Schizophrenia Research 170 (2016) 235–244

Contents lists available at ScienceDirect

Schizophrenia Research

journal homepage: www.elsevier.com/locate/schres

# NeuN$^+$ neuronal nuclei in non-human primate prefrontal cortex and subcortical white matter after clozapine exposure

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ARTICLE INFO

*Article history:*
Received 15 October 2015
Received in revised form 24 December 2015
Accepted 28 December 2015
Available online 6 January 2016

*Keywords:*
Schizophrenia
White matter
Antipsychotic
Clozapine
Flow cytometry
Monkey

ABSTRACT

Increased neuronal densities in subcortical white matter have been reported for some cases with schizophrenia. The underlying cellular and molecular mechanisms remain unresolved.
We exposed 26 young adult macaque monkeys for 6 months to either clozapine, haloperidol or placebo and measured by structural MRI frontal gray and white matter volumes before and after treatment, followed by observer-independent, flow-cytometry-based quantification of neuronal and non-neuronal nuclei and molecular fingerprinting of cell-type specific transcripts.
After clozapine exposure, the proportion of nuclei expressing the neuronal marker NeuN increased by approximately 50% in subcortical white matter, in conjunction with a more subtle and non-significant increase in overlying gray matter. Numbers and proportions of nuclei expressing the oligodendrocyte lineage marker, OLIG2, and cell-type specific RNA expression patterns, were maintained after antipsychotic drug exposure. Frontal lobe gray and white matter volumes remained indistinguishable between antipsychotic-drug-exposed and control groups. Chronic clozapine exposure increases the proportion of NeuN$^+$ nuclei in frontal subcortical white matter, without alterations in frontal lobe volumes or cell type-specific gene expression. Further exploration of neurochemical plasticity in non-human primate brain exposed to antipsychotic drugs is warranted.


## 1. Introduction

Schizophrenia, a major psychiatric disorder significantly impacting quality of life, is commonly treated with antipsychotic drugs but many patients show insufficient responses to current treatments (Lieberman et al., 2005; Swartz et al., 2007). Therefore, the pursuit of new schizophrenia treatments should start, among other approaches, with detailed explorations of transcriptomes (Feher et al., 2005; Girgenti et al., 2010; Iancu et al., 2012; Middleton et al., 2002) and synaptic proteomes (Ji et al., 2009; Ma et al., 2009) in the brains exposed to typical dopamine $D_2$-preferential antagonists and atypical antipsychotic drugs with broader receptor profiles. Changes in cell composition of the antipsychotic-drug-exposed brain have also been reported. Long-term exposure of non-human primates to two widely prescribed antipsychotics, haloperidol and olanzapine, resulted in 8–11% brain weight reduction and volume loss affecting gray and white matter, decreased astro- and oligodendrocyte numbers (Konopaske et al., 2008), together with a 10.2% increase in neuronal densities (Konopaske et al., 2007).

These findings are also of interesting from the viewpoint of the interstitial white matter neurons (WMN), a cell type residing in subcortical white matter of the adult brain. The large majority of WMN are considered remnants of the subplate, a transient structure important for connectivity formation during early development (Kanold, 2004; Kostovic et al., 2011). Interestingly, more than 15 studies have examined postmortem brain tissue and reported supernormal WMN numbers and densities in prefrontal, cingulate and medial or lateral temporal cortex of subjects diagnosed with schizophrenia (Akbarian et al., 1993a; Akbarian et al., 1996; Akbarian et al., 1993b; Anderson et al., 1996; Eastwood and Harrison, 2003, 2005; Ikeda et al., 2004; Joshi et al., 2012; Kirkpatrick et al., 1999; Kirkpatrick et al., 2003; Rioux et al., 2003; Yang et al., 2011). While negative findings have also been published (Beasley et al., 2002; Beasley et al., 2009) most research on this

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http://dx.doi.org/10.1016/j.schres.2015.12.016
0920-9964/

topic indicates that WMN alterations could affect a subset of up to 25% of patients with schizophrenia (Connor et al., 2009). It remains unclear whether increased numbers of WMN in patients with schizophrenia marks a subtype of schizophrenia and if treatment with antipsychotic medication in vivo plays any role. So far, medication-induced changes in WMN never have been explored in a controlled, prospective study. This is both surprising, given the potential importance of antipsychotic drugs, which are widely prescribed to millions of patients, and anticipated, as controlled studies on drug-mediated effects very difficult to conduct on human brain.

Here, we designed an integrative study on 26 macaque monkeys subjected to 6 months of oral intake of haloperidol and clozapine, followed by MRI-based in vivo neuroimaging with scans before and after antipsychotic drug exposure, followed by automated quantification of neuron to glia (non-neuron) ratios in frontal gray and white matter and cell-type specific molecular fingerprinting (Fig. 1). We choose haloperidol and clozapine because these drugs are extensively prescribed prototypes representing conventional antipsychotics primarily acting as dopamine $D_2$ receptor antagonists (haloperidol) or atypical antipsychotics with broader receptor profiles (clozapine). Furthermore, clozapine is generally considered of superior therapeutic efficiency compared to many of the typical or atypical antipsychotics (Meltzer, 2013; Wenthur and Lindsley, 2013). We report increased proportions of nuclei expressing the neuronal phenotypic marker for '*Neuronal Nuclei*' (NeuN) (Mullen et al., 1992) after clozapine exposure, affecting subcortical white matter and more subtle changes in overlying cortex, without affecting cortical volumes or cell type specific gene expression.

## 2. Materials and methods

### 2.1. Animals and antipsychotic drug treatments

26 young adult and drug-naïve rhesus macaques (12 female, 14 male) were randomly assigned to one of the three treatment groups: haloperidol (4 mg/kg/day), clozapine (5.2 mg/kg/day), or vehicle (Table 1). Using previously established protocols (Lidow et al., 1997; Lidow and Goldman-Rakic, 1994, 1997), monkeys were administered antipsychotic drugs orally for six months, mixed with powdered sugar and given in peanut butter or fruit treats. Monkeys received standard enrichment, including social enrichment, human interaction, variety in diet, and age-appropriate objects as directed by the Animal Welfare Act and the Wake Forest University Policy for Non-human Primate Environmental Enrichment. Animal care procedures strictly followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Wake Forest Health Sciences. All 26 animals completed the treatment.

### 2.2. MRI/neuroimaging

*In vivo* imaging studies were conducted on altogether 18 of the 26 animals. Scans from one animal were excluded for technical reasons, leaving 5 controls, 7 haloperidol- and 5 clozapine-treated animals for imaging analyses. Each animal was scanned twice, immediately before the first drug (or vehicle) treatment and again 2 weeks before necropsy. Drug treatment continued until the day of necropsy. Images were acquired on a General Electric 3.0 Tesla Signa MR unit (GE Healthcare Systems) with a human GE quadrature lower extremity coil. During the scanning procedure, the monkeys were anesthetized with 6 mg/kg i.m. of Telazol® (Aveco) and 0.05–0.1 mg/kg i.m. of Acepromazine (Ayerst), and placed in a MRI-compatible head holder. Following a conventional sagittal scout scan (TR = 500 ms, TE = 12 ms, flip angle = 90°), a three-dimensional Inversion Recovery Prepared $T_1$-weighted spoiled gradient echo (SPGR) protocol was used to acquire structural brain images (TR = 14 ms, TI = 300 ms, TE = 3 ms, flip angle = 15°, number of averages = 4, acquisition matrix = 256 × 256). The SPGR scan contained 124 contiguous transaxial slices of 0.5 mm thickness (with no inter-slice gap) through the entire brain.

### 2.3. Image processing and analysis

Image segmentation and analysis routines were performed using standard statistical parametric mapping techniques along with customized scripts in MATLAB 6.5 (Mathworks). The 124 transaxial slices from the SPGR scan were converted into Analyze format and resized to yield isotropic voxels of 0.47 $mm^3$. The image volumes associated with each animal were oriented to the anterior-posterior commissural plane to eliminate slice angle bias.

### 2.4. Image analysis

Skull stripping and tissue-type segmentation was performed, using tools from the Oxford Centre for Functional Magnetic Resonance Imaging of the Brain's Software Library (FMRIB; www.fmrib.ox.ac.uk/fsl). The brains were first extracted using FMRIB's Brain Extraction Tool(Smith, 2002). The output binary mask was adjusted in FSL View.

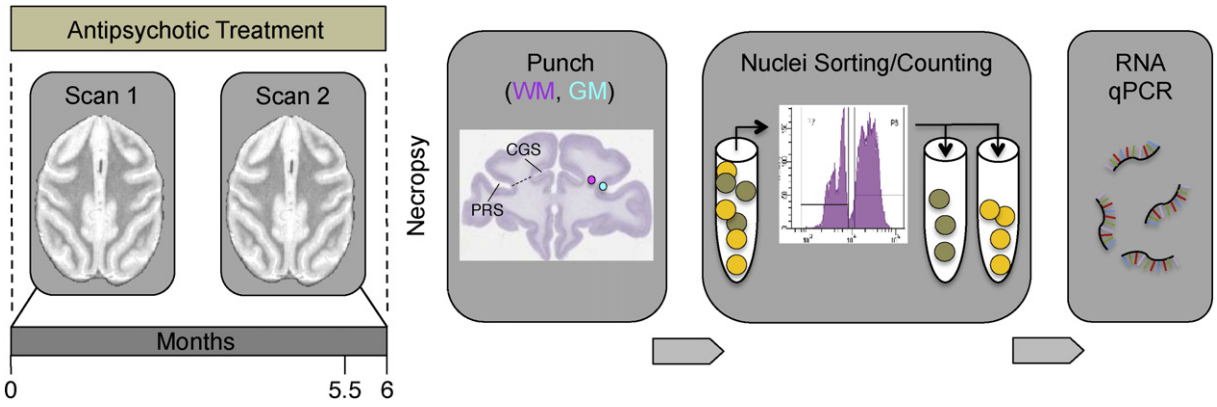


**Fig. 1.** Time line and sequential order of experiments. Monkeys received daily haloperidol and clozapine for 6 months, with in vivo neuroimaging (structural MRI) scans first before, and then again at 5.5 months after begin of treatment. After necropsy, punches from frontal subcortical white matter (WM), and from overlying cortex (GM, gray matter), were obtained for extraction and fluorescence-activated sorting, separation and counting of immunotagged nuclei, followed by PCR-based quantification of RNA extracted from sorted cell-type specific ($NeuN^+$ and $NeuN^-$) nuclei. Nissl-stained coronal image from brainmap.org. PRS = principal sulcus, CGS = cingulate sulcus.