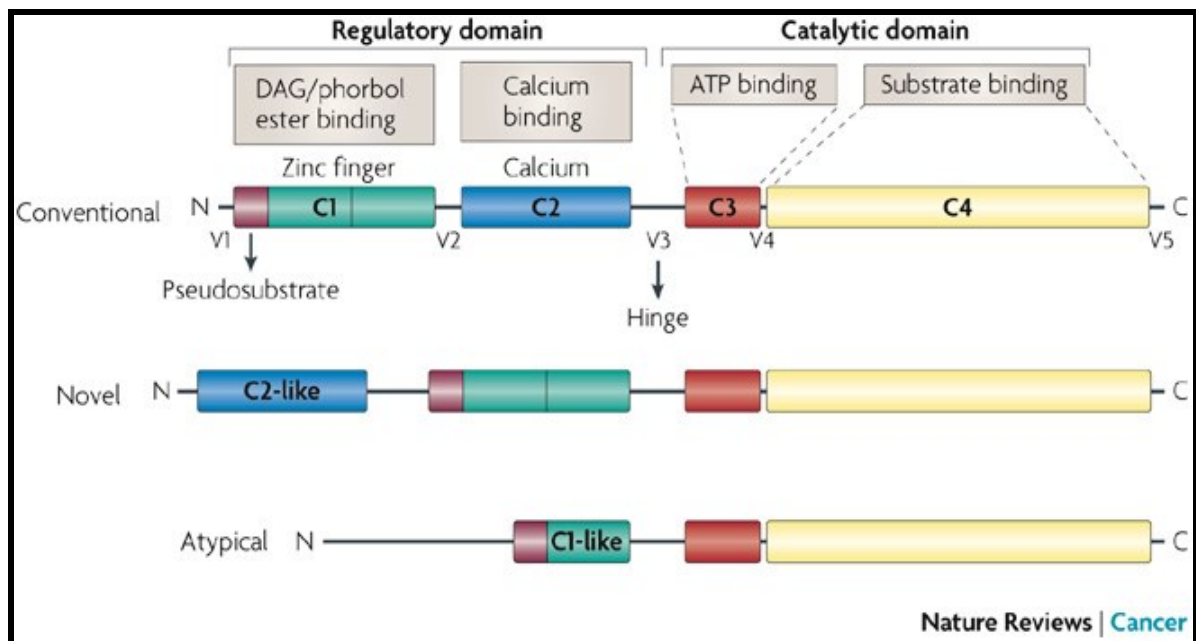
The three subclasses are:

- 1) conventional PKCs (cPKCs) that contains both a C1 domain that functions as a DAG binding site and a C2 domain that binds  $\text{Ca}^{2+}$ ;
- 2) novel PKCs (nPKCs) are structurally similar to the cPKCs, except that they lack a  $\text{Ca}^{2+}$  binding site but can be stimulated by DAG;
- 3) atypical PKCs (aPKCs) also lack a  $\text{Ca}^{2+}$  -binding C2 domain and have an atypical C1 domain that makes them insensitive to DAG but responsive to other lipids such as arachidonic acid and ceramide.

The group of conventional PKC isoforms is the most highly studied and best understood subclass. The PKC family transduces cell signals by

promoting lipid hydrolysis (17). Many known membrane receptors transmit intracellular signals through activation of PKC (18).

An external signal activates a G-ProteinCoupled Receptor (GPCR), which activates the GTPase enzyme phospholipase C (PLC). PLC cleaves phosphoinositol-4,5-bisphosphate (PIP2) into DAG and inositol-1,4,5-trisphosphate (IP3). IP3 interacts with a calcium channel in the endoplasmic reticulum (ER) thereby triggering the release of Ca<sup>2+</sup> into the cytoplasm. The increase in Ca<sup>2+</sup> levels activates cytoplasmic PKC which translocates to the membrane and is anchored to the plasma membrane via DAG and phosphatidylserine (PS). Activated PKC isoforms phosphorylate protein substrates in the membrane and cytosol to propagate signals throughout the cell that culminate in various biological and cancer-related phenotypes.



**Figure 1:** Schematic representation of protein kinase C isozyme structure and classification

Many known membrane receptors transmit intracellular signals through activation of PKC (Kenny et al., 2007; Lee and Bell, 1991). One known pathway for PKC activation is described in (Koivunen et al., 2006). An external signal activates a G-ProteinCoupled Receptor (GPCR), which activates the GTPase enzyme phospholipase C (PLC). PLC cleaves phosphoinositol-4,5-bisphosphate (PIP<sub>2</sub>) into DAG and inositol-1,4,5-4 trisphosphate (IP<sub>3</sub>). IP<sub>3</sub> interacts with a calcium channel in the endoplasmic reticulum (ER) thereby triggering the release of Ca<sup>2+</sup> into the cytoplasm. The increase in Ca<sup>2+</sup> levels activates cytoplasmic PKC which translocates to the membrane and is anchored to the plasma membrane via DAG and phosphatidylserine (PS). Activated PKC isoforms phosphorylate protein substrates in the membrane and cytosol to propagate signals throughout the cell that culminate in various biological and cancer-related phenotypes.

PKC is a member of the larger group of serine/threonine kinases that contain protein kinases G and A, among others and is widely expressed throughout the body. The family of PKCs is made up of ten different isoforms, classified into three groups based on their regulatory domains. The conventional PKCs ( $\alpha$ , $\beta$ I, $\beta$ II and  $\gamma$ ) require diacylglycerol, calcium, and phospholipids for activation. Finally, the atypical PKCs ( $\zeta$ , $\iota$ / $\lambda$ ) require anionic phospholipids for activation instead of either calcium or diacylglycerol.

Within the brain, PKC interacts with neurotransmitters via several mechanisms, interacting with ion channels and increasing the vesicle pool (19,20) As a result, PKC activity increases the release of many neurotransmitters, including dopamine (21), norepinephrine (22), and glutamate (23). PKC regulates both D2R and DAT through phosphorylation. The N-terminus of DAT contains a series of serines that are phosphorylated by PKC (24). Removal of these serines either via mutation to non-

phosphorylatable alanines or truncation of the N-terminus abolishes the amphetamine-stimulated efflux of dopamine through DAT without altering the normal uptake process. D2R is phosphorylated by PKC on the third intracellular loop causing internalization and desensitization of the receptor (25). Additionally, PKC  $\beta$  activity is required for amphetamine-stimulated dopamine efflux through the use of specific PKC $\beta$  inhibitors (26).

## 2. MATERIALS AND METHODS

### 2.1 Cell cultures

#### 2.1.1 MDSCs (*muscle derived stem cells*)

Primary cultures of MDSCs were prepared from newborn mice wild type and PKC $\beta$  null (2–3 days) as described in Brini *et al.* Adipogenic conversion was observed both in the first preplating (15 min) and in the replating of non-rapidly adherent cells. For this reason, the first pool of adherent cells was used for all experiments. The viable cells obtained were counted using the Trypan blue exclusion assay and were seeded at a density of  $10 \times 10^5$  cells per square centimeter for *in vitro* expansion in Dulbecco's modified Eagle's medium (DMEM) supplemented with glucose 25 mM for 5 days. At day 5, APDs (clozapine, olanzapine; quetiapine, risperidone and aripiprazole (250 mM)) were added for the following 3 days. The DMEM without the addition of high glucose (low glucose) was used as a negative control.

#### 2.1.2 ADSCs (*Adipose-derived stem cells*)

ADSCs were extracted from human adipose tissues of five healthy female patients undergoing cosmetic surgery procedures, following guidelines from the Clinic of Plastic Surgery, University of Padova. Adipose tissues were digested with 0.075% collagenase (type 1A; Sigma–Aldrich, Milan, Italy) in modified Krebs–Ringer buffer for 60 min at 37 °C followed by 10 min with 0.25% trypsin. The viable cells obtained were counted using the Trypan blue exclusion assay and seeded at a density of  $1 \times 10^6$  cells per square centimeter for *in vitro* expansion in DMEM supplemented with

glucose 25 MM after 5 days of expansion. At day 5, APDs were added for the following 3 days. DMEM without the addition of high glucose (low glucose) was used as a negative control.

### ***2.1.3 Morphological analysis***

Morphological cell analyses to determine the extend of preadipocyte differentiation were performed using a phase-contrast microscope at a 10x magnification, counting cells in the field of vision, randomly chosen. Five fields were counted. Main morphological criteria for differentiation were an increasing number and size of visible lipid droplets as well as a change in morphology from elongated contours to a round shape.

### ***2.1.4 Oil Red O staining***

To determine the Adipose Differentiation Oil Red O staining was performed according to a method by Zacarias et al. Monolayer cultures were washed with PBS and fixed with cold 10% paraformaldehyde and incubated for five hours at 4 °C. Oil Red O working solution (0.5 g in 100 ml isopropanol) was added to culture flasks for two hours at room temperature. After washing, stained cells were kept in 10% paraformaldehyde and examined by light microscopy.

### ***3.1.4 Microscopic analysis of PKC translocation***

The cells were seeded before transduction onto 24 mm glass coverslips and allowed to grow to 50% confluence. At this stage, images of PKC- $\beta$  translocation were obtained with digital imaging system, at 24 h after infection with adenovirus PKC- $\beta$ -green fluorescent protein chimera. At 24 h after infection, the medium was changed from DMEM and 10% fetal calf

serum to Krebs–Ringer buffer. Images were recorded using a digital imaging system based on a Zeiss Axiovert 200 (Arese, Milan, Italy) fluorescence microscope equipped with a back-illuminated charge coupled device camera (Roper Scientific, Trenton, NJ, USA), excitation and emission filterwheels (Sutter Instrument Company, Novato, CA, USA) and piezoelectric motoring of the z-stage (Physik Instrumente, GmbH & Co., Germany) for rapid focusing in the z-plane. The data were acquired and processed using the MetaMorph analyzing program (Universal Imaging Corporation, Downingtown, PA, USA). This allows the direct monitoring of fluorescence intensity. A high-resolution, three-dimensional reconstruction of the distribution of a green fluorescent protein chimera can be obtained with the technique of digital image restoration, also called deconvolution or deblurring. The graph indicates the plasma membrane of PKC- $\beta$ –green fluorescent protein expressed as the increase in fluorescence ratio with respect to time zero (calculated as a ratio of plasma membrane/cytosol average fluorescence). Averaging results are representative of at least three independent experiments.

## **2.2 Animals**

Animals employed in all procedures were male mice at the age of 4-6 weeks. Prior to any experimental procedures, mice were either group-housed or isolated randomly (depending upon the experimental protocols). They were kept under constant conditions: 12/12 h light /dark cycle (light on 0800 hours), temp (24 ° C) and humidity (54 –60%) in our laboratory facility.

In all cases, mice were fed with 12 g of high fat feed (60% of fat) obtained from Harlan Laboratories(lotto: 222140).

The weight of the animals was monitored every day.

Daily access to feed, water and Clozapine was ad libitum. In all procedures, the mice were left under specified conditions to acclimatize for at least three weeks before conducting any experimental procedures. Mice were properly handled by the experimenter and made familiar to gavage administration during the specified periods. All experiments employed were done in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the local council of animal care (University of Ferrara).

#### Groups of animals

A: male mice C57BL/6J – control group

B: male mice C57BL/6J – treated with Clozapine (Haxal AG, Germany)

C: male mice PKC $\beta$  null - control group

D: male mice PKC $\beta$  null - treated with Clozapine (Haxal AG, Germany)

E: male mice C57BL/6J - treated with the pharmacological inhibitor of PKC $\beta$  (LY33351, Eli Lilly & Co. (Indianapolis, IN)).

D: male mice C57BL/6J- co-administration of Clozapine and the pharmacological inhibitor of PKC $\beta$

## 2.3 Drugs

Drugs and chemicals employed in this dissertation were scientifically proven quality and from known sources.

Clozapine (Hexal AG, Germany) was administrated orally, diluted in drinking water (25mg of Clozapine in 100ml of drinking water). Twice a week



the water containing the Clozapine was changed. The long – term treatment with this drug had a maximum duration of five months.

Pharmacological Inhibitor of PKC $\beta$  (LY33351- Eli Lilly & Co.,Indianapolis, IN)

Ruboxistaurin was submitted for approval in Europe in the second quarter of 2006. The agent is also in phase II studies for the treatment of diabetic maculopathy (macular retinopathy) in Japan. Data from a phase III, 3-year study of ruboxistaurin in patients with moderate to severe diabetic retinopathy showed that ruboxistaurin markedly reduced the risk of sustained vision loss compared with placebo. This multicentre, randomised study, named PKC-DRS2 (Protein Kinase C-Diabetic Retinopathy Study 2), was conducted at 70 clinical sites and involved 685 patients with diabetic retinopathy. The agent is also in a phase II study in the US, Canada and Europe in patients with clinically significant macular oedema.

## **2.4 Treatments**

### ***2.4.1 Long-term treatment with Clozapine and Weight gain monitoring***

Male mice of the age of 4-6 weeks were used for all the experiments of weight monitoring. The animals of the treated group with Clozapine, have had as only source of water the Clozapine diluted in drinking water, always at the same concentration during the experiment.

The first data obtained in our laboratory are referred to a three month treatment and then repeated with five month duration due to verify if the weight gain and the motor phenotype persist after long periods of treatment.

Treatment with the pharmacological inhibitor was performed using the technique of oral dose (Gavage).

#### ***2.4.2 Oral Dosing (Gavage) in Mice***

In order to administer the pharmacological inhibitor of PKC $\beta$  oral-gavage was employed daily for duration of three months. During this period of time the mice were also orally treated with Clozapine.

In order to obtain valid data on monitoring the weight gain, the control group of mice was treated by using the oral-gavage with drinking water and induce the same levels of stress in all the groups of animals.

This Standard Operating Procedure (SOP) describes the procedure of orally dosing rats and mice. This SOP follows the UBC and CCAC guidelines for acceptable oral dose volumes in rodents (UBC Animal Care Guidelines).

#### **Materials**

- Correctly sized feeding needles (see chart below)
- Appropriate sized syringes
- Solution to be administered

#### **Procedure**

1. Calculate the maximum volume that can be administered to the animal. Oral dosing volumes should not exceed 10ml/kg.
2. Prior to performing the oral dosing procedure, measure the distance from the oral cavity to the end of the xyphoid process (caudal point of the sternum) with the feeding needle on the outside of the restrained animal. This will be how far the needle will be inserted into the esophagus.

3. With the feeding needle attached to the filled syringe, slide the end of the feeding needle along the roof of the animal's oral cavity towards the animal's left side. The feeding needle should slide down the esophagus with gravity alone. There should be no resistance when passing the feeding needle. The gavage needle may rotate clockwise slightly as it passes the epiglottis and into the esophagus. The animal may gag when the needle is passed; this is normal. If there is any resistance or if the animal struggles excessively remove the feeding needle, ensure you have good restraint on the animal and attempt to pass the needle again.
4. Do not at any time force the needle down the esophagus, this may cause tears to the esophagus, injury to the animal, or you may inadvertently force the needle down the trachea.
5. Once the feeding needle is in to the premeasured distance, slowly inject the solution to minimize the fluid coming back up the esophagus. There should be no resistance while injecting – the needle will be in the distal esophagus, not the stomach.
6. Remove the feeding needle in the opposite direction from insertion and return the animal to its cage.
7. Monitor the animal for potential complications

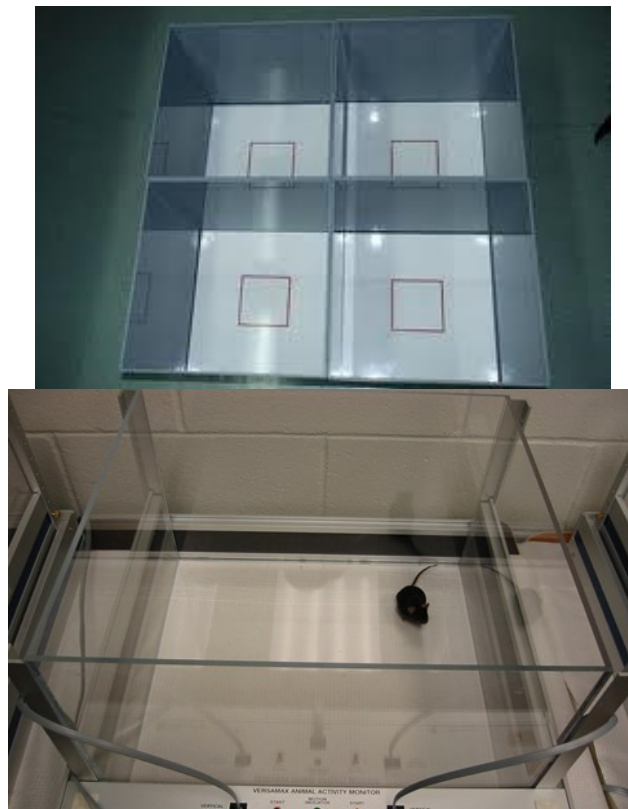


**Figure 2:** *Oral Gavage in mice.*

## 2.5 Motor Activity

### 2.5.1 Open Field Measurement

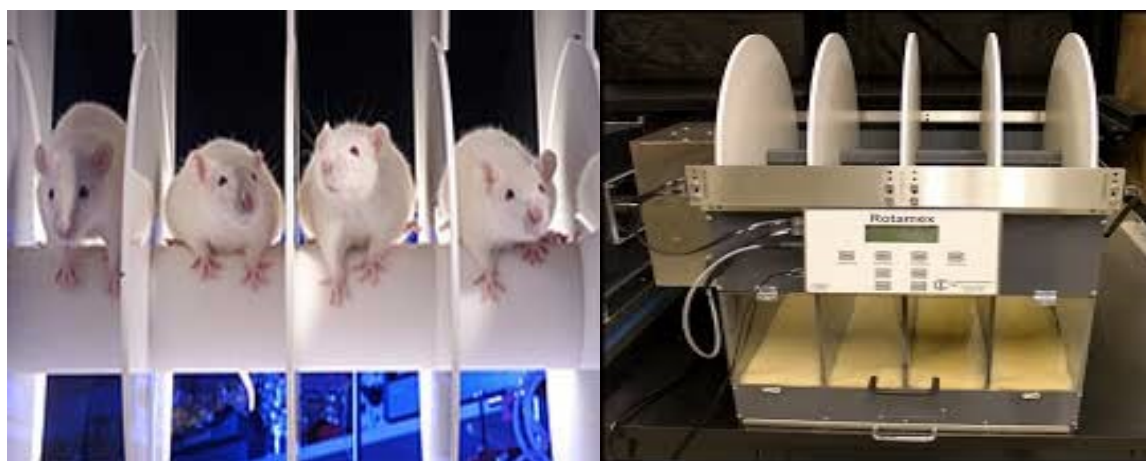
To examine a motor response thought to be related to the negative effect of clozapine tests of measurement of motor activity Horizontal and vertical locomotion of mice were assessed using an open-field box (Fig.). In the open field boards, the behaviour was first videotaped and later offline monitored using a dose-based program. In the activity box, locomotion was monitored using a fully automated computer controlled photocells (Process control motility test 302000, TSE, Technical and Scientific Equipment). Interruptions of horizontal and vertical light beams due to mouse's movement were registered automatically. These data were then later converted to digital values, expressed as distance travelled in meter or number of rearing.



**Figure 3:** Schematic presentation of open-field box (A) and activity box (B)

### **2.5.2 Rotarod test**

The akinetic/bradykinetic effects of Clozapine on mice behaviour were evaluated through the Rotarod test, mice were placed on a 6 cm diameter rod (Model 720A, IITC/Life Science Instruments, Woodland Hills, CA) that was rotating at 10 revolutions per minute. The amount of time spent on the rod before falling off was measured in two consecutive trails with a 2 min break between trials. Data represent the average time spent on the rod during these trials.



**Figure 4:** *Rotarod Test*

### **2.5.3 Bar Test**

This motor test was applied to mice, due to evaluate the Akinetic phenotype or catalepsy induced by the treatment of Clozapine.

Catalepsy is manifested as a physical condition usually with catatonic schizophrenia, characterized by suspension of sensation, muscular rigidity, fixity of posture, and often by loss of contact with the environment.



**Figure 5:** *bar test*

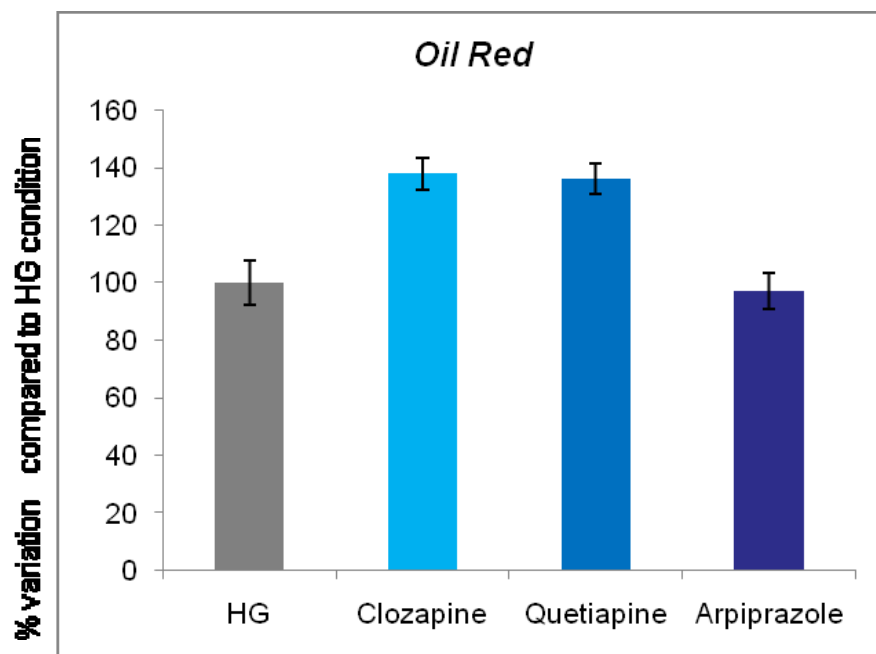
#### ***2.5.4 Drag test***

This test measures the ability of the animal to balance its body posture with the forelimbs in response to an externally imposed dynamic stimulus (backward dragging). It gives information regarding the time to initiate and execute (bradykinesia) a movement. Animals were gently lifted from the tail leaving the forepaws on the table, and then dragged backwards at a constant speed (about 20 cm/s) for a fixed distance (100 cm). The number of steps made by each paw was recorded. Five to seven determinations were collected for each animal.

### 3. RESULTS

#### 3.1 Clozapine induces Differentiation in vitro

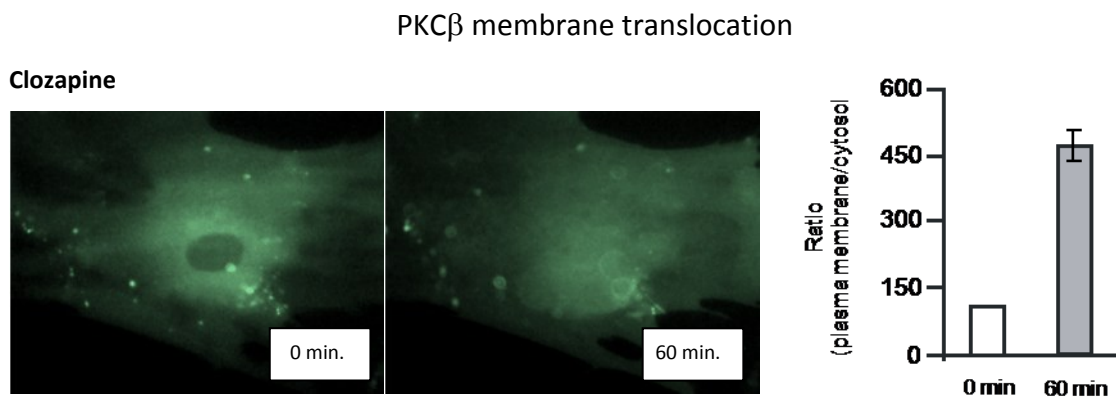
Adipose derived stem cells have been evaluated in terms of lipid production after treatment with different APDs. The results obtained with oil red quantification are reported in term of percentage variation compared to the high-glucose medium set to 100%.



**Graphic 1:** APDs are able to increase the total lipid production on ADSCs: Clozapine and Quetiapine show a clear difference in terms of lipid production. Aripiprazole induces lipid production comparable to that observed in untreated cells

### 3.2 APDs induce PKC $\beta$ activation in Vitro

The endogenous PKC $\beta$  is mostly cytosolic under low-glucose conditions. Incubation for 1h with Clozapine induced notable translocation of the kinase into the plasmamembrane, it's typical localization after activation. The ratio of the detected signal in cytoplasm when compared to plasmamembrane showed a kinetic directly related to Clozapine treatment.

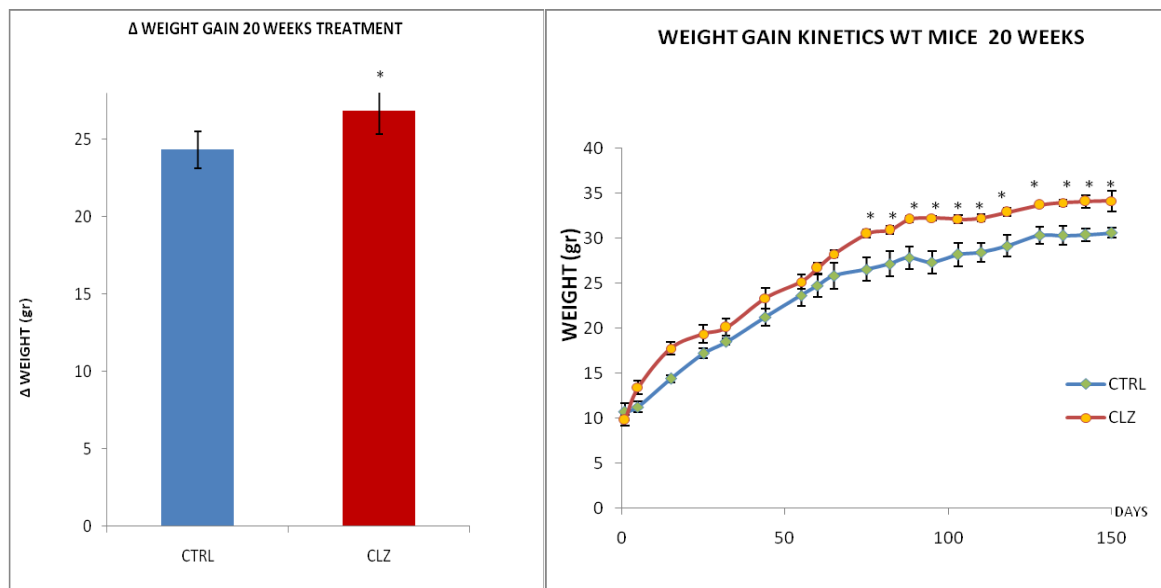


**Figure 6:** PKC $\beta$  membrane translocation.



### 3.2.1 Weight gain monitoring of wild type mice treated for three months with Clozapine

Wild type mice were treated for three months with Clozapine. Daily monitoring of the weight during this period of time revealed a significant weight gain of the animals. The results are expressed as  $\Delta$  of weight gain and kinetics during the experiment.



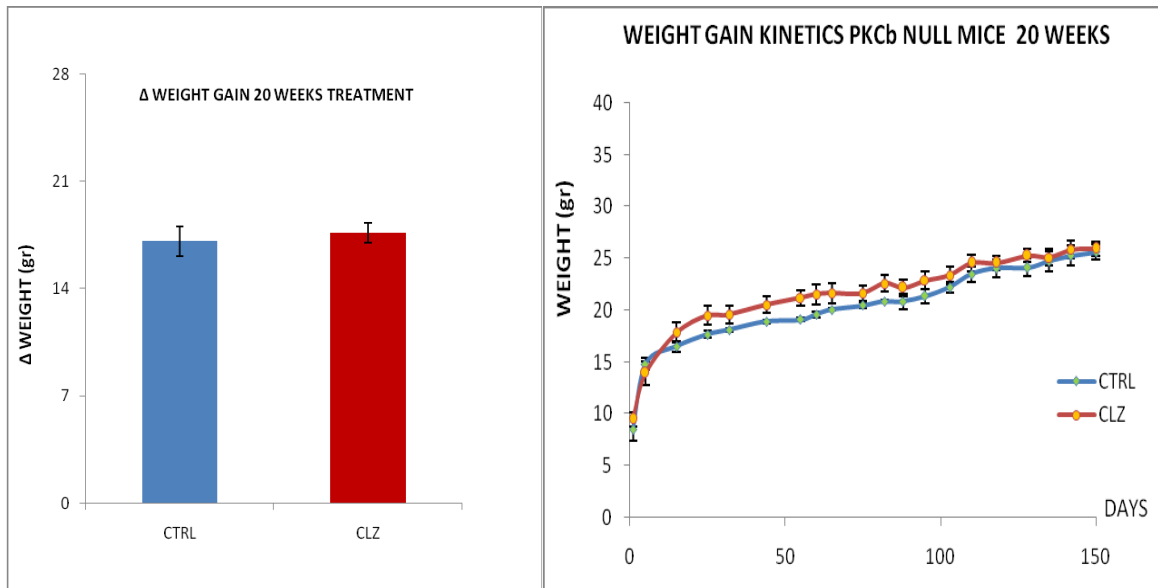
*Clozapine chronic treatment in WT mice:*

- i) *treated mice present a significative major  $\Delta$  weight gain against controls*
- ii) *Traces of weight gain kinetics during the chronic administration of Clozapine for 12 weeks, present significative differences only the last 2 weeks of the experiment*

**Graphic 2:** *Weight gain in wild type mice during 20 weeks of treatment with clozapine*

### 3.2.2 Weight gain monitoring of PKC $\beta$ null mice treated for three fine with Clozapine

The data shown are referred to PKC $\beta$  null mice treated for three months with Clozapine. No significant weight gain in the treated mice was demonstrated.



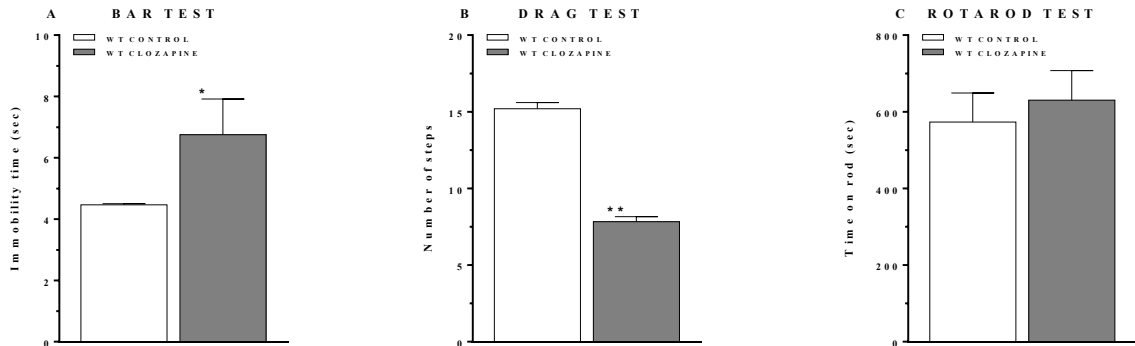
*Clozapine chronic treatment in PKC $\beta$  null mice:*

- i. treated and untreated mice have no significant variance in weight gain*
- ii. Traces of weight gain kinetics during the chronic administration of Clozapine for 12 weeks treatment. Treated animals present no significant differences vs controls*

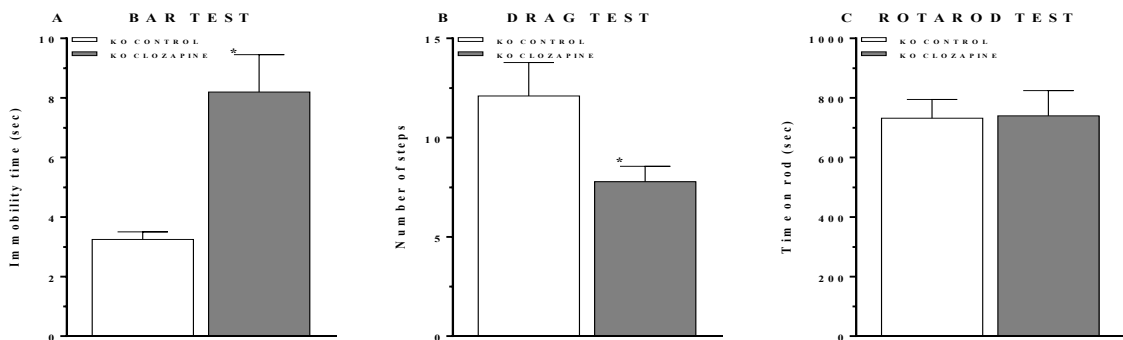
**Graphic 3:** Weight gain in PKC $\beta$  mice during 20 weeks of treatment with clozapine

### 3.2.3 Motor phenotype of the mice treated with Clozapine

#### Motor Activity of wild type mice treated with clozapine



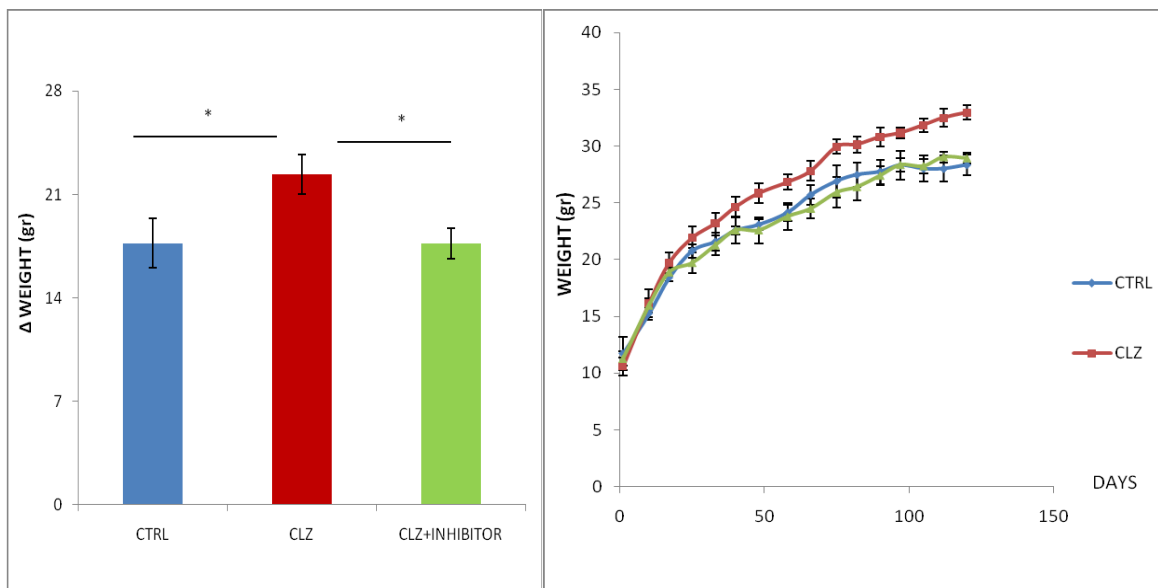
#### Motor Activity of PKC $\beta$ null mice treated with clozapine



**Graphic 4:** Effect of chronic administration of CLOZAPINE in the bar (A), drag (B) and rotarod (C) tests in mice. Data are expressed as time on bar (A), number of steps (B) and time on the rod (C) and are means  $\pm$  SEM of 4-5 determinations per group. \* $p < 0.05$ , \*\* $p < 0.01$  significantly different from control

### 3.3 Co-treatment of Clozapine and the Pharmacological inhibitor of PKC $\beta$

The results indicate that the inhibition of PKC $\beta$  protects against the weight gain during the treatment with Clozapine. Motor tests are exhibit to prove that the antipsychotic effect of Clozapine persists even with the administration of the pharmacological inhibitor.

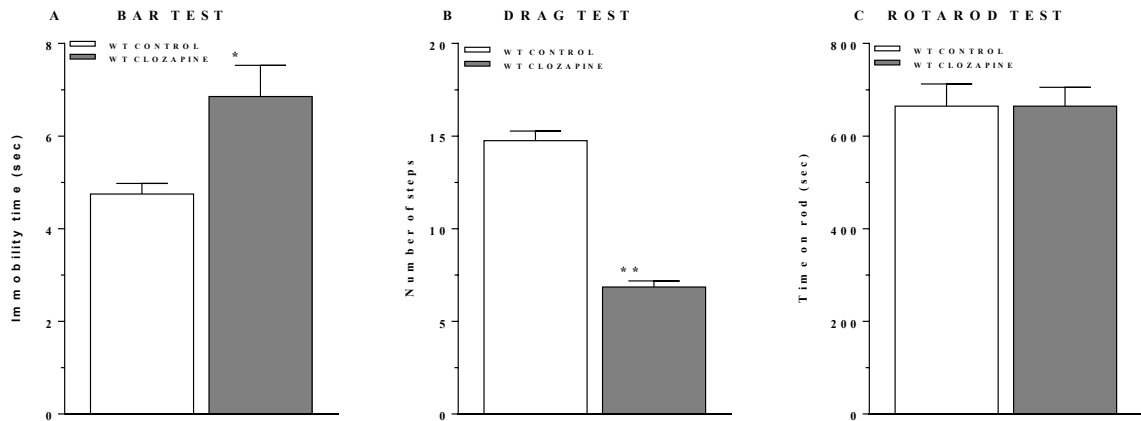


\* $p < 0.05$  to CTRL, • $p < 0.05$  to CLZ

- i)  $\Delta$  of the weight gain in mice treated with Clozapine and co- administration of the Pharmacological inhibitor of PKC $\beta$  (of LY-333,351)
- ii) Weight gain kinetics, significance: \* expressed to controls, • expressed to clozapine

**Graphic 5:** Co-treatment of Clozapine and the Pharmacological inhibitor of PKC $\beta$

**Motor Activity of wild type mice treated with Clozapine and the pharmacological inhibitor**



**Graphic 6:** *Effect of chronic administration of CLOZAPINE in the bar (A), drag (B) and rotarod (C) tests in mice. Data are expressed as time on bar (A), number of steps (B) and time on the rod (C) and are means  $\pm$  SEM of 10-17 determinations per group. \* $p < 0.05$ , \*\* $p < 0.01$  significantly different from control*

## 4. DISCUSSION

Atypical antipsychotics (APDs) are currently used in clinical practice for a variety of mental disorders (schizophrenia, bipolar disorder, severe behavioral disorder) presenting as common side effects the weight gain, obesity, lipid abnormalities and diabetes.

Our previous studies in vitro on adipogenic events, show that in cell cultures of human pre-adipocytes and rat muscle-derived stem cells, where APDs (clozapine, olanzapine, quetiapine, risperidone and aripiprazole) were added in the presence of high glucose, presented an increase in lipid accumulation and an enhancement of pre-adipocyte differentiation.

Our data show that Protein Kinase C isoform  $\beta$  (PKC  $\beta$ ) has a crucial role in the metabolic pathways leading to the neo-differentiation of adipose cells during APDs treatment. Through pharmacological inhibitor (bisphosphonate) and molecular silencing we have prevented the APDs-induced lipid accumulation, providing its direct involvement into this process.

Clozapine can active adipogenesis through PKC- $\beta$  translocation to plasma membrane and activation.

Based on these data, in vivo experiments were carried on. A pharmacological inhibitor (LY333531-mesylate) of PKC- $\beta$  is oral administered in wild type mice treated with clozapine. Our study indicates that the mice treated with clozapine and in addition with the inhibitor present in the behavioral tests decreased time spent resting and reaching distances.

In the last three months, our experiments in vivo, are focused in PKC- $\beta$  -/- mice, treated with clozapine, so we can monitor their behavior and weight gain and compare it to our control animals. At present, our data support the previous findings, showing that the PKC- $\beta$  knock out mice

treated with clozapine and those not treated present the same rate of weight gain. These findings underline the crucial role of PKC- $\beta$  protein in weight gain, when APDs are administrated in animal models.

Atypical antipsychotics (APDs) are currently used in clinical practice for a variety of mental disorders (schizophrenia, bipolar disorder, severe behavioral disorder) presenting as common side effects the weight gain, obesity, lipid abnormalities and diabetes.

Our previous studies in vitro on adipogenic events, show that in cell cultures of human pre-adipocytes and rat muscle-derived stem cells (ADSCs), where APDs (clozapine, olanzapine, quetiapine, risperidone and aripiprazole) were added in the presence of high glucose, presented an increase in lipid accumulation and an enhancement of pre-adipocyte differentiation.

Our data show that Protein Kinase C isoform  $\beta$  (PKC  $\beta$ ) has a crucial role in the metabolic pathways leading to the neo-differentiation of adipose cells during APDs treatment. The pharmacological inhibition and the molecular silencing of PKC- $\beta$  prevent the APDs-induced lipid accumulation, providing its direct involvement into this process.

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