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ORIGINAL INVESTIGATION

Assessing appetitive and consummatory phases of ethanol self-administration in C57BL/6J mice under operant conditions: regulation by mGlu5 receptor antagonism

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Abstract
Rationale The development of mouse models of ethanol consumption and ethanol-seeking behavior is of particular importance in understanding the underlying mechanisms of drug abuse because these models can enable an analysis of an effect of specific genotype on drug-seeking behavior and the interaction of potential therapeutics with genotype. However, there are some limitations with present models, notably the inability to examine appetitive and consummatory behavior separately.
Materials and methods In the present study, C57BL/6 mice were trained to self-administer 10% ethanol in a modified operant protocol that allowed a clear delineation of consummatory and appetitive phases. The utility of this procedure was confirmed with the use of the metabotropic glutamate 5 (mGlu5) receptor antagonist 3-[(2-methyl-1,3-thiazol-4-yl) ethynyl]-pyridine (MTEP).
Results Limited-access consumption during the dark phase of the light–dark cycle with intermittent access (every second or third day) led to a high level of consumption by the mice. MTEP caused a dose-dependent decrease in both the consumption of ethanol and the appetitive response for ethanol. Furthermore, this effect was unrelated to any effect of MTEP on locomotor activity.
Conclusions The model provides a useful paradigm for examining both the appetitive and consummatory phases of ethanol consumption in mice; furthermore, the data indicate mGlu5 receptors are involved in both phases.

Keywords mGlu5 · MTEP · Ethanol self-administration · Mice

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Introduction

The development of mouse operant models of drug-seeking behavior was of particular benefit in understanding the underlying mechanisms of drug abuse because these can enable us (using genetically modified mice) to analyze the effect of specific genotype on drug-seeking behavior and the interaction of potential therapeutics with genotype (Chiamulera et al. 2001; Elmer et al. 2002; Navarro et al. 2001; Szumlinski et al. 2004). However, the development of mouse operant models of ethanol self-administration has been somewhat problematic. Although mice have been trained to self-administer ethanol intravenously using operant techniques (Grahame et al. 1998; Kashkin et al. 2002), a more appropriate model would involve oral ethanol self-administration. Thus, mice were trained to preferentially respond for oral ethanol using a sucrose-fade technique (Middaugh et al. 2000b; Olive et al. 2000; Risinger et al. 1999). Whereas this technique is amenable to pharmacological manipulation, a disparity between levels of responding and actual levels of consumption was observed (when examined; Middaugh et al.

2000a,b). More frequently, consumption of ethanol is examined separately using a two-bottle preference test (measurement of volumes consumed of ethanol solution vs water; Rhodes et al. 2005; Roberts et al. 2000, 2001; Ryabinin et al. 2003; Stephens et al. 2005). This method, however, does not allow analysis of the pattern of consumption (without the use of lickometer or similar equipment) and the latency to ethanol-seeking behavior cannot be examined; therefore, there is some room for refinement in these models.

The metabotropic glutamate 5 (mGlu5) receptor is one of the family of eight G-protein-coupled glutamate receptors within the central nervous system and retina (Conn and Pin 1997; Hermans and Challiss 2001). At a biochemical level, mGlu5 receptors have been associated with phosphoinositide hydrolysis and activation of phospholipase C and with the stimulation of adenylate cyclase and the inhibition of voltage-operated calcium channels (Conn and Pin 1997; Hermans and Challiss 2001). At a behavioral level, mGlu5 receptors appear to have a role in stress and anxiety-like responses (Brodkin et al. 2002b; Busse et al. 2004; Klodzinska et al. 2004), depressive-like behavior (Pilc et al. 2002; Tatarczynska et al. 2001), and spatial memory formation (Balschun and Wetzel 2002; Lu et al. 1997). The mGlu5 receptor also appears to mediate, at least in part, the reinforcing properties of a number of drugs of abuse because the mGlu5 antagonist MPEP [2-methyl-6-(phenylethynyl)-pyridine] decreased cocaine and nicotine self-administration (Kenny et al. 2005; Paterson et al. 2003) and morphine- and cocaine-induced place preference (Aoki et al. 2004; McGeehan and Olive 2003).

MPEP was also shown to decrease self-administration of ethanol in rats and mice (Hodge et al. 2006; Olive et al. 2005; Schroeder et al. 2005) and prevented the reinstatement of ethanol-seeking behavior induced by olfactory cues in rats (Bäckstrom et al. 2004). We have recently demonstrated that MTEP (3-[(2-methyl-1,3-thiazol-4-yl) ethynyl]-pyridine), a mGlu5 receptor antagonist with greater selectivity and bioavailability than MPEP (Anderson et al. 2002; Cosford et al. 2003), caused a decrease in ethanol self-administration by two strains of alcohol-preferring rats: the inbred alcohol-preferring and the Fawn-Hooded (Cowen et al. 2005b) rats. However, to date the effect of MTEP on ethanol self-administration has not been determined in mice. Therefore, using some novel modifications of recent operant procedures (Hodge et al. 2006; Middaugh et al. 2000b; Sharpe and Samson 2001) and our own experience using operant procedures in rats (Cowen et al. 2005a,b; Lawrence et al. 2006; Liang et al. 2006), in the present study, we have developed a protocol that enables the quantification of both the appetitive and consummatory phases of ethanol consumption in C57BL/6J mice. In so doing, we also validated this technique for neuropharmacological intervention by determining the effect of MTEP on both phases in mice.

Materials and methods

All experiments were performed in accordance with the Prevention of Cruelty to Animals Act, 1986, under the guidelines of the National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia.

Animals

Male C57BL/6J mice (n=18) were obtained from the breeding colony at the Howard Florey Institute, University of Melbourne. This colony is maintained using mice F1 stock or B6 mice obtained from the Animal Resource Centre, Western Australia (rederived annually using mice from The Jackson Laboratory). The mice were 4 months of age at the commencement of experimental procedures and the average weight of the mice at the beginning was 25±1 g. Mice were used either to examine the effect of MTEP on appetitive and consummatory behavior, followed by locomotor behavior (n=8) or locomotor activity alone (n=10). Mice used for operant sessions had unlimited access to both water and standard chow, apart from during the operant sessions, and were group-housed (4–5/cage) under a reversed 12-h light/dark cycle (lights off at 10:30 A.M.). Operant sessions commenced 2–4 h after the onset of the animal's dark phase, corresponding to their peak time of ethanol consumption under ad libitum conditions (Middaugh et al. 2000b; Rhodes et al. 2005; Ryabinin et al. 2003). Mice were trained under operant conditions every second or third day because preliminary tests and previous reports (Olive et al. 2000) indicated that intermittent access enhanced consumption of ethanol by mice. Mice used for locomotor activity alone had unlimited access to both water and standard chow, except during analysis of locomotor activity, and were housed under a standard light/dark cycle (lights on at 7:00 A.M.).

Operant ethanol self-administration

The effect of MTEP on the appetitive and consummatory phases of ethanol consumption was examined using operant chambers supplied by Med Associates (St Albans, VT, USA). Each chamber was housed individually in sound-attenuation cubicles, with a fan to provide airflow and mask external noise. The chambers were connected to a computer running Med-PC IV software (Med Associates) to record lever responses and receptacle licks. Two ultrasensitive retractable mouse levers (ENV-312M) were placed on

either sides of a mouse liquid receptacle (ENV-303LP); the levers could be inserted into the chambers during the operant session and retracted at the end of the operant session. A cue light was placed above each lever. A variable speed pump (PHM-100VS) delivered fluid directly to the liquid receptacle from a 5-ml syringe via Silastic tubing. Fluid consumption from the receptacle was monitored using a lickometer controller (ENV-250) controlling a capacitance contact sensor connected to the receptacle and grounded on the grid floor of the operant chamber; thus, delivery of fluid could be triggered either in response to lever pressing or licks at the receptacle.

The mice underwent two 1-h habituation sessions in the operant chambers and a further two (days 3 and 4) when a 16% (w/v) sucrose solution was present in the receptacle. Mice were then trained to self-administer sucrose under an fixed-ratio (FR)-1 schedule, such that one lever press on the active lever led to the activation of the cue light (3 s) and delivery of 10 μl of 16% sucrose solution into the receptacle. After four sessions with 16% sucrose, the session length was reduced to 30 min and the mice were trained to self-administer ethanol using a sucrose-fade procedure (Middaugh et al. 2000b; Samson et al. 1988) according to the following sequence (percent sucrose and percent ethanol: v/v, days): 16/0, 8 days; 12/12, 2 days; 8/12, 2 days, 4/12, 1 day; 0/12 for the remainder of these sessions. Data (i.e., responses and receptacle licks) were collected into 6×5 min time bins. Once responding became stable over sessions, with preferential responding for ethanol compared with responding on the nonreinforced lever, the experimental protocol was modified, allowing for a separate examination of consummatory and appetitive behavior. The effect of MTEP was examined in these two distinct experimental phases.

Phase 1: effect of MTEP on consummatory behavior

During sessions in phase 1, mice (n=8) gained access to neither lever, but each receptacle was filled with 650 μl of ethanol solution before the commencement of the 30-min session. Consumption was monitored using the lickometer and recording commenced before placing the mice in the operant chambers because the onset of consumption was very rapid. Initial tests with a range of ethanol concentrations (5, 10, and 20%) led to the choice of 10% ethanol for all further experiments as this stimulated a relatively high rate of consumption in the first 10 min of the access session (see Results). Licks at the receptacle were used to control ethanol delivery; thus, five licks at the receptacle caused 25 μl of 10% ethanol solution to be delivered directly into the receptacle, approximately maintaining the volume within the receptacle (average lick volume 5.6 μl). The volume of ethanol solution consumed was verified by comparing the volume within the receptacle at the beginning and end of the access session. Once consumption patterns were stable (<20% variation across three sessions), MTEP (5–40 mg/kg, i.p.) or vehicle was administered 20 min before the operant session. This time point was chosen based on previous works demonstrating the efficacy of this compound in mice and rats (Brodkin et al. 2002a; Busse et al. 2004; Cowen et al. 2005b). At least one nontreatment session occurred before MTEP was tested again at a different dose, and the mice received each dose of MTEP in a semirandom (nonsequential) manner. The numbers of licks per session and licks/5-min time bin were recorded.

Phase 2: effect of MTEP on appetitive behavior

At the commencement of each 30-min operant session in phase 2, both levers were inserted but the receptacle was empty. Mice (n=8) were trained to respond to gain access to 650 μl of 10% (v/v) ethanol solution at FR-10 for two sessions and then FR-20 for the remainder of the operant sessions. A stimulus light above the active lever indicated (1 s) when the lever press requirement was made. After the lever press requirement was made, the active lever was withdrawn and no further lever presses could be made. However, the nonreinforced lever remained inserted throughout the operant session as a measure of general activity. Training continued until the latency to gain access to ethanol remained stable (<10% variation across sessions). MTEP (5–20 mg/kg, i.p.) or vehicle was administered 20 min before the operant session. At least one nontreatment session occurred before MTEP was tested again at a different dose, and the mice received each dose of MTEP in a semirandom manner. The latency until the mice gained access to the ethanol was recorded, and so were the number of licks per session and the number of the responses on the nonreinforced lever.

Locomotor activity

To confirm that the effect of MTEP was a specific effect on ethanol consumption and not due to sedation or some other locomotor effect, the effect of MTEP on locomotor activity was analyzed. For this experiment TruScan Photobeam Activity Monitors were used. The arenas are of clear polymethyl methacrylate (25×25×40-cm high) with the photobeam sensor ring in the X–Y plane 2.2 cm above the floor pan. The TruScan system beam spacing is 1.52 cm, allowing for a spatial resolution of 0.76 cm. The sampling interval was 100 ms and light at the level of the floor pan was 90 lux. Once the mice were placed in the locomotor cells the experimenter left the experimental area. MTEP-experienced mice (n=8) were habituated in these locomotor cells in 3×30 min-sessions over 3 days. Mice received

MTEP (20–40 mg/kg, i.p.) or vehicle on alternate days in a semirandom manner, 20 min before being placed in the locomotor cells. The total distance moved was recorded automatically for each 30-min session. To confirm that tolerance to the effect of MTEP had not occurred, a second group of mice (n=10), naïve to both ethanol and MTEP, were habituated in the locomotor cells in 3×30-min sessions over 3 days. Mice then received vehicle on two separate days and MTEP (20 mg/kg, i.p.) on the last experimental day 20 min before being placed in the locomotor cells.

Statistics

A significance level of p=0.05 was used throughout. The relationship between ethanol consumption and lickometer contacts was analyzed using analysis of covariance. In general, the effect of MTEP on appetitive and consummatory behavior was analyzed using ANOVA or repeated measures ANOVA as appropriate, with Student–Newman–Keuls post hoc tests. However, the effect of MTEP on latency to access ethanol was analyzed using Friedman's nonparametric ANOVA on ranks. Locomotor activity was measured using a one-way repeated measures ANOVA. Where results were negative (p>0.05), power analyses were performed to confirm a type II error was not made (accept null hypothesis when the samples were in fact different). One mouse did not respond during the initial phases of the training and so data from this mouse was not included in the analysis of the relevant sections (i.e., where the data sets were not complete).

Drugs

MTEP was dissolved in DMSO before being diluted in dH_2O and delivered at a volume of 10 ml/kg (maximum concentration of DMSO 5%). For doses of 20 mg/kg and above, 5% 1,2-propanediol was also used. Although vehicles with and without propanediol were tested on the mice, only the data obtained using the 5% 1,2-propanediol-containing vehicle are presented because this was the potentially more aversive. There was, however, no difference in the data obtained with the two vehicles.

Results

Using a prefilled receptacle with lick-induced delivery of ethanol, varying the concentration of ethanol altered the pattern of ethanol consumption (Fig. 1a). Ten percent ethanol solution was chosen to further characterize consummatory behavior because this induced a relatively high level of consumption in the first 5–10 min. With access to a 10% ethanol solution in the receptacle, the mice made an average of 121±22 licks at the receptacle per 30-min session, corresponding to an average intake of 2.6±0.3 g/kg (average lick volume 5.6±0.4 μl). There was a highly significant correlation between the number of licks made at the receptacle and the ethanol consumed using a single value per animal (R=0.980, R^2=0.960, F(2,6)=0.002, p=0.002) or two data points per animal (one data point missing; R=0.832, R^2=0.692, F(3,12)=6.740, p=0.011).

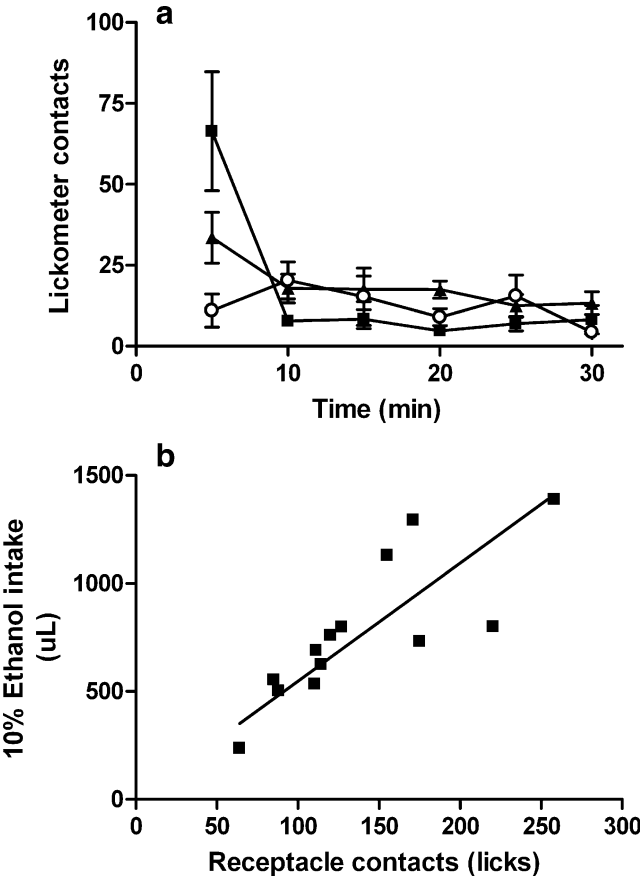

Fig. 1 **a** Altering the concentration of ethanol available at the receptacle led to differential consummatory behavior, with progressively lower licks at the receptacle (particularly within the first 5 min) for 20% (*open circles*), 10% (*closed triangles*), and 5% (*closed squares*) ethanol solutions. Data are mean±SEM (n=7). **b** A significant correlation (p<0.001) was observed between total volume consumed and the number of licks made at the receptacle. Two data points per mouse and (n=7) are presented, except in one instance when multiple data was unavailable from one mouse

Effect of MTEP on consummatory and appetitive behavior

In phase 1, MTEP caused a marked reduction in consumption of ethanol by the C57BL/6J mice, which was significant at 20 and 40 mg/kg [F(4,26)=6.355, p=0.001; Fig 2a]. Time course analysis indicated this effect of MTEP predominantly occurred within the first 10 min of the access session [F(4,144)=16.494, p<0.001; Fig. 2b]. In

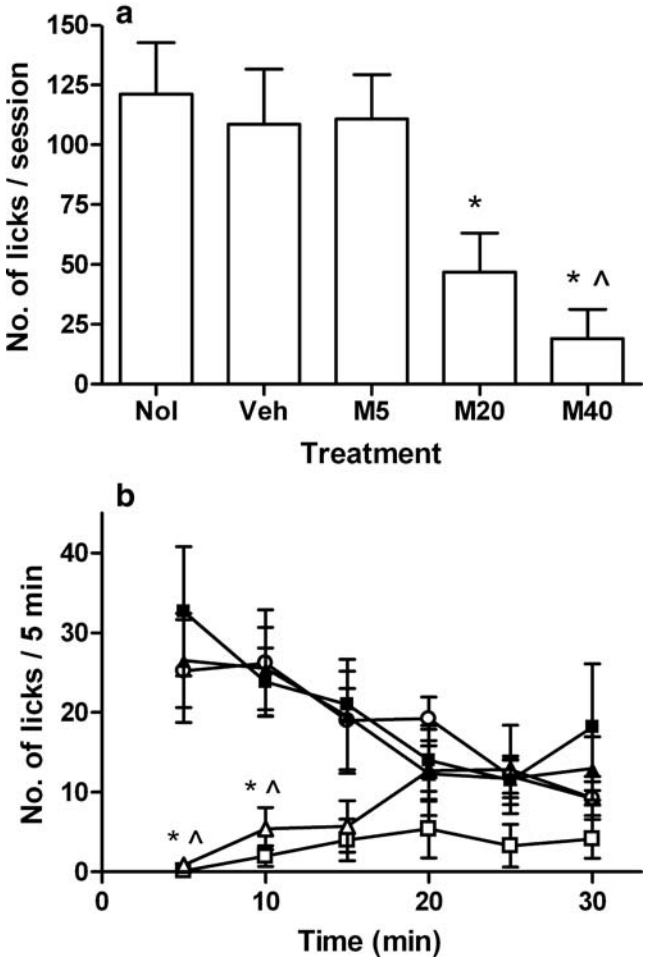

Fig. 2 Effect of MTEP (5–40 mg/kg, i.p.) on ethanol consumption as determined using a lickometer during a 30-min access session. Average volume/lick was 5 μl. **a** MTEP caused a significant decrease ($p<0.05$) in ethanol consumption at 20 mg/kg (*M20*) and 40 mg/kg (*M40*) compared with vehicle (*Veh*, *caret*) and noninjection (*NoI*, *asterisk*) days (*M5*, 5 mg/kg MTEP). **b** MTEP at 20 mg/kg (*open triangles*) and 40 mg/kg (*open squares*) significantly ($p<0.05$) reduced consumption predominantly in the first 10 min of access. *Asterisks*: significantly different to noninjection (*closed squares*) days. *Carets*: significantly different to vehicle (*closed triangles*) treatment. MTEP (5 mg/kg i.p.) (*open circles*) had no effect on the consumption of ethanol. Data are mean±SEM ($n=8$)

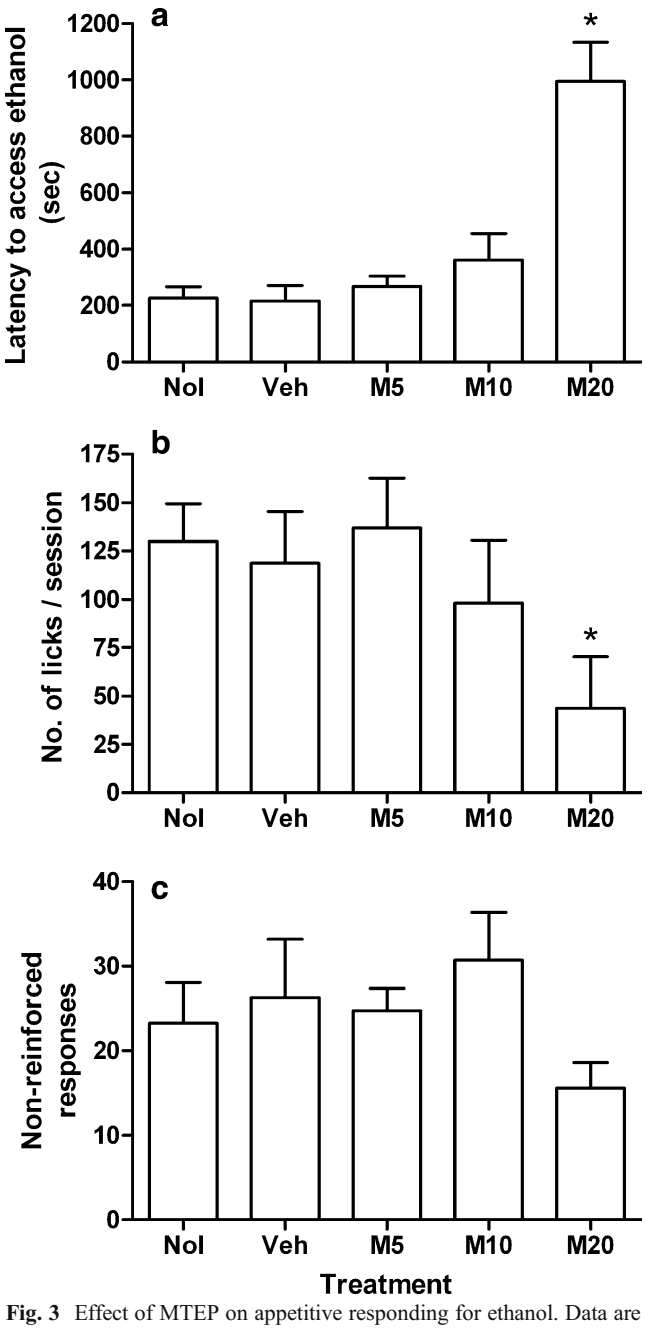

Fig. 3 Effect of MTEP on appetitive responding for ethanol. Data are mean±SEM ($n=8$). *Asterisk*: MTEP caused a marked increase in the time taken to meet the lever press requirement (compared with both vehicle and noninjection days) such that the time taken to access the ethanol was significantly ($p<0.05$) increased (**a**) and a significant decrease in ethanol consumption as determined using a lickometer (**b**). MTEP had no effect on the number of times the nonreinforced lever was pressed (**c**)

phase 2, the mice learned to respond at FR-20 to gain access to ethanol over a period of approximately nine sessions, with the latency to access ethanol decreasing to an average of 215±43 s. MTEP caused a significant increase in the latency to access ethanol [Friedman's test $\chi^2(4)=16$, $p=0.003$; Fig. 3a] with a significant decrease in the number of times the receptacle was accessed [$F(4,24)=4.916$, $p=0.005$; Fig. 3b]. There was no significant effect of MTEP on nonreinforced responding at the highest dose tested [20 mg/kg; $F(4,24)=1.627$, $p=0.2$; Fig. 3c]. Power analysis indicated that for a difference of 15 with a residual standard deviation of eight, power was 0.818.

Effect of MTEP on locomotor activity

In ethanol- and MTEP-experienced mice, MTEP caused a significant reduction in locomotor activity in terms of distance moved [$F(4,24)=12.999$, $p<0.001$; Fig. 4a], but post hoc analysis indicated this was only at the highest

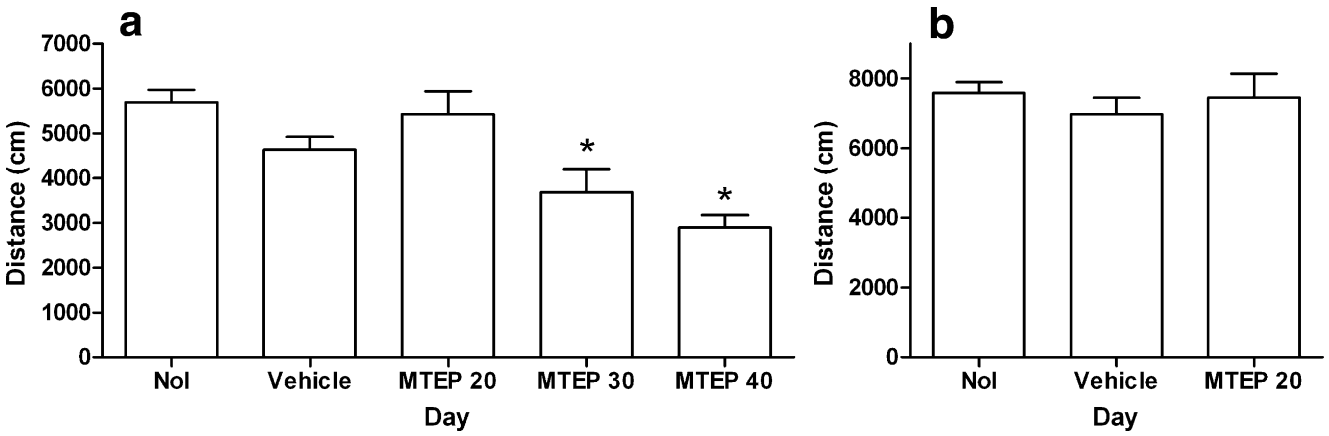

Fig. 4 Effect of MTEP on locomotor activity during a 30-min session. Data are mean±SEM (n=8–10). *Asterisk*: in ethanol- and MTEP-experienced mice, MTEP caused a significant (p<0.05) reduction in distance moved (**a**) compared with both noninjection (*NoI*) and vehicle (*Veh*) treatment days, but only at the highest doses used (30 and 40 mg/kg). **b** MTEP (20 mg/kg, i.p.) had no significant effect on distance moved by ethanol- and MTEP-naive mice

doses tested (30 and 40 mg/kg, i.p.). The dose of MTEP effective at reducing both consummatory and appetitive responding for ethanol (20 mg/kg, i.p.) had no significant effect on locomotor activity in ethanol- and MTEP-experienced mice. In the separate cohort of ethanol- and MTEP-naïve mice, MTEP (20 mg/kg i.p.) had no significant effect on distance moved [$F(2,18)=0.410$, $p=0.669$; Fig. 4b] over the same time period. Power analysis indicated that for a difference in means of 2,600 (using the same relative ratio as that observed with 30 mg/kg of MTEP in MTEP-experienced mice), with an expected standard deviation of 980, power was 1.000.

Discussion

The present study represents an advance in oral operant ethanol self-administration models in mice by clearly delineating between appetitive and consummatory phases in consumption using off-the-shelf components. Although consumption as determined using a lickometer has been measured previously (Boyce-Rustay and Risinger 2003; Middaugh et al. 2000b; Mittleman et al. 2003; Risinger et al. 2000), licks do not appear to have been used to deliver ethanol or water. Therefore, removing the lever press requirement during this phase represents a simplification of the procedure that may more adequately reflect simple consummatory behavior. In contrast, the delayed access induced with a FR-20 lever press requirement allowed a robust effect on appetitive responding to be observed. We note that whereas temporally resolved consumption can be determined by attaching a lickometer controller to a home cage ethanol/water bottle, this method would preclude analysis of appetitive behavior.

The amount of ethanol consumed (2.6 g kg^{-1} $session^{-1}$) appears to be one of the highest levels of ethanol consumption observed in mice without further dietary manipulations (i.e., food or water restriction, or sucrose added to the solution; Finn et al. 2005; Middaugh and Kelley 1999; Mittleman et al. 2003; Ryabinin et al. 2003). We attribute this high consumption to a combination of intermittent access (every second or third day) and access during the early dark phase, factors that have both been shown to enhance ethanol consumption in mice (Middaugh et al. 2000b; Olive et al. 2000; Rhodes et al. 2005; Ryabinin et al. 2003), but never previously in combination (as far as we can ascertain). However, at the present time, we cannot conclude unequivocally which of the components of the training regime contributed to this high level of ethanol consumption.

The separation of the appetitive and consummatory phases of ethanol consumption in the present study in mice parallels, and is built upon, the work of Samson and colleagues on rats (Czachowski et al. 2001; Samson et al. 1998, 1999; Sharpe and Samson 2001). In these previous studies, an increasingly elevated response requirement led to access to a sipper tube so that both appetitive (ethanol-seeking) and consummatory (ethanol-drinking) behavior could be analyzed. These two aspects were differentially amenable to pharmacological manipulation; thus, the opioid antagonist naloxone (Sharpe and Samson 2001) and acamprosate (Czachowski et al. 2001) diminished consumption but not appetitive responding, whereas the cannabinoid CB1 receptor antagonist SR141716A also decreased appetitive responding (Freedland et al. 2001). Interestingly, our previous work with rats has suggested that acamprosate may decrease the motivational relevance of ethanol-associated cues (Cowen et al. 2005a), suggesting that there are aspects

of motivation to consume (certainly cue-induced motivation) that are separate to the innate or trained motivation that can be assessed using an appetitive-consummatory paradigm (Czachowski et al. 2001). In the present study, licks at a receptacle rather than at a sipper tube (Samson et al. 1998, 1999) were used, although a retractable sipper tube for mice is now available through the same company (Med Associates) that supplied the other components of our operant system. The sipper tube could therefore also be connected to a lickometer controller to provide the same level of temporal resolution of consumption as in the present study.

In the present study, the mGlu5 antagonist MTEP caused a significant and dose-dependent decrease in both consummatory and appetitive phases of ethanol consumption. MTEP had no effect on responding on the nonreinforced lever, nor did it affect locomotor activity at the lowest dose that caused a significant decrease in consumption; thus, the effect of MTEP was specific to ethanol consumption. In general, these results are in line with previous studies demonstrating a significant decrease in ethanol consumption and ethanol-seeking behavior using the mGlu5 receptor antagonist MPEP (Hodge et al. 2006; Olive et al. 2005) and also the prevention of cue-induced reinstatement of ethanol-seeking behavior (Bäckstrom et al. 2004). The present studies confirm and extend these findings by demonstrating that MTEP, an mGlu5 receptor antagonist with greater selectivity and bioavailability than MPEP (Anderson et al. 2002; Cosford et al. 2003), also affected both appetitive and consummatory phases of ethanol consumption. Interestingly, in contrast with our study in rats (Cowen et al. 2005b), MTEP decreased ethanol-seeking behavior and consumption at a dose that did not affect locomotor activity, confirming the locomotor-modifying effects of MTEP are mediated via a separate mechanism (whether at the level of the receptor and/or at a more neuroanatomical level) to its effect on drug-seeking behavior, at least in mice.

In summary, we have developed a protocol that allows the analysis of consummatory and appetitive phases of ethanol consumption in mice. Access to an ethanol solution during the dark phase of the light–dark cycle with intermittent access (every second or third day) led to a high level of consumption by the mice and ethanol consumption was significantly correlated with receptacle contacts. The mGlu5 receptor antagonist MTEP significantly reduced both the consumption of ethanol and the appetitive response for ethanol by these mice. Further modifications of this protocol may, however, be possible; for example, removing the sucrose-fade training and increasing the lever press requirement in the appetitive phase to provide a paradigm akin to a progressive ratio requirement or break-point analysis (Czachowski and Samson 2002). Moreover, the appetitive responding phase that we have developed is also clearly amenable to an extinction–reinstatement schedule. In conclusion, we have developed and pharmacologically verified a robust method for analysis of consummatory and appetitive phases of ethanol self-administration under operant conditions in mice. This paradigm will prove useful in the characterization of genes involved in the motivation to consume ethanol.

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