NO synthase-positive striatal interneurons are decreased in schizophrenia

S. Fritzen, M. Lauer, A. Schmitt, T. Töpner, A. Strobel, K.-P. Lesch, A. Reif

Abstract The gaseous messenger NO has repeatedly been suggested to play a role in the pathogenesis of psychoses. Following a pilot study, we investigated whether the number of nitrinergic neurons in the putamen of patients suffering from schizophrenia, bipolar disorder or major depression is altered. Post-mortem striatum sections of 15 brains from patients with either disease were examined by NADPH-diaphorase staining, which selectively labels NO synthase-positive interneurons. Quantification of these cells revealed significantly lower numbers of NO synthase-containing neurons in the putamen of schizophrenic patients. Our results suggest that striatal nitrinergic interneurons are involved in the pathophysiology of at least some forms of schizophrenia, such as e.g. catatonic schizophrenia.

1. Introduction

Nitric oxide (NO) is a gaseous neurotransmitter thought to be implicated in a variety of higher CNS functions. At a molecular level, NO acts as a second messenger of the NMDA receptor in the hippocampus, whereas in the cortex and the basal ganglia, nitrosylation or cGMP-mediated mechanisms are important (Snyder and Ferris, 2000). Furthermore, it nitrosylates monoamine transporters (Kiss and Vizi, 2001). Due to these interactions, NO is a promising candidate molecule in the pathogenesis of endogenous psychoses, both on the genetic (Reif et al., 2006) as well as on the histopathological level (Bernstein et al., 2005). In the human brain, NO is predominantly formed by neuronal NO-Synthase (NOS-I). Initial histochemical studies already indicated an involvement of NOS-I in schizophrenia: by means of the NOS-specific NADPH-diaphorase staining (NADPHd), Akbarian and colleagues demonstrated maldistribution of NOS-positive cells in the frontal and temporal lobes (Akbarian et al., 1993a,b). Other regions in which dysfunction of nitrinergic neurotransmission was found include the brain stem (Karson et al., 1991), the vermis (Karson et al., 1996) and nuclear regions (Bernstein et al., 1998; Garcia-Rill et al., 1995). The striatum has also repeatedly been suggested in the pathogenesis of schizophrenic psychoses. Holt et al. (1999) reported that cholinergic striatal

Abbreviations: Bip, bipolar disorder; DMSO, dimethylsulphoxide; ic, internal capsule; MD, major depression; NADPHd, nicotinamide adenine dinucleotide phosphate diaphorase; NC, nucleus caudatus; NMDA, N-methyl-D-aspartate; NO, nitric oxide; NOS, nitric oxide synthase; PMI, post-mortem interval; Pu, corpus putamen; SMRI, Stanley Medical Research Institute; Sz, schizophrenia.

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interneurons are decreased in schizophrenic patients. Neuropathological studies have revealed altered and decreased in number (Lauer et al., 2005). In the striatum of schizophrenic subjects are morphologically altered and decreased in number (Lauer et al., 2005). In the present study, we sought to verify our initial findings in a larger sample of schizophrenic patients as well as extending the investigation to affective psychoses, under the a priori hypothesis that NADPHd positive cells are reduced in number in schizophrenic patients as compared to controls. Furthermore, the effect of potentially intervening variables was investigated for by means of multiple regression analysis.

2. Experimental procedures

2.1. Subjects

Frozen unfixed sections of the human striatum were obtained from the Stanley Foundation Neuropathology Consortium (Torrey et al., 2000). The collection consisted of 7 slices per brain, collected from 5 subjects suffering from schizophrenia, 15 from bipolar disorder (Bip; 11 thereof featuring signs of psychosis), 15 from major depression (MD) without psychotic features and 15 controls. All groups were matched for age, sex, race, post-mortem interval (PMI) and hemispheric side. Demographic information and medical data including lifetime use of psychotropic medications, history of drug abuse and last prescribed medication were provided by the Stanley Medical Research Institute (SMRI). All experiments were performed blinded to the diagnosis. All experimental procedures were in accordance with the Declaration of Helsinki, and complied with the ethical guidelines of the University of Würzburg.

2.2. NADPH-diaphorase staining

For detection of NOS-I expressing cells in the striatum, seven sections of each brain were processed for NADPHd labelling (Johannes et al., 2003). After 7 min of fixation in 4% paraformaldehyde, slices were washed 3×5 min in Tris–HCl buffer (pH 7.4). Afterwards they were transferred into the staining solution containing DMSO (1.2%, Sigma, Germany), nitro blue tetrazolium (0.4 mg/ml, Sigma), NADPH (2 mg/ml, Sigma) and Triton-X (0.3%, Sigma), dissolved in Tris–HCl. Each slice was incubated overnight under light protection in 1 ml of the staining solution. Subsequently, the slices were washed, air-dried and finally covered with VitroClud (R. Langenbrinck, Germany). Artifacts due to shrinkage were avoided since no aggressive dehydration procedure by passages in ascending grades of alcohol was performed.

2.3. Quantification

Human brain sections were examined semi-quantitatively to compare between different diagnostic entities using a brightfield Leica microscope (DMRBE, Wetzlar, Germany). Microphotographs were taken with a digital camera mounted on the microscope. Cell counts in the putamen were performed in a systematic random manner (Fig. 1A, B); as all counted particles were of comparable size, no Abercrombie correction was applied. By combination of tube length, eyepiece (10×/25) and objective (10/0.30), a microscopic field of 1 mm² (equivalent to a sampling volume of 0.014 mm³), thickness of slices 14 μm, was defined. While the first field to be counted was chosen randomly, further fields followed in a defined distance of 2 mm.

2.4. Statistics

The average number of NADPHd labelled cells is reported as means±SEM NOS-positive cells/mm² putamen (equivalent to 0.014 mm³). Multiple regression analyses were performed to assess whether NADPHd cell number is influenced by potentially confounding variables (age at death, gender, brain pH and weight, days of storage, post-mortem interval (PMI), and brain hemisphere; Table 1) in all subjects. As

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Brain hemisphere (n right)</th>
<th>Age at death (yrs)</th>
<th>pH</th>
<th>PMI (h)</th>
<th>Storage (days)</th>
<th>Onset of disease (age)</th>
<th>Disease duration (yrs)</th>
<th>Neuroleptic treatment (lifet ime quantity of fluphenazine or equivalent; mg)</th>
<th>Received APs (n)</th>
<th>Severity of alcohol use (n)</th>
<th>Suicide (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>48±10</td>
<td>6.2±0.2</td>
<td>23±9</td>
<td>338±234</td>
<td>n.a</td>
<td>n.a.</td>
<td>n.a.</td>
<td>15</td>
<td>1.0±1.0</td>
<td>n.a.</td>
</tr>
<tr>
<td>Schizophrenia</td>
<td>6</td>
<td>44±13</td>
<td>6.1±0.2</td>
<td>33±14</td>
<td>621±233</td>
<td>23±8</td>
<td>21±11</td>
<td>52266±62061</td>
<td>1</td>
<td>1.9±1.7</td>
<td>4</td>
</tr>
<tr>
<td>Bipolar disorder</td>
<td>8</td>
<td>42±12</td>
<td>6.1±0.2</td>
<td>32±16</td>
<td>620±172</td>
<td>21±8</td>
<td>20±10</td>
<td>20820±24015</td>
<td>3</td>
<td>2.8±1.8</td>
<td>9</td>
</tr>
<tr>
<td>Major depression</td>
<td>6</td>
<td>46±9</td>
<td>6.1±0.2</td>
<td>24±11</td>
<td>434±290</td>
<td>34±13</td>
<td>13±11</td>
<td>n.a.</td>
<td>15</td>
<td>1.9±2.0</td>
<td>7</td>
</tr>
</tbody>
</table>

Every diagnostic group comprised of 9 males and 6 females. Severity of alcohol abuse was graded qualitatively by the SMRI from 0 to 5. APs, antipsychotics; n.a., not applicable.
alcohol is known to inhibit NO production, a dichotomized measure of severity of alcohol abuse was also considered as predictor in the multiple regression analysis. Furthermore, the influence of onset and duration of disease as well as the cumulative lifetime dosage of fluphenazine was examined (in patients only). Thereafter, differences between schizophrenic patients and controls, as well as between all diagnostic groups were determined by ANOVA. According to our a priori hypothesis of a reduced number of NADPHd-positive cells in schizophrenia, we employed a one-tailed level of significance of 5% for the comparison between schizophrenic patients and controls. For all other tests, a two-tailed level of significance of 5% was used.

3. Results

NADPHd staining resulted in reliably stained striatal interneurons in every brain and every slice. All NADPHd-positive neurons displayed a homogeneous staining of cell somata and dendrites (Fig. 1C–E) enabling quantification. Multiple regression analysis, incorporating age at death, gender, brain pH and weight, days of storage, PMI, hemisphere, and severity of alcohol abuse yielded no significant effects of these variables on NADPHd cell number (all subjects: all single predictors $p \geq 0.417$, total model $p = 0.953$). Similarly, onset and duration of disease as well as of total lifetime quantity of fluphenazine had no significant influence on NADPHd cell number in patients (all single predictors $p \geq 0.325$, total model $p = 0.764$). Thus, these potentially confounding variables did not seem to have a major impact on the number of NOS-positive cells in the putamen and therefore, these covariates were not further controlled for.

We then examined whether the number of nitrinergic interneurons is altered in the putamen of patients with psychiatric disorders. Neuron counts in all three diagnostic groups were performed as described and compared to controls. First, we tested our a priori hypothesis that schizophrenic patients displayed a significant reduction in the amount of NADPHd-positive cells by means of ANOVA. This indeed was the case, as NADPHd labelled cells were significantly decreased by 20% in the putamen of schizophrenic patients ($1.78 \pm 0.19$ cells/mm$^2$ vs. $2.21 \pm 0.09$ cells/mm$^2$ in controls; $p = 0.037$; Fig. 2). Partial $\eta^2$ as a measure of explained variance was substantial.
hoc ANOVA (all psychotic features, subjects have additionally been categorized subjects (1.84±0.15 cells/mm² vs. 2.18±0.10 cells/mm²; NADPHd positive cells as compared to non-psychotic symptoms displayed a significant decrease in the amount of MD, CRTL) of psychotic features. Patients showing psychotic in two groups, absence (11 Bip, 15 Sz) vs. presence (4 Bip, 15 controls. We succeeded to replicate our initial finding that reduced in number. The present study extended our previous examination to a set of 15 schizophrenic subjects, compared to patients suffering from affective psychoses as well as controls. We succeeded to replicate our initial finding that NOS-positive neurons are significantly reduced in schizophrenia, but not in affective disorders and healthy subjects. NADPHd-positive cells have been counted by a “systematic random” method as described. Each bar represents the means of 15 subjects, and each dot the means of 7 putaminal sections in one individual. There was no difference in the mean number of stained cells between major depression/bipolar disorder and controls, but a significant reduction of NADPHd expressing cells in the putamen of schizophrenic subjects. The line represents a cut-off value of 1.5 NADPHd labelled cells/mm², below which no control subjects were found. 3 of the 4 bipolar subjects below the threshold suffered from psychotic symptoms. *, p<0.05 (Student’s t-test).

(0.127), i.e. about 13% of the variation in the number of NADPHd labelled cells was explained by the presence vs. absence of Sz. No significant changes were detectable in patients with MD (2.15±0.13 cells/mm²) and Bip (1.99±0.24 cells/mm²) in post-hoc ANOVA (all p>0.05). As 73% of Bip patients displayed also psychotic features, subjects have additionally been categorized in two groups, absence (11 Bip, 15 Sz) vs. presence (4 Bip, 15 MD, CRTL) of psychotic features. Patients showing psychotic symptoms displayed a significant decrease in the amount of NADPHd positive cells as compared to non-psychotic subjects (1.84±0.15 cells/mm² vs. 2.18±0.10 cells/mm²; p=0.029).

4. Discussion

Evidence is accruing that NO plays a role in the pathophysiology of neuropsychiatric disorders, such as vascular dementia, Alzheimer’s disease and Huntington’s disease, which show pathophysiological changes in NO metabolism (for critical reviews see Akyol et al., 2004; Bernstein et al., 2005). Especially in schizophrenia there is increasing support for an involvement of NOS (summarized also in Bernstein et al., 2005). Accordingly we showed in a pilot study (Lauer et al., 2005) that at least in some patients putaminal nitrinergic neurons are either morphologically abnormal or reduced in number. The present study extended our previous examination to a set of 15 schizophrenic subjects, compared to patients suffering from affective psychoses as well as controls. We succeeded to replicate our initial finding that NOS-positive neurons are significantly reduced in schizophrenia by 20%; this also held true for subjects with psychotic features in general. Medication effects were not evident from the provided data, and also substance abuse did not seem to influence the results.

Recently, a study employing MRI scanning demonstrated that total putaminal volume is unchanged in schizophrenic patients, however putaminal white matter is reduced and grey matter is increased (Tamagaki et al., 2005). This correlates well with our previous finding that the number of neurons is increased in the putamen of schizophrenics (Beckmann and Lauer, 1997). Considering this, the reduction in NOS positive neurons demonstrated in the present study is even more pronounced. The implications thereof are as yet elusive, however a hypothetical picture can be envisaged: We showed that two functional NOS1 promoter polymorphisms are associated with schizophrenia as well as psychopathological, neuropsychological, and electrophysiological measures (Reif et al., 2006). These regulate the expression levels of alternative first exons, both of which are present in the putamen at high levels (Reif et al., 2006), with the at-risk variation resulting in an approximately 30% decrease in expression (Saur et al., 2004). Thus, a genetic predisposition towards decreased putaminal NOS expression might further aggravate the reduction in NOS-I striatal interneurons as both mechanisms impact on the synaptic transmission in basal ganglia circuits. NO modulates the firing patterns of dopaminergic striatal neurons (West and Grace, 2000). It has been suggested that NO signalling integrates input from prefrontal cortex and the dopaminergic nigrostriatal system thereby influencing striatal output. The fine-tuning of dopaminergic afferents to the striatum is most probably accomplished by balancing tonic and phasic firing of dopaminergic neurons (West et al., 2002): Loss of nitrinergic neurons will lead to an impaired tonic dopaminergic striatal output as regulated by glutamatergic cells, probably due to disinhibition of the dopamine transporter, and furthermore, impaired NO signalling might result in glutamatergic dysfunctioning of corticostriatal afferents. Consequently, a disturbance of NO functioning will eventually result in the disruption of the glutamatergic control of striatal dopamine output, thereby contributing to the etiopathology of schizophrenic psychoses. Besides the dominating dopaminergic system, other neurotransmitter systems like the cholinergic system however are dysfunctional as well: Holt et al. (1999) e.g. showed a deficit in cholinergic interneurons in the striatum of schizophrenic patients, which was predominant in the ventral striatum (Holt et al., 2005). As those neurons are excitable upon NO release (Centonze et al., 2001), which in vivo appears to be due to NOS-I (Buchholzer and Klein, 2002), a decrease in NOS-positive neurons might also further promote cholinergic deficits. Together, the reported reduction of NADPHd-positive neurons in the putamen of schizophrenics thus fits well into current concepts of dysbalanced intrastriatal neurotransmission and warrants further investigations.

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References


