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Sensitivity of Hypocretin System to Chronic Alcohol Exposure: A Human and Animal Study

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Abstract—Human heroin addicts and mice administered morphine for a 2 week period show a greatly increased number of hypothalamic hypocretin (Hcrt or orexin) producing neurons with a concomitant reduction in Hcrt cell size. Male rats addicted to cocaine similarly show an increased number of detectable Hcrt neurons. These findings led us to hypothesize that humans with alcohol use disorder (AUD) would show similar changes. We now report that humans with AUD have a decreased number and size of detectable Hcrt neurons. In addition, the intermingled melanin concentrating hormone (MCH) neurons are reduced in size. We saw no change in the size and number of tuberomammillary histamine neurons in AUD. Within the Hcrt/MCH neuronal field we found that microglia cell size was increased in AUD brains. In contrast, male rats with 2 week alcohol exposure, sufficient to elicit withdrawal symptoms, show no change in the number or size of Hcrt, MCH and histamine neurons, and no change in the size of microglia. The present study indicates major differences between the response of Hcrt neurons to opioids and that to alcohol in human subjects with a history of substance abuse.Published by Elsevier Ltd on behalf of IBRO.

Keywords: hypocretin, melanin concentrating hormone, histamine human, alcohol, microglia.

INTRODUCTION

There has been a global increase in the consumption of alcohol, one of the most commonly abused drugs (Abrahao et al., 2017, Manthey et al., 2019). Alcohol misuse, a leading risk factor for deaths and disability, (Collaborators, 2018, Barberia-Latasa et al., 2022) can lead to physical dependence with associated organ damage, including brain damage (Li, 2008). Multidisciplinary studies in brains of humans with chronic alcohol use have reported significant structural changes, including brain atrophy and neuronal loss in cortical as well as subcortical regions (Harper and Blumbergs, 1982, Harper and Kril, 1985, Jernigan et al., 1991, Pfefferbaum et al., 1992, Kril et al., 1997, Rosenbloom et al., 2003, Cardenas et al., 2005, Sullivan and Pfefferbaum, 2005, Chanraud et al., 2011). Chronic alcohol consumption selectively damages certain brain regions and neuronal populations while sparing others, indicating specific vulnerabilities (Kril and Harper, 1989, Halliday et al., 1992, Baker et al., 1994, Cullen et al., 1997, Harding et al., 1997, Kril et al., 1997, Harding et al., 2000). Microglial cells, key components of the innate immune response in the brain (Eggen et al., 2013), are also greatly affected by alcohol exposure and are thought to play a role in alcohol use disorder (AUD) and alcohol neurotoxicity (Mayfield et al., 2013, Henriques et al., 2018, Erickson et al., 2019).

Damage to the hypothalamus, a structure classically involved with ingestive behavior and reward processing (Anand and Brobeck, 1951, Hoebel and Teitelbaum, 1962, Hetherington and Ranson, 1983), has been also associated with alcohol consumption (Marfaing-Jallat et al., 1970, Wayner et al., 1971). Animal studies have shown sensitivity to chronic alcohol exposure in several hypothalamic neuronal populations, including the hypocretin (Hcrt) cells, suggesting a causal relation with the development and maintenance of alcohol use disorder (AUD) (Harding et al., 1996, Leibowitz et al., 2003, Lawrence et al., 2006, Chang et al., 2007, Chang et al., 2010, Morganstern et al., 2010, Barson et al., 2015, Zimatkin and Phedina, 2015). In addition, dysregulation of the Hcrt system after exposure to other drugs of abuse like opioids or cocaine has been recently reported (Thannickal et al., 2018, James et al., 2019, Matzeu

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and Martin-Fardon, 2021). In the present study we aimed to determine the number and size of hypothalamic Hcrt, melanin concentrating hormone (MCH) and histamine neurons as well as microglia in the brains of humans with AUD and examine if chronic intermittent ethanol (CIE) vapor exposure in an animal model would produce similar changes.

EXPERIMENTAL PROCEDURES

All procedures were approved by the Institutional Animal Care and Use Committee of the University of California at Los Angeles, the Veterans Administration Greater Los Angeles Health Care System and The Scripps Research Institute, La Jolla, California.

Human studies

Hypothalamic tissue. AUD was determined by MC, a neuropathologist, on the basis of the patients' medical records. HIPAA restrictions prevented our access to some details. Subject characteristics are presented in Table 1. Brains were fixed in 10% buffered formalin in PBS. The hypothalamus was cut into 40 μm sections. Immunostaining was then performed to detect neuropeptides Hcrt and MCH. Histamine neurons were identified by the presence of histidine decarboxylase (HDC) and the microglia cell population was marked with ionized calcium binding adaptor molecule-1 (Iba-1) (Ito et al., 1998).

Hcrt, MCH, HDC and Iba-1 immunostaining for human with AUD and control brains. Hcrt, MCH, HDC and Iba-1 immunostaining was performed as in our earlier reports (Thannickal et al., 2000, Thannickal et al., 2007, John et al., 2013). We used antigen retrieval to maximize Hcrt, MCH, HDC and Iba-1 detection. Sections were treated with 0.5% sodium borohydride in PBS for 30 min and washed with PBS, incubated for 30 min in 0.5% H_2O_2 for blocking of endogenous peroxidase activity, followed by 10 mM sodium citrate (pH 8.5) solution for 30 min at 80 °C in a water bath. The sections were cooled to room temperature in sodium citrate. After thorough washing with PBS, the sections were placed for 2 h in either 1.5% normal goat serum (NGS for Hcrt, MCH and HDC) or 1% rabbit serum (NRS for Iba-1) in PBS containing 0.25% Triton X (PBST) and incubated for 72 h at 4 °C with either rabbit anti-Hcrt-1 (H-003-30, Phoenix Pharmaceuticals, USA, Lot# 01651-11, 1:10000), rabbit anti-MCH (Cat No. H-070-47, Phoenix Pharmaceuticals, USA, Lot# 01629-5, 1:20000), rabbit anti-HDC (Cat No. EUD2601, OriGene Technologies, Lot#17106, 1:5000) or goat anti-Iba-1 antibody (ab5076, Abcam, USA, Lot # GR3322398-1, 1:5000) PBST, 1% NGS or 1% NRS. Sections were then incubated for 120 min in a secondary antibody (biotinylated goat anti-rabbit IgG or rabbit anti-goat IgG, 1:400, Vector Laboratories, Burlingame, CA) in PBST, avidin–biotin complex (120 min, ABC Elite Kit, 1:300, Vector Laboratories, in PBS) and developed with the diaminobenzidine tetrahydrochloride (DAB) method, which consisted of tissue immersion in 0.02% DAB and 0.03% hydrogen peroxide for 8 min.

A common practice in histology laboratories that use the DAB method to develop immunohistochemical procedures, is to periodically examine the tissue sections "under the microscope" during the developing process and remove the sections from the DAB solution once neurons are visualized. However, this approach can add variability to the end result. Therefore, we previously standardized our DAB method and established an 8-minute optimal developing time and used this precise duration in all of our studies. Furthermore, all developing solutions were prepared in one container, homogenized and aliquoted in the respective developing wells. All developing procedures were performed with room lights off and the wells containing tissue were wrapped with aluminum foil to further protect them from any light exposure. Wells were agitated at 55 rpm and all developing solutions were used only once. All procedures were done on coded tissue so that the individual performing the staining was unaware of the condition.

Table 1. Summary of human control and AUD patients

Control	Age	Sex	Cause of death
C-1	57	F	Septic shock
C-2	50	F	Pulmonary embolism
C-3	57	M	Renal infection
C-4	27	F	Congenital heart defect
C-5	41	M	Myocardial infraction, aortic aneurism
C-6	47	M	Congestive heart failure
AUD			
AUD-1	42	M	AUD, liver cirrhosis
AUD-2	52	M	AUD, liver cirrhosis
AUD-3	33	M	AUD, hepatitis, pancreatitis
AUD-4	50	F	AUD, hepatitis

Animal studies

Subjects. Sixteen male Wistar rats (Charles River, Wilmington, MA, USA) weighing 150–175 g at the beginning of the study, were housed two per cage in a temperature- and humidity controlled vivarium on a reverse 12 h/12 h light/dark cycle with *ad libitum* access to food and water. The animals were given at least 1 week to acclimate to the housing conditions and handling before experimental manipulations were conducted.

Chronic intermittent alcohol (ethanol [EtOH]) vapor exposure in rats. This is a reliable method for inducing alcohol dependence in rodents and allows the experimenter to control the dose, duration and pattern of exposure. It was shown that this model causes rats to exhibit somatic and motivational signs of withdrawal (O'Dell et al., 2004, Gilpin et al., 2008, Vendruscolo and Roberts, 2014, Matzeu et al., 2018, Matzeu and Martin-Fardon, 2020, Varodayan et al., 2022). Half of the rats (n = 8) were made dependent by chronic intermittent EtOH (CIE) vapor exposure. The other half (n = 8), the nondependent group, was exposed only to alcohol-free

air. Cage size and airflow was identical in both groups. During 3-week dependence induction, the rats underwent daily cycles of 14 h exposure (ON) and 10 h OFF. Blood Alcohol Levels, (BLAs), during vapor exposure ranged between 150 and 250 mg%, measured with a blood analyzer (GC-headspace, Agilent Technologies, Santa Clara, CA, USA). Control animals were left undisturbed except to measure their BLAs during the last 15 min of vapor exposure.

Processing tissue from rats. On the 21st day of CIE vapor exposure, at 8 hour abstinence (i.e., 8 h after the EtOH was turned off) all animals were deeply anesthetized with CO_2 and transcardially perfused with 0.3 l of heparinized (1000 units/l) phosphate buffered saline (PBS, 0.1 M, pH 7.4) followed by 0.7 l of 4% paraformaldehyde in phosphate buffer (PBS, 0.1 M, pH 7.4). The brains were removed and coded. All subsequent procedures were performed by an investigator blind to the procedures performed prior to sacrifice. Brains were immersed in PBS with 20% sucrose and then transferred to 30% sucrose solution for cryoprotection. Seventy-two hours later, brains were frozen and cut into 40 μm coronal sections using a sliding microtome (American Optical, US). Each section was placed in one well of a 5-well tray containing PBS, and immunohistochemical procedures were performed immediately. The remaining tissue was transferred to a cryoprotectant solution and stored at −20 °C.

Immunohistochemistry. All immunohistochemical procedures were performed by sequential incubation of free-floating sections, as in our previous reports (McGregor et al., 2005, McGregor et al., 2011, McGregor et al., 2017). The following antibodies were used to identify neuronal groups; Hcrt with rabbit anti-Hcrt-1 (H-003-30, Phoenix Pharmaceuticals, USA, Lot# 01651-11, 1:10000), MCH with rabbit anti-MCH (Cat No. H-070-47, Phoenix Pharmaceuticals, USA, Lot# 01629-5, 1:10000) and HDC with rabbit anti-HDC (EUD 2601, Acris Antibodies, Germany, Lot# HS1859, 1:3000). Detection of Iba-1 was performed with rabbit anti-Iba-1 (Cat No. 019-19741, WAKO Chemicals, USA, Lot# LKH4160, 1:10000). Prior to Iba-1 immunostaining, an antigen retrieval procedure was performed, as in the human studies, by incubating the sections in 10 mM sodium citrate (pH 8.5) at 80 °C for 30 min. The sections were then cooled to room temperature in sodium citrate and washed with PBS.

All hypothalamic sections were first incubated for 30 min in 0.5% H_2O_2, followed by primary antibody for 72 h at 4 °C in PBST, corresponding biotinylated secondary antibody in PBST (1:400, Jackson ImmunoResearch, West Grove, PA, USA), standard ABC (1:300, Vector Laboratories, Burlingame, CA, USA in PBS) and developed with the diaminobenzidine tetrahydrochloride (DAB) method, which consisted of tissue immersion in 0.02% DAB and 0.03% hydrogen peroxide for 8 minutes.

Data collection

General procedures in rat and human tissue. The number, distribution and size of $Hcrt^+$, MCH^+, HDC^+ and Ibal-1^+ cells were assessed using a Nikon Eclipse 80i microscope with three axis motorized stage, video camera, Neurolucida interface and Stereoinvestigator software (MicroBrightField Corp.). Cell counting was performed using either 40x or 60x objective and cell size was determined using the Neurolucida Nucleator probe. All counting and cell measurements were performed by a trained histologist, always blind to the experimental condition. In every case, the same individual counted both the experimental and control tissue. Only neurons with an identifiable nucleus were counted. Microglia were recognized by their projections from the cell body.

Human tissue. $Hcrt^+$, MCH^+ and HDC^+ cell number, distribution and size were determined using matching sections as in our prior studies (Thannickal et al., 2000, John et al., 2013, Thannickal et al., 2018). Iba-1^+ cell distribution and size was performed in 4 different sites within the Hcrt/MCH neuronal field by placing 1 mm^2 squares (500 μm × 500 μm) at each sampling site.

Rat tissue. Quantification of $Hcrt^+$, MCH^+ and HDC^+ cells was done bilaterally on every fifth section of the hypothalamic tuberomammillary region. Ibal-1^+ quantification was performed unilaterally in the middle of the Hcrt/MCH neuronal field by placing a square (1000 μm × 1000 μm) dorsal to the fornix, with the lower corners of the square equidistant from the center of the fornix. This is near the center of the Hcrt/MCH cell distributions.

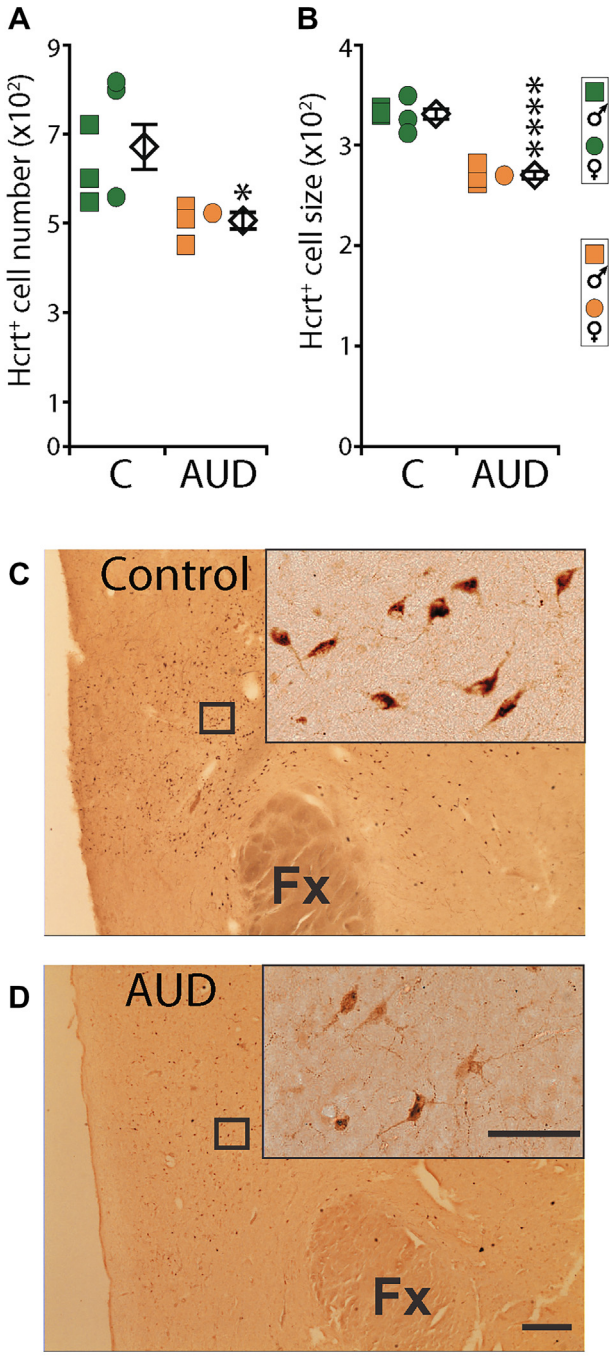
Statistical analysis

All statistical tests were done using SYSTAT version 13. $Hcrt^+$, MCH^+, HDC^+ and microglia cell number and size in humans and animals were analyzed using unpaired *t*-tests. Statistical significance was set at $P < 0.05$.

RESULTS

Human studies

Humans with alcohol use disorder have a decrease in the number and in the size of their Hcrt producing neurons. MCH neurons were smaller than controls. HDC neurons did not exhibit changes. There was an average 23.9% decrease in the number of detectable $Hcrt^+$ neurons in human AUD relative to matched human controls (Fig. 1a; $p = 0.038$, $t = 2.474$, df = 8). In addition, $Hcrt^+$ cells were 18.5% smaller in the AUD brains (Fig. 1b; $p = 0.00022$, $t = 8.782$, df = 8).). The number of intermingled MCH^+ neurons was unchanged in AUD brains compared to controls (Fig. 2a), but showed a significant reduction in the cell size (10.58%) (Fig. 2b; $p = 0.011$, $t = 3.301$, df = 8). In contrast, the



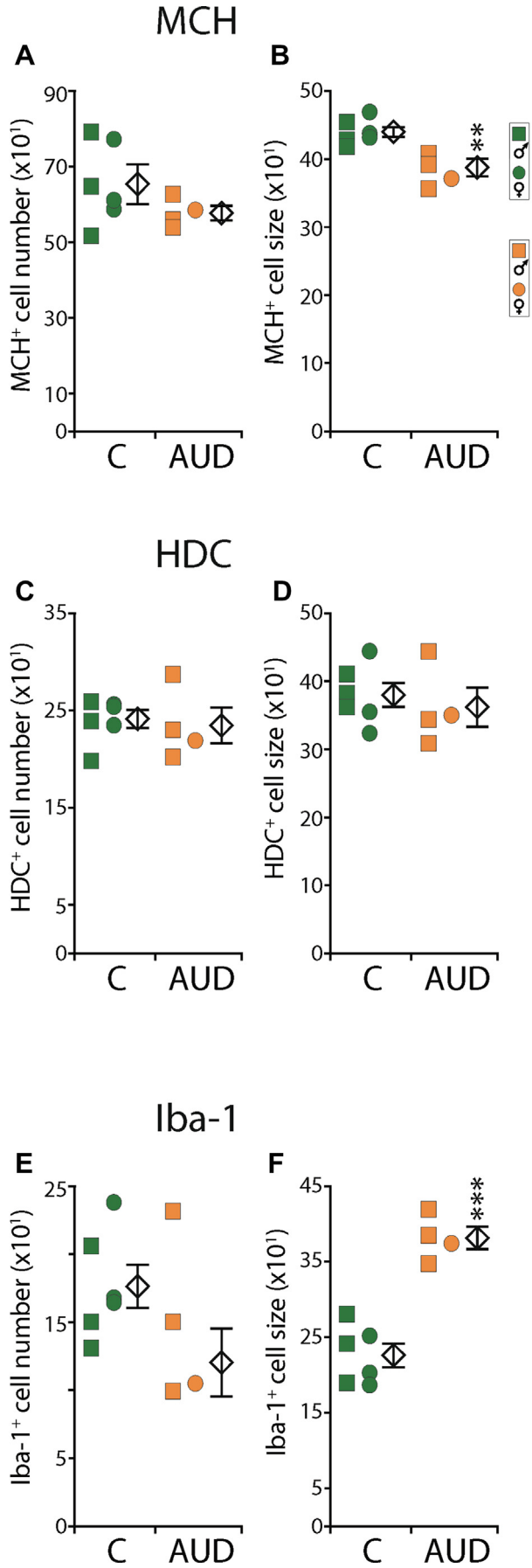


Fig. 1. Humans with AUD have a decreased number and size of hypocretin (orexin) neurons. (a) There was a 23.9% decrease in the number of detectable hypocretin neurons in human AUD (n = 4) relative to human controls (n = 6). This difference was significant (p = 0.038, t = 2.47 df = 8). **(b)** The hypocretin cells were 18.5% smaller in the AUD brains (p = 0.00002, t = 8.78, df = 8). Graphs show individual values for each patient indicating the biological sex as follows; male patients are represented with squares, female patients are represented with circles. Average group data is indicated with rhombus. All error bars in the figures are standard error of the mean (SEM). **(c**, **d)** Representative examples of immunohistochemically labeled hypocretin cells in control and AUD brains. Inserts are at higher (×20) magnification photomicrographs of the selected area (black square) for each condition. Cal bar. 500 μm and 100 μm respectively. Fx, fornix. ****p = 0.00002, *p = 0.038.

number of HDC$^+$ neurons (Fig. 2c) and their size (Fig. 2d) remained comparable in AUD and control brains.

Humans with alcohol use disorder have comparable microglia cell numbers to controls, but their cell size is greatly increased. Microglia cell number was comparable in both conditions (Fig. 2e), whereas the cell size was significantly increased in AUD brains (69%) (Fig. 2f; $p = 0.0001$, $t = 6.841$, df = 8).

Animal studies

Hypocretin, MCH, HDC and microglia. The number and distribution of Hcrt$^+$, MCH$^+$, HDC$^+$ neurons and Iba-1$^+$ cells was assessed in animals subjected to 3 weeks of CIE vapor or air exposure. All neuronal groups were distributed within the hypothalamus as previously described in the rat. Iba-1$^+$ cells were observed homogeneously distributed in this same region.

Animals that were exposed to alcohol vapor did not show a significant change in the number (Fig. 3a) or size (Fig. 3b) of Hcrt$^+$ neurons. Visually, Hcrt neurons were indistinguishable in animals exposed to alcohol (Fig. 3c) and control (Fig. 3d). MCH neuronal numbers (Fig. 4a) and size (Fig. 4b) also were unchanged after alcohol treatment. In addition, there was no difference in the number and size of HDC$^+$ neurons (Fig. 4c, d) and of Iba-1$^+$ cells (Fig. 4e, f) after CIE alcohol treatment. Comparison between human and animal analysis for all systems are summarized in Tables 2 (cell number) and 3 (cell size).

DISCUSSION

Hcrt, MCH and HDC

Humans with AUD experience profound shifts in behavior and cognition with deleterious effects on their lives (Bernard et al., 2021, Creupelandt et al., 2021, Mistarz et al., 2021, Pervin and Stephen, 2021, Duan et al., 2022, Kumar et al., 2022). These symptoms are thought to be a product of alcohol neurotoxicity and metabolic insults caused by thiamine deficiency (Ridley et al.,

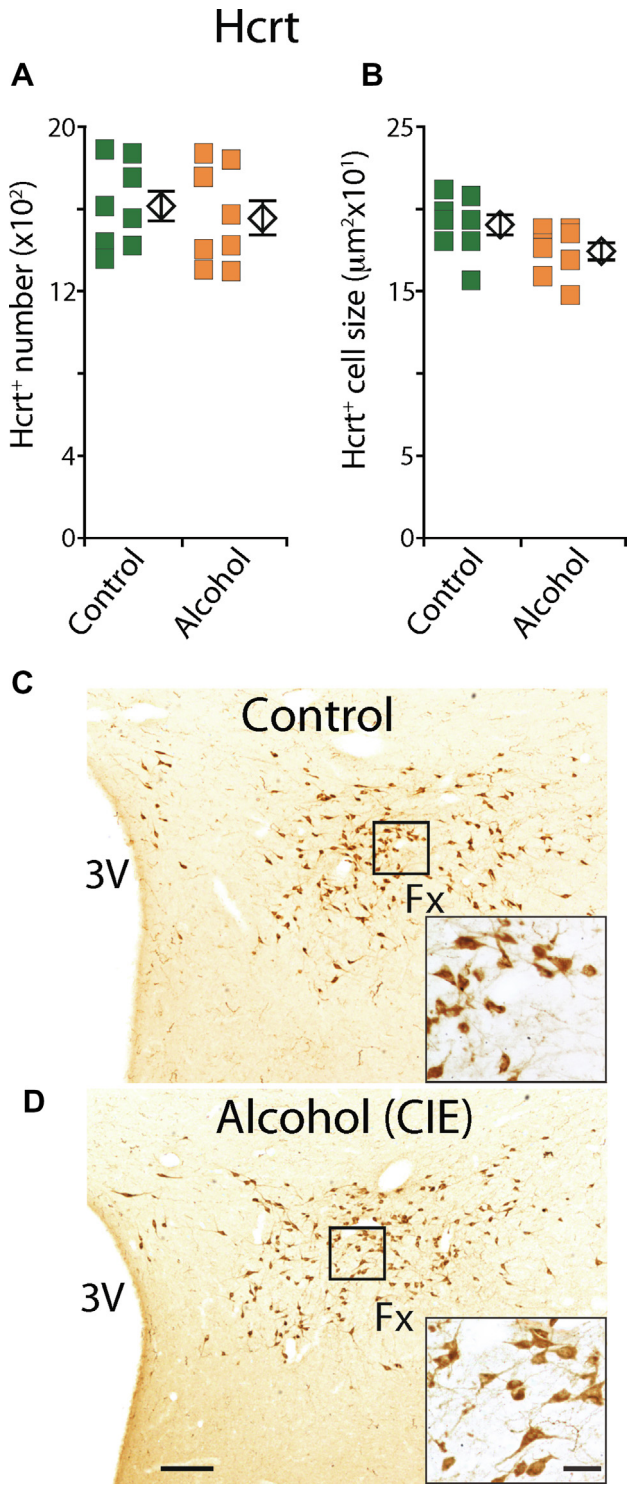


Fig. 3. In rats, Hcrt neuronal number and size remains unchanged after alcohol exposure. (a, b) There was no significant change in the average number (**a**) or size (**b**) of Hcrt expressing neurons in animals exposed to alcohol ($n = 8$) relative to controls ($n = 8$). All data is displayed in two columns because of the extensive overlap between individual animals. **(c, d)** Representative examples of immunohistochemically labeled hypocretin cells in control and alcohol exposed brains. Inserts are at higher (×20) magnification photomicrographs of the selected area (black square) for each condition. Cal bar. 200 µm and 40 µm respectively. Fx, fornix; 3 V, third ventricle.

Fig. 2. Humans with AUD have smaller MCH neurons and increased microglia size. Histamine neurons remain unchanged. (a) There was no significant change in the number of MCH expressing neurons in human AUD ($n = 4$) relative to human controls ($n = 6$). **(b)** The MCH neurons were 10.58% smaller compared to controls. This difference was significant ($p = 0.011$, $t = 3.3$, df = 8). **(c, d)** There was no significant difference in the number or size of HDC expressing neurons in AUD ($n = 4$) compared to control ($n = 6$) humans. **(e)** AUD brains ($n = 4$) showed no significant difference in the number of Iba-1 expressing cells in the hypothalamic regions analyzed compared to controls ($n = 6$). **(f)** Shows a significant increase in the size of Iba-1 expressing cells (69%) in these same regions ($p = 0.0001$, $t = -6.84$, df = 8). Graphs show individual values for each patient indicating the biological sex as follows; male patients are represented with squares, female patients are represented with circles. ***$p = 0.0001$, **$p = 0.011$.

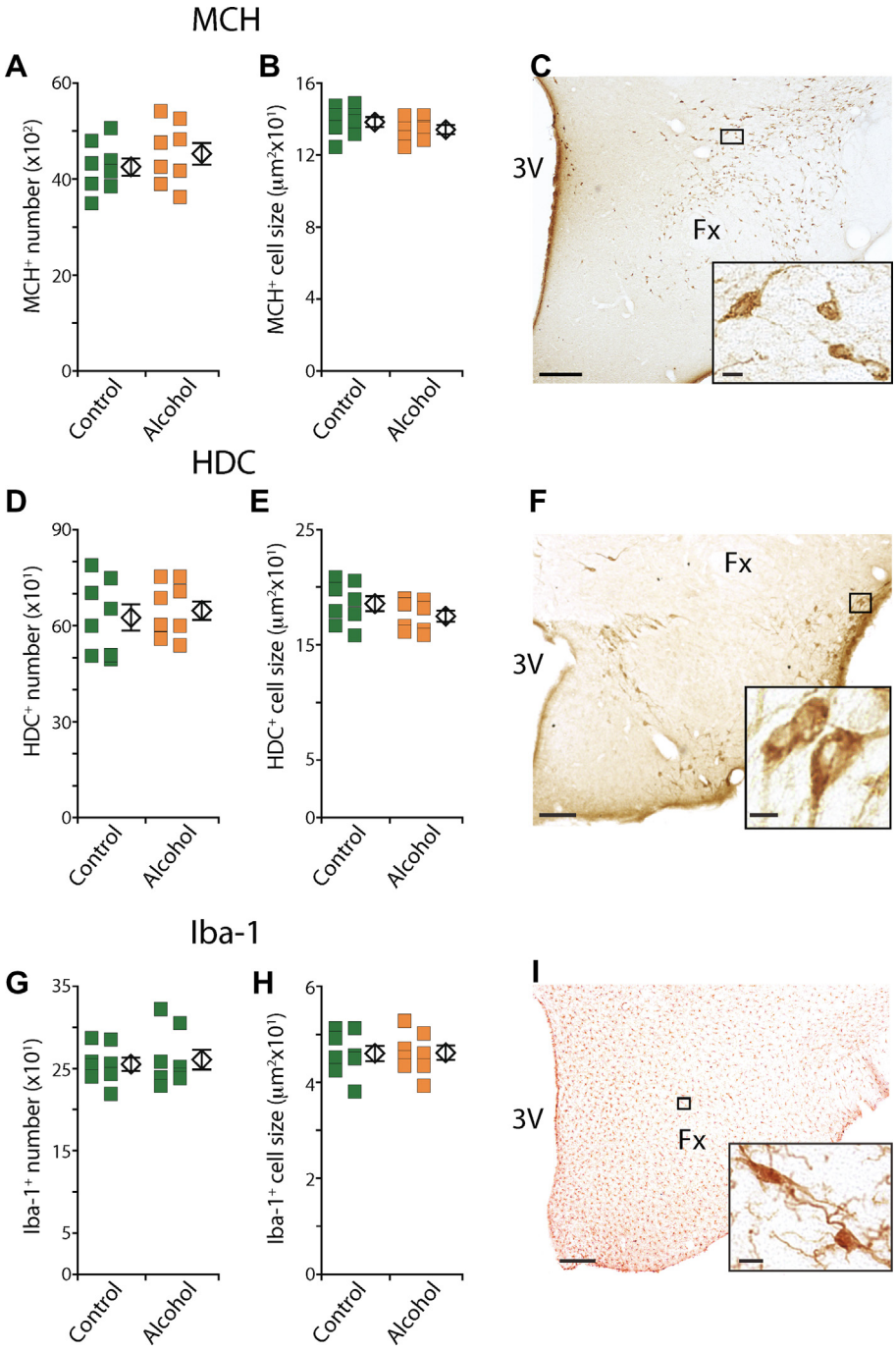


Fig. 4. In rats, MCH, HDC and microglia cell number and size remain unchanged after alcohol exposure. (a, b) No difference was observed in the average number (**a**) or size (**b**) of MCH expressing neurons in animals exposed to alcohol (n = 8) relative to controls (n = 8). (**c**) Representative examples of immunohistochemically labeled MCH cells in a control brain. Inserts are at higher (×40) magnification photomicrographs of the selected area (black rectangle). Cal bar. 250 μm and 10 μm respectively. **(d, e)** The average number (**d**) and size (**e**) of HDC$^+$ cells in hypothalamic tuberomammillary region was comparable between control animals (n = 8) and animals exposed to alcohol (n = 8). **(f)** Representative examples of immunohistochemically labeled HDC cells in a control brain. Inserts are at higher (×40) magnification photomicrographs of the selected area (black square). Cal bar. 125 μm and 10 μm respectively. **(g, h)** There was no change in the number (**e**) or size (**f**) of Iba-1$^+$ cells after alcohol exposure (n = 8 per condition). All data is displayed data in two columns because of the extensive overlap between individual animals. **(i)** Representative examples of immunohistochemically labeled Iba-1 cells in a control brain. Inserts are at higher (×40) magnification photomicrographs of the selected area (black square). Cal bar. 250 μm and 10 μm respectively. Fx, fornix; 3 V, third ventricle.

2013, Wiegmann et al., 2020) resulting from poor nutrition and inadequate vitamin absorption (Sechi and Serra, 2007).

In our present study we found a significant reduction in the number and size of Hcrt expressing neurons in human AUD patients and a significant decrease in the size of intermingled MCH neuronal population. We also observed that the number and size of histamine neurons remained unchanged in AUD brains, suggesting that chronic alcohol selectively affects different hypothalamic populations. On the other hand, in our experimental animal model all these neuronal groups remained unaffected by exposure to alcohol.

Our result of a reduced number of Hcrt neurons in AUD patients is in contrast to a recent brief report (Kudriavova et al., 2019), which indicated that humans with AUD have no change in the number of Hcrt expressing neurons. Size was not reported. There are several possible explanations for this discrepancy. For example, Kudriavova and Gavrilov did not describe any details of the patients used for the study including their age. This is a critical factor in determining and comparing Hcrt neuronal populations across individuals, since Hcrt cell numbers decrease with age in both humans and rodents (Kessler et al., 2011, Hunt et al., 2015). For the current study we chose age matched AUD patients and controls (Thannickal et al., 2000, Volkow and Wise, 2005). Other parameters that can affect Hcrt neuronal number and that were not detailed by the prior authors are the cause of death, underlying neurological disorders, pathological conditions, and diagnostic criteria.

The reduction of the number of Hcrt neurons that we observe in AUD patients should not be assumed to be the result of neuronal death, since we have shown that in 24% of neurons capable of producing Hcrt the level of Hcrt peptides will fall below the detection levels under baseline conditions (McGregor et al., 2017). Thus, it is possible that the decrease in Hcrt$^+$ cell numbers could be related to a change in the amount

Table 2. Summary of human and animal changes in neuronal numbers compared to control conditions for all signaling systems analyzed.

System	Human AUD	Rats CIE
Hcrt	Decrease	No Change
MCH	No Change	No Change
HDC	No Change	No Change
Ibal-1	No Change	No Change

Table 3. Summary of human and animal changes in neuronal size compared to control conditions for all signaling systems analyzed.

System	Human AUD	Rats CIE
Hcrt	Decrease	No Change
MCH	Decrease	No Change
HDC	No Change	No Change
Ibal-1	Increase	No Change

of Hcrt synthesis rather than neuronal death. In this regard, animal studies have reported reduction of Hcrt mRNA levels with chronic alcohol consumption (Morganstern et al., 2010).

Our animal model of alcohol exposure did not mirror the results obtained in the human studies, suggesting that nutritional or other changes linked to human AUD may be responsible for the changes we observe. In addition, it is possible that the EtOH exposure protocol used in the present work might not result in significant changes in the Hcrt and MCH systems compared to other protocols. We selected our protocol because this level of exposure results in the expression of somatic and motivational signs of alcohol withdrawal. Similarly the dose and duration of opioid exposure used in prior work from this laboratory was selected based on the expression of opioid withdrawal symptoms (McGregor et al., 2022).

The decrease in Hcrt cell number in humans with AUD reported here markedly contrasts with our recent finding of greatly increased Hcrt neuronal number in human heroin addicts and morphine treated mice (Thannickal et al., 2018) and the similar findings of increased numbers of Hcrt neurons in rats after cocaine exposure (James et al., 2019). However, we observed that Hcrt neuronal cell body size in patients with AUD is reduced to a similar extent to that which we reported in human heroin addicts and mice exposed to opioids (22%) (Thannickal et al., 2018). We also observed a reduction in the cell body size of intermingled MCH neuronal population in human AUD brains compared to control. Cellular shrinkage has also been reported for other neuronal populations after exposure to alcohol as well as other drugs of abuse. For example, the superior frontal and motor cortices showed decreased neuronal size in AUD humans (Harper and Kril, 1989, Kril and Harper, 1989). Ventral tegmental area dopamine neurons shrink after exposure to morphine (Sklair-Tavron et al., 1996, Chu et al., 2007) as do frontal cortex and hippocampal neurons after methamphetamine administration (Kuczenski et al., 2007).

In contrast we did not observe differences in the cell number or size in HDC expressing neurons in human AUD compared to control brains. This is in agreement with prior work in humans indicating selective neuronal vulnerabilities to chronic alcohol. For example, the number and size of the pigmented neurons of the locus coeruleus (Halliday et al., 1992), the cholinergic nucleus basalis (Cullen et al., 1997) and the serotonin neurons in the dorsal raphe nucleus (Baker et al., 1996) remain unchanged after alcohol exposure whereas arginine-vassopressing expressing neurons in the supraoptic and paraventricular hypothalamic nuclei (Harding et al., 1996), as well as neurons in the superior, cingulate (Harper and Kril, 1989) and superior association frontal cortices (Kril et al., 1997) experience reduction in number and size in AUD.

Microglia

Microglia, resident immune cells of the brain are essential for neuronal function (Tremblay et al., 2011, Salter and Beggs, 2014, Henriques et al., 2018) and can quickly respond to homeostatic disturbances including alcohol exposure (He and Crews, 2008, Eggen et al., 2013). Upon stimulation, these cells convert from a default highly branched surveillant profile (Nimmerjahn et al., 2005) to the more amoeboid, migratory profile (Kettenmann et al., 2011) with phagocytic capacity (Ransohoff and Cardona, 2010). Increase in Iba-1, protein constitutively expressed in all microglia (Imai et al., 1996, Ahmed et al., 2007), mediates morphological changes in these cells (Imai et al., 1996, Sasaki et al., 2001) and is widely used as an immunohistochemical marker to identify this process (Vinet et al., 2012). Prior reports have indicated significant fluctuations in the levels of Iba-1 immunoreactivity in brains of AUD human patients with strong regional differences within the brain. For example the prefrontal cortex (PFC) and cingulate cortex show a significant increase in Iba-1 immunoreactivity. But Iba-1 immunoreactivity in the ventral tegmental area (VTA), the midbrain and amygdala does not differ from human controls (He and Crews, 2008, Rubio-Araiz et al., 2017), whereas in the hippocampus of AUD patients Iba-1 is reduced (Korbo, 1999). In the present study we found a significant increase in the size of microglia cell bodies within the hypothalamic sector that contains the Hcrt/MCH neuronal populations in human AUD brains. This indicates the transition of microglia from the surveillance phenotype to the amoeboid/phagocytic state. This shift in the morphology of microglia has been previously associated with neuroinflamation and impaired neuronal function (Yang et al., 2014) and thus might be a contributor to the changes we observe in the Hcrt and MCH signaling systems. On the other hand, we did not observe changes in the microglia population in this hypothalamic region of rats exposed to alcohol. Since microglia can rapidly deactivate following alcohol exposure (Ahlers et al., 2015), it is possible that microglial activation could return to baseline level within the abstinence period (8 h) before sacrifice. It has been reported that morphological alteration of this cellular type depends on the recent presence of alcohol (Sanchez-Alavez et al., 2019). Another possibility is that microglial cells in this hypothalamic region might not be affected by alcohol exposure, similar to what has been

previously described in animals for other sectors of the brain including the dentate gyrus (DG), the cerebellum and the nucleus accumbens core (Bajo et al., 2016, Sanchez-Alavez et al., 2019, Siemsen et al., 2020).

Morphological changes in the brain are correlated with drug ingestion in humans and rodents and may play a key role in the development of addictive behaviors. The study of anatomical changes in the brain of drug users has received renewed attention recently since the discovery of the increase number in Hcrt neurons following drug exposure (Thannickal et al., 2018, James et al., 2019, Matzeu and Martin-Fardon, 2021). Our current study indicates major differences between the response of Hcrt neurons to opioids and that to alcohol.

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