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Effects of combined brief etomidate anesthesia and postnatal stress on amygdala expression of Cl⁻ cotransporters and corticotropin-releasing hormone and alcohol intake in adult rats

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Abstract

Early life stressors, including general anesthesia, can have adverse effects on adult neural and behavioral outcomes, such as disruptions in inhibitory signaling, stress responsivity and increased risk of psychiatric disorders. Here we used a rat model to determine the effects of combined exposure to etomidate (ET) neonatal anesthesia and maternal separation on adult amygdala expression of genes for corticotropin-releasing hormone (*Crh*) and the chloride co-transporters *Nkcc1* and *Kcc2*, as well as ethanol intake. Male and female Sprague-Dawley rats were subjected to 2 h of ET anesthesia on postnatal days (P) 4, 5, or 6 followed by maternal separation for 3 h on P10 (ET + SEP). During the P91-P120 period rats had daily 2 h access to three 0.05% saccharin solutions containing 0%, 5%, or 10% ethanol, followed by gene expression analyses. The ET + SEP group had increased *Crh* mRNA levels and *Nkcc1/Kcc2* mRNA ratios in the amygdala, with greater increases in *Nkcc1/Kcc2* mRNA ratios in males. A moderate increase in 5% ethanol intake was evident in the ET + SEP males, but not females, after calculation of the ratio of alcohol intake between the last week and first week of exposure. In contrast, control males tended to decrease alcohol consumption during the same period. A brief exposure to ET combined with a subsequent episode of stress early in life induced significant alterations in expression of amygdala *Crh, Nkcc1* and *Kcc2* with greater changes in the Cl⁻ transporter expression in males. The possibility of increased alcohol intake in the exposed males requires further confirmation using different alcohol intake paradigms.

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Brief summary:

Neonatal rat exposure to etomidate followed by a single episode of maternal separation results in long-term increases in amygdala *Nkcc1/Kcc2* mRNA ratio and *Crh* mRNA levels.

Keywords

Etomidate; Stress; Developing brain; NKCC1/KCC2; Alcohol intake

As many as 1.5 million children per year in the U.S. alone are exposed to general anesthesia during the first year of life [1]. Many retrospective epidemiological studies have found that such general anesthesia exposure is associated with later learning disabilities, long-term memory impairment, and attention-deficit/hyperactivity disorder (ADHD) [2–10]. Although establishing a causal role for general anesthesia in such deficits can be challenging, strong support from animal research raises serious concerns that general anesthetics administered during early life may alter subsequent neurodevelopmental trajectories. The development of translational strategies to mitigate these effects is impeded by an incomplete understanding, even in rodent models, of the full range of physiological functions altered by early life anesthetic exposure, as well as the mechanisms of such alterations [11].

In a recent series of publications, we have shown that in neonatal rats, administration of general anesthetic agents that share the ability to enhance GABA type A receptor ($GABA_AR$) activity, such as sevoflurane, propofol and etomidate, induce not only behavioral deficiencies, but also neuroendocrine abnormalities that are reminiscent of those induced by neonatal exposure to stress [12–17]. These neuroendocrine abnormalities, which are more robust in males, include increased anxiety-like behavior and exacerbated corticosterone responses to stress. In addition, the rats have elevated corticotropin-releasing hormone (*Crh*) mRNA levels in the hypothalamus, as well as up- and down-regulated hypothalamic mRNA levels of the Cl^- transporters Na^+-K^+-$2Cl^-$ (*Nkcc1*) and K^+-$2Cl^-$ (*Kcc2*), respectively [13–17].

Normally, an elevated NKCC1/KCC2 ratio supports depolarizing (excitatory) GABAAR signaling in immature neurons, while developmental reduction in the NKCC1/KCC2 ratio, mainly due to increases in neuronal-specific KCC2, forms the basis for the shift toward inhibitory $GABA_AR$ signaling later on [18–20]. The GABA-initiated depolarization and resulting Ca^{++} influx in immature neurons regulate a wide spectrum of developmental processes from gene expression to synapse formation [18–20]. Obviously, an early-in-life anesthesia-induced delay in $GABA_AR$ signaling transition to inhibitory [13,16,17] may affect numerous $GABA_AR$ signaling-regulated functions in the body. One example may be dysregulated stress responsivity because of impaired $GABA_AR$-mediated inhibitory control of CRH-secreting neurons [21,22]. Impaired NKCC1/KCC2 maturation and the resulting shift in $GABA_AR$ signaling toward excitatory, as well as dysregulated stress systems, have been implicated in development of psychiatric conditions, including male-predominant autism spectrum disorders (ASD) and schizophrenia, as well as increased ethanol intake in adulthood [23–27]. Of special interest, clinical studies report a high level of co-occurrence

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of ASD with ADHD, with the latter being significantly increased in those who had early in life exposure to general anesthesia.

Given that amygdala dysfunction may play a role in ADHD and ASD etiology [28–30], and that CRH and GABAergic neurotransmission in the amygdala are involved in regulation of alcohol consumption in rodents [31,32], in the present study we used rats to test whether relatively brief neonatal exposure to ET combined with a single episode of maternal separation affects the expression *of Nkcc1, Kcc2* and *Crh* in the amygdala of adult rats, as well as their alcohol intake. This anesthesia regimen, which was also used in our recently published study [13], may be of significant translational value, as many infants who require exposure to general anesthesia are also exposed to stressors, such as surgery, diseases, pain, and/or psychosocial stress.

Materials and methods

Animals

All experimental procedures were approved by the University of Florida Institutional Animal Care and Use Committee (Gainesville, FL). Breeding pairs of Sprague-Dawley rats were purchased from Charles River and bred at the University of Florida animal care facility. Prior to the alcohol consumption studies (see below) rats were housed under controlled illumination (12-h light/dark, lights on at 0700) and temperature (23–24 °C) with free access to food and water. Within 24 h of delivery, litters were culled to 12 pups. At the age of 21 days, pups were weaned and housed in sex-matched groups of two for the rest of the study. At the beginning of anesthesia with etomidate, the pups were well nourished, as judged by their stomachs being full of milk (detectable through the transparent abdominal wall). To control for litter variability, pups from the same litter were used for different experimental conditions. Multiple sets of rats were used in the experiments. The data reported in this study were collected from 94 male and 87 female rats, obtained from a total of 27 litters.

Experimental groups

The experimental groups and number of animals per group are described in Table 1. During etomidate anesthesia and maternal separation, rat pups were kept in a temperature-controlled chamber (+37 °C) with a continuous supply of 30% oxygen in air. Gas monitoring was performed using a calibrated Datex side stream analyzer (Datex-Ohmeda, Helsinki, Finland), which sampled from the interior of the chamber. On either P4, P5, or P6, the etomidate group (ET) received 8 mg/kg of etomidate intraperitoneally for induction of anesthesia followed by second injection of etomidate (4 mg/kg, intraperitoneally) 50 min after the first administration. At these doses of ET, the pups did not exhibit a righting reflex for 2 h. We have shown previously that this ET administration regimen induces a depth of anesthesia that is in a similar range to that induced by 2.1% sevoflurane [13,15]. As a control for the period of maternal separation during ET anesthesia, rats in the Sep condition were separated from the dams for 120 min in a temperature-controlled chamber with a continuous supply of 30% oxygen in air without exposure to anesthesia. To simulate postanesthesia stress, half of the rats in the ET and Sep groups were subjected to maternal separation for 3 h at P10 (the etomidate plus maternal separation group (ET + SEP) and the maternal

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separation at P4, 5 or 6 plus maternal separation at P10 (Sep + SEP) group, respectively). To assess the effects of maternal separation alone, a separate group of rats was subjected to maternal separation for 3 h at P10 only (the SEP group). Finally, a group of control rats was subjected to animal facility rearing only (the Con group; i.e., neither ET nor separation).

Alcohol consumption studies

Ethanol intake was assessed between P91 and P120 using a modified drinking-in-the-dark model with three bottles containing 0.05% saccharine, 5% ethanol in 0.05% saccharine, and 10% ethanol in 0.05% saccharine [33]. For this experiment, the rats were individually housed in a designated room with a reverse light-dark cycle (lights off at 0800 and on at 2000). This ensured the rats would be in their active (dark) cycle when the alcohol was available. The rats underwent a one week habituation period to this new environment. During this time they did not receive any alcohol and had access to water and food *ad libitum.* Following the habituation period, the rats were given 2 consecutive days of 24-h access to the three bottles, for a total of 48 h. Ethanol access always began 3 h after the start of the dark cycle and fresh bottles were exchanged after 24 h. This was termed the acquisition period. After the acquisition period the rats had a 24 h “off day” on which no alcohol was provided, before beginning the experimental period. For the experimental period the rats were given 2-h access to the three solutions for 30 consecutive days, again beginning 3 h after the start of the dark cycle. The bottles were placed on the rats’ cages each day for 2 h, with the position of the 3 bottles randomized across days. Bottles were weighed before and after each drinking period and weights were recorded. Rats were weighed weekly in order to account for growth when calculating grams of ethanol consumed/kilograms of body weight. After observing the normal drinking habits of the rats, we determined that amounts greater than 35 grams were not plausible and could therefore be deemed a “leak day”. Less than 1 percent of the consumption data met this criterion, and they were not included in the data analysis. The rats had water ad lib outside the 2h/day when the 3 bottles were on the cage.

Analyses of mRNA levels for Nkcci, Kcc2 and Crh

Three weeks after finishing the alcohol intake study, rats were deeply anesthetized with sevoflurane and decapitated. Whole brains were removed and immediately put in a prefrozen rat brain matrix. The amygdala regions were dissected from the coronal slices, according to the atlas of Paxinos and Watson [34]. All the tissues were immediately submerged in RNAlater solution (Invitrogen, Carlsbad, CA) and kept overnight at 4°C, followed by long-term storage at −20 °C. *Crh, Nkcci*, and *Kcc2* mRNA in the amygdala were analyzed via real-time reverse transcription-PCR (qRT-PCR) in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). RNA was extracted from the samples using an RNeasy Plus Kit (Qiagen, Valencia, CA, USA), reverse transcribed with a high-capacity cDNA reverse transcription kit (Bio-Rad Laboratories, Hercules, CA, USA), and analyzed via qRT-PCR using an Applied Biosystems StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA). Oligonucleotide primers and Taqman probes specific for the above genes were obtained from Applied Biosystems (Carlsbad, CA, USA): *Crh* (Rn01462137_m1), *Nkcc1* (Rn00582505_m1), *Kcc2* (Rn00592624_m1). Data were normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) mRNA

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(Rn01775763_g1). Gene expression was calculated using the CT method and data presented as relative fold change from that of control animals.

Statistical analysis

For each condition, 0%, 5%, and 10% w/v ethanol, intake was indexed by three measures. For the first and last week of exposure, intake (grams) on each day was normalized to body weight (kilograms) then averaged for each animal (i.e. mean daily intake on the first or last 7 days, g/kg bodyweight). Additionally, the ratio of each of these measures was taken (mean daily intake last 7 days/mean daily intake first 7 days, referred to as the intake ratio). All measures were summarized as mean ± SEM. Outliers (intake > 35 g in one day) were recorded as missing before measure construction.

ANOVA or Welch ANOVA (one-way) was used to assess mean differences for each intake measure across combined treatment and separation groups (Con, Sep, ET, SEP, Sep + SEP, ET + SEP), separately by sex. Welch ANOVA and the separate analyses by sex were conducted due to violations of the homogeneity of variance assumption for comparisons between sexes and across treatment conditions for some outcomes. ANOVA (one-way) was used to analyze gene expression data. Bonferroni or Tamhane (for unequal variances) tests were used for post-hoc multiple comparisons. $P < 0.05$ was considered statistically significant. All analyses were conducted using SPSS v. 24 (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp.).

Results

mRNA levels of Nkcc1, Kcc2 and Crh in the amygdala

In male rats, there were statistically significant group differences in amygdala *Nkcc1* mRNA levels ($F_{(5,21)}$ =5.971, P = 0.001; Fig. 1A), and Bonferroni post-hoc comparisons showed that the ET + SEP group had elevated *Nkcc1* mRNA levels (P = 0.002 vs Con; P = 0.005 vs Sep; and P = 0.015 vs SEP). There were also statistically significant group differences in amygdala *Kcc2* mRNA levels ($F_{(5,21)}$ = 11.283, P < 0.001; Fig. 1B). The Bonferroni post-hoc tests showed that the ET + SEP group had reduced levels of *Kcc2* mRNA (P = 0.002 vs Con; P < 0.001 vs Sep; and P = 0.023 vs SEP). In addition, the Sep + SEP group had decreased levels of *Kcc2* mRNA in the amygdala compared to the Sep group (P < 0.001) and the Con group (P = 0.015). Moreover, the ET group had decreased the amygdala levels of *Kcc2* mRNA when compared to the Sep group (P = 0.007). Finally there were statistically significant group differences in the resulting *Nkcc1/Kcc2* mRNA ratio ($F_{(5,21)}$ = 4.611, P = 0.005; Fig. 1C). Post-hoc tests showed that the *Nkcc1/Kcc2* ratio was higher in the ET + SEP group (P = 0.021 vs Con; P = 0.019 vs Sep; P = 0.02 vs SEP).

In female rats, there were no statistically significant group differences in amygdala *Nkcc1* mRNA levels ($F_{(5,22)}$ = 1.562, P = 0.212; Fig. 1D). There were statistically significant group differences in amygdala *Kcc2* mRNA levels ($F_{(5,22)}$ = 5.837, P = 0.001; Fig. 1E), with Bonferroni post-hoc tests showing that the ET + SEP group had decreased Kcc2 mRNA levels (P = 0.043 vs Con; P = 0.001 vs Sep; and P = 0.022 vs SEP). There were also statistically significant group differences in amygdala *Nkcc1/Kcc2* mRNA ratios ($F_{(5,22)}$ =

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6.073, $P = 0.001$; Fig. 1F). Post-hoc tests showed that the ET + SEP group had higher Nkcc1/Kcc2 mRNA ratios ($P = 0.004$ vs Con; $P = 0.003$ vs Sep; $P = 0.003$ vs SEP; and $P = 0.014$ vs ET). Finally, the ET + SEP-induced increase in *Nkcc1/Kcc2* mRNA ratio was greater in males than in females ($t_{(8)} = 2.955$, $P = 0.0183$, Fig. 1C,F).

In male rats, there were statistically significant group differences in amygdala *Crh* mRNA levels ($F_{(5,24)} = 14.157$, $P < 0.001$; Fig. 1G), with Bonferroni post-hoc tests showing that male rats in the ET + SEP group had increased amygdala *Crh* mRNA levels ($P < 0.001$ vs each other experimental group). There were also statistically significant group differences in amygdala *Crh* mRNA levels in female rats ($F_{(5,24)} = 10.569$, $P < 0.001$; Fig. 1H). Post-hoc tests showed that rats in the ET + SEP group had elevated *Crh* mRNA levels ($P < 0.001$ vs Con and Sep; $P = 0.001$ vs SEP and Sep + SEP; and $P = 0.045$ vs ET). The ET + SEP-induced increases in *Crh* mRNA levels in the amygdala were similar in male and female rats ($t_{(8)} = 1.716$, $P = 0.125$, Fig. 1G,H).

5% Alcohol, 10% alcohol and saccharine intake

In male rats, there were no statistically significant group differences in mean intake of 5% alcohol in the first 7 days ($F_{(5,88)} = 2.174$, $P = 0.064$; Fig. 2A) or last 7 days ($F_{(5,88)} = 1.627$, $P = 0.161$; Fig. 2B). To determine whether early life exposure to etomidate and the stress of maternal separation changed alcohol consumption across the 30 day exposure to alcohol, we compared the ratio of alcohol consumption between the last and first week (the intake ratio). There were statistically significant group differences for the intake ratio (Welch $F_{(5,40)} = 7.484$, $P < 0.001$; Fig. 2C). The Tamhane post-hoc tests showed that compared to the Con group, only the ET+SEP group ($P < 0.001$) had a higher intake ratio.

In female rats, there were no statistically significant group differences in mean intake during first 7 days ($F_{(5,81)} = 0.867$, $P = 0.507$; Fig. 2D), last 7 days ($F_{(5,81)} = 1.426$, $P = 0.224$; Fig. 2E) or in intake ratio ($F_{(5,81)} = 0.315$, $P = 0.903$; Fig. 2F).

*T*here were no statistically significant group differences in mean intake of 10% alcohol or saccharine during the first 7 days, last 7 days or in intake ratio in male and female rats. Also, there were no statistically significant group differences in mean body weight of male rats during the first 7 days ($F_{(5,88)} = 0.515$, $P = 0.764$), last 7 days ($F_{(5,88)} = 0.353$, $P = 0.879$) or in their the body weight ratio (mean body weight last 7 days / mean body weight first 7 days) ($F_{(5,88)} = 0.880$, $P = 0.498$). Similarly, there were no statistically significant group differences in mean body weight of female rats during the first 7 days ($F_{(5,81)} = 0.285$, $P = 0.92$), last 7 days ($F_{(5,81)} = 0.695$, $P = 0.629$) or in their body weight ratio ($F_{(5,81)} = 0.185$, $P = 0.968$).

Discussion

Neonatal exposure to general anesthesia can cause a range of adverse neural and behavioral outcomes in adulthood. Here we report the novel findings that early life exposure to a brief ET anesthesia combined with a single episode of maternal separation induces adult upregulation of *Nkcc1* and downregulation of *Kcc2* Cl^- co-transporter expressions in the amygdala in a sex-dependent manner, suggesting a fundamental change in functioning of the

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main inhibitory neurotransmitter GABA. Amygdala expression of *Crh*, one of principal hormones of the stress response system, was also significantly enhanced in adult rats neonatally exposed to ET and maternal separation. In addition to profound differences in gene expression, adult male (but not female) rats exposed to ET plus maternal separation exhibited opposite changes in consumption of 5% alcohol compared to their unexposed counterparts (an increase and decrease, respectively, across 30 days of access).

The developmental down-regulation of NKCC1 and up-regulation of KCC2 determines the ontogenetic shift in $GABA_A$R signaling from depolarizing/stimulatory to inhibitory. This shift occurs at older ages in male compared to female pups [35–37], suggesting that this may be one of reasons for the sex-specific (and more prominent in males) developmental effects of GABAergic anesthetics. Our current findings, together with previously published data [13,17], demonstrate that GABAergic anesthetics administered to neonatal rats may not only exacerbate $GABA_A$R-mediated depolarization/stimulation at the time of exposure of neonatal rats to anesthesia, but may also induce long-term impairment in the transition of $GABA_A$R signaling to inhibitory that persists into adulthood. Considering that similar molecular mechanisms, such as an increased NKCC1/KCC2 ratio and impaired $GABA_A$R-mediated inhibition, may be involved in early life environmental stressor-induced dysregulation of stress response systems and neurobehavioral abnormalities [38–40], it is plausible that developmental effects of early life anesthesia and environmental stressors may be additive or synergistic. The concept of the cumulative effects of early life anesthesia and subsequent environmental stressor(s) is supported by our current findings in the amygdala and our recently published data on the effects of etomidate or sevoflurane in combination with a subsequent single episode of maternal separation in the hypothalamus and hippocampus, respectively [13,16]. These findings may offer a mechanistic explanation of the observation that human patients may develop anesthesia-related abnormalities even though the typical anesthesia duration in humans, when normalized to the life span, is much shorter than that shown to induce developmental deficiencies in rodents [41]. The environmental stressors, such as diseases or psychosocial stress, may contribute to developmental outcomes of early life anesthetic exposure in humans. In future studies, it will be important to determine whether significant developmental effects can be detected in animals exposed to even shorter anesthetic durations when post-anesthesia stress regimens more closely model the stressful conditions human subjects may experience.

Although it was not robust, a significant increase in 5% alcohol intake across 30 days of access in male, but not female, rats exposed to ET plus maternal separation could be detected when compared to their control counterparts that exhibited an opposite trend in alcohol consumption. Several factors, including type of anesthetic, duration of anesthesia, and strength of stressor, may contribute to this specific effect. Etomidate is a general anesthetic that shares with propofol and sevoflurane the property of enhancing $GABA_A$R activity. Unlike propofol and sevoflurane, however, ET is known to disrupt adrenal synthesis of corticosterone by inhibiting 11-β-hydroxylase [42]. Hence, ET may activate the HPA axis via reduced negative feedback by corticosterone. In addition, ET-induced 11-β-hydroxylase inhibition increases substrate availability for neuroactive steroid synthesis, which, by enhancing $GABA_A$R depolarization/stimulation, may further increase CRH production [42, 43]. Both increased CRH and corticosterone levels have been linked to greater intake of

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drugs of abuse, particularly alcohol [44]. Because of expected lower corticosterone levels with ET, due to the anesthetic-caused inhibition of the corticosterone producing enzyme, 11-β-hydroxylase, the effect on alcohol intake might not be as robust as it might have been with other anesthetics. A comparison of the alcohol intake-inducing effects of ET and other GABAergic anesthetics may provide a more complete understanding of the role of neonatal anesthesia in predisposition to alcohol consumption later in life.

Expression and function of CRH in the amygdala has been linked to alcohol intake, particularly in the presence of stressors [45, 46]. Hence, it is possible that the increase in amygdala *Crh* expression in rats exposed to ET plus maternal separation was causally linked to the increase in alcohol intake. Notably, however, the increased alcohol intake was only evident in male rats, whereas the increase in *Crh* expression was evident in both sexes. In contrast, the increase in amygdala *Nkcc1/Kcc2* ratio was evident only in males, suggesting that it might be more closely related to the change in alcohol intake. Indeed, increased amygdala excitability (the expected outcome of a greater *Nkcc1/Kcc2* ratio) would be expected to be anxiogenic, which should promote alcohol drinking [46]. In future studies it will be important to determine whether the changes in expression of these genes and in levels of their respective proteins are localized to specific subregions of the amygdala, as well as to other distinct brain regions. Of direct relevance to this latter point, recent studies have linked downregulated KCC2 and the consequent shift toward more excitatory $GABA_AR$ signaling in the ventral tegmental area (VTA) to increased alcohol self-administration, while a KCC2 agonist administered into the VTA reduced alcohol consumption [26, 27].

Although neonatal anesthesia with ET and subsequent maternal separation induced greater changes in the alcohol intake ratio in males, females in general, independent of treatment condition, exhibited greater mean alcohol intake compared to males (Fig. 2). Greater alcohol consumption by adult female rats is consistent with data in the literature [47, 48]. Vetter-O'Hagen et al. [49] suggested that greater alcohol intake by adult female rats can be explained by the suppressive role of the male sex hormone testosterone on ethanol intake in adult male rats, as castration increases ethanol intake and testosterone replacement is sufficient to return ethanol intake to pre-castration levels. As $GABA_AR$ signaling plays a crucial role in regulating the function of steroid hormones in general, and sex steroids in particular [50], investigation of effects of developmental exposure to GABAergic anesthetics on adult sex hormones is an important topic of future work.

In summary, a combination of relatively brief ET anesthesia and a single episode of maternal separation during early postnatal development induced significant alterations in expression of amygdala *Nkcc1, Kcc2* and *Crh*, as well as moderate increase in alcohol intake in adulthood, with greater changes in males.

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Highlights

- Subsequent stress potentiated developmental effects of neonatal etomidate in rats
- The effects included altered expressions of Ch transporters and *Crh* in the amygdala
- Exposed and unexposed rats exhibited opposite changes in alcohol consumption
- The effects were sex-specific with greater changes in males

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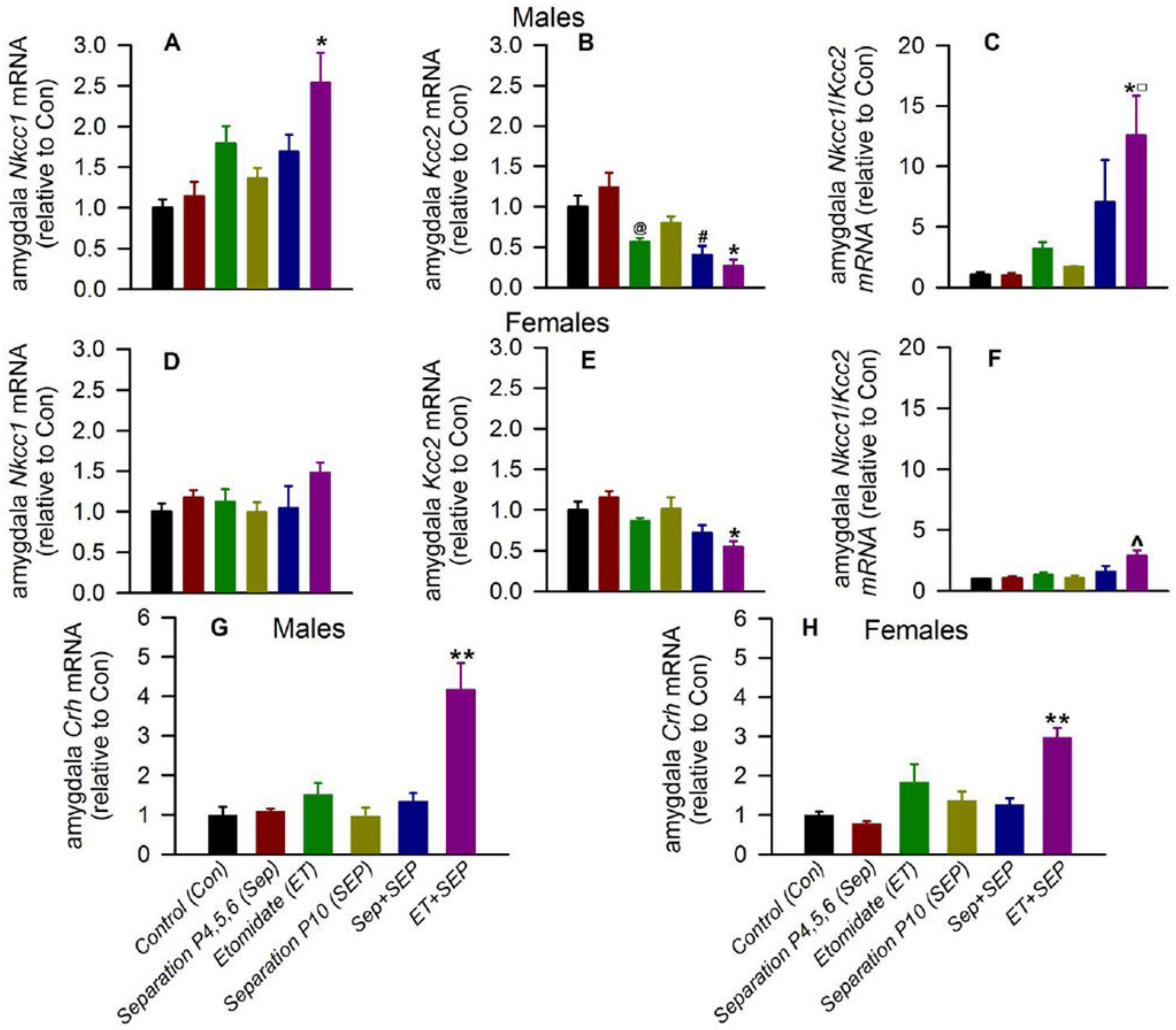

Figure 1.

Anesthesia with etomidate (ET) for 2 h on postnatal days (P) 4, 5 or 6 followed by maternal separation for 3 h at P10 induced alterations in amygdala levels of *Nkcc1, Kcc2* and *Crh* mRNA in adult rats. Amygdala tissue samples were collected at P>120 for qRT-PCR analyses. Shown are levels of *Nkcc1* mRNA (A) and *Kcc2* mRNA (B), and the resulting *Nkcc1/Kcc2* mRNA ratios (C) in male rats. The respective data for female rats are shown in (D) *Nkcc1* mRNA levels, (E) *Kcc2* mRNA levels and (F) the resulting *Nkcc1/Kcc2* mRNA ratios. Data normalized against control are means ± SEM from 4-5 rats per treatment group. *P < 0.05 vs. Con, Sep, and SEP; #P < 0.05 vs. Con and Sep; @P < 0.05 vs. Sep; ^P < 0.05 vs Con, Sep, SEP, and ET; □P < 0.05 vs ET + SEP females). (G,H) Shown are levels of *Crh* mRNA in males (G) and females (H). Data normalized against control are means ± SEM from 4-5 rats per treatment group. **P < 0.05 vs. all experimental groups; Color coding in Fig. 1G, H is applicable to the entire figure.

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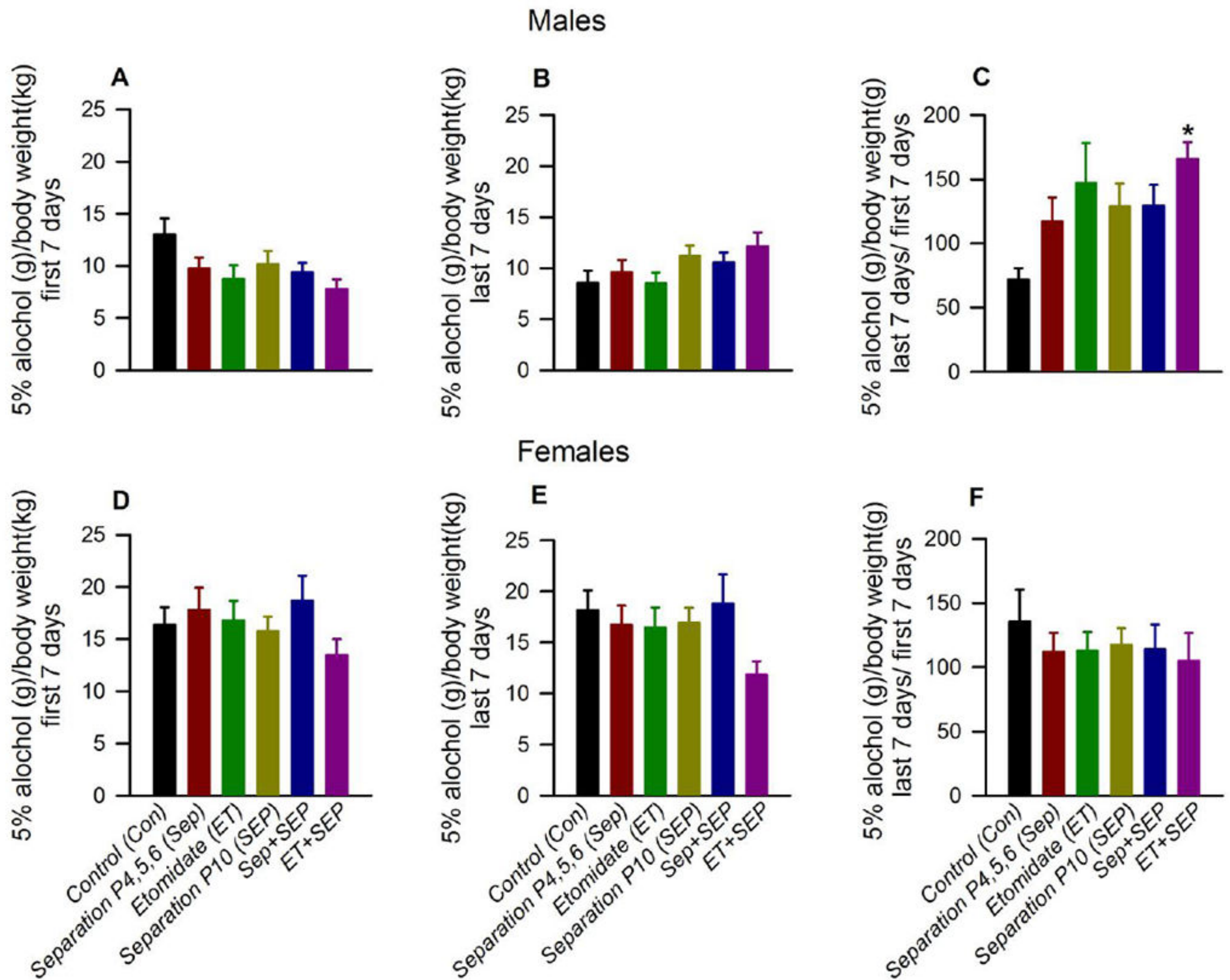

Figure 2.

Anesthesia with etomidate (ET) for 2 h at postnatal days (P) 4, 5 or 6 followed by maternal separation for 3 h at P10 led to an increase in 5% ethanol intake in male, but not female, adult rats. Shown are: (A) mean daily 5% ethanol intake first 7 days [ethanol intake (grams) on each day was normalized to body weight (kilograms) then averaged for each animal]; (B) mean daily intake last 7 days; and (C) the intake ratio (mean daily intake last 7 days / mean daily intake first 7 days, in g ethanol / kg body weight) for male rats. (D-F) show respective data for female rats: (D) mean daily intake first 7 days; (E) mean daily intake last 7 days; and (F) the intake ratio. Data are means ± SEM from 13-16 rats per experimental group. *$P < 0.05$ vs. the Con group. Color coding in Fig. 2D-F is applicable to the entire figure.

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Table 1.

Group Number	Treatment	Number of animals per group	
		male	female
1	Facility rearing only (the Con group)	16	16
2	Maternal separation for 120 min at P4, 5 or 6 (the Sep group)	15	15
3	Anesthesia with Etomidate for 120 min at P4, 5 or 6 (the ET group)	16	14
4	Maternal separation for 180 min at P10 only (the SEP group)	16	15
5	Maternal separation for 120 min at P4, 5 or 6 plus maternal separation for 180 min at P10 (the Sep + SEP group)	16	14
6	Anesthesia with Etomidate for 120 min at P4, 5 or 6 plus maternal separation for 180 min at P10 (the ET + SEP group)	15	13