Hippocampal Subfield Volumes in Abstinent Men and Women with a History of Alcoholism

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Abstract

Aims. Alcohol use disorder (AUD), also often referred to as alcoholism, has been associated with abnormalities in hippocampal volumes, but these relationships have not been fully explored with respect to gender or sub-regional volumes.

Methods. Using Magnetic Resonance Imaging at 3 Tesla, we obtained brain images from 67 alcoholic (ALC) participants (31 women) and 63 nonalcoholic control (NC) participants (30 women). We used Freesurfer 6.0 to segment the hippocampus into 12 regions. These were imputed into mixed models to examine the relationships of brain volume with alcoholism group, gender, age, drinking history, and memory.

Results. The ALC group had approximately 5% smaller CA1, hippocampal tail, and molecular layer regions than the NC group. Age was negatively associated with volumes for the ALC group in the hippocampal tail, subiculum, and presubiculum. The relationships for delayed and immediate memory with hippocampal tail volume differed for ALC and NC groups: Higher scores were associated with smaller volumes in the ALC group, but larger volumes in the NC group. Length of sobriety was associated with decreasing CA1 volume in women (0.02% per year) and increasing volume size in men (0.03% per year).

Conclusions. These findings confirm and extend evidence that alcoholism, gender, and age differentially impact volumes of component parts of the hippocampus. The differential relationships of volume to age and memory could indicate a distinction in the impact of alcoholism on the function of the hippocampal tail. Further, the course of abstinence on CA1 volume differed for men and women.

Short Summary

Hippocampal subfield volumes were obtained from MRI scans of men and women with and without a history of alcohol use disorder (AUD). The CA1, hippocampal tail, and molecular layer were approximately 5% smaller in association with AUD. Further relationships were observed between the volumetric measures with gender, age, and memory.

Introduction

Magnetic resonance imaging (MRI) has been used extensively to study morphological changes in the brain associated with alcohol use disorder (AUD), a widespread and harmful condition (Nutt *et al.*, 2010; Grant *et al.*, 2015) that is commonly referred to as alcoholism. Because memory impairments are associated with long-term chronic alcoholism, one neuroanatomical focus of investigation has been the hippocampus (Bühler and Mann, 2011; Staples and Mandyam, 2016). Moreover, findings from previous research have shown that the largest subcortical volume loss observed in the brains of chronic alcoholics was in the hippocampus (Fein and Fein, 2013), and a meta-analysis (Wilson *et al.*, 2017) found a negative association between total hippocampal volume and degree of alcohol use (clinical vs. subclinical).

Although results of MRI studies have suggested gender-specific susceptibility of the brain to alcohol abuse (Hommer *et al.*, 2001; Ruiz and Oscar-Berman, 2013; Sawyer *et al.*, 2016, 2017, 2018, 2019; Mosher Ruiz *et al.*, 2017; Seitz *et al.*, 2017; Rivas-Grajales *et al.*, 2018; Oscar-Berman *et al.*, 2019), few studies have examined gender differences in the relationship between hippocampal volume and alcoholism. Instead, researchers have assessed alcoholism-related volumetric abnormalities in men (Laakso *et al.*, 2000; Beresford *et al.*, 2006; Chanraud *et al.*, 2007; Ozsoy, Durak, and Esel, 2013; Lee *et al.*, 2016), or in alcoholic (ALC) groups comprised of a combination of men and women (Agartz *et al.*, 1999; Kühn *et al.*, 2014; Zahr *et al.*, 2019). In studies that did analyze alcoholic men (ALCm) and alcoholic women (ALCw) separately, investigators observed lower hippocampal volumes in the ALCm and in the

ALCw, but they did not find significant interactions between gender and diagnostic group (Agartz *et al.*, 1999; Zahr *et al.*, 2019).

Similarly, the relationships observed between hippocampal volume and cognitive ability also have been inconsistent. It has been widely reported that lesions to the hippocampus result in memory deficits (Squire and Zola, 1997; Clark, Zola, and Squire, 2000). Positive correlations between hippocampal volume and memory performance also have been reported in clinical populations such as patients with Alzheimer's disease or amnesic disorders (Petersen *et al.*, 2000; Barber *et al.*, 2001; Kopelman *et al.*, 2001; Mungas *et al.*, 2002), but this positive correlation has not been systematically observed in healthy populations (Van Petten, 2004). In the context of AUD, some research has indicated the presence of functional deficits with lower hippocampal volumes. For example, decreases in hippocampal gray matter volume in alcohol dependent men have been associated with executive functioning deficits as measured by the Trail Making Test Part B, an assessment of visual-conceptual and visual-motor tracking skills (Chanraud *et al.*, 2007). However, among participants with AUD in these earlier studies, smaller volumes of the anterior portion of the hippocampus were not significantly associated with memory impairment (Sullivan *et al.*, 1995).

The inconsistent findings relating volume and function may be impacted by the distinct functions of hippocampal subfields and the processing streams that exist within the hippocampus (Figure 1). Traditionally, hippocampal subfields have been defined as the subiculum, dentate gyrus, and *cornu ammonis* regions (CA1 through CA3). Researchers have focused on two major neural pathways, as follows: First, major input to the hippocampus originates in entorhinal cortex and proceeds along a trisynaptic circuit (1) to the dentate gyrus, then (2) to CA2 or CA3, and

finally, (3) to CA1 (and then, subiculum), which serve as outputs of the hippocampus. Second, the entorhinal cortex additionally has a direct connection to CA1, among other regions. All the input connections from CA1, CA2, CA3, and subiculum are made within an area called the molecular layer (also known as *stratum lacunosum moleculare*, or SLM).

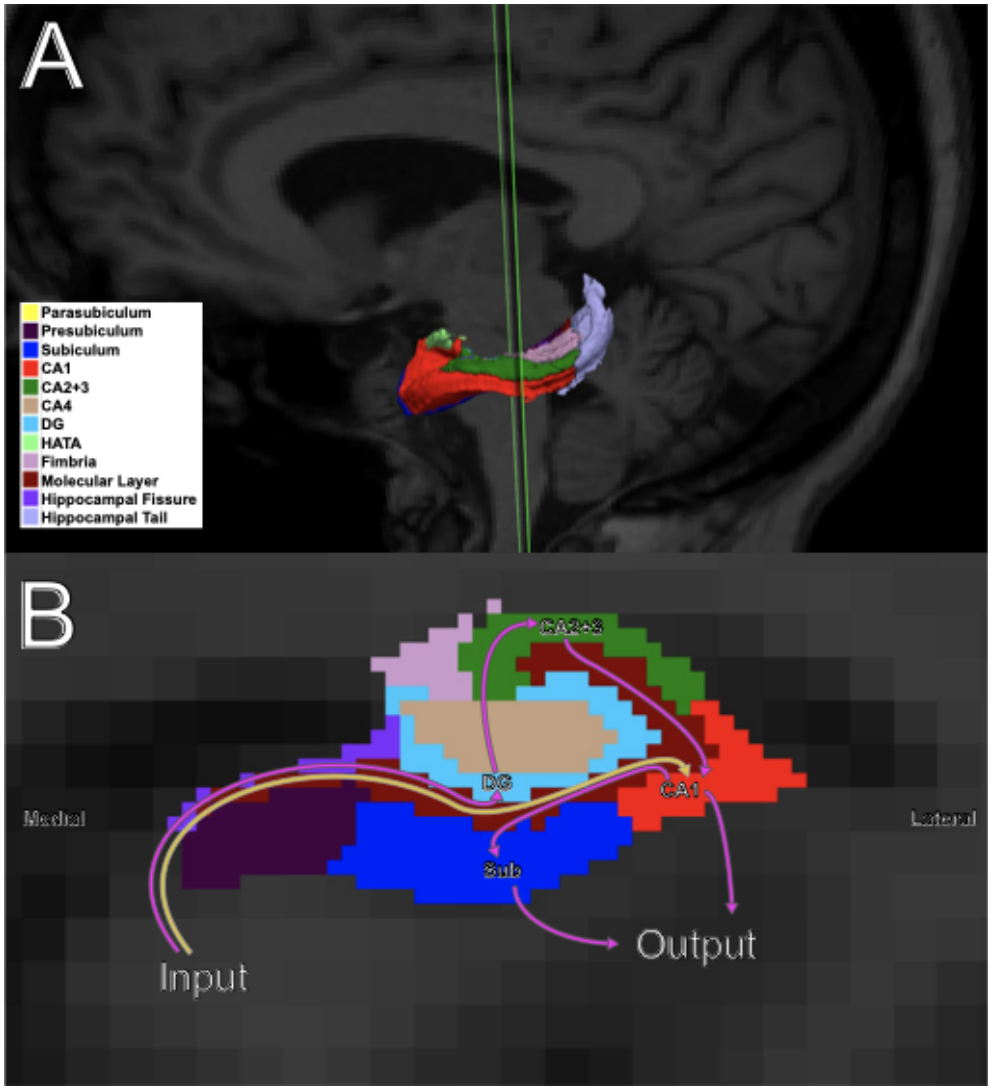

Figure 1. Hippocampal subfield segmentation (Iglesias et al. 2015) of the left hippocampus shown in three dimensions in A (lateral view) and cross-section in B (coronal plane).

A. This three-dimensional model of the left hippocampus depicts its segmentation into 12 subfields (although not all subfields are visible from the angle presented). B. This coronal section through the hippocampus illustrates subfields that are visible in the slice, and it shows the two major hippocampal neural pathways originating in the entorhinal cortex and as described in the text: The pink arrows indicate the trisynaptic circuit; the yellow arrow shows the direct circuit. Abbreviations: CA1 = cornu ammonis 1; CA2+3 = cornu ammonis 2 and 3; CA4 = cornu ammonis 4; DG = dentate gyrus; HATA = hippocampal-amygdaloid transition area; Sub = subiculum.

In the context of this network, specific memory functions have been associated with some of these regions. The molecular layer is the point of entry for information, including the direct pathway from entorhinal cortex that is theorized to contain information regarding presently experienced stimuli (Levy, 1989; Siekmeier *et al.*, 2007). The dentate gyrus has been shown to play a role in distinguishing different contexts (pattern separation) (Aimone, Deng, and Gage, 2011). For the structures involved in the trisynaptic pathway, the CA2 and CA3 regions have been ascribed, among other things, to encoding (Eldridge *et al.*, 2005), learning, and early retrieval of verbal information (Mueller *et al.*, 2011), and disambiguation and encoding of overlapping representations (Newmark *et al.*, 2013). The output fields, CA1 and subiculum, are thought to compare current context with remembered contexts, or mental time travel (Bartsch *et al.*, 2011). Their projections include regions implicated in addictive disorders: the prefrontal cortex, amygdala, nucleus accumbens, and ventral tegmental area. This processing stream is repeated along the axis of the hippocampus, described in the human as anterior to posterior, and in rodent models as ventral to dorsal. The anterior (or ventral) portion has been considered to involve a wide variety of contexts including stress, emotion, and affect (Fanselow and Dong, 2010), while the posterior (or dorsal) portion has been demonstrated to have involvement in spatial processing (Poppenk *et al.*, 2013). White matter fibers that carry the projections of the hippocampus include the fimbria (Schultz and Engelhardt, 2014).

In a sample of men with ongoing AUD, Lee et al. (2016), reported smaller volumes of the left presubiculum, bilateral subiculum regions, and fimbria, but in studies that have included women, significant differences in interactions of AUD and gender have not been detected for hippocampal subfield volumes (Kühn *et al.*, 2014; Zahr *et al.*, 2019). Research also has

supported reports of smaller AUD-related volumes in subiculum, CA1, CA4, or other parts of the hippocampus (dentate gyrus, hippocampal-amygdaloid transition area [HATA], and the fimbria) (Lee *et al.*, 2016; Zahr *et al.*, 2019), along with CA2 and CA3, which showed significant normalization two weeks following withdrawal (Kühn *et al.*, 2014). Additionally, effects of age were found for the fimbria and the hippocampal fissure, while a significant interaction between AUD diagnosis and age was seen in CA2 and CA3 regions. However, in neither the Lee et al.(2016) nor the Kühn et al. (2014) studies, was memory assessed, and Zahr et al. (2019) found no significant memory impairment associated with reduced hippocampal subfield volumes.

The present study assessed the impact of gender on hippocampal subfield volumes, and the relationships between memory and volumes of these regions in abstinent individuals with a history of AUD. Examining the impact of alcoholism on individual subfield volumes and their interactions with gender and memory can further elucidate which components of this brain network are associated with the problems contributing to, and resulting from, AUD.

Methods

Participants

Participants provided written informed consent approved by the Institutional Review Boards of the participating institutions. A total of 67 ALC (31 women) and 63 NC participants (30 women) were included in analyses (Table 1), as detailed in the Supplemental Methods. Exclusion criteria included left-handedness, serious medical and psychiatric conditions, and extremely large or small hippocampal subfield brain volumes (+/- 4 standard deviations).

Psychiatric criteria were assessed through an interview schedule. Memory was assessed with the Wechsler Memory Scale (Wechsler, 1997). Drinking history was obtained through a questionnaire (Cahalan, Cisin, and Crossley, 1969) that provided measures of Duration of Heavy Drinking (DHD; years), Daily Drinks (DD; ounces of ethanol per day), and Length of Sobriety (LOS; years). ALC participants drank heavily (over 21 drinks per week) for at least five years, met criteria for alcohol abuse or dependence, and had a minimum of four weeks of abstinence prior to testing.

MRI Acquisition and Analyses

As described in the Supplemental Methods, T1-weighted MRI scans were obtained using a 3 Tesla Siemens Trio system, with 1x1x1.5 mm voxels. The 12 hippocampal subfield volumes were obtained with FreeSurfer 6.0. Mixed model analyses were conducted using R. Volume was entered as the dependent variable, and 'group,' 'gender,' 'region,' and 'age' were the independent factors. We examined the interaction of group-by-gender-by-region to assess how the impact of alcoholism differed for men and women. Also, we examined the interaction of group-by-gender-by-age to assess abnormal aging for men as compared to women. Following significant effects, additional models were constructed to examine relationships with memory measures, and with drinking history (DHD, DD, and LOS).

Results

Participant Characteristics

The participants consisted of 67 ALC (31 women) and 63 NC (30 women) with a mean age of 52 years (Table 1). Between-group (NC vs. ALC) differences in sex distribution and age were not significant (95% CI of mean difference = [5.4, -3.4] years). The ALC participants had 0.9 fewer years of education ($t(114.74) = -2.49$, $p < 0.05$) and 8.1 points lower FSIQ scores ($t(127.99) = -3.07$, $p < 0.01$).

	Alcoholic Women N=31		Alcoholic Men N=36		Nonalcoholic Control Women N=30		Nonalcoholic Control Men N=33	
Measure	Mean	SD	Mean	(SD)	Mean	(SD)	Mean	(SD)
Age	54.5	12	51.2	10.8	53.0	15.8	50.5	12.3
Education	14.7	1.8	14.1	1.9	15.3	2.6	15.4	2.5
FSIQ	103.1	14.5	104.1	16.6	110.8	15.1	112.7	14.7
IMI	107.3	17.3	102.6	14.9	113.0	16.1	110.5	15.4
DMI	110.2	16.9	104.8	14.1	115	12.5	114.2	17.4
DHD	13.7	5.6	17.5	9.1	0	0	0	0
DD	8.8	7.2	11.8	6.8	0.3	0.3	0.2	0.2
LOS	10.9	11.9	3.9	6.2	2.2	7.4	2.1	7.9

Table 1. Participant Characteristics.

Means and standard deviations (SD) are provided for the participant characteristics of Alcoholic and Nonalcoholic Men and Women. DMI was not obtained from one alcoholic man and one control man. IMI was not obtained from one alcoholic man and one control man. Abbreviations: FSIQ = Wechsler Adult Intelligence Scale Full IQ; DMI = Delayed Memory; IMI = Immediate Memory; DHD = Duration of Heavy Drinking; DD = Daily Drinks; LOS = Length of Sobriety.

Subfield Volumes, Group, Gender, and Age

Mixed-model analyses of normalized subfield volumes revealed significant three-way interactions (Table S2) for group-by-region-by-age ($F(11, 5366.83) = 3.02$, $p < 0.01$) and gender-by-region-by-age ($F(11, 5366.83) = 2.50$, $p < 0.01$). No significant group-by-gender

interactions were observed. Group differences in means, and in slopes (for age), are reported below.

Mean differences between groups revealed significantly smaller (Bonferroni adjustment of family-wise $p < 0.05$ for 12 regions: $p < 0.0042$) CA1, hippocampal tail, and molecular layer volumes in the ALC group compared to the NC group, with marginal significance ($p = 0.0046$) for subiculum (Figure 2 and Table S2). The ALC group had 5.18%, 5.08%, 4.80%, and 3.70% smaller volumes in the CA1, molecular layer, hippocampal tail regions, and subiculum, respectively.

Significant gender differences (with analyses including ALC and NC participants together) were found for the subiculum, presubiculum, CA1, and molecular layer regions. Women had 8.43%, 4.99%, 4.47%, and 3.30% larger volumes in the presubiculum, subiculum, molecular layer, and CA1.

The impact of age on subfield volumes significantly interacted with group. For the ALC group, age was associated with significantly smaller hippocampal tail, subiculum, presubiculum, and molecular layer volumes (-0.52, -0.45, -0.58, and -0.47, respectively; all percent/year); and these relationships were significantly more negative than those observed for the NC group (-0.17, -0.03, -0.04, and -0.17; all percent/year). The impact of age on subfield volumes also significantly interacted with gender. For women, age was associated with 0.4 percent/year significantly lower CA1 volumes, a rate significantly less negative than that observed for men (1.0 percent/year; not significant).

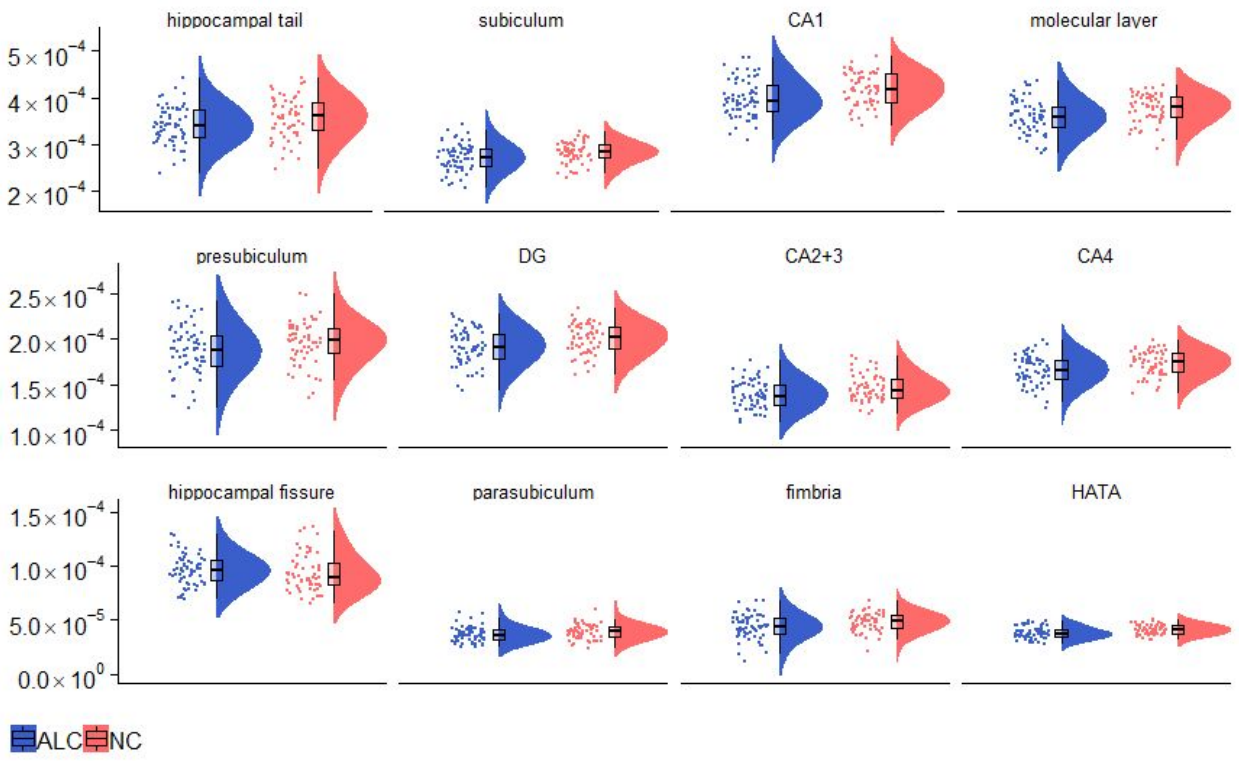

Figure 2. Regional volumes (percentage of estimated total intracranial volume).

Half violin raincloud plots (Allen *et al.*, 2019) show hippocampal subfield volumes for the nonalcoholic (NC) and alcoholic (ALC) groups. See Table S1 for mean and standard deviation values. Abbreviations: CA1 = cornu ammonis 1; CA2+3 = cornu ammonis 2 and 3; CA4 = cornu ammonis 4; DG = dentate gyrus; HATA = hippocampal-amygdaloid transition area; Sub = subiculum.

Subfield Volumes and Memory

Following our main analyses, we assessed the relationship of memory scores and volume. We found significant interactions (Figure 3, Figure S1, Figure S2, Table S3, and Table S4) for group-by-region-by-IMI and for group-by-region-by-DMI. Slopes were divided by the grand mean for each region and are thus presented as percent volume/IMI or DMI unit. The comparison of slopes for the relationship of memory with subfield volume between the ALC and

NC women was not significant, but it was significant for men, as follows.

Immediate Memory Index. Following the identification of significant group effects for CA1, hippocampal tail, and molecular layer, and of marginal significant group effects in the subiculum ($p = 0.0046$), we found that associations of IMI with those regions differed for ALC and NC groups in the hippocampal tail ($t(594.84) = -3.51$, $p < 0.001$). In ALC participants, the volumes decreased by 0.12%, while for NC they increased by 0.13% with each point of IMI.

Delayed Memory Index. Also in the hippocampal tail, a significant interaction of ALC and NC participants was observed between DMI and subfield regions (Table S4). In ALC participants, the volumes were 0.14% lower with each unit of DMI, while for NC participants, volumes were 0.20% higher with each unit of DMI, relationships that differed significantly from each other ($t(599.16) = -4.55$, $p < 0.0001$).

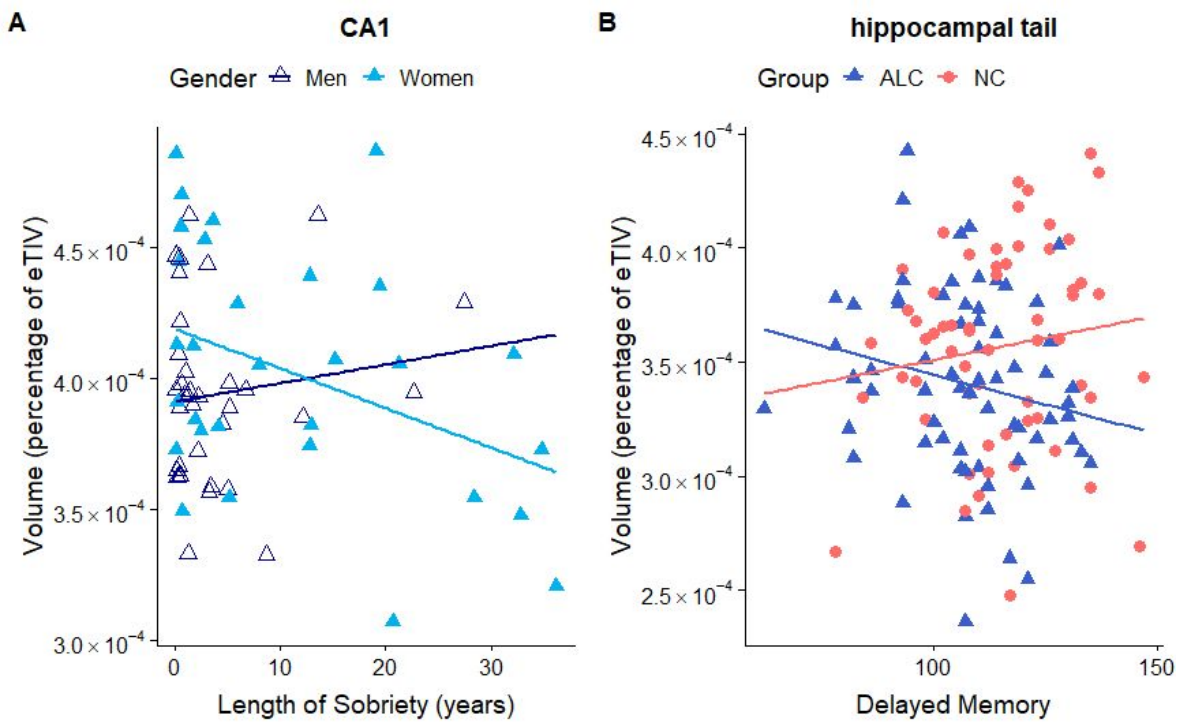

Figure 3. Distinct relationships of subfield volume for alcoholic (ALC) and nonalcoholic control (NC) groups.

A. For ALC men, CA1 Volumes (percentage of eTIV) were positively associated with Length of Sobriety, while for ALC women, a negative relationship was observed.

B. For the ALC group, Delayed Memory was associated with lower volumes, while for the NC group, a positive relationship was observed.

Abbreviations: CA1 = cornu ammonis 1; eTIV = estimated total intracranial volume

Subfield Volumes and Drinking History

For the ALC group, we examined the relationships of DHD, DD, and LOS to volumes, and the interactions of those drinking history measures. Only results with LOS were significant. Table S5 shows that the LOS-by-gender-by-region interaction was significant ($F(11, 2474.63) = 1.94$, $p = 0.03$). Sobriety was associated with smaller volumes in women and larger volumes in

men in the CA1 ($t(257.95) = -2.493$, $p = 0.01$). CA1 volumes increased by 0.03% per year and decreased by 0.02% per year of sobriety in ALCm and ALCw, respectively.

Discussion

Results of this study confirmed findings of smaller volumes of hippocampal subfields in association with alcoholism (Kühn *et al.*, 2014; Lee *et al.*, 2016; Zahr *et al.*, 2019). Compared to the NC group, the ALC participants exhibited significantly smaller volumes in the CA1, subiculum (with marginal significance), molecular layer, and hippocampal tail. Smaller volumes in these regions could result in abnormal neural processing, coincident with impairments of distinct mental functions. We also found significant associations between the volumes of individual subfields with age, memory, gender, and measures of drinking history. In the sections below, we discuss each of the significant hippocampal subregions, followed by a discussion of the influence of participant characteristics on the findings.

Alterations in hippocampal subfield volumes have implications both for downstream targets and for hippocampal processing of upstream inputs. That is, fewer neurons in the CA1 and subiculum may result in weaker output from the hippocampus, and smaller dendritic arborizations in the molecular layer may cause the hippocampus (particularly anterior regions) to be less effective in distinguishing between distinct contextual inputs, thereby resulting in forms of overgeneralization (Berkers, Klumpers, and Fernández, 2016). Both types of abnormality (input and output) could have severe consequences for emotion and motivation or sensitivity to reward. For example, the hippocampus projects to medial prefrontal areas, which are involved in conflict detection (Thierry *et al.*, 2000). Weak or insufficiently precise contextual signals from

hippocampus may bias these cortices towards greater sensitivity to emotional signals, such as those received from the amygdala. Elevated or overgeneralized processing of conflict may, in turn, bias the motivational and reward systems, particularly the nucleus accumbens, towards the use of poor coping strategies, including excessive drinking and other forms of self-medication. Abnormal inputs also can bias processing. For example, since the amygdala sends strong projections to the hippocampus (Wang and Barbas, 2018), those signals may modulate the hippocampus more strongly if it has fewer neurons or smaller dendritic arborizations. This could act synergistically with weak contextual representations, thereby reinforcing their emotional valence. In other words, overgeneralized contextual representations might be more susceptible to "somatic markers" (Foster *et al.*, 2017), as well as other forms of associative learning (Berkers *et al.*, 2016), which would increase the vulnerability to contextual signals that motivate drinking.

Subiculum

Both output regions of the hippocampus (CA1 and subiculum) were reduced in volume, which could be related to two cognitive corollaries: (1) an abnormal ability to distinguish a currently experienced context from other similar previously learned contexts (Smith and Bulkin, 2014; Barrientos and Tiznado, 2016) and (2) higher susceptibility to emotion-driven actions (Bergado, Lucas, and Richter-Levin, 2011). That is, following a history of AUD, a given experienced context might be more likely to match a previous context in which drinking occurred.

The role of context in triggering alcohol craving is well established, and context has behavioral and treatment implications (Zironi *et al.*, 2006; Crombag *et al.*, 2008). Evidence

suggests that social support may be helpful for circumventing specific contexts entirely (Beattie and Longabaugh, 1999), which would avoid the aforementioned overgeneralizing activity of an impaired CA1 and subiculum. The subiculum in particular has been implicated in the prediction of future rewards (Vorel *et al.*, 2001), perhaps due to its projections (along with projections of CA1) to the nucleus accumbens and ventral tegmental area. Likewise, the CA1 has been shown to play a role in mental processes involving envisioning the self in the future and past (Bartsch *et al.*, 2011). Therefore, the abnormalities we observed in these structures in association with alcoholism could negatively impact motivation through the impairment of both future processing and reward prediction.

Molecular Layer

The molecular layer also was smaller in the ALC group. This region includes the apical dendrites of hippocampal pyramidal cells, and is traditionally viewed as an input region to the subfields of the hippocampus (Van Strien, Cappaert, and Witter, 2009). Smaller volumes of the molecular layer could suggest a relationship of AUD with hippocampal inputs or with local computational processes of the hippocampus (as opposed to connectivity exiting the structure) and could lead to outcomes that are similar to those resulting from smaller CA1 and subiculum volumes. In the molecular layer, entorhinal cortex inputs are thought to convey information from the current sensory context, while intra-hippocampal projections to the molecular layer are thought to represent a memory representation of the current context (Hasselmo, 2005; Vago and Kesner, 2008). With reduced volume of this circuitry in the molecular layer, the quality of the processing at the meeting of these two streams of input could be impacted. Therefore, it could

result in a reduced ability to compare previously experienced contexts with the current experience, and might lead the hippocampus to overgeneralize the present context to previously experienced high-valence contexts. In turn, this could bias the system to addictive behaviors, i.e., a similar outcomes to those discussed regarding reductions in CA1 and subiculum volumes.

Hippocampal Tail

While the anterior hippocampus has been associated with stress, emotion, and affect (Fanselow and Dong, 2010), reductions in cells and cell processes in the posterior tail could involve many elements of spatial processing (Poppenk *et al.*, 2013). This possibility is congruent with previous findings of alcohol-mediated alterations of spatial processing (Fein *et al.*, 2006; Kopera *et al.*, 2012; Oscar-Berman *et al.*, 2014), which in rats, has been hypothesized to occur through alterations of hippocampal place cells (Matthews, Simson, and Best, 1996). In our ALC group, hippocampal tail volume was negatively associated with both immediate and delayed memory scores, in contrast to the positive association observed in the NC group. One possible interpretation for this finding involves regional compensation within the brain, as has been suggested previously (Chanraud *et al.*, 2013; Chanraud and Sullivan, 2014). That is, for ALC participants with smaller hippocampal tail volumes, the extent of structural impairment might be sufficient to necessitate a shift to using other structures for the same function. Once this shift occurs, the new structures may provide good compensation for learning — but this shift may occur more often for the more extreme cases of hippocampal perturbation. In contrast, for ALC participants with mild hippocampal impairments, the brain may continue to rely on the impaired structures instead of shifting to an alternative compensatory region; relying on the impaired

structures could result in impaired memory performance. (A metaphor that may assist in understanding is as follows: One may continue using a somewhat functioning toaster and make low quality toast; with a broken toaster one may switch to the oven broiler and make better toast.) Taken together, this explanation would fit with the observed tendency for larger hippocampal volumes to be associated with worse performance in the ALC group, and is consistent with findings that report how medial prefrontal cortex may compensate by increasing its efficiency for learning and memory after substantial hippocampal dysfunction (Krasne, Fanselow, and Zelikowsky, 2011).

Influence of Participant Characteristics

While our results of smaller volumes of the CA1, subiculum, and molecular layer in ALC subjects are in agreement with past findings (Kühn *et al.*, 2014; Lee *et al.*, 2016; Zahr *et al.*, 2019), our results did not indicate significant reductions in other regions that were reported in those studies, including CA2+3, CA4, HATA, and fimbria. However, abstinence durations for the ALC participants examined in those papers were generally shorter than in the present study (an average of 7.1 years). Therefore, results reported in the earlier studies could reflect both acute effects of alcohol consumption and long-term sequelae of chronic alcohol use. For the regions that are consistent across those studies and ours (CA1, molecular layer, and subiculum), the combined results support the view that they are vulnerable to chronic long-term AUD.

Gender differences also contribute to divergent findings across studies. As a proportion of head size, total hippocampal volumes are larger in women than in men (Agartz *et al.*, 1999; Perlaki *et al.*, 2014), a finding we support in more detail with evidence from subfield volumetric

analyses (subiculum, presubiculum, CA1, and molecular layer). Although the present study and the results reported by Agartz et al. (1999) and Zahr et al. (2019) did not show significant interactions between gender and diagnostic group, gender differences in volumes may be elucidated further by considering quantity and duration of alcohol consumption, and LOS. In the present study, CA1 volume was related to LOS differently for men and for women. In men, CA1 volume increased with LOS, suggesting recovery over time. However, in women, the volumes continued to decline with sobriety, even after statistically accounting for age. This might indicate an AUD-related abnormality in another biological system, as ALCw may be more susceptible to liver injury and heart disease, and have been reported to display lower drinking thresholds for systemic damage (Vatsalya *et al.*, 2016). Thus, a brain abnormality could continue to result in reduced health over a long period, even following abstinence, explaining the divergent gender effects of LOS. Additionally, in mice, chronic ethanol administration induces astrocyte activation in a number of hippocampal regions, including the CA1, but exclusively in female mice (Wilhelm *et al.*, 2016). Despite recovery from chronic ethanol intoxication, female mice exhibited altered astrocyte neurochemistry and function. Astrocytes are involved in neural health and vascular coupling, so abnormalities could have substantial neural consequences. While further studies are needed to translate these results to human populations, this study suggests that women could be more vulnerable to life-long astrocyte inflammation-mediated brain damage, including in the hippocampus and associated regions (Wilhelm *et al.*, 2016).

Age, another factor that individuates the impact of alcoholism, was more negatively associated with hippocampal volumes in our ALC group than in our NC group for the subiculum, presubiculum, hippocampal tail, and molecular layer regions; in the ALCw, age was negatively

associated with CA1 volume. Zahr and colleagues (2019) found smaller hippocampal volumes in older ALC individuals than in their NC group, but for the CA2+3 subfield, not the regions we report. However, there is a long-running hypothesis discussing how age may be associated with greater volume losses in ALC groups than NC groups (Pfefferbaum *et al.*, 1992), but findings have not been entirely consistent.

In summary, we observed smaller input (molecular layer) and output (CA1) regions, abnormalities that could be related to distorted context processing. We also found significant effects of alcoholism group, gender, age, and LOS: (1) the volume of the molecular layer was related to gender and group; (2) the CA1 was related to gender, group, and age; and (3) the hippocampal tail, subiculum, and presubiculum were related to the interaction of group and age; (4) age was more negatively associated with volumes for the ALC group than the NC group in the hippocampal tail, subiculum, presubiculum, and molecular layer regions; (5) CA1 volume was related to LOS differently for men and for women; and (6) in the ALC group, hippocampal tail volume was negatively associated with both immediate and delayed memory scores, in contrast to the positive association observed in the NC group.

Limitations

In the present study, the automated subfield labeling procedure that we employed relied upon a probabilistic atlas, rather than borders defined by image contrast at high resolution. Additionally, we used a cross-sectional design, whereas a longitudinal cohort may be better able to show how the observed abnormalities were related to pre-existing risk factors for AUD or consequences of AUD. Finally, we did not consider several other factors that could influence our

findings (see Oscar-Berman et al. (2014) for review). For example, we did not consider participant characteristics such as: family history of alcoholism (Cardenas *et al.*, 2005); smoking (Durazzo *et al.*, 2013; Luhar *et al.*, 2013); or body mass index (Pfefferbaum et al., 2009; Gazdzinski et al., 2010a).

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Supplemental Methods

Participants

The study originally included 146 participants: 73 abstinent chronic alcoholic (ALC) adults (33 ALCw; 40 ALCm) and 73 nonalcoholic control (NC) adults (34 NCw; 39 NCm), who were recruited locally through online and print advertisements. Participants provided written consent for participation in the study, which was approved by the Institutional Review Boards at the Boston VA Healthcare System, Massachusetts General Hospital, and Boston University School of Medicine. Exclusion criteria for participants included left-handedness, Korsakoff's syndrome, HIV, hepatic disease, head injury with loss of consciousness greater than 15 minutes, stroke, seizures unrelated to alcoholism, schizophrenia, Hamilton Rating Scale for Depression (HRSD) (Hamilton, 1960) score over 16, and illicit drug use (except marijuana) greater than once a week within the past five years. Sixteen individuals were excluded from the study for the following reasons: Three ALC participants (1 woman) were excluded for illicit drug use, and another three (1 woman) for brain lesions and/or head trauma. Six NCm were excluded for binge drinking, head trauma, and invalid or incomplete scan data. Two NCw were excluded for claustrophobia and brain lesions, and another two NCw were identified as outliers, with brain hippocampal region volumes +/- 4 standard deviations, and removed from further analyses. A total of 67 ALC (31 women) and 63 NC participants (30 women) were included in data analyses (Table 1).

Diagnostic criteria for study exclusion were based on a medical history interview, the

HRSD, and a computer-assisted, shortened version of the Computerized Diagnostic Interview Schedule (DIS) (Robins *et al.*, 2000). The DIS provides diagnoses of lifetime psychiatric illnesses as defined by criteria established by the American Psychiatric Association. Full-Scale IQ and cognitive performance were measured through the Wechsler Adult Intelligence Scale-IV (WAIS-IV) and the Wechsler Memory Scale-IV (WMS-IV) (Wechsler, 1997). Incomplete WMS scores were obtained from one NCm and two ALCm and were excluded from the memory analyses.

Drinking history was assessed using Duration of Heavy Drinking (DHD), i.e., years of consumption of 21 drinks or more per week, and Length of Sobriety (LOS) scale, which measures abstinence duration. The amount, type, and frequency (ounces of ethanol per day, roughly corresponding to daily drinks; DD) of alcohol use was measured for the last six months during which the participant drank alcohol (Cahalan *et al.*, 1969). Criteria for ALC participants included at least five years of previous alcohol abuse or dependence, and a minimum of four weeks of abstinence prior to testing. Compared to the ALCw, the ALCm had greater periods of heavy drinking and shorter periods of abstinence, which are consistent with national trends (Dawson and Archer, 1992). Two NCw and one NCm had no prior history of drinking, whereas the remaining NC participants were social drinkers.

MRI Acquisition and Analysis

MRI scans were obtained at the Martinos Center for Biomedical Imaging at Massachusetts General Hospital on a 3 Tesla Siemens (Erlangen, Germany) MAGNETOM Trio Tim scanner with a 32-channel head coil. Image acquisitions included two T1-weighted

multiecho MPRAGE scans collected for volumetric analysis that were averaged to aid in motion correction (TR = 2530 ms, TE = 1.79 ms, 3.71 ms, 5.63 ms, 7.55 ms [root mean square average used], flip angle = 7 degrees, field of view = 256 mm, matrix = 256 x 256, slice thickness = 1 mm with 50% distance factor, 176 interleaved sagittal slices, GRAPPA acceleration factor = 2; voxel size = 1.0 mm x 1.0 mm x 1.5 mm).

Scans were analyzed using an automated hippocampal segmentation method in FreeSurfer (https://surfer.nmr.mgh.harvard.edu), which has shown high reliability and agreement with manual segmentations (Fung *et al.*, 2019). Brain reconstructions were manually inspected and errors were corrected. Volumes of the hippocampal subfields (12 per hemisphere) were calculated using the hippocampal subfields subroutines for Freesurfer 6.0 (Iglesias *et al.*, 2015) (which omits the alveus due the poor reliability of the segmentation). These 12 volumes were used to define the extent of the hippocampus. Estimated total intracranial volume was taken from the segmentation volume estimate (Buckner *et al.*, 2004).

Statistical Analyses

All statistical analyses were performed using R version 3.4.0 (R Core Team, 2016). We used hierarchical linear models (Bates *et al.*, 2015) to investigate the impact of several variables on regional hippocampal volumes (data and code are available at https://gitlab.com/kslays/moblab-hippocampus). Because brain volumes vary with head size, we used normalized volume values (i.e., volume of each participant divided by total intracranial volume). Left and right hemisphere values of corresponding regions were averaged. Participants with outlier volumes (greater than or less than four standard deviations) within any region were

removed from the analysis. We visually confirmed that other regression assumptions were satisfied (normality, equality of variance, homogeneity of regression), and set thresholds for multicollinearity (Pearson correlations among predictors were < 0.5) and influence (Cook's D < 1.0).

For our primary analysis, volume was entered as the dependent variable, and 'group,' 'gender,' 'region,' and 'age' were the independent factors (all fixed effects). In order to account for multiple observations per subject, individual subject effects were specified as random intercepts. Thus, the effect of 'region' was evaluated as a within-subjects measure.

Interactions were included to assess the primary aims of this project. We examined the interaction of group-by-gender-by-region to assess how the impact of alcoholism differed for men and women, and how gender differences impacted certain regions in comparison to others. Also, we examined the interaction of group-by-gender-by-age to assess abnormal aging for men as compared to women. The four way interaction of group-by-gender-by-region-by-age was examined to confirm homogeneity of regression slopes. All non-significant (i.e., $p > 0.05$) interactions (except lower-order interactions included in higher-order interactions) were removed from the model, and a subsequent model was examined; this process of interaction removal was repeated until all of the remaining highest-order interactions were significant.

Following significant interactions, we evaluated mean differences within each region using Bonferroni correction. All 12 hippocampal subfields were examined with an adjusted family-wise *p*-value of 0.004, and results were reported as percent differences (i.e., mean difference/mean). Significant regions were then used as our a priori regions for our secondary aims. For interactions of group-by-gender, the comparisons of ALCm vs. NCm and ALCw vs.

NCw were planned, while for region-by-group, only ALC vs. NC comparisons were planned. For age interactions, the effect of age for each of the subgroups was assessed, and the slope differences were compared in the same manner as mean differences.

To address our secondary aims, we started with the model that resulted from our primary aims. We then investigated interactions of group-by-gender with separate models for two memory measures from the WMS-IV: the immediate memory index (IMI) and the delayed memory index (DMI); these interactions were assessed in conjunction with each score's interaction with each region. We applied the same procedure used in the primary analyses to examine heterogeneity of regression slopes and to remove extraneous interactions. Following significant interactions, we examined the significance of each score's slope in the same six regions as for the primary analyses, and the same slope differences as for the primary analyses. The WMS-IV Index Scores and WAIS-IV Composite Scores were analyzed in separate ANOVA models, each of which contained between-subjects factors of group and gender, and a group-by-gender interaction term.

To investigate how alcohol-drinking history (DD, DHD, and LOS) impacted volume, we conducted an analysis with only the ALC participants included. We examined interactions of each of these three measures with gender-by-region in a single model. As with the primary and secondary aims, we tested for heterogeneity of regression slopes, and removed interactions as dictated by significance levels. Following significant interactions, we examined the significance of each score's slope in the same six regions as for the primary analyses, and we compared the slopes for men to the slopes for women.

Supplemental Tables and Figures

	ALC Women (N=31)		ALC Men (N=36)		NC Women (N=30)		NC Men (N=33)	
Hippocampus Region	Mean	(SD)	Mean	(SD)	Mean	(SD)	Mean	(SD)
Hippocampal tail*	0.000338	(0.000039)	0.000340	(0.000044)	0.000357	(0.000034)	0.000357	(0.000050)
Subiculum	0.000278	(0.000034)	0.000264	(0.000026)	0.000288	(0.000020)	0.000278	(0.000024)
CA1*	0.000402	(0.000046)	0.000392	(0.000036)	0.000424	(0.000035)	0.000414	(0.000035)
Hippocampal fissure	0.000098	(0.000013)	0.000095	(0.000014)	0.000101	(0.000017)	0.000087	(0.000014)
Presubiculum	0.000194	(0.000028)	0.000180	(0.000025)	0.000203	(0.000020)	0.000189	(0.000024)
Parasubiculum	0.000037	(0.000007)	0.000036	(0.000007)	0.000040	(0.000005)	0.000038	(0.000007)
Molecular layer*	0.000365	(0.000040)	0.000350	(0.000034)	0.000383	(0.000028)	0.000372	(0.000032)
DG	0.000194	(0.000021)	0.000188	(0.000018)	0.000201	(0.000015)	0.000200	(0.000018)
CA2+3	0.000140	(0.000015)	0.000137	(0.000017)	0.000146	(0.000013)	0.000147	(0.000015)
CA4	0.000167	(0.000017)	0.000163	(0.000015)	0.000174	(0.000012)	0.000173	(0.000015)
Fimbria	0.000045	(0.000010)	0.000041	(0.000013)	0.000049	(0.000007)	0.000047	(0.000009)
HATA	0.000039	(0.000005)	0.000037	(0.000006)	0.000041	(0.000005)	0.000040	(0.000004)
Whole hippocampus	0.002199	(0.000219)	0.002129	(0.000198)	0.002307	(0.000144)	0.002256	(0.000184)

Table S1. Regional Volumes (proportion of estimated total intracranial volume).

Means and standard deviations (SD) are provided for the hippocampal regional volumes of Alcoholic (ALC) and Nonalcoholic (NC) men and women. Abbreviations: CA1 through 4 = cornu ammonis 1 through 4; DG = dentate gyrus; HATA = hippocampal-amygdaloid transition area. *Indicates regions where ALC < NC, $p < 0.004$.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
group	1.3E-09	1.3E-09	1	78.97	4.11	0.05
region	1.8E-06	1.6E-07	11	5366.83	519.09	<0.01
age	7.4E-09	7.4E-09	1	78.97	23.77	<0.01
gender	1.3E-09	1.3E-09	1	78.97	4.18	0.04
group:region	6.2E-09	5.7E-10	11	5366.83	1.82	0.05
group:age	2.7E-09	2.7E-09	1	78.97	8.81	<0.01
region:age	5.4E-08	4.9E-09	11	5366.83	15.59	<0.01
region:gender	1.2E-08	1.1E-09	11	5366.83	3.41	<0.01
age:gender	6.3E-10	6.3E-10	1	78.97	2.01	0.16
group:gender	2.8E-10	2.8E-10	1	78.97	0.88	0.35
group:region:age	1.0E-08	9.4E-10	11	5366.83	3.02	<0.01
region:age:gender	8.6E-09	7.8E-10	11	5366.83	2.50	<0.01
group:region:gender	2.3E-09	2.0E-10	11	5366.83	0.66	0.78
group:age:gender	3.9E-10	3.9E-10	1	78.97	1.25	0.27

Table S2. Analysis of variance for model including group, gender, and age.

The analysis of variance obtained from the mixed model significant indicated significant group-by-region-by-age and gender-by-region-by-age interactions for volumes. Abbreviations: Sum Sq = sums of squares; Mean Sq = mean square; NumDF = numerator degrees of freedom; DenDF = denominator degrees of freedom; Pr(>F) = probability > F (i.e., *p* value).

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
group	3.5E-10	3.5E-10	1	75.01	1.12	0.29
region	4.9E-07	4.5E-08	11	5274.22	144.84	<0.01
IMI	3.3E-11	3.3E-11	1	75.01	0.11	0.75
gender	1.3E-09	1.3E-09	1	75.01	4.05	0.05
age	6.7E-09	6.7E-09	1	75.01	21.82	<0.01
group:region	6.1E-09	5.5E-10	11	5274.22	1.80	0.05
group:IMI	8.0E-11	8.0E-11	1	75.01	0.26	0.61
region:IMI	5.9E-10	5.4E-11	11	5274.22	0.17	1.00
region:gender	9.3E-09	8.4E-10	11	5274.22	2.73	<0.01
gender:age	5.7E-10	5.7E-10	1	75.01	1.85	0.18
region:age	4.3E-08	3.9E-09	11	5274.22	12.68	<0.01
group:gender	1.0E-11	1.0E-11	1	75.01	0.03	0.85
group:age	1.2E-09	1.2E-09	1	75.01	3.74	0.06
group:region:IMI	7.0E-09	6.3E-10	11	5274.22	2.06	0.02
region:gender:age	6.8E-09	6.2E-10	11	5274.22	2.01	0.02
group:gender:age	1.7E-11	1.7E-11	1	75.01	0.06	0.81
group:region:gender	1.2E-09	1.1E-10	11	5274.22	0.37	0.97

Table S3. Analysis of variance for model including Immediate Memory Index.

The analysis of variance obtained from the mixed model indicated a significant group-by-region-by-IMI interaction for volumes. Abbreviations: Sum Sq = sums of squares; Mean Sq = mean square; NumDF = numerator degrees of freedom; DenDF = denominator degrees of freedom; Pr(>F) = probability > F (i.e., *p* value).

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
group	5.5E-10	5.5E-10	1	73.62	1.80	0.18
region	4.6E-07	4.2E-08	11	5276.69	136.07	<0.01
DMI	7.5E-14	7.5E-14	1	73.62	0.00	0.99
gender	1.4E-09	1.4E-09	1	73.62	4.64	0.03
age	6.8E-09	6.8E-09	1	73.62	22.22	<0.01
group:region	9.0E-09	8.2E-10	11	5276.69	2.67	<0.01
group:DMI	2.2E-10	2.2E-10	1	73.62	0.71	0.40
region:DMI	7.4E-10	6.8E-11	11	5276.69	0.22	1.00
region:gender	1.1E-08	9.9E-10	11	5276.69	3.23	<0.01
gender:age	6.9E-10	6.9E-10	1	73.62	2.27	0.14
region:age	4.4E-08	4.0E-09	11	5276.69	13.15	<0.01
group:gender	1.1E-11	1.1E-11	1	73.62	0.04	0.85
group:age	1.1E-09	1.1E-09	1	73.62	3.63	0.06
group:region:DMI	1.0E-08	9.4E-10	11	5276.69	3.07	<0.01
region:gender:age	8.4E-09	7.6E-10	11	5276.69	2.49	<0.01
group:gender:age	1.9E-11	1.9E-11	1	73.62	0.06	0.80
group:region:gender	1.3E-09	1.2E-10	11	5276.69	0.38	0.97

Table S4. Analysis of variance for model including Delayed Memory Index.

The analysis of variance obtained from the mixed model indicated a significant group-by-region-by-DMI interaction for volumes. Abbreviations: DMI = Delayed Memory Index; Sum Sq = sums of squares; Mean Sq = mean square; NumDF = numerator degrees of freedom; DenDF = denominator degrees of freedom; Pr(>F) = probability > F (i.e., *p* value).

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
gender	8.2E-13	8.2E-13	1	36.00	0.00	0.96
region	4.4E-07	4.0E-08	11	2474.63	129.65	<0.01
DHD	4.1E-11	4.1E-11	1	36.00	0.13	0.72
DD	3.2E-10	3.2E-10	1	36.00	1.04	0.31
LOS	2.4E-10	2.4E-10	1	36.00	0.77	0.38
age	2.0E-09	2.0E-09	1	36.00	6.35	0.02
gender:region	3.6E-09	3.3E-10	11	2474.63	1.06	0.39
gender:DHD	1.3E-12	1.3E-12	1	36.00	0.00	0.95
region:DHD	2.2E-09	2.0E-10	11	2474.63	0.65	0.79
gender:DD	1.9E-11	1.9E-11	1	36.00	0.06	0.81
region:DD	3.7E-09	3.4E-10	11	2474.63	1.09	0.37
gender:LOS	7.0E-10	7.0E-10	1	36.00	2.28	0.14
region:LOS	7.3E-09	6.7E-10	11	2474.63	2.15	0.01
gender:age	1.5E-10	1.5E-10	1	36.00	0.49	0.49
region:age	1.4E-08	1.2E-09	11	2474.63	3.97	<0.01
gender:region:DHD	1.8E-09	1.6E-10	11	2474.63	0.52	0.89
gender:region:DD	3.8E-09	3.5E-10	11	2474.63	1.12	0.34
gender:region:LOS	6.6E-09	6.0E-10	11	2474.63	1.94	0.03
gender:region:age	4.5E-09	4.1E-10	11	2474.63	1.32	0.21

Table S5. Analysis of Variance for model including drinking history (DHD, DD, and LOS) of the alcoholic (ALC) group.

The analysis of variance obtained from the mixed model indicated a significant gender-by-region-by-LOS interaction for volumes, for the ALC group. Abbreviations: DHD = duration of heavy drinking; DD = daily drinks; LOS = length of sobriety; Sum Sq = sums of squares; Mean Sq = mean square; NumDF = numerator degrees of freedom; DenDF = denominator degrees of freedom; Pr(>F) = probability > F (i.e., *p* value).

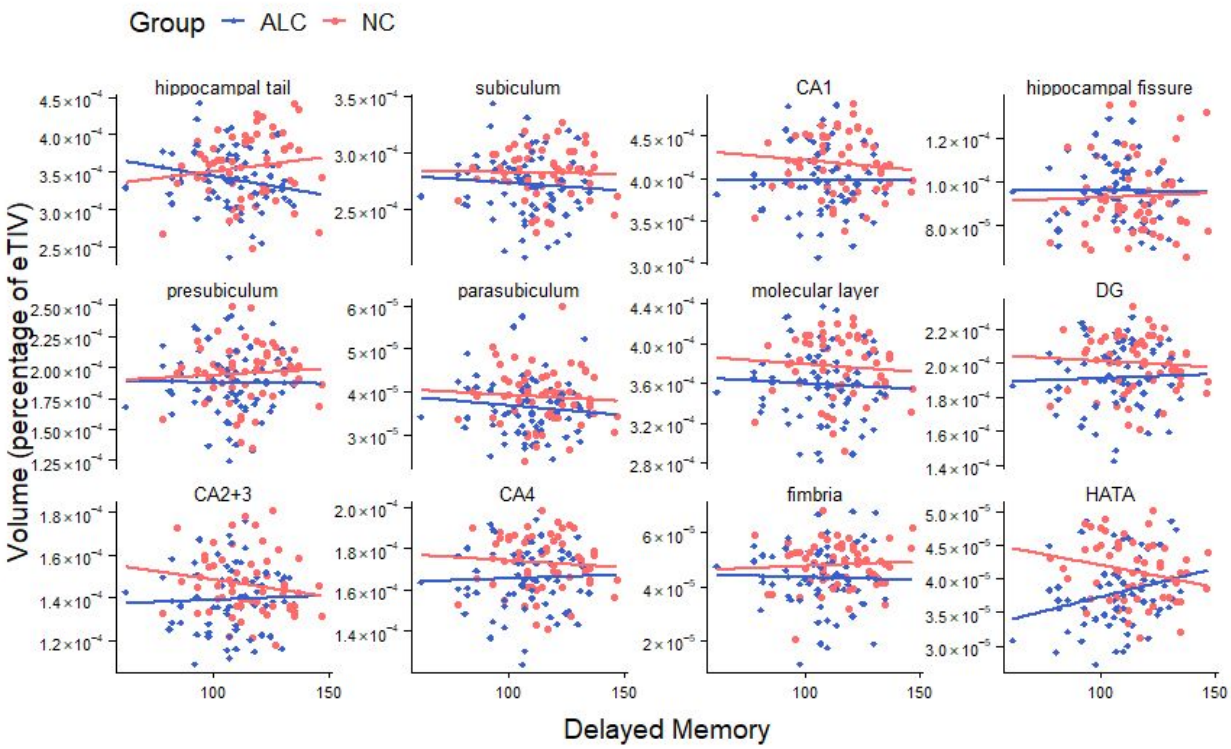

Figure S1. Relationships of volume with delayed memory for alcoholic (ALC) and nonalcoholic control (NC) groups.

As described in Figure 3, for the ALC group, Delayed Memory was associated with smaller hippocampal tail volumes (percentage of eTIV), while for the NC group, a positive relationship was observed. This figure shows the relationships for all 12 regions. Abbreviations: CA1 = cornu ammonis 1; CA2+3 = cornu ammonis 2 and 3; CA4 = cornu ammonis 4; DG = dentate gyrus; HATA = hippocampal-amygdaloid transition area; Sub = subiculum.

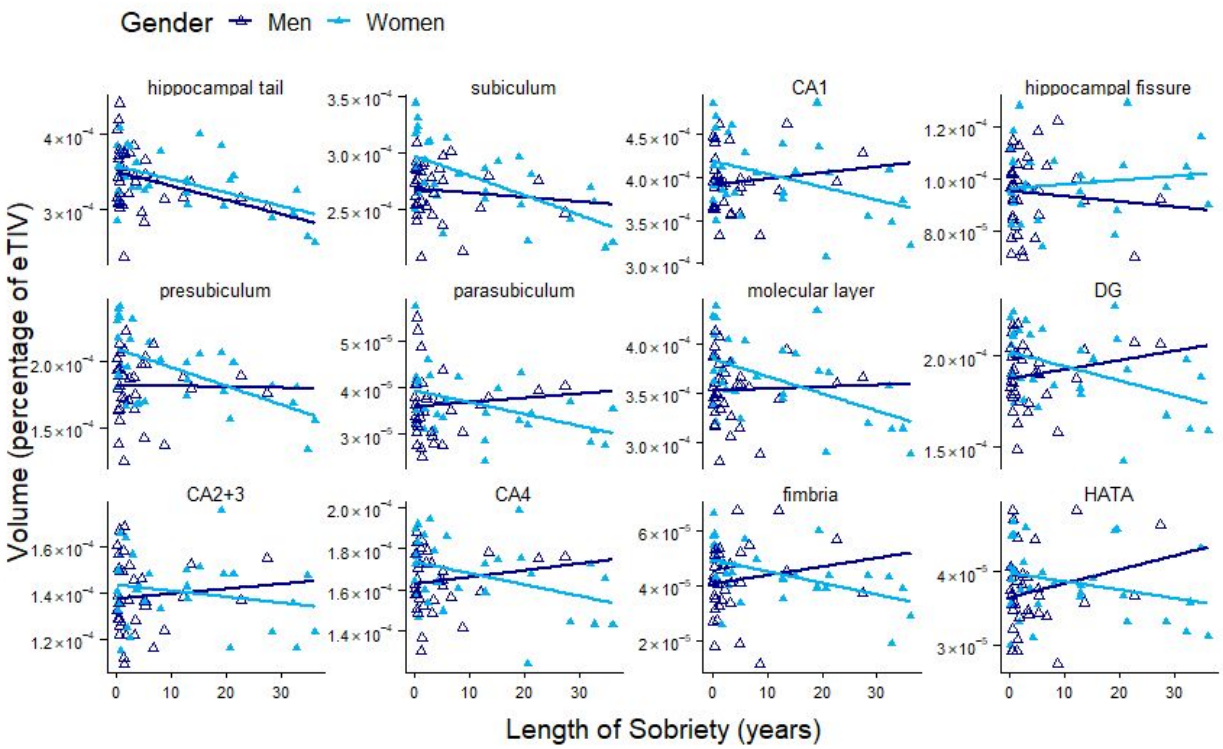

Figure S2. Relationships of volume with length of sobriety for alcoholic (ALC) men and women.

As described in Figure 3, for ALC men, CA1 volumes (percentage of eTIV) were positively associated with Length of Sobriety, while for ALC women, a negative relationship was observed. This figure shows the relationships for all 12 regions. Abbreviations: CA1 = cornu ammonis 1; CA2+3 = cornu ammonis 2 and 3; CA4 = cornu ammonis 4; DG = dentate gyrus; HATA = hippocampal-amygdaloid transition area; Sub = subiculum.

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