Neuronal Changes in the Cerebral Cortex of the Rat Following Alcohol Treatment and Thiamin Deficiency

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Abstract. The contribution of thiamin deficiency to the pathology of alcohol-related brain damage is still unclear. This study used a model of prolonged alcohol abuse in which animals were subjected to a brief period of mild thiamin deficiency. The episode of thiamin deficiency was early (after 4 weeks), in the middle (after 15 weeks) or late (after 26 weeks) in their 28 week alcohol treatment period. A control group of animals fed no alcohol and maintained on a thiamin-replete diet was used for comparison. The brains were removed and sectioned in the coronal plane at 50 μm intervals. Successive serial sections were stained with cresyl violet for Nissl substance and immunohistochemically with antibodies to the calcium-binding proteins parvalbumin and calbindin. These calcium-binding proteins identify the majority of GABA-containing neurons in the cerebral cortex. The number of cells in the Fr1 region of the cerebral cortex was quantitated. A significant loss of Nissl-stained neurons was identified from the early group, while a loss of parvalbumin-immunoreactive neurons was seen in the early and middle groups. No loss of neurons was identified from the late group. In addition, no loss of calbindin-immunoreactive neurons was seen. This study represents the first report of cortical neuronal loss in an animal model of alcohol abuse and thiamin deficiency. Moreover, the results imply that thiamin deficiency is integrally involved in the pathogenesis of alcohol-related cortical neuronal loss.

Key Words: Alcohol toxicity; Calcium-binding proteins; Frontal cortex; Rat; Thiamin deficiency.

INTRODUCTION

Brain damage after a single episode of thiamin deficiency has been documented in both humans (1, 2) and animals (3, 4). Chronic Wernicke–Korsakoff syndrome (WKS) pathology may develop after a single or repeated episodes of thiamin deficiency (5). However, clinical documentation of an episode of acute Wernicke’s encephalopathy is not always identifiable in a proportion of those alcoholics who develop chronic WKS (6, 7). This has lead a number of authors to suggest that chronic WKS pathology may result from repeated subclinical episodes of thiamin deficiency (5, 6). Such an hypothesis has serious implications for the study of alcohol-related brain damage. It suggests that some, or maybe all, alcoholics have episodic “subclinical” thiamin deficiency. In such patients the classical clinical symptoms of thiamin deficiency may not be apparent, but the eventual outcome of this compromised metabolism would be cell death and ultimately chronic WKS pathology. Bowden (8) takes the hypothesis further by suggesting that some of the neuropsychological deficits reported in detoxified alcoholics are due to undetected WKS. He finds evidence to support his theory in pathological studies which have, on retrospective examination of clinical notes, found that the most common of the so-called “classical triad” of symptoms described in patients with chronic WKS is impaired mentation (7, 9). As a corollary to this, it can be proposed that those alcoholics without clinically identifiable WKS pathology may have some degree of cell damage which has been caused by subclinical thiamin deficiency.

Further evidence for subclinical thiamin deficiency among alcoholics comes from a survey of the thiamin status of homeless men in Sydney (10). Fifteen percent had a deficient thiamin status (measured by the thiamin pyrophosphate effect), 21% had a marginal thiamin status and the mean daily thiamin intake of the whole group was less than the recommended daily intake.

These findings highlight the difficulties, in retrospective pathological studies, of knowing whether alcoholics were thiamin-deficient or not. Thus, it is difficult to examine the role of alcohol toxicity and thiamin deficiency in alcohol-related brain damage. The use of an animal model allows these two factors to be varied at will.

This study explores the contribution of thiamin deficiency to alcohol-related brain damage using an animal model which parallels the drinking patterns of many alcoholics. Rats were chronically fed an alcohol-containing diet and then subjected to a brief period of mild thiamin deficiency. The frontal region 1 (Fr1) of the cerebral cortex was studied as cortical neuronal loss from the frontal cortex (Brodmann’s area 8) has been identified in human alcoholics (11). In addition, immunohistochemistry and size differentiation was used to identify subsets of cortical neurons for analysis.

MATERIALS AND METHODS

Animal Model

Forty-eight male Wistar rats were housed separately in a room air conditioned to 23 ± 2°C and 50% relative humidity with a 12 hour light and dark cycle. They had free access to pelleted Lab Chow (Barastoc, Australia) and tap water. At 18 weeks of

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age they were randomly divided into four groups. Their mean weight was 422 ± 46 g. At the end of the experimental period 17 rats were randomly chosen from the group of 48 for neuropathological examination.

**Control Group (n = 5):** Rats had free access to Lab Chow and tap water throughout the entire period of the experiment. No thiamin supplementation of their diet was made.

**Experimental Groups (n = 4 in each group):** The three experimental groups were given a 20% v/v solution of ethanol as their only drinking fluid. They had free access to Lab Chow, except for the period of thiamin deficiency when they were fed a powdered thiamin-free diet (diet number 903027, ICN Biochemicals, Costa Mesa, CA). Daily intraperitoneal injections of 50 µg/kg pyrithiamine hydrobromide (Sigma Chemicals, St. Louis, MO) in saline were given for 10 days to induce thiamin deficiency. When piloerection, rolling gait, anorexia and weakness of the hindlimbs were present the animals were injected intraperitoneally with 100 mg/kg of thiamin hydrochloride twice at 24 hour intervals to reverse the symptoms. They were then returned to their lab chow and ethanol diet until the end of the experimental period (28 weeks). **Experimental group 1** (early) was made thiamin-deficient after 4 weeks on alcohol. **Experimental group 2** (middle) was made thiamin-deficient after 15 weeks on alcohol. **Experimental group 3** (late) was made thiamin-deficient after 26 weeks on alcohol. At the end of the 28 week experimental period, all experimental and control animals were fed a lab chow and water diet until they were sacrificed at 82 weeks of age. Behavioral testing was performed during this period. The results of these studies will be published elsewhere.

**Preparation of Tissue**

After weighing, animals were anesthetized with an intraperitoneal injection of thiopentone. Five hundred units of heparin was injected into the left ventricle of the heart. Animals were transected into small pieces first with 100 ml phosphate buffered saline (pH 7.4) and then with fixative (4% paraformaldehyde, 0.5% glutaraldehyde, 0.2% picric acid in phosphate buffer, pH 7.4). After 10 minutes the flow rate of fixative was reduced and allowed to continue until 500 ml was used. The brain was removed immediately, weighed, and immersed in fixative for a further 24 hours and then into 0.1 M Tris HCl buffer with 0.01% sodium azide (pH 7.4) until required.

The cerebellum and brainstem were separated from the cerebrum by a horizontal cut through the midbrain and sectioned midsagittally. A block of tissue 2 mm thick was processed for paraffin embedding. Ten micrometer thick sections were cut and stained with hematoxylin and eosin and cresyl violet (12).

The cerebral hemispheres were cryoprotected in 30% sucrose in 0.1 M Tris HCl. Serial 50 µm sections were cut on a freezing microtome (Lietz Instruments). Successive sections were stained using cresyl violet for Nissl substance (12), and immunohistochemically with antibodies to parvalbumin and calbindin.

**Immunohistochemistry**

Antibodies to the calcium-binding proteins parvalbumin and calbindin were used. These proteins occur in mutually exclusive populations of GABA-containing neurons in the cerebral cortex and together represent almost all GABAergic neurons (13). Both proteins have a role in buffering Ca²⁺ and Mg²⁺ ions and are therefore directly related to the excitability of the neuron (13).

All immunohistochemistry was performed on free-floating tissue sections using the method described by Halliday and Türk (14). The tissue was washed with gentle agitation in 50% ethanol and in 50% ethanol containing 3 ml/100 ml of hydrogen peroxide. Non-specific binding sites were blocked using 10% normal horse serum in 0.1 M Tris HCl with 0.01% sodium azide for 20 minutes with gentle agitation.

Primary antibody incubation was performed for 48 hours at 4°C at a dilution of 1:10,000 for monoclonal mouse anti-parvalbumin (Sigma) and 1:2,000 for monoclonal mouse anti-calbindin (Sigma) in 0.1 M Tris HCl with 0.01% sodium azide and 1% normal horse serum. Secondary antibody (biotinylated horse anti-mouse IgG, Vector Laboratories, Burlingame, CA) incubation was performed at room temperature for 1 hour at a dilution of 1:200. Tertiary complex (avidin DH and biotinylated horseradish peroxidase complex, Vector Laboratories) incubation was performed at room temperature for 1 hour at a concentration of 100 in 0.1 M Tris HCl with gentle agitation.

Visualization was performed using twice-filtered 0.6 mg/ml 3,3'-diaminobenzidine (DAB, Sigma) in 0.1 M Tris HCl for 10 minutes, and the reaction product was developed by adding 25 µl of 1:10 hydrogen peroxide for a further 6 minutes. Sections were mounted onto slides, dehydrated and coverslipped using a permanent mounting medium (DPX).

The specificity of the antibodies was previously tested (Sigma). The specificity of the immunohistochemical reaction was tested by substituting normal sera for the primary antibodies. No peroxidase reaction product was noted in any of these sections.

**Quantitation**

Quantitation was performed on the Fr1 of the cerebral cortex at the level of the genu of the corpus callosum (1.2 mm anterior to the bregma) (Fig. 1a, b). The region was identified with the aid of the rat brain atlas of Paxinos and Watson (15) and Zilles and Wree (16). The Fr1 region was chosen for quantitation as it is granular cortex which receives input from a number of thalamic and hypothalamic nuclei, the basal forebrain, locus ceruleus and raphe nuclei and thus resembles Brodmann’s area 8 in the human cerebral cortex. Neuronal loss from area 8 in humans has been shown to occur in alcoholics with and without thiamin deficiency (11). For the Nissl-stained sections, cells in two strips of cortex perpendicular to the pial surface and spanning the entire thickness of the cortical mantle were quantified using Magellan software on a NEC computer and a microscope with a camera lucida attachment to visualize the computer screen (17). The number of large (>20 µm diameter), medium (10–20 µm) and small (<10 µm) neurons was determined at a magnification of ×200 using a calibrated marker. The area of each strip was measured and the number of cells per square millimeter was calculated.

The number of parvalbumin- and calbindin-immunoreactive cells was determined in the entire Fr1 region. The boundaries of the region were drawn at a magnification of ×40 and the cells in each size class were counted at a magnification of ×200. The number of immunopositive cells per square millimeter was calculated.
Statistics

Analysis of data was performed using the statistics package SuperANOVA (Abacus Concepts Inc., Berkeley, CA) on a Macintosh IIci computer. Comparisons between groups for each size class for each strain were performed using the one-way analysis of variance and Fisher's protected t-test.

RESULTS

The Animal Model

All experimental animals had symptoms of thiamin deficiency and these were reversed by the administration of thiamin. No animal experienced seizures, had observable opisthotonus or died during the thiamin-deficient period.

Population Statistics and Neuropathological Examination

The mean body weight and brain weight for each group is listed in Table 1. There was no significant difference between the control and experimental groups in either of these weights.

Nissl-stained sections at 1 mm intervals from throughout the forebrain and hematoxylin and eosin-stained sections of the cerebellum and brainstem were examined microscopically. No observable neuropathological abnormalities were detected in any of the sections. In particular, necrosis and hemorrhages, which have been reported previously in models of thiamin deficiency, were not seen.

Neuronal Counts

Nissl: Small, medium and large Nissl-stained cells in the Fr1 region of five control animals and four animals in each of three experimental groups were counted (Table 2). A significant reduction in the number of neurons was found for each of the size classes in experimental group 1 (early). Proportionally, this loss was greatest from the large size class (73.3%), followed by the medium (51.1%) and the small (13.2%). Overall, the loss of neurons was 18.9%. In absolute terms, the loss was greatest from the small size class as 86.9% of cortical neurons are small. There was no significant loss of Nissl-stained neurons...
from any size class in either experimental group 2 (middle) or 3 (late).

**Parvalbumin:** Immunoreactive cells were seen throughout the cerebral cortex (except for layer I) and striatum (Fig. 2a). Cells were round or multipolar and most were less than 10 μm in diameter (small). Large neurons were rarely immunopositive. Dense fiber staining was seen in all cortical layers (except layer I), and this was most marked in layers V and VI. At higher power many immunopositive fibers and terminals could be seen (Fig. 2a, b). Stained dendrites were observed for up to 20 μm from the cell body and some of these were seen to branch close to the cell body. In addition, the outline of unstained cells could be distinguished by the stained terminals surrounding them (Fig. 2b, arrows).

The number of parvalbumin-immunoreactive neurons in each size class was determined (Table 3). Too few large neurons were stained to allow statistical comparison. Qualitatively, there was no difference in the number of large cells in any of the four groups studied.

Approximately 11% of all cortical neurons are parvalbumin-immunoreactive. These consist mainly of small and medium-sized cells where 10.8% and 22.8%, respectively, of that cell class are immunopositive.

A significant reduction in the number of parvalbumin-immunoreactive neurons was seen from both the early and middle groups (Table 3). The reduction of parvalbumin-positive cells in the early group reaches significance in total cell count, but fails (0.07) in each of the two size classes. A loss of neurons was noted from both the small and medium-size classes in the middle group. The magnitude of change (30–40%) is the same in both experimental groups and there is no significant difference between groups 1 and 2. No change was found in the number of immunoreactive neurons in the late group.

**Calbindin:** Calbindin-immunoreactive cells were seen throughout the cortex (Fig. 3a). The density of cells was greatest in the upper layers (II and III). There were only occasional immunopositive cells in layer IV and none in layer I. Cells were exclusively small (<10 μm diameter) and the cell bodies were round with few stained processes (Fig. 3b). Diffuse fiber staining was present predominantly in layers II and III (Fig. 3a). A few vertically projecting fibers were stained in the upper layers. These could be seen extending 50–60 μm in length. A minor population of the immunoreactive cells contained darker reaction product (Fig. 3b). The proportion of these was similar throughout the cortex.

The number of calbindin-immunoreactive cells in each group was determined (Table 4). There was no significant difference between the number of cells in the control group and any of the experimental groups. In addition, the percentage of cells which stained darkly was also determined. This proportion was not significantly different from the control group in any of the experimental groups. The percentage of Nissl-stained cells which are calbindin-immunoreactive is similar in the control group and experimental groups 2 and 3. The percentage is greater (not significantly) in the early group, but this is due to there being fewer Nissl-stained cells in this group rather than

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**Table 2: Mean Number of Neurons in Nissl-stained Sections**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Experimental 1</th>
<th>Experimental 2</th>
<th>Experimental 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Count*</td>
<td>% Stain</td>
<td>Count*</td>
<td>% Stain</td>
</tr>
<tr>
<td>Small</td>
<td>800 (26)</td>
<td>86.9</td>
<td>694 (28)</td>
<td>92.9</td>
</tr>
<tr>
<td>Medium</td>
<td>92 (9)</td>
<td>10.0</td>
<td>45 (2)</td>
<td>6.0</td>
</tr>
<tr>
<td>Large</td>
<td>30 (6)</td>
<td>3.3</td>
<td>8 (2)</td>
<td>1.1</td>
</tr>
<tr>
<td>Total</td>
<td>921 (31)</td>
<td>100</td>
<td>747 (67)</td>
<td>100</td>
</tr>
</tbody>
</table>

* Number/mm² (SEM).
** p value determined using Fisher's protected t-test following ANOVA compared to controls.

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**Table 3: Mean Number of Parvalbumin-immunoreactive Cells**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Experimental 1</th>
<th>Experimental 2</th>
<th>Experimental 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Count*</td>
<td>% Nissl</td>
<td>Count*</td>
<td>% Nissl</td>
</tr>
<tr>
<td>Small</td>
<td>86 (8)</td>
<td>10.8</td>
<td>61 (1)</td>
<td>8.8</td>
</tr>
<tr>
<td>Medium</td>
<td>21 (2)</td>
<td>22.8</td>
<td>14 (1)</td>
<td>31.1</td>
</tr>
<tr>
<td>Total</td>
<td>107 (8)</td>
<td>11.6</td>
<td>76 (1)</td>
<td>10.2</td>
</tr>
</tbody>
</table>

* Number/mm² (SEM).
** p value determined using Fisher's protected t-test following ANOVA compared to controls.
Fig. 2. (a) Parvalbumin-immunoreactive neurons in the Fr1 region of a control animal. Bar = 100 μm. The boxed region is seen at a higher power in (b). Arrows indicate unstained cells surrounded by immunoreactive terminals. Bar = 50 μm.

Fig. 3. (a) Calbindin-immunoreactive neurons in the Fr1 region of the cerebral cortex. Bar = 100 μm. (b) A higher power view of immunoreactive neurons. Bar = 50 μm.
a real increase in the number of calbindin-immunoreactive cells.

**DISCUSSION**

Previous pathological studies on animal models of alcohol-related brain damage have concentrated on either thiamin deficiency or alcohol, but have rarely used them in combination. Animal models of alcohol-related brain damage have identified a number of neuronal abnormalities. Quantitative studies show that the density of hippocampal granule cells is reduced by 20% (18) and 37% (19) compared to controls following prolonged alcohol consumption. Both studies allowed an alcohol-free period before quantitation was performed. Similarly, hippocampal pyramidal and cerebellar Purkinje and granule cells are reduced in number (18, 20, 21). Golgi studies of hippocampal pyramidal cells (22) and cerebellar Purkinje cells (23, 24) have revealed a reduction in dendritic arborization in response to alcohol exposure. The cerebral cortex has not been studied in animal models of alcohol abuse.

A recent study on the progression of the clinical symptoms of thiamin deficiency in alcohol-treated rats found that those animals fed a combination of a thiamin-deficient diet and alcohol developed symptoms more rapidly than those fed a thiamin-deficient diet or alcohol alone (25). In addition, the authors showed pathological changes in the brainstem of a number of the rats in the thiamin-deficient and thiamin-deficient plus alcohol groups. No cortical pathology was identified and no quantitative pathological analyses were performed.

The animal model used in this study has a number of advantages over previously published work. Authors concerned with the effects of thiamin deficiency have produced models of “end-stage” disease in which the animals are severely debilitated (25-27). The animals developed clinical signs of thiamin deficiency such as ataxia, piloerection, hypothermia and loss of righting reflex, but in addition, many suffered generalized sensory-evoked seizures and were moribund at the time of harvest. While this may resemble the situation in people who die with acute WKS, it is not a good model of the chronic disease. In addition, although epileptic seizures are a hallmark of alcoholic withdrawal syndrome (28) and are more common among alcoholics than the general population (29), it is not a symptom specifically associated with WKS (30).

Much has been published on the effects of seizures on specific neuronal populations in humans and in experimental models (31, 32). The onset of pathological lesions in the brain of thiamin-deficient animals coincides with the onset of seizures (33). Therefore, the pathology may result from the seizures rather than from the thiamin deficiency. By using a more moderate model of thiamin deficiency it is believed that a closer approximation to the human disease, WKS, is achieved.

**TABLE 4**

<table>
<thead>
<tr>
<th>Count*</th>
<th>% Nissi</th>
<th>% Dark</th>
<th>p**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>128 (11)</td>
<td>16.0</td>
<td>10.8</td>
</tr>
<tr>
<td>Experimental 1</td>
<td>152 (6)</td>
<td>21.9</td>
<td>11.2</td>
</tr>
<tr>
<td>Experimental 2</td>
<td>147 (10)</td>
<td>19.5</td>
<td>10.0</td>
</tr>
<tr>
<td>Experimental 3</td>
<td>122 (15)</td>
<td>16.0</td>
<td>15.5</td>
</tr>
</tbody>
</table>

* Number/mm² (SEM).
** p value determined using Fisher's protected t-test following ANOVA compared to controls.

The episode of thiamin deficiency in these animals was varied during their alcohol-treatment period: early, middle or late. The results suggest that there is a time-dependent relationship between thiamin deficiency and neuronal damage. Those animals who were made thiamin-deficient immediately before the end of the alcohol-treatment period (26 weeks) did not show any significant neuronal loss either in the total neuronal population (Nissl-stained) or in any of the neuronal subpopulations. Conversely, the early group (thiamin-deficient after 4 weeks on an alcohol-containing diet) showed a significant neuronal loss from all size classes of the Nissl-stained neurons and of parvalbumin-containing neurons. The middle group (thiamin-deficient after 15 weeks on an alcohol-containing diet) showed a significant loss of parvalbumin-containing neurons, but not of Nissl-stained neurons. Therefore appears that there is a lag period between the thiamin deficiency and any observable neuronal loss. Furthermore, it appears that continued alcohol consumption is necessary for this neuronal damage to be manifested as there is no neuronal loss apparent from experimental group 3 despite an episode of thiamin deficiency. These animals were withdrawn from their alcohol treatment shortly after the thiamin-deficient period and allowed to survive for a further 35 weeks before sacrifice.

These findings may be explained on the basis of compromised metabolism. Thiamin deficiency in combination with alcohol abuse may cause a disruption of the metabolism of the cell which is, at first, manifested as impaired function, but which ultimately results in cell death. Evidence for this hypothesis is gained from the observation that in the middle group there is a reduction in the number of parvalbumin-containing neurons without an overall loss of Nissl-stained neurons. Although the loss is small enough to be masked within the population variance, it may be that there is an alteration in the parvalbumin protein within the cell. This may be a reduction in the amount of parvalbumin or a change in the structure so that it is no longer recognized by the antibody. Parvalbumin is a calcium-binding protein and is involved in the regulation of intracellular calcium levels. Disruption of such vital homeostatic mechanisms, and thus of the neurotransmitter and second messenger systems which
depend on calcium, may facilitate cell death. Such a mechanism has been proposed as a possible explanation for the loss of parvalbumin-containing neurons in ischemia (34). Other mechanisms of cellular damage secondary to thiamin deficiency should also be considered. Lactate accumulation (32), decreased energy levels (35) and reduced transketolase activity (36) have all been suggested as possible mechanisms of cell damage in thiamin deficiency.

Alternatively, if the reduction in the number of parvalbumin-immunoreactive cells in the middle group is truly a cell loss and not a loss of immunoreactivity, then this would suggest that those neurons which contain parvalbumin are damaged first. This would be possible as the magnitude of reduction (40% of parvalbumin-immunoreactive cells which are 11.6% of Nissl-stained cells) could be masked in the standard deviation of the total cell count and therefore no loss of Nissl-stained cells would be identifiable. If the loss of parvalbumin-containing cells precedes the loss of other neuronal populations, then this would give valuable insight into the mechanism of damage. Parvalbumin-containing cells also contain GABA and a reduction in GABAergic neurotransmission may result in the death of other cells due to a loss of inhibition resulting in overexcitation. This loss of cells could be amplified over time because the original cells lost are involved in the regulation of others.

In summary, it appears that a brief period of thiamin deficiency in animals fed an alcohol-containing diet for a prolonged period is sufficient to cause neuronal damage. Furthermore, as neuronal damage was only identified in two of the three experimental groups it appears that the timing of the thiamin-deficient period relative to the alcoholic period is crucial. Such findings lend weight to the "biochemical lesion" theory of the WKS. In addition, it implies that thiamin deficiency is an important contributor to alcohol-related brain damage, and consequently the thiamin status of alcoholic patients should be considered of utmost importance in their management.

ACKNOWLEDGMENTS

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