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Elevated Synaptophysin I in the Prefrontal Cortex of Human Chronic Alcoholics

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Abstract

Convergent lines of evidence suggest potentiation of glutamatergic synapses after chronic ethanol exposure, and indicate that the presynaptic effect hereof is on modulators of synaptic strength rather than on executors of glutamate release. To address this hypothesis in the context of ethanol dependence in humans, we used semiquantitative immunoblotting to compare the immunoreactivities of synaptophysin I, syntaxin 1A, synaptosome-associated protein 25, and vesicle-associated membrane protein in the prefrontal and motor cortices between chronic alcoholics and control subjects. We found a region-specific elevation in synaptophysin I immunoreactivity in the prefrontal cortex of alcoholics, but detected no significant differences between the groups in the immunoreactivities of the other three proteins. Our findings are consistent with an effect of repeated ethanol exposure on modulators of synaptic strength but not on executors of glutamate release, and suggest a role for synaptophysin I in the enduring neuroplasticity in the prefrontal cortical glutamate circuitry that is associated with ethanol dependence.

Keywords

alcoholics; prefrontal cortex; synaptophysin I; glutamate

INTRODUCTION

Ethanol dependence in humans is associated with persistent alterations in glutamatergic neurotransmission in the prefrontal cortex (PFC) (reviewed in: Goldstein and Volkow, 2002; Kalivas and O'Brien, 2008). Basal activity in this region is decreased in alcoholics (reviewed in: Goldstein and Volkow, 2002), indicative of increased synaptic strength (reviewed in: Turrigiano and Nelson, 2004), that is, of net synaptic potentiation. Consistent with this observation, animal studies show that chronic ethanol treatment (CET) results in elevated numbers of ethanol-sensitive *N*-methyl-D-aspartate (NMDA) receptor subunits (Hendricson et al., 2007; Roberto et al., 2006), and enlargements of dendritic spines and

associated postsynaptic densities at glutamatergic synapses (Carpenter-Hyland and Chandler, 2006; Carpenter-Hyland et al., 2004; Hendricson et al., 2007). However, the presynaptic effect of CET appears to vary (Hendricson et al., 2007; Roberto et al., 2006), indicative of an influence on modulators of synaptic strength rather than on executors of glutamate release.

Synaptophysin I (SYPI), a major synaptic vesicle protein (Takamori et al., 2006), appears to be involved in determination of synaptic strength (reviewed in: Evans and Cousin, 2005), and several lines of evidence implicate this protein in release-independent potentiation of cortical glutamatergic synapses: SYPI localizes largely to glutamatergic termini in the rat cerebral cortex (Bragina et al., 2007), it plays a role in NMDA receptor-mediated long-term potentiation but not depression without affecting glutamate release (Janz et al., 1999; McMahon et al., 1996), and it is involved in activity-dependent synapse formation at glutamatergic synapses (Tarsa and Goda, 2002). The *N*-ethylmaleimide-sensitive factor attachment proteins (SNAREs), syntaxin 1A (SYX1A), synapto-some-associated protein 25 (SNAP-25), and vesicle-associated membrane protein (VAMP) mediate membrane fusion and are essential for synaptic vesicle exocytosis (reviewed in: Jahn and Scheller, 2006).

To, in the context of ethanol dependence in humans, address the hypothesis that repeated ethanol exposure affect modulators of synaptic strength but not executors of glutamate release, we used semi-quantitative immunoblotting to compare the immunoreactivities of SYPI, SYX1A, SNAP-25, and VAMP in the prefrontal and motor cortices between chronic alcoholics and control subjects; the motor cortex (MC) was included to test for regional specificity. In support hereof, we found a region-specific elevation in SYPI immunoreactivity in the PFC of alcoholics, and no significant differences between the groups in the immunoreactivities of the other three proteins. Our results on SYPI suggest a role for this protein in the ethanol dependence-associated enduring neuroplasticity in the prefrontal cortical glutamate circuitry.

SUBJECTS AND METHODS

Subjects

This study was approved by Stockholm's ethic vetting board. Postmortem samples from the PFC and MC, Brodmann's areas nine and four, respectively, of 14 human chronic alcoholics and 14 control subjects were obtained from the New South Wales Tissue Resource Center. Cases were collected by qualified pathologists under full ethical clearance and with informed, written consent from the next of kin. All subjects were male and Caucasian. Alcoholics met DSM-IV criteria; they did not have Wernicke-Korsakoff's syndrome or liver cirrhosis and had no history of poly drug abuse. Control subjects had no history of drug abuse. Individual demographic and clinical data are given in Table I.

Immunoblotting

Tissue extracts were prepared as described earlier (Okvist et al., 2007); in short, by solubilization of powdered tissue (approx. 30 mg) in 1:3 w/v 4% SDS buffer and sonication. DC assay (Bio-Rad) was used for determination of protein concentration. Aliquots containing 70 µg of total protein were heated at 95°C for 5 min and resolved by SDS-PAGE on 10% tricine gels as described elsewhere (Yakovleva et al., 2007). Proteins were transferred onto nitrocellulose membranes (Schleicher and Schuell, Germany) at 4°C and stained with MemCode™ (Pierce). Blots were probed with a cocktail of the following mouse monoclonal antibodies: anti-SYPI (cat. # S5768, Sigma) at 1:100,000; anti-SYX1A (cat. # MAB336, Chemicon) at 1:2,000,000; anti-SNAP-25 (cat. # MAB331, Chemicon) at 1:1,000,000; and anti-VAMP (cat. # MAB333, Chemicon) at 1:3000 and incubated with a

peroxidase-conjugated goat antimouse secondary antibody (cat. # 170-6516, Bio-Rad) at 1:25,000. All blots were made in duplicates. Blots were developed in ECL (Amersham, UK). Films were digitized using a scanner. Densitometric analysis was performed in Image Gauge (V3.12) (Fujifilm, Japan). To enable data normalization, reference samples consisting of pooled cerebellar extracts from control subjects were loaded onto four wells: one on the left edge, one in the middle, and two on the right edge. SYPI, SYX1A, SNAP-25, and VAMP optical densities (ODs) were expressed as ratio to MemCode™ OD.

Statistical analysis

Data normality was examined with Kolmogorov-Smirnov test. Group differences were investigated either with Student's *t*-test or Fisher's exact test. Correlations of SYPI, SYX1A, SNAP-25, and VAMP immunoreactivities with age, postmortem interval (PMI), brain pH, and storage time were examined with Pearson's correlation. Covariate influence was assessed with analysis of covariance (ANCOVA). Significance was set to $P < 0.05$. Statistical analysis was performed in Statistica (V8.0) (StatSoft, Sweden).

RESULTS

SYPI, SYX1A, SNAP-25, and VAMP immunoreactivities were quantified by immunoblotting. All four proteins migrated as single bands of the predicted molecular weights, that is, 38, 32, 25, and 16 kDa, respectively (Fig. 1a). Their respective linear ranges of detection were determined using a blot made with serial dilutions of a reference sample (10–140 µg of total protein load), and linear dependency analysis of each protein's OD on MemCode™ OD (Fig. 1b). OD values outside of the linear ranges were excluded from the analysis; a total of 7% of the values with no bias towards any one group, that is, alcoholics and control subjects, or protein (Fisher's exact test, $P > 0.05$ in both cases). A representative blot is shown in Figure 1c.

SYPI immunoreactivity was increased by 32% in the prefrontal cortex of human chronic alcoholics as compared to control subjects (Student's *t*-test, $P < 0.01$) whereas in the MC; it did not differ significantly between the groups (Student's *t*-test, $P > 0.05$) (Fig. 2). SYX1A, SNAP-25, and VAMP immunoreactivities did not differ significantly between alcoholics and control subjects in either region (Student's *t*-test, $P > 0.05$ in all cases) (Fig. 2). With the exception of the immunoreactivity of SYPI, which correlated negatively with age in the PFC (Pearson's correlation, $r = -0.4$, and $P < 0.05$), the protein immunoreactivities did not correlate with age, PMI, brain pH, and storage time (Pearson's correlation, $P > 0.05$ in all cases). Analysis of covariance (SYPI/age) marginally improved the significance of the difference (ANCOVA, $F(1, 25) = 13$, $P < 0.01$).

The main body of the population was smoking (Table I). To assess the influence of nicotine abuse on SYPI immunoreactivity in the PFC, subgroup analysis of smokers was therefore performed. Essentially the same results were obtained, as when all subjects were included into the analysis: SYPI immunoreactivity was increased by 28% in the PFC of smoking alcoholics as compared to smoking control subjects (Student's *t*-test, $P < 0.05$).

The sample toxicology screen was incomplete (Table I). This parameter was therefore not correlated against. However, to assess the influence of apparent benzodiazepine use on SYPI immunoreactivity in the PFC, the three alcoholics who had traces of these medications in their blood at the time of death were excluded, and the data were reanalyzed. Basically the same results were obtained, as when all subjects were included into the analysis: SYPI immunoreactivity was increased by 34% in the PFC of alcoholics as compared to control subjects (Student's *t*-test, $P < 0.05$).

DISCUSSION

In this article, by the use of semiquantitative immunoblotting, we detected a significant increase in SYPI immunoreactivity in the PFC of human chronic alcoholics as compared to control subjects whereas in the MC; it did not differ significantly between the groups. SYX1A, SNAP-25, and VAMP immunoreactivities did not differ significantly between alcoholics and control subjects in either region.

Attention was given to confounders, that is, age, PMI, brain pH, and storage time, identified in previous studies on presynaptic proteins in human postmortem brain tissue (Eastwood and Harrison, 2001; Halim et al., 2003). Covariate analysis showed that none of these parameters interfered with the interpretation of the results. Subgroup analysis of smokers and exclusion analysis revealed that nicotine abuse and apparent benzodiazepine use, respectively, did not interfere with the interpretation of the result on SYPI in the PFC. However, due to the low number of nonsmokers in our sample population we could not assess the influence of comorbidity with nicotine abuse on this result. Even so, it is likely to mirror the situation in ethanol dependence, given that 80% of alcoholics are also heavy smokers (Batel et al., 1995), and that there is a strong correlation between the severity of nicotine and ethanol dependence (Dani and Harris, 2005). The relative smallness of our sample population/groups is in itself an important limitation, which makes replication in a larger study necessary to enable evaluation of the biological significance of our findings.

The increase in SYPI immunoreactivity in the PFC of chronic alcoholics as compared to control subjects is consistent with an effect of repeated ethanol exposure on modulators of synaptic strength. The absence of significant differences in the immunoreactivities of the SNARE proteins, SYX1A, SNAP-25, and VAMP between alcoholics and control subjects are in agreement with no apparent effect of chronic ethanol exposure on the glutamatergic release machinery. Our results on the SNARE proteins also coincide well with the lack of an effect on glutamate release in SYPI deficient mice (McMahon et al., 1996). Altogether, these findings support the initial hypothesis, and warrant further investigation thereof.

The absence of a significant difference in SYPI immunoreactivity in the MC between alcoholics and control subjects, suggests that the effect of chronic ethanol exposure on SYPI immunoreactivity in the PFC is region specific. It is tempting to speculate that it is also circuitry specific: alcoholism is suggested to affect selected brain circuits (Sullivan and Pfefferbaum, 2005), SYPI colocalizes to a higher extent with the vesicular glutamate transporter 1 (VGLUT1) (95%) than with VGLUT2 (30%) in the rat cerebral cortex (Bragina et al., 2007), and VGLUT1 and VGLUT2 are more associated with the cognitive and motor circuits, respectively (Fremeau et al., 2004). In view hereof, our results on SYPI suggest a role for this protein in the ethanol dependence-associated enduring neuroplasticity in the prefrontal cortical glutamate circuitry.

If not an adaption to chronic ethanol exposure, the elevated SYPI immunoreactivity in the PFC of alcoholics may result from a genetic distinction between alcoholic and nonalcoholic individuals. This alternative is viable because it has been shown in vitro that SYPI expression is regulated mainly at the level of translation initiation (Daly and Ziff, 1997), which may explain why it was not identified in an array study on genes differentially expressed in the PFC of alcoholics and control subjects (Liu et al., 2006). However, based on the apparent decrease in basal activity in the PFC of alcoholics (reviewed in: Goldstein and Volkow, 2002), the seeming potentiation of glutamatergic synapses after CET (Carpenter-Hyland and Chandler, 2006; Carpenter-Hyland et al., 2004; Hendricson et al., 2007; Roberto et al., 2006) and the evidence of a role for SYPI in determination of synaptic

strength (reviewed in: Evans and Cousin, 2005), e.g., potentiation of glutamatergic synapses (Janz et al., 1999; Tarsa and Goda, 2002), we consider this option less probable.

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Fig. 1.

(a, b) Determination of the linear ranges of detection of synaptophysin I (SYPI), syntaxin 1A (SYX1A), synaptosome-associated protein 25 (SNAP-25), and vesicle-associated membrane protein (VAMP) by semiquantitative immunoblotting. (a) Image of blot made with serial dilutions of a reference sample (10–140 µg of total protein load per well). Dashed area: bands which optical densities (ODs) were linearly dependent on MemCode™ OD as shown in b. (b) Linear ranges of SYPI, SYX1A, SNAP-25, and VAMP ODs as derived from linear dependency analysis of each protein's OD on MemCode™ OD ($r > 0.95$ in all cases). (c) Representative immunoblot. 12A = alcoholic number 12; 12C = control subject number 12, etc.

Fig. 2.

Comparison of SYPI, SYX1A, SNAP-25, and VAMP immunoreactivities in the prefrontal and motor cortices between human chronic alcoholics and control subjects. PFC = prefrontal cortex; MC = motor cortex. Results for alcoholics are presented as percentage of those for control subjects. Horizontal bars represent group means and standard error of the mean. *n* for each protein/ structure, alcoholics/control subjects: SYPI/PFC, 14/14; SYPI/MC, 13/12; SYX1A/PFC, 12/14; SYX1A/MC, 12/11; SNAP-25/PFC, 14/14; SNAP-25/MC, 14/13; VAMP/PFC, 14/14; and VAMP/MC, 10/13. (***P* < 0.01).

TABLE I

Individual demographic and clinical data

Subject no.	Age (y)	PMI (h)	Brain (pH)	Storage time (months)	Smoking history (yes/no)	Cause of death	Toxicologic findings (at time of death)
Control							
1	34	20.5	6.73	52	Yes	Acute exacerbation of asthma	NA
2	78	6.5	6.2	30	No	Dehydration and adenocarcinoma of the lung and rectum with multiple metastases	None
3	63	72	6.9	36	Yes	Coronary-artery atherosclerosis	None
4	82	23.5	6.4	46	NA	Sepsis	None
5	38	13.5	6.26	127	Yes	ACSVD	None
6	69	16	6.6	34	Yes	ACSVD	Paracetamol and CO
7	56	24	6.53	83	Yes	Coronary-artery atheroma	NA
8	59	20	6.56	81	Yes	Coronary thrombosis	None
9	56	25	6.1	32	NA	CAD	Codeine, Morphine and Naproxen
10	56	37	6.76	38	Yes	LV scarring, hypertension and cardiomegaly	None
11	82	36	6.24	55	No	Myocardial infarction	NA
12	44	50	6.6	40	Yes	CAD	None
13	61	24	6.52	83	Yes	CAD	NA
14	53	16	6.84	50	No	Dilated cardiomyopathy	Lignocaine and Sotalol
Mean and SD	59 ± 15	27 ± 16	6.5 ± 0.2	56 ± 28			
Alcoholic							
1	34	8.5	6.61	98	Yes	Hanging	Alcohol
2	77	20	6.34	97	Yes	Lobular pneumonia	None
3	79	48	6.34	87	Yes	CAD	Temazepam
4	39	24	6.56	78	Yes	Aortic sclerosis	NA
5	70	33.5	6.24	74	Yes	Respiratory failure	None
6	56	45	6.51	73	NA	BEV	Alcohol
7	59	24	6.57	71	No	Cardiomyopathy	None
8	56	22	6.52	71	Yes	CAD and UGH	None
9	56	15	6.66	67	NA	CAD and emphysema	Nordiazepam
10	81	36	6.44	58	Yes	Sepsis	None
11	44	15	6.48	51	No	CAD	Diazepam and Nordiazepam

Subject no.	Age (y)	PMI (h)	Brain (pH)	Storage time (months)	Smoking history (yes/no)	Cause of death	Toxicologic findings (at time of death)
12	52	45.5	6.78	45	Yes	Lobar pneumonia	None
13	62	49	6.49	44	Yes	CAD	Sertraline
14	53	57	6.75	40	Yes	CAL	NA
Mean and SD	58 ± 14	32 ± 16	6.5 ± 0.2	68 ± 19			

Abbreviations: NA, not available; ACSVD, atherosclerotic cardiovascular disease; BEV, bleeding esophageal varices; CAD, ischaemic heart disease; CAL, chronic airflow limitation; CO, carbon monoxide; LV, left ventricular; PMI, postmortem interval; UGIH, upper gastrointestinal hemorrhage.