Early drug use is associated with increased substance use disorders later in life (Dawson et al., 2008) and drugs such as nicotine, alcohol, or cannabis are likely tested before heavier substances such as psychostimulants or opioids (Degenhardt et al., 2009). Adolescent alcohol use may increase the risk of alcohol use disorder (AUD) in adulthood by disturbing the normal brain development during this sensitive period (Crews et al., 2007; Spear, 2000), but the underlying neurobiological mechanisms are unclear.

Opioids have been implicated in the mechanism of action of alcohol and in propensity for AUD (Drews and Zimmer, 2010). The effectiveness of opioid antagonists to reduce alcohol intake in both animals and humans further supports opioid involvement (Vengeliene et al., 2008). Previous studies have shown that acute and long-term exposure to alcohol affects expression and levels of opioid peptides in a number of brain areas in animals (Chang et al., 2010; Cowen and Lawrence, 2001; Jarjour et al., 2009; Lam et al., 2008; Marinelli et al., 2006; Palm et al., 2012; Schulz et al., 1980) and humans (Bazov et al., 2013). However, most studies have been done in adult subjects and very little is known about alcohol’s acute and long-lasting effects on opioid peptides in the adolescent brain. To study this, animal models of adolescent drinking are of great importance.

The age window identified as adolescence in rodents is between postnatal day 28 and 50 (Spear, 2000), and studies show that alcohol intake (Daoura et al., 2011; García-Burgos et al., 2009) and the response to alcohol is significantly altered in comparison with adults (Spear, 2000). Furthermore, we have previously shown that alcohol intake during adolescence leads to altered dopamine response in

**Key Words:** Adolescence, Amygdala, Cingulate Cortex, Endogenous Opioids, Ethanol.

**Background:** Endogenous opioids are implicated in the mechanism of action of alcohol and alcohol affects opioids in a number of brain areas, although little is known about alcohol’s effects on opioids in the adolescent brain. One concern, in particular when studying young animals, is that alcohol intake models often are based on single housing that may result in alcohol effects confounded by the lack of social interactions. The aim of this study was to investigate short- and long-term alcohol effects on opioids and the influence of housing conditions on these effects.

**Methods:** In the first part, opioid peptide levels were measured after one 24-hour session of single housing and 2-hour voluntary alcohol intake in adolescent and adult rats. In the second part, a model with a cage divider inserted during 2-hour drinking sessions was tested and the effects on opioids were examined after 6 weeks of adolescent voluntary intake in single-and pair-housed rats, respectively.

**Results:** The effects of single housing were age specific and affected Met-enkephalin-Arg^6^-Phe^7^- (MEAP) in particular. In adolescent rats, it was difficult to distinguish between effects induced by alcohol and single housing, whereas alcohol-specific effects were seen in dynorphin B (DYNB), beta-endorphin (BEND), and MEAP levels in adults. Voluntary drinking affected several brain areas and the majority of alcohol-induced effects were not dependent on housing. However, alcohol effects on DYNB and BEND in the amygdala were dependent on housing. Housing alone affected MEAP in the cingulate cortex.

**Conclusions:** Age-specific housing- and alcohol-induced effects on opioids were found. In addition, prolonged voluntary alcohol intake under different housing conditions produced several alcohol-induced effects independent of housing. However, housing-dependent effects were found in areas implicated in stress, emotionality, and alcohol use disorder. Housing condition and age may therefore affect the reasons and underlying mechanisms for drinking and could potentially affect the outcome of a number of end points in research on alcohol intake.
adulthood (Palm and Nylander, 2014) and induces effects on opioid peptide levels (Palm et al., 2013). Despite this, in the literature to date, little attention has been given to voluntary alcohol intake during adolescence and its neurobiological consequences. Further studies are therefore warranted and the first part of this study aimed to increase knowledge about alcohol-induced effects on opioid peptide levels during adolescence by studying the age-specific effects of 1 session of voluntary alcohol intake during adolescence or adulthood. This part also included a comparison between single- and group-housed animals, as the social environment during adolescence largely influence the normal development of rats. Animal models of alcohol intake often involve single housing of the animals and this is of great concern for adolescent alcohol intake models because social isolation during this period have been shown to alter behavior and neurochemistry (Fone and Porkess, 2008) and increase alcohol intake in adulthood (Becker et al., 2011; Chappell et al., 2013). Social isolation in adolescent rats has also been shown to affect opioid peptide levels, particularly Met-enkephalin-Arg^6^Phe^7^ (MEAP), in several brain areas (Granholm et al., 2014). Alcohol intake models based on social isolation during adolescence may therefore produce alcohol-induced effects confounded by the lack of social environment. The hypothesis was that single housing alone would produce effects and that these would be different between adolescents and adults. Indeed, this was also seen in the results of the first part of the study where it was particularly difficult to separate the effects of single housing and alcohol in the adolescent rats.

Thus, the aim of the second part was to test a model of prolonged adolescent voluntary alcohol intake and evaluate the effects on opioid peptide levels under conditions of single housing and pair housing, to separate effects of social environment and alcohol.

**MATERIALS AND METHODS**

**Animals**

All animals were kept in standard type IV cages (59 x 38 x 20 cm) with wood chip bedding and a wooden house, in a temperature- (22 ± 1°C) and humidity- (50 ± 10%) controlled room with a reversed 12-hour light/dark cycle, with lights off at 06:00 hour. They had ad libitum access to pellet food (Type R36; Lantmännen, Kimstad, Sweden) and water. Water access was restricted only during the 2-hour alcohol sessions in part 1 of the study. All animal experiments were performed under a protocol approved by the Uppsala Animal Ethical Committee and followed the guidelines of the Swedish Legislation on Animal Experimentation (Animal Welfare Act SFS1998:56) and the European Communities Council Directive (86/609/EEC).

**Experimental Outline**

**Part 1.** Three-week-old (N = 40) or 9-week-old (N = 40) male Wistar rats (RccHan:WI; Harlan Laboratories B.V., Horst, the Netherlands) were group housed, that is, 3 or 4 per cage, upon arrival at the animal facility. At 4 or 10 weeks old, respectively, the rats were divided into subgroups that were single housed for 24 hours (N = 30 from each age) or continuously group housed (N = 10 from each age). Twenty of the single-housed rats from each age group were given access to 20% (v/v) alcohol for one 2-hour session. Ten single-housed rats of each age were kept as water controls, and 10 group-housed rats of each age were kept as housing controls. The rats were sacrificed directly after the 2-hour session.

**Part 2.** Three-week-old male Wistar rats (RccHan:WI; Harlan Laboratories B.V.) were pair housed (N = 20) or single-housed (N = 20) upon arrival. Ten of the single-housed and 10 of the pair-housed rats were assigned to the alcohol-drinking group, and the other rats were assigned water. Before alcohol sessions began, the wooden houses were removed, and the cages were divided to keep the rats from drinking from each other’s bottles. The divider was made from see-through plastic with a mesh wire to allow for tactile contact. Water controls and single-housed rats were also given dividers at the start of each session. The alcohol-drinking rats were given a choice between 20% (v/v) alcohol or water, and the water-drinking rats had access to 2 bottles of water. Access was given for three 2-hour sessions per week, Tuesday, Wednesday, and Thursday, for 6 weeks, about 3 hours into the dark phase of the light/dark cycle. At 10 weeks of age, the rats were sacrificed immediately at the end of a 2-hour session.

**Alcohol Intake**

Alcohol solutions were made from 96% ethanol (Solveco Etanol A 96%; Solveco AB, Rosersberg, Sweden) and tap water. Intake was measured at the end of each session by weighing the bottles, and intake was calculated in grams of pure alcohol per kilogram body weight (g/kg). For weekly intake, an average intake for each animal per week was calculated followed by the median of this intake in each group. Preference for alcohol was calculated as the percentage (%) of the total fluid intake drunk from the alcohol bottle.

**Dissection**

The rats were sacrificed by decapitation. The brain and pituitary were removed, and in the adult rats, the pituitary gland was further divided into the neurointermediate and anterior lobes. The hypothalamus was removed from the brain, which was then placed in a cooled matrix (ASI Instruments, Inc., Warren, MI). Coronal sections were made by manually slicing with razor blades. From these sections, 9 structures were dissected: medial prefrontal cortex, nucleus accumbens, caudate putamen, hippocampus, amygdala, substantia nigra, ventral tegmental area, and periaqueductal gray. In part 2, the cingulate cortex was also dissected. All tissues were immediately frozen on dry ice and stored in −80°C.

**Peptide Analysis**

Tissue extraction, purification of extracts, and analysis of the opioid peptides with specific radioimmunoassays were done according to previously described protocols (Christensson-Nylander et al., 1985; Nylander et al., 1997). Antiserum for the dynorphin B (DYNB) and MEAP peptides was generated in rabbits and used in a final dilution of 1:250,000 for DYNB (113+) and 1:80,000 for MEAP (90:3DII). The antisera have been thoroughly characterized and shown to be valid as selective markers for prodynorphin and proenkephalin, respectively, (e.g., Christensson-Nylander et al., 1985; Nylander et al., 1997). The MEAP antiserum shows no cross-reactivity with Leu-enkephalin or prodynorphin-derived peptides. Cross-reactivity for the DYNB antiserum was 1% with DYNB 29 and 100% with dynorphin 32. No cross-reactivity has been found with proenkephalin- or proopiomelanocortin-derived peptides. For
the beta-endorphin (BEND) assay commercially available antiserum was used, according to the protocol provided by the manufacturer (Peninsula Laboratories LLC, San Carlos, CA). Antibody bound peptides in the DYNB and BEND assays were separated from free peptides by adding 50 μl goat-anti-rabbit IgG and 50 μl normal rabbit serum (Peninsula Laboratories LLC). In the MEAP assay, separation was performed by adding charcoal suspension.

Statistical Analysis

Statistical analyses were performed using Statistica 12 (StatSoft Inc., Tulsa, OK). Differences were considered statistically significant at \( p < 0.05 \).

Alcohol or fluid intake was not normally distributed, as shown by the Shapiro–Wilk’s test (\( W < 0.95 \) suggests skewed distribution) and nonparametric statistics were used. For analysis of intake over time, Friedman analysis of variance (ANOVA) followed by Wilcoxon matched pairs test was used. For differences between 2 groups, the Mann–Whitney \( U \)-test was used.

Body weights and opioid peptide data were normally distributed and for alcohol- or housing-induced effects factorial ANOVA was used followed by Fisher’s least significant difference (LSD) test. In part 1, age-specific effects were investigated in combination with experimental group (i.e., group housing, single housing, or single housing and alcohol drinking). In part 2, housing conditions (pair or single housing) in combination with drinking (alcohol or water) were investigated.

RESULTS

Body Weight

Part 1. Adolescent animals weighed 125 ± 3 g (mean ± SEM) and adults weighed 324 ± 2 g at the time of the 1 session of single housing and single housing and alcohol intake. There were no differences between experimental groups.

Part 2. A general increase in body weights was found in all groups, \( F(12, 432) = 3.942, p < 0.001 \), but no differences between groups were found (see Fig. S1). At the beginning of the experiment, the rats weighed 86 ± 1 g and at the end they weighed 323 ± 4 g.

Alcohol Intake

Part 1. The adolescent rats had a significantly higher intake during a single session of alcohol access than the adult rats (\( U = 64.0; p < 0.001 \)). Adolescent drinking ranged from 0.6 to 3.6 g/kg/2 h, with a median intake of 1.7 g/kg/2 h. Adult drinking ranged from 0.6 to 2.0 g/kg/2 h, with a median of 0.9 g/kg/2 h.

Part 2. There were no differences in alcohol intake or preference between single-and pair-housed rats during any of the 6 weeks of alcohol access (Table 1). Alcohol intake over time decreased in both the single-housed (\( \chi^2 = 21.1; p < 0.001 \)) and the pair-housed rats (\( \chi^2 = 15.5; p = 0.008 \)), while the alcohol preference fluctuated in the pair-housed group (\( \chi^2 = 15.9; p = 0.007 \)) (Table 1). In the single-housed group, there was a trend toward an increase in preference over time (\( \chi^2 = 10.4; p = 0.06 \)).

Total fluid intake decreased over time in all groups (\( \chi^2 = 114; p < 0.001 \)), from a median intake of 26.9 to 13.4 g/kg/2 h. A difference in total fluid intake was found only in week 3 between pair-housed water- and alcohol-drinkers (\( U = 18.0; p = 0.02 \)). Pair-housed water-drinkers had a median intake of 15.5 g/kg/2 h and alcohol-drinkers 18.9 g/kg/2 h. No other differences in total fluid intake were found.

Effects of Single Housing and Alcohol Intake on Opioid Levels

Part 1. Immunoreactive (ir) levels of the 3 peptides in the brain areas measured can be found in Table S1 for the adolescent rats and Table S2 for the adult rats. In general,
adolescent rats had lower levels of peptides than adult rats, with the exception of ir BEND in the nucleus accumbens, where adolescent levels were higher than in adults.

**Dynorphin B**—A main effect of experimental group (i.e., group housing, single housing or single housing and alcohol intake) was found in the amygdala, $F(2, 68) = 13.4, p < 0.001$ (Fig. 1A). Different responses between experimental groups in the 2 age groups were seen as interaction effects in the striatum, $F(2, 73) = 4.42, p = 0.02$ (Fig. 1B). Single housing induced decreased levels in the amygdala of both adolescent and adult rats (Fig. 1A), and in the striatum of adult rats (Fig. 1B). Alcohol (i.e., as compared to single-housed water-drinking rats) increased levels in the amygdala of both adolescent and adult rats (Fig. 1A), and in the striatum of adult rats (Fig. 1B). Main effects of age were found in the pituitary, $F(1, 68) = 29.1, p < 0.001$, hypothalamus, $F(1, 72) = 71.1, p < 0.001$, medial prefrontal cortex, $F(1, 65) = 74.9, p < 0.001$, striatum, $F(1, 73) = 6.70, p = 0.01$, hippocampus, $F(1, 74) = 35.7, p < 0.001$, amygdala, $F(1, 68) = 26.6, p < 0.001$, substantia nigra, $F(1, 71) = 30.5, p < 0.001$, and ventral tegmental area, $F(1, 71) = 19.6, p < 0.001$ (Tables S1 and S2).

Met-Enkephalin-Arg^6^Phe^7^—Main effects of experimental group were found in the hypothalamus, $F(2, 72) = 12.7, p < 0.001$, medial prefrontal cortex, $F(2, 72) = 3.96, p = 0.02$, hippocampus, $F(2, 74) = 9.28, p < 0.001$, and amygdala, $F(2, 59) = 6.11, p = 0.004$ (Fig. 2A–D). Different responses between experimental groups in the 2 age groups were seen as interaction effects in the hypothalamus, $F(2, 72) = 5.77, p = 0.005$, medial prefrontal cortex, $F(2, 72) = 9.03, p < 0.001$, and pituitary, $F(2, 65) = 4.91, p = 0.01$ (Fig. 2A,B,E). Single housing induced increases in MEAP in the medial prefrontal cortex, hippocampus, and pituitary of the adolescent animals (Fig. 2B,C,E). In the adult rats, effects of single housing were seen as increases in levels of the hypothalamus and hippocampus (Fig. 2A,C). Alcohol increased MEAP in the amygdala of adolescent rats (Fig. 2D), and induced decreases in MEAP levels in the hypothalamus, medial prefrontal cortex, and hippocampus of the adult rats (Fig. 2A–C). Gradual changes in levels, from group housing to single housing with alcohol, were seen in the pituitary of adult rats (Fig. 2E). Main effects of age were found in the hypothalamus, $F(1, 72) = 28.3, p < 0.001$, medial prefrontal cortex, $F(1, 72) = 61.4, p < 0.001$, hippocampus, $F(1, 74) = 63.8, p < 0.001$, amygdala, $F(1, 66) = 33.3, p < 0.001$, substantia nigra, $F(1, 72) = 16.8, p < 0.001$, and ventral tegmental area, $F(1, 64) = 52.4, p < 0.001$ (Tables S1 and S2).

**Beta-Endorphin**—Main effects of experimental group were found in the nucleus accumbens, $F(2, 71) = 6.11, p = 0.02$, and amygdala, $F(2, 73) = 3.33, p = 0.04$ (Fig. 3A,B). A group effect was also seen in the anterior lobe of the pituitary, $F(2, 37) = 4.03, p = 0.03$, for the adult rats (Table S2). Different responses between experimental groups in the 2 age groups were seen as interaction effects in the nucleus accumbens, $F(2, 71) = 4.14, p = 0.02$ (Fig. 3A). Single housing decreased BEND levels in the amygdala of adult rats (Fig. 3B). Alcohol induced an increase in the nucleus accumbens of adolescent rats (Fig. 3A). Main effects of age were found in the pituitary, $F(1, 72) = 61.0, p < 0.001$, hypothalamus, $F(1, 73) = 7.36, p = 0.008$, nucleus accumbens, $F(1, 71) = 52.2, p < 0.001$, amygdala, $F(1, 73) = 9.05, p = 0.004$, and periaqueductal gray area, $F(1, 72) = 18.1, p < 0.001$ (Tables S1 and S2).

**Part 2.** Ir levels of the 3 peptides in the brain areas measured can be found in Table S3.

**Dynorphin B**—Main effects of alcohol were found for levels in the anterior lobe of the pituitary, $F(1, 32) = 28.8, p < 0.001$, the whole pituitary, $F(1, 28) = 10.8, p = 0.003$, and nucleus accumbens, $F(1, 34) = 5.15, p = 0.03$, where alcohol reduced levels in the anterior lobe of the pituitary of the pair-housed (Fig. 4A) and single-housed rats (Fig. 4B).
Alcohol also decreased levels in the whole pituitary of the single-housed rats (Fig. 4B). Differences in response to alcohol between the pair- and single-housed groups were found in levels of the amygdala, $F(1, 31) = 5.60, p = 0.02$, where a slight decrease was found in pair-housed rats (Fig. 4A) as opposed to a slight increase in the single-housed rats (Fig. 4B).

**Met-Enkephalin-Arg⁶Phe⁷**—Main effects of alcohol were found for levels in the whole pituitary, $F(1, 26) = 6.60, p = 0.02$, and nucleus accumbens, $F(1, 30) = 5.32, p = 0.03$, with significant increases in the pituitary of both pair- and single-housed rats (Fig. 4), and a significant increase in the nucleus accumbens of the single-housed rats (Fig. 4B). A main effect of housing was found in the cingulate cortex, $F(1, 34) = 7.27, p = 0.01$, where higher levels were found in the single-housed water-drinking rats compared to the pair-housed water-drinking rats (Fig. 5).

**Beta-Endorphin**—Main effects of alcohol were found for levels in the anterior lobe of the pituitary, $F(1, 35) = 9.14, p = 0.005$, the whole pituitary, $F(1, 33) = 4.19, p = 0.049$, and the ventral tegmental area, $F(1, 27) = 5.72, p = 0.02$, with a significant decrease in the anterior lobe of the

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**Fig. 2.** Met-enkephalin-Arg⁶Phe⁷ (MEAP) levels (fmol/mg tissue) in the (A) hypothalamus, (B) medial prefrontal cortex, (C) hippocampus, (D) amygdala, and (E) pituitary after short-term drinking in the adolescent and adult animals. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (ANOVA followed by Fisher's LSD test). G = group-housed water-drinking; SW = single-housed water-drinking; SE = single-housed ethanol-drinking.
pair-housed rats (Fig. 4A). A decrease was also seen in the amygdala of the single-housed rats (Fig. 4B). In the pair-housed rats, the amygdala response to alcohol was in the opposite direction, resulting in an interaction, $F(1, 35) = 6.41, p = 0.02$ (Fig. 4).

**DISCUSSION**

This study is, to our knowledge, the first to investigate effects of short- and long-term adolescent voluntary alcohol intake on endogenous opioids and the influence of single housing on these effects. The major findings were (i) age-specific short-term alcohol- and housing-induced effects, (ii) long-term effects of adolescent drinking, including housing-dependent effects in the amygdala, and (iii) changes in the cingulate cortex after long-term single housing.

**Short-Term Single Housing, Alcohol Intake, and Age-Specific Effects**

Short-term single housing, that is, 24 hours, mainly affected regions involved in the stress response, that is, pituitary, hypothalamus, amygdala, hippocampus, and cortex regions (Watts, 2000), which suggests that this is an acute stressor in both ages. A large impact of single housing on MEAP levels is in agreement with recent findings (Granholm et al., 2014). An increase in MEAP levels as seen here in the early stages of single housing could be an indicator of an activated stress-adapting system. Enkephalins have been suggested to enhance the activity of the mesolimbic dopaminergic system during stress and reinforce the positive emotional state, decrease anxiety and thereby result in better adaptation to stress (Przewlocki, 2002).
DYNB levels were lower after single housing in the amygdala in both adolescent and adult rats but only adults had lower levels in the striatum. The striatum has been hypothesized to be involved in the shift from initial drug use to compulsive use and addiction (Everitt and Robbins, 2013), and lower levels of dynorphin have been proposed to increase alcohol preference (Racz et al., 2013) and to confer vulnerability to addiction (Yuferov et al., 2009). The amygdala has also been proposed to play an important role in AUD, and dynorphin is involved in stress response in this area (Land et al., 2008). Decreased levels in DYNB after 24-hour single housing indicate a change in the responsiveness of this system, which in turn may affect the response to later drug intake as well as the vulnerability to substance use disorders.

The age-specific effects of single housing on both DYNB and MEAP levels may partly explain some of the differences in alcohol response and intake seen in adolescents compared to adults (Daoura et al., 2011; García-Burgos et al., 2009; Spear, 2000). In line with this, higher alcohol intake in the adolescent animals was also observed in the current study.

A finding of particular interest was the difficulty to distinguish specific alcohol-induced effects in the adolescent animals, that is, without confounding effects by single housing. The alcohol effects noted were increases in DYNB and MEAP in the amygdala and an increase in BEND in the nucleus accumbens. The effects on DYNB in the amygdala were similar in both ages, indicating that the response to alcohol is independent of age in this area. Overall, alcohol-specific effects were mainly observed in adult rats and, generally, alcohol intake counteracted the housing effects. Such a pattern was not evident in the adolescent animals, showing that responses to alcohol in combination with single housing are age specific.

Alcohol intake in adults generally induced increases in DYNB levels and decreases in MEAP and BEND levels, which is opposite to findings of increased enkephalins (Marinelli et al., 2006; Mendez et al., 2010; Seizinger et al., 1983) and endorphins (Jarjour et al., 2009; Lam et al., 2008) after acute injections of alcohol. However, the results may well be in line with the idea of increased endogenous opioid activity in response to acute alcohol (Drews and Zimmer, 2010), but a 2-hour drinking session may be too long to assess these effects. That is, if the rats drink most of their intake during the beginning of the session, degradation of released peptides (Hallberg et al., 2005) would account for the decreases seen in MEAP, whereas release of BEND to the blood stream would account for the decreases seen in the pituitary (Gianoulakis, 1987). Compensatory release of dynorphin in response to increased dopamine can account for the later increase seen in DYNB (Drews and Zimmer, 2010). Moreover, the acute alcohol-induced effects may also depend on the mode of intake. Most studies used intraperitoneal administration (Jarjour et al., 2009; Lam et al., 2008; Marinelli et al., 2006; Mendez et al., 2010; Seizinger et al., 1983), whereas this study used voluntary intake, which is known to cause different effects than forced intake (Spanagel, 2003). As single housing per se changed peptide levels, it is important to note that the motivation to drink may relate to the separation from cage mates and that the effects induced by alcohol are effects in an individual already affected by housing conditions.

**Long-Term Single Housing and Alcohol Intake**

Effects of long-term single housing during adolescence were seen as increased MEAP in the cingulate cortex. Interestingly, the cingulate cortex plays a role in generation of emotional states and executive control of the influence of these states on behavioral selection (Etkin et al., 2011). Changes in this area may have implications for social deficits associated with several psychiatric disorders and may also be involved in the escalation of drug intake that occurs in the addiction cycle (Perry et al., 2011). Antagonism of delta-opioid receptors in the cingulate cortex has been shown to produce anxiety-like behavior in mice (Narita et al., 2006) and patients with borderline personality disorder show greater mu-opioid receptor activation in response to sustained sadness (Prosin et al., 2010). These results point toward important regulatory functions of the endogenous opioid system in this area, and the changes seen herein indicates that lack of social interactions disturb the normal regulation and contribute to vulnerability to addiction.

BEND in the amygdala was affected by both short- and long-term single housing, but the response was different depending on the duration of the single housing. Interestingly, the amygdala is involved in both social interactions and AUD. For example, the rodent amygdala is important in social play (Vanderschuren and Trezza, 2014) and mu-opioid receptor density is increased in the basolateral amygdala after social isolation during early adolescence, and this effect is persistent even after social housing (Van den Berg et al., 1999). The amygdala is also implicated in emotional memory and BEND impairs memory formation in the amygdala (Quirarte et al., 1998). Furthermore, a role for opioids in
amygdala in relation to AUD has been proposed and the alcohol-induced results in the single-housed animals are in line with the proposed hypothesis of increased ligands for the kappa-opioid receptor and decreased ligands for the mu-receptor in AUD (Kissler et al., 2014), although the increase in DYNB in the single-housed animals was not statistically significant.

When comparing the long-term alcohol drinking in pair- and single-housed animals, no differences were found in alcohol intake, but there was a trend towards an increase in preference over time in the single-housed rats. The results are consistent with a previous study that showed no differences in intake during adolescence between single-and pair-housed animals (Doremus et al., 2005). Other studies of single housing/isolation rearing have mainly focused on adult alcohol intake, where most studies show an increased intake in the isolation-reared groups (Becker et al., 2011; Chappell et al., 2013). In the future, it would therefore be of interest to continue the alcohol intake further into adulthood to see whether the single-housed animals will escalate their intake.

Interestingly, the alcohol-induced effects on BEND and DYNB levels in the amygdala were clearly dependent on housing condition. The differential response in in pair-and single-housed animals suggests that amygdala is sensitive to housing conditions and that there are underlying differences in this brain area between animals reared under different social conditions. However, many of the long-term alcohol-induced effects were independent of housing condition, showing that single housing may not always produce confirmed results. Increased levels of DYNB in the nucleus accumbens may be an adaptation response to the increased MEAP and fits with the hypothesis of increased dynorphin activity in the course of the addiction cycle (Drews and Zimmer, 2010). The decrease in BEND in the ventral tegmental area also suggests adaptation mechanisms to prolonged alcohol intake (Drews and Zimmer, 2010). Changes in the pituitary may reflect a modulated stress axis in response to repeated alcohol intake (Koob and Volkow, 2010). The consequences of adolescent voluntary alcohol intake for endogenous opioids in nondependent and non-prefering animals are to our knowledge not known and the effects seen herein need to be further investigated to interpret the functional relevance of changes induced by adolescent drinking.

**Methodological Discussion**

For the long-term drinking in this study, pair-housed rats were used as social controls. The practical reason was that pair-housed rats could easily be separated by a divider during alcohol intake, to allow for individual intake measurement. Using a divider is a simple and cost-effective solution of separating rats in their home cage. The wire mesh lets the rats keep part of the tactile, as well as auditory, visual, and olfactory contact, with their cage mate. It has previously been shown that contact through a mesh barrier, similar to the one used in this study, is sufficient for social reward-conditioned place preference in adolescent rats (Peartree et al., 2012). Furthermore, the short-term presence of the divider gave the rats plenty of time to play during the rest of the day. This is important as play behavior is critical for the normal development of rats (Vanderschuren and Trezza, 2014). Furthermore, differences in morphine consumption between pair-housed and single-housed rats have been shown and, interestingly, these differences can be abolished by short daily sessions of social interactions, suggesting that social interaction can attenuate isolation-induced changes in endogenous opioid activity levels (Raz and Berger, 2010). However, common controls in isolation studies are group-housed rats and there are some differences between group and pair housing. For example, the response to novelty has been shown to differ between adolescent male rats that were housed 2 or 3 per cage (Zakharova et al., 2012). It is therefore possible that the effects of housing on opioids could be different depending on the control group used, something to bear in mind when comparing the short sessions from part 1 with the long-term drinking in part 2.

**CONCLUSIONS**

The results show that age and housing conditions are important factors in studies of alcohol-induced effects on the endogenous opioid system. The model of prolonged voluntary adolescent alcohol intake under different housing conditions tested herein proved useful in separating housing- and alcohol-induced effects and revealed interaction effects in areas related to emotionality. Thus, variation in age and housing may lead to different underlying mechanisms for alcohol intake and may therefore affect vulnerability to AUD and studies of potential therapeutic agents.

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Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Body weights (g) (mean ± SEM) of the 4 groups of the long-term drinking rats in part 2 throughout the experiment.

**Table S1.** Mean ir dynorphin B (DYNB), Met-enkephalin-Arg⁶Phe⁷ (MEAP), and beta-endorphin (BEND) levels (fmol/mg tissue) ± SEM in the dissected brain areas in the adolescent group-housed, single-housed, and ethanol-drinking rats.

**Table S2.** Mean ir dynorphin B (DYNB), Met-enkephalin-Arg⁶Phe⁷ (MEAP), and beta-endorphin (BEND) levels (fmol/mg tissue) ± SEM in the dissected brain areas in the adult group-housed, single-housed and ethanol-drinking rats.

**Table S3.** Mean ir dynorphin B (DYNB), Met-enkephalin-Arg⁶Phe⁷ (MEAP), and beta-endorphin (BEND) levels (fmol/mg tissue) ± SEM in the dissected brain areas in the pair-housed and single-housed water- and ethanol-drinking rats.