Upregulation of the initiating step of the kynurenine pathway in postmortem anterior cingulate cortex from individuals with schizophrenia and bipolar disorder

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ABSTRACT

Upregulation of the kynurenine pathway has been associated with several etiologies of psychosis, an indication that increased levels of pathway intermediates might be involved in eliciting some psychotic features. In schizophrenia, tryptophan 2,3-dioxygenase (TDO2) was previously identified in postmortem frontal cortex as the enzyme likely responsible for the reported increase in pathway activity in the brain. For this follow-up study of postmortem anterior cingulate gyrus, we have found evidence of increased TDO2 activity at three different levels of regulation: mRNA, protein, and metabolic product. The results were unaffected by neuroleptic status or smoking history. To make the distinction between mental disorders with psychosis and those without, this study included patients with bipolar disorder and major depression. Compared to the control group, the HPLC, RT-PCR, and immunohistochemistry results show significant elevation of (1) kynurenine in schizophrenia (1.9-fold, P = 0.02), and in bipolar disorder (1.8-fold, P = 0.04), primarily in the bipolar subgroup with psychosis (2.1-fold, P = 0.03); (2) TDO2 mRNA in schizophrenia (1.7-fold; P = 0.049); and (3) the immunohistochemistry values for the density of TDO2-positive white matter glial cells in schizophrenia (P = 0.01) and in major depression (P = 0.03) as well as the density and intensity of glial cells (in both gray and white matter) stained for TDO2 in bipolar disorder (P = 0.02). Unlike the results for schizophrenia and bipolar disorder, the increase in TDO2 protein in the major depression group was not associated with an increase in kynurenine concentration.

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1. Introduction

Activation of the kynurenine pathway appears to be a common feature of disorders involving psychotic manifestations. Previous studies of schizophrenia have demonstrated a significant elevation in the metabolites kynurenine and kynurenic acid in postmortem pre-frontal cortex samples (Schwarcz et al., 2001), an elevation in kynurenic acid in the CSF (Erhardt et al., 2003) and, in the postmortem frontal cortex, an elevation in tryptophan 2,3-dioxygenase (TDO2; Miller et al., 2004a) which catalyzes the first step in the kynurenine pathway (Fig. 1). In addition, increased levels of kynurenine

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pathway metabolites have been found in brain samples of patients with AIDS dementia (Heyes et al., 1998; Bara et al., 2000) and with Huntington’s disease (Guidetti et al., 2000), though not in the CSF of Huntington’s patients (Heyes et al., 1991). Pellagra and, in its severe form, psychotic pellagra activate TDO2 through loss of feedback inhibition by niacin (Badawy and Evans, 1976; Cho-chung and Pitot, 1967, 1968). An animal model of porphyria, a heme disorder that can elicit psychosis in humans, also shows kynurenine pathway upregulation (Morgan and Badawy, 1992).

Whether specific kynurenine pathway metabolites might be associated with particular symptomatology is not clear. Both 3-hydroxykynurenine and quinolinate are neurotoxins (Guidetti and Schwarz, 1999), though the levels of quinolinate expected to result from a less than 2-fold increase in pathway activation in schizophrenia (Miller et al., 2004a; Schwarz et al., 2001) may not be high enough to cause substantial neuronal stress (Obrenovitch and Urenjak, 2003). In contrast to quinolinate, the metabolite kynurenic acid is considered to be generally neuroprotective (Foster et al., 1984; Moroni, 1999; Stone, 1993). It is perhaps through its action as an antagonist of the nicotinic alpha-7 receptor (Erhardt et al., 2001; Hilmas et al., 2001) that kynurenic acid would be expected to cause phenotypic changes seen in schizophrenia, such as disrupted auditory gating (Freedman et al., 2003; Shepard et al., 2003). Alternatively, the accumulation of these or other intermediates may result in psychotomimetic products from non-traditional reactions catalyzed by enzymes that are not considered to be part of the kynurenine pathway.

In this study, we expanded the scope of prior results (Miller et al., 2004a) by including four diagnostic groups (schizophrenia, bipolar disorder, major depression, and controls), by examining another brain region (anterior cingulate) that has been implicated in the limbic system circuitry considered important for psychosis (Heckers and Goff, 2002) and by carrying out three types of analyses for each brain sample: RT real-time PCR for TDO2; immunohistochemistry for TDO2 protein, and HPLC analyses for the metabolites kynurenine and kynurenic acid.

2. Results

2.1. RT real-time PCR measurement for TDO2 mRNA

RT real-time PCR was used to determine the GAPDH values, both to normalize the TDO2 results and to screen out the RNA samples that were extensively degraded (Miller et al., 2004b). The percent error for triplicate analyses of the sample set was ±12% for the GAPDH PCR. In approximately one third of the samples (17/54), the RNA quality was not suitable for inclusion in the data set based on the GAPDH value (Experimental procedures).

RT real-time PCR for TDO2 in the remaining samples revealed a significant, 1.7-fold elevation in the schizophrenia group as compared to controls (P = 0.049; Fig. 3 lower panel) and no significant difference between the other groups and controls. Restricting the bipolar group to bipolar with psychosis (n = 6) did not increase the difference in TDO2 seen for that group relative to controls. The percent error for quadruplicate analyses of the sample set was ±21% for the TDO2 PCR.

There was no significant correlation between TDO2 mRNA and the downstream product of its activity, kynurenine, in any of the groups.

2.2. HPLC measurement of kynurenine and kynurenic acid

The results for the sample set show that kynurenine was elevated in the schizophrenia group (1.9-fold, P = 0.02) and in the bipolar group (1.8-fold, P = 0.04) as compared to control values (Fig. 3, upper panel). Within the bipolar group, the majority of the patients (10/14) were diagnosed as having psychotic symptoms. Fig. 2 illustrates that the kynurenine values were 2.1-fold elevated in the bipolar-with-psychosis group (P = 0.03) as compared to controls and not significantly elevated in the bipolar-without-psychosis group (1.3-fold, P = 0.36). The percent error in the kynurenine analysis, including the sectioning variation, was ±22%.

Although kynurenic acid exhibited the same inter-group pattern as kynurenine (Fig. 3, upper panels), the difference in
Kynurenic acid between controls and cases was not significant. With respect to the diagnosis of psychosis in bipolar disorder, the kynurenic acid was not significantly elevated in the bipolar-with-psychosis group (1.5-fold, P = 0.21) and equal to that of the controls in the bipolar-without-psychosis group (Fig. 2). The percent error in the kynurenic acid analysis, including the sectioning variation, was ± 11%.

The correlation between kynurenine and kynurenic acid values within each group was significant: for schizophrenia, r = 0.80, P = 0.0001; for bipolar disorder, r = 0.54, P = 0.0001; for major depression, r = 0.65, P = 0.0001; and for controls, r = 0.38, P = 0.001. As compared to controls, each of the patient groups was lower in the ratio of kynurenic acid/kynurenine; however, this difference trended to significance only in the schizophrenia group, for which the ratio was 32% lower than in the control group (P = 0.09).

### 2.3 Immunohistochemistry for TDO2

The sections used for immunohistochemistry were not immediately adjacent to those used for the RNA analyses but were acquired from the same region of the contralateral formalin-fixed hemisphere. Neurons, glial cells, and vessels were immunostained in the brains of the controls and the disease groups. In all sample groups, TDO2-A stained vessels strongly but rarely stained both neurons and glial cells strongly but rarely stained vessels (Figs. 4A–D). The staining of neurons and glial cells was cytoplasmic (Figs. 4A–D). The immunopositive glial cells were identified as astrocytes based on morphological criteria: round vesicular nuclei, no evident nucleoli, scanty cytoplasm, and no Nissl substance. In addition, the identification of astrocytes was corroborated by immunopositivity of a subgroup of these cells for GFAP as revealed on double labeling (Fig. 4). However, it should be noted that not all TDO2-immunopositive astrocytes were GFAP-positive.

**Fig. 2** – Upper panel: kynurenine is significantly elevated in bipolar patients with psychosis as compared to controls. The difference between bipolar patients without psychosis and controls is not as large. Lower panel: kynurenic acid is not significantly elevated in bipolar patients with or without psychosis compared to controls.

**Fig. 3** – Increase in kynurenine and TDO2 mRNA in disorders involving psychosis. Shown are the median values (solid horizontal line in box), 95% confidence limits (whiskers) and the outlier values for schizophrenia, major depressive disorder, controls and bipolar disorder (bipolar with and without psychosis grouped as one). Upper panel: kynurenine is significantly increased in schizophrenia compared to controls. The RT-PCR fold values for TDO2 are presented as values normalized for the housekeeping gene GAPDH.
None of the TDO2-immunopositive cells could be identified as microglia. In large vessels, the luminal and abluminal lining of the vessel wall and endothelial cell cytoplasm were stained. The entire wall of capillaries was also stained. There were statistically significant differences between the two antibodies for each examined parameter \( (P \leq 0.0001, \text{data not shown}) \).

The findings of the immunohistochemical ratings for the disease and control groups are displayed in Table 1.

2.3.1. Controls versus schizophrenia
A statistically significant increase \( (P = 0.01) \) in the density score for TDO2-A-immunopositive astroglial cells in the white matter was found for the schizophrenia group compared to the control group \( \text{(WM-glia-density, Table 1)} \). This increase was also reflected in the significant increase of the compound value \( (\text{intensity} \times \text{density}, P = 0.01) \) in Table 1 \( \text{(WMGLIA)} \). However, there was no significant difference for the intensity score of TDO2-A-immunopositive cells in the white matter. In the gray matter of the cerebral cortex, there were no significant differences in the density and intensity scores or the compound value of labeled glial cells between both groups. The compound value summed for gray and white matter glial cells \( \text{(GLIA in Table 1)} \) was significantly increased in the schizophrenia group \( (P = 0.01) \). There were no significant differences in the density and intensity scores of neurons or vessels in the gray matter between the two groups. There was a trend towards statistical significance \( (P = 0.06) \) for the intensity score and compound value of cortical glial cells when using the anti-TDO2-B antibody \( \text{(results not shown in tabular form)} \).

2.3.2. Controls versus bipolar disorder
With the anti-TDO2-A antibody, the compound value for glial cells, from the gray and white matter summed was significantly increased in the bipolar group \( \text{(Table 1, GLIA, } P = 0.02) \). However, no significant changes between the control and the bipolar group were found for any of the other evaluated parameters. There was a trend towards statistical significance \( (P = 0.06) \) for the intensity score of cortical and white matter glial cells when using the anti-TDO2-B antibody \( \text{(results not shown in tabular form)} \).

2.3.3. Controls versus major depression
In the depression group, there was a significant increase \( (P = 0.03) \) in the density score of TDO2-A-immunopositive glial cells in the white matter \( \text{(Table 1, WM-glia-density, } P = 0.03) \) and a trend towards statistical significance \( (P = 0.06) \) for the compound value of white matter glial cells \( \text{(Table 1, WMGLIA)} \). There was also a significant increase in the compound value for glial cells, from the gray and white matter summed up, in the group of patients with depression \( \text{(Table 1, GLIA, } P = 0.02) \). No significant changes were seen when using the TDO2-B antibody.

2.4. Psychosis in bipolar disorder
There was a significant difference between controls \( (n = 14) \), bipolar patients without psychosis \( (n = 4) \), and bipolar patients with psychosis \( (n = 11) \) for the compound value of total glial cells summed for gray and white matter \( \text{(data not shown in Tabular Form)} \).
tabular form). The compound value for glia averaged 5.00 (SEM: 0.55) in controls and 6.18 (SEM: 0.42) \((P = 0.04)\) in bipolar patients with psychosis.

2.5. Effect of demographic and postmortem variables on the experimental results

None of the demographic or postmortem variables evaluated (Experimental procedures) were confounding for the kynurenine pathway measurements in any of the four diagnostic groups, i.e., the statistical assumptions of ANCOVA were not satisfied with respect to linearity, homogeneity, and direction of the correlations in each group. Nevertheless, a set of the demographic and postmortem variables previously considered important in postmortem studies of brain tissue from controls and/or those with mental illness were selected for further analysis. These included brain pH, PMI (postmortem interval), age, sex, smoking history, and neuroleptic use (Table 2). Multiple linear regression analysis was carried out for the effect of these variables on the kynurenine pathway measures.
in the control group. The analysis confirmed that where a significant difference exists between cases and controls (Table 2), the direction and/or degree of the correlation between that confounding variable and the experimental results could not cause the differences found in the kynurenine pathway measures (Table 3). The coefficient of greatest magnitude was found for the correlation between pH and the TDO2 RNA measurement ($r = 0.69$), but that effect did not reach statistical significance. To determine if this pH effect would reach significance if the group was expanded to include all diagnostic categories, multiple linear regression was carried out for the same set of variables across the entire sample set. The correlation coefficient for effect of pH on TDO2 mRNA in the entire sample set was 0.17, $P = 0.29$. Neuroleptic lifetime dose (Torrey, 1988) was analyzed within the schizophrenia group, and the regression coefficient was 0.00 with respect to dose (Torrey, 1988) was analyzed within the schizophrenia group. Smoking was associated with a non-significant lowering of the kynurenine pathway measures, but this was a trend ($P = 0.09$) only for kynurenic acid (Table 4C).

### 3. Discussion

This study of anterior cingulate samples adds new data to support the conclusion that kynurenine pathway activation in the brain is greater in schizophrenia. The additional finding of increased kynurenine in the group of bipolar patients with psychosis and not in the major depression group indicates that increased levels of pathway metabolites may fit in the broader context of behavioral disorders that are associated with psychosis. In the psychotic disorders we evaluated here, activation of the pathway appears to occur at the first enzymatic step. The evidence for this in schizophrenia is threefold: the elevation of TDO2 mRNA, the elevation of immunohistochemistry signal for TDO2 protein in white matter glia, and the elevation in the first major product of the pathway, kynurenine (Fig. 3). The evidence for activation of the first enzymatic step in bipolar disorder with psychosis is two-fold: the elevation in the immunohistochemistry signal for TDO2 protein in glia and the elevation in kynurenine. In the major depression group, the TDO2 protein was increased in white matter glia, but this did not result in increased kynurenine or kynurenic acid levels. These data do not directly address flux through the pathway, however, which may affect the initial pathway precursor without significant changes in pathway intermediates. It is known that activation of kynurenine synthesis through pharmacological intervention is associated with the onset of symptoms of depression (Capuron et al., 2003), and the likely mediator of this effect is depletion of tryptophan (Capuron et al., 2003; Smith et al.,

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**Table 2 – Major demographic and postmortem variables**

<table>
<thead>
<tr>
<th>Sample set</th>
<th>Subset for RNA</th>
<th>pH</th>
<th>Subset for RNA</th>
<th>PMI (h)</th>
<th>Subset for RNA</th>
<th>Age (years)</th>
<th>Subset for RNA</th>
<th>Sex ratio (M/F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schizophrenia</td>
<td>6.11 ± 0.075</td>
<td>6.09 ± 0.085</td>
<td>37.3 ± 4.0*</td>
<td>37.6 ± 4.9*</td>
<td>43.8 ± 3.7</td>
<td>45.1 ± 4.3</td>
<td>2.00</td>
<td>3.00</td>
</tr>
<tr>
<td>Depression</td>
<td>6.18 ± 0.068</td>
<td>6.15 ± 0.078</td>
<td>27.8 ± 3.0</td>
<td>27.1 ± 3.7</td>
<td>45.9 ± 2.6</td>
<td>47.5 ± 2.8</td>
<td>1.50</td>
<td>1.75</td>
</tr>
<tr>
<td>Bipolar disorder</td>
<td>6.16 ± 0.060</td>
<td>6.31 ± 0.040</td>
<td>31.2 ± 3.8</td>
<td>26.6 ± 3.0</td>
<td>41.8 ± 3.2</td>
<td>39.3 ± 5.3</td>
<td>1.50</td>
<td>1.00</td>
</tr>
<tr>
<td>Bipolar disorder with psychosis</td>
<td>6.17 ± 0.07</td>
<td>6.32 ± 0.05</td>
<td>24.9 ± 2.11</td>
<td>25 ± 1.53</td>
<td>43.2 ± 3.7</td>
<td>40.8 ± 6.0</td>
<td>1.8</td>
<td>1.33</td>
</tr>
<tr>
<td>Controls</td>
<td>6.28 ± 0.064</td>
<td>6.33 ± 0.061</td>
<td>26.1 ± 3.8</td>
<td>25.3 ± 2.6</td>
<td>48.6 ± 2.9</td>
<td>49.4 ± 3.2</td>
<td>1.33</td>
<td>1.40</td>
</tr>
</tbody>
</table>

* Mean ± standard error of the mean for continuous variables.

**Table 3 – Continuous demographic and postmortem variables: determining the effect on kynurenine pathway measures by multiple linear regression analysis in the controls**

<table>
<thead>
<tr>
<th>Log fold value TDO2</th>
<th>Kynurenine</th>
<th>Kynurenic acid</th>
<th>TDO2-positive glia, density × intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMI 0.00, $P = 0.61$</td>
<td>0.01, $P = 0.51$</td>
<td>0.00, $P = 0.8$</td>
<td>0.03, $P = 0.79$</td>
</tr>
<tr>
<td>pH 0.69, $P = 0.14$</td>
<td>$-0.10, P = 0.77$</td>
<td>0.01, $P = 0.97$</td>
<td>0.54, $P = 0.87$</td>
</tr>
<tr>
<td>Age 0.00, $P = 0.96$</td>
<td>0.01, $P = 0.26$</td>
<td>0.01, $P = 0.28$</td>
<td>$-0.05, P = 0.53$</td>
</tr>
</tbody>
</table>

* Units are log (pmol/10 mg tissue).
enzymes with higher activity per mole of enzyme for purified mammalian TDO2 tetramer is approximately 3.5 to 9 times that of IDO (Littlejohn et al., 2000; Ren et al., 1996; Schutz and Feigelson, 1972). Thus, increased TDO2 expression would be expected to have a much greater impact on the rate of product formation, whereas increased IDO expression would have a more profound effect on the extent of tryptophan depletion.

The data were analyzed to determine the potential confounding effect of postmortem and demographic variables, and the results illustrated that, where there was a significant difference in those variables between groups, the difference was in a direction opposite to the findings for the kynurenine pathway results. Thus, the brain pH was lower in the schizophrenia samples, yet to the extent that there is a pH effect in the control group (Table 3), lower pH corresponds to higher not lower TDO2 values. The male/female ratio was lower in the schizophrenia group than in the control group (Table 2); however, it exerted no effect on the data in the disease groups (Table 3).

Smoking history is also of great concern in studies of schizophrenia, in that more schizophrenics tend to smoke, and they smoke more cigarettes per day than normal controls (Hughes et al., 1986). The tendency of a positive smoking

Table 4 – Categorical demographic variables

A. Comparison of kynurenine pathway median values* between males and females

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
<th>Males</th>
<th>Females</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Log TDO2 fold value</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>−0.068</td>
<td>−0.266</td>
<td>P = 0.34</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All groups</td>
<td>−0.063</td>
<td>0.063</td>
<td>P = 0.94</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B. Comparison of kynurenine pathway mean values between smokers and non-smokers

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>No</th>
<th>Yes</th>
<th>No</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Log TDO2 fold value</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>−0.306</td>
<td>−0.036</td>
<td>P = 0.16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All groups</td>
<td>−0.075</td>
<td>0.040</td>
<td>P = 0.13</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C. The kynurenine pathway mean values grouped by neuroleptic on/off status at death

<table>
<thead>
<tr>
<th></th>
<th>On</th>
<th>Off</th>
<th>On</th>
<th>Off</th>
<th>On</th>
<th>Off</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Log TDO 2 fold value</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neuroleptic schizophrenia</td>
<td>0.06</td>
<td>0.20</td>
<td>P = 0.31</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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* Values are not normally distributed by gender in sample set; compared with Mann–Whitney test.

b In control group: males, N = 5; females, N = 5; in all groups combined: males, N = 23; females, N = 14.

c Units are log (pmol/10 mg tissue).

d In control group: males, N = 7; females, N = 6; in all groups combined: males, N = 33; females, N = 21.

e Smoking status known for 55% of control group and 54% of all samples combined in the subset available for RNA analysis; control group: positive smoking history, N = 4; negative smoking history, N = 1; all groups combined: positive smoking history, N = 11; negative smoking history, N = 9.

f Units are log (pmol/10 mg tissue).

g Smoking status known for 50% of control group, 54% of all samples combined in the subset available for RNA analysis; control group: positive smoking history, N = 2; negative smoking history, N = 1; all groups combined: positive smoking history, N = 1; negative smoking history, N = 9.

h For TDO2 mRNA subset, N = 3 off neuroleptics, N = 5 on neuroleptics; for complete set available for metabolite assay and immunohistochemistry, N = 3 off neuroleptics, N = 9 on neuroleptics.

i Units are log (pmol/10 mg tissue).
history to be associated with slightly lower kynurenine pathway measures (Miller et al., 2004a) was also observed for this data set (Table 4B), an indication that increased smoking was not causing the differences observed between cases and controls.

Neuroleptic treatment is necessarily an important issue in studies of schizophrenia. Including drug-free patients is not preferable to studying treated patients, since schizophrenic individuals that can safely exist in the drug-free state may have a milder form of the disease. What is known about the effect of neuroleptics on the kynurenine pathway comes from work in animals (Schwarcz et al., 2001), demonstrating a decrease in kynurenic acid with neuroleptic treatment. In the data we report here, the extent of neuroleptic treatment did not have an effect on the data. Furthermore, being on neuroleptics at the time of death corresponds to lower values for the kynurenine measures than does being off neuroleptics at the time of death, a difference that trends toward significance for kynurenic acid (Table 4C).

As seen in the prior study of frontal cortex tissue (Miller et al., 2004a), there was no correlation between the TDO2 mRNA and other measures of TDO2 function, despite the fact that the components show similar inter-group trends (Fig. 3). As the mRNA and immunohistochemistry studies were performed on contralateral sections by necessity (one hemisphere was fixed in formalin and the other was frozen), this is one likely explanation for the lack of correlation between those two measures. However, the mRNA and the metabolites were extracted from the same tissue block. The picture that emerges from these data is that although each of the measured kynurenic components exists at a higher steady-state level in schizophrenia, the oscillations that exist at that level in each component may not be synchronous. In fact, correspondence of the measures across individuals would not be the predicted outcome if any feedback loops exist between RNA expression, protein levels, and metabolic products.

The basis for kynurenine pathway upregulation in schizophrenia and psychotic bipolar disorder may not reside within the TDO2 gene itself, but rather with one of the many factors that control the expression of TDO2. One of the most common forms of regulation in biological systems is feedback regulation, and the downstream products that have been shown to regulate TDO2 activity and expression are niacin or its congeners (Badawy and Evans, 1976; Cho-chung and Pitot, 1967, 1968; Scherer and Kramer, 1972). In searching for the most likely initiating factor, there are data available in the clinical and epidemiological literature that can be brought to bear. Numerous clinical studies of niacin effects in individuals with schizophrenia have been carried out for the purpose of studying a blunting of the niacin flush response reported for that population (Horrobin, 1980; Rybakowski and Weterle, 1991; Waldo, 1999; Ward et al., 1998), including one study showing a blunting of the flush response only in acute first-episode patients (Smesny et al., 2005). Cutaneous flushing induced by either niacin ingestion or skin patches is mediated by niacin stimulation of prostaglandin E2 (PGE2) release (Saareks et al., 1999) and/or prostaglandin D2 (PGD2) release (Morrow et al., 1989). Of the two prostaglandins, PGE2 is thought to be an important mediator in rheumatoid arthritis (McCoy et al., 2002), a disease with a relatively low incidence in the schizophrenic population (Oken and Schulzer, 1999). Therefore, it seems possible that the common denominator in these clinical phenomena and in the upregulation of TDO2 is the diminished effect of niacin, perhaps through control of its steady state at a low level or reduced signal transduction via the niacin receptor.

The significant difference that we have found in TDO2-negative immunopositive cells is specific for astrocytes, a cell type that has been shown in culture to preferentially process kynurenine to kynurenic acid rather than quinolinic acid (Guillemin et al., 2001). A negative effect of kynurenine pathway upregulation on mental status could theoretically be mediated by the kynurenic acid branch of the pathway (Fig. 1). Kynurenic acid and kynurenic acid are highly correlated in each of the sample groups, but most strikingly in the schizophrenia group \( r = 0.8, P = 0.0001 \), replicating a previous report of a higher degree of correlation between the two metabolites in schizophrenia than in controls (Schwarcz et al., 2001). However, the higher degree of correlation does not necessarily equate to a proportionately higher flux along the kynurenic acid branch versus other branches of the pathway, as the ratio of moles kynurenic acid per mole of kynurenine tends to be lower in the patient population than in the controls. Indeed, the steady-state level of kynurenic acid was not significantly higher for the patients we report here, although a significant difference was found in a study by others of the pre-frontal cortex in a larger sample size (Schwarcz et al., 2001). The inhibition of the nicotinic alpha-7 and NMDA receptors by kynurenic acid (Erhardt et al., 2001; Hilmas et al., 2001; Shepard et al., 2003; Stone, 1993) would be two potential consequences and are of interest in light of reports for dysfunction of both systems in schizophrenia. Downregulation of nicotinic function has been linked to deficient auditory processing, seen in both schizophrenic humans (Freedman et al., 2003; Leonard et al., 1996) and in animal models (Luntz-Leybman et al., 1992; Stevens et al., 1998). Although psychotic symptoms have been related to drugs that antagonize the NMDA receptor (Javitt and Zukin, 1991), most of those same drugs have effects on catecholamines as well (Miller et al., 1992; Swanson and Schoepp, 2003) which are known to be psychotomimetic. Therefore, NMDA antagonism by kynurenic acid may not be sufficient to cause psychosis, and there are no data linking a deficiency in nicotinic alpha-7 receptor function with psychotomimetic effects. Thus, the question of what additional downstream metabolites or byproducts show an increase proportional to kynurenine remains an important question for future studies of metabolic changes in psychosis.

4. Experimental procedures

4.1. Tissue samples for study

Tissue from the anterior cingulate of 12 patients with schizophrenia, 14 with bipolar disorder, 14 with major depression, and 14 controls was available for study. The demographics of each individual and postmortem variables of the samples are listed in Table 2. All subjects used for this study were Caucasians. The cause of death, methods of diagnosis, and acquisition of the samples...
from which our sample set was derived have been described previously (Torrey et al., 2000). The tissue samples were processed as previously described for RNA and immunohistochemistry (Miller et al., 2004a). All analyses were carried out in a blinded manner, with respect to knowledge of the diagnostic category of each sample group.

4.2. RNA preparation and analysis

Many aspects of the RNA handling and cDNA generation have been previously described in detail (Miller and Yolken, 2003; Miller et al., 2004a). Briefly, approximately 200 mg tissue was homogenized and total RNA extracted using the Qiagen RLT buffer and subsequent RNase columns (Qiagen, Stanford, CA). The RNA was evaluated for its major components by electrophoresis on the Agilent BioAnalyzer Nano 6000 chip (Agilent, Palo Alto, CA) as described elsewhere (Miller et al., 2004b). Prior to electrophoresis (but not prior to the RT reactions, see below), it was necessary to concentrate the RNA approximately 10-fold in a ISS110 SpeedVac (ThermoSavant, Holbrook, NY).

4.3. RT reactions to generate cDNA

The amount of RNA added to each RT reaction was calculated based on the \( A_{260} \) value of the RNA dissolved in 0.125 mM Tris EDTA buffer, pH 8. The RNA was not treated with Dnase 1, as Dnase 1 has been shown to have a negative effect on RNA (Ivarsson and Weijdegard, 1998). Total RNA (100 ng) was reverse transcribed using random hexamers in a SUPERSCRIPT First-Strand Synthesis System (Gibco-BRL-Invitrogen, Carlsbad, CA) cDNA reaction as described elsewhere (Miller et al., 2004a).

4.4. PCR

4.4.1. Standard curves

The template for the PCR to generate each standard curve was purified from several prior PCRs of the gene product, using the QIAquick column purification method. The purified double-stranded DNA product was quantified to \( A_{260} \) in 0.125 mM Tris EDTA buffer, pH 8. Serial dilutions were made, and triplicate PCRs were carried out at each dilution.

4.4.2. Real-time PCR of cDNA samples

One microliter of cDNA product (equivalent to 1.25 ng RNA starting material) was used per 25 \( \mu \)l reaction mix (final volume) in each tube. Stratagene Brilliant Green master mix (24 \( \mu \)l) was used for all reactions, containing a hot-start Taq polymerase, glycerol, dimethylsulfoxide (DMSO), dNTPs, and SYBR Green dye. Between each sample addition, the pipettor was cleaned (tap water aspirated into the barrel, followed by ethanol) before using it to aspirate the cDNA sample. The PCR controls were run at the end of the sample additions, using the same pipettor cleaned as above, but 1 \( \mu \)l water (Rnase-, Dnase-free water, Sigma Chemical Company, St. Louis, MO) was added instead of the sample.

All PCR primers were selected using the BeaconDesigner 2.0 program (Premier Biosoft International, Palo Alto, CA). The primers used for the GAPDH PCR were GTCAAGGCCTGA-GAACGGGAAG (forward) and GTGAAAGCCGACATTGGACTC (reverse), \( T_m = 58^\circ C \). The primers used for the TDO2 PCR were CATGGCTGGAAAGAACTC (forward) and CTGAAAGTCTCCTGTAT-GAC (reverse), \( T_m = 50^\circ C \). TDO2 is so designated because the acronym TDO was first given to the gene associated with Trichodentoosseus Syndrome. The primer pairs for TDO2 did not give a PCR product from genomic DNA that is of the same size as the cDNA product (span intronic regions). The primer pair for GAPDH gave a PCR product from genomic DNA of the same size as that derived from cDNA. The primers for GAPDH actually span an intron but will react with the processed pseudogenes for GAPDH that are in genomic DNA.

The Brilliant SYBR Green QPCR real-time kit (Stratagene, La Jolla, CA) and Stratagene MX 4000 (96-well format) were used for PCR in the following protocol: [10 min 95\(^\circ\)C] × 1 cycle then [15 s 95\(^\circ\)C, 30 s at annealing temperature, 1 min 30 s extension 72\(^\circ\)C] × 40 to 47 cycles. Following the last cycle, the instrument was programmed to carry out a melting temperature analysis, to confirm the presence of one product and the absence of primer-dimers (lower melt temperature) in the analysis. The standard curves run with each PCR represented serial dilutions of gel-purified PCR product.

4.5. HPLC

Many of the published methods for kynurenine extraction involve the addition of acid, including TCA, to precipitate protein and other cellular debris prior to HPLC analysis (Holmes, 1988; Pfefferkorn et al., 1986; Schwarz et al., 2001). However, the TCA precipitation method has been reported to degrade tryptophan (Bloxam and Warren, 1974; Ravikumar et al., 2000) and appeared to result in the generation of kynurenic acid from tryptophan in our preliminary studies. As acid hydrolysis has been shown to alter the composition of tissue extracts (Berlett, 1991), we elected to further examine the relevance of such potential artifacts to our work by monitoring the conversion of radiolabeled tryptophan to radiolabeled kynurenic acid. To do so, we added 20% TCA to 100 mM potassium phosphate buffer (pH 7.0) containing 100 \( \mu \)M FeSO\(_4\) (within the range reported for rat and human brain; Jurczuk et al., 2003; Ogg et al., 1999; Zecca et al., 2001), 25 mM ascorbate (slightly more than levels estimated to be present in neurons; Rice, 2000) and 45 pmol/\( \mu \)l \(^{14}\)C-tryptophan (PerkinElmer Life and Analytical Sciences, Boston, MA) slightly higher than the concentration estimated to be present in human brain (Korpi et al., 1986). The tryptophan was labeled with \(^{14}C\) on the third carbon of the side chain to a specific activity of 50 Ci/mol.

The TCA-treated and untreated standard was analyzed on a C-18-reverse phase column (3 \( \mu \), 80 mm \( \times \) 4.6 mm), mobile phase: 250 mM zinc acetate, 50 mM sodium acetate, pH adjusted to 6.2 with acetic acid, then brought to 4.5% acetonitrile, at 1ml/min at room temperature and coupled to both a UV detector and a scintillation detector. The results showed an approximately 1.6% conversion of the \(^{14}C\)-tryptophan to radiolabeled kynurenic acid, the appearance of numerous other unidentified degradation products and a substantial reduction in the size of the tryptophan peak (Fig. 5). The kynurenic acid was not derived from N-formyl kynurenine contamination of the tryptophan standard, as the chromatogram of the untreated standard showed one major peak and no minor peaks. As a 1.6% conversion of endogenous tryptophan in the tissue, estimated to be approximately 1000 pmol tryptophan per 10 mg tissue (Korpi et al., 1986) would significantly affect the results, a filtration method was evaluated for metabolite extraction. The effect of filtration on kynurenine and kynurenic acid standards was studied using perethylen sulfone (PES) Vivasin columns (Vivasience, Hanover, Germany), size exclusion 5-kDa columns. The samples were analyzed by injection on a reverse-phase (C-18) HPLC column (3 \( \mu \), 80 mm \( \times \) 4.6 mm), mobile phase: 250 mM zinc acetate, 50 mM sodium acetate, pH adjusted to 6.2 with acetic acid, then brought to 4.5% acetonitrile. Kynurenic acid was measured with a UV detector with the wavelength set at 365 nm. Kynurenic acid was detected with a fluorescence detector, wavelength set at 344-nm excitation and 398 nm for detection of the fluorescence.
kynurenine absorbs at 365 nm but tryptophan does not absorb at that wavelength.

This filtration method resulted in no production of kynurenine from tryptophan-containing buffer, and the recovery of kynurenine and kynurenic acid added to tissue using this method was consistently 70% and 65%, respectively. Filtration of tryptophan standards did not result in the appearance of detectable kynurenine in the eluate. The sample set was then extracted using the filtration method, and all values were corrected for recovery. Duplicate 20 to 40 mg sections were obtained from each anterior cingulate sample, weighed, and homogenized in 4 volumes of MPER detergent (Pierce Chemical Co., Rockford, IL). Tissue homogenates (130 μl) were spin filtered as above and the flow through was diluted 1:1 with deionized water for HPLC analysis (100 μl injection volume) using the above column and mobile phase conditions.

4.6. Antibody generation for immunohistochemistry

Polyclonal antibodies were generated to two regions of the enzyme TDO2 as previously described (Miller et al., 2004a). TDO2-A was a polyclonal antibody generated by injecting rabbits with a peptide composed of a sequence found near the N-terminus of TDO2: GSEEDKSQTGVNRAS. TDO2-B was a polyclonal antibody generated by injecting rabbits with a peptide composed of a sequence found in a more central region of TDO2: RVPYNRRHYRDNFKGEEN. The Western blots of cortical tissue for these antibodies have been previously published (Miller et al., 2004a).

4.7. Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed and paraffin-embedded 5-μm-thick sections as previously described. Briefly, deparaffinized, rehydrated sections underwent antigen retrieval using the DAKO target retrieval solution (DAKO Cyto- mation, Carpinteria, CA, USA, No: S1700). Sections were treated with 3% H2O2 for 5 min to block endogenous peroxidase followed by protein block (0.25% casein in PBS containing carrier protein and NaNO3, DAKO code X0909). The primary antibodies were applied at concentrations of 1:300 for TDO2-A and 1:250 for TDO2-B for 30 min and 2 h, respectively. Sections were incubated with the secondary antirabbit antibody (conjugated with horseradish peroxidase enzyme-labeled polymer) for 30 min. The reaction product was visualized using 3,3′-diaminobenzidine chromogen (liquid DAB+ included in the kit) for 5 min. Then the sections were counterstained with Gill 2 hematoxylin (Richard-Allan Scientific, Kalamazoo, MI, USA).

As negative control, the primary antibody was omitted and replaced with normal rabbit serum (code X0903, DakoCyto- mation, Carpinteria, CA, USA).

For immunohistochemical identification of astrocytes, double labeling for GFAP and TDO2-A and TDO2-B was carried out using the DAKO EnVision Doublestain System, AP/HRP,DAB and Fast Red (code K1395, DakoCyto- mation, Carpinteria, CA, USA) as described previously (Miller et al., 2004a).

4.8. Evaluation of the immunohistochemical stains

On each immunohistochemically stained section, neurons, glial cells, and blood vessels were analyzed separately in the gray and white matter. Morphological criteria were used to identify the cells. Astrocytes were distinguished from neurons based on the presence of round vesicular nuclei, no evident nucleoli, scanty cytoplasm, and no Nissl substance. In addition, astrocytes were identified by their GFAP immunoreactivity. However, only a small number of cortical astrocytes are GFAP-positive (Weis et al., 1993).

For each cell type (neurons, glia) or structure (vessels), the staining intensity was rated as follows: (0) no staining, (1) weak staining, (2) moderate staining, and (3) strong staining. The density (% of positive cells/structures relative to the density of the total population of that specific cell type/structure) of stained cells or structures was rated as follows: (0) no cell/structure stained, (1) low density (<25%), (2) moderate density (25 to 50%), and (3) high density (>50%). The intra-rater reliability for this method of scoring was determined to be 96.5% for repeated analyses of a sample set of 10 on two different days. For each single cell (i.e., neurons and glia) and structure (vessel), compound values were calculated by multiplying the staining intensity with the density scores. Finally, sums were calculated for the gray matter by adding the compound values of neurons, glia, and vessels, and for the white matter by adding the compound values of glia and vessels. In addition, sums were calculated for the compound values (i.e., density x intensity) of glial cells and...
vessels, separately, by adding the respective values for the gray and white matter.

The differences between the control and the diagnostic groups were assessed using the non-parametric Mann–Whitney test (Statistical Package for the Social Sciences, SPSS).

4.9. Statistical analyses

4.9.1. Statistical analysis of the group RT-PCR, HPLC, and immunohistochemistry experimental results

The mean and standard error of the mean (SEM) for the log values of the metabolites and transcript number were calculated for each sample group, and, as the values were normally distributed, the data in each group were compared by Student’s t test (SigmaStat, Jandel Scientific, San Rafael, CA).

The threshold value for suitable RNA quality based on the housekeeping gene GAPDH was determined from a histogram (bin number = 9) of the bimodal frequency distribution of the RT-PCR values for GAPDH. The cut off was determined by calculating the mean of the lowest population and adding one standard deviation, yielding the value of approximately 20,000 copies per ng total RNA, i.e., very similar to the GAPDH value at the quality cut off threshold obtained from a previous data set of frontal cortex tissue (Miller et al., 2004b). This value corresponds to approximately 25% of the number of GAPDH transcripts per cell determined for fresh mammalian tissue from other sources (Weiss and Sunde, 2001). Three electropherogram measures were also applied in evaluating the RNA data: the RIN program, the 28S/18S ratio, and the ratio of the 18S peak to the highest peak in the tRNA to 18S baseline (Miller et al., 2004b). The latter method was used to confirm most of the poor quality samples (13/17) and all but three of the better quality GAPDH samples as such (34/37). The other two electropherogram measures were used to confirm the quality of the remaining 7 samples.

The percent error calculation for the RT-PCR and HPLC analytical methods were derived from Standard Error of the Mean divided by the mean for replicate analyses. For the kynurenine and kynurenic acid measures, this error included section to section variability incurred when sectioning duplicate, adjacent regions of tissue. For the TDO2 RT-PCR analysis, these results are derived from one RNA extraction and subsequent RT reaction; thus, the error reflects replicate PCR results from the same cDNA.

For the immunohistochemistry data, non-parametric statistical procedures, such as contingency tables (Chi-square), Mann–Whitney U test and the Kruskal–Wallis one-way analysis of variance were used to assess differences in the variables between the four diagnostic groups.

4.9.2. Statistical analyses for confounding variables

The demographic and postmortem variables analyzed included age, sex, side of the examined hemisphere, suicide status, smoking at time of death, postmortem interval (PMI), brain pH, cerebellar granular cell layer necrosis, rate of death, fixation time of tissue, lifetime antipsychotic intake (in fluphenazine mg. equivalents), data on intake of 1st and 2nd generation antipsychotic generic drugs, mood stabilizer generic drugs, lithium, antidepressant generic drugs, and anticholinergic generic drugs. Correlations between these data and the experimental data were determined where appropriate. For the continuous data, a Pearson correlation was performed for each of the four sample groups. In order to evaluate the potential influence of these variables on the non-continuous immunohistochemistry data, some of the continuous variables were grouped according to their scaling properties prior to determining the correlation coefficient (Spearman Rank correlation). The statistical assumptions for applying ANCOVA, such as significant correlations, homogeneity, and linearity of the regression lines, were tested.

In addition, t tests were performed to determine differences between the sample groups for the most common confounders (Johnston et al., 1997; Leonard et al., 1993; Mirmics et al., 2000; Preece and Cairns, 2003), i.e., age, postmortem interval, and brain pH (Table 2). Multiple linear regression analysis was carried out to determine the respective correlation coefficients for effect of these variables (i.e., age, postmortem interval, and brain pH) on each kynurenine pathway measure (Table 3). The assumptions for the analysis were alpha value for power = 0.05; residuals standardized to flag values above 2;5; normality test, P value to reject = 0.05; constant variance test, P value to reject = 0.05; and standardized regression coefficients were used. The Student’s t test or the Mann–Whitney Rank Order test was performed to assess the differences in the experimental data (Table 4) when grouped according to the categorical demographic variables (gender, smoking history, and neuroleptic on/off status at death).

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