Reductions in cholesterol and synaptic markers in association cortex in mood disorders


**Objectives:** Cholesterol forms an integral part of cell membranes and is a major component of myelin. Furthermore, cholesterol also plays a vital role in the development, function and stability of synapses. While low serum cholesterol has previously been associated with mood disorders, cholesterol levels have yet to be quantified within the brain in these disorders. The aim of this study was to quantify sterol levels in the brains of patients with major psychiatric disorders and further to relate these levels to markers of myelin and synapses.

**Methods:** Samples of visual association cortex were obtained postmortem from subjects with bipolar disorder (BPD), major depressive disorder (MDD) and schizophrenia (SCZ) and from controls (all n = 15). Concentrations of brain cholesterol, its precursors lathosterol, desmosterol and lanosterol and its metabolite 24S-hydroxycholesterol were determined by gas-liquid chromatography. Immunoreactivity for myelin basic protein (MBP), synaptophysin and VAMP was quantified by enzyme-linked immunosorbent assay.

**Results:** Cholesterol levels were 13% lower in MDD (p = 0.018) and 10% lower in BPD (p = 0.052) compared with controls. Cholesterol precursor or metabolite concentrations did not differ between groups. Synaptophysin immunoreactivity was 20% lower in BPD (p = 0.025) and VAMP immunoreactivity 37% lower in MDD (p = 0.032) and 45% lower in BPD (p = 0.009). MBP immunoreactivity was not altered in any disorder.

**Conclusions:** Our data suggest that lower brain cholesterol levels and a reduction in synapses may be features of mood disorders.
The functional significance of low cholesterol levels is unclear. Cholesterol is an integral part of cell membranes. Increasing evidence suggests that this molecule has a multitude of functions and may play a role in the regulation of signalling molecules and the modulation of receptor function, as well as acting as a precursor of steroid hormones (19, 20). Recent studies have suggested that cholesterol may be involved in the development, function and stability of synapses (21). A key event in neurotransmitter release from synaptic terminals is the formation of the soluble N-ethyl-maleimide-sensitive factor attachment protein receptor (SNARE) complex. The SNARE complex consists of three main proteins: VAMP/synaptobrevin, SNAP-25 and syntaxin. SNARE-dependent exocytosis of synaptic vesicles occurs at cholesterol-rich ‘rafts’ within plasma membranes (22, 23). Furthermore, the ability of VAMP to interact with its SNARE complex partners is modified by the synaptic-associated protein synaptophysin. Recent investigations have identified synaptophysin as a specific cholesterol-binding protein that also plays an important role in the retrieval of synaptic vesicle proteins during membrane recycling (24).

Within the brain cholesterol is largely synthesized in situ, with little transfer of cholesterol or its precursors from plasma (25). The majority of brain cholesterol is present within myelin, with much of the remaining pool being found within the plasma membranes of neurones and glial cells. Central nervous system cholesterol homeostasis involves de novo synthesis or uptake using membrane transport systems or specific ligands, with excess cholesterol being converted to 24S-hydroxycholesterol (24S-OH-Chol), which is then released into the systemic circulation (26). As cholesterol is not able to cross the blood–brain barrier it is important to investigate levels in the brain itself. In this study concentrations of cholesterol, the cholesterol precursors lathosterol, desmosterol and lanosterol, the cholesterol metabolite 24S-OH-Chol and the plant-derived sterols campesterol and sitosterol were determined in the visual association cortex of patients with BPD, major depressive disorder (MDD), SCZ and healthy controls. The plant-derived sterols campesterol and sitosterol are exclusively of dietary origin and are found in significant amounts in the brain only if the blood–brain barrier is disrupted. As much of the cholesterol present in the brain is contained within myelin, immunoreactivity for myelin basic protein (MBP) was also quantified. Furthermore, in response to recent evidence highlighting the important influence of cholesterol on synaptic function, immunoreactivity for the synaptic vesicle-associated protein synaptophysin, which binds to cholesterol, and VAMP, a vesicular member of the SNARE protein complex, was also quantified in the same cases.

Materials and methods

Patients

Frozen samples of visual association cortex (BA 19) were obtained from the Stanley Foundation Neuropathology Consortium, MD, USA. Choice of this region was based primarily on tissue

<table>
<thead>
<tr>
<th>Variable</th>
<th>Controls</th>
<th>SCZ</th>
<th>BPD</th>
<th>MDD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Demographic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age at death in years (mean±SD)</td>
<td>48.1±10.7</td>
<td>44.2±13.1</td>
<td>42.3±11.7</td>
<td>46.4±9.3</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>9/6</td>
<td>9/6</td>
<td>9/6</td>
<td>9/6</td>
</tr>
<tr>
<td><strong>Histological</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Postmortem interval in hours (mean±SD)</td>
<td>23.7±9.9</td>
<td>33.7±14.6</td>
<td>32.5±16.1</td>
<td>27.5±10.7</td>
</tr>
<tr>
<td>Brain hemisphere (right:left)</td>
<td>7.8</td>
<td>6.9</td>
<td>8.7</td>
<td>6.9</td>
</tr>
<tr>
<td><strong>Clinical</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cause of death</td>
<td>13a:2b</td>
<td>8a:2b:4c:1d</td>
<td>4a:1b:9c:1d</td>
<td>7a:7c:1d</td>
</tr>
<tr>
<td>Duration of disorder in years (mean±SD)</td>
<td>0±0</td>
<td>21.3±11.4</td>
<td>20.1±9.7</td>
<td>12.7±11.1</td>
</tr>
<tr>
<td>Antipsychotic dose in mg (minimum; median; maximum)</td>
<td>0; 0; 0</td>
<td>35,000; 200,000</td>
<td>7,500; 60,000</td>
<td>0; 0; 0</td>
</tr>
<tr>
<td>Past alcohol/drug abuse or dependence (no:yes)</td>
<td>13:2</td>
<td>12:3</td>
<td>12:3</td>
<td>12:3</td>
</tr>
<tr>
<td>Current alcohol/drug abuse or dependence (no:yes)</td>
<td>15:0</td>
<td>12:3</td>
<td>11:4</td>
<td>12:3</td>
</tr>
<tr>
<td>Antidepressant treatment (no:yes)</td>
<td>15:0</td>
<td>10:5</td>
<td>7:8</td>
<td>3:12</td>
</tr>
<tr>
<td>Mood stabilizer treatment at death (none:lithium:other)</td>
<td>15:0</td>
<td>12:2:1</td>
<td>5:4:6</td>
<td>13:2:0</td>
</tr>
<tr>
<td>Death by suicide (no:yes)</td>
<td>15:0</td>
<td>11:4</td>
<td>6:9</td>
<td>8:7</td>
</tr>
</tbody>
</table>

SCZ = schizophrenia; BPD = bipolar disorder; MDD = major depressive disorder. Neuroleptic dose is lifetime neuroleptic dose in fluphenazine milligram equivalent dose. Cause of death is categorized under the following headings: a = cardiopulmonary; b = accident; c = suicide; d = other.
availability, however, there is mounting evidence for visual processing deficits in psychiatric disorders (27). The sample consisted of 60 subjects (15 healthy controls, 15 schizophrenics, 15 BPD and 15 MDD). Diagnoses were made according to Diagnostic and Statistical Manual of Mental Disorders (DSM) IV criteria. Detailed case summaries were provided on demographic, clinical and histological information (Table 1). All brains underwent clinical neuropathological examination and none demonstrated evidence of neurodegenerative changes or other pathological lesions. Tissue was available from one hemisphere of each brain, with approximately equal numbers sampled in a random manner from each side.

Sterol analysis
Following rapid thawing, grey matter was dissected and homogenized in 10 volumes of ice-cold phosphate-buffered saline. Sterols were extracted from grey matter homogenates as previously described (26). Cholesterol levels were quantified by gas chromatography-flame ionization. Lathosterol, desmosterol, lanosterol, campesterol, sitosterol and 24S-OH-Chol levels were determined by combined gas-liquid chromatographic-mass spectrometry analysis. Sterol concentrations were calculated from standard curves using 5α-cholestane, epicoprostanol or racemic 24R,S-OH-Chol as internal standards. Identification of all sterols was proved by comparison with full scan mass spectra of authentic compounds.

Enzyme-linked immunosorbent assay (ELISA)
Monoclonal antibodies reactive with MBP (SMI94, Sternberger Monoclonals, Lutherville, MA, USA, 1:250), synaptophysin (EP10, 1:10) and VAMP (SP10, 1:10) were used to quantify synaptic- and myelin-associated proteins by ELISA as previously described (28–30). Grey matter homogenates were diluted to 60 µg protein/mL in distilled water. Duplicate samples were then serially diluted over a 128-fold range and dried onto 96-well ELISA plates. Non-specific binding was blocked and the plates were incubated with primary antibody overnight at 4°C. Each plate also contained control wells in which tissue culture-conditioned media was substituted for the primary antibody. The plates were further incubated with peroxidase-conjugated secondary antibody and then with 2,2’-azino-di-3-ethylbenzthiazoline substrate for 30 min. The optical density of each well was determined at 405 nm. The mean optical density of each sample was then plotted against the protein concentration (Softmax; Molecular Devices, Sunnyvale, CA, USA) and the linear portion of the curve determined for each sample. The mean linear range for the antibodies used was 16-fold for MBP, 32-fold for synaptophysin and 48-fold for VAMP. To compare immunoreactivity between samples the amount of protein required to give an optical density reading of 0.5 was used. Samples were run two or three times, on different days, and mean values used for analysis. Between-run correlations ranged between 0.88 and 0.95.

Immunoblotting
Immunoblotting studies were performed to confirm the specificity of the antibodies. Brain homogenate (30 µg per lane) from control and BPD subjects was separated on 15% SDS polyacrylamide gels. Following transfer to nitrocellulose, blots were incubated with monoclonal antibodies against synaptophysin (EP10, 1:10), MBP (SMI94, Sternberger Monoclonals, 1:2000) or VAMP (SP10, 1:10) and bands detected with enhanced chemiluminescence (Fig. 1).

Statistical analysis
Measures of brain cholesterol, the cholesterol precursors lathosterol, desmosterol and lanosterol, the cholesterol metabolite 24S-OH-Chol, the plant-derived sterols campesterol and sitosterol, MBP, synaptophysin and VAMP were compared between groups. As our primary objective was to look for differences between the control group and each of the three patient groups separately, analysis of covariance (ANCOVA) analyses were run using diagnoses as contrasts. Normal distributions were

Fig. 1. Immunoblotting studies indicate bands at the expected molecular weights for synaptophysin (EP10), myelin basic protein (MBP) and VAMP (SP10) in representative, bipolar disorder (BPD) and control (CON) subjects. Vertical scale represents molecular weight in kilodaltons.
confirmed by Kolmogorov–Smirnov tests. The demographic and histological variables listed in Table 1 were considered to be potential confounders of group differences if they differed between the psychiatric groups and the control group according to t-tests. In addition, for each measure a forward selection procedure with 5% inclusion threshold was employed to identify further demographic and histological variables that could be shown empirically to predict the outcome. Analyses were adjusted for potential confounders and empirical predictors identified in this way. As low serum cholesterol levels have been associated with an increased risk of suicide (2–5) and furthermore, as suicide may influence myelin and synaptic protein levels within the brain (29), suicide was included as a factor in the analyses irrespective of whether this variable was identified as a confounder or not.

The presence of a relationship between cholesterol concentrations and immunoreactivity for MBP, synaptophysin and VAMP was tested using Spearman’s rank correlation both overall and within each of the diagnostic groups separately. The presence of a relationship between each of the variables measured and lifetime antipsychotic dose was tested using Spearman’s rank correlation. All analyses were carried out in SPSS version 11 (SPSS Inc., Chicago, IL, USA).

Results
Sterol analysis
Summaries of levels of brain cholesterol, the cholesterol precursors lathosterol, desmosterol and lanosterol, the cholesterol metabolite 24S-OH-Chol, the plant-derived sterols campesterol and sitosterol are shown in Table 2. Mean postmortem interval for the schizophrenic group was significantly higher than for controls (p = 0.038). No group differences were detected for the remaining demographic and histological variables listed in Table 1. Thus all analyses were adjusted for suicide and postmortem interval. Planned comparisons between each of the individual psychiatric groups and the control group, using diagnoses as contrasts, indicated that levels of cholesterol were lower by 13% in the MDD group (p = 0.018) and by 10% in the BPD group (p = 0.052) compared with controls (Fig. 2). This finding was not statistically significant following correction for multiple comparisons. Lathosterol, desmosterol, lanosterol, 24S-OH-Chol, campesterol or sitosterol concentrations did not differ between the psychiatric disorder groups and the control group.

Overall, cholesterol levels were 12% lower in the psychiatric patients who did not die by suicide [n = 25, 98.2 ± 23.2 (mean ± SD)] compared

Table 2. Observed mean (±SD) concentrations (µg/mg wet weight) of cholesterol, lathosterol, desmosterol, lanosterol, 24S-hydroxycholesterol (24S-OH-Chol), campesterol and sitosterol within the visual association cortex in schizophrenia (SCZ), bipolar disorder (BPD) and major depression (MDD) and controls (CON)

<table>
<thead>
<tr>
<th>Dx</th>
<th>Cholesterol</th>
<th>Lathosterol</th>
<th>Lanosterol</th>
<th>Desmosterol</th>
<th>24S-OH-Chol</th>
<th>Campesterol</th>
<th>Sitosterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON (n = 15)</td>
<td>116.5 ± 26.0</td>
<td>117.9 ± 39.8</td>
<td>19.1 ± 7.5</td>
<td>69.1 ± 17.7</td>
<td>105.6 ± 31.3</td>
<td>22.5 ± 8.5</td>
<td>24.5 ± 9.1</td>
</tr>
<tr>
<td>SCZ (n = 15)</td>
<td>106.9 ± 20.6</td>
<td>124.5 ± 40.0</td>
<td>18.8 ± 7.6</td>
<td>67.3 ± 17.7</td>
<td>108.3 ± 22.2</td>
<td>17.9 ± 10.0</td>
<td>21.6 ± 10.5</td>
</tr>
<tr>
<td>BPD (n = 15)</td>
<td>104.8 ± 17.2</td>
<td>116.2 ± 36.0</td>
<td>18.4 ± 4.3</td>
<td>75.6 ± 61.7</td>
<td>100.1 ± 39.7</td>
<td>21.0 ± 11.4</td>
<td>25.2 ± 11.3</td>
</tr>
<tr>
<td>MDD (n = 15)</td>
<td>100.9 ± 28.3*</td>
<td>101.7 ± 36.7</td>
<td>17.8 ± 6.3</td>
<td>58.2 ± 15.8</td>
<td>94.0 ± 30.4</td>
<td>21.6 ± 11.8</td>
<td>25.6 ± 13.3</td>
</tr>
</tbody>
</table>

*Analysis of individual measures indicated a significant effect of diagnosis on cholesterol concentration (MDD, p = 0.018).
with the patients that did (n = 20, 111.7 ± 18.7, ANOVA, F = 4.264, p = 0.045). We were able to further subdivide cause of death into non-violent suicide (overdose) and violent suicide (other methods) for each of the psychiatric cases in this study. Brain cholesterol concentrations did not differ between psychiatric patients who died by violent suicide (n = 10, 116.4 ± 312.5) and patients who died by non-violent suicide (n = 10, 107.1 ± 33.2). No correlation was observed between cholesterol levels and lifetime antipsychotic dose.

Enzyme-linked immunosorbent assay

Sex was found to be an empirical predictor of both synaptophysin (F = 8.843, p = 0.004) and VAMP (F = 7.326, p = 0.009) immunoreactivity. Furthermore, postmortem interval was found to predict MBP immunoreactivity (r² = 0.075, p = 0.034). For MBP group comparisons were adjusted for suicide and postmortem interval, while for synaptophysin and VAMP comparisons were also adjusted for sex. Planned comparisons of synaptophysin immunoreactivity between each of the individual psychiatric groups and the control group indicated 20% lower levels in BPD (p = 0.025). Furthermore, VAMP immunoreactivity was lower by 45% in BPD (p = 0.009) and by 37% in MDD (p = 0.032) compared with controls (Fig. 2). No significant relationship was observed between VAMP, synaptophysin or MBP immunoreactivity and lifetime antipsychotic dose.

No statistically significant correlations were observed between brain sterol levels and MBP, synaptophysin or VAMP immunoreactivities when all cases were pooled, or in control or disease groups individually.

Discussion

Cholesterol levels were 13% lower in the visual association cortex in MDD and 10% lower in BPD, compared with controls. While sterol levels have recently been quantified in the brain of patients with Alzheimer’s disease (31), this is the first study to have examined cholesterol levels within the brain in psychiatric disorders. Low serum cholesterol levels have previously been reported in patients with affective disorders (9) and BPD (8, 10).

Brain cholesterol levels were slightly lower in those psychiatric patients who did not die by suicide compared with the patients that did. This appears to be at odds with previous studies describing an association between suicidality and low serum cholesterol levels (2–5). However, while some epidemiological studies have previously reported an association between low serum cholesterol levels and death by suicide within the general population (14, 15), others have found either no association (16) or a positive correlation (17, 18). Furthermore, it should be stressed that the relationship between brain levels and the serum levels quantified in previous investigations is not yet fully understood. Indeed there is evidence from rodent studies that peripheral and brain cholesterol may be independently regulated (32, 33).

We cannot rule out an effect of medication on these findings. However, we did not observe any correlation between sterol levels and lifetime antipsychotic dose. Furthermore, previous studies have indicated that in patients medicated with carbamazepine, imipramine, clozapine or olanzapine, serum cholesterol levels are increased or unchanged, rather than reduced (34–37).

Levels of the cholesterol precursors lanosterol, lathosterol and desmosterol were not altered in any of the psychiatric groups studied. We also found no differences in levels of the plant sterols campesterol and sitosterol between groups. These plant sterols provide a useful marker of dietary cholesterol absorption. Moreover, we did not find altered levels of the cholesterol metabolite 24S-OH-Chol in any of the psychiatric disorders investigated. Altered levels of 24S-OH-Chol have previously been described in Alzheimer’s disease (31) and may be indicative of anomalous cholesterol metabolism. While we found no evidence for altered levels of cholesterol precursors or metabolites in this study, we cannot rule out anomalous cholesterol processing. Brain cholesterol recycling is thought to involve apolipoprotein E (apoE), along with membrane transport proteins such as members of the low-density lipoprotein receptor family (38). While levels of apoE, the principal carrier of free cholesterol in the brain, have yet to be measured in depressed patients, a recent study found increased levels of apoE in the prefrontal cortex in BPD compared with healthy controls (39).

The majority of brain cholesterol is present within myelin. Due to the slow turnover of myelin, cholesterol localized within the myelin sheath is effectively immobilized. In this study we found no correlation between cholesterol levels and immunoreactivity for MBP. While this could reflect the use of different techniques for quantification, we suggest that the reduced cholesterol levels observed in MDD are not a consequence of an overall loss of cortical myelin. Furthermore, as MBP levels did not differ between groups, we feel it is unlikely that the reduction in cholesterol results from an unequal amount of white matter being included in the cortical grey matter homogenates during the
dissection process. Cholesterol particles are secreted by glial cells and are taken up by neurones utilizing a receptor-mediated process (40). There is increasing evidence for a reduction in the density of glial cells in the cerebral cortex in psychiatric disorders (41–43). We hypothesize that reduced numbers of glial cells could influence synaptic function and plasticity by means of diminished cholesterol transfer.

In the light of recent evidence that cholesterol plays a role in the development, function and stability of synapses, we also looked for a possible relationship between the synaptic-associated protein synaptophysin and the SNARE protein VAMP and sterol levels. No overall correlation was observed between measures of cholesterol and either of these proteins. However, in this study we found that immunoreactivity for VAMP was significantly lower by 45% in BPD and by 37% in MDD, compared with controls. We also noted that synaptophysin immunoreactivity was lower by 20% in the BPD group compared with controls. Therefore, we cannot rule out the possibility of an association between low cholesterol concentrations and reduced synaptic density or function in mood disorder patients. While the distribution of synaptic proteins has not yet been fully examined in visual association cortex in major psychiatric disorders, reductions in synaptophysin immunoreactivity have been previously described in SCZ in a second visual association region, BA 20 (44). Furthermore, of two previous studies of the primary visual cortex, one described a loss of synaptophysin protein and mRNA in female schizophrenic patients (45), while the other detected no change in this disorder (46). To our knowledge, VAMP levels have not previously been investigated in BPD. However, reductions in VAMP immunoreactivity have been reported in the frontal cortex in patients with SCZ compared with controls (47).

In this study we observed a reduction in cholesterol levels in the visual association cortex in MDD, with a similar trend in BPD, compared with controls. Cholesterol levels were found to be lowest in mood disorder patients. We also observed lower levels of the synaptic markers synaptophysin and VAMP in mood disorder patients. We suggest that lower brain cholesterol levels and a reduction in synapses may be pathophysiological features of mood disorders.

Acknowledgements

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References
