Research report

The high-affinity niacin receptor HM74A is decreased in the anterior cingulate cortex of individuals with schizophrenia

Christine L. Miller, Jeannette R. Dulay

1. Introduction

The niacin-signaling cascade has been of interest in schizophrenia research since early observations of the parallels between schizophreniform psychosis and the mental alterations seen in pellagra [4]. During the numerous trials of niacin therapy for schizophrenia that were carried out in the mid-1900s, it was observed that many individuals with schizophrenia exhibited a blunting of the flush response that normally occurs in response to niacin ingestion [15,16]. This phenomenon was proposed as a diagnostic test by Horrobin [17], wherein the response can be measured following topically applied niacin or its derivative, methyl-nicotinate. The results of subsequent studies more or less confirmed that the epidermal flushing seen in the majority, but not all, schizophrenic subjects is of lesser magnitude, delayed onset and/or shorter duration than in normal controls [36,18,51,53,26,35] with the exception of a study by Fiedler et al. [12]. One group has reported an age-dependent difference in flush, such that the younger, recent onset patients exhibited a blunted response relative to age-matched controls, whereas the age-matched controls for the chronic patients showed a decreased response relative to their younger counterparts, rendering the difference between controls and cases non-significant in the older group [43]. The potentially confounding role of antipsychotic medication was addressed by a study of unmedicated schizophrenic subjects which determined that the blunted flush response was prevalent independent of medication status [41], although the potentially confounding effect of neuroleptics is worthy of continued study [50].

The recent discovery of the G-protein coupled niacin-responsive receptors, HM74A and HM74, led to a greater understanding of the basis for the flush response [23,55,45,7,32]. HM74 has a low-affinity for niacin, whereas HM74A exhibits high affinity and is thought to be the prime-mediator for the stimulation of prostaglandin synthesis by niacin. Neither receptor is responsive to nicotinamide [23,55]. Binding of niacin activates cyclooxygenase to synthesize prostaglandins E2 and D2 (Fig. 1), the latter being the agents responsible for the vasodilation of the epidermal capillaries and hence, flushing of the skin [23,10]. Increases in prostaglandin E2 are thought to be associated with rheumatoid arthritis [25], a disease exhibiting a low comorbidity with schizophrenia [33]. Thus, a defect in the niacin-signaling cascade would be consistent with both the blunted niacin flush response and the relatively low incidence of rheumatoid arthritis in individuals with schizophrenia.
During conditions of dietary insufficiency of niacin, de novo synthesis of NAD from tryptophan occurs via the kynurenine pathway [5,42]. The kynurenine pathway has been shown to be upregulated in individuals with schizophrenia as well as other forms of psychosis [38,29,30,1]. Several possible causes for this activation are under investigation, including substrate activation and feedback inhibition by small molecule regulators that act through allosteric interaction with enzymes or through receptors. When dietary niacin is sufficient in the periphery, it is utilized to form NAD and thereby regulates a host of biochemical pathways, including those involving energy transfer, gene-silencing and by extension, apoptosis [37,13,3]. The function of niacin in the brain has not yet been studied. Although no transcripts for HM74A were detected in two previous studies of whole brain homogenates of mouse and human [55,44], since that time, HM74A (GPR109a) expression in brain has been demonstrated by others [48], including the anterior cingulate area of the mouse brain (Allen Brain Institute: http://brain-map.org/welcome.do;jsessionid=F170A95BFBE706A5EAABAC3EF423E2DB8, GPR109a, sagittal position 2400).

Using RT-QPCR and Western blots, we have now measured the expression of the niacin-responsive HM74A and HM74 receptors in postmortem brain tissue of schizophrenic patients, bipolar patients (whose flush is similar to controls [35]) and normal controls. The anterior cingulate region was chosen for study, as it is a key region in the limbic circuitry thought to be disrupted in psychosis [14].

2. Materials and methods

2.1. Tissue samples for study

Tissue from the anterior cingulate of 12 patients with schizophrenia, 14 with bipolar disorder and 14 controls was provided from the Neuropathology Consortium set by the Stanley Medical Research Institute (SMRI) brain bank in Bethesda, MD. The tissue was acquired with the consent of next of kin and in accordance with Institutional Review Board procedures for postmortem tissue. All subjects were matched for race (all were Caucasian). The demographics for each diagnostic group have been reported previously [48] and are summarized for each group in Table 1. The tissue samples were processed as previously described [29,30] and the sample identifiers when listed in the figures correspond to SMRI codes. This sample set has been studied in other laboratories using similar techniques to those presented below [46].

Table 1: Demographic and postmortem information [mean ± S.D.] for the samples of this study

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Age (year)</th>
<th>Gender</th>
<th>PMI</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>14</td>
<td>48.6 (10.9)</td>
<td>6F, 8M</td>
<td>26.1 (9.3)</td>
<td>6.29 (0.24)</td>
</tr>
<tr>
<td>Bipolar</td>
<td>14</td>
<td>41.8 (12.0)</td>
<td>5F, 9M</td>
<td>31.2 (14.4)</td>
<td>6.16 (0.22)</td>
</tr>
<tr>
<td>Schizophrenia</td>
<td>12</td>
<td>43.2 (12.8)</td>
<td>4F, 8M</td>
<td>37.3 (13.8)</td>
<td>6.11 (0.26)</td>
</tr>
</tbody>
</table>

* All individuals were Caucasian. More details of this sample set have been presented previously [28,49] and are available online [http://www.stanleyresearch.org/programsbrain_collection.asp] under the heading SMRI consortium tissue chart.

Fig. 1. Model of the relationship between the kynurenine pathway (right side of figure, see [6]) and the signaling cascade initiated by the HM74A niacin-responsive receptor (left side of figure) that results in the flush response. Interactions with receptors are indicated by box arrows and metabolite flux via enzyme activity is indicated by thin arrows. Key enzymes are boxed and identified with HUGO nomenclature. Note that mammals are not thought to form niacin de novo, and therefore the action of NaPRT must be unidirectional towards NAD from niacin [6]. The HM74A stimulation of arachidonic acid release via PLA2 has been demonstrated by Tang et al. [47]. HM74A stimulation can activate cyclooxygenase in some macrophage cell types in a PLA2-independent manner [21]. The receptors for PGE2 [34] and HM74A [56,52] negatively regulate lipolysis (left side of figure) that results in the flush response. Interactions with receptors are indicated by the box arrows and metabolite flux via enzyme activity is indicated by thin arrows. Key enzymes are boxed and identified with HUGO nomenclature. Note that mammals are not thought to form niacin de novo, and therefore the action of NaPRT must be unidirectional towards NAD from niacin [6].
cycles-to-threshold for pMCH in the anterior cingulate preparation was 43, and the dissociation curves were sharp and uniform in all samples, corresponding to the peak derived from the short cDNA product rather than the longer genomic DNA product. Thus, the abundance of the transcripts of interest, HM74A and HM74 (for SYBR uptake, approximately 33 cycles-to-thresholds for the sample set; for the TagMan assay, approximately 37 cycles-to-threshold) is well above the level at which DNA contamination (if present) would exert an effect.

2.3.2. Tagman® QPCR

All amplicons were 20 μl final volume, containing AmpliTaq® Gold 2.0® master mix (Roche Molecular Systems, Pleasanton, CA), 20× custom primers and probe, and cDNA equivalent to 1.25 ng RNA starting material using the following amplification program: 95 °C for 10 min, 56 °C (for 15 s, 60 °C for 1 min). The Ct for the HM74A and HM74 reactions were of a similar range (for both 33–41 cycles) and for the GAPDH reactions (cDNA diluted 1:20; in above SYBR uptake above), 28–31 cycles. QPCR standard curves were linear for both Ct ranges (r² = 0.99, efficiency 97.5% in the former to r² = 0.99, efficiency 101% in the latter).

The primers and probes were optimized in a custom synthesis by Applied Biosystems (Foster City, CA), designed to match the coding region for the peptide epitopes recognized by the antibodies for HM74A and HM74. As these are regions of mismatch between the two genes, the PCR was specific (at least four nucleotides of mismatch per probe). For HM74A, the primers were forward, AGTCCACCTCCTGAGAGAGGAT; reverse, GGTATGCAACCTGTCAGAGT; reporter, ACCGTATCTGCTGATGTC. The specificity of the PCR for HM74A was confirmed by a nested PCR, using the products of the first cycle as the template for the second cycle.

This region was selected from antigenicity and surface exposure profiles visualized with the program Prot Pep (DANstar, Milwaukee, WI). The antibody for HM74A lies in a region of 225% mismatch between HM74A and HM74, with no matched region+ residues. One antibody was generated to the HM74 receptor in the carboxy-terminal region (residues 361–378) with no overlap to the HM74 receptor antisera (TNSSHKSKGHGCHQFES). The HM74A and HM74 antibodies were studied in their antisera and affinity-purified forms.

2.4. Antibody generation for Western blots

Four antibodies were used as probes in this study, a commercially available affinity-purified antibody (HM74ab) that, according to the manufacturer, was generated to an undisclosed sequence corresponding to half of HM74A and HM74, available through Abcam (ab12610) (Cambridge, MA); two polyclonal antibodies generated to an undisclosed sequence corresponding to half of HM74A and HM74; and the affinity-purified antibody and thus the antisera form was used for the quantification analyses. The antibodies were affinity-purified antibody and thus the antisera form was used for the quantification analyses.

The antibody for HM74A in the antisera form yielded a more linear standard curve in the quantitative measurement of the Western blots than did the affinity-purified antibody and thus the antisera form was used for the quantitative Western blots, HM74A antisera (1:500), affinity-purified HM74 (1:500) and the HM74B (1:5000) Western blots were performed using the ECL procedure (5% milk-based blocking agent 1 h rocking at room temperature, primary antibody incubation in TBS-0.1% Tween rocking overnight at 4 °C, secondary anti-rabbit antibody 1:2500 incubation for 1 h at room temperature, with appropriate washes in between). The developed films were optically scanned into tif files, and the negative generated (more signal equates to whiter band). Adjacent to the tif document contrast and brightness was carried out to optimize the linearity of the signal strength in the standard curve generated from the 0.4 mg, 0.2 mg and 0.1 mg aliquots of an anterior cingulate sample homogenate. Quantification of the antibody-positive bands was carried out by drawing a boundary that conformed to the contours of each band and using standard imaging software to calculate the luminosity volume (mean lumens × pixels) within the boundaries of each band.

A mild stripping buffer (100 mM glycine, pH 2.7) at 60 °C in a roller flask was used prior to re-probing. The blotting procedure was then repeated with subsequent antibodies, and the quantification carried out as described above. A maximum of five probes were carried out per blot. A rabbit anti-HM74 antibody was utilized in one set of blots that were subsequently probed with GAPDH. The HM74A and HM74 antibodies were used in the same set of blots, followed by staining for GAPDH. The HM74B Abcam Western blot with the Abcam antibody was carried out in duplicate, from aliquots of homogenates of the same section. The HM74A and HM74 Western blots were carried out in duplicate from aliquots of homogenates derived from adjacent sections of anterior cingulate. Thus the variation in the results for these receptors incorporates sectioning variability.

2.6. Statistical analyses

2.6.1. Statistical analysis of the experimental results

The values for each group (expressed as fold-values) were determined from each standard curve, and the confidence intervals for the significant results are presented in the figures (error bars). For HM74, the major demographic and postmortem variables that were analyzed for effect (see Section 3). Multiple linear regression analysis was carried out to determine the respective contribution coefficients for effect of the variables previously identified as showing a trend to differ between the groups (i.e. age, postmortem interval, brain pH, and lifetime neuroleptic dose) on each experimental measure. The assumptions for the multiple linear regression analyses were: alpha value for power = 0.05; residuals standardized to flag values above and below normal range; effect to reject = 0.05; constant variance test, p-values to reject = 0.05; standardized regression coefficients were used. ANCOVA analysis was not applied because the requirement for constancy of direction of effect across diagnostic groups was not satisfied for the potentially confounding variables. The Student’s t-test or the Mann–Whitney Rank Order test, was performed where appropriate to assess the differences in the experimental data when grouped according to the categorical demographic variables (gender, smoking history, and neurologic on/off status at death). Clustering of categorical effects in individual samples was tested for to rule out additive effects in the diagnostic groups.

2.6.2. Statistical analyses for confounding variables

For the positive experimental findings, the major demographic and postmortem variables that were analyzed for effect (see Section 3). Multiple linear regression analysis was carried out to determine the respective contribution coefficients for effect of the variables previously identified as showing a trend to differ between the groups (i.e. age, postmortem interval, brain pH, and lifetime neuroleptic dose) on each experimental measure. The assumptions for the multiple linear regression analyses were: alpha value for power = 0.05; residuals standardized to flag values above and below normal range; effect to reject = 0.05; constant variance test, p-values to reject = 0.05; standardized regression coefficients were used. ANCOVA analysis was not applied because the requirement for constancy of direction of effect across diagnostic groups was not satisfied for the potentially confounding variables. The Student’s t-test or the Mann–Whitney Rank Order test, was performed where appropriate to assess the differences in the experimental data when grouped according to the categorical demographic variables (gender, smoking history, and neurologic on/off status at death). Clustering of categorical effects in individual samples was tested for to rule out additive effects in the diagnostic groups.

3. Results

3.1. RT-QPCR

SYBR uptake PCR for HM74A was carried out using gene-specific primers as part of the preliminary investigation of between-group differences in transcript levels of the niacin receptor genes. The majority of the schizophrenia and bipolar samples amplified to threshold before the control group for both gene transcripts (Fig. 2). Quantitative comparisons were carried out after normalizing to the QPCR value of the housekeeping gene GAPDH and excluding the poor quality RNA samples [30,28]. Of the remaining samples (N = 11 in the controls, N = 7 in the bipolar group and N = 8 in the
The full-length HM74 protein is approximately 45 kDa, as seen for the primary band recognized by the antibodies HM74A, HM74 and HM74ab (Fig. 3A). The full-length GAPDH protein is approximately 35 kDa (Fig. 3A). Specificity of the affinity-purified antibody for HM74A is demonstrated in Fig. 3B, showing strong blocking of the antibody with the HM74A sequence, but no blocking of the antibody by a peptide with the corresponding HM74 sequence or with an unrelated peptide sequence. The blocking experiments were carried out under lower stringency conditions than the blots shown in Fig. 3A, and exhibit non-specific labeling of a higher MW band that served as a control for antibody, protein, and peptide conditions (Fig. 3B). The expression in different tissues is presented in Fig. 3C (left panel), to demonstrate abundant expression in the spleen, a tissue that has previously been shown to express this receptor at a high level [55] and as compared to the spleen, a relative lack of expression of this receptor in two brain regions, the caudate and the corpus collicus (right panel). Aggregate results for a set of paired blots of Western blots carried out for the entire sample set (N = 40) for HM74A, HM74, HM74ab and GAPDH are shown in Fig. 3D. Standard curves for a test sample loaded at three dilutions on the same blots as in Fig. 3D are shown in Fig. 4.

The mean value for the full-length HM74A protein was significantly reduced in the schizophrenia group, 0.55-fold ± 0.33 relative to the control group mean (p = 0.004). For purposes of controlling for variability in yield of protein, all subsequent values are reported as normalized to GAPDH protein. When normalized to GAPDH, HM74A protein was 0.56-fold ± 0.36 relative to controls, p = 0.016, but not significantly different in the bipolar group (0.96-fold ± 0.50, p = 0.83), as compared to the mean values in controls (Fig. 5A). The schizophrenia group was also significantly decreased relative to the bipolar group for HM74A (Fig. 5A). The protein recognized by the less specific antibody, HM74ab, was also significantly reduced in the schizophrenia group, 0.52-fold (±0.62) that of controls, p = 0.038, not in the bipolar group (0.91-fold ± 0.48, p = 0.59) and the results were highly correlated with the specific antibody for HM74A in the schizophrenia group and bipolar groups (r = 0.83, p = 0.001 and r = 0.78, p = 0.001, respectively), and less so in the control group (r = 0.52, p = 0.06). The low-affinity niacin receptor (HM74) did not exhibit a significant difference between the individuals with schizophrenia or bipolar disorder versus controls (Fig. 3), although in the bipolar group there was a trend for an increase in HM74 protein (1.3-fold, p = 0.09).

The correlation between the high-affinity HM74A receptor protein and the low affinity HM74 receptor protein was high and significant (r = 0.95, p = 0.001; r = 0.68, p = 0.008; r = 0.91, p = 0.001; in the schizophrenia, bipolar and control groups, respectively). Nevertheless, the ratio of HM74A to HMA74 protein was significantly reduced in the schizophrenia group (0.58-fold ± 0.19 that of controls, p = 0.001). The ratio was also decreased in the bipolar group, 0.78-fold ± 0.33 relative to that of controls (p = 0.04), resulting from the trend for an increase in the low affinity HM74 receptor mentioned above. There was a non-significant, negative correlation between the protein for HM74A and the RT-QPCR for HM74A transcript in all groups. The correlation between the protein for HM74 and the RT-QPCR for HM74 transcript was of low magnitude, and though positive for the schizophrenia and control groups, was also not significant in any group.

The proportion of transcript represented by full-length protein was significantly decreased for HM74A in the schizophrenia group (0.17-fold ± 0.12 the mean value for the controls, p = 0.006), but not
Fig. 3. Western blots and total protein stain results, illustrating the specificity of each reagent and examples of the results for each sample in the sample set. (A) Examples of blots of PAGE lanes loaded with 0.2 mg brain tissue homogenate, showing the bands labeled, when probed with antibody HM74A affinity purified (lane A'), antibody HM74A-antisera (lane A), affinity-purified antibody for HM74 (lane B), affinity-purified antibody HM74ab (lane ab), and the affinity-purified antibody for GAPDH (gap). (B) Peptide-blocking results (using peptide for the affinity-purified antibody for HM74, showing unblocked antibody, antibody blocked (blocked unspecific 1) with the homologous peptide sequence in HM74 (LKKKLIENFQANVCIS), antibody blocked (blocked unspecific 2) with an unrelated peptide (TSNNHKKHCQEPAS) and antibody blocked (blocked specific) with the specific peptide (LKKKLPQNGGANLCSS). The washing conditions in these experiments were less stringent than those used for the blots shown in A and in C, D (below). A parallel set of peptide-blocking experiments demonstrated the specificity of the HM74 antibody for its peptide epitope (data not shown). (C) Representative Western blots to illustrate both positive and negative controls for the HM74A antibody. Expression is detected in the tissue for which prior work by others had shown significant expression at the mRNA level (the spleen [5]) and in the anterior cingulate from our sample set (positive controls), but may not be expressed in all brain regions (e.g. caudate and corpus callosum samples as negative controls). These individuals were not part of the sample set used to compare diagnostic cases and controls. (D) The Western blot results for each antibody (HM74A, HM74, HM74ab, GAPDH) are aligned for each sample (for all antibodies, the whiter the signal, the more protein is present). Two blots (left panel and right panel) were necessary to cover the entire sample set for each antibody hybridization. The diagnosis is noted above each lane (S = schizophrenia; B = bipolar). Duplicate sets of each were analyzed (top panel set and lower panel set). The lower panel represents replicate analyses run for each antibody using a section of anterior cingulate tissue immediately adjacent to the sample section used for the top panel.
Fig. 4. Representative standard curves for each antibody ECL signal (from top panel of blots in Fig. 3D). Only three standards were used for each blot, as it was necessary to include the samples, MW markers and standards within 26-lanes without placing a sample or standard at the edge of the gel, where less than optimal electrophoresis can occur. The standard curves (as above) were used to calculate the relative amount present (in mg tissue equivalents) for each antibody-responsive band in each sample. The data for the mg equivalent of HM74A and HM74ab were then normalized to the GAPDH mg equivalent values for quantitative comparisons. The open circles represent data points from the first blot of the sample set and the closed circles from the second blot of the sample set.

Tic status at death was not related to the experimental results (median HM74A QPCR in schizophrenia subjects on neuroleptics at death = 1.8-fold that of controls; in schizophrenia subjects off neuroleptics at death = 1.9-fold that of controls). A positive smoking history was associated with an increase in the transcript for HM74A in the controls (p = 0.04), but this effect was in the opposite direction and not significant in the schizophrenia group (p = 0.433). The effect of gender in the control group was a non-significant increase (p = 0.359) in the female group versus the males, and in the schizophrenia group, the difference was also not significant, though somewhat higher in the males than females (p = 0.857).

Confounding effects on HM74A protein levels
A combined examination of age, PMI, and brain pH parameters in multiple linear regression analysis revealed no significant effect (p < 0.05) on the experimental data (Table 2A), though the effect of pH trended to significance (p < 0.10) in the schizophrenia group (not in the control group). The regression values for pH (Table 2A) were attributable to a subset (N = 2) in the schizophrenia group at the high end of the pH range, illustrating a lack of homogeneity of effect. Comparing the experimental results for HM74A within the range where pH has no effect in either group (Table 2B; pH 5.8–6.4; N = 10 controls and N = 10 individuals with schizophrenia), yielded essentially the same result as comparisons within the full sample group, i.e. a significant decrease in the high-affinity niacin receptor in the schizophrenia group as compared to controls (0.48-fold ± 0.29 the mean value of the control group, p = 0.018, for the HM74A protein). The difference in HM74ab values did not reach significance in this subset.

The categorical variables were evaluated by t-test comparisons of the protein data within each diagnostic category when grouped by the categorical variable of concern. No significant additive effects were found within each group for the categorical variables combined. The effect of gender was not significant for any of the experimental variables, and to the extent that there was a difference, it was in the direction opposite to the difference between the groups, i.e. there were more males in the schizophrenia group versus controls (1.5-fold) and males tended to have higher levels of HM74A protein, not less. Similarly, the effect of smoking was not significant for any of the experimental results (Table 3). To the

Table 2A
Multiple linear regression analysis of the major demographic and postmortem continuous variables: coefficients for effect on measures HM74A and HM74ab

<table>
<thead>
<tr>
<th>Group</th>
<th>pH a</th>
<th>PMI</th>
<th>Age</th>
<th>Lifetime Neuroleptic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HM74A</td>
<td>HM74ab</td>
<td>HM74A</td>
<td>HM74ab</td>
</tr>
<tr>
<td>Controls</td>
<td>0.190, p = 0.85</td>
<td>0.850, p = 0.34</td>
<td>-0.01, p = 0.64</td>
<td>-0.002, p = 0.92</td>
</tr>
<tr>
<td>Schizophrenia</td>
<td>0.750, p = 0.22</td>
<td>1.72, p = 0.09</td>
<td>0.004, p = 0.69</td>
<td>0.012, p = 0.49</td>
</tr>
</tbody>
</table>

= 12 individuals with schizophrenia. The mean pH, PMI, and age are shown in Table 1. The multiple linear regression analysis of these variables was not significant in this group for effect on HM74A or HM74ab (overall p = 0.37 and p = 0.28, respectively).

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Table 2B

Multiple linear regression analysis of the major demographic and postmortem continuous variables: coefficients for effect on measures HM74A and HM74ab (subset with pH ≤ 6.4).

<table>
<thead>
<tr>
<th>Group</th>
<th>pH</th>
<th>PMI</th>
<th>Age</th>
<th>Lifetime Neuroleptic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controlsb</td>
<td>-0.64, p = 0.71</td>
<td>0.040, p = 0.98</td>
<td>0.036, p = 0.35</td>
<td>0.029, p = 0.40</td>
</tr>
<tr>
<td>Schizophreniaa</td>
<td>-0.49, p = 0.61</td>
<td>1.2, p = 0.38</td>
<td>0.007, p = 0.52</td>
<td>0.024, p = 0.31</td>
</tr>
</tbody>
</table>

"a" For this subset of the control group, the mean pH, PMI, and age were 6.17, 27.9 h, and 50 years, respectively. The multiple linear regression analysis of these variables was not significant in this group for effect on HM74A or HM74ab (overall p = 0.73 and p = 0.44, respectively).

"b" For this subset of the schizophrenia group, the mean pH, PMI, age, and lifetime neuroleptic dose were 6.02, 45.7 h, 45 years, and 17,995 fluphenazine mg equivalents, respectively. The multiple linear regression analysis of these variables was not significant in this group for effect on HM74A or HM74ab (overall p = 0.79 and p = 0.95, respectively).

Fig. 5. The significantly lower expression of high-affinity niacin receptor protein in the schizophrenia group: (A) when quantified with the antibody for specific for HM74A, (B) the results for HM74A antibody when case samples are grouped by neuroleptic status at death (ON or OFF neuroleptics for >2 weeks). The schizophrenia group ON neuroleptics at death was significantly lower in HM74A than the bipolar group ON neuroleptics at death. The median is indicated by the horizontal line within each box. All data were normalized to GAPDH protein present for each sample on the blot. Note that the mean brain pH of the two groups ON neuroleptics at death were essentially the same (6.17 for the schizophrenia subset and 6.20 for the bipolar subset). In the schizophrenia subset OFF neuroleptics, one patient was never medicated, one was off neuroleptics for 1 year prior to death and the other was off medication for more than 2 weeks prior to death. In the bipolar subset OFF neuroleptics, three patients had never been medicated with neuroleptics, one was off neuroleptics for >1 year and one was off neuroleptics for >2 weeks.

472 extent that there was an effect on the HM74A and HM74ab results when grouped by smoking history, the cases and controls exhibited opposite trends, and thus the criteria for a confounding variable are not fulfilled. However, bearing in mind the importance of the potential effect of smoking on gene expression, the non-smoking cases were compared to the non-smoking controls in the sample set. In the schizophrenia non-smoking group, the HM74A protein was 0.31-fold ± 0.13 the mean level of the controls (p = 0.006) and the HM74ab protein was 0.14-fold ± 0.10 the mean level of the controls (p = 0.001). The bipolar group did not show a significant difference with controls when the comparison was limited to non-smokers.

Neuroleptic lifetime dose was found to have no effect in the multiple linear regression analysis of the continuous variables (Table 2).

450 To evaluate the impact of neuroleptic status at death (“on” vs. “off”), bipolar patients on neuroleptics at death were compared to controls (1.06-fold ± 0.60 the level of the control group, p = 0.80 for HM74A and 0.98-fold ± 0.42 controls, p = 0.91 for the protein recognized by HM74ab) and to the schizophrenia group on neuroleptics at death.

444 The schizophrenia group on neuroleptics at death was significantly lower for HM74A, 0.50-fold ± 0.39 the level of the bipolar group on neuroleptics (Fig. 5B, p = 0.043), and trending to a lower value for HM74ab, 0.50-fold ± 0.70 (p = 0.06).

4 Discussion

The results of this study demonstrate that the protein for the high-affinity niacin receptor is significantly down-regulated in the anterior cingulate cortex of individuals with schizophrenia, and suggest that the peripheral deficits previously reported for their response to niacin are likely to extend to the brain, with as yet uncharacterized consequences. Specifically, the possible relevance of the down regulation of the high-affinity niacin receptor to the upregulation of the kynurenine pathway in schizophrenia [38,29,30] must be considered.

Niacin in the diet renders de novo synthesis of NAD from tryptophan unnecessary and thus negative regulation of the kynurenic pathway by niacin would be predicted. Although NAD and NADP are effective feedback regulators of kynurenine synthesis, niacin exerts only a slight degree of inhibition of catalysis [11] by tryptophan dioxygenase (TDO2), one of two enzymes in mammals that initiate kynurenine synthesis and the primary enzyme activated under conditions of niacin depletion. Rather than regulation at the catalytic level, regulation at the level of gene expression could occur for the enzymes in the kynurenine pathway that lead to the production of NAD. The most likely candidate is TDO2, based on prior work show-

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ing that only expression of TDO2 is upregulated in the schizophrenia [29]. Negative regulation of gene expression via nuclear receptors responsive to pathway components is a recognized feature of some enzymatic pathways, e.g., the bile acid ligands of the farnesoid X nuclear receptor regulate gene expression of enzymes in the biosynthetic pathway of bile acids in the liver [9]. Studies in cell culture and animal models will be necessary to explore the effect of agonists of the HM74A receptor on components of the kynurenine pathway and to determine whether regulation is exerted through gene transcription.

The cycles-to-threshold values were quite different between the GAPDH RT-QPCR and that for the niacin receptor transcripts. Although this shows, as expected, a markedly different abundance of expression, there is no literature demonstrating that this difference renders GAPDH inappropriate as a normalizing factor. To the contrary, the less standard deviation in the assay for the internal standard the less error induced into the results, and that is the case for GAPDH as compared to other transcripts [28]. Postmortem, the degradation rates of all species of mRNA varies by factors other than abundance [28,72,75]. In addition, differential degradation likely occurs due to differences in sequence, structure and cellular or tissue location of the nucleotides.

The lack of correspondence between the level of HM74A transcription and level of protein product in each diagnostic group was notable, and has been reported in studies of another class of receptors in postmortem human anterior cingulate cortex [22]. Possible causes of these findings include: (1) the transcript is alternatively spliced and the bulk of the excess transcript goes to an as-yet-identified product; (2) the transcripts, as measured, include endogenous antisense transcripts of the genetic locus, which are not distinguished from sense expression when using generic priming methods (oligo-dT or random hexamer priming for reverse transcription); (3) a polymorphism in the genomic sequence that leads to a failure at the translational stage; and/or (4) the mRNA synthesis is upregulated due to low levels of the protein through increased degradation or a decrease in production of the final protein product. The possibility of the discrepancy being caused by a genetic polymorphism is currently being addressed by SNP studies of HM74A in schizophrenia versus controls. Antisense expression, alternative splicing and increased degradation of the protein will also be pursued in future studies.

In studies of a brain disorder such as the work we present here, the use of postmortem human brain tissue to elucidate disease-associated changes obviously holds great promise when compared to studies of peripheral tissues, or to the use of non-invasive techniques that attempt to indirectly measure the same receptors in living patients. However, the positive aspects of results from postmortem brain are somewhat offset, though certainly not negated, by the inability to completely control for premortem and postmortem variables. The potentially confounding variable that could not be examined in both cases and controls was obviously the use of neuroleptics. The bipolar group on neuroleptics at death was not different from the control group, but the schizophrenia group on the same range of neuroleptic class at death as the bipolar group (first and second generation antipsychotics, including clozapine) was significantly lower in HM74A protein than both the controls and the bipolar group (Fig. 5B). Analysis of the effect of specific neuroleptics, and dose of that neuroleptic prior to death, was not possible due to the small sample size. Nevertheless, if a profound effect was exerted by neuroleptic use, the bipolar group would be expected to show a decrement in the HM74A receptor as compared to controls, whereas no difference was found (Fig. 5B). The potential for interaction between neuroleptics and the niacin receptor should continue to be explored, however, in regards to possible therapeutic effects. Not all of the therapeutic benefit of atypical neuroleptics can be explained by the profile of their catecholamine and indoleamine receptor interactions [39,40].

In conclusion, one important implication of the data we present here is that early clinical studies by Hoffer [16] reporting in a notable degree of success through treatment of unmedicated patients with niacin, but inconsistently replicated in follow-up work by others [20], should now be re-evaluated in the context of the limitations imposed by a deficient receptor. That there could be a beneficial effect of niacin agonists, particularly on negative symptoms, is supported by reports that the mood-enhancing effects of a ketogenic diet were associated with elevations in an agonist of the niacin receptor (β-hydroxybutyrate [31]), that sudden cessation of niacin following therapy for high lipid levels can be associated with the onset of negative psychiatric features [24] and that the depression associated with pellagra is profound [4]. The possibility that a deficiency in the high-affinity niacin receptor is a core feature of many individuals with schizophrenia provides a basis for research into more potent receptor agonists and therapies that might significantly increase expression of the fully functional protein.

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