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HALOPERIDOL AND CLOZAPINE DECREASE S100B RELEASE FROM GLIAL CELLS

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Abstract—Recent meta-analyses showed consistently elevated levels of S100B in serum and cerebrospinal fluid of schizophrenic patients. This finding has been attributed to glial pathology because S100B is produced by astrocytes and oligodendrocytes. However, S100B may be likewise associated with schizophrenia-related disturbances in glial cell as well as adipocyte energy supply and glucose metabolism. The influence of antipsychotic drugs on S100B levels remains unclear, and some studies have suggested that treatment with these drugs may actually contribute to the elevated S100B levels observed in schizophrenic patients. In this study, we explored the effects of the typical antipsychotic haloperidol and the atypical prototype drug clozapine on the release of S100B by astrocytic C6 cells and oligodendrocytic OLN-93 cells. Because of the association between schizophrenia and disturbances in energy metabolism, we assessed the effects of these drugs under basal condition (BC) compared to serum and glucose deprivation (SGD). We found that treatment of C6 and OLN-93 cells with haloperidol and clozapine reduced the release of S100B from C6 and OLN-93 cells under BC and SGD *in vitro* at a tissue concentration corresponding to the assumed therapeutic dose range of these drugs. These data suggest that elevated levels of S100B in bodily fluids of schizophrenic patients are normalized rather than increased by the effects of antipsychotic drugs on glial cells.

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Abbreviations: BC, basal condition; C6, an astroglial cell line from rat; DAPI, a nuclear staining with 4′,6-diamidine-2′-phenylindole dihydrochloride; DMEM, Dulbecco's modified Eagle's medium; D2, D3 and D4, dopamine receptors; EDTA, ethylenediaminetetraacetic acid; M2 and M4, muscarinic acetylcholine receptors; OLN-93, an oligodendrocytic cell line from rat; PBS, phosphate buffered saline; PI, Propidium Iodide; RPMI, Roswell Park Memorial Institute medium; SGD, serum and glucose deprivation; S100B, member of a family of proteins that are 100% soluble in ammonium sulphate at neutral pH; v/v, volume per volume; 5HT1A and 5HT2A, serotonin receptors.

Key words: astrocytes, oligodendrocytes, clozapine, haloperidol, S100B, schizophrenia.

S100B is synthesized by and released from astrocytes and oligodendrocytes (Steiner et al., 2007, 2008b). It acts as a dose-dependent growth factor for neurons and glial cells and affects synaptogenesis as well as dopaminergic and glutamatergic neutrotransmission (Tramontina et al., 2006b; Rothermundt et al., 2007; Liu et al., 2008). Moreover, S100B plays an important role in brain development (Tiu et al., 2000) and regulates both the maturation of cortical astrocytes (Raponi et al., 2007) and the transformation of multipotent oligodendrocyte precursor cells to slowly proliferating pro-oligodendrocytes (Deloulme et al., 2004). Recent meta-analyses showed that levels of S100B in the blood of schizophrenic patients with acute exacerbations and deficit symptoms were consistently elevated (Schroeter et al., 2009; Schroeter and Steiner, 2009). This finding supports the accumulating evidence for astrocyte- and oligodendrocyte-related pathologies in schizophrenic patients (Bernstein et al., 2009). Indeed, a previous post-mortem study reported higher densities of S100B-positive cells, which were mainly astrocytic, in cortical brain regions of patients with paranoid schizophrenia along with a loss of S100B-positive glia, which were mainly oligodendrocytic, in the adjacent white matter regions (Steiner et al., 2008a).

Despite these findings, whether elevated S100B serum concentrations exclusively reflect schizophrenia-related glial pathologies remains unclear. Antipsychotic drugs and non-glial cellular sources of S100B may also influence concentrations of S100B in bodily fluids. In support of the idea that antipsychotic drugs may affect S100B levels, cross-sectional clinical studies have shown both increased (Wiesmann et al., 1999; Schroeter et al., 2003; Schmitt et al., 2005; Ling et al., 2007; Qi et al., 2009) and decreased levels of S100B in blood of patients taking antipsychotic medication (Gattaz et al., 2000). In contrast to these findings, Rothermundt et al. (2004) reported that compared to age- and sex-matched healthy controls, schizophrenic patients had significantly increased levels of S100B in serum both upon admission and after 12 or 24 weeks of treatment with risperidone or flupenthixol, and the level of S100B in serum from these patients did not change between these time points. Recently, Steiner et al. (2009) and Ling et al. (2007) observed higher levels of S100B in schizophrenic patients at baseline than after 6 or 12 weeks of treatment, suggesting that antipsychotic medication could decrease S100B levels in schizophrenic patients. Few studies have used animal models or glial cell cultures to address the

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ability of antipsychotic drugs to modulate S100B production (Dean et al., 2006; Quincozes-Santos et al., 2008).

Schizophrenia is associated with certain haplotypes of the S100B gene that are associated with increased S100B expression (Liu et al., 2005), suggesting that systemic processes involving non-glial cell types that express S100B, such as adipocytes or vascular endothelial cells, may also contribute to the observed increase in concentrations of S100B in the blood of schizophrenic patients (Zimmer et al., 1997; Netto et al., 2006; Steiner et al., 2007). These findings are supported by recent studies showing that levels of S100B in the blood are correlated with adipose tissue mass as well as adipokine levels in healthy subjects (Steiner et al., 2010a,b) and insulin resistance in schizophrenic patients (Steiner et al., 2010b). This relationship between S100B and metabolism may prove important for future research on the significance of elevated levels of S100B in schizophrenic patients because obesity, metabolic syndrome and type 2 diabetes are more prevalent in schizophrenic patients and their first-degree relatives than in healthy subjects (Thakore, 2005). Such schizophrenia-associated disturbances in glucose metabolism may likewise affect glial cells and their release of S100B. This consideration is in line with the finding of elevated cerebrospinal fluid glucose levels (Holmes et al., 2006; Huang et al., 2007), and impaired insulin receptor signaling in postmortem dorsolateral prefrontal cortex tissue from schizophrenia cases (Zhao et al., 2006). An intact energy supply in astrocytes is critical in maintaining the balance of the glutamate-glutamine system, which seems to be disturbed in schizophrenic patients (Stone et al., 2007). Interestingly, previous cell culture experiments have demonstrated that an impaired astrocytic glucose supply and a hypoglutamatergic state may result in up-regulation of glial S100B release (Gerlach et al., 2006; Tramontina et al., 2006a; Nardin et al., 2007).

As discussed above, it is unclear whether antipsychotic drugs normalize the increased levels of S100B observed in schizophrenic patients or instead contribute to increased S100B production. We thus explored the effects of the typical antipsychotic haloperidol and the atypical prototype drug clozapine on glial S100B release and cell viability. Astrocytic C6 and oligodendrocytic OLN-93 glial cultures were tested because S100B is synthesized by astrocytes and oligodendrocytes but not by human microglia (Steiner et al., 2007). These cells provide suitable model systems for these studies because they express neurotransmitter receptors that are modulated by haloperidol and clozapine. To account for the potential interference of schizophrenia-related disturbances in glial energy supply, we analyzed the effects of these drugs on S100B production in cells cultured under basal condition (BC) compared to serum and glucose deprivation (SGD).

EXPERIMENTAL PROCEDURES

Cell culture

C6 glioma cell cultures were prepared as previously described (Steiner et al., 2008b): Briefly, cryopreserved C6 cells were thawed, resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum, 1% L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin (growth medium), and transferred to culture flasks. After 3 days, cells were removed from the flasks by mild trypsinization (5 min; trypsin/EDTA: 0.05%/0.002%) and replated on ∅35 mm Petri dishes (50,000 cells/dish). After 6 days, experiments were performed.

Oligodendroglial OLN-93 cells were obtained from the Richter-Landsberg lab and cultured as previously described (Richter-Landsberg and Heinrich, 1996; Steiner et al., 2008b). Cryopreserved OLN-93 cells were thawed, resuspended in DMEM supplemented with 10% fetal calf serum, 50 U/ml penicillin and 50 μg/ml streptomycin (growth medium), and transferred to culture flasks. After 1 week, cells were removed from the flasks by mild trypsinization (5 min; trypsin/EDTA: 0.05%/0.002%) and replated in ∅35-mm Petri dishes (50,000 cells/dish). After 3 days, the concentration of fetal calf serum in the growth medium was reduced to 0.5%, and experiments were performed 72 h later.

Given the therapeutic ranges of haloperidol (plasma levels of 5–20 ng/ml with 10- to 30-fold higher levels in brain tissue) (Kornhuber et al., 1999; Zhang et al., 2007) and clozapine (plasma levels of 100–600 ng/ml with 10- to 24-fold higher levels in brain tissue) (Baldessarini et al., 1993; Weigmann et al., 1999; Zhang et al., 2007), the effects of antipsychotic medication were analysed by adding vehicle (phosphate buffered saline), haloperidol (0.1 or 1 μg/ml) or clozapine (1 or 10 μg/ml) (Sigma, Taufkirchen, Germany) to the cell culture systems for 6 or 24 h. All cultures were incubated at 37 °C in a humidified atmosphere under 5% CO_2 in air (normoxic conditions) for the duration of the experiment. Fifteen dishes were used for each experimental setting. All cultures were kept at 37 °C in a humidified atmosphere under 5% CO_2 in air (normoxic conditions) for the duration of the experiment. For SGD, the normal growth medium was replaced with serum- and glucose-free DMEM for 6 or 24 h. In BC control cultures, the normal growth medium was replaced with DMEM containing serum and glucose.

Immunocytochemistry

C6 and OLN-93 cells were washed twice with phosphate buffered saline (pH 7.4), fixed for 30 min in 4% buffered paraformaldehyde and incubated at room temperature for 3 h with a 1:500 dilution of one of the following rabbit polyclonal antibodies: 5HT1A, 5HT2A, D2, D3, D4, M2 or M4 receptor (Abcam, Cambridge, UK).

Following incubation with these primary antibodies, cells were washed three times for 5 min with phosphate buffered saline and incubated with a 1:500 dilution of an Alexa Fluor 546 goat anti-rabbit IgG secondary antibody for 3 h (Molecular Probes, Göttingen, Germany). Specimens were examined using a fluorescence microscope (Imager.M1, Zeiss, Jena, Germany). Negative controls, which were incubated with buffer instead of primary antibodies, were free of specific immunostaining.

Protein extraction and S100B assay

Media were removed from the cultures and centrifuged at 4000×*g* at 4 °C. Supernatants were frozen at −80 °C until further analysis. Cells were scraped from the surface of the culture dishes. The resulting cell suspensions were harvested in 300 μl sodium phosphate buffer (50 mM, pH 7.4) containing protease inhibitors (Complete MiniTM, Roche, Mannheim, Germany) and centrifuged at 2000×*g*. The cell pellets were homogenized at 4 °C by performing ten strokes with a Potter–Elvehjem glass-Teflon homogenizer at 600 rpm. The homogenates were stored at −80 °C until further analysis. Protein was measured with the BCATM protein assay (Pierce, Rockford, IL) according to the manufacturer's instructions and using bovine serum albumin as a standard.

S100B concentrations were measured by immunoluminometric sandwich assay using directly coated magnetic microparticles