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Upregulation of *NRG-1* and *VAMP-1* in Human Brain Aggregates Exposed to Clozapine

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Abstract

Growing genetic evidence has implicated a role for neuregulin-1 (NRG-1) in schizophrenia pathogenesis as well as alterations in SNAP receptor (SNARE) proteins at both gene and protein levels in post-mortem investigations. In relation to a potential therapeutic mechanism for atypical antipsychotic medications, clozapine has been shown to increase both NRG-1 levels and synaptic markers in rodents. As evidence continues to mount for a potential restoration in connectivity by antipsychotic medications being a mode of efficacy we chose to examine the effects of the atypical antipsychotic clozapine and the typical antipsychotic haloperidol on NRG-1 and SNARE protein transcripts in a human brain aggregates exposed to plasma levels chronically for a period of three weeks. At the end of this exposure period we performed quantitative real-time PCR to investigate the mRNA levels of *NRG-1, VAMP-1* and *SNAP-25*. Overall we found that clozapine had the ability to upregulate *NRG-1* (+3.58 fold change) and *VAMP-1* (+1.92) while *SNAP-25* remained unchanged. Changes for haloperidol exposed aggregates were below our cut-off of +1.5. Overall the results of our investigation lend further support to atypical antipsychotic medications having the potential to increase levels of neurotrophic and synaptic markers such as *NRG-1* and *VAMP-1*, the former being a strong candidate susceptibility gene for schizophrenia. In the absence of frank neuronal loss in schizophrenia, restoration of neuronal and synaptic functions by atypical antipsychotics in the brains of schizophrenics maybe a key mechanism of therapeutic efficacy by re-establishing normal connectivity and functioning.

Keywords

Clozapine; Haloperidol; VAMP-1; Aggregates; Synaptic; NRG1

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Introduction

Neuregulin-1 (NRG-1) is a trophic factor that contains an epidermal growth factor domain and mediates it's effects via stimulating ErbB receptor tyrosine kinases (Mei and Xiong 2008). To date several studies have shown an association for the gene for NRG-1 and schizophrenia (Li et al 2006; Liu et al 2005; Petryshen et al 2005; Stefansson et al 2003; Stefansson et al 2002). NRG-1's known role in neurodevelopment and synaptic plasticity add strength to its candidacy as a susceptibility gene for schizophrenia, with a growing body of evidence pointing to altered brain connectivity within the disorder. Indeed, some of the most consistent post-mortem brain observations in schizophrenia include reductions in synaptic proteins and mRNAs (Eastwood and Harrison 2005; Eastwood and Harrison 2006; Fatemi et al 2001; Halim et al 2003; Honer and Young 2004; Mukaetova-Ladinska et al 2002; Sawada et al 2005; Thompson et al 2003; Webster et al 2001; Young et al 1998). In relation to antipsychotic medications and NRG-1, a recent study has demonstrated that NRG-1 levels were elevated in peripheral blood lymphocytes in first-episode schizophrenic subjects following treatment with the atypical antipsychotics risperidone or quietapine (Zhang et al 2008). This effect of clozapine on NRG-1 expression is supported by an animal study demonstrating increased *NRG-1* in the rat PFC following chronic clozapine treatment (Wang et al 2008). Interestingly, clozapine has also been shown to increase levels of synaptic genes in the brains of rodents (Critchlow et al 2006; Kontkanen et al 2002) as well as promoting neurogenesis following chronic exposure (Halim et al 2004; Kodama et al 2004).

Within our laboratory we have developed and continue to use an *in vitro* human fetal brain aggregate system containing similar proportions of neurons and glial cells to those typically found in the cerebral cortex (Trillo-Pazos et al 2004). This model has been characterized as having cell types for all major neurotransmitter systems with a number of different transcripts altered in this model following chronic cortisol exposure (Salaria et al 2006). The aim of this investigation was to assess gene expression of *NRG-1* in clozapine exposed human brain aggregates using quantitative real-time PCR (qRT-PCR) and relate changes in *NRG-1* expression to two SNAP receptor (SNARE) related genes, synaptosomal associated protein-25 (SNAP-25) and vesicle-associated membrane protein-1 (VAMP-1) in human brain aggregates exposed to clozapine. These synaptic related genes were chosen on the basis of being found to be reduced in brains of patients with schizophrenia, with *SNAP-25* confirmed as being contained within a chromosomal hotspot for schizophrenia at 20p12.3-11 in a recent meta-analysis (Corradini et al 2009). For comparison we included aggregates exposed to the typical antipsychotic haloperidol. Results for both clozapine and haloperidol exposed brain aggregates were compared to non-exposed control human brain aggregates.

Materials and Methods

Human fetal brain tissue was procured by Advanced Bioscience Resources (ABR, Almeda), with ethical committee approval from University of California, San Diego IRB. Brain tissue was collected from terminations at 14-18 weeks gestation. This gestation period was chosen due to neurons being largely differentiated at this stage of development and because previous investigations have revealed survival rates for neurons and glia to be optimal within this window (Trillo-Pazos et al 2004). Detailed methodology for culturing human brain aggregates has been described previously (Trillo-Pazos et al 2004). Briefly, human fetal brain tissue were mechanically disaggregated, filtered through a 200μM pore filter and processed to a single cell suspension. Cells were then seeded at 10^6 cells/ml in DMEM-F12 containing 5% human serum, 0.5% glutamine (200mM) and 0.1% gentamicin sulphate (all solutions from Sigma, USA) in 24 well culture plates (Corning, NY) that were previously coated with 0.75% Noble Agar (Sigma, USA). Culture media was replaced every 3-4 days and tissue allowed to mature to four weeks, at which time cells formed a well circumscribed spherical brain aggregate.

Exposure of brain aggregates to clozapine and haloperidol

12 aggregates were utilized per exposure condition. Mature, four week old aggregates were exposed to haloperidol [500nM] and clozapine [2μM] (compounds from Sigma, USA) for three weeks. These doses represent high end plasma concentrations for medications seen in patients with schizophrenia (Facciola et al 1999; Roh et al 2001; Zoccali et al 2003). Due to both compounds being relatively insoluble in water, ethanol was used as a solubilizing agent prior to being serially diluted in culture media. The final concentration of ethanol exposed to cells being minimal at ~1:20,000. This same concentration was given to the control aggregates without the presence of drug as a vehicle. Drug and control media were replenished every 48 hours to maintain bioactivity of compounds. Toxicity was monitored by a standardized cytotoxicity kit (Promega, USA) that measures the production of lactate dehydrogenase (LDH) within the media.

RNA extraction

Following completion of the exposure period aggregates from each condition were pooled together and total RNA was extracted using a standard Trizol® method (*Invitro*gen, CA) (Chomczynski and Sacchi 1987) followed by treatment with DNase I to remove any contaminating DNA. RNA quality was checked via the use of a spectrophotometer at A260/280nm and banding of RNA checked using standard gel electrophoresis. Both yield and quality of RNA were found to be of a high standard.

qRT-PCR

cDNA was reverse transcribed from extracted RNA using the SuperScript™ III RT system (Invitrogen, Carlsbad, CA). cDNA was serially diluted and served as template in a qRT-PCR using master mix and gene-specific validated Taqman assays from Applied Biosystems, Foster City, CA. The instrument utilized was the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Transcripts investigated included NRG-1 (Hs00247641_m1), VAMP-1 (Hs01051303_g1), and SNAP-25 (Hs00938958_m1). For each gene of interest we loaded 20ng of cDNA, with experiments run in triplicate. We normalized cycle thresholds to the housekeeping gene GAPDH (Applied Biosystems, Foster City, CA). In addition, no (-) template control samples were included to rule out reagent contamination. The reaction conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes, followed by 50 cycles of 95°C for 15 seconds, and 60°C for 1 minute.

Results

LDH

No differences in LDH levels between clozapine and haloperidol exposed aggregates versus control non-exposed aggregates was seen, indicating no toxicity associated with administration of either compound.

qRT-PCR

Results were recorded as cycle threshold (Ct) values and mean Ct values were analyzed according to the $2^{-\Delta\Delta Ct}$ method to arrive at fold changes of expression (Livak and Schmittgen 2001). As a cut-off we chose a fold change of 1.5 as demonstrating differential expression. We observed an increased expression for *NRG-1* in clozapine exposed aggregates (+3.58 fold change) as well as an increase *VAMP-1* (+1.92) with *SNAP-25* (0.99) being relatively unchanged. No changes in expression that met our cutoff criteria were observed for *NRG-1* (+1.31), *VAMP-1* (-0.772) or *SNAP-25* (0.97) haloperidol treated aggregates versus controls. No differences in *GAPDH* Ct values were observed between experimental conditions and controls using a student's t-test ($p>0.10$).

Discussion

Our results demonstrate that *NRG-1* and *VAMP-1* expression were upregulated in clozapine exposed human brain aggregates compared to controls. These effects were not seen in haloperidol exposed brain aggregates. Our results provide further evidence for a potential therapeutic mode of action of atypical antipsychotics such as clozapine in restoring neurotrophic and synaptic connectivity in brains of schizophrenic patients. In support of this, increased *NRG-1* expression has been observed in the rodent PFC following chronic exposure to clozapine together with increased NRG-1 in peripheral blood lymphocytes in first-episode patients after treatment with current atypical antipsychotics risperidone and quietapine. Of interest, this latter study demonstrated reduced NRG-1 expression in patients prior to receiving medication which was then reversed with clozapine. Upregulation of NRG-1 was also seen two weeks post-treatment corresponding to known therapeutic lag for antipsychotics. In further relation to NRG-1, it has been observed recently that a double knockout mice for NRG-1/ErbB4 demonstrated decreased dendritic spine density versus control animals, an effect that was reversed with administration of clozapine (Barros et al 2009).

The ability of clozapine to upregulate *SNAP-25* and *VAMP-1* at the mRNA level are in contrast to decreases in SNARE mRNA and proteins found in a number of different brain regions in schizophrenia, in particular the PFC. While some post-mortem studies have reported increased mRNA and/or protein changes for *VAMP-1* and *SNAP-25* (Scarr et al 2006; Sokolov et al 2000) these increases maybe related to the long term effects of antipsychotic treatment in upregulating synaptic transcripts and/or proteins in the brains of these patients. *VAMP-1* has also been found to be reduced in both schizophrenia and bipolar disorder within a combined microarray analysis of the Stanley Foundation's brain collections (Higgs et al 2006).

In conclusion, the findings of our investigation support a potential mode of therapeutic action for atypical antipsychotics via increased neurotrophic and synaptic support within the schizophrenic brain where these elements seem to be compromised. Further work in this *in vitro* model will focus on the repetition of this experiment, including protein quantitation of our candidates and characterization of other synaptic and neurotrophic changes following exposure of our model to various psychotropic medications.

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