Electrically Evoked Synaptosomal Amino Acid Transmitter Release in Human Brain in Alcohol Misuse

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Key Words

Brain damage • Excitotoxicity • Cirrhosis

Abstract

Severe chronic alcohol misuse leads to neuropathological changes in human brain, with the greatest neuronal loss in the dorsolateral prefrontal cortex. In this region, GABA_A receptors are selectively upregulated, and show altered subunit expression profiles only in alcoholics without comorbid disease, whereas glutamate_{NMDA} subunit expression profiles are selectively downregulated only in alcoholics with comorbid cirrhosis of the liver. To determine whether these outcomes might be conditional on synaptic transmitter levels, evoked release was studied in well-characterized synaptosome suspensions preloaded with L-[³H]glutamate and [¹⁴C]GABA and stimulated electrically (± 10 V contiguous square waves, 0.4 ms, 100 Hz, 1.5 min) with and without Ca²⁺. Stimulation elicited brief peaks of both radioisotopes that were larger in the presence of Ca²⁺ ions ($p < 0.01$). A repeat stimulus evoked a second, smaller ($p < 0.01$) peak. Ca²⁺-dependent L-[³H]glutamate release, but not [¹⁴C]GABA release, was higher overall in alcoholics than in controls ($p < 0.05$). With comorbid cirrhosis, L-[³H]glutamate release showed a graded response, whereas [¹⁴C]GABA release was lowest in noncirrhotic alcoholics. Release patterns did not differ be-

tween cortical regions, or between males and females. Neither age nor postmortem interval was a significant confounder. The released transmitters may differentially alter receptor profiles on postsynaptic cells.

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Introduction

Severe chronic alcohol misuse has neuropathological, cognitive, and behavioral effects on human subjects, but the underlying mechanisms are not well understood [1]. Selective neuronal loss in the cerebral cortex is mostly confined to the dorsolateral prefrontal gyrus [2, 3]. When chronic alcoholic subjects are compared with nonalcoholic controls, this region expresses GABA_A receptors with a different subunit profile, consistent with reduced protection against excitotoxicity [4–6]. Microarray analyses of over 50,000 mRNA transcripts have been performed on dorsolateral prefrontal cortex tissue samples from chronic alcoholics and controls [7–9]. Transcripts coding for several synaptic proteins differed by 40% (1.4-fold) or more between the case groups, suggesting that synaptic transmission may be affected by chronic alcohol intake.

Synaptosomes prepared from autopsy brain show good rates of respiration stimulatory by depolarization [10, 11], indicative of the generation of a membrane potential [12, 13]. Synaptosome profiles contain mitochondria and vesicles, which shows that they are pinched off rather than burst and resealed: probable glial fragments are largely empty [10], and indices of glial function are markedly reduced as compared with neuronal activities [14]. Synaptosomes from human autopsy brain can be sub-fractionated to prepare functional synaptic vesicles [15]; both plasma-membrane and vesicular transport functions persist for long periods post-mortem [14, 15]. Alcoholics without comorbid disease express GABA_A receptors in the dorsolateral prefrontal cortex that differ from controls in pharmacology and subunit profile, consistent with reduced protection against excitotoxicity [4, 6]. In alcoholics with cirrhosis, GABA_A sites are little affected but glutamate-NMDA receptors markedly altered [16]. It is problematic to model regional cortical pathology in lissencephalic animals, and it is difficult to induce cirrhosis experimentally with alcohol in laboratory animals, even non-human primates (~20% of baboons develop fibrosis after 5 years on a diet in which 50% of their caloric intake is ethanol) [17].

In previous work, we developed a release protocol for amino acid transmitters from autopsy brain using chemical stimulation of synaptosomes [18]. However, the released amino acids originated predominately from the cytoplasmic compartment, because the Ca²⁺-dependent component was minor. Changing the electrochemical gradient across the plasma membrane will reverse plasma-membrane carriers [19]. In contrast, electrical stimulation induces Ca²⁺-dependent exocytosis via rapid transmembrane fluxes in potential that mimic physiological inputs [20].

The present study investigated the use of electrical stimulation to trigger transmitter release from synaptosomes prepared from human autopsy brain tissue. The aim was to evaluate the effects of chronic alcohol misuse on the dynamics of neurotransmitter release, to contribute to an understanding of the pathogenesis of alcohol-related brain damage. Preliminary reports of the work have been presented [21, 22].

Materials and Methods

Human Tissue Preparation

Unfixed frozen tissue samples were obtained from the Queensland Brain Bank and the NSW Tissue Resource Centre in collaboration with the Australian Brain Bank Network. All cases

had a full autopsy including macroscopic and microscopic examination of the brain and liver. Informed written consent was obtained for all autopsies, which were performed by authorized pathologists. The University of Queensland Medical Research Ethics Committee approved the consent, tissue retrieval, and research protocols (clearance No. H/09/Biochem/NHMRC/98). For each case, information on general health, diet, alcohol intake, medication, and the presence of alcohol-related diseases such as cirrhosis of the liver was available from medical records and autopsy reports. No subject had clinical or pathological evidence of neurological or psychiatric disease (apart from alcoholism) prior to death. According to the reports, no patient was a polysubstance or intravenous drug user, and none had been prescribed neuroactive medication. Cases were divided into groups depending on alcohol intake and the presence of complicating disease. Full liver pathology, which differentiated cirrhotic and noncirrhotic participants, was available in every case. Controls were teetotal or had low alcohol intake (<20 g of ethanol per day), whereas alcoholics consumed more than 80 g of ethanol per day. All alcoholics had been misusing ethanol for most of their adult lives, and in almost all instances where information was available had been drinking up until the time of death: mean alcohol-misuse durations were 38 years for noncirrhotic and 40 years for cirrhotic alcoholics. Full consumption details were not available in every case, but where they were, the average daily consumption by alcoholics without comorbid disease was 13 standard drinks (130 g ethanol); it was 25 standard drinks (250 g ethanol) for alcoholics with cirrhosis. At autopsy, one hemisphere was fixed in formalin for pathological examination, while the other was dissected in the mortuary to minimize the delay to cryoprotection. Tissue samples (1–5 cm³) were dissected from various cortical regions, placed individually into small plastic bags, and immediately immersed in ice-cold 0.32 M sucrose. The samples were brought to the laboratory, frozen slowly, and stored at –80°C until required. These procedures are optimal for the preservation of tissue components [11]. Clinical details and causes of death are listed in table 1.

Synaptosome Preparation

The method previously described was used with minor modifications [23]. In brief, tissue samples were rapidly thawed in 0.32 M sucrose at 37°C, immediately placed in 10 vol. of ice-cold 0.32 M sucrose, and homogenized with a motor-driven Teflon-glass homogenizer at 500 rpm for 8 strokes. The homogenate was centrifuged at 1,400 g for 5 min (Beckman J2–21 centrifuge; JA20 rotor; Beckman Coulter P/L, Gladesville, N.S.W., Australia). The supernatant was removed, overlaid onto a cushion of 1.2 M sucrose, and centrifuged at 33,000 g for 30 min. The interface material was removed and diluted with ice-cold 0.32 M sucrose to the original suspension volume. This suspension was layered onto a cushion of 0.8 M sucrose and centrifuged at 33,000 g for 30 min. The supernatant was aspirated and discarded, and the pellet dispersed in ice-cold Ca²⁺-free perfusion buffer.

Perfusion Buffer

Two Krebs/HEPES perfusion buffers were used: (1) Ca²⁺-containing buffer, PBC: 10 mM HEPES, 118 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 2.5 mM CaCl₂, 10 mM glucose, pH 7.4; gassed with 95% O₂/5% CO₂; (2) Ca²⁺-free buffer, PB0: same composition as PBC, except that CaCl₂ was omitted and 0.5 mM EGTA included.

Preloading Labeled Transmitter

To determine the optimal time for transmitter uptake, 10 μCi of L-[^3H]glutamate or [^{14}C]GABA (each 1 μM) was added to a synaptosome preparation (~ 1 mg protein/ml in PBC). The suspension was divided into subsets that were each incubated at 37°C. At various time points, the assay was terminated by filtration onto Whatman GF/B filters, followed by three rapid washes with ice-cold 0.9% NaCl to remove incubation medium, using a cell harvester (Brandel M24, Gaithersburg, Md., USA). Filters were air-dried and placed into scintillation vials to which 4 ml of scintillation fluid was added (Packard Emulsifier-Safe; PerkinElmer P/L, Glen Waverley, Vic., Australia); radioactivity was determined in a scintillation counter with on-board quench correction. Time courses of tracer uptake are shown in figure 1a, b. It may be seen that the uptake of labeled neurotransmitter reached a maximum at 20 min. This incubation time was chosen for subsequent studies of release.

In the optimized protocol for preloading neurotransmitters, synaptosomes were resuspended in prewarmed PB0 (0.5 ml; final protein concentration 1 mg/ml) and incubated with 1 μM of L-[^3H]glutamate or 1 μM of [^{14}C]GABA for 20 min at 37°C, then placed on ice for 10 min before use.

Electrically Evoked Release

Aliquots of preloaded synaptosomes (160 μl) were transferred to the chambers of the superfusion apparatus (Brandel S600) housed in a 37°C room. A feature of the apparatus is that the cylindrical chambers are arrayed vertically such that buffer enters from below and leaves at the top, with both entry and exit ports protected by retaining screens. This ensures that the synaptosome suspension is constantly gently irrigated to prevent it from compacting at the base of the chamber, which is critical with these delicate subcellular organelles. Ring electrodes are fitted at the top and bottom of the perfusion chambers, which have minimal dead-space to ensure optimal response to stimulation. The chambers were perfused at a rate of 0.5 ml/min with perfusion buffer; the buffer reservoir was constantly gassed with 95% O_2 /5% CO_2 throughout the experiment. After a 20-min washout, fractions of perfusate were collected every 1.5 min on the built-in collector. Release of tracer was triggered by stimuli applied to the chamber electrodes from an electrical stimulator (manufactured in the SCMB workshop). Current was monitored routinely with a Digital Multimeter (Digitech QM 1535; Jaycar Electronics, Woolloongabba, Qld., Australia). Electrical stimulation was applied as contiguous rectangular pulses of alternating positive and negative polarity, 0.4 ms duration, 100 Hz. By immediately reversing the polarity of the stimulus, the duration of depolarization event and the risk of anodic corrosion of the electrodes are both minimized.

At the end of the session, the perfusion buffer was replaced with 0.25 M perchloric acid (HClO_4) to release residual radioactivity from the tissue. This final sample was neutralized with KOH to precipitate KClO_4 and clarified by brief centrifugation before further processing. Each fraction was mixed with 4 ml of scintillation fluid for the determination of radioactivity, as described above.

Data Analysis

Data are expressed as a fractional release rate (FRR) in which the amount of radioactivity in each fraction was divided by the synaptosomal content of radioactivity prior to its collection. The

Table 1. Case information on subjects

No.	Sex	Age years	PMI, h	Cause of death
<i>Nonalcoholic subjects</i>				
1	F	66.58	56.77	Peripheral ischemia
2	F	75.97	24.43	Heart disease
3	F	81.22	21.43	Intracranial hemorrhage
4	F	43.17	18.50	Pulmonary embolism, venous thromboses
5	F	57.42	9.75	Cardiac failure
6	F	87.09	21.50	Pulmonary edema, cardiac failure
7	F	32.86	48.42	Pulmonary thromboembolism, breast carcinoma
8	F	78.36	4	Ischemic heart disease
9	M	70.57	26	Cardiac and renal failure, broncho-pneumonia
10	M	86.42	15.15	Myocardial infarction, ischemic heart disease
11	M	85.12	24.50	Pneumonia, ischemic heart disease
12	M	82.80	46.83	Cardiorespiratory arrest
13	M	24	24	Electrocution, myocardial ischemia
14	M	37	11	Pulmonary embolism
Average		65 ± 22	25 ± 15	
<i>Alcoholic subjects</i>				
<i>Alcoholics without comorbid disease</i>				
1	F	70	17	Cardiac arrhythmia
2	F	64	58	Peritonitis, pneumonia
3	F	28.22	49.42	Pneumonia
4	F	44.99	16	Alcohol-related disorder
5	F	46	27	Pulmonary congestion and epilepsy
6	F	46	5	Respiratory distress syndrome
7	M	18.77	30.50	Alcohol toxicity
8	M	59.08	15	Hanging
9	M	51.24	7.50	Acute pneumonia, cardiac failure
10	M	29.15	24.50	Pulmonary edema, cardiomyopathy
11	M	56	48	Renal failure, ischemic heart disease
12	M	66.99	46	Pancreatic carcinoma
13	M	66	11.5	Renal failure, chronic anemia
Average		50 ± 16	27 ± 18	
<i>Cirrhotic alcoholics</i>				
1	F	74.62	20.25	Renal failure
2	F	37	14	Not stated
3	M	55.55	58.08	Multisystem organ failure due to pulmonary hypertension
4	M	63.40	26.5	Upper gastrointestinal hemorrhage
5	M	48.48	14.67	Renal failure
Average		56 ± 14	27 ± 18	
Average values for age and PMI are means $\pm$ standard deviation.				

Fig. 1. Transmitter preloading and release paradigms. **a** L-[³H]glutamate uptake time course. **b** [¹⁴C]GABA uptake time course. Radiolabeled transmitters were each applied to synaptosome suspensions at 1 μ M as described in the 'Methods' section. Uptake of both substrates reached a maximum by $\sim$ 20 min; this time was used for preincubations in assays of release. Representative time courses are shown; each assay was performed three times with similar results. Ordinates show radioactivity accumulated in the synaptosome residue obtained at each time point ('Methods'). The drop in L-[³H]glutamate content after 20 min may have come about because its concentration in the medium was sufficient to activate presynaptic receptors and elicit rerelease of vesicular transmitter. **c** The standard superfusion paradigm employed showing the application and duration of stimuli S1 and S2 and how the AUC values of the evoked release peaks P₁ and P₂ were determined. FRR was calculated as described in the 'Methods' section. The delay from initiation of stimulation to the peak of release reflects the dead space in the collection lines. **d** Average sizes of release peaks. Mean AUC values for P₁ and P₂ are shown, averaged across both areas, all subjects, the two transmitters, and both Ca²⁺ concentrations; the order of perfusion with the two Ca²⁺ concentrations was randomized across experiments. To rectify the non-normal distribution of AUC values, a Kleczkowski transformation [24] was applied (see 'Methods'), given by $\kappa(A)_{ij} = \log_e(A_{ij} + 14.919)$, where A_i is the AUC of the i^{th} peak elicited by stimulus S_j . An ANOVA was performed on the transformed values; least-squares mean and SEM values from the ANOVA were converted back to the FRR scale for presentation. P₁ AUC averaged 46% of P₂ AUC (main effect for peak significant, $F_{1,30} = 221.928$, $p < 0.001$). ■ = 1st release peak; □ = 2nd release peak; values are means $\pm$ SEM on the FRR scale. **e** Comparison of L-[³H]glutamate and [¹⁴C]GABA release. Overall, L-[³H]glutamate release was about double [¹⁴C]GABA release (main effect for amino acid, $F_{1,30} = 233.069$, $p < 0.001$). The L-[³H]glutamate P₂ AUC averaged 39% of the corresponding P₁ AUC, whereas [¹⁴C]GABA P₂ AUC averaged 56% of the [¹⁴C]GABA P₁ AUC (peak $\times$ amino acid interaction significant, $F_{1,30} = 82.016$, $p < 0.001$). Key as in **d**.

synaptosomal content of radioactivity was calculated by summing the radioactivity released in all subsequent fractions, including the radioactivity released by HClO_4 .

$$\text{FRR for fraction } x_i = \frac{\text{dpm in fraction } x_i}{\text{dpm in fraction } x_i + \text{all subsequent fractions}}$$

Synaptosomes were electrically stimulated during the collection of the 4th (S_1) and 16th (S_2) fractions (fig. 1c). This elicited the release of brief pulses of radioactivity, signified as P_1 and P_2 (fig. 1c). When stimuli were applied, the sum of the increased FRR values minus the interpolated baseline values gave the 'stimulus-evoked release peak' as the area under the curve (AUC; fig. 1c–e). To balance the order of stimulation in buffers containing the two Ca^{2+} ion concentrations, duplicate samples of synaptosomes were superfused in parallel (the apparatus has six superfusion chambers that can be fed independently from different buffer reservoirs).

Peak AUC data were compiled and plotted using the computer programs Excel (Microsoft P/L, North Ryde, N.S.W., Australia) and GraphPad Prism (Graphpad Software, San Diego, Calif., USA). Statistical analysis was performed with the Statistica v9.0 (StatSoft, Tulsa, Okla., USA) and SPSS v18.0 (IBM, Somers, N.Y., USA) software packages. Preliminary tests showed that AUC data distribution deviated significantly from normal in the number of instances. Normal probability plots were significantly nonlinear (Shapiro-Wilks tests, $p < 0.01$), indicating a positive skewness, and plots of peak AUC values obtained at different Ca^{2+} concentrations were nonparallel. Kleczkowski transformations [24] stabilized the variances and generated co-parallel normal probability plots. Separate analyses were done on the Ca^{2+} -dependent component of stimulus-coupled release (v.i.). These values were obtained by subtracting the peak AUC obtained in PB0 from the AUC obtained in PBC for a given transmitter and stimulus. A separate Kleczkowski transform was calculated for these difference peaks. Statistical tests were performed on transformed values; least-squares mean and SEM values were converted back to the original scale of measurement for presentation.

Following regression analysis (v.i.), age at death and postmortem interval (PMI) were tested as continuous predictors in analyses of covariance, but no term containing these factors, alone or in combination, was significant in any analysis. Moreover, the groups did not differ significantly on either factor by analysis of variance (ANOVA), and no pairwise comparison was significant ($p > 0.1$). It was thus appropriate to use ANOVAs, together with relevant post-hoc tests where necessary, to assess differences in stimulus-evoked transmitter release [25]. Exact probabilities are quoted down to $p = 0.001$; significance was accepted at $p < 0.05$.

Results

Stimulus Repetition

To demonstrate that electrically evoked release retains a key characteristic of exocytosis and did not result from compromise of the synaptic membrane, a repeat stimulus of equal strength was applied after a brief recovery period. It may be seen (fig. 1c) that the second stimulus elicited a second response peak, albeit somewhat attenuated

(fig. 1d). The attenuation was more marked for glutamate than for GABA (fig. 1e).

Characterization of the Electrical Stimulation Parameters

Due to the limited availability of human tissue, only two voltage settings were tested. Because repeated stimuli led to attenuation of the release peak (above), these tests were carried out on synaptosomes that were only stimulated once (S_1). Figure 2a, b illustrates that the size of both the L-[^3H]glutamate and [^{14}C]GABA release peaks was larger at 10 V. Consistent with the present data, previous work had shown that the response elicited from rat synaptosomes by ~ 10 V was maximal [26], so this intensity was selected for subsequent work. Figure 2c, d shows the effect of varying the S_1 stimulation time on release. Doubling the duration of a 10-volt stimulus from 1.5 min to 3 min did not significantly increase the AUC of the release peak. The shorter stimulation time was used in all subsequent experiments.

Effect of Ca^{2+}

Perfusion with a physiological concentration of Ca^{2+} ions significantly enhanced transmitter release (fig. 3a). The mean increase was 89% overall (fig. 3 legend). [^{14}C]GABA release was not as strongly induced by electrical stimulation in either perfusion medium (fig. 3b), and neither was the net increase due to Ca^{2+} as great (fig. 3b, legend). The Ca^{2+} -dependent peak size was used as an index of synaptic-vesicle exocytosis.

Effects of Age and PMI

The effects of age at death and PMI on Ca^{2+} -dependent stimulus-coupled transmitter release were assessed by linear regression. Separate analyses were performed on transforms of the P_1 and P_2 difference peaks. The only significant regression was P_2 L-[^3H]-glutamate release on age (fig. 3c, legend). It may be seen (fig. 3c–f) that the P_1 transforms for both transmitters gave quite shallow and nonsignificant slopes against either factor. Analysis of covariance can be problematic if the slopes of regressions on the covariate are heterogeneous [25]. In any case, covariance analyses showed that no term containing a covariate (main effect or interaction) was significant (see above). Further analyses were conducted by ANOVA on P_1 difference peaks only.

Chronic Alcohol Misuse and Vesicular Release

Overall Ca^{2+} -dependent synaptosomal release of L-[^3H]glutamate in response to electrical stimulation was

Fig. 2. Optimization of stimuli. To minimize potential confounds, only one stimulus was applied to control synaptosomes superfused with Ca^{2+} -containing medium. **a, b** Intensity. Stimulus parameters were kept constant (see 'Methods'), except that current was applied at ± 5 V (squares, dashed lines) or ± 10 V (circles, solid lines) for 1.5 min. **c, d** Duration. Parameters were kept constant except that 10-volt stimuli were applied for 1.5 min (circles, solid lines) or 3 min (squares, dashed lines). **a, c** L-[^3H]glutamate. **b, d** [^{14}C]GABA. Representative release assays are shown; each assay was performed three times with similar results.

Fig. 3. Ca^{2+} dependence of release. **a** Mean AUC values for peaks elicited at the two Ca^{2+} ion concentrations were derived from the ANOVA used in figure 1d, e, averaged across both areas, all subjects, both transmitters, and peaks P_1 and P_2 . The overall mean peak AUC in the presence of 2.5 mM Ca^{2+} ion concentration (PBC) was 189% of that in the absence of Ca^{2+} ions (PB0; main effect for Ca^{2+} significant, $F_{1,30} = 181.375$, $p < 0.001$). $\square$ = Release peak elicited in 0 mM Ca^{2+} ; $\blacksquare$ = release peak elicited in 2.5 mM Ca^{2+} ; values are means $\pm$ SEM on the FRR scale. **b** Comparison of the extent of enhancement of L-[^3H]glutamate and [^{14}C]GABA release by Ca^{2+} . L-[^3H]glutamate release AUC in PBC averaged 203% of the AUC in PB0, whereas [^{14}C]GABA release AUC in PBC averaged 175% of the AUC in PB0 ($\text{Ca}^{2+} \times$ amino acid interaction significant, $F_{1,30} = 23.670$, $p < 0.001$). Key as in **a**. **c-f** Regressions on potential confounds. To delineate the vesicular component of release, peaks evoked in PB0 were subtracted from peaks evoked in PBC by the same stimulation event (either S1 or S2). To normalize these data, a different Kleczkowski transformation [24] was applied, given by $\kappa(\Delta)_{ij} = \log_e(\Delta_{ij} + 25.194)$, where Δ_i is the AUC

of the i^{th} difference (PBC – PB0) peak elicited by stimulus S_j . Values were regressed on the patient's age at death (**c, d**) or on the interval from death to autopsy (PMI; **e, f**). Scatterplots show individual $\kappa(\Delta)_{ij}$ values from each area (dorsolateral prefrontal cortex, DLPFC; primary motor cortex, PMC) of subjects undifferentiated for alcoholic status or liver pathology. Circles = DLPFC P_1 ; squares = PMC P_1 ; triangles = DLPFC P_2 ; inverted triangles = PMC P_2 . **c, e** L-[^3H]glutamate. **d, f** [^{14}C]GABA. Solid lines = regressions averaged across areas in P_1 ; dashed lines = regressions averaged across areas in P_2 . Regression statistics: L-[^3H]glutamate on age, P_1 : $r^2 = 0.045$, $p = 0.24$; L-[^3H]glutamate on age, P_2 : $r^2 = 0.216$, $p = 0.007$; [^{14}C]GABA on age, P_1 : $r^2 = 0.084$, $p = 0.11$; [^{14}C]GABA on age, P_2 : $r^2 = 0.003$, $p = 0.77$; L-[^3H]glutamate on PMI, P_1 : $r^2 = 0.007$, $p = 0.65$; L-[^3H]glutamate on PMI, P_2 : $r^2 = 0.007$, $p = 0.65$; [^{14}C]GABA on PMI, P_1 : $r^2 = 0.011$, $p = 0.57$; [^{14}C]GABA on PMI, P_2 : $r^2 = 0.056$, $p = 0.19$. No other regression, including those restricted to a single cortical area or to a single group of subjects, achieved statistical significance.

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Fig. 4. Effect of alcohol misuse on Ca^{2+} -dependent release. An ANOVA restricted to P_1 difference (PBC – PB0) peak transforms $[\kappa(\Delta)]_{il}$ values showed that electrical stimulation elicited vesicular L-[^3H]glutamate release more strongly than it elicited vesicular [^{14}C]GABA release (main effect for amino acid significant, $F_{1,30} = 54.426$, $p < 0.001$). **a** Overall comparison between controls and alcoholics undifferentiated by comorbid cirrhosis. Release peak AUC values were averaged across brain regions. Ca^{2+} -dependent stimulus-evoked L-[^3H]glutamate release was greater in combined alcoholic than in nonalcoholic subjects, but [^{14}C]GABA release was not (alcoholic misuse status $\times$ amino acid interaction significant, $F_{1,30} = 4.889$, $p = 0.035$). Post-hoc testing by the Newman-Keuls method showed that the difference between groups ap-

proached significance for L-[^3H]glutamate release, $p = 0.065$, but not for [^{14}C]GABA release, $p = 0.37$. In a separate ANOVA where subjects were further divided by gender, profiles did not vary significantly between males and females (not shown; alcoholic status $\times$ sex $\times$ amino acid interaction not significant, $F_{1,28} = 0.568$, $p = 0.46$). In fact, no term involving gender was significant in that ANOVA, $p > 0.45$. **b, c** Release peak AUC values in each brain region. Regional profiles did not vary significantly from the overall pattern (alcohol-misuse status $\times$ amino acid $\times$ area interaction not significant, $F_{1,30} = 0.329$, $p = 0.57$). □ = Nonalcoholic control subjects; ■ = alcoholic subjects. Values are means $\pm$ SEM on the FRR scale.

stronger in alcohol-misusing subjects than in controls (fig. 4a). In contrast, [^{14}C]GABA release trended lower in alcoholics, although the effect was not significant (fig. 4a). The net effect would be to change the excitatory:inhibitory input to postsynaptic neurons. This pattern was not restricted to the dorsolateral prefrontal cortex (fig. 4b, c): the enhancement in L-[^3H]glutamate release was as strong in the relatively spared primary motor cortex. This lack of regional specificity also applied to the following analyses (fig. 5 legend).

Interactions with Cirrhosis and Gender

Subjects were further divided by the presence of comorbid liver cirrhosis and/or by gender. This produced contrasting effects with the two transmitters. The release of L-[^3H]glutamate showed an increasing trend, with cirrhotic alcoholics $>$ noncirrhotic alcoholics $>$ controls (fig. 5a). The data on cirrhotic alcoholics must be treated with caution because of the sample size. No pairwise difference was significant by post-hoc testing (fig. 5 legend), but independent confirmation of the trend was seen when male and female subjects were analyzed separately

(fig. 5b, c); both sexes showed the same graded response. In contrast, [^{14}C]GABA release was markedly attenuated in alcoholics without comorbid disease but significantly enhanced in cirrhotic alcoholics (fig. 5a legend). The greater number of noncirrhotic alcoholics underpinned the overall reduction in [^{14}C]GABA release in alcoholics (fig. 4a). The data on cirrhotic alcoholics must be treated with caution because of the sample size, but it is noteworthy that the same trends were seen in independent analyses of male and female subjects (fig. 5b, c). The lack of male-female differences also applied to the previous analyses (fig. 4 legend).

Discussion

The primary accomplishment of the present study was to develop and optimize a paradigm to study the Ca^{2+} -dependent stimulus-evoked release of amino acid neurotransmitters from synaptosomes prepared from human brain obtained at autopsy. Short trains of electrical pulses elicited brief pulses of preloaded L-[^3H]glutamate

Fig. 5. Ca^{2+} -dependent release in subjects divided by alcohol misuse status and cirrhosis. An ANOVA restricted to P_1 difference (PBC – PB0) peak transforms $[\kappa(\Delta)_{il}]$ values was performed. **a** Release peak AUCs averaged across brain regions. L-[^3H]glutamate release was graded by disease severity, whereas the pattern [^{14}C]GABA release was biphasic (group $\times$ amino acid interaction significant, $F_{2,29} = 3.476$, $p = 0.044$). Post-hoc testing by the Newman-Keuls method showed that no pairwise difference between groups was significant for L-[^3H]glutamate release ($p > 0.15$); [^{14}C]GABA release differed between alcoholics without comorbid disease and alcoholics with cirrhosis ($p = 0.036$) but on no other

between-group pairwise comparison ($p > 0.15$). Regional profiles did not vary significantly from the overall pattern (not shown; group $\times$ amino acid $\times$ area interaction not significant, $F_{2,29} = 0.651$, $p = 0.53$). **b, c** In a separate ANOVA, subjects were further divided by gender. Profiles did not vary significantly between males and females (group $\times$ sex $\times$ amino acid interaction not significant, $F_{2,26} = 0.248$, $p = 0.78$). In fact, no term involving gender was significant in the ANOVA, $p > 0.25$. $\square$ = Nonalcoholic control subjects; $\square$ with diagonal lines = alcoholics without comorbid disease; $\blacksquare$ = alcoholics with cirrhosis. Values are means $\pm$ SEM on the FRR scale.

and [^{14}C]GABA. The manner in which the autopsy tissue was collected, frozen, and thawed is critical for the preparation of viable synaptosomes [10, 11]. The data shown here suggest that electrically evoked transmitter release was a physiological response rather than the result of lysis of the synaptosomes. The release peaks were self-limiting (no increase in peak duration with a 3-min stimulus compared with a 1.5-min stimulus), and a second stimulus after a rest period evoked an additional release peak. These responses also confirm that the synaptosomes had developed membrane potentials upon incubation, as we have noted previously [18]. Radiolabeled transmitters enter synaptosomes via plasma membrane Na^+ -coupled transporters [19], and may then transit to synaptic vesicles [27]. Both uptake processes are energy dependent, because the transmitters are taken up against concentration gradient [19, 27]. We have previously shown that the plasma-membrane glutamate and GABA transporters, and the intrasynaptosomal vesicular glutamate transporter, are functional in human synaptosomes prepared in the manner used here [14, 15, 28].

A component of electrically evoked release was dependent on the presence of Ca^{2+} ions, which is considered to be an essential feature of vesicular stimulus-secretion

coupling [29]. The release peak elicited in Ca^{2+} -free medium was smaller, but not zero. It is possible that electrical stimulation induced Ca^{2+} release from intracellular stores, or that the sequestering of Ca^{2+} by EGTA in the Ca^{2+} -free buffer was incomplete. However, the most likely source of the Ca^{2+} -free peak is reversal of the plasma-membrane transporters [30]. Our previous work showed that ionic concentration-effect curves for glutamate and GABA transport are much steeper in human than in rat synaptosomes [31], which suggests that changes in transmembrane ionic gradients will more markedly increase reverse transport in human preparations. Background non- Ca^{2+} -dependent release hindered development of a vesicular exocytosis paradigm based on K^+ -induced depolarization [32]. Attempts to wash out the intrasynaptosomal cytoplasmic pool of radiolabel with a pulse-chase protocol, or to circumvent transporter reversal by utilizing 4-aminopyridine as the stimulus, proved unsatisfactory [33]. Depolarization-induced increase in respiration, which is likely to track closely with depolarization-evoked vesicular transmitter release, shows a more marked decline post-mortem than does transmitter uptake [18, 31]: vesicular exocytosis is a multi-component process, whereas uptake requires the integrity of a single protein

complex [19]. This may have aided the current paradigm, where there was only modest decline in Ca^{2+} -dependent release with increasing postmortem delay, because the radiolabel would only be taken up by viable synaptosomes.

The first L-[^3H]-glutamate release peak was higher in chronic alcoholics than in controls. This might reflect a difference in glutamate concentration in the nerve-ending cytoplasm. The concentrations of small molecular weight components can change markedly after death [18]: the levels achieved in synaptosomes after ex vivo incubation depend on reestablishment of membrane potentials coupled with metabolic processes supported by active respiration and glucose metabolism [34, 35]. We found no differences in radiolabeled glutamate or GABA uptake between severe alcoholic cases and controls in previous studies [36]. Enhanced L-[^3H]-glutamate release in chronic alcoholics did not comport with cellular neuropathology. Rather than mirroring or underpinning localized neuronal loss, it may obliquely reflect the widespread loss of dendritic spines in the brain of the chronic alcoholic [37], because surviving, more robust, synaptic boutons would retain functional transporters that could take up L-[^3H]-glutamate. Glutamate concentration is elevated in the brains of rats chronically administered ethanol [38, 39]. These studies were carried out on whole brain or brain slices, and did not distinguish the vesicular and metabolic compartments. The effect of chronic alcohol administration on the level of glutamate within nerve terminals was not determined.

Cirrhosis is a surrogate for alcohol misuse severity in that its prevalence correlates strongly with consumption [40], and this was true for the subjects in this study (see 'Methods'). The manifestation of glutamate-NMDA receptors in alcoholics is markedly affected by comorbid disease. Noncirrhotic alcoholics vary minimally from controls, whereas cirrhotic alcoholics differ from both of these groups in showing changes in receptor-binding parameters indicative of reduced receptor efficacy, and lower subunit transcript expression levels [16, 41, 42]. Reduced subunit transcript expression was found in both cortical regions. These observations are consonant with the finding that glutamate release increased with cirrhosis/severity, because it would be expected that increased availability of glutamate in the synapse would lead to downregulation of its receptors on the post-synaptic neuron [43]. In contrast, several studies have shown that chronic ethanol administration causes a significant decrease in the K^+ -induced release of L-[^3H]-glutamate and endogenous L-glutamate from rat brain terminals [39, 44]. As well as possible species differences and variations

in release paradigms [45], this mode of eliciting release results in significant reversal of the glutamate transporter (see above) [30].

The densities of GABA_A receptors in human alcoholics depend critically on cirrhosis of the liver. B_{MAX} is significantly higher in noncirrhotic alcoholics than in controls, but does not differ between cirrhotic alcoholics and controls [5], or in hepatic encephalopathy patients who had acquired cirrhosis through alcohol misuse but abstained from alcohol for at least 6 months prior to death [46]. Chronic ethanol administration reportedly does not alter GABA concentrations in synaptosomes isolated from rat frontal cortex [47]. In the present study, [^{14}C]GABA release did not differ between cirrhotic alcoholics and controls, consistent with the GABA_A B_{MAX} data in those subjects noted above, although this must be treated with caution due to the limited availability of cases. The reduced synaptosomal [^{14}C]GABA release data in alcoholics without comorbid disease is consistent with the significant upregulation of GABA_A receptors in the same subjects.

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