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LIMITED INFLUENCE OF OLANZAPINE ON ADULT FOREBRAIN NEURAL PRECURSORS *IN VITRO*

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Abstract—We evaluated the activity of the atypical antipsychotic drug olanzapine on differentiation and gene expression in adult neural precursor cells *in vitro*. Neural precursors obtained from forebrain subventricular zone (SVZ)-derived neurospheres express a subset (13/24) of receptors known to bind olanzapine at high to intermediate affinities; in contrast, all 24 are expressed in the SVZ. In the presence of 10 nM, 100 nM or 1 μM olanzapine, there is no significant change in the frequency of oligodendrocytes, neurons, GABAergic neurons and astrocytes generated from neurosphere precursors. In parallel, there is no apparent change in cell proliferation in response to olanzapine, based upon bromodeoxyuridine incorporation. There are no major changes in cytological differentiation in response to the drug; however, at one concentration (10 nM) there is a small but statistically significant increase in the size of glial fibrillary acidic protein–labeled astrocytes derived from neurosphere precursors. In addition, olanzapine apparently modulates expression of one serotonin receptor—5HT2A—in differentiating neurosphere cultures; however, it does not modify expression of several other receptors or schizophrenia vulnerability genes. Thus, olanzapine has a limited influence on differentiation and gene expression in adult neural precursor cells *in vitro*.

Key words: neurospheres, olanzapine, neurogenesis, gliogenesis, stem cells, cell proliferation.

The effect of psychotropic drugs on the generation of new neurons in the adult brain has attracted a great deal of interest in the last 5 years (reviewed by Duman, 2004). New neurons—if generated in significant numbers and integrated into existing circuits—might influence functional capacity, perhaps providing a previously unimagined therapeutic mechanism (Arango et al., 2001; Wakade et al., 2002; Santarelli et al., 2003; Duman, 2004; Kodama et al., 2004). While such arguments are controversial for several reasons (reviewed by Rakic, 2002; Brooks, 2002; Kempermann et al., 2004; see also Wang et al., 2004), one fundamental question remains unanswered: do any of the drugs act directly on neural precursors thought to generate new neurons and glia in the adult brain. To address this question, we adapted the "neurosphere" assay (Weiss et al., 1996), a standard approach for isolating, propagating and manipulating neural precursors *in vitro* from neurogenic regions *in vivo*, to examine the biological activity of the atypical neuroleptic olanzapine on these multipotent precursors.

Enhanced generation or differentiation of oligodendrocytes, neurons, or astrocytes in the mature brain might underlie the recently reported increase in the proportion of subcortical white matter following chronic olanzapine therapy in schizophrenic patients (Lieberman et al., 2005). Several studies in adult rodents report that chronic olanzapine administration is correlated with a modest increase in bromodeoxyuridine (BrdU) incorporation in the forebrain subventricular zone (SVZ) where resident neural precursors are found (Wakade et al., 2002), as well as in the anterior cortex and striatum (Kodama et al., 2004; Wang et al., 2004). The identity and fate of these cells are uncertain; apparently, most are not neurons and do not survive (Wang et al., 2004). It remains unknown whether olanzapine, acting via cognate receptors (Bymaster et al., 1996, 2001, 2003; Bymaster and Falcone, 2000), modulates proliferation or differentiation of adult precursors that normally give rise to limited numbers of new neurons (reviewed by Gage, 2000). Thus, we characterized receptor expression and evaluated directly the activity of olanzapine on adult forebrain neural precursor cells *in vitro* to better define olanzapine's potential influence on adult neurogenesis.

Our data indicate that olanzapine has little detectable influence on differentiation or proliferation of adult SVZ-derived neural precursors *in vitro*, despite expression of multiple receptors with known affinities in neurospheres and their progeny. Thus, correlations between chronic olanzapine and increased frequency of mitotically labeled cells in the adult brain are unlikely to reflect direct activity of the drug on this class of forebrain neural precursors.

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Abbreviations: ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; BrdU, bromodeoxyuridine; D1-5, dopamine receptors 1–5; Gapdh, glyceraldehydephosphate dehydrogenase; GFAP, glial fibrillary acidic protein; HEPES, 4-(2-hydroxyethyl)-1-piperzineethanesulfonic acid; H1, histamine receptor 1; M1-5, muscarinic acetylcholine receptors 1 through 5; PRODH, proline dehydrogenase; RGS4, regulator of G-protein signaling 4; RT-PCR, reverse transcriptase polymerase chain reaction; SVZ, subventricular zone; 5HT, 5-hydroxytryptamine.

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Nevertheless, indication of a dosage-specific increase in the size of glial fibrillary acidic protein (GFAP) -labeled astrocytes generated from olanzapine-exposed neurosphere precursors, as well as an absolute expression change for one serotonin receptor—5-hydroxytryptamine (5HT) 2A—suggests that olanzapine may modulate, *modestly*, differentiation and gene expression in neural precursors or their progeny, perhaps resulting in differing abilities of any newly generated forebrain neurons or glia to respond to neurotransmitters or psychotropic drugs.

EXPERIMENTAL PROCEDURES

Animals

The UNC-CH Department of Laboratory Animal Medicine maintained the adult male CF-1 mice (Charles River Laboratories, Inc., Wilmington, MA, USA; adult >7 weeks of age) used in this study. Procedures involving animals were approved by the UNC-CH Institutional Animal Care and Use Committee, and met or exceeded all international and U.S. National Institutes of Health guidelines. Every possible precaution was taken to minimize animal suffering, and the minimal number of animals was used for this study. Mice were killed by rapid cervical dislocation, brains and meninges immediately dissected, and brain tissue transferred to ice-cold solution Hanks' balanced salt solution, 1 mM HEPES, for further dissection.

Preparation, harvesting and differentiation of neurospheres

An approximate 1 mm band of tissue containing the SVZ was dissected from coronal brain slices through the anterior lateral ventricle. This tissue was transferred to dissociation medium (Hyaluronidase/Trypsin with DNAse in Neurobasal-A medium; Gibco/Invitrogen, Carlsbad, CA, USA) for 20 min at 37 °C, gently triturated, incubated in the same medium for 16 min, triturated, further digested, and then stopped with trypsin inhibitor. Dissociated cells were filtered through a 40 μm pore size membrane, spun at 700 r.p.m. for 10 min, and resuspended in Neurobasal-A (B27 and N2 supplements/1% penicillin/streptomycin). Cells were plated into 24 well dishes at 5000 cells/well; recombinant human epidermal growth factor and recombinant human fibroblast growth factor (Promega, 20 ng/ml) were added to promote precursor proliferation.

After 9–14 days, subsets of neurospheres (see below) were transferred to Trizol (Invitrogen, Carlsbad, CA, USA) for mRNA extraction for reverse transcriptase polymerase chain reaction (RT-PCR), or selected as individuals and plated singly in laminin/poly-D-lysine-coated chambers of eight well glass chamber slides (LabtekII) for differentiation. For differentiation, spheres were grown for 7 days in Neurobasal-A media (B27 and N2 supplemented, 1% penicillin/streptomycin, and 1% fetal bovine serum) with or without olanzapine. For BrdU incorporation, we added 5 μg/ml of BrdU (Sigma, St. Louis, MO, USA) to the differentiation medium (Haskell and LaMantia, 2005), 24 h after the initial plating of neurospheres, and fixed and stained the cells 24 h later. A delay of 24 h from the initial plating was chosen to ensure that all BrdU incorporation occurred after olanzapine was present in treated cultures, and not in mitotically active neurosphere cells that were in S-phase at the time of plating. We analyzed a total of 164 neurospheres in different conditions, and counted 11,091 cells labeled with a variety of markers.

Olanzapine was provided as a pure compound by Eli Lilly Company (Indianapolis, IN, USA), diluted to 10 mM in 1N HCL/sterile water, and diluted in culture medium to 10 nM, 100 nM and 1 μM. Media and drug were replaced on the 3rd and 5th day following plating. There are no reliable data on the stability of olanzapine in medium; however, the estimated half-life after single doses in patients is between 24 and 30 h, with plasma concentrations of 15 ng/ml 75 h after a single dose (Kassahun et al., 1997; Grothe et al., 2000). Systemic metabolic mechanisms in humans or rodents where olanzapine turnover may be significantly more rapid (Kapur et al., 2003) may be less relevant *in vitro*. The two primary enzymes thought to degrade olanzapine—cytochrome P450 (CYP) 1A2 and flavin mono-oxygenase 3 (Callaghan et al., 1999)—are predominantly found in the liver. Moreover, two of the initial concentrations used here are 10–100 times greater than that estimated to remain in plasma after 75 h in humans, and the affinities of many of the relevant receptors are 1–100 nM. Our cultures are most likely exposed to significant concentrations of olanzpine throughout the 48-hour intervals over which medium was changed and drug refreshed; nevertheless, we cannot be sure that the starting concentrations are maintained over these intervals. Thus, our olanzapine treatment regimen may reflect a pulsatile dosing regimen with peak concentrations of 10 nM, 100 nM and 1 μM available at 1, 3 and 5 days *in vitro*, with some decline in the intervening 48 h.

RT-PCR from neurospheres and neurosphere-derived cultures

Primers for the dopamine, serotonin, muscarinic acetylcholine, GABA and histamine receptors known to bind olanzapine with moderate to high affinity (Bymaster et al., 1996, 2001, 2003; Bymaster and Falcone, 2000) were standardized for annealing temperature, GC content and amplicon length (Table 1). When possible, primers spanned exon/intron boundaries. Each primer set, when used to probe cDNA pools from whole P7, P14, and P60 mouse brain (36 cycles, standard conditions used throughout this study), yielded a single amplicon whose electrophoretic mobility was equivalent to that of the predicted amplicon size (Fig. 1). These amplicons were further verified as cDNA-specific with no-RT controls, and validated by direct DNA sequencing.

Freshly dissected adult SVZ, neurospheres after the initial generation period, or differentiated neurosphere-derived cultures (7 days) were homogenized in Trizol, for mRNA collection. This fraction treated with DNAse (DNA Free, Ambion Inc., Austin, TX, USA) and reverse-transcribed (ImpromptII RT enzyme, Promega Corp., Madison, WI, USA) to yield cDNA. cDNA samples of equivalent starting concentrations were probed to obtain accurate PCR-based comparisons of untreated, differentiated, and olanzapine-treated samples. Concentrations were standardized after quantitative determination of glyceraldehydephosphate dehydrogenase (Gapdh) cycle threshold (Ct; ABI *Taq*-Man quantitative PCR). Quantitative PCR established that an additional gene Hmbs (hydroxymethlybilane synthase) was present in the same proportion as Gapdh, indicating that both genes are stable under the experimental conditions described here, and thus suitable for standardizing cDNA concentrations. These cDNA pools of standard concentration from multiple neurosphere experiments under control and olanzapine-exposed conditions were probed in duplicate for the full set of genes shown in Fig. 1. We analyzed only the absolute detection, or lack thereof, of each transcript in the various samples. Consistent results were obtained for all genes across multiple samples and duplicate experiments. We assessed at least two independent experiments for each gene, and found complete agreement for either expression or lack thereof, in each instance.

Fixation and immunostaining of neurosphere-derived cultures

Differentiated cultures were rinsed with 0.1 M phosphate-buffered saline, fixed for 30 min with 4% paraformaldehyde, rinsed, and immunostained for neuronal and glial markers. We used