IMMUNOREACTIVITY OF cAMP RESPONSE ELEMENT BINDING PROTEIN IS NOT ALTERED IN THE POST-MORTEM CEREBRAL CORTEX OR CEREBELLUM OF ALCOHOLICS

MEGUMI YAMAMOTO*, SABINE POHLI, NURIA DURANY, HIROKI OZAWA, TOSHIKAZU SAITO, KARL W. BOISSL, ROBERT ZÖCHLING, PETER RIEDERER, JOBST BÖNING and MARIO E. GÖTZ

Clinical Neurochemistry, Department of Psychiatry and Psychotherapy, University of Würzburg, Füchsleinstrasse 15, 97080 Würzburg, Germany,
1 Department of Neuropsychiatry, Sapporo Medical University, S.1, W. 16, Chuo-ku, Sapporo, 060-8543 Japan and
2 State Hospital of Psychiatry and Neurology, Mauer, A-3362 Mauer, Austria

(Received 28 March 2000; in revised form 19 June 2000; accepted 8 July 2000)

Abstract — We examined amounts of cAMP response element binding protein (CREB) and its phosphorylated form in post-mortem frontal and temporal cortices and cerebella from alcoholics and controls by immunoblotting. No significant differences were observed in the levels of these proteins in each brain region, suggesting that the assumed neuroadapations to chronic ethanol intake may not be reflected by quantitative alterations of CREB in alcoholic brain.

INTRODUCTION

Altered intracellular signal transduction in alcoholic brain has been proven by many neurochemical investigations in the last 20 years. Ethanol acts specifically on certain G proteins in the synaptic cell membrane (Hoffman and Tabakoff, 1990) and altered adenosine-3',5'-cyclic-monophosphate (cAMP) signalling was observed in ethanol-treated animals and alcoholics (Saito et al., 1987, 1994; Wand et al., 1993). The cAMP response element binding protein (CREB) is a transcription factor of particular interest as a downstream target in the cAMP signalling pathway. CREB is involved in regulation of genes essential for neuronal function and production of important proteins, such as brain-derived neurotrophic factor (BDNF) (Zafra et al., 1992). In addition, CREB links immediate synaptic events to long-term neuronal changes suggested to occur in the addicted brain (Nestler and Duman, 1995; Koob et al., 1998).

Increased adenylyl cyclase (AC) activity via activation of stimulatory G protein (Gs) is induced by acute exposure to ethanol, whereas chronic exposure to ethanol attenuates AC activity through inactivation of Gs in cells and tissues (Saito et al., 1987; Chung et al., 1989; Ravin, 1993). Previous studies with post-mortem human brains reported decreased amount and functions of the G proteins in the cerebral cortex of alcoholics (Ozawa et al., 1993, 1994). Moreover, a reduced level of the type I adenylyl cyclase (AC-I) has been found in the temporal cortex of alcoholics (Hashimoto et al., 1998). These findings suggest a disturbance of the cAMP signal transduction mediated by the dysfunctional G proteins and the impaired particular AC isoform in alcoholic brain.

Regarding the effect of ethanol exposure on downstream events of cAMP signalling, it was reported that chronic treatment with ethanol resulted in an attenuation of the phosphorylation of CREB and its CRE binding activity in rat brain (Yang et al., 1996, 1998a,b). These observations suggest the possibility that the impaired cAMP signalling, including reduced CREB-dependent gene transcription, may be involved in the pathophysiology of alcoholism. Nonetheless, very few studies have directly investigated post-second messenger signalling pathways in alcoholic brain. In the present study, we examined the amounts of total CREB and the phosphorylated form of this protein by immunoblotting in homogenate preparations from post-mortem frontal and temporal cortices, and cerebella obtained from alcoholics and age-matched controls. The same brain preparations were also analysed with antibodies to neurofilament 200 (NF-200) and glial fibrillary acidic protein (GFAP) in order to examine the selectivity of results with respect to the general level of neuronal or glial protein expression respectively.

MATERIALS AND METHODS

Subjects and brain sources

Post-mortem brain tissues were obtained from seven alcoholics and eight controls who were matched with respect to age and post-mortem interval and confirmed to be free of both psychiatric disease and substance abuse according to their records. The procedure used for acquisition, clinical diagnosis, dissection, storage, and distribution of brain material in our brain bank system was previously described in detail (Gsell et al., 1993). Diagnosis for alcoholic patients was established using DSM-IV criteria (303.90) (American Psychiatric Association, 1994). All alcoholic patients examined in the present study were current abusers and the time from last drinking to death was 1–5 days. Other detailed characteristics of the patients and control subjects are given in Table 1.

Homogenate preparation

Whole-cell homogenates for immunoblotting were prepared from post-mortem frontal and temporal cortices and cerebellum, as previously described with minor modifications (Yamamoto-Sasaki et al., 1999). In brief, brain tissue was homogenized in a buffer containing 20 mM HEPES, 0.25 M sucrose, 0.3 mM phenylmethylsulphonyl fluoride, 1 mM dithiothreitol, 1 mM EGTA, 1 mM MgCl₂, 5 nM calyculin A,
TABLE 1. Detailed characteristics of subjects

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Diagnosis</th>
<th>Age (years)</th>
<th>Sex</th>
<th>PMDT (h/min)</th>
<th>Analysed area</th>
<th>Cause of death</th>
<th>Medications at time of death</th>
<th>Days after cessation of drinking</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>Alcoholic</td>
<td>54</td>
<td>M</td>
<td>23.00</td>
<td>F, T, Ce</td>
<td>Hypopharyngeal cancer</td>
<td>Carbamazepine, nitrofurantoin, vitamin B complex</td>
<td>1</td>
</tr>
<tr>
<td>A2</td>
<td>Alcoholic</td>
<td>45</td>
<td>M</td>
<td>40.50</td>
<td>F, T, Ce</td>
<td>Unknown</td>
<td>Unknown</td>
<td>1</td>
</tr>
<tr>
<td>A3</td>
<td>Alcoholic</td>
<td>51</td>
<td>M</td>
<td>8.50</td>
<td>F, T, Ce</td>
<td>Bronchial cancer</td>
<td>Haloperidol, carbamazepine, morphine, biperidin, oxazepam</td>
<td>1</td>
</tr>
<tr>
<td>A4</td>
<td>Alcoholic</td>
<td>66</td>
<td>M</td>
<td>5.50</td>
<td>F, T, Ce</td>
<td>Colonic cancer</td>
<td>Morphine, haloperidol, natrium picosulphate</td>
<td>5</td>
</tr>
<tr>
<td>A5</td>
<td>Alcoholic</td>
<td>61</td>
<td>M</td>
<td>11.00</td>
<td>F, T, Ce</td>
<td>Bronchial cancer</td>
<td>Amantadin</td>
<td>3</td>
</tr>
<tr>
<td>A6</td>
<td>Alcoholic</td>
<td>70</td>
<td>M</td>
<td>17.00</td>
<td>F, T, Ce</td>
<td>Sepsis, renal failure</td>
<td>None</td>
<td>1</td>
</tr>
<tr>
<td>A7</td>
<td>Alcoholic</td>
<td>67</td>
<td>M</td>
<td>17.50</td>
<td>F, T, Ce</td>
<td>Bronchial pneumonia</td>
<td>Theophylline, flunitrazepam, isoprenaline</td>
<td>1</td>
</tr>
<tr>
<td>C1</td>
<td>Control</td>
<td>82</td>
<td>M</td>
<td>24.00</td>
<td>F, Ce</td>
<td>Infirmitiy</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>C2</td>
<td>Control</td>
<td>75</td>
<td>M</td>
<td>24.00</td>
<td>F, T, Ce</td>
<td>Myocardial infarction</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td>Control</td>
<td>62</td>
<td>M</td>
<td>15.00</td>
<td>F, T, Ce</td>
<td>Heart failure</td>
<td>Heparin, corticosteroids, diazepam</td>
<td></td>
</tr>
<tr>
<td>C4</td>
<td>Control</td>
<td>67</td>
<td>M</td>
<td>20.00</td>
<td>F, T, Ce</td>
<td>Heart failure</td>
<td>Digitalis, amphotericin, vancomycin, mexiletine, dopamine</td>
<td></td>
</tr>
<tr>
<td>C5</td>
<td>Control</td>
<td>61</td>
<td>M</td>
<td>18.00</td>
<td>F, T, Ce</td>
<td>Myocardial infarction</td>
<td>Nifedipine, nitroglycerine, acetylsalicylate</td>
<td></td>
</tr>
<tr>
<td>C6</td>
<td>Control</td>
<td>55</td>
<td>M</td>
<td>24.00</td>
<td>F, T, Ce</td>
<td>Haemorrhagic pancreatitis</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>C7</td>
<td>Control</td>
<td>30</td>
<td>M</td>
<td>24.00</td>
<td>F, T, Ce</td>
<td>Heart failure</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>C8</td>
<td>Control</td>
<td>81</td>
<td>M</td>
<td>19.30</td>
<td>T, Ce</td>
<td>Mesenteric venous embolism</td>
<td>None</td>
<td></td>
</tr>
</tbody>
</table>

PMDT, post-mortem delay time; F, frontal cortex; T, temporal cortex; Ce, cerebellum.
Age: 59.14 ± 3.53 years (F, T, Ce) in alcoholics, 61.71 ± 6.30 years (F), 61.57 ± 6.23 years (T) and 64.13 ± 5.97 years (Ce) in controls.
Post-mortem interval: 17.57 ± 4.44 h (F, T, Ce) in alcoholics, 21.29 ± 1.39 h (F), 20.61 ± 1.34 h (T) and 21.04 ± 1.23 h (Ce) in controls.
There were no significant differences in age and post-mortem interval between alcoholics and controls in the three brain regions examined. Values are means ± SEM.

and 1 μM okadaic acid (pH 7.4) and divided into aliquots of appropriate size. The whole-cell homogenates were stored at –80°C until required. Protein concentrations were determined by the Coomassie blue binding method using bovine serum albumin (BSA) as a standard (Bradford, 1976).

Gel electrophoresis and immunoblotting

The proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to nitrocellulose membranes for subsequent immunoblotting as previously described. In brief, cortical homogenates were immunobotted for CREB (30 μg used) (Dowlatshahi et al., 1999; Yamamoto-Sasaki et al., 1999), for GFAP and NF-200 (30 μg used) (Haug et al., 1996), respectively. Homogenates were subjected to SDS-PAGE with 10% (for CREB proteins and GFAP) or 4–12% (for NF-200) polyacrylamide gels at 125 V for 2 h. Proteins were transferred to nitrocellulose membranes at 30 V for 75 min at room temperature. The membranes were blocked in 5% bovine serum albumin (BSA)/Tris-buffered saline (TBS) buffer for 1 h at room temperature and then incubated in 3% BSA/TBS buffer overnight at 4°C with either phosphorylated CREB antiserum (1:1000 dilution) or total CREB antiserum (1:2500 dilution) (both Upstate Biotechnology), GFAP antiserum (1:5000 dilution) or NF-200 antiserum (1:5000 dilution) (both from Sigma). Membranes were washed and incubated with secondary antibody, anti-rabbit Ig HRP-linked F(ab′)2 (Amersham) diluted to either 1:1000 for CREB or 1:10 000 for NF-200 and GFAP in 5% BSA/TBS buffer for 1 h at room temperature. Immunoreactive bands were detected with the enhanced chemiluminescence (ECL) system (Amersham) and analysed by laser densitometry (NIH 1.55 image analysis system). Linearity of our immunoblots was tested using control human cortical homogenate. In the immunoblots using the standard homogenate, the immunoreactivities showed linearity within the ranges 5–75 μg for CREB proteins and 10–50 μg for GFAP and NF-200.

Statistical analyses

Results are given as the means ± SEM. The data were analysed by Welch’s t-test and values of P < 0.05 were taken to indicate statistical significance of differences between groups. The effects of age and post-mortem interval on each immunoreactivity were determined by Spearman’s rank order correlation analysis.

RESULTS

As a factor which might have an effect on the immunoreactivities detected, correlation between age or post-mortem interval and immunoreactivities of CREB, NF-200 or GFAP were analysed by Spearman’s rank order analysis using 15 subjects. Neither age nor post-mortem delay showed any significant effect on the measured immunoreactivities in both cortical regions and cerebellum (P > 0.10 in all cases).

CREB proteins

Figure 1 shows representative immunoblots of CREB proteins, NF-200 and GFAP at either expected molecular weight in frontal cortex from alcoholics and control subjects. Immunoblotting with the anti-CREB and the anti-phosphorylated CREB antibodies yielded two bands at apparent molecular weights of 43 kDa and a non-specified band at 55 kDa (Dowlatshahi et al., 1999; Yamamoto-Sasaki et al., 1999). The two inducible bands with relative molecular weight of 43 kDa...
may be splice variants of CREB, i.e. CREBα and CREBδ, which differ in sequence by 14 amino acids (Ginty et al., 1993). Both immunoreactive bands were included in the quantification by densitometry.

Results obtained by densitometry are shown in Fig. 2. Neither phosphorylated CREB nor total CREB immunoreactivity showed any significant difference in frontal and temporal cortices between alcoholics and controls (frontal cortex: $P = 0.60$ for phosphorylated CREB and $P = 0.32$ for total CREB, respectively; temporal cortex: $P = 0.13$ for phosphorylated CREB and $P = 0.18$ for total CREB, respectively). There were also no significant differences in the levels of both CREB proteins in cerebellum between these two groups ($P = 0.88$ for phosphorylated CREB and $P = 0.90$ for total CREB, respectively).

**NF-200 and GFAP**

The levels of NF-200 and GFAP (see Fig. 1) also showed no significant change in both cortical regions and cerebellum between alcoholics and controls (frontal cortex: $P = 0.31$ for NF-200 and $P = 0.25$ for GFAP, respectively; temporal cortex: $P = 0.81$ for NF-200 and $P = 0.78$ for GFAP, respectively; cerebellum: $P = 0.40$ for NF-200 and $P = 0.90$ for GFAP, respectively) (Fig. 2).

**DISCUSSION**

The effect of the post-mortem interval must be considered as a factor which could affect neurochemical parameters investigated using post-mortem tissues. We previously demonstrated that post-mortem delays of up to 96 h in the human hippocampus had no significant effect on the detection of immunoreactivities of phosphorylated and total CREB (Yamamoto-Sasaki et al., 1999). In the present study, human brains with post-mortem intervals of <40.5 h were used, and Spearman’s rank order analysis revealed no effect of post-mortem delay on the examined immunoreactivities of the CREB proteins.
We have also measured the immunoreactivities of the NF-200 and GFAP as indicators of general glial and neuronal changes in the samples used. Influence of such a general cell alteration in the brain preparations on the results can be ruled out, since immunoblotting studies failed to show any significant differences in NF-200 and GFAP levels between alcoholics and control subjects in each brain region examined.

Concerning the effect of chronic ethanol treatment on cAMP response element (CRE)–DNA binding activity of brain CREB, it was found that chronic ethanol treatment had no effect on the CRE–DNA binding activity in rat cerebellum (Yang et al., 1996), but it was also observed that chronic ethanol exposure impaired CRE–DNA binding activity in rat striatum (Yang et al., 1998b). On the other hand, it was reported that acute or chronic ethanol treatment has no significant effect on CRE–DNA binding activities in the rat cortex, whereas both CRE–DNA binding activity and the expression of CREB target, i.e., the protein level of BDNF, are decreased in the same region during ethanol withdrawal with peak reductions at 24 h after cessation of chronic ethanol intake (Pandey et al., 1999).

It was thus a logical consequence to look for possible alterations in expression of the CREB protein and CREB phosphorylation state after chronic ethanol exposure in animals and human subjects.

Effects of chronic ethanol intake on the levels of brain CREB were previously investigated in experimental animals. It was observed that there were no significant changes in the levels of total or phosphorylated CREB, but induction of CREB phosphorylation by an acute ethanol challenge was markedly attenuated (50%) in the rat striatum after chronic exposure to ethanol (Yang et al., 1998a). It was also reported that chronic ethanol exposure resulted in a 25% decrease in the level of phosphorylated CREB and in a 50% reduction in the induction of CREB phosphorylation by a subsequent acute ethanol challenge in the granule cell layer of the rat (Yang et al., 1998b).

It was also demonstrated that the attenuated CREB phosphorylation was associated with reduction in the levels of the catalytic subunit of protein kinase A (PKA) and calcium/calmodulin-dependent kinase IV (CaMKIV), which are known to phosphorylate CREB on Ser-133 (Yang et al., 1998b).

Our present study has found no significant differences in the levels of phosphorylated or total CREB in the frontal and temporal cortices, nor in cerebella of alcoholics relative to control subjects. In the previous study using rat cerebellum, Yang et al. (1998b) also reported that levels of protein phosphatase 1 (PP-1), which dephosphorylates the phosphorylated CREB, did not alter in the nuclear extracts of cerebellum after chronic and acute ethanol exposure. These authors, however, did not use inhibitors of PP-1 in the preparation of nuclear extracts, whereas we did in the preparation of homogenates. This could explain the difference in the level of phosphorylated CREB between these two studies. Another potential reason for the discrepancy between the results in rat and human subjects is that of species differences in the expression ratio of granule cells to Purkinje cells in cerebellum. Furthermore, we have used whole-cell homogenates for immunoblotting to avoid loss of the CREB proteins during preparation, and this may have a bearing on the results obtained. Future studies concerning alterations in levels and activities of PKA and CaMKIV in human alcoholic brain could provide more information on this point.

In most alcoholic patients examined in the present study, the time from cessation of drinking to death was very short, and it is thus possible to consider the subjects as having been in the withdrawal state. The unchanged amounts of CREB proteins observed in alcoholic cortices and cerebella in the present study seem to be inconsistent with the previous finding in rat cortex showing decreased CRE–DNA binding activity during ethanol withdrawal (Pandey et al., 1999). However, it has been suggested that phosphorylation of CREB at Ser-133 induces a conformational change of the protein from an inactive to an active form that specifically stimulates the transcriptional activity without affecting its DNA binding properties (Gonzalez et al., 1991). Therefore, the reduction of CRE–DNA binding activity observed in the rat cortex is unlikely to be caused by the alteration in the levels of CREB proteins, and it is possible that the unaltered level of phosphorylated CREB in our present study may compensate the disturbed adenyl cyclase activity and levels of the enzymes in alcoholic brain (Saito et al., 1987; Ravin, 1993; Hashimoto et al., 1998).

Both CREB gene and AC-I gene knockout animals show impaired spatial and long-term memory, indicating that cAMP signalling plays an essential role in the mechanism of neural plasticity (Bourchuladze et al., 1994; Wu et al., 1995). Addiction is characterized by the compulsive use of a drug, which is presumably due to certain adaptive changes that arise in neurons of specific brain areas (Nestler and Duman, 1995; Self and Nestler, 1995). Our observations show that neural adaptation to chronic ethanol intake in human cerebral cortex is not directly reflected by a change in the level of CREB and its phosphorylation. However, an unchanged protein level does not always imply an intact protein function. It might be possible that the neuroadaptation to chronic ethanol exposure in the cortical regions of alcoholics will become manifest as altered function of the CREB when the conditioned stimulus, i.e., ethanol, is given, as demonstrated in animal brain (Yang et al., 1998a, b), and the alterations in brain CREB physiology might change expression of genes that are dependent on CREB for transcription. This proposition should be elucidated by further studies on the functional quality of the CREB in alcoholic brain.

**Acknowledgements** — This study was supported by grants from the German Ministry for Research and Technology [BMBF(01EB9410)] and the Ministry of Education, Science and Culture (Japan) to T. Saito.

**REFERENCES**


