Adverse effects of antipsychotics on micro-vascular endothelial cells of the human blood brain barrier.

Ekramy Elmorsy*[2], Laila M. Elzalabany[2], Hany M. Elsheikha[†] , and Paul A. Smith*[1]

**School of Life Science, University of Nottingham Medical School, Queens Medical Centre, Nottinghamshire, NG7 2UH, UK*

[2]*Departments of Forensic Medicine and Clinical Toxicology, Faculty of Medicine, Mansoura University, Egypt*

[†] *School of Veterinary Medicine and Science, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire, LE12 5RD, UK*

[1] *To whom correspondence should be addressed at School of Life Science, University of Nottingham Medical School, Queens Medical Centre, Nottinghamshire, NG7 2UH, UK,*

Fax: (0115)823-0135. E-mail: paul.a.smith@nottingham.ac.uk

Key words: Antipsychotics; Blood brain barrier; apoptosis; Evans blue, endothelium; ROS

Abstract

Although the mechanisms of action of antipsychotics (APs) on neuronal function are well understood, very little is known about their effects on cells of the blood brain barrier (BBB); one function of which is to limit the access of these amphiphilic compounds to the central nervous system. To address this question we have investigated the cytological and functional effects of four APs: chlorpromazine (CLP), haloperidol (HAL), risperidone (RIS) and clozapine CLZ), at concentrations typical of high therapeutic dosage on a human brain microvascular endothelial cell (HBMEC) model of the BBB. At ~10μM all four APs impaired the ability of HBMECs to reduce MTT which was followed by decreased Trypan blue exclusion and increased Lactate dehydrogenase release. These effects were associated with oxidative stress which was partly reversed by incubation in 10mM glutathione. At their EC_{50} concentrations for MTT reduction, all four APs disrupted cellular ultrastructure and morphology. HAL, CPZ and CLZ increased Caspase -3, -8 and -9 activity, chromatin condensation and fragmentation, data indicative of apoptosis. These events were associated with decreased transcytosis of Evans blue and increased transendothelial potential difference and electrical resistance of this BBB model. These findings suggest that at high therapeutic concentrations, CPZ and CLZ are likely to incur cytoxic effects and apoptosis of BBB endothelia with an impairment of barrier functionality. Such events may underlie the

aetiology of neuroleptic associated cerebral oedema and neuroleptic malignant syndrome.

1. Introduction

Antipsychotics (APs) are widely prescribed to manage psychoses that occur with schizophrenia and bipolar disorder (Miyamoto *et al*., 2005). Initially thought to achieve their therapeutic outcome by blockade of central dopaminergic and serotonergic pathways, however, APs are now known to involve a wider range of receptor targets which may, at higher doses, impart advantageous as well as deleterious behavioural (Putten et al, 1991) and neurological sequelae (Wiklund *et al*., 2010). Indeed, it is well established that at high doses, members of the typical class of APs, such as the phenothiazine chlorpromazine (CPZ) can be cytotoxic for different types of cell in culture (Dwyer *et al*., 2003) as well as inhibiting the proliferation of both normal and cancer cells (Nordenberg at al 1999). Whereas, other typical antipsychotics, which have different chemical structures, such as haloperidol (HAL) a butyrophenone, still have a similar clinical outcome as CPZ but do not, however, always have the same neurotoxic effects that are associated with high dosages of phenothiazines (Munyon *et al*.,1987; Galili-Mosberg *et al*., 2000).

The mechanisms that underlie AP neurotoxicity remain unclear. They may be related to their direct actions as ligands on dopaminergic and serotonergic receptors (McGrath and Neifeld, 1985), or indeed other receptor classes such as sigma receptors (Vilner *et al*., 1995). Their neurotoxicity may result from disruptive actions on mitochondria and oxidative respiration (Dudani and Gupta, 1987) or occur via disruption of

cellular cholesterol metabolism (Dujovne and Zimmerman, 1969; Wiklund *et al*., 2010). However, any *in-vivo* appreciation of the neurological functional sequelae of AP usage, whether with normal or overdosage is confounded by the fact that the access of these drugs to the CNS and their associated pharmacokinetics, are subject to the transport properties of the blood brain barrier (BBB).

The BBB controls the transport of metabolites, drugs and chemicals between the blood and brain compartments, in doing so it acts to both protect, but also maintain, the interstitial environment of the CNS (Abbott 2013, Abbott et al 2010; Wilhem et al 2011). Anatomically, the structure of the BBB comprises of a monolayer of brain micro-vascular endothelial cells joined by tight and adherent junctions (Weiss *et al*., 2009; Abbott et al 2010). This physical arrangement ensures that the endothelial cells act to restrict the movement of microscopic particles (e.g. bacteria) and hydrophilic molecules into the cerebrospinal fluid (CSF) (Bernas *et al*., 2010; Abbott 2013, Abbott et al 2010). Once this barrier is breached, the CNS is exposed to potential insult by hazardous solutes and pathogens circulating in the blood (Abbott et al 2010). In fact the BBB is functionally predisposed to prevent the entry of amphiphilic compounds such as APs into the CNS, which raises the question as to the mechanism by which these drugs actually gain access to the CNS and affect their therapeutic action (Abbott 2013, Abbott et al 2010; Wilhem et al 2011). This is a particularly pertinent problem since endothelial cells of the BBB possess

members of the ABC transporter family such as P-glycoprotein (Cordon-Cardo et al., 1989; Abbott et al 2010) whose predominant function is to actively pump amphiphilic compounds out of cells. One possible mechanism by which APs accumulate in the CNS is that these drugs inhibit, or are not substrates, for P-glycoprotein of the BBB endothelial cells. Alternatively the membrane permeability of the APs may be sufficiently high such that their passive influx exceeds the capacity of P-glycoprotein to extrude them the . Another further mechanism by which APs may gain access to the CNS is that the drugs themselves are cytotoxic to the cells of the BBB with the resultant impairment in the barrier function allowing them access to the CNS.

It is unknown is to what extent that AP, especially at dosages at the higher end of their therapeutic window, affect the function of cells of the BBB, a phenomena that will have consequences for transport of these drugs across the BBB, their associated pharmacokinetics, as well as the overall effect of APs on CNS function and their clinical outcome. APs, at concentrations similar to those measured in the serum of highly dosed patients, are already established to induce gross permeability changes in the BBB; for example its permeability to $FeCl_3$, mannitol and inulin are all enhanced by acute administration of 5μM CPZ (Pardridge *et al*., 1973; Ben-Shachar *et al*., 1994), a phenomenon indicative of a BBB breach at a drug concentration similar to that occasionally measured in human serum (3μM, 1000ng/ml; Van Putten et al, 1991), and.

Since it is possible to isolate and culture the different cell types that constitute the BBB (Bernas *et al*., 2010) the primary aim of this study was to investigate the cellular and functional effects of two typical APs (CPZ and HAL) and two atypical APs: risperidone (RIS) and clozapine (CLZ), at the higher end of therapeutic levels, as well as those expected with over dosage, on a model system of the BBB. In particular, we wished to determine to what extent these drugs affect cell viability, programmed cell death, barrier permeability and to investigate putative mechanisms behind these processes.

2. **RESULTS**

We first investigated the concentration dependence for the effect of two typical (CPZ and HAL) and two atypical (RIS and CLZ) APs on the viability of human brain microvascular endothelial cells (HBMECs).

2.1 Antipsychotics impair HBME cell viability

Figure 1 show the relationships between drug concentrations and reduction of MTT and exclusion of trypan blue for HBMECs for 4 different time points (4hrs, 24hrs, 48hrs and 72hrs). At concentrations of AP at 10 μM or less, all the drugs significantly inhibited the ability of the cell population to reduce MTT relative to control ($p<0.001$, ANOVA). However, even at concentrations in excess of 0.3-1mM and with extended incubation times of 72hrs none of the drugs abolished MTT reduction, with ~ 20-30% of the cell population apparently resistant to the action of the APs. The inhibitory effects of the APs on the cells ability to reduce MTT were mirrored by a decreased ability of the cells to exclude trypan blue; data which indicates that a loss of plasma-membrane integrity of the cells is associated with their redox capability. For both the MTT and trypan blue exclusion assays, the concentration-effect relationships for HAL and RIS were best described by a monophasic relationship (equation 1, Fraction = 0). For CPZ and CLZ their concentration-effect relationships on MTT were best described by a biphasic relationship (equation 1) with the IC_{50} for the highest affinity components given in Figure 2. The fraction of the high affinity components were 0.29 (0.14 to 0.44, 95% CI) and 0.34 (0.27 to

0.41, 95% CI) for CPZ and CLZ respectively. A monophasic equation (as compared to a biphasic using the F-test) best described the relationship between the concentrations of these drugs and the exclusion of trypan blue. Over the 72hr incubation period neither the drugs nor vehicle control produced a significant change in total cell count: cells excluding trypan blue + cells containing trypan blue. This data inundates that the proliferation rate of the cells was slow enough as not to be affected by the time courses of the experimental paradigms employed.

For both RIS and HAL, a significant release of LDH (ANOVA) occurred at all concentrations tested, with a greater release at higher concentrations (data not shown). However, even after 24hrs incubation with either drug at 1mM, only around 60% of the cell population released LDH; data again indicative of a population of cells resistant to the cytotoxic effects of these drugs. The concentration-effect relationship between either RIS or HAL and the release of LDH was readily described by the monophasic form of equation 1 with the IC_{50} given in Figure 2. CPZ and CLZ produced similar effects on the release of LDH, with the data best described by the monophasic form of equation 1 with the IC_{50}s given in Figure 2.

Figure 2 clearly demonstrates that both CLZ and CPZ develop a greater effect and potency with the MTT assay than on trypan blue exclusion at prolonged exposure times; an observation that suggests that a decrease in cellular redox status precedes the loss of cell integrity.

To further clarify the temporal effects of the APs, the relationships between the percentage viability with 10μM of drug were plotted against the time of exposure (Fig. 3). For all four APs tested, both MTT reduction and TB were significantly and negatively correlated with incubation time for a fixed drug concentration ($r<-0.95$, $p<0.05$ for all cases; Pearson one-tailed). The greatest drug effect was always demonstrated with the MTT assay ($p<0.001$, for each drug tested ANOVA); data again suggestive that a decrease in cellular redox status precedes the loss of cell plasma membrane integrity.

2.2 Antipsychotics condense chromatin

Cell visualised with transmission electron microscopy (TEM) revealed that vehicle treated cells appeared normal (Fig. 4), whilst cells treated with antipsychotics had degraded organelles, vacuolated cytoplasm, nuclear breakdown, increased electron density and chromatin condensation; observations consistent with apoptosis. Qualitatively, these apoptotic effects appeared to be the most prominent in CPZ and CLZ treated cells, but least in those treated with RIS and HAL. The induction of apoptosis by the APs was supported by the observation of heterogeneous DAPI staining of the nuclei. After 24hrs incubation at their EC_{50}s for viability, the APs led to ~50% of the nuclei to become heterogeneously stained: 50±2%, 51±0.3%, 46±2% and 46±0.5% for CPZ, HAL, RIS and CLZ respectively.

2.3 Antipsychotics-induce DNA breakage

The TM and TD components of the Comet assay from cells treated with vehicle or APs are shown in Figure 5. Although there was no significant effect for any drug on the TM (ANOVA), the TD component was significantly increased by 7.6% with CPZ ($p<0.001$; ANOVA) and 3.2% with CLZ ($p<0.01$; ANOVA). Although we observed Gamma-Histone H2AX staining within DAPI labelled nuclei with all treatments the actual amount of staining, quantified by the number of immunofluorescent spots per cell, was almost identical between the APs and the vehicle control ($p=0.112$, Chi^2 test; data not shown).

2.4 Antipsychotics and the cell cycle

There was no discernible effect of the APs on the number of cells in the G0/G1 phase or the G2/G1 ratio of the cell cycle.

2.5 Antipsychotics activates Caspase-3, -8 and -9

Figure 6 shows, with the exception of RIS, the APs caused a significant increase in the activity of Caspases-3, -8 and -9 relative to the vehicle control (ANOVA).

2.6 Antipsychotics impair the transport properties of confluent monolayers.

When applied for 72hrs at their EC_{50} concentration for viability, both CLZ and CPZ greatly enhanced the permeability of Evans blue across HBMEC monolayers (Fig. 7). In fact, an enhanced flux of Evans blue was observed even after 2hr incubation with the drugs at these concentrations. Microscopic inspection of the endothelia monolayers revealed widespread

disruption of monolayer structure and continuity, data which suggests these drug concentrations are too aggressive for the BBB to maintain functionality. In response to this situation, the experiments were repeated, but with the monolayers incubated at half the EC_{50} for each respective AP. Under these conditions, the permeation of Evans blue after 72hr incubation with AP was significantly decreased by both CPZ and CLZ ($p<0.01$, $p<0.001$ respectively; Kruskal Wallis Dunns multiple comparison test to control) but not by RIS or HAL. Figure 7 shows that for both APs and vehicle, both the TEER and TEPD significantly increased in magnitude with incubation time ($p<0.05$ for TEER; $p<0.01$ for TEPD; Spearman Rank correlation for all cases). Moreover all four APs significantly increased both TEER ($p<0.01$ for all cases) and TEPD ($p<0.01$ for all cases with the exception of CPZ $p>0.05$; Figure 7) relative to the values measured for vehicle (2-way ANOVA, Dunnett's multiple comparison test in each instance).

2.7 Antipsychotics cause oxidative stress.

Figure 8 illustrates that, with the exception of RIS, the APs, along with antimycin A the positive control, at their lowest concentration tested (<10μM) all significantly ($p<0.001$, ANOVA) increased oxidative damage, indicative of ROS generation, relative to that produced with the vehicle control. At their EC_{50}s for cell viability, all four APs significantly increased ROS generation relative to vehicle control ($p<0.001$, ANOVA). To determine if the production of ROS and the resultant oxidative damage was responsible in part for the decrease in viability observed on exposure

of the HBMECs to the APs, cells were co-incubated with 10mM glutathione (GSH); a reductant known to protect against oxidative stress and its associated cellular damage. With the exception of CLZ, 10mM glutathione significantly ($p<0.05$ in all cases, Unpaired t-test), but only partially, reversed the decrease in MTT reduction produced by the APs at both the lowest concentration tested and their EC_{50} concentration for viability. However, in no case was 100% viability restored. 10mM glutathione also reversed the DNA damage produced by CLZ at its EC_{50} for viability as indicated by the Comet assay (Fig 5).

3. DISCUSSION

The primary aim of this study was to investigate the cellular and functional effects of four antipsychotics (APs): two typical, CPZ and HAL, and two atypical, RIS and CLZ, on a HBMEC model of the BBB, in particular at concentrations similar to those seen with therapeutic over dosage in man as well as those commonly used in animal models. At concentrations of ~10μM and greater, all four drugs were found to adversely affect HBMEC function, with CPZ and CLZ the most potent, HAL and RIS lesser so. All four drugs impaired the ability of HBMECs to reduce MTT, an effect paralleled by a loss of plasma membrane integrity with increased uptake of trypan blue and leak of lactate dehydrogenase. At their EC_{50} concentrations for these effects, all four APs disrupted cellular ultrastructure and morphology. However, the same concentrations of drugs did not affect DNA breakage (except CPZ) as indicated by the alkali

Comet assay, nor did they affect Gamma-Histone H2AX staining or the cell cycle. HAL, CPZ and CLZ, but not RIS, did increase the activity of Caspases -3, -8 and -9 as well as promoting chromatin condensation and fragmentation, data indicative of apoptosis. These cytotoxic effects were associated with changes in the transendothelial permeability and electrical properties of a BBB model. The cytotoxic effects of these drugs were in part mediated by oxidative stress since their action was partially blocked with glutathione, a reductant well established to protect against oxidative stress associated cytoxicity (Slater et al 1995).

Mechanisms of cytotoxicity

Phospholipidosis and bioenergetic impairment are two established mechanisms by which antipsychotics induce cytotoxicity. Amphiphilic compounds such as antipsychotics can promote phospholipidosis (Halliwell, 1997), as well as interact directly with cell membrane sterols (Dujovne and Zimmerman, 1969; Erik *et al*., 2010) either of which could induce the cytotoxic changes we observed.

It is also well-established that antipsychotics can impair cellular bioenergetics (Dwyer *et al*., 2005). These effects need not be secondary to that of phospholipidosis; since, CPZ, HAL, RIS and CLZ are known to affect mitochondrial function by an inhibition of Complex I of the electron transport chain, with IC_{50}'s of ~35μM for CPZ and HAL, ~65 μM for RIS and >200μM for CLZ in isolated mitochondrial preparations (Burkhardt *et al*., 1993; Modica-Napolitano *et al*., 2003). CPZ can also impair succinate-

cytochrome C reductase activity as well as acting as a mitochondrial uncoupler (Modica-Napolitano *et al*., 2003). Indeed the inhibition of Complex I within neurons of the cortex by neuroleptics has previously been proposed to partly explain the extrapyramidal symptoms seen with this class of drugs (Maurer & Möller, 1997).

A consequence of mitochondrial function disruption is the induction of apoptosis. The fact that in our study the APs led to nuclei heterogeneously stained with DAPI, chromatin condensation, as well as degraded organelles and vacuolated cytoplasm, supports an apoptotic rather than necrotic mechanism of AP cytotoxicity. An idea also supported by the ability of HAL, CPZ and CLZ, but not RIS, to activate the caspases -3, -8 and -9; enzymes whose activation is strongly associated with the apoptotic cascade (Ola *et al*., 2011). RIS may induce apoptosis via a non-caspase pathway.

The observation that the APs always had greater effect on MTT reduction than on TBE; suggests the cytotoxic effect is initially precipitated by impairment in the cellular redox status. This idea is consistent with a mitochondrial target of the APs and is supported by the following explanation of the conundrum as to why ~30-40% of any cell population may be explained by the fact that cells at certain stages of the cell cycle (Finkel and Hwang, 2009) are endowed with different metabolic profiles: during the G1 phase, energy production occurs predominantly via mitochondrial aerobic respiration, whereas during S and G2 phases it

occurs predominantly by aerobic glycolysis (Schieke *et al*., 2008). Our cell cycle analyses demonstrated that the populations of HBMECs we used were not fixed at any particular stage of cell cycle but had cells in GO/G1, S and G2. Since Complex I of the mitochondrial electron transport chain is suggested to a major target for the APs we used, this notion implies that cells in the S1 and G2 phases (~40% present study) whose metabolic flux is predominantly glycolytic would be resilient to the cytotoxic effects of these drugs, where as those in the G1\G0 phase of cell cycle (~60% present study) would be prone. Since these percentages of cell cycle phase are almost identical to the respective proportion of cells resilient (~40%) or adversely affected (~60%) by the antipsychotics, is strong evidence in support of the cell cycle phase and its associated bioenergetic state affecting the cytotoxicity of antipsychotics in this HBMEC of the BBB.

Comparative potency of the antipsychotics

After 72hr exposure, CPZ had the most potent affect of the four APs on MTT reduction, with an IC_{50} of <1μM (~1μM at 48hr) compared to 2μM for CLZ, 48μM for HAL and 56μM for RIS. This data implies that, for CPZ and CLZ at least, cytoxic effects of the BBB may occur with concentrations of these AP at the higher end of their clinical therapeutic window: 300 ng/ml (1μM) for CPZ and 700 ng/ml (2μM) for CLZ. The fact that the ability of both CLZ and CPZ to inhibit MTT reduction was biphasic in nature suggests that at least two sites of action exist for these two drugs. Interestingly the IC_{50}s for the low affinity sites of these APs at 50μM and 200μM for CPZ

and CLZ respectively are similar in magnitude to those estimated for HAL and RIS; a finding which suggests that all four of these APs may share a common, as yet undefined, low affinity target of action. When fitting the biphasic relationship between MTT and the concentration of CPZ or CLZ it was assumed that the fractional contribution of this component was constant and it was the affinity, IC_{50}, that changed with time. However, it is more likely that the IC_{50} for the high affinity component is actually constant and it is the fractional contribution of this component that increased with time; an idea consistent with either intracellular accumulation of the drug or\and the aggregation over time of its molecular action. The observation that neither the loss of LDH or exclusion of trypan blue did not have a high affinity component unlike MTT for either CPZ or CLZ strongly suggests that the temporal decrease in MTT observed with these two APs at low concentrations reflects a gradual impairment of redox potential with drug exposure as demonstrated in figure 3 for these two drugs. Whereas for HAL and RIS, who lack this high affinity component, a higher concentration of drug is needed to do induce cytotoxicity via the low affinity site which just happens to be just happens to be the associated with a more rapid onset of action. The fact that trypan blue exclusion, an indicator of cellular integrity, lags behind the decrease in MTT by between 12 to 24 hours (fig.3) suggests that this is the period of time it takes to complete the apoptotic pathway which culminates in cell death and trypan blue uptake. Indeed similar length delays between cytotoxic induction and completion of apoptosis has been

described in other cellular systems e.g. 18hr for MPP^+ induced apoptosis in neuroblastoma (Fall & Bennett 1999).

A similar ranked order of cytotoxic potency for these APs is reported for the phaemocytochroma cell line, PC12 after 48hr exposure; with IC_{50}s of 60 μM and 110μM for CPZ and CLZ respectively, and ~200μM for both RIS and HAL (Dwyer *et al*., 2003). In endothelial cells of the human umbilical vein (HUVECs) after 72hr exposure, CPZ was shown to be slightly less cytotoxic with an IC_{50} of 25μM, whereas HAL had no effect up to 400 μM (Wiklund *et al*., 2010). It thus appears that HBMECs are exquisitely sensitive to CLZ and CPZ compared to HAL and RIS. Interestingly, the IC_{50} values for the cytotoxic effects of CPZ is similar to the value reported for its direct inhibitory effect on the activity of Complex I in isolated rat brain mitochondria: IC_{50} of <5μM (Rosenfeld *et al*., 2011). Together, this evidence suggests that for CPZ to achieve a potency in HBMECs similar to that observed for its inhibition of Complex I in isolated mitochondria the drug must have easy access to the cytosol. Moreover, a predominantly mitochondrial effect of these drugs explains why a decrease in redox status, as monitored by the MTT assay, appears to be the primary event in the cytotoxic cascade described here.

The generation of ROS with the production of oxidative stress and damage is known to occur with agents that inhibit mitochondrial electron transport. This fact is exemplified by our observation with Antimycin A which blocks complex II of the mitochondrial electron transport chain; an action well

documented to lead to ROS production. Our findings support this notion and indeed corroborate previous literature that demonstrates increased oxidative stress by APs such as HAL and CPZ in the CNS *in-vivo* (Reinke et al 2004). Elevated ROS can act as a trigger for apoptosis (Slater et al 1995) as well as having direct cytotoxic effects on a variety of cellular proteins and processes. Our observation that exogenously supplied glutathione partly alleviates the decrease in MTT observed with the APs, as well as preventing DNA breakage as indicated by the Comet assay, is strong evidence for a role for oxidative stress in the mechanism of AP cytotoxicity. It is of note that only CPZ affected DNA breakage, an observation that presumably relates to the fact that it was the most potent drug studied.

One question is to how these drugs accumulate to high enough concentrations to be mitochondrial poisons since endothelial cells of the BBB possess members of the ABC transporter family such as P-glycoprotein (Cordon-Cardo et al., 1989): proteins which act to pump amphiphilic compounds, like APs out of the cell and in the case of endothelial cells, out across the BBB (Hembury et al., 2008; Hemmelmann et al., 2012). Our data suggests that the drugs we used are either not transported by P-glycoprotein or they inhibit its activity. Alternatively, these APs may possess passive membrane permeability so high such that their rate of influx is larger than, and overwhelms that, of the P-glycoprotein efflux pathway; a scenario already demonstrated for CPZ in

BBB models (Seelig, 2007). Although these APs at ~30μM can stimulate the ATPase activity of P-glycoprotein and are perhaps substrates for transportation (Boulton et al., 2002), the observation that at similar concentrations they actually inhibit the ability of this protein to extrude the amphiphilic molecule, RH123 from cells (Wang et al., 2006) strongly suggest APs are in fact inhibitors of this particular ABC transporter. Such an action will contribute to their relatively high potency of cytotoxicity due to the inability of the cell to extrude them and prevent their intracellular accumulation.

Another factor of consideration in the cellular dynamics of these drugs is that they all have ionisation constants (pKa's) > 7.4 (Lombardo *et al*., 2004) which means they will accumulate in the relatively acidic environment of the cytosol. Finally, all four drugs possess halogen moieties within their molecular structures, a structural feature that enhances their membrane permeability and potency (Gerebzoff et al 2004).

At their EC_{50}'s for cell viability we have shown that the antipsychotics are capable of severely disrupting cell integrity and function of endothelial cells of the BBB. Even at concentrations within the upper range of therapeutic doses, let alone overdose, a significant decrease in cell viability was noted; affects exacerbated for CLZ and CPZ after an extended period of incubation. The in-vivo consequences are numerous; at excessively high concentrations of APs subsequent damage to the BBB

would make it leaky, not just to blood solutes and components with ensuing extravasation (Pardridge *et al*., 1973; Ben-Schachar *et al*., 1994), but also to the APs themselves with the various pathological consequences this implies. Indeed, we have demonstrated that prolonged exposure at high drug concentrations induce cytoxic actions on the HMBECs of the BBB with a concomitant loss of its integrity and a subsequent inability to act as a physical barrier to the passage of the drugs into the brain. Whereas at the lower doses of APs we tested, more akin to those seen at the higher therapeutically-relevant serum concentrations in man: 0.2-1μM for CPZ,.15-2μM for CLZ, we saw an associated decrease in transepithelial transport and enhanced transepithelial electrical resistance Since the transcellular flux of Evans blue occurs via an energy-dependent transcytosic process (Abbott 2013), its decrease with the lower doses of CLZ and CPZ is consistent with the ability of these drugs to decrease the energy state of the cells, as indicated by the MTT assay.

Regardless of whether the APs produce a breakdown in the endothelium of the BBB, impair their bioenergetics or/and wreak oxidative stress damage, any of these phenomena may contribute to the associated neuropathologies observed with long term AP therapy such as neuroleptic associated cerebral oedema (Jellinger 1977) and neuroleptic malignant syndrome (Karagianis et al 1999). Since apoptosis was observed with even the lowest concentrations of drug employed at the shortest incubation time suggest that a bolus of AP in-vivo may be sufficient to

illicit catastrophic damage to the BBB. Whether over dosage with the APs used within this study, or elevated levels outside their therapeutic window affect the endothelial integrity of other microvasculature within the body with its concomittant sequelue are unknown.

4. **Experimental Procedure**

Chemical and reagents. 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) and DAPI were purchased from Promega (Corp., Madison, Wisconsin, USA). CLZ was purchased from Abcam (Cambridge, MA, USA). All other reagents, including CPZ, HAL and RIS were purchased from Sigma-Aldrich (Poole, UK) unless stated otherwise. All stock solutions of drugs were made in ethanol (vehicle).

4.1 Cells and tissue culture conditions

For this study we used primary human brain microvascular endothelial cells (HBMECs), a fundamental element of the BBB. Cells were used from passage 21-23 and were grown and maintained as previously described (Elsheikha *et al*., 2013). When confluent, our HBMECs demonstrated an endothelial morphology with features all typical of a functional endothelium constructed from this cell type (Wong *et al*., 2004; Colgan *et al*., 2008; Allen and Bayraktutan, 2009; Wilheml et al 2011): transendothelial electrical resistances (TEER) of ~100Ωcm^{-2}; transendothelial potential differences (TEPD) of ~1mV; and an impermeability of albumin conjugated Evans blue. The fact that a TEPD

was observed supports the idea that our monolayers were a homogenous tight barrier with the absence of any gaps to produce an electrical leakage, since these would dissipate the TEPD; even though the TEER appear to be relatively low to those measured in other cellular (200Ωcm^{-2}; Wilhelm et al 2010) and ex-vivo (>1000Ωcm^{-2}; Abbot et al 2010) models of the BBB. Furthermore, the observation the monolayers significantly decreased the passive flux of Evans blue across the transwell also argues for a continuum of tight junctions between cells that limits paracellular flux.

4.2 Cytotoxicity assays:

Fluorescent microscopy was conducted with a Leica DM5000 (Leica, B Wetzlar, Germany) with images collected and processed with a Leica DFC420 camera and Leica application suite v3.8 software. Since APs have long half-lives, especially in the cases of overdose where their metabolism is changed from 0th to 1st order kinetics (Ereshefsky, 1999), we performed experiments at four different time points to mimic this: 4hrs, 24hrs, 48hrs and 72hrs post treatment. These longer time periods of incubation were further warranted due to the time-dependence of AP cytotoxicity, often taking at least 48hrs to achieve a maximum effect (Erik *et al*., 2010). The lowest concentrations of CPZ and CLZ used in our experiments are near to the top end of therapeutic serum levels for CPZ and CLZ: 0.2-1μM (40-300 ng/ml) and 1-2μM (300-700 ng/ml) respectively, and are similar to those measured in overdosed individuals:

~3μM for CPZ (~1000ng/ml; Van Putten et al 1991) and ~5μM for CLZ (~1500ng/mL; Chang et al 1997). For HAL and RIS the lowest concentrations of drugs used were about 70-80 times therapeutic serum levels: 5-135nM (2-50ng/ml) for HAL and 50-160nM (20-65 ng/ml) for RIS, although the lowest values we used in the present study are similar to those measured in highly/over dosed individuals: ~ 0.7μM for HAL (~270 ng/ml; Chang et al 1994) and for RIS ~0.5-0.8μM (~220 to 320 ng/mL ng/ml; Titier et al 2003, Nishikage et al 2002). We used these higher concentrations to accelerate and evoke detectable effects of the APs within the time frame of cell division as not to be confounded by affects on cell proliferation. Furthermore, the concentrations used allowed us to mimic overdose scenarios that are clinically experienced.

4.2.1 MTT assay

The MTT assay relies on cellular reduction of the MTT substrate to produce a blue formazan product. As such it is a facile indicator of cell viability that results from the product of cell number and redox status. For this assay we followed the manufacturer protocol (CellTiter 96 Non-Radioactive Cell Proliferation Assay; Promega, UK). Briefly, cells were seeded at $1x10^4$ cells per well in 96-well plastic plates (Nunc) and incubated overnight at 37°C under humidified 5% CO_2 conditions. To assess chronic effects cells were then incubated for a 4, 24, 48 or 72hrs in the presence of drug or its vehicle. The original drug and vehicle measurements were corrected via subtraction of a blank (media with MTT reagents but without cells). The

MTT absorbance value is expressed as a percent of the vehicle control (defined as 100%). Each experiment was performed in triplicate, where n represents the number of experiments (triplicates) performed.

4.2.2 Lactate dehydrogenase assay

Plates for this assay were prepared as for MTT, but with lysis by 2% Triton X-100 the positive control. All measurements were corrected via subtraction of a blank (media with reagents but without cells). Leakage of LDH was calculated as a percentage of the Triton X-100 positive control. Since LDH release was unaffected by vehicle we did not correct for it. Due to degradation of LDH in the media over extended incubation times only 2 time points (4 and 24hrs) were assayed for its release. Each experiment was done in triplicate, where n represents the total number of experiments (triplicates) performed.

4.2.3 Trypan blue exclusion assay

To aid comparison with the MTT assay and determine the number of viable cells we harvested both attached and floating cells for this assay. As the vehicle did not affect the TBE assay we have not corrected for it. Results are expressed as % viability compared to control. Each experiment was done in triplicate per treatment, where n represents the number of experiments (triplicates) performed.

4.3 Genotoxicity and apoptotic assays

To investigate nuclear/DNA toxic effects of the APs a variety of techniques were used. For these protocols, drug treatment was achieved by incubation of the cells for 24hrs at the EC_{50} concentration obtained from the respective MTT assay for the APs tested: CPZ, 78μM; HAL, 330μM; RIS, 300μM and CLZ, 153μM (see Figs. 1 & 2). For vehicle control, the same volume of ethanol was added as used for drug.

4.3.1 Caspase Assay

To determine the pathway(s) by which APs, may induces apoptosis we evaluated the activities of caspases 3, 8 and 9 after treatment with the APs. These were determined using a BD ApoAlert caspase fluorescent assay kits (Clontech Laboratories, Palo Alto, CA).

4.3.2 Ultrastuctural damage

Nuclear changes indicative of apoptosis were assessed by staining with DAPI and measuring the proportion of cells with chromatin condensation and nuclear fragmentation. Nuclei were considered to have a healthy phenotype when their fluorescence was homogeneously bright, whereas apoptotic nuclei were identified by condensed chromatin at their periphery or a fragmented morphology. For analysis, at least 300 cells from 10 different fields of each treatment were counted and scored blind. Each treatment was repeated at least 3 times. The number of the homogenous (healthy) nuclei was quantified as a percentage of the total nuclei for each

experiment. Transmission Electron Microscopy (TEM) was also used to study the structural integrity of cell nuclei.

4.3.3 Comet assay

The alkaline Comet assay is a sensitive method that can detect both single and double stranded DNA breaks. Its "tail" parameters are the most frequently used to describes DNA damage, with the tail moment (TM) and tail DNA (TD) considered the most accurate markers of such damage (De Boeck *et al*., 2000). After treatment with APs, cells were harvested in ice-cold PBS and exposed to 30% ethanol. The infranatant was mixed with agarose and plated onto slides. After lysis, the samples underwent electrophoresis (30 minutes at 300 mA) and stained with propidium iodide (1mg/ml). Cells were imaged by fluorescent microscopy at 546nm excitation and 640nm emission. Images were analysed by Comet score IV software (Perspective Instruments, UK).

4.3.4 Gamma-Histone H2AX

Gamma-Histone H2AX (γH2AX) is another marker for double stranded DNA breaks (Lowndes and Toh, 2005). For γH2AX staining, cells were grown on glass coverslips for 24 hrs and then treated with APs. Inmunohistochemistry was performed with a Anti-H2AX mouse primary antibody (phospho-Ser139, #05-636; Millipore, Billerica, MA) and an AlexaFluor 594nM tagged fluorescent secondary antibody (Invitrogen, Eugene, OR). Samples were examined with fluorescent microscopy using

dual excitation and emission filter pairs of 360nm and 470nm for DAPI, and 546nm and 640nm for H2AX. A minimum of 100 nuclei per sample were examined and the number of γH2AX foci per nuclei counted.

4.3.5 Cell cycle analysis

Cells were cultured in T25 flasks for 24 hrs and then treated. Both floating and adherent cells were harvested and pelleted by centrifugation. DNA content stained by propidium iodide was analysed by FACScan with 495nm excitation and 600nm emission (Beckman Coulter, High Wycombe, UK). Data were analysed with Weasel software version 2.7.4 (Walter & Eliza Analysis Software). Doublets and cellular aggregates are a common problem of DNA analysis, where summation of the individual G1-phase DNA contents is often recorded as a single event which leads to overestimation of the G2/M population (Nunez, 2001). To overcome this problem we only selected the area that contained single cells. These experiments were repeated in triplicate.

4.4 Measurement of reactive oxygen species

We used the 3',7'-dichlorodihydrofluorescein diacetate (DCFDA) assay to detect products of oxidative stress that result from the presence of reactive oxygen species (ROS). Cells were cultured in 96-well plates and treated for 24hrs with APs at either at its EC_{50} for viability or the lowest concentration tested. Cells were then washed with PBS and incubated with 100μM DCFDA in Hanks for 45 minutes at 37°C. Ethanol was used for

vehicle control, Antimycin A (10μM for 30 minutes) as the positive control and wells with non-stained cells were used as blank. Cells were then washed with PBS and their emitted fluorescence at 535nm measured with 485nm excitation. The drug and vehicle measurements were corrected via subtraction of the blank. The oxidative products due to thepresence of ROS are expressed as a percent of the vehicle control (defined as 100%). Each experiment was performed in triplicate, where n represents the number of experiments (triplicates) performed.

4.5 Functional assays

To assess the functional outcome of the antipsychotics and also to confirm that our HBMEC cultures suitably modelled the transport properties of the BBB endothelium, we utilized three assays of endothelial function: transcellular transport of Evans blue, transendothelial electrical resistance (TEER) and transendothelial potential difference (TEPD). For these studies, cells were grown to confluence on semi permeable membrane 24-well inserts (area 0.6 cm^2; Millicell, Millipore Corporation). Cells were incubated with APs for 72hrs at either the full or half of the respective MTT EC_{50} concentration. For controls, wells with confluent cells were incubated with either vehicle alone or with just media with no additions. To assess the transcellular permeability, Evans blue (0.05 mg ml^{-1}) labelled albumin (4%) was added to the culture media in the well insert. The passage of labelled albumin across the monolayer was determined by the increase in absorbance at 620 nm of the infranatent after 3 hours.

To measure the TEER and TEPD of the endothelial monolayer, we used a WPI EVOM resistance meter (WPI, UK). This was prepared and calibrated in accord with the manufacturer's instructions. Controls with wells but without cells did not restrict the movement of Evans Blue nor did they possess a TEPD. However, blank wells do have a TEER which is in series with that of the monolayer, this was corrected for by subtraction.

4.6 Statistical analysis

The D'Agostino-Pearson omnibus normality test was always sused to check if data was Gaussian distributed. For multiple comparisons on Gaussian distributed data, one way ANOVA test was used with either Tukey's multiple comparisons or Dunnett's multiple comparisons post-tests, whereas for non-normal data Kruskall Wallis test with Dunnett's multiple comparisons post-test was used; for counts, Chi^2 was used. Either Pearson r or Spearman Rank was used to correlate variables depending on the data type and its statistical distribution.

Concentration-response relationships were constructed from single concentration experiments. The concentration-response relationships were quantified by fitting the data with the following biphasic equation:

$$Y = \frac{\text{Span x Fraction}}{(1 + ([D]/IC_{50\_1})^{h1})} + \frac{\text{Span x (1-Fraction)}}{(1 + ([D]/IC_{50\_2})^{h2})}$$

Where Y is the magnitude of drug effect normalised as a percentage of the control effect; [D] is the concentration of drug; IC_{50_i} is the drug concentration that produces half-maximal effect (50%) and h_i is an index of slope; for high affinity sites, i=1, and for low affinity sites, i=2. Span is the range of the drug effect (Maximum - minimum) and Fraction is the relative proportion of the concentration relationship described by the high affinity component. For the situation where only a monophasic, low affinity, relationship was observed, Fraction was set to zero. The IC_{50} values are quoted with 95% confidence intervals. When data was best described by a monophasic relationship, the EC_{50} of the effect is equal to the IC_{50}, however, when the data was best described by a biphasic relationship the EC_{50} of the effect was measured empirically.

These procedures were all performed using PRISM 6 (GraphPad Software Inc., San Diego, CA). Statistical significance is defined as $P < 0.05$, and is indicated in the figures as *** for $p<0.001$, ** for $p<0.01$ and * for $p<0.05$.

ACKNOWELDGEMENTS

Financial support to EE was provided by the Ministry of Higher Education of Egypt. PAS thanks the University of Nottingham for research facilities.

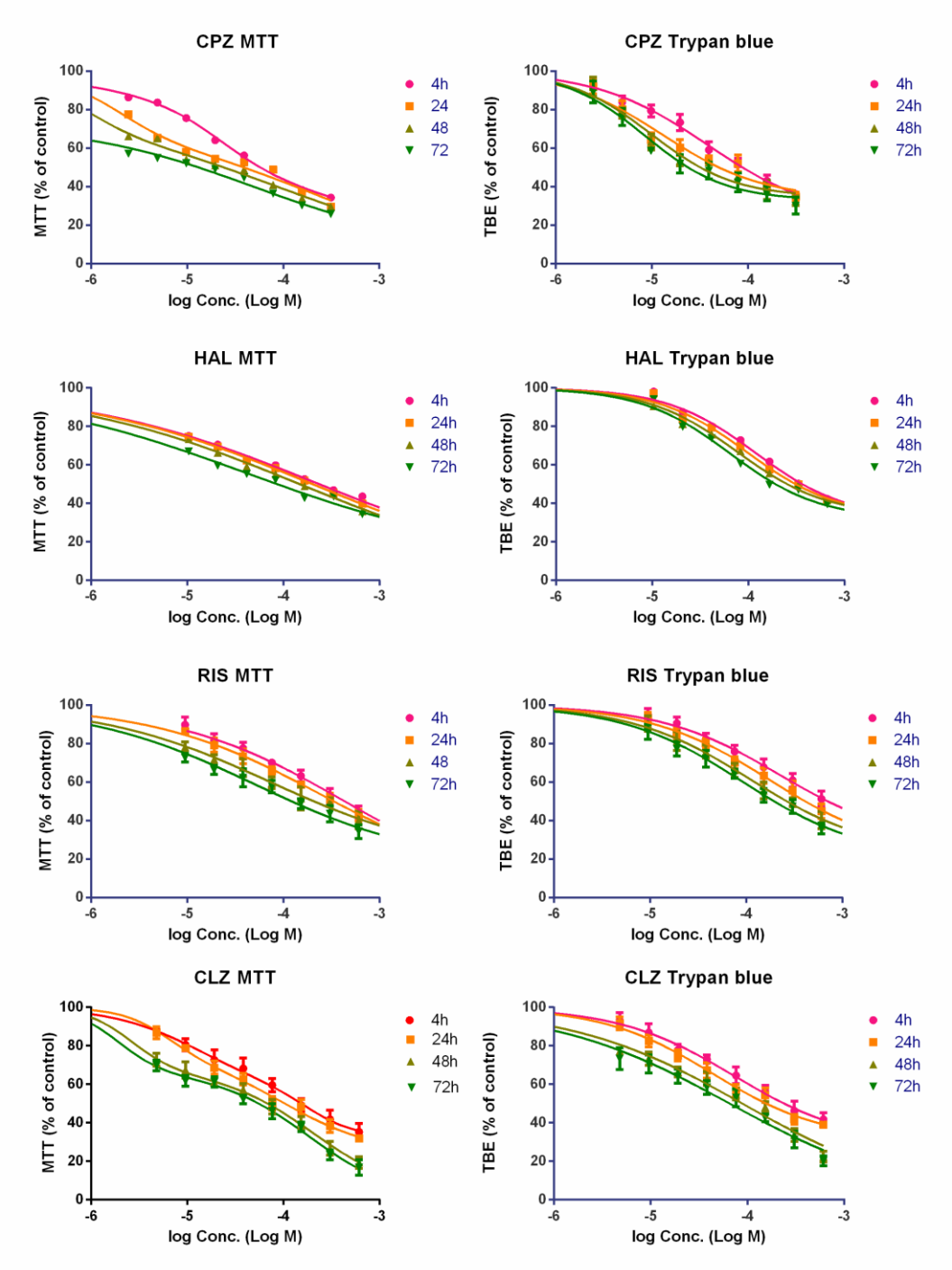


FIG. 1. Concentration-effect relationships for the effect of chlorpromazine (CPZ), haloperidol (HAL), risperidone (RIS) and clozapine (CLZ) on the reduction of MTT and exclusion of trypan blue (TBE). Data is measured at 4 different time points (●, 4 hr; ■, 24hr; ▲, 48hr and ▼, 72hr), and is corrected for vehicle and background. The lines are all best fits of equation 1 to the data with IC_{50}s given in figure 2. Data shown as means ± S.E.M (*n*=9) for each time point.

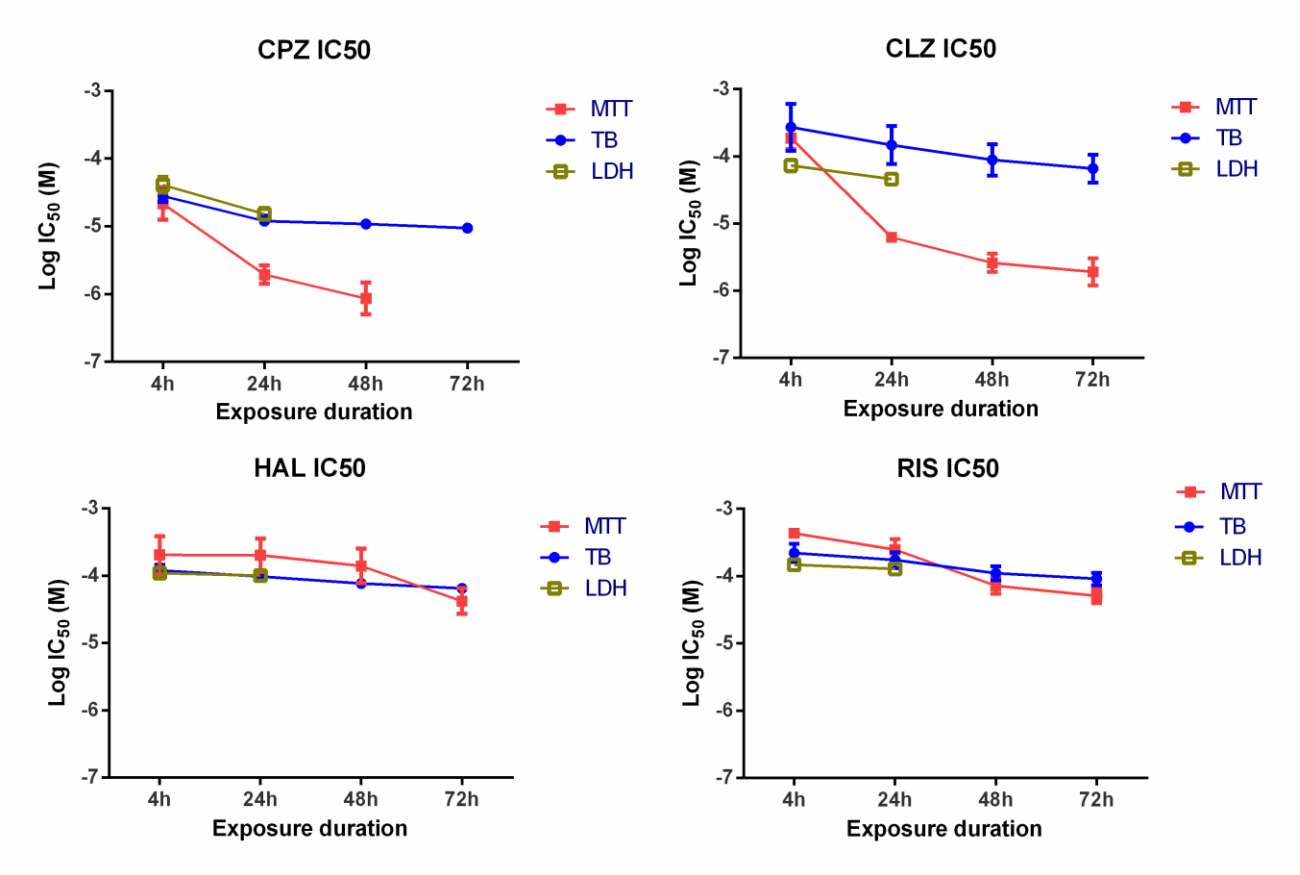


FIG. 2. The effect of incubation time (exposure duration) on the IC_{50} values determined for the reduction of MTT (■), uptake of trypan blue (●) and release of LDH (□), for the four different antipsychotics as indicated. Data is shown as the best fit value (symbol) with 5-95% confidence intervals (bars) of equation 1 to data like that shown in Figure 1. Note that CPZ and CLZ appear to be the most cytotoxic with the smallest values of IC_{50}, whereas RIS and HAL appear to be the least cytotoxic. The 72hr time point for CPZ is excluded due to its value being ambiguous: a $pIC_{50} < 8$.

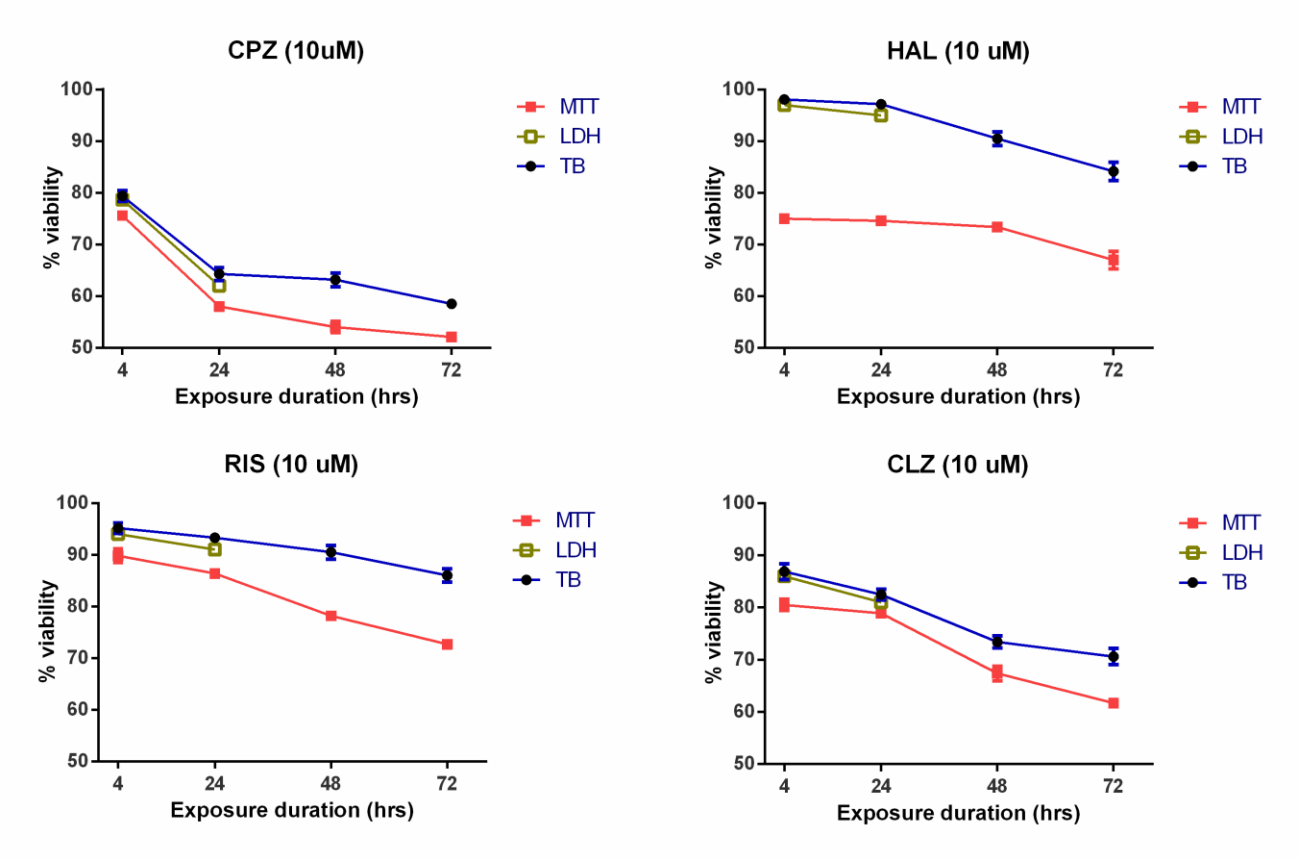


FIG. 3. Time courses of HBMEC viability measured with three different assays for the four APs all at 10μM. The least viability was demonstrated with the MTT assay (■), followed by LDH (□) and trypan blue exclusion (●). Data are shown as means (± S.E.M; *n*=9). Note inflated Y-scale.

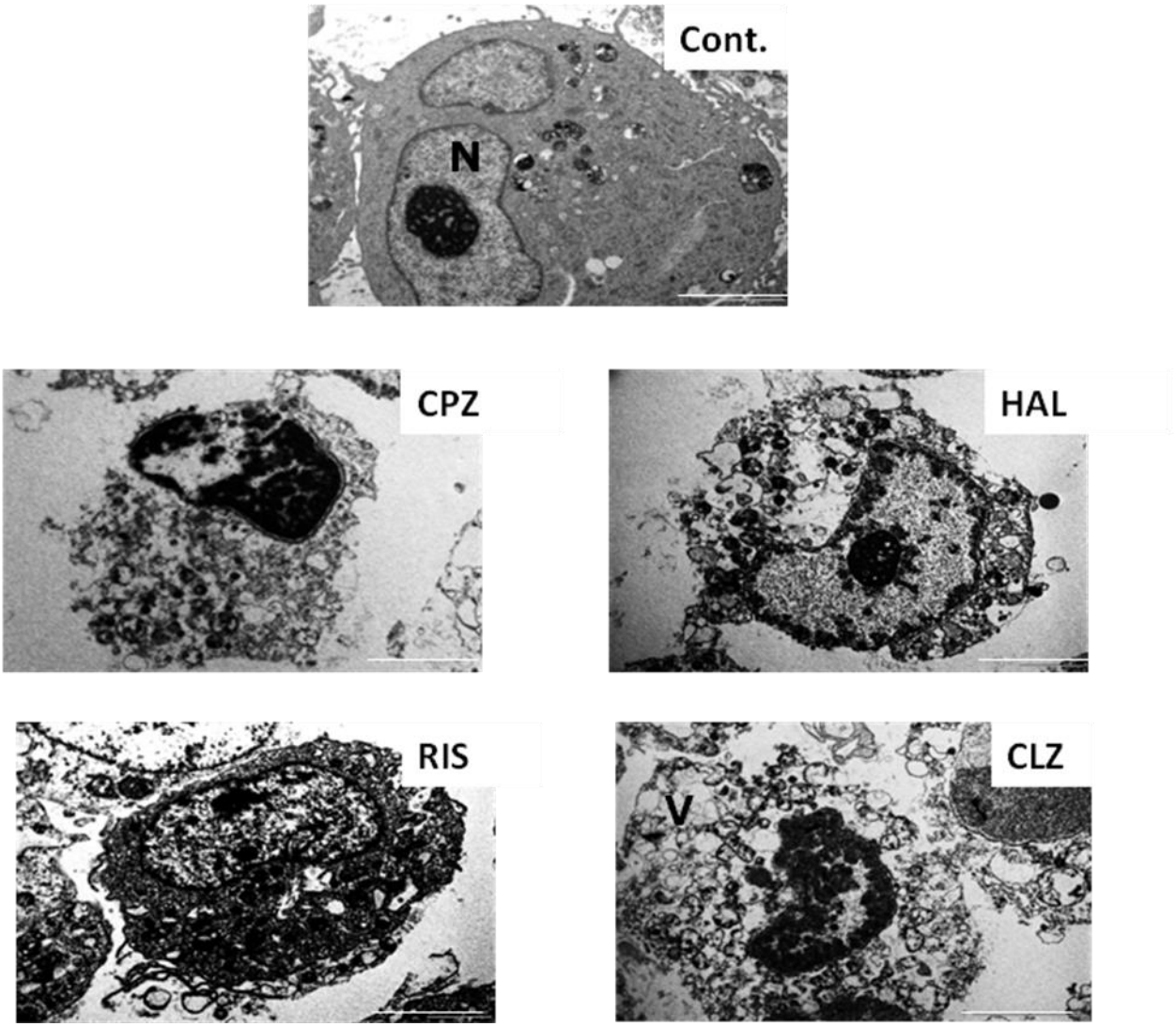
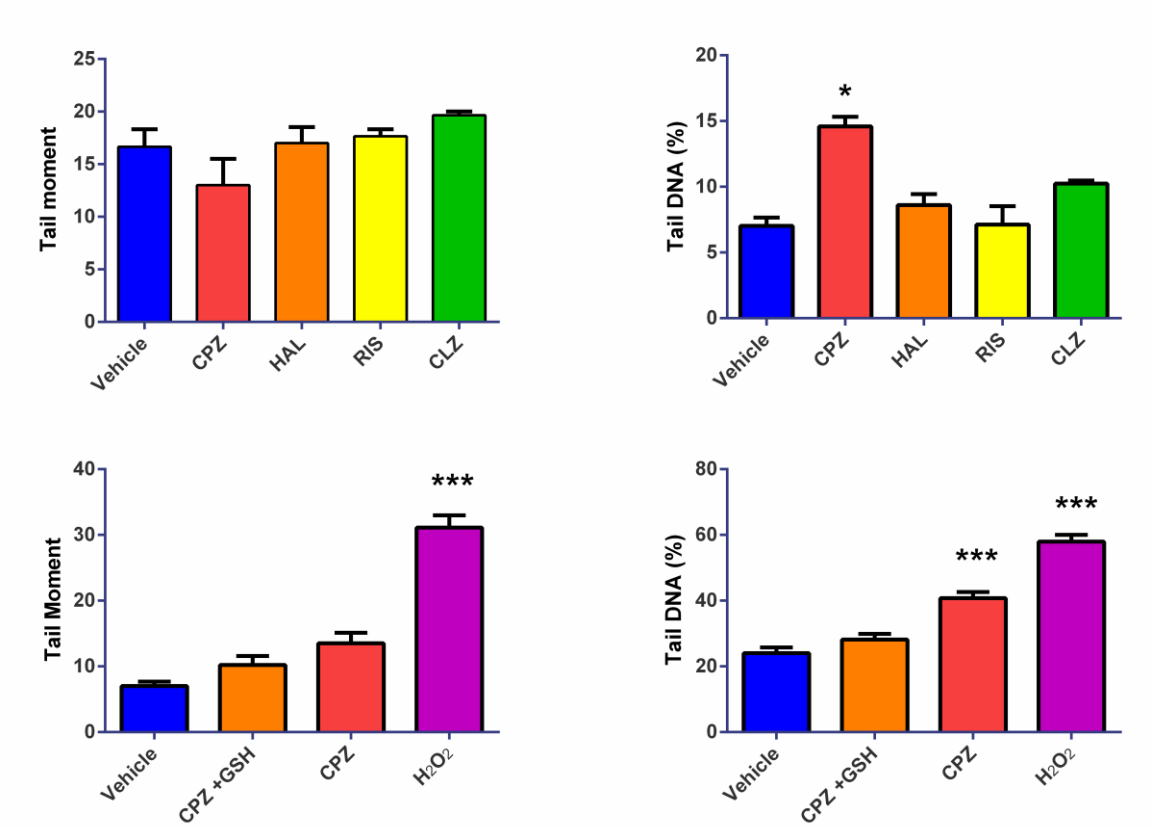


FIG. 4. Representative transmission electron microscopy images of HBMECs of BBB treated with antipsychotic at its EC_{50} concentration for cell viability or vehicle as indicated. Control cell (Cont.) treated with vehicle (2.5 µl ml^{-1} ethanol) appear to possess a normal nucleus and cytoplasm. Whereas cells treated with the drugs all demonstrate a degraded vacuolated cytoplasm (V in VLZ image), nuclear breakdown, increased electron density and chromatin condensation. Scale bar = 2 µm.



FI

G. 5. Tail parameters determined from the Comet test (alkaline method) for HBMECs of BBB after 24hr incubation with antipsychotics. Top panels, comparison of tail moment (left panel) and tail DNA (right panel) for the four antipsychotics indicated at their EC_{50} for viability. Note that only CPZ elicited DNA breakage. Lower panels, comparison of tail moment and tail DNA for CPZ at its EC_{50} for viability, in the presence and absence of 10mM glutathione (GSH). Note how 10mM glutathione protects against DNA breakage by CPZ. Also shown is the effect of the positive control for DNA brakeage, 25 μM H_20_2 for 1hr. Statistical significance is all relative to vehicle control. Data is shown as means (± S.E.M; *n*=3).

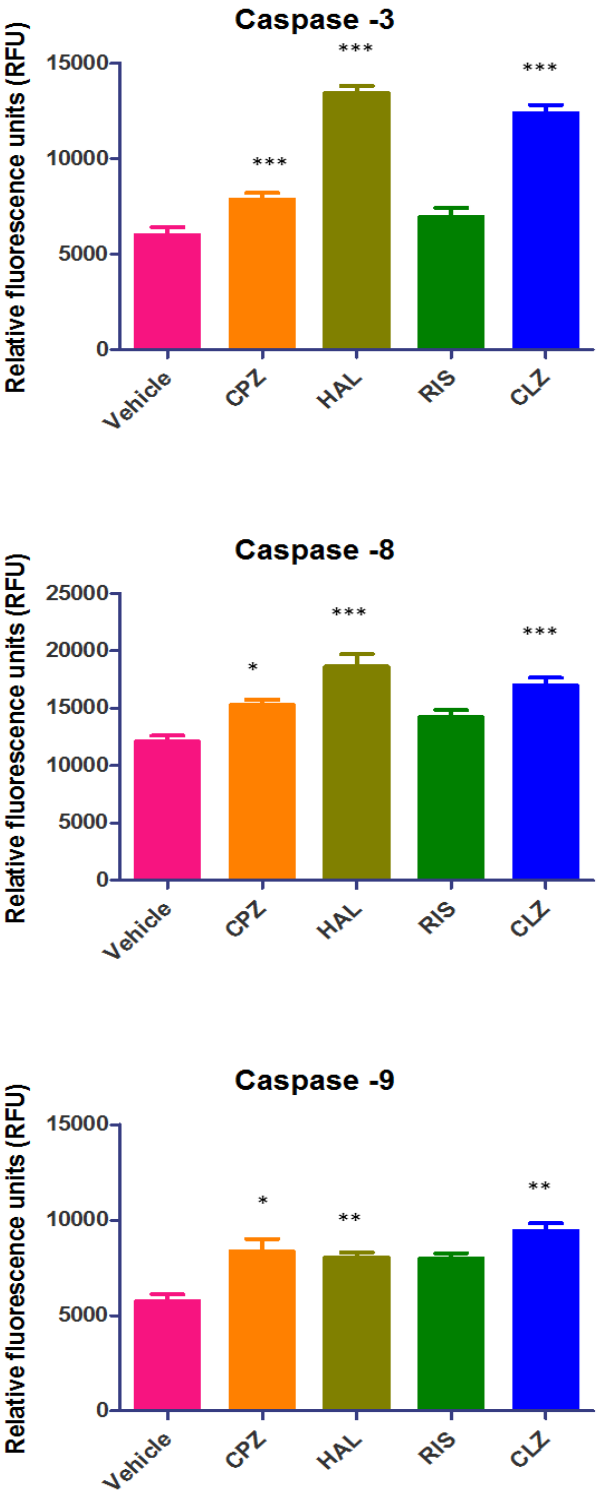


FIG. 6. The effects of antipsychotics on the activities of caspase-3, -8 and -9 measured in HBMECs of the BBB. Cells were incubated for 24hr at the viability EC_{50} for the APs indicated. Statistical significance is relative to vehicle. Values are means ± S.E.M (n=3).

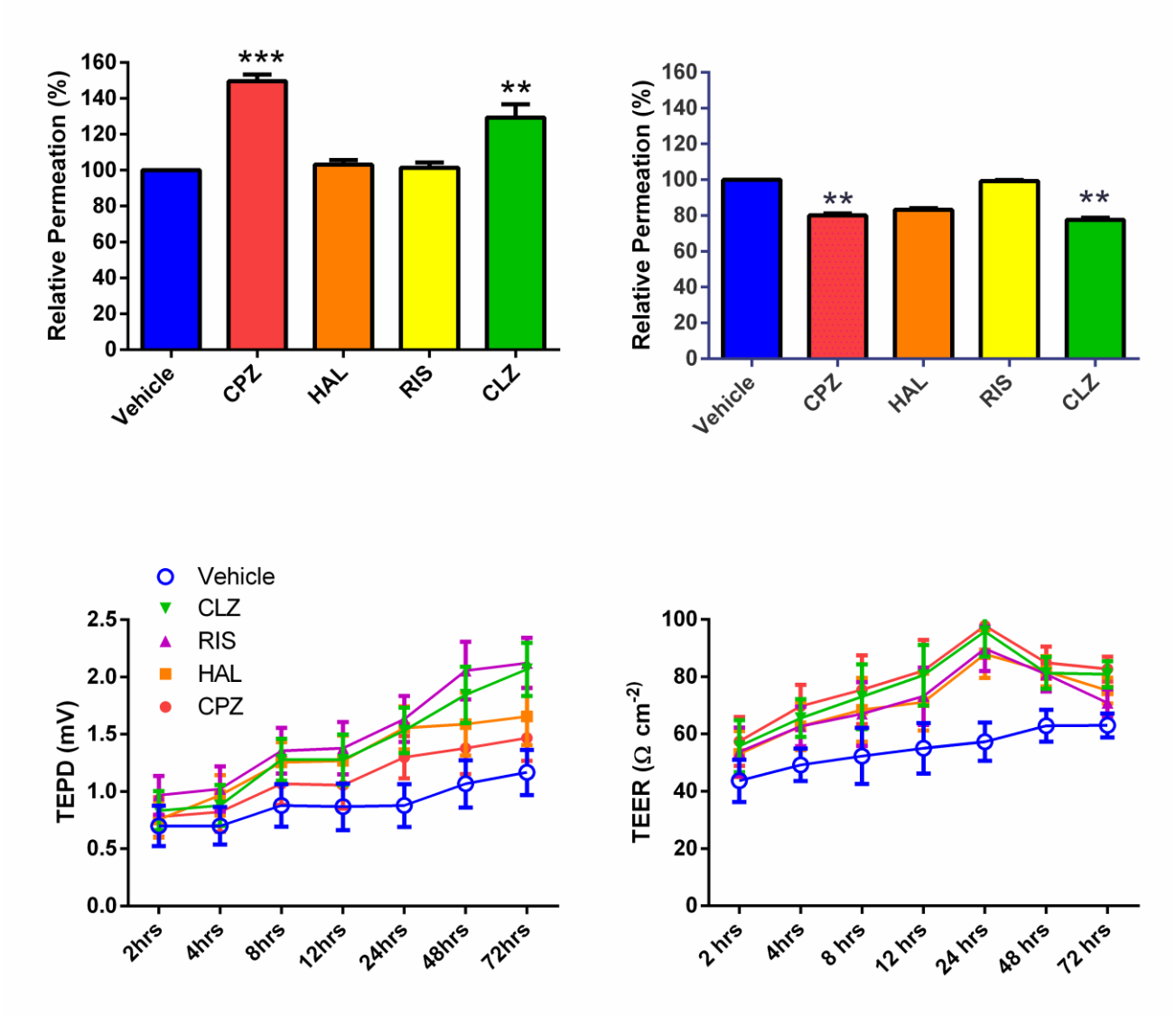


FIG. 7. Top panels: Permeation of Evans blue across endothelial monolayers relative to vehicle control after 72hr incubation at the viability EC_{50} (left) and half that value (right) for the APs indicated. Statistical significance is relative to vehicle control. Values are means ± S.E.M (n=5). Bottom two panels: The relationships between the effects of antipsychotics on the transendothelial potential difference (TEPD) and transendothelial electrical resistance (TEER) of HBMECs as a function of incubation time: Vehicle (O), CLZ (▼), RIS (▲), HAL (■) and CPZ (●). Cells were incubated at half the viability IC_{50} for the APs indicated. Both TEPD and TEER were positively correlated with time and were all significantly larger than vehicle. Values are means (± S.E.M; n=3).

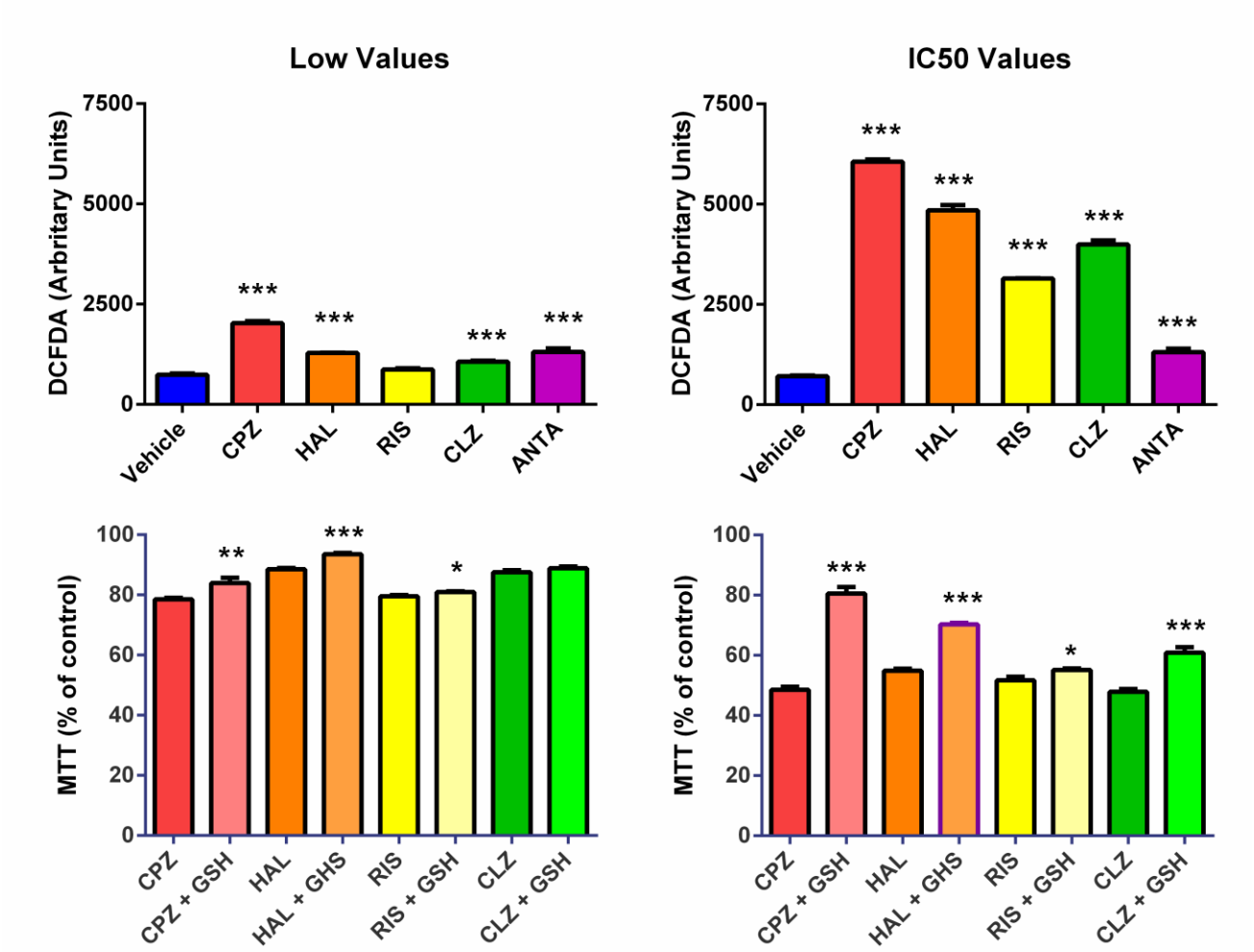


FIG. 8. Top panels: Production of ROS as indicated by DCFDA fluorescence after 24hr incubation in APs at their lowest concentrations tested (<10μM, left) and at their EC_{50}s for viability (right), ANTA is the response to 2μM Antimycin A, the positive control. With the exception of RIS, at the lowest concentrations tested the APs significantly increased ROS relative to vehicle control ($p<0.001$, ANOVA). At their EC_{50}s the APs all significantly increased ROS relative to vehicle control ($p<0.001$, ANOVA). Note the ~ 4 fold increase in ROS production when the higher concentration of drugs was used. Values are means ± S.E.M (n=3-4). Bottom panels: MTT reduction in the absence and presence of 10mM GSH after 24hr incubation at the lowest concentrations of AP tested (<10μM, left) and at their EC_{50}s for viability (right). With the exception of CLZ at its lowest concentration, 10mM glutathione (GSH) significantly ($p<0.05$ in all cases, Unpaired t-test with and without GSH) reversed the decrease in MTT reduction produced by the APs at both their lowest and EC_{50} concentrations. Values are means (± S.E.M; n=9).

REFERENCES

Abbott, N. J. (2013). Blood–brain barrier structure and function and the challenges for CNS drug delivery. *Journal of inherited metabolic disease*, **36**, 437-449..

Abbott, N. J., Patabendige, A. A., Dolman, D. E., Yusof, S. R., & Begley, D. J. (2010). Structure and function of the blood–brain barrier. Neurobiology of disease **37**, 13-25.

Allen, C. L., and Bayraktutan, U. (2009). Antioxidants attenuate hyperglycaemia-mediated brain endothelial cell dysfunction and blood-brain barrier hyperpermeability. *Diabetes Obes. Metab.* **11**, 480-490.

Ben-Shachar, D., Livne, E., Spanier, I., Leenders, K.L., and Youdim, M.B. (1994). Typical and atypical neuroleptics induced alteration in blood-brain barrier in brain $^{59}FeCl_3$ uptake. *J.Neurochem.* **62**, 1112-1118.

Bernas, M. J., Cardoso, F. L., Daley, S. K., Weinand, M. E., Campos, A. R., Ferreira, A. J. G., and Brito, M. A. (2010). Establishment of primary cultures of human brain microvascular endothelial cells to provide an in vitro cellular model of the blood-brain barrier. *Nature Protoc.* **5(7)**, 1265-1272.

Boulton, D. W., DeVane, C. L., Liston, H. L., and Markowitz, J. S. (2002). *In vitro* P-glycoprotein affinity for atypical and conventional antipsychotics. *Life Sci.* **71(2)**, 163-169.

Burkhardt, C., Kelly, J. P., Lim, Y. H., Filley, C. M., and Parker, W. D. (1993). Neuroleptic medications inhibit complex I of the electron transport chain. *Ann.Neurol.*, **33(5)**, 512-517.

Chang, W. H., Shieh, Y. S., Liu, H. C., Jann, M. W., & Chien, C. P. (1994). Plasma reduced haloperidol/haloperidol ratios in schizophrenic patients treated with high dosages of haloperidol. *European Neuropsychopharmacology*, *4*(2), 119-126.

Chang, W. H., Lin, S. K., Lane, H. Y., Hu, W. H., Jann, M. W., & Lin, H. N. (1997). Clozapine dosages and plasma drug concentrations. *Journal of the Formosan Medical Association* *96*(8), 599.

Colgan, O. C., Collins, N. T., Ferguson, G., Murphy, R. P., Birney, Y. A., Cahill, P. A., and Cummins, P. M. (2008). Influence of basolateral condition on the regulation of brain microvascular endothelial tight junction properties and barrier function. *Brain Res.* **1193**, 84-92.

Cordon-Cardo, C., O'Brien, J. P., Casals, D., Rittman-Grauer, L., Biedler, J. L., Melamed, M. R., and Bertino, J. R. (1989). Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. *Proc. National Acad. Sci.* **86(2)**, 695-698.

De Boeck, M., Touil, N., De Visscher, G., Vande, P. A., and Kirsch-Volders, M. (2000). Validation and implementation of an internal standard in Comet assay. *Mutat. Res.* **469**, 181–197.

Dudani, A.K., and Gupta, R.S. (1987). Effect of chlorpromazine and fluoperazine on cytoskeletal components and mitochondria in cultured mammalian cell. *Tissue Cell* **19**, 183–196.

Dujovne, C.A., and Zimmerman, H.J. (1969). Cytotoxicity of phenothiazines on Chang liver cells as measured by enzyme leakage. *Proc. Soc. Exp.Biol. Med.* **131**, 583–587.

Dwyer, D.S., Lu, X.H., and Bradley, R.J. (2003). Cytotoxicity of conventional and atypical antipsychotic drugs inrelation to glucose metabolism. *Brain Res.* **971**:31–39.

Dwyer, D.S., Donohoe, D., Lu, X.H., and Aamodt, E.J. (2005). Mechanistic connections between glucose/lipid disturbances and weight gain induced by antipsychotic drugs. *Int. Rev. Neurobiol.***65**,211–247.

Elsheikha, H. M., McKinlay, C. L., Elsaied, N. A., and Smith, P. A. (2013). Effects of Neospora caninum infection on brain microvascular endothelial cells bioenergetics. *Parasites & vectors* ***6*****(1)**, 24.

Ereshefsky, L. (1999). Pharmacologic and pharmacokinetic considerations in choosing an antipsychotic. J. Clin. Psychiatry 60 Suppl **10**:20-30.

Fall, C. P., & Bennett, J. P. (1999) Characterization and time course of MPP^+induced apoptosis in human SH-SY5Y neuroblastoma cells. *Journal of neuroscience research* **55(5),** 620-628.

Finkel, T., and Hwang, P. M. (2009). The Krebs cycle meets the cell cycle: Mitochondria and the G1–S transition. *Proc.National. Acad.Sci. USA* **106(29),** 11825-11826.

Galili-Mosberg, R., Gil-Ad, I., Weizman, A., Melamed, E., and Offen, D. (2000). Haloperidol-induced neurotoxicity-possible implications for tardive dyskinesia. *J. Neural Transm.* **107**, 479–490.

Gerebtzoff, G., Li-Blatter, X., Fischer, H., Frentzel, A., & Seelig, A. (2004). Halogenation of drugs enhances membrane binding and permeation. *ChemBioChem*, *5*(5), 676-684.

Halliwell, W.H. (1997). Cationic amphiphilic drug-induced phospholipidosis. *Toxicol.l Pathol.* **25(1)**, 53-60.

Hemmelmann, M., Metz, V. V., Koynov, K., Blank, K., Postina, R., and Zentel, R. (2012). Amphiphilic HPMA-LMA copolymers increase the transport of Rhodamine 123 across a BBB model without harming its barrier integrity. *J. Control. Release* **163**, 170-177.

Hembury, A., and Mabondzo, A. (2008). Endothelin-1 reduces P-glycoprotein transport activity in an in vitro model of human adult blood–brain barrier. *Cell. Mol. Neurobiol.* **28(7)**, 915-921.

Jellinger, K. (1977). Neuropathologic findings after neuroleptic long-term therapy. *Neurotoxicology*, *25*.

Karagianis, J. L., Phillips, L. C., Hogan, K. P., & LeDrew, K. K. (1999). Clozapine-associated neuroleptic malignant syndrome: two new cases and

a review of the literature. *The Annals of pharmacotherapy*, *33*(5), 623-630.

Lombardo, F., Obach, R. S., Shalaeva, M. Y., & Gao, F. (2004). Prediction of human volume of distribution values for neutral and basic drugs. 2. Extended data set and leave-class-out statistics. *J. Med. Chem*, ***47*(5)**, 1242-1250.

Lowndes, N. F., and Toh, G. W. (2005). DNA repair: the importance of phosphorylating histone H2AX. *Curr. Biol.* **15(3)**, 99-102.

Maurer, I., & Möller, H. J. (1997). Inhibition of complex I by neuroleptics in normal human brain cortex parallels the extrapyramidal toxicity of neuroleptics. In *Detection of Mitochondrial Diseases* (pp. 255-259). Springer US.

McGrath P. C., and Neifeld J. P. (1985) Inhibition of human neuroblastoma by dopamine antagonists. *Surgery* **98**, 135–141.

Miyamoto S, Duncan G. E, Marx C. E, and Lieberman J. A. (2005). Treatments for schizophrenia: a critical review of pharmacology and mechanisms of action of antipsychotic drugs. *Mol. Psychiatry* **10**,79–104.

Modica-Napolitano, J. S., Lagace, C. J., Brennan, W. A., and Aprille, J. R. (2003). Differential effects of typical and atypical neuroleptics on mitochondrial function in vitro. *Arch. Pharm. Res.* **26(11)**, 951-959.

Munyon W.H., Salo R., and Briones D.F. (1987). Cytotoxic effects of neuroleptic drugs. Psychopharmacology **91**, 182–188.

Nishikage, H., Nakanishi, T., Takamitsu, Y., & Yamamoto, J. (2002). Sequential changes in the plasma concentration of risperidone following intentional overdose. *Clinical neuropharmacology*, *25*(6), 307-309.

Nordenberg, J., Fenig, E., Landau, M., Weizman, R., and Weizman, A. (1999). Effects of psychotropic drugs on cell proliferation and differentiation. *Biochem.Pharmacol.* **58(8)**, 1229-1236.

Nunez, R. (2001). DNA measurement and cell cycle analysis by flow cytometry. *Curr. Issues Mol. Biol.* **3**, 67-70.

Ola, M. S., Nawaz, M., and Ahsan, H. (2011). Role of Bcl-2 family proteins and caspases in the regulation of apoptosis. *Mol. Cell. Biochem.* **351(1)**, 41-58.

Pardridge, W. M., Crawford, I. L., and Connor, J. D. (1973). Permeability changes in the blood-brain barrier induced by nortriptyline and chlorpromazine. *Toxicol. Appl. Pharmacol.* **26(1)**, 49-57.

Reinke, A., Martins, M. R., Lima, M. S., Moreira, J. C., Dal-Pizzol, F., & Quevedo, J. (2004). Haloperidol and clozapine, but not olanzapine, induces oxidative stress in rat brain. *Neuroscience letters*, *372*(1-2), 157-160.

Rosenfeld, M., Brenner-Lavie, H., Ari, S. G. B., Kavushansky, A., & Ben-Shachar, D. (2011). Perturbation in mitochondrial network dynamics and in complex I dependent cellular respiration in schizophrenia. *Biological psychiatry*. ***69*****(10)**, 980-988.

Schieke, S. M., McCoy Jr, J. P., and Finkel, T. (2008). Coordination of mitochondrial bioenergetics with G1 phase cell cycle progression. *Cell Cycle* **7(12)**, 1782-1787.

Seelig, A. (2007). The role of size and charge for blood–brain barrier permeation of drugs and fatty acids. *J. Mol. Neurosci.* **33(1)**, 32-41.

Titier, K., Bouchet, S., Pehourcq, F., Moore, N., & Molimard, M. (2003). High-performance liquid chromatographic method with diode array detection to identify and quantify atypical antipsychotics and haloperidol in plasma after overdose. *Journal of Chromatography B*, ***788*****(1)**, 179-185.

Slater, A. F., Stefan, C., Nobel, I., & Van Den Dobbelsteen, D. J. (1995). Signalling mechanisms and oxidative stress in apoptosis. *Toxicology letters*, *82*, 149-153.

Van Putten, T., Marder, S. R., Wirshing, W. C., Aravagiri, M., & Chabert, N. (1991). Neuroleptic plasma levels. *Schizophrenia bulletin*, *17*(2), 197-216.

Vilner, B. J., de Costa, B. R., and Bowen, W. D. (1995). Cytotoxic effects of sigma ligands: sigma receptor-mediated alterations in cellular morphology and viability. *J. Neurosci.* **15**, 117–134.

Wang, J. S., Zhu, H. J., Markowitz, J. S., Donovan, J. L., and DeVane, C. L. (2006). Evaluation of antipsychotic drugs as inhibitors of multidrug resistance transporter P-glycoprotein. *Psychopharmacology* **187(4)**, 415-423.

Weiss, N., Miller, F., Cazaubon, S. and Couraud, P.O. (2009). The blood–brain barrier in brain homeostasis and neurological diseases. *Biochem. Biophys. Acta* **1788**, 842–857.

Wiklund, E. D., Catts, V. S., Catts, S. V., Ng, T. F., Whitaker, N. J., Brown, A. J., and Lutze-Mann, L. H. (2010). Cytotoxic effects of antipsychotic drugs implicate cholesterol homeostasis as a novel chemotherapeutic target. *Int. J. Cancer* **126**(1), 28-40.

Wilhelm, I., Fazakas, C., & Krizbai, I. A. (2011). In vitro models of the blood-brain barrier. *Acta Neurobiol Exp* (Wars), **71**(1), 113-128.

Wong, D., Dorovini-Zis, K., and Vincent, S. R. (2004). Cytokines, nitric oxide, and cGMP modulate the permeability of an in vitro model of the human blood–brain barrier. *Exp. Neurol.* **190(2)**, 446-455.