Neuroscience 207 (2012) 167–181

DISRUPTIONS IN SEROTONERGIC REGULATION OF CORTICAL GLUTAMATE RELEASE IN PRIMATE INSULAR CORTEX IN RESPONSE TO CHRONIC ETHANOL AND NURSERY REARING

G. M. ALEXANDER,[a1] J. D. GRAEF,[b1] J. A. HAMMARBACK,[c] B. K. NORDSKOG,[c] E. J. BURNETT,[b] J. B. DAUNAIS,[d] A. J. BENNETT,[d] D. P. FRIEDMAN,[d] S. J. SUOMI[e] AND D. W. GODWIN[c]*

[a]*Department of Neurobiology, Duke University Medical Center, Bryan Research Building, Durham, NC 27710, USA*

[b]*Neuroscience Program, Wake Forest University School of Medicine, Medical Center Blvd., Winston Salem, NC 27157, USA*

[c]*Department of Neurobiology and Anatomy, Wake Forest University School of Medicine, Medical Center Blvd., Winston Salem, NC 27157, USA*

[d]*Department of Physiology and Pharmacology, Wake Forest University School of Medicine, Medical Center Blvd., Winston Salem, NC 27157, USA*

[e]*Laboratory of Comparative Ethology, Eunice Kennedy Shriver National Institute, of Child Health and Human Development, NIH, Animal Center, PO Box 529, Poolesville, MD 20837, USA*

Abstract—Early-life stress has been shown to increase susceptibility to anxiety and substance abuse. Disrupted activity within the anterior insular cortex (AIC) has been shown to play a role in both of these disorders. Altered serotonergic processing is implicated in controlling the activity levels of the associated cognitive networks. We therefore investigated changes in both serotonin receptor expression and glutamatergic synaptic activity in the AIC of alcohol-drinking rhesus monkeys. We studied tissues from male rhesus monkeys raised under two conditions: Male rhesus monkeys (1) "mother reared" (MR) by adult females (*n*=9) or (2) "Nursery reared" (NR), that is, separated from their mothers and reared as a separate group under surrogate/peer-reared conditions (*n*=9). The NR condition represents a long-standing and well-validated nonhuman primate model of early life stress. All monkeys were trained to self-administer ethanol (4% w/v) or an isocaloric maltose–dextrin control solution. Subsets from each rearing condition were then given daily access to ethanol, water, or maltose–dextrin for 12 months. Tissues were collected at necropsy and were further analyzed. Using real time RT-PCR we found that ethanol-naive, NR monkeys had lower AIC levels of $5\text{-}HT_{1A}$ and $5\text{-}HT_{2A}$ receptor mRNA compared with ethanol-naive, MR animals. Although NR monkeys consumed more ethanol over the 12-month period compared with MR animals, both MR and NR animals expressed greater $5\text{-}HT_{1A}$ and $5\text{-}HT_{2A}$ receptor mRNA levels following chronic alcohol self-administration. The interaction between nursery-rearing conditions and alcohol consumption resulted in a significant enhancement of both $5\text{-}HT_{1A}$ and $5\text{-}HT_{2A}$ receptor mRNA levels such that lower expression levels observed in nursery-rearing conditions were not found in the alcohol self-administration group. Using voltage clamp recordings in the whole cell configuration we recorded excitatory postsynaptic currents in both ethanol-naive and chronic self-administration groups of NR and MR monkeys. Both groups that self-administered ethanol showed greater glutamatergic activity within the AIC. This AIC hyperactivity in MR alcohol-consuming monkeys was accompanied by an increased sensitivity to regulation by presynaptic $5\text{-}HT_{1A}$ receptors that was not apparent in the ethanol-naive, MR group. Our data indicate that chronic alcohol consumption leads to greater AIC activity and may indicate a compensatory upregulation of presynaptic $5\text{-}HT_{1A}$ receptors. Our results also indicate that AIC activity may be less effectively regulated by 5-HT in ethanol-naive NR animals than in NR monkeys in response to chronic ethanol self-administration. These data suggest possible mechanisms for increased alcohol seeking and possible addiction potential among young adults who had previously experienced early-life stress that include disruptions in both AIC activity and serotonin system dynamics. **© 2012 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: anterior insula, ethanol, serotonin, stress, $5\text{-}HT_{1A}$.

The anterior insular cortex (AIC) has recently become an area of interest in both anxiety (Paulus and Stein, 2006) and addiction (Goldstein et al., 2009; Naqvi and Bechara, 2009) because of its unique role as both an integrator and evaluator of internal, homeostatic information from the external environment (Craig, 2002; Goldstein et al., 2009; Paulus and Stein, 2006). Insular hyperactivity has been shown in anxiety-prone individuals, patients with general social phobias (Lorberbaum et al., 2004; Simmons et al., 2006; Stein et al., 2007), and in anticipatory anxiety (Chua et al., 1999; Nitschke et al., 2006). Smokers with brain damage involving the insula are reportedly more likely to quit smoking (Naqvi et al., 2007), and neuroimaging studies have shown insular activation in response to cravings for cigarettes, food, and cocaine (Bonson et al., 2002; Pelchat et al., 2004; Wang et al., 2007). Additionally, a study by Contreras et al. (2007) suggested that drug-craving in rats was eliminated by focal inactivation of the insula as assessed by condition place preference paradigm. These findings provide evidence for the important role of the AIC in networks determining both anxiety and addiction potential.

[1] These authors contributed equally to this manuscript.

*Corresponding author. Tel: +1-(336)-716-9347; fax: +1-(336)-716-4534.
E-mail address: dgodwin@wakehealth.edu (D. W. Godwin).

Abbreviations: ACSF, artificial cerebrospinal fluid; AIC, anterior insular cortex; APV, 2-amino-5-phosphonopentanoic acid; BEC, blood ethanol concentration; EPSC, excitatory postsynaptic current; IEI, inter-event interval; MR, mother reared; NBQX, 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide; NR, nursery reared; PPR, paired-pulse ratio; sEPSC, spontaneous EPSC; 8-OH-DPAT, (±)-8-hydroxy-2-dipropylaminotetralin hydrobromide.

0306-4522/12 $36.00 © 2012 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2012.01.027

Disruptions of the serotonin system have also been implicated in both anxiety and addiction disorders. For example, studies have demonstrated reduced 5-HT_{1A} receptor binding in the insula of patients with social anxiety disorder (Lanzenberger et al., 2007) and depression (Bhagwagar et al., 2004; Sargent et al., 2000). Furthermore, rhesus monkeys exposed to early-life stress have been reported to have reduced 5-HT_{1A} receptor binding throughout the majority of the brain (Spinelli et al., 2010), and monkeys exposed to this rearing condition exhibit behavioral and chemical indications of high anxiety, including increased frequency of serious aggression, decreased complexity of social behavior, lower social rank achieved, increased alcohol consumption, and increased adrenocorticotropin releasing hormone (ACTH) and cortisol levels in response to acute stress (Barr et al., 2009; Higley et al., 1991, 1996a,b; Suomi, 1997). Similarly, decreased 5-HT_{2A} receptor binding in several cortical structures, including the insula, has been shown in patients with obsessive-compulsive disorder (OCD) (Perani et al., 2008). Based on the involvement of the 5-HT system in behavioral abnormalities observed in animals exposed to early life stress and the involvement in psychopathology, as evidenced by specific serotonin reuptake inhibitors (SSRIs) and other 5-HT-acting agents being the most widely prescribed psychotropic medications (Golden, 2004), we chose to investigate the 5-HT system in our primate model. We investigated 5-$HT_{1A,\ 2A,\ 1B,\ \text{and}\ 1D}$ in cortical brain tissue. Of these receptors, 5-HT_{1A} and 5-HT_{2A} are most heavily expressed in cortex. The 5-HT_{1A} receptor is expressed on presynaptic terminals where it regulates glutamate and 5-HT release and is also active postsynaptically, whereby it hyperpolarizes pyramidal neurons. By contrast, 5-HT_{2B} receptors induce a net depolarization of pyramidal neurons (for review, see Andrade, 2011).

It has also been suggested that disruptions of the serotonergic system may be involved in the development of alcoholism (Johnson, 2004; Verheul et al., 1999). Serotonin receptor expression has been shown to be altered in animals and humans exposed to chronic ethanol, as well as in humans with mood disorders (Heinz et al., 2004; López-Figueroa et al., 2004). Additionally, previous data from our group has demonstrated ethanol-induced alterations in 5-HT_{1A} receptor expression in specific regions of the hippocampus in both cynomolgus and rhesus macaques (Davenport et al., 2005, 2007). Indeed, SSRIs have been shown to decrease spontaneous ethanol consumption in rodents (Kelai et al., 2003; Maurel et al., 1999). More specifically, stimulation of 5-HT_{1A} receptors decreases ethanol intake (McKenzie-Quirk and Miczek, 2003; Kelai et al., 2006), and chronic ethanol consumption has been shown to alter 5-HT_{1A} receptor expression in the brain as well as increase receptor sensitization (Kelai et al., 2008; Nevo et al., 1995; Storvik et al., 2009). These findings suggest that altered serotonin function may be a common factor contributing to both mood disorders and alcohol addiction potential (Lesch, 2005). One mechanism by which serotonin regulates cortical function is through modulation of glutamatergic signaling, suggesting that serotonergic regulation of cortical hyperexcitability could be involved in mechanisms of mood disorders and alcohol addiction potential. We therefore focused on AIC as an important region to examine the regulation of neural excitability by serotonin and how infant rearing conditions and chronic drinking may affect serotonergic function in insular cortex.

We used nursery-reared nonhuman primates as a model to determine possible mechanisms by which early-life stress might lead to later alcohol-drinking behavior. Nursery rearing in nonhuman primates has been shown to result in behavioral and neurochemical changes later in life consistent with affective disorders, and these animals have been reported to consume greater quantities of ethanol relative to mother-reared (MR) animals (Harlow et al., 1959; Harlow and Harlow, 1965; Harlow and Suomi, 1974; Seay and Harlow, 1965; Suomi, 1997; Shannon et al., 1998; Machado and Bachevalier, 2003; Cirulli et al., 2009; Higley et al., 1991). Consistent with this, a parallel study using the MR and nursery-reared (NR) ethanol-naive monkeys that were also used in the current study reported altered socioaffective behavior in NR monkeys compared with MR monkeys (Corcoran et al., 2011). This model thus provides a unique resource to understand neurobiological changes similar to those that may occur in human children exposed to early-life stress and how these changes may contribute to the development of addictive behaviors later in life.

Using this model, we hypothesized that chronic ethanol exposure and nursery rearing may disrupt serotonin receptor expression and function in AIC. To test this hypothesis, we assessed serotonin receptor gene expression by measuring mRNA levels in AIC tissue isolated from control and chronic ethanol consuming MR and NR animals. In addition, we hypothesized that chronic self-administration of ethanol would disrupt AIC network activity and that serotonin regulation of excitatory signaling would be significantly altered. We further tested the hypothesis that nursery rearing or chronic ethanol consumption in adulthood would produce a hyperexcitable state in the prefrontal cortical network in response to reduced serotonergic regulation. We found an interaction between nursery rearing and chronic alcohol consumption that resulted in a synergistic enhancement of serotonin receptor expression and altered AIC excitability.

EXPERIMENTAL PROCEDURES

Experimental subjects

Eighteen male rhesus monkeys housed at the Wake Forest University Primate Center were used in the study. All animals were born at the Laboratory of Comparative Ethology at the National Institute of Child Health and Human Development and transferred to Wake Forest University School of Medicine in two cohorts as adolescents. For testing, the animals were initially acclimated to individual housing ($76 \times 60 \times 70$ cm^3) for 3 months before undergoing training for self-administration. Throughout the study period, monkeys were maintained at a free-feeding weight on a diet of Primate Food pellets (Research Diets Inc., New Brunswick, NJ, USA) supplemented with fresh produce.

Manipulation of early rearing environment

Each subject experienced one of two early rearing environments: (1) rearing by an adult female in a social group comprised of multiple mother–infant dyads and two adult males (MR group; $n=9$). These subjects lived from birth to approximately 7 months of age in social groups housed in indoor–outdoor cages. Over the course of the first 7 months the diet of the MR subjects consisted of breast milk and weaning to chow and water. (2) Nursery-reared animals (NR group; $n=9$) that were separated from their mothers within 24 h of birth, moved to a neonatal nursery, and reared under surrogate-peer-reared conditions using procedures based on those developed at the University of Wisconsin Harlow Primate Laboratory (see Novak and Sackett, 1997 for detailed description and comparison of nursery-rearing conditions; Shannon et al., 1998). Briefly, infants were housed in an incubator containing an inanimate surrogate equipped with a spring to allow rocking motion, covered with a heating pad, and encased in thick fleece. Infants were fed formula (50:50 Similac:Primilac) and weaned to solid foods and water over the course of 6 months. Beginning at 14 days, infants were moved to one quadrant of a cage within a room of the nursery and allowing for visual, auditory, and olfactory contact with other monkeys. At approximately 37 days of age, daily 2-h socialization periods were given in which four infants were united in a play cage. Animals from both rearing groups experienced identical environments after weaning at 6–7 months of age.

Ethanol self-administration

Monkeys were divided into four experimental groups by separating both the MR and NR monkeys into control and ethanol groups. Thus, the four experimental groups included NR controls ($n=5$), MR controls ($n=5$), NR ethanol ($n=4$), and MR ethanol ($n=4$). All monkeys were trained to operate a drinking panel and consume water under scheduled pellet deliveries (i.e. schedule-induced polydipsia; Falk, 1971) followed by 4% w/v ethanol (ethanol group), as previously described (Vivian et al., 2001; Grant et al., 2008). The daily amount of ethanol increased in a stepwise fashion over three 30-day epochs from 0.5 to 1.5 g/kg. During this same induction period, the control group self-administered maltose–dextrin under identical conditions. At the conclusion of this induction period the animals were then allowed free access to 4% ethanol (w/v) and water (ethanol group) or isocaloric maltose–dextrin and water (control group) for 22 h each day for 12 consecutive months. All of the animals in the first cohort ($n=8$) had access to ethanol and water, whereas the second cohort ($n=10$) had access to a maltose–dextrin solution and water. During the 22-h self-administration period, animals were allowed to self-administer a banana pellet diet rationed into three meals per day supplemented with daily fresh produce. The study design was such that all animals began the 22-h self-administration portion of the paradigm between 5 and 6 years of age, which corresponds to young adulthood in rhesus macaques (Lewis, 1997; Watts and Gavan, 1982). Blood samples were obtained for analysis of blood ethanol concentration (BEC) through awake venipuncture of the femoral or saphenous vein every 5 days throughout the 22-h self-administration portion of the drinking paradigm. Blood samples were collected just before lights out at 6:00 PM, approximately 6 h following the onset of the daily session. Samples were stored at −20 °C until the end of the study period at which point they were assayed simultaneously. BEC was determined by gas chromatograph (Agilent GC 7890A, Agilent Technologies, Santa Clara, CA, USA).

Both cohorts exhibited a progressive increase in body weight over the entire study period. No significant difference was observed in body weights at the end of the study period between cohorts (data not shown). Monkeys were necropsied immediately following the final 22 h access period. During necropsy, monkeys were perfused through the heart with ice-cold artificial cerebrospinal fluid (ACSF) containing the following (in mM): 124 NaCl, 5 KCl, 2 $MgSO_4$, 2 $CaCl_2$, 23 $NaHCO_3$, 3 NaH_2PO_4, 10 glucose (pH 7.4, osmolarity 290–300 mOsm) oxygenated with 95% O_2:5% CO_2. A tissue block containing the AIC was isolated and immediately immersed in oxygenated (95% O_2:5% CO_2), ice cold sucrose-substituted ACSF containing the following (in mM): 220 sucrose, 12 $MgSO_4$, 10 glucose, 2 KCl, 1.5 NaH_2PO_4, 26 $NaHCO_3$, 0.2 $CaCl_2$ (pH 7.4, osmolarity 290–300 mOsm). Vibratome sections for *in vitro* electrophysiology were made from the tissue block. In addition, a portion of the AIC tissue block was snap frozen in liquid nitrogen and stored at −80 °C until tissue was collected from each animal and gene expression analyses could be performed on all samples simultaneously.

Real-time RT-PCR

After removal from storage, tissue was immediately homogenized in TriREAGENT (Molecular Research Center, Inc, Cincinnati, OH, USA) using a Powergen 125 Tissue Homogenizer (Fisher Scientific, Hampton, NH, USA). Total RNA was isolated as described in the manufacturer's protocols. Absence of DNA contamination was verified in the purified RNA preparation by performing cDNA synthesis in the absence of reverse transcriptase (−RT control) followed by RT-PCR (see below). As an added precaution, RNA was further purified using Qiagen RNeasy spin columns (Qiagen, Valencia, CA, USA) to remove possible contaminants including genomic DNA. RNA concentration was measured using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and quality was assessed by electrophoresis in 1% agarose formaldehyde gels. cDNA was synthesized using the SuperScript III First Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) for real-time PCR with random hexamer primers as per manufacturer's instructions. Real-time PCR was performed using the 5′-exonuclease method (TaqMan, Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed on an ABI 7300 thermal cycler (Applied Biosystems) using 2 ng of amplified sample cDNA with TaqMan specific gene assays, as described by TaqMan Universal PCR Master Mix (Applied Biosystems) protocols. The cycling parameters were as follows: 50 °C for 4 min followed by a DNA polymerase activation step at 95 °C for 10 min and a two-temperature PCR of 40 cycles at 95 °C for 15 s (denaturing step) followed by 55 °C for 1 min (annealing step). Relative expression levels for the 5-HT receptors, GAPDH, and actin were quantified according to the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001). Each gene/animal was run in triplicate and normalized to actin gene expression. Data are represented as the group mean±SEM. One tissue sample from the MR ethanol group was excluded from analysis because of contamination during the mRNA isolation process.

Electrophysiology

The tissue block was sectioned on a vibratome (model OTS 4000, Electron Microscopy Sciences, Fort Washington, PA, USA) at 400 μm, and slices containing the intermediate and lateral agranular insula areas (Iai and Ial, respectively) and precentral opercular cortex (PrCo) of AIC (Carmichael and Price, 1994) were maintained in oxygenated, warm (34 °C) ACSF of the same composition as the perfusion ACSF, for at least 1.5 h before being transferred to another chamber for recordings (Harvard Apparatus, Holliston, MA, USA). Slices were recorded using "blind" recording techniques from 400 μm slices, as previously described for recordings from primate tissue (Alexander et al., 2006). For whole cell patch recordings, slices were submerged in oxygenated (95% O_2, 5% CO_2) ACSF at room temperature. Oxygenated ACSF was perfused at a flow rate of 2 ml per min. Patch pipettes (5–10 MΩ) were pulled from borosilicate glass (Sutter Instruments, Novato, CA, USA) with a PC-10 vertical puller (Narishige International

USA, Inc., East Meadow, NY, USA) and were filled with an internal solution containing the following (in mM): 100 Gluconic acid, 100 CsOH, 10 NaCl, 10 HEPES, 20 TEA-Cl, 1 EGTA, 4 ATP (pH 7.3 with 2 N CsOH, Osmolarity 270–290 mOsm). The calculated liquid junction potential was +7 mV with this internal solution, and membrane potential measurements were corrected for the junction potential post hoc. Resting membrane potential measurements were made 1–2 min following establishment of whole-cell configuration. Cellular activity was acquired with an AxoClamp 2B amplifier (Axon Instruments, Union City, CA, USA), digitized with a Digidata 1322 (Axon Instruments), and analyzed using pCLAMP 9.0 software (Axon Instruments). To acquire cells for voltage clamp, patch pipettes were advanced “blind” through tissue in bridge mode until encountering a cell, a >1 GΩ seal was formed, the membrane ruptured to allow whole cell access, and the amplifier was then switched to single electrode voltage clamp mode. Pharmacological agents used in electrophysiology included picrotoxin (Sigma-RBI, St. Louis, MO, USA), DL-2-amino-5-phosphonopentanoic acid (APV; Sigma-RBI), 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX; Tocris Bioscience, Ellisville, MO, USA), and (±)-8-hydroxy-2-dipropylaminotetralin hydrobromide (8-OH-DPAT; Tocris Bioscience).

Statistical analyses

A two-way ANOVA was used to assess differences in ethanol consumption behavior between MR and NR animals, with months of drinking and rearing group as factors. Unpaired *t*-tests were used to assess differences in gene expression within each group, and a two-way ANOVA was used to test for interactions between rearing condition and ethanol exposure on gene expression levels. Unpaired *t*-tests were used to compare physiological measures between groups of animals, and paired *t*-tests or Wilcoxon rank tests were used to compare responses to agonist administration within groups. All consumption analyses were performed using SAS 9.1 (SAS Institute, Cary, NC, USA). The remaining analyses were performed using Prism 5 (GraphPad Software, La Jolla, CA, USA) Data were considered significant if $P<0.05$.

RESULTS

Alcohol self-administration

A two-way ANOVA revealed a main effect of rearing condition on ethanol intake. NR monkeys consumed significantly more ethanol than MR monkeys ($F(1,6)=54.51$, $P<0.01$; Fig. 1A). The average daily intake for MR monkeys was 2.2±0.03 g/kg, and the average daily intake for NR monkeys was 2.9±0.03 g/kg. These intake values surpass the NIAAA definition of a heavy drinker (>4 drinks/day or >14 drinks/week where 0.25 g/kg equals one drink) and thus, these animals should be considered an appropriate model of human heavy drinking. Further analyses also uncovered a significant effect of time ($F(365,2179)=5.47$, $P<0.0001$) and an interaction between rearing and time ($F(365,2179)=1.29$, $P<0.01$).

For BEC analyses, blood samples were collected just before lights out at 6:00 PM to allow for as much day-time drinking as possible with the aim being to obtain BEC values reflective of the maximum intake for a particular day. Whereas some animals drank the bulk of their daily intake during the day, as has been the case in previous cohorts, in this cohort, the two heaviest drinkers often consumed more than half their ethanol after 6:00 PM, drinking throughout the night and into the following morning.

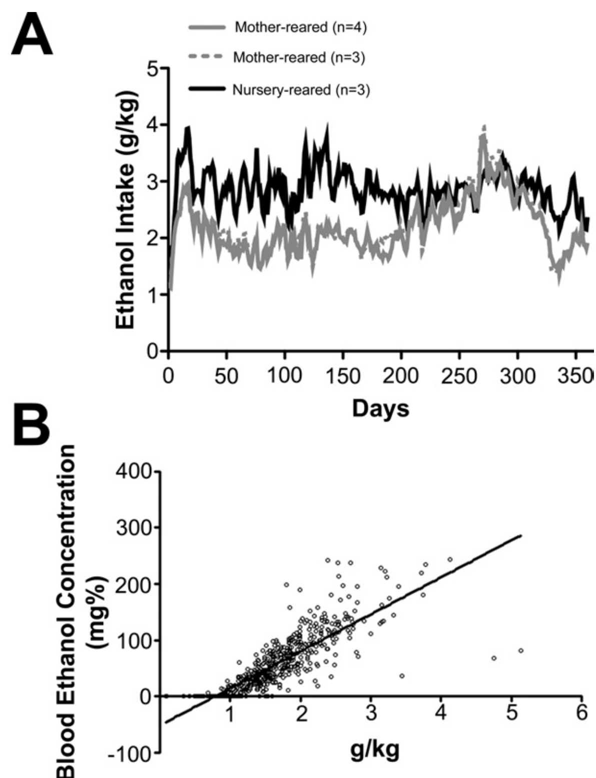

Fig. 1. (A) Daily ethanol intake (g/kg) during 12 mon of unlimited access to ethanol (4% w/v) in MR and NR monkeys. Two data sets are plotted for MR monkeys: one set with all four MR alcohol-exposed monkeys used in the study and one set with three MR alcohol-exposed monkeys that were used for gene expression studies. Data are smoothed using weighted average of nine nearest data points. In comparing consumption of NR monkeys with each set of MR, NR monkeys consumed significantly greater ethanol than either set of MR monkeys throughout the study period (two-way repeated measures ANOVA, $P<0.01$). (B) Blood ethanol concentrations during 22-h free access and corresponding ethanol consumption at the time of sampling (sample taken 6 h post session start time) revealing a significant correlation ($r^2=0.67$, $P<0.0001$).

That said, the BEC data showed that MR animals had significantly higher average BECs during the 22-h self-administration period than NR animals ($P<0.001$) despite greater daily intake in the NR group. Using the data acquired from the 6:00 PM samples, MR monkeys had an average BEC of 69 mg/dl, ranging from 0 to 196 mg/dl, whereas NR monkeys obtained an average BEC of 49 mg/dl, ranging from 0 to 170 mg/dl. Data acquired from a 5-h metabolism study in which all animals were given a standard dose of 1.0 g/kg ethanol showed that both ethanol-naive and ethanol-experienced MR and NR animals attained similar peak BECs (~100 mg/dl) but that the NR monkeys metabolized ethanol at a higher rate, thus clearing it more quickly regardless of ethanol exposure history ($P=0.02$). A manuscript describing this finding is in preparation. The higher rate of metabolism and large amount of drinking after 6:00 PM by the heaviest drinkers are both consistent with the lower BECs seen in the 6:00 PM samples. It is also worth noting that although the correlation between drinking amounts and BECs, while highly statis-

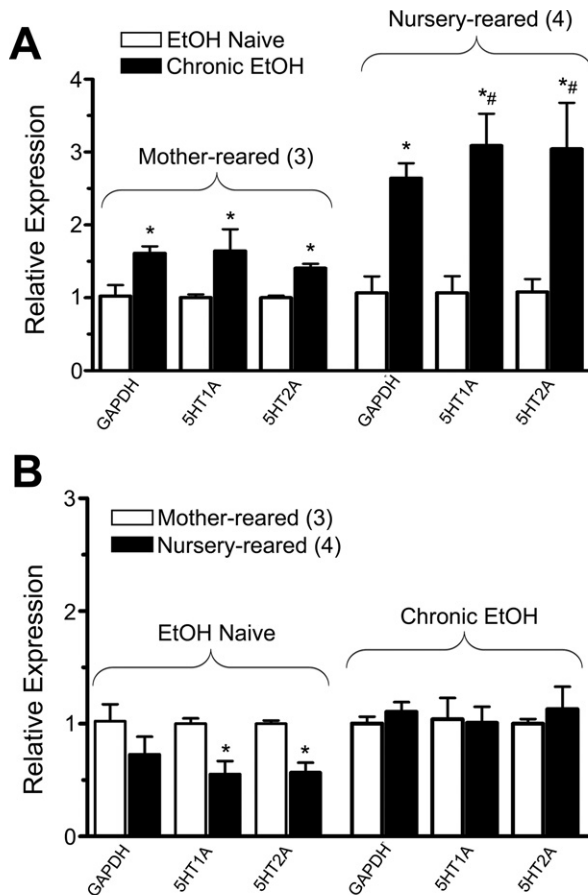

Fig. 2. Relative mRNA expression levels of GAPDH and various 5-HT receptor subtypes for ethanol naive and ethanol exposed MR or NR monkeys. (A) Comparison of ethanol's effects on MR and NR groups reveals a significant increase in GADPH, 5-HT_{1A}, and 5-HT_{2A} mRNA levels (n=3, * P<0.05 unpaired t-test). Chronic ethanol significantly upregulated GAPDH, 5-HT_{1A}, and 5-HT_{2A} mRNA levels in the NR monkeys (n=4, * P<0.05 unpaired t-test), with a significant interaction between chronic ethanol exposure and rearing conditions for 5-HT_{1A} and 5-HT_{2A} mRNA levels (# P<0.05, two-way ANOVA). (B) When comparing rearing conditions, nursery rearing alone produced a significant decrease in expression of both 5-HT_{1A} and 5-HT_{2A} when compared with MR, ethanol-naive monkeys. No differences were seen between MR and NR animals chronically exposed to ethanol.

tically significant (P<0.0001), revealed an r^2 of only 0.67 (Fig. 1B), indicating that factors other than total daily intake *per se* influenced BECs taken at 6:00 PM.

For the remainder of this study, we examined cellular and molecular properties of AIC tissues isolated from these animals. However, for the gene expression studies, tissue from one MR monkey was not collected. The rearing effect on alcohol intake remained, however, despite the removal of this animal from the drinking data analysis (n=3 MR, n=4 NR) (F(1,5)=31.83, P<0.01; Fig. 1A).

Chronic ethanol self-administration alters serotonin receptor gene expression

We found that chronic ethanol self-administration and nursery rearing differentially regulated mRNA levels of serotonin receptors and GAPDH in AIC tissues (Fig. 2). Because levels of GAPDH mRNA, which are typically used to normalize mRNA levels, differed significantly among groups we normalized serotonin receptor mRNA levels to that of actin, an endogenous control that has been shown to be a stable reference gene in comparing human prefrontal brain tissues of chronic alcoholics and controls (Johansson et al., 2007). Actin mRNA expression levels showed no significant differences among the four groups tested. To probe for a main effect of ethanol exposure on gene expression, we compared relative mRNA expression levels between animals self-administering ethanol (n= three MR monkeys and four NR monkeys) and ethanol-naive controls (n=three MR monkeys and four NR monkeys) with rearing conditions grouped. We found that chronic ethanol-consuming animals had significantly greater mRNA levels of 5-HT_{1A} (P<0.05, unpaired t-test) and 5-HT_{2A} (P<0.05, unpaired t-test). In contrast, mRNA levels of 5-HT_{1B} and 5-HT_{1D} did not differ between animals that chronically self-administered alcohol and controls not exposed to alcohol (data not shown). Thus, ethanol exposure produced a main effect on serotonin receptors.

We then investigated whether or not rearing conditions affected gene expression in monkeys exposed to ethanol. To that end, we divided the monkeys into four groups based on ethanol exposure and rearing condition and assessed relative change in gene expression for one variable at a time. We examined the effect of ethanol exposure on each of the MR and NR groups (Fig. 2A), then we examined the effect of rearing for each of ethanol-naive and chronic ethanol self-administration groups (Fig. 2B). The control groups for each of these comparisons were either ethanol-naive or MR animals, so changes in gene expression were assessed relative to their appropriate control group for all comparisons. In comparing the effect of ethanol exposure, we found that MR animals chronically exposed to ethanol had significantly greater expression of GAPDH, 5-HT_{1A}, and 5-HT_{2A} mRNA (n=3, P<0.05, unpaired t-test) (Fig. 2A). For the NR group, ethanol monkeys had significantly greater levels of GAPDH, 5-HT_{1A}, and 5-HT_{2A} mRNA than ethanol-naive animals (n=4, P<0.05, unpaired t-test) (Fig. 2A). Furthermore, using raw ΔCt values we found a significant interaction between ethanol exposure and rearing condition, suggesting that chronic ethanol self-administration had a significantly greater impact on the transcriptional upregulation of 5-HT_{1A} (F(1,10)=4.93, P<0.05; two-way ANOVA) and 5-HT_{2A} (F(1,10)=5.92, P<0.05; two-way ANOVA) mRNA in the NR group. This enhancement in NR animals was not because of higher levels of ethanol consumption because no statistical correlation between gene expression and average daily ethanol intake was observed (data not shown).

Nursery rearing alone alters serotonin receptor gene expression

In comparing the effect of rearing condition, we found that NR monkeys showed significantly lower expression levels of 5-HT_{1A} and 5-HT_{2A} mRNA among ethanol-naive animals (P<0.05, unpaired t-test) (Fig. 2B). However, among animals that chronically self-administered ethanol, these

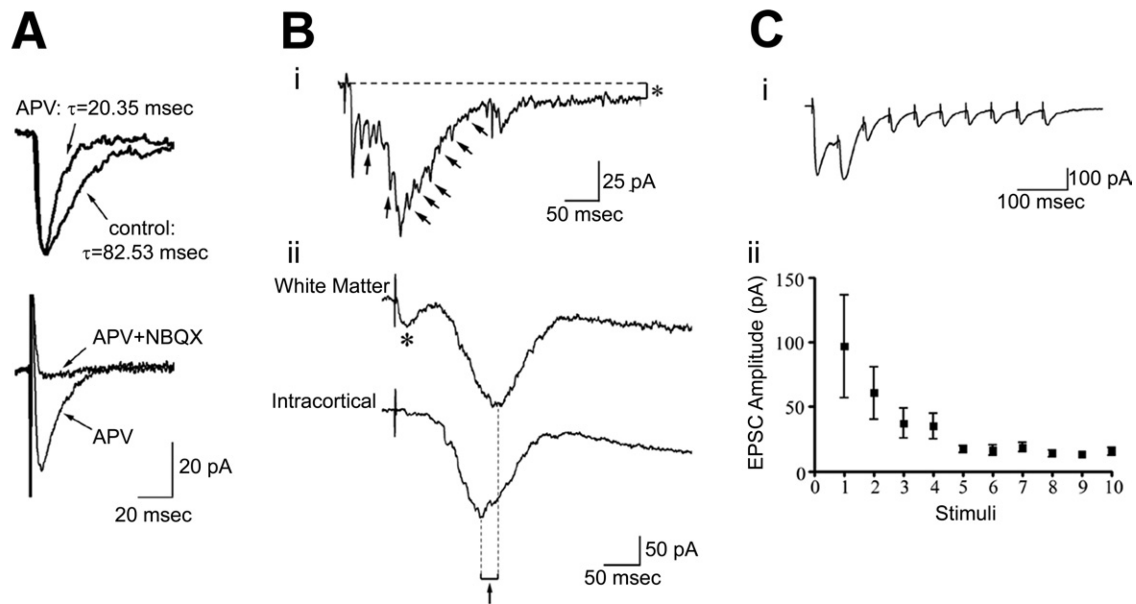

Fig. 3. Synaptic properties of AIC neurons upon white matter or intracortical electrical stimulation in MR control animals. (A) EPSCs consisted of APV-sensitive NMDA component and NBQX-sensitive AMPA/kainate component. (B) Electrical stimulation evoked a rapid synaptic response followed by a delayed synaptic barrage. (Bi) Single sweep showing white matter-evoked short-latency synaptic response followed by delayed synaptic barrage. Arrows show individual synaptic responses that comprise the barrage response. Asterisk shows persistent inward current resulting from synaptic stimulation. Inward current persisted for ~1500 ms. (Bii) Averaged sweeps showing white matter stimulation-evoked and intracortical stimulation-evoked responses from the same neuron. Asterisk shows short-latency synaptic response that was elicited by white matter stimulation but not intracortical stimulation. Arrow shows difference in latency to onset of synaptic barrage arising from white matter stimulation and intracortical stimulation. (C) Short-term plasticity of white matter stimulation evoked synaptic responses showing frequency-dependent depression for an individual neuron (i) and for the population (n=6 cells; ii).

differences between MR and NR monkeys were not apparent (P>0.05) (Fig. 2B).

Synaptic properties of AIC neurons

Altered activation of certain brain circuits is likely to occur following either chronic ethanol exposure or early-life stress. We hypothesized that the alterations in gene expression we observed may either contribute to, or be homeostatically regulated in response to, increased neuronal excitability in AIC neurons. To begin testing this hypothesis, we initially sought to characterize the synaptic and membrane properties of neurons in Iai, Ial, and PrCo of AIC (Carmichael and Price, 1994) because these properties of monkey AIC neurons are unknown. We therefore performed whole-cell recordings from deep-layer neurons and placed bipolar stimulating electrodes in both the white matter adjacent to the recording electrode and more superficial mantle of the adjacent cortex.

In the presence of the $GABA_A$ receptor blocker picrotoxin (100 μM), we examined the composition of the white matter-evoked excitatory postsynaptic currents (EPSCs). We found that bath application of the NMDA receptor blocker DL-APV (100 μM) produced an EPSC with faster decay kinetics than the baseline EPSC (i.e. sharper), and subsequent bath application of the AMPA/kainate receptor inhibitor NBQX (1 μM) abolished the remaining synaptic response. These findings indicate that the glutamatergic receptors that contribute to the EPSC within the monkey AIC include NMDA and AMPA/kainate receptors (Fig. 3A).

In a portion of the recordings (six cells of 21 studied from MR control animals), in addition to the short-latency synaptic response, a second, polysynaptic response was elicited with increased stimulus intensity (>350 μA). This response had a longer latency to onset with a peak amplitude approximately 100–150 ms following stimulation (Fig. 3Bi). This longer-latency response likely arose from activation of intracortical circuitry because direct cortical stimulation elicited a polysynaptic response of similar profile but with a peak response amplitude occurring approximately 20 ms earlier than the peak response amplitude elicited by white matter stimulation (Fig. 3Bii). Furthermore, synaptic stimulation sufficient to elicit the putative polysynaptic response was followed by an inward current that persisted for >1500 ms (Fig. 3Bi). This response may be due to activation of metabotropic glutamate receptors because inward currents of a similar duration have been described in other brain regions in both monkeys and rodents (Anwyl, 1999), and mGluR1 has been shown to be expressed on dendritic spines of principal cells within monkey prefrontal cortex (Muly et al., 2003).

We then examined short term synaptic plasticity of intermediate layer cells upon stimulation of the white matter tract. We found that paired stimuli did not consistently result in paired-pulse depression or facilitation, yielding a mean paired-pulse ratio (PPR) of 0.99±0.06 (n=12 cells) with an interstimulus interval of 100 ms and a stimulus intensity of that which produced ~50% of the

maximal EPSC amplitude (Table 1). However, synaptic responses showed depression upon delivery of a train of 10 synaptic stimuli at 10 Hz (n=6 cells) (Fig. 3C), supporting the conclusion that this synapse shows synaptic depression.

Chronic alcohol self-administration and nursery rearing alter spontaneous excitatory synaptic activity

We next compared membrane and synaptic properties of AIC neurons from the four groups of animals (Table 1). AIC neurons from animals exposed to chronic ethanol had a significantly more depolarized mean membrane potential relative to neurons from control monkeys ($P<0.01$, unpaired t-test). Furthermore, this depolarized membrane potential was associated with a significant reduction in membrane resistance in the ethanol-exposed animals ($P<0.05$, unpaired t-test). We then compared evoked synaptic responses. Comparison of the EPSC decay time yielded no significant difference between controls and ethanol-exposed monkeys. However, in comparing paired synaptic stimulations with an interstimulus interval of 100 ms, we found that ethanol-exposed monkeys showed a significantly reduced PPR. In other words, while control animals showed no facilitation or depression upon paired stimulation, monkeys that chronically self-administered ethanol showed paired-pulse depression, reflecting an increased probability of synaptic glutamate release (Zucker and Regehr, 2002). Furthermore, electrical stimulation of either the white matter or intracortical areas elicited delayed synaptic barrages, defined as those synaptic responses with a peak amplitude occurring 100–150 ms after stimulation, in significantly more cells from animals exposed to chronic ethanol (92%, or 23 of 25 cells studied) than control animals (29%, or 6 of 21 cells studied), consistent with increased excitability of AIC circuits following chronic ethanol consumption.

AIC neurons displayed a considerable degree of spontaneous excitatory synaptic activity. We quantified the spontaneous EPSC (sEPSC) amplitude, frequency, and decay kinetics in the presence of APV and pictotoxin in AIC neurons from control and ethanol-exposed animals. We found no difference in sEPSC decay time or amplitude. However, we did find significantly lower sEPSC interevent intervals (IEIs) among ethanol-exposed animals, indicating a higher frequency of recorded sEPSCs. Dividing the control and ethanol-exposed groups into MR and NR yielded a significant effect of rearing among ethanol-naive animals and a significant effect of ethanol-exposure among MR animals (Fig. 4, Table 1). Specifically, we found a significantly greater sEPSC frequency (reduced IEI) in AIC neurons from MR monkeys that chronically self-administered ethanol as compared with MR control monkeys (Fig. 4, $P<0.05$, unpaired t-test). In addition, we found significantly lower sEPSC IEIs among NR control animals compared with MR control animals, indicating NR monkeys exhibit a higher frequency of spontaneous AIC activity (Fig. 4, $P<0.05$, unpaired t-test).

Table 1. Membrane and synaptic response properties for AIC neurons acutely isolated from control (ethanol naive) and ethanol-exposed animals divided into two rearing conditions

	Membrane potential (mV)	Membrane resistance (MΩ)	EPSC decay (ms)	Paired pulse ratio	sEPSC amp (pA)	sEPSC IEI (ms)	sEPSC decay (ms)
Mother-reared control	−66.1±0.8 (7 cells, 3 animals)	295.7±35.0 (12 cells, 3 animals)	21.5±3.0 (7 cells, 3 animals)	1.05±0.07 (7 cells, 3 animals)	3.1±0.7 (7 cells, 3 animals)	194.9±27.6 (7 cells, 3 animals)	3.1±0.7 (7 cells, 3 animals)
Nursery-reared control	−70.0±1.8 (8 cells, 3 animals)*	228.7±29.6 (9 cells, 3 animals)	26.2±4.8 (5 cells, 3 animals)	0.95±0.12 (5 cells, 3 animals)	7.6±1.0 (6 cells, 3 animals)	145.6±11.7 (6 cells, 3 animals)*	7.6±0.8 (6 cells, 3 animals)
Mother-reared EtOH	−58.4±2.4 (11 cells, 4 animals)#	177.2±23.3 (12 cells, 4 animals)#	23.9±3.6 (13 cells, 4 animals)	0.85±0.06 (11 cells, 4 animals)#	7.4±1.3 (5 cells, 3 animals)	106.7±25.2 (5 cells, 3 animals)#	7.0±1.4 (5 cells, 3 animals)#
Nursery-reared EtOH	−60.8±1.9 (8 cells, 4 animals)#	235.1±61.0 (7 cells, 3 animals)	22.3±2.8 (8 cells, 4 animals)	0.93±0.03 (5 cells, 4 animals)	6.7±1.9 (2 cells, 2 animals)	111.1±10.7 (2 cells, 2 animals)	6.5±2.6 (2 cells, 2 animals)

Values represent mean±SEM. Parenthetical numbers represent number of cells studied.
* Between rearing conditions.
# between ethanol treatment. $P<0.05$, unpaired t-test.

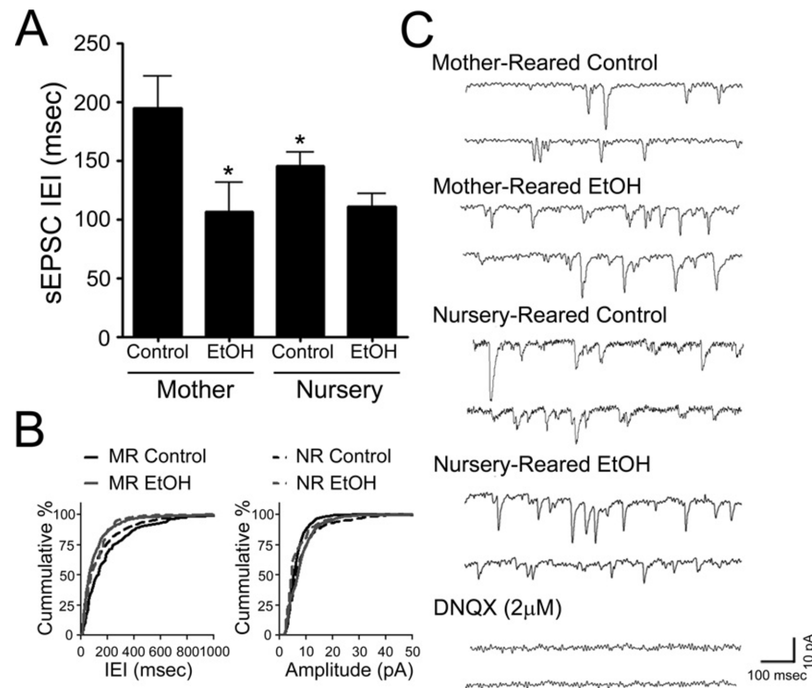

Fig. 4. AMPA/kainate mediated spontaneous EPSCs recorded from AIC neurons in MR and NR control and chronic ethanol-consuming monkeys. (A) MR animals that chronically self-administered ethanol showed significantly greater frequency (lower IEI) of sEPSCs as compared with MR ethanol-naive controls (* $P<0.05$). NR ethanol-naive monkeys also showed significantly lower sEPSC IEIs (higher frequency) as compared with MR ethanol-naive controls (* $P<0.05$). (B) Cumulative IEI and amplitude histograms for the population of cells in each group of animals. (C) Example traces for all four groups are also shown, along with a trace demonstrating that these sEPSC events could be eliminated with the AMPA/kainate receptor blocker DNQX in an MR control animal.

Serotonin differentially modulates AIC glutamatergic synaptic activity in animals that chronically consumed ethanol

Guided by our findings of differential expression of AIC 5-HT_{1A} and 5-HT_{2A} receptor mRNA among animals exposed to various rearing conditions and alcohol exposure, we hypothesized that AIC neurons from each of these groups of animals would show varying responses to agonists for these receptors. Serotonin, released from axon terminals arising from the raphe nuclei, has been shown to affect transmission of several neurotransmitters through heterosynaptic activation of serotonin receptors on neighboring axon terminals (Fink and Göthert, 2007). For example, heterosynaptic activation of serotonin receptors on presynaptic glutamatergic neurons has been shown to modulate glutamate release (Calcagno et al., 2006; Gołembiowska and Dziubina, 2002; Maura et al., 1998). We therefore performed whole-cell recordings from AIC neurons and examined both white matter-stimulation-evoked EPSCs and sEPSCs before and in the presence of bath applied 8-OH-DPAT, a 5-HT_{1A} receptor agonist. Because of the limited number of animals available, we focused our study on MR animals and 5-HT_{1A} receptors. We isolated acute slices from animals that chronically self-administered ethanol and from ethanol-naive animals and assessed the differential response magnitude of AIC neurons to 8-OH-DPAT bath application.

We found that bath application of 8-OH-DPAT (2 μM) reduced the amplitude of white matter-evoked EPSCs recorded from AIC neurons for both control animals (78.0±4.3% of baseline EPSC amplitude, $n=6$; Fig. 5A) and monkeys that self-administered ethanol (51.3±5.9% of baseline EPSC amplitude, $n=8$; Fig. 5B). However, the magnitude of reduction of the EPSC amplitude was significantly greater for animals exposed to chronic ethanol than for control animals ($P<0.05$, unpaired *t*-test). Paralleling the increased magnitude of EPSC inhibition by 8-OH-DPAT in ethanol-exposed animals, we also found a significant increase in the PPR (baseline=0.82±0.09; 8-OH-DPAT=1.00±0.14; $P<0.05$, Wilcoxon rank test), indicating that a portion of this inhibition was through a presynaptic site of action. By contrast, neurons from control animals, which showed only a moderate decrease in EPSC amplitude in response to 8-OH-DPAT, also failed to show a significant change in PPR (baseline=0.97±0.25; 8-OH-DPAT=0.95±0.32; $P>0.05$) (Fig. 5C), suggesting that the reduction in EPSC amplitude was independent of activation of presynaptic 5-HT_{1A} receptors. These findings indicate that 5-HT_{1A} receptors exerted their effects through heterosynaptic mechanisms that reduced presynaptic glutamatergic excitatory transmission in ethanol-naive monkeys. Taken together, these findings suggest that animals exposed to chronic ethanol have increased presynaptic 5-HT_{1A} receptor function, possibly mediated by increased receptor expression in AIC.

DISCUSSION

In this study, we examined the effects of chronic ethanol self-administration on serotonergic modulation of glutamate transmission in the AIC of MR and NR rhesus mon-

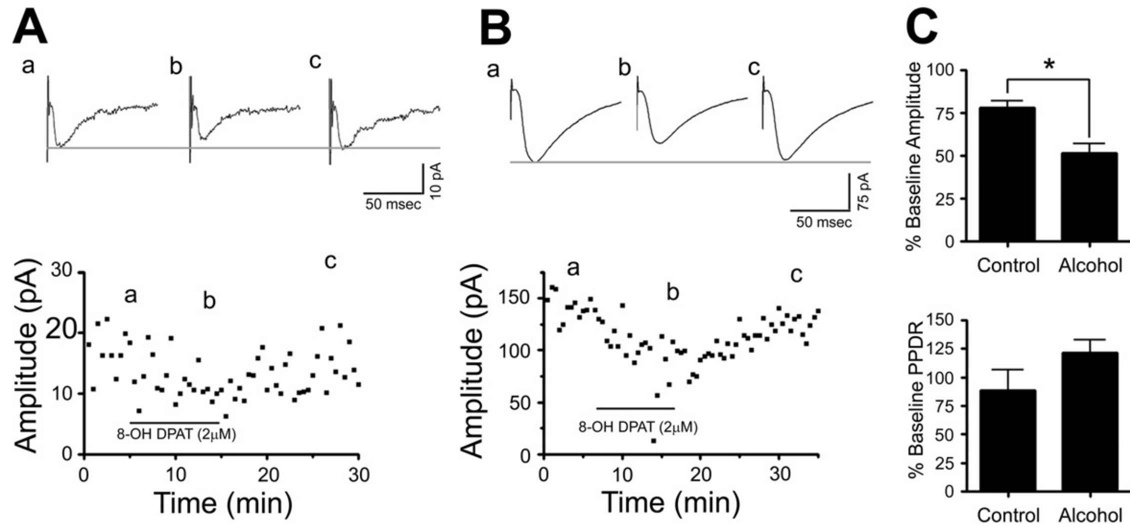

Fig. 5. The 5-HT_{1A} receptor agonist 8-OH DPAT reduced the amplitude of white matter-evoked EPSCs in layer 4/5 AIC neurons. (A, B) Time course of the effect of 8-OH DPAT (2 μM) on EPSC amplitude recorded from neurons from control MR monkeys (A) or ethanol-exposed MR monkeys (B). In (A) and (B), the graph shows the time course of the drug effect in which the letters refer to the corresponding traces shows above, which were taken during baseline (a), peak drug effect (b) and following recovery (c). (C) 8-OH DPAT reduced the amplitude of EPSCs from ethanol-exposed MR monkeys significantly more than EPSCs from MR control monkeys. The magnitude of the decreased EPSC amplitude is paralleled by the magnitude of the increase in the paired pulse depression ratio (PPDR) (* $P<0.05$, Wilcoxon rank test).

keys to identify possible mechanisms underlying the neural adaptations resulting from early-life stress and how they relate to later drinking behavior. The major results of this study are (1) NR monkeys self-administered more ethanol over the course of the study period than MR monkeys; (2) nursery rearing alone resulted in a significantly higher sEPSC frequency in AIC neurons, and (3) lower 5-HT_{1A} and 5-HT_{2A} mRNA levels in the AIC; (4) chronic ethanol and nursery rearing together produced a significant enhancement of serotonergic receptor mRNA expression; and (5) chronic ethanol significantly enhanced sEPSC frequency in AIC neurons, resulting in a more depolarized resting membrane potential. Chronic ethanol also increased the level of 5-HT_{1A} mRNA, which may reflect an upregulation of presynaptic 5-HT_{1A} heteroreceptors (Fig. 6). Based on the role of AIC in motivation and decision making, our findings are consistent with the hypothesis that AIC hyperactivity produced by early-life stress may play a role in the reorganization of networks associated with anxiety and alcohol use disorders. Based on our findings, we propose a model of AIC synaptic function altered by early-life stress and alcohol exposure, whereby hyperexcitable network states are promoted, at least in part, because of modified serotonergic receptor expression and function, causing altered AIC excitability (Fig. 6).

Chronic ethanol self-administration alters AIC

Acute ethanol exposure has been shown to decrease miniature EPSCs (mEPSCs) and persistent spiking activity of rodent cortical neurons (Moriguchi et al., 2007; Tu et al., 2007; Woodward and Pava, 2009), but chronic ethanol exposure has been shown to increase sEPSC and mEPSC frequency in hippocampal and amygdalar neurons (Hendricson et al., 2007; Läck et al., 2007; Roberto et al., 2004). Here, we report an increase in presynaptic glutamate release in ethanol-exposed monkeys as evidenced by (1) an increased sEPSC frequency in AIC neurons, (2) a greater ability to evoke large, polysynaptic barrages in postsynaptic neurons from animals chronically exposed to ethanol, and (3) a significant increase in evoked paired-pulse depression. Although no studies have investigated PPRs in cortical neurons following chronic ethanol exposure, it has been shown that human alcoholics exhibit an increase in synaptophysin I in prefrontal tissue, consistent with enhanced synaptic strength (Henriksson et al., 2008). Based on the short latency to evoked excitatory postsynaptic potentials (EPSPs) following stimulation of the white matter tract adjacent to the recording pipette in AIC, the EPSPs likely arose from afferent glutamatergic projections to AIC, which include the extended amygdala and entorhinal cortex, among other areas (Turner et al., 1980; Insausti et al., 1987; Augustine, 1996; Reynolds and Zahm, 2005). Therefore, our findings are consistent with a model in which chronic ethanol exposure produces either an increase in the excitability of the afferent neurons to AIC or an increase in glutamate release at the axon terminal of these afferent neurons. Because our paired pulse results are derived from white matter stimulation, they support the latter interpretation (Fig. 6). Furthermore, we found that neurons from animals exposed to chronic ethanol had significantly depolarized resting membrane potentials and decreased membrane resistance, both of which would contribute to increased excitability.

Serotonin control of presynaptic release (i.e., 5-HT_{1A} receptor mediated heterosynaptic reduction of axonal excitability and/or synaptic glutamate release) in AIC, which appears to be enhanced after chronic ethanol consumption, may represent a strategy by which the brain compen-

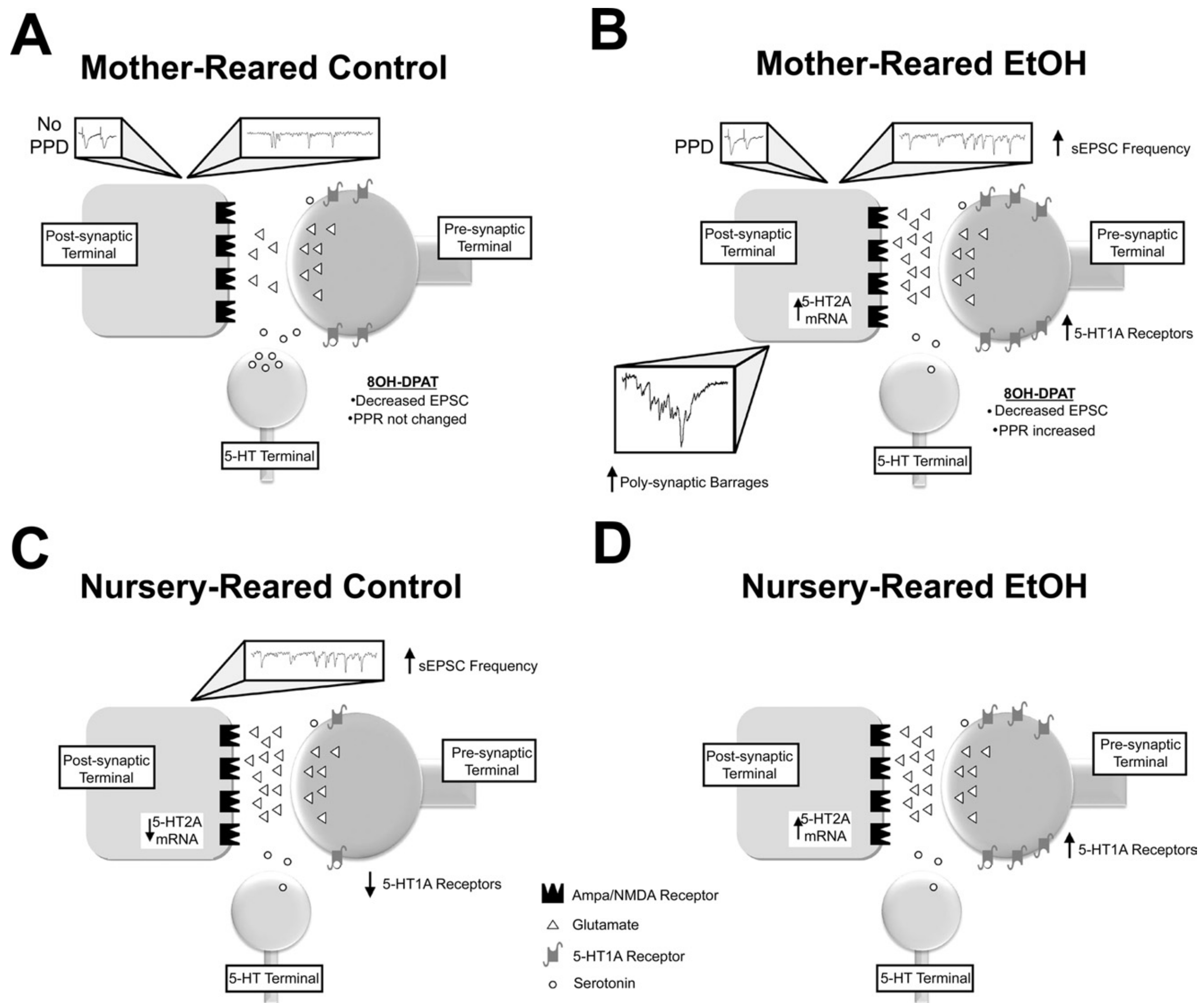

Fig. 6. Proposed model of synaptic changes in animals exposed to alcohol and/or nursery rearing based on experimental findings. (A, B) Chronic exposure to alcohol may increase synaptic strength and expression of 5-HT_{1A} and 5-HT_{2A} receptors when compared with MR controls. Nursery rearing may also increase synaptic strength but decrease expression of 5-HT_{1A} and 5-HT_{2A} receptors. (D) Chronic alcohol exposure may increase 5-HT_{1A} and 5-HT_{2A} receptor expression in nursery-reared monkeys, returning serotoninergic tone closer to baseline levels.

sates for the ethanol-induced increases in synaptic strength and neuronal excitability. While this suggestion is a parsimonious conclusion based on our observations, further investigation will be needed to determine if the altered serotonergic modulation of glutamate release is a compensatory CNS response or a direct result of chronic ethanol exposure. A parallel study using brain tissue from the same monkeys revealed no difference in tissue content of 5-HT or a metabolite of 5-HT, 5-HTIAA; therefore, the effect of ethanol intake on the 5-HT system is likely at the receptor level and not in the levels of available ligand (Huggins et al., in press).

Activation of 5-HT_{1A} receptors has been shown to reduce excitatory synaptic responses in several brain areas by presynaptic mechanisms (Schmitz et al., 1995, 1998, 1999; Cheng et al., 1998; Murakoshi et al., 2001; Torres-Escalante et al., 2004). Here, we have demonstrated similar presynaptic inhibition by 5-HT_{1A} receptors in a monkey model. In slices from ethanol-naive animals, we found a modest reduction in evoked EPSC amplitude and no significant change in PPR in response to 8-OH-DPAT application. In contrast, in slices from animals that chronically self-administered ethanol, we found decreased EPSC amplitude and a significant increase in PPR. The lack of significant change in PPR in the ethanol-naive monkeys is consistent with decreased expression or function of 5-HT_{1A} receptors on presynaptic terminals in AIC; accordingly, reduced magnitude of glutamate release suppression may have been below the threshold for detecting changes in PPR. Alternatively, these effects may be explained by the predominant, proximal axonal location (near the axon hillock) of 5-HT_{1A} receptors on glutamatergic cortical neurons (Azmitia et al., 1996; DeFelipe et al., 2001; Czyrak et al., 2003; Wedzony et al., 2007).

In addition to 5-HT$_{1A}$ receptors, 5-HT$_{2A}$ receptors are also densely expressed in cortical neurons (Amargós-Bosch et al., 2004; Santana et al., 2004) and have been shown to play a role in enhancing EPSCs in rat cortical neurons (Aghajanian and Marek, 1997). We found that chronic ethanol consumption increased 5-HT$_{2A}$ receptor mRNA levels. Activation of 5-HT$_{1A}$ has been shown to reduce serotonin release (Amargós-Bosch et al., 2004), so it is possible that increased 5-HT$_{1A}$ receptor expression decreased serotonin levels in the synaptic cleft, prompting a compensatory increase in postsynaptic 5-HT$_{2A}$ receptor expression. Indeed, paradigms of learned helplessness and chronic stress have been shown to similarly reduce serotonin levels, resulting in higher 5-HT$_{2A}$ receptor densities in rodent prefrontal cortex (Ferretti et al., 1995; Dwivedi et al., 2005).

Alterations because of chronic ethanol consumption in NR monkeys

Early-life stress caused by maternal separation is an established model of future disruptions in social and emotional behavior. In nonhuman primates, nursery rearing has been shown to produce several alterations in physiology and behavior, including increased anxiety and social aggression, decreased social competence, disruptions in sleep-wake patterns, reduced HIAA CSF levels, and increased ethanol consumption (Higley et al., 1996a,b; Barrett et al., 2009). Notably, both increased stress reactivity and sleep disorders associated with early adverse rearing conditions may play a role in the development of alcohol use disorders (Stein and Friedmann, 2005; Barr et al., 2009). Studying the nursery-rearing model is valuable for understanding the neurodevelopmental adaptations that may occur in children exposed to early-life stress who develop addictive behaviors later in life. In this study, both MR and NR animals consumed sufficient volumes of alcohol to classify them as heavy drinkers, and the NR animals consumed modestly, yet significantly, more alcohol than MR animals.

Among ethanol-naive monkeys, we have shown an increase in glutamatergic transmission in the AIC of NR monkeys, as demonstrated by increased sEPSC frequency. Similar to ethanol-exposed monkeys, these findings may indicate either an increase in the excitability of the afferent neurons to AIC or an increase in glutamate release at the axon terminal of these afferent neurons. Furthermore, this increased excitation was correlated with a decrease in 5-HT$_{1A}$ receptor mRNA levels. This reduction is supported by studies demonstrating reduced 5-HT$_{1A}$ receptor binding in brains of rhesus monkeys exposed to early-life stress (Spinelli et al., 2010) as well as reduced 5-HT$_{1A}$ receptor binding in the insula of patients with social anxiety disorder (Lanzenberger et al., 2007) or depression (Bhagwagar et al., 2004; Sargent et al., 2000).

In addition to the rearing-dependent differences among ethanol-naive animals, we also found a significant interaction between rearing and ethanol consumption, indicating significantly greater increases in the gene expression levels of 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors among ethanol-exposed NR animals. These results may suggest an enhanced sensitivity of the serotonin system to stressors in animals that experience early adverse rearing conditions.

The role of the AIC and serotonin in anxiety and addiction potential

The AIC has bidirectional connections with the extended amygdala and entorhinal cortex (Turner et al., 1980; Insausti et al., 1987; Augustine, 1996; Reynolds and Zahm, 2005), receives additional input from the lateral hypothalamus and mediodorsal thalamus (Allen et al., 1991), and projects to the ventral striatum (Haber et al., 1995; Chikama et al., 1997), anterior cingulate (Vogt and Pandya, 1987), and orbital frontal cortices (Ongür and Price, 2000). These inputs and outputs place the AIC in a position to generate conscious motivations by evaluating current and future internal states based on potential interactions with external stimuli (Craig, 2002). Chronic alterations in either the homeostatic set points that drive these internal representations or the processes by which present and future states are evaluated could then affect the activation of cortical neurons encoding these internal representations. Accordingly, insular hyperactivity has been demonstrated in anxiety-prone individuals (Simmons et al., 2006). Rodent models have also shown that early-life stress leads to decreased expression or function of 5-HT receptors later in life (Oreland et al., 2009; Gartside et al., 2003), increased aggression and behaviors consistent with psychopathology (Huot et al., 2001; Ladd et al., 2000) as well as increased propensity for high voluntary ethanol intake (Gustafsson and Nylander, 2006; Roman and Nylander, 2005; for review, see Veenema, 2009). Our results demonstrating increased AIC glutamatergic transmission in NR ethanol-naive monkeys as well as MR ethanol-exposed monkeys are consistent with previous reports in rodents and support a role for the AIC in psychopathology and alcohol seeking (Goldstein and Volkow, 2002; Hyman et al., 2006; Jentsch and Taylor, 1999). We conclude that early-life stress, induced by nursery rearing, resulted in altered expression of components of the 5-HT system and altered excitability of AIC circuits, which rendered the monkeys susceptible to anxiety-like psychopathology, characterized by several behavioral features, including increased alcohol consumption (Barr et al., 2009; Lesch, 2005).

Limitations

Our study was limited in scope for several reasons. First, the number of animals used in this study was limited to the minimum required to reach a level of statistical significance. Second, the amount of alcohol animals consumed by MR and NR monkeys classifies the monkeys as heavy drinkers, and although NR monkeys did consume significantly more alcohol that MR monkeys, the magnitude of the increased consumption was only modest. Thus, the differences we found in gene expression, electrophysiological response to 5-HT challenge, and excitability of AIC neurons between MR and NR animals exposed to alcohol were likely underestimates. Third, while analysis of mRNA provides useful information regarding how nursery rearing or ethanol exposure affects serotonin receptor expression

at the transcriptional level, protein levels measure functional outcome of alterations in transcription, translation, and modifications. Therefore, future studies should assess protein levels of serotonin receptors in the AIC following exposure to nursery rearing and alcohol exposure. Our group has assessed 5-HT_{1A} protein expression in the hippocampus of the same animals as those used in the current study and found that ethanol and nursery rearing induced changes in 5-HT_{1A} protein expression in hippocampus, although the direction of the change depended on the specific hippocampal region (Davenport et al., 2005, 2007). Fourth, because of limited tissue availability, we did not study serotonergic function in other brain areas, so we cannot exclude the possibility that similar changes occur elsewhere in the brain. Fifth, because our recordings were performed blind and cesium was used in the internal solution, we cannot be certain of the neuronal subclass of each recorded neuron.

Our findings demonstrating enhanced presynaptic inhibition of glutamate release through increased 5-HT_{1A} receptor function may be only one mechanism by which the CNS may adapt to aberrant AIC activity. Indeed, pharmacologically targeting 5-HT_{1A} receptors has been shown to be a promising treatment in reducing both alcohol consumption and anxiety (Schreiber et al., 1993; McKenzie-Quirk and Miczek, 2003; Kelai et al., 2006). Buspirone, a partial 5-HT_{1A} receptor agonist, has also been shown to be an effective treatment in alcoholics with comorbid anxiety (Malec et al., 1996). Additionally, FG 5974, a drug with both 5-HT_{1A} receptor agonist and 5-HT_{2A} receptor antagonist properties significantly decreased ethanol self-administration in rats (Roberts et al., 1998), which complements our results showing increased AIC gene expression of both inhibitory 5-HT_{1A} and excitatory 5-HT_{2A} receptors. These results support the idea of specific, brain regional roles of distinct serotonin receptor subtypes in the development and maintenance of alcohol dependence within certain populations of patients. Continuing to identify both the direct and adaptive changes as a result of chronic ethanol exposure will provide a valuable framework for the successful treatment of alcoholism. Furthermore, identifying the neurodevelopmental changes following nursery rearing will aid in understanding the mechanism by which individuals who experience early life stress would later develop addictive behaviors.

Acknowledgments—We thank Dr. Satoru Hayasaka for statistical support. These studies were supported by NIH grants AA014106 and AA015568 (D.P.F.), AA016748 (J.B.D.), AA016852, AA017056, EY018159, and the Tab Williams Family Fund (D.W.G.).

REFERENCES

Aghajanian GK, Marek GJ (1997) Serotonin induces excitatory postsynaptic potentials in apical dendrites of neocortical pyramidal cells. Neuropharmacology 36:589–599.

Alexander GM, Carden WB, Mu J, Kurukulasuriya NC, McCool BA, Nordskog BK, Friedman DP, Daunais JB, Grant KA, Godwin DW (2006) The native T-type calcium current in relay neurons of the primate thalamus. Neuroscience 141:453–461.

Allen GV, Saper CB, Hurley KM, Cechetto DF (1991) Organization of visceral and limbic connections in the insular cortex of the rat. J Comp Neurol 311:1–16.

Amargós-Bosch M, Bortolozzi A, Puig MV, Serrats J, Adell A, Celada P, Toth M, Mengod G, Artigas F (2004) Co-expression and *in vivo* interaction of serotonin1A and serotonin2A receptors in pyramidal neurons of prefrontal cortex. Cereb Cortex 14:281–299.

Andrade R (2011) Serotonergic regulation of neuronal excitability in the prefrontal cortex. Neuropharmacology 61:382–386.

Anwyl R (1999) Metabotropic glutamate receptors: electrophysiological properties and role in plasticity. Brain Res Brain Res Rev 29:83–120.

Augustine JR (1996) Circuitry and functional aspects of the insular lobe in primates including humans. Brain Res Brain Res Rev 22:229–244.

Azmitia EC, Gannon PJ, Kheck NM, Whitaker-Azmitia PM (1996) Cellular localization of the 5-HT1A receptor in primate brain neurons and glial cells. Neuropsychopharmacology 14:35–46.

Barr CS, Dvoskin RL, Gupte M, Sommer W, Sun H, Schwandt ML, Lindell SG, Kasckow JW, Suomi SJ, Goldman D, Higley JD, Heilig M (2009) Functional CRH variation increases stress-induced alcohol consumption in primates. Proc Natl Acad Sci U S A 106:14593–14598.

Barrett CE, Noble P, Hanson E, Pine DS, Winslow JT, Nelson EE (2009) Early adverse rearing experiences alter sleep-wake patterns and plasma cortisol levels in juvenile rhesus monkeys. Psychoneuroendocrinology 34:1029–1040.

Bhagwagar Z, Rabiner EA, Sargent PA, Grasby PM, Cowen PJ (2004) Persistent reduction in brain serotonin1A receptor binding in recovered depressed men measured by positron emission tomography with [11C]WAY-100635. Mol Psychiatry 9:386–392.

Bonson KR, Grant SJ, Contoreggi CS, Links JM, Metcalfe J, Weyl HL, Kurian V, Ernst M, London ED (2002) Neural systems and cue-induced cocaine craving. Neuropsychopharmacology 26:376–386.

Calcagno E, Carli M, Invernizzi RW (2006) The 5-HT(1A) receptor agonist 8-OH-DPAT prevents prefrontocortical glutamate and serotonin release in response to blockade of cortical NMDA receptors. J Neurochem 96:853–860.

Carmichael ST, Price JL (1994) Architectonic subdivision of the orbital and medial prefrontal cortex in the macaque monkey. J Comp Neurol 346:366–402.

Cheng LL, Wang SJ, Gean PW (1998) Serotonin depresses excitatory synaptic transmission and depolarization-evoked Ca2+ influx in rat basolateral amygdala via 5-HT1A receptors. Eur J Neurosci 10:2163–2172.

Chikama M, McFarland NR, Amaral DG, Haber SN (1997) Insular cortical projections to functional regions of the striatum correlate with cortical cytoarchitectonic organization in the primate. J Neurosci 17:9686–9705.

Chua P, Krams M, Toni I, Passingham R, Dolan R (1999) A functional anatomy of anticipatory anxiety. Neuroimage 9:563–571.

Cirulli F, Francia N, Berry A, Aloe L, Alleva E, Suomi SJ (2009) Early life stress as a risk factor for mental health: role of neurotrophins from rodents to non-human primates. Neurosci Biobehav Rev 33:573–585.

Contreras M, Ceric F, Torrealba F (2007) Inactivation of the interoceptive insula disrupts drug craving and malaise induced by lithium. Science 318:655–658.

Corcoran CA, Pierre PJ, Haddad T, Bice C, Suomi SJ, Grant KA, Friedman DP, Bennett AJ (2011) Long-term effects of differential early rearing in rhesus macaques: behavioral reactivity in adulthood. Dev Psychobiol, doi: 10.1002/dev.20613.

Craig AD (2002) How do you feel? Interoception: the sense of the physiological condition of the body. Nat Rev Neurosci 3:655–666.

Czyrak A, Czepiel K, Maćkowiak M, Chocyk A, Wedzony K (2003) Serotonin 5-HT1A receptors might control the output of cortical glutamatergic neurons in rat cingulate cortex. Brain Res 989:42–51.

Davenport AT, Daunais JB, Grnat KA, Friedman DP (2005) The effect of chronic ethanol administration on serotonin 5HT1A-receptor density in the hippocampus of male cynomolgus macaques. Research Society on Alcoholism Annual Meeting Abstract.

Davenport AT, Szeliga KT, Moser VM, Daunais JB, Friedman DP (2007) Effect of chronic ethanol on 5HT1A and serotonin transporter densities in the hippocampus and raphe of mother-reared vs peer-reared macaques. Research Society on Alcoholism Annual Meeting Abstract.

DeFelipe J, Arellano JI, Gómez A, Azmitia EC, Muñoz A (2001) Pyramidal cell axons show a local specialization for GABA and 5-HT inputs in monkey and human cerebral cortex. J Comp Neurol 433:148–155.

Dwivedi Y, Mondal AC, Payappagoudar GV, Rizavi HS (2005) Differential regulation of serotonin (5HT)2A receptor mRNA and protein levels after single and repeated stress in rat brain: role in learned helplessness behavior. Neuropharmacology 48:204–214.

Falk JL (1971) The nature and determinants of adjunctive behavior. Physiol Behav 6:577–588.

Ferretti C, Blengio M, Gamalero SR, Ghi P (1995) Biochemical and behaviour changes induced by acute stress in a chronic variate stress model of depression: the effect of amitriptyline. Eur J Pharmacol 280:19–26.

Fink KB, Göthert M (2007) 5-HT receptor regulation of neurotransmitter release. Pharmacol Rev 59:360–417.

Gartside SE, Johnson DA, Leitch MM, Troakes C, Ingram CD (2003) Early life adversity programs changes in central 5-HT neuronal function in adulthood. Eur J Neurosci 17:2401–2408.

Golden RN (2004) Making advances where it matters: improving outcomes in mood and anxiety disorder. CNS Spectr 9(6 Suppl 4):14–22.

Goldstein RZ, Craig AD, Bechara A, Garavan H, Childress AR, Paulus MP, Volkow ND (2009) The neurocircuitry of impaired insight in drug addiction. Trends Cogn Sci 13:372–380.

Goldstein RZ, Volkow ND (2002) Drug addiction and its underlying neurobiological basis: neuroimaging evidence for the involvement of the frontal cortex. Am J Psychiatry 159:1642–1652.

Gołembiowska K, Dziubina A (2002) Inhibition of amino acid release by 5-HT1B receptor agonist in the rat prefrontal cortex. Pol J Pharmacol 54:625–631.

Grant KA, Leng X, Green HL, Szeliga KT, Rogers LS, Gonzales SW (2008) Drinking typography established by scheduled induction predicts chronic heavy drinking in a monkey model of ethanol self-administration. Alcohol Clin Exp Res 32:1824–1838.

Gustafsson L, Nylander I (2006) Time-dependent alterations in ethanol intake in male Wistar rats exposed to short and prolonged daily maternal separation in a 4-bottle free-choice paradigm. Alcohol Clin Exp Res 30:2008–2016.

Haber SN, Kunishio K, Mizobuchi M, Lynd-Balta E (1995) The orbital and medial prefrontal circuit through the primate basal ganglia. J Neurosci 15:4851–4867.

Harlow HF, Harlow MK (1965) The effect of rearing conditions on behavior. Int J Psychiatry 1:43–51.

Harlow HF, Suomi SJ (1974) Induced depression in monkeys. Behav Biol 12:273–296.

Harlow HF, Zimmermann RR (1959) Affectional responses in the infant monkey; orphaned baby monkeys develop a strong and persistent attachment to inanimate surrogate mothers. Science 130:421–432.

Heinz A, Goldman D, Gallinat J, Schumann G, Puls I (2004) Pharmacogenetic insights to monoaminergic dysfunction in alcohol dependence. Psychopharmacology (Berl) 174:561–570.

Hendricson AW, Maldve RE, Salinas AG, Theile JW, Zhang TA, Diaz LM, Morrisett RA (2007) Aberrant synaptic activation of N-methyl-D-aspartate receptors underlies ethanol withdrawal hyperexcitability. J Pharmacol Exp Ther 321:60–72.

Henriksson R, Kuzmin A, Okvist A, Harper C, Sheedy D, Garrick T, Yakovleva T, Bakalkin G (2008) Elevated synaptophysin I in the prefrontal cortex of human chronic alcoholics. Synapse 62: 829–833.

Higley JD, Hasert MF, Suomi SJ, Linnoila M (1991) Nonhuman primate model of alcohol abuse: effects of early experience, personality, and stress on alcohol consumption. Proc Natl Acad Sci U S A 88:7261–7265.

Higley JD, Suomi SJ, Linnoila M (1996a) A nonhuman primate model of type II alcoholism? Part 2. Diminished social competence and excessive aggression correlates with low cerebrospinal fluid 5-hydroxyindoleacetic acid concentrations. Alcohol Clin Exp Res 20:643–650.

Higley JD, Suomi SJ, Linnoila M (1996b) A nonhuman primate model of type II excessive alcohol consumption? Part 1. Low cerebrospinal fluid 5-hydroxyindoleacetic acid concentrations and diminished social competence correlate with excessive alcohol consumption. Alcohol Clin Exp Res 20:629–642.

Huggins KN, Mathews TA, Locke JL, Szeliga KT, Friedman DP, Bennett AJ, Jones SR (in press) Effects of early life stress on drinking and serotonin system activity in rhesus macaques: 5-hydroxyindoleacetic acid in cerebrospinal fluid predicts brain tissue levels. Alcohol Int Biomed J, in press.

Huot RL, Thrivikraman KV, Meaney MJ, Plotsky PM (2001) Development of adult ethanol preference and anxiety as a consequence of neonatal maternal separation in Long Evans rats and reversal with antidepressant treatment. Psychopharmacology (Berl) 158:366–373.

Hyman SE, Malenka RC, Nestler EJ (2006) Neural mechanisms of addiction: the role of reward-related learning and memory. Annu Rev Neurosci 29:565–598.

Insausti R, Amaral DG, Cowan WM (1987) The entorhinal cortex of the monkey: II. Cortical afferents. J Comp Neurol 264:356–395.

Jentsch JD, Taylor JR (1999) Impulsivity resulting from frontostriatal dysfunction in drug abuse: implications for the control of behavior by reward-related stimuli. Psychopharmacology (Berl) 146:373–390.

Johansson S, Fuchs A, Okvist A, Karimi M, Harper C, Garrick T, Sheedy D, Hurd Y, Bakalkin G, Ekström TJ (2007) Validation of endogenous controls for quantitative gene expression analysis: application on brain cortices of human chronic alcoholics. Brain Res 1132:20–28.

Johnson BA (2004) Role of the serotonergic system in the neurobiology of alcoholism: implications for treatment. CNS Drugs 18: 1105–1118.

Kelai S, Aïssi F, Lesch KP, Cohen-Salmon C, Hamon M, Lanfumey L (2003) Alcohol intake after serotonin transporter inactivation in mice. Alcohol Alcohol 38:386–389.

Kelai S, Hanoun N, Aufrère G, Beaugé F, Hamon M, Lanfumey L (2006) Cannabinoid-serotonin interactions in alcohol-preferring vs. alcohol-avoiding mice. J Neurochem 99:308–320.

Kelai S, Renoir T, Chouchana L, Saurini F, Hanoun N, Hamon M, Lanfumey L (2008) Chronic voluntary ethanol intake hypersensitized 5-HT1A autoreceptors in C57BL/6J mice. J Neurochem 107:1660–1670.

Läck AK, Diaz MR, Chappell A, DuBois DW, McCool BA (2007) Chronic ethanol and withdrawal differentially modulate pre- and postsynaptic function at glutamatergic synapses in rat basolateral amygdala. J Neurophysiol 98:3185–3196.

Ladd CO, Huot RL, Thrivikraman KV, Nemeroff CB, Meaney MJ, Plotsky PM (2000) Long-term behavioral and neuroendocrine adaptations to adverse early experience. Prog Brain Res 122:81–103.

Lanzenberger RR, Mitterhauser M, Spindelegger C, Wadsak W, Klein N, Mien LK, Holik A, Attarbaschi T, Mossaheb N, Sacher J, Geiss-Granadia T, Kletter K, Kasper S, Tauscher J (2007) Reduced serotonin-1A receptor binding in social anxiety disorder. Biol Psychiatry 61:1081–1089.

Lesch KP (2005) Alcohol dependence and gene×environment interaction in emotion regulation: is serotonin the link? Eur J Pharmacol 526:113–124.

Lewis DA (1997) Development of the prefrontal cortex during adolescence: insights into vulnerable neural circuits in schizophrenia. Neuropsychopharmacology 16(6):385–398.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. Methods 25:402–408.

López-Figueroa AL, Norton CS, López-Figueroa MO, Armellini-Dodel D, Burke S, Akil H, López JF, Watson SJ (2004) Serotonin 5-HT1A, 5-HT1B, and 5-HT2A receptor mRNA expression in subjects with major depression, bipolar disorder, and schizophrenia. Biol Psychiatry 55:225–233.

Lorberbaum JP, Kose S, Johnson MR, Arana GW, Sullivan LK, Hamner MB, Ballenger JC, Lydiard RB, Brodrick PS, Bohning DE, George MS (2004) Neural correlates of speech anticipatory anxiety in generalized social phobia. Neuroreport 15:2701–2705.

Machado CJ, Bachevalier J (2003) Non-human primate models of childhood psychopathology: the promise and the limitations. J Child Psychol Psychiatry 44:64–87.

Malec TS, Malec EA, Dongier M (1996) Efficacy of buspirone in alcohol dependence: a review. Alcohol Clin Exp Res 20:853–858.

Maura G, Marcoli M, Tortarolo M, Andrioli GC, Raiteri M (1998) Glutamate release in human cerebral cortex and its modulation by 5-hydroxytryptamine acting at h 5-HT1D receptors. Br J Pharmacol 123:45–50.

Maurel S, De Vry J, Schreiber R (1999) Comparison of the effects of the selective serotonin-reuptake inhibitors fluoxetine, paroxetine, citalopram and fluvoxamine in alcohol-preferring cAA rats. Alcohol 17:195–201.

McKenzie-Quirk SD, Miczek KA (2003) 5-HT1A agonists: alcohol drinking in rats and squirrel monkeys. Psychopharmacology (Berl) 167:145–152.

Moriguchi S, Zhao X, Marszalec W, Yeh JZ, Narahashi T (2007) Effects of ethanol on excitatory and inhibitory synaptic transmission in rat cortical neurons. Alcohol Clin Exp Res 31:89–99.

Muly EC, Maddox M, Smith Y (2003) Distribution of mGluR1alpha and mGluR5 immunolabeling in primate prefrontal cortex. J Comp Neurol 467:521–535.

Murakoshi T, Song SY, Konishi S, Tanabe T (2001) Multiple G-protein-coupled receptors mediate presynaptic inhibition at single excitatory synapses in the rat visual cortex. Neurosci Lett 309:117–120.

Naqvi NH, Bechara A (2009) The hidden island of addiction: the insula. Trends Neurosci 32:56–67.

Naqvi NH, Rudrauf D, Damasio H, Bechara A (2007) Damage to the insula disrupts addiction to cigarette smoking. Science 315:531–534.

Nevo I, Langlois X, Laporte AM, Kleven M, Koek W, Lima L, Maudhuit C, Martres MP, Hamon M (1995) Chronic alcoholization alters the expression of 5-HT1A and 5-HT1B receptor subtypes in rat brain. Eur J Pharmacol 281:229–239.

Nitschke JB, Sarinopoulos I, Mackiewicz KL, Schaefer HS, Davidson RJ (2006) Functional neuroanatomy of aversion and its anticipation. Neuroimage 29:106–116.

Novak MF, Sackett GP (1997) Pair-rearing infant monkeys (Macaca nemestrina) using a "rotating-peer" strategy. Am J Primatol 41(2):141–149.

Ongür D, Price JL (2000) The organization of networks within the orbital and medial prefrontal cortex of rats, monkeys and humans. Cereb Cortex 10:206–219.

Oreland S, Pickering C, Gökturk C, Oreland L, Arborelius L, Nylander I (2009) Two repeated maternal separation procedures differentially affect brain 5-hydroxytryptamine transporter and receptors in young and adult male and female rats. Brain Res 1305(Suppl):S37–S49.

Paulus MP, Stein MB (2006) An insular view of anxiety. Biol Psychiatry 60:383–387.

Pelchat ML, Johnson A, Chan R, Valdez J, Ragland JD (2004) Images of desire: food-craving activation during fMRI. Neuroimage 23:1486–1493.

Perani D, Garibotto V, Gorini A, Moresco RM, Henin M, Panzacchi A, Matarrese M, Carpinelli A, Bellodi L, Fazio F (2008) *In vivo* PET study of 5HT(2A) serotonin and D(2) dopamine dysfunction in drug-naive obsessive-compulsive disorder. Neuroimage 42:306–314.

Reynolds SM, Zahm DS (2005) Specificity in the projections of prefrontal and insular cortex to ventral striatopallidum and the extended amygdala. J Neurosci 25:11757–11767.

Roberto M, Schweitzer P, Madamba SG, Stouffer DG, Parsons LH, Siggins GR (2004) Acute and chronic ethanol alter glutamatergic transmission in rat central amygdala: an *in vitro* and *in vivo* analysis. J Neurosci 24:1594–1603.

Roberts AJ, McArthur RA, Hull EE, Post C, Koob GF (1998) Effects of amperozide, 8-OH-DPAT, and FG 5974 on operant responding for ethanol. Psychopharmacology (Berl) 137:25–32.

Roman E, Nylander I (2005) The impact of emotional stress early in life on adult voluntary ethanol intake-results of maternal separation in rats. Stress 8:157–174.

Santana N, Bortolozzi A, Serrats J, Mengod G, Artigas F (2004) Expression of serotonin1A and serotonin2A receptors in pyramidal and GABAergic neurons of the rat prefrontal cortex. Cereb Cortex 14:1100–1109.

Sargent PA, Kjaer KH, Bench CJ, Rabiner EA, Messa C, Meyer J, Gunn RN, Grasby PM, Cowen PJ (2000) Brain serotonin1A receptor binding measured by positron emission tomography with [11C]WAY-100635: effects of depression and antidepressant treatment. Arch Gen Psychiatry 57:174–180.

Schmitz D, Empson RM, Gloveli T, Heinemann U (1995) Serotonin reduces synaptic excitation of principal cells in the superficial layers of rat hippocampal-entorhinal cortex combined slices. Neurosci Lett 190:37–40.

Schmitz D, Gloveli T, Empson RM, Draguhn A, Heinemann U (1998) Serotonin reduces synaptic excitation in the superficial medial entorhinal cortex of the rat via a presynaptic mechanism. J Physiol 508:119–129.

Schmitz D, Gloveli T, Empson RM, Heinemann U (1999) Potent depression of stimulus evoked field potential responses in the medial entorhinal cortex by serotonin. Br J Pharmacol 128:248–254.

Schreiber R, Opitz K, Glaser T, De Vry J (1993) Ipsapirone and 8-OH-DPAT reduce ethanol preference in rats: involvement of presynaptic 5-HT1A receptors. Psychopharmacology (Berl) 112:100–110.

Seay B, Harlow HF (1965) Maternal separation in the rhesus monkey. J Nerv Ment Dis 140:434–441.

Shannon C, Champoux M, Suomi SJ (1998) Rearing condition and plasma cortisol in rhesus monkey infants. Am J Primatol 46:311–321.

Simmons A, Strigo I, Matthews SC, Paulus MP, Stein MB (2006) Anticipation of aversive visual stimuli is associated with increased insula activation in anxiety-prone subjects. Biol Psychiatry 60:402–409.

Spinelli S, Chefer S, Carson RE, Jagoda E, Lang L, Heilig M, Barr CS, Suomi SJ, Higley JD, Stein EA (2010) Effects of early-life stress on 5-HT1A receptors in juvenile rhesus monkeys measured by PET. Biol Psychiatry 67:1146–1153.

Stein MB, Simmons AN, Feinstein JS, Paulus MP (2007) Increased amygdala and insula activation during emotion processing in anxiety-prone subjects. Am J Psychiatry 164:318–327.

Stein MD, Friedmann PD (2005) Disturbed sleep and its relationship to alcohol use. Subst Abus 26:1–13.

Storvik M, Häkkinen M, Tupala E, Tiihonen J (2009) 5-HT(1A) receptors in the frontal cortical brain areas in Cloninger type 1 and 2 alcoholics measured by whole-hemisphere autoradiography. Alcohol Alcohol 44:2–7.

Suomi SJ (1997) Early determinants of behavior: evidence from primate studies. Br Med Bull 53:170–184.

Torres-Escalante JL, Barral JA, Ibarra-Villa MD, Pérez-Burgos A, Góngora-Alfaro JL, Pineda JC (2004) 5-HT1A, 5-HT2, and GABAB receptors interact to modulate neurotransmitter release probability in layer 2/3 somatosensory rat cortex as evaluated by the paired pulse protocol. J Neurosci Res 78:268–278.

Tu Y, Kroener S, Abernathy K, Lapish C, Seamans J, Chandler LJ, Woodward JJ (2007) Ethanol inhibits persistent activity in prefrontal cortical neurons. J Neurosci 27:4765–4775.

Turner BH, Mishkin M, Knapp M (1980) Organization of the amygdalopetal projections from modality-specific cortical association areas in the monkey. J Comp Neurol 191:515–543.

Veenema AH (2009) Early life stress, the development of aggression and neuroendocrine and neurobiological correlates: what can we learn from animal models? Front Neuroendocrinol 30:497–518.

Verheul R, van den Brink W, Geerlings P (1999) A three-pathway psychobiological model of craving for alcohol. Alcohol Alcohol 34:197–222.

Vivian JA, Green HL, Young JE, Majerksy LS, Thomas BW, Shively CA, Tobin JR, Nader MA, Grant KA (2001) Induction and maintenance of ethanol self-administration in cynomolgus monkeys (*Macaca fascicularis*): long-term characterization of sex and individual differences. Alcohol Clin Exp Res 25:1087–1097.

Vogt BA, Pandya DN (1987) Cingulate cortex of the rhesus monkey: II. Cortical afferents. J Comp Neurol 262:271–289.

Wang Z, Faith M, Patterson F, Tang K, Kerrin K, Wileyto EP, Detre JA, Lerman C (2007) Neural substrates of abstinence-induced cigarette cravings in chronic smokers. J Neurosci 27:14035–14040.

Watts ES, Gavan JA (1982) Postnatal growth of nonhuman primates: the problem of the adolescent spurt. Hum Biol 54:53–70.

Wedzony K, Chocyk A, Kolasiewicz W, Maćkowiak M (2007) Glutamatergic neurons of rat medial prefrontal cortex innervating the ventral tegmental area are positive for serotonin 5-HT1A receptor protein. J Physiol Pharmacol 58:611–624.

Woodward JJ, Pava MJ (2009) Effects of ethanol on persistent activity and up-states in excitatory and inhibitory neurons in prefrontal cortex. Alcohol Clin Exp Res 33:2134–2140.

Zucker RS, Regehr WG (2002) Short-term synaptic plasticity. Annu Rev Physiol 64:355–405.

(Accepted 13 January 2012)
(Available online 21 January 2012)