Morphological Abnormalities in Nitric-Oxide-Synthase-Positive Striatal Interneurons of Schizophrenic Patients

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Key Words
Schizophrenia · Neuropathology · Nitric oxide synthase · Interneurons, striatal · Putamen · Nicotinamide adenine dinucleotide phosphate diaphorase

Abstract
Schizophrenia has been suggested to be a neurodevelopmental disorder, and nitric-oxide-synthase (NOS)-positive neurons were shown to be involved in distorted cortical development in schizophrenia. Here we investigated whether nitrinergic neurons in the striatum of schizophrenic patients also display abnormalities regarding distribution or morphology. To do so, postmortem putaminal sections of schizophrenic subjects were examined by means of nicotinamide adenine dinucleotide phosphate diaphorase (NADPHd) staining and NOS immunohistochemistry. NOS-positive neurons were counted and analyzed morphologically. Abnormalities regarding morphology or number of NOS-containing neurons could be found in the putamen of schizophrenics (n = 3), but not controls (n = 5). Neurons were either of abnormal size and branching pattern, or they were markedly reduced (130 ± 44 vs. 54 ± 62 NADPHd-positive somata/mm³ putamen; p < 0.0001). Striatal nitrinergic interneurons might thus be involved in the pathogenesis of at least some forms of schizophrenia. Studies on larger samples are however needed to further corroborate this finding.

Introduction
The pathophysiology of schizophrenic disorders is still only poorly understood. While it seems to be established that genetic factors play an important role, the identification of relevant genes is still in the beginning and has probably been hampered by the genetic heterogeneity of schizophrenic psychoses. Likewise, neuropathological findings are inconclusive, yet there is a growing body of evidence arguing that schizophrenia constitutes a neurodevelopmental disorder [1]. Underlying pathologies might include deviant neuronal migration [2–4] or morphological abnormalities of distinct neuronal populations [5, 6]. These disparate findings probably further point to an etiological and pathophysiological heterogeneity of schizophrenic psychoses and might reflect varying gene × gene or gene × environment interactions.

Several converging lines of evidence suggest that the striatum is probably involved in the pathogenesis of...
schizophrenic psychoses [7–10], and, in a previous report, cholinergic striatal interneurons were found to be decreased in schizophrenia [11]. We could previously show that the striatal volume, when corrected for total hemisphere volume, is increased in schizophrenic patients [10] and that this is paralleled by an increased number of neurons in the nucleus caudatus/nucleus accumbens complex. However, the molecular basis for striatal pathology still remains elusive.

The gaseous messenger molecule nitric oxide (NO) can be found abundantly in striatal interneurons, where it has been suggested to balance the dopaminergic tone of the striatum. It acts as a second messenger of glutamate [12], thus linking two transmitter systems crucially involved in the pathogenesis of schizophrenia. Furthermore, several studies argued for an involvement of the NO-producing enzyme NO synthase type I (NOS-I) in schizophrenia: Akbarian et al. [3, 4] e.g. could show a malformation of NOS-positive cells in the frontal and temporal lobes. Hence, it seems worthwhile to search for pathological alterations of striatal NOS-positive neurons. To do so, we have established a morphological classification of nitrergic striatal interneurons [13] based on the nicotinamide adenine dinucleotide phosphate diaphorase (NADPHd) staining method, which represents neuronal NOS-I. In the present pilot investigation, we examined whether NADPHd-positive neurons in the putamen of schizophrenic patients show abnormalities compared to healthy controls. According to the findings of Akbarian et al. [3, 4], as well as animal studies [14–17], we hypothesized that either decreased cell counts or abnormal NADPHd-positive neurons are present in the striatum of schizophrenic patients.

**Methods**

Three brains from schizophrenic subjects were obtained by the New Brain Collection of the Department of Psychiatry, University of Würzburg (D. Senitz, Neurobiological Laboratory). The investigation complied with the ethical guidelines of the University of Würzburg. Tissue was collected at autopsy and was free of significant macroscopic pathological alterations as evidenced by hematoxylin-eosin, Nissl, Palmgren, Heidenhain, periodic acid-Schiff and van Gieson staining. Five brains were used as controls; clinical data on patients and controls are specified in table 1. Tissue processing and NADPHd histochemistry followed a protocol described in detail elsewhere [13]. Briefly, 75-μm-thick slices of the putamen were incubated for 21 h in a staining solution containing 1.2% dimethylsulfoxide, 0.4 mg/ml nitro blue tetrazolium, 2 mg/ml NADPH and 0.3% Triton-X (all chemicals were from Sigma Chemicals, Deisenhofen, Germany). More than 10 slices of each case were investigated, and approximately 1,000 neurons of each brain were examined. Neurons were classified according to a previously published classification system [13].

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<th>Table 1. Clinical data on the used cases</th>
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HPD = Haloperidol; PMI = postmortem interval; Sz = schizophrenia.
A three-dimensional stereological method [18] was used for counting the density of NADPH-positive neurons. At least 10 microscopic fields per slice were selected randomly of complete rostral, intermediate and caudal slices of the putamen, and all NADPH-positive neurons within this volume of 0.157 mm³ were counted. Every NADPHd-stained soma was counted in each microscopic field. All examinations were done blinded for diagnosis.

Tissue sections were also stained immunohistochemically for NOS-I. Following blocking, mounted sections were incubated overnight with rabbit anti-NOS-I polyclonal antibody (1:750, Chemicon, Calif., USA). For the detection of the NOS-I antibody, a biotinylated goat antirabbit IgG antibody was applied (1:400, Vector Laboratories, Burlingame, Calif., USA) in combination with the avidin-biotin-peroxidase complex (Vector Laboratories) and 3,3'-diaminobenzidine (Roche, Mannheim, Germany).

**Results**

We first examined whether NADPHd-positive neurons are morphologically abnormal. To do so, NADPHd staining was performed in 3 schizophrenic and 5 control brains. Approximately 1,000 neurons per brain were examined and compared to a previously established classification [13]. In schizophrenic brains only, two types of morphologically abnormal cells could be found (fig. 1), which were markedly different from all other NADPHd-positive cells described in our or other classification systems: whereas the first type was larger than any other nitrinergic cell observed in control brains (>600 μm), the other cell type was the smallest NOS-positive neuron in our sample (diameter 150 μm including the whole visible dendritic field). The giant cell type had a pear-shaped soma, 3–4 thick and slightly curled dendrites whose branching level was intermediate; the shape was asymmetric and neurons appeared bizarre. The small cells were entirely different, displaying a round, medium-sized soma, 4 thick and short dendrites which hardly branched. These neurons were however observed only in 2 of the 3 brains of schizophrenic patients (subjects P and Q, table 1).

To assess whether nitrinergic interneurons are also altered in number, neuron counts in schizophrenic and control subjects were performed by means of NADPHd, which has the advantage that the staining is very robust compared to immunolabeling, thus preventing common problems with quantitative immunohistochemistry cell counts. In 2 of the 3 brains from schizophrenic subjects (subjects P and R, table 1), the number of NADPHd-positive interneurons in the putamen was markedly decreased (fig. 2; fig. 3b, d, f), and thus the mean number of NOS-positive neurons in schizophrenic subjects was lowered from 130 ± 44 to 54 ± 62 NADPHd-positive somata/mm³ putamen (fig. 2; p < 0.0001, Mann-Whitney U test: sum of ranks in a, 410; sum of ranks in b, 718; lower 95% confidence limit 110 vs. 28 cells/mm³, upper 95% confidence limit 150 vs. 80 cells/mm³). This was selective for the putamen and not due to staining artifacts, as neighboring anatomical structures, i.e. the caudate nucleus, were stained normally. The reduction of NADPHd-stained neurons was paralleled by a substantial decrease in NOS-I immunohistochemistry (fig. 3a–d), arguing against breakdown of the enzymatic NADPH conversion but for an actual decrement of NOS-I enzyme. No rostro-caudal gradient was evident for the reduction in NOS-positive neurons.
Discussion

Our findings suggest an involvement of nitrinergic interneurons in the pathophysiology of schizophrenic disorders; however, due to the very limited sample size, they should be regarded as preliminary, and further studies are clearly warranted. In each of the 3 investigated brains, pathological findings were present, although they were different, as neurons were abnormal either in morphology or in number. This could make a case for schizophrenia being a heterogeneous disorder with varying underlying pathologies and thus diverging findings, as suggested in previous pathoanatomical studies [11]. Psychopathologically, case P had marked catatonic features without remission, while Q displayed both paranoid as well as catatonic symptoms, with a bipolar, yet chronic course. Case R had chronic paranoid delusions and hallucinations. Thus, the different findings on striatal pathology in the present study might reflect different pathologies resulting in distinct schizophrenia subtypes, as abnormal cells were only present in patients with catatonic features. Due to the small sample size, of course no firm conclusions can be drawn on this topic; however, it is intriguing to speculate that abnormalities of striatal interneurons could be associated with specific schizophrenia types, such as catatonia. Further studies linking schizophrenia subtypes, or endophenotypes, to histopathological findings are clearly needed to untangle the heterogeneity of the schizophrenic spectrum. Variables to be considered at least should include the criterion of remission and bipolarity.

Alternatively, the heterogeneous findings in the present study might reflect different stages of pathology. This hypothesis can be derived from the fact that morphologically abnormal neurons could not be found in the brain with the smallest number of NADPHd-positive neurons. A trajectory from morphological abnormality to degeneration and ultimately cell death provides a possible explanation for this finding, which however cannot be deduced from the present examination and should be tested in further studies. Of course we cannot rule out that psychotropic medication had an impact on NADPHd-positive neurons. Arguing against this, it was shown that administration of atypical antipsychotics in rats did not alter the number of NOS-positive striatal neurons [19]; comparable studies with typical antipsychotics are not available. As all of our patients have been treated with typical antipsychotics, we can thus not exclude the possibility of drug-induced neuronal abnormalities, especially when methodological problems comparing animal studies with human data are taken into account. Most interestingly, a recent study demonstrated that haloperidol-induced tardive dyskinesia was associated with decreased NOS activity in an animal model, yet enzyme expression or neuronal morphology have not been assessed [20–22]. Thus, if the reduction of NOS-positive neurons is due to treatment with typical neuroleptics and not connected to schizophrenia per se, it might however contribute to the pathophysiology of extrapyramidal side effects.

Given that the morphological and numerical abnormalities found in the present studies are not due to medication, how can these findings be incorporated in the
conceptual framework of the pathogenesis of schizophrenia? Evidence for an involvement of NO therein arises from three lines of evidence. First, in the cortex of the frontal and temporal lobes malformations of nitrinergic neurons have already been described in schizophrenic patients [3, 4], although mRNA measurements yielded quite contradictory results [23]. However, in agreement with the findings of Akbarian, reduced NOS activity in the prefrontal cortex was found, although NOS-I and NOS-III protein levels were normal [24]. Also in the hypothal-
amiss, a reduction of NOS-I immunoreactive neurons could selectively be demonstrated in schizophrenics in the right paraventricular nucleus [25]. In contrast to those studies, an increase – or no change – in NOS-I levels was demonstrated in the cerebellum by varying methods [26– 28]. Whether this reflects a compensatory mechanism or whether this is not related to striatal pathology is as yet unclear, as data from the striatum and cerebellum are derived from different brains. In future studies, both regions should thus be investigated simultaneously.

Second, NO is critically involved in the modulation of striatal output. NO, generated by NADPH-d-positive interneurons, is supposed to balance phasic and tonic striatal dopamine transmission [12], thereby linking glutamatergic input from the (pre-)frontal cortex and dopaminergic nigrostriatal system. As this important modulatory role of NO seems to depend on behavioral arousal, nitrinergic striatal interneurons integrate sensory information from the prefrontal cortex and motor activity, a circuitry which might well be disturbed at least in some forms of schizophrenia with psychomotor symptoms, i.e. catatonia. Given the apparent complexity of NO/dopamine/glutamate interactions in the striatum, disruption or dysbalance of the nitrinergic system will have an impact on striatal output, as already shown pharmacologically [29, 30] and behaviorally [31, 32].

The third line of evidence comes from the role of NO in neurodevelopment and its differential expression pattern in the developing brain [33, 34]. In the rodent striatum, NOS-positive neurons can be found from day E18 on, reaching their peak number 3–4 weeks postnatally. Nitrinergic neurons during brain development can be found in patches together with tyrosine-hydroxylase-containing nerve cells, further highlighting the close interplay between NO and dopaminergic transmission. Interestingly, several risk factors for schizophrenic psychosis could be shown to have an impact on NOS, at least in animals. In utero influenza virus infections, an established risk factor for schizophrenic psychosis, result in significant decreases in NOS expression [14]; hippocampal lesioning, another schizophrenia model, also reduces the number of NOS-positive neurons in the prefrontal and entorhinal cortex [15]. Postnatal stress in the form of nonhandling, which was shown to mimic behavioral disturbances that resemble schizophrenia, results in decreased NOS expression [16, 35]. Finally, neonatal NOS inhibition resulted in amphetamine and phencyclidine hypersensitivity along with deficits in prepulse startle inhibition [17]. Together, these findings converge to the notion that impairment of the nitrinergic pathway during brain development and maturation might contribute to the pathogenesis of schizophrenia.

In conclusion, we thus suggest that abnormalities of striatal nitrinergic interneurons, as found in the present study, might have a role in at least some forms of schizophrenic disorders, yet further studies (which are under way in our laboratory) with larger sample sizes are needed to corroborate this result.

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References


