NO LOSS OF SOMATOSTATIN-IMMUNOREACTIVE NEURONS IN THE HIPPOCAMPAL DENTATE HILUS OF ALCOHOL-WITHDRAWAL-KINDLED RATS

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Abstract — The neuropeptide somatostatin has been suggested to play a role in seizure genesis, electrical kindling and the neurotoxic effects of alcohol. The purpose of the present experiment was to study somatostatin-immunoreactive (SS-IR) neurons in hippocampus during alcohol-withdrawal kindling. Alcohol-withdrawal kindling was performed by subjecting male Wistar rats to seven weekly episodes consisting of 2 days of severe alcohol intoxication and 5 days of alcohol withdrawal. Then the kindled animals (multiple withdrawal group) and a single withdrawal group, which was fed isocalorically with the kindled animals during episodes 1-7, were exposed to 4 days of severe alcohol intoxication (episode 8). During the following withdrawal, the seizure activity was observed 9-15 h after last alcohol dose, in order to subdivide the animals from these two groups into groups with and without seizures. Subsequently, SS-IR neurons were visualized immunocytochemically and counted in the hilus of the dentate gyrus (hippocampus). The number of SS-IR neurons per unit area of the hilus was neither affected by a single nor by multiple episodes of alcohol withdrawal. We therefore concluded that a loss of these neurons is not involved in the development of alcohol-withdrawal-kindled seizures.

INTRODUCTION

In an animal paradigm for alcohol-withdrawal kindling, we have demonstrated that alcohol withdrawal seizures are kindled by repeated episodes of withdrawal from 2 days of severe alcohol intoxication (Clemmesen and Hemmingsen, 1984; Ulrichsen et al., 1992, 1995). The mechanisms underlying alcohol-withdrawal kindling are presently obscure. The neuropeptide somatostatin has been suggested to be involved in seizure genesis and kindling processes as well as in the neurotoxic effects of alcohol. For instance, seizures induced by either systemic kainic acid administration or by intermittent focal electrical stimulation in the hippocampus result in a marked reduction of the number of somatostatin-immunoreactive (SS-IR) neurons in the dentate gyrus of the hippocampal formation (Sloviter, 1987; Spérk et al., 1992; Pérez et al., 1995). In addition, a decreased number of SS-IR neurons in hippocampus was found in humans with cryptogenic temporal lobe epilepsy suggesting a seizure-inhibiting role of somatostatin in humans (de Lanerolle et al., 1989; Robbins et al., 1991). Somatostatin may also counteract kindling processes, since intrahippocampal injections of somatostatin antibody facilitate electrical kindling (Monno et al., 1993). In agreement with this finding, Spérk et al. (1992) showed that convulsions in picrotoxin-kindled rats were inhibited by intrahippocampal injections of somatostatin and facilitated by intrahippocampal injections of antisomatostatin serum. Clinical studies have shown that the cerebrospinal fluid levels of somatostatin are decreased in alcoholics with an early onset of their drinking behaviour and in patients with dementia associated with alcoholism (Koponen et al., 1990; Roy et al., 1991), suggesting that dysfunction of the cerebral somatostatin system may play a role in alcoholism. This speculation is supported by experimental research. Thus, Andrade et al. (1992) detected a reduced number of SS-IR neurons in the dentate hilus of hippocampus after chronic alcohol administration. In

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addition, biochemical studies have shown that alcohol intoxication affects the brain levels of somatostatin (Fuhrmann et al., 1986; Barrios et al., 1990), although these studies are not in agreement.

The evidence linking somatostatin to seizures, kindling and the neurotoxic effects of alcohol makes it reasonable to speculate that this neurotransmitter could be involved in the mechanisms underlying alcohol-withdrawal kindling. Thus, the purpose of the present experiment was to investigate the role of somatostatin neurons in this process.

**MATERIALS AND METHODS**

*Animals and intoxication procedure*

Male Wistar rats (45–50 days old with a body weight of 180–200 g; from Møllegaard, Køge, Denmark) were housed in a room with a 12 h light/dark cycle (lights on at 07:00) and had free access to food pellets and water. During each intoxication period (see below), alcohol was administered five times a day between 08:00 and 24:00 by intragastric intubation (Majchrowicz, 1975). Before each alcohol administration, the degree of alcohol intoxication was assessed by using the following rating scale: (0) neutrality (no signs of intoxication); (1) sedation (reduced muscle tone, dulled appearance and slow locomotor activity, but no impairment of gait or coordination); (2) ataxia 1 (slight gait impairment and slight motor incoordination, but able to elevate abdomen and pelvis); (3) ataxia 2 (clearly impaired staggering gait and impaired motor coordination with some elevation of abdomen and pelvis); (4) ataxia 3 (slowed righting reflex, heavily impaired motor coordination, but no elevation of abdomen and pelvis); (5) loss of righting reflex (LRR) (inability to right itself when placed on its back, other reflexes still present); (6) coma (no signs of movement; no response to pain stimuli; no blinking reflex; spontaneous breathing).

The alcohol dose was adjusted individually to the degree of intoxication. Neutral rats received 5–7 g/kg whereas animals with LRR received 0–1 g/kg. The aim was to reach an intoxication level of 3–5 (ataxia 2–LRR) within 8 h and thereafter maintaining this level for the rest of the intoxication period. The alcohol solution consisted of: ethanol (200 g/l), sucrose (300 g/l) and multivitamin mixture (4 ml/l) in Ringer’s solution; the sucrose being added in order to prevent hypoglycaemia and ketosis (Hemmingsen and Chapman, 1980). Control animals received an isocaloric amount of sucrose instead of alcohol and the same amount of water and food as the alcohol-treated rats. Blood alcohol concentration (BAC) was not measured in the present experiment, but it has previously been shown that, at an intoxication level of 3–4 (ataxia 2–ataxia 3), the corresponding BAC is ~3–4 g/l at the second day of intoxication (Ulrichsen et al., 1992, 1995) and slightly higher the following days due to the development of tolerance (Majchrowicz and Hunt, 1976).

Alcohol-withdrawal kindling was performed by subjecting the experimental animals [multiple withdrawal (MW) group, n = 72] to seven episodes, each consisting of 2 days of alcohol intoxication followed by 5 days of alcohol withdrawal (Clemmesen and Hemmingsen, 1984; Ulrichsen et al., 1992, 1995). Following the seventh intoxication period, the animals were allowed to recover for 11 alcohol-free days. Subsequently, the animals were challenged by 4 days of severe alcohol intoxication and observed 9–15 h into the withdrawal phase in order to detect any spontaneous seizure activity. In order to contrast the long-term (i.e. eight episodes) versus the acute effects of alcohol withdrawal on seizure activity, we used a single withdrawal group (SW) (n = 30) which were given alcohol only during the 4-day intoxication period at the end of the study. Prior to alcohol exposure, this group was fed under the same conditions as the control group. Seizure activity was rated openly by three observers. Animals so intoxicated that they had lost their righting reflex at the beginning of the seizure observation were excluded from the behavioural studies.

In order to verify that the animals were in fact showing clinical signs of alcohol withdrawal during the alcohol-withdrawal kindling regimen, the animals were rated openly 16 h into withdrawal during episodes 2 and 5 as previously described (Ulrichsen et al., 1986). Briefly, the three individual items ‘intentional tremor’, ‘rigidity’ and ‘hyperactivity/irritability’ were each scored on a 4-level scale (0–3) and the sum of these scores (0–9) was used as a quantitative
The number of SS-IR neurons was counted in the following groups of animals: (C) isocalorically fed controls; (SW−) animals fed isocalorically for 7 weeks and then challenged with a single 4 day alcohol intoxication period. During the subsequent withdrawal reaction, these animals did not develop seizures; (SW+) as group SW−, but these animals developed spontaneous withdrawal seizures; (MW−) animals subjected to seven episodes, each of which comprised alcohol intoxication and withdrawal and later challenged with a 4-day alcohol intoxication period. During the subsequent withdrawal phase, these animals did not develop seizures; (MW+) as group MW−, but in this group the animals developed spontaneous withdrawal seizures.

Six animals from these groups were randomly selected for the histological studies, except for group MW+ in which all the five animals (see above) were selected. In order to avoid a possible loss of neurons due to seizures per se, animals from group MW+ and SW+ were perfused with paraformaldehyde (see below) within 30 min after the seizure. In addition, we observed the animals from the two multiple episode groups (MW− and MW+) during the seventh withdrawal episode in order to exclude animals with seizures during this episode from the histological analysis. No seizures, however, were observed during this withdrawal episode. Observations of earlier episodes were not relevant, as Clemmesen and Hemmingsen (1984) showed that spontaneous seizures do not occur during the first five to six weekly episodes of alcohol-withdrawal kindling.

The rats used for the histological studies were anaesthetized with halothane and perfused transcardially with 0.05 M phosphate-buffered saline containing 0.02% KCl (KPBS, pH 7.4) to which heparin (15,000 IU/I) was added, until the blood was washed out. Then perfusion continued for 15 min with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Subsequently, the brains were immediately removed and post-fixed overnight in the same fixative. On day 2, the brains were placed for 24 h in a cryoprotective solution of 20% sucrose diluted in KPBS. On day 3, an avidin–biotinylated horseradish peroxidase immunocytochemical procedure was performed for visualization of SS-IR neurons (Mikkelsen et al., 1993). Free-floating coronal sections (50 μm) were incubated in a mouse monoclonal antiserum raised against the tetradecapeptide somatostatin (kindly provided by NOVO Nordic, Denmark; diluted 1:4000) for 16 h. As secondary antibody, we used a biotinylated rabbit anti-mouse IgG (#354, Dakopatts, Denmark; diluted 1:600). The sections were subsequently incubated for 60 min in an avidin–biotin–horseradish peroxidase complex (Vector Elite Kit™; diluted 1:125) and reacted for peroxidase activity by incubation for 20 min with a solution of 0.125% diaminobenzidine. Finally, the sections were rinsed for 2 x 10 min in distilled water before being mounted on pregelatinized glass slides and covered slips in Pertex (Sigma, St Louis, MO, USA).

The specificity of the immunocytochemical reaction was tested either by omission of the primary antiserum or by adding 12 nmol somatostatin (# S-9129, Sigma). In both cases, staining of SS-IR neurons was completely absent.

SS-IR neurons in the dorsal hippocampus were counted in the hilus of the left and right dentate gyrus (magnification × 25). The hilus was defined as the area surrounded by the dentate granular layer and situated between this layer and the CA3 region of hippocampus (Fig. 1A). Only cells with intact perikaryal contours were included in the study. The area of the hilus from which the cells were counted was measured using a microscope coupled to a commercially available computer-based image analysis system [a Macintosh computer with a scion frame grabber and the image analysis program IMAGE (version 1.59), developed by Wayne Rasband, NIH, MD, USA], in order to determine the number of SS-IR neurons per unit area of hilus (areal density). Areal densities were expressed as neurons/mm² and determined in three sections from each animal by one of the authors (D.W.), who was blind as to which group the animals belonged to.

Statistical analysis

Two-tailed t-tests were used to assess whether the expected numbers of SS-IR neurons in the dentate hilus across groups were different. The following strategy was chosen: First, we tested whether there were differences between: (1) the two multiple episode groups (MW− vs MW+);
(2) the two single episode groups (SW− vs SW+). In both cases, the seizure and the non-seizure groups were pooled, if the differences were not significant, and subsequently the data from the two pooled groups were compared to the control group. If, on the other hand, significant differences were found, the two groups in question were compared individually with the control group. As these statistical tests were carried out as simultaneous tests in several steps, the levels of significance in these analyses were reduced according to the Bonferroni method (Grove and Andreasen, 1982) from 5% (which was the overall level of significance, see below) to 2.5%.

Analysis of convulsive behaviour was done by the $\chi^2$ test for a $2 \times 2$ table (Altman, 1991). We wanted to test the hypothesis that the number of withdrawal seizures was not affected by repeated alcohol withdrawal (H$_0$: group SW = group MW).

The overall level of significance was set to 5%. Data are presented as mean ± SD.

**Ethics**

The study was approved by the Danish Animal Experiment Inspectorate, Ministry of Justice.

**RESULTS**

During the alcohol-withdrawal kindling regimen, the number of animals was reduced from 72 to 42 because of overdosage of alcohol (see above). In the subsequent challenge episode (episode 8) additionally four animals in this group (group MW) died, while three of the 30 animals in the single episode group (group SW) died. A net weight increase during episodes 1–8 of 35 ± 11% and 47 ± 10% was detected in group MW and group SW, respectively. During the kindling regimen, the daily alcohol dose per episode (episodes 1–7, $n$ = 7 episodes) was 13.2 ± 1.2 g/kg/day/episode, whereas the mean intoxication score per episode was 3.3 ± 0.1/
Table 1. Areal density of somatostatin-immunoreactive neurons in the dentate hilus of hippocampus

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of animals</th>
<th>Density of somatostatin neurons (neurons per mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>117 ± 14</td>
</tr>
<tr>
<td>SW− (single episode without seizures)</td>
<td>5</td>
<td>118 ± 13</td>
</tr>
<tr>
<td>SW+ (single episode with seizures)</td>
<td>6</td>
<td>113 ± 14</td>
</tr>
<tr>
<td>MW− (multiple withdrawal without seizures)</td>
<td>6</td>
<td>124 ± 11</td>
</tr>
<tr>
<td>MW+ (multiple withdrawal with seizures)</td>
<td>4</td>
<td>123 ± 28</td>
</tr>
</tbody>
</table>

Areal cell densities were determined in three sections from each animal as described in the text. Data represent the means ± SD. No significant group differences were found.

Discussion

The multiple episode alcohol administration paradigm used in the present study leads to an increase in convulsive behaviour during withdrawal from 2 days of alcohol intoxication (Clemmesen and Hemmingsen, 1984). We recently showed that blocking the withdrawal reactions in episodes 1–9 with either phenobarbital (Ulrichsen et al., 1992) or diazepam (Ulrichsen et al., 1995) prevents this augmented seizure activity, when the animals were observed during the withdrawal reactions in episodes 10–13, in which the administration of phenobarbital and diazepam was terminated and both drugs were metabolized. It was concluded that the increased convulsive withdrawal behaviour in episodes 10–13 was caused by kindling-like processes during the withdrawal phases in episodes 1–9 (Ulrichsen et al., 1995). The present results show that loss of SS-IR neurons in the dentate hilus is not involved.
in alcohol-withdrawal kindling. Exposing rats to much longer alcohol intoxication (6 months or more), Andrade et al. (1992) found that the density of SS-IR neurons in the dentate hilus was reduced by 30%. In agreement with this finding, studies in which the same alcohol paradigm was used demonstrated cell death of both granule cells in fascia dentata and pyramidal cells in CA1 and CA3 of the hippocampal formation (Cadete Leite et al., 1988, 1989; Brandao et al., 1995). In addition, alcohol-induced cell death has been demonstrated in frontal cortex (Cadete Leite et al., 1990). As to the effects of chronic alcohol intoxication on the SS-IR cells in the dentate hilus, the negative results of the present study are in disagreement with those of Andrade et al. (1992). The discrepancies are most likely due to the much shorter duration of the alcohol intoxication periods during the alcohol-withdrawal kindling in the current investigation. Andrade et al. (1992) on the other hand, reported that no loss of SS-IR cells in the dentate hilus occurred during the withdrawal reaction, a finding which is in consensus with the unchanged density of these neurons in the alcohol-withdrawal kindled rats in the present investigation. There is evidence to suggest that alcohol-induced death of granule cells in the fascia dentata continues after the alcohol administration is terminated (Cadete Leite et al., 1988), and therefore degeneration of these cells may theoretically play a role in alcohol-withdrawal kindling.

Neuronal death due to a noxious stimulus may be undetected for a few days if brain sections are analysed by light microscopy (Johansen and O’Hare, 1989; Sperry et al., 1992). Hence we cannot rule out that the 4 days of severe alcohol intoxication in episode 8 actually led to degeneration of hilar SS-IR neurons in the fascia dentata, as the animals were killed within 16 h after the last alcohol dose. Similarly, we cannot draw any conclusions as to whether alcohol withdrawal seizures, like seizures induced by kainic acid or focal electrical stimulation (Sloviter 1987; Sperry et al., 1992; Pérez et al., 1995), result in loss of hippocampal somatostatin-containing neurons. These uncertainties would have been clarified if we had performed the histological analyses at a later time. The main purpose of the present investigation was, however, to test whether alcohol-withdrawal kindling led to loss of hilar SS-IR neurons. As the kindling regimen was terminated 15 days before the histological analyses, any loss of SS-IR neurons due to alcohol-withdrawal kindling would have been detected at this time. If, on the other hand, we had done the cell counting a few days into the withdrawal reaction, the kindling effects on the SS-IR neurons could have been obscured by a potential cell loss induced simply by the withdrawal seizures in episode 8. As to the seizure risk of a given rat withdrawing from chronic alcohol intoxication, the present study does not suggest that a genetically altered density of somatostatin-containing neurons in the dentate hilus could be a predisposing factor, as there were no differences in the number of SS-IR neurons between the single withdrawal animals with and without seizures (SW+ vs SW−) or between the kindled animals with or without seizures (MW+ vs MW−) in the current study.

Differences in cerebral hydration across groups could effect the volumetric changes which occur during the histological processing, and thus lead to false conclusions regarding the areal densities of the hilar SS-IR in the different groups. The cerebral state of hydration, however, is not affected by 4 days of severe alcohol intoxication (Hemmingsen and Jørgensen, 1980) and it is therefore unlikely that the volumetric changes in the alcohol-treated groups during the histological processing were different from control levels.

The decreased seizure activity in the MW group in the present investigation is in disagreement with the current literature including several studies from our own laboratory. Thus, other researchers have demonstrated that repetition of alcohol dependence is accompanied by increased convulsive withdrawal behaviour in both humans (Brown et al., 1988; Lechtenberg and Worner, 1992; Booth and Blow, 1993) and rodents (Branchey et al., 1971; Walker and Zornetzer, 1974; Baker and Cannon, 1979; Becker and Hale, 1993; Becker, 1994), while only the negative results of Goldstein (1974) to our knowledge disagree with these findings. Previous alcohol-withdrawal kindling studies conducted in our own laboratory showed that the convulsive withdrawal behaviour was augmented during repeated alcohol withdrawal (Clemmensen and Hemmingsen, 1984; Ulrichsen et al., 1992, 1995), a result which is in accordance with biochemical studies where the same paradigm was used, in which we detected the convulsive withdrawal behaviour in order to divide the animals into those with seizures and
those without seizures (Ulrichsen et al., 1988; Clemmesen et al., 1988). Due to the inconsistency of the current results with the previous alcohol-withdrawal kindling studies, we recently tried to reproduce the behavioural findings of the present experiment. In this study, we found no significant difference between the seizure activity in the MW and the SW groups as seizures were detected in six out of 25 of the MW animals (24%) and five out of 19 of the SW animals (26%) (J. Ulrichsen et al., unpublished results). In the present study, the intoxication score was slightly higher in the SW group (3.6) than in the MW group (3.3), a factor which may have caused the seizure activity to decrease in the latter group. We have therefore concluded that there is no true difference in the seizure activity between alcohol-withdrawal-kindled rats (group MW) and non-kindled rats (group SW) withdrawing from 4 days of alcohol intoxication, and that the significant decrease in the convulsive behaviour of the MW group in the present study represents a type 1 statistical error. This conclusion is in agreement with the Goldstein (1974) results, but is still in disagreement with the remaining literature showing increased seizure activity following alcohol-withdrawal kindling (Branchey et al., 1971; Walker and Zornetzer, 1974; Baker and Cannon, 1979; Clemmesen and Hemmingsen, 1984; Brown et al., 1988; Lechtenberg and Worner, 1992; Ulrichsen et al., 1992; Booth and Blow, 1993; Becker and Hale, 1993; Becker, 1994; Ulrichsen et al., 1995). In our previous studies (Clemmesen and Hemmingsen, 1984; Ulrichsen et al., 1992, 1995), the animals were exposed to the same alcohol-withdrawal kindling paradigm as in the present study, but the seizure activity was assessed during withdrawal from 2 days of alcohol intoxication, while the animals in the present investigation were exposed to withdrawal from 4 days of severe alcohol intoxication. While withdrawal from 2 days of severe alcohol intoxication is a subconvulsive stimulus in non-kindled rats (Clemmesen and Hemmingsen, 1984; Ulrichsen et al. 1992, 1995), withdrawal from 4 days of severe alcohol intoxication probably represents the maximal severity of alcohol withdrawal which can be obtained in rats, and seizure activity normally occurs in 25–35% of the animals (Majchrowicz, 1975; Majchrowicz and Hunt, 1976). These observations have led us to speculate that alcohol-withdrawal kindling in rats only produces subtle alterations in the cerebral neurosystems resulting in a latent hyperactivity of the brain. This hyperactivity does not produce behavioural alterations per se, but can be demonstrated if the alcohol-withdrawal-kindled rats are challenged with a relatively weak stimulus such as withdrawal from 2 days of severe alcohol intoxication. If, on the other hand, the rats are challenged with a much stronger withdrawal reaction, as in the present experiment, the accompanying cerebral hyperactivity is so intense that it far exceeds the kindling-induced neuroexcitability, which therefore remains undetected. This hypothesis may be extended to cover other rodents. Thus, in alcohol-withdrawal-kindled mice, an augmented convulsive withdrawal behaviour was detected following 16 h of severe alcohol intoxication (Becker and Hale, 1993; Becker, 1994), but not after 3 days of severe alcohol intoxication (Goldstein, 1974). An alternative and more simple explanation for the behavioural results of the present experiments could be that the alcohol-withdrawal-kindling-induced alterations may have faded during the 11 days recovery period following episode 7, as in our previous studies we only exposed the animals to 5 day intervals between intoxication–withdrawal episodes. In disagreement with this theory, however, we recently found facilitation of electrical kindling 33 days after alcohol-withdrawal kindling was performed, indicating that alcohol-withdrawal kindling induces irreversible changes in the brain (J. Ulrichsen et al., unpublished results).

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