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Effects of benzodiazepine treatment on cortical $GABA_A$ and muscarinic receptors: Studies in schizophrenia and rats

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ABSTRACT

Changes in cortical γ-aminobutyric acid A ($GABA_A$) receptors and muscarinic receptors have been reported in schizophrenia, a disorder treated with antipsychotic drugs and benzodiazepines. As there is a reported functional relationship between the GABAergic and cholinergic systems in the human central nervous system we have investigated whether there are changes in the $GABA_A$ and muscarinic receptors in the cortex of subjects from APD-treated subjects with schizophrenia and whether changes were different in subjects who had also received benzodiazepine treatment. We failed to show any strong correlations between changes in $GABA_A$ and muscarinic receptors in the CNS of subjects with schizophrenia. We showed that subjects with schizophrenia treated with benzodiazepines had lower levels of muscarinic receptors; which was not the case in rats treated with APDs, benzodiazepines or a combination of both drugs. Further, the benzodiazepine binding site, but not the muscimol binding site, was decreased in the parietal cortex of subjects with schizophrenia independent of benzodiazepine status at death. These data would therefore support our previously stated hypotheses that changes in the cortical cholinergic and GABAergic systems are involved in the pathophysiology of schizophrenia.

1. Introduction

The γ-aminobutyric acid A receptor (GABAR) in the human central nervous system consists of a combination of sub-units which have been separated into families based on sequence homogeneity (Sieghart, 2006); the nomenclature for these sub-unit families being α, β, γ, δ, ε, π and θ. The GABAR has a GABA/muscimol binding site and, in receptors containing αβγ subunits, a benzodiazepine (BZs) binding site (Sieghart, 2006). The binding site for GABA crosses the interface between α- and β-subunits (Olsen and Tobin, 1990), whilst BZs bind to opposing faces of the α- and γ-subunits (Smith and Olsen, 1995). Thus, changes in the binding of muscimol and BZs in the CNS from subjects with schizophrenia support the notion that levels of GABARs are affected as part of the pathophysiology of the disorder (Costa et al., 2004).

Changes in GABAR density in the CNS from subjects with schizophrenia, as indicated by muscimol and BZ binding, appear complex. [^{3}H] muscimol binding has been consistently shown to be increased in the cortex and hippocampus of subjects with schizophrenia (Benes et al., 1992, 1996, 1997; Benes, 1995; Dean et al., 1999; Deng and Huang, 2006; Hanada et al., 1987). By contrast, levels of radioactive BZ binding in those regions have been reported as increased, unaltered or decreased in schizophrenia (Kiuchi et al., 1989; Pandey et al., 1997; Reynolds and Stroud, 1993; Squires et al., 1993). Similarly, findings on GABAR sub-units differ, with one study reporting no change in levels of cortical α, β or γ sub-unit mRNA (Akbarian et al., 1995) and another showing a marked (52%) reduction in γ2 short, but not γ2 long, sub-unit mRNA in the superior frontal gyrus (Huntsman et al., 1998) from subjects with schizophrenia.

Neuroimaging studies, which have focussed on measuring BZ binding sites in the CNS of subjects with schizophrenia, have failed to show changes in the levels of BZ binding in subjects with schizophrenia (Abi-Dargham et al., 1999; Asai et al., 2007; Ball et al., 1998; Busatto et al., 1997; Verhoeff et al., 1999). This discrepancy prompted the suggestion that the differences between postmortem and neuroimaging studies were a result of drug treatment before death in subjects with schizophrenia from whom CNS tissue was collected postmortem (Asai et al., 2007). The authors based this assumption on the fact that their study involved either antipsychotic drug (APD) naive subjects or subjects that had not received APDs for up to 1 year prior to scanning. However, this hypothesis must be interpreted with caution because the neuroimaging studies were not completed using benzodiazepine naive subjects, rather the subjects had not received benzodiazepines for between 3 weeks (Abi-Dargham et al., 1999) and 3 months (Busatto et al., 1997) before scanning. Thus,

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it is not possible to discount the effects of BZ treatment as a confounding factor in the neuroimaging studies.

The idea that drug treatment before death can affect levels of GABAR is supported by one postmortem study that shows radioactive BZ binding is only increased in subjects with schizophrenia that were not taking APDs at death (Pandey et al., 1997). These data suggest that APD treatment acts to reduce the level of GABAR in the CNS, a finding supported by a number of studies in rats that have reported decreased GABAR after such treatment (Dean et al., 2001; Farnbach-Pralong et al., 1998). However, there are other studies showing APD treatment does not affect (Wong et al., 1996) or increases (Frey et al., 1987, 1989; Huffman and Ticku, 1983; See et al., 1989, 1990; Skilbeck et al., 2007) levels of GABARs. To add to this complex picture there is a limited amount of data from rats that suggest that changes in GABAR after APD treatment are regionally selective (McLeod et al., 2008; Zink et al., 2004) and of a temporal nature, the latter being suggested following the demonstration of an up-regulation of [^{3}H]muscimol binding after acute treatment but an up-regulation of [^{3}H]flunitrazepam binding after chronic treatment with APDs (Skilbeck et al., 2007).

Importantly, in clinical practice BZs are usually prescribed in conjunction with APDs early in a psychotic episode (Alexander et al., 2004) and may be given more chronically to control increased levels of anxiety or sleep disturbance (Haw and Stubbs, 2007). Thus it is significant that we have shown different changes in [^{3}H]muscimol and [^{3}H]flumazenil binding in rats following treatment with APDs, BZs or a combination of these drugs (McLeod et al., 2008). These findings highlight the usefulness of animal models in determining the possible outcomes of the polypharmacy used in the treatment of schizophrenia. At a CNS functional level, animal studies have revealed interactions between central GABAergic and muscarinic receptors (CHRMs) (Aramakis et al., 1997; Buhl et al., 1998; Dickson and Alonso, 1997), which is significant as both GABARs and CHRMs (Crook et al., 2001; Dean et al., 2002; Mancama et al., 2003; Zavitsanou et al., 2004) are altered in the cortex of subjects with schizophrenia. Whilst there is no evidence from studies in animals that changes in CHRMs are simply due to APD treatment (Crook et al., 2001; Han et al., 2008; See et al., 1990; Zavitsanou et al., 2007) it is important to note that there appears to be no studies to determine if combined APD and BZ treatment regime could affect levels of GABAR and CHRMs. However, current data could indicate that functional interactions between GABAR and CHRM1 may have caused these receptors to change in the cortex of subjects with in schizophrenia. If correct, this would be important as both of these receptors are critical in maintaining cognitive functioning in the mammalian CNS (Mohler, 2007; Raedler et al., 2007) and the combined dysfunction of these receptors could be contributing to cognitive deficits of schizophrenia (Mohler, 2007; Raedler et al., 2007). This notion is supported by recent proof-of-principle studies showing improvement in cognitive deficits in subjects with schizophrenia after treatment with either CHRM1/4 (Shekhar et al., 2008) or GABAR sub-unit (Lewis et al., 2008) selective agonists.

To begin to address whether there may be associated changes in GABARs and CHRM1 in schizophrenia, we decided to determine if there were concomitant changes in these receptors in the cortex of subjects with the disorder. We also wanted to better understand the possible impact of APD and BZ polypharmacy on cortical GABARs and CHRM1 and thus we measured levels of CHRM1 in the cortex of subjects with schizophrenia treated with APDs with (SBZ+-), or without (SBZ−) BZs. In addition, we measured cortical CHRM1s in rats treated with APD, BZ or a combination of both drugs because we already have data on GABAR (McLeod et al., 2008) from rats treated in this manner.

2. Materials and methods

2.1. Materials

[^{3}H]Flumazenil, [^{3}H]muscimol and [^{3}H]pirenzepine were obtained from Perkin Elmer Australia Pty Ltd, Melbourne, Australia. SR-95531 and (±)-3-Quinuclidinyl Xanthene-9-Carboxylate Hemioxalate (QNX), diazepam, clonazepam and paraformaldehyde were obtained from Sigma Aldrich Pty. Ltd., Castle Hill, New South Wales, Australia. [^{3}H]Microscales® were obtained from Amersham Biosciences Australia Pty. Ltd., Sydney, Australia. TRIS and hydrochloric acid (HCl) were obtained from AJAX Chemicals Pty. Ltd., Auburn, New South Wales, Australia.

2.2. Tissue collection

2.2.1. Collection of human tissue

Approval to collect postmortem human CNS was given by the Ethics Committee of the Victorian Institute of Forensic Pathology, whilst the approval for this particular study was obtained from the North-Western Health Care Human Ethics Committee.

Brodmann's area (BA) 9 (the region of the CNS on the lateral surface of the frontal lobe including the middle frontal gyrus superior to the inferior frontal sulcus) and BA 40 (the lateral surface of the parietal lobe including primarily the supramarginal gyrus surrounding the posterior segment of the lateral fissure) was taken from 20 subjects with schizophrenia. Tissue was also collected from the same CNS regions from 10 subjects with no history of a psychiatric or neurological disorder (Controls). We choose to study BA9 because we have previously shown that [^{3}H]muscimol binding was increased (Dean et al., 1999) and [^{3}H]pirenzepine binding was decreased (Crook et al., 2001; Dean et al., 2002) in that region. By contrast we showed that [^{3}H]pirenzepine binding was not altered in BA 40 from subjects with the disorder schizophrenia (Dean et al., 2002). From these data we postulated that the molecular cytoarchitecture of BA9 was compromised by the pathophysiology of schizophrenia, whereas BA40 may have been spared and that any changes in receptor density in our current study would be limited to BA9.

Diagnosis was made, by consensus, after a case history review using the Diagnostic Instrument for Brain Studies (DIBS) (Hill et al., 1996), which allowed diagnoses according to Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) criteria (Roberts et al., 1998). Ten of the subjects with schizophrenia in this study had measurable blood BZs at death (SBZ+-), whilst 10 subjects had no history of receiving BZs and no measurable blood levels of those drugs at death (SBZ-).

Using the information in the DIBS, duration of illness (DOI) was calculated as the time from first contact with a psychiatric service until time of death. The final recorded APD dose (FRADD) was converted to chlorpromazine equivalents (Foster, 1989; Woods, 2003) and lifetime exposure to APDs (LEAP) was calculated as kilogram chlorpromazine equivalents (Knable et al., 2002). Similarly, the final recorded doses and blood levels of BZs were converted to diazepam equivalents (Ashton, 2007). In cases where death was witnessed, the time between death and autopsy was taken as the postmortem interval (PMI). When death was not witnessed, tissue was taken only from individuals who had been seen alive up to 5 h before being found dead, with the PMI determined as the interval half way between the donor being found dead and last seen alive to autopsy. In all cases, cadavers were refrigerated within 5 h of being found. Agonal hypoxia was measured via brain pH as described previously (Kingsbury et al., 1995), as this variable is now a better indicator of tissue quality than is the PMI (Torrey et al., 2000). For this study, RNA integrity number values were not measured as such data give no added value above CNS pH with regard to protein integrity in postmortem CNS (Stan et al., 2006).

2.2.2. Animal studies: treatment regime and CNS collection

After permission from the University of Melbourne Animal Ethics and Experimentation Committee, 6-week-old male Sprague–Dawley rats (100–150 g; $n=5$ per group) were treated for 12 days via intraperitoneal (IP) injection with vehicle (dimethyl sulfoxide: DMSO), diazepam (3.0 mg/kg/day), haloperidol (1.0 mg/kg/day) or a combination of diazepam and haloperidol at the same concentration (McLeod et al., 2008). The treatment period was selected to reflect the period of acute use of BZs in the treatment of schizophrenia (Alexander et al., 2004). The animals were maintained on a 12 h light cycle with free access to food and water. Rats were sacrificed 48 h after the last drug treatment by decapitation, the CNS removed, frozen in isopentane on dry ice and stored at −70 °C until required.

2.3. In situ radioligand binding and single-point saturation analysis

For human CNS, 15×20 μm serial coronal sections were cut at −20 °C with a microtome-cryostat (Leica CM 1900) and thaw-mounted on gelatin-chrom alum-coated slides. For rats, tissue blocks containing the rat frontal cortex (Interaural 9.20 mm; Bregma 0.20 mm (Paxinos and Watson, 1986)) were used, with 5×20 μm serial coronal sections being cut at −20 °C and thaw-mounted on gelatin-chrom alum-coated slides.

For both human and rat studies radioligand binding was measured using single-point saturation analysis which provides a good estimate of receptor density (Rodbard, 1981); to ensure saturation of available binding sites at a single concentration of radioligand, all radioligands were at greater than three times the concentration of their binding K_d. Under the binding conditions used in our study, [^{3}H]muscimol would bind to the GABA binding site, whilst [^{3}H]flumazenil would bind to the BZ binding site on the GABAR (Sieghart, 2006). [^{3}H]pirenzepine would predominantly bind to the CHRM1 (Scarr and Dean, 2008). To measure [^{3}H]muscimol binding, tissue sections were incubated with 100 nM [^{3}H]muscimol in 50 mM Tris-citrate assay buffer (pH 7.1) (Dean et al., 1999) in the absence (three sections: total binding (TB)) or presence (two sections: non-specific binding (NSB)) of 10^{-6} M of the GABAR antagonist SR-95531 for 60 min at room temperature. For [^{3}H]flumazenil, tissue sections were incubated with 12 nM radioligand alone (three sections: