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Antipsychotics differentially regulate insulin, energy sensing, and inflammation pathways in hypothalamic rat neurons

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ABSTRACT

Introduction: Second generation antipsychotic (AP)s remain the gold-standard treatment for schizophrenia and are widely used on- and off-label for other psychiatric illnesses. However, these agents cause serious metabolic side-effects. The hypothalamus is the primary brain region responsible for whole body energy regulation, and disruptions in energy sensing (e.g. insulin signaling) and inflammation in this brain region have been implicated in the development of insulin resistance and obesity. To elucidate mechanisms by which APs may be causing metabolic dysregulation, we explored whether these agents can directly impact energy sensing and inflammation in hypothalamic neurons.

Methods: The rat hypothalamic neuronal cell line, rHypoE-19, was treated with olanzapine (0.25–100 uM), clozapine (2.5–100 uM) or aripiprazole (5–20 uM). Western blots measured the energy sensing protein AMPK, components of the insulin signaling pathway (AKT, GSK3β), and components of the MAPK pathway (ERK1/2, JNK, p38). Quantitative real-time PCR was performed to determine changes in the mRNA expression of interleukin *(IL)-6, IL-10* and brain derived neurotrophic factor (*BDNF*).

Results: Olanzapine (100 uM) and clozapine (100, 20 uM) significantly increased pERK1/2 and pJNK protein expression, while aripiprazole (20 uM) only increased pJNK. Clozapine (100 uM) and aripiprazole (5 and 20 uM) significantly increased AMPK phosphorylation (an orexigenic energy sensor), and inhibited insulin-induced phosphorylation of AKT. Olanzapine (100 uM) treatment caused a significant increase in IL-6 while aripiprazole (20 uM) significantly decreased IL-10. Olanzapine (100 uM) and aripiprazole (20 uM) increased BDNF expression.

Conclusions: We demonstrate that antipsychotics can directly regulate insulin, energy sensing, and inflammatory pathways in hypothalamic neurons. Increased MAPK activation by all antipsychotics, alongside olanzapine-associated increases in *IL-6*, and aripiprazole-associated decreases in *IL-10*, suggests induction of pro-inflammatory pathways. Clozapine and aripiprazole inhibition of insulin-stimulated pAKT and increases in AMPK phosphorylation (an orexigenic energy sensor) suggests impaired insulin action and energy sensing. Conversely, olanzapine and aripiprazole increased *BDNF*, which would be expected to be metabolically beneficial. Overall, our findings suggest differential effects of antipsychotics on hypothalamic neuroinflammation and energy sensing.

1. Introduction

Antipsychotics (APs) are the gold-standard treatment for schizophrenia and are increasingly being prescribed both on- and off-label for other psychiatric conditions. However, these medications cause serious metabolic side effects. In turn, patients with schizophrenia have a two-fold increase in standardized mortality ratio from cardiovascular disease (Hennekens et al., 2005). Although the liability for metabolic side

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effects differs between APs (Newcomer, 2005), it has recently become clear that no single AP is devoid of this risk, particularly in younger individuals who are AP-naïve (Correll et al., 2009). Additionally, it is found that regardless of class or agent, APs increase risk of diabetes 2–3 fold, above and beyond the risk that is conferred by the illness of schizophrenia itself (Rajkumar et al., 2017).

The mechanisms of AP-induced metabolic dysregulation are unclear, but are understood to occur at least in part through the central nervous system (Kowalchuk et al., 2018). In addition, the question has arisen whether APs could be causing metabolic perturbations by directly impact brain insulin signalling (Kowalchuk et al., 2017). Insulin receptors are expressed at high levels in many brain areas, where signalling pathways (mediated through AKT, GSK3β, and the MAPKs) play a significant role in neuronal growth, memory, and energy regulation. Brain studies examining AP administration in vitro and in rodents in vivo have variably suggested upregulation of pathways downstream of the insulin receptor (i.e. AKT phosphorylation/ activation; GSK phosphorylation /deactivation), which is difficult to reconcile with the adverse metabolic effects of APs observed clinically. However, many in vitro studies have not been conducted under conditions of insulin stimulation, and thus fail to directly test effects of APs on insulin response. Insulin stimulation has been used when investigating AP effects in peripheral tissues, with variable results (Del Campo et al., 2018; Engl et al., 2005). *in vivo* rodent data on central insulin signalling is similarly difficult to interpret as most APs cause acute elevations in blood glucose and insulin (Boyda et al., 2010; Smith et al., 2014). Hyperglycemia and hyperinsulinemia has been shown to increase central AKT and GSK-3 phosphorylation, even in the face of insulin deficiency or disrupted insulin action (Clodfelder-Miller et al., 2005; Smith et al., 2014). Thus, it is difficult to determine if changes in activation of these proteins are due to the AP or secondary to the elevations in blood glucose. There is also the possibility that APs upregulate central insulin signalling but impair alternate energy sensing signals such as AMP-activated protein kinase (AMPK), which has been associated with AP-induced glucose dysregulation (Ikegami et al., 2013; Martins et al., 2010).

Alternatively, inflammation is also an area of interest as neuroinflammation is considered a causal factor of metabolic disease (Thaler and Schwartz, 2010). However, clinical studies evaluating AP effects on inflammation are highly inconclusive due to the many confounds which can influence inflammatory state and which are associated with the illness of schizophrenia (i.e. smoking, diet, obesity) (Tourjman et al., 2013). The heterogeneity among studies examining effects of APs on insulin and inflammatory pathways may also be attributable to a differential response of brain regions to AP exposure. Only a limited number of studies have examined APs effects on insulin signalling or energy sensing specifically in the hypothalamus (Kowalchuk et al., 2018), which is the primary brain region involved in whole body energy regulation. In addition, the hypothalamus is composed of highly heterogenous neuronal populations, and the limited in vivo papers available have examined the entire hypothalamus (Obuchowicz et al., 2006; Zhang et al., 2014). Thus, we do not know how APs may act on specific cell types. Finally, as these were whole-body experiments, we do not know if the effects seen are due to the direct impact of APs on the hypothalamus, or occur in response to feedback from other tissues signalling to this brain region.

In the present study, we set out to elucidate the direct effects of different APs (clozapine, olanzapine, aripiprazole) on insulin signalling, energy sensing and inflammation in the hypothalamus. In order to circumvent the confounding variables present in clinical studies, and to avoid changes in adiposity or acute hyperglycemia observed in rodents administered APs, we chose to employ an invitro hypothalamic cell model. We hypothesized that APs would differentially impair insulin signalling, upregulate AMPK, and induce inflammation, according to their clinical metabolic liability (clozapine = olanzapine > aripiprazole).

2. Methods and materials

2.1. Cell culture and reagents

Rat hypothalamic neurons were immortalized using SV-40 T-antigen as previously described (Belsham et al., 2004; Gingerich et al., 2009). The immortalized rat hypothalamic embryonic neuronal cell line, rHypoE-19, was selected as it has been previously characterized to express the insulin receptor, essential components of the insulin signalling pathway, and cytokine receptors (Dhillon et al., 2012; Mayer and Belsham, 2009; Nazarians-Armavil et al., 2013). The cell line also expresses neuropeptide-Y (NPY) and agouti-related peptide (AgRP), key orexigenic regulators of satiety and energy homeostasis.

2.2. Antipsychotic dosing

The APs olanzapine and clozapine were chosen as they are highly effective therapeutically but also confer the highest metabolic risk. Clozapine is additionally the only AP with an indication for treatment-resistant schizophrenia (Hasan et al., 2012). Aripiprazole was investigated as it is a newer class of AP which also acts as a partial D2 receptor agonist and clinically is associated with a lower risk of weight gain (Bak et al., 2014).

100 μM of olanzapine was the highest concentration chosen to treat the rHypoE-19 neurons, based on previous studies showing changes in insulin signalling (pAKT and pGSK3β) in skeletal muscle cells (Engl et al., 2005) and changes in BDNF in neuroblastoma cells (Lee et al., 2010) with this concentration. Clinically, clozapine doses are about 10 times higher in comparison to olanzapine. However, 1000 uM of clozapine induced cell death within a few minutes of exposure. 100 uM of clozapine was thus used for western blot analysis, as cells remained intact during the experiments (i.e. 15 min of exposure). However, during the RT-qPCR experiments, cells were exposed to the drugs for 24 h; 100 uM and 50 uM of clozapine induced cell death after 24 h. Thus, 20 μM of clozapine was used for RT-qPCR experiments. 50 uM aripiprazole was initially chosen as this concentration has been previously shown to increase AMPK phosphorylation in in vitro studies (Takami et al., 2010). However, 50 μM of aripiprazole caused cell death after 15 min; thus, 20 μM of aripiprazole was used, a concentration which has previously been shown to increase BDNF and pGSK3β protein expression in vitro (Park et al., 2013).

2.3. Western blot analysis

Prior to treatment, cells were serum starved for 1 h in low-glucose DMEM (MilliporeSigma, Oakville, ON, Canada) with added 1% penicillin, then treated with either olanzapine, clozapine, aripiprazole (Toronto Research Chemicals, Toronto ON), or DMSO, with and without insulin (10 nM) for 15 min. 15 min was chosen as this is when the greatest effect of insulin-stimulated pAKT and glucose-stimulated pAMPK has previously been established in immortalized hypothalamic rodent neurons (Mayer and Belsham, 2010; McFadden et al., 2013).The selected insulin dose has been well-established to activate the insulin signalling pathway in immortalized hypothalamic neurons (Mayer and Belsham, 2009; Nazarians-Armavil et al., 2013). After a 15-minute treatment, protein was harvested using 1X cell lysis buffer (Cell Signalling Technology, Danvers, MA, USA), 1% protease inhibitor, 1% phosphatase inhibitor, and 1% PMSF (MilliporeSigma, Oakville, ON, Canada). Protein was then quantified using the Pierce BCA Protein Assay Kit (ThermoFisher, USA). 25ug of total protein was subjected to 8% SDS–PAGE, and transferred onto 0.22 lm polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Mississauga, ON, Canada). Protein blots were blocked with 5% milk in TBS-T for 1 h, and incubated overnight at 4 °C with primary antibodies. The blots were probed for phospho-AKT (ser473), AKT, phospho-AMPK (Thr172), AMPK, phospho-p44/42 (ERK1/2) (Tyr204), p44/42 (ERK1/2), phospho-JNK (Thr183/Tyr185),

JNK, phospho-P38, P38 (CST), phospho- GSK3β(Ser9), and GSK3β (MilliporeSigma, Oakville, ON, Canada), which were diluted in either 5% milk in 1 × TBST or 5% BSA in 1 × TBST (MilliporeSigma, Oakville, ON, Canada). After incubation with primary antibody, blots were washed in 1X TBS with 0.1% Tween, and incubated with anti-rabbit secondary (1:7500, Cell Signalling Technology Inc.) for 1 h at room temperature. Blots were imaged using chemiluminescence on the Kodak Image Station 2000R, using ECL Select Detection reagent (GE Healthcare Life Sciences, Pittsburgh, PA, USA). Densitometry was performed using Image J64 software; phosphorylated protein was normalized to total protein.

2.4. RT-qPCR

Cells were grown to 70% confluency, and treated with olanzapine, clozapine, aripiprazole, or vehicle for 4 or 24 h. RNA was isolated using PureLink RNA Kit with on-column PureLink DNase DNase (Ambion, Streetsville, Ontario, Canada), and RNA concentration and purity were measured using the Nanodrop 2000c spectrophotometer. 1 µg of RNA was used to synthesize cDNA with the high capacity cDNA reverse transcription kit (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). qRT-PCR was then performed using qRT-PCR master mix (Platinum SYBR Green qPCR SuperMix-UDG with ROX; Applied Biosystems, Life Technologies) with gene specific primers for *IL6*, *IL10* and *BDNF* (Table 1). Samples were run as triplicates on an Applied Biosystem Prism 7900 H T machine. qRT-PCR data analysis was performed using standard curve method and normalized to the reference gene, histone 3A.

2.5. Statistical analysis

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) and presented as mean ± SEM. One- or two-way ANOVA were used with Tukey's *post hoc* test when appropriate. Statistical significance is considered as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ (Table 2).

3. Results

3.1. Antipsychotics induce changes in insulin signalling and energy sensing

3.1.1. AKT & GSK3β

Phosphorylation of AKT is a key step in the canonical insulin signalling pathway; GSK3β is downstream of AKT and phosphorylation inhibits GSK3β action (Clodfelder-Miller et al., 2005). To determine if APs impair insulin response, the rHypoE-19 cells were treated with one of the three APs, with and without insulin, for 15 min. As a positive control, insulin stimulation alone significantly increased pAKT protein expression compared to vehicle. Clozapine (100 µM) treatment significantly decreased insulin-stimulated pAKT by 2.7-fold (clozapine + insulin vs. insulin), however, clozapine had no effect on pAKT protein expression without insulin stimulation (Fig. 1B). Insulin stimulated pAKT was no longer significantly increased with aripiprazole (5 and 20 uM) co-treatment (aripiprazole + insulin vs. aripiprazole) (Fig. 1C). Olanzapine (100 – 0.25 uM)) had no effect on pAKT with or without insulin stimulation in the rHypoE-19 neurons (Fig. 1A).

Table 1
Primer sequences.

Gene	Primer Sequence
Histone 3A	F: CGC TTC CAG AGT GCA GCT AAT R: ATC TTC AAA AAG GCC AAC CAG AT
IL6	F: GTG GCT AAG GAC CAA GAC CA R: GGT TTG CCG ACT AGA CCT CA
IL10	F: AGC ACT GCT ATG TTG CCT GCT CTT R: TGA CTG GGA AGT GGG TGC AGT TAT
BDNF	F: GGT CAC AGC GGC AGA TAA A R: GCA GCC TTC CTT CGT GTA A

Table 2
Summary of the effects of olanzapine (OLA), clozapine (CLO), and aripiprazole (ARI) on the protein and mRNA levels in rHypoE-19 neurons. Effects are indicated for antipsychotic versus DMSO, except for pAKT. For pAKT, analysis is run comparing the AP to the DMSO, as well as comparing the AP + insulin (INS) to INS alone. The concentrations in uM are listed in brackets.

	Protein/Gene of Interest		OLA	CLO	ARI
PROTEIN	pAKT	AP vs. DMSO	–	–	–
		AP + INS vs. INS	–	↓ (100)	–
		AP vs AP + INS	–	–	↓ (5, 20)
	pGSK3β		–	–	–
	pAMPK			↑ (20)	↑ (20)
	pERK1/2		↑ (100)	↑ (100)	–
	pJNK		↑ (100)	↑ (100, 20)	↑ (20)
	p-p38		–	–	↑ (5)
mRNA	BDNF		↑ (100)	–	↑ (20)
	IL-6		↑ (100)	–	–
	IL-10		–	–	↓ (20)

3.1.2. AMPK

In the hypothalamus, AMPK acts as an energy sensor activated by increases in the AMP/ATP ratio when fuel is low, to activate anabolic processes and enhance orexigenic signals (Ronnett et al., 2009). Clozapine (20 µM) and aripiprazole (20 µM and 5 µM) significantly increased pAMPK protein expression by 1.4 and 3-fold, respectively, compared to the vehicle after 15 min of treatment (Fig. 1B/C). Olanzapine (100 – 0.25 uM) had no effect on pAMPK protein in the rHypoE-19 neurons (Fig. 1A).

3.2. Antipsychotics induce changes in MAPKs and inflammatory mediators

3.2.1. MAPKs

The mitogen activated protein kinases (MAPKs) extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun amino-terminal kinases (JNK), and p38 are involved in a diverse array of cell signalling responses, such as cell growth, proliferation, and apoptosis. They are also involved in inflammatory signalling cascades (Cargnello and Roux, 2011). After 15 min, olanzapine (100 µM) treatment significantly increased pERK1/2 protein expression by 2.8-fold and pJNK protein expression by 4.3-fold compared to vehicle, but left p-p38 unchanged (Fig. 2A). Clozapine (100 µM) also increased pERK1/2 protein expression by 3.8-fold and pJNK protein expression by 1.7-fold without changing p-p38 levels (Fig. 2B). Aripiprazole increased pJNK (20 uM) and p-p38 (5 uM) by 1.5-fold, without changing protein levels of pERK1/2 (Fig. 2C).

3.2.2. IL6, IL10, BDNF

The genes examined were selected based on reports separately linking changes in interleukin (IL)-6 (Borovcanin et al., 2017), *IL10* (Xiu et al., 2014), and brain derived neurotrophic factor (BDNF) (Pandya et al., 2013) with the illness of schizophrenia, AP action, and regulation of metabolic homeostasis via the central nervous system. After 24 h, olanzapine (100 µM) treatment increased *BDNF* and *IL6* mRNA expression in the rHypoE-19 neurons by 1.6 and 1.5-fold, respectively (Fig. 3A). After 4 h, aripiprazole (20 uM) also increased *BDNF* mRNA expression by 5.5-fold, while also causing a 6-fold decrease in *IL10* mRNA expression (Fig. 3C). Clozapine had no effect on *IL6, IL10,* or *BDNF* mRNA expression in the rHypoE-19 neurons after 4 or 24 h of exposure (Fig. 3B).

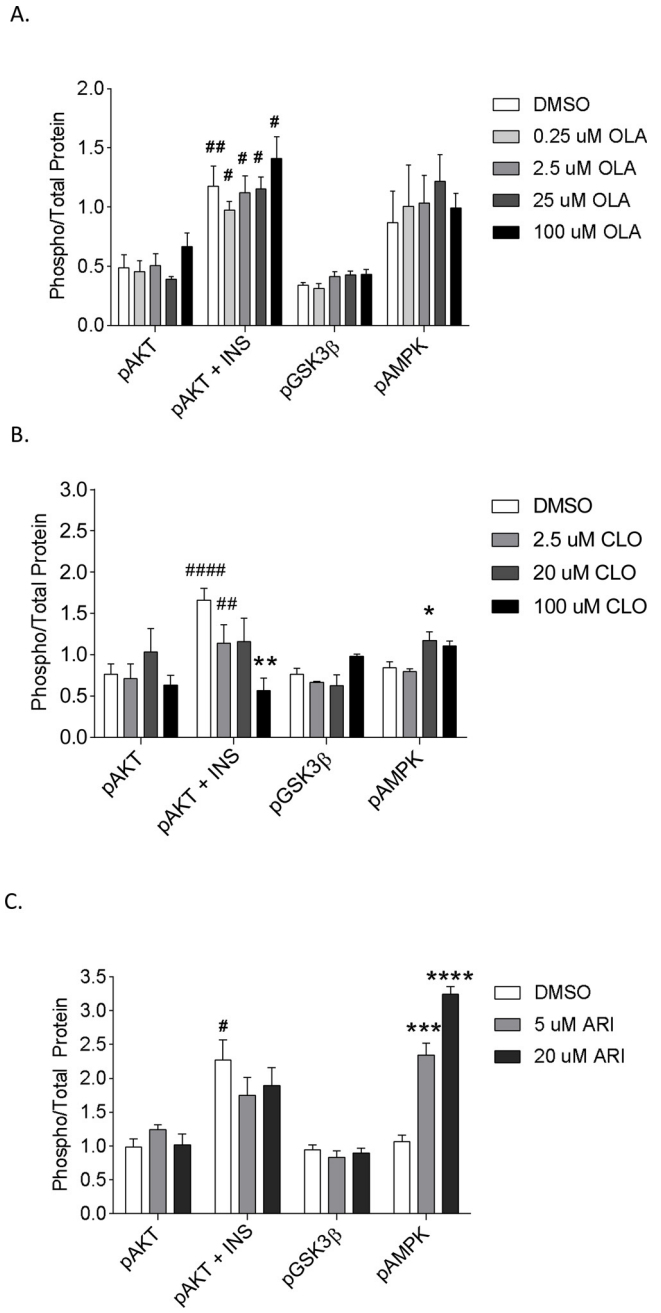

Fig. 1. Effects of antipsychotics on insulin signalling pathways: rHypoE-19 neurons were treated with OLA (A), CLO (B), or ARI (C) with and without INS for 15 min, followed by protein isolation and Western blot analysis of proteins related to insulin signalling/energy sensing (AKT, GSK3β, AMPK). Phospho-proteins were normalized to total proteins as a loading control and expressed as mean ± SEM. *refers to effect of AP vs. DMSO: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ****$p < 0.0001$; # refers to effect of INS stimulation relative to no stimulation (across treatment groups): #$p < 0.05$; ## $p < 0.01$; ####$p < 0.0001$; n = 3-8. Legend: OLA: olanzapine; CLO: clozapine; ARI: aripiprazole; INS: insulin.

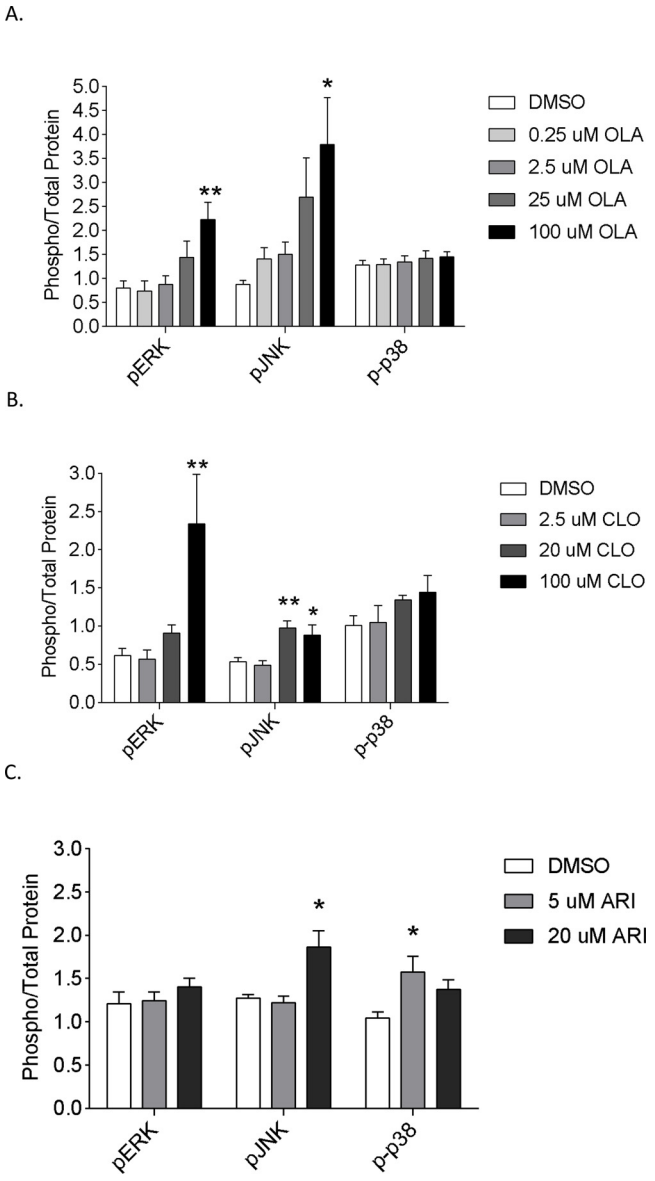

Fig. 2. Effects of antipsychotics on the MAPK pathway. rHypoE-19 neurons were treated with OLA (A), CLO (B), or ARI (C) with and without INS for 15 min followed by protein isolation and Western blot analysis of the MAPK proteins (ERK1/2, JNK, p38). Phospho-proteins were normalized to total proteins as a loading control and expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$; n = 4-8. Legend: OLA: olanzapine; CLO: clozapine; ARI: aripiprazole.

4. Discussion

This study provides the first evidence that antipsychotics (AP)s can directly impact insulin signalling, AMPK, MAPKs, and inflammatory mediators in hypothalamic neurons. Given that the hypothalamus is a key regulator of whole body energy homeostasis, direct action of APs on the hypothalamus could account in part for metabolic dysregulation, such as the insulin resistance and obesity, observed in patients taking APs. AP-induced hypothalamic dysregulation is also a concern as it has been proposed that an intrinsic metabolic dysregulation is observed in the illness of schizophrenia prior to AP treatment (Freyberg et al., 2017). In keeping with this observation, impaired AKT and GSK3β signalling is postulated to be involved in schizophrenia (Emamian, 2012). Previous in vitro studies found that olanzapine and clozapine increased pAKT in neuronal-like cells (Lu et al., 2004; Lu and Dwyer, 2005; Roh et al., 2007), thus suggesting a mechanism by which APs may modulate schizophrenia pathology. However, these studies did not use hypothalamic neurons, and did not measure insulin-stimulated pAKT (leaving the possibility that APs may impair central insulin response). In fact, in our study we found that APs may specifically impair insulin-mediated action in the hypothalamus, where clozapine and aripiprazole inhibited insulin-stimulated phosphorylation of AKT. Without insulin stimulation, there was no effect of any APs on pAKT protein levels, which is in agreement with our in vivo work in rodents

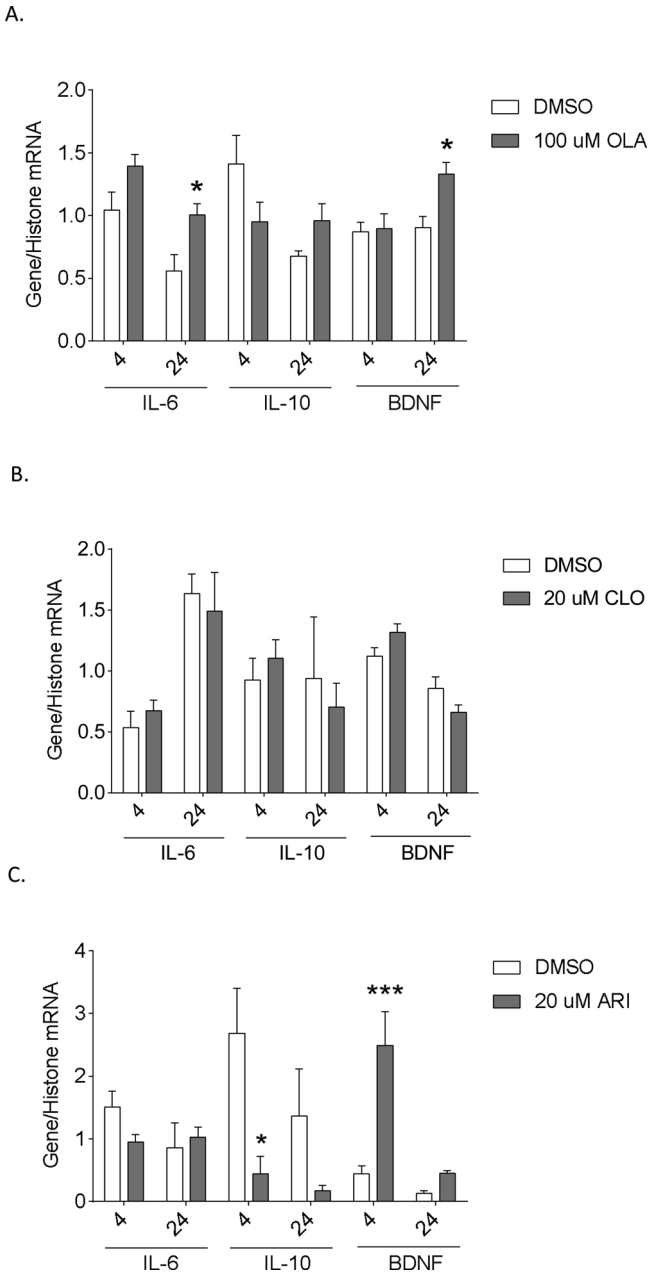

Fig. 3. Effects of antipsychotics on inflammation and BDNF. rHypoE-19 neurons were treated with OLA (A), CLO (B), or ARI (C) with and without INS for 15 min for 4 or 24 h, followed by qRT-PCR to measure mRNA expression of *IL-6, IL-10,* and *BDNF*. Relative mRNA expression is normalized to histone 3 A and expressed as mean ± SEM.*$p < 0.05$, *** $p < 0.001$; n = 4. Legend: BDNF; brain derived neurotrophic factor; OLA: olanzapine; CLO: clozapine; ARI: aripiprazole.

demonstrating that CNS-mediated impairments in glucose homeostasis by APs may require the presence of central insulin stimulation (Kowalchuk et al., 2017). In addition, despite indications in the literature that APs upregulate pGSK3β, no AP increased pGSK3β in this study. The difference could be because previous studies on GSK3β examined brain such as the cortex, suggesting a potential differential effect by brain region.

Though not a part of the canonical insulin signalling pathway, hypothalamic AMPK is influenced by insulin (as well as other energy signals) to sense energy levels (Ronnett et al., 2009). Thus, there is also the possibility that APs impair alternate energy sensing signals to insulin such as AMPK. An emerging body of research suggests that AP-induced activation of central AMPK may be responsible for glucose dysregulation and increased food intake (Ikegami et al., 2013; Martins et al., 2010). Olanzapine and clozapine have been found to increase pAMPK in the rodent hypothalamus, and studies have linked this induction to AP antagonism of the Histamine-1 (H1) receptor (He et al., 2014; Kim et al., 2007; Lian et al., 2014). In accordance with this hypothesis, in our study clozapine increased pAMPK in the rHypoE-19 neurons. In addition, we reported increased pAMPK with aripiprazole, which was not hypothesized as aripiprazole does not have prominent H1 antagonism activity. However, our findings are in agreement with aripiprazole -induced increase in pAMPK in PC12 cells reported by Takami et al. (2010), which was determined to be H1 independent (Takami et al., 2010).

The MAPKs are also involved in insulin signalling, along with cellular processes such as cell survival, differentiation, apoptosis, and neuronal plasticity. ERK1/2 is primarily involved in cell differentiation and survival. At least one study has reported decreased protein levels of ERK1/2 in the prefrontal cortex of schizophrenia patients, suggesting dysregulation of this pathway in schizophrenia (Yuan et al., 2010). We found that olanzapine and clozapine increased pERK1/2. This is consistent with literature looking at other brain areas, where chronic clozapine and olanzapine administration have previously been shown to increase pERK1/2 in the pre-frontal cortex of rats (Browning et al., 2005; Lu et al., 2004). In addition, acute olanzapine or clozapine treatment of PC12 or Hela cells has been reported to increase pERK1/2 (Aringhieri et al., 2017; Lu and Dwyer, 2005). Interestingly, the D2 antagonists haloperidol and raclopride have also been found to increase ERK phosphorylation in the striatum, suggesting that D2 receptor blockade may contribute to AP-induced increases in ERK phosphorylation (Valjent et al., 2004). As ERK1/2 has been implicated in neuronal plasticity and connectivity, there exists the possibility that this action of APs on ERK1/2 could have beneficial effects on psychopathology.

JNK is another MAPK, however unlike ERK1/2 it is more involved in cellular processes related to stress, such as apoptosis, inflammation, and cytokine production (Kyriakis and Avruch, 2012). Interestingly, *MAP2K7,* the gene for the kinase which phosphorylates JNK, has recently been functionally associated with schizophrenia (Winchester et al., 2012) suggesting a link between JNK and schizophrenia. Phosphorylation of JNK protein was upregulated by olanzapine, clozapine, and aripiprazole, making AP induction of JNK the most consistent finding from this study. These findings suggest that APs may be causing stress or inflammation to result in increased JNK activation. Hypothalamic JNK is also implicated in peripheral glucose regulation (Belgardt et al., 2010), systemic insulin resistance (Tsaousidou et al., 2014), as well as direct cellular regulation of hypothalamic insulin receptor substrate proteins (Aguirre et al., 2000), thus AP-induced upregulation of JNK could contribute to metabolic adverse-effects. Finally, p-p38, another MAPK involved in inflammation and stress, remained unchanged by olanzapine and clozapine, which is similar to findings by Kyosseva et al. (2001) who found that p38 levels remained unchanged frontal cortex, brainstem, hippocampus, or cerebellum in the chronic PCP rat model of schizophrenia with AP treatment (Kyosseva et al., 2001).

The activation of the MAPKs suggests that APs may be influencing inflammation. This is also supported by our findings demonstrating that olanzapine increased the mRNA levels of pro-inflammatory cytokine *IL6* after 24 h of treatment, while aripiprazole decreased anti-inflammatory cytokine *IL10*. Taken together, these data suggest that olanzapine and aripiprazole can directly induce a pro-inflammatory response in hypothalamic neurons. This also supports idea the that hypothalamic inflammation may be involved in AP-induced dysregulation of central energy homeostasis, as both cytokines are involved in energy regulation (Nakata et al., 2017; Welc and Clanton, 2013). In contrast to previous studies suggesting that clozapine may have proinflammatory properties in the periphery (Kluge et al., 2009), in our

hypothalamic cell model clozapine did not impact any of the examined inflammatory markers. The discrepancy may be explained by a differential effect based on tissue or brain region. In addition, in clinical studies, illness and lifestyle factors (i.e. smoking, poor dietary habits, inactivity), and other co-morbidities (Tourjman et al., 2013) make it challenging to determine the direct role of antipsychotics on inflammation.

BDNF is of particular interest as decreases in this neurotrophic factor has consistently been linked to the underlying etiology of schizophrenia (Pandya et al., 2013). Olanzapine and aripiprazole both increased *BDNF*. This result is supported by the literature, as past studies in other cell types and in rodents found increases in *BDNF* with olanzapine (Bai et al., 2003; Parikh et al., 2004; Park et al., 2013). The increase in BDNF expression is also particularly interesting in relation to the induction of ERK1/2 seen with the APs, as BDNF is known to elicit neuronal survival pathways via ERK1/2 activation (Almeida et al., 2005).

It is important to discuss the drug concentrations used in this study. Unlike in vivo studies, where dopaminergic (D2) occupancy can be used to establish clinically relevant drug dosing, there is little guidance to determine clinically relevant in vitro drug dosing. That being said, the concentrations of olanzapine, clozapine, and aripiprazole used in this study are higher than concentrations observed clinically in plasma (Kapur et al., 2003). However, reports indicate that these antipsychotics can accumulate in brain tissue at concentrations 10- to 30-fold higher compared to plasma (Aravagiri et al., 1999; Zhang et al., 2007), and the doses used here are in alignment with, or lower than, concentrations used by other in vitro studies (Ardizzone et al., 2001; Engl et al., 2005; Lee et al., 2010; Takami et al., 2010). In addition, the timing is another potential limitation of the study, as other timepoints could potentially result in different effects. However, the selected timing was based off previously determined maximal induction of insulin-stimulated pAKT and pAMPK in immortalized hypothalamic cell line (Mayer and Belsham, 2010; McFadden et al., 2013).

Aside from the individual findings of APs on the proteins and cytokines of interest, an interesting finding from this study is the highly differential effects between APs. Though D2 antagonism is considered a shared feature across all APs, the drugs differ in their binding affinities for other neurotransmitter systems (serotonergic, adrenergic, histaminergic, muscarinic). Among the agents we studied, olanzapine and clozapine would be predicted to be more similar to each other based on clinical metabolic liability, as compared to aripiprazole which carries a lower metabolic propensity and acts as a partial D2 agonist. Unexpectedly, clozapine and aripiprazole were more similar to each other each (impairing insulin-stimulated pAKT and inducing pAMPK), as compared to olanzapine which did not impact these pathways. Conversely, with respect to inflammation, olanzapine and aripiprazole, but not clozapine, induced pro-inflammatory marker expression. While these findings did not necessarily align with our working hypothesis, they are however in keeping with emerging data that suggests that no single AP is completely devoid of metabolic risk (Correll et al., 2009; Rajkumar et al., 2017), including findings that aripiprazole in healthy volunteers induces insulin resistance after only nine days of administration (Teff et al., 2013).

5. Conclusions

This study is a first step towards disentangling the proposed association between AP-induced metabolic effects, therapeutic response, and schizophrenia (which may be associated with underlying disturbances in insulin signalling or inflammatory pathways). We have found that the APs olanzapine, clozapine, and aripiprazole can directly regulate insulin signalling, AMPK, MAPKs, and inflammatory mediators in rat hypothalamic neurons. These direct effects of APs on the hypothalamus could contribute to the detrimental metabolic side-effects of these drugs. We have also found that these effects do not necessarily align with the known clinical metabolic risk for each APs (i.e. clozapine = olanzapine > aripiprazole). This finding however, adds credence to the idea that APs such as aripiprazole, which were initially developed in hope of avoiding metabolic adverse effects, are not free of metabolic risk. Differences between our findings and existing literature examining effects of APs on pathways of metabolism and inflammation also highlight the possible differential effects based on different brain regions and cell types studied, as well as other confounding factors related to illness and metabolic comorbidity which are difficult to avoid in clinical populations and in vivo rodent models. Finally, we found potentially beneficial effects of these medications on neurons, such as induction of BDNF and ERK1/2, which underscores the complexity of these effects and the possibility that the action of these drugs on metabolism may be both therapeutically beneficial and metabolically adverse.

Taken together, these findings support further research to examine centrally mediated mechanisms underlying metabolic adverse effects of APs, as well as investigations to determine how schizophrenia pathophysiology interplays with the AP-induced changes in insulin, energy sensing, and inflammatory pathways in specific brain regions. As prescription of APs continues to increase, this line of research has the ultimate goal to reduce adverse metabolic side-effects and enhance therapeutic effects.

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Conflicts of interest

MH received consultant fees from Alkermes. All other authors declare that they have no conflicts of interest.

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References

Aguirre, V., Uchida, T., Yenush, L., Davis, R., White, M.F., 2000. The c-jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of ser(307). J. Biol. Chem. 275, 9047–9054.

Almeida, R.D., Manadas, B.J., Melo, C.V., Gomes, J.R., Mendes, C.S., Grãos, M.M., et al., 2005. Neuroprotection by BDNF against glutamate-induced apoptotic cell death is mediated by ERK and PI3-kinase pathways. Cell Death Differ. 12, 1329–1343.

Aravagiri, M., Teper, Y., Marder, S.R., 1999. Pharmacokinetics and tissue distribution of olanzapine in rats. Biopharm. Drug Dispos. 20, 369–377.

Ardizzone, T.D., Bradley, R.J., Freeman, A.M., Dwyer, D.S., 2001. Inhibition of glucose transport in PC12 cells by the atypical antipsychotic drugs risperidone and clozapine, and structural analogs of clozapine. Brain Res. 923, 82–90.

Aringhieri, S., Kolachalam, S., Gerace, C., Carli, M., Verdesca, V., Brunacci, M.G., et al., 2017. Clozapine as the most efficacious antipsychotic for activating ERK 1/2 kinases: role of 5-HT2A receptor agonism. Eur. Neuropsychopharmacol. 27, 383–398.

Bai, O., Chlan-Fourney, J., Bowen, R., Keegan, D., Li, X.-M., 2003. Expression of brain-derived neurotrophic factor mRNA in rat hippocampus after treatment with antipsychotic drugs. J. Neurosci. Res. 71, 127–131.

Bak, M., Fransen, A., Janssen, J., Van, Os J., Drukker, M., 2014. Almost all antipsychotics result in weight gain: a meta-analysis. PLoS One 9.

Belgardt, B.F., Mauer, J., Wunderlich, F.T., Ernst, M.B., Pal, M., Spohn, G., et al., 2010. Hypothalamic and pituitary c-jun N-terminal kinase 1 signaling coordinately regulates glucose metabolism. PNAS 107, 6028–6033.

Belsham, D.D., Cai, F., Cui, H., Smukler, S.R., Salapatek, A.M.F., Shkreta, L., 2004. Generation of a phenotypic array of hypothalamic neuronal cell models to study

complex neuroendocrine disorders. Endocrinology 145, 393–400.
Borovcanin, M.M., Jovanovic, I., Radosavljevic, G., Pantic, J., Minic Janicijevic, S., Arsenijevic, N., et al., 2017. Interleukin-6 in schizophrenia-Is there a therapeutic relevance? Frontiers Media SA. Front. Psychiatry 8, 221.
Boyda, H.N., Tse, L., Procyshyn, R.M., Honer, W.G., Barr, A.M., 2010. Preclinical models of antipsychotic drug-induced metabolic side effects. Trends Pharmacol. Sci. 31, 484–497.
Browning, J.L., Patel, T., Brandt, P.C., Young, K.A., Holcomb, L.A., Hicks, P.B., 2005. Clozapine and the mitogen-activated protein kinase signal transduction pathway: implications for antipsychotic actions. Biol. Psychiatry 57, 617–623.
Campo, A., Del, Bustos, C., Mascayano, C., Acuña-Castillo, C., Troncoso, R., Rojo, L.E., 2018. Metabolic syndrome and antipsychotics: the role of mitochondrial fission/fusion imbalance. Front. Endocrinol. (Lausanne) 9, 144.
Cargnello, M., Roux, P.P., 2011. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. Microbiol. Mol. Biol. Rev. 75, 50–83.
Clodfelder-Miller, B., De, Sarno P., Zmijewska, A.A., Song, L., Jope, R.S., 2005. Physiological and pathological changes in glucose regulate brain Akt and glycogen synthase kinase-3. J. Biol. Chem. 280, 39723–39731.
Correll, C.U., Manu, P., Olshanskiy, V., Napolitano, B., Kane, J.M., Malhotra, A.K., 2009. Cardiometabolic risk of second-generation antipsychotic medications during first-time use in children and adolescents. JAMA 302, 1765–1773.
Dhillon, S.S., Gingerich, S., Virtanen, C., Belsham, D.D., 2012. Gene array analysis of embryonic- versus adult-derived hypothalamic NPY-expressing cell lines. Mol. Cell. Endocrinol. 358, 116–126.
Emamian, E.S., 2012. AKT/GSK3 signaling pathway and schizophrenia. Front. Mol. Neurosci. 5, 33.
Engl, J., Laimer, M., Niederwanger, A., Kranebitter, M., Starzinger, M., Pedrini, M., et al., 2005. Olanzapine impairs glycogen synthesis and insulin signaling in L6 skeletal muscle cells. Mol. Psychiatry 10, 1089–1096.
Freyberg, Z., Aslanoglou, D., Shah, R., Ballon, J.S., 2017. Intrinsic and antipsychotic drug-induced metabolic dysfunction in schizophrenia. Front. Neurosci. 11, 432.
Gingerich, S., Wang, X., Lee, P.K.P., Dhillon, S.S., Chalmers, J.A., Koletar, M.M., et al., 2009. The generation of an array of clonal, immortalized cell models from the rat hypothalamus: analysis of melatonin effects on kisspeptin and gonadotropin-inhibitory hormone neurons. Neuroscience 162, 1134–1140.
Hasan, A., Falkai, P., Wobrock, T., Lieberman, J., Glenthoj, B., Gattaz, W.F., et al., 2012. World Federation of societies of biological psychiatry (WFSBP) guidelines for biological treatment of schizophrenia, part 1: update 2012 on the acute treatment of schizophrenia and the management of treatment resistance. World J. Biol. Psychiatry 13, 318–378.
He, M., Zhang, Q., Deng, C., Wang, H., Lian, J., Huang, X.-F., 2014. Hypothalamic histamine H1 receptor-AMPK signaling time-dependently mediates olanzapine-induced hyperphagia and weight gain in female rats. Psychoneuroendocrinology 42, 153–164.
Hennekens, C.H., Hennekens, A.R., Hollar, D., Casey, D.E., 2005. Schizophrenia and increased risks of cardiovascular disease. Am. Heart J. 150, 1115–1121.
Ikegami, M., Ikeda, H., Ishikawa, Y., Ohsawa, M., Ohashi, T., Kai, M., et al., 2013. Olanzapine induces glucose intolerance through the activation of AMPK in the mouse hypothalamus. Eur. J. Pharmacol. 718, 376–382.
Kapur, S., VanderSpek, S.C., Brownlee, B.A., Nobrega, J.N., 2003. Antipsychotic dosing in preclinical models is often unrepresentative of the clinical condition: a suggested solution based on in vivo occupancy. J. Pharmacol. Exp. Ther. 305, 625–631.
Kim, S.F., Huang, A.S., Snowman, A.M., Teuscher, C., Snyder, S.H., 2007. Antipsychotic drug-induced weight gain mediated by histamine H1 receptor-linked activation of hypothalamic AMP-kinase. Proc Natl Acad Sci U S A 104, 3456–3459.
Kluge, M., Schuld, A., Schacht, A., Himmerich, H., Dalal, M.A., Wehmeier, P.M., et al., 2009. Effects of clozapine and olanzapine on cytokine systems are closely linked to weight gain and drug-induced fever. Psychoneuroendocrinology 34, 118–128.
Kowalchuk, C., Teo, C., Wilson, V., Chintoh, A., Lam, L., Agarwal, S.M., et al., 2017. In male rats, the ability of central insulin to suppress glucose production is impaired by olanzapine, whereas glucose uptake is left intact. J. Psychiatry Neurosci. 42, 424–431.
Kowalchuk, C., Castellani, L., Chintoh, A., Remington, G., Giacca, A., Hahn, M., 2018. Antipsychotics and glucose metabolism: how brain and body collide. Am. J. Physiol. Metab. https://doi.org/10.1152/ajpendo.00164.2018.
Kyosseva, S., Owens, S.M., Elbein, A.D., Karson, C.N., 2001. Differential and region-specific activation of mitogen-activated protein kinases following chronic administration of phencyclidine in rat brain. Neuropsychopharmacology 24, 267–277.
Kyriakis, J.M., Avruch, J., 2012. Mammalian MAPK Signal transduction pathways activated by stress and inflammation: a 10-year update. Physiol. Rev. 92.
Lee, J.G., Cho, H.Y., Park, S.W., Seo, M.K., Kim, Y.H., 2010. Effects of olanzapine on brain-derived neurotrophic factor gene promoter activity in SH-SY5Y neuroblastoma cells. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 34, 1001–1006.
Lian, J., Huang, X.-F., Pai, N., Deng, C., 2014. Betahistine ameliorates olanzapine-induced weight gain through modulation of histaminergic, NPY and AMPK pathways. Psychoneuroendocrinology 48, 77–86.
Lu, X.-H., Dwyer, D.S., 2005. Second-generation antipsychotic drugs, olanzapine, quetiapine, and clozapine enhance neurite outgrowth in PC12 cells via PI3K/AKT, ERK, and pertussis toxin-sensitive pathways. J. Mol. Neurosci. 27, 43–64.
Lu, X.-H., Bradley, R.J., Dwyer, D.S., 2004. Olanzapine produces trophic effects in vitro and stimulates phosphorylation of Akt/PKB, ERK1/2, and the mitogen-activated protein kinase p38. Brain Res. 1011, 58–68.
Martins, P.J.F., Haas, M., Obici, S., 2010. Central nervous system delivery of the antipsychotic olanzapine induces hepatic insulin resistance. Diabetes 59, 2418–2425.
Mayer, C.M., Belsham, D.D., 2009. Insulin directly regulates NPY and AgRP gene expression via the MAPK MEK/ERK signal transduction pathway in mHypoE-46 hypothalamic neurons. Mol. Cell. Endocrinol. 307, 99–108.
Mayer, C.M., Belsham, D.D., 2010. Central insulin signaling Is attenuated by long-term insulin exposure via insulin receptor substrate-1 serine phosphorylation, proteasomal degradation, and lysosomal insulin receptor degradation. Endocrinology 151, 75–84.
McFadden, S.A., Menchella, J.A., Chalmers, J.A., Centeno, M.-L., Belsham, D.D., 2013. Glucose responsiveness in a novel adult-derived GnRH cell line, mHypoA-GnRH/GFP: involvement of AMP-activated protein kinase. Mol. Cell. Endocrinol. 377, 65–74.
Nakata, M., Yamamoto, S., Okada, T., Yada, T., 2017. AAV-mediated IL-10 gene transfer counteracts inflammation in the hypothalamic arcuate nucleus and obesity induced by high-fat diet. Neuropeptides 62, 87–92.
Nazarians-Armavil, A., Menchella, J.A., Belsham, D.D., 2013. Cellular Insulin Resistance Disrupts Leptin-Mediated Control of Neuronal Signaling and Transcription. http://dx.doi.org.myaccess.library.utoronto.ca/101210/me2012-1338.
Newcomer, J.W., 2005. Second-generation (atypical) antipsychotics and metabolic effects: a comprehensive literature review. CNS Drugs 19, 1–93.
Obuchowicz, E., Marcinowska, A., Drzyzga, Ł, Wójcikowski, J., Daniel, W.A., Herman, Z.S., 2006. Effect of chronic treatment with perazine on lipopolysaccharide-induced interleukin-1β levels in the rat brain. Naunyn. Schmiedebergs Arch. Pharmacol. 373, 79–84.
Pandya, C.D., Kutiyanawalla, A., Pillai, A., 2013. BDNF-TrkB signaling and neuroprotection in schizophrenia. Asian J. Psychiatry 6, 22–28.
Parikh, V., Khan, M.M., Mahadik, S.P., 2004. Olanzapine counteracts reduction of brain-derived neurotrophic factor and TrkB receptors in rat hippocampus produced by haloperidol. Neurosci. Lett. 356, 135–139.
Park, S.W., Lee, C.H., Cho, H.Y., Seo, M.K., Lee, J.G., Lee, B.J., et al., 2013. Effects of antipsychotic drugs on the expression of synaptic proteins and dendritic outgrowth in hippocampal neuronal cultures. Synapse 67, 224–234.
Rajkumar, A.P., Horsdal, H.T., Wimberley, T., Cohen, D., Mors, O., Børglum, A.D., et al., 2017. Endogenous and antipsychotic-related risks for diabetes mellitus in young people with schizophrenia: a Danish population-based cohort study. Am. J. Psychiatry 174, 686–694.
Roh, M.-S., Seo, M.S., Kim, Y., Kim, S.H., Jeon, W.J., Ahn, Y.M., et al., 2007. Haloperidol and clozapine differentially regulate signals upstream of glycogen synthase kinase 3 in the rat frontal cortex. Exp. Mol. Med. 39, 353–360.
Ronnett, G.V., Ramamurthy, S., Kleman, A.M., Landree, L.E., Aja, S., 2009. AMPK in the brain: its roles in energy balance and neuroprotection. J. Neurochem. 109 (Suppl), 17–23.
Smith, G.C., McEwen, H., Steinberg, J.D., Shepherd, P.R., 2014. The activation of the Akt/PKB signalling pathway in the brains of clozapine-exposed rats is linked to hyperinsulinemia and not a direct drug effect. Psychopharmacology (Berl.) 231, 4553–4560.
Takami, G., Ota, M., Nakashima, A., Kaneko, Y.S., Mori, K., Nagatsu, T., et al., 2010. Effects of atypical antipsychotics and haloperidol on PC12 cells: only aripiprazole phosphorylates AMP-activated protein kinase. J. Neural Transm. 117, 1139–1153.
Teff, K.L., Rickels, M.R., Grudziak, J., Fuller, C., Nguyen, H.-L., Rickels, K., 2013. Antipsychotic-induced insulin resistance and postprandial hormonal dysregulation independent of weight gain or psychiatric disease. Diabetes 62, 3232–3240.
Tourjman, V., Kouassi, É, Koué, M.-È, Rocchetti, M., Fortin-Fournier, S., Fusar-Poli, P., et al., 2013. Antipsychotics' effects on blood levels of cytokines in schizophrenia: a meta-analysis. Schizophr. Res. 151, 43–47.
Tsaousidou, E., Paeger, L., Belgardt, B.F., Pal, M., Wunderlich, C.M., Brönneke, H., et al., 2014. Distinct roles for JNK and IKK activation in agouti-related peptide neurons in the development of obesity and insulin resistance. Cell Rep. 9, 1495–1506.
Valjent, E., Pages, C., Herve, D., Girault, J.-A., Caboche, J., 2004. Addictive and non-addictive drugs induce distinct and specific patterns of ERK activation in mouse brain. Eur. J. Neurosci. 19, 1826–1836.
Welc, S.S., Clanton, T.L., 2013. The regulation of interleukin-6 implicates skeletal muscle as an integrative stress sensor and endocrine organ. Exp. Physiol. 98, 359–371.
Winchester, C.L., Ohzeki, H., Vouyiouklis, D.A., Thompson, R., Penninger, J.M., Yamagami, K., et al., 2012. Converging evidence that sequence variations in the novel candidate gene MAP2K7 (MKK7) are functionally associated with schizophrenia. Hum. Mol. Genet. 21, 4910–4921.
Xiu, M.H., Yang, G.G., Tan, Y.L., Chen, D.C., Tan, S.P., Wang, Z.R., et al., 2014. Decreased interleukin-10 serum levels in first-episode drug-naïve schizophrenia: relationship to psychopathology. Schizophr. Res. 156, 9–14.
Yuan, P., Zhou, R., Wang, Y., Li, X., Li, J., Chen, G., et al., 2010. Altered levels of extracellular signal-regulated kinase signaling proteins in postmortem frontal cortex of individuals with mood disorders and schizophrenia. J. Affect. Disord. 124, 164–169.
Zhang, G., Terry, A.V., Bartlett, M.G., Bartlett, M.G., 2007. Sensitive liquid chromatography/tandem mass spectrometry method for the simultaneous determination of olanzapine, risperidone, 9-hydroxyrisperidone, clozapine, haloperidol and ziprasidone in rat brain tissue. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 858, 276–281.
Zhang, Q., He, M., Deng, C., Wang, H., Huang, X.-F., 2014. Effects of olanzapine on the elevation of macrophage infiltration and pro-inflammatory cytokine expression in female rats. J. Psychopharmacol. 28, 1161–1169.